THE UNIVERSITY *of* ADELAIDE

INVESTIGATING THE ROLE OF ARABIDOPSIS PLASMA MEMBRANE INTRINSIC PROTEIN AtPIP2;1 IN SEED GERMINATION

In partial fulfilment of the requirements for the degree of

Doctor of Philosophy

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Abstract

Soil salinity can cause osmotic stress during seed germination by limiting water uptake and causing NaCl accumulation. Exposure to salinity stress during seed imbibition can alter germination percentage, slow the germination rate and seedling growth. Subsets of membrane intrinsic proteins called aquaporins contribute to salinity stress tolerance in plants, and some isoforms of these proteins could have roles in helping seeds to germinate in saline conditions. Aquaporins have multiple roles in many physiological processes, such as solute transport, hydraulic conductance, signalling, development and adjusting to changes in the environment.

Recent studies have revealed that some plant aquaporins are candidates for being involved in both water and Na^+ transport. For example, *Arabidopsis thaliana* Plasma Membrane Intrinsic (PIP) proteins AtPIP2;1 and AtPIP2;2 transported both water and univalent cations (Na^+ and K^+) when tested in heterologous systems. To explore whether AtPIP2;1 influenced Na^+ transport in plants, *Atpip2;1* loss of function mutants and wild type Columbia-0 seed were germinated on media containing 50 mM NaCl. The wild type Arabidopsis seed appeared to germinate earlier than the seed from the *Atpip2;1* mutant lines. This preliminary observation led to the hypothesis that there may be a link between the function of AtPIP2;1 and factors that influence seed germination percentage in Arabidopsis. To test this hypothesis, wild type Arabidopsis, two *Atpip2;1* mutant lines, a *Atpip2;1*Atpip2;2* mutant line, and two transgenic lines overexpressing AtPIP2;1 (35S::AtPIP2;1) and a null control line were propagated in controlled conditions and seed was harvested from each of these seven genotypes. The seed was used to test whether the seven genotypes differed in germination percentage and seed area, weight and Na^+ and K^+ content when imbibed in control or saline treatment. This research aims to investigate the role of an aquaporin protein, AtPIP2;1, in seeds in the model plant *Arabidopsis thaliana*. A series of exploratory experiments were carried to explore possible roles for this protein in seed germination, with a particular focus in germination under salt stress.

In saline conditions, seed from *Atpip2;1* mutant lines and lines overexpressing AtPIP2;1 germinated slower than wild type and null control lines, respectively. Fifty hours after sowing, the seedlings of one mutant *Atpip2;1* line weighed more than wild type, and seedlings of both 35S::AtPIP2;1 overexpression lines weighed less than null controls. When imbibed in saline conditions, *Atpip2;1* mutant line seed contained more K^+ than wild type seed. Whereas seed

from lines overexpressing AtPIP2;1 contained more K^+ than null line seed when imbibed for 30 h in water then transferred to a solution containing 75 mM NaCl from 30 to 50 h after sowing, but not when imbibed in the saline treatment from 0 to 50 h. In conclusion, the potential for these observations to be a consequence of AtPIP2;1 transport of water, ions or hormones, or linked to protein-protein interactions and signaling roles are discussed. Options for future experiments targeted at distinguishing which aspects of AtPIP2;1 functions are important in contributing to optimal seed germination in saline treatment are considered.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by

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Phan Thi Thanh Hoai

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List of Publications

Journal publication

- Phan T.T. Hoai, Stephen D. Tyerman, Nicholas Schnell, Matthew Tucker, Samantha A. McGaughey, Jiaen Qiu, Michael Groszmann and Caitlin S. Byrt**. Deciphering aquaporin regulation and roles in seed biology.** *Journal of Experimental Botany*. doi:10.1093/jxb/erz555. 28 February 2020

Conference poster

- Hoai Phan Thi Thanh, Jiaen Qiu, Matthew Gilliham, Caitlin S. Byrt. **Roles of Aquaporin water and ion transport during seed germination.** *Grains Research & Development Corporation.* 20-21 Feb 2018. Adelaide, Australia.

- Phan T. T. Hoai, Samantha A. McGaughey, Jiaen Qiu, Matthew Tucker, Nicholas Schnell, Stephen D. Tyerman, Caitlin S. Byrt. **Roles for aquaporins in seed water relations.** *ARC Centre of Excellence in Plant Energy Biology Annual Forum.* 3 -6 June 2019. Canberra, Australia.

- Phan Thi Thanh Hoai, Caitlin S. Byrt, Jiaen Qiu, Stephen D. Tyerman. **Exploring aquaporin roles in seed development and germination.** *National Meeting of the Australia and New Zealand Society for Cell and Developmental Biology Conference.* 2 – 4 October 2019. Adelaide, Australia.

Oral presentation

- Hoai T. T. Phan, Matthew Gilliham, Jiaen Qiu, Caitlin S. Byrt. **Testing whether AtPIP2;1 function influences seed imbibition and germination.** *ARC Centre of Excellence in Plant Energy Biology Annual Forum.* 11 – 13 September 2018. Perth, Australia.

- Hoai T. T. Phan, Jiaen Qiu, Stephen D. Tyerman, Caitlin S. Byrt. **Analysing Aquaporin roles in seed development and germination.** *The ComBio2019 - Australian Society of Plant Scientists Conference.* 26 – 29 November 2019. Melbourne, Australia.

Media presentation

- Hoai T. T. Phan. **Tiny holes in seed membranes impact food security**. *Virtual Three Minute Thesis (3MT®) competition, The University of Adelaide.* 17 July 2020. https://vimeo.com/439306261.

Chapter 1

Introduction and Literature review

1.1 Aquaporins in plants

The term 'aquaporin' refers to 'water-pore', represented by 'aqua' and 'porin', respectively. Aquaporins (AQPs) are Membrane Intrinsic Proteins (MIPs) capable of providing rapid passive water transport across cell membranes and they play critical roles in whole plant water relations (Chaumont and Tyerman, 2017). Besides these well-known roles, AQPs can also conduct a wide range of nonpolar solutes (urea, glycerol), and unconventional permeants, such as the nonpolar gases (carbon dioxide, nitric oxide), the polar gas (ammonia), the reactive oxygen species (hydrogen peroxide), cations (sodium, potassium) and the metalloids antimonite, arsenite, boron and silicon (Chaumont and Tyerman, 2017; Li *et al.*, 2014). AQPs have different permeability which are affected by the regulation and drive of the phosphorylation, heteromerization, pH, Ca^{2+}, pressure, solute gradients, temperature and nutritional conditions (Maurel *et al.*, 2008; Tyerman *et al.*, 1999). The concept that plant AQPs may have roles in ion transport has emerged relatively recently, and it is not yet clear how AQP ion transport might influence plant physiology (Tyerman *et al.*, 2021).

Inspired by the discoveries of Yool et al. (1996) that the human aquaporin 1 protein (HsAQP1) could also transport ions in addition to water, Zhao (2013) tested whether ionic conductance could be detected when AtPIP2;1 was expressed in *Xenopus laevis* oocytes. Zhao and colleagues (2013) observed that AtPIP2;1, which is a plant aquaporin with 93.4% identity to HsAQP1, could transport ions when expressed in heterologous systems. The team observed that AtPIP2;1 was permeable to Na^+ when expressed in yeast *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes indicating that AtPIP2;1 influenced transport of Na^+ across membranes (Byrt *et al.*, 2017). AtPIP2;1 was also observed to influence K^+ transport (Qiu *et al.*, 2020). However, there are yet to be any reports revealing AtPIP2;1 transport of ions *in planta*. The observations of aquaporin ion transport in heterologous systems revealed the possibility that AQPs may participate in physiological processes not only related to water homeostasis and neutral substrate transport, but also related to nutrient acquisition and non-selective cation channel (NSCC) mechanisms (Tyerman *et al.*, 2021). This study specifically focused on the roles of Arabidopsis plasma membrane intrinsic protein *AtPIP2;1* during the germination period.

Aquaporin structures

The structure of AQPs is similar in many eukaryotes and includes a circular α-helical group arranged to make a narrow pore of about 3 Å to let solutes through from the inside

(intracellular) to the outside (extracellular) of the cell and vice versa (Chaumont and Tyerman, 2017). The AQP standard structure can be described as a monomer including six tilted (crossing angles between 25 and 40°) membrane spanning α-helices (H1 - H3 and H4 - H6) and two re-entrant short α-helices (HB and HE) forming two halves of the basic shape, with five interconnecting loops (LA - LE) that collectively form a right-handed α-helical bundle (Fig. 1.1A). The two re-entrant alpha-helices HE and HB are in the centre of AQPs and carry an Asn-Pro-Ala (NPA) motif. The NPA motif is thought to be involved in preventing the transmembrane movement of H^+ and other ions (Luang and Hrmova, 2017). Four AQP monomers assemble as tetramers in membranes, with each monomer functioning as a transporting channel, and there is a central channel in the middle (Chaumont and Tyerman, 2017; Verkman, 2013) (Fig. 1.1B).
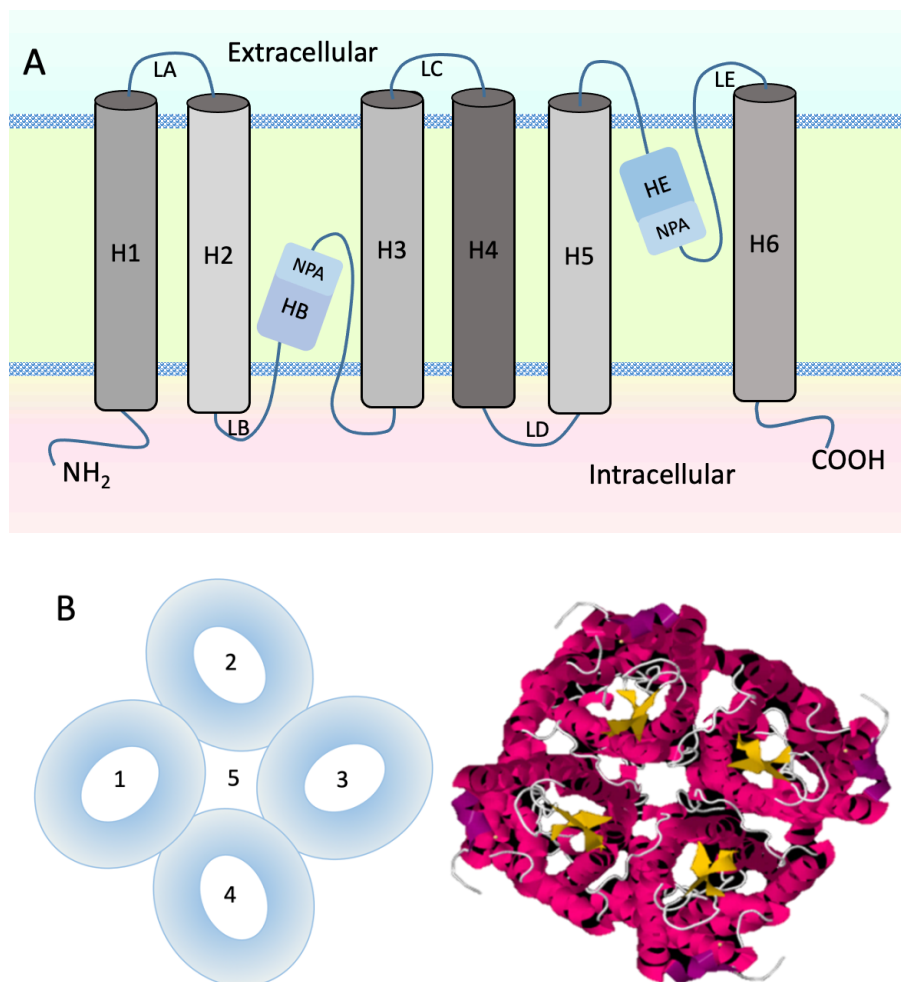

Figure 1.1: Basic membrane topography of the aquaporin monomer and tetramer.

A. Six trans-membrane α-helical segments are labelled H1 – H6, are connected by five loops (LA to LE). N- and C-terminal domains are localized in the cytosol. Two short helical segments in loop B and loop E with HE and HB labels are NPA

motifs (Asn-Pro-Ala) (Adapted from Chaumont and Tyerman, 2017). All segments are folded halfway into the membrane from opposite sides forming a single aqueous pore with high selectivity. B. Four monomer AQPs (1 - 4) assemble into a tetramer with a central channel in the middle (5). The model on the right is an example of AtPIP2;1 from SWISS MODEL https://swissmodel.expasy.org/repository/.

Aquaporin isoforms

The number of aquaporins isoform differs between organisms; from one or two isoforms in microorganisms to 13 isoforms in human and many in plants (Azad *et al.*, 2021; Chaumont and Tyerman, 2017; Day *et al.*, 2014; Gomes *et al.*, 2009; Kitagawa *et al.*, 2011). In *Arabidopsis thaliana*, rice (*Oryza sativa L.*), flax (*Linum usitatissimum*) and barley (*Hordeum vulgare L.*), there are 35 (Johanson *et al.*, 2001), 33 (Sakurai *et al.*, 2005), 51 (Shivaraj *et al.*, 2017) and over 40 (Hove *et al.*, 2015) AQP encoding genes, respectively.

In plants, AQP isoforms are classified into five sub-families in both dicots and monocots based on sequence homology. They are plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) (Johanson and Gustavsson, 2002), and the largely uncharacterised X intrinsic proteins (XIPs) (Danielson and Johanson, 2008). There are three other AQP subfamilies which have been identified: the GlpF-like intrinsic proteins (GIPs), the hybrid intrinsic proteins (HIPs) found in the moss *Physcomitrella patens* (Danielson and Johanson, 2008; Gustavsson *et al.*, 2005), and the uncategorized X intrinsic proteins (XIPs), which have been found in a wide variety of non-vascular and vascular plants (Bienert *et al.*, 2011; Danielson and Johanson, 2008). The types of AQPs in each species may differ and not all species have a member from each of all five of the above sub-families. For example, in the model plant *Arabidopsis thaliana,* 35 MIP (major intrinsic protein) homologues are divided into 13 plasma membrane intrinsic proteins (PIPs), which can be further split into PIP1 (five isoforms) and PIP2 (eight isoforms) subfamilies according to their sequence identity. PIP2 proteins have a shorter N terminal extension and a longer C terminal end containing putative phosphorylation sites compared with PIP1 proteins. The TIPs contain 10 subfamily members split into 5 groups i.e. TIP1, TIP2, TIP3, TIP4 and TIP5. The NIPs include nine subfamilies from NIP1 to NIP7. There are three SIPs and no XIPs in *Arabidopsis thaliana* (Fig. 1.2) (Bezerra-Neto *et al.*, 2019; Quigley *et al.*, 2001).

Aquaporins are distributed in almost every parts of organisms. In plants, they present in cell plasma membrane, vacuole, ER membrane; both vegetative and reproductive growths including important organs including : root, stem, leaf, petal, egg, pollens. The majority of plant AQPs fall into the PIPs and the TIPs subfamilies which have been reported to be localized both at the plasma membrane and at intracellular membranes (Gattolin *et al.*, 2009; Mizutani *et al.*, 2006), and these subfamilies are thought to have the most important roles in transcellular and intracellular water transport (Shivaraj *et al.*, 2017). The PIPs are highly expressed in organs and tissues where there are large fluxes of water, such as vascular tissues, guard cells and suberized endodermis and bundle sheath cells (Chaumont and Tyerman, 2014; Cintia *et al.*, 2017). Water flux is also key in seeds, where influx of water triggers germination, but the role of AQPs in seed germination is yet to be clearly understood (Vander Willigen *et al.*, 2006).

Figure 1.2. Phylogenetic analysis of the complete set of 35 different MIPs (aquaporins) encoded in the Arabidopsis genome.

Arabidopsis thaliana MIPs are divided into four distinct subfamilies: PIPs, TIPs, NIPs, and SIPs. Similar proteins within a subfamily, with a maximum of 30% divergence, are clustered in monophyletic groups. The first and the last digit in the protein name identify the group and the individual protein reference, respectively. The figure is adapted from Johanson et. al (2001).

1.2 Seeds and the process of germination

Seeds play an essential role in human life because they are a staple food for both humans and animals. Half of the worldwide energy intake per capita is provided by seeds, therefore research in the area of seed biology is important for ensuring the worlds energy needs continue to be met (Bewley, 1997). Seeds are also the primary means of transferring and maintaining genetic information to the next generation. Seeds in the plant kingdom differ significantly in size and anatomy. For example, *Arabidopsis thaliana* seed is relative small, approximately 500 μm in length (Muller *et al.*, 2006), about one tenth of the length of a rice seed, and the longest seeds in the world are 30-centimetre-long palm tree seeds. For a new plant to be produced from a seed, it must undergo germination. Germination is controlled by cellular processes, such as mRNA transcription and translation, cell extension, cell cycle activation, mechanisms of reparation and organelle reassembly (Rosental *et al.*, 2014). As the seed takes up water during imbibition many biochemical processes occur resulting in the production of enzymes that react with substrates within the seed and trigger the germination process. Metabolic activity generates the building units needed to translate proteins, for cell growth and division, and for radicle protrusion (Rosental *et al.*, 2014).

The pathways for water uptake when seeds imbibe and germinate may differ in different plant species. For the model plant *Arabidopsis thaliana*, and for agriculturally relevant crops such as rice *(Oryza sativa L.)*, wheat *(Triticum aestivum)* and barley (*Hordeum vulgare)*, the relative importance of AQP function in seed imbibition and germination is actively studied. It has been suggested that AQPs may not have a role in initial water uptake during imbibition, but their function may be needed in early germination (Gattolin *et al.*, 2011). For example, when imbibing Arabidopsis seeds were treated with mercury, an AQP inhibitor, there was little difference in water uptake but the treatment reversibly delayed radicle emergence and testa rupture (Vander Willigen *et al.*, 2006). However, a possible role for plasma membrane intrinsic proteins (PIPs) in rice germination is supported by the observation that a reduction in *OsPIP1;3*

transcript by gene silencing reduced seed germination percentage relative to normal seed (Chaumont and Tyerman, 2017; Lutts *et al.*, 2016).

Seeds in a mature dry condition can serve as a storable food or serve as the next generation of plants. In this stage, seeds are dormancy, which is an innate seed property associated with environmental conditions to germinate. Mature seeds can be categorized into several types in terms of water content and their germinating ability after maturation. An orthodox seed can desiccate to a low water content and be stored for a period of time without an effect on germination percentage whereas recalcitrant seeds contain a higher water content and perish during desiccation and storage. Another group is considered to be a combination between orthodox and recalcitrant, called intermediate seeds. These seeds can desiccate for storage but the lifespan is reduced, especially when they are preserved at low temperature (Pammenter and Berjak, 2000). Recalcitrant seeds are desiccation intolerant and become damaged if dried to 40% of the seed water content (Benech-Arnold and Sanchez, 2004; Obroucheva *et al.*, 2017). To develop into a new seedling, the orthodox seeds need to absorb water, a process called imbibition.

Imbibition is the process of water absorption by dry seed during germination process. It is a very important pre-germination event that initiates the biochemical processes leading to germination of the seed. Seed imbibition involves water absorption into a quiescent dry seed and starts immediately when the dry seeds contact water. Water absorption initiates energy metabolism, regulation of oxidative status, DNA repair, cell cycle activation, energy reserve mobilization and modification of hormonal status (Lutts *et al.*, 2016). Water influx during seed imbibition is also thought to occur mainly via a small opening called the micropyle (Manz *et al.*, 2005).

There are three phases of imbibition (Fig. 1.3). In Phase I water is rapidly and passively absorbed into the dry tissues of the seed mainly through apoplastic spaces. The rate of water uptake is thought to remain static in Phase II, where metabolic activity increases in preparation for next phase. Phase III has an increase in water uptake which occurs in association with the appearance of the embryonic axes and radicle protrusion (Bewley, 1997; Fenner and Thompson, 2005; Lutts *et al.*, 2016; Vander Willigen *et al.*, 2006).

Phase I of germination involves water absorption or imbibition. In this initial process, water influx is rapidly driven by the very low water potential (a matrix potential) in dry seed (Obroucheva *et al.*, 2017). Seeds need to reach a certain moisture content to begin germination. For example, barley seed needs around 35% to 45% greater moisture content than that of their

initial dry weight, whereas for pea seeds, after ~6 h imbibition, they increase their weight up to or over 130% of their initial weight in preparation for germination (Fettell *et al.*, 2010; Veselova and Veselovsky, 2006). In Arabidopsis, imbibing seed during this phase change in size and shape (Robert *et al.*, 2008). Testa of Arabidopsis carries volcano-shaped cell wall structures that quickly release mucilage covering the seed. This layer is composed mainly of rhamnogalacturonan pectins with smaller amounts of homogalacturonan, cellulose, xyloglucan, xylan, arabinan and galactan and cellulose arranged in an outer water soluble layer and an inner layer covalently bound to the testa by cellulose microfibrils (Haughn and Western, 2012; Macquet *et al.*, 2007; Voiniciuc *et al.*, 2016). The mucilage is very hydrophilic and delays water loss, it can adhere to surfaces for seed dispersal and aid in germination in osmotically stressed environments, such as saline environments (Yang *et al.*, 2010).

Phase I
Imbibition

- Seed swelling
- Volume change
- Shape change
- Solute leakage

Phase II
Metabolism Activation

- Rehydration
- Leakage of cellular solutes
- Resumption of respiration
- Oxygen consumption
- Activation of amino acid metabolism
- Activation of Kreb's cycle enzymes
- DNA reparation and protein synthesis
- Mitochondria reparation
- New mitochondria form
- mRNAs are synthesized and produce proteins
- Diffusion of substrates and small metabolites
- Reassembly of organelles through entire seeds

Phase III
Germination

- Cell divide
- Testa rupture
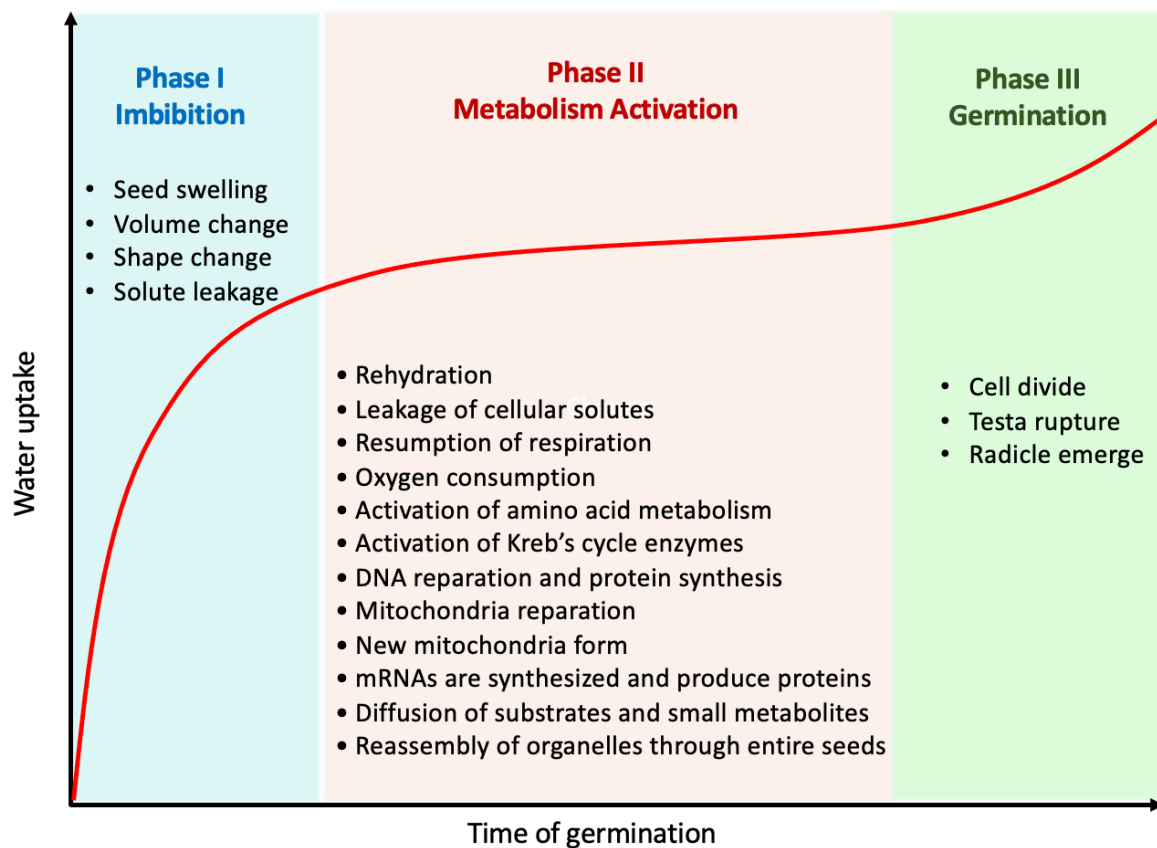- Radicle emerge

Water uptake

Time of germination

Figure 1.3: Seed water uptake during germinating phases and major events associated with imbibition and germination.

The time for water uptake and germination events to be completed varies from several hours to many weeks, depending on the plant species and the germination conditions (Adapted from Bewley, 1997; Weitbrecht *et al.*, 2011).

Phase II of germination is where metabolic activity increases; the embryo has imbibed water and is swollen. During this stage, hormones that stimulate enzyme activity are produced by the embryo that break down complex substances such as starch and proteins to simple sugars and amino acids, providing energy to the growing embryo. Other activities simultaneously take place in the seed such as restoration of cellular integrity, mitochondrial and DNA repair, and respiration (He and Yang, 2013). For orthodox seeds, this can be a reversible process, which means seeds imbibe water until the end of this phase, then if unfavourable conditions occur, such as drought, seeds can desiccate and become dormant again (Lutts *et al.*, 2016). This phase ends when the seed coat begins to rupture and the radicle emerges. This is also the first sign of germination visible to the naked eye.

The chemical processes involved in Phase II, according to Bewley (1997) and Weitbrecht et al. (2011), include: the rehydration of cells and organelles, the imbibitional leakage of cellular solutes (Crowe and Crowe, 1992; Van Steveninck and Ledeboer, 1974), resumption of respiration leading to oxygen consumption, the activation of amino acid metabolism and Kreb's cycle enzymes, DNA reparation and protein synthesis, mitochondrial reparation and mitochondria genesis, synthesis of mRNAs, protein synthesis, diffusion of substrates and small metabolites along with reassembly of organelles throughout the entire seed.

In Phase III of germination, the embryo grows quickly and is easy to be observed. The radicle appears first, followed by other primary roots and then the hypocotyl or coleoptile protrudes. The activity of Phase II enzymes results in the transfer of sugars and amino acids stored in the seed to the growing embryo (Bewley *et al.*, 2013; Fenner and Thompson, 2005)

The diameter of seeds changes during imbibition but the extent to which the diameter increases depends on the seed coat and the ability to absorb water. After 3 h of imbibition, wild-type Columbia seeds of Arabidopsis generally have a 10% and 5% increase in their short and long diameters, respectively, and there is little change after this point (Preston *et al.*, 2009; Robert *et al.*, 2008). Measurement of changes in short and long diameters, or maximum cross sectional areas do not fully represent isodiametric changes. True volume changes could be determined using a specific gravity bottle or pycnometer (Fasina, 2010; Tang and Sokhansanj, 1993). The shape of the seed changes during imbibition due to water uptake and intrinsic metabolic activities. Observations were made by Robert et al. (2008) that in seeds of Arabidopsis mutants with altered ethylene signaling (*ctr1-1, eto1-1, etr1-1, ein2-1*), that the shape of the mutant seed differed from wild type, specifically, the values for curvature at the poles were greater.

Between apical and base poles, there was significant differences in curvature, the value of the curvature decreased in both poles with the largest change seen in the mutant seed.
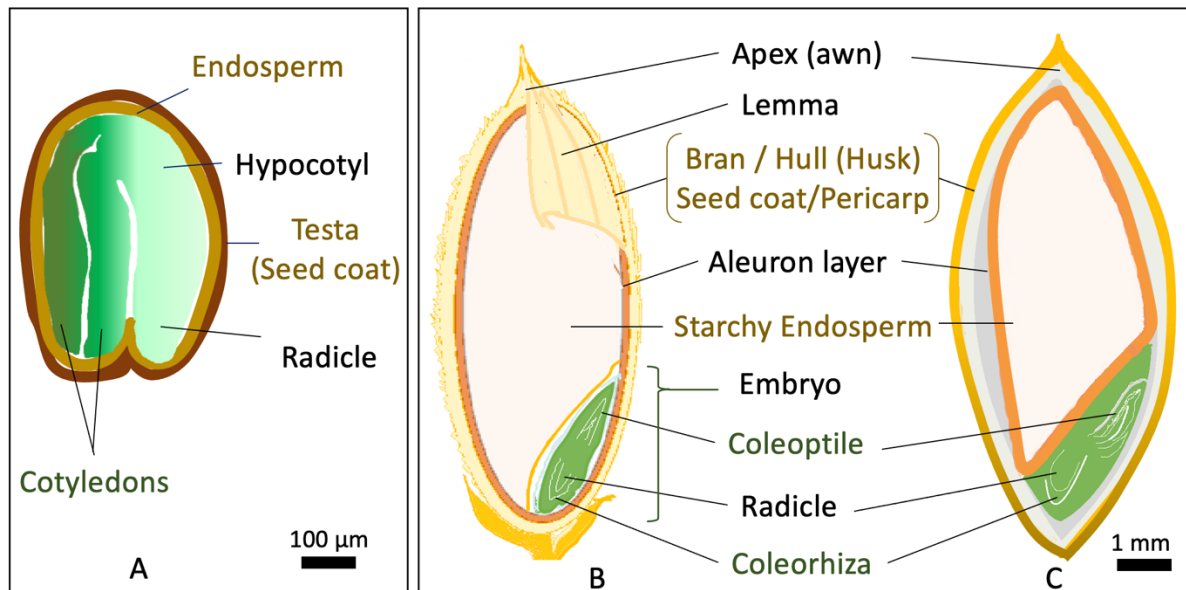

Figure 1.4: Seed anatomy of *Arabidopsis thaliana* (A), rice *Oryza sativa L.* (B) and barley *Hordeum vulgare L.* (C). Bar scales were applied for each frame. (Adapted from FAO, 1995; Muller *et al.*, 2006; Sýkorová *et al.*, 2009).

Seeds from different plants can be diverse in anatomy and appearance, however they have features in common such as the seed coat (testa), endosperm which contains nutrients, the apical meristem or cotyledon that become the leaves later and the radicle that becomes the first root (Figure 1.4). The emergence of the radicle and the root tip out of seed coat is the first visible step in the germination process. Then the hypocotyl or coleoptile protrudes from the seed coat with a main role of protecting the emerging leaf. Next, the first leaf emerges; its green colour is usually visible through the hypocotyl or coleoptile. From this time, a plant can photosynthesise if there is available water, carbon dioxide and sunlight, and it no longer needs the energy stored in the endosperm. After seed germination, water is no longer absorbed primarily by the testa and is instead mainly absorbed through the root.

The time from imbibition to germination depends on many factors such as species. For example, the first rupture of the seed coat in *Arabidopsis thaliana* may be 35 hours after imbibition (Muller *et al.*, 2006; Vander Willigen *et al.*, 2006), and He et al. (2013) showed that radicle emerge was approximately 48 - 50 hours after imbibition for barley and rice. Moreover, the germination time of each species is also affected by other environmental conditions: light

quality, temperature, moisture, aeration (O_2 and CO_2), the presence of any chemical inhibitor substances (in the laboratory this can be mercury, NaHS, NaF) (Veselova and Veselovsky, 2006), in nature there can be biochemical inhibitors and soil mechanical impedance, and interactions between these factors (Baudouin *et al.*, 2016; Hadas, 2004; Imaizumi *et al.*, 2017; Ishibashi *et al.*, 2017).

Radicle elongation from the embryo marks the ending of germination and the beginning of seedling growth. Although water uptake and transport are important in these processes it is not clear whether AQP function only becomes important after seedling growth begins, or at an earlier stage. There are two key processes that need to be coordinated for the initiation of radicle growth (Bewley, 1997). The first process occurs at the late stage of seed germination, osmotic potential decreases due to the accumulation of solutes during the hydrolysis of polymers in radicle cells creating a driving force for greater water uptake and leading to an increase in turgor and cell extension. The other process involves enzymes and hormones such as xyloglucan endotransglycosylases, auxin and abscisic acid influencing cell wall extensibility. This process enhances the elongation of the radicle cell wall (Bewley, 1997; Nonogaki, 2019). The driving force for water uptake can be disrupted if the soil environment is saline. Salinity stress reduces the rate of water uptake by seeds, causing inhibition of germination and root elongation (Werner and Finkelstein, 1995).

It is possible that cell osmotic potential remains unchanged during imbibition, but the cell wall in the radicle becomes weaker, and therefore the turgor potential of the radicle cells is adequate to drive extension (Bewley, 1997; Steinbrecher and Leubner-Metzger, 2017). The restriction from the surrounding structures may have little or even no effect on radicle cell extension in some seeds, but in seeds with a tough seed coat there is a need to reduce the toughness of the seed coat for germination to be completed. The elongating cells in the radicle require water influx to maintain turgor as they grow. The pathway of water into elongating radicle cells involves transport across cell plasma membranes and this may involve AQPs.

1.3 Aquaporins and water transport in seeds and seedlings

The role of AQPs in germination has been tested but the results are not conclusive. Water uptake into seeds is thought to be almost exclusively through the micropyle, however for water to enter the vasculature, AQPs could be involved (Maurel *et al.*, 1997; Vander Willigen *et al.*, 2006). For example, α-TIPs are expressed in the membrane of protein storage vacuoles in seeds

of many plant species (Maurel *et al.*, 1997). PIP2;4 showed predominant expression in roots, PIP2;6 and PIP2;8 expressed mainly in young and emerging leaves respectively (Da Ines, 2008). PIP2 is expressed in root hair zone and functions as a water channel to control cellular osmoregulation, transcellular water transport, or performs other roles in cell growth.

Transcript analysis of some AQPs is consistent with a possible function during early seedling growth (Footitt *et al.*, 2019; Hoai *et al.*, 2020; Klepikova *et al.*, 2016; Vander Willigen *et al.*, 2006). For example, expression of AQPs *PIP1;1, PIP2;1* and *TIP1;1* in pea (*Pisum sativum* L.) was detected in expanding cotyledons and in the seed coat during development and germination. These genes were also expressed in roots and shoots of seedlings but the expression of *NIP1;1* was observed only in the seed coat (Schuurmans *et al.*, 2003). This could mean NIP1;1 has a particular role in seeds, but this remains to be tested.

In Arabidopsis, TIPs are thought to contribute to water transport during seed germination and maturation. For instance, AQPs of the α-TIP sub class (TIP3) were found to be expressed in the membrane of protein storage vacuoles in Arabidopsis seeds (Höfte *et al.*, 1992), and expression decreased as the seedling became more established (Postaire *et al.*, 2007). Using fluorescent protein tagging of their genomic sequences and confocal microscopy, two isoforms, AtTIP3;1 and AtTIP3;2, were implicated in water transport during imbibition because they were the only two TIPs detected in the embryo in early seed germination (Gattolin *et al.*, 2011). Fluorescent protein tagging of TIP3;1 and confocal microscopy revealed bright fluorescence in dry seeds and during imbibition. The fluorescence continued during radicle emergence and throughout the emergence of the radicle and lasted several days post emergence (Gattolin *et al.*, 2011).

The roles of AQPs in germination and in hypocotyl or coleoptile growth remain to be established. In the beginning of imbibition, seeds experience the most rapid and extreme changes in tissue hydration relative to the entire process of germination. Water diffuses into the apoplasm and must cross cell membranes for the commencement of increased metabolic activity. It is not clear whether AQPs are important in this intracellular water transport process.

Involvement of AQPs in seed germination

Tonoplast intrinsic protein (TIP) roles in seeds have been investigated (Footitt *et al.*, 2019; Gattolin *et al.*, 2011), but PIPs may also play a role during imbibition and early germination. Putative roles for AQPs have been studied by applying treatments with inhibitors of AQP

function to germinating seeds. For example, an AQP blocker mercury which is non-specific, and functions as a strong metabolic inhibitor (Zhang and Tyerman, 1999) was applied to seeds, and the results reported by Willigen *et al.* (2006) indicated that mercury treatments altered germination percentage but did not alter water uptake in Arabidopsis seeds during imbibition. Hence, the authors suggested that AQPs do not play a role during imbibition. However, Frick and colleagues (2013) investigated the effect of treatments of 0.5 mM $HgCl_2$ on the rate of water transport through a *Spinacia oleracea* AQP called SoPIP2;1, and their data indicated that mercury did not inhibit all AQPs, indicating that tests with mercury may not be diagnostic for AQP function.

When mercury was applied to seeds as 5 μM $HgCl_2$, it decreased the speed of Arabidopsis seed germination, delaying it by 8 – 9 hours in relation to maternal seed coat (testa) rupture and 25–30 h for radicle emergence (Vander Willigen *et al.*, 2006). Mercury is thought to bind three out of four cysteine residues on AQP tetramers (Frick *et al.*, 2013). In a report by Veselova et al. (2006) a compound containing mercury (p-chloromercuribenzoate) was used as a blocker of water channels. Seed germination ability was estimated by counting the number of normal seedlings after 5 days of germination. The authors reported that when the content of the putative inhibitor increased the water uptake decreased but the germination percentage improved. NaF (100 μM) is an inhibitor of phosphatase, and treatments with NaF result in more rapid water uptake in high quality seeds of *Pisum sativum L.* pea. When air dried, those seeds subjected to accelerated ageing (Veselova and Veselovsky, 2006). Use of inhibitors alone to test AQP roles in imbibition and germination has not given conclusive results, perhaps because these inhibitors are non-specific and may inhibit metabolism and other processes (Zhang and Tyerman, 1999).

Rather than using putative AQP inhibitors, a genetics approach using Arabidopsis mutants with altered AQP function was pursued here as a strategy to investigate the role of AQPs in seed imbibition, germination and early seedling growth. This approach also was conducted by Footitt et al (2019) on tonoplast intrinsic proteins (TIPs) during dormancy cycling and during germination under normal and water stress conditions. In the current study seeds with altered aquaporin abundance including seeds from loss-of-function *Atpip2;1* mutants and *Atpip2;1-Atpip2;2* double mutants, and seeds relatively high PIP2;1 abundance (overexpress PIP2;1 lines) and wild type Arabidopsis Col-0 were utilised in a series of experiments involving assessing germination in saline and non-saline conditions.

Stress, signalling, aquaporins and germination

Abiotic stresses and hormones influence seed germination, and seed germination responses to environmental stresses. Stresses such as soil salinity can affect germination by decreasing water uptake and causing NaCl accumulation in seeds in sufficient amounts to inhibit the metabolic processes required for growth (Ayers, 1952). A reduction in seed water uptake and an increase in accumulation of NaCl in seeds can lead to a slower rate of radicle emergence and a decrease in the percentage of germination success. The responses of plants to salt stress involve abscisic acid (ABA), a plant hormone that regulates numerous aspects of seed dormancy, plant growth, development, and stress responses, and in mutant plants that fail to accumulate ABA more rapid germination has been observed (Gonzalez-Guzman *et al.*, 2002).

ABA and GA play crucial roles as phytohormones in mediating light and temperature-induced transition from seed dormancy establishment to seed germination (Yan and Chen, 2020). ABA promotes dormancy establishment during seed maturation and inhibits seed germination, while GA promotes seed germination (Finch-Savage and Leubner-Metzger, 2006). It has been shown that treatments with H_2O_2 can increase ABA content and gibberellic acid (GA) catabolism in cotton seeds and that this was associated with improved cotton seed germination (Kong *et al.*, 2017). Recently Footitt et al. (2019) found that AtTIP3;1 and AtTIP3;2 modulated responses to abscisic acid in seed influencing germination percentage. AtPIPs may also have a role in seed germination. AtPIP2;1 has been shown to be permeable to H_2O_2 (Rodrigues *et al.*, 2017). This indicates that it is possible that AtPIP2;1 could be involved in facilitating the transport of H_2O_2 linked to increases in ABA in seed during germination in saline conditions. It is also possible that AtPIP2;1 is involved in water and Na^+ transport (Byrt *et al.*, 2017; Qiu *et al.*, 2020) in seed during germination in saline treatment. AtPIP2;2 was also reported to be permeable to Na^+ when expressed in heterologous systems, and cation conductance associated with AtPIP2;1 and AtPIP2;2 was inhibited by low pH and by divalent cations (Mohamad *et al.*, 2017). Treatments with NaCl were reported to lead to differential phosphorylation of AtPIP2;1 and AtPIP2;2 (Boursiac *et al.*, 2005), and mutants lacking AtPIP2;2 were reported to have lower osmotic hydraulic conductivity than wild type plants (Boursiac *et al.*, 2005; Javot *et al.*, 2003).

Soil salinization is one of the main abiotic stresses impacting plant productivity. Salt accumulation inhibits plant growth and reduces the ability of roots to uptake water and nutrients, leading to osmotic or water-deficit stress (Hanin *et al.*, 2016; Munns and Tester, 2008; Tester and Davenport, 2003). To cope with salt stress, plants have evolved two key types

of tolerance mechanisms based on either limiting the entry of salt by the roots, or controlling its concentration and distribution within leaves and cells (Garcia de la Garma *et al.*, 2015; Hanin *et al.*, 2016). Shoots and seeds are considered as the final stops for water and nutrient flow inside plants, and the concentration of Na^+ in these tissue parts tend to be affected by the plant growing conditions (Reid and Smith, 2000; Tester and Davenport, 2003).

Towards testing whether AtPIP2;1 ionic conductance was physiologically relevant *in planta* two Arabidopsis mutant lines lacking AtPIP2;1 were sourced with the intention to test whether these *Atpip2;1-1* and *Atpip2;1-2* lines differed in tissue ion accumulation relative to wild type (Col-0) lines. Whilst germinating seed from this plant material on plates containing half strength Murashige and Skoog media with an extra 50 mM NaCl added, Dr Jiaen Qiu observed that the germination success of the *Atpip2;1* mutants appeared to be lower than wild type (see appendix Figure S1). This prompted the conception of a project with the goal to gain quantitative data to determine whether the apparent differences in *Atpip2;1* mutant seed germination relative to wild type lines in saline conditions was statistically significant. Further research was designed to establish the role of AQPs such as AtPIP2;1 during seed germination.

This thesis comprises six chapters as follows:

Chapter 1 provides general information about the knowledge gap, research objectives and hypothesis.

Chapter 2 is a published review that summarizes the expression of Arabidopsis aquaporins in seeds during development and germination.

Chapter 3 was written in conventional thesis format, describes the research materials and methodology used in the study including the development of methodology used to generate the data reported in Chapters 4 and 5. Preliminary data is incorporated in Chapter 3 towards demonstrating the outcome of the different methods trialled and developed.

Chapter 4 is written in a manuscript article format in preparation for submitting to a journal at a later date. Additional information about the creation of the material used for the experiments reported in this chapter will be included at the stage of submitting for publication and collaborators who shared initial samples of their transgenic seed lines are acknowledged in the methods section and will be included as co-authors in the submission. This chapter reports on analysis of wild type Columbia 0 seed, loss-of-function *Atpip2;1* mutant seed and lines overexpressing AtPIP2;1 2x35S::AtPIP2;1 seed during germination in water or in saline conditions, where the data included is in terms of germination percentage, seed weight and seed Na^+ content.

Chapter 5 is written in manuscript format. This chapter presents the data of overexpression UBI:nGFP:PIP2;1 lines. Localisation of gene and other characterisations have been mentioned.

Chapter 6 is written in a conventional thesis format. This chapter includes general discussion and conclusions of the study.

1.4 Research gaps, aims and hypothesis

Research gaps

- The roles of AQPs in seed imbibition and early seedling vigour are unclear. Hypothetically AQPs could be involved in transporting key solutes, such as water and nutrients, and/or they could have roles in signalling during seed germination.

- When parent Arabidopsis plants are grown in saline relative to control conditions, the seed may produce differ in Na^+ content, and this feature may be influenced by the function of AQPs involved in Na^+ and K^+ transport in the parent plant, such as AtPIP2;1, and consequently parent plant AQP function could influence the subsequent generation of seed germination success.

- Early germination processes may or may not require plasma membrane intrinsic proteins, such as AtPIP2;1 function, and loss or increase of AtPIP2;1 function could potentially influence seed phenotypes such as seed size, seed weight, germination percentage or ion content.

Aims, objectives and hypothesis of the project

The project aim is to test whether there is a role for AtPIP2;1 during seed germination and early seedling vigour. The hypothesis is that AtPIP2;1 has a role in seed imbibition and germination processes. The sub-aims were to:

- Test whether there were differences in germination between *Atpip2;1* loss-of-function mutants, and AtPIP2;1 over-expression lines and wild-type (Columbia-0) control lines; and check for differences in seed weight and swelling during germination.

- Analyse the expression and localisation of AtPIP2;1 in an over-expression line.

- Measure Na^+ and K^+ ion content in Arabidopsis seed of wild type Col-0, mutants lacking AtPIP2;1 and AtPIP2;1 overexpression lines after parent plants had been grown in control or saline treatments.

- Investigate the ion content in seed of wild type (Columbia-0), mutant *pip2;1* and/or *pip2;2* lines and AtPIP2;1 overexpression lines when they were imbibed in either saline or control treatment.

Hypothesis of the project: Optimal germination of Arabidopsis seeds may involve AtPIP2;1.

Chapter 2

Deciphering aquaporin regulation and roles

in seed biology

Statement of Authorship

Title of Paper	Deciphering aquaporin regulation and roles in seed biology
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Name of Principal Author (Candidate)	Phan Thi Thanh Hoai		
Contribution to the Paper	Designed and performed experiments; gathered, analyzed and interpreted data; and wrote the manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	9/7/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

   i. the candidate's stated contribution to the publication is accurate (as detailed above);
   ii. permission is granted for the candidate in include the publication in the thesis; and
   iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Contributed to gathering transcript data information		
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Signature		Date	9/7/2021
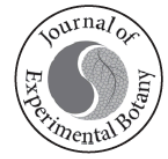
EXPERT VIEW

Deciphering aquaporin regulation and roles in seed biology

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Abstract

Seeds are the typical dispersal and propagation units of angiosperms and gymnosperms. Water movement into and out of seeds plays a crucial role from the point of fertilization through to imbibition and seed germination. A class of membrane intrinsic proteins called aquaporins (AQPs) assist with the movement of water and other solutes within seeds. These highly diverse and abundant proteins are associated with different processes in the development, longevity, imbibition, and germination of seed. However, there are many AQPs encoded in a plant's genome and it is not yet clear how, when, or which AQPs are involved in critical stages of seed biology. Here we review the literature to examine the evidence for AQP involvement in seeds and analyse Arabidopsis seed-related transcriptomic data to assess which AQPs are likely to be important in seed water relations and explore additional roles for AQPs in seed biology.

Keywords: Development, germination, imbibition, water transport, hormones, signalling.

Introduction

Aquaporins (AQPs) are channel proteins belonging to the membrane intrinsic protein (MIP) family that can facilitate the passive influx and efflux of water and often other solutes across membranes mediated by osmotic or solute gradients (Chaumont and Tyerman, 2017). AQP members are present across all taxonomic kingdoms, but the different types of AQPs are particularly diverse in land plants (Box 1). In angiosperms, AQP family sizes range from 30 to >100 members in some polyploid species (Groszmann *et al.*, 2017). During the evolution of seed plants, members of the expanding AQP family were recruited into processes associated with seed development, maturation, and germination (Obroucheva, 2013). The earliest studies implicating a role for AQPs in seeds were published in the early 1990s (Johnson *et al.*, 1990; Höfte *et al.*, 1992), and these studies reported on the high abundance of tonoplast intrinsic proteins (TIPs; present in seed storage tissues). Since then, our understanding has progressed to recognize broader AQP networks and the importance of hormonal regulation of AQPs during seed development. In particular how abscisic acid (ABA) and TIP interplay influence the timing of germination (Box 2).

Box 1. Aquaporin subfamilies and localization trends in seeds

Plant membrane intrinsic proteins (MIPs) are traditionally classified into seven subfamilies based on sequence similarity, but up to 13 subfamilies are recognized in the plant kingdom (Chaumont and Tyerman, 2017)(Laloux *et al.*, 2018). Five of these are found in seed plants, which include the two largest groups of the plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs) that generally localize to the plasma membrane (PM) and vacuolar membranes (i.e. the tonoplast), respectively. Seed plants also have Nodulin 26-like intrinsic proteins (NIPs), small intrinsic protein (SIPs), and X intrinsic proteins (XIPs) which predominantly localize to the PM and endoplasmic reticulum (ER). The remaining eight subfamilies include the GlpF-like intrinsic proteins (GIPs) and hybrid intrinsic protein (HIP), which are found only in older land plant lineages (e.g. mosses), while the MIPs A–E occur only in green algae, and the large intrinsic proteins (LIPs) are exclusive to diatoms. The subcellular localization and function of these eight subfamilies of aquaporins remain to be determined.

Of the seed plant aquaporins, the most abundant transcripts present in the developing seed as a whole are those encoding members of the PIP, TIP, and SIP subfamilies. Within these subfamilies, plant AQPs display a high multiplicity of isoforms along with diversity in their subcellular localizations, at the PM, tonoplast, and various other intracellular compartments. Some members also exhibit exceptional dual localization in distinct cell compartments, or polarized or domain-specific localization at the PM or tonoplast, respectively (Chaumont and Tyerman, 2017). An example of such atypical dual localization occurs during seed development, where some members of the TIP subfamily co-localize to both the tonoplast and plasma membrane (Gattolin *et al.*, 2011). The Arabidopsis genome encodes 10 TIP isoforms, further classified into five subgroups: three γ-TIP (TIP1), three δ-TIP (TIP2), the seed-specific α- and β-TIPs (TIP3;1 and TIP3;2), one ε-TIP (TIP4;1), and one ζ-TIP (TIP5;1) (Johanson *et al.*, 2001).

The importance of water relations in seed biology

Water relations during the development and maturation of seeds

Seed development commences at the point of fertilization and progresses through three key stages (Box 3). During these three stages, there are substantial changes in the water content of the developing seeds. There is a relatively high initial water content, ~90%, during the early embryo morphogenesis (EEM) stage, which then decreases to ~7% later during the maturation stage. The dry weight of the developing seed increases during development from nearly 0 μg to ~22 μg, and the fresh weight increases from ~13 μg to 42 μg and then decreases during the latter stage of development to ~23 μg (Fig. 1) (Baud *et al.*, 2002). In general, seed development requires both solute influx and water efflux. Since AQPs can transport more than just water, it is possible that there may be roles for AQPs in each of these processes (Zhou *et al.*, 2007; Béré *et al.*, 2017). For instance, a subset of plant AQPs have recently been shown to facilitate ion transport across membranes (Byrt *et al.*, 2017). Two Arabidopsis AQPs, AtPIP2;1 and AtPIP2;2, are capable of conducting monovalent cations when expressed in heterologous systems (Byrt *et al.*, 2017; Mohamad *et al.*, 2017). It is therefore possible that AQPs could facilitate nutrient ion transport in addition to transporting water and other neutral solutes required during seed development.

Water relations during seed imbibition and germination

Successful well-timed seed germination is critical for the life strategy of plants and essential for agricultural productivity. At maturity, seeds enter a period of dormancy, a strategy

evolved to ensure germination proceeds in the most favourable of environmental conditions (Nonogaki, 2014, 2017, 2019). Dormancy can be an undesirable trait in crop species, which require rapid uniform germination after sowing. This has resulted in selection of low dormancy levels during crop domestication that sometimes problematically result in pre-harvest sprouting and a reduction in harvest quality (Rodríguez *et al.*, 2015). In favourable conditions, which include sufficient soil moisture, seed germination commences with imbibition. Water predominantly enters the seed through either the micropylar region, the hilum (prior attachment point of the seed stalk or funiculus), or the lens (Pietrzak *et al.*, 2002; Manz *et al.*, 2005; Koizumi and Kano, 2016; Munz *et al.*, 2017). The initial phase of imbibition is driven by the water potential gradient between the dry seed and the external environment. The rehydration of the endosperm and embryo triggers a recommencement of metabolic activity and initiates processes that progressively lead to further increases in embryo water content. This is followed by a phase of low water influx but high metabolic activity where signals to break dormancy are potentially triggered, and the seed prepares for radicle protrusion. The third phase involves further water influx predominantly into the embryo, promoted by endogenous osmotica. Water enters the cell to drive cellular expansion, across both the plasma membrane and tonoplast, therefore enabling the radicle to pierce both the loosened endosperm and testa to finalize germination (Takahashi *et al.*, 2004; Obroucheva *et al.*, 2017; Steinbrecher and Leubner-Metzger, 2017; Nonogaki, 2019).

Arabidopsis, like most angiosperms, produce orthodox seeds that are essentially dehydrated at seed abscission with only ~11% water content. This physiological state improves longevity of seed and subsequently the probability of successful progression to the next generation. Upon exposure to sufficient moisture,

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Box 2.   Key developments revealing potential roles for aquaporins in seed biology in relation to hormone signalling processes during seed development and germination and in related cell biology processes

Abscisic acid (ABA) and gibberellic acid (GA) are two major phytohormones that can act antagonistically to regulate seed dormancy and germination status (Finkelstein *et al.*, 2008; Rodríguez-Gacio *et al.*, 2009; Liu and Hou, 2018; Nonogaki, 2019). ABA is responsible for inducing seed dormancy during maturation and also prevents quiescent seed from transitioning from embryonic to germination growth in response to imbibition (Finkelstein *et al.*, 2008). GA breaks dormancy and stimulates germination by antagonistically suppressing ABA, and helps radical protrusion by weakening tissue surrounding the embryo, increasing embryonic growth potential (Ogawa *et al.*, 2003; Stamm *et al.*, 2017).

ABA has signalling roles in both early and late stages of seed development, and ABA activation targets include aquaporins

Early in seed development, the timing of endosperm cellularization is influenced by ABA, which in turn influences seed size (Cheng *et al.*, 2014), and later in development ABA influences dormancy and transport processes associated with desiccation. Differences in the transcriptional regulation of TIP, PIP, and NIP aquaporins were observed by Chauffour *et al.* (2019) when comparing wild-type Arabidopsis seed relative to seeds that were ABA deficient.

Hormonal signals coordinate cell wall loosening with water uptake and turgor pressure-regulating mechanisms during embryo growth and radicle emergence

An evaluation of the evolutionary conservation of molecular, biochemical, and biomechanical mechanisms associated with germination was reported by Steinbrecher and Leubner-Metzger (2017). Following water uptake into dry seed, a turgid state must be reached to enable the cell expansion step that initiates embryo axis growth. Endosperm weakening and embryo and radicle growth are induced by GA and ethylene, and inhibited by ABA, and reactive oxygen species are implicated in signalling coordination. ABA and hydrogen peroxide signals are linked to both changing cell wall properties and regulating solute flux. AQP transport of solutes could be important in this water flux. Solute influx is an important feature of cell expansion, enabling sufficient pressure to build up in the later stages of germination for the radicle to puncture the seed coat (Takahashi *et al.*, 2004; Steinbrecher and Leubner-Metzger, 2017).

Aquaporins can facilitate hydrogen peroxide transport across cell membranes to mediate ABA signalling

Our general knowledge of plant cell signalling was advanced by the demonstration by Rodrigues *et al.* (2017) that aquaporin transport of hydrogen peroxide was required to enable ABA-induced hydrogen peroxide accumulation. This was demonstrated in guard cells, and further tests are needed to reveal whether aquaporin transport of hydrogen peroxide is also associated with ABA signalling in seed germination.

Aquaporins have ABA-regulated roles in seed sensing of the environment which contribute to the regulation of dormancy and germination in Arabidopsis

Roles for TIP3;1, TIP3;2, and TIP4;1 in regulating germination were revealed by Footitt *et al.* (2019) following their investigation of TIP loss-of-function and overexpressing transgenic Arabidopsis lines. These three TIPs differ in sensitivity to ABA, and modification of their function alters the base water potential required for germination and regulation of the timing of germination.

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both water content and weight of the Arabidopsis seed showed a rapid increase during the first 4 h of imbibition, followed by a more gradual progression thereafter (Fig. 2). On average, an individual Arabidopsis dry seed weighs ~20 µg, increasing to ~97 µg, after 4 h of imbibition in water. The initial water content of dry seed increased during imbibition, from ~11% to >82% within the first 24 h. A similar rate of water accumulation has been reported in the orthodox seed of broad bean (from 10% to 40% in 6 h, then to 75% at 24 h) (Obroucheva *et al.*, 2017).

Involvement of AQPs in seed water relations during germination

In many species, the uptake of water through AQPs can be inhibited by mercury (presented as HgCl$_2$), which can be alleviated by reducing agents such as DTT and β-mercaptoethanol. Mercury is thought to inhibit AQP activity through direct inhibitory binding of the protein. However, mercury suppression may also arise indirectly through inhibiting respiration

> **Box 3.   Stages in *Arabidopsis thaliana* seed development**
>
> Arabidopsis seed development can be categorized into three stages. The first stage of seed development is early morphogenesis, which occurs after the double fertilization event in the embryo sac and subsequent cell divisions. The second stage is the maturation phase, where the embryo cells go through a period of cellular expansion and differentiation concomitant with accumulation of storage products in the embryo, including proteins and lipids (requiring the synthesis of nitrogen and carbon storage compounds). The final stage is termed late maturation, and this is where the embryo becomes metabolically quiescent and tolerant to desiccation (Baud *et al.*, 2002; Weijers and Jürgens, 2005; Dubreucq *et al.*, 2010). There are substantial changes in the water and solute content of seed during these processes, which to some extent are likely to be controlled through the regulation of aquaporins.
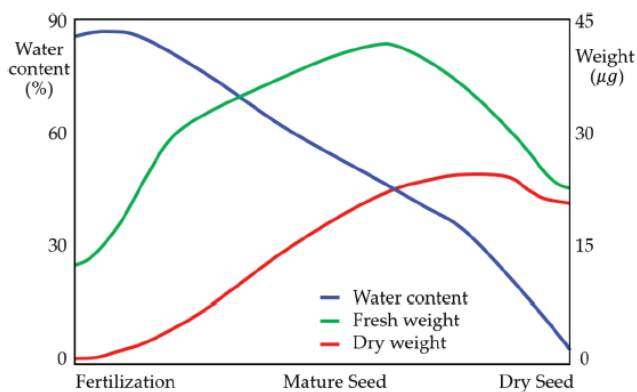

Fig. 1. Graphical model representing general trends in Arabidopsis seed water content and weight during development, from fertilization to mature seed. This model is based on data reported by Baud *et al.* (2002) for the Wassilewskija (Ws) accession. Different Arabidopsis accessions may vary in exact water content during these stages; however, the general trend is likely to remain similar to the depicted model. Reproduced from Baud *et al.* (2002). An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. Plant Physiology and Biochemistry 40, 151–160. Copyright © 2002 Elsevier Masson SAS. All rights reserved.

Fig. 2. Changes in wild-type Arabidopsis (Columbia-0) seed water content and seed weight during imbibition and germination from dry seed. Water content %=(imbibed seed weight–dry seed weight)×100 dry seed weight, where the dry seed weight was measured after seeds were dried at 65 °C for 72 h. 'Wet seed' represents seed weight after 5 min imbibition in water. 'Germination' is the time at which 50% of imbibed seed displayed radicle protrusion of the seed coat. Error bars represent the SD; at some time points, the error bars are so tight as to be obscured by the symbols (*n*=5–10 replications, with 100–300 seeds per replication).

and acidification of cytoplasmic pH, which can also inhibit some AQPs (Zhang and Tyerman, 1999; Tournaire-Roux *et al.*, 2003). Applications of such pharmacological agents has implicated AQPs as having an important role in water relations during seed germination across a range of species including; Arabidopsis (Vander Willigen *et al.*, 2006), broad bean (Novikova *et al.*, 2014; Obroucheva *et al.*, 2017), tomato (Jain *et al.*, 2008), pea (Veselova and Veselovsky, 2006), and the arboreal species *Aesculus hippocastanum* (Obroucheva *et al.*, 2017) and *Plathymenia reticulata* (Cardoso *et al.*, 2015). Although these observations implicate AQPs as being involved in germination, it is difficult to ascertain to what extent given that mercury and other inhibitory compounds may have side effects, and may not work as effective blockers for all AQP isoforms (Frick *et al.*, 2013). More direct evidence comes in the form of mutant and transgenic studies. In rice, *OsPIP1;3* knock-down lines show slow and reduced germination efficiency (only ~70% of the seed germinated) under optimal conditions and as little as ~8% under water-stressed conditions. On the other hand, *OsPIP1;3* overexpression lines showed enhanced germination efficiency under water-stressed conditions relative to the germination efficiency obtained in optimal conditions. These phenotypes
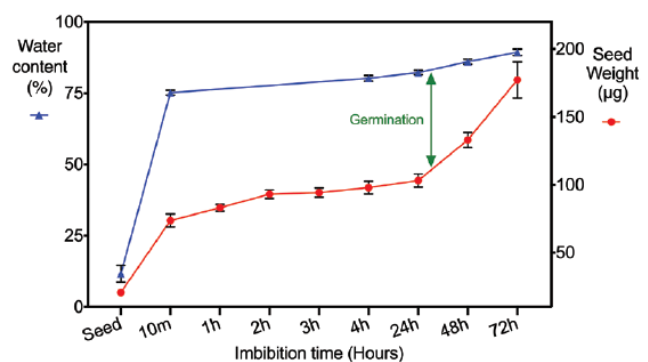
were directly linked with changes in the water uptake capacity of the embryo (Liu *et al.*, 2007). Similar effects were observed in Arabidopsis mutants, and overexpression lines of seed-expressed TIPs including AtTIP3;1, AtTIP3;2, and AtTIP4;1. In Arabidopsis, these TIPs were shown to be important in both seed germination events and prolonging seed longevity (Mao and Sun, 2015; Footitt *et al.*, 2019).

Additional evidence can be gleaned from studies of altered AQP expression in the context of drought tolerance, where improved germination rates are reported for transgenic tobacco, Arabidopsis, and rice seed, when exposed to osmotic stress (Yu *et al.*, 2005; Peng *et al.*, 2007; Hu *et al.*, 2012; Zhou *et al.*, 2012; Liu *et al.*, 2013). AQPs are also implicated in the increased germination performance and water uptake of spinach and *Brassica napus* osmo-primed seed (Gao *et al.*, 1999; Chen *et al.*, 2013; Lechowska *et al.*, 2019), with osmo-priming being a pre-sowing treatment used to improve germination performance and seedling establishment in cultivated plant that involves controlled imbibition, for example by soaking in a polyethylene glycol (PEG) solution.

The timing of AQP functionality during germination may differ between species. For Arabidopsis and *A. hippocastanum*, the involvement of AQPs is possibly not necessary for water
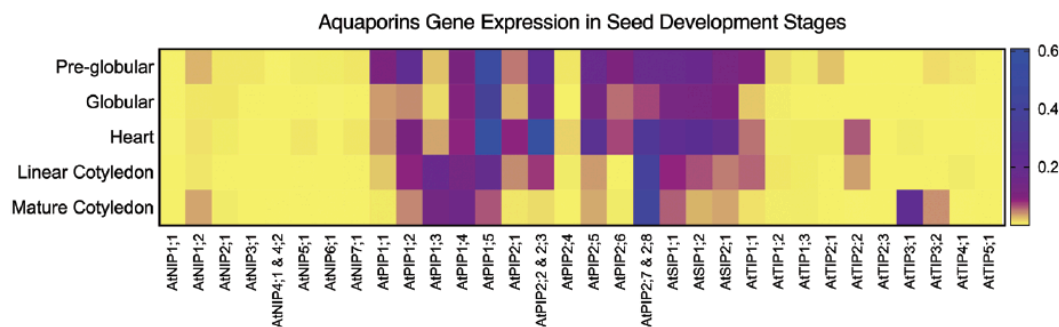
Fig. 3. A comparison of transcript abundance of all 35 Arabidopsis aquaporins over the course of seed development. The data show that a subset of AtAQPs are highly expressed in developing seed, with these showing differential patterns during the course of development. Data were sourced from seedgenenetwork.net, initially published by Belmonte et al. (2013). Transcript abundance is represented as ratios relative to *GAPDH*, a housekeeping gene (Czechowski et al., 2005).

uptake during the initial phase of germination (Obroucheva et al., 2017; Vander Willigen et al., 2006). In contrast, in seed from other species (i.e. pea, tomato, rice, and the legume *P. reticulata*), the importance of AQP activity is evident from the initial phase of germination, becoming more prominent in the emergence phase (Veselova and Veselovsky, 2006; Liu et al., 2007; Jain et al., 2008; Cardoso et al., 2015). The importance of AQP activity is more evident under water-stressed conditions, such as where PEG8000 was used to decrease water potential during germination (Liu et al., 2007; Footitt et al., 2019), a state not always examined when assessing AQP involvement in germination.

Regulation of Arabidopsis aquaporins in seeds

The role of AQPs in seed biology has been most extensively studied in the model species Arabidopsis. Several of the plant AQP subfamilies have been implicated in seed development, with the most noteworthy currently being the TIPs (Gattolin et al., 2011; Chaumont and Tyerman, 2017). Three of the TIP gene isoforms (*TIP2;1*, *TIP2;2*, and *TIP1;1*) are expressed in distinct maternal seed tissues, but not in embryos, whereas *TIP3;1* and *TIP3;2* were detected in the endosperm and embryo of developing and mature seeds (Gattolin et al., 2011). During seed maturation and germination, *TIP3;1* and *TIP3;2* localize to both protein storage vacuoles and the plasma membrane (Box 1), and have a key role in controlling seed dormancy and response to stress and ABA signals (Gattolin et al., 2011; Footitt et al., 2019). A recent investigation of the rate of radicle emergence in Arabidopsis lines where *TIP3;1*, *TIP3;2*, and *TIP4;1* are either overexpressed, or have been disrupted by a T-DNA insertion, revealed that these three TIPs are implicated in mechanisms that influence dormancy status and the timing of germination under variable soil conditions (Footitt et al., 2019). These same TIPs have also been implicated in increasing seed longevity (Mao and Sun, 2015).

To explore which other types of AQPs may be important in Arabidopsis seed development and germination, we analysed transcriptome data focusing on three key stages: seed

development, imbibition, and germination. Data were gathered from publicly available databases: seed development data was sourced from http://seedgenenetwork.net (Belmonte et al., 2013), imbibition data from the Arabidopsis eFP Browser http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Nakabayashi et al., 2005), and germination data from both the Arabidopsis eFP Browser http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Narsai et al., 2011) and the Nottingham Seed eFP Browser http://ssbvseed01.nottingham.ac.uk/efp_browser/efpWeb.cgi (Dekkers et al., 2013).

AQP expression during seed development

Seed transcript analysis revealed AQP-encoding transcripts in different seed tissues and throughout all stages of seed development (Fig. 3). For example, *AtPIP1;4* and *AtSIP1;1* transcripts were abundant throughout seed development; *AtPIP* (*1;5*, *2;2/2;3*, and *2;5*) were present in all stages but were more abundant early in seed development; *AtPIP2;7/2;8* were more abundant in later than in early stages; and *AtTIP3;1* and *AtTIP3;2* transcripts levels were abundant in mature cotyledons. A similar analysis in rice (*Oryza sativa*) revealed that *OsPIP2;1* and *OsTIP3;1* transcripts were abundant in developing grain, which matched trends in protein abundance tested by labelling grain sections with anti-AQP antibodies (Hayashi et al., 2015). The authors proposed that OsPIP2;1 and OsTIP3;1 have roles contributing to water efflux and dry matter accumulation processes in developing grains after the mid-grain filling stage.

There is no vasculature in the Arabidopsis seed, with the vasculature terminating at the funicular–chalazal seed coat boundary. The nutrients required to support the developing seed are unloaded at this junction, with the seed coat providing a symplastic extension for delivery into the seed across an apoplastic step from the maternal to filial generation (Stadler et al., 2005; Chen et al., 2015). The Arabidopsis seed has at least three apoplastic borders: between the outer integument and inner integument; between the inner integument and endosperm; and between the endosperm and embryo. For each apoplastic border membrane, transporters are required for the efflux into the apoplast then uptake back to the symplast (Stadler et al., 2005). Genes and proteins
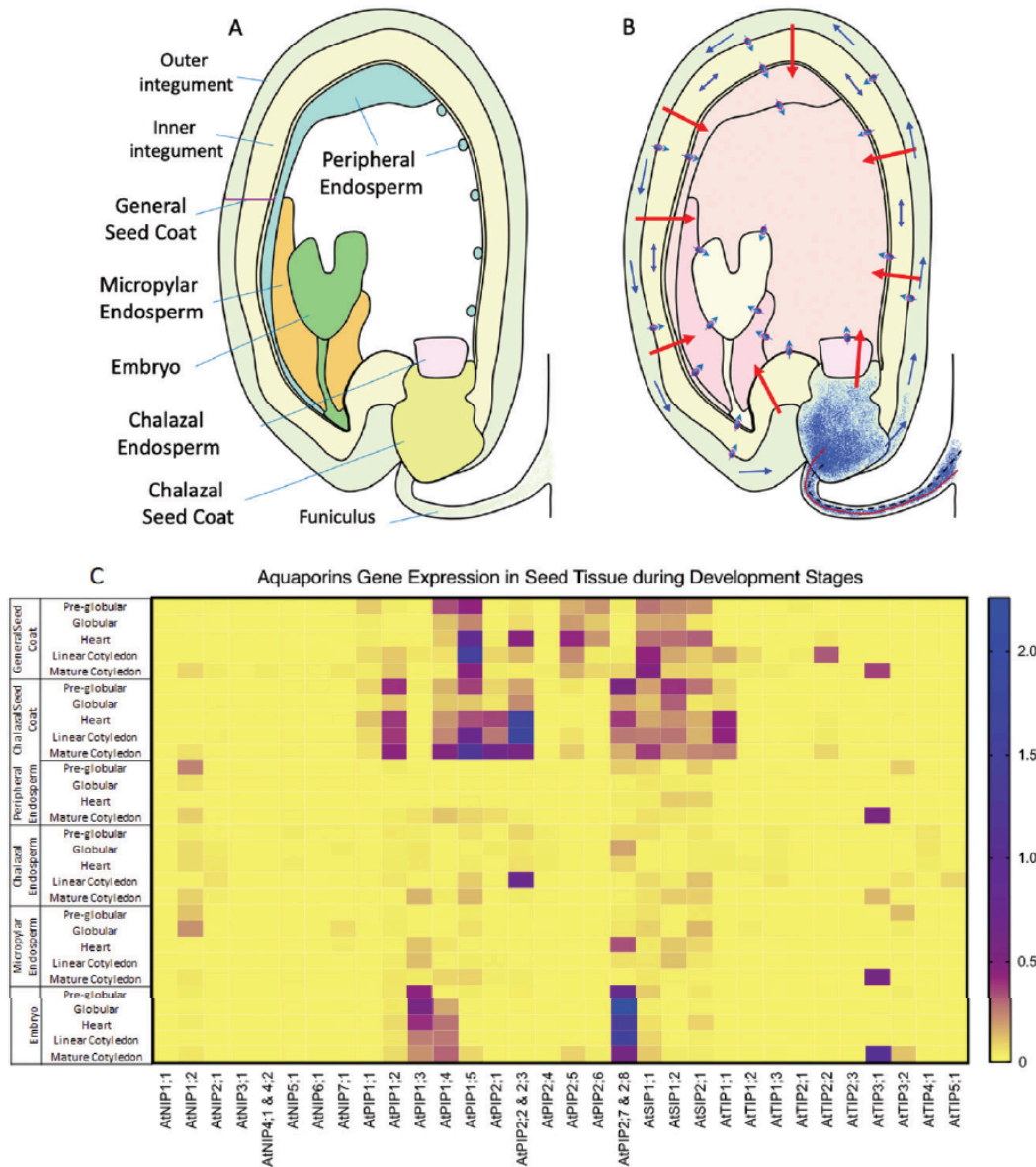
Fig. 4. (A) Map of Arabidopsis seed at the heart stage indicating the names of each tissue. (B) Model showing the symplastic pathway in a developing Arabidopsis seed and the location in the pathway where there are likely to be membrane transport steps. Purple circles indicate the likely locations of membrane transport proteins (Chen *et al.*, 2015). Blue shading indicates symplastic continuity, and influx of solutes and water from the vascular bundle of the funiculus. Movement within the outer integument and inner integument (dark blue arrows) is symplastic. A double line between the inner integument and endosperm represents the location of the maternal–filial boundary. Centripetal transport (red arrows) from the outer integument toward the endosperm occurs over two barriers to symplastic continuity, requiring both efflux and influx steps (Stadler *et al.*, 2005). The dashed blue and dark red lines within the funiculus represent phloem and xylem tissues within the vasculature; water and nutrients are imported via the vasculature as seed development progresses and water must also be recycled back to the parent plant (Zhang *et al.*, 2007; Chan *et al.*, 2016). (C) Aquaporin transcript abundance in specific cell types during the course of seed development. The data enable us to compare changes in Arabidopsis aquaporin expression levels as seeds progress through development. The data were sourced from seedgenenetwork.net initially published by Belmonte *et al.* (2013). Transcript abundance is represented as ratios relative to, *GAPDH*, a housekeeping gene (Czechowski *et al.*, 2005).

involved in sugar, nitrate, and amino acid transport have been observed to have coordinated patterns of expression within various seed compartments, enabling a feeding pathway of these essential nutrients from the outer integument to the endosperm and embryo (Chen *et al.*, 2015; Tegeder and Hammes, 2018; Tegeder and Masclaux Daubresse, 2018). It is likely that AQPs also have roles in facilitating solute transport across the apoplastic borders of the integuments and endosperm (Fig. 4A, B).

We examined AQP transcript abundance derived from specific tissues of the seed during development (Fig. 4). This revealed that transcripts for several *AtPIP* genes (*1;2, 1;4, 1;5, 2;1, 2;2/2;3*, and *2;7/2;8*), *AtSIP* genes (*1;1* and *1;2*), and *AtTIP1;1* were abundant in the chalazal seed coat at the position
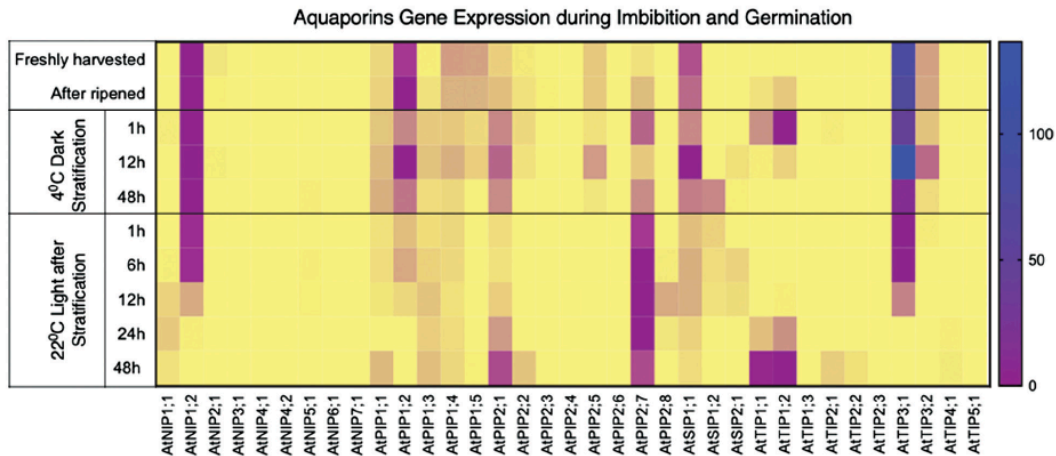
Fig. 5. Aquaporin transcript abundance in Arabidopsis seeds during seed imbibition (under 4 °C stratification) and then after transfer to standard light conditions. Transcriptomic surveys were made on freshly harvested seed, after-ripened seed (2 weeks dark dry conditions), and then at several time points during dark stratification imbibition and through to light growth conditions. Germination occurred at 24 h into the 22 °C light period. Data were retrieved from expression.latrobe.edu.au/agriseqdb, initially published by Narsai *et al.* (2017). Transcripts are presented as a ratio relative to the control housekeeping gene, GAPDH (Czechowski *et al.*, 2005).

of vasculature unloading, and transcripts for *AtPIP* genes (*1;5, 2;2/2;3, 2;5*, and *2;6*) and *AtSIP* genes (*1;1, 1;2*, and *2;1*) were detected in the seed coat. Transcripts from *AtPIP1;3* were detected from the point of endosperm cellularization, *AtNIP1;2* in the micropylar tissues, and peripheral endosperm tissues at the early stages of development, and once again later at the mature cotyledon stage, at which point the *AtTIP3;1* transcript level was notably high. *AtPIP2;7/2;8* transcripts were most abundant relative to the other AQP transcripts in the micropylar endosperm and chalazal endosperm. In the embryo, *AtPIP1;3, PIP1;4*, and *AtTIP3;1* transcripts were abundant. These spatial and temporal changes in AQP transcript abundance during seed development are likely to be important in endosperm and embryo development, and further research is required to elucidate possible functional roles.

AQP expression during seed imbibition and germination

AQPs are putatively involved in the rapid water intake that occurs in many types of seeds prior to germination. Transcription of candidate AQPs would occur during the final stages of seed development, with the protein product stored, because the mature seed is not metabolically active when in a dried state. Candidate AQP genes include *AtPIP1;3, PIP1;4, PIP2;7/2;8, AtTIP3;1*, and *TIP3;2*, which are all expressed in the embryo as the seed matures (Fig. 4). This is consistent with data showing that the AtTIP3;1 and AtTIP3;2 proteins accumulate during seed maturation and are abundant in dry seed, with seed of the *tip3;1 tip3;2* double mutant having reduced viability and germination rates (Mao and Sun, 2015; Footitt *et al.*, 2019).

For rapid translation of AQP proteins, AQP mRNAs may be stored in dry seed, ready for immediate translation upon initial rehydration. Candidates include *AtNIP1;2, PIP1;2, SIP1;1, TIP3;1*, and *TIP3;2*, because these transcripts are abundant in dry seed (Fig. 5). The transcript levels for each of these candidates is lower during imbibition and germination relative to in dry seed, consistent with their potential involvement in the initial germination processes. After the initial germination process, the AQP transcripts of greatest abundance then change to an alternative set, which includes *AtPIP2;1, PIP2;7, TIP1;1, TIP1;2*, and *TIP4;1*. The abundance of transcripts for this set increases at or immediately after germination, indicating that this set of AQPs are candidates for driving the rapid cellular expansion associated with post-germination seedling growth, as reported previously for TIP4;1 (Footitt *et al.*, 2019).

Hormone signalling in seeds and implications for aquaporin regulation

AQP regulation at transcriptional and post-translational levels is influenced by plant hormones, and responds to developmental and stress cues (Maurel et al., 1995, 2015; Bastian *et al.*, 2010; Chaumont and Tyerman, 2014; Kapilan *et al.*, 2018; Kuromori *et al.*, 2018). Hormone-driven regulatory responses also control AQP regulation during seed development and germination (Box 2). For example, seed dormancy, inhibition of germination, and stimulation of germination can be manipulated by exogenous applications of ABA or gibberellin (GA; Müller *et al.*, 2006; Finkelstein *et al.*, 2008). The transcriptional response to such treatments includes changes in the abundance of seed-expressed AQPs (Fig. 6). ABA treatment increases expression levels of *AQP* genes normally induced during early imbibition (*AtTIP3;2, TIP3;1, NIP1;2*, and *SIP1;1*), while suppressing those normally expressed leading up to germination (*AtTIP1;1, PIP2;1*, and *PIP2;7*; Figs 5, 6A). Up-regulation of *AtTIP3;2* and *TIP3;1* and repression of *AtPIP2;7* were observed in response to ABA in a dose-dependent manner. The inverse relationship occurred upon GA treatment, with an up-regulation of *PIP2;7* and down-regulation of *AtTIP3;2* and *TIP3;1* (Fig. 6B).
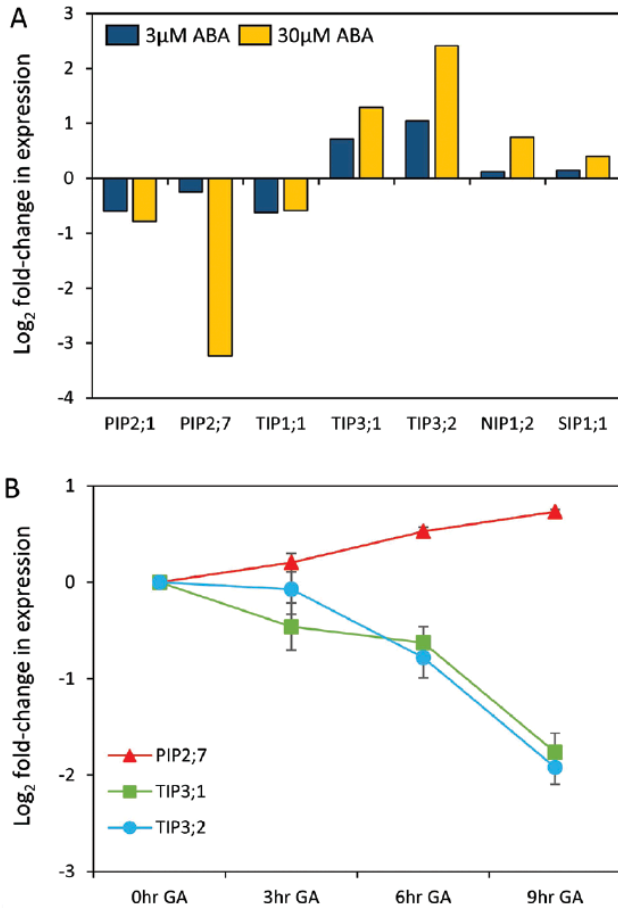
Fig. 6. The response of Arabidopsis AQPs expressed during seed germination to exogenous application of (A) ABA or (B) GA. (A) Wild-type Columbia seed was imbibed for 24 h in either water or water+ABA, at two concentrations, and transcript levels measured were measured after 24 h of imbibition. (B) GA-deficient *ga1-3* mutant seed were imbibed in either water or water+5 µM GA and sampled over a 9 h period. Expression levels were first standardized to *GAPDH*. Changes in expression between treatments represent log$_2$ fold change difference of (A) ABA treatment versus water control, or (B) GA treatment versus water control at a given time point then standardized to the a 0 h time point. Data were retrieved using the Arabidopsis eFP browser (Winter *et al.*, 2007) initially published by Goda *et al.* (2008).

Using loss-of-function mutants and overexpression lines, Footitt *et al.* (2019) demonstrated the importance of *TIP3;1* and *TIP3;2* for seed germination and dormancy in response to ABA and environmental conditions. In general, *TIP3;1* positively regulated germination and *TIP3;2* negatively regulated germination. A third TIP not normally expressed pre-germination, *AtTIP4;1*, was found to be elicited by stress, and reported to be functionally important in responding to water deficit (Footitt *et al.*, 2019). These findings indicate that the regulation of AQP functions in seeds may be important for agronomic-related traits such as germination success in challenging environments and pre-harvest sprouting.

The trends in *PIP2;7* expression reveal a possible role in seed germination. For example, transcripts encoding PIP2;7 were abundant in the mature embryo during seed development (Fig. 4C); they increased during the later stages of imbibition leading up to germination (Fig. 5); there was an antithetical response to ABA and GA treatments (Fig. 6), and a reciprocal relationship with *TIP3;1* and *TIP3;2*. Following these observations, we suggest that further investigation of the role of *AtPIP2;7* in seed germination is warranted.

Hydrogen peroxide (H_2O_2) is an important signalling molecule that mediates many signalling processes in plants, including photosynthesis, photorespiration, stomatal movement, the cell cycle, senescence, and stress responses (Mhamdi and Van Breusegem, 2018; Smirnoff and Arnaud, 2019). Moreover, H_2O_2 acts as an important signalling molecule regulating seed dormancy and germination upstream of ABA and GA. Hydrating seeds release H_2O_2 and nitric oxide (NO), which triggers ABA catabolism and GA biosynthesis in both Arabidopsis and crop species (Liu *et al.*, 2010; Leymarie *et al.*, 2012; Wojtyla *et al.*, 2016). The low rate at which H_2O_2 diffuses through biological membranes can be enhanced via certain AQP isoforms (Bienert and Chaumont, 2014), and this is important in pathogen responses (Tian *et al.*, 2016). Future testing for AQP roles in H_2O_2 transport in seed tissues during germination will require the careful use of loss-of-function mutants, gain-of-function transgenics, and inhibitors.

The signalling pathways regulating AQP function in seeds may involve cyclic nucleotide-related signalling (Maurel *et al.*, 1995; Bastian *et al.*, 2010). For example, cyclic nucleotide signalling influenced the phosphorylation state of a seed TIP when expressed in *Xenopus laevis* oocytes, and these changes in protein phosphorylation influenced the water transport activity (Maurel *et al.*, 1995). More recently, cGMP was implicated in GA signalling in barley aleurone cells (Bastian *et al.*, 2010).

Aquaporin regulation in seeds and implications for agronomic traits

AQP function is associated with seed dormancy and responses to environmental stresses (McGaughey *et al.*, 2018; Footitt *et al.*, 2019). This means that AQP function is potentially a target for manipulating the timing of germination, responses to environmental stress conditions, and pre-harvest sprouting traits. Robust uniform germination is essential for vigorous early seedling establishment and subsequent growth (Finch-Savage and Bassel, 2016). Manipulating AQPs may improve the effect of germination-enhancing practices such as osmo-priming, as well as providing flexibility in sowing practices by allowing efficient germination in soils with suboptimal moisture content. Altering AQP function in mature seeds may be a pathway towards limiting problems such as pre-harvest sprouting which can lead to yield losses of 20–50% in wheat (Ali *et al.*, 2019).

Concluding remarks

Membrane transport steps are required for water and nutrient transport into seeds during development and presumably for water transport out of seeds as seeds become increasingly desiccated when approaching maturity. The transport of water and important nutrients across plasma and tonoplast membranes of seeds cells is likely to involve AQPs. Transcripts encoding PIPs and TIPs are abundant in seed, with distinct expression patterns during development and germination stages that implicate the involvement of numerous isoforms at different stages of seed development. Further research is required to explore post-translational modification of AQPs in seed and determine which kinases and phosphatases are involved, and how hormonal changes interact with these regulatory processes. We suggest that AQPs present an intriguing target for engineering improvement in germination uniformity, resilience of germination to environmental stress, and manipulation of pre-harvest sprouting traits of crop seed.

Funding

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Chapter 3

Trialling methods for assessing seed germination

3.1 Introduction

Planning steps for devising experiments to compare germination of seed from different genotypes or lines must give consideration to factors which can confound the interpretation of results (Bezerra-Neto *et al.*, 2019). This means that when comparing seed from different genotypes in germination assays the seed must be from parent plants and environments that are as uniform as possible. Seed from different genotypes could differ in their germination trends simply because of factors that are unrelated to the genetics of the seed. The maternal environment is a key factor that influences the release of primary dormancy. For example, seed from plants that experienced a shorter photoperiod might have a lower germination percentage than seed from a plant that experienced a longer photoperiod (Fernández et al., 2019; Penfield, 2017b).

Genes and environment influence seed germination success and timing. Genetic and epigenetic factors have been reported to influence seed size, weight and germination in chickpea (Rajkumar et al., 2020). Genetic differences between varieties and the age of seed have been reported to influence the timing of seed germination success in plants including crops such as sunflower (*Helianthus*) (see Haferkamp et al. (1953) and Seiler (1998)).

Environmental conditions impact germination as these conditions influence:

- The moisture content of orthodox seeds, oily seeds, non-oily seeds and recalcitrant seed (Liu *et al.*, 2019; Roberts and Ellis, 1989).
- The aeration of the environment that the seed is exposed to, what the ideal O_2 percentage for germination is, commonly >20% (Ahmed *et al.*, 2018; Bradford *et al.*, 2007; Ozbingol *et al.*, 1998).
- The temperature optimum which is dependent on variety but generally is not below 5°C and over 35°C (Bradford *et al.*, 2007; Liu *et al.*, 2019; Ozbingol *et al.*, 1998).
- Light is also a critical regulator of seed germination, particularly in small-seeded plants where the light conditions interact with phytochromes influencing induction of seed germination (Seo *et al.*, 2009).

The chemistry of the environment that seeds germinate in is incredibly important. Chemical factors such as the presence of heavy metals, nutrients and carbon dioxide have significant and complicated influences on germination (Ahmed *et al.*, 2018; Di Salvatore *et al.*, 2008; Osuna *et al.*, 2015). An additional point of note in relation to germination conditions is the interaction

between chemical and biological factors (Dalling *et al.*, 2011; Liu *et al.*, 2021), which impacts features of the substrates the seeds are exposed to during imbibition and germination stages (Liu *et al.*, 2013c; Ozbingol *et al.*, 1998) (Figure 3.1). Therefore, it is important to ensure that when seeds from different genotypes are used in comparative germination assays that the seed has come from parent plants that were exposed to equivalent conditions and that the seed itself experiences equivalent conditions, otherwise variation in germination might be due to factor others than genotypic differences between the lines.
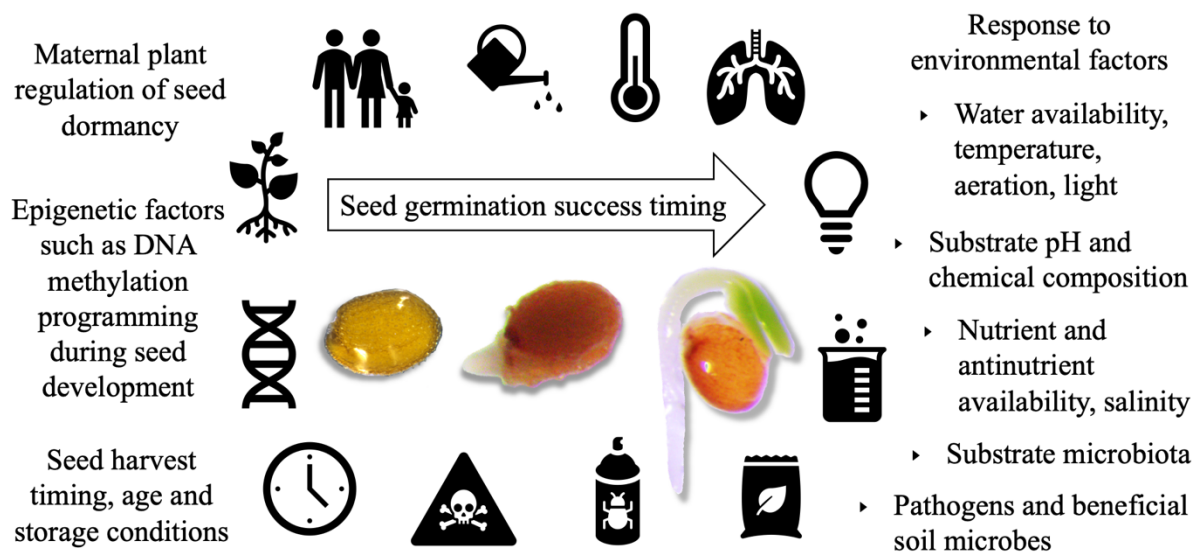
Figure 3.1. Creative representation of factors influencing germination such as the influence of maternal plant factors and the influence of environmental conditions.

Examples of key factors to consider when assessing seed germination include maternal environment, epigenetic factors (Fernández *et al.*, 2019; Penfield, 2017a; Rajkumar *et al.*, 2020), seed age (Haferkamp *et al.*, 1953; Seiler, 1998), and environmental conditions (Roberts and Ellis, 1989) such as moisture (Roberts and Ellis, 1989), temperature (Liu *et al.*, 2019), aeration (Bradford *et al.*, 2007; Ozbingol *et al.*, 1998), light and the chemical (Ahmed *et al.*, 2018; Di Salvatore *et al.*, 2008; Osuna *et al.*, 2015) and biological (Dalling *et al.*, 2011; Liu *et al.*, 2021) features of the substrate seeds are exposed to during imbibition and germination.

3.2 Materials and methods

3.2.1 Plant materials

Experiments were performed at the Plant Research Centre, Waite campus, the University of Adelaide. Plant material included *Arabidopsis thaliana* Columbia-0 (Col-0) ecotype background, two AtPIP2;1 loss-of-function mutant alleles (*Atpip2;1-1* and *Atpip2;1-2*), a double PIP2;1 and PIP2;2 loss-of-function mutant, an overexpression line driven by a ubiquitin promoter, UBI:nGFP::PIP2;1, and two overexpression lines driven by double 35S promoters, 2x35S::PIP2;1.

Seven different Arabidopsis genotypes were used in this study including: wildtype (Columbia 0), two single gene knock-out mutants *Atpip2;1-1* and *Atpip2;1-2*, a double mutant *Atpip(2;1*2;2),* a sodium transporter mutant *Athkt1-1,* and three different overexpression lines, two 35S::AtPIP2;1 lines and a UBI:nGFP::PIP2;1. Details of each genotype are included below.

Wild type - Columbia 0

Arabidopsis thaliana ecotype Columbia - 0 (Col-0) was obtained from the Gilliham Laboratory stock source, which had originally been sourced from the Arabidopsis Biological Resource Centre (ABRC).

Loss-of-function/Knockout mutant lines Atpip2;1-1 (pip2;1-1) and Atpip2;1-2 (pip2;1-2)

These two knockout mutants are T-DNA knockout lines (SALK lines), ordered from ABRC to characterize the candidate gene function *in planta*. The mutant *Arabidopsis* T-DNA insertion *pip2;1-1* (6AAS98 line, Amaze) (Wisman *et al.*, 1998) and *pip2;1-2* (SM_3_35928) lines were obtained from the Nottingham Arabidopsis Stock Centre (Da Ines *et al.*, 2010). Plasma Membrane Intrinsic (PIP) aquaporin PIP2;1 is 'AT3G53420' in the *Arabidopsis thaliana* genome.

Double loss-of-function/knockout mutant Atpip(2;1*2;2)

The double mutant *Atpip(2;1*2;2)* had Columbia 0 ecotype background, was generated by crossing *Atpip2;1* (SM_3_35928) and *Atpip2;2* (SAIL_169A03) single mutants (Ceciliato *et al.*, 2019; Da Ines *et al.*, 2010; Javot *et al.*, 2003; Qing *et al.*, 2016), and was kindly donated by Dr Anton Schäffner (Institute of Biochemical Plant Pathology, German Research Centre for

Environmental Health). In the single *Atpip2;1* mutant, the *Atpip2;2* is thought to be upregulated (Da Ines, 2008), hence the double mutant *Atpip(2;1*2;2)* was tested.

Mutant Athkt1-1

Arabidopsis thaliana genotype *sos3-1 hkt1-1* were crossed to Col-0 gl1, and F_2 plants were genotyped to identify *Athkt1-1* homozygotes. PCR analysis to genotype At*hkt1-1* and HKT1 followed Rus et al. (2001). Seeds were kindly donated by Associate Professor Stuart Roy from the University of Adelaide.

Overexpression lines 35S::AtPIP2;1

The 2x35S::AtPIP2;1 (lines 7, 21 and null segregant) seeds from overexpression transgenic lines were kindly donated by Dr Michael Groszmann from The Australian National University. The null-2x35S::AtPIP2;1 seeds did not contain the 2x35S promoter and were PCR tested to confirm that they were nulls by checking for presence or absence of the 2x35S promoter. The null segregant material was used as an ideal control in experiments assessing the phenotype of the *35S::AtPIP2;1* material. Dr Groszmann previously tested the transcript levels of *AtPIP2;1* in these lines and observed that Line 7 had ~30-fold greater transcript level that wild type Col-0 and Line 21 had ~12-fold greater transcript levels than wild type Col-0; both lines are single copy inserts of the transgene (personal communication Dr Michael Groszmann 12th December 2020). Seeds were chosen from T2 segregation lines selected on antibiotics. For results of hygromycin tests for these seeds, the expected result would be 75% positive for the transgene and from those that are positive there would be 1/3rd homozygous and 2/3[rd]s heterozygous. For the current study, T3 lines that were positive for the transgene were selected to generate seed and a null line was propagated as a control. For later experiments described in chapters 4 and 5, seed from T3 generation that were positive for the transgene and confirmed to be homozygous were used. To confirm the presence of the transgenes, seeds were germinated in media containing hygromycin B at a concentration of 15 µg mL^{-1} (Ref: #H0125, Melford Laboratories Ltd.) and gDNA was collected to run PCR to double check the presence or absence of the transgene. There were two independent lines of 2x35S::AtPIP2;1 (lines 7 and 21), and the same protocol was applied to each line.

Overexpression lines UBI:nGFP::PIP2;1

An overexpression line of AtPIP2;1 which included an n-terminal green fluorescent protein (GFP) was driven by a ubiquitin promoter. This promoter was generated using an pUBI-GFP_DEST vector (Figure 3.2), to make UBI::nGFP:PIP2;1 expressing Arabidopsis lines. These seeds were kindly shared by Professor Steve Tyerman. The material was created by Dr Chuang Wang whilst working in the Tyreman Laboratory at the University of Adelaide, where the seed available was T2 seeds.
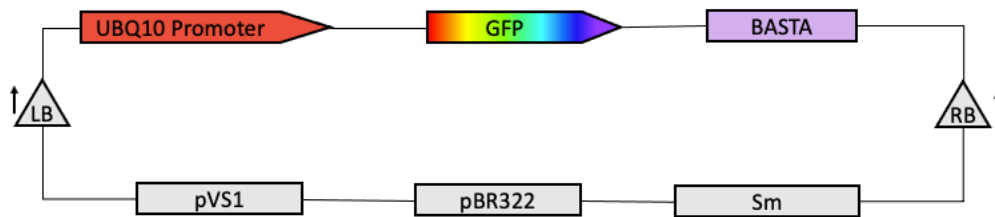
Figure 3.2: Schematic representation of the pUBI-Dest vector used to prepare a plasmid for generating UBI:nGFP::PIP2;1 overexpression Arabidopsis lines.

The transgenic material was designed to over-express UBI:nGFP::PIP2;1 under the control of the Arabidopsis ubiquitin-10 promotor (At4g05320; 634 bp immediately preceding the ATG start codon). In addition, the fragment carries the gene phosphinothricin-N-acetyltransferase that confers resistance to the herbicide Basta (nGFP constructs carry resistance to the antibiotic hygromycin) embedded in 5'- and 3'- regulatory elements of the nopaline synthase. The vector backbone contains the pVS replication sequence for maintenance in *Agrobacterium tumefaciens*, the pBR322 origin for high copy number in *Escherichia coli*, and the aminoglycoside-3-adenyltransferase gene for resistance against streptomycin or spectinomycin in bacteria (Adapted from Grefen *et al.*, 2010).

The *Arabidopsis thaliana* ubiquitin-10 gene promotor (PUBQ10) is expected to facilitate moderate expression in nearly all tissues of Arabidopsis, the vector set should result in moderate levels of expression of intrinsic membrane and soluble proteins in Arabidopsis, with a temporal stability after transient transformation that is apparently limited only by the maintenance of the plants. Experiments involving transformation using vectors derived from

this vector have been reported and transgene expression in guard cells, epidermal cells and root hairs was described (Grefen *et al.*, 2010).

To confirm the insertion of nGFP tagged AtPIP2;1 in this material, three methods were used: (1) Basta resistance selection on soil (Section 3.2.2.3), (2) Confocal microscope screening to observe presence or absence of GFP in seedlings (Section 3.2.4) and (3) PCR confirmation of the presence of the transgene in gDNA from plants (Section 3.2.5).

3.2.2 Plant growth conditions

3.2.2.1 Growth conditions for bulking up seed using plants grown in soil, and in controlled conditions

Seeds were initially sown on a petri dish containing sterilized half strength Murashige and Skoog (½ MS), pH 7.0 medium with 1% (w/v) phytagel (WXBD1420V, Sigma-Aldrich, Germany). Half strength MS was autoclaved at 121°C for 20 min before pouring into petri dishes. Seeds were sterilized (see 3.2.6.1) and stratified in the dark at 4°C in for 72 hours. Imbibed seeds were incubated in long-day growth chambers for 14 days. The growth chamber was at 19°C - night and 22°C – day with light irradiance was 100 - 120 μmol m^{-2} s^{-1} at the plant height following duration of 16 h light and 8 h dark. The humidity was maintained between 60 - 75 %. At 2-weeks-old, seedlings were transferred to pots, one plant per pot, 4 - 5 pots as replicates per genotype. Pot sizes were 7.5 cm x 6.0 cm, and 6.0 cm in height, containing 170 grams sterilized mixture of cocopeat based soil and 'Debco seed & cutting premium germinating mix' at a 1:1 ratio. Prior to seedling transplantation, 15 pots were placed on a tray and soaked with 2 litres of water containing of Imidacloprid at a concentration of 0.1 g liter^{-1} (Confidor 200 SC Insecticide, Bayer Cropscience Pty Ltd) and *Bacillus Thuringiensis* 0.1 mL liter^{-1} (BTI Larvicide 1200 ITU mg^{-1}, Amgrow Pty Ltd) to control blackflies and fungus gnats. Young seedlings were covered with a plastic transparent lid (Smoult, Kersbrook, SA, Australia) for the first 4 weeks to maintain a high humidity for optimal growth. All plants were randomly distributed within trays and their position changed every week to reduce positional biases within the growth chamber (such as potential uneven light distribution and air flow variation). Plants were watered every 2 - 3 days with reverse osmosis (RO) treated water to maintain optimal water level for plant development and reproduction. When plants started flowering each plant was covered by a plastic transparent floral sleeve 20 cm x 50 cm until seeds were harvested.

To create a resource of seed where parent plants had all been growing in saline conditions, the process involved transferring seedlings when they had 4 - 5 real leaves, at 22 days after sowing, into conditions where 100 mL of sodium chloride solution was applied with each pot. There were several treatment solutions trialled, including treatments with water and NaCl at 0 mM, 5 mM, 25 mM, 50 mM and 75 mM of NaCl (water, 5 mM NaCl, 25 mM, 50 mM and 75 mM salt treatments respectively). As expected, treatment with water and NaCl at 5 mM NaCl had less severe effects on plant growth and seed formation relative to the higher NaCl treatments. For example, mutant *Athkt1* struggled to grow during the vegetative phase and tended to die after 10 weeks whilst in the reproductive growth phase, when plants were grown in the higher saline concentration treatments, hence *Athkt1* plants did not produce seeds under the 75 mM NaCl treatment (Figure S18). Presumably Na^+ slowly accumulated in seeds during growth under the water and NaCl at 5 mM NaCl treatment without obvious stress to the plants, whereas the other treatments caused obvious stress to the plants, therefore seed from the water and NaCl at 5 mM NaCl treatment was used to test the germination percentage in different media. NaCl was purchased from AnalaR NORMAPUR® ACS (Catalogue Number 27810.364, Reagent (specification as per) European Pharmacopoeia analytical reagent, VWR International LTD).

3.2.2.2 Harvesting plant material

- For the seed: When the first siliques of parent plants were brown and dry (about three months old), plant watering was ceased to allow drying out, which took 2 weeks at room temperature (22ºC – 24ºC) on drying shelves. Seeds from yellow brown siliques were harvested by gently tapping the dried plants onto a sheet of A3 paper. Seeds were filtered through a fine strainer to remove all residue tissues. Seeds were collected and enclosed in 2 mL Eppendorf centrifuge tubes before being stored in a dark room at 22ºC.

- For the ion (Na^+, K^+) measurement in tissue samples: the whole parent shoots including leaves, stems and siliques but not containing seeds were collected and stored for later measurements. There were 25 growth assays in total, which were number followed the order of assay.

3.2.2.3 BASTA selection of transgenic plants in soil grown conditions

Transgenic seed with a 'bar' selection marker, which is the BASTA resistance gene, were selected for by a process that involved the application of the herbicide glufosinate ammonium

$C_5H_{15}N_2O_4P$, MW 198.16 g (BASTA; Bayer Crop Science, Australia). The influence of this herbicide application should kill the non-transformants but have no effect on positive transgenic plants which carry the BASTA resistance gene.

Seed was sprinkled into sterilized and saturated cocopeat soil (Section 3.2.2.1) evenly and sparsely. Wild type Col-0 and 'no spray' pots of transgenic plants were planted as controls. Seed was stratified in a cold 4ºC dark room for 3 days under high humidity before exposure to long-day growth chamber conditions which are described in section 3.2.2.1. Reverse osmosis (RO) water was refilled every 2 – 3 days during this period. Once the seedlings had several true leaves and were healthy, the BASTA treatment was applied. At 21 days after sowing, seedlings were sprayed thoroughly with herbicide 120 mg L^{-1} glufosinate ammonium (C5H15N2O4P, MW 198.16 g; BASTA; Bayer Crop Science, Australia) solution. Then this process was repeated two times every three days (24 and 27 days after sowing). The BASTA working solution used for spraying was at a concentration of 120 mg L^{-1} and stored at 4ºC in the dark.

At 34 days after sowing, the number of weak yellowish and dead plants as well as healthy plants in each tray, was recorded to determine the proportion of plants with BASTA resistance (Figure 3.14 A). Plants that survived which were healthy and green from 34 days after sowing were considered to be transgenic plants, harbouring the BASTA resistance gene courtesy of the UBI:nGFP::PIP2;1 vector. Transgenic lines that had the highest percentage positive transgenic seedlings were chosen for propagation to generate seed for the subsequent experiments. The surviving plants were transferred to a pot of cocopeat soil and they continued growing until mature seeds were produced and then harvested. Plant tissue was harvested, gDNA extracted from leaves, and a PCR assay was used to confirm the insertion of the transgene (see section 3.2.5).

3.2.3 Assessing seed coat mucilage production

Arabidopsis seed releases mucilage, which can influence properties such as flotation potential (Saez-Aguayo *et al.*, 2014). Arabidopsis seed mucilage and cell wall properties can also influence seed germination (Mulller *et al.*, 2013). To check for any differences in mucilage properties in the germplasm used for this project, the mucilage produced by each genotype was visualised by Ruthenium red staining.

3.2.3.1 Solution preparation

Volumes of 100 mL of 50 mM Ethylenediamine tetra acetic acid (EDTA) were prepared: 1.861 g EDTA ($C_{10}H_{16}N_2O_8$ laboratory reagent, MW 292.2438 g mol^{-1}, www.chemsupply.com.au) was added into water and topped up to 100 mL with water and pH adjusted to between 8 and 9 with NaOH. To make 1 litre of 0.01% ruthenium red (RR) (ProSciTech (PST) Pty Ltd, C075) solution (w/v): 0.1 g Ruthenium red was added into 1 L water. The solution was stored in the dark (wrapped in aluminium foil) at 4°C. The solution can only be used if the colour is bright pink.

3.2.3.2 Ruthenium red staining protocol

Whole mature seeds were completely desiccated and stored for at least 4 weeks before the staining experiments. After 10 min soaking in RR solution, individual seed was observed under 50X magnification using a Zeiss Stemi 2000 microscope with an attached AxioCam ERc 5s camera.

For observing the adherent inner layer only, the seed was pre-hydrated in 50 mM EDTA (Arsovski *et al.*, 2009). About 20 seeds for each sample were treated in 50 mM EDTA for 2 hours with orbital shaking (400 rpm) followed by 1 hour in RR solution with shaking at 20°C. The solution was then removed and seed was washed with MQ water; seeds were observed under 32X magnification using a stereo microscope (Nikon SMZ25 stereo microscope with fluorescence and DS-Ri1 CCD camera). Three biological replicates (seed from three independent parent plants) for every plant genotype were used in this experiment.

3.2.4 Phenotyping transgenic plants harbouring the overexpression construct UBI:nGFP::PIP2;1 using laser scanning confocal microscopy

Sterilized seeds from transgenic plants harbouring the contruct of UBI:nGFP::PIP2;1 were germinated on petri dishes with ½ MS and stratified for 72 hours in dark cold (4°C) condition. Seeds were then transferred to a long-day growth cabinet (See section 3.2.2.1).

At 10 days after sowing, seedlings were placed on slides in a water droplet and under a coverslip. Seedlings were scanned using a Nikon A1R laser scanning confocal microscope to visualize the GFP signal. A standard FITC filter set to visualise GFP was used with the excitation at 488 nm and dichroic mirror 505 nm and barrier filter 520 nm.

Although the expression of UBI:nGFP::PIP2;1 attributable to the stably transformed construct UBI:nGFP::PIP2;1 was visible in several parts of seedlings such as leaf, root, hypocotyl, meristem; the root specifically was chosen for further scanning because fluorescence in this tissue was clear and most convenient to confirm that the material was transgenic.

The number of transgenic plants relative to nulls was recorded, and a collection of positive transformant seedlings was kept and grown to propagate additional seeds for subsequent experiments.

3.2.5 Molecular methods for genotyping Arabidopsis lines

3.2.5.1 Plant genomic DNA extraction

There were two genomic DNA (gDNA) extraction methods used in this project depending on the downstream applications. A rapid extraction protocol was used to quickly screen seed material from lines harbouring the overexpression construct 2x35S::AtPIP2;1 that involved using a Thermo Scientific Phire Plant Direct PCR kit (F130WH, Thermo Scientific™ Phire™ Plant Direct PCR Kit), however this procedure has lower quality and purity requirements for the gDNA than the other method used. The higher quality gDNA extraction for confirming the genotyping of transgenic plants involved the Edward's protocol (1991). gDNA was extracted from leaves, was used as a template for Polymerase Chain Reaction (PCR) to confirm the knockout or overexpress genes.

Phire Plant Direct gDNA isolation

A simple method used for gDNA extraction from mature dry Arabidopsis seed was used. Arabidopsis seeds (~10 seeds) were put into a 1.5 mL Eppendorf microcentrifuge tube (kept at 4ºC) and homogenised with sterilized steel tweezers at 22°C. An amount of 20µL "Dilute Buffer" (Thermo Scientific™ Phire™ Plant Direct PCR Kit) was added directly into the tube. The tube was shaken well by a brief vortex and then incubated at 22°C for 3 min. Care was taken to make sure that the seed sample was covered with the dilution buffer. The gDNA extracted can be used directly or stored at -20°C. Before using the sample, the tube was centrifuged briefly and 1.0 µL of the supernatant was used as a template for a 10 µL PCR reaction. This method is not suitable for downstream applications that require high quality gDNA.

Edward's gDNA extraction

A gDNA extraction method following Edward et al. (1991) was used to extract DNA quickly from a limited amount of tissue. In brief, a small fresh green leaf was squeezed with a pellet pestle (Z359947, Sigma-Adrich) in a 2 mL Eppendorf tube at 22°C, and 400 µL of cold Edwards extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added. The tube was vortexed briefly (5 seconds) and centrifuged at 13000 rpm (18928 relative centrifugal force, g-force) for 5 minutes in a benchtop microcentrifuge (Microfuge 16, Beckman Coulter, US). Then, 300 µL supernatant was decanted into a fresh microfuge tube and 300 µL of isopropanol was added for DNA precipitation. Tubes were gently shaken for 5 seconds and left in a rack for 5 minutes before being centrifuged at 13000 rpm for 5 min at 22°C. A DNA pellet was collected at the bottom of the tube, then 500 µL ethanol (70-75%) was used to wash the pellet. Tubes were centrifuged at 13000 rpm for 5 mins and all wash solution was removed. The pellet was allowed to dry on the bench at 22°C before being dissolved in 100 µL DNase-free water and stored at –20ºC until further use. This gDNA was used for PCR in subsequent analyses at 1.0 µL each.

For frozen leaves and tissues: plant tissues were ground in liquid nitrogen prior to following the above listed steps.

3.2.5.2 Application of the Polymerase Chain Reaction (PCR) for genotyping

Direct PCR amplification

Table 3.1: The composition of a Phire Plant Direct PCR

PCR components	Final Conc.
2X Phire Plant PCR Buffer	1X
Phire Hot Start II DNA Polymerase	0.5 µL
Forward primer (PMDC 32 2x35S F)	0.5 µL
Reverser primer (AtPIP2;1 CDS R)	0.5 µL
Template (gDNA)	1.0 µL
PCR grade water top	up to 10.0 µL

Thermo Scientific™ Phire™ Plant Direct PCR Kit was used to perform PCR directly from Arabidopsis seeds without prior DNA purification. This PCR was used to check the genotype of seeds from lines 7 and 21 harbouring the overexpression construct 2x35S::AtPIP2;1 and involved using mature dry seeds. A reaction of Phire plant direct PCR was assembled as presented in Table 3.1 with primers recorded in Section 3.2.5.3. The PCR cycling conditions are detailed in Table 3.2.

Table 3.2: The Phire Plant Direct PCR cycling conditions

Cycle steps	Temperature	Time	Cycles
Initial denaturation	98ºC	5 min	1
Denaturation	98ºC	8 s	
Annealing	59ºC	10 s	40-43
Extension	72ºC	30 s	
Final extension	72ºC	30 s	1
Hold	10ºC	-	-

Standard PCR amplification

Table 3.3: The composition of a standard PCR

PCR components	Final Conc.
10x Kapa Taq Buffer + dye + 1.5 mM Mg	1X
10 mM dNTP Mix (ATGC)	200 μM
5 U/μL Kapa Taq DNA polymerase	0.5 U μL^{-1}
Forward primer (0.5 uM)	0.5 μL
Reverser primer (0.5 uM)	0.5 μL
Template (gDNA)	1.0 μL
PCR grade water top	up to 10.0 μL

PCR was employed in this project for genotyping purposes mainly to identify insertions in putative transgenic plants. Standard PCRs using KAPA Taq PCR Kit (KK1021, 250 U, Sigma-Aldrich) were assembled as indicated in Table 3.3. Specific primers for PCR were ordered from Geneworks (Adelaide, Australia) and details of the primers are recorded in section

3.2.5.3. The PCR cycling conditions were similar to those shown in Table 3.4. The cycling conditions were modified as appropriate for each PCR, for example, the annealing temperature was adjusted according to each primer's particular denaturing optimum and the extension time was adjusted according to the expected product length.

Table 3.4: The standard PCR cycling conditions

Cycle steps	Temperature	Time	Cycles
Initial denaturation	95ºC	3 min	1
Denaturation	95ºC	30 s	
Annealing	57ºC	30 s	30
Extension	72ºC	30 s	
Final extension	72ºC	1 min	1
Hold	10ºC	-	-

Annealing temperature depends on the primer Tm value

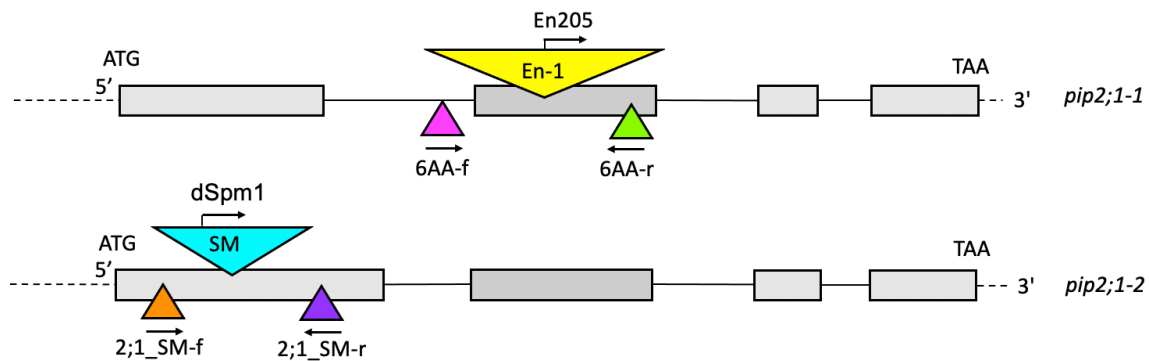## 3.2.5.3 Primers for PCR

Figure 3.3: Schematic representation of the AtPIP2;1 gene with En-1 and dSpm1 insertions relevant to Atpip2;1-1 and Atpip2;1-2 mutants, respectively. Boxes represent the exons, the lines represent the introns, and the dashed lines indicate the 5' and 3' regions of the gene, arrows indicate the orientation of primers. ATG start codon, TAA/TGA stop codon (Da Ines, 2008).

The positions of the T-DNA insertions in mutant lines lacking functional *Atpip2;1* were verified by sequencing and arrows indicate the orientation of the T-DNA left border. The En-1 element is located in the 2nd exon after base 849 (base 1 is the adenine of the start codon). En205 denotes En-1-specific oligonucleotides which means that the oligonucleotide binds 205 bp away from the left border of the En-1 element.

For *Atpip2;1-2*, the nonautonomous defective Spm (dSpm1) element in SM lines is located in the first exon. The fragment SM_3_35928 JIC SM has 117bp and was inserted at chr3 C/19805250-198053660.

PCR was used to differentiate between homozygous mutants, heterozygous mutants and wild-type plants. Two PCR reactions were set up for each plant. PCR reaction 1 contained PIP2, En-1-specific oligonucleotide 6AA-En205 F and 6AA R. PCR reaction 2 contains two PIP2s; oligonucleotide 1 specifically 6AA F and 6AA R. In homozygous mutant plants, only PCR product 1 with 563 bp length was produced (both alleles have a En-1 element). In wild-type Col-0 plants only PCR product 2 of 314 bp length was produced. In heterozygous mutant plants resulted in both PCR product 1 and 2. TAA and TGA identify the start and stop codons of PIP2;1.

Mutant Atpip2;1-1

For the knockout mutant *Atpip2;1-1* line 6AAS98 (En-1) Amaze, the sequences of 3 primers (Da Ines, 2008; Tissier *et al.*, 1999) used were:

- Forward primer 1: 6AA F

Sequence: 5'-GCTTGTTGTACCTTAATTCTCAAGT-3'.

- Forward primer 2: 6AA-En205 F), an insert mutant specific primer

5' – 3' sequence: GGTGCTTGTGTTATGTTATGTTGA from Amaze (En) collection.

- Reverse primer: 6AA R

Sequence: 5'-AGAGAAGACGGTGTAGACAAGAAC-3'.

PCR reaction 1: 6AA-En205 F and 6AA R identified both homozygous (Hom) and Heterozygous (Het) *Atpip2;1-1* mutant by showing 563 bp band on electrophoresis gel.

PCR reaction 2: 6AA F and 6AA R identified heterozygous (Het) *Atpip2;1-1* and Columbia-0 (Col-0) by producing 314 bp band on electrophoresis gel.

Mutant Atpip2;1-2

For the knockout mutant *Atpip2;1-2*, the insertion is SM_3_35928 JIC SM. This is a SM transposon line carrying a single defective Spm (dSpm1) transposon element as a stable insertion in the genome. This insertion contains sequence of the original DNA transformants and a kanamycin marker.

3 primers were used on the PCR as following (Da Ines, 2008).

- Forward primer 1: 2;1_SM F (SM35928 F)

Sequence: 5'- AACATATAACGTTGGCAAAAA - 3'.

- Forward primer 2: dSpm1 F, an insert mutant specific dSpm1 primer from JIC SM collection

Sequence: 5' - CTTATTTCAGTAAGAGTGTGGGGGTTTTGG - 3'.

- Reverse primer: 2;1_SM R (SM35928 R)

Sequence: 5' - TTAATTTCGAATAAACAAACCA - 3'.

The 1st pair primer 2;1_SM F and 2;1_SM R identified heterozygous (Het) *Atpip2;1-2* and Columbia-0 (Col-0) by producing > 900 bp band on electrophoresis gel.

The 2nd pairs are dSpm1 F + 2;1_SM R identified both Homozygous (Hom) and Het *Atpip2;1-2* mutant by showing >500 bp band on electrophoresis gel.

Double mutant Atpip2;1*2;2

Double mutant *Atpip2;1*2;2* is the result of crossing *pip2;1* and *pip2;2* mutant lines, the homozygosity was checked for both mutants using primers in Table 3.5.

To confirm the genotype of *Atpip2;1*2;2* double mutant line, PCR checks were completed testing for loss of *Atpip2;1-2* using primers (1 and 2), (3 and 4) listed in Table 3.5. To confirm the absence of *Atpip2;2* in *Atpip2;1*2;2* double mutant lines, two PCRs were used. One PCR in which the homozygous double mutant plant produced no product when using pair primers (5 and 6) and another where the PCR produced a product that had the size >600 bp when using primers (7 and 8). A heterozygote would produce a PCR product in any pair of primers (1 and 2) or (5 and 6).

In brief, homozygous double mutant *Atpip2;1*2;2* plants produced no product when using primers (1 and 2) or (5 and 6) while using primers (3 and 4) produced a PCR product had the

size >900 bp or using primers (7 and 8) produced a PCR product had the size >600 bp on an electrophoresis gel.

Table 3.5: Primers used to amplify double mutant *Atpip2;1*2;2* gene coding sequences from gDNA derived from Arabidopsis leaves

Order	Primers	Primer sequences	TM (°C)	Size of CDS
1	SM35928 F	5'-AACATATAACGTTGGCAAAAA-3'	57.9	>500 bp
2	SM35928 R	5'-TTAATTTCGAATAAACAAACCA-3'	56.0	
3	Spm 32 F	5'-CTTATTTCAGTAAGAGTGTGGGG TTTTGG-3'	66.8	>900 bp
4	SM35928 R	5'-TTAATTTCGAATAAACAAACCA-3'	56.0	
5	Sail_LB3	5'-TAGCATCTGAATTTCATAACCAA TCTCGATACAC-3'	67.7	>900 bp
6	Sail_169A03 R	5'-TTATAGATTACGGCAGCTCCG-3'	62.5	
7	Sail_169A03 F	5'-CAACCATAAGCCTACCAAAAGG-3'	62.4	>600 bp
8	Sail_169A03 R	5'-TTATAGATTACGGCAGCTCCG-3'	62.5	

Overexpression lines 2x35S::AtPIP2;1

There were two independent lines of 2x35S::AtPIP2;1; lines 7 and 21 produced with the plasmid vector PMDC32, schematically displayed in Figure 3.4. The same protocol was applied for each line. There were two pairs of primers used in this PCR experiment (Table 3.6).
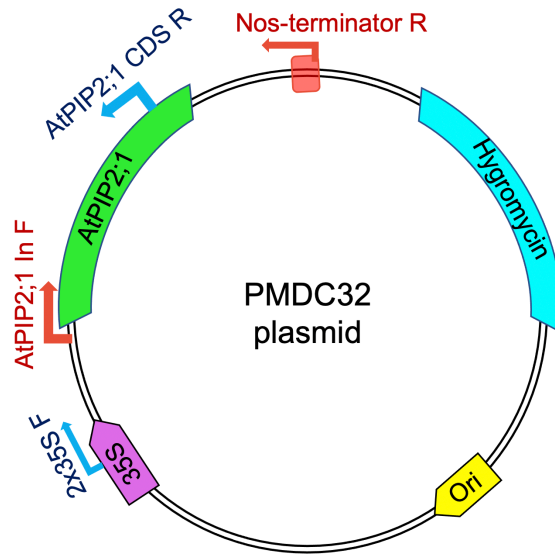
Figure 3.4: Schematic representation of the overexpression construct 2x35S::PIP2;1 using PMDC32 as the vector backbone.

Primers InF1 and Nos-terminator R1 were used in the PCR reaction to generate the PCR product '1', in which AtPIP2;1 InF1 is a forward primer located in the middle of the AtPIP2;1 CDS, and Nos-terminator R1 is a reverse primer located in the backbone of pMDC32.

Product 2 was amplified by 2x35S F and AtPIP2;1 CDS R and the band from this product was equivalent to approximately 980 bp in length. This PCR was used for additional confirmation, when some of the samples from plants which appeared to be positive based on their growth in antibiotic media (~95%) but did not return a band in attempts to amplify PCR product 1.

Table 3.6: Primers used to amplify 2x35S:PIP2;1 gene coding sequences from gDNA derived from Arabidopsis leaves

	Primers	Primer sequences	T_M (°C)	Size of CDS (bp)
1	AtPIP2;1 In F	5'-ACTGACCCCAAACGTAGTGC-3'	65.4	~500 bp
	Nos-terminator R	5'-CAAGACCGGCAACAGGATT-3'	63.4	
2	PMDC 32 2x35S F	5'-TCATTTGGAGAGGACCTCGA-3'	63.3	~980 bp
	AtPIP2;1 CDS R	5'-TTAGACGTTGGCAGCACTTCT-3'	65.0	

Overexpression UBI:nGFP::PIP2;1 lines

The UBQ10 promotor fragment was a 634 bp genomic DNA fragment immediately upstream of the ATG and was amplified from Arabidopsis genomic DNA using forward primer (5'-gcgaagcttGTCGACGAGTCAGTAATAAACG-3') and reverse primer (5'-gcgctcgagCTGTTAATCAGAAAAACTCAG-3' (Grefen *et al.*, 2010). Primers used were forward primer Ubi:64p F and reverse primer AtPIP2;1 CDS R with sequences of 5'-GTCGTCCTCGTCTTTTCTTCT-3' and 5'-TTAGACGTTGGCAGCACTTCT-3' respectively. The PCR product was expected to be 1982 bp in size. Schematic representation of the pUBI-Dest vector used to prepare a plasmid for generating UBI:nGFP::PIP2;1 overexpression Arabidopsis lines presented in Figure 3.2 (see also Chapter 5 Figure 1).

Actin house-keeping gene

Primers Actin 2 F and Actin 2 R, kindly shared by Dr Jiaen Qiu were used to check template quality of gDNA.

Sequences of forward primer AtActin2 F: 5'-TGAGCAAAGAAATCACAGCACT-3'

Sequences of reverse primer AtActin 2 R: 5'-CCTGGACCTGCCTCATCATAC-3'

When the template quality was satisfactory then a PCR product would be amplified using these primers which is expected to be 300 bp in the size.

3.2.5.4 Agarose gel electrophoresis

Prior to each run the gel tank was cleaned and sprayed with RNase Zap before 1 x TAE buffer was transferred into it as running buffer. Agarose Molecular Grade (Cat. No. BIO-41025, Bioline, Meridian Bioscience) (g mL^{-1}) was dissolved at 1% into 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) using a microwave. SyBR-safe (0.005% (v/v)) (Invitrogen, Mulgrave, Australia) was added to the cooling gel to visualise DNA. PCR product was mixed with 6 X purple gel loading dye (New England Biolabs, USA) and loaded into the gel. Loading 3μL of 1Kb DNA ladder (N3232S, New England Bio Labs, Inc) into the gel was for indicating the product size. The gels were run at 100 Volts for 30 – 40 min. The gels were visualised with a UV transilluminator (ChemiDoc™ Touch with UV Trans Illumination) using standard Filter-SYBR Safe settings.

3.2.6 Germination assays on ½ MS media

3.2.6.1 Seed sterilization

Chlorine gas sterilization

Samples of approximately 20 (± 5) mg of mature seed of each genotype were put into 2 mL Eppendorf centrifuge tubes. Tubes were opened and placed in a rack, inside a small desiccator. To a volumes of 100 mL bleach (5.25%) in a small beaker, 3 mL of hydrochloric acid (HCl, 32%) acid was carefully added. Seed samples in their tubes and the beaker of bleach and HCl were placed in a sealed desiccator for 2 – 4 hours to allow sterilization of the seed by the chlorine gas produced.

The opened tubes were placed in a sterilized laminar flow cabinet for 2 hours or overnight to disperse any trace amounts of chlorine gas in a sterile environment. Seeds in their tubes were sealed, then stored at 22ºC ready for germination assays.

Ethanol and bleach sterilization of seed

Samples of 100 – 500 seeds were placed in 2 mL centrifuge tubes, and 1 mL of ethanol 75% (v/v) (Reagent grade, www.chemsupply.com.au) was added, samples were vortexed for 5 seconds or shaken well to make sure all seed were exposed to the ethanol solution and tubes were incubated for 5 min at 22°C.

To removal the ethanol from the seeds, 1 mL of 10% (v/v) sodium hypochlorite solution containing 8% available chlorine (Fisher Scientific, UK #S/5040/21) was added, and samples vortexed for 5 seconds then incubated for 5 minutes at 22°C in the sodium hypochlorite solution. Samples were centrifuged in their tubes for 30 seconds at 8000 rpm (7155 g-force).

The liquid solution was taken out then samples were washed using 1 mL sterile Milli-Q (MQ) water, vortexed for 5 seconds then spun in a mini centrifuge (FC5306, OHAUS) for 30 seconds. This process was repeated 4 times to fully wash the ethanol and sodium hypochlorite from the seeds.

Seed was then used for germination assays immediately or left on filter paper (diameter 110mm, Whatman, Life Sciences) to dry for 2 hours and stored in sterilized petri dishes for later use depending on the experiment. Each genotype used for comparisons was treated with equivalent sterilisation, washing and subsequent steps.

All steps were performed in a sterile laminar flow hood to prevent microbial contamination.

3.2.6.2 Germination assays

3.2.6.2.1 Selection of transgenic plants on solid ½ MS Hygromycin media

Half strength MS media at 55ºC – 60ºC was prepared for the addition of appropriate antibiotics for selection. For 35S promoter transgenic lines, hygromycin B solution (H385, Phyto Technology Laboratories, US) was added to achieve a concentration of 15 µg mL^{-1}. Hygromycin B ($C_{20}H_{37}N_3O_{13}$, MW 527.5 g mol^{-1}) is an aminoglycoside antibiotic derived from *Streptomyces hygroscopicus* that interferes with the translation step of polypeptide synthesis of prokaryotes and eukaryotes (Brodersen *et al.*, 2000). It inhibits peptide chain elongation by preventing elongation-factor EF-2 dependent translocation (Gonzalez *et al.*, 1978).

Sterilised seeds were dispersed on selective media and petri dishes were sealed by 3M micropores tape. Seeds were stratified for 3 days in the dark at 4ºC before they were illuminated under continuous light (100-120 µmol m^{-2} s^{1}) for 6 h at 22ºC to stimulate germination.

Seed plates were wrapped in aluminium foil (dark) and incubated for 2 days at 22ºC. Seedlings were then grown for 24 – 48 h at 22ºC in a 16-h light, 8-h dark regime with continuous light (100-120 µmol m^{-2} s^{-1}).

Following the 48-h light period, transformed seedlings were identified visually. Hygromycin B-resistant seedlings had long hypocotyls and green cotyledons, whereas non-resistant seedlings had short hypocotyls and green cotyledons. Seedlings identified as positive transgenics based on this test were carefully transferred to another plate containing ½ MS media, 1% phytagel without antibiotics for further growth in long day growth room, for up to 1 week before being transplanted to soil pots (Harrison *et al.*, 2006). To confirm whether these seedlings were positive transformants containing the genes of interest, leaf tissue was harvested. gDNA was extracted (section 3.2.5.1) and a standard PCR test was performed using primers designed to amplify a fragment specific to the binary vector which is incorporated into the plant genome (section 3.2.5 for further details).

3.2.6.2.2 Assessing seed from parent plants grown in control and saline treatments and germination occurred in control and saline treatments

The approach to germination scoring initially followed methods reported by Joosen *et al.* (2010) which involved germinating seed on blue germination paper to help with visualising germination stages (Blue Blotter Paper FT-2-383-200350 Grade 194, Sartorius Stedim Biotech, Anchor Paper Company, http://www.seedpaper.com), where the number of germinated seed was counted by the GERMINATOR tool. Modifications to the approach were required to optimise germination scoring. Between 150 – 200 Arabidopsis seeds for each plant were germinated on square petri dishes (120 x 120 x 17 mm, Z617679, Sigma-Aldrich, Germany) containing 50 mL ½ MS media; media at pH 7.0, 1% (w/v) phytagel (WXBD1420V, Sigma-Aldrich, Germany) was autoclaved at 121°C for 20 min, with or without addition of 20 mM NaCl or 75 mM NaCl. In preliminary trials a salt treatment of 20 mM NaCl was tested and the difference in germination between treatments with and without 20 mM NaCl were unclear, so higher concentrations, 50 mM and 75 mM NaCl were selected for treatments (Ahmed *et al.*, 2020; Clemens *et al.*, 1983; Nanda *et al.*, 2019; Nasri *et al.*, 2016; Ravindran *et al.*, 2020; Sarabi *et al.*, 2016; Thomas *et al.*, 1995; Wankhade and Sanz, 2013; Yao *et al.*, 2021). Four separate plants of each genotype were sown on 4 plates (representative of 4 replications, where each replication is seed from a different parent plant). Plates were sealed with 3M micropore tape and placed in a sealed transparent box to maintain optimal moisture conditions. Stratification was applied in the dark at 4°C for 72 hours to let seeds synchronize before germinating. Seed was then incubated to long-day growth chamber conditions (Section 3.2.2.1). All procedures described above were performed in a laminar flow hood to prevent microbial contamination.

Seeds of Col-0, *Atpip2;1-1* and *Atpip2;1-2* mutants from parents grown under control treatments (water) and treatment where parent plants were watered with water and NaCl at 5 mM NaCl were visualised with a binocular microscope at 24 hours post-stratification and were classified as 'germinated' when their endosperm ruptured (germination *sensu stricto*) and when the radicle is visible (Bewley, 1997). Subsequent to the 24-hour timepoint, seeds were further monitored for germination 'success' at 12-hour intervals for up to 4 days, then at 24 hour intervals for the 5, 6 and 9 day time points after stratification or until no change in scores was observed.

Successful seed germination was assigned when the seed endosperm ruptures and any of the following plant organs: cotyledons and/or hypocotyl and/or radicle is visible. Seeds lacking cotyledons and/or hypocotyl and/or radicle were regarded as 'not-germinated'.

Seed germination data are represented as percentages of germinated seeds (% germination = (germinated seeds x 100) / total sown seeds) as a function of incubation time post-stratification (Bewley *et al.*, 2013).

Time to reach 50% germination (T_{50}) was calculated according to the formula of Coolbear et al. (1984)

$$T_{50} = t_i + \left[\frac{\left(\frac{N+1}{2}\right) - n_i}{n_i - n_j} \right] . (t_i - t_j)$$

where N is the final number of germinated seed and n_j are the total number of germinated seeds by adjacent counts at times t_i and t_j, respectively where $n_i < N / 2 < n_j$. (Santo *et al.*, 2019).

3.2.6.2.3 Assessing the germination percentage of PIP2;1 loss of function mutants and overexpression lines grown in water (non-saline) or saline treatments.

Seeds of both mutant lines (loss of PIP2;1 expression) and PIP2;1 overexpression lines were assessed in the germination percentage assays. Seeds were placed in rows in petri dishes which contained ½ MS (section 3.2.2.1) with or without NaCl treatment (½ MS or ½ MS + 75 mM NaCl). Descriptions of the germination media, growth chamber conditions and observations of germination were provided in Section 3.2.2.1. There were 6 experiments conducted from different batches of plants.

3.2.7 Testing seed weight during imbibition in water and saline treatments

3.2.7.1 Seed number counting

Imbibing seeds at around 24 – 36 hours after the start of imbibition were scatted evenly on glass slides and images captured by a stereo zoom microscope (C-10x Widefield eyepiece, Nikon SMZ-800). The illumination settings were adjusted to minimise reflections and increase contrast between seed coat and background. Captured images were used to count the total number of seed in each sample by ImageJ software.

3.2.7.2 Seed moisture content

See Chapter 4 (manuscript) for the description of the methods used for seed moisture content analysis.

3.2.7.3 Trials of open mesh fabric and 20 mM NaCl treatments

Samples of 10-15 mg mature seed were placed on a 30 x 30 mm mesh with 250 μm open pores, 430 μm thickness (Sefar 05-250/29x139, Sefar Pty Ltd Filtration and Metal Mesh; http://www.sefa.com.au). Mesh and seed were placed on 11.5 x 11 cm blue germination test paper (see section 3.2.6.2.2) and covered by 12 x 12 cm petri dished in 22ºC and long day conditions (section 3.2.2.1). There were 3 salt treatments: 46 hours in MQ water (Water), first 30 hours in water then transferred to 20 mM NaCl from 30 to 46 hours (Water-Salt), and 46 hours in 20 mM NaCl (Salt) (see Figure 3.5). After soaking for 15 minutes, the meshes carrying seed were taken off the germination paper and placed on paper tissue to absorb excess water for 1 minute then weight was recorded. The meshes were then returned to the petri dish, for the subsequent exposure and measurement times.

The weighing of seed was repeated at 30, 45 minutes and 1, 2, 3, 4, 24, 30, 46 hours of imbibition. The weight of imbibing seed was calculated by determining the weight at each timepoint minus the weight of the mesh. Data are represented as a fresh seed weight and percentage weight increase over time. A fresh seed weight was calculated as the average of seed in a sample, specifically the weight of the sample divided by the number of seed. Percentage weight increases over time was calculated as:

$$\%increase = \frac{(weight\ of\ seed - initial\ seed\ weight)}{initial\ seed\ weight} \times 100.$$

In these methods, we aimed the change in seed weight when exposed to water or saline water within 15, 30, 45 min and 1, 2, 3, 4, 24, 30 and 50 h after sowing. Seeds were not stratified in a cold and dark room for 48 h as in the germination experiment, where dormancy needed to be completely eliminated before exposure to light and warmer temperatures. Therefore seed/seedling age was not the same for seeds in the germination and weight / ion experiments as seed in the germination test had a longer exposure time to water than seed in other experiments.

Previous germination and growth studies have subjected plants to a range of salt treatments: 20 mM NaCl (Ahmed *et al.*, 2020; Clemens *et al.*, 1983; Sarabi *et al.*, 2016; Wankhade and Sanz, 2013), 50 mM NaCl (Nasri *et al.*, 2016), 75 mM NaCl (Ravindran *et al.*, 2020), 100 mM NaCl (Thomas *et al.*, 1995; Yao *et al.*, 2021), 150 mM NaCl (Nanda *et al.*, 2019), 200 mM NaCl (Liu *et al.*, 2013a; Tsugane *et al.*, 1999; Yang *et al.*, 2010), 250 mM NaCl (Santo *et al.*, 2019; Wang *et al.*, 2021; Werner and Finkelstein, 1995), 150, 200, 250, 300 and 350 mM NaCl (Peng *et al.*, 2021) and 400 mM NaCl (Thomas *et al.*, 1995). In preliminary experiments, a moderate salt treatment with 20 mM NaCl added to water during the germination stage was tested. However, this treatment is mild relative to treatments used in the literature (Ahmed *et al.*, 2020; Sarabi *et al.*, 2016), which are often around 50 mM NaCl to 150 mM NaCl (Nanda *et al.*, 2019; Nasri *et al.*, 2016; Peng *et al.*, 2007; Ravindran *et al.*, 2020; Thomas *et al.*, 1995; Yao *et al.*, 2021). Therefore, in subsequent experiments, the concentration of salt was increased to 75 mM NaCl (Figure 3.5).
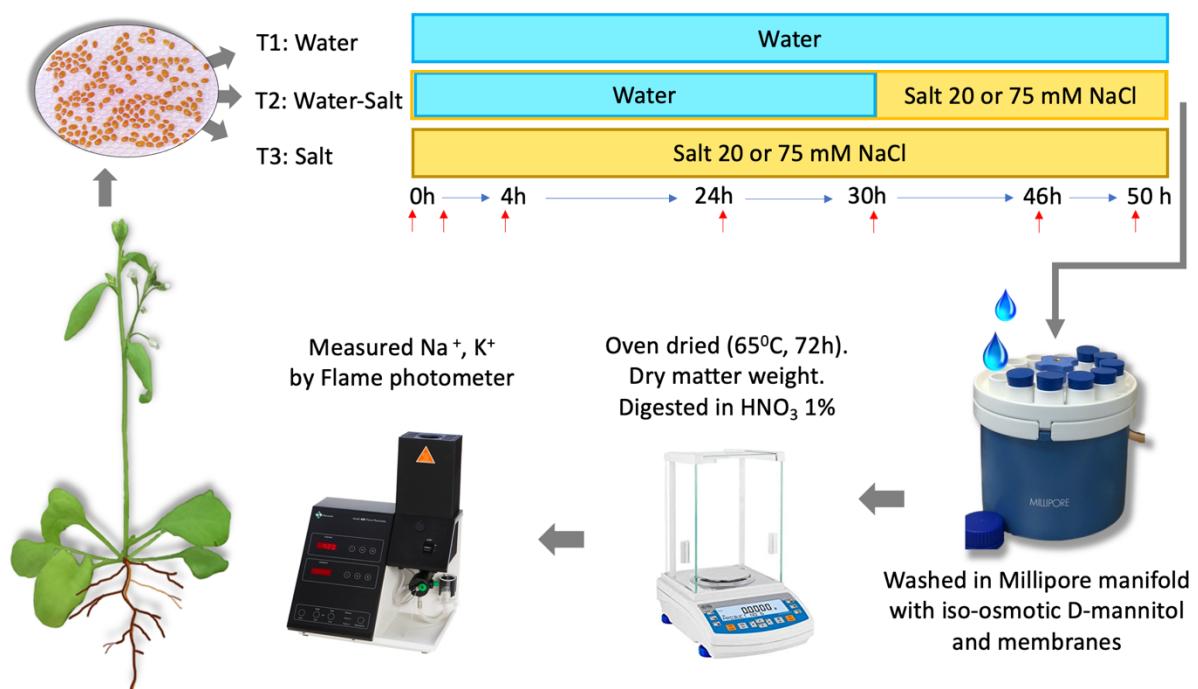
Figure 3.5. Illustration of experiments conducted to measure the seed weight and Na$^+$ and K$^+$ content during imbibition and germination in three treatments: Water, Water-Salt and Salt. The salt treatment was either 20 mM NaCl or 75 mM NaCl depending on the experiment and this is listed in each respective results figure legend. The red arrows indicate the time when seeds were weighed (dry seed is at 0 h). The final harvest time was either 46 or 50 h, depending on the experiment, as indicated in each respective results figure legend.

3.2.7.4 Fine, open mesh fabric and 75 mM NaCl treatments

The procedure was similar to the above description (section 3.2.7.3) but with some alterations as the previous design did not provide sufficient accuracy in the weight results (the weight of seeds did not change or changed very little compared to previous measurements). Mature seeds were placed on finer mesh, which was a 30 x 30 mm Sefar mesh with 75 μm opening pores, 93 μm thickness (Sefar 03-75/34x136, Sefar Pty Ltd Filtration and Metal Mesh; http://www.sefa.com.au). There were 3 salt treatments: 50 hours in MQ water (Water), 30 hours in water followed by transfer to 75 mM NaCl for 30 to 50 hours (Water-Salt) and 50 hours in 75 mM NaCl (See section 3.2.7.3). The concentration of 75 mM NaCl was chosen because previous studies indicated that this concentration is sufficiently stressful so as to influence Arabidopsis germination and seed ion content, relative to standard non-saline treatments, but is not so severe as to completely prevent germination (Mcdowell *et al.*, 2013; Nasri *et al.*, 2016; Ravindran *et al.*, 2020). After soaking seed for 15 minutes, meshes with seeds were taken off germination paper and placed on paper tissue to absorb all the excess water present on the seed for 30 seconds. All seed were picked out of the mesh and the weight was recorded. The weight of the seed was recorded at 1, 4, 30 and 50 hours of imbibition.

Data are represented as fresh seed weight and percentage weight increase over time (section 3.2.7.3) as specified in respective figures.

3.2.8 Measurement of Na^+ and K^+ content in seeds and plant tissues

3.2.8.1 Ion content measurements for germinating seeds and seedlings

Preliminary experiments to test seed ion content for Atpip2;1 mutants and wild type Col-0 where the parents had been watered with either water or water and NaCl at 5 mM NaCl

Mature seed (10 – 15 mg) from parents grown in different water regimes (water or water and NaCl at 5 mM NaCl) were harvested after 2 weeks and air dried in 22ºC. All samples were then oven dried at 65ºC for 72 hours before recording the dry weight. The dried seed was put in a 2 mL Eppendorf tube and digested in 1% HNO_3 (Nitric acid 69%, AnalaR analytical reagent, BDH Laboratory Supplies, England) at 80ºC for 4 hours. Blank samples contained 1% HNO_3 but not included dry seed, which were treated the same with samples. An aliquot of the filtered supernatant was taken for dilution and Na^+ and K^+ analysis was performed using an

Atomic Absorption Spectrophotometer (AAS; Shimadzu AA-7000) following the manufacturer's instructions.

Analysis of Na⁺ and K⁺content in seed imbibed in water, 20 mM NaCl or water followed by transfer to 20 mM NaCl after 30 hours nylon mesh filter and simple seed washing approach

Seeds were weighed (10 – 15 mg), distributed to a thin layer on nylon mesh with 250 μm opening pores and placed on filter papers in petri dishes as described in section 3.2.7.3. A 25 mL of MQ water or 20 mM NaCl solution was supplied to respective petri dishes for three different imbibing experiments: 1) seeds exposed to MQ water for 46 hours (Water), 2) seeds exposed to MQ water from 0 – 30 hours and then transferred to 20 mM NaCl from 31 – 46 hours (Water-Salt), 3) seeds exposed to 20 mM NaCl for 46 hours (Salt). Time was recorded upon addition of solutions to ensure consistent seed exposure time. Plates were sealed by micropore tape and incubated in long-day chambers under the conditions described in section 3.2.2.1.

After imbibing treatments, seeds with mesh were harvested and washed in 5 mL MQ water through a simple washing funnel. Seeds were then oven dried at 65°C for 72 hours before the dry weight was recorded. Dried seeds were digested in 1% (v/v) HNO_3 at 80°C for 4 h before Na^+ and K^+ analysis was performed using Atomic Absorption Spectrometry (AAS) as above.

Na^+ and K^+ content in seed imbibed in water treatment or 75 mM NaCl treatment or water followed by transfer to 75 mM NaCl solution after 30 hours treatment with seed rapidly washed on a vacuum manifold by iso-osmotic solutions

There were some adjustments in terms of material and washing methods compared to the experiment described in above sections. A finer mesh with 75 μm opening pores was used in order to remove all water on the mesh for more accurate weighing (see section 3.2.7.4). Meshes were placed on 11.5 x 11 cm blue germination paper (see section 3.2.6.2.2) inside a 12 x 12 cm petri dish in 22ºC and long day conditions. When harvesting after 50 hours imbibition, up to 12 seed samples were collected. Seeds were placed on 25 mm diameter nitrocellulose membrane filters (0.45 μm pores, MF-Millipore Tm, Merck Millipore Ltd, Tullagreen, Carrigtwohill, Co. Cork, Ireland) and put in a Millipore Manifold vacuum system (XX2702550 Merck Millipore 1225 Sampling Vacuum Manifold, USA) to control the rate that the solution passed through the filter. Seeds were washed by 5 mL D-mannitol solution, that had the same

isosmotic concentration with water with 75 mM NaCl added (140 mOsmol kg^{-1} measured using a Fiske 210 Micro-Sample Osmometer freezing-point, Advanced Instruments, USA). The washing process happened within one minute per sample to prevent excessive ion leakage and to ensure uniformity of the wash process between samples. Seeds were oven dried at 65°C for 72 hours before the dry weight was recorded. Dried seeds were digested in 1% (v/v) HNO_3 acid at 80°C for 4 h. The seed Na^+ and K^+ contents in samples were measured using a Sherwood 420 flame photometer (Sherwood, Cambridge, UK) following the manufacturers' instructions.

3.2.8.2 Measurement of Na^+ and K^+ contents in plant tissues

Na^+ and K^+ content in plant tissues when grown in salt treatments using the AAS

Tissues including all leaves, stems and siliques of a plant were pooled into a paper bag and dried at 65°C for 72 hours. Then dry matter samples were weighted (range between 150 – 1200 mg) and put into 50 mL Falcon tubes. Blank samples contained 1% HNO_3 but no dry tissues. Samples were digested by adding 30 – 40 mL of 1% HNO_3 acid and heated at 80°C in 2 – 4 hours. An aliquot of the supernatant was filtered through a 0.2 µm filter (Filtropur S0.2, Sarstedt, Germany. #83.1826.001) and taken for dilution before measuring Na^+ and K^+ using the AAS as above.

Na^+ and K^+ content in plant tissues using flame photometer

Pooled tissue samples were oven dried at 65°C for 72 hours. Then samples were ground into homogenous small pieces. 200 mg of sample were digested in 22 mL of 1% HNO_3 in 50 mL Falcon tubes. Blank samples contained 1% HNO_3 but no dry tissues. Samples were heated at 80°C for 4 hours. Supernatant was filtered, diluted before measuring potassium and sodium content using a Sherwood 420 flame photometer (Sherwood, Cambridge, UK) following the manufacturers' instructions.

3.2.9 Measuring seed size during swelling

3.2.9.1 Increases in seed maximum cross sectional area (MCS area) based on video footage of three genotypes Col-0; *Atpip2;1-1* and *Atpip2;1-2* mutants

Seeds were placed on ½ MS media in a round petri dish (92 x 16 mm with cams, wwwsarstedt.com, Germany) containing 10 mL ½ MS media. The area recorded was 5 mm x 6.5 mm encompassed up to 12 seeds within frame with a Nikon C-S4A light microscope (Nikon Japan) and the ED plan 1.5 x objective lens WD45 (Nikon Japan). Seeds were placed to get the maximum cross sectional (MCS) area, (ie projected seed MCS area in images as a surrogate for seed volume assuming isodiametric swelling) then seeds were captured with a SCC-B1391P colour camera (Samsung) at 2X magnification and recorded with IC CAPTURE 2.0 software (The Imagine Source, USA). Video was recorded continuously for 3 days with images taken at one-minute intervals. The period was subsequently adjusted to 2, 3, 5 and 10 minutes in later experiments. All captured images were stacked to make short videos in AVI format to examine the imbibition process. Images at certain time points were extracted and seed MCS was determined using ImageJ software (National Institute of Health, USA). The images were calibrated using a micrometre scale.

3.2.9.2 Changes in seed MCS area using Image Capture at certain time points during imbibition and germination

Maximum 35 seeds of one plant were placed gently on petri dish as describe in 2.9.1. The orientation of seeds was adjusted using the microscope in order to view the largest MCS area on images. Seed was from four plants of each genotype.

After placing seeds in plates, images were taken at 1, 15, 30, 45 minutes and 1, 2, 3, 4, 24, 30 and 48 hours after sowing. Lids of Petri dish were taken off to capture images then put on again in the interval time of measurements. Images were analysed as above (section 3.2.9.1). Data is presented as the average MCS area of a seed (mm^2/ seed) and percentage of MCS area increase over time, calculated as:

$$\%increase = \frac{(MCS\ area\ of\ seed - Initial\ MCS\ area\ of\ seed)}{Initial\ MCS\ area\ of\ seed} \times 100.$$

3.2.10 Data analysis

One-way analysis of variance; Two-way analysis of variance, Fisher's LSD multiple comparison test; t-test were used. All data was compiled in Microsoft Excel and all analyses were performed using Graphpad Prism 9. A $p < 0.05$ was considered as significant. Data on graphs are presented as mean $\pm$ SEM.

The one-phase association analysis by Prism, using the Non-lin regression fitted equation: $Y=Y_0 + (Plateau-Y_0) * (1-exp(-K*x))$. Where Y_0 is the Y value when X (time) is zero. It is expressed in the same units as Y, Plateau is the Y value at infinite times, expressed in the same units as Y. K is the rate constant, expressed in reciprocal of the X axis time units. If X is in minutes, then K is expressed in inverse minutes. Tau is the time constant, expressed in the same units as the X axis. It is computed as the reciprocal of K. Half-time is in the time units of the X axis. It is computed as $ln(2)/K$. Span is the difference between Y0 and Plateau, expressed in the same units as your Y values.

3.3 Results

3.3.1 Confirming the genotype of mutant and transgenic lines

3.3.1.1 Genotyping mutant *Atpip2;1* lines

Knockout mutant Atpip2;1-1

In order to detect the T-DNA insertion mutation in collections of lines where some material contained the mutation and other material was wild-type diagnostic PCRs were used to genotype mutant *Atpip2;1-1* lines. Primers 6AA F and 6AA R were used to detect wild type Col-0; while primers 6AA-En205 F and 6AA R were employed to detect *Atpip2;1-1*. As expected, the products in lanes 2 – 7 (Figure 3.6) had gDNA of mutant *Atpip2;1-1* as a template and no products were detected, whereas a band ~ 500 bp was amplified in lanes 9 – 14, suggested that the T-DNA insertion was present (Figure 3.6 A). In contrast, control Col-0 in lane 8 produced a band ~300 bp as expected, but not in lane 15 (Col-0) as the primers were specific for the mutant *Atpip2;1-1*.

This test was repeated in a second seed batch shown in Figure 3.6 B, lane 3 had a band at ~300 bp whereas lane 5 – 8 had products >500 bp. This confirmed the homozygous genotype of *Atpip2;1-1* material used for experiments to study germination and imbibition.

Additional information about the diagnostic primers and their expected products is listed in Table S3.
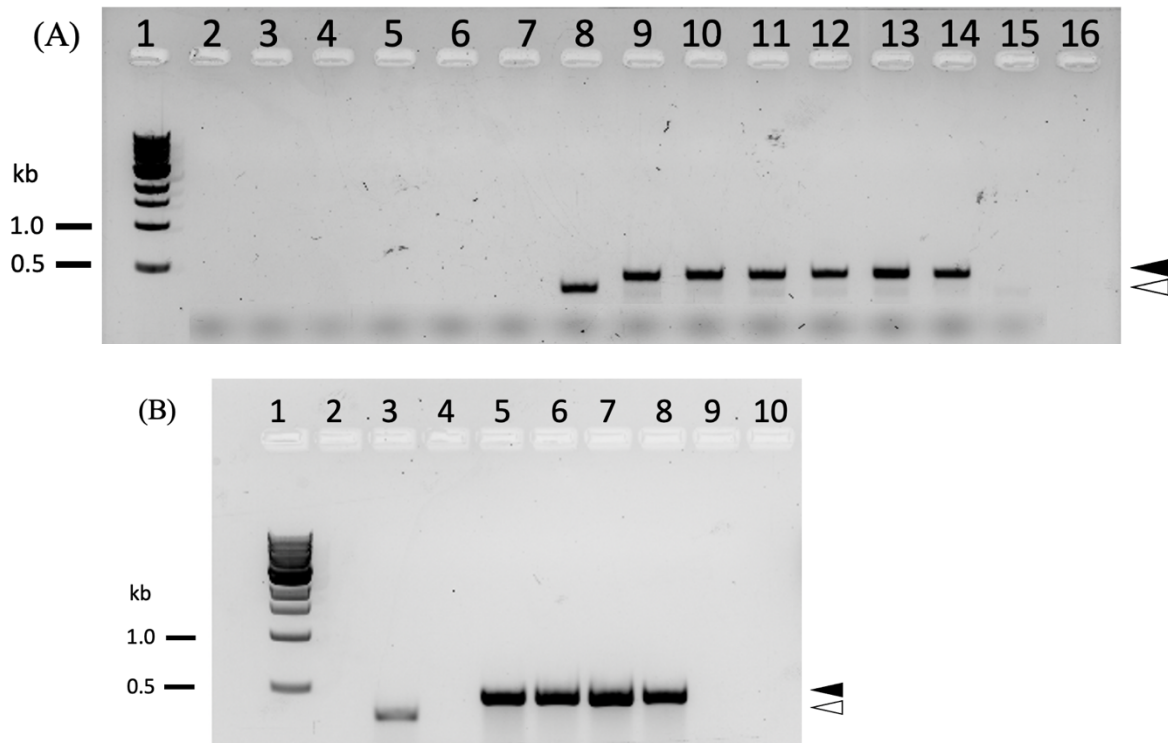
Figure 3.6 Testing for the presence of *Atpip2;1-1* in *Arabidopsis thaliana* candidate mutant lines.

(A) Lane 1 contained 3µl of 1Kb DNA ladder, lanes 2 – 7 and 9 – 14 contained PCR products from PCR where the template was DNA from *Atpip2;1-1* mutant plants; lanes 8 and 15 contain PCR product where Col-0 DNA template was used as a control; lane 16 was no-template control. PCR products in lane 2 – 8 were from reactions using primers 6AA F and 6AA R designed to amplify the native *PIP2;1* gene of WT Col-0 and heterozygous AtPIP2;1 T-DNA alleles, lanes 9 – 15 had primers 6AA-En205 F and 6AA R designed to amplify the T-DNA insertion of PIP2;1 knock-out. Black triangle indicates the expected size of the T-DNA fragment amplification for the *Atpip2;1-1* mutant. The open triangle indicates the expected size of the wild type *AtPIP2;1* product.

(B) Lane 1 contained 3µl of 1Kb DNA ladder, lanes 2, 10 had water used as template, lanes 3, 4 contained Col-0 templates, lanes 5 – 9 contained *Atpip2;1-1* templates. Lanes 3 and 9 used 6AA F and 6AA R primers, lanes 4 – 8 had primers 6AA-En205 F and 6AA R. Black triangle indicates the expected size of the T-DNA fragment amplification for the *Atpip2;1-1* mutant. Open triangle indicates the expected size of the wild type *AtPIP2;1* product.

Knockout mutant Atpip2;1-2

Diagnostic PCRs were used to genotype mutant *Atpip2;1-2* line: The mutant *Atpip2;1-2* plants were confirmed if they did not yield any PCR product when using primers SM35928 F and SM35928 R, whereas with the same reaction of wild type Col-0 amplified to products at around 900 bp. In contrast, the knockout mutant *Atpip2;1-2* had PCR products ~ 600 bp when using primers dSpm1 F and SM35928 R whereas Col-0 template did not get any band (Figure 3.7).
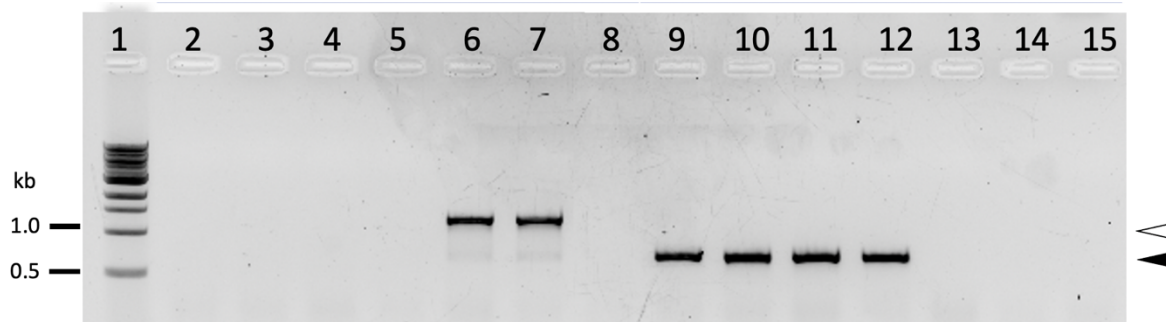
Figure 3.7: Genotyping *Atpip2;1-2 Arabidopsis thaliana* mutant line candidates in agarose electrophoresis gel.

Lane 1 was 3μl of 1 Kb ladder. Lanes 2 – 5 and 9 – 12 were *Atpip2;1-2* templates, lanes 6 – 7 and 13 – 14 contained Col-0 templates, lane 8 contained no-template control and lane 15 had water. Primers SM35928 F and SM35928 R were used in lanes 2 – 8 designed to amplify the native *PIP2;1* of WT Col-0 and heterozygous T-DNA alleles. Spm 32 F and SM35928 R primers were in lane 9 – 15 designed to amplify the PIP2;1 knock-out T-DNA insertion. Black triangle indicates the expected size of the T-DNA fragment amplification for the *Atpip2;1-2* mutant. The open triangle indicates the expected size of the *wild type AtPIP2;1* product.

Additional checks for *Atpip2;1-2* and *Atpip2;1-1* lines involved using the primer pair Spm 32 F and SM35928 R.

Diagnostic PCRs used to genotype mutant *Atpip2;1-2* and *Atpip2;1-2* lines: Lanes 3 – 5 had a band of ~600 bp, suggesting all *Atpip2;1-2* mutant lines had T-DNA successfully inserted. No band was amplified in Col-0 lanes 2 and 6, which were included as a negative control (no T-DNA insertion). Lanes 7 – 9 did not have products using Spm 32 F and SM35928 R primers because the primers are designed against *AtPIP2;1-1*'s gDNA as a template (Figure 3.8). Therefore, we confirmed *Atpip2;1-1* and *Atpip2;1-2* mutant insertions by confirming the presence of the T-DNA insertion fragments.
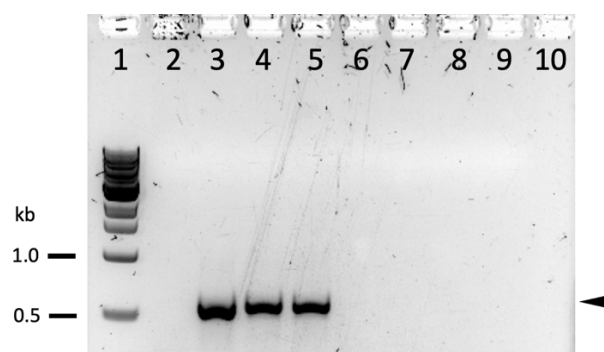
Figure 3.8: Testing genotypes for *Atpip2;1-1* and *Atpip2;1-2 Arabidopsis thaliana* mutant lines using Spm 32 F and SM35928 R primers.

Lane 1 was 3µl of 1 Kb ladder. Lanes 2 and 6 were Col-0 template and non-template controls, lanes 3 – 5 contained *Atpip2;1-2* templates, lanes 7 – 10 contained mutant *Atpip2;1-1* templates, lane 10 was water (no-template-control). Lanes 2 – 10 had products from PCRs using Spm 32 F and SM35928 R primers which specifically amplified the *Atpip2;1-2* mutant T-DNA insertion. Black triangle indicates the expected size of the T-DNA fragment amplification for the *Atpip2;1-2* mutant.

3.3.1.2 Genotyping overexpression 2x35S::AtPIP2;1 material using hygromycin selection

The number of seedlings with long hypocotyls and green cotyledons after growing on antibiotic media with 50 µg ml^{-1} hygromycin in ½ MS media was recorded. The percentage of transformed seedlings was calculated by determining the number of hygromycin B – resistant seedlings relative to total germinated seeds.

The seed of Col-0 and a null segregant line derived from segregation of 35S::AtPIP2;1 line 7 and 21 germinated on selective media and as expected the non-transgenic material had very short hypocotyls (Figure 3.9 A) whereas the overexpression lines were resistant to the hygromycin and had long hypocotyls after incubating in the dark for 2 days. The plants turned vigour green after 48 – 72 hours exposure to long-day conditions (Figure 3.9 B). The percentage of long hypocotyl seedlings is summarised in Table 3.7.
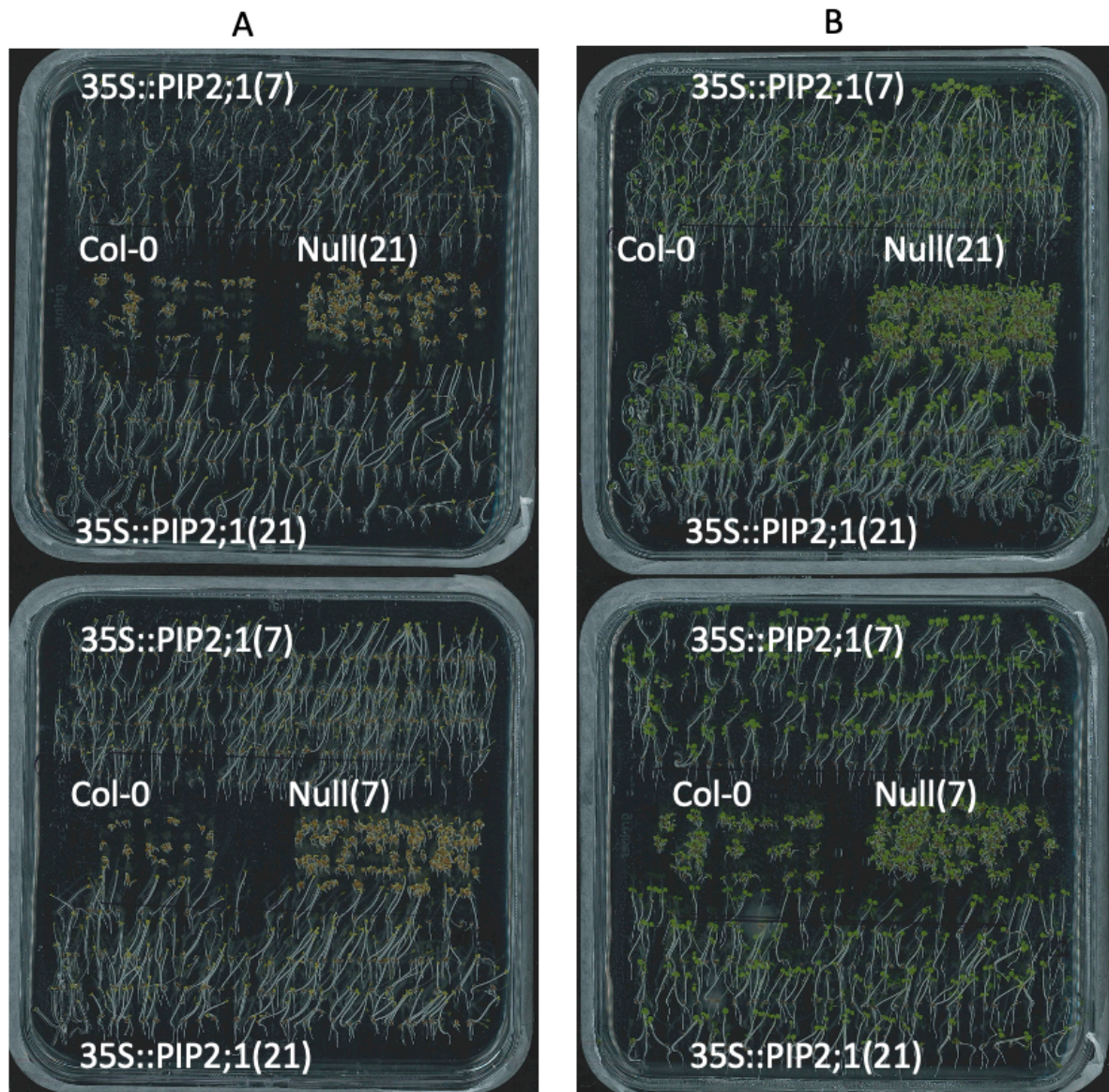
Figure 3.9: Seedling development of wild type Col-0, transgenic 2x35S::AtPIP2;1 overexpression lines and its null on hygromycin media. A, Plates were scanned after 2 days of incubation in dark conditions following stratification. B. Plates scanned at 5 days after stratification.

The hygromycin tests were conducted on multiple batches of seed (referred to as growth batch 25/10/2019 (HP10) and 06/02/2019 (HP15)), and these tests confirmed which seeds were from heterozygous T2 parents. The range of % confirmed transgenic plants based on the long hypocotyl test varied from 50% to 99%. The homozygous plant (Hom) is expected to have ~100% long hypocotyls, the heterozygous plant (Het) should have approximately 75% and the null transgenic material (Null of 2x35S::AtPIP2;1 was used as a control for physiological

experiments, null) had 0% of seedlings with long hypocotyls. The plants which had at least 95% hypocotyls were transferred to ½ MS media and soil pots to grow and harvest seeds for further experiments. Plants numbered 2, 13, 15-18 and 23 (Table 3.7) were grown until they produced mature seeds. A PCR test was also used to check the genotype of these plants.

From Table 3.7, there were some plants that had 100% seed with short hypocotyls such as plant number 31, 32 and 33. It is suggested that these plans were null of 35S::AtPIP2;1 lines and used them as control genotypes after running PCR to confirm their 'null' genotype.

Table 3.7: The percentage of seedlings that had long hypocotyls and green cotyledons after treatment with hygromycin and growth in dark conditions.

# Plant	Growth batch	Genotype, replication	Parent growth treatment	Percent of long hypocotyl seedlings (%)	Conclusion
1	6/2/19	2x35S::AtPIP2;1 OE_21-U4	Water	95	Hom
2	6/2/19	2x35S::AtPIP2;1 OE_7-U6	Water	95	Hom
3	6/2/19	2x35S::AtPIP2;1 OE_21-U2	Water	85	Het
4	6/2/19	2x35S::AtPIP2;1 OE_7-U1	Water	80	Het
5	6/2/19	2x35S::AtPIP2;1 OE_21-U3	Water	75	Het
6	6/2/19	2x35S::AtPIP2;1 OE_21-U5	Water	75	Het
7	6/2/19	2x35S::AtPIP2;1 OE_21-U6	Water	75	Het
8	6/2/19	2x35S::AtPIP2;1 OE_7-U5	Water	75	Het
9	6/2/19	2x35S::AtPIP2;1 OE_21-U1	Water	70	Het
10	6/2/19	2x35S::AtPIP2;1 OE_7-U3	Water	60	Het
11	6/2/19	2x35S::AtPIP2;1 OE_7-U4	Water	50	Het
12	6/2/19	2x35S::AtPIP2;1 OE_7-U2	Water	*0*	*Null*
13	25/10/19	2x35S::AtPIP2;1 OE_21-S1	5mM NaCl	99	Hom
14	25/10/19	2x35S::AtPIP2;1 OE_21-S2	5mM NaCl	99	Hom

15	25/10/19	2x35S::AtPIP2;1 OE_21-U2	Water	98	Hom
16	25/10/19	2x35S::AtPIP2;1 OE_21-S3	5mM NaCl	97	Hom
17	25/10/19	2x35S::AtPIP2;1 OE_21-S4	5mM NaCl	95	Hom
18	25/10/19	2x35S::AtPIP2;1 OE_21-U5	Water	95	Hom
19	25/10/19	2x35S::AtPIP2;1 OE_21-S5	5mM NaCl	90	Het
20	25/10/19	2x35S::AtPIP2;1 OE_21-U1	Water	90	Het
21	25/10/19	2x35S::AtPIP2;1 OE_21-U3	Water	85	Het
22	25/10/19	2x35S::AtPIP2;1 OE_21-U6	Water	85	Het
23	25/10/19	2x35S::AtPIP2;1 OE_7-S4	5mM NaCl	85	Hom
24	25/10/19	2x35S::AtPIP2;1 OE_7-S2	5mM NaCl	79	Het
25	25/10/19	2x35S::AtPIP2;1 OE_21-U4	Water	75	Het
26	25/10/19	2x35S::AtPIP2;1 OE_7-U2	Water	75	Het
27	25/10/19	2x35S::AtPIP2;1 OE_7-S3	5mM NaCl	65	Het
28	25/10/19	2x35S::AtPIP2;1 OE_7-U3	Water	65	Het
29	25/10/19	2x35S::AtPIP2;1 OE_7-S1	5mM NaCl	60	Het
30	25/10/19	2x35S::AtPIP2;1 OE_7-U4	Water	50	Het
31	25/10/19	2x35S::AtPIP2;1 OE_7-S5	5mM NaCl	*0*	*Null*
32	25/10/19	2x35S::AtPIP2;1 OE_7-U1	Water	*0*	*Null*
33	25/10/19	2x35S::AtPIP2;1 OE_7-U5	Water	*0*	*Null*

3.3.1.3 Genotype overexpression 2x35S::AtPIP2;1 by PCR tests to genotype transgenic lines and null lines

PCR on plants ranked as positive transgenics based on hygromycin selection media tests

Plants ranked as transgenic had long hypocotyls when grown in hygromycin selection media (Section 3.2.6.2) and their progeny were examined by diagnostic PCR with primers referred to as AtPIP2;1 In F and Nos-terminator R.

Towards ensuring that the genotypes of transgenic and null lines were confirmed the material which had been tested using growth on selective media was subjected to PCR tests. From the

gel image in Figure 3.10, clear bands were observed in lanes 3, 5 – 10, 12 and 14 with the expected length ~500 bp following PCR using AtPIP2;1 In F and Nos-terminator R primers whereas lane 2 had Col-0 (plant #16) template and no product detected. This revealed that the plants with the expected ~500bp product size were successful transgenic lines which could be used for later experiments.

Lane 4 contained the template of 35S::AtPIP2;1 line 7 (plant #12), but no product was amplified indicating this line was a null, and for this line the seedlings had short hypocotyls when grown in hygromycin media. This was selected to be a negative control null line for experiments to study germination.

Lanes 11 and 13 had template DNA from 35S::AtPIP2;1 line 21 (plant #7 and #25), which were seedlings with 75% long hypocotyls detected when grown in hygromycin media. However, there was no band detected in the PCR. From this result, we double checked the 35S insertion by running a second PCR with alternative primer sets to confirm the genotype of negative and positive plants.
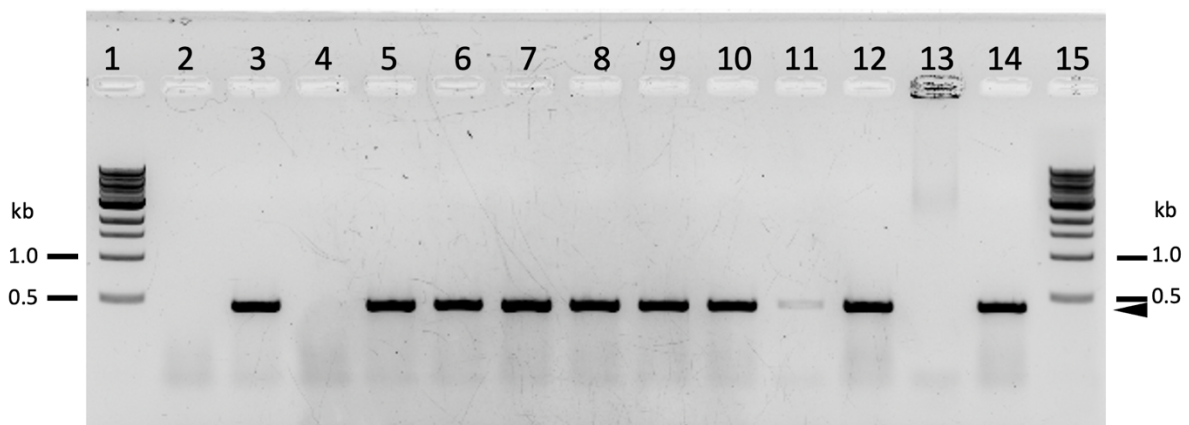
Figure 3.10: 2x35S::AtPIP2;1 overexpression (OE) lines 7 and 21 in *Arabidopsis thaliana* background tested using primers AtPIP2;1 In F and Nos-terminator R which designed to amplified 2x35S::PIP2;1 OE insertion.

Lane 1 was 3µl of 1 Kb ladder. Lanes 2 had Col-0 template, lanes 3 – 8 contained 2x35S::AtPIP2;1 OE (7) templates, lanes 9 – 14 contained 2x35S::AtPIP2;1 OE (21) templates. Black triangle indicates the size of the expected fragment from the 2x35S::AtPIP2;1 overexpression lines.

Re-confirming 35S::PIP2;1 transgenic and null lines by PCR using primers PMDC 32 2x35S F and Atpip2;1 CDS R

A second set of diagnostic primers was used to check the genotype of transgenic and null lines shown in Table 3.8. Lane 2, 4, 5 and 6 had expected bands ~ 900 bp in size whereas lanes 3, 7 and 8 did not have bands of this size (Figure 3.11). This confirmed the genotype of the transgenic and null material tested, as listed in Table 3.9.

Table 3.8: Follow up PCR using primers PMDC 32 2x35S F and *Atpip2;1* CDS R to check the genotype of transgenic and null lines.

#Lane in Figure 3.6	#Lane in Figure 3.5	gDNA (#tube)	+/- treatment	% long hypocotyl
1		Ladder 1Kb		
2	3	2x35S::AtPIP2;1 OE#7 U1 (#19)	+	95%
3	4	Null of 35S (7) (#12)	-	0%
4	11	2x35S::AtPIP2;1 OE#21 U3 (#7)	-	75%
5	12	2x35S::AtPIP2;1 OE#21 U4 (#28)	+	95%
6	13	2x35S::AtPIP2;1 OE#21 U5 (#25)	-	75%
7	2	Col-0 (#16)	-	0%
8		No-template control	-	
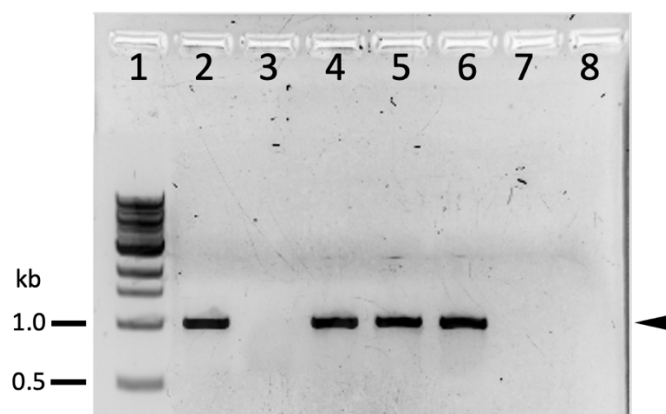
Figure 3.11: PCR product outcome for testing 35S::AtPIP2;1 line 7 and 21 *Arabidopsis thaliana* lines using primers PMDC 32 2x35S F and Atpip2;1 CDS R which were designed to amplify 2x35S::PIP2;1 OE insertion fragments. Black triangle indicates the size of the expected fragment from the 2x35S::AtPIP2;1 overexpression lines.

Table 3.9: Summary of hygromycin media test and PCR genotyping on 2x35S::AtPIP2;1 overexpression line material.

Lines	% long hypocotyl in hygromycin media	PCR Product 1	PCR Product 2	Conclusion
2x35S::AtPIP2;1_7-U1	80	+	+	+
2x35S::AtPIP2;1_7-U2	0	Negative	Negative	Negative
2x35S::AtPIP2;1_7-U3	60	+	n/a	+
2x35S::AtPIP2;1_7-U4	50	+	n/a	+
2x35S::AtPIP2;1_7-U5	75	+	n/a	+
2x35S::AtPIP2;1_7-U6	95	+	n/a	+
2x35S::AtPIP2;1_21-U1	70	+	n/a	+

2x35S::AtPIP2;1_21-U2	85	+	n/a	+
2x35S::AtPIP2;1_21-U3	75	Negative	+	+
2x35S::AtPIP2;1_21-U4	95	+	+	+
2x35S::AtPIP2;1_21-U5	75	Negative	+	+
2x35S::AtPIP2;1_21-U6	75	+	n/a	+

The material prepared for physiological tests was checked by three approaches to confirm the genotypes. This involved growth on selective media and two diagnostic PCRs and the results are summarised in Table 3.9. The null line for 35S plants, which were negative for the transgene in both PCR tests and had no seedlings with long hypocotyls when grown on hygromycin selection media were also progressed through the subsequent experiments as an appropriate control. The genotype of candidate transgenic plants as well as lines still ranked as having homozygous seed were confirmed. Seeds from confirmed homozygous and null plants were grown to maturity to bulk up the seeds, available for these lines. The next generation of plants were also confirmed in relation to their genotype by PCR using primers PMDC 32 2x35S F2 and Atpip2;1 CDS R2. An example is illustrated in Table 3.10 and Figure 3.12.

Table 3.10: The concentration of gDNA for PCR genotyping for 2x35S::AtPIP2;1 overexpression line progeny

#Lane	DNA Tube #	Genotype		Nanodrop ng/ul	Gel Band
1	3µl Ladder 1K (Biolab)				-
2	H20				-
3	G21-02	Col-0 – 2 – 4		47.41	-
4	G21-01	#21- 24 – 9		94.85	+
5	G21-04	#07- 34 – 2	2x35S::AtPIP2;1_7-U1	83.88	+
6	G21-05	#21- 09 – 3	2x35S::AtPIP2;1_21-S3	96.91	+
7	G21-07	#21- 09 – 2	2x35S::AtPIP2;1_21-S3	104.71	+
8	G21-08	#07- 14 – 7	2x35S::AtPIP2;1_7-U6	85.99	+
9	G21-09	#07- 14 – 6	2x35S::AtPIP2;1_7-U6	99.93	+

10	G21-10	#07- 14 – 8	2x35S::AtPIP2;1_7-U6	155.06	+
11	G21-12	Col-0 (2) – 3		86.15	-
12	G21-11	#07- 14 – 9	2x35S::AtPIP2;1_7-U6	82.51	+
13	G21-13	#07- 34 – 1	2x35S::AtPIP2;1_7-U1	141.58	+
14	G21-15	#21- 09 – 1	2x35S::AtPIP2;1_21-S3	109.29	+
15	G21-16	#21- 24 – 6	2x35S::AtPIP2;1_21-U2	94.88	+
16	G21-17	#21- 24 – 7	2x35S::AtPIP2;1_21-U2	120.82	+
17	G21-18	#21- 24 – 8	2x35S::AtPIP2;1_21-U2	115.32	+
18	G21-19	#21- 24 – 1	2x35S::AtPIP2;1_21-U2	132	+
19	G21-20	#21- 24 – 2	2x35S::AtPIP2;1_21-U2	87.68	+
20	G21-21	#21- 24 – 3	2x35S::AtPIP2;1_21-U2	123.16	+
21	3µl Ladder 1K (Biolab)				-
22		H20			-
23	G21-03	Col-0 (2) 1-5		85.38	-
24	G21-22	#21- 24 – 4	2x35S::AtPIP2;1_21-U2	139.34	+
25	G21-23	#21- 24 – 5	2x35S::AtPIP2;1_21-U2	105.85	+
26	G21-24	#21- 03 – 1	2x35S::AtPIP2;1_21-S1	130.05	+
27	G21-25	#21- 03 – 2	2x35S::AtPIP2;1_21-S1	145.15	+
28	G21-26	#21- 03 – 3	2x35S::AtPIP2;1_21-S1	177.72	+
29	G21-27	#21- 03 – 4	2x35S::AtPIP2;1_21-S1	171.55	+
30	G21-28	#07- 14 – 1	2x35S::AtPIP2;1_7-U6	146.25	+
31	G21-06	Col-0 (2) – 6		89.15	-
32	G21-29	#07- 14 – 2	2x35S::AtPIP2;1_7-U6	155.03	+
33	G21-30	#07- 14 – 3	2x35S::AtPIP2;1_7-U6	190.44	+
34	G21-31	#07- 14 – 4	2x35S::AtPIP2;1_7-U6	143.74	+
35	G21-32	#07- 14 – 5	2x35S::AtPIP2;1_7-U6	91.86	+
36	G21-33	#07- 10 – 1	2x35S::AtPIP2;1_7-S4	70.26	+
37	G21-34	#07- 10 – 2	2x35S::AtPIP2;1_7-S4	64.57	+
38	G21-35	#07- 10 – 3	2x35S::AtPIP2;1_7-S4	72.85	+
39	G21-36	#07- 10 – 4	2x35S::AtPIP2;1_7-S4	103.81	+
40	G21-14	Col-0 – 2 – 5		229.22	-
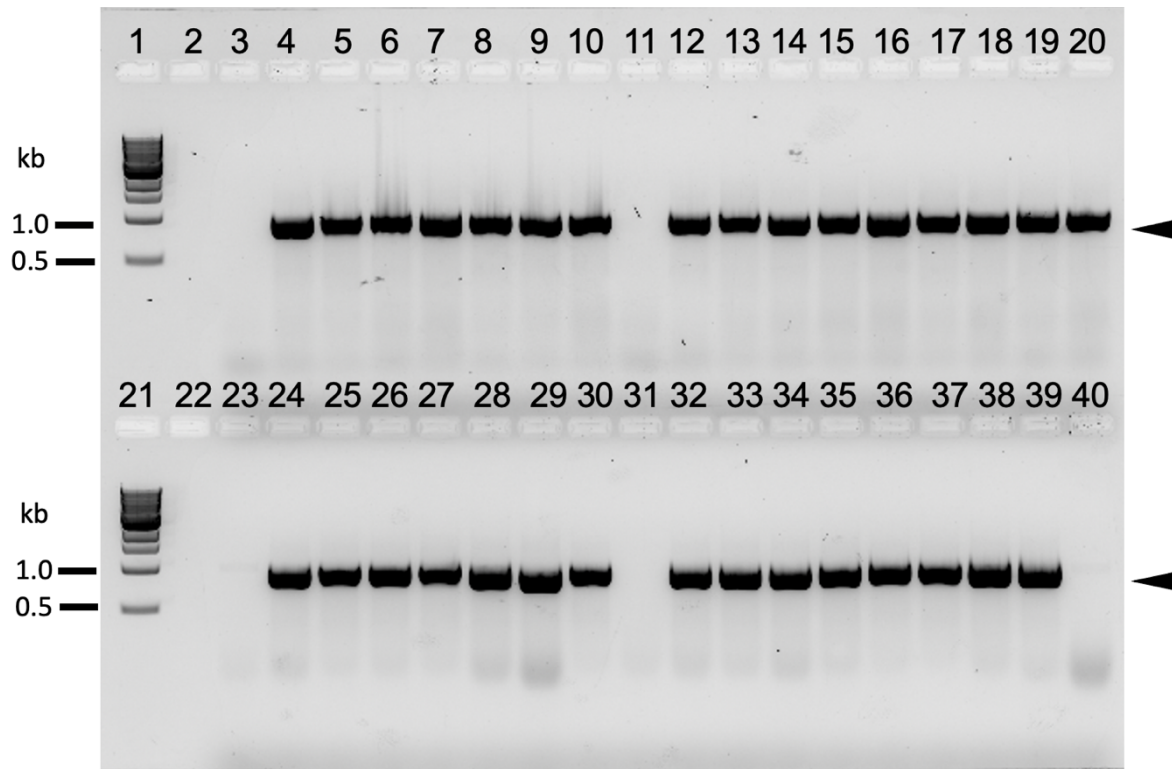
Figure 3.12: Testing the genotype of 35S::AtPIP2;1 lines 7 and 21 *Arabidopsis thaliana* lines using primers PMDC 32 2x35S F and Atpip2;1 CDS R which were designed to amplify 2x35S::PIP2;1 OE insertion fragments before collecting seed for subsequent experiments. Black triangle indicates the size of the expected fragment from the 2x35S::AtPIP2;1 overexpression lines.

Phire Hot Direct and Gradient PCR tests to genotype seeds

To rapidly confirm the genotype of seed from 2x35S::AtPIP2;1 OE lines and define the proper amplification cycles and temperature for annealing primers, PCR was performed using Phire Hot Direct PCR and gradient PCR using seeds from the null plants of the 2x35S::AtPIP2;1 lines and seed of confirmed transgenic 2x35S::AtPIP2;1 OE lines.

Figure 3.13: Electrophoresis of overexpression 2x35S::AtPIP2;1 OE PCR products generated from DNA extracted directly from seed in order to confirm null and Col-0 material.

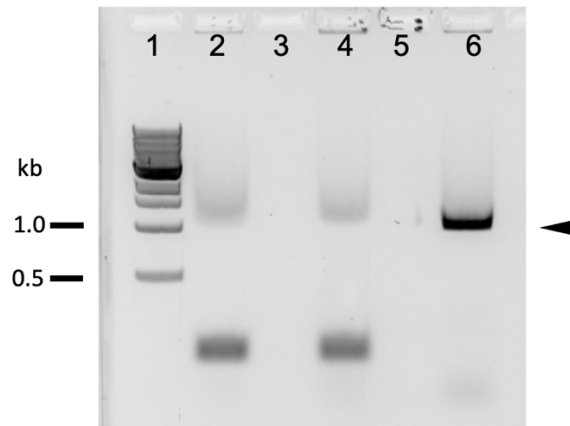Lane 1 contained 1Kb ladder. Lane 2, 3, 4, 6 was gDNA of Null 2x35S::AtPIP2;1, no-template control, Col-0 and 2x35S::AtPIP2;1 respectively. Primers were PMDC 32 2x35S F and Atpip2;1 CDS R which were designed to amplify 2x35S::PIP2;1 OE insertion fragments. Black triangle indicates the size of the expected fragment from the 2x35S::AtPIP2;1 overexpression lines.

As expected, the materials that had been identified as being nulls and the wild-type Col-0 plants exhibited smeared bands of different sizes to the single band identifying the overexpression line. The overexpression 2x35S::AtPIP2;1 lines had a band at ~900 kb (Figure 3.13). This method helped to confirm the genotype, however there was the potential for problems in terms of the purity of gDNA and the temperature could have been adjusted to optimise the amplification. The outcome was deemed sufficiently clear given the many earlier tests confirming the genotype of this material.

3.3.1.4 Genotyping UBI:nGFP::PIP2;1 plant material

Selection of transgenic plants in soil using BASTA spray

BASTA spray treatment of control and UBI:nGFP::PIP2;1 seedlings killed the non-transgenic seedlings but not the transgenic seedlings. After two spray treatments at 27 days after sowing, some seedlings turned yellowish and stopped growing. They continued to dry out and died around 34 days after sowing, at the stage of the third spray (Figure 3.14). Those plants that died were considered non-transgenic individuals and parents could be heterozygous.

Plants that had over 95% of their seedlings survive after the three sprayings were possible homozygous lines.
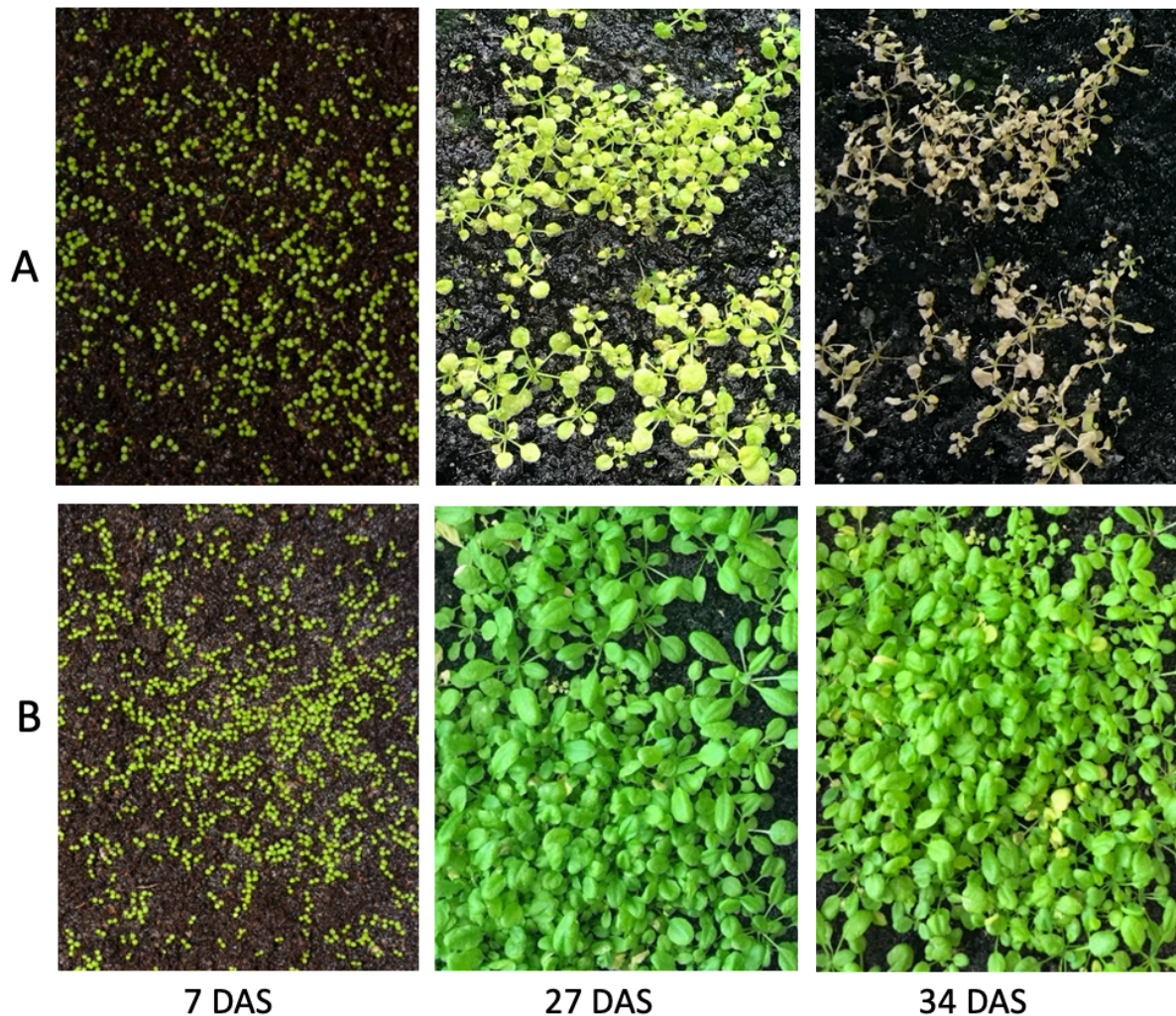
Figure 3.14: Seedlings of UBI:nGFP::PIP2;1 plants at 7, 27 and 34 days after sowing (DAS) with BASTA treatment applied on 21, 24 and 27 DAS. (A) Not-transgenic plants. (B) Positive UBI:nGFP::PIP2;1 material.

Confocal microscope scanning to check the genotype of transgenic lines

To confirm the expression of the transgene in the UBI:nGFP::PIP2;1 overexpress line, seedlings at about 7 – 10 days old were investigated using a confocal microscope, with wave lengths of 480 nm and 500 – 520 nm, used for excitation and emission respectively. Seedlings displaying strong GFP fluorescence were clearly distinguished from those with background autofluorescence alone. The GFP was detected in root tips at the meristem zone and in the mature zone in the endodermis and stele (Figure 3.15). For the wild-type Col-0 genotype, a

light green autofluorescence in the root can be seen, but the expression was very weak, a faint yellow colour and not easily detected in the root caps or stele (Figure 3.15 a – c).

Seedlings with obvious expression of GFP were returned to Petri dishes and grown for a further 2 weeks in a long-day growth room and then transferred to soil for growing, until they produced seeds.
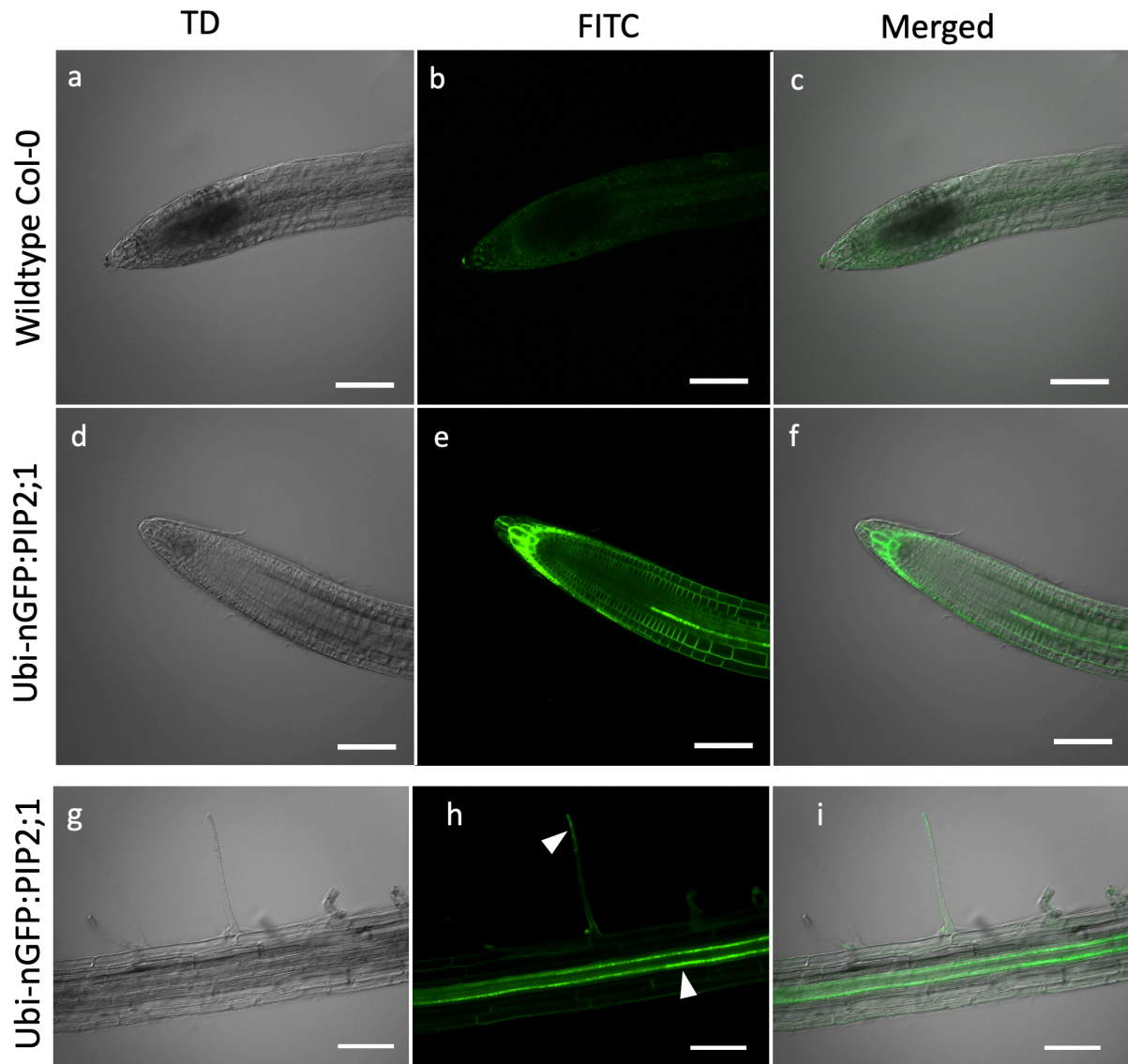
Figure 3.15: Root tips from seedlings of wild type Col-0 (a-c) displayed some background autofluorescence, but limited fluorescence compared to UBI:nGFP::PIP2;1 material in root tips (d-f) and the mature root zone of UBI:nGFP::PIP2;1(g-i). Scale bars = 50 µm.

Confirming the genotype of UBI:nGFP::PIP2;1 material by Polymerase Chain Reaction

Genomic DNA samples from plants which survived from BASTA spray and those that had passed the green fluorescence scanning test using the confocal microscope were extracted and used for PCR using forward primer Ubi:64p f and reverse primer AtPIP2;1 CDS r. The gel images revealing the PCR results and the list of all plants are summarised in Figure 3.16 and Table 3.11 respectively. From the result of the genotyping tests, it was possible to conclude that there were some plants that survived the BASTA tests even though the transgene insertion might not have been successful, such as plants in lane 25 and 27. In contrast most of the confocal scanned plants which were clearly expressing GFP also produced an expected diagnostic band >1500 bp confirming the genotype. Plants ranked as positive for the transgene in both selection tests and PCR tests, UBI:GFP::PIP2;1 lines 7 and 18 were chosen for subsequent experiments to study the germination of later generations of seed.

Figure 3.16: Testing UBI:nGFP::PIP2;1 *Arabidopsis thaliana* material (A-B) by primers Ubi:64p f and AtPIP2;1 CDS r to amplify nGFP tagged PIP2;1 insertions and c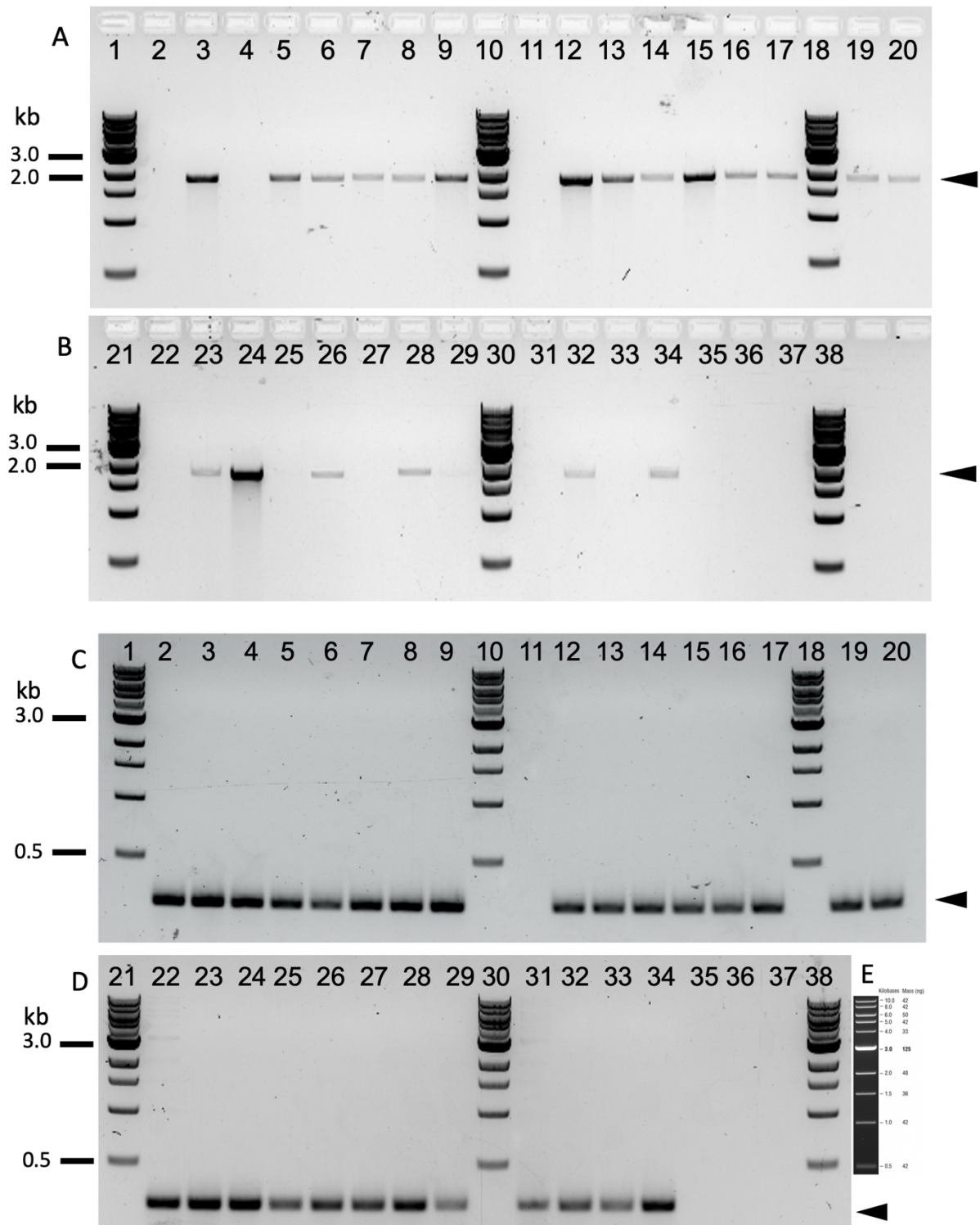hecking the DNA quality by amplifying the housekeeping gene Actin2 in the same samples (C-D) by primers Actin 2 F and Actin 2 R. Black triangle indicates

the size of the expected fragment from the 2x35S::AtPIP2;1 overexpression lines.
(E) Ladder 1 Kb scale.

Table 3.11: Summary of the genotyping analysis for the overexpression UBI:GFP::PIP2;1 material.

Lane	Genotype (#line) - #pot	Treatments	PCR band (Yes or No)
1	Ladder 1kb		
2	Col–0 - 2	PCR	No
3	UBI:nGFP::PIP2;1 (7) - 4	Fluorescence	Yes
4	UBI:nGFP::PIP2;1 (7) - 6	Fluorescence	No
5	UBI:nGFP::PIP2;1 (7) - 2	Randomly selected from plate for checking	Yes
6	UBI:nGFP::PIP2;1 (9) - 1	Fluorescence	Yes
7	UBI:nGFP::PIP2;1 (6) - 1	Fluorescence	Yes
8	UBI:nGFP::PIP2;1 (9) - 2	Fluorescence	Yes
9	UBI:nGFP::PIP2;1 (7) - 5	Fluorescence	Yes
10	Ladder 1kb		
11	No gDNA (H$_2$O)		No
12	UBI:nGFP::PIP2;1 (7) - 1	Fluorescence	Yes
13	UBI:nGFP::PIP2;1 (8) - 1	Fluorescence	Yes
14	UBI:nGFP::PIP2;1 (6) - 2	Fluorescence	Yes
15	UBI:nGFP::PIP2;1 (7) - 4	Fluorescence	Yes
16	UBI:nGFP::PIP2;1 (7) - 3	Fluorescence	Yes
17	UBI:nGFP::PIP2;1 (8) - 2	Fluorescence	Yes
18	Ladder 1kb		
19	UBI:nGFP::PIP2;1 (8) - 3	Fluorescence	Yes
20	UBI:nGFP::PIP2;1 (6) - 3	Fluorescence	Yes
21	Ladder		
22	Col-0 - 5	PCR	No
23	UBI:nGFP::PIP2;1 (9) - 3	Fluorescence	Yes
24	UBI:nGFP::PIP2;1 (7) - 4	BASTA	Yes
25	UBI:nGFP::PIP2;1 (7) - 3	BASTA	No
26	UBI:nGFP::PIP2;1 (7) - 1	BASTA	Yes
27	UBI:nGFP::PIP2;1 (2) - 1	BASTA	No
28	UBI:nGFP::PIP2;1 (18) - 3	BASTA	Yes
29	UBI:nGFP::PIP2;1 (18) - 4	BASTA	Yes
30	Ladder 1kb		

31	Col-0 – 1	PCR	No
32	UBI:nGFP::PIP2;1 (2) - 3	BASTA	Yes
33	UBI:nGFP::PIP2;1 (7) - 13	Randomly selected from plate for checking	No
34	UBI:nGFP::PIP2;1 (18) - 2	BASTA	Yes
35	No gDNA (H$_2$O)		No
36	Empty		
37	Empty		
38	Ladder 1kb		

3.3.2 Mucilage extrusion from seed coats

As soon as Arabidopsis seed makes contact with water, it starts to release a viscous mucilage layer that surrounds the seed coat. It is thought that mucilage may help dispersal, germination and adaptation to stress (Tsai *et al.*, 2021; Witztum *et al.*, 1969; Young and Evans, 1973). Hypothetically mucilage could influence germination, hence it was important to check if the *Atpip2;1* mutants of interest differed in mucilage extrusion relative to Col-0 (Chen *et al.*, 2018). The mucilage layer can be stained (pink) using a ruthenium red solution and the shape of the mucilage can reveal if there are differences in mucilage extrusion (Figure 3.17). This cloud shape of reddish-pink ruthenium red stained mucilage that was reported to have an acidic and polysaccharide composition consisting of pectin, cellulose, homogalacturonan, arabinans, and galactans (Arsovski *et al.*, 2009; Zhao *et al.*, 2017). The structure of extruded seed mucilage in all genotypes comprised two distinct layers: a cloud-like outer layer and an inner unstained layer (Figure 3.17) (Phan *et al.*, 2016; Zhao *et al.*, 2017). Therefore, seed needs to be shaken and hydrated in 50 mM EDTA for staining. There was no significant difference apparent in the outer and inner layers of *Atpip2;1* mutants relative to wild type seed.
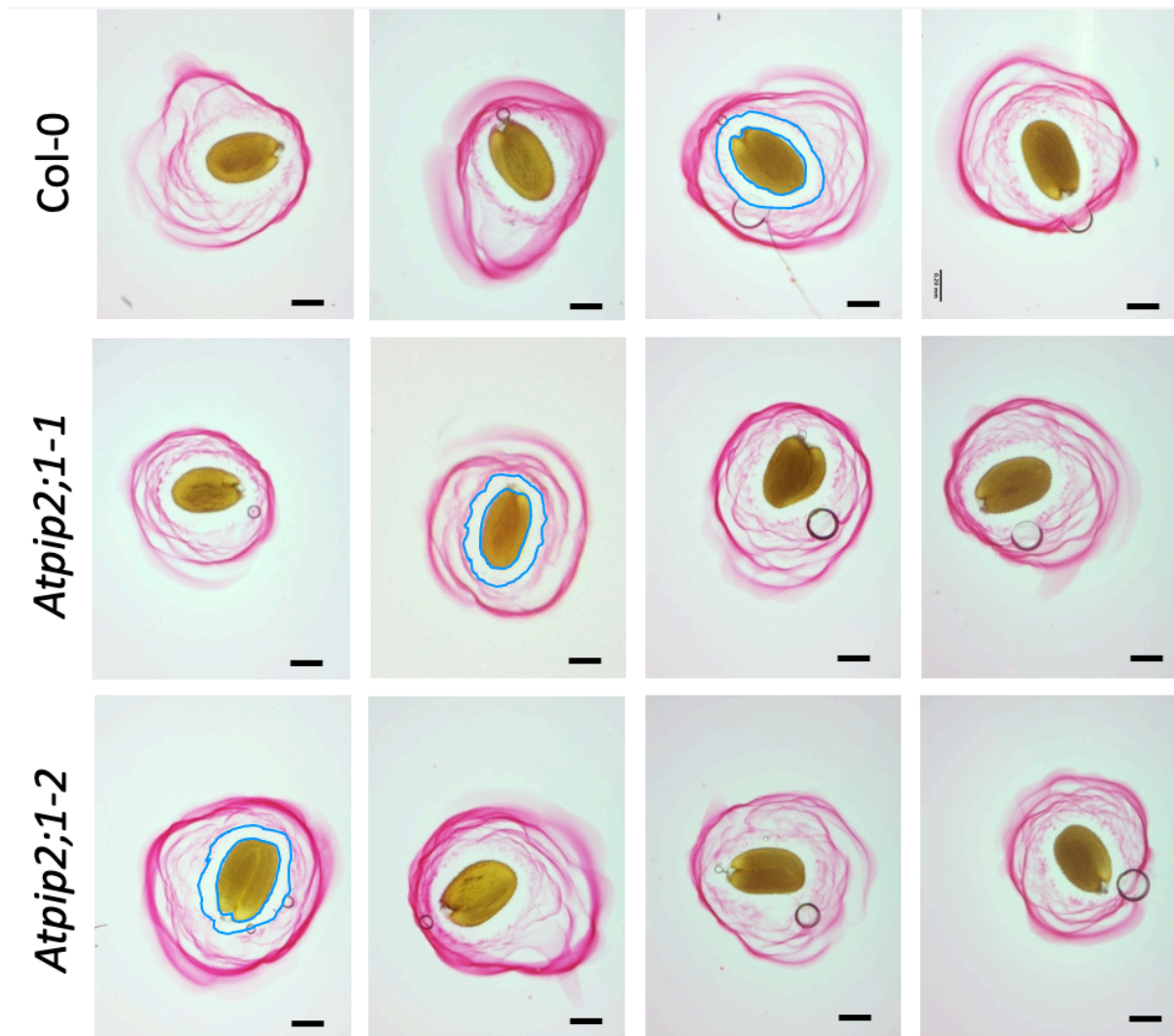
Figure 3.17: Images of mucilage layers for wild-type Col-0, *Atpip2;1-1* and *Atpip2;1-2* seeds at 15 minutes following ruthenium red solution staining. Scale bars = 20 μm. Blue outline marks the inner layers of mucilage on examples from each genotype. Bars = 20 μm.

To further investigate the seed coat and check for differences in the density of inner mucilage layer structures, seeds of wild-type Col-0, *Atpip2;1-1, Atpip2;1-2* and *Athkt1* mutants were pre-hydrated in 50 mM EDTA 2 hours before stained in ruthenium red solution; this solution is used to extract the adherent mucilage from seed (Zhao *et al.*, 2017); see Figure 3.18. The HKT1 loss-of-function mutants were examined because they were used as controls in later germination tests. Seed was shaken using an orbital shaker at 400 rpm in both solutions because the shaking process tended to break apart the outer layer and let the ruthenium red dye penetrate the thick inner pectin layer which can be seen as a pink stained capsule of mucilage in Figure

3.18, where the area is highlighted using an overlay of two blue circles. Seed images were captured under a stereo microscope.

Col-0 *Atpip2;1-1* *Atpip2;1-2* *Athkt1*

Figure 3.18: Ruthenium red staining of wild-type Col-0, *Atpip2;1-1, Atpip2;1-2* and *Athkt1* seeds. Seeds were shaken at 400 revolutions per minutes in 50 mM EDTA for 2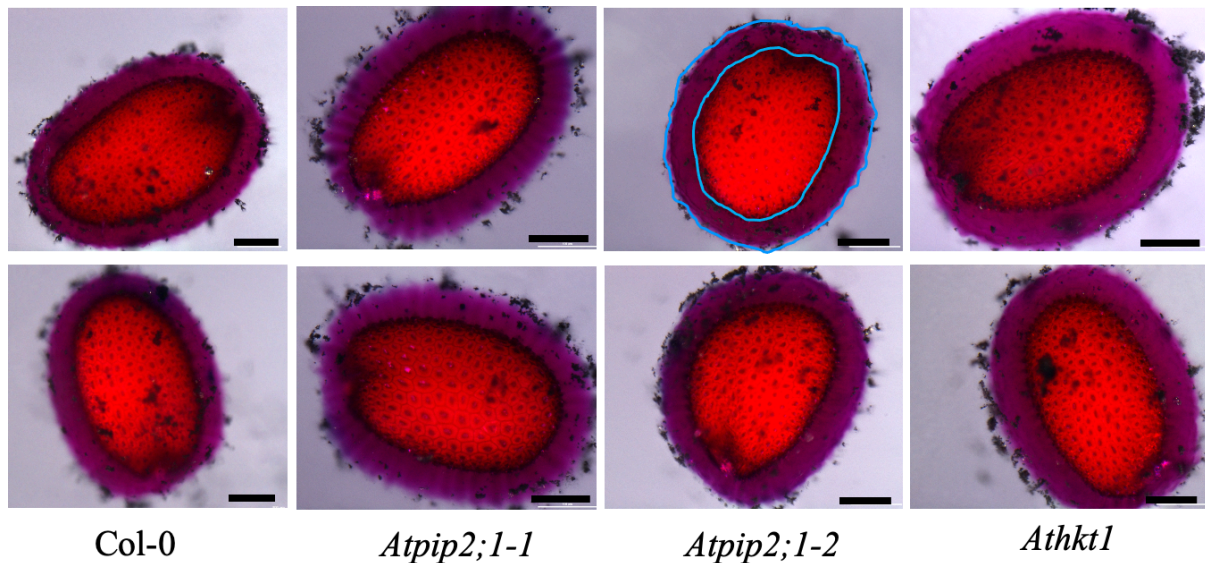 hours and then shaken whilst being stained with ruthenium red for 1 hour and then imaged. Blue outline marks the inner layers of mucilage on one *Atpip2;1-2* image as an example. Scale bars = 100 μm.

The appearances of mucilage inner layers for the genotypes tested were similar. Each seed has a thick dark reddish-pink gel capsule, fully covering the seed coat. This layer strongly adhered to the seed coat and would have been formed during seed coat differentiation in epidermal cells (Haughn and Western, 2012). In the Arabidopsis seed coat, the seed coat mucilage secretory cells are scattered across the surface of the seed coat (Phan *et al.*, 2016; Western *et al.*, 2000; Zhao *et al.*, 2017). These cells contain large quantities of pectinaceous mucilage in the upper tangential corners of the cell. The mucilage is secreted from a volcano-shaped cytoplasm pocket when the seed is hydrated (Arsovski *et al.*, 2009). No difference in the mucilage secretion and structure could be detected between these genotypes; hence any differential effect of mucilage in relation to seed imbibition or germination would not be expected.

3.3.3 UBI:nGFP::PIP2;1 fluorescence in imbibing seed

From 30 hours after sowing, the expression of UBI:nGFP::PIP2;1 was obvious from the GFP fluorescence in radicle and the seed embryo. Fluorescence of GFP could be observed in the

embryo at 48 hours after sowing (HAS), in the hypocotyl outer cells and in the cotyledons (Figure 3.19).
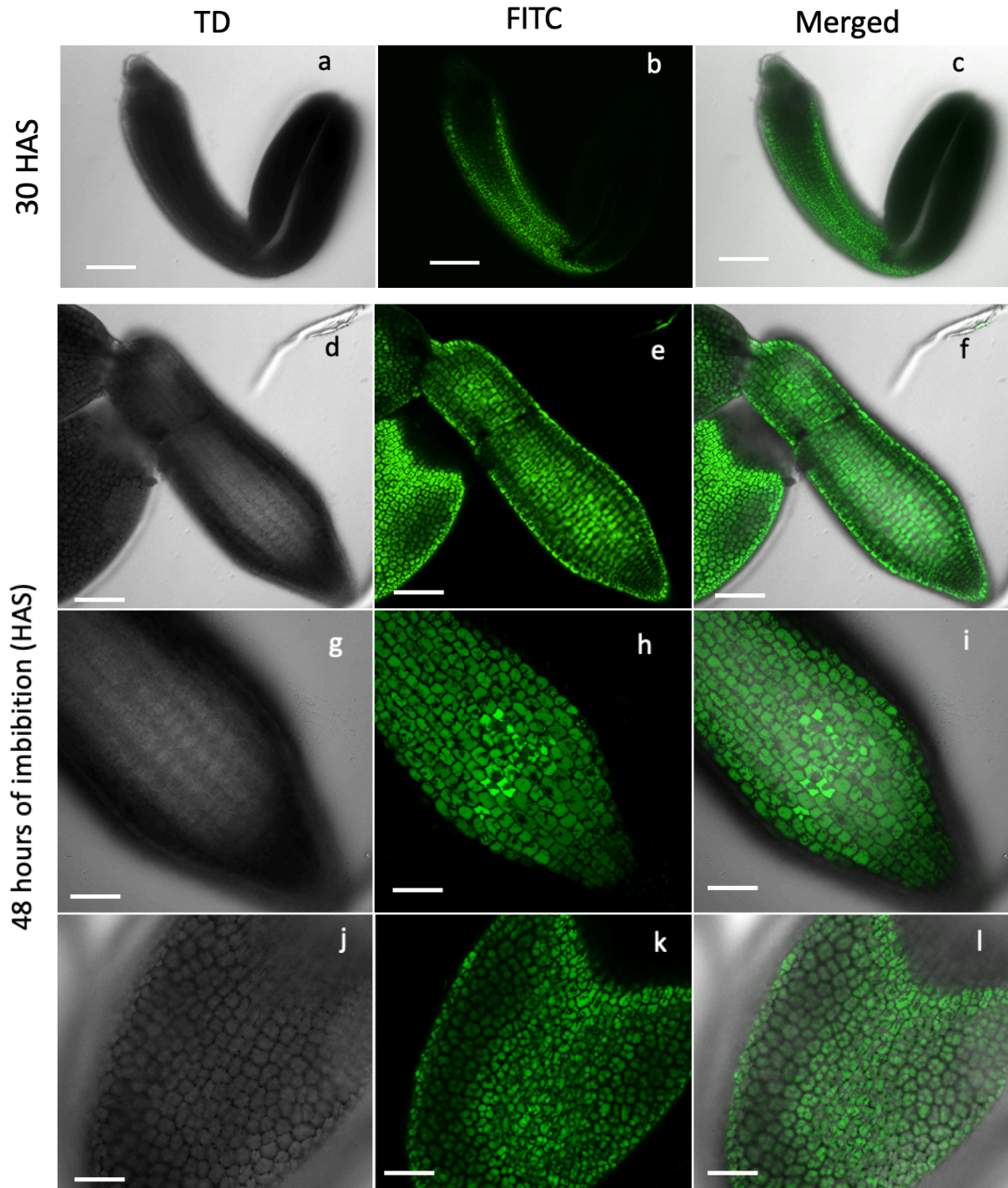
Figure 3.19: Investigating the expression of nGFP::PIP2;1 in embryos. Images were captured under Transmitted Detector (TD), FITC (488nm) and merged channels from 30 hours after sowing (HAS) (Panels–a - c) and 48 hours after sowing (Panel d – l). Scale bars = 50 µm.

At 72 HAS, over 50% of seeds from the transgenic lines had germinated. In the root, GFP fluorescence was observed in epidermal and root tip cells (Figure 3.20 A) and GFP fluorescence was observed in the cotyledons (Figure 3.20 B). Radicles and cotyledons protruded out of the seed coat. Autofluorescence from tissue caused a background green seedling colour. This is clearly seen in Figure 3.20 C where meristem fluorescence is shown in a purple colour in Cy5 channel and guard cells are evident. At 5 - 7 days after sowing, UBI:nGFP::PIP2;1 was observed clearly in roots, particularly in the root tips and the stele (Figure 3.20 D), consistent with previous reports analyzing transcript data that revealed high *Atpip2;1* expression in root tips and stele tissues (McGaughey *et al.*, 2018).
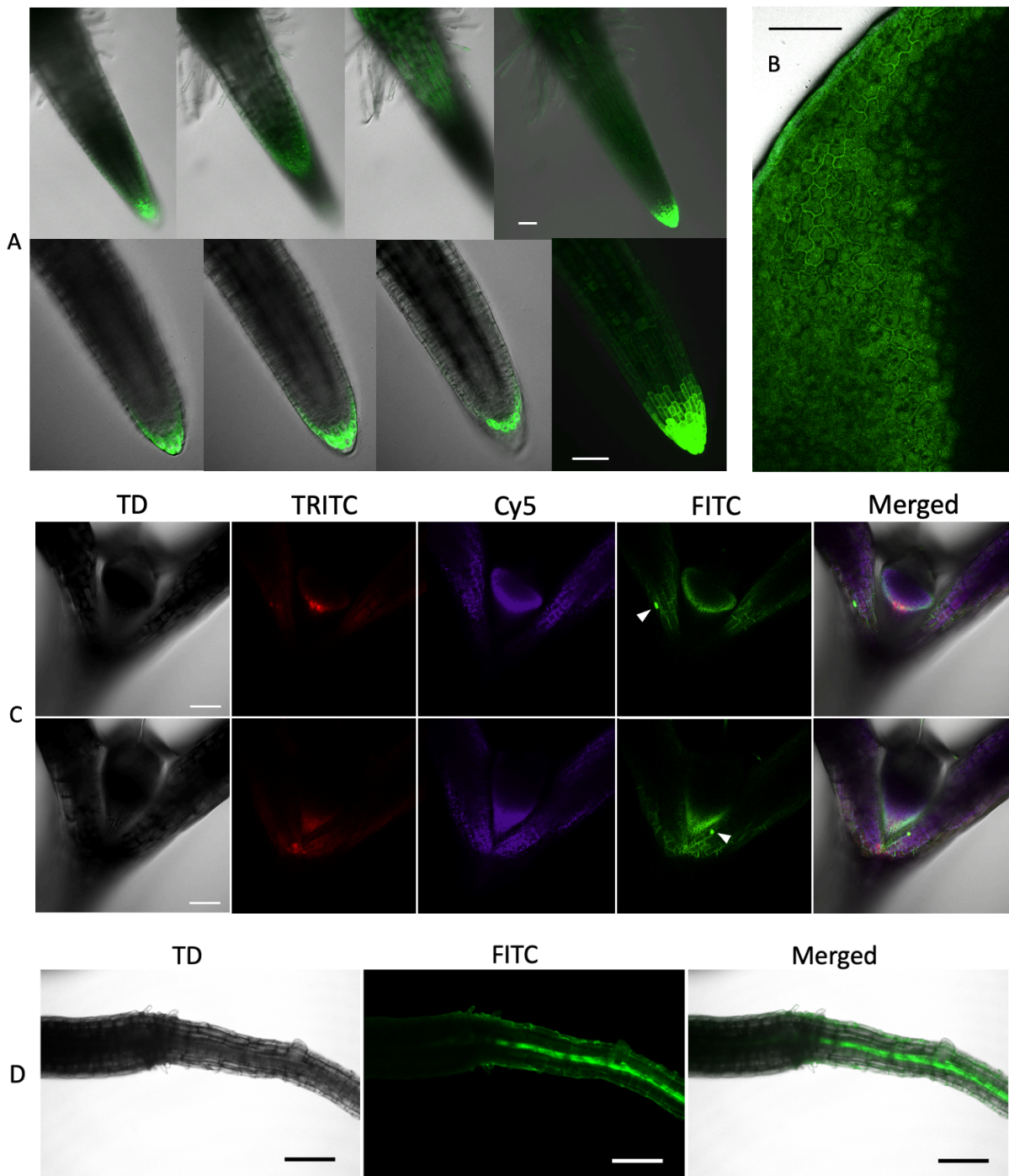
Figure 3.20: Assessing the expression of UBI:nGFP::PIP2;1 in imbibing seeds from 60 hours to 7 days after sowing. A. Four root tip Z-series examples. B. Four cotyledon Z-series examples; Scale bars = 50 µm at 60 hours imbibition. C. Shoot apical meristem at 72 hours imbibition under TD, TRITC (561nm), Cys 5 (640 nm), FITC (488nm) and merged channels. White arrows point to stomata. Scale bars = 100µm. D. Root stele and epidermis under TD, FITC and merged channels of 7 days old seedling. Scale bars = 100µm.

3.3.4 Assessing seed germination percentage

3.3.4.1 Preliminary trials to explore germination trends in two independent *Atpip2;1* mutant lines and Col-0

In preliminary tests of germination for AtPIP2;1 loss of function mutant lines, it was unclear if the germination percentage differed to that of wild type when imbibed in control or saline media, as there was high variability between replicates (Figure 3.21). For example, for mutant *Atpip2;1* seed from plants grown in water treatments (water grown) and germinated in non-saline treatments (½ MS), the germination percentage varied from 72 – 97% germination success at three days after sowing (DAS) (Figure 3.21 A).

In these preliminary tests, there were six independent biological replications for each genotype (meaning seed from six independent parent plants for each line), each replicate included ~100 seeds. There were two growing treatments applied for parents: Water grown (grown in soil moistened with water) and Saline grown (grown in soil with water water and NaCl at 5 mM NaCl) and seeds germinated in two media treatment ½ MS and ½ MS + 50 mM NaCl.

The germination percentage of seeds from saline treated parents appeared to be reduced relative to that of control parents at 3 DAS; and the germination percentage of *Atpip2;1-2* seed placed in saline media was lower than that of the same seed placed in control media at 3 DAS.

The seed from *Atpip2;1-2* knock out mutants grown in water treatments had higher germination percentage in non-saline media at 3 DAS relative to the seed from this line from saline treated parents and seed from this line germinated in saline treatments (Figure 3.21 A).

At nine DAS, all genotypes achieved a relatively high germination percentage under control treatment, ½ MS media, and there appeared to be more variation in germination percentage observed in saline treatments relative to non-saline treatments.

Seed of Col-0 grown in water treatment and seed germinated in water had higher germination percentage than that from plants grown in saline treatment (Figure 3.21 B).
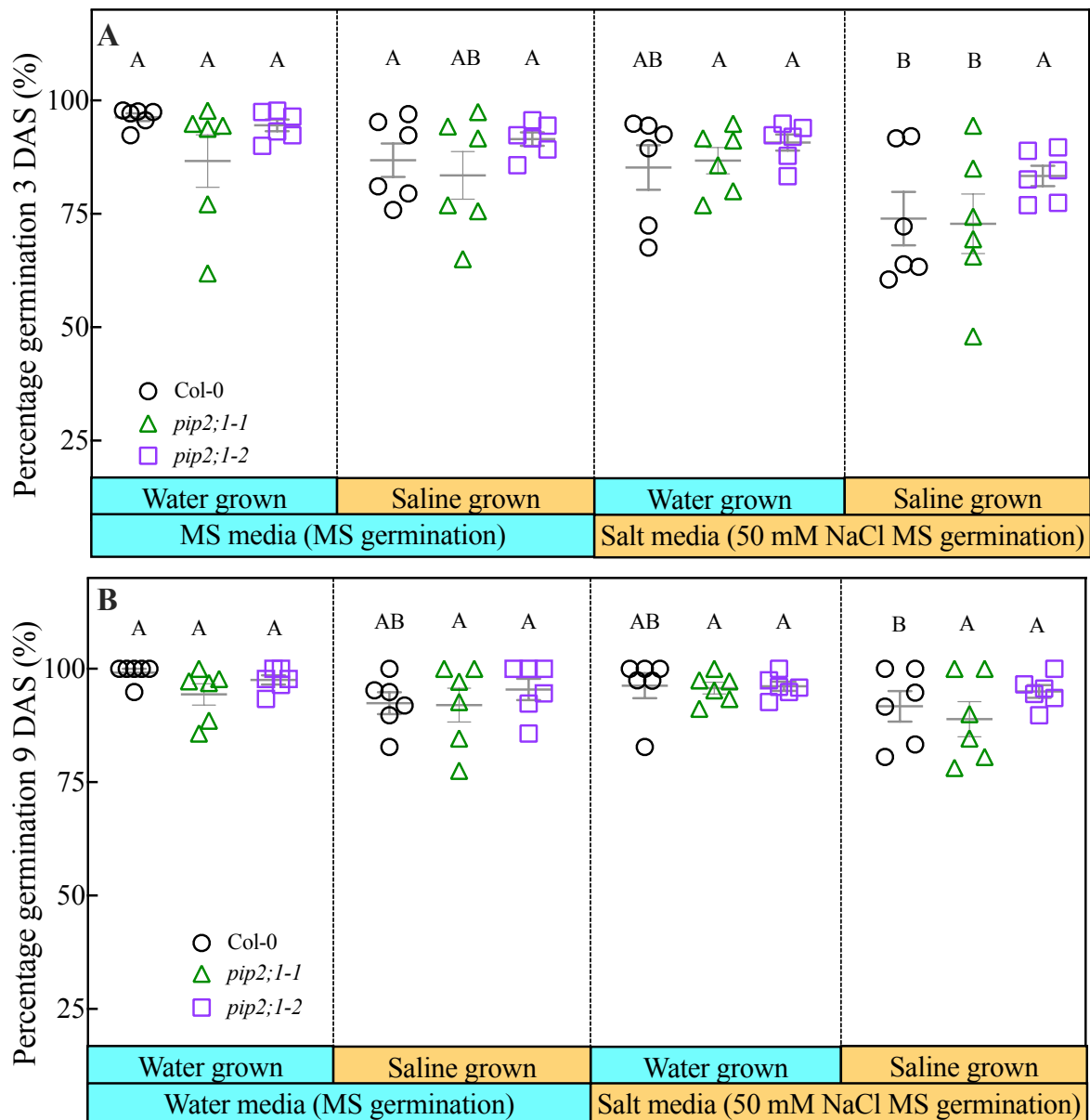
Figure 3.21: Evaluation of successful seed germination in wild type Col-0, mutants *Atpip2;1-1* and *Atpip2;1-2* under water treatment and 50 mM NaCl treatment. **A – B,** Quantification and statistical analysis of germination percentage of seed from three genotypes of parents grown in two treatments: water grown parent (water) and salt grown parent (water and NaCl at 5 mM NaCl) at 3 DAS (A) and nine DAS (B). Analysis by two-way ANOVA and LSD post-tests. Data represents mean ± SEM with n = 6 independent plants (replications) and 150 – 300 seeds from each plant.

3.3.4.2 Assessing the germination percentage of *Atpip2;1* loss of function mutants and AtPIP2;1 overexpression lines

Germination success over time was assessed for both knock-out mutant and overexpression lines. The overexpression lines included the UBI:nGFP::PIP2;1 lines and 2x35S::AtPIP2;1 lines. In this experiment, material was a mixture of heterozygous and homozygous lines as the final genotyping had not been completed at this stage, and asterisks have been added for those lines that are heterozygous (*).

Mutant lines lacking a major Na^+ transporter (AtHKT1) (Davenport *et al.*, 2007) were used as a comparison because PIP2;1 has also been shown to transport Na^+ (Qiu *et al.*, 2020). Therefore, comparing mutants lacking PIP2;1 with mutants lacking a gene that is well known to function as a Na^+ transporter (HKT1) may be a good comparison. The purpose was assessment of whether there was any similarities in phenotypes while *Athkt1* mutants were found to accumulate Na^+ in floral tissues (An *et al.*, 2017). At 3 DAS, the percentage seed germination of *Athkt1* (lacking the HKT1 sodium transporter) from parents grown in saline treatment and germinated in saline media had significantly lower germination percentage than *Athkt1* seeds from parent plants grown in water and germinated in ½ MS media (Figure 3.22). Seed from the *Athkt1* line had delayed germination compared to *Atpip2;1-1*, UBI:nGFP::PIP2;1* and heterozygote 2x35S::AtPIP2;1 OE line 7 and 21 seed from plants grown in saline treatment. The *Atpip2;1-2* seed from plants grown in water and germinated on MS media had higher germination percentage than *Atpip2;1-2* seed from plants grown in salt treatment and germinated in saline media. Col-0 seed from plants grown in saline treatment and germinated on saline media had lower germination percentage than Col-0 seed from plants grown in water treatment (Table S4).

The germination percentage at 9 DAS was not different between the genotypes that were germinated in ½ MS media, whether they had parent plants supplied with water or saline water (Figure 3.22 B). Percentage of *Athkt1* seed from salt grown parents and germinated in saline media was significantly lower to Col-0, *Atpip2;1-1*, UBI:nGFP::PIP2;1* , 2x35S::AtPIP2;1 OE (7)* and (21)* in the same treatments. *Athkt1* seed from salt grown parents had lower germination percentage than *Athkt1* seed from water grown parents whether germinated in saline or non-saline media (Table S4). The statistical analysis results relevant to this dataset are presented in Table S4 because there are too many differences to clearly present the differences using symbols within Figure 3.22.
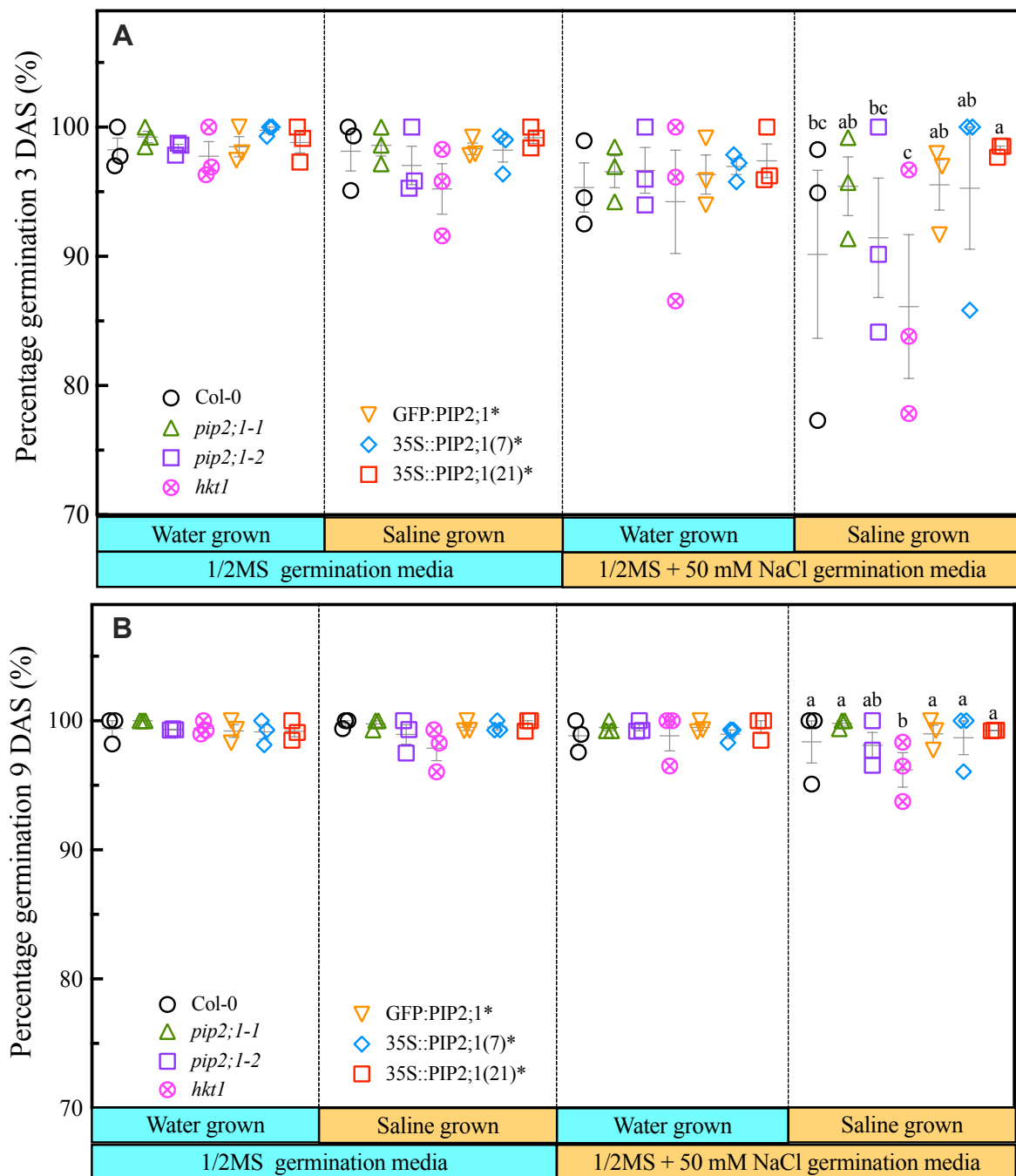
Figure 3.22: Evaluation of successful germination, expressed as percentages between 7 genotypes under treatments where seed was germinated in ½ MS or in ½ MS + 50 mM NaCl media. A – B: Quantification and statistical analysis of germination percentage of seed from parent grown in water (water grown) and water and NaCl at 5 mM NaCl (saline grown) at 3 days after sowing (DAS) (A) and 9 DAS (B). Analysis by two-way ANOVA and LSD post-tests. Data are mean ± SEM, with n = 3 which included three individual parent plant biological replications and 150 – 300 seeds from each plant in a replication.

3.3.5 Testing seed weight during imbibition in water and saline treatments

3.3.5.1 Trials of nylon mesh drainage and 20 mM NaCl solution treatments for seed weight assays

At the time of method development for assessing seed weight the seed of the overexpression lines UBI:nGFP::PIP2;1 and 2x35S::AtPIP2;1 were still heterozygous (annotated with *). Here the method development steps are reported and the final experiments using homozygous seed are included in Chapter 4 and Chapter 5.

Dry seed weight was in the range of 16 – 21 µg per seed and there were no differences between genotypes except for the 2x35S::AtPIP2;1 OE (21)* lines, which had greater seed weight than the other lines (Figure 3.23 A). After 15 min exposure to water and 20 mM NaCl solution, 2x35S::AtPIP2;1 OE (21)* had the heaviest seed weight, followed by UBI:nGFP::PIP2;1*. Col-0, *Atpip2;1-1, Atpip2;1-2, Athkt1* and 2x35S::AtPIP2;1 OE (7)*. These seeds were not significantly different in seed weight (Table S5a, b).

At 4 hours into imbibition, 2x35S::AtPIP2;1 OE (21)* had the heaviest seed weight in both treatments. In water treatment, UBI:nGFP::PIP2;1* had higher seed weight than *Col-0, Atpip2;1-1, Atpip2;1-2, Athkt1* and 2x35S::AtPIP2;1 OE (7)*. In salt treatment, UBI:nGFP::PIP2;1* had higher seed weight than Col-0, *Atpip2;1-1* and 2x35S::AtPIP2;1 OE (7)*. The weight of 2x35S::AtPIP2;1 OE (21)* seed imbibed in water was heavier than the weight of seed from this line imbibed in 20 mM NaCl (Table S5a, b).

At 24 HAS, the difference in seed weight for seed from the collection of lines imbibed in the salt treatment was not different except for UBI:nGFP::PIP2;1* which had higher weight compared to *Atpip2;1-2* seed. Under water treatment, 2x35S::AtPIP2;1 OE (21) and UBI:nGFP::PIP2;1* seed weight were higher than that of Col-0, *Atpip2;1-1, Atpip2;1-2, Athkt1* and 2x35S::AtPIP2;1 OE (7)*. Comparison of seed weights between treatments revealed that seed under water treatment increased faster than seed weight gain under 20 mM NaCl treatment (Table S5a, b).

At 30 hours after sowing the seed from *Atpip2;1-1, Athkt1*, UBI:nGFP::PIP2;1* and 2x35S::AtPIP2;1 OE (7)* in the salt treatment weighed less than that of seed from each respective line under water treatment. Seed of 2x35S::AtPIP2;1 OE (21)* weighed more than the seed of the other lines (except UBI:nGFP::PIP2;1*) in both water and salt treatment and

UBI:nGFP::PIP2;1* seed in water treatment weighed more than seed of Col-0, *Atpip2;1-1, Atpip2;1-2, Athkt1* and 2x35S::AtPIP2;1 OE (7)* (Table S5a, b).

At 46 HAS the seed weight of each genotype in water was heavier than that of seed in salt treatment and water-salt transfer treatment. Under salt treatment 2x35S::AtPIP2;1 (21)* seed weighed more than Col-0 and *Atpip2;1-1, Atpip2;1-2* mutant seed. Under water-salt transfer treatment seed from 2x35S::AtPIP2;1 (21)* weighed more than Col-0 and *Atpip2;1-1* mutant seed. Under water treatment 2x35S::AtPIP2;1 (21)* seed weighed more than seed from Col-0, *Atpip2;1* mutants and *Athkt*. The UBI:nGFP::PIP2;1* had higher percentage seed weight than that of *Atpip2;1-1*. The seed weight of 2x35S::AtPIP2;1 (7)* was lower compared to that of 2x35S::AtPIP2;1 OE (21)* in all treatments (Figure 3.23) (Table S5a, b).
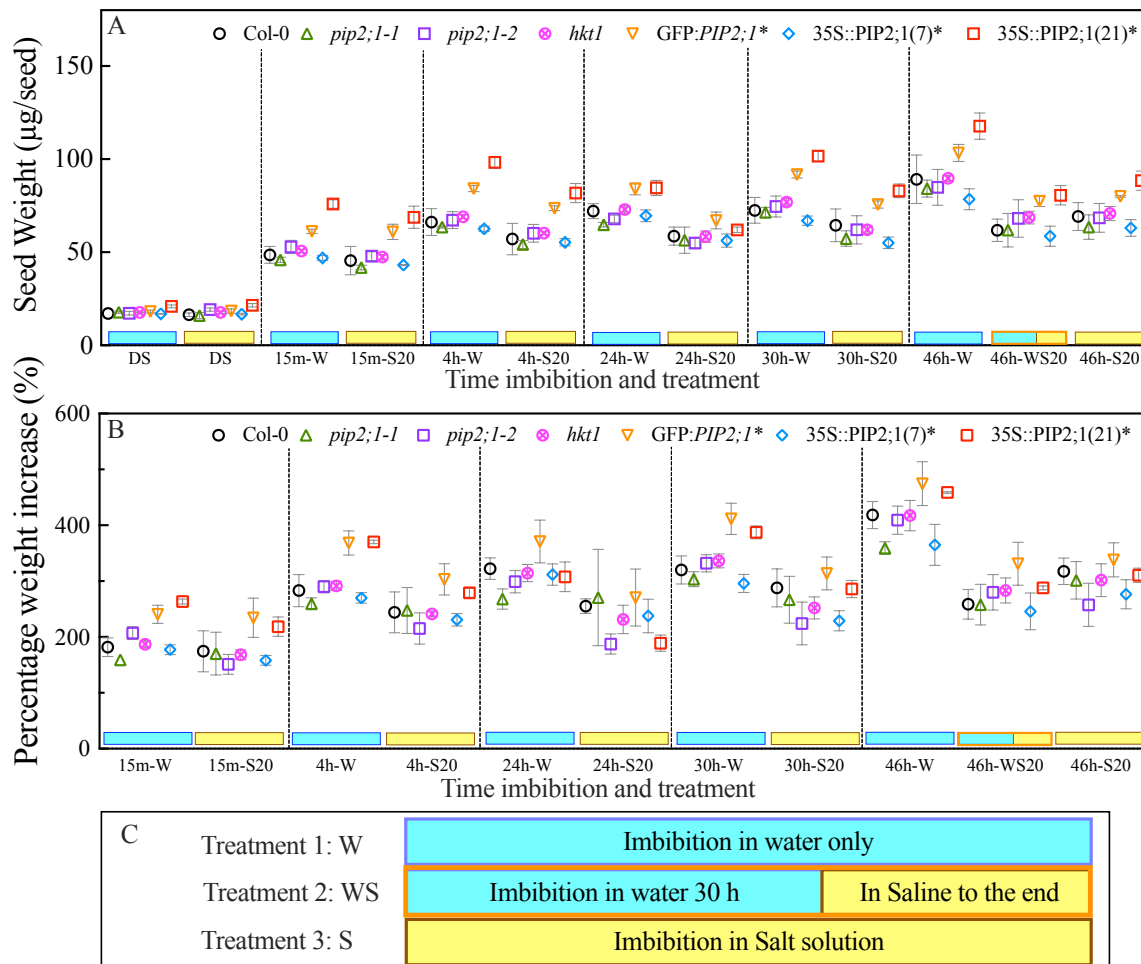

Figure 3.23: Seed weight and percentage weight change during imbibition.

There were three different media treatments: W (water): seed were imbibed in water for 46 hours; WS20 (water-salt): seed was imbibed in water for 30 hours before being transferred to 20 mM NaCl solution for 16 hours; S20 (Salt): Seed

was imbided in water with 20 mM NaCl added for 46 hours. Seed weight measurements were conducted at dry seed, 15 minutes, 4 hours, 24 hours, 30 hours and 46 hours after sowing. Data are mean ± SEM for approximately 350 - 500 seeds which came from three independent plants for each genotype.

During imbibition the percentage weight increase for seeds from all lines subjected to salt treatment was lower than the percentage weight increase for each respective line under the control water treatment (Figure 3.23 A, Table S5 b and d). The statistical analysis results for this dataset are presented in Table S5 because there are too many differences to clearly present the differences using symbols within the figure. At 15 min into imbibition the percentage gain in seed weight was not different between the treatments except for seed of *Atpip2;1-2* which had lower weight gain in the salt treatment. In water treatment 2x35S::AtPIP2;1 OE (21)* and UBI:nGFP::PIP2;1* seed had a greater increase in percentage weight increase relative to seed from Col-0, *Atpip2;1-1, Athkt1* and 2x35S::AtPIP2;1 OE (7)*.

At 4 and 30 HAS, under control water treatment, the percentage weight increase for seed from Col-0, *Atpip2;1-1, Atpip2;1-2* and 2x35S::AtPIP2;1 OE (7)* lines was less than that of UBI:nGFP::PIP2;1* and 2x35S::AtPIP2;1 OE (21)* seed. There were no differences between seed weight gains for the genotypes when seed was exposed to the 20 mM NaCl treatment with the exception of seed from UBI:nGFP::PIP2;1* which had greater weight gains than seeds of the At*pip2;1-2* mutant and 2x35S::AtPIP2;1 OE (7)* line. There were differences in seed weight gain for *Atpip2;1-2, Athkt1*, UBI:nGFP::PIP2;1* and 2x35S::AtPIP2;1* OE lines 7 and 21 when seed was imbided in the salt treatment relative to the water treatment, specifically the salt treatment limited seed weight gain (Table S5c, d).

At 46 HAS, the seed for all genotypes imbided in water had significantly higher percentage of weight increase compared to the respective seed in salt or water-salt transfer treatments. There was no difference between genotypes under salt and water-salt transfer treatment but for the water treatment the seed from overexpression lines UBI:nGFP::PIP2;1* and 2x35S::AtPIP2;1 OE (21)* had greater percentage of increase in seed weight than the seed from the other genotypes (Table S5c, d).

3.3.5.2 Changes of seed weight using fine nylon mesh drainage and treatments with water or 75 mM NaCl treatment or transfer from water to 75 mM NaCl treatment

The weight of dry seed did not differ between genotypes but weight of seed imbibed under water treatment gained more weight than seed under 75 mM NaCl treatment (Figure 3.24 A, Table S6). At 50 HAS, seed of 2x35S::AtPIP2;1 OE (21)* had significantly more weight than that of Col-0 and *Atpip2;1*2;2* under both water or salt treatments (Figure 3.24 A).

The percentage of seed increase compared to dry seed also showed higher increase in water treatment relative to salt treatment (Table S6). The increase of *Atpip2;1*2;2* was lower than Col-0, *Athkt1* and 2x35S::AtPIP2;1 OE (21) in either water or water transfer to salt treatment (Figure 3.24 B).

Additional replications of this experiment revealed similar results and this data is included in Figure S7 and S8.
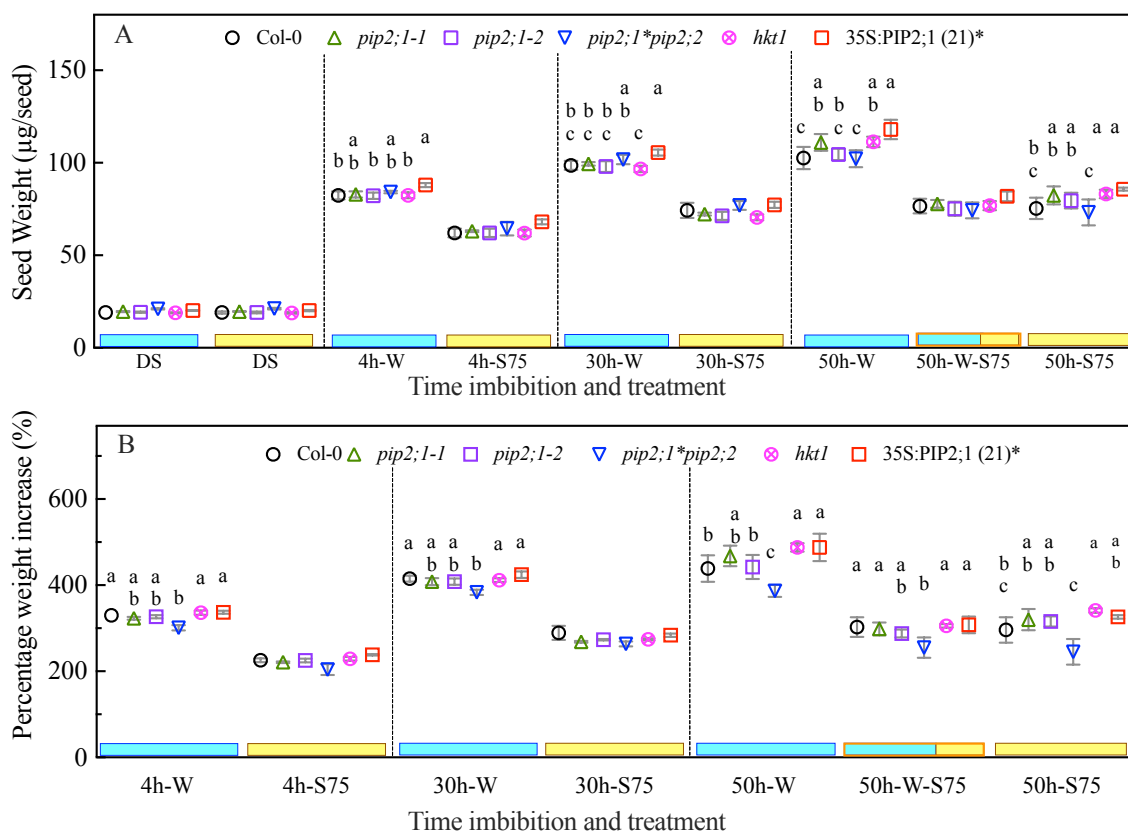
Figure 3.24: Seed weight (panel A) and weight percentage (panel B) changes during imbibition in three treatments. W (water): seed were imbibed in water for 50 hours; W-S75 (water-salt): seed was imbibed in water in 30 hours before being transferred to 75 mM NaCl solutions for a further 20 hours; S75 (Salt): Seed was

exposed to 75 mM NaCl for 50 hours. Measurements were conducted at dry seed before sowing (DS), 4 hours (4h), 30 hours (30h) and 50 hours (50h) of imbibition. Different lowercase letters indicate significant differences within a treatment group between genotypes, absence of letters indicates a non-significant difference (p < 0.05). Data are mean ± SEM where seed is from four independent plants for each genotype /treatment.

3.3.6 Measurement of Na^+ and K^+ content in seed and seedlings

3.3.6.1 Preliminary experiments to test seed ion content for *Atpip2;1* mutants and wild type Col-0 where the parent had been watered with either water or water with NaCl added

As expected, watering parent plants with water and NaCl at 5 mM NaCl resulted in seed accumulating more Na^+ than the seed from parent plants watered without additional NaCl (Figure 3.25). There were no differences in seed Na^+ content for seed from the two *Atpip2;1* mutant lines and Col-0 where the seed had all been harvested from parent plants grown in water treatment. For seed from parent plants grown in water and NaCl at 5 mM NaCl the *Atpip2;1-1* seed had higher Na^+ than seed from Col-0 plants, but *Atpip2;1-2* seed did not differ in seed Na^+ relative to Col-0 seed. The seed K^+ content was not significantly different between the genotypes and treatments except for the K^+ content of *Atpip2;1-1* seed, which was lower in seed from parent plants grown in water relative to the content in seed from parent plants grown with the saline treatment.
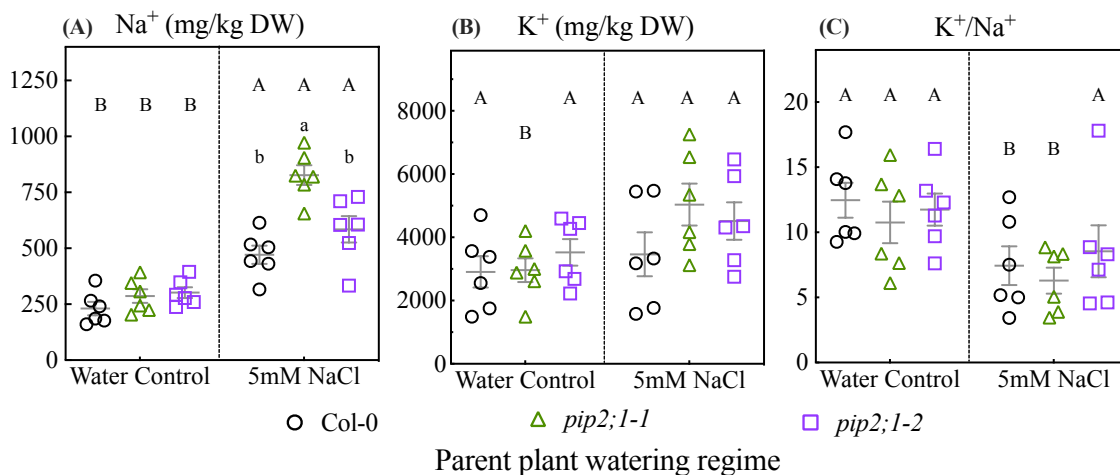
Figure 3.25: Seed Na^+ (A) and K^+ (B) content where seed is from parents grown under standard treatment, watered with pure water, or treatment where the parent

plants were cultivated on water and NaCl at 5 mM NaCl, and data for seed ratio K^+/Na^+ ratio (C). Mature seeds were collected after plants had been dry for two weeks, then seed was dried further in an oven at 65°C for 4 days. 3-5 mg of dry seed were weighed and digested in 1% HNO_3 at 80°C for 2 hours and analysed by AAS. Data are mean ± SE (n = 6). Different lowercase letters indicate significant differences within a treatment group between genotypes, absence of letters indicates a non-significant difference; different uppercase letters indicate significant differences between treatments for a particular genotype (p < 0.05). For additional methods information see section 3.2.2 and 3.2.8.

In another repeat experiment similar results were observed (Figure S9): There was no difference between genotypes in terms of seed Na^+ and K^+ content for seed from parent plants grown in standard treatment and in seed from parent plants grown in water and NaCl at 5 mM NaCl and higher seed Na^+ content was observed for all genotypes relative to the respective seed from plants grown in water treatment. The seed from the *Atpip2;1-1* again had higher seed Na^+ than Col-0 when parent plants for each genotype were watered with water and NaCl at 5 mM NaCl.

The Na^+ and K^+ content in shoots of parent plants was analysed where shoot tissues included leaves, stems and siliques. As expected, the water and NaCl at 5 mM NaCl treatment led to a significant increase in the shoot Na^+ content relative to the parent plants grown in water alone, but no differences in shoot K^+ content were observed (Figure S9). The ratio of K^+/Na^+ was generally higher in shoots of plants grown in water treatment relative to that of plants grown in saline treatment, but there were only statistically significant differences for the Col-0 genotype (Figure S9 C).

3.3.6.2 Seed Na^+ content was influenced by the level of salinity that the parent plant experienced

As expected, the seed Na^+ content was greater when the parent had been watered with water and NaCl at 5 mM NaCl compared to seed from parent plants grown in water treatment (Figure 3.26). The *Athkt1* mutant was used as a comparison for several experiments as this plant represents a mutant known to differ from Col-0 in shoot and floral tissue Na^+ accumulation (An *et al.*, 2017; Sunarpi *et al.*, 2005), but the *Athkt1* mutant struggled to grow in the saline stress conditions involving watering with 75 mM NaCl (Figure 3.29 C), and the plants could

not produce seeds (see full data in Figures S11, S17 and S18). The two mutant *Atpip2;1* lines had lower seed Na^+ compared to Col-0 whereas the 2x35S::AtPIP2;1 OE line 7 had higher seed Na^+ compared to Col-0 and the mutant *Atpip2;1-2* (Figure 3.26). This may be due to different levels of overexpression within the lines, as expression differences were observed previously by Dr Michael Groszmann who generated the material.

The seed K^+ was not significantly different between genotypes, with the exception of 2x35S::AtPIP2;1 OE line 7 which had lower seed K^+ content than Col-0, *Atpip2;1-1, Atpip2;1-2* under water treatment. 2x35S::AtPIP2;1 OE (7) also had lower seed K^+ content than Col-0 in the 25 mM NaCl treatment (Figure 3.26, Table S10).

The water content of seed was 4-5.5% and there were no differences between genotypes, with the exception of seed from *Atpip2;1-2* which had greater percentage of seed water than seed from the 2x35S::AtPIP2;1 line 7 in water treatment. Seed from parent plants grown in saline treatments (25 and 50 mM NaCl) had lower seed moisture content relative to seed under control treatment (Figure 3.26, Table S10).
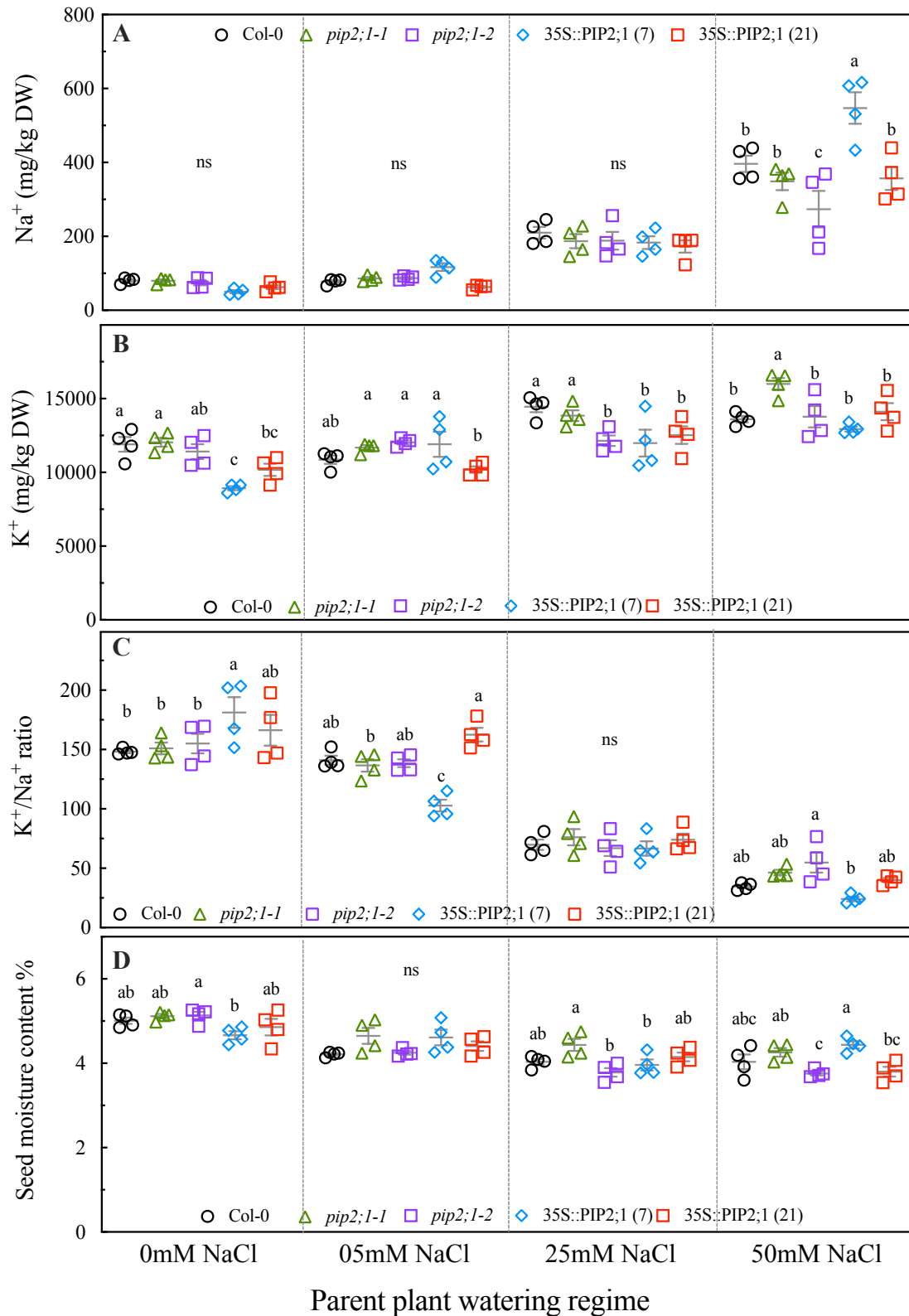
Figure 3.26: Seed Na$^+$, K$^+$ and K$^+$/Na$^+$ ratio and seed moisture content for fresh dry seed from parent plants watered with water and NaCl at 0 mM, 5 mM, 25 mM or 50 mM NaCl.

A-C, Na$^+$ and K$^+$ content and the ratio of these ions in seed from five genotypes of interest tested where the parent plants were grown in different treatments and seeds were collected when mature and dry.

D, Moisture content of seed (%) = (fresh dry seed weight – oven dried seed weight) x 100/ fresh dry seed weight. Seeds were weighed, dried in an oven temperature at 65°C for 72 hours and then dried seeds were weighed. Data from Col-0, Mutant *Atpip2;1-1*, *Atpip2;1-2*, 35S::AtPIP2;1-7 and 35S::AtPIP2;1-21 was collected (*Athkt1* data was omitted from this analysis due to the inability of parent plants to tolerate salinity treatments). Different lowercase letters indicate significant differences within a treatment group between genotypes, "ns" indicates no significant difference ($p < 0.05$). Data points are means and SEM (n = 4 plates per treatment, 600 - 900 seeds per genotype in each plate). This experiment was repeated two times. Significant differences determined by two-way ANOVA and Fisher's LSD pair-wise tests.

3.3.6.3 Testing Na$^+$ content in seed imbibed in water or 20 mM NaCl or water followed by transfer to 20 mM NaCl at 30 hours, using nylon mesh filters and a simple seed washing approach

As expected, the seeds that were imbibed and germinated under saline treatments (20 mM NaCl and water followed by transfer to 20 mM NaCl from 30 hours) had greater Na$^+$ contents compared to the respective seed imbibed and germinated in water (Figure 3.27). There were no differences in Na$^+$ content between genotypes when seeds were imbibed in water for 46 hours. There were differences in seed Na$^+$ content between genotypes when seeds were exposed to saline treatment from 30 - 46 hours and for 46 hours of imbibition. In particular, Col-0 (WT) and *Athkt1-1* had higher seed Na$^+$ content than that of the other genotypes tested in salt treatments, while seed Na$^+$ content of *Athkt1-1* was the only genotype with high Na$^+$ content in the water to saline treatment. The mutant *Atpip2;1-2* had greater Na$^+$ content in seed than that of the 2x35S::AtPIP2;1 OE lines.
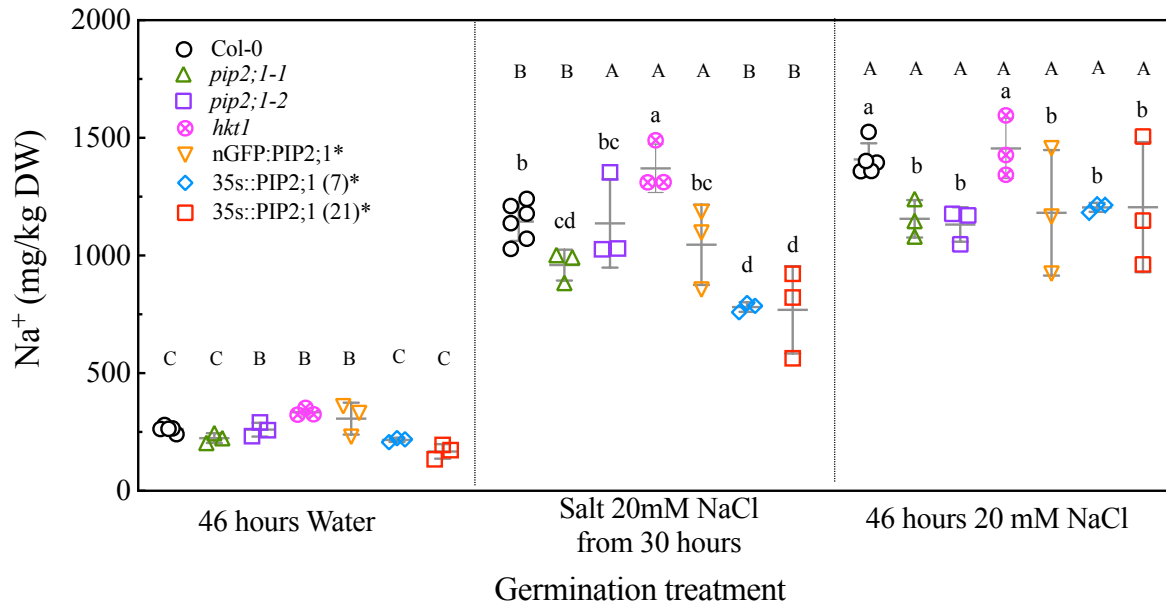
Figure 3.27: Sodium (Na^+) content of seed when imbibed for 46 hours in one of three treatments: water for 46 hours, or water for 30 hours then 20 mM NaCl from 30 to 46 hours or 20 mM NaCl for 46 hours. Different lowercase letters indicate significant differences between genotypes within a treatment, absence of letters indicates a non-significant difference; different uppercase letters indicate significant differences between treatments for a particular genotype ($p < 0.05$). Data are mean ± SE (n = 3).

3.3.6.4 Analysis of Na^+ and K^+ content in seed imbibed in water or 75 mM NaCl or water followed by transfer to 75 mM NaCl after 30 hours

There were some significant differences in seed Na^+ content between genotypes following salt treatment and water transfer to salt treatment but not in seed subjected to the water treatment (Figure 3.28 A). Seed Na^+ in the *Atpip2;1-2* mutant, *Atpip2;1*2;2* double mutant and 2x35S::AtPIP2;1 OE (21)* were lower than Col-0 and *Athkt1* seed Na^+ content in the water to salt transfer treatment. *Athkt1* had the highest seed Na^+ content in the transfer from water to 75 mM NaCl treatment.

The K^+ content for Col-0 and *Atpip2;1*2;2* double mutant seed were higher than that of *Atpip2;1-2* and *Athkt1* genotypes germinated in water treatment. In water transfer to salt treatment after 30 hours imbibition Col-0 had higher K^+ content in seed than *Athkt1*. There

was no significant difference between seed K^+ content for genotypes germinated in 75 mM NaCl (Figure 3.28 B).

The K^+/Na^+ ratio in germinated seed did not differ in saline and transfer to saline treatment. In water *Athkt1* seed had the lowest K^+/Na^+ ratio followed by *Atpip2;1-1, Atpip2;1-2* and 2x35S::AtPIP2;1 OE (7)* (Figure 3.28 C).
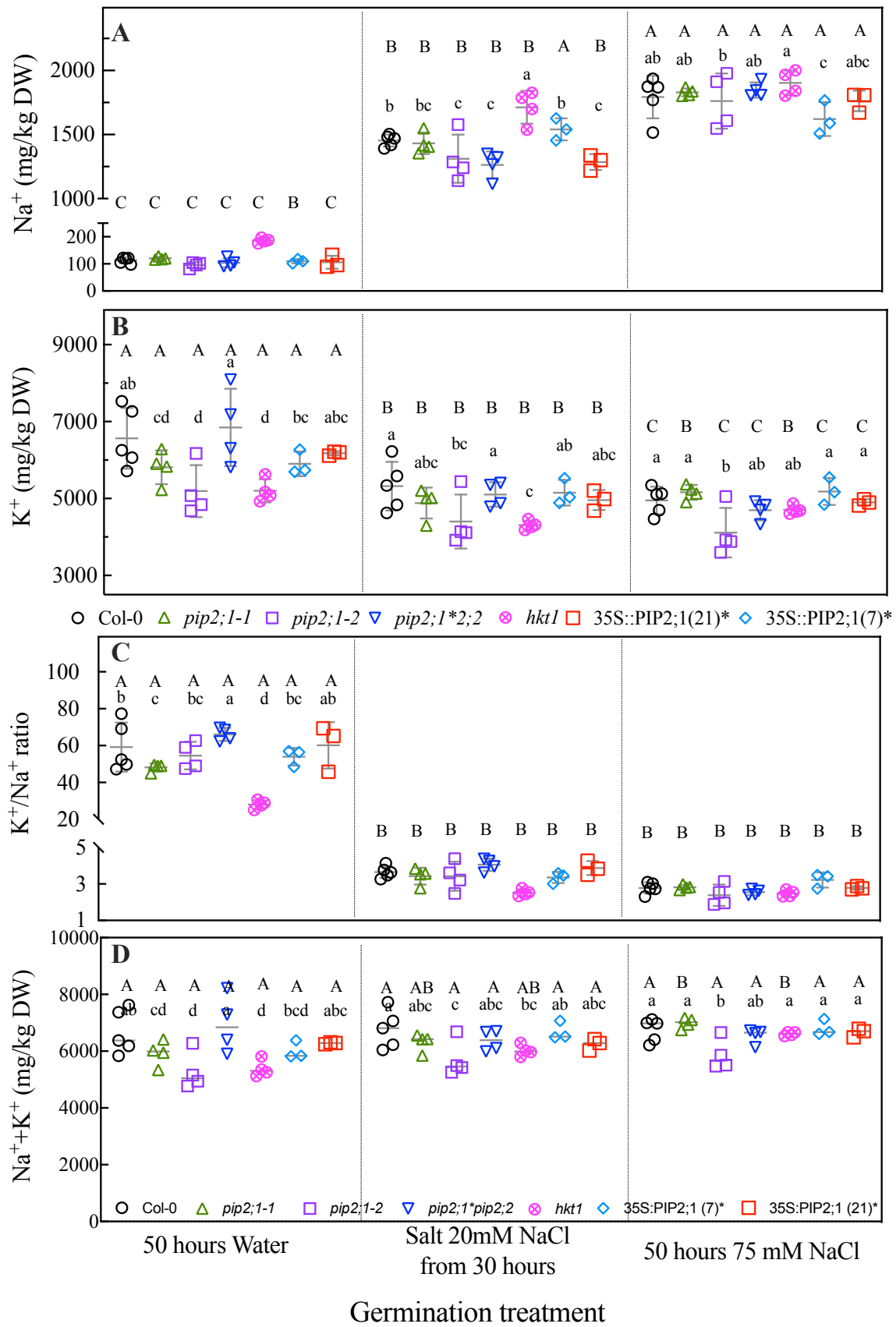
Figure 3.28: The content of seed Na^+ and K^+, K^+/Na^+ ratio and total Na^+ and K^+ in seed following imbibition and germination. There were three treatments: Water: 50 hours ; Salt: 75 mM NaCl for 50 hours; and Water-Salt which involved imbibition in water for 30 hours then transfer to 20 mM NaCl for a further 20 hours. Data are the mean of values from seed from 4 plants for each genotype ± SEM. At some timepoints the error bars are so tight as to be obscured by the symbols. Significant differences determined by two-way ANOVA and Fisher's LSD pair-wise tests. Different lowercase letters indicate significant differences between genotypes within a treatment, absence of letters indicates a non-significant difference; different uppercase letters indicate significant differences between treatments for a particular genotype ($p < 0.05$). Ion content units: mg per 1 kg dry weight (mg/kg DW).

3.3.7 Measurement of Na^+ and K^+ content in plant tissues

3.3.7.1 Na^+ and K^+ content in plant tissues when grown in salt stress conditions using atomic absorption spetrometry (AAS)

The seed Na^+ content of parents grown in water treatment was significantly lower than that of seeds grown in saline treatment (Figure 3.29 A, C, Table S14). In the same growing conditions, *Athkt1* seed had significantly higher Na^+ content than Col-0, *Atpip2;1-1* and *Atpip2;1-2* indicating that the lines lacking HKT1 were unable to prevent Na^+ accumulation in above ground tissues. The *Athkt1* plants died following the 75 mM NaCl treatment, so there was no seed available from these treatments for further analysis.

In contrast the seed K^+ of *Athkt1* parent plants grown in water and NaCl at 5 mM NaCl was lower than Col-0, *Atpip2;1-1* and *Atpip2;1-2* (Figure 3.29 B). Similar trends were observed in parents grown in 25 and 50 mM NaCl (Figure 3.29 D).
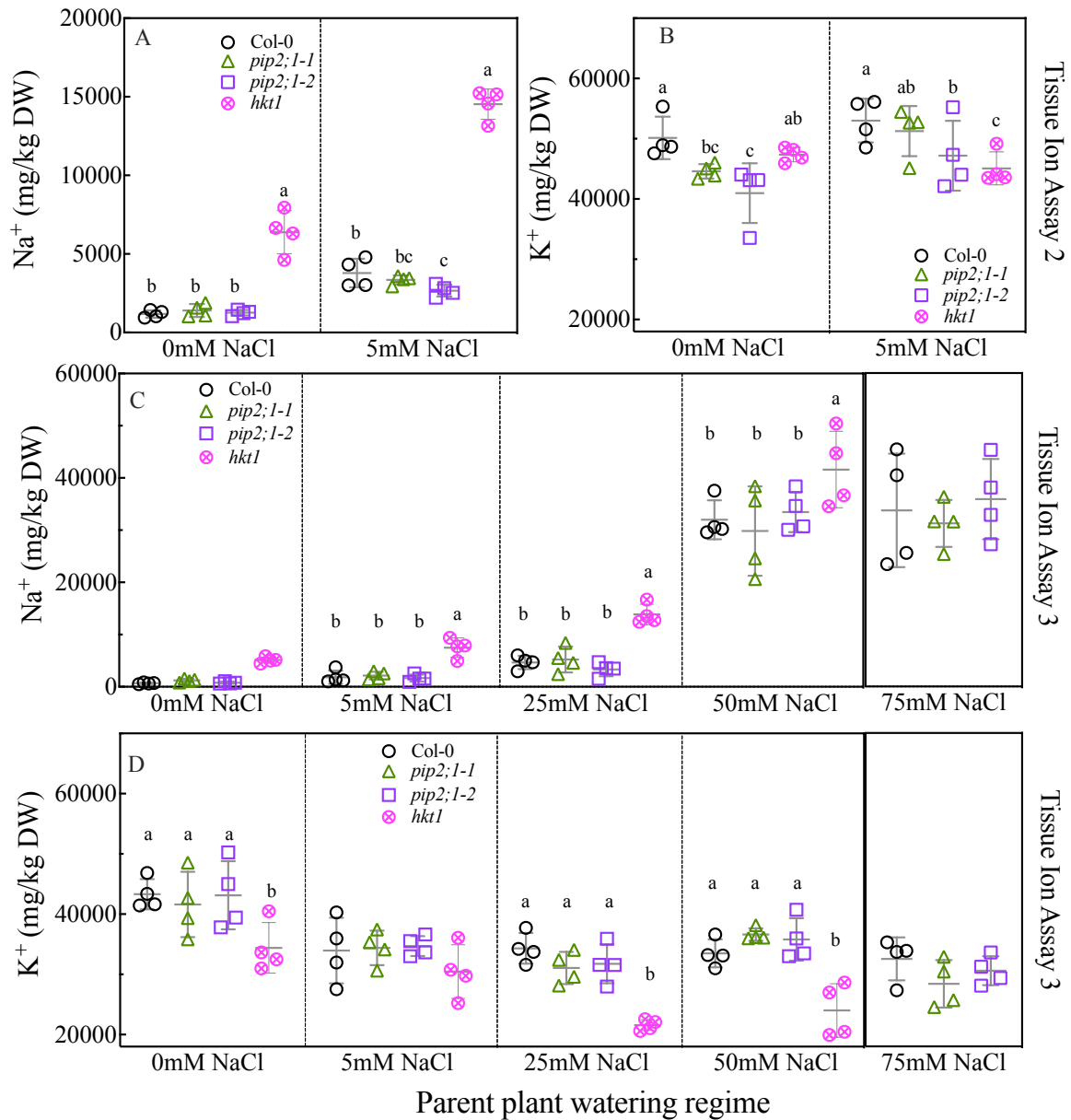
Figure 3.29: Na$^+$ and K$^+$ content in tissue of Arabidopsis grown in control treatment (water, 0mM NaCl) or treatment where they were watered with water and NaCl at 5 mM NaCl (A-B) or a series of 0, 5, 25 50 or 75 mM NaCl solution (C-D) in independent growth batches (each batch was an assay or an experimental replication). Tissue ion assay 2 including 4 genotypes grown in two water treatments water and 5 mM NaCl. Tissue ion assay 3 including 4 genotypes grown in 5 water regimes, which includes water, 5, 25, 50 and 75 mM NaCl. *Athkt1* did not produce seed in the 50 mM NaCl water regime and plants died in the 75 mM NaCl water regime. Different lowercase letters indicate significant differences

between genotypes within a treatment, absence of letters indicates a non-significant difference ($p < 0.05$).

The K^+/Na^+ ratios of Col-0 were higher than that of mutant *Atpip2;1-1* and *Athkt1* in water treatment (Figure S12). In saline treatments only *Athkt1* had significantly lower K^+/Na^+ ratios than Col-0 and *Atpip2;1-1* and *Atpip2;1-2* (Figure S12 A, Table S14*)*. As expected, the ratios of K^+/Na^+ were lower for plants of all genotypes when grown in saline treatment relative to water (control) treatment (Figure S12 C-D, Table S14). In particular, the Col-0 and the two mutants *Atpip2;1* lines had significantly lower seed K^+/Na^+ ratios for the control treatment compared to treatment with water and NaCl at 5, 25 or 50 mM NaCl; and between samples from the water and NaCl at 5 mM NaCl treatment relative to treatments with 25 and 50 mM NaCl, but there were no differences between samples from the 25 and 50 mM NaCl treatments (Figure S12, table S14). For *Athkt1*, there were no differences in the ratios of seed K^+/Na^+ when parents were grown in saline or water treatments (Table S14). The total Na^+ and K^+ contents of parents treated with 50 mM NaCl (65000 - 70000 mg/kg dry weight) were significantly higher than the total Na^+ and K^+ contents for the plants grown in the three other treatments: water (control), 5 and 25 mM NaCl, where the total amounts were around 35000 - 42000 mg/kg dry weight.

3.3.7.2 Na^+ and K^+ content in homogenised above ground plant tissues from loss-of-function lines and overexpression lines of AtPIP2;1

Plants were watered with water or water and NaCl at 5 mM NaCl comparing Col-0, *Atpip2;1-1, Atpip2;1-2, Athkt1*, UBI::nGFP::PIP2;1*, null and 2x35S::AtPIP2;1 OE (7)* and (21)*. The Na^+ and K^+ content in homogenised above ground plant tissues was determined (Figure 3.30). The Na^+ for *Athkt1* plants was the highest of all the genotypes tested in both water and saline treatments, except when compared to 2x35S::AtPIP2;1 OE (21)* under water treatment; and in water and NaCl at 5 mM NaCl treatment, Col-0 had the lowest Na^+ content of the genotypes tested (Figure 3.30 A, Table S14).

The tissue K^+/Na^+ ratios in *Athkt1* were lower than that of WT Col-0 in both saline and water treatments, however the total Na^+ and K^+ content in tissue were not significantly different (Figure S13 A).
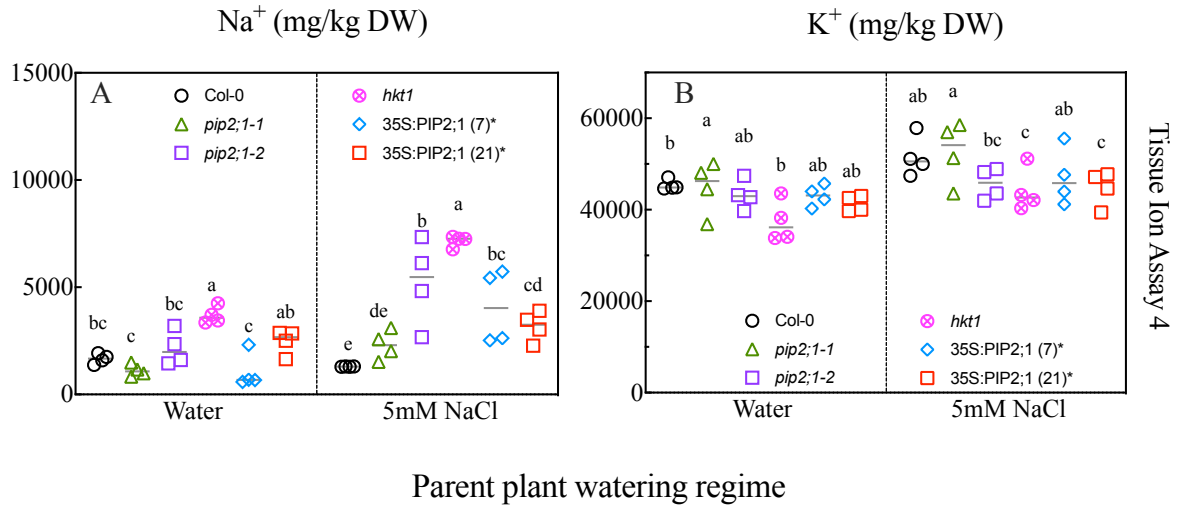
Figure 3.30: Na$^+$ and K$^+$ content in tissues of Arabidopsis grown in water and water and NaCl at 5 mM NaCl (5 mM NaCl) treatments in the growth assay 4. Data is mean +/- SEM with n = 4. Significant differences determined by two-way ANOVA and Fisher's LSD pair-wise tests. Different letters indicate significant differences between genotypes within a treatment (p < 0.05). Ion content units: mg per 1 kg dry weight (mg/kg DW).

3.3.8 Seed swelling during imbibition and germination

3.3.8.1 Increases in seed maximum cross sectional area (MCS area) based on video footage of three genotypes Col-0; *Atpip2;1-1* and *Atpip2;1-2* mutants

The images of seeds were extracted from short videos at a series of time points then used to measure the seed MCS areas as shown in Figure 3.31.
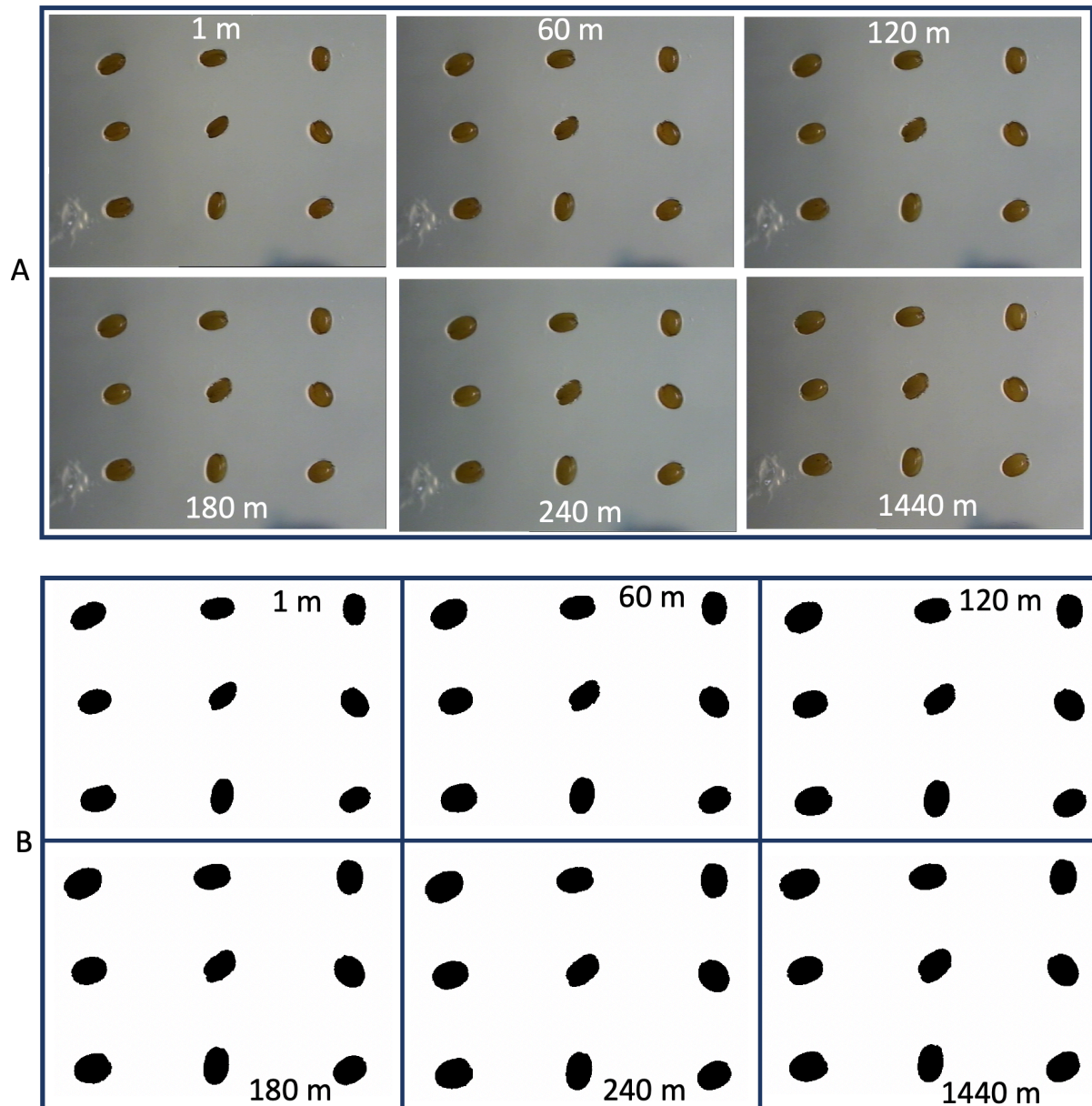
Figure 3.31: Images of seed swelling extracted from video footage from 1, 60, 120, 180, 240 and 1440 min (A) and MCS area measurement images in ImageJ (B).

Experiment included 4 seeds from each of 3 genotypes using video footage. Images of seed at beginning of experiment 0 hour, then 1, 2, 3, 4, 24 and 30 hours were used to analyse the seed MCS area and percentage MCS area increase of three genotypes Col-0, *Atpip2;1-1* and *Atpip2;1-2* mutant lines.

The experiments to explore seed swelling were conducted using seed from parent plants grown in water treatment or water and NaCl at 5 mM NaCl treatment. Nineteen video footage files

were collected and in each video 4 seeds x 3 genotypes were represented, individual graphs are displayed in Figure S15.

Seed MCS area was determined to be 13-15 mm^2/seed at the beginning of measurement (0 hour) and there was no significant difference between genotypes at this stage (Figure 3.32 A, C). In water treatment the MCS area increase of *Atpip2;1-1* and *Atpip2;1-2* mutant seed appeared smaller compared to Col-0 seed during imbibition but this was not statistically significant. There were no differences in saline grown seed in both seed MCS area or the percentage increase between three tested lines (Figure 3.32 C-D).
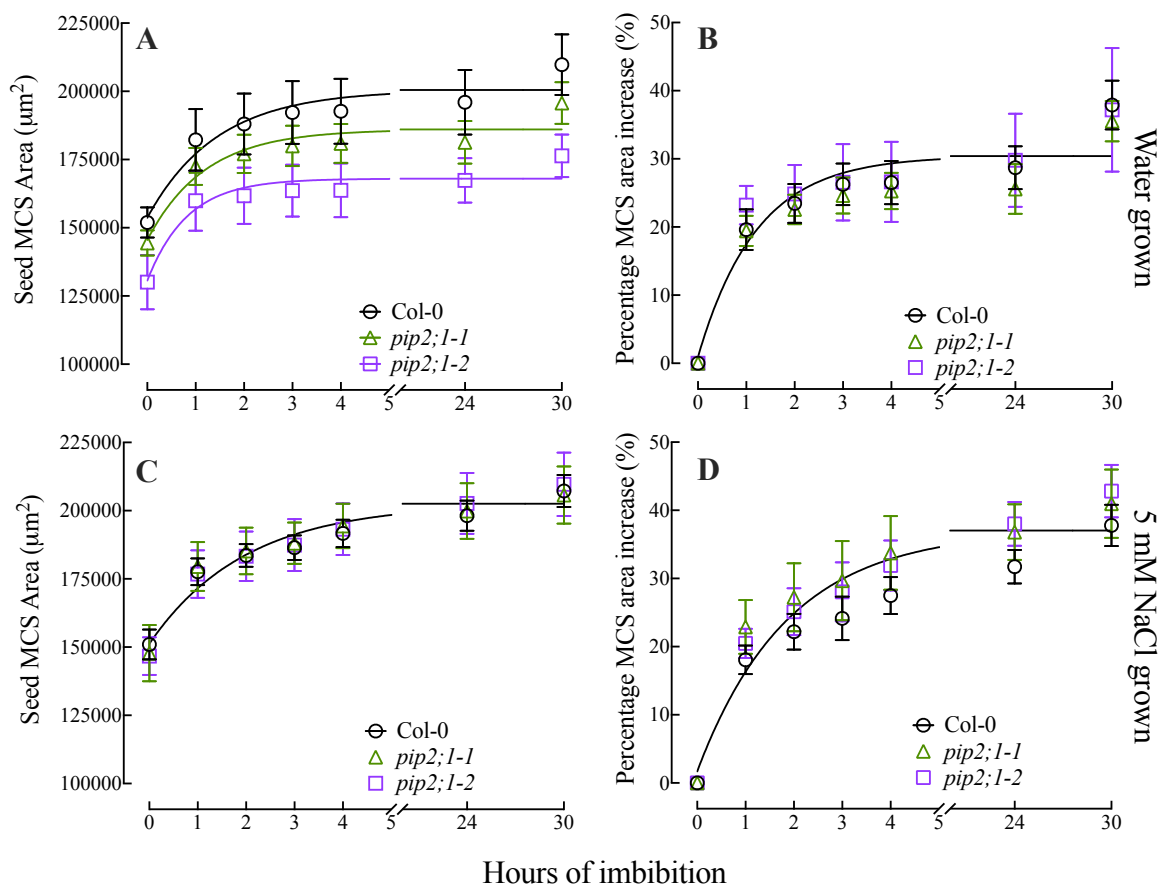
Figure 3.32: Seed MCS area and percentage MCS area increase during imbibition.

Seeds from parent plants grown in water treatment (A-B) and seeds from parent plants grown in water and NaCl at 5 mM NaCl (5 mM NaCl) treatment (C-D). % increase = (MCS area at time t – MCS area at beginning at 2 min) *100%/MCS area at 2 min. Data are mean ± SEM. The experiment was repeated three times, four biological replicates (seed samples from four independent parent plants per genotype) were included each time with similar results observed for each repeat.

Exponential regressions of MCS versus time ($Y = Y_0 + (Plateau-Y_0)*(1-exp(-K*X))$) where k is the rate constant (hour^{-1}); For panel A, different curves are required to fit the data; while in panels B, C, D, single exponential time courses adequately fit all the data in each case (Prism F test)) (see method section 3.2.10).

3.3.8.2 Changes in seed MCS area using Image Capture at nominated time points during imbibtion and germination

Water uptake into a dry seed involves three phases: an initial imbibition phase with high water uptake (Phase I), transition phase with low-to-negligible water uptake (Phase II) and germination phase when radicles protrude from seed coat and start rapid uptake of water (Phase III) (Bewley, 1997; Hadas, 2005). When the seed absorbs water, the seed volume will increase as well as the seed MCS area (Figure S16). The MCS area for mature seed of the eight genotypes tested ranged from 11.0 to 12.5 mm^2 seed^{-1} (Figure 3.33). The increase in MCS was rapid once seed contacted water, particularly from 1 to 4 hours from first contact with water. From 4 hours to 30 hours of imbibition the increase was slower, most likely because the seed were in the transition phase of imbibition.

There were significant differences in seed MCS area for 2x35S::AtPIP2;1 OE (21)* relative to Col-0 and mutants *Atpip2;1-1* and *Atpip2;1-2* from dry seed to imbibing seed at 30 hours after sowing. In contrast, the overexpression line 2x35S::AtPIP2;1 (7) did not differ to Col-0 and mutants (Table S17).

There were no significant differences in the increase of seed MCS area in percentage (Table S17). The change in percentage MSC area was about 60 – 70 % in total at 30 hours of imbibition relative to the start of imbibition (Figure 3.33 and Table S22).
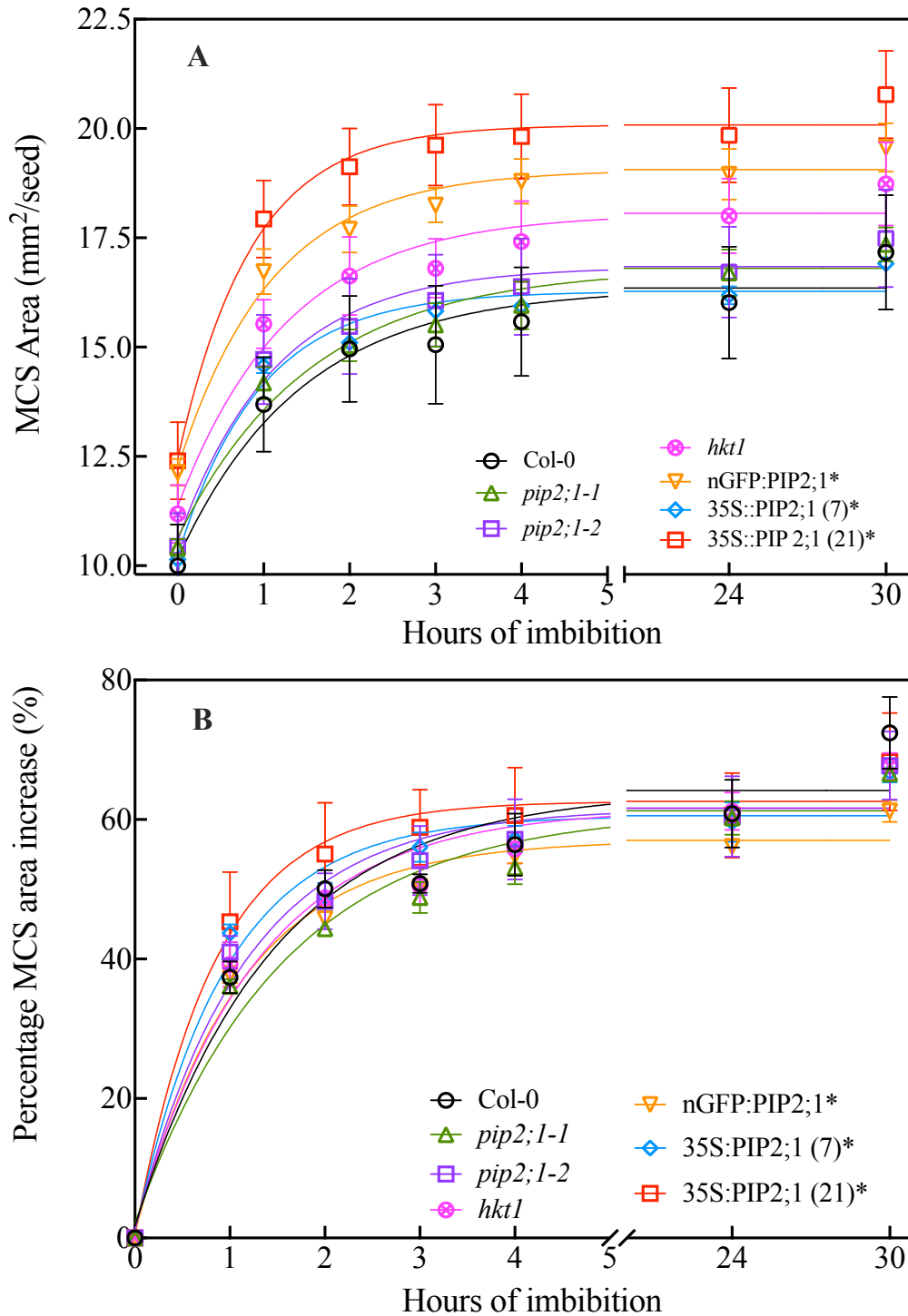
Figure 3.33: Seed MCS area and percentage MCS area increase during imbibition for eight selected genotypes. Seed from parent plants grown in water treatment were sown on ½ MS media without stratification. There were ~ 30 seeds on each plate. Data are mean ± SEM with independent biological replication involving use of seed from five independent plants per genotype (n = 5). Exponential regressions of MCS versus time $(Y=Y_0 + (Plateau-Y_0)*(1-exp(-K*X)))$ where k is the rate

constant (hour^{-1}); For panel A, different curves were required to fit the data; while in panels B, C, D, single exponential time courses adequately fit all the data in each case (Prism F test) (see method section 3.2.10).

3.4 Discussion

3.4.1 Genotyping mutant and transgenic lines of interest

The steps to confirm the genotype of the mutant material was straight forward for the knock-out lines *Atpip2;1-1, Atpip2;1-2* and *Atpip2;1*2;2* because the mutant lines were homozygous (Figure 3.6 – 3.8). This meant that PCR tests were sufficient to confirm the genotype of these lines.

The genotyping process for the over expression (OE) lines 2x35S::AtPIP2;1 (7), 2x35S::AtPIP2;1 (21) and UBI:nGFP::PIP2;1 was complicated because these plants were segregating from T2 generation parents (Table 3.7). The seeds were genotyped using several methods to make sure that the materials used for final experiments were homozygous seeds (Table 3.8 - 9). However, during the course of obtaining homozygous material trial experiments were completed to refine the phenotyping methods.

Under confocal microscope scanning, samples from the transgenic line UBI:nGFP::PIP2;1 exhibited bright fluorescence in roots (Figure 3.15). The fluorescence in the area of the endodermis was expected based on previous data from http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi which indicates high AtPIP2;1 transcript abundance in endodermis cells. There was a pattern in the cell fluorescence in the outermost sections of cells in the root indicating that there may have been polar AtPIP2;1 localization in some cells but additional higher resolution images are needed to further explore this. Polar localisation for aquaporins has been reported previously for AtNIP5;1 and AtNIP6;1 which were found in epidermal root cells and displayed polar localization to the outer membrane face (Wang *et al.*, 2017b).

3.4.2 Phenotyping methodology development

The methods developed as part of this body of research were designed to enable measurement of seed weight, ion content and cross-sectional area. The cross-sectional area being easy to assess rapidly during imbibition using image analysis. Previous literature was used as a guide to determine potential suitable approaches (Colmer *et al.*, 2020; Coolbear *et al.*, 1984; Fasina, 2010; Goertz and Coons, 1991; Hou and Romo, 1998; Joosen *et al.*, 2010; Nelson, 2002;

Panuccio *et al.*, 2014; Sarabi *et al.*, 2016; Steiner *et al.*, 2019; Tang and Sokhansanj, 1993). However, the tiny size of Arabidopsis seed, which normally are about 0.6 millimetres in length (Robert *et al.*, 2008), meant that many of the methods used previously for larger seed were not suitable for Arabidopsis seed. During the process of methodology development, several methods were applied and the understanding derived from these trials were subsequently used in the final methods, adopted to minimise variation from technical errors. After finishing each experiment, the results were analysed to determine the standard deviation for a given genotype exposed to a given treatment, if the standard deviation was too large it might be that the method used led to unacceptable variation between technical or biological replicates, hence the method was revised. The reasons why variation may have occurred, and how methods could be improved and what aspects of the methods needed to change are discussed. The modifications and improvements to the methodology are systematically summarised in Table 3.12.

Table 3.12: Summary of methodology tested, modifications to the methods, identification of problems and improvements in outcomes

Experiment	Main features and modifications	Factors potentially influencing variation
Methods for measuring seed weight		
1	Open mesh fabric used for seed washing and 20 mM NaCl treatments applied as salt stress	The mesh thickness limited the ease by which the excess water could be removed by blotting. It is possible that the seeds did not retain an even amount of water at each time of weighing because the mesh was thick. CV% of Col-0 at 30h in water treatment was 9.8%.
2	Fine mesh fabric used for seed washing and 20 mM NaCl treatments applied as salt stress	One possible option to eliminate water retained on mesh and seeds involved using finer mesh, which helped in absorbing all excess water. A concentration of 20 mM NaCl may not have been high enough to have a clear influence on changes in seed weight. CV% of Col-0 at 30h in water treatment was 8.3%.
3	Fine mesh fabric used for seed washing and 75 mM NaCl treatments applied as salt stress treatment. Seed was taken and weighted separately from mesh.	The final method enabled greater repeatability because the coefficient of variance was lower relative to experiments 1 and 2. The CV% of Col-0 at 30h in water treatment was 3.8 – 5.0 %
Methods for measuring seed Na$^+$, K$^+$ content		
1	Treatments involved imbibing seed in water treatment for 46 hours, or 20 mM NaCl treatment for 46 hours or water for 30 hours then transfer to 20 mM NaCl	The results from repeated experiments revealed different patterns between genotypes, e.g Col-0 and *Atpip2;1-1* were highest for seed Na$^+$ content for some experiments, then lower in subsequent repetitions. This led to concern that the washing step, involving pouring water though seed on the mesh might cause ion leakage. Potentially MQ water could cause

	from 31 - 46 hours treatment. MQ water was used for washing and a mesh used for drainage. Ion content was measured using AAS (See section 3.2.8.1).	ion efflux because of the gradient of ion concentration. CV% of Col-0 seed Na^+ in water treatment was 8.5 % There were problems with the AAS during repeats of this experiment where repeats of standards throughout the experiment generated lower values than measurements of initial standards, referred to as 'drift', so it was necessary to switch to using a flame photometer which was more reliable but less sensitive.
2	Treatments were water for 50 hours (W) or 75 mM NaCl for 50 hours (S) or water for 30 hours and then transfer to 75 mM NaCl from 31 - 50 hours (WS). Seed were washed using D-mannitol solution isosmotic to 75 mM NaCl, and wash solution was rapidly removed using a manifold vacuum and membranes. Ion content was measured by Flame Photometer.	This procedure was regarded as being highly repeatable, and was adopted for the final experiments, because the CV% of Col-0 seed Na^+ in water was relatively low, 3.9 %, compared to the earlier method trialled where the CV was 8.5%.
Na^+ and K^+ content in plant tissues		
1	Na^+ and K^+ content in above ground plant tissues measured where plants were	In these experiments, the analysed tissues were from the whole above ground plant and the supernatant was filtered and diluted before measurements. The variation in the size of whole plants was problematic because packing large plants into a tube with the same amount of 1%

	grown in saline treatment and samples analysed using the AAS	HNO_3 for digestion could mean that the digestion is less effective relative to digestion of smaller plants. This could have influenced the extraction of Na^+ and K^+ from the tissue of plants of different sizes. CV% was 20.73 %. There were also problems with drift of the measures of AAS standards (see above).
2	Na^+ and K^+ content in plant tissues measured using flame photometer	An alternative approach was trailed involving measuring on content using a flame photometer. Samples were filtered or centrifuged to remove all residual tissues which may otherwise block the equipment. CV% of Col-0 was 10.93 %
Seed MCS area		
1	Measuring increases in seed maximum cross sectional (MCS) area based on video footage for three genotypes Col-0; *Atpip2;1-1* and *Atpip2;1-2* mutants	For this approach the number of seeds was limited to less than 12 seeds per run while the time for each video recording was approximately 5 days in total which limited the number of samples that could be included. In later experiments, 9 genotypes were used, so the number of experimental batches were too many to record all genotypes and repeats. Air humidity greatly affected the recording process of the seed MCS region. Initially, the petri dish was sealed by micropore tape to prevent water loss, but the microscope light caused condensation under the lid. The images were blurry which reduced accuracy of the measurements. To remove the condensation issues, a small hole (5 x 8 milimeter) was made in the lid of the petri dish to perform the recording process. However, because of the long recording process the loss of water in the agar, caused the images to become out of focus and the image quality was affected, e.g the image was not captured exactly at the maximum of the cross-sectional

		area and was blurry. The duration of footage capture meant that seeds tended to end up lying in the wrong direction, e.g. standing up instead of lying down, because germinated seed moved and this meant that the MCS area was unreliable.
2	Changes in seed MCS area using Image Capture under microscope at certain time points during imbibition and germination	This approach was less time-consuming relative to the first approach. Images were captured at certain time points (1, 4, 24, 30 h) after sowing. These time points were deemed informative from the previous series experiments that involved generating continuous video footage. With this method, more seeds, more genotypes, and greater replication was possible. Each image had about 30 seeds, and a maximum of 12 samples could be captured in 1 h interval. The air humidity was more easily controlled because the petri dishes were contained in a sealed and transparent container, allowing light to pass through but maintaining a constant humidity throughout the experiment.

3.4.3 Determining imbibition and germination phenotypes

3.4.3.1 There was no obvious difference in mucilage patterns in *Atpip2;1* mutant and control lines.

The pattern of seed coat mucilage for Arabidopsis was consistent with previous results reporting staining of seed with ruthenium red and with and without vigorous shaking (Haughn and Western, 2012; Macquet *et al.*, 2007; Phan *et al.*, 2016). When seeds were wetted, they quickly become surrounded by a gelatinous mucilage. Later, a thick cellulose-rich cell wall was extruded that adhered to the seed coat. The outer layer was extruded immediately after coming in contact with water, and this layer was loose and broke in water after shaking (Figure 3.17). The potential influence of mucilage on water uptake could be linked to the increase of seed weight in the first 15 min after sowing of seed (Figure 3.23). Most of the inner layer of mucilage remained attached to the seeds after shaking (Figure 3.18), this layer was present throughout the duration of imbibition of the seed. There were similarities in the mucilage pattern and the timing of release of the outer and inner layers of mucilage in mutant lines and wild type. Therefore, it was expected that the seed coat and mucilage might have similar influences on the dynamics of seed germination in this material in later measurements and was judged not to be a confounding factor in the experiments.

3.4.3.2 Germination percentage differed in material varying in AtPIP2;1 abundance

To score germination, methods from Joosen *et al.* (2010) were applied in which seeds were germinated on blue germination paper (see section 3.2.6.2.2) and images captured by a Nikon camera (section 3.2.7.1). The number of germinated seeds were recorded by the GERMINATOR tool. However, the contrast of images was not sufficient to distinguish between the colour of imbibing seed coats, newly emerged roots and germinated seeds. Moreover, the light reflection on mucilage caused mistakes in counting ungerminated and germinated seeds by ImageJ (Figure S19). Therefore, manual scoring of germination using a binocular microscope was applied during subsequent experiments. The results were accurate but costly in terms of labour. It might be helpful in future to apply tools such as the GERMINATOR or GerminaQuant (Joosen *et al.*, 2010; Marques *et al.*, 2015) for scoring

germination; but these tools would require significant improvements in capacity to capture images with high contrast between seed coat and emerging roots, and require elimination of problematic light reflections in images. Having well characterised control lines that are known to differ in germination percentage would also be useful for experiments ranking germination. For example, transgenic overexpression of Arabidopsis *MYB44* results in sensitivity to ABA during seed germination (Nguyen *et al.*, 2012). Therefore, having both knock-out mutant (*myb44)* and overexpression of MYB44 with the CaMV35S promoter in its mutant background (MYB44/*myb44*) clearly tested the influence on germination percentage, in which overexpression MYB44/*myb44* had significantly lower percentage of germination (15-20%) relative to both WT and mutant *myb44* (48%) (Nguyen et al., 2012). Another example is an experiment of osmotic stress on germination of seed from Liu et al. (2015). The author used a group of genotypes including mutant *Atdsptp1* (higher seed germination percentage, better root elongation under osmotic stress relative to WT); overexpression AtDsPTP1-OE (inhibited seed germination and root elongation); and its complemented line, DsPTP1-Com, (resembles the wild type and rescues DsPTP1-OE under osmotic stress). Those lines had similar high germination percentage under control treatment (>97%) but behaved differently under osmotic stress (330 - 440 mM mannitol), in which Col-0 and DsPTP1-Com performed similarly and significantly differently to mutant and overexpression lines (Liu *et al.*, 2015).

For some halophyte species, treatment of parent plants with saline solutions improves germination percentage of their seed; for example Guo *et al* (2020) reported that seeds from *Suaeda salsa* plants watered with saline water exhibited better seedling emergence and progeny fitness. This is not the case for Arabidopsis, which is no surprise since it is classified as a glycophyte. For Arabidopsis, seeds germinated in saline treatment (e.g. 50 mM NaCl) have been reported to reduce Arabidopsis final seed successful germination percentage and delay germination (Nasri *et al.* (2016), similar to my results (Figure 3.22, table S4).

Whether seed lacked AtPIP2;1 or had greater AtPIP2;1 abundance relative to wild type, the seed germination did not differ in non-saline treatment, such as in water or ½ MS at the stage of three days after sowing (Figure 3.22). However, seeds with greater abundance of AtPIP2;1 (2x35S::AtPIP2;1 OE (21)*) had a higher germination percentage than seeds of Col-0 (WT) and loss-of-function *Atpip2;1-2* when the parent plants were grown in saline treatment (5 mM NaCl) and when the seed was germinated in saline treatment (½ MS + 50 mM NaCl) (Figure 3.22). By the stage of nine days after sowing, when the radicle emerged from success germinated seedlings, these differences were no longer detected, indicating that potentially

whatever benefit there was from having high AtPIP2;1 abundance was not relevant by the stage that the seeds reached nine days after sowing. These tests reveal that overexpression of AtPIP2;1 has the potential to improve germination outcomes for Arabidopsis seed when the parent plants have experienced salt stress and the seeds produced are germinated in saline treatment, at least within the first three days of sowing. These sets of experiments did not reveal germination percentage differences between the loss-of-function *Atpip2;1* mutants and wild type Col-0. In the future it would be ideal to explore whether there are roles for aquaporins, such as AtPIP2;1 in contributing to the mechanisms that enable seed priming to alleviate salinity stress in germinating seeds (Ibrahim, 2016).

3.4.3.3 The timing of seed water uptake was consistent with previous studies

Seed weight gain during imbibition was regarded as being indicative of seed water uptake during imbibition, prior to germination (Bewley, 1997; Hoai *et al.*, 2020; Obroucheva *et al.*, 2017). As expected, the majority of water uptake into the seed happened at the beginning of imbibition, leading to the Arabidopsis seed weight increasing quickly after 4 hours sowing; the weight of all genotypes increased 250 – 350 % relative to dry seeds (Figure 3.23 and 3.24). Data reported for cactus pear also revealed that seeds took up 80% of the water within the first three hours of imbibition (Orozco-Segovia *et al.*, 2007). For Arabidopsis seed, the initial water content increased from ~11% to >82% within the first 24 h during imbibition (Hoai *et al.*, 2020). In previous studies, when seed has been treated with mercury, which can inhibit the transport function of some aquaporins (Jain *et al.*, 2008; Liu *et al.*, 2013a), there was the potential that this treatment could have limited aquaporin water permeability therefore resulting in lower percentages of seed germination than that of non-treated seed (Cardoso *et al.*, 2015; Vander Willigen *et al.*, 2006). However, as mercury has also been reported to increase water permeability for some aquaporins and to inhibit respiration (Zhang and Tyerman, 1999), the use of mercury treatments on seeds provide limited insight into aquaporin roles in seed water uptake, and a genetic approach using loss of function or transgenic overexpression lines has the potential to be more informative (Footitt *et al.*, 2019; Frick *et al.*, 2013).

It is not expected that all aquaporins have roles during seed imbibition, but there is the likelihood that some aquaporins play roles in imbibition and possible that many factors could interfere with these roles. Examples of aquaporins reported to be involved in imbibition stages are Arabidopsis TIP3;1, TIP3;2 and TIP4;1, and the function of these TIPs is influenced by

ABA signals (Footitt et al., 2019; Gattolin et al., 2011). Other potential candidate aquaporins that may be involved in imbibition based on transcript abundance include AtPIP2;1 and AtPIP2;7 (Hoai et al., 2020; Narsai et al., 2017). The function of aquaporins in seed imbibition and germination are likely to be distinctive in every plant species, and the importance of water uptake in each germination stage in different plant species are likely to diverge. Another proposal from previous studies suggests that the role of water uptake in the early germination phase may not be critical in *Arabidopsis thaliana* or *Aesculus hippocastanum* (Obroucheva, 2013; Vander Willigen *et al.*, 2006). However, it was important for pea and tomato in the initial phase of germination to increase the hydration in seeds (Jain *et al.*, 2008; Schuurmans *et al.*, 2003).

3.4.3.4 Relationships between AtPIP2;1 function and water uptake in seedlings

Hypothetically AtPIP2;1 could alter the water influx of germinating seed or seedlings. Previous studies have shown that there is a link between AtPIP2;1 activity in root and shoot hydraulic conductivity of Arabidopsis (Chaumont and Tyerman, 2014; Javot *et al.*, 2003; Sorieul *et al.*, 2011) and rice (Ding *et al.*, 2019; Meng and Fricke, 2017). Comparisons of knock-out mutant *Atpip2;1* and overexpression lines AtPIP2;1 reported here revealed differences in seed weight, percentage weight increase (Figure 3.23, 3.24, table S5), and the seed MCS area during imbibition (Figure 3.33). These data indicated the influence of aquaporin PIP2;1 during water uptake of seed imbibition and germination stages. However, there are other factors that affect water uptake in seed such as temperature, humidity, and nutrient availability (Baskin, 2014; Dubreucq *et al.*, 2010).Water uptake into Arabidopsis seedlings could differ in saline relative to control treatments. AtPIP2;1 function could be implicated in this water influx because saline treatment tend to reduce calcium availability while calcium can influence AtPIP2;1 function by inhibiting AtPIP2;1 ionic conductance (Byrt *et al.*, 2017; Cabanero *et al.*, 2006; McGaughey *et al.*, 2018; Mohamad *et al.*, 2017).

The seed water uptake of all genotypes was reduced under salt stress from 4 - 50 hours imbibition (Figure 3.24 and table S4). It is likely that water efflux occurred when seeds, initially imbibed in water for 30 hours, were transferred to the saline treatment because of the osmotic difference, and consistent with this hypothesis in some cases seed weight decreased (Figure 3.24). However, the germination process did not cease, but was delayed (Chapter 4). This type of delay could cause a reduction in germination uniformity in saline field conditions and late germination of grains, which can limit optimal plant development and root patterning (Riaz *et*

al., 2018; Zörb *et al.*, 2019). Saline conditions can also be toxic to germinating seeds (Khajeh-Hosseini *et al.*, 2003)

The weight of seed from the 2x35S::AtPIP2;1 OE (21)* line was greater than that of Col-0 seed in the control treatment but not in the saline treatments (Figure 3.24). This prompted consideration that hypothetically in the control treatment a greater quantity of water could be taken up by the transgenic seed in a shorter time due to having a greater abundance of AtPIP2;1 in seed cell membranes. To test this, it would be necessary to use approaches that enable better resolution of the movement of water into or out of the seed, from the different genotypes in the control and saline treatments. Potentially in the future, seed hydration in the *Atpip2;1* mutant lines and AtPIP2;1 overexpression lines could be better detected using deuterated water and detection of NMR signals (Di Nola *et al.*, 1991).

3.4.3.5 *Atpip2;1* mutants and overexpressing AtPIP2;1 lines differed in seed and tissue Na$^+$ and K$^+$ content

As expected, mature plants grown in conditions where they were exposed to salt stresses throughout vegetative and reproductive development stages (watered with 25, 50, 75 mM NaCl) accumulated more Na$^+$ and less K$^+$ in both their seed and tissues relative to plants grown in control conditions (Figure 3.26 and 3.29). Previous reports also indicated that Na$^+$ content in flowers, stems and rosette leaves of 5 weeks old plants was greater when plants were exposed to salt treatment (100 mM NaCl) compared to plants in the control water treatment (An *et al.*, 2017). When exposed to saline treatments, plants take up Na$^+$ and distribute the Na$^+$ between different tissues and organs (Maathuis *et al.*, 2014; Munns and Tester, 2008).

The Na$^+$ ion has been described as being toxic due to inhibition of enzyme activity that requires K$^+$, and excess Na$^+$ can also cause osmotic stress (Maathuis *et al.*, 2014). Previous studies have reported that AtPIP2;1 can permeate Na$^+$ and K$^+$ (Qiu *et al.*, 2020). Therefore, *Atpip2;1* mutants have presumably lost the function of a membrane protein that can facilitate some Na$^+$ and K$^+$ transport. A previous report stated that a membrane protein that facilitates Na$^+$ transport is AtHKT1 (An *et al.*, 2017). AtHKT1 is a Na$^+$ transporter that influences Arabidopsis salt tolerance and was reported to be involved in reducing floral Na$^+$ content (An *et al.*, 2017). The mutant *Athkt1,* which lacks the function of the AtHKT1 sodium transporter, was used as a comparison for testing seed phenotypes, and the results revealed that the seed Na$^+$ and tissue Na$^+$ accumulation were similar when comparing between Col-0, *Atpip2;1-1* and *Atpip2;1-2* in

control treatment, but *Athkt1* accumulated notably more Na^+ (Figure 3.26, 3.29, Table S6). This is consistent with results of Arabidopsis from An et al. (2017) where the mutant *Athkt1* contained significantly more Na^+ in stems, flowers and rosette leaves compared to Col-0. In other species like alfalfa, the Na^+ content differed when plants grown in salt stress treatment from 30 mM NaCl up to 150 mM NaCl, with increasing tissue Na^+ content coinciding with increasing salt treatments, while the inversed trend occurred for K^+ content (Li *et al.*, 2010).

Heterozygous AtPIP2;1 overexpression lines 2x35S::AtPIP2;1 OE (7)* and (21)* had a tendency to accumulate more Na^+ and less K^+ in their tissues than wild type controls but the differences were not significant (Figure 3.30, Table S6). This could be because they were still segregating so some of the lines may not have had AtPIP2;1 overexpression.

The function of AtPIP2;1 alone may not be the only factor influencing the plant phenotypes. For example, potentially altering the abundance of PIP2;1 in the mutant and overexpression lines may alter the level of other PIP proteins, e.g. PIP1 (Liu, 2015) and this disruption to PIP protein regulation could have influenced the phenotype of the plants.

3.5. Conclusion

Following testing various approaches for measuring seed weight, area and ion content, final methods were identified that minimised variance for control genotypes (summarised in Table 3.12). A key limitation of the data was that for the trial experiments, to develop the methods only seed from heterozygous lines were available. Whilst methods were being trialled, sufficient seed from homozygous mutant and transgenic AtPIP2;1 overexpression lines became available to examine more definitively whether the abundance of AtPIP2;1 was influencing seed germination, weight, area or ion content. Reporting of the outcomes of applying the final chosen methods to the homozygous material are included in Chapter 4 and Chapter 5.

Chapter 4

Arabidopsis plasma membrane Intrinsic Protein AtPIP2;1 has a salinity conditional effect on seed germination

ABSTRACT

Dynamic changes in aquaporin gene expression occurs during seed germination. For example, the abundance of *Arabidopsis thaliana PIP2;1* transcripts increases in the order of 30-fold within 24 hours of seed imbibition. To investigate whether AtPIP2;1 may be involved in processes that influence seed germination, wild type Columbia-0, loss-of-function *Atpip2;1* mutants and *Atpip2;1-Atpip2;2* double mutants, and transgenic over-expression 2x35S::AtPIP2;1 lines and null controls were grown. Seed from parent plants propagated in controlled treatment (grown in water) was used to test percentage of germination, seed maximum cross sectional (MCS) area, seed weight and seed Na^+ and K^+ content in control (water) and saline (75 mM NaCl) treatments. Seed from mutant lines lacking functional PIP2;1 and/or PIP2;2 proteins, and transgenic lines overexpressing PIP2;1 had delayed germination in saline treatment relative to wild type and null line seed, respectively. Exposure to saline treatment during germination resulted in mutant lines lacking AtPIP2;1 having greater seed weight and less Na^+ than wild type, whereas lines over-expressing AtPIP2;1 had reduced seed weight and greater seed K^+ content than null control line seed. Loss of function and overexpression of AtPIP2;1 could have triggered altered regulation of other aquaporins involved in germination in the mutant and transgenic lines, respectively, and this could have led to the delayed germination, or the delayed germination could be due to differences in AtPIP2;1 contributions to water or ion transport, or signaling. Future research to determine which types of aquaporin functions are key in influencing germination will aid in resolving how different aquaporins influence germination success in saline soils.

INTRODUCTION

Seed germination involves many different cellular activities, such as hydration, mRNA transcription and translation, cell extension, cell cycle activation, mechanisms of reparation and organelle reassembly, and these processes are influenced by hormone signalling and environmental factors (Bewley et al., 2013; Rosental et al., 2014). The first stage of germination is imbibition, which triggers the metabolic activity required to generate the resources for translating proteins, for cell growth and division, and subsequent radicle protrusion (Rosental et al., 2014). Imbibition involves water absorption into a quiescent, dormant and dry seed. Water absorption initiates energy metabolism, regulation of oxidative

status, DNA repair, cell cycle activation, energy reserve mobilization and modification of hormonal status (Lutts et al., 2016). Water influx during seed imbibition is thought to occur mainly via a small opening called the micropyle (Manz *et al.*, 2005). Water also moves across the membranes within imbibing seeds via aquaporins (Bewley, 1997; Fenner and Thompson, 2005; Lutts *et al.*, 2016; Vander Willigen *et al.*, 2006).

Aquaporins belong to an extensive ancient family of major intrinsic proteins (MIPs). They functionate in facilitating passive movement of water and other solutes across membranes in plants and animals. Plant aquaporins can be subdivided into subfamilies according to their sequence homology (Abascal *et al.*, 2014; Chaumont *et al.*, 2001; Tyerman *et al.*, 2021). Seed embryonic axes are known to be equipped with aquaporins (Obroucheva, 2013). Tonoplast Intrinsic Proteins (TIPs) are thought to provide water inflow to enlarging vacuoles in maturing seeds and during vacuole transformation to protein-storage vacuoles, and water outflow from water displacement by deposited proteins (Maurel *et al.*, 1997; Zhang *et al.*, 2007; Zhou *et al.*, 2007). Plasma membrane Intrinsic Protein (PIP) isoforms have been implicated in influencing the seed germination responses to osmotic stress. For example, silencing of *OsPIP1;1* and *OsPIP1;3* reduced seed germination and over-expression of *OsPIP1;3* promoted germination under water-stress conditions (Liu *et al.*, 2007). The roles of OsPIPs in germination have been associated with their responses to nitric oxide and reactive oxygen species signalling (Guha *et al.*, 2021; Matsunami *et al.*, 2021).

In maturing Arabidopsis seed embryos, the transcripts of six aquaporins are notably more abundant than the transcripts of the remaining 35 aquaporins, these include: *PIP1;3, PIP1;4, PIP2;7/2;8, TIP3;1* and *TIP3;2*, while the most abundant aquaporin transcripts in dry seed are *NIP1;2, PIP1;2, SIP1;1* and *TIP3;2* (Hoai *et al.*, 2020). In particular *TIP3;1* and *TIP3;2* accumulate during seed maturation, and these two isoforms along with *AtTIP4;1* have roles influencing germination (Footitt *et al.*, 2019). TIP3 isoforms were shown to act antagonistically to modulate the response of Arabidopsis seed germination to ABA where *TIP3;1* and *TIP4;1* are up-regulated during seed germination under osmotic stress conditions (Footitt *et al.*, 2019). Analysis of Arabidopsis lines lacking or overexpressing TIP3 and TIP4 isoforms revealed that TIPs have roles in sensing of water stress, soil temperature and moisture content (Footitt *et al.*, 2019). During germination *TIP3;1* transcript abundance decreases and transcripts for *PIP2;1, PIP2;7, TIP1;1, TIP1;2* and *TIP4;1* become more abundant indicating that this set of aquaporins are candidates for being involved in the rapid cellular expansion that occurs during post-germination growth (Hoai *et al.*, 2020).

Individual aquaporin isoforms can have multiple roles (Tyerman *et al.*, 2021). For example, AtPIP2;1 has been reported to be involved in transport of water, H_2O_2 and CO_2 in guard cells, and in roots. It also has been reported to be involved in lateral root emergence regulated by auxin as well as proposed as a candidate for having a non-selective cation channel function (Bienert and Chaumont, 2014; Byrt *et al.*, 2017; Grondin *et al.*, 2015; Groszmann *et al.*, 2017; Péret *et al.*, 2012; Rodrigues *et al.*, 2017; Verdoucq *et al.*, 2014; Wang *et al.*, 2016a). Regulation of the expression of *AtPIP2;1* has been observed to be influenced by changes in abscisic acid, ethylene, H_2O_2 and salicylic acid signalling, and AtPIP2;1 permeability has been reported to be influenced by changes in pH and calcium (McGaughey *et al.*, 2018; Qing *et al.*, 2016). These observations reveal that the potential role for AtPIP2;1 in other aspects of plant physiology, such as in seed germination, may be complex. During seed development, dormancy and germination, aquaporins are differentially regulated (Belmonte et al., 2013; Footitt et al., 2019; Hoai et al., 2020; Narsai et al., 2017). When aquaporin water permeability was inhibited using mercury in germinating Arabidopsis seed, the water uptake accompanying expansion and growth was restricted (Vander Willigen *et al.*, 2006). Although aquaporins are expected to contribute to the regulation of water flow in seeds the complement of different roles that aquaporins play during seed germination is yet to be resolved (Gattolin *et al.*, 2011; Hoai *et al.*, 2020; Vander Willigen *et al.*, 2006).

Aquaporins can help germinating seeds to adjust to unfavourable conditions in the environment in which the seed is germinating, such as salinity (Footitt *et al.*, 2019; Liu *et al.*, 2013a). Soil salinity is one of the most common environmental stresses influencing plant performance, particularly seed development and germination (Ayers and Hayward, 1948). Soil salinity can affect seed germination by decreasing water uptake into seed and by causing seed to accumulate NaCl. This decrease in water uptake and accumulation of NaCl can lead to a slower rate of germination, a reduced proportion of successful seed germination and limit early seedling growth. Plants grown in saline treatments experience osmotic stress and stress caused by excess Na^+ and Cl^- ions (Munns and Tester, 2008). Similarly, salinity stress can prevent or delay seed germination either by osmotic effects or ion toxicity (Nasri *et al.*, 2016). Excess salt in the soil inhibits seed water absorption from the external environment (Wang *et al.*, 2021). Previous analysis of the Arabidopsis eFP Browser data (http://bar.utoronto.ca/) revealed that during seed imbibition and germination aquaporins from the PIP2 subfamily are expressed from early imbibition to 48 hours of imbibition, particularly the PIP2;7 and PIP2;1 aquaporins (Hoai *et al.*, 2020). These PIPs are candidates for having roles in water and hydrogen peroxide

permeation, but also monovalent cation permeation that may depend on posttranslational modifications (Byrt *et al.*, 2017; Groszmann *et al.*, 2021; Qiu *et al.*, 2020); while the role of PIP2;7 in Na^+ transport remains unclear (Groszmann *et al.*, 2021; Mohamad *et al.*, 2017). PIPs may also be involved in signalling (Bienert and Chaumont, 2014; Dynowski *et al.*, 2008; Hooijmaijers *et al.*, 2012; Tian *et al.*, 2016). Here we test whether seed lacking AtPIP2;1 and seed over-expressing AtPIP2;1 differ in their percentage of successful germination in control and saline treatments, relative to wild type and null control lines, respectively.

MATERIALS AND METHODS

Plant materials and growth conditions

Wild type *Arabidopsis thaliana* 2x35S::AtPIP2;1-0 seed was sourced from the Arabidopsis Biological Resource Centre (ABRC). T-DNA insertion knockout mutant lines (SALK lines), *Atpip2;1-1* (6AAS98 Amaze) *and Atpip2;1-2* (SM_3_35928) were originally from the Nottingham Arabidopsis Stock Centre and sourced from the Arabidopsis Biological Resource Centre (ABRC) (Da Ines et al., 2010). The *pip2;1/pip2;2* double mutant *Atpip(2;1*2;2)* was generated by crossing *Atpip2;1* (SM_3_35928) and *Atpip2;2* (SAIL_169A03) single mutants (Ceciliato *et al.*, 2019). The 2x35S::AtPIP2;1 (lines 7, 21 and null) overexpression seeds were kindly donated by Dr Michael Groszmann from The Australian National University.

All experiments were performed on stable T3 transgenic plants or confirmed homozygous mutant lines. All mutant and transgenic lines and the wild type (Col-0) plants were grown simultaneously in controlled conditions at the Plant Research Centre, Waite Campus, University of Adelaide, to generate comparable seed for subsequent experiments. Seeds of all genotypes were sterilized with chlorine gas and sown on half strength Murashige and Skoog, pH 7.0 medium with 1% phytagel (½ MS). Seeds were then stratified in the dark at 4°C in for 72 hours to let seeds synchronize before germinating. Imbibed seeds were incubated in long-day growth chambers with temperatures between 19°C – 22°C, continuous light intensity of 90 – 130 μmol m^{-2} s^{-1} and 16 h light/8 h dark for 14 days. At 2-weeks-old, seedlings were transferred to pots (1 per pot, 4 -5 pots per genotype). Pots were 7.5 cm x 6.0 cm and 6.0 cm in height, containing 170 grams autoclaved mix of 'coco peat 6:4' and 'Debco seed & cutting premium germinating mix' at 1:1 ratio. Prior to seedling transplant, 15 pots were placed on a tray and soaked with 2 litres of Imidacloprid 0.1 g liter[-1] (Confidor 200 SC Insecticide, Bayer

Cropscience Pty Ltd) and *Bacillus thuringiensis* 5 mL liter^{-1} (BTI Larvicide 1200 ITU mg-1, Amgrow Pty Ltd). Plants were watered every 2 – 3 days with reverse osmosis water. Young seedlings were covered with a plastic transparent lid for the first 4 weeks to maintain a high humidity for optimal growth. All plants/pots were randomly distributed within trays and their position changed every week to reduce positional biases within the growth chamber (potential for uneven light distribution and air flow variation). When plants grew higher and started flowering, each plant was covered by a plastic transparent floral sleeves 20 cm × 50 cm. After siliques turned dark brown plants were placed in a drying area for 2 weeks at 25°C for desiccation. Seed was collected and stored in enclosed 2 mL centrifuge tubes in the dark, at 22°C ready for seed experiments, which were conducted within 3 months from harvesting.

Seed germination assays

Seeds were sterilized with chlorine gas for 2 hours and kept in enclosed 2 mL tubes at 22°C. 150 – 200 seeds per line were sown on petri dishes (12 × 12 cm) containing ½ MS media, or ½ MS with an additional 75 mM NaCl. The Na$^+$ and K$^+$ concentration in ½ MS is 0.104 mM and 10.03 mM, respectively (Murashige and Skoog, 1962). The concentration of 75 mM NaCl was chosen because previous studies indicated that this concentration is sufficiently stressful so as to influence Arabidopsis germination and seed ion content, relative to standard non-saline conditions, but is not so severe as to completely prevent germination (Mcdowell *et al.*, 2013; Nasri *et al.*, 2016; Ravindran *et al.*, 2020).

Seed of four individual plants from each genotype was sown onto four plates (representative of 4 replications). Plates were sealed with micropore tape and placed in a sealed transparent box to maintain optimal moisture conditions. Stratification and growth chamber conditions were as described above.

Seeds were visualised with a binocular microscope at 24 hours post-stratification and were classified as 'germinated' when their endosperm ruptured (germination *sensu stricto*). Subsequent to the 24-hour timepoint, seeds were further monitored for germination 'success' at 12-hour intervals for up to 4 days, then at 24 hour intervals for the 5, 6 and 9 day time points after stratification or until no change in scores were observed.

Successful seed germination was assigned when the seed clearly showed any of the following plant organs: cotyledons and/or hypocotyl and/or radicle. Seeds lacking cotyledons and/or hypocotyl and/or radicle were regarded as 'ungerminated'.

Seed germination data are represented as percentages of germinated seeds (% germination = (germinated seeds × 100) / total sown seeds) as a function of incubation time post-stratification (Bewley *et al.*, 2013).

Time to reach 50% germination (T_{50}) was calculated according to the formula of Coolbear et al. (1984)

$$T_{50} = t_i + \left[\frac{\left(\frac{N+1}{2}\right) - n_i}{n_i - n_j} \right] . (t_i - t_j)$$

where N is the final number of germinated seed and n_i, n_j are the total number of germinated seeds by adjacent counts at times t_i and t_j, respectively where $n_i < N / 2 < n_j$.

Monitoring changes in seed weight during imbibition

Seeds were weighed (~15 mg) and placed on pre-prepared 25 mm diameter plastic mesh with 75 µm opening pores and 93 µm in thickness (Sefar mesh 03-75/34 x136; Sefar Pty Ltd Filtration and Metal Mesh; http://www.sefa.com.au). The mesh was laid on two layers of 11x11cm blue germination paper (Blue Blotter Paper; Anchor Paper Company, http://www.seedpaper.com) in a 12 x 12 cm petri dish. MilliQ (MQ) water or 75 mM NaCl solution (25 mL) was poured into the Petri dishes and seeds spread as a thin layer on the mesh. Time was recorded upon addition of solutions to ensure consistent seed exposure time.

At 30 minutes, 1 hour, 4 hours, 8 hours and 30 hours after sowing, the plastic mesh containing seeds were collected and placed on thick layer of tissue paper to absorb all the excess water on seed. A very thin and flat spatula was used to collect all the seeds from the plastic mesh and weight was recorded by an electronic semi-micro balance with 0.00001 g resolution (EX125D-OHAUS, Gerard Scientific). Seeds were then placed back on the plastic mesh and filter paper for subsequent exposure times.

Data are represented as a fresh seed weight and percentage weight increase over time.

A fresh seed weight was calculated from the average of 15 mg seed. The number of seed was counted by using ImageJ on captured images (Nikon SMZ-800) at x magnification.

Percentage weight increase over time was calculated as:

$$\%increase = \frac{(weight\ of\ seed - initial\ seed\ weight)}{initial\ seed\ weight} \times 100.$$

Sodium and potassium content in seeds

Seeds for each genotype were weighed (15 mg), distributed on plastic mesh (75 μm pores) and placed on filter papers in petri dishes as described above. MQ water or 75 mM NaCl (25 mL each) were supplied to respective petri dishes for three different imbibing experiments: 1) seeds exposed to MQ water for 50 hours (Water), 2) seeds exposed to MQ water from 0 – 30 hours and then transferred to 75 mM NaCl solution (following Mcdowell *et al.*, 2013; Ravindran *et al.*, 2020) from 31-50 hours (Water-Salt), 3) seeds exposed to 75 mM NaCl for 50 hours (Salt). The selection of the 30 h timepoint for the treatment involving moving seed from water to 75 mM NaCl was on the basis that *Atpip2;1* expression would be expected to double in wild type Col-0 seed between 24 h and 48 h after imbibition, based on previous observations by Klepikova et al (2016) available via http://bar.utoronto.ca/eplant/. Plates with imbibing seed were sealed with micropore tape and placed in long-day chambers set to condition described in the previous section.

After imbibing treatments, seeds were harvested and washed using 5 mL iso-osmotic D-mannitol solution iso-osmotic with 75 mM NaCl (140 mOsmol kg^{-1} measured using a Fiske 210 Micro-Sample Osmometer, Fiske Advanced Instruments, USA). Seeds were placed on 25 mm diameter nitrocellulose membrane filters with 0.45 μm pores (MF-Millipore Tm, Merck Millipore Ltd, Tullagreen, Carrigtwohill, Co. Cork, Ireland) and put in a Millipore Manifold vacuum (XX2702550 Merck Millipore 1225 Sampling Vacuum Manifold, USA) to control the rate that the solution passed through the filter. The washing process happened within one minute per sample to prevent excessive ion loss and ensure uniformity of the wash process between samples. Seeds were oven dried at 65°C for 72 hours before the dry weight was recorded. Dried seeds were digested in 1% (v/v) nitric acid at 80°C for 4 h. The seed Na$^+$ and K$^+$ contents in samples from the seeds were measured using a Sherwood 420 flame photometer (Sherwood, Cambridge, UK) following the manufacturers' instructions.

Statistical analysis

For data analysis the genotype collections were split into two; genotype collection 1 consisted of wild type Columbia 0 (Col-0), *Atpip2;1-1* and *Atpip2;1-2* mutants, and the double *Atpip2;1*2;2* mutant*; and genotype collection 2 consisted of 2x35S::AtPIP2;1 overexpression (OE) line 7, 2x35S::AtPIP2;1 OE line 21 and a null segregant line of 2x35S::AtPIP2;1 which was used as a control because it had been through the same tissue culture process as the two

OE lines. There were three experimental replications for each experiment and the same patterns were observed for each replication. Data was analysed using GraphPad Prism (version 9). The statistical analyses were two-way ANOVA Fisher LSD multiple comparison test, where $p < 0.05$ was considered as significant. Data on graphs are presented as mean +/- SEM.

RESULTS

Percentage seed germination of Arabidopsis lines differing in the abundance of PIP2;1 and PIP2;2

Seed of Arabidopsis Columbia-0 (WT), two *Atpip2;1* lines and one *Atpip2;1*Atpip2;2* loss of function mutant line, and two 2x35S::AtPIP2;1 overexpression (OE) lines and one null of 2x35S::AtPIP2;1 transgenic lines were propagated in water treatment for use in experiments to assess whether the germination of seed from these genotypes differed in control and saline treatments. Seed germination was recorded over 9 days (216 hours) of post-stratification. WT (Col-0), *Atpip2;1* mutants, *Atpip2;1*pip2;2* double mutants, 2x35S::AtPIP2;1 OE lines and a null of 2x35S::AtPIP2;1 seeds were germinated on ½ MS media (control treatment) or ½ MS + 75 mM NaCl treatment (salt – saline treatments). At 24 hours in the control treatment, Col-0 had a significantly higher percentage of germinated seeds (29.5%) compared to the percentage of germinated seed from mutants *Atpip2;1-1* and *Atpip2;1*2;2*, which were in the range of 15.1% - 17.8 %, Figure 1A-B, E-F). At 48 hours, the germination of the null of 2x35S::AtPIP2;1::PIP2;1 in ½ MS was higher compared to all genotypes but not to Col-0, reaching ~98% (Figure 1B, G, H).

In the saline treatment, germination was delayed relative to control treatment, with the 75 mM NaCl treatments resulting in ~ 1% germination at the 24 h timepoint (Figure 1C-D) contrasting to the controls at 24 h where germination ranged between 15-35% across all genotypes (Figure 1A-B). *Atpip2;1-1* consistently had the lowest percentage of germination in salt treatment from 48 hours to 5 days after stratification (Figure 1C-D). The percentage of germination of all genotypes after 9 days of exposure to ½ MS media was over 96.7% (SE = 0.42%), whereas for the 75 mM NaCl treatment the total average germination of all genotypes was reduced to 93.8% (SE = 0.61%).

A non-significant difference in percentage of germination was observed in genotype collection 1 (2x35S::AtPIP2;1-0 (WT), two *Atpip2;1* lines and one *Atpip2;1*pip2;2* loss of function mutant) when grown on control treatment across all time points, whereas under saline

conditions, a reduction in germination compared to 2x35S::AtPIP2;1-0 WT seeds was observed at all measured time points for *Atpip2;1-1*, and at 72 hours for *Atpip2;1-2*. The *Atpip2;1*pip2;2* double mutant line did not have significant changes in germination compared to 2x35S::AtPIP2;1-0 WT seeds whereas the *Atpip2;1-2* mutant had a significantly lower percentage of germination compare to WT in the saline treatment (Figure 1E, G, I).

In genotype collection 2 (two 2x35S::AtPIP2;1 OE lines and null control), the 2x35S::AtPIP2;1 OE seeds (lines 7 and 21) exhibited a significant reduction in percentage of germination relative to the 2x35S::AtPIP2;1 null seed in both control and saline treatments at all measured time points (Figure 1F, H, J).

For the control treatment there were no significant differences between genotypes in both genotype collections for the time taken for 50% of seed to germinate (T_{50}), which was at 29 – 33 hours post-stratification. However in the salt treatment, *Atpip2;1-1* and *Atpip2;1-2* had longer T_{50} times compared to WT (51, 44 and 34 hours, respectively). The OE lines 2x35S::AtPIP2;1 7 and 21 also had longer T_{50} times compared to the null of the 2x35S::AtPIP2;1 (40, 42 and 35 hours, respectively). All genotypes were slower to achieve 50% seed germination in saline than the control treatment (Figure 1K - L).

Key: Genotype collection 1

- ○ Col-0
- △ pip2;1-1
- □ pip2;1-2
- ▽ pip2;1*2;2

Key: Genotype collection 2

- ○ Null:35S::PIP2;1
- ◇ 35S::PIP2;1(7)
- □ 35S::PIP2;1(21)
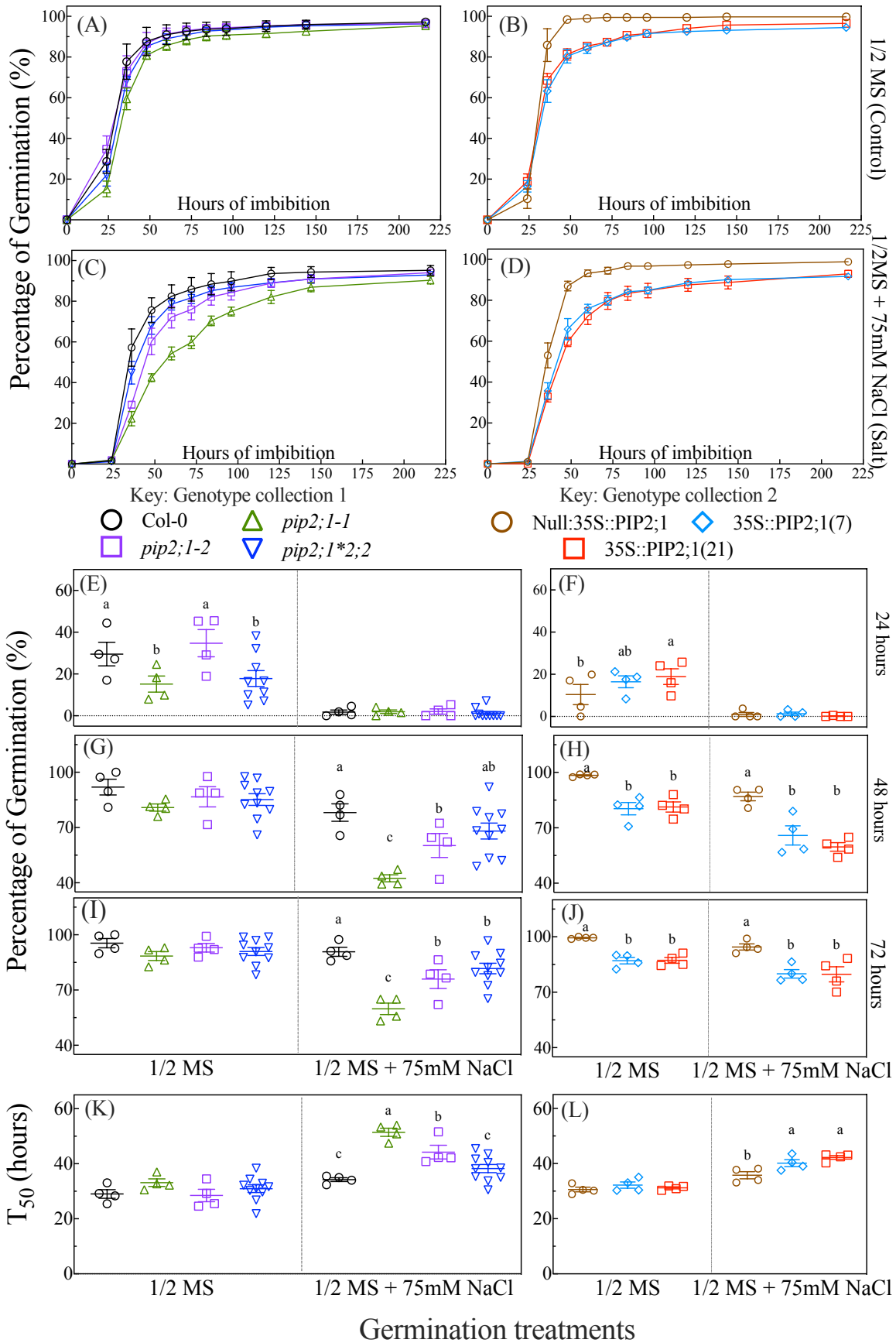
Figure 1. Cumulative germination success and time to fifty percent germination (T_{50}) of Arabidopsis lines differing in PIP abundance in the presence or absence of 75 mM NaCl.

Percentage of seed germinated under control treatment (½ MS) or saline treatment (½ MS + 75 mM NaCl). All the seed with endosperm rupture were recorded at intervals between 0 and 216 hours, upon exposure to 0 mM (A) or 75mM (B) added NaCl treatments. Statistical analysis of percent germination data at individual time points after stratification: 24 hours (panels C-D), 48 hours (panels E-F) and 72 hours (panels G-H) and time to reach fifty percent germination T_{50} in hours (panels I-J). Data was divided into two groups for analysis; genotype collection 1 consisting of Col-0 (○), Mutant *Atpip2;1-1* (△), *Atpip2;1-2* (□), double mutant *Atpip2;1*2;2* (▽), and genotype collection 2 consisted of 2x35S::AtPIP2;1 OE 7 (◇), 2x35S::AtPIP2;1 OE 21 (□) and null of 2x35S::AtPIP2;1 (○). Each panel includes percent seed germination in ½ MS or ½ MS + 75 mM NaCl. Data are means (±SEM) from n = 4 – 10 plates, with 150 – 200 seeds per genotype per plate. Entire experiment were repeated 3 times. Significant difference determined by two-way ANOVA and Tukey's HSD pair-wise tests at significant level 0.05. Different letters indicate a statistical difference between genotypes, absence of letters indicates a non-significant difference. Absence of error bars indicates SE is smaller than the symbol.

Changes in seed weight during imbibition in control and saline treatments

Changes in seed weight and maximum cross sectional (MCS) area (ie projected seed MCS area in images as a surrogate for seed volume assuming isodiametric swelling) during imbibition are indicative of seeds accumulating water and swelling. We observed increases in seed weight and MCS area over time for all tested genotypes in control (½ MS) and saline (½ MS + 75mM NaCl) treatments (Figure S1; Figure S2; Figure S3; Figure S4). The initial seed weight of the various genotypes fell within 17 – 20 µg (n.s.d.). However, the rate of seed/seedlings weight increase differed among genotypes during imbibition and germination over 50 hours.

The *Atpip2;1-1* mutant had a seedling weight greater than that of the Col-0 control and the *Atpip2;1*2;2* double mutant at 50 h of imbibition in 75mM NaCl (Figure 2B). In the water and the Water-Salt transfer treatments, differences were also observed between *Atpip2;1-1* and

Atpip2;1-2 mutant lines, with *Atpip2;1-1* having a higher seedling weight in both treatments after 50h of imbibition (Figure 2B). The weight of the 2x35S::AtPIP2;1 null line increased faster than the weight of the 2x35S::AtPIP2;1 OE lines in all imbibition treatments (Figure 2C).

Seed MCS area increased upon imbibition in the control treatment (½ MS) in all lines tested, with the largest increase observed in genotype collection 1 and occurring within 30 mins of imbibition, increasing from 115,000 – 120,000 to 140,000 – 155,000 μm^2 (Figure S2; Figure S3). The rate of MCS area increase slowed down after 30 min through to the total of 30 h of imbibition, with seeds reaching MCS area of ~180,000 $\mu m ^2$ (Figure S2A).

Seeds of genotype collection 2 had statistically different dry seed sizes (ranging from 11 - 12.5 mm^2), with 2x35S::AtPIP2;1 OE seeds having a smaller seed size than the null control (Figure S3B). In the water treatment the 2x35S::AtPIP2;1 OE lines had statistically significant reduction in water accumulation rates, relative to null control (Figure S3B). At 30 h imbibition the seed MCS area of *Atpip2;1*2;2* was significantly smaller than that of the *Atpip2;1-2* mutant line (Figure S3). The influence of the saline treatments was more evident for seed weight than for seed MCS area (Figure S3C, D; Figure S4). This may be because MCS area was measured from two-dimensional images and thus did not represent seed swelling in all directions.
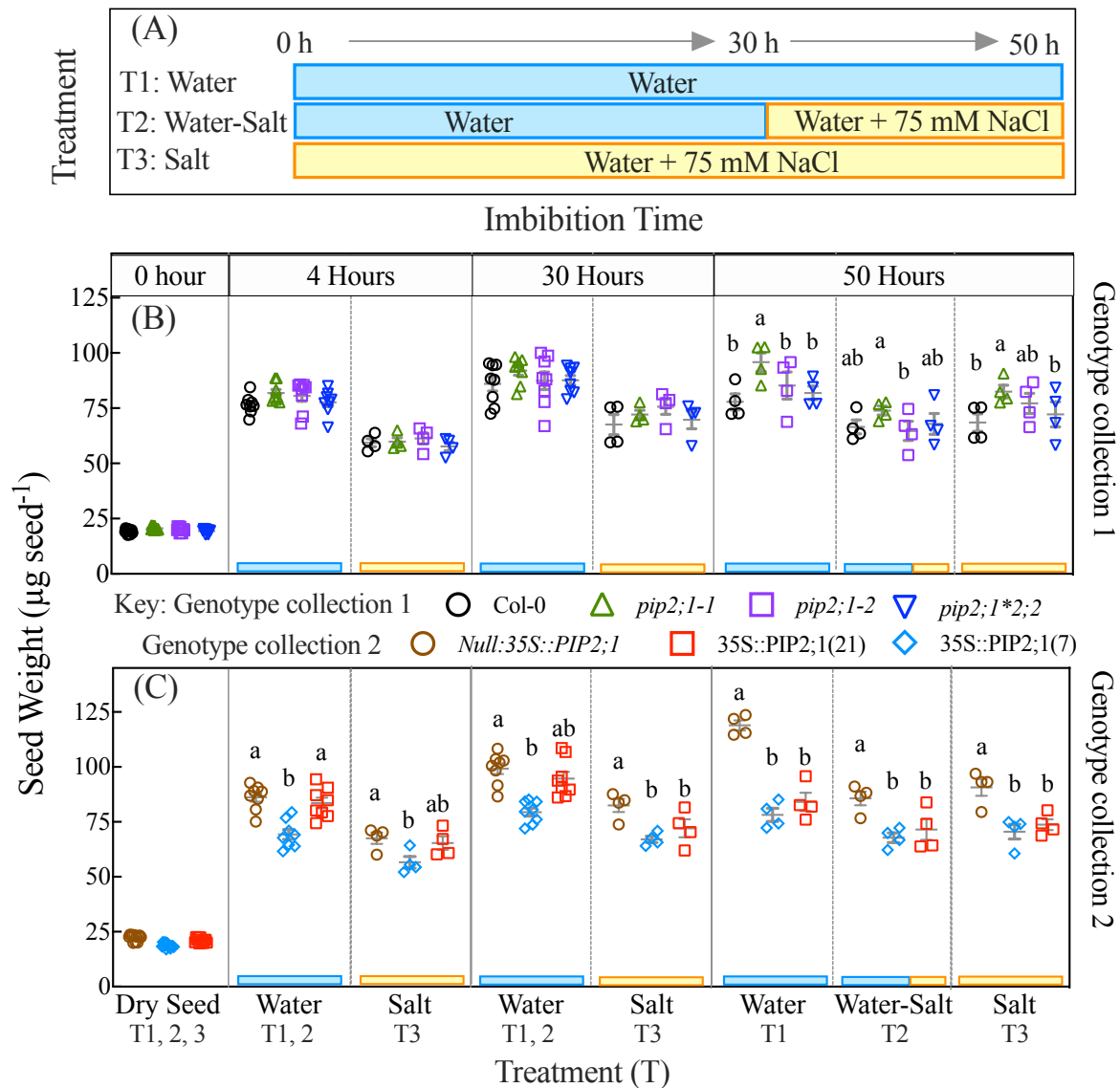
Figure 2. Changes in seed weight over time during imbibition in the control or saline treatment or treatment that changed from water to saline during the course of 50 hours of imbibition.

A. Summary of the experimental set up consisting of 3 treatments: 50-hour imbibition in water only (water; T1); 30-hour imbibition in water followed by 20 hours in water containing 75 mM NaCl solution (50 h total, Water-Salt; T2) and 50 hour imbibition in 75 mM NaCl solution (salt; T3).

B-C. Seed weight increases in two genotype collections over 50 h of imbibition.

Dry seed weight data is the average of 600-900 seeds used per genotype for each experimental replicate (plates), n = 4 – 8 plates per condition. Data are means and standard error of the mean. The experiment was repeated 3 times and showed the same patterns. Significant difference determined by two-way ANOVA and

Fisher's LSD pair-wise tests, $p < 0.05$. Different letters indicate a statistical difference between genotypes, absence of letters indicates a non-significant difference.

Sodium and potassium content in seeds with altered PIP2;1 and PIP2;2 abundance
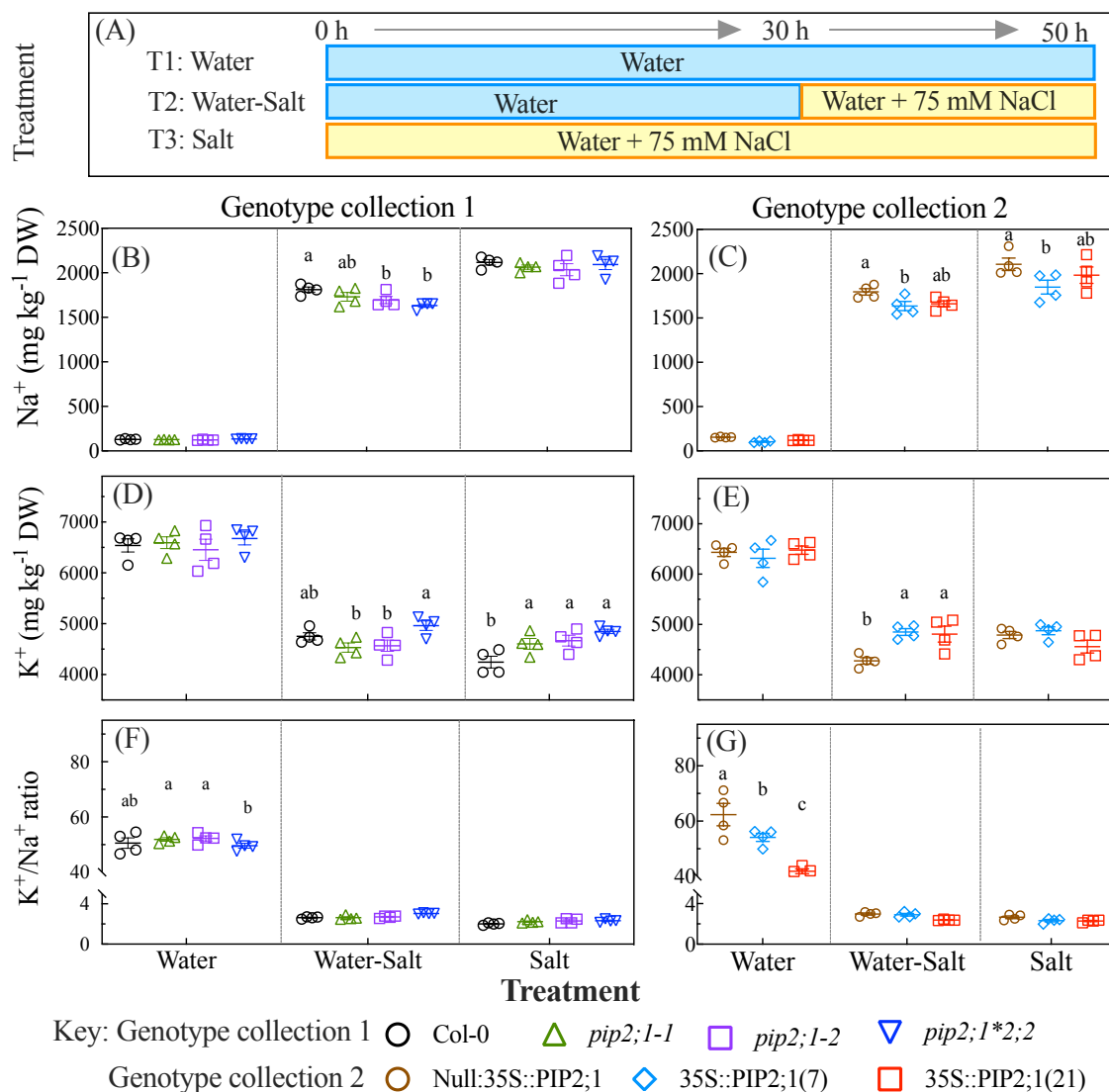
Figure 3. Seed Na$^+$ and K$^+$ content for seven genotypes including loss-of-function mutants and AtPIP2;1 over-expression lines exposed to three treatments: Water for 50 h (Water); 75 mM NaCl solution for 50 h (Salt); or 30 h in water then transferred to a solution containing 75 mM NaCl from 30 to 50 h (Water-Salt) treatments; Na$^+$

content (panels B, C) and K^+ content (panels D, E) and the K^+/Na^+ ratio in seed (panels F, G) following the three treatments (represented in panel A). At some timepoints, the error bars are so tight as to be obscured by the symbols. Significant difference determined by two-way ANOVA and Fisher's LSD pair-wise tests, $p < 0.05$. Ion concentration unit: mg per 1 kg dry weight (mg kg^{-1} DW).

Seeds of AtPIP2;1 loss-of-function mutants and over-expression lines and their respective controls were germinated in three different treatments which were: Water (T1), Water-Salt transfer (T2) and Salt (T3) (Figure 3). As expected, the seeds exposed to saline solutions (T2 and T3) had higher Na^+ and lower K^+ content (per unit dry weight) , compared to seeds germinated in the water treatment (T1) (Figure 3B - E).

There were no differences between the genotypes in relation to the Na^+ or K^+ content of the seeds when germinated in the water (T1) treatment (Figure 3B - E), although the *Atpip2;1*pip2;2* mutant seed did have a lower K^+/Na^+ ratio than the seed for the two *Atpip2;1* mutant lines. Differences in Na^+ and K^+ content were observed between the genotypes for seeds germinated in Water-Salt transfer (T2) and Salt (T3) treatments, and for dry seed prior to imbibition (Figure 3B - E; Figure S5). More specifically, the Na^+ content in Col-0 seeds was higher than that of the *Atpip2;1-2* and *Atpip2;1*2;2* double mutant seed in the Water-Salt transfer (T2) treatments, but there were no differences in Na^+ content between these three genotypes when germinated in the Salt (T3) treatment (Figure 3B). The 35S::AtPIP2;1 null seeds had a higher Na^+ content than 2x35S::AtPIP2;1-7 OE seeds when germinated on the Water-Salt (T2) and the Salt (T3) treatments, and also in dry seed prior to imbibition (Figure 3C; Figure S5).

The K^+ content in seeds of the *Atpip2;1*Atpip2;2* double mutant was higher than that of the *Atpip2;1-1* and *Atpip2;1-2* mutant lines when germinated on the Water-Salt (T2) treatment, but each mutant had a similar K^+ content to the Col-0 control, whereas when germinated solely on Salt (T3), *Atpip2;1 and Atpip2;1*Atpip2;2* mutant seeds had a higher K^+ content than Col-0 control seeds (Figure 3D).

Both 2x35S::AtPIP2;1 OE lines had a higher K^+ content compared to null controls when germinated on the Water-Salt treatment (T2), and there was no difference in K^+ content in these three genotypes when germinated on Salt (T3, Figure 3E)

There were no differences in the K^+/Na^+ ratio in the seed from the Salt (T3) treatment and the treatment involving transfer from water to saline treatment (ratio was 2 -3), but the K^+/Na^+

ratio was higher in the *Atpip2;1-1* and *Atpip2;1-2* mutant lines (51.8, and 52.2 respectively) relative to the *Atpip2;1*2;2* double mutant (49.6) in the water (T1) treatment (Figure 3F). The null 35S::AtPIP2;1 had the largest K^+/Na^+ ratio (62.3) followed by the 2x35S::AtPIP2;1 OE line 7 (54.1) and 2x35S::AtPIP2;1 OE line 21 had the smallest K^+/Na^+ ratio (41.8) (Figure 3F - G).

Comparing measures of seed germination in lines with modified PIP2;1 and PIP2;2 abundance

In the control treatment, the trends in germination and ion accumulation were similar in the *pip2;1* and *pip2;1*pip2;2* mutant lines relative to wild type (WT; Col-0) and similar in the 2x35S::AtPIP2;1 OE lines relative to nulls (Figure 4); but seed weight was higher in the *pip2;1* mutant line than WT and seed weight was lower in the 2x35S::AtPIP2;1 OE line than the null (Figure 4). In the saline treatment, the single *Atpip2;1* mutant line had reduced percentage of germination compared to WT and the 2x35S::AtPIP2;1 OE lines had lower percentages of germination than null controls. This reveals that a decrease or increase in abundance of AtPIP2;1 was associated with a reduced percentage of germination. The *Atpip2;1*Atpip2;2* double mutant and the 2x35S::AtPIP2;1 OE lines had higher K^+/Na^+ ratios relative to their respective controls indicating that disruption of AtPIP2;1/AtPIP2;2 function influenced ion homeostasis in Arabidopsis seed. In germinating wild type Col-0 seed the transcripts of AtPIP2;1 and AtPIP2;7, followed by AtPIP2;2 were observed to be most abundant relative to the other AtPIP2s (Groszmann *et al.*, 2021; Figure S7). However, the relative abundance of transcripts coding for each of the 35 Arabidopsis aquaporins in the mutant and transgenic lines is yet to be determined, and may not necessarily correspond to the protein abundance for each of the respective aquaporins (Liu, 2015), revealing that it may be necessary to test the abundance of the complement of aquaporins in the various genotypes tested to determine the extent to which disruption of AtPIP2;1 function indirectly influences germination due to differential regulation of other aquaporins.

Relative to control [1]	Percentage germination at 72h	T_{50}	Weight at 50h	Na^+	K^+	K^+/Na^+
Dry seed						
pip2;1	n/a	n/a	n/a	n.s.d	n.s.d	n.s.d
pip2;1*2;2	-	-	-	-	-	-
35S::PIP2;1	n/a	n/a	n/a	↓	↓	↑
Imbibition in control conditions						
pip2;1	n.s.d	n.s.d	↑	n.s.d	n.s.d	n.s.d
pip2;1*2;2	n.s.d	n.s.d	n.s.d	n.s.d	n.s.d	n.s.d
35S::PIP2;1	↓	n.s.d	↓	n.s.d	n.s.d	↓
Imbibition in control-saline transfer conditions						
pip2;1	-	-	n.s.d	↓	n.s.d	n.s.d
pip2;1*2;2	-	-	n.s.d	↓	n.s.d	n.s.d
35S::PIP2;1	-	-	↓	↓	↑	n.s.d
Imbibition in saline conditions						
pip2;1	↓	↑	↑	n.s.d	↑	n.s.d
pip2;1*2;2	↓	n.s.d	n.s.d	n.s.d	↑	n.s.d
35S::PIP2;1	↓	↑	↓	↓	n.s.d	n.s.d

[1] For mutant *Atpip2;1* and double mutant *Atpip2;1*2;2,* control is Col-0.
For overexpression 2x35S::PIP2;1, control is null of 2x35S::PIP2;1.

Dry seed   Imbibition   Germination

Figure 4. Summary of comparisons for lines lacking PIP2;1 and PIP2;2 and lines over expressing PIP2;1 relative to controls. Data trends for ion content for dry seed and physiological parameters measured during imbibition including ion content, seed percentage of germination, time to reach fifty percent germination (T_{50}) and seedling weight are represented by either red downward arrows or green upward arrows for mutant relative to Col-0 and transgenic lines relative to null of 2x35S::AtPIP2;1, or not-statistically-different (n.s.d) if no differences were observed. "n/a" and "-" mean "not applicable" and "not tested" respectively.

DISCUSSION

Atpip2;1 loss-of-function mutants and AtPIP2;1 overexpression line seed both exhibited delayed germination relative to controls when imbibed in saline treatment

In Arabidopsis and in cereals, the transcript abundance of various genes encoding for PIP1s, PIP2s and TIPs increase rapidly in seed during imbibition and germination indicating that there are potential roles for multiple aquaporin isoforms from different subfamilies implicated in germination (Matsunami *et al.*, 2021). One example is *AtPIP2;1*, transcript abundance during the time course of imbibition increases between 12 and 48 hours after the start of imbibition, during the time period when germination occurs which is typically around 24 hours after the start of imbibition (Hoai *et al.*, 2020 and Figure S6). Here the function of AtPIP2;1 in seed germination was explored using *Atpip2;1* loss-of-function mutant lines, AtPIP2;1 over-expression lines and their respective control lines. Disruption of wild type PIP2;1 abundance, by either loss of PIP2;1 or increased PIP2;1 abundance, influenced seed germination, seed weight and seed Na^+ and K^+ content when imbibing in the saline treatment (Figure 4). At the stage of 50 hours after stratification, the seed from mutant and over-expression lines did not differ from controls in germination percentage when imbibed in water, but when imbibed in saline treatment, germination percentage of seed from mutant and over-expression lines was delayed relative to wild type and null controls, respectively (Figure 1). This reveals that AtPIP2;1 is likely to have a role in enabling seed to achieve optimal germination success when subjected to saline treatment.

Relative to the changes in weight of wild type control seed, the weight gain during imbibition was either greater or less for seed with reduced or increased PIP2;1 expression, respectively (Figure 2). The loss or overexpression of PIP2;1 also resulted in reduced seed Na^+ content and greater seed K^+ content in mutant and transgenic lines relative to controls (Figure 3). Hypothetically, greater abundance of PIP2;1 in seed might be expected to be associated with more rapid water uptake during imbibition and loss of PIP2;1 might be expected to limit water uptake during imbibition. It might be expected that water uptake would be reflected in seed weight increases but the trends in seed weight observed were not consistent with this hypothesis. Instead lines over-expressing AtPIP2;1 had reduced weight and loss-of-function mutants had greater weight gain during imbibition (Figure 2). This indicates that the putative role for AtPIP2;1 in seed germination may not be related to influencing water permeability.

AtPIP2;1 is permeable to water, CO_2, H_2O_2 and ions, and altered regulation of AtPIP2;1 could influence permeability to different substrates in different ways (Qiu *et al.*, 2020). AtPIP2;1 function is known to be influenced by differences in hormones and signalling molecules (Daszkowska-Golec, 2011; Wojtyla *et al.*, 2016). Moreover, regulation of AtPIP2;1 is known to influence the regulation of other aquaporins (Liu, 2015). Therefore, there may be a range of other potential factors that could explain why germination of seed with altered AtPIP2;1 abundance is delayed in saline treatment. These hypothetical factors have been categorised as follows:

- Transport of either Na^+ or K^+ via AtPIP2;1 could be important in seed germination in conditions of salt stress (Qiu *et al.*, 2020).

- The contribution of AtPIP2;1 to signalling processes, such as distribution of H_2O_2 signals, could be important in seed germination (Groszmann *et al.*, 2021).

- Disruption of AtPIP2;1 could cause differential regulation of other aquaporins which may influence seed germination (Da Ines, 2008).

Implications for seed germination associated with AtPIP2;1 water and ion permeability

Hypothetically changing the abundance of AtPIP2;1 in seed could influence either seed swelling, or radicle cell expansion, during seed imbibition or germination. Previous studies have demonstrated using either heterologous expression systems or whole Arabidopsis plants that AtPIP2;1 influences water flux, therefore it is feasible that AtPIP2;1 may also influence water flux in germinating seeds. For example, when AtPIP2;1 was expressed in *Xenopus laevis* oocytes those oocytes swelled more rapidly than control oocytes when transferred to a hypo-osmotic solution due to AtPIP2;1 facilitating water influx into the oocyte (Byrt *et al.*, 2017; Mahdieh *et al.*, 2008; Prado *et al.*, 2019; Qiu *et al.*, 2020; Wang *et al.*, 2016a). Differences in relative water flux in *Atpip2;1* and *Atpip2;2* loss-of-function mutant plant rosettes relative to wild type controls has also been reported using deuterium as a tracer (Da Ines *et al.*, 2010). The Da Ines et al. (2010) study of Arabidopsis rosette water flux revealed that loss of AtPIP2;1 and AtPIP2;2 in different tissues and cells contributed in an integrated fashion to the reduced water flux in intact plants. Here seed swelling of lines with varying AtPIP2;1 or AtPIP2;1 and AtPIP2;2 abundance was investigated by measuring changes in seed area over time when seed was imbibing in water or in saline treatments (Figure S2 and Figure S3). The seed area of

Atpip2;1*pip2;2 mutant lines was similar to that of wild type and *Atpip2;1* mutant lines during the first four hours of imbibition, but at 8 and 30 hours imbibition the relative increase in area of the *Atpip2;1*pip2;2* mutant was less than that of wild type and *Atpip2;1* lines (Figure S2). The influence of these PIP2s may have potentially differed in different cell types within the swelling seed during the period of imbibition and germination. Hypothetically poorly optimised swelling between different cell types caused by disrupted AtPIP2;1 abundance could have limited the germination efficiency of the mutant lines (Figure 1).

The abundance of *AtPIP2;1, AtPIP2;2* and *AtPIP2;7* in germinating Arabidopsis Columbia-0 seed increased after 3 days soaking in water. The transcript level of these genes were very high in roots, while in hypocotyls, cotyledons and the shoot apical meristem, the transcripts levels were different (Figure S6) (Klepikova *et al.*, 2016). This result matches with the finding of salt treatment (100 mM NaCl) caused delay in emergence of lateral roots in Arabidopsis seeds because of the possible induce thickening of the Casparian strip (Duan *et al.*, 2013).

The relationship between seed weight and seed MCS area was positive at the zero time point (dry seed) and at timepoints of 4 and 30 hours into imbibition, under water and saline treatments (Figure S4). These relationships were fitted with linear regression lines ($Y=B_0 + B_1*X$, where Y is, seed weight (µg/seed), X is the seed MCS area (µg^2/seed), B_0 is the intercept of dry seed weight, B_1 is the slope of the lines). For values from seed measurements taken at the zero time point (Figure 4S.A), a single linear line of MCS versus time adequately fits all the data in each case (Prism F test), and at the later time points (Figure 4S.B) a single linear line of MCS versus time adequately fits all the data collected for water and salt treatments. However, different linear lines were required to fit the data for measurements taken following the water to 75 mM salt treatments. There were significant differences in the linear regression lines between 0 mM and 75 mM for P2 but not for P1. Lighter seed weight in the samples from the 75 mM NaCl treatment compared to 0 mM NaCl treatment was observed at the same area, or at the same seed weight the area is larger in the 75 mM NaCl.

There was a delay in the time to reach 50% germination associated with the 75mM NaCl treatment in both the single mutant *pip2;1* and overexpression line but not in the double mutant *Atpip2;1*2;2* compared to wild type Col-0. Germination percentage of each single mutant *pip2;1* was lower than wild type Col-0, but there was a recovery of germination percentage in the double mutant *Atpip2;1*2;2* compared to wild type Col-0 at 48 hours imbibition. Previous studies have revealed that salt stress delays seed germination. For example, Bafoil *et al.* (2019)

revealed that seed in 50 mM NaCl had delayed testa rupture relative to seed in conditions without NaCl treatment.

AtPIP2;1 responses to seed signalling could have influenced seed germination

Hydrogen peroxide has multiple signalling roles in seeds including influencing seed germination and responses to stress such as salt stress, and AtPIP2;1 is permeable to H_2O_2 (Dynowski *et al.*, 2008; Groszmann *et al.*, 2021; Hooijmaijers *et al.*, 2012; Wojtyla *et al.*, 2016). Hypothetically AtPIP2;1 H_2O_2 permeability could influence how seed germination responds to stress and this could have been a factor influencing the differences in germination in the mutant and transgenic seed in saline treatments (Figure 1). Seed dormancy and germination are known to be regulated by complex signalling processes that involve other phytohormones and these signals may also influence AtPIP2;1 function in seed. Germination is influenced by the abundance of ABA, while gibberellins (GA) and ethylene can restrict the effect of ABA on dormancy and instead GA and ethylene promote germination. Nitric oxide (NO) is proposed to be implicated in the cross talk between ABA and ethylene in seed where NO treatments also tend to promote germination (Arc *et al.*, 2013b; Li *et al.*, 2015), and potentially some aquaporins may be permeable to NO (Wang and Tajkhorshid, 2010). Ethylene treatment was reported to influence the C-terminal phosphorylation of AtPIP2;1 which can influence the proportion of AtPIP2;1 in cell plasma membranes and this impact upon water and ion transport (Qing *et al.*, 2016; Qiu *et al.*, 2020). This means that where there is potentially greater ethylene accumulation under salt stress this could influence abundance and function of AtPIP2;1 (Wilson *et al.*, 2014).

Auxin is a plant growth hormone which acts as an adaptable trigger for controlling many developmental processes (Vanneste and Friml, 2009). Auxin plays a critical role in Arabidopsis seed dormancy that is independent of ABA but requires the ABA signaling pathway (Liu *et al.*, 2013b). Previously Peret et al. (2012) reported that lateral root emergence is delayed or slowed when AtPIP2;1 abundance is either reduced or increased, in Arabidopsis loss-of-function or overexpression lines, respectively and that auxin signalling influences AtPIP2;1 contribution to hydraulic conductivity to regulate lateral root emergence. Auxin signalling also influences directionality in relation to root growth (Hu *et al.*, 2021; Vanneste and Friml, 2009). The analysis of AtPIP2;1 roles in germination here reveals a potential trend for influencing

radicle emergence which is reminiscent of the observations for roles of AtPIP2;1 in influencing lateral root emergence, where AtPIP2;1 loss-of-function and overexpression lines both have delayed root emergence. To further study whether AtPIP2;1 has a role in radicle emergence it would be ideal to capture high resolution images of radicle emergence in the loss-of-function *Atpip2;1* and AtPIP2;1 overexpression lines relative to controls in non-saline and saline treatments.

Disruption of AtPIP2;1 and the potential for differential regulation of other aquaporins which influence seed germination

There are 35 aquaporin encoding genes in Arabidopsis and transcripts of around half of these genes have been detected in seeds during either seed development or germination, but the trends in aquaporin gene transcript abundance differ during different stages of development and phases of germination (Hoai *et al.*, 2020). For example, the abundance of transcripts coding for AtNIP1;2, PIP1;2, and TIP3;1 are particularly high in dry seed, whereas transcripts coding for PIP2;1, PIP2;7, TIP1;1, TIP1;2, and TIP4;1 increase either at or immediately after germination (Footitt *et al.*, 2019; Hoai *et al.*, 2020). At the stage of 24 h after the start of imbibition *AtPIP1;2* and *AtPIP2;7* transcripts were most abundant, and by 48 h *AtPIP1;2, AtPIP2;7* and *AtPIP2;1* transcript abundance was notably higher than that of other AtPIPs (Groszmann *et al.*, 2021). This indicates that different aquaporins are required during seed development relative to seed germination, and furthermore it is possible that different aquaporins are required during different phases of seed germination.

The three phases of germination can be summarised as follows (Bewley, 1997; Weitbrecht *et al.*, 2011): Phase I involves seed swelling; volume and shape change and solute leakage (Crowe and Crowe, 1992; Niemann *et al.*, 2013; Simon and Harun, 1972; Simon and Mills, 1983; Van Steveninck and Ledeboer, 1974). In seeds such as white spruce (*Picea glauca* (Moench) Voss) seed K^+ loss during imbibition has been reported, indicating the potential for there to be a role in membrane proteins permeable to K^+ in this process (Reid *et al.*, 1999). Phase II involves rehydration; solute leakage; resumption of respiration and oxygen consumption; activation of amino acid metabolism and Kreb's cycle; mRNA, DNA and protein synthesis; formation of new mitochondria; substrate and small molecule diffusion as well as organelle assembly. Phase III involves cell division; testa rupture and radicle emergence. Previous analysis of aquaporin roles in these phases by Willigen et al. (2006) led to the conclusion that aquaporin functions

are unlikely to be involved in Phase I, and are more likely to be involve in Phase III. Potentially TIP3s might be the only AQPs involved in mediating seed water uptake in Phase I, but if this was the case they would need to be located in the plasma membrane to contribute (Gattolin *et al.*, 2011). Multiple PIP and TIP1 coding transcripts are abundant during germination, but some polypeptides do not become detectable until approximately 60 h post germination, well into phase III of water transport in the seed (Gattolin *et al.*, 2011). In the context of seed weight changes during imbibition and germination, which may be indicative of solute flux changes, differences between loss of function mutant lines and wild type were not observed until 50 h after imbibition, where one of the two *Atpip2;1* loss of function mutant lines exhibited greater seed area than wild type controls (Figure 2B). Whereas for AtPIP2;1 overexpression lines differences were observed 4 h after imbibition, and the transgenic overexpression line seed did not gain weight as quickly as null control line seed (Figure 2C). It is difficult to determine which phases of seed germination AtPIP2;1 may play a role in when reviewing the differences in the timing onset of phenotypic differences for mutant and overexpression line material relative to controls.

As there are hundreds of proteins that could potentially interact with AtPIP2;1 the implications of loss of AtPIP2;1 function or overexpression of AtPIP2;1 could potentially influence the function of many other proteins. For example, 400 potential AtPIP2;1 and AtPIP2;2 interacting proteins were reported by Bellati et al (2016) and their analysis revealed TIP2;2 as a potential interacting partner, revealing that PIP abundance could impact TIP regulation, and TIPs including TIP3;1, TIP3;2 and TIP4;1 were previously reported to significantly influence Arabidopsis seed germination in response to stress (Footitt *et al.*, 2019). In cereals, such as barley and rice, some of the trends in aquaporin regulation in seeds are similar to those in Arabidopsis, for example there are key TIPs such as HvTIP3;1 and OsTIP3;1 that are abundant in seeds, and OsTIP3;1 like AtTIP3;1 is influenced by ABA signalling (Han *et al.*, 2018; Takahashi *et al.*, 2004; Utsugi *et al.*, 2015). Transcripts of rice PIPs including PIP1;1, PIP1;2, PIP1;3 and PIP2;8 were reported to increase in abundance in germinating seed in response to nitric oxide treatments, further implicating PIPs in the processes that influence seed germination (Liu *et al.*, 2007). Loss of PIP1;1 and PIP1;3 function in rice led to reduced seed germination and PIP1;3 overexpression promoted seed germination under water-stressed conditions (Liu *et al.*, 2007). Therefore, changes in the abundance of PIP2;1 and PIP2;2 may influence the regulation of other aquaporins during seed germination, especially in the case of the double *Atpip2;1*pip2;2* mutant where there may be differences in the abundance of other

PIPs relative to the abundance of various PIPs in the single mutant lines. The potential for different regulation of other aquaporins in the single and double PIP2 mutants studied here could in part explain why there were different trends in the phenotypes of these different mutant lines. For example, the germination, weight and ion content data for the double mutant was more similar to wild type than the data for the single *Atpip2;1* mutant (Figure 1, 2, 3).

Narrowing in on AtPIP2;1 roles in seed germination

There is definitely a role or possibly multiple roles for AtPIP2;1 in seed germination, but the exact nature of this role or roles remains unclear. The role could be related to membrane transport or related to signalling. For example, the rose (*Rosa*) RhPIP2;1 serves as a key player in orchestrating trade-off between growth and stress survival (Zhang *et al.*, 2019). The next steps towards determining AtPIP2;1 role(s) in seed germination could involve:

- Testing whether *Atpip2;1* mutants and AtPIP2;1 overexpression lines have delayed germination in non-saline osmotic stress, such as treatments involving germination tests in solutions containing sorbitol, mannitol or polyethylene glycol (PEG) (Liu *et al.*, 2013c). This may help resolve whether the role of AtPIP2;1 in germination in saline conditions relates to sensing osmotic stress or contributing to osmotic adjustment.

- Testing whether there are differences in the flux of Na^+ and K^+ from the radicle of *Atpip2;1* mutants and AtPIP2;1 overexpression lines relative to controls, by using approaches such as microelectrode ion flux estimation (Shabala *et al.*, 2003).

- Testing the extent to which germination of *Atpip2;1* mutants and AtPIP2;1 overexpression lines and control lines change if their seed are exposed to hormone treatments during germination to explore the potential interaction between AtPIP2;1 regulation in response to hormones and regulation of germination.

- Testing the transcript abundance of the complement of Arabidopsis aquaporin encoding genes during seed imbibition and germination in *Atpip2;1* mutants and AtPIP2;1 overexpression lines to determine the extent to which the regulation of other aquaporins changes when the abundance of AtPIP2;1 is disrupted.

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SUPPLEMENTARY FIGURES
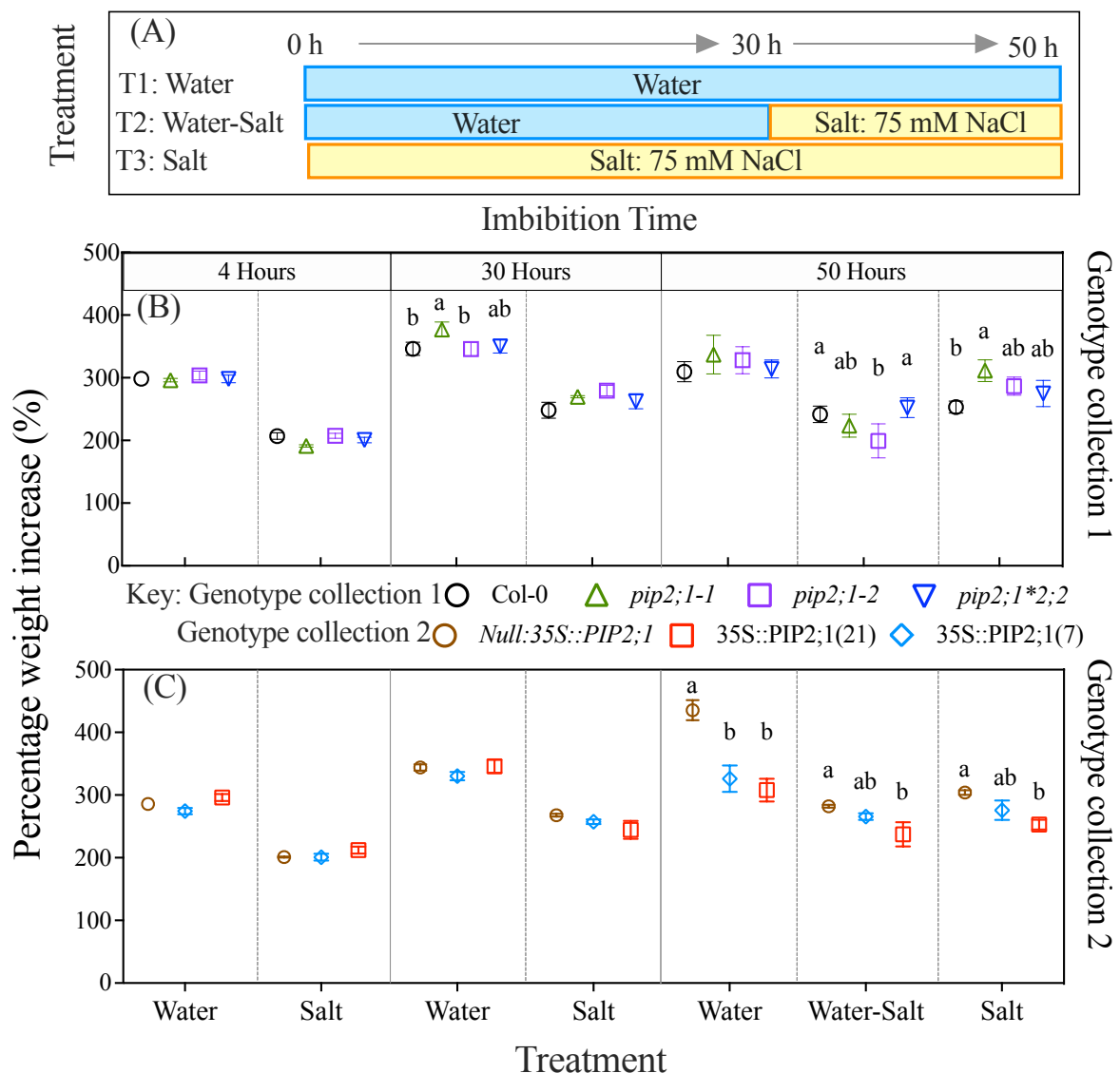
Figure S1. Seed weight increase in percentage relative to fresh dry seed.

A, Summary of the experimental set up with 3 treatments: Treatments consisted of 50-hour imbibition in water only (Water; T1); 30-hour imbibition in water followed by 20 hours in 75 mM NaCl solution (50 hour total, Water-Salt; T2) and 50 hour imbibition in 75 mM NaCl solution (Salt; T3). B-C, Relative increase in seed weight compared to the fresh dry seed weight during 50 hours of imbibition in two genotype collections. Genotype collection 1 consisted of Col-0 (○), Mutant *Atpip2;1-1* (△), *Atpip2;1-2* (□), double mutant *Atpip2;1*2;2* (▽), and genotype collection 2 consisted of 2x35S::AtPIP2;1 OE 7 (◇), 2x35S::AtPIP2;1 OE 21 (□) and null of 2x35S::AtPIP2;1 (○).
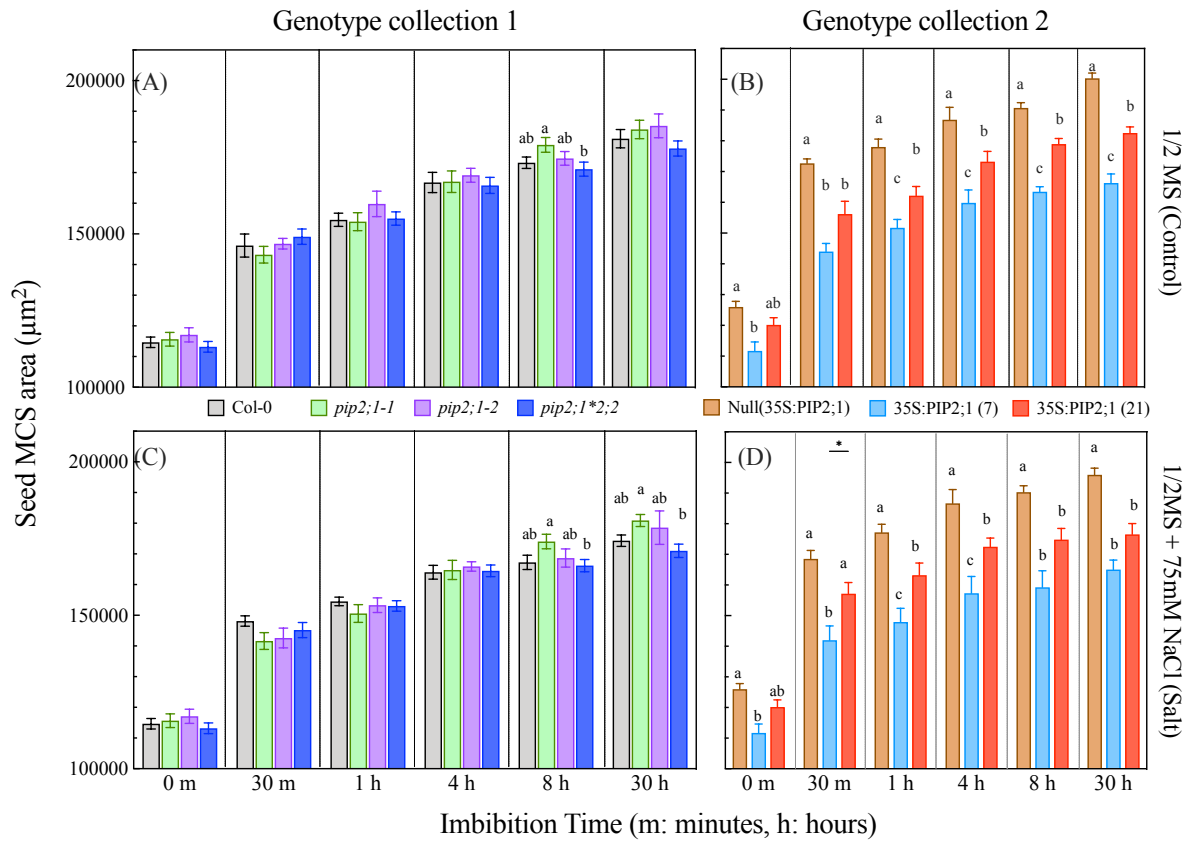
Figure S2: Seed MCS area increase upon imbibition in control (½ MS - panels A and B) and saline treatment (½ MS + 75 mM NaCl - panel C and D).

Data are means (±SE) from n=4-8 plates, with 200 seeds per genotype per plate. At some time points the error bars are so tight as to be obscured by the symbols. Significant difference determined by two-way ANOVA and Fisher's LSD pairwise tests.
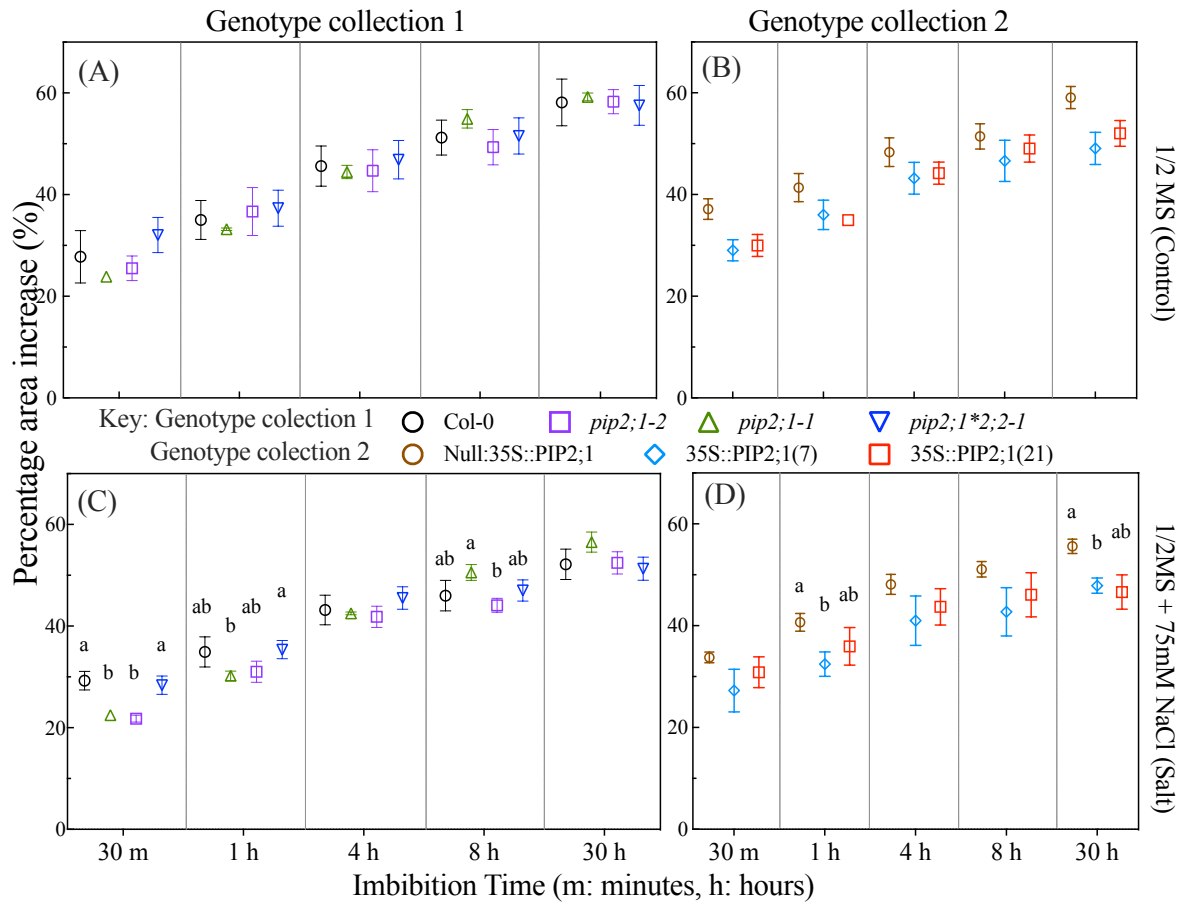
Figure S3. Changes in percentage of imbibing seed MCS area over time compared to seed which had been imbibing for two minutes.

Data is divided into two groups: Genotype collection 1 consisted of Col-0 (O), Mutant *Atpip2;1-1* (△), Atpip*2;1-2* (□), double mutant *Atpip2;1*2;2* (▽), and Genotype collection 2 consists of 2x35S::AtPIP2;1 OE 7 (◇), 2x35S::AtPIP2;1 OE 21 (□) and null of 35S::AtPIP2;1 (O).
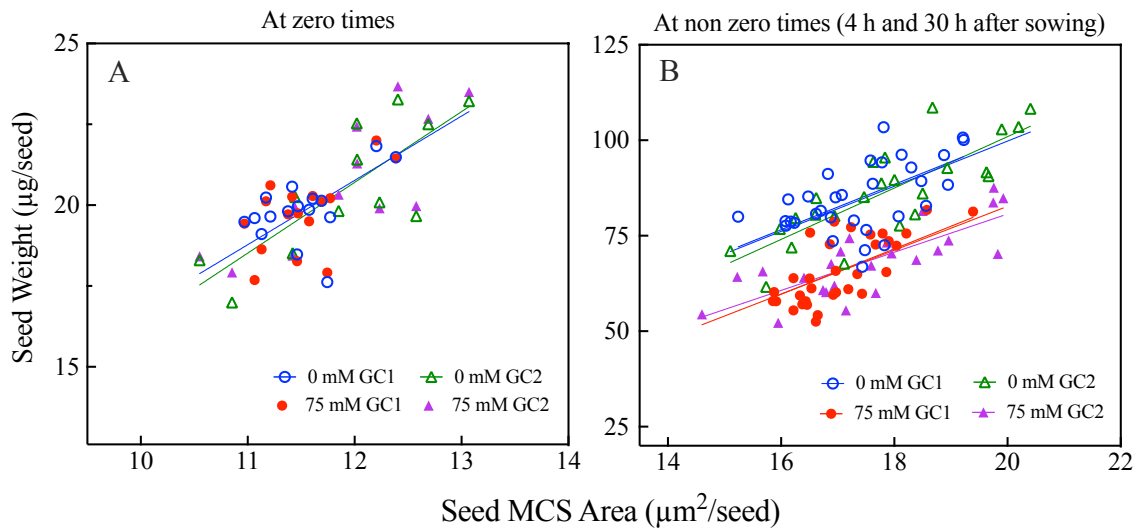
Figure S4. Positive relationships between seed MCS area and its weight change from dry seed to 30 hours imbibition of two genotype collections. Genotype collection 1 (GC1) consists of WT Col-0, mutant *Atpip2;1-1*, *Atpip2;1-2*, double mutant *Atpip2;1*2;2*, and genotype collection 2 (GC1) includes 2x35S::AtPIP2;1 OE 7, 2x35S::AtPIP2;1 OE 21 and null of 35S::AtPIP2;1. Data at zero times (0 h) (Figure S4A) and at non zero times (4 and 30 h) (Figure S4B) after the start of imbibition are shown. As seed swells during imbibition, it is expected that both the weight and seed area would increase, so the positive relationship was expected. The seed area measures that were captured represent maximum cross-sectional (MCS) rather than total seed area because images of seed swelling were captured from an aerial view which does not capture vertical swelling, hence it was expected that the R^2 values would be less that 1.
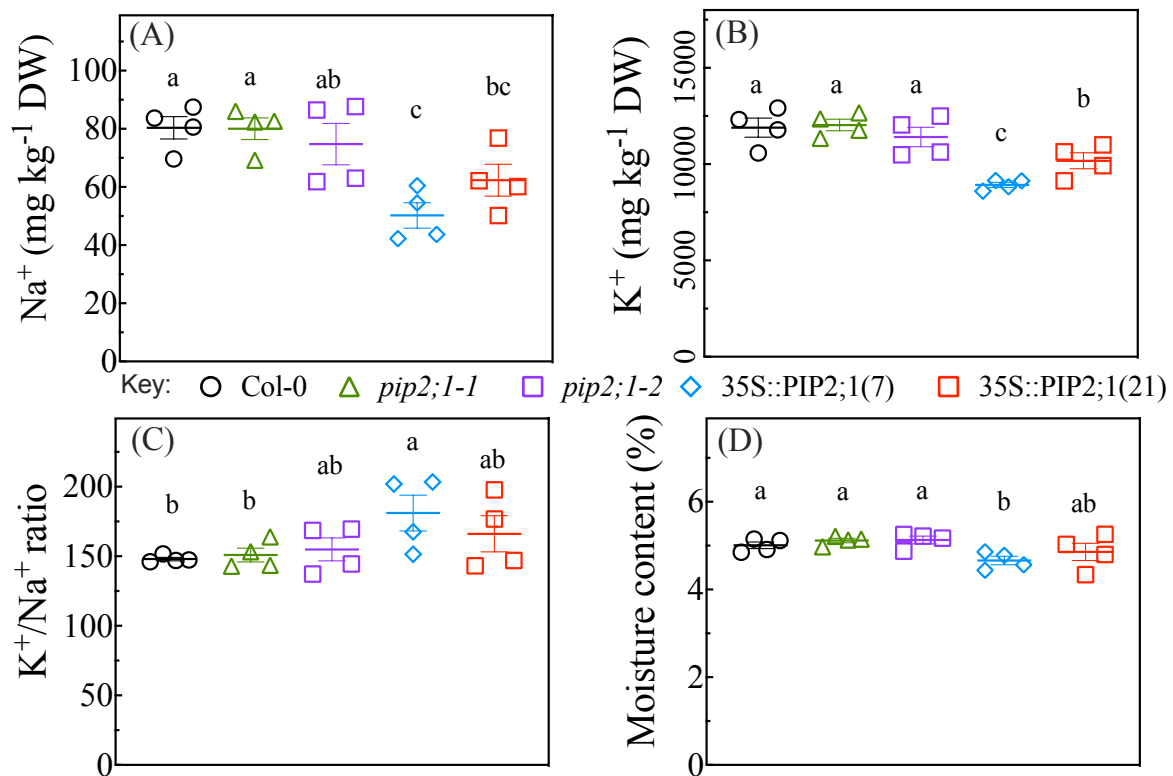
Figure S5. The K⁺ and Na⁺ content and moisture content of fresh dry seed grown in control (water) conditions.

A-C, The K⁺ and Na⁺ ion content and the ratio of ions in seed of five genotypes where parent plants were grown in control conditions and seeds were collected when mature and dry. D, Moisture content of seed (%) = (fresh dry seed weight – oven dried seed weight) x 100/ fresh dry seed weight. Seeds were weighed, dried in an oven temperature at 65°C in 72 hours and then dried seeds were weighed. Data for Col-0 (O), Mutant *Atpip2;1-1* (△), Atpip*2;1-2* (□), 2x35S::AtPIP2;1 OE 7 (◇) and 2x35S::AtPIP2;1 OE 21 (□). Data points are means and s.e.m. (n = 4 plates per condition, 600 - 900 seeds per genotype in each plate). The experiment was repeated 3 times. Significant difference determined by two-way ANOVA and Fisher's LSD pair-wise tests. This seed is a different batch of seed relative to seed in Figure 3.

Days after soaking	Dry	1	2	3		SAM	Hypocotyl	Cotyledons	Root	Root without apex	Root with apex
AtPIP2;1	2.09 ± 0.31	1.85 ± 0.85	66.08 ± 0.21	148.83 ± 1.59		16.16 ± 5.71	194.34 ± 21.5	95.11 ± 24.12	432.09 ± 2.41	232.34 ± 8.57	432.97 ±179.54
AtPIP2;2	1.72 ± 0.44	1.43 ± 0.62	18.45 ± 1.47	137.47 ± 1.68		6.88 ± 2.06	51.66 ± 1.22	108.29 ± 5.53	758.83 ± 34.2	537.79 ± 16.72	774.5 ±298.93
AtPIP2;7	0.52 ± 0.1	106.07 ± 12.97	145.97 ± 0.33	196.36 ± 19.72		203.23 ± 64.68	79.59 ± 20.02	238.05 ± 2.43	265.14 ± 8.67	253.92 ± 7.59	453.93 ± 59.5

Figure S6. Summary of previously reported endogenous transcript abundance of *AtPIP2;1, AtPIP2;2* and *AtPIP2;7* (Gene ID: AT3G53420, AT2G37170 and At5g05290 respectively) in germinating Arabidopsis Columbia-0 seed. There are five PIP1 and eight PIP2 encoding genes in Arabidopsis and of the eight PIP2s the transcripts of AtPIP2;1 and AtPIP2;7 were most abundant at 48 hrs into germination (see Figure 9 of Groszmann et al. (2021). Transcript data values are from Klepikova et al (2016) available via http://bar.utoronto.ca/eplant/.

Chapter 5

Characterisation of overexpression Arabidopsis UBI:nGFP::PIP2;1 seed during germination and under saline conditions

ABSTRACT

Green fluorescent protein (GFP) is widely used as a molecular tool to evaluate protein expression and localization (Chalfie *et al.*, 1994; Luu *et al.*, 2012; Monici, 2005; Zimmer, 2002). In Arabidopsis, GPF labelled AtPIP2;1 overexpression lines UBI:nGFP::PIP2;1 could be used as a tool to explore how AtPIP2;1 overexpression influences the phenotype of Arabidopsis plants, where the localisation of AtPIP2;1 can also be reviewed by imaging GFP fluorescence. Here the expression of UBI:nGFP::PIP2;1 driven by the Ubiquitin-10 gene promotor (PUBQ10) was checked in putative transgenic lines using BASTA spray, laser scanning confocal microscopy and polymerase chain reaction (PCR) assays. Homozygous plants were used to localize the AtPIP2;1 protein in germinating seed by laser scanning confocal microscopy. In imbibing seed UBI:nGFP::PIP2;1 expression was clearly observable from 30 hours after sowing in root tips, root stele, developing stems, and developing guard cells, and was especially abundant in root cell membranes. Seeds of lines overexpressing UBI:nGFP::PIP2;1 exhibited delayed germination relative to wild type (WT, Col-0) lines, and had longer T_{50} in saline treatment (75 mM NaCl) compared to WT. Criteria related to AtPIP2;1 contribution to water, ion and H_2O_2 transport in transgenic seeds during imbibition and germination were considered in this study, along with the potential influence of the fusion of the nGFP.

Keywords: **GFP, fluorescent protein, aquaporins, germination seed, seedlings, saline treatments.**

INTRODUCTION

Location of aquaporins in the plant at the cell, tissue and organ level can depend on the particular type of aquaporin as well as developmental changes and in response to stress. The modification of an overexpressed protein by tagging for visualisation may also affect the function of the protein (Ikeda et al., 2002; Liu et al., 2020; Weill et al., 2019; Zhang et al., 2014). This may be important particularly for UBI:nGFP::PIP2;1, in relation to the phenotyping under control and saline stress treatments. UBI:nGFP::PIP2;1 introduces green fluorescent protein (GFP) which emits fluorescence and has been used previously as a tool to explore the location of aquaporins in plants either under native promoter or overexpression promoter control (Igawa *et al.*, 2013; Luu *et al.*, 2012; Teuscher and Ewald, 2018; Wang *et al.*, 2017b; Zhou *et al.*, 2012). The fluorescence is a type of luminescence (emissions of photon or light energy), in which a molecule absorbs a photon (light), transitions to an excited state, then emits the energy and releases the photon but at a lower frequency and longer wavelength than the frequency and wavelength of the photon it absorbed due to loss of energy during the process (Monici, 2005).

GFP was also used as a tool to isolate proteins in female and male gametes (Igawa *et al.*, 2017). In Arabidopsis, *pDD45::sGFP::PIP2a* is an overexpressed GFP-PIP2;1 driven by the promoter DD45 (Igawa *et al.*, 2013; Steffen *et al.*, 2007), and the transgenic plants expressing this construct were used to identify the localisation in pistils during fertilization (Igawa et al., 2017). It is possible that overexpression of PIP2;1_GFP could change cell membrane hydraulic conductivity in transgenic lines relative to control plants, and the presence of excess PIP2;1 in overexpression lines could alter PIP2;1 interactions with other PIPs influencing the trafficking to the plasma membrane (PM) (Sorieul et al., 2011). Another possibility is that the effect of greater PIP2;1 abundance in the overexpression line nGFP PIP2;1 influenced water flux in seed as a result of there being a different proportion of PIP2;1 with varying states of posttranslational modifications (PTM) as previously PTMs have been shown to influence PIP2;1 osmotic permeability (see Supplementary 7 data of Prado *et al.*, 2019).

The localisation of other aquaporins as well as the roles they play in plant development have also been. For example, AtNIP5;1 and AtNIP6;1 were localized mainly in the PM of epidermal root cells and displayed polar localization in the PM of root cells when observed as fusions with the ProNIP5;1:sGFP-NIP5;1 promoter (Wang *et al.*, 2017b). The polar localization was determined using the ratio of GFP signals, that revealed the NIP5;1 and NIP6;1 preferential localization on the outermost, soil-facing side of the root cells versus the stele-side portions in

the cross sections. Developmental differences in aquaporin localisation using NIP2;1::GFP, and NIP2;1 promoter–β-glucuronidase fusions were reported by Mizutani et al. (2006) who observed that the NIP2;1-GFP was detected in young roots, but not in leaves, stems, flowers or siliques. The authors found that NIP2;1-GFP mainly localised to the endoplasmic reticulum membrane in Arabidopsis roots.

Recent studies investigating protein function with and without tags revealed that tagged protein sometimes behaves differently, relative to the untagged protein. For example, adding fluorescent tags to proteins by creating fusion proteins can influence the native form of the protein and may impair protein activity, reduce stability, disrupt interactions with binding partners, cause altered targeting and or cause unnatural topology (Dave *et al.*, 2016; Terpe, 2003; Weill *et al.*, 2019). The effect of GFP protein tagging at either the carboxy terminus (C′) or amino terminus (N′) of the polypeptide chain for a range of proteins expressed in budding yeast *Saccharomyces cerevisiae* were different to the effects of expressing just the protein without the tag (Weill *et al.*, 2019). They revealed that the localisation of the protein of interest was different in relation to subcellular localization. The localisation of rat AQP6 proteins when a green fluorescence protein (GFP) tag was added to the N-terminus redirected the protein to the plasma membranes of transfected mammalian cells, rather than being in internal membrane locations, and changed the anion conductance properties (Ikeda *et al.*, 2002). In a study of GFP tagging of a heparin protein, a glycosaminoglycan which interacts with a number of proteins mediating diverse patho-physiological processes, the authors revealed that the heparin-GFP tagging reduced the binding affinity of Roundabout homolog 1 and the regulation process was through a different kinetic mechanism relative to heparin without GFP binding (Zhang *et al.*, 2014). In rice, *GFP-OsPIP1;3* expressed in *Xenopus* oocytes or rice protoplasts showed an altered localization in terms of the proportion in the endoplasmic reticulum relative to the plasma membrane and significantly changed water permeability in oocytes (Liu *et al.*, 2020). Therefore, attaching tagged GFP to the PIP2;1 protein may influence the function and or the localisation of this aquaporin. In the present work, seeds of overexpression lines of transgenic Arabidopsis which had GFP tagged to the N-terminal tail of AtPIP2;1 were utilised to explore the location of PIP2;1 by a laser scanning confocal microscope and check the influence of this protein overexpression during germination in salt stress and control treatments.

MATERIALS AND METHODS

Materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (WT, Col-0) was derived from the University of Adelaide Gilliham laboratory stock, which had originated from the Arabidopsis Biological Resource Centre (ABRC).

UBI:nGFP::PIP2;1 is an overexpression line of PIP2;1 which included an N-terminal green fluorescent protein (GFP) (Chalfie *et al.*, 1994) and was driven by a Ubiquitin promoter, which was generated using an pUBI-GFP_DEST vector (Figure 1), to make UBI:nGFP::PIP2;1 expressing Arabidopsis lines. These lines were kindly shared by Professor Steve Tyerman. The material was created by Dr Chuang Wang whilst working in the Tyreman Laboratory at the University of Adelaide, where the seed available belonged to the T2 generation.

The *Arabidopsis thaliana* Ubiquitin-10 gene promotor (PUBQ10) is expected to facilitate moderate expression in nearly all tissues of Arabidopsis, and the construct should result in moderate levels of expression of intrinsic membrane proteins in Arabidopsis, with a temporal stability after transient transformation that is apparently limited only by the maintenance of the plants (Grefen *et al.*, 2010; Norris *et al.*, 1993). Experiments involving transformation using constructs derived from this vector have been reported previously and transgene expression in guard cells, epidermal cells and root hairs was described (Grefen *et al.*, 2010). To confirm the insertion of UBI:nGFP tagged AtPIP2;1 in this material, three methods can be used: Basta resistance selection on soil, laser confocal microscope screening to observe presence or absence of GFP in seedlings and PCR confirmation of the presence of the transgene in gDNA from plants.
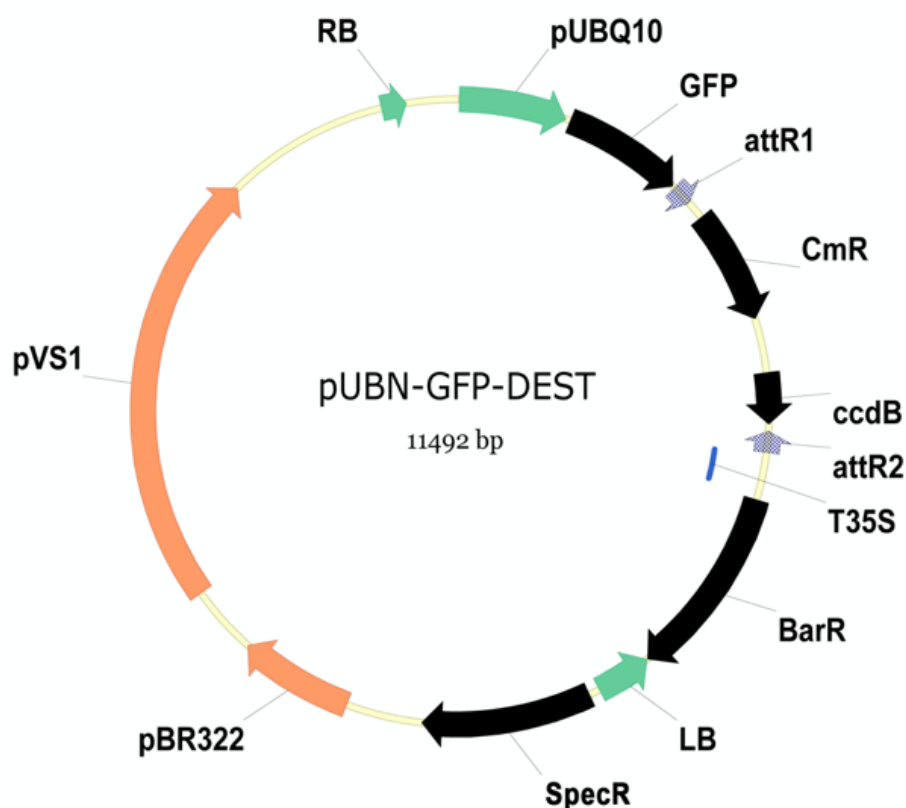
Figure 1. Plasmid map of UBI:nGFP::PIP2;1 expression vector, following https://www.ruhr-uni-bochum.de/botanik/Resources/pdf/pUBN-GFP-Dest.pdf

Information about the ubiquitin-10 promoter is reported by Grefen et al. (2010). The abbreviations used to refer to this promoter vary in the literature and common abbreviations include UBI, UBN and UBQ10 as previous studies use either pUBN or pUBI to refer to the ubiquitin promoter. Throughout this thesis the abbreviation UBI is used. *Aequoria victoria* Green Fluorescent Protein (2x35S::AtPIP2;1); right border (RB); left border (LB); chloramphenicol acetyltransferase confers resistance to chloramphenicol (CmR); attR1/R2 are recombination sites for the reaction to insert the fragment of interest; *ccdB* is a bacterial toxin that poisons DNA gyrase so that *E.coli* containing the *ccdB* gene cannot propagate in standard strains; t2x35S::AtPIP2;135S terminator T35S; the T-DNA carries the gene phosphinothricin-N-acetyltransferase that confers resistance to the herbicide Basta (BarR); the stability replicon protein of the *Pseudomonas* plasmid (pVS1), the pBR322 origin for high copy numbers in *E.coli* and the aminoglycoside-3-adenyltransferase gene for resistance against streptomycin (SpecR) or spectinomycin in bacteria (pBR322).

Arabidopsis plants were cultivated at 19°C night, 22°C day temperature, in an incubator under long day conditions (16 h light, 8 h dark), continuous light irradiance was 100 - 120 µmol m^{-2} s^{-1} and 70% relative humidity.

UBI:nGFP::PIP2;1 localisation by laser scanning confocal microscope

To observe the localization and expression time of UBI:nGFP::PIP2;1 in seeds during germination, samples of seed from at least 4 independent transgenic plants were germinated on agar plates containing half-strength Murashige and Skoog (½ MS) Basal Medium (M542 PhytoTechnology Laboratories, US) (Section 3.2.2.1). Imbibing seeds at 12, 24, 36, 48, 72 hours after sowing were popped out of the testa (seat coat), using needles under a microscope (this is because the testa obscures observation of fluorescence). Seed was mounted on slides inside of a MQ water drop, covered by a coverslip and immediately scanned. The fluorescence of fluorescent proteins in transgenic UBI:nGFP::PIP2;1 imbibing seed was imaged by confocal laser scanning microscopy using a Nikon A1R Laser Scanning Confocal with DS-Ri1 CCD camera and a multiline argon ion and helium/neon gas lasers (Carl Zeiss). Sequential scanning and laser excitation was used to capture fluorescence via the Nikon A1R Laser Scanning Confocal from GFP (fluorophore FITC: excitation = 488 nm, emission = 525–575 nm), and chlorophyll autofluorescence (fluorophore Cy5: excitation = 561 nm, emission = 595–645 nm). Emission from natural fluorescence called autofluorescence was also recorded. This endogenous autofluorescence could be due to pyridine nucleotides NAD(P)H, flavin coenzymes, lipo-pigments, redox and their derivatives present in organisms and cells (Monici, 2005; Wagnieres *et al.*, 1998). However, the brightness of autofluorescence differs from fluorescence of GFP.

To ensure that true GFP signals were imaged rather than endogenous auto-fluorescent molecules in cells, the present of fluorescence in both wild type (WT) Col-0 and overexpression lines UBI:nGFP::PIP2;1 were first checked, together. The UBI:nGFP::PIP2;1 expressing seeds were scanned to confirm the positive expression of the transgene fragment. GFP fluorescence collected at 525 – 575 nm emission after excitation with 488 nm light. Then wild type Col-0 seed was scanned using the laser channel to capture the autofluorescence with the lowest fluorescence in images. These settings were determined to control the autofluorescence of each assay. Using the same settings used to scan Col-0, the UBI:nGFP::PIP2;1 seeds were scanned to make sure that most of the autofluorescence would be removed from the samples. Seed of UBI:nGFP::PIP2;1 had clear green fluorescent colour, visible on cell membranes in the roots, hypocotyls, and cotyledons, whereas Col-0 only had a

dull yellow colour visible at the same age and location in seeds indicating that autofluorescence was not interfering with the analysis of the UBI:nGFP::PIP2;1 seed at the stages tested.

Seed germination percentage, changes in seed weight and maximum cross sectional (MCS) area during imbibition and seed Na^+ and K^+ content assays

Seeds of Col-0 and UBI:nGFP::PIP2;1 independent lines 7 and 18 were used for these experiments. Methodology for these measurements was described in the Materials and Methods section of Chapter 3.

Data analysis

There were three or more experimental replications for each experiment and the same patterns were observed for each replication. Data was analysed using GraphPad Prism (version 9). The statistical analyses were two-way ANOVA, with Fisher LSD multiple comparison post test, where $p < 0.05$ was considered as significant. Data on graphs are presented as mean +/- SEM.

RESULTS

Timing of UBI:nGFP::PIP2;1 detection in imbibed seed

Visualisation of GFP expression relative to endogenous auto-fluorescence in UBI:nGFP::PIP2;1 and Col-0 control lines is represented in Figure 2. I observed the emission of imbibing seed during a course that included timepoints of 24, 36, 48 and 72 hours after sowing. At the stage of 24 hours after sowing seeds of UBI:nGFP::PIP2;1 OE and Col-0 both emitted a similar weak fluorescence in the radicle and cotyledons (Figure 2, panels a-d). This indicates that the fluorescence at that stage was likely to be autofluorescence and the PIP2;1 tagged GFP was possibly not expressed in the seeds at this early stage.

The next step involved testing the difference in the fluorescence between Col-0 and transgenic UBI:nGFP::PIP2;1 OE lines from 36 hours after sowing, which revealed fluorescence in the radicles (Figure 2.g-h). At 48 hours after sowing the green fluorescence expanded from radicle to cotyledons (Figure 2.k-l), and the bright green colour covered the whole seed but there was no clear specificity in relation to the cells in which expression was detected. This result is not surprisingly since expression of nGFP::PIP2;1 was under a constitutive promoter, not under its native promoter. At 72 hours after sowing, the expression of UBI:nGFP::PIP2;1 was distributed

in many parts of the imbibed seed, specifically in the root cap, root epidermis, root stele and cotyledon membranes and especially in guard cells (Figure 2.o-p)
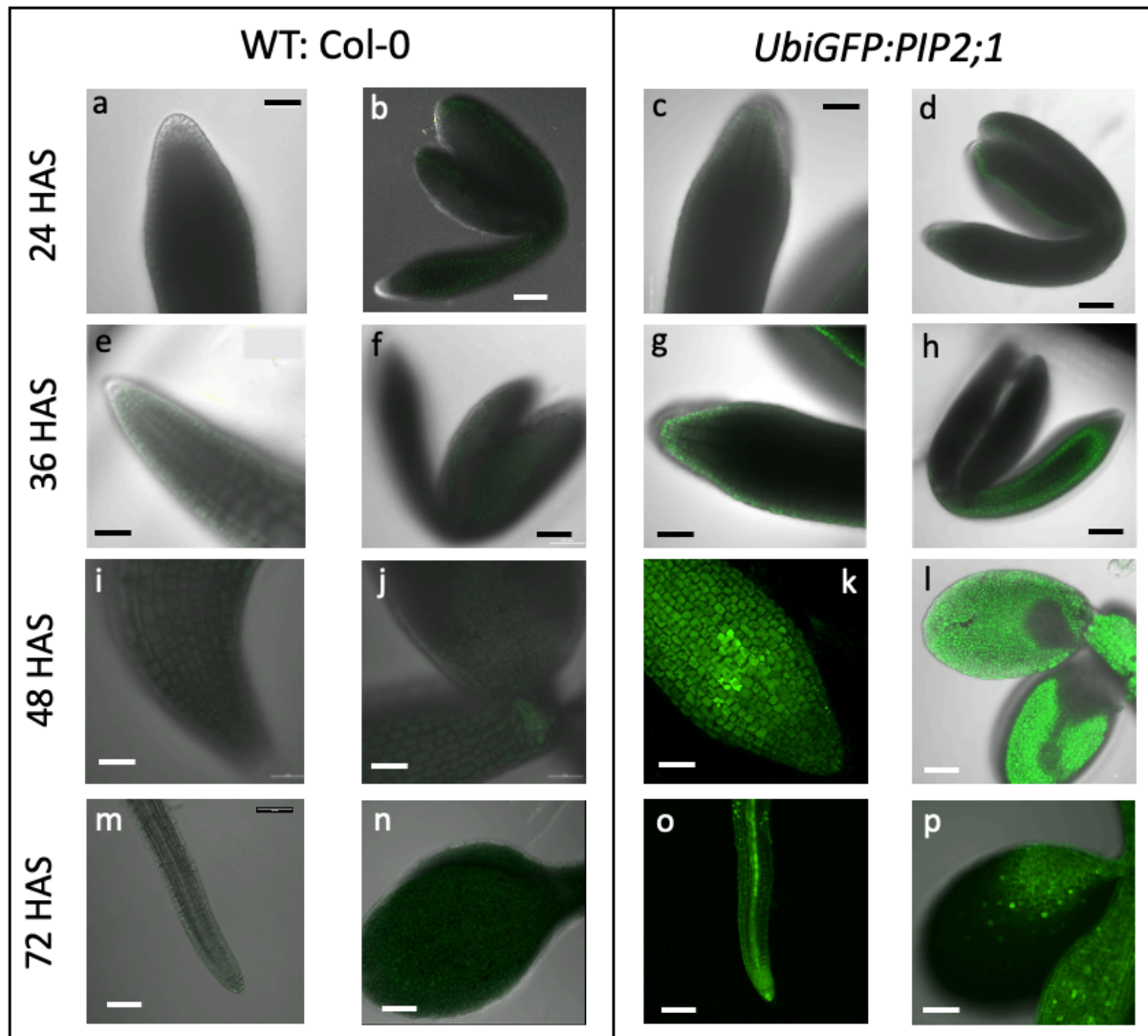
Figure 2. Images of Col-0 and UBI:nGFP::AtPIP2;1 imbibing seeds under laser scanning confocal microscope at 24, 36, 48, 72 hours after sowing (HAS).

Green color in the images were the GFP fluorescence collected at 525 – 575 nm emission after excitation with 488 nm light. Four or more seeds and seedlings for each genotype and time were studied. Panels a, c, e, g, i, k, m and o show the root hypocotyls, panels b, d, f and h show the whole seeds, panels j, l n and p show the cotyledons. Panels a, c, e, g, i – k and p have bars = 50μm. Panels b, d, f, h and l – o have bars = 100μm.

Localisation of UBI:nGFP::PIP2;1 protein in seedlings

The green fluorescence stood out in many regions of the seedlings. As can be seen in Figure 3, the fluorescence in the root tip, root stele, root epidermis, epidermal hypocotyl, cotyledons including the guard cells was strong. The expression of UBI:nGFP::PIP2;1 could be seen in structures consistent with cell membranes in all parts of the seedling, but higher magnification images of each different part of the seedling would be needed to confirm the localisation in each specific tissue or cell type along with use of a specific plasma membrane marker.
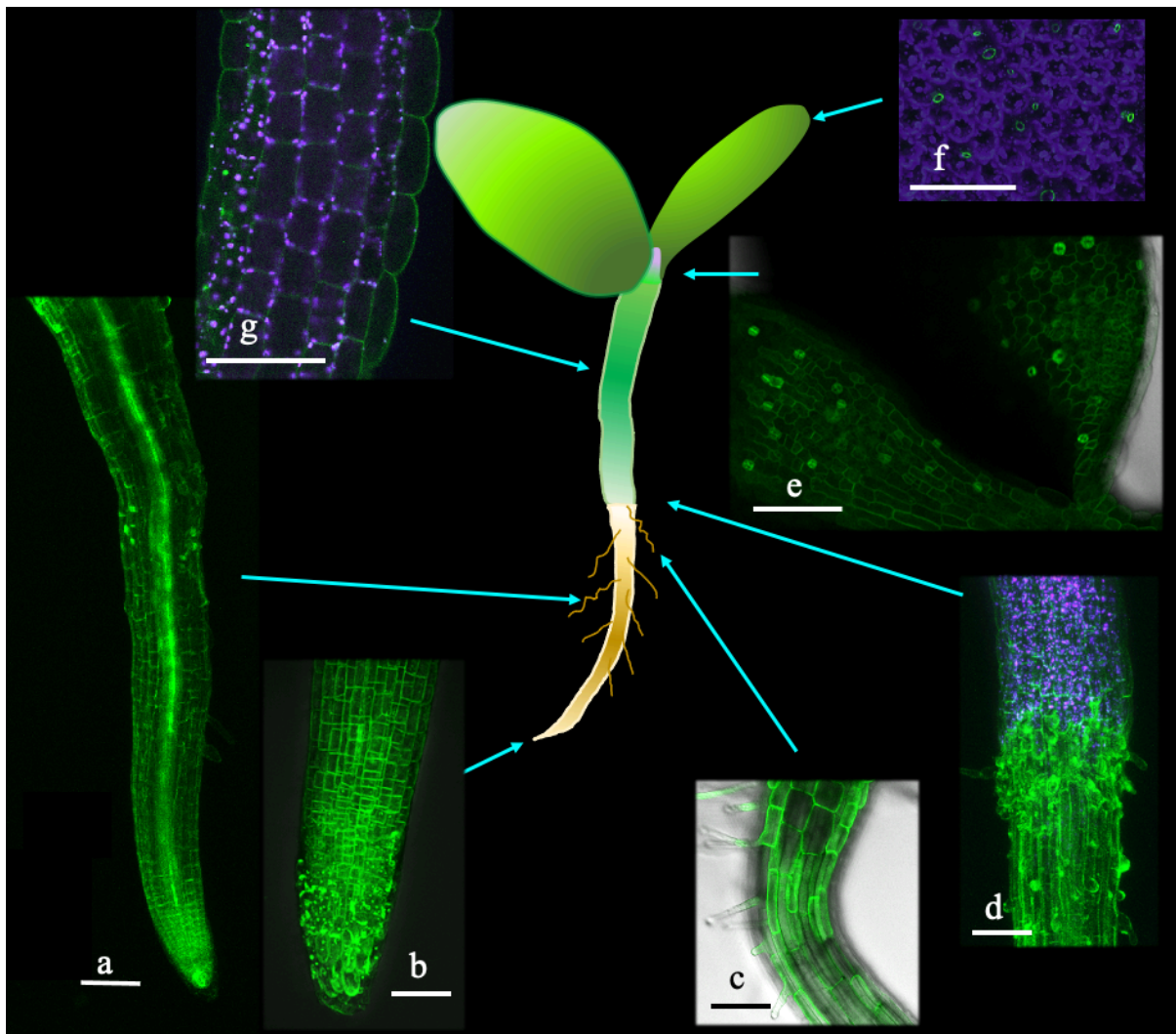
Figure 3: Summary of UBI:nGFP::PIP2;1 detection in various tissue regions of transgenic seedlings sowing in ½ MS media.

Scale bars = 100μm. Green color in the images was the GFP fluorescence collected at 525 – 575 nm emission after excitation with 488 nm light. Purple colour indicates chlorophyll autofluorescence collected at 595 – 645 nm emission after excitation

with 561 nm light. Panels (a) and (b) show roots with root tips, panel (c) is root hairs and epidermal cells, panel (d) is the junction between radicle (root - below) and hypocotyl (stem - above), panel (g) is zoomed in on the hypocotyl, panel (e) and (f) are cotyledon images with guard cells brightly fluorescing.

Seed germination in ½ MS compared with ½ MS + 75 mM NaCl

As expected, the germination of all genotypes was delayed in saline treatment (½ MS + 75 mM NaCl) relative to ½ MS (Figure 4 a - b). At 24 hours after sowing, the germination percentage of Col-0 (30%) was higher than UBI:nGFP::PIP2;1 lines (17.5%). Saline conditions inhibited the germination percentage of the three genotypes, with percentages germination close to 0% at 24 h after sowing (Figure 4 c). At 36 hours after sowing, the germination percentage of the three genotypes was about 78% in ½ MS control treatment and there were significant differences between genotypes. In the saline treatment, over 58% of the Col-0 seed had germinated, which was higher than that of UBI:nGFP::PIP2;1 lines at this time (33 – 45%) (Figure 4 d).

From 72 hours until 216 hours (9 days) after sowing, the germination and growth rate of the three genotypes on ½ MS treatment increased quickly and 97.5 – 99 % of seed had germinated within 9 days. Subtle differences between Col-0-0 and UBI:nGFP::PIP2;1 OE were observed; specificalCol-0Col-0 had a higher germination percentage than both UBI:nGFP::PIP2;1 line 7 and 18 after sowing at 72 hours and 6 days (Figur– 4 e - g). The time when 50 percent of seed had germinated (T_{50}) for the three genotypes was not different and ranged from 29 – 30.5 hours on ½ MS treatment. In saline treatment, the T_{50} was delayed to between 34.2 – 43.5 hours, and the value for the UBI:nGFP::PIP2;1 line 7 (43.5 hours) was higher than the value for Col-0 (34.2 hours) (Figure 4 h).
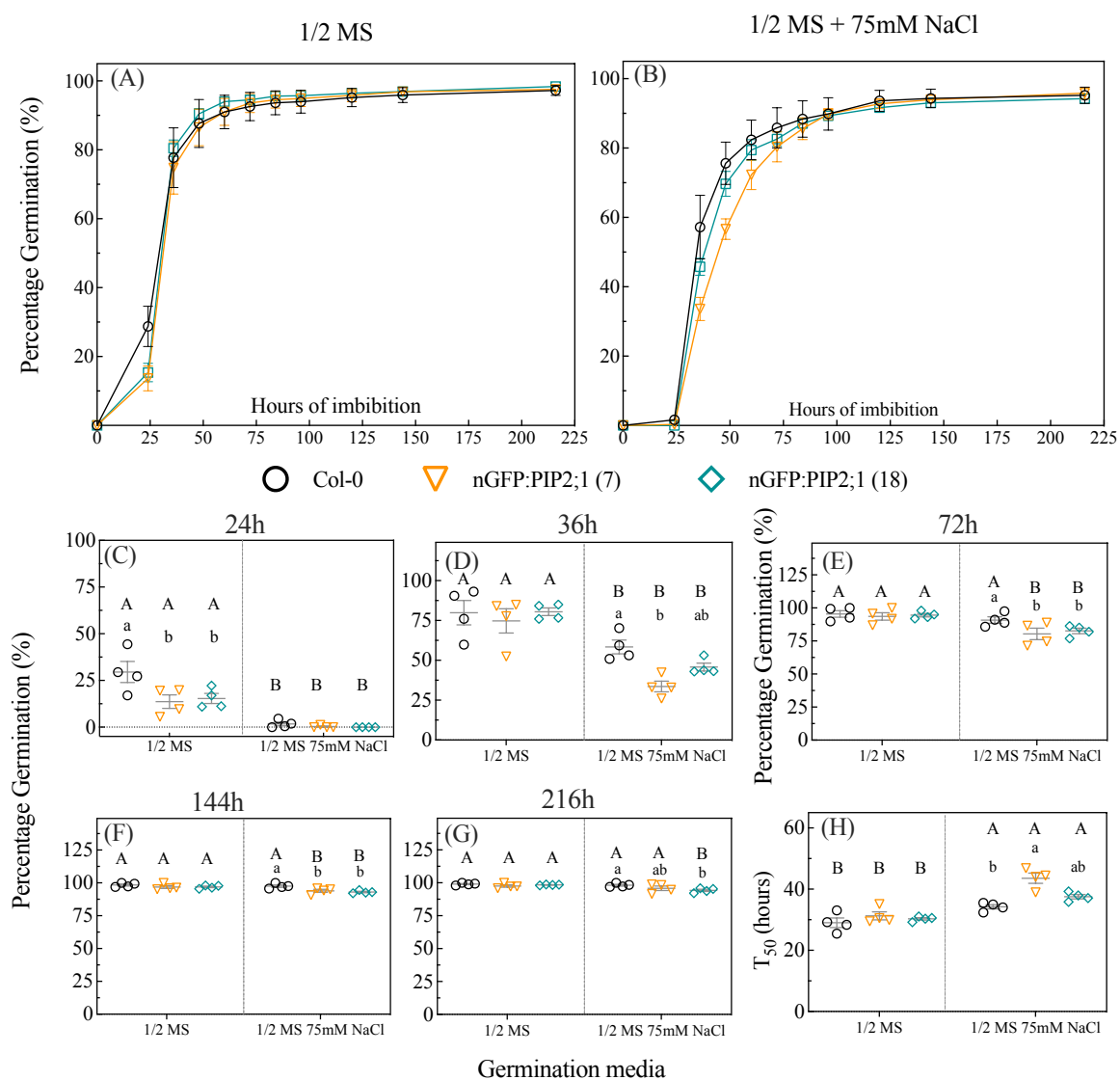
Figure 4. Cumulative germination success and time to fifty percent germination (T_{50}) for Arabidopsis Col-0 compared to UBI:nGFP::PIP2;1 lines in the presence or absence of saline. Percentage of seed germinated under control treatment (½ MS) or saline treatment (½ MS + 75 mM NaCl). All the seed with successful endosperm rupture were recorded over time (0 - 216 hours) upon exposure to ½ MS (A) or ½ MS + 75mM NaCl (B) treatments. Statistical analysis of percent germination data at individual time points after sowing: 24 hours, 36h, 72h, 6 days, 9 days (panels C - G), and time to reach fifty percent germination (T_{50}) in hours (panel H). Each panel includes percent seed germination in ½ MS or ½ MS + 75 mM NaCl. Data are means ± SEM from four different plates, where each plate included seed from different parent plants for each genotype. 150 – 200 seeds were

in a plate per genotype, and assays were repeated three times. Significant difference was determined by two-way ANOVA and Fisher's LSD tests at significant level p < 0.05. Different lowercase letters indicate significant differences within a treatment group between genotypes, absence of letters indicates a non-significant difference; different uppercase letters indicate significant differences between treatments for a particular genotype.

Changes in seed weight and the percentage weight of UBI:nGFP::PIP2;1 compared to WT Col-0 during imbibition and germination

Dry seed weight was not different between genotypes, however once seeds were exposed to moisture, they started to gain weight rapidly and differences between genotypes emerged (Figure 5B, S20). After imbibition for 4 hours in water, the UBI:nGFP::PIP2;1 (18) seed increased in weight more rapidly relative to Col-0 but despite a similar pattern to control, there were no significant differences between genotypes in salt treatment at the 4 h timepoint (Figure 5, S20).

At 30 hours after sowing the seed weight of genotypes was lower in salt treatment than under water treatment. The UBI:nGFP::PIP2;1 lines had higher weight compared to WT Col-0 in both water and salt treatments. This was also the case at 50 hours after sowing in all treatments including the water transfer to saline treatment. The UBI:nGFP::PIP2;1 (18) seed weight was significantly different compared to Col-0 under only water transfer to saline treatment.

The percentage increase of seed weight of the three genotypes at 4 h was about 200% in saline treatment and 250 - 300% in water. UBI:nGFP::PIP2;1 (7) had a lower percentage increase compared to Col-0 and UBI:nGFP::PIP2;1 (18) at 4 hour imbibition (Figure 5C, S20). At 30 hours after sowing, the percentage of seed weight increase was different between water and saline treatments but not between genotypes. At 50 hours after sowing, both UBI:nGFP::PIP2;1 lines had higher percent weight increase compared to WT Col-0 in water treatment (T1) for all treatments. The UBI:nGFP::PIP2;1 (18) but not the UBI:nGFP::PIP2;1 (7) line exhibited a higher percentage weight increase as compared to WT Col-0 in both the T2 and T3 treatments.
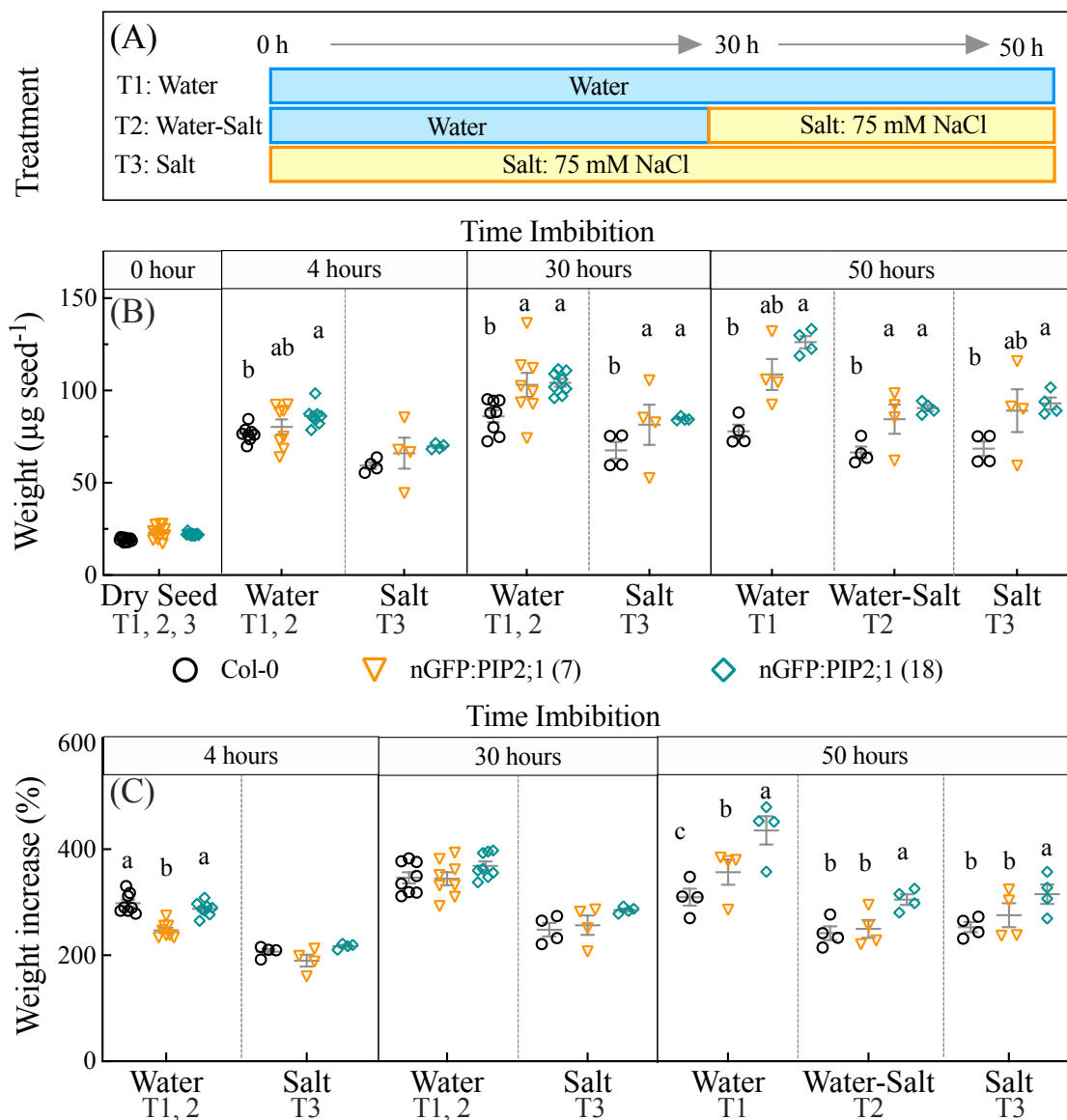
Figure 5. Changes in seed weight over time during imbibition in water treatment or saline treatment or treatment that changed from water to saline during the course of 50 hours of imbibition.

A, Summary of the experiment set up with three treatments: Treatments consisted of 50-hour imbibition in water only (Water; T1); 30-hour imbibition in water followed by 20 hours in 75 mM NaCl solution (50 hour total, Water-Salt; T2) and 50 hour imbibition in 75 mM NaCl solution (Salt; T3).

B-C, Progress of seed weight gain in Col-0 and UBI:nGFP::PIP2;1 over 50 h of imbibition.

Dry seed weight data is the average for all seed used in the experiment. Data are means ± SEM. 600 – 900 seeds per genotype in each plate, n = 4 – 8 plates per treatment, each plate had seed from an individual parent plant. The experiment was repeated three times. Significant differences were determined by two-way ANOVA and Fisher's LSD tests, $p < 0.05$. Different letters mean statistical differences between genotype, and where there are no letters, this is indicating there were not any significant differences. Two-way ANOVA comparisons of an individual genotype between treatments were presented in Figure S20.

Measuring the maximum cross sectional (MCS) area of swelling seeds

The seed MCS area at the beginning of the experiment (0 min into imbibition) ranged from 11.5 - 12 mm^2 per seed and the values were not significantly different between the three genotypes. The MCS areas increased after 30 min through to 30 hours in both ½ MS and ½ MS + 75 mM NaCl (Figure 6).

There were no differences in the MCS area and percentage change in MSC area between genotypes when germinated on ½ MS + 75 mM NaCl (Figure 6B, D). On ½ MS UBI:nGFP::PIP2;1 line (18) seed had larger MCS areas than Col-0 seed over time from between 30 mins and 30 hours of imbibition (Figure 6A). At 30 hours after sowing both UBI:nGFP::PIP2;1 lines had significantly larger seed MCS area than WT Col-0 but there was no significant difference in percentage increase in MCS of ½ MS (Figure 6C).

At 30 hours of imbibition, the MCS areas of seeds on ½ MS were significant larger than those on ½ MS + 75 mM NaCl (Figure 6, S21).
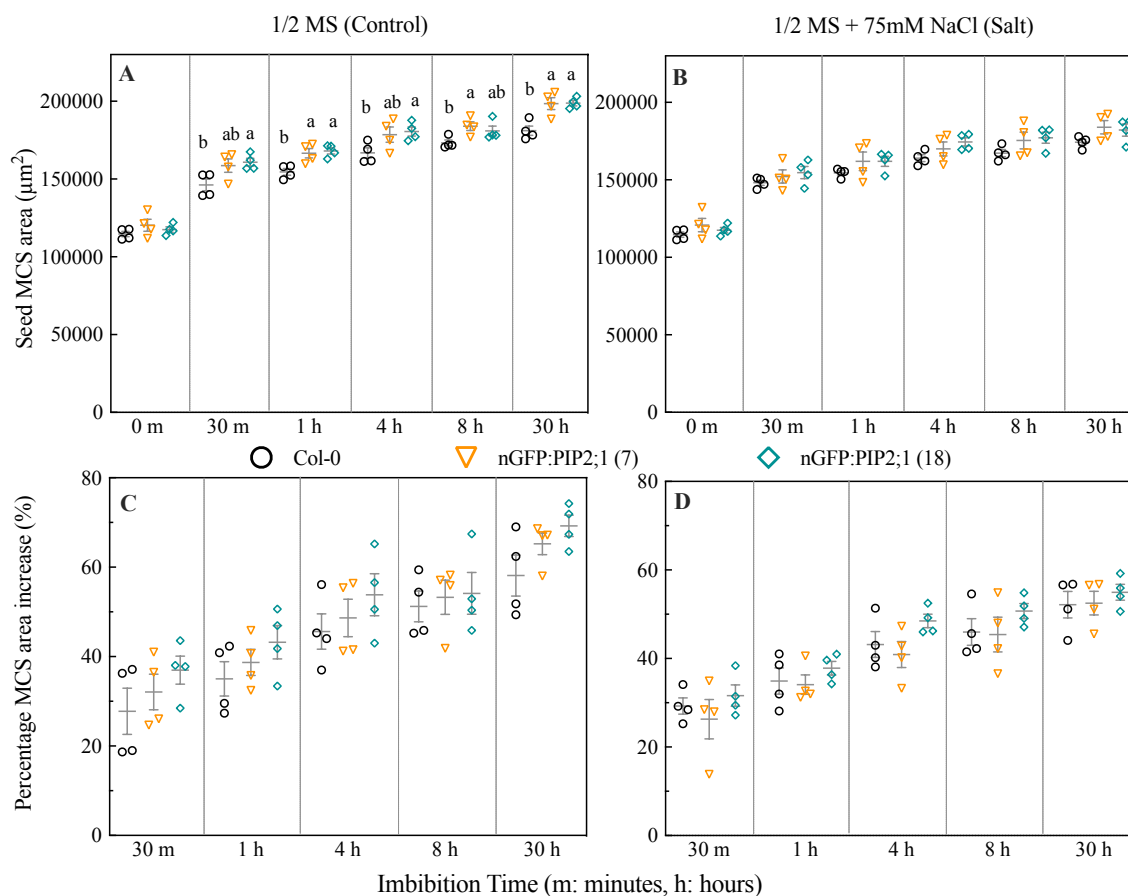
Figure 6. Seed MCS area increase upon imbibition on control treatment (½ MS media, panels A and B) and saline treatment (½ MS + 75 mM NaCl, panels C and D).

Data are means ± SEM of values from seed from n = 4 - 8 plates, with 200 seeds per genotype per plate. For each genotype, seeds from four independent parent plants were tested. At some time points the error bars are so tight as to be obscured by the symbols. Significant differences were determined by two-way ANOVA and Fisher's LSD pair-wise tests, $p < 0.05$. Different letters indicate statistical differences between genotypes, where there are no letters, significant differences were not measured. Two-way ANOVA comparisons of an individual genotype between treatments are presented in Figure S21.

Analysis of the Na$^+$ and K$^+$ content in germinating seeds of UBI:nGFP::PIP2;1 compared to WT Col-0.

As expected, the Na$^+$ content in seed germinated in saline (water + 75 mM NaCl) and water transfer to saline treatments was greater than that of seed germinated in water treatment, however differences in the values between different genotypes were not detected in any of the treatments tested: water, saline and water to saline treatments (Figure 7B). The K$^+$ content of seed subjected to water treatment was higher compared to the saline and the saline to water transfer treatments. There were no significant differences in the seed K$^+$ between genotypes in water and water-to-saline transfer treatments; but in the saline treatment after 50 hours, the seed K$^+$ was greater in both UBI:nGFP::PIP2;1 lines compared to Col-0 (Figure 7B).

The seed K$^+$/Na$^+$ ratios were higher in the water treatment compared to the water-to-saline transfer treatment and saline treatment at 50 hours (Figure 7C). In water treatment, Col-0 and Ubi:nGFP::PIP2;1 (7) had higher K$^+$/Na$^+$ ratios than Ubi:nGFP::PIP2;1 (18) (Figure 7C). The total of seed Na$^+$ plus K$^+$ content in the three treatments ranged from 6300 to 7200 mg/kg dry weight. There were no differences in total seed Na$^+$ plus K$^+$ between genotypes in water and water-to-saline transfer treatments but in the saline treatment after 50 hours the total seed Na$^+$ plus K$^+$ was higher in both UBI:nGFP::PIP2;1 lines compared to Col-0 (Figure 7D).
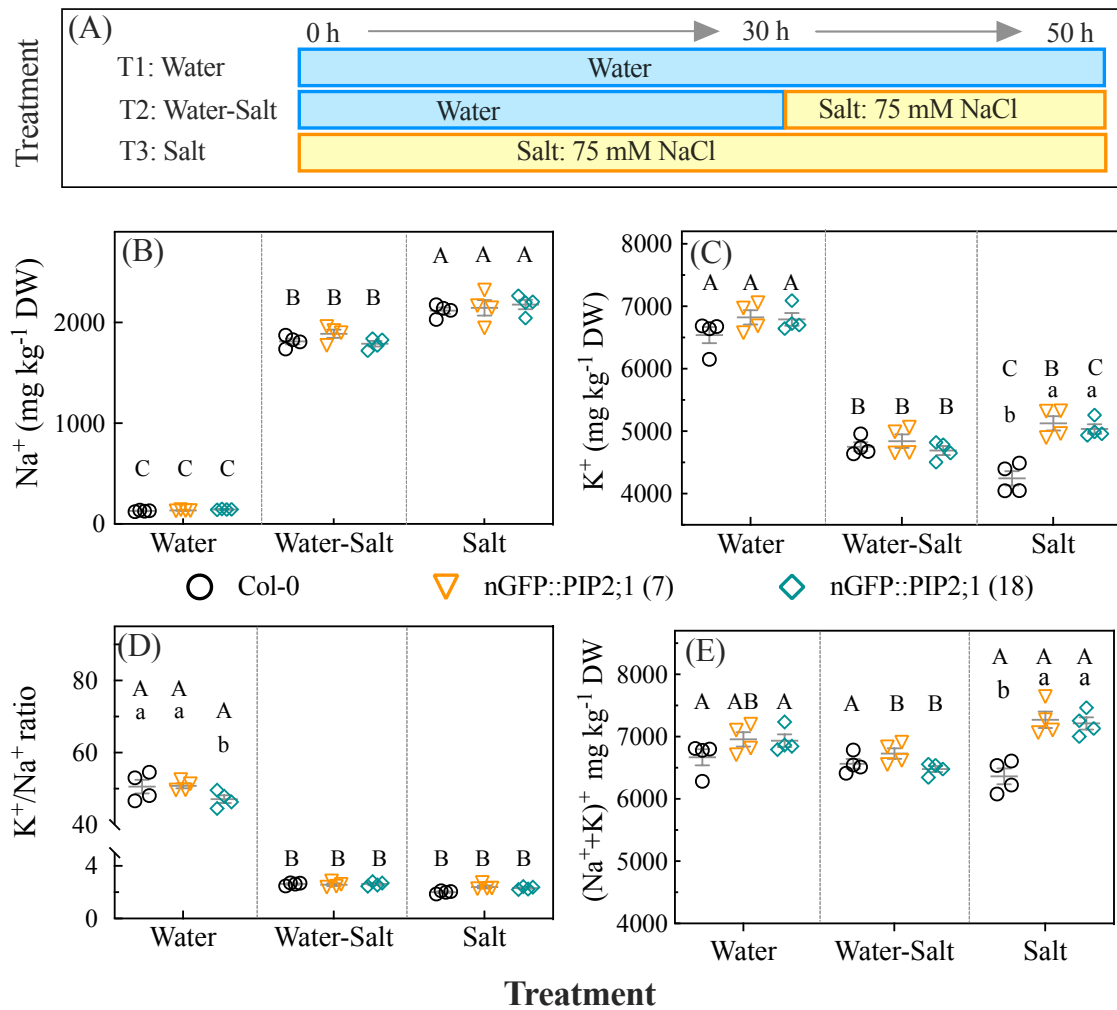
Figure 7. Na$^+$ and K$^+$ content of seed from Col-0 and AtUBI:nGFP::PIP2;1 genotypes following three treatments during imbibition.

Control treatment (Water); 75 mM NaCl treatment for 50 hours (Salt); and treatment for 30 hours in water then transfer to 75 mM NaCl from 30 to 50 hours (Water-Salt) treatment (panel A). Na$^+$ content (panel B) and K$^+$ content (panel C), the K$^+$/Na$^+$ ratio in seed (panel D) and the total Na$^+$ and K$^+$ content (panel E) following the three treatments. Data are means ± SEM of 4 biological replications. At some timepoints the error bars are so tight as to be obscured by the symbols. Significant differences were determined by two-way ANOVA and Fisher's LSD pair-wise tests. Different lowercase letters indicate significant differences within a treatment group between genotypes, absence of letters indicates a non-significant difference; different uppercase letters indicate significant differences between treatments for a particular genotype ($p < 0.05$).

DISCUSSION

Localisation of PIP2;1 during seed imbibiton and germination and in young seedlings

The localization and abundance of Arabidopsis nGFP::PIP2;1 constitutively expressed using the ubiquitin promoter in seeds during seed imbibition, germination and in young seedlings was investigated. In these lines, GFP fluorescence in seeds was detected following 36 hours of imbibition. The fluorescence was observed in hypocotyls, root tips, meristems and leaves (Figure 2). At the timepoint of 24 hours after the start of imbibition, fluorescence was not obvious in UBI:nGFP::PIP2;1 indicating that it was between 24 and 36 hours that translation of nGFP::PIP2;1 occurred control of the constitutive UBI promoter (Figure 2 d relative to 2 h). Previous reports suggest that the expression of native promoter PIP2;1 in seed significantly increased around day 2 and 3 after soaking compared to dry seed and compared to 1 day after soaking seed; this was based on assessment of the activity of the native promoter (Hoai *et al.*, 2020; Klepikova *et al.*, 2016; Narsai *et al.*, 2017). This indicates that although the nGFP::PIP2;1 was driven here by the UBI promoter rather than the native *Atpip2;1* promoter it is likely that the timing of the increase in abundance of PIP2;1 in the transgenic seed follows a similar trend in germinating seed as the endogenous increase in PIP2;1 abundance in wild-type Arabidopsis.

Given that the abundance of PIP2;1 is low in the first phase of imbibition (see section 1.2 in Chapter 1 for description of phases), it is possible that there is not a critical role for PIP2;1 in water uptake at this stage, and it is likely that water is distributed within the seed by either non-aquaporin mediated pathways or other aquaporins (Orozco-Segovia *et al.*, 2007). For example, the properties of lipid components in membranes might influence water uptake and distribution more than aquaporins (Wang *et al.*, 2017b), or it could be that other aquaporins are key to water uptake and distribution at this stage, such as TIPs (Footitt et al. 2019) or PIP2;7 and NIP1;2 (Hoai *et al.*, 2020).

Changes in PIP2;1 abundance and the potential to influence seed water uptake at different stages

Water influx from the environment to dry seed during imbibition is critical for germination and is thought to occur by several pathways including penetration directly through the micropyle and diffusion through the testa from the outside to the inside. The pattern of water uptake can be generally divided into 3 phases (Bewley, 1997) and may not be identical for every species. In the *Opuntia Tomentosa* seed 80% of the water uptake occurred during the first 3 h of imbibition, and after 72 h the hydration curves were nearly asymptotic so only a minimal amount of water continued to be taken up for an additional 3-7 days (Orozco-Segovia et al., 2007). When the radicle protrudes from the seed coat and the endosperm, the young root can take up water and nutrients for seedling growth (Muller *et al.*, 2006). At this time, when radicles protruded from the Arabidopsis seed, the expression of UBI:nGFP::PIP2;1 was strongly detected (Figure 3). The green fluorescence was abundant in almost all of the cell membranes of the seedlings. In the roots the expression of GFP was bright in the tips, epidermis, steles and root hairs which play key roles in the water and nutrient transport pathways needed to deliver these resources to above ground parts. A previous study reported that the root hydraulic conductivity for wild type Col-0 relative to Col-0 overexpressing PIP2;1-GFP driven by a double *CaMV35S promoter* was similar (Sorieul *et al.*, 2011). Further experiments to explore the influence of UBI:nGFP::PIP2;1 on hydraulic conductivity would be required in the future to test whether there were differences in hydraulic conductivity in the transgenic lines relative to controls.

Following observation of cross sections of epidermal root cells previous reports suggested that the abundance of PIP2;1 was greater on the soil side of the cell membranes in three- or four-day old seedlings than the stele side (Wang *et al.*, 2017b). In the material tested here, we did not study cross sections of the roots but the tendency for UBI:nGFP::PIP2;1 to orientate towards the soil face in root was observed (Figure 3.15 – Chapter 3). In future experiments, it would be interesting to unequivocally determine whether there is polar localisation of PIP2;1 in the UBI:nGFP::PIP2;1 material or whether use of the UBI promoter causes higher expression in all sections of root cell membranes.

PIP2;1 trafficking trends under salt stress conditions

Previous studies have reported that in general, plant aquaporins are found to localize in the plasma membrane, endoplasmic reticulum, vacuoles, plastids and, in sometimes in membrane compartments interacting with symbiotic organisms, and that aquaporin proteins have narrow pores of about 3 Å that enhance water diffusion across membranes to maintain cellular water balance (Maurel *et al.*, 2015; Maurel *et al.*, 2008). For AtPIP2;1, specifically previous studies have revealed that PIP2;1 is mainly located in the plasma membrane (PM), but that there are cycles of PIP2;1 movement between the PM and the trans-Golgi network (TGN) regulated by clathrin-mediated endocytosis (Ueda et al., 2016).

Salt treatment (100 mM NaCl) has been previously shown to influence trafficking of AtPIP2;1; specifically there was enhanced cycling of nGFP::PIP2;1 between PM and TGN in saline relative to control conditions (Luu *et al.*, 2012). Salt stress treatments triggered retention of AtPIP2;1 in the endoplasmic reticulum, and this was associated with reduced hydraulic conductivity (Sorieul *et al.*, 2011). Salt stress decreased the fluorescence of GFP-PIP2;1 at the PM and increased it in the vacuolar lumen as shown by staining of the vacuolar membrane (Ueda *et al.*, 2016). The authors hypothesied that AtPIP2;1-GFP retained in the ER might interact with other PIPs to hamper their trafficking to the plasma membrane. Cycling of nGFP::PIP2;1 and influence of PIP2;1 overexpression on the regulation of other aquaporins were not tested in the present study, but it is likely that a similar phenomenon could be present in the material studied here as was observed by Sorieul et al. (2011) and Ueda et al. (2016) and that therefore the seed germination, weight, area and ion content differences (Figure 4, 5, 6, 7) in the transgenic line seed relative to Col-0 seed could be a result of a combination of differences in AtPIP2;1 abundance as well as differential regulation of other aquaporins.

A previous study revealed that in non-stressed conditions very little PIP2;1 accumulation was observed in the vacuole, but under salt stress conditions, an increase in PIP2;1 cycling between PM and TGN was reported and notable quantities of PIP2;1 became internalised from the PM and directed to the vacuolar lumen (Ueda *et al.*, 2016). In the present study, we observed nGFP::PIP2;1 in structures that are consistent with nGFP::PIP2;1 being localised in the plasma membrane of cells in germinating seed and young seedlings in standard conditions, but further tests using staining with a plasma membrane marker are required to check the localisation (Figure 3). In the future it would be interesting to repeat these experiments and include treatments with saline solutions to see whether the trends in PIP2;1 abundance and localisation change. Ideally this would be completed where nGFP::PIP2;1 was driven by the native

AtPIP2;1 promoter rather than the UBI promoter to give a better idea of how endogenous regulatory mechanisms respond to changes in salinity.

Germination, seed weight and ion content phenotypes for UBI::nGFP::PIP2;1 lines

In the control treatment (½ MS) the germination percentage of UBI:nGFP::PIP2;1 was lower than Col-0 at 24 h after sowing, but did not differ at 24 h or 72 h (Figure 4). This indicates that by this stage of germination, there were no significant differences between the transgenic and wild type lines in relation to germination percentage in the control treatment indicating that overexpression of PIP2;1 had no influence on the overall germination percentage in control treatment. Whereas in the saline treatment, at both 36 and 72 h after sowing, Col-0 germination was faster than that of UBI:nGFP::PIP2;1 seed indicating that nGFP::PIP2;1 overexpression hampered germination in the saline treatment (Figure 4 d, e, f, g and h) or it may be a consequence of the nGFP. In this case, the energetic penalty associated with transgene overexpression of UBI:nGFP::PIP2;1 might require more time and energy to synthesise protein and this could impose a delay on germination and slow T_{50} in the saline treatment (Figure 4h, S20).

The trends in weight over time for the transgenic lines relative to Col-0 revealed that the transgenic lines gained more weight earlier than Col-0 in the control treatment. This could mean that transgenic seed was rapid swelling which was not as optimally controlled in the transgenic lines relative to controls; which could have limited the efficiency of germination (Figure 5). These observations indicate that disruption of endogenous PIP2;1 regulation during germination by overexpressing nGFP::PIP2;1 hinders the capacity of seed to germinate in the saline treatment.

The seed MCS area of OE UBI:nGFP::PIP2;1 and Col-0 were similar in the saline treatment, but in the control treatment seed area of the transgenic lines tended to be greater than that of wild type (Figure 6). It is unclear why the area and weight data trends differ (Figure 5 and 6), but previous studies have indicated that at different stages of Arabidopsis seed imbibition the dimensions such as the long diameter relative to short diameter, area, perimeter and volume might increase to different proportions (Robert *et al.*, 2008). Specifically, it is possible that the long diameter changes more between 3 and 24 h imbibition than the short diameter changes (see Figure 3 of Robert *et al.*, 2008). Potentially disruption of endogenous PIP2;1 function in

germinating seeds limits the ability of the seed to optimally control the uptake of solutes as the radicle emerges and the distribution of solutes within the germinating seed and that this changes the relationship between seed area and weight during germination in the transgenic lines relative to wild type lines.

The seed Na^+ and K^+ of OE UBI:nGFP::PIP2;1 and Col-0 were similar in the control treatment but in the saline treatment the transgenic lines had greater K^+ content than wild type (Figure 7). PIP2;1 is actually more permeable to K^+ than Na^+ (Qiu et al. 2020). Potentially increasing the abundance of PIP2;1 in the transgenic lines altered the trend in management of K^+ flux during seed germination. In the future, it would be interesting to measure whether there are differences in Na^+ and K^+ flux in these transgenic and control lines using microelectrode ion flux estimation (MIFE) to determine whether there are differences in the exchange of these ions between germinating seed radicle and surrounding solution in material overexpressing PIP2;1 (Shabala *et al.*, 2003).

Chapter 6

General discussion

6.1 Assessing Arabidopsis seed phenotypes towards unravelling aquaporin roles in seed biology

Assessment of Arabidopsis seed phenotypes is challenging, given the small size of Arabidopsis seed. It is also challenging to resolve the role of aquaporins in seed given that there are many aquaporin encoding genes expressed at different stages of seed development and germination (Hoai *et al.*, 2020; Klepikova *et al.*, 2016; Obroucheva, 2013; Shiota *et al.*, 2006) and because aquaporins are multifunctional (Chaumont and Tyerman, 2014; Gomes *et al.*, 2009; Maurel *et al.*, 2015; Postaire *et al.*, 2007; Tyerman *et al.*, 2021). To explore the function of AtPIP2;1 in Arabidopsis seeds, it was necessary to adapt previously reported methods for assaying seed phenotypes and use a genetic approach involving loss-of-function mutants and transgenic over-expression lines. The approaches to develop methods and the outcomes of the assays to resolve the phenotypes of the Arabidopsis mutant and transgenic lines, along with future directions are discussed in this chapter.

6.2 Optimising methods for assessing changes in Arabidopsis seed weight and area during imbibition and germination

The dimensions and density of crop seeds have successfully been determined using a pycnometer (Fasina, 2010; Nelson, 2002; Tang and Sokhansanj, 1993). A pycnometer has been used to gather measurements from seeds of crops such as barley, corn, cotton, sorghum, peanut, rice, wheat and sunflower (Nelson, 2002). The weight and dimensions of crop seeds from many varieties were recorded by Nelson (2002) and these assays revealed seed weights from 2 – 650 milligrams per seed, seed length dimensions from 1.6 to 17.4 millimetres, seed width dimensions from 1.3 to 9.0 millimetres. Relative to the crop plants, seeds of Arabidopsis are in the order of 80 times lighter in weight, at around 0.02 milligrams (Hoai *et al.*, 2020), and much smaller in dimensions than the smallest seed from previous studies; Arabidopsis dimensions are on average 0.4 and 0.6 millimetres in width and length, respectively (Robert *et al.*, 2008). The small size of Arabidopsis seeds makes them difficult to work with, particularly when the number of seed available is limited which can be the case when working with mutant and transgenic lines where seed from each line needs to be bulked up and is being used for a range of phenotypic tests. To address this challenge methods to assess seed weight and dimensions for this study involved determining seed weight using scales that measured to five decimal

places and dimensions captured using image analysis (see section 3.3.5, 3.3.8 and Table 3.12 in Chapter 3).

Video recording methods are commonly used to detect size changes for *Xenopus laevis* oocytes in experiments to determine water permeability characteristics when oocytes are expressing aquaporin proteins of interest (Byrt *et al.*, 2017). The same recording system was used in the present work to trace the change of seed maximum cross sectional (MCS) area during imbibition. The video-based methods were modified because trial experiments revealed problematic variation between replicates which may have been associated with changes in image clarity and focus over time during the recording. Image clarity was lost after 12 hours recording during the night when the experiments were run for 5 consecutive days. This was a consequence of water evaporating and condensing on the upper lid, the lack of focus meant that the edges of those seeds could not be discerned. To solve the problem, a hole with dimension of 6 x 8 mm was pierced through the upper lid and seeds were planted in the same area. This solved the condensation problem and created a new problem. The holes were not covered during the 5 days of the experiment due to continuous filming, therefore under the microscope light and 22°C conditions, water evaporated which influenced the thickness of the media, causing shrinking. Hence, the position of the seed changed during experiments and caused images to shift out of focus. Finally, seeds were kept in petri dishes and enclosed in transparent boxes, and images were taken at target timepoints determined from video footage from trial experiments. With these method revisions the coefficient of variation (CV) for repeated tests within the experiments was reduced from over 15% to under 5% (Table 3.12) providing confidence in the reliability and repeatability of the data.

From previous studies using pycnometers to measure seed density, the CVs were different between species; for example, soybean, field corn, and sunflower ranged from 3.3%, 7.7% and 12.5% respectively (Nelson, 2002). As the incorrect seed density can introduce significant errors in process calculations when comparing grain and legume seed density using a pycnometer and an envelope density analyzer (Fasina, 2010), reducing the CV to under 5.0% in my experiments compares favourably to pycnometry on other types of seed (Nelson, 2002). In future research, if Arabidopsis seed numbers were more available, measuring density and seed size using a liquid pycnometer and the transmitted light from the slide scanning function of a flatbed scanner and particle analysis of the resulting images (Herridge *et al.*, 2011) should be considered.

6.3 Potential implications of tagging AtPIP2;1 with nGFP in relation to seed phenotypes

Relative to the other seven PIP2-type genes in Arabidopsis the *AtPIP2;1* gene is notable for having transcripts detectable in most cells and tissues. This gene has particularly high transcript levels detected in roots, guard cells, leaves, shoot apical meristems and stem epidermal cells (Klepikova *et al.*, 2016; Winter *et al.*, 2007). Previous analysis of *AtPIP2* promoter::GUS fusion lines by Da Ines (2008) revealed that most PIP2 genes exhibited expression in roots, leaves and flowers, but there were differences in their expression pattern, with GUS activity for AtPIP2;1, AtPIP2;2 and AtPIP2;7 being particularly high in roots and leaves, and in root hairs (see Supplementary Figure 1). The Da Ines (2008) study also revealed that when one *AtPIP* gene is knocked out then others may be upregulated to compensate which can complicate interpretation of data from phenotyping mutant knock out lines.

The work of Da Ines did not include analysis of seeds. There is little information about PIP2;1 localisation and abundance in seeds during imbibition. Previous studies from Igawa *et al* (2013, 2017) reported that expression of GFP-PIP2;1 driven by DD45 promoters (pDD45::GFP-AtPIP2;1) in Arabidopsis revealed GFP-AtPIP2;1 in boundary cells of female gametes, cell membranes of sperm and egg cells during fertilisation. The authors observed GFP-PIP2;1 in plasma membranes of male and female gametes during double fertilisation (Igawa *et al.*, 2017; Igawa *et al.*, 2013). However, similar to the challenge faced in the present study where the construct used to express nGFP::AtPIP2;1 included the UBI promoter rather than the native promoter, the construct used by Igawa et al. (2017) was not driven by the native promoter. Therefore, analysis of the patterns in GFP might not reveal what occurs in wild type plants. Localizations of PIP2;1 in germinating seeds and seedlings using fusions of *AtPIP2;1* with fluorescent tags driven by the native *AtPIP2;1* promoter are needed to characterise the location of AtPIP2;1 in seed. This type of experiment would help provide a better understanding of whether there are associations between AtPIP2;1 abundance and areas of the seed that are key in relation to the rapid growth stages during germination.

The seed of UBI::nGFP::PIP2;1 did not express the tagged GFP in the 24 hours following sowing, but GFP could be detected from 36 hours after the start of imbibition (Figure 3.19 - Chapter; Figure 2 - Chapter 5). The timing of detection of nGFP::PIP2;1 is similar to the timing of upregulation of expression of *AtPIP2;1* during imbibition based on transcript abundance reported by Groszman et al. (2021), Hoai et al. (2020) and Klepikova et al. (2016). It is unclear

if this is a coincidence or whether there are mechanisms influencing the up-regulation of *AtPIP2;1* expression at this stage that are not dependent on the activity of the native promoter.

In young UBI:nGFP::PIP2;1 seedlings, the assessment immediately post-germination fluorescent revealed that fluorescent signals were observed in root regions, specifically root tips, in the stele potentially in the vicinity of endodermal cells, in the vegetative meristem and in guard cells (Figure 3 - Chapter 5). *AtPIP2;1* transcript abundance is also high in roots, stems and vegetative meristems in seedlings (Groszmann *et al.*, 2021), and in vascular bundles (Alexandersson *et al.*, 2005; Rodrigues *et al.*, 2017). This reveals that AtPIP2;1 may have a role at the stage of seedling early vigour, which could be important for supporting optimal seedling development.

Interpreting whether or not data from the UBI:nGFP::PIP2;1 material might relate to what normally happens in wild type Arabidopsis is problematic not just because the construct was not driven by the native promoter, but also because the nGFP tag may influence PIP2;1 function. The influence of the promoter or the tag could have contributed to why the phenotypic trends for the 2x35S::AtPIP2;1 lines and UBI:nGFP::PIP2;1 differed, for example the timing of changes in seed weight during imbibition at 4 and 30 h timepoints (Figure 2 - Chapter 4 and Figure 5 - Chapter 5). The UBI and 35S promoters may have caused differences in the level of expression of *AtPIP2;1* in the different lines. The nGFP may interact or interfere with sections of the AtPIP2;1 protein, such as the movement of the n-terminal region, or with the interactions between AtPIP2;1 and other proteins (Weill *et al.*, 2019), and this could cause differences in phenotype for the material tested.

Previous concerns have been elaborated about the "GFP effect" on the biological activity of tagged proteins (Baens *et al.*, 2006; Koelsch *et al.*, 2013; Zhang *et al.*, 2014). For example, GFP tagging reportedly led to reduced binding affinity between interacting partners (Zhang *et al.*, 2014). GFP is quite large (>20kD), and tagging a protein of this size onto another protein, in this case PIP2;1, at either -N or -C terminals might disrupt interactions either within the monomer or the tetrameric assembly, or with other proteins (Rana *et al.*, 2018). The phenotypic results were not always similar in the different material assessed that had overexpression of PIP2;1 (nGFP and 2x35S), and the potential interference of the tag could be a factor that influenced the differences in the phenotypes and the total expression may have been different since the two promoters are different.

6.4 AtPIP2;1 may have multiple roles in seed germination in saline conditions

PIP2;1 is a candidate for regulating water transport in seed during germination

The adaptation of seed germination to environmental stress influences the successful establishment of a plant (Ibrahim, 2016; Laetitia and Christian, 2008; Liu *et al.*, 2019; Nguyen *et al.*, 2021; Srivastava *et al.*, 2021; Zhang *et al.*, 2010). Salinity is a major abiotic stress that impacts plant development because the ionic and/or oxidative stress decreases the potential for seed germination and the osmotic stress delays seedling growth (Longo *et al.*, 2021; Munns and Tester, 2008). Osmotic stress can affect starch synthesis and energy production processes (level of adenosine triphosphate - ATP) achieved through seed respiration (Bewley *et al.*, 2013), resulting in reduced germination percentage and causing delay in the timing of germination. The most common responses of plants to the reduction of osmotic potential are a delay in the initial germination and a reduction in the germination percentage (Steiner *et al.*, 2019). Our knowledge about the mechanisms of water and ion (e.g. Na^+ and/or K^+) transport in imbibing and germinating seeds in conditions of salt or osmotic stress is limited. It is conceivable that PIP2;1 water and/or ion transport could be involved in seed germination responses to different environmental conditions (Qiu *et al.*, 2020). For example, hypothetically aquaporins that can function as non-selective monovalent ion channels could be involved in mechanisms such as contributing to ion efflux during imbibition.

When the concentration of NaCl in the soil solution around seeds increases, there is an associated decrease in the water potential gradient between the seeds and their surrounding media (Bewley *et al.*, 2013; Zhang *et al.*, 2010). Seeds exposed to high concentrations of NaCl tend to have poor germination success, with final percentages of germination of seeds treated with high salt concentrations tending to be much lower than that of seed treated with water (Laetitia and Christian, 2008). In many studies, salinity stress negatively affects seed germination, either osmotically through reduced water absorption or ionically through the accumulation of Na^+ and Cl^-, causing an imbalance in the nutrient uptake and toxicity effects (Ahmed *et al.*, 2020; Carles *et al.*, 2002; Steiner *et al.*, 2019; Werner and Finkelstein, 1995; Yang *et al.*, 2019; Zeng *et al.*, 2021). Saline conditions had negative effects on germination percentage and delayed germination in rice (Xu *et al.*, 2017; Zeng *et al.*, 2021), wheat (Lethin *et al.*, 2020), quinoa (Panuccio *et al.*, 2014) cowpea (Ravelombola *et al.*, 2017) and sugar beet (Wang *et al.*, 2017a). The influence on the trends in seed germination varied according to the

change in NaCl osmotic potential, for example Cokkizgin (2012) reported that NaCl negatively impacted seed germination of *Phaseolus vulgaris L* and that seed was sensitive to the different osmotic potentials associated with salt treatments.

In the present study, decreases in percentages germination in the salt stress treatment were observed relative to percentages from seed germinated in water, and I observed that seed with a different abundance of PIP2;1 (mutant or overexpression lines) behaved differently compared to controls in the salt treatment. This may be due to seeds developing an osmotically enforced 'dormancy' (Llanes *et al.*, 2016). A previous example indicating an adaptive strategy seeds deploy to prevent germination under harsh environmental conditions is evident from the observation that 80% of the seeds of *Arabidopsis thaliana* mutants *abi5-5* and *abi5-2*, as well as *abi4-1*, germinated on 175 mM NaCl, whereas control seeds did not germinate at this salt concentration (Carles *et al.*, 2002). This implicates ABA signalling in this dormancy response. Two weeks after the germination stage, more than half of all the *abi* mutant seedlings that had germinated in 175mM NaCl had not survived (Carles *et al.*, 2002). The delay in germination of Arabidopsis under saline stress was likely to be due to an increase in the external osmotic stress which adversely affected the water absorption by seeds. The seed needed to reach an adequate level of hydration during the imbibition phase in order to support the reactivation of seed metabolic processes and growth of the embryonic axis (Bewley *et al.*, 2013), and the seeds exposed to saline stress required more time to adjust the internal osmotic potential in accordance with the external environment (Munns and Tester, 2008).

Seed germination responses to saline conditions have been explored in many previous studies, however, the roles of genes that display sodium-responsive changes in expression in seeds is an area where there are limited previous studies. Resolving the key transcription factors that are involved in these processes is an important step to understanding the regulation of seed germination responses to stress. Previously zinc finger type transcriptional regulators and mitogen-activated protein kinases such as ZAT6 (AT5G04340.1) and MPK6 (AT2G43790.1) have been shown to be important in regulating Arabidopsis seed germination under salinity and osmotic stresses, and associations between phytohormones like ABA and GA and transcriptional regulators such as MYB44 that influence seed germination have been revealed (Liu *et al.*, 2013c; Nguyen *et al.*, 2012). In the present work analysis of the phenotypes of lines with varying abundance of AtPIP2;1 in control and salt-stressed treatment reveal a potential role for AtPIP2;1 in seed germination in saline treatments. Exploring which key transcription factors and protein kinase signalling pathways are influencing *AtPIP2;1* regulation during seed

germination in saline conditions would be an interesting direction for future research. Particularly considering that differential phosphorylation of AtPIP2;1 is expected to influence whether AtPIP2;1 functions more as a water channel or as a cation channel and this could be important in relation to AtPIP2;1 contributing to optimal seed germination (Prak *et al.*, 2008; Qiu *et al.*, 2020). This may be a key area for future research when looking at the ABA responses in the *pip2* mutants.

Hypothetically AtPIP2;1 could function as a monovalent cation channel in imbibing seed (Byrt *et al.*, 2017; McGaughey *et al.*, 2018; Qiu *et al.*, 2020). In saline treatment, when AtPIP2;1 is knocked-out or over-expressed it might be that this leads to differences in Na^+ and K^+ transport in mutant or transgenic seed relative to controls. The germination percentage and T_{50} of mutants *Atpip2;1-1, Atpip2;1-2* and *Atpip2;1*2;2* did not differ in the control treatment, but fewer mutant seeds germinated in saline treatment relative to controls and germination of the mutant seed was delayed in 75 mM NaCl after 3 days sowing. These differences could be due to the potential influence of different AtPIP2;1 associated to water or ion transport trends or responses to hormone regulation during the germination process that involve AtPIP2;1 or a combination of both these factors. The germination percentage of all genotypes after 9 days of sowing was similar (Figures 3.21- 3.22), revealing that the influence of loss-of-function PIP2;1 was no longer a factor, and that the mutant seeds presumably managed to adopt other mechanisms to compensate for the loss of PIP2;1. Similarly in a recent study, the percentage of germination of Col-0 was lower in salt (75, 100, and 125 mM NaCl) treatments in the 2 and 3 days after sowing relative to transgenic lines over-expressing a glucose-6-phosphate dehydrogenase (G6PD), but the germination of all tested genotypes including Col-0 were not different by 4 days after sowing (Jin *et al.*, 2021). Col-0 seeds tend to achieve over 95% germination after 4 days of sowing even if there is a delay in germination in the 1-3 days after sowing in 50 and 100 mM NaCl salt treatments (Yang *et al.*, 2019). This reveals that the catch up in the germination of the loss-of-PIP2;1-function material relative to Col-0 which occurred may be a feature that is not associated just with the material tested in the present study.

PIP2;1 and monovalent ion transport in germinating seed

Seed Na^+ and K^+ content was similar in knock out mutants *Atpip2;1-1 Atpip2;1-2, Atpip2;1*2;2* and overexpression UBI:nGFP::PIP2;1 seeds relative to Col-0 seeds, and similar in overexpression 2x35S::AtPIP2;1 seed compared to seed from the relevant null control line (Figure 3 - Chapter 4 and Figure 7 – Chapter 5). However, the Na^+ content was lower in mutant

seed exposed to the transfer treatment that involved moving the seed from water to 75 mM NaCl after 30 hours of imbibition relative to Col-0 and lower in 2x35S::AtPIP2;1 seed relative to the null seed (Figure 3 - Chapter 4). This may indicate that AtPIP2;1 needs to be at wild-type level to contribute to optimal solute uptake in imbibing seed when the seed experiences a sudden change in osmolality, such as the change associated with transfer from water to 75 mM NaCl from 30 – 50 hours imbibition.

Seed K^+ content increased in the saline treatment for the mutant seed and UBI:nGFP::PIP2;1 seed, relative to their respective controls; but for the overexpression 2x35S::AtPIP2;1 seed differences in K^+ content were only observed between the transgenic and control seeds in the conditions involving transfer from water to 75 mM NaCl (Figure 3 - Chapter 4 and Figure 7 – Chapter 5). Previously Prak et al. (2008) reported that NaCl treatment induced an intracellular accumulation of At-PIP2;1 which was associated with differential C-terminal site phosphorylation. AtPIP2;1 forms differing in their phosphorylation at Ser_{283} were observed to differ in accumulation in distinct intracellular structures. Hypothetically, the amount of AtPIP2;1 and the amounts of AtPIP2;1 with different phosphorylation states could differ in the mutant and transgenic lines (Qiu *et al.*, 2020). This means that the mutant and transgenic lines might have less AtPIP2;1 present in the plasma membrane in a state that permits ion permeation, and this could influence Na^+ and K^+ content in imbibing seed. If this was the case it might explain in part why the loss of-function mutants had less Na^+ that wild type during germination when they were exposed to salt at 30 hours into imbibition. This time is expected to be the start of the upregulation of *PIP2;1* expression and increase in PIP2;1 abundance (Figure 2 - Chapter 5).

If a greater abundance of AtPIP2;1 has potential to facilitate greater uptake of Na^+ into seeds in saline treatment then for the overexpression 2x35S::AtPIP2;1 lines one would expect greater seed Na^+ content than that of the null control lines, but this is not what was observed (Figure 3 - Chapter 4). The seed Na^+ was lower and seed K^+ was higher in the null lines than the transgenic lines (Figure 3 - Chapter 4). Potentially the salt treatment could result in different regulation of AtPIP2;1 phosphorylation state depending on the abundance of AtPIP2;1 and this could complicate the relationship between AtPIP2;1 abundance and associated ion transport contribution. The abundance and phosphorylation status of PIP2;1 were reported to change when Arabidopsis roots were exposed to salt (Boursiac *et al.*, 2005; Sutka *et al.*, 2005). Salt stress increased the trafficking of AtPIP2 isoforms between the plasma membrane and intracellular compartments, reducing the abundance of PIP2 proteins on the plasma membrane,

and reducing the hydraulic conductivity of salt-stressed root cells. In the present study, the seeds from parent plants grown under saline watering regimes had higher seed Na^+ and higher Na^+ content in samples of above-ground shoot and leaf tissues than plants in control treatment (Figure 3.25, 3.26, 3.29, 3.20). As expected, the *Athkt1* genotype accumulated higher Na^+ in seed and shoots than all other lines because this mutant lacks the important HKT1 mechanism for limiting root-to-shoot Na^+ accumulation in saline treatment. The mutant and transgenic lines tended to accumulate more Na^+ in their above ground tissues than wild type Col-0 in the saline treatment (Figure 3.30 in Chapter 3), indicating that there is potential that AtPIP2;1 contributes in some way to influencing Na^+ transport in Arabidopsis. However, it is unclear whether these differences relate to the likelihood that there were hydraulic conductance differences, given the previous observations reported by Boursiac et a. (2005) and Sutka et al. (2005), or whether it relates to AtPIP2;1 ion transport features (Qiu *et al.*, 2020).

AtPIP2;1 may influence the regulation of other AQPs during seed imbibition and germination.

Changing the regulation of one aquaporin is expected to have feed-back effects in relation to the regulation of other aquaporins. For example, expression of PIP2-type aquaporins could regulate the PIP1-type aquaporins in root and leaves of Arabidopsis (Liu, 2015). When Arabidopsis seed germinates in stressful conditions the transcript abundance of genes coding for aquaporins may differ relative to when seed germinates in favorable conditions. For example, an ABA treatment triggered a decrease in *PIP2;1* and *PIP2;7* transcript abundance in germinating seed while GA triggered an increase in *PIP2;7* (Goda *et al.*, 2008; Hoai *et al.*, 2020). Hypothetically, the mutant and transgenic lines studied here that had different AtPIP2;1 abundance might also have different AtPIP2;7 abundance if differential regulation of AtPIP2;7 occurs to compensate for disrupted AtPIP2;1. AtPIP2;7 contributes to hydraulic conductivity in Arabidopsis and is important in stress responses. For example in Arabidopsis, PIP2;7 protein interactions with a tryptophan-rich sensory protein/translocator (TSPO) was reported to be important in fine-tuning the water permeability of the endoplasmic reticulum and Golgi membranes in planta (Hachez *et al.*, 2014). The interaction of TSPO versus AtPIP2;7 is stress responsive and associated with AtPIP2;7 phosphorylation state (Hachez *et al.*, 2014). ABA can decrease the relative phosphorylation level of AtPIP2;7 by up to 26% within 5 min of incubation of plant tissues in 50 μM ABA and can result in a 60% decrease after 30 min of incubation, which is predicted to cause gating, meaning closure of the water-transport of

AtPIP2;7, and the implications are linked with ABA's role in ameliorating the effect of drought (Kline *et al.*, 2010).

Hypothetically seeds may require specific changes in the regulation of aquaporin transcripts to support appropriate osmoregulation as they progress through germination. In relation to seed priming processes, if dry seeds were hydrated for a certain time then dehydrated again and stored those primed seeds may have different abundance of aquaporins (such as PIP2;1) if they were then subsequently used in germination experiments. Previous studies of aquaporin expression during seed osmo-priming in Spinach (*Spinacia oleracea*) revealed associations between aquaporins and enhanced germination performances associated with priming (Chen *et al.*, 2013). Further research is required to understand whether aquaporins are involved in seed priming mechanisms and to explore whether it is relevant in field conditions. In brief, the disruption or enhancement of AtPIP2;1 may cause differential regulation of other aquaporins that influence seed germination via water and ion transport. Temperature is also a key factor that may influence *AtPIP2;1* regulation in seed. For example, Arabidopsis seed imbibed for 96 hours at 4°C relative to 22°C had differences in the transcript levels of *AtPIP2;1* (Yamauchi *et al.*, 2004). Seed imbibed at 22°C had very high transcript levels of *AtPIP2;1* whereas seed imbibed at 4°C had relatively low *AtPIP2;1* transcript levels, even not significantly different to that of dry seeds. This could mean that transcript levels differed in seed subjected to stratification (for the germination tests) (Figure 1 – Chapter 4, Figure 4 – Chapter 5) and relative to seed that was not subjected to stratification (relevant to the weight, MCS area and ion content experiments) (Figure 2, 3, S2 – Chapter 4, Figure 5, 6, 7 – Chapter 5). Further experiments would be required to test whether the different approaches used for the various phenotyping assays resulted in different levels of *AtPIP2;1* expression in the material tested.

Changes in seed weight and seed MCS for seed differing in PIP2;1 abundance

A dry quiescent seed goes through three phases of absorbing water to reach the germination stage and develop into a seedling (Figure 1.3) (Bewley, 1997). Measurements revealing the increase of seed weight during the germination of Arabidopsis seed observed here was as expected relative to the defined Phases (See Fig. 2 from Hoai *et al.*, 2020). The observations reported in Chapters 3, 4 and 5 in relation to seed weight and seed MCS area changes during imbibition and germination were consistent with the published reports. The linear correlations of seed weight and seed MCS were positive during these stages (Figure S4 – Chapter 4). The water uptake during imbibition, revealed by weight and area changes, influence whether seed

will germinate (Nee *et al.*, 2017). Within four hours of imbibition, seed weight doubled and seed MCS area increased to over 60% of that for the dry seed; this change represents the first phase of imbibition (Figure 3.24, 3.33). It is followed by smaller increases in weight and area up until seed germination, which is achieved by the stage of 50 hours after sowing. Water uptake into seed was slower in saline treatment than in water due to the influence of the salinity or osmotic potential, causing negative water potentials, and hindering seed water uptake (Cokkizgin, 2012; Steiner *et al.*, 2019; Yang *et al.*, 2010). In the second phase of imbibition, although seed continues to absorb water, it may lose weight as it uses up starch stores in the cotyledons for producing energy, as it is preparing for germination, and of course the seedling cannot photosynthesise at this stage (Bewley *et al.*, 2013). Therefore, the seed weight and seed MCS area increased slowly from 4 hours after sowing (HAS) to 50 HAS (Figure 3.24) and the seed MCS area increased just about 15% at 30 HAS compared to 4 HAS (Figure 3.33). In water or saline treatments, seedling weight for the 2x35S::AtPIP2;1 OE (21)* line was more than double that of the mutant *Atpip2;1*2;2* line, indicating the possibility that the abundance of PIP2;1 in the transgenic line relative to the mutant influenced the magnitude of water uptake during seed imbibition and germination (Figure 3.24). However, there were not clear differences between the mutant and transgenic lines and their respective wild type and null control lines. Further investigation involving measuring hydraulic conductance in these seedlings is needed to check whether the abundance of AtPIP2;1 influenced the seedling water uptake.

6.5 Phytohormone regulation and seed germination in saline conditions

The potential interactions between hormone signalling and roles for AtPIP2;1 in seeds was discussed in Chapter 4 (Discussion section, page 142). In this section, a model to represent the potential interactions will be presented and future directions in relation to resolving the relationships between hormone signaling and AtPIP2;1 regulation in seeds will be discussed (Figure 6.1).

ABA

GA

Auxin

Nitric oxide

Ethylene

H_2O_2

Wild type
(Col-0)

Loss of function
Atpip2;1 mutant

$T_{50} = 29\text{-}31$ h

2x35S::PIP2;1
over-expression

Control

0   10   20   30   40   50

Hours to achieve germination

Saline

Nitric oxide

GA

ABA   Auxin

Ethylene H_2O_2

Wild type (Col-0) $T_{50} \sim 34$ hrs

Loss of function *Atpip2;1* mutant $T_{50} \sim 44\text{-}51$ hrs
Reduced K^+

2x35S::PIP2;1 over-expression $T_{50} \sim 40\text{-}42$ hrs
Increased K^+

- Differential regulation of multiple aquaporins due to altered PIP2;1 abundance and altered PIP2;1 response to changes in signalling
- Potential for altered cell-to-cell permeability to water, monovalent ions and hydrogen peroxide
- Disruption of AtPIP2;1 impacting successful control of hydraulic conductivity to support radicle emergence

AtPIP2;1 responds to auxin signals influencing root growth

AtPIP2;1 is permeable to H_2O_2

Ethylene changes trigger differential phosphorylation of AtPIP2;1 S280/S283 which alters localisation, water and ionic conductance

Figure 6.1 Summary of hours to achieve germination for wild type (Columbia-0), loss of AtPIP2;1 mutant lines and overexpression (2x35S::AtPIP2;1) lines in control (water) and saline conditions in the context of expected differences in signalling molecule trends. Expected trends in signalling molecule change in control and saline conditions in Arabidopsis are represented by upward pointing triangles for expected increases and downward pointing triangles for expected decreases in the saline conditions relative to the control conditions and these are

presented over the time in which seeds imbibe and begin to germinate following previously published reports investigating changes in these key signalling molecules in Arabidopsis seed during imbibition and germination (Arc *et al.*, 2013a; Bethke *et al.*, 2006; Choudhary *et al.*, 2020; Corbineau *et al.*, 2014; Daszkowska-Golec, 2011; El-Maarouf-Bouteau and Bailly, 2008; Jung and Park, 2011; Millar *et al.*, 2006; Molassiotis *et al.*, 2010; Péret *et al.*, 2012; Prak *et al.*, 2008; Qing *et al.*, 2016; Qiu *et al.*, 2020; Rodrigues *et al.*, 2017; Sanz *et al.*, 2015; Tao *et al.*, 2015; Vishal and Kumar, 2018; Wojtyla *et al.*, 2016). This is a hypothetical model.

Control of seed germination is orchestrated by the phytohormones gibberellic acid (GA) and abscisic acid (ABA) whose endogenous levels are regulated by the environmental signals such as light, temperature, salinity, drought etc. GA promotes germination while ABA represses seed germination (Llanes *et al.*, 2016). The production of GA and ABA by seeds is controlled in response to environmental signals such as light, water potential, temperature, concentration of nitrate or ion toxicity (sodium, chloride) (Bewley *et al.*, 2013; Nonogaki, 2019; Rodríguez-Gacio *et al.*, 2009). Hormone levels in seeds are determined mainly by metabolism – biosynthesis and catabolism. Another critical factor, which could significantly affect hormone responses in seeds is the transport of hormones and their precursors from or to different cells and tissues within seeds.

There are previous studies reporting on the interactions between hormone signalling and seed germination. The Arabidopsis endosperm is a single cell layer surrounding the embryo, and it synthesizes and continuously releases ABA towards the embryo via four AtABCG transporters which were believed to be involved in controlling seed germination and in preventing germination in drought and salinity conditions (Kang *et al.*, 2015). The loss of function of a leucine-rich repeat protein, LRRop-1, in Arabidopsis seed influenced germination in relation to the response to ABA-mediated abiotic stress, and led to an increase in germination percentage in 75 mM NaCl media (Ravindran *et al.*, 2020). There was also the example presented in section 6.3 regarding associations between phytohormones like ABA and GA and transcriptional regulators such as by MYB44 that influence seed germination (Liu *et al.*, 2013c; Nguyen *et al.*, 2012).

The germination process is regulated not only by ABA and GA but also by more complex interactions between other hormones and their regulation during germination. In the mutant

and transgenic material studied here, there may be differential regulation of multiple aquaporins due to altered PIP2;1 abundance and/or altered PIP2;1 response to changes in signalling. For example, H_2O_2 rapid systemic signals could occur via PIP2;1, which could influence seed germination (see Figure 2D, Fichman *et al.*, 2021). When plants were treated with salt (NaCl) and H_2O_2, it was reported that PIP2;1 phosphorylation at two C-terminal sites (Ser_{280} and Ser_{283}) were affected and this influenced intracellular AtPIP2;1 accumulation (Prak *et al.*, 2008). Ethylene also was found to regulate water transport via the C-terminal phosphorylation of AtPIP2;1 (Qing *et al.*, 2016). Also seed of Medicago treated with ethylene differed in the relative salt tolerance of the germinating seed, boosting seed tolerance to salt stress (Wang *et al.*, 2021). AtPIP2;1 putatively interacts with MDR4 auxin efflux transporter (ABCB4), and auxin influences seed germination speed (Wang *et al.*, 2016b). This positions AtPIP2;1 to potentially be influenced by many different pathways of hormone signals (Figure 6.1).

6.6 Conclusion and future directions

Alterations in the abundance of PIP2;1 influenced Arabidopsis seed germination in saline conditions. This could be associated with a range of complex factors and future research is needed to determine which of these factors may be most important (Figure 6.2).
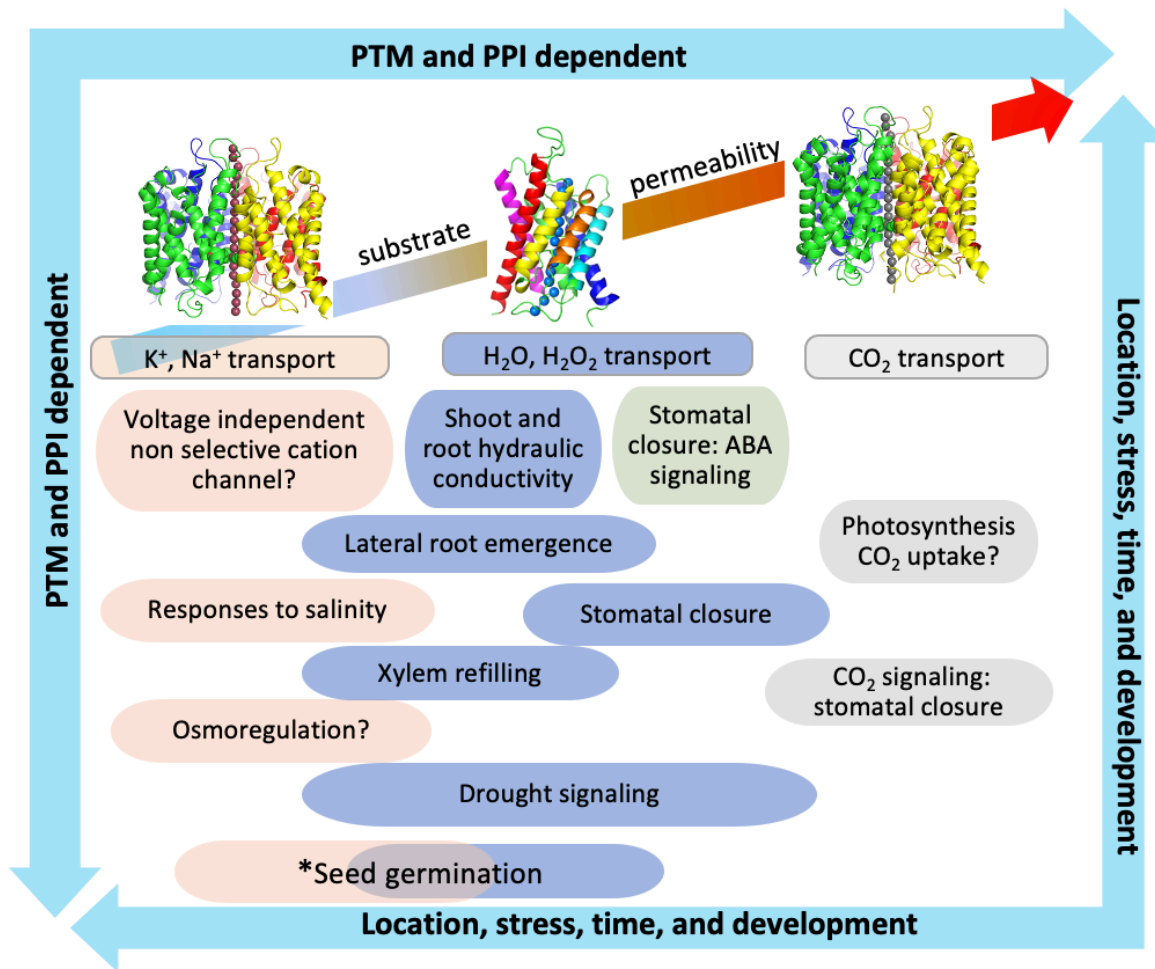
Figure 6.2 Complexity in determining AtPIP2;1 role(s) in seed germination in the context of the multidimensional functionality of AtPIP2;1. AtPIP2;1 is complex because this protein has the ability to transport monovalent cations, water, hydrogen peroxide and carbon dioxide and the transport is dependent on post translational modifications (PTMs) and/or protein-protein interactions (PPIs). The various known functions and putative functions for AtPIP2;1 are indicated under each transport mode in the figure. It is not known if simultaneous transport of different substrates can occur but this might be relevant to some functions (e.g. signaling) in relation to ion conductance and/or H_2O_2 permeability. Structures indicated are for AtPIP2;1 modelled on AtPIP2;4 (6qim (187) (Wang *et al.*, 2020)) obtained from Swiss-Model P43286. Also shown are the trajectories for the pores through the central pore of the tetramer and the intrasubunit pore (water pore) obtained from Porewalker (2009) drawn with PyMol with a cutaway view to make the pores more visible. Transmembrane helices are presented in different colours and a single protomer is shown in the centre image. This figure was adapted with

permission from Tyerman et al. (2021) Annual Review of Plant Biology to include
*seed germination as a development stage where AtPIP2;1 function is important.

Atpip2;1-1, *Atpip2;1-2* and *Atpip2;1*2;2* mutants lack either AtPIP2;1, or AtPIP2;1 and AtPIP2;2, and seed from these lines had lower percentages of seed germination than Col-0 when germinated in 75 mM NaCl but they did not differ from Col-0 when germinated in water. The overexpression lines including 2x35S::AtPIP2;1 and UBI:nGFP::PIP2;1 also had lower percentages of germination compared to null and WT Col-0 controls. Additional experiments are needed to determine whether the trends observed are related to AtPIP2;1 membrane transport, response to hormones or interactions with other proteins. Future experiments could include germination tests in the presence of ABA, GA and the use of ROS sensors to check for differences in H_2O_2 signalling towards gaining greater understanding of the role of AtPIP2;1 in seed germination. Potentially this could be completed efficiently using a set-up involving automation of germination experiments. For example, using robot technologies such as 'ScreenSeed' technology which was recently reported as a promising approach to monitor seed germination of Arabidopsis, which involves automated image analysis and captures radicle protrusion (Merieux *et al.*, 2021). Adoption of this type of approach may improve the efficiency of phenotyping seed, and enable the scaling-up of the size of experiments to avoid any human error in measurement collection.

It would be ideal in future experiments to include controls involving germination tests that include mannitol treatments (isosmotic relative to the NaCl treatments). It would also be ideal to test seed ion content following germination experiments where other monovalent ions such as K^+ or Li^+ were included in the media and testing of accumulation of these elements in the seed during imbibition and germination time-courses. Seeds could also be moved from media containing these ions to a media without these ions, and micro-electrode ion flux could be used to see if different amounts of ions could be detected in the media following the transfer of the seed (Shabala *et al.*, 2003). These types of assays could help reveal the potential role for AtPIP2;1 in influencing seed influx or efflux of ions during germination.

Studying GFP in UBI:nGFP::PIP2;1 seedlings exposed to various salt and osmotic stress treatments would be useful for determining whether changes in localisation of GFP show similar trends to those reported previously by Prak et al (2008). This would enable greater understanding of whether trends are controlled by post-translational mechanisms or whether the native *AtPIP2;1* promoter is important in this level of regulation. Transcriptomic and

proteomic analysis to explore the abundance of all aquaporins in the mutant and transgenic lines during imbibition and germination would also be useful for exploring whether differences in the abundance of AtPIP2;1 influence the regulation of other aquaporins towards understanding the role of AtPIP2;1 in seed germination.

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Appendix
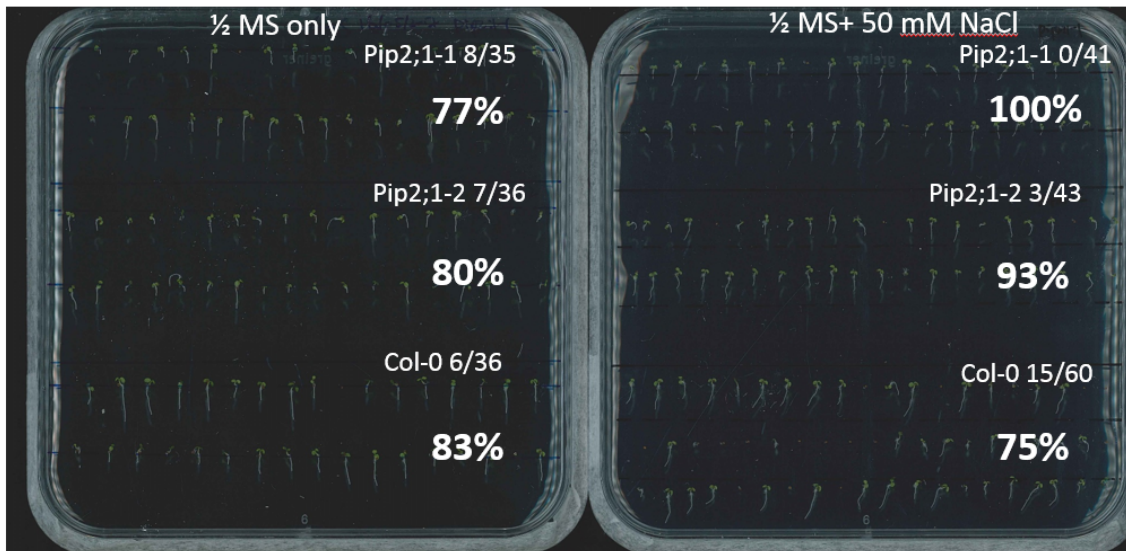
Figure S1: Percent germination in control ½ MS (Murashige and Skoog, 1962), pH 7.0 and saline ½ MS, pH 7.0, (added 50 mM NaCl) conditions (Jiaen et al. 2017, unpublished data, personal correspondence).
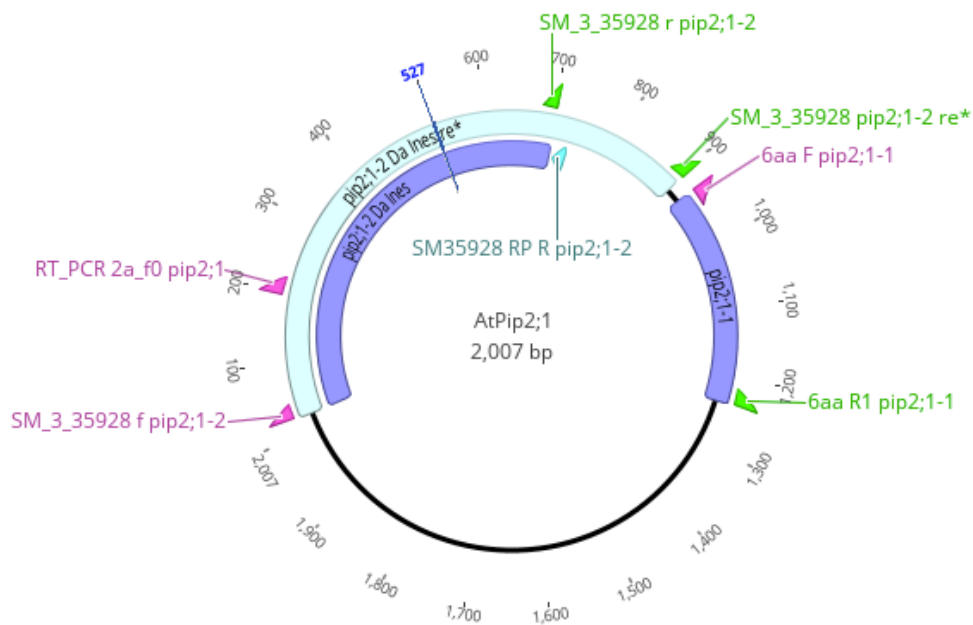
Figure S2: Map of Atpip2;1 Chr3:19803657..19805663, with the length 2007 bp presented by Genious Prime. 6AA F/R are the pip2;1-1 primers of T-DNA insertions. SM_3_3598 F/R are the pip2;1-2 primers of T-DNA insertion.

Table S3. Primer information. Melting temperature (T$_m$); Calculated using the link from www.thermofisher.com

Transgenic lines	T-DNA accession name	Primer name	Sequence	Tm (°C)	Length Product	PCR product homo-zygous transgenic	PCR product Hetero-zygous / non-inserted transgenic or WT
Atpip2;1-1	6AAS98 Amaze	6AA F	5'-GCTTGTTGTACCTTAATTCTCAAGT-3'	62.6	~314 bp	0	1
		6AA R	5'-AGAGAAGACGGTGTAGACAAGAAC-3'	64.8			
		6AA-En205 F	5'-GGTGCTTGTGTTATGTTATGTTGA-3'	62.8	~563 bp	1	0
		6AA R	5'-AGAGAAGACGGTGTAGACAAGAAC-3'	64.8			
Atpip2;1-2	SM_3_35928 JIC SM	SM35928 F	5'-AACATATAACGTTGGCAAAAA-3'	57.9	>900 bp	0	1
		SM35928 R	5'-TTAATTTCGAATAAACAAACCA-3'	56.0			
		Spm 32 F	5'-CTTATTTCAGTAAGAGTGTGGGGTTTTG G-3'	66.8	>500 bp	1	0
		SM35928 R	5'-TTAATTTCGAATAAACAAACCA-3'	56.0			
Atpip2;1 pip2;2	SM_3_35928 JIC SM	SM35928 F	5'-AACATATAACGTTGGCAAAAA-3'	57.9	>900 bp	0	1
		SM35928 R	5'-TTAATTTCGAATAAACAAACCA-3'	56.0			

and		Spm 32 F	5'-CTTATTTCAGTAAGAGTGTGGGGTTTTGG-3'	66.8	>500 bp	1	0
	Sail_169A03	SM35928 R	5'-TTAATTTCGAATAAACAAACCA-3'	56.0			
		Sail_LB3	5'-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3'	67.7	>600 bp	1	0
		Sail_169A03 RP R	5'-TTATAGATTACGGCAGCTCCG-3'	62.5			
		Sail_169A03 LP F	5'-CAACCATAAGCCTACCAAAAGG-3'	62.4	>900 bp	0	1
		Sail_169A03 RP R	5'-TTATAGATTACGGCAGCTCCG-3'	62.5			
35S::PIP2;1	35S PMDC32 plasmid	AtPIP2;1 InF1	5'-ACTGACCCCAAACGTAGTGC-3'	65.4	>500 bp	1	0
		Nos-terminator R1	5'-CAAGACCGGCAACAGGATT-3'	63.4			
		2x35S F	5'-TCATTTGGAGAGGACCTCGA-3'	63.3	~980 bp	1	0
		AtPIP2;1 CDS R	5'-TTAGACGTTGGCAGCACTTCT-3'	65.0			
UBI:nGFP::PIP2;1	pUB-Dest	Ubi:64p F1	5'-GTCGTCCTCGTCTTTTCTTCT-3'	62.7	~1982 bp	1	0
		AtPIP2;1 CDS R	5'-TTAGACGTTGGCAGCACTTCT-3'	65.0			
House-keeping gene Actin 2		AtActin 2 F	5'-TGAGCAAAGAAATCACAGCACT-3'	63.8	~300 bp	1	1
		AtActin 2 R	5'-CCTGGACCTGCCTCATCATAC-3'	65.1			

Table S4. Results of two-way ANOVA comparing values for germination percentage for each genotype from different parents growth treatments and germination treatments. Data is shown in Figure 3.22

Uncorrected Fisher's LSD	At 3 days after stratification		At 9 days after stratification	
	Summary	Individual P Value	Summary	Individual P Value
Col-0				
ControlMedia-WaterParent vs. ControlMedia-SaltParent	ns	0.9745	ns	0.6848
ControlMedia-WaterParent vs. SaltMedia-WaterParent	ns	0.4038	ns	0.5644
ControlMedia-WaterParent vs. SaltMedia-SaltParent	*	0.0234	ns	0.2877
ControlMedia-SaltParent vs. SaltMedia-WaterParent	ns	0.4219	ns	0.3275
ControlMedia-SaltParent vs. SaltMedia-SaltParent	*	0.0252	ns	0.1441
SaltMedia-WaterParent vs. SaltMedia-SaltParent	ns	0.1418	ns	0.6235
pip2;1-1				
ControlMedia-WaterParent vs. ControlMedia-SaltParent	ns	0.8533	ns	0.8077
ControlMedia-WaterParent vs. SaltMedia-WaterParent	ns	0.4422	ns	0.5993
ControlMedia-WaterParent vs. SaltMedia-SaltParent	ns	0.2769	ns	0.8322
ControlMedia-SaltParent vs. SaltMedia-WaterParent	ns	0.5588	ns	0.7776
ControlMedia-SaltParent vs. SaltMedia-SaltParent	ns	0.3655	ns	0.9748
SaltMedia-WaterParent vs. SaltMedia-SaltParent	ns	0.7471	ns	0.7535
pip2;1-2				
ControlMedia-WaterParent vs. ControlMedia-SaltParent	ns	0.6971	ns	0.699
ControlMedia-WaterParent vs. SaltMedia-WaterParent	ns	0.6178	ns	0.8661
ControlMedia-WaterParent vs. SaltMedia-SaltParent	*	0.0497	ns	0.2106
ControlMedia-SaltParent vs. SaltMedia-WaterParent	ns	0.9124	ns	0.579
ControlMedia-SaltParent vs. SaltMedia-SaltParent	ns	0.112	ns	0.3838
SaltMedia-WaterParent vs. SaltMedia-SaltParent	ns	0.1381	ns	0.1566
hkt1				
ControlMedia-WaterParent vs. ControlMedia-SaltParent	ns	0.4704	ns	0.1169
ControlMedia-WaterParent vs. SaltMedia-WaterParent	ns	0.315	ns	0.5502
ControlMedia-WaterParent vs. SaltMedia-SaltParent	**	0.0014	**	0.0015
ControlMedia-SaltParent vs. SaltMedia-WaterParent	ns	0.775	ns	0.3258
ControlMedia-SaltParent vs. SaltMedia-SaltParent	*	0.0112	ns	0.0876
SaltMedia-WaterParent vs. SaltMedia-SaltParent	*	0.023	**	0.0085
UBI:nGFP::PIP2;1*				
ControlMedia-WaterParent vs. ControlMedia-SaltParent	ns	0.9632	ns	0.7663
ControlMedia-WaterParent vs. SaltMedia-WaterParent	ns	0.5386	ns	0.7728
ControlMedia-WaterParent vs. SaltMedia-SaltParent	ns	0.3988	ns	0.8208
ControlMedia-SaltParent vs. SaltMedia-WaterParent	ns	0.5694	ns	0.9932
ControlMedia-SaltParent vs. SaltMedia-SaltParent	ns	0.4248	ns	0.6007

SaltMedia-WaterParent vs. SaltMedia-SaltParent	ns	0.8177	ns	0.6067
35S::PIP2;1(7)*				
ControlMedia-WaterParent vs. ControlMedia-SaltParent	ns	0.6594	ns	0.6947
ControlMedia-WaterParent vs. SaltMedia-WaterParent	ns	0.4208	ns	0.8583
ControlMedia-WaterParent vs. SaltMedia-SaltParent	ns	0.2014	ns	0.6423
ControlMedia-SaltParent vs. SaltMedia-WaterParent	ns	0.7143	ns	0.5683
ControlMedia-SaltParent vs. SaltMedia-SaltParent	ns	0.3991	ns	0.3926
35S::PIP2;1(21)*				
ControlMedia-WaterParent vs. ControlMedia-SaltParent	ns	0.9143	ns	0.5821
ControlMedia-WaterParent vs. SaltMedia-WaterParent	ns	0.6846	ns	0.7571
ControlMedia-WaterParent vs. SaltMedia-SaltParent	ns	0.8713	ns	0.9565
ControlMedia-SaltParent vs. SaltMedia-WaterParent	ns	0.6076	ns	0.809
ControlMedia-SaltParent vs. SaltMedia-SaltParent	ns	0.7875	ns	0.6199
SaltMedia-WaterParent vs. SaltMedia-SaltParent	ns	0.8069	ns	0.7989

Table S5. Results of two-way ANOVA comparing values for seed weight at nominated time points, data is showed in Figure 3.23. W, WS20, S20 are the abbreviations of treatments following water treatment for 46 hours, water in 30 hours then transfer to salt 20 mM NaCl treatment and salt 20 mM NaCl treatment for 46 hours, respectively.

a. Compare seed weight between genotypes

Seed weight during imbibition and germination						
Dry seed	Col-0	*pip2;1-1*	*pip2;1-2*	*hkt1*	UBI:nGFP:: PIP2;1*	35S:: PIP2;1 (7)*
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	ns	ns	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	ns	
35S::PIP2;1(21)*	ns	ns	ns	ns	ns	ns
15m-W						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	**	***	ns	*		
35S::PIP2;1(7)*	ns	ns	ns	ns	**	
35S::PIP2;1(21)*	****	****	****	****	**	****
15m-S20						
pip2;1-1	ns					

pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	*	**	*	*		
35S::PIP2;1(7)*	ns	ns	ns	ns	**	
35S::PIP2;1(21)*	***	****	**	**	ns	****
4h-W						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	***	****	***	**		
35S::PIP2;1(7)*	ns	ns	ns	ns	****	
35S::PIP2;1(21)*	****	****	****	****	**	****
4h-S20						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	*	**	*	*		
35S::PIP2;1(7)*	ns	ns	ns	ns	**	
35S::PIP2;1(21)*	***	****	***	***	ns	****
24h-W						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	**	****	***	*		
35S::PIP2;1(7)*	ns	ns	ns	ns	**	
35S::PIP2;1(21)*	**	****	***	*	ns	**
24h-S20						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	ns	ns	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	ns	
35S::PIP2;1(21)*	ns	ns	ns	ns	ns	ns
30h-W						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	****	****	***	**		
35S::PIP2;1(7)*	ns	ns	ns	*	****	
35S::PIP2;1(21)*	****	****	****	****	*	****
30h-S20						

pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	**	*	*		
35S::PIP2;1(7)*	ns	ns	ns	ns	**	
35S::PIP2;1(21)*	**	****	**	**	ns	****
46h-W						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	*	**	**	*		
35S::PIP2;1(7)*	ns	ns	ns	ns	***	
35S::PIP2;1(21)*	****	****	****	****	*	****
46h-WS20						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	*	*	ns	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	**	
35S::PIP2;1(21)*	**	**	ns	ns	ns	***
46h-S20						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	*	ns	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	**	
35S::PIP2;1(21)*	**	***	**	**	ns	***

b. Compare seed weight between treatments

Time	Dry Seed	15 min	4 h	24h	30h	46h	46h	46h
Treatment vs. treatment		W - S20	W - S20	W - S20	W - S20	W - WS20	W - S20	WS20 - S20
Col-0	ns	ns	ns	*	ns	**	*	ns
pip2;1-1	ns	ns	ns	ns	*	*	*	ns
pip2;1-2	ns	ns	ns	*	ns	ns	ns	ns
hkt1	ns	ns	ns	**	*	*	*	ns
UBI:nGFP::PIP2;1*	ns	ns	ns	**	*	**	*	ns
35S::PIP2;1(7)*	ns	ns	ns	*	ns	*	ns	ns
35S::PIP2;1(21)*	ns	ns	**	****	**	***	**	ns

c. Compare percentage of seed weight between genotypes

15m-W	Col-0	pip2;1-1	pip2;1-2	hkt1	GFP:PIP2;1*	35S::PIP2;1(7)*
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	*	**	ns	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	*	
35S::PIP2;1(21)*	**	***	*	**	ns	**
15m-S20						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	ns	*	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	*	
35S::PIP2;1(21)*	ns	ns	ns	ns	ns	ns
4h-W						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	**	****	**	**		
35S::PIP2;1(7)*	ns	ns	ns	ns	***	
35S::PIP2;1(21)*	**	****	**	**	ns	***
4h-S20						
pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	ns	*	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	ns	
35S::PIP2;1(21)*	ns	ns	ns	ns	ns	ns
24h-W						
pip2;1-1	*					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	***	**	*		
35S::PIP2;1(7)*	ns	ns	ns	ns	*	
35S::PIP2;1(21)*	ns	ns	ns	ns	*	ns
24h-S20						
pip2;1-1	ns					
pip2;1-2	ns	*				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	ns	*	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	ns	
35S::PIP2;1(21)*	ns	*	ns	ns	*	ns

30h-W

pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	***	***	**	**		
35S::PIP2;1(7)*	ns	ns	ns	ns	****	
35S::PIP2;1(21)*	*	**	*	ns	ns	***

30h-S20

pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	ns	*	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	*	
35S::PIP2;1(21)*	ns	ns	ns	ns	ns	ns

46h-W

pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	**	ns	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	**	
35S::PIP2;1(21)*	ns	*	ns	ns	ns	*

46h-WS20

pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	ns	ns	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	*	
35S::PIP2;1(21)*	ns	ns	ns	ns	ns	ns

46h-S20

pip2;1-1	ns					
pip2;1-2	ns	ns				
hkt1	ns	ns	ns			
UBI:nGFP::PIP2;1*	ns	ns	*	ns		
35S::PIP2;1(7)*	ns	ns	ns	ns	ns	
35S::PIP2;1(21)*	ns	ns	ns	ns	ns	ns

d. Compare percentage of seed weight between treatments

Time	Dry Seed	15 min	4 h	24h	30h	46h	46h	46h
Treatment vs. treatment		W - S20	W - S20	W - S20	W - S20	W - WS20	W - S20	WS20 - S20
Col-0	ns	ns	ns	ns	***	*	ns	ns

pip2;1-1	ns	ns	ns	ns	*	ns	ns	ns
pip2;1-2	*	*	*	**	**	***	ns	*
hkt1	ns	ns	ns	*	**	**	ns	ns
UBI:nGFP::PIP2;1*	ns	*	*	**	***	**	ns	ns
35S::PIP2;1(7)*	ns	ns	ns	*	**	*	ns	ns
35S::PIP2;1(21)*	ns	**	**	**	***	***	ns	ns

Table S6. Results of two-way ANOVA comparing seed weight between treatments for each genotype, data is showed in Figure 3.24. W, WS75, S75 are the abbreviations of treatments: water treatment, water in 30 hours then transfer to salt 75mM NaCl treatment and salt 75mM NaCl treatment, respectively.

Time	Seed weight during imbibition and germination					
	Dry Seed	4 h	30h	50 h	50 h	50 h
Treatment vs. treatment		W - S75	W - S75	W - WS75	W - S75	WS75 - S75
Col-0	ns	****	****	****	****	ns
pip2;1-1	ns	****	****	****	****	ns
pip2;1-2	ns	****	****	****	***	ns
*pip2;1*pip2;2*	ns	****	****	****	****	ns
hkt1	ns	****	****	****	****	ns
35S:PIP2;1 (21)*	ns	****	****	****	****	ns
Time	% seed weight increase relative to dry seed					
		4 h	30h	50 h	50 h	50 h
Treatment vs. treatment		W - S75	W - S75	W - WS75	W - S75	WS75 - S75
Col-0		****	****	****	****	ns
pip2;1-1		****	****	****	****	ns
pip2;1-2		****	****	****	****	ns
*pip2;1*pip2;2*		****	****	****	****	ns
hkt1		****	****	****	****	ns
35S:PIP2;1 (21)*		****	****	****	****	ns
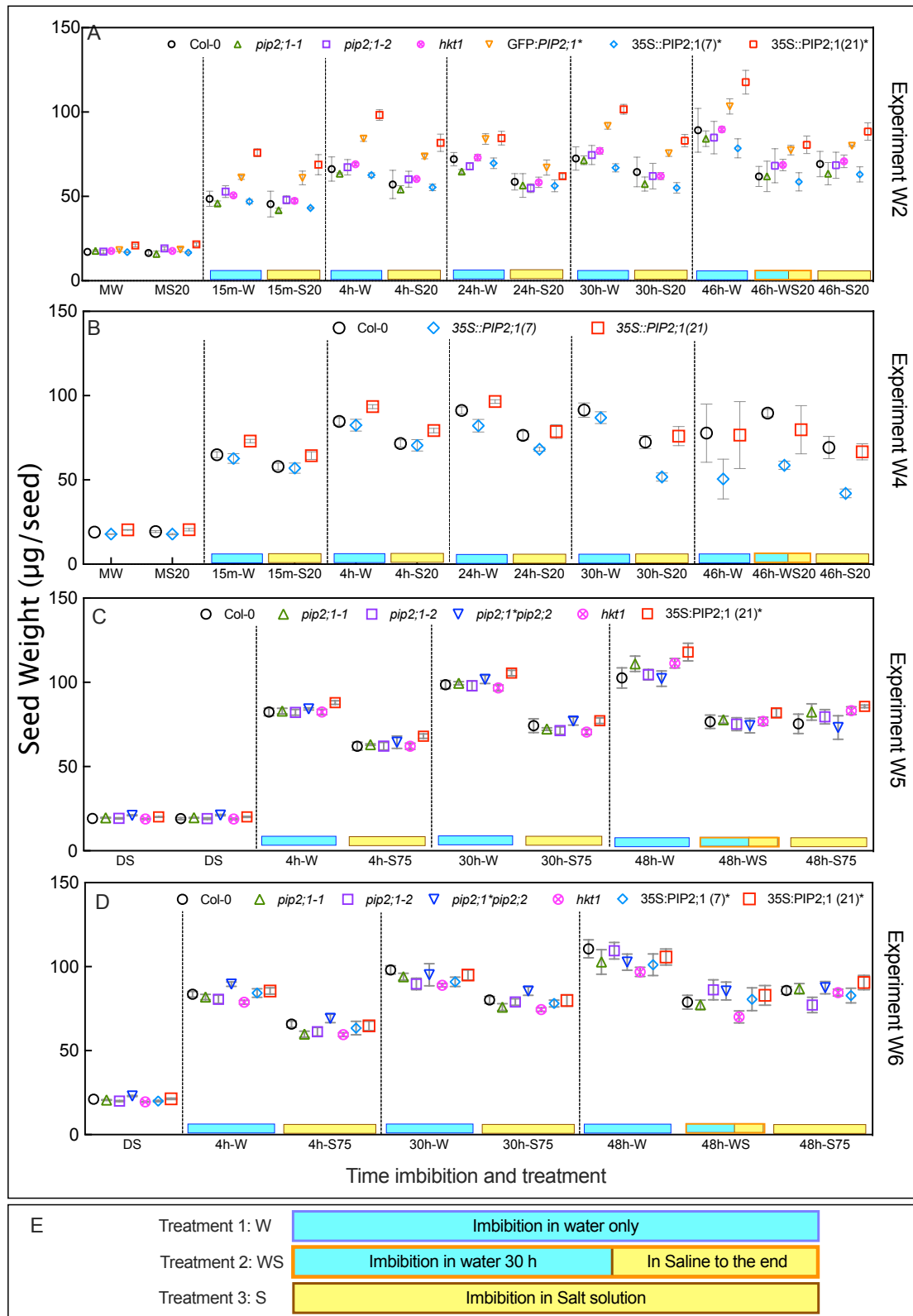
Figure S7: The seed weight during imbibition and germination in 3 treatments at biology replications. Each replication was 8 plants per genotype from a separated grown batch. Data are mean ± SEM. Absence of error bars indicates SEM is smaller than the symbol.
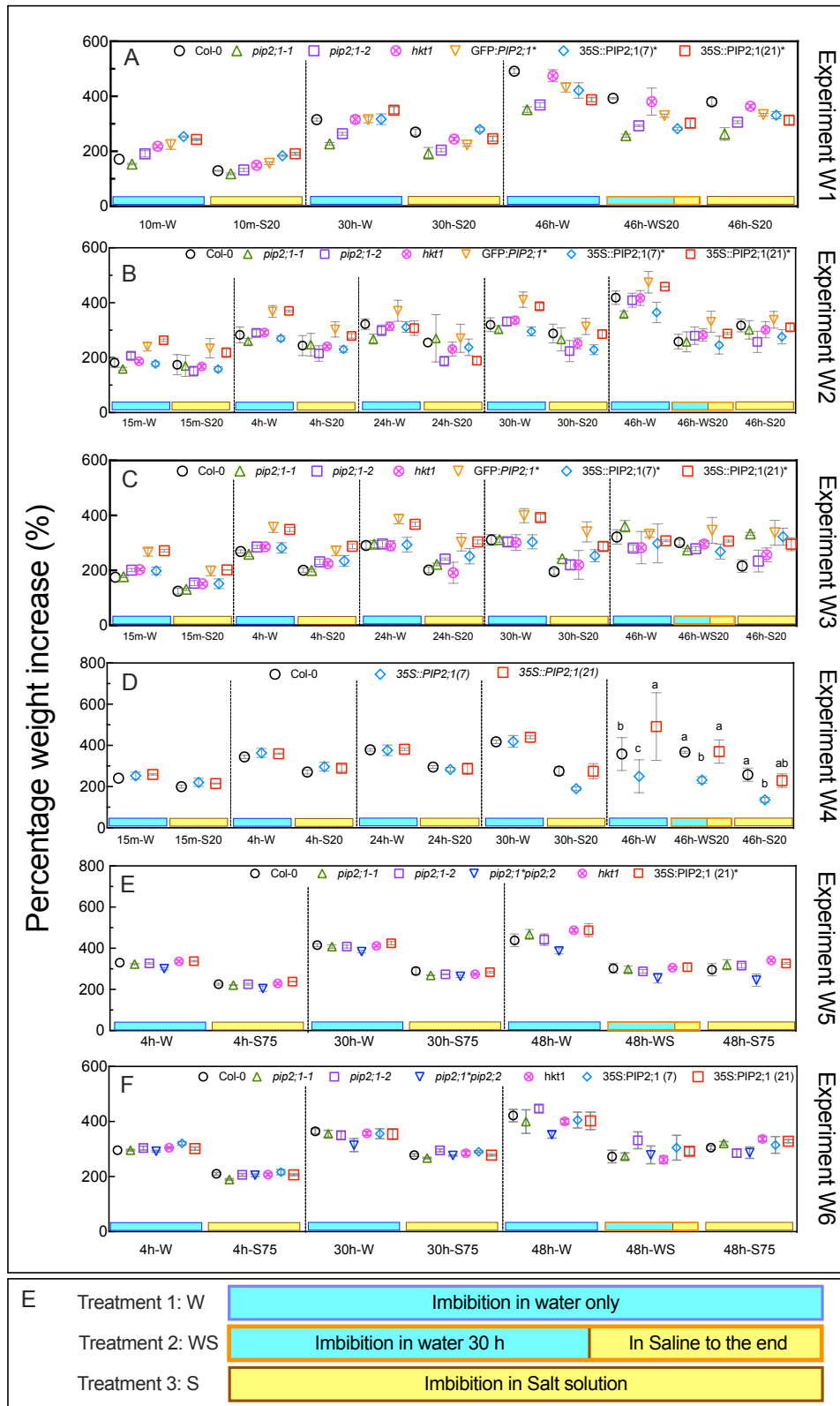
Figure S8: The percentage seed weight increase during imbibition and germination in 3 treatments at biology replications. Each replication was 3 - 12 plants per genotype from a separated grown batch. Data are mean ± SEM. Absence of error bars indicates SEM is smaller than the symbol.
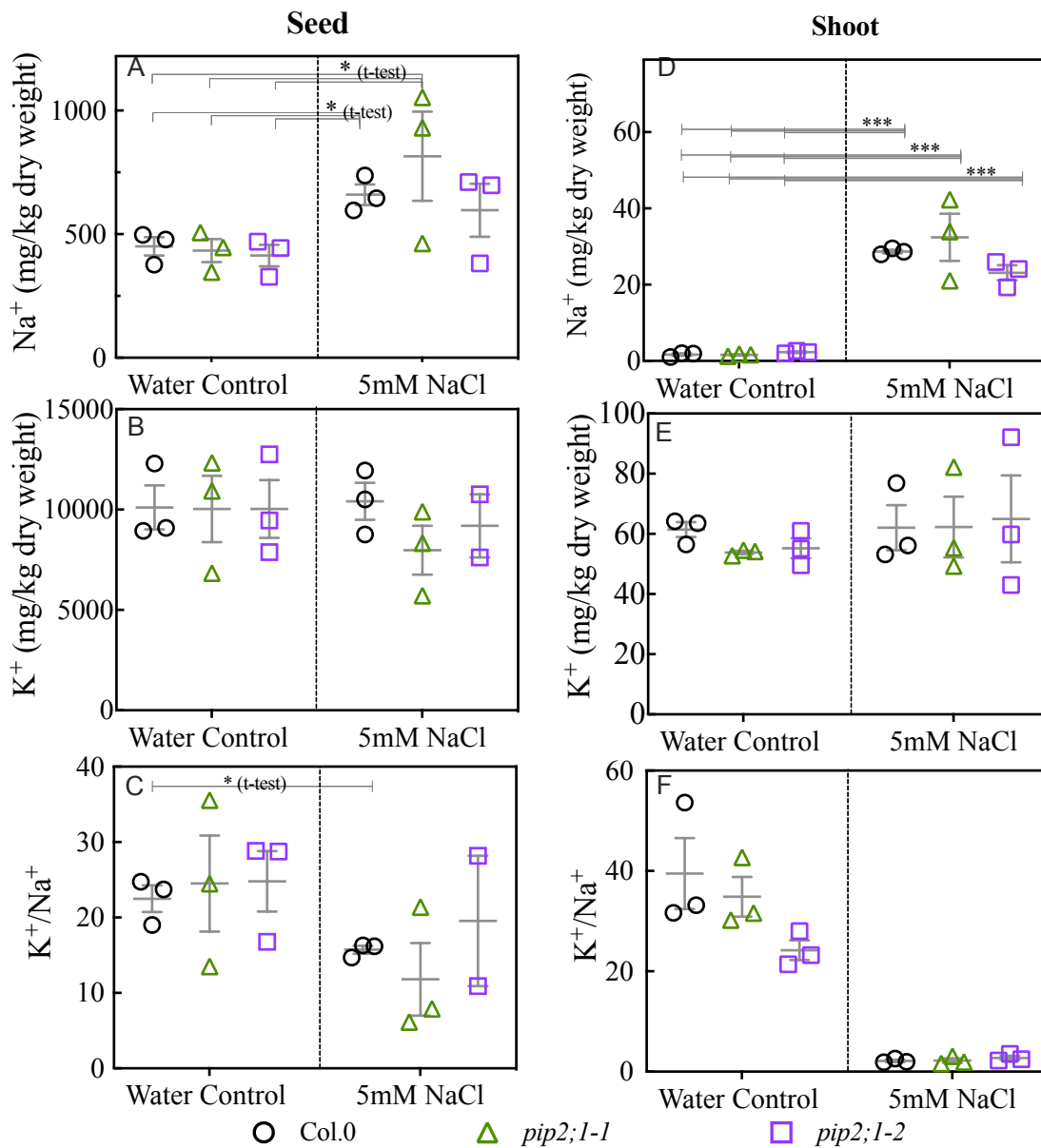
Figure S9: Na$^+$ and K$^+$ content and ratio of K$^+$ and Na$^+$ in seed and shoot from parents grown in different treatments.

Under standard treatment (water grown) and water and NaCl at 5 mM NaCl (5 mM NaCl grown) treatment (panels A-C); and shoot Na+ and K+ content and ratio of K+ and Na+ of the parent plants in each treatment (panels D-F). Mature seeds were collected after plants had been dry for 2 weeks, then seed was further dried in an oven at 65ºC for 4 days. 3-5 mg of dry seed was digested in 1% HNO3 at 80ºC for 2 hours and analysed by ASS. For additional methods information see section 3.2.2

and 3.2.8. Data are mean ± SE (n = 6). For some data points the error bars are smaller than the symbols.

Table S10. Results of two-way ANOVA comparing values of Na^+, K^+, Na^++K^+ and ratios K^+/Na^+ for each genotype between 4 treatments (plants grown in Water (0), 5, 25 and 50 mM NaCl solution. Data is presented in Figure 3.26

Genotype	Treatment pairs	Na^+	K^+	K^+/Na^+	%water in seed
Col-0	0mM NaCl vs. 05mM NaCl	ns	ns	ns	****
	0mM NaCl vs. 25mM NaCl	****	***	****	****
	0mM NaCl vs. 50mM NaCl	****	*	****	****
	05mM NaCl vs. 25mM NaCl	****	****	****	ns
	05mM NaCl vs. 50mM NaCl	****	***	****	ns
	25mM NaCl vs. 50mM NaCl	****	ns	**	ns
pip2;1-1	0mM NaCl vs. 05mM NaCl	ns	ns	ns	**
	0mM NaCl vs. 25mM NaCl	***	**	****	***
	0mM NaCl vs. 50mM NaCl	****	****	****	****
	05mM NaCl vs. 25mM NaCl	***	**	****	ns
	05mM NaCl vs. 50mM NaCl	****	****	****	*
	25mM NaCl vs. 50mM NaCl	****	**	**	ns
pip2;1-2	0mM NaCl vs. 05mM NaCl	ns	ns	ns	****
	0mM NaCl vs. 25mM NaCl	***	ns	****	****
	0mM NaCl vs. 50mM NaCl	****	***	****	****
	05mM NaCl vs. 25mM NaCl	***	ns	****	**
	05mM NaCl vs. 50mM NaCl	****	*	****	**
	25mM NaCl vs. 50mM NaCl	**	*	ns	ns
35S::PIP2;1 (7)	0mM NaCl vs. 05mM NaCl	*	****	****	ns
	0mM NaCl vs. 25mM NaCl	****	****	****	****
	0mM NaCl vs. 50mM NaCl	****	****	****	ns
	05mM NaCl vs. 25mM NaCl	*	ns	***	***
	05mM NaCl vs. 50mM NaCl	****	ns	****	ns
	25mM NaCl vs. 50mM NaCl	****	ns	****	**
35S::PIP2;1 (21)	0mM NaCl vs. 05mM NaCl	ns	ns	ns	**
	0mM NaCl vs. 25mM NaCl	***	***	****	****
	0mM NaCl vs. 50mM NaCl	****	****	****	****
	05mM NaCl vs. 25mM NaCl	***	***	****	ns
	05mM NaCl vs. 50mM NaCl	****	****	****	***
	25mM NaCl vs. 50mM NaCl	****	*	**	*

Figure S11. Seed Na$^+$ and K$^+$ content, K$^+$/Na$^+$ ratio and moisture content of fresh dry seed grown in water treatment included *hkt1* which died in 50 mM NaCl treatment (added Athkt1 compared to figure 3.26).

A-C, Na$^+$ and K$^+$ ion content and the ration of K$^+$/Na$^+$ in seed of five genotypes where parent plants were grown in control treatment and seeds were collected when

mature and dry. D, Moisture content of seed (%) = (fresh dry seed weight – oven dried seed weight) x 100/ fresh dry seed weight. Seeds were weighed, dried in an oven temperature at 65°C in 72 hours and then dried seeds were weighed. Data for Col-0 (◯), Mutant *Atpip2;1-1* (△), Atpip*2;1-2* (☐), 2x35S::AtPIP2;1 OE 7 (◇), 2x35S::AtPIP2;1 OE 21 (☐) and *Athkt1* (⊗). Data points are means and SEM (n = 4 plates per condition, 600 - 900 seeds per genotype in each plate). The experiment was repeated 3 times. Significant difference determined by two-way ANOVA and Fisher's LSD pair-wise tests.
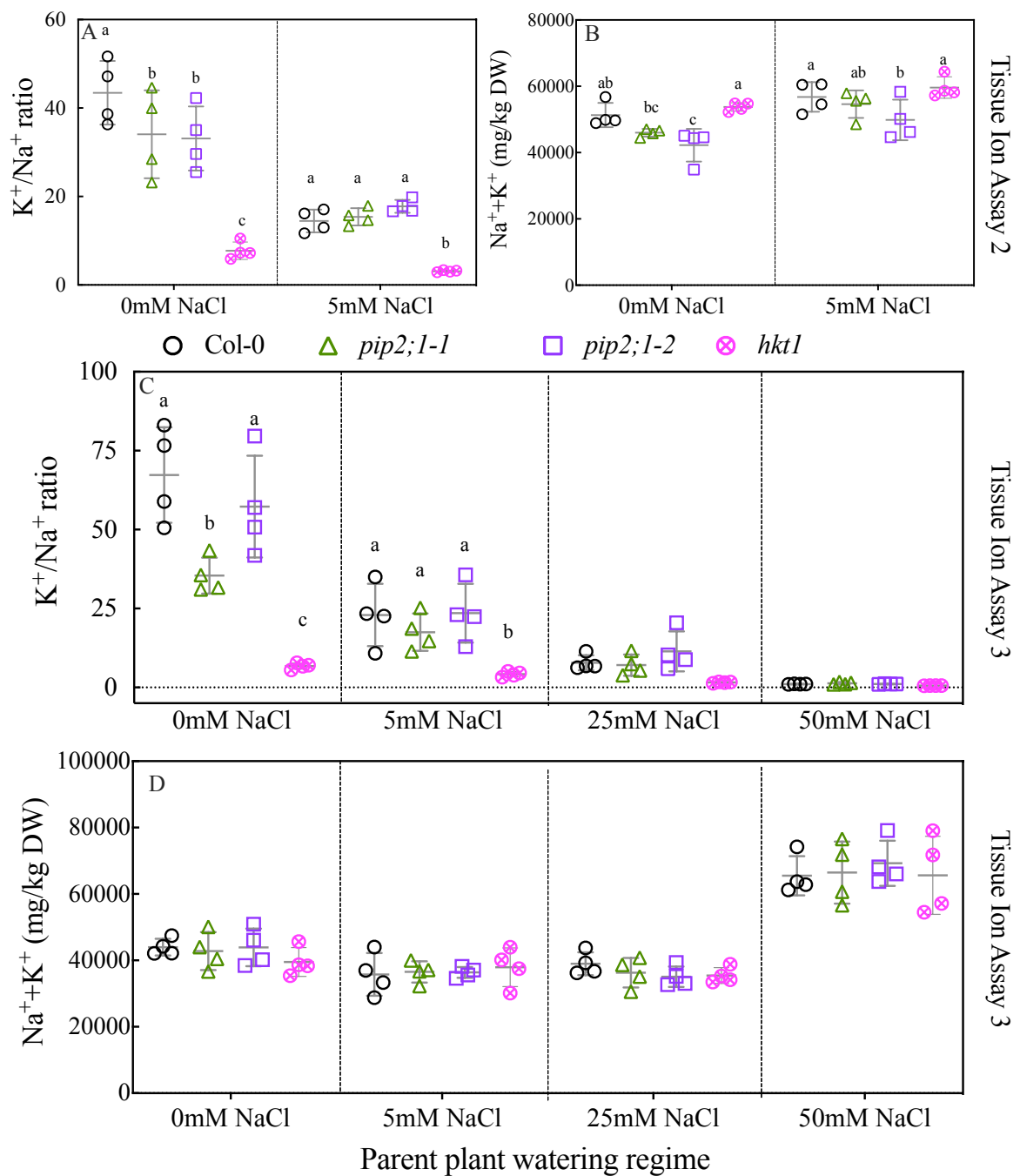
Figure S12. K$^+$/Na$^+$ ratio and total Na$^+$ and K$^+$ content in tissue of plants grown in different salt treatments, supporting figure 3.29. There were 2 independent experiments called tissue ion assay 2 and 3. Data is mean +/- SEM, statistical analysis presented in table S14.

Figure S13. K$^+$/Na$^+$ ratio and total Na$^+$ and K$^+$ content in tissue of plants grown in different salt treatments in the growth assay 4 (A, B) and 5 (E, F) and the Na$^+$, K$^+$ content in plant tissue of growth assay 5 (C, D), supporting Figure 3.30. Data is mean +/- SEM, statistical analysis presented in table S14.

Table S14. Results of two-way ANOVA comparing values for each genotype between treatments (p < 0.05). Stars indicate significant differences, 'ns' indicates nonsignificant difference. Data is showed in Figures 3.29, 3.30, S12 and S13.

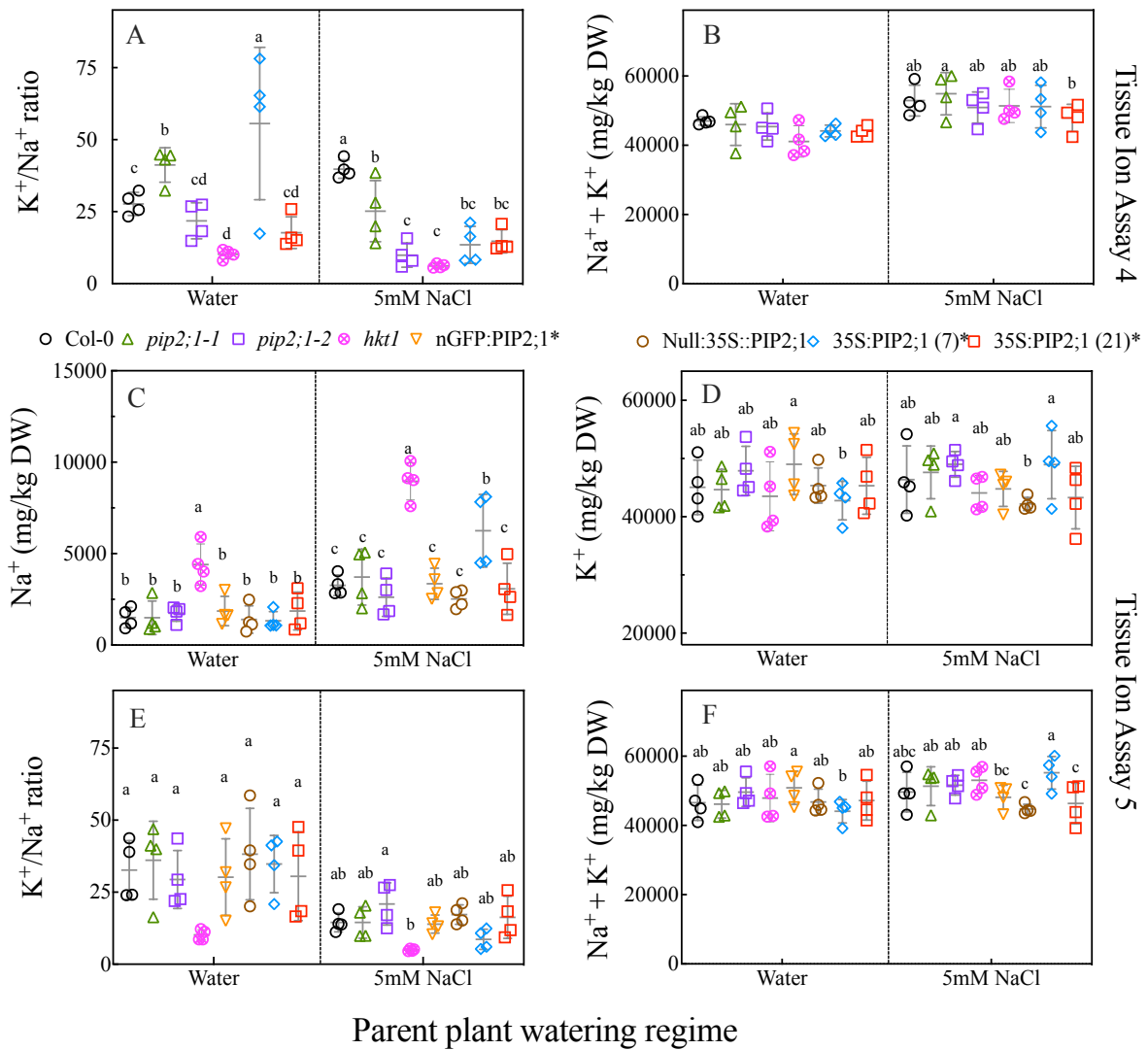*Tissue Ion Assay 3: Statistical analysis for data from plants grown in different water regimes for ion content values for each key genotype*

Genotype	Treatment pairs	Na$^+$	K$^+$	K$^+$/Na$^+$	(K$^+$) + (Na$^+$)
Col-0	Water vs. Salt5mM	ns	***	****	*
	Water vs. Salt25mM	ns	***	****	ns
	Water vs. Salt50mM	****	***	****	****
	Salt5mM vs. Salt25mM	ns	ns	**	ns
	Salt5mM vs. Salt50mM	****	ns	****	****
	Salt25mM vs. Salt50mM	****	ns	ns	****
pip2;1-1	Water vs. Salt5mM	ns	**	***	ns
	Water vs. Salt25mM	ns	***	****	ns
	Water vs. Salt50mM	****	ns	****	****
	Salt5mM vs. Salt25mM	ns	ns	*	ns
	Salt5mM vs. Salt50mM	****	ns	**	****
	Salt25mM vs. Salt50mM	****	*	ns	****
pip2;1-2	Water vs. Salt5mM	ns	**	****	ns
	Water vs. Salt25mM	ns	****	****	*
	Water vs. Salt50mM	****	**	****	****
	Salt5mM vs. Salt25mM	ns	ns	*	ns
	Salt5mM vs. Salt50mM	****	ns	****	****
	Salt25mM vs. Salt50mM	****	ns	*	****
hkt1	Water vs. Salt5mM	ns	ns	ns	ns
	Water vs. Salt25mM	***	****	ns	ns
	Water vs. Salt50mM	****	***	ns	****
	Salt5mM vs. Salt25mM	**	**	ns	ns
	Salt5mM vs. Salt50mM	****	*	ns	****
	Salt25mM vs. Salt50mM	****	ns	ns	****

Tissue Ion Assay 2, 4 and 5: Statistical analysis for data from plants grown in treatment where they were in water treatment and water and NaCl at 5 mM NaCl treatment (5 mM NaCl)

	Genotype	Na$^+$	K$^+$	K$^+$/Na$^+$	(K$^+$) + (Na$^+$)
Tissue Ion Assay 2	Col-0	****	ns	****	ns
	pip2;1-1	***	*	****	**
	pip2;1-2	*	*	***	*
	hkt1	****	ns	ns	*

Tissue Ion Assay 4	Genotype	Na⁺	K⁺	K⁺/Na⁺	(K⁺) + (Na⁺)
	Col-0	ns	ns	ns	ns
	pip2;1-1	ns	*	*	**
	pip2;1-2	****	ns	ns	ns
	hkt1	****	*	ns	**
	35S:PIP2;1 (7)*	****	ns	****	*
	35S:PIP2;1 (21)*	ns	ns	ns	ns

Tissue Ion Assay 5	Genotype	Na⁺	K⁺	K⁺/Na⁺	(K⁺) + (Na⁺)
	Col-0	*	ns	**	ns
	pip2;1-1	**	ns	**	ns
	pip2;1-2	ns	ns	ns	ns
	hkt1	****	ns	ns	ns
	UBI:nGFP::PIP2;1*	*	ns	*	ns
	Null:35S::PIP2;1	ns	ns	**	ns
	35S:PIP2;1 (7)*	****	*	***	**
	35S:PIP2;1 (21)*	ns	ns	*	ns

Seed from water grown parents
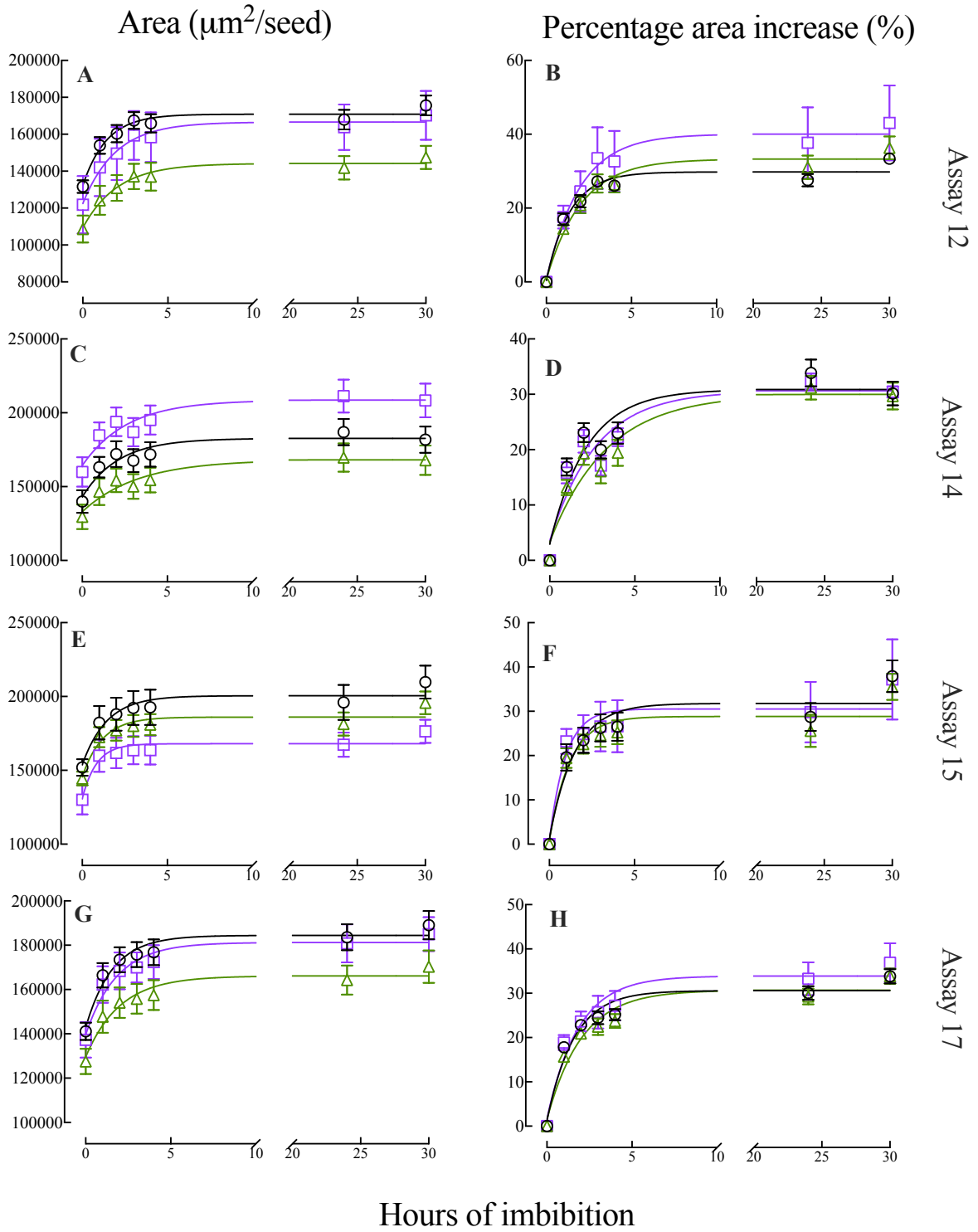
Seed from saline grown parents
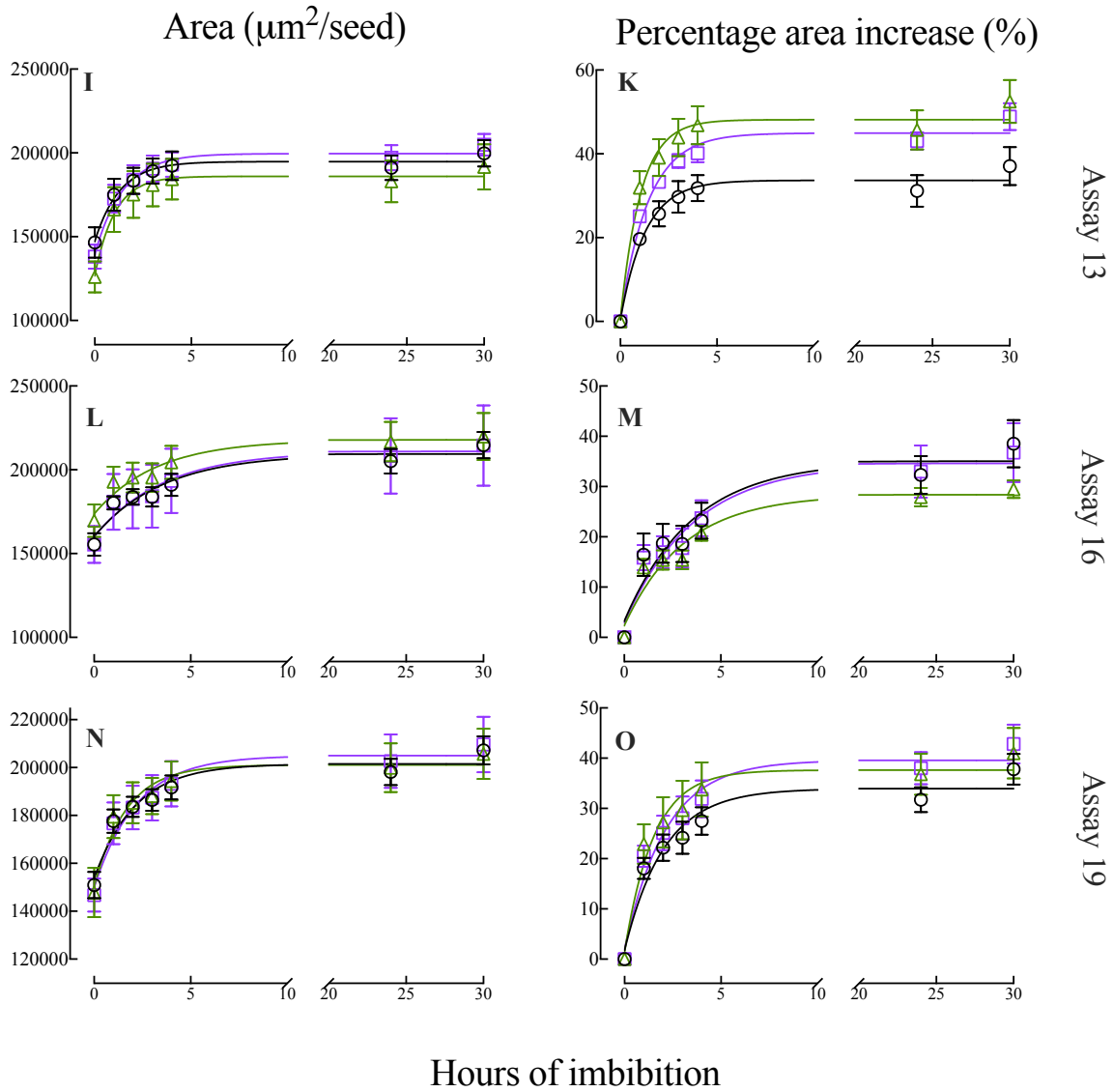
Figure S15: Seed MCS area extracted from video footage (19 individual videos during 2017 - 2018). Seeds of Col-0, *Atpip2;1-1* and *Atpip2;1-2* mutants were from parents grown under water (panels A-H) and water and NaCl at 5 mM NaCl regime (panels I-O). Left figures are the MCS area and the right figures are the percentage of MCS area with the assay repetition number shown on the right hand y-axis. Lines are non-linear regression fit. Data are mean +/- SEM of 4 seeds.

MCS Area (mm²/seed)   Percentage MCS area increase (%)

Hours of imbibition
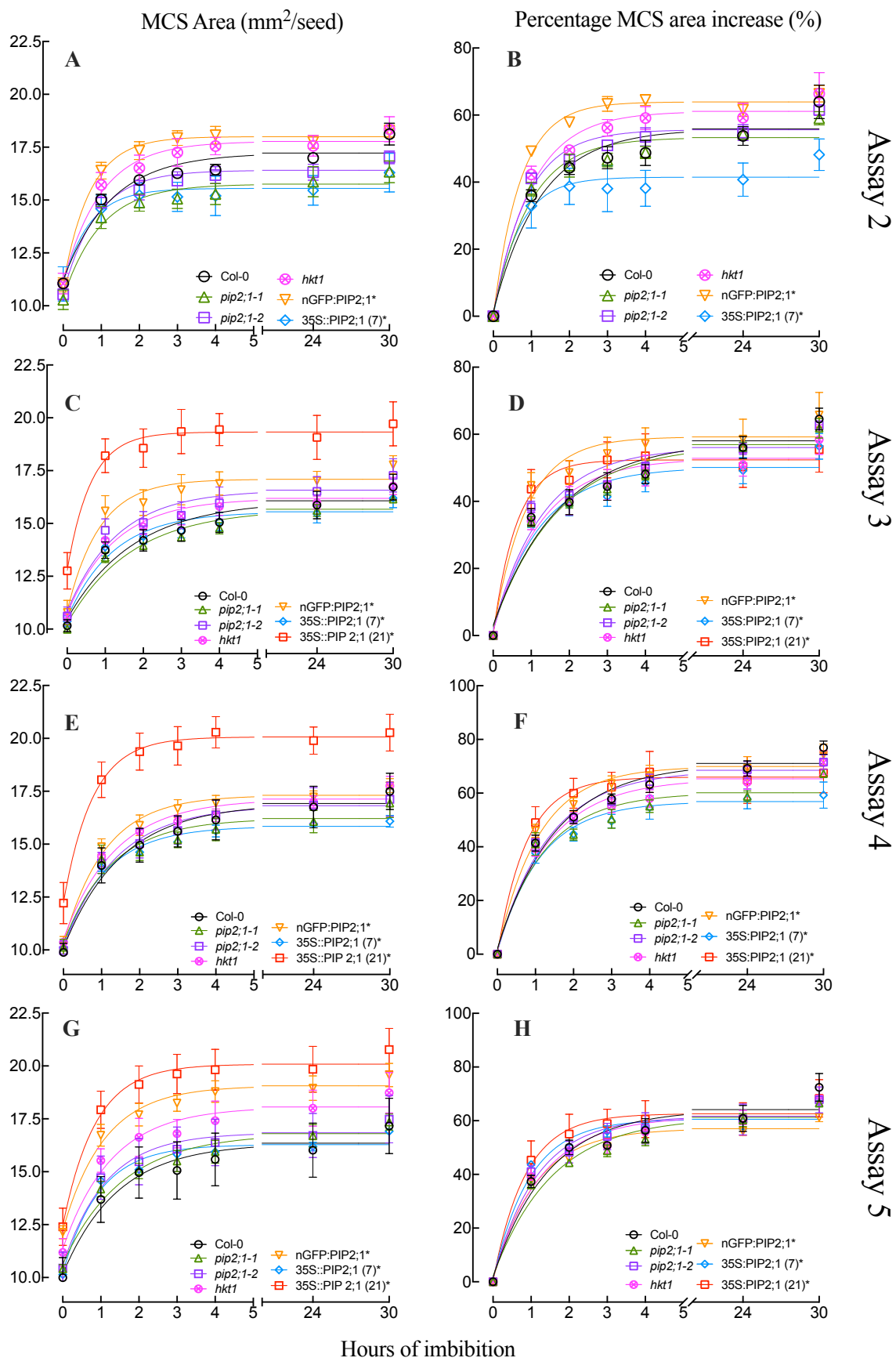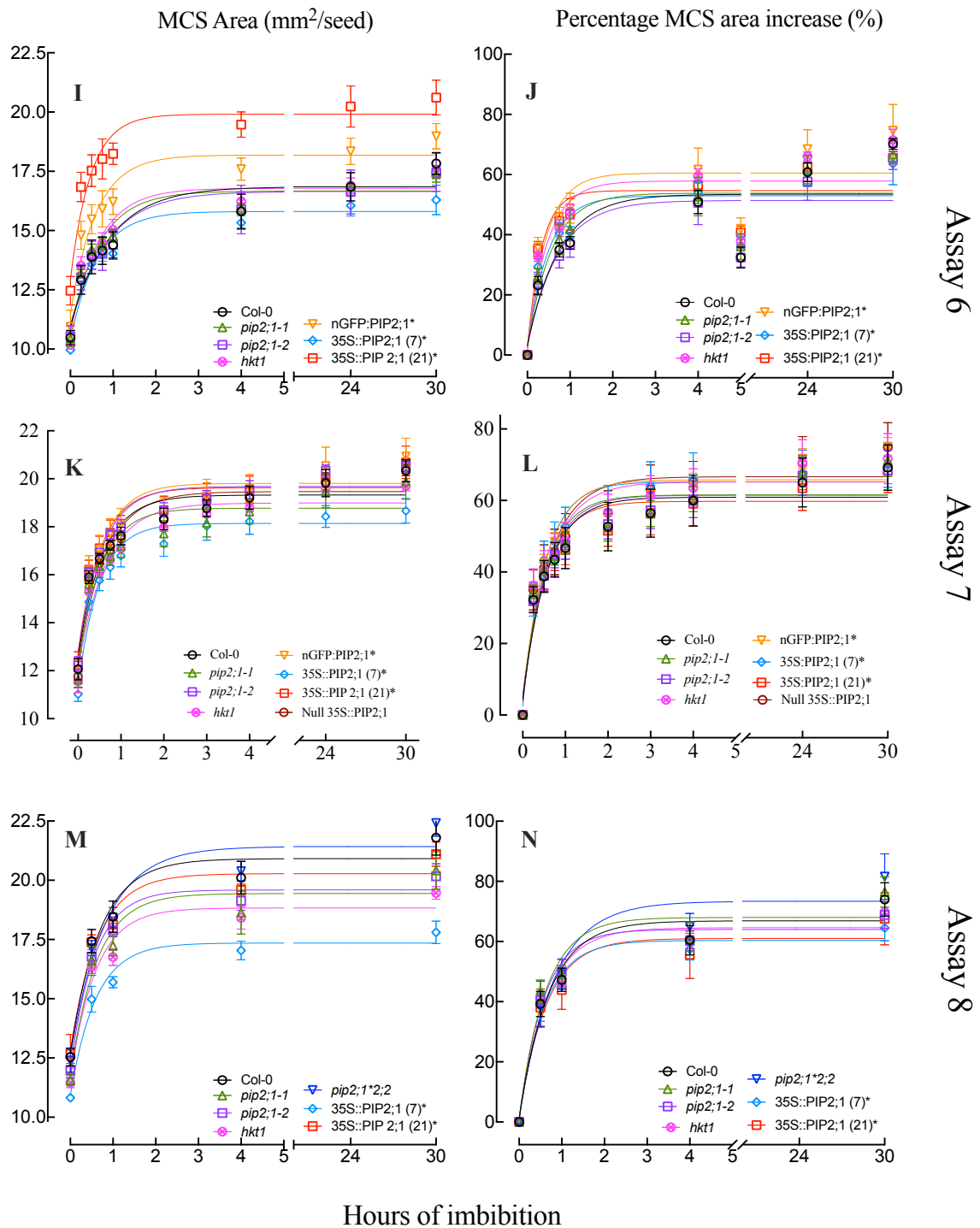
Figure S16: Seed MCS area (panels A, C, E, G, I, K, M) and percentage increase in seed MCS area (panels B, D, F, H, J, L, N). Data is mean +/- SEM of n = 4. Each graph was a biological replication with an individual experiment from different plant growths. Growth replications were numbered from 2 to 5 in 2019 and 6 – 8 in 2020.

Tables S17: Results of one-way ANOVA comparing seed MCS area and percentage of seed MCS area between genotypes, data is present in Fig. 3.34.

| Pairs of genotype comparisons	Seed MCS area					Percentage of seed MCS area			
Time imbibition	0h	1h	4h	24h	30h	1h	4h	24h	30h
Col-0 vs. *pip2;1-1*	ns	ns	ns	ns	ns	ns	ns	ns	ns
Col-0 vs. *pip2;1-2*	ns	ns	ns	ns	ns	ns	ns	ns	ns
Col-0 vs. *hkt1*	ns	ns	ns	ns	ns	ns	ns	ns	ns
Col-0 vs. nGFP:PIP2;1*	*	*	*	*	ns	ns	ns	ns	ns
Col-0 vs. 35S::PIP2;1 (7)*	ns	ns	ns	ns	ns	ns	ns	ns	ns
Col-0 vs. 35S::PIP2;1 (21)*	*	**	**	**	*	ns	ns	ns	ns
pip2;1-1 vs. *pip2;1-2*	ns	ns	ns	ns	ns	ns	ns	ns	ns
pip2;1-1 vs. *hkt1*	ns	ns	ns	ns	ns	ns	ns	ns	ns
pip2;1-1 vs. nGFP:PIP2;1*	ns	*	*	ns	ns	ns	ns	ns	ns
pip2;1-1 vs. 35S::PIP2;1 (7)*	ns	ns	ns	ns	ns	ns	ns	ns	ns
pip2;1-1 vs. 35S::PIP2;1 (21)*	*	**	**	*	*	ns	ns	ns	ns
pip2;1-2 vs. *hkt1*	ns	ns	ns	ns	ns	ns	ns	ns	ns
pip2;1-2 vs. nGFP:PIP2;1*	ns	ns	ns	ns	ns	ns	ns	ns	ns
pip2;1-2 vs. 35S::PIP2;1 (7)*	ns	ns	ns	ns	ns	ns	ns	ns	ns
pip2;1-2 vs. 35S::PIP2;1 (21)*	*	**	*	*	*	ns	ns	ns	ns
hkt1 vs. nGFP:PIP2;1*	ns	ns	ns	ns	ns	ns	ns	ns	ns
hkt1 vs. 35S::PIP2;1 (7)*	ns	ns	ns	ns	ns	ns	ns	ns	ns
hkt1 vs. 35S::PIP2;1 (21)*	ns	*	ns	ns	ns	ns	ns	ns	ns
nGFP:PIP2;1* vs. 35S::PIP2;1 (7)*	*	ns	*	*	*	ns	ns	ns	ns
nGFP:PIP2;1* vs. 35S::PIP2;1 (21)*	ns	ns	ns	ns	ns	ns	ns	ns	ns
35S::PIP2;1 (7)* vs. 35S::PIP2;1 (21)*	*	**	**	**	**	ns	ns	ns	ns
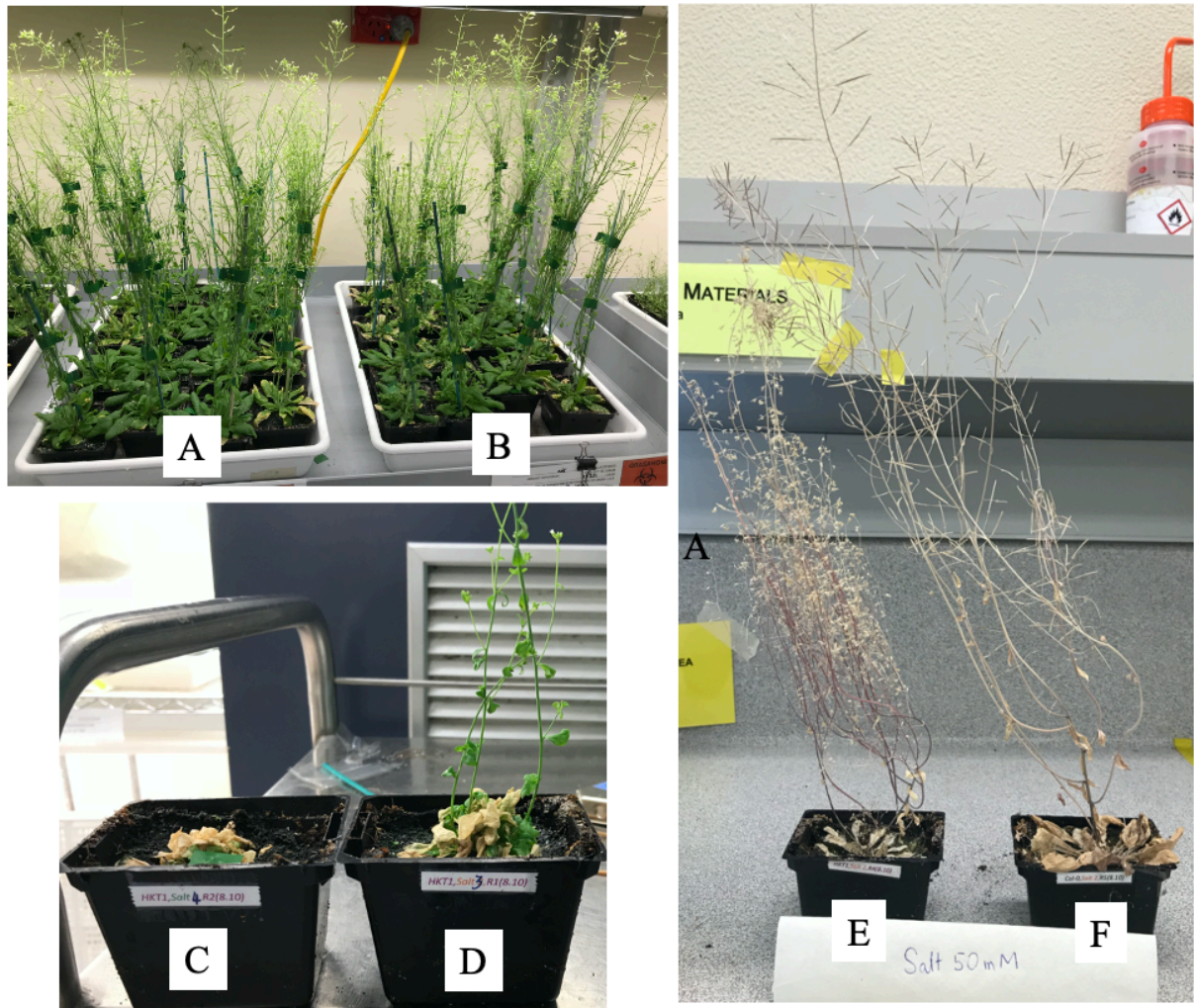
Figure S18: Arabidopsis plants grown for experiments involving different saline treatments.

A: Tray watering with water (Water, control). Plants at 8 weeks after sowing.

B: Tray watering with water and NaCl at 5 mM NaCl. Plants at 8 weeks after sowing.

C: Pot of Athkt1 watered with water containing 75 mM NaCl. Plant at 8 weeks after sowing.

D: Pot of Athkt1 watered with water containing 50 mM NaCl. Plant at 8 weeks after sowing.

E: Pot of Athkt1 watered with water containing 50 mM NaCl; 16 weeks after sowing. The plant did not produce siliques and seeds.

F: Pot of Col-0 watered with water containing 50 mM NaCl. This plant was ready to harvest at 16 weeks after sowing and produced seeds.
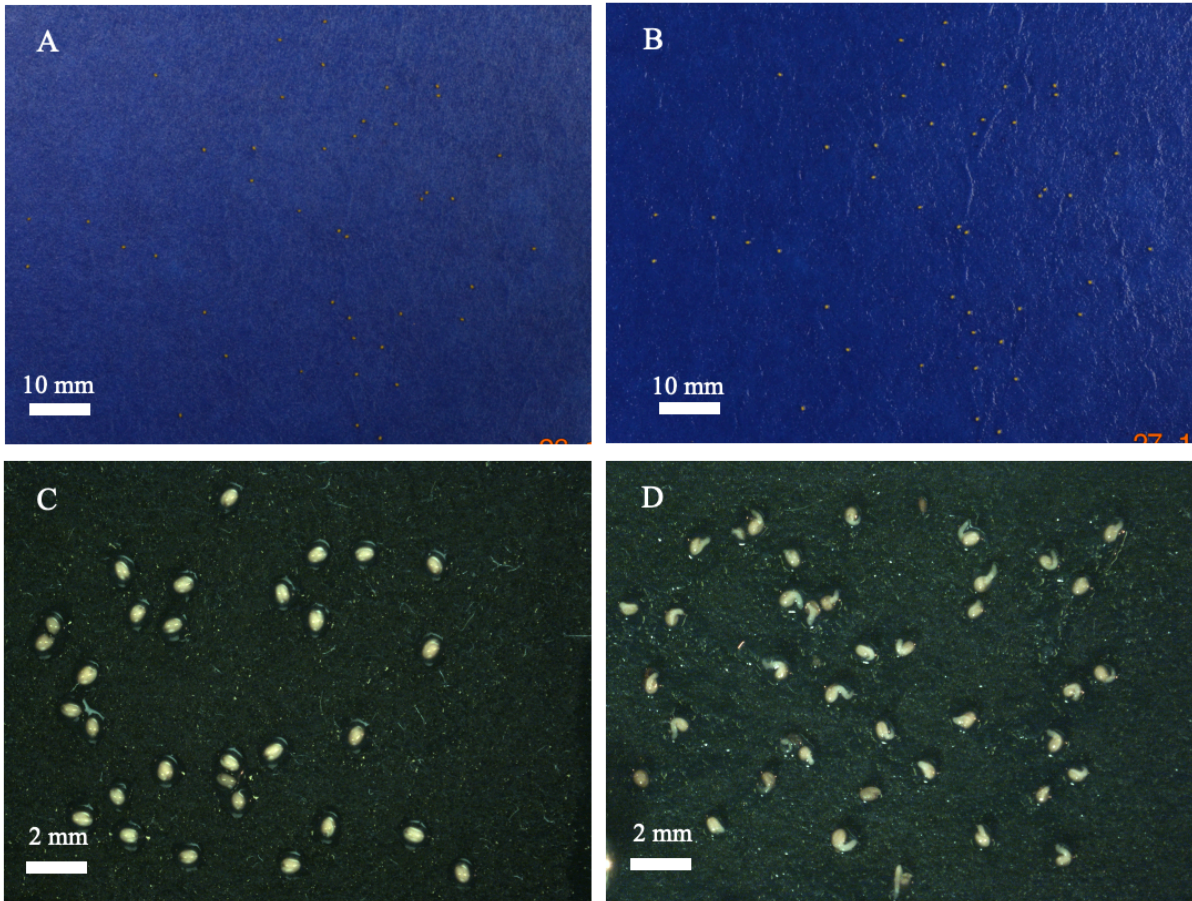
Figure S19: Arabidopsis seed on blue germination paper (Blue Blotter Paper FT-2-383-200350 Grade 194, Sartorius Stedim Biotech, Anchor Paper Company, http://www.seedpaper.com). Dry seeds on dry blue germination paper (A). Imbibing seeds 2 hours after the start of imbibition (B) and 25 hours after the start of imbibition (C) in water. Germinated seeds at 48 hours in water (D).

Table S20: Two way-ANOVA statistical analysis for seed weight and percentage of seed weight data. Comparison for individual genotypes between water treatment for 50 hrs, and water in 30 hrs then transfer to salt 75mM NaCl treatment and salt 75 mM NaCl treatment for 50 hrs (supporting data for Figure 5 – Chapter 5).

a, Seed weight

Col-0	Dry seed	Water 4h	Salt 4h	Water 30h	Salt 30h	Water 50h	Water-Salt 50h
Water 4h	****						
Salt 4h	****	**					
Water 30h	****	ns	****				
Salt 30h	****	ns	ns	**			
Water 50h	****	ns	**	ns	ns		
Water-Salt 50h	****	ns	ns	**	ns	ns	
Salt 50h	****	ns	ns	**	ns	ns	ns

| nGFP:PIP2;1 (7)	Dry seed	Water 4h	Salt 4h	Water 30h	Salt 30h	Water 50h	Water-Salt 50h
Water 4h	****						
Salt 4h	****	*					
Water 30h	****	****	****				
Salt 30h	****	ns	*	***			
Water 50h	****	****	****	ns	***		
Water-Salt 50h	****	ns	**	**	ns	***	
Salt 50h	****	ns	***	*	ns	**	ns

nGFP:PIP2;1 (18)	Dry seed	Water 4h	Salt 4h	Water 30h	Salt 30h	Water 50h	Water-Salt 50h
Water 4h	****						
Salt 4h	****	**					
Water 30h	****	***	****				
Salt 30h	****	ns	*	**			
Water 50h	****	****	****	***	****		
Water-Salt 50h	****	ns	**	*	ns	****	
Salt 50h	****	ns	***	ns	ns	****	ns

b, Percentage seed weight

Col-0	Water 4h	Salt 4h	Water 30h	Salt 30h	Water 50h	Water-Salt 50h
Salt 4h	****					
Water 30h	***	****				
Salt 30h	**	*	****			
Water 50h	ns	****	*	**		

	Water 4h	Salt 4h	Water 30h	Salt 30h	Water 50h	Water-Salt 50h
Water-Salt 50h	**	ns	****	ns	***	
Salt 50h	**	*	****	ns	**	ns

nGFP:PIP2;1 (18)	Water 4h	Salt 4h	Water 30h	Salt 30h	Water 50h	Water-Salt 50h
Salt 4h	**					
Water 30h	****	****				
Salt 30h	ns	**	****			
Water 50h	****	****	ns	****		
Water-Salt 50h	ns	**	****	ns	****	
Salt 50h	ns	****	***	ns	****	ns

nGFP:PIP2;1 (7)	Water 4h	Salt 4h	Water 30h	Salt 30h	Water 50h	Water-Salt 50h
Salt 4h	****					
Water 30h	****	****				
Salt 30h	ns	***	****			
Water 50h	****	****	***	****		
Water-Salt 50h	ns	****	***	ns	****	
Salt 50h	ns	****	**	ns	****	ns

Table S21: Two way-ANOVA statistical analysis of seed MCS area for comparison of individual genotypes between ½ MS and ½ MS + 75 mM NaCl treatments (supporting Figure 6 – Chapter 5).

Seed MCS Area	Imbibition time				
Genotype	30 min	1 hour	4 hours	8 hours	30 hours
Col-0	ns	ns	ns	ns	*
nGFP::PIP2;1 (7)	ns	ns	*	ns	***
nGFP::PIP2;1 (18)	ns	ns	ns	ns	***

% Seed MCS Area	Imbibition time				
Genotype	30 min	1 hour	4 hours	8 hours	30 hours
Col-0	ns	ns	ns	ns	*
nGFP::PIP2;1 (7)	ns	ns	*	ns	***
nGFP::PIP2;1 (18)	ns	ns	ns	ns	***

Table S22: (Support Figures 3.32 -3.33) One-phase association analysed results from Prism F tests:

a. For Figure 3.32A: A seed MCS area increase during imbibition, seeds from parent plants grown in water treatment with exponential regressions of MCS versus time are shown ($Y=Y_0 + (Plateau-Y_0)*(1-exp(-K*X))$).

Comparison of Fits			
Null hypothesis	One curve for all data sets		
Alternative hypothesis	Different curve for each data set		
P value	<0.0001		
Conclusion (alpha = 0.05)	Reject null hypothesis		
Preferred model	Different curve for each data set		
F (DFn, DFd)	5.662 (6, 75)		
Different curve for each data set	**Col-0**	*pip2;1-1*	*pip2;1-2*
Best-fit values			
Y0	153645	145490	130643
Plateau	200479	186015	167996
K	0.6964	0.8422	1.206
Tau	1.436	1.187	0.8289
Half-time	0.9953	0.8230	0.5745
Span	46834	40524	37353

b. For Figure 3.32B: Percentage MCS area increase during imbibition, seeds from parent plants grown in water treatment with exponential regressions of MCS versus time are shown ($Y=Y_0 + (Plateau-Y_0)*(1-\exp(-K*X))$).

Comparison of Fits			
Null hypothesis	One curve for all data sets		
Alternative hypothesis	Different curve for each data set		
P value	0.9489		
Conclusion (alpha = 0.05)	Do not reject null hypothesis		
Preferred model	One curve for all data sets		
F (DFn, DFd)	0.2708 (6, 75)		
Different curve for each data set	**Col-0**	*pip2;1-1*	*pip2;1-2*
Best-fit values			
Y0	1.157	0.7648	0.6245
Plateau	31.77	28.82	30.53
K	0.6810	0.8365	1.063
Tau	1.468	1.195	0.9405
Half-time	1.018	0.8286	0.6519
Span	30.61	28.06	29.90

c. For Figure 3.32C: A seed MCS area increase during imbibition, seeds from parent plants grown in water and NaCl at 5 mM NaCl treatment with exponential regressions of MCS versus time are shown ($Y=Y_0 +$ (Plateau-Y_0)*(1-exp(-K*X))).

Comparison of Fits	
Null hypothesis	One curve for all data sets
Alternative hypothesis	Different curve for each data set
P value	0.9961
Conclusion (alpha = 0.05)	Do not reject null hypothesis
Preferred model	One curve for all data sets
F (DFn, DFd)	0.1014 (6, 159)

Different curve for each data set	Col-0	*pip2;1-1*	*pip2;1-2*
Best-fit values			
Y0	153614	150086	149770
Plateau	201506	201057	204960
K	0.4747	0.6062	0.4575
Tau	2.107	1.650	2.186
Half-time	1.460	1.143	1.515
Span	47892	50971	55190

d. For Figure 3.32D: Percentage MCS area increase during imbibition, seeds from parent plants grown in water and NaCl at 5 mM NaCl treatment with exponential regressions of MCS versus time are shown (($Y=Y_0 + (Plateau-Y_0)*(1-exp(-K*X))$)).

Comparison of Fits			
Null hypothesis	One curve for all data sets		
Alternative hypothesis	Different curve for each data set		
P value	0.2553		
Conclusion (alpha = 0.05)	Do not reject null hypothesis		
Preferred model	One curve for all data sets		
F (DFn, DFd)	1.311 (6, 159)		
Different curve for each data set	**Col-0**	***pip2;1-1***	***pip2;1-2***
Best-fit values			
Y0	1.725	1.310	1.962
Plateau	33.93	37.63	39.58
K	0.4886	0.6659	0.4705
Tau	2.047	1.502	2.125
Half-time	1.419	1.041	1.473
Span	32.20	36.32	37.62

e. For Figure 3.33.A: Seed MCS area increase during imbibition for eight selected genotypes. Seed from parent plants grown in water treatment were sown on ½ MS media without stratification. Exponential regressions of MCS versus time are shown ($Y=Y_0 + (Plateau-Y_0)*(1-\exp(-K*X))$).

Comparison of Fits	Global (shared)
Null hypothesis	One curve for all data sets
Alternative hypothesis	Different curve for each data set
P value	<0.0001
Conclusion (alpha = 0.05)	Reject null hypothesis
Preferred model	Different curve for each data set
F (DFn, DFd)	9.230 (18, 126)

Different curve for each data set	Col-0	pip2;1-1	pip2;1-2	hkt1	nGFP:PIP2;1*	35S::PIP2;1 (7)*	35S::PIP2;1 (21)*
Best-fit values							
Y0	10.16	10.64	10.56	11.35	12.23	10.22	12.44
Plateau	16.35	16.80	16.84	18.06	19.06	16.28	20.08
K	0.6954	0.6436	0.8798	0.7801	0.9115	1.048	1.168
Tau	1.438	1.554	1.137	1.282	1.097	0.9539	0.8564
Half-time	0.9968	1.077	0.7878	0.8885	0.7604	0.6612	0.5936
Span	6.191	6.166	6.277	6.707	6.826	6.056	7.645

f. For Figure 3.33.B: Percentage seed MCS area increase during imbibition for eight selected genotypes. Seed from parent plants grown in water treatment were sown on ½ MS media without stratification. Exponential regressions of MCS versus time are shown ($Y=Y_0 + (Plateau-Y_0)*(1-exp(-K*X))$)

Comparison of Fits	Global (shared)
Null hypothesis	One curve for all data sets
Alternative hypothesis	Different curve for each data set
P value	0.2478
Conclusion (alpha = 0.05)	Do not reject null hypothesis
Preferred model	One curve for all data sets
F (DFn, DFd)	1.230 (18, 126)

Comparison of Fits							
Different curve for each data set	Col-0	pip2;1-1	pip2;1-2	hkt1	nGFP:PIP2;1*	35S:PIP2;1 (7)*	35S:PIP2;1 (21)*
Best-fit values							
Y0	1.706	2.118	1.170	1.522	0.7932	0.8010	0.3132
Plateau	64.15	61.26	61.61	61.61	57.03	60.55	62.61
K	0.6920	0.6430	0.8719	0.7873	0.9109	1.047	1.185
Tau	1.445	1.555	1.147	1.270	1.098	0.9547	0.8442
Half-time	1.002	1.078	0.7950	0.8804	0.7609	0.6617	0.5851
Span	62.45	59.14	60.44	60.09	56.24	59.75	62.30

Supplementary Figure 1: Figures from Da Ines (2008).

(A)

(B)

Supplementary Figure 1: This figure reveals Oliver Da Ines analysis of PIP2::GUS activity of all eight Arabidopsis PIP2s in vegetative tissues. It is included for examination purposes for further information but will be removed from the final thesis. Da Ines included these images in Figure 8 (A) and Figure 11 (B) of his PhD Dissertation, available via https://edoc.ub.uni-muenchen.de/12029/1/Da_Ines_Olivier.pdf (Da Ines, 2008). Da Ines wrote that "Distinct tissues of 8 to 13 day-old seedlings grown in vitro were analyzed and photographed. For expression in leaves, plants were grown in soil. (a, f, k, p, u, z, ae, aj) Eight to Thirteen-day-old seedlings; arrows point to hypocotyl; (b, g, l, q, v, aa, af, ak) section of the root hair zone of 2-week-old seedlings; arrows designate endodermis (c, h, m, r, w, ab, ag, al) root tip of 2-week-old seedlings; (d, i, n, s, x, ac, ah, am) leaves of 3 week-old plants grown in soil; (e, j, o, t, y, ad, ai, an) flowers of 5- to 6-week-old plants grown in soil.", and "Expression in root hairs of 2-week-old seedlings expressing PIP2 promoter::GUS constructs: (a) PIP2;1, (b) PIP2;2,"

Supplementary Data 1: Data of pUBN-GFP-Dest Sequence with the length at 11492 bp (Grefen *et al.*, 2010) derived from https://www.ruhr-uni-bochum.de/botanik/Resources/gb/pUBN-GFP-Dest.gb

```
LOCUS       pUBN-GFP-DEST      11492 bp    DNA     circular    30-SEP-
2010
SOURCE   ORGANISM  REFERENCE   1  (bases 1 to 10818)
  AUTHORS   Grefen,C., Donald,N., Hashimoto,K., Kudla,J., Schumacher,K. and
            Blatt,M.R.
   TITLE     A ubiquitin-10 promoter-based vector set for fluorescent
protein
            tagging facilitates temporal stability and native protein
            distribution in transient and stable expression studies
  JOURNAL   Plant J. 64 (2), 355-365 (2010)
   PUBMED    20735773
FEATURES             Location/Qualifiers
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                      /label=LB
     misc_feature     11052..11200
                      /label=RB
     terminator       3123..3331
                      /label=T35S
     CDS              3381..4531
                      /label=BarR
     promoter         1..639
                      /label=pUBQ10
     CDS              4931..5941
                      /label=SpecR
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                      /label=attR1
     CDS              2628..2933
                      /label=ccdB
     misc_recomb      complement(2974..3098)
                      /label=attR2
     CDS              1628..2305
                      /label=CmR
     exon             666..1382
                      /label=GFP
     rep_origin       7482..10075
                      /label=pVS1
     rep_origin       6392..7072
                      /label=pBR322
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      121 aacaactttg cgtgtaaaca acgctcaata cacgtgtcat tttattatta gctattgctt
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260

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 661 ctagtatggt gagcaagggc gaggagctgt tcaccggggt ggtgcccatc ctggtcgagc
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5461 gcgcgaactg caatttggag aatggcagcg caatgacatt cttgcaggta tcttcgagcc
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