Investigating factors that affect grapevine fruit set during abiotic stress

By

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Investigating factors that affect grapevine fruit set during abiotic stress

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Abstract

In angiosperms pollen tubes play a crucial role in sexual reproduction in delivering male gametes to female tissue for fertilization. Any type of impairment in pollen tube growth (PTG) might lead to the poor fruit set. Poor fruit set leads to partially developed berries and poor yields in grapevine. Salinity is a major environmental factor that constrains optimal fruit set. However, whether PTG is restricted in the style during saline conditions is still unknown.

PTG relies upon many co- ordinated processes including cytoskeletal rearrangements, vesicle trafficking, signal transduction pathways and pollen–pistil interactions. Various chemical factors are known to affect PTG, including gamma-aminobutyric acid (GABA) which at low concentrations guides the pollen tube to the ovary but at high concentrations inhibit pollen tube growth. GABA concentration also increases in plant tissues under stress, including salinized conditions.

The transport of ions across various pollen tube membranes is crucial for PTG; the proteins responsible for the ion movement across grapevine pollen are unknown. ALMTs/QUAC (Aluminum activated malate transporters/Quick activating anion channels) were found in Arabidopsis pollen tubes and are candidates for the movement of Cl^-. Recently it was discovered that anion currents through ALMT are gated by GABA. Here, the link between ALMTs and GABA and its role in controlling PTG under stress is explored.

Pollen performance and its potential role in poor fruit set under saline conditions was explored using Shiraz (BVRC17) vines. Pollen tube length and growth rate for salt treated vines was found to be significantly less in first 4 hours as compared to untreated pollen grains when grown in *in vitro* conditions on pollen germination media (PGM). Pollen grains were treated with exogenously-applied GABA (1-100 mM) using the *in vitro* pollen assay; length increased after 1-5 mM GABA and then decreased after 20-100 mM GABA treatment. An analogue of GABA (Muscimol) was inhibitory to PTG and an antagonist of GABA binding in mammalian $GABA_A$ receptors (Bicuculline) was stimulatory adding more evidence for the role of GABA in regulating PTG.

In order to check the GABA levels GABase assay was done and GABA levels were found to be nearly 2 fold increase in salt stressed flowers as compared to control flowers which may be contributing to the reduced fruit set through stunted pollen tube growth.

GABA concentration in tissue was examined using a GABase assay and found to be nearly 2 fold increased in salt stressed flowers as compared to control flowers which was hypothesized to be contributing to the reduced fruit set through stunted pollen tube growth. Gene

expression levels were examined for GABA shunt enzymes in control and salt treated flowers but were not significantly different.

ALMT expression in flowers, pollen grains and pollen tubes was examined. Five *ALMT* were found in flowers (*Vv ALMT 7, 9-like, 9-like_2, 10* and *13*), two in pollen tubes (*Vv ALMT 9-like* and *10*) and one in pollen grains (*Vv ALMT 10*). Ion transport by Vv ALMT 9-like was found to be GABA sensitive and therefore is a prime candidate for transducing GABA signals in pollen tubes which regulate PTG under standard and salinized conditions.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any University or any other tertiary institution. One of the figures in the thesis from Chapter 4 is included in an article published in a high impact journal. Other than this it contains no material previously published or written by another person, except where due references has been made in the text. I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint- award of this degree.

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Satwinder Kaur Date

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Abbreviations

ACA- Auto inhibited Ca^{2+} ATPases

ADF- Actin Depolymerizing Factor

ALMTs- Aluminum Activated Malate Transporters

ATP- Adenosine triphosphate

Ca^{2+}- Calcium ion

Ca^{2+}-CAM- Ca^{2+}- Calmodulin

CaCA- Ca^{2+} cation Antiporter

$CaNO_3$- Calcium Nitrate

$CaCl_2$- Calcium Chloride

CER- Controlled Environment Room

C:N- Carbon: Nitrogen

˚C- Degree celsius

CHX- Cation Hydrogen exchanger

CNGC- Cyclic Nucleotide Gated ion channel

CNS- Central Nervous System

DAO- Diamine Oxidase

ETC- Electron Transport Chain

FAO- Food and Agriculture Organization

GABA- γ- Amino butyric Acid

GABA-T- GABA-Aminotransferase

GAD-Glutamate Decarboxylase

GAT-GABA transporter

GHB- γ- hydroxybutyric acid

GLR- Glutamate receptor channel

H_2O_2- Hydrogen Peroxide

IBA- Indole Butyric acid

LGOs- Live Green ovaries

LN-Liquid Nitrogen

mm- Millimeter

mM- Milli Molar

MES- 4- Morpholineethanesulphonic acid

meq l^{-1}- milliequivalent per litre

Mg^{2+}- Magnesium

$MgSO_4$- Magnesium Sulphate

NA- niflumic acid

Na- Sodium

NAD- Nicotinamide adenine dinucleotide

NG- non germinated

NO_3^-- Nitrate ion

NPPB- 5-nitro-2-(3-phenylpropylamine)-benzoic acid

PAs- Polyamines

PAO- Polyamine Oxidase

%- Percent

PG- pollen germination

PTG- pollen tube growth

PTL- pollen tube length

PGM- pollen germination media

PO_4^{-}Phosphate ion

Pop 2- Pollen pistil 2

Put -Putrescine

QUAC- Quickly activating anion channel

R-type- Rapid type

ROS- Reactive Oxygen species

SEM- standard error mean

SLAC/ SLAH- Slowly activating anion channel

SO_4^{-}Sulphate

SSADH- Succinic semialdehyde dehydrogenase

SSR- Succinic semialdehyde reductase

Spm- Spermine

Spd- Spermidine

TRIS- Tris- (hydroxymethyle) aminomethane

µm- micro meter

µM- micro Molar

Chapter 1 Introduction

In angiosperms pollen tube plays an important role in sexual reproduction. Any kind of impairment in pollen tube growth leads to reduced fertilization, which reduces fruit set (Bouquet and Danglot 1996; Dantas *et al*. 2005; Kelen and Demirtas 2003; Mullins *et al*. 1992). As such, pollen tube growth (PTG) is observed to be an important determinant of fruit set in many plants including grapevine (Bouquet and Danglot 1996; Dantas *et al*. 2005; Ebadi *et al*. 1995a; Kelen and Demirtas 2003; Mullins *et al*. 1992). There are many abiotic factors influencing the growth of pollen tube like water deficit, temperature (cold and heat) and salinity (Dupuis and Dumas 1990; Fang *et al*. 2009; Porch and Jahn 2001; Stone 2001). In grapevine, poor fruit set can lead to higher levels of coulure (excessive shedding of young ovaries) and millerandage (excessive number of small berries mixed with sparse number of fully sized berries) and consequently poor yields (Dry *et al*. 2010), which can decrease wine quality (Walker *et al*. 2010). Pollen tubes are guided by several biological and chemical factors from the stigma through the style until it reaches the ovary to deliver male gametes and accomplish fertilization. GABA or γ-aminobutyric acid (GABA) is one such chemical factor which can control the PTG with gradients in GABA concentration thought to guide the pollen tube the ovary in *Arabidopsis thaliana*, whilst at high concentrations GABA inhibits pollen tube growth (Palanivelu *et al*. 2003). Interestingly, stress events, including salinity, increase GABA concentration in plant tissues (Akçay *et al*. 2012; Bai *et al*. 2013; Baki *et al*. 2000; Renault *et al*. 2010; Xing *et al*. 2007), and as such GABA is potentially a key regulator of grapevine fruit set under stress. Although it has been reported that GABA plays an important role in pollen tube guidance along the stylar region (Palanivelu *et al*. 2003), the actual mechanism underpinning this regulation is still obscure. Ion fluxes across the pollen tube plasma membrane are known to have a key role in regulating pollen tube length (Brewbaker and Kwack 1963; Breygina *et al*. 2009; Hedrich 2012; Malhó *et al*. 2000; Schiøtt *et al*. 2004; Steer 1989; Steinhorst and Kudla 2013). It has recently been discovered that ALMT/QUAC (Aluminium activated malate transporter/ quickly activating anion channels) can facilitate anion fluxes (Cl^-, NO_3, SO_4^{2-} etc.) across the plasma membrane (Pineros *et al*. 2008), and also they have a putative GABA binding motif (Ramesh *et al*. 2015). In *Arabidopsis thaliana* pollen tubes *ALMT12* is transcribed (Gutermuth *et al*. 2013; Pina *et al*. 2005) and in other tissues this protein is associated with NO_3^- and Cl^- transport (Meyer *et al*. 2010). As it is known that GABA is responsible for guiding pollen tubes, and *ALMT*s are present in pollen tubes, and are responsible for transporting a variety of anions (Cl^- and NO_3)

essential for PTG, it is possible that GABA can exert control upon PTG through ALMT channel proteins.

1.1 Objectives of the Research

Objectives of this research were to:

1) to explore if GABA concentration in flowers increases during salt stress
2) to explore if salt stress regulates the expression of gene/s responsible for GABA accumulation.
3) to find if GABA has any effect on Pollen germination (PG) and pollen tube growth (PTG) in grapevine?
4) To explore which ALMT are expressed in grapevine pollen tubes and may be responsible for the GABA effect upon pollen tube growth?

1.2 Linking statement

The research in this thesis is presented in chapters, including a research article prepared for eventual submission to the *Journal of Experimental Botany* (JXB).

- Chapter 1 comprises the introduction to the thesis.
- Chapter 2 is a review of literature about soil salinity, its impact on grapevine production especially on fruit set, the role of pollen tubes in reproduction, the role of different ion channels particularly R-type (Rapid-type) anion channels, an introduction to GABA and the potential role of ALMTs in GABA perception and signalling.
- Chapter 3 is a short chapter describing the optimization of an *in vitro* pollen germination assay as a way to assess the effect of GABA on PG and PTG.
- Chapter 4 contains the research article which explores pollen performance in salt stressed conditions, the role of exogenous GABA on PG and PTG using the *in vitro* assay, the effect of applied pharmacological drugs (muscimol and bicuculline) on pollen tube growth, a quantification of endogenous GABA levels upon salt stress, the effect of salt and GABA application on grapevine fruit set, an exploratory study for the possible gene/s responsible for altering GABA concentration in reproductive tissue, and an examination of the ALMT gene members present in grapevine flowers, pollen grains and pollen tubes. This chapter advances our understanding of the role of GABA in regulating PG and PTG through ALMT proteins channels.

- Chapter 5 is the general discussion about the work from the two chapters 3 and 4. It gives an idea about the research gaps and possible experiments to fill those gaps in near future.

Chapter 2 Literature Review

2.1 Grapevine production and uses

Grapevine (*Vitis vinifera* L.) is native to Europe as well as East and Central Asia and appears to have originated ~65 million years ago. It belongs to the order *Vitales* and Family *Vitaceae*. It is known for its various uses since ancient times (Lacirignola and Digiaro 1999). According to FAO in 2011 (http://faostat.fao.org) the total global area under grapevine production is 7.5 million ha with China being the largest producer followed by Italy, the United States, France, Spain, Portugal, Romania, China, Argentina, Australia and Chile. Australia is a top ten grapevine producing country with an area of 163,951 ha under grapevine cultivation. Australia is the fourth largest exporter of wine contributing approximately 750 million litres to the international market annually. In Australia, the Riverland in South Australia produces about 448 million litres, which is 58% of national production, followed by Riverina in New South Wales with 30%, and Sunraysia in Victoria with 12% of Australia's total. According to FAO (Food and Agriculture organization), approximately 71% of the world grape production is used as wine, 27% as a fresh fruit and 2% as a dried fruit.

2.2 Salinity in Australia

Soil salinity can be defined as the accumulation of excessive salt concentrations in the soil solution to an extent that inhibits plant growth, this occurs for grapevine around ~20 mM NaCl or an electrical conductivity of ~2 dSm^{-1}. Around the world about 800 million hectares of land is considered as saline (FAO, 2008). Many Australian soils were also found to be saline to some degree (Northcote and Srene 1972). According to the national land and water resources audit (2000), about 5.7 million hectares of land is prone to develop dryland salinity which may rise to 17 million hectares by 2050. Dryland salinity is a natural type of salinity which leads to the accumulation of salts in the surface and ground water. In Australia the dryland salinity is due to the ancient stores of salts which are released due to weathering of rocks or was carried in from the surrounding oceans in the form of rainfall, with salt being trapped in the landscape long ago. These salt stores are distributed widely across the semi-arid and arid parts of the Australia, stretching in a huge arc from the North Tropics down south adjacent to the Great Dividing Range, broadening and sweeping across the Murray Darling basin to take in the Riverina and Mallee regions of New South Wales, Victoria and South Australia (Salinity fact sheet CSIRO; http://www.csiro.au/files/files/pmyj.pdf). Exposure of land due to removal of vegetation and poor agricultural practices further aggravates the

situation. With the introduction of drip irrigation salinity has become a major concern in the field of Agriculture. This is a common problem in semi-arid to arid areas of the world (Israel, Egypt, Saudi Arabia and Australia). In Australia, before 1960s furrow irrigation was the main type of irrigation which was used to irrigate the vineyards but after the introduction of drip irrigation system there was an increase in the use of regional ground water accompanied by considerable salt accumulation (Christen *et al*. 2007).

2.2.1 Salinity and grapevine

In Australia, large parts of grape growing areas are prone to water logging and salt accumulation (Fitzpatrick *et al*. 1993). Generally, salinity inhibits plant growth in two phases, firstly by limiting water uptake through an osmotic or water-deficit effect arising from relatively high solute concentration in the soil. Secondly, ion uptake where it injures cells through ionic stress effects (Munns 2005). Grapevine varieties are ranked moderately sensitive to salt stress by most researchers (Hawker and Walker 1978; Maas and Hoffman 1977; Shani *et al*. 1993; Walker *et al*. 2002). In grapevines, a yield loss occurs from 1.5dS/m and above and there is about a 10% loss in yield when salinity is 2.5dS/m or above (Christen *et al*. 2007). With increasing salinity, a reduction in shoot growth, bunch number, berry weight, loss in yield and an increase in Cl^- and Na^+ and acid concentration was found (Prior *et al*. 1992; Walker *et al*. 1996). During salt stress both Na^+ and Cl^- ions accumulate but grapevines are commonly more sensitive to Cl^- toxicity compared to Na^+ - as more Cl^- accumulates in shoots (Walker *et al*. 2004). The accumulation levels of the ions greatly varies with the type of cultivar depending upon their exclusion capacity for salts (Walker *et al*. 2010). Overall, the inhibition of grapevine growth during salt stress can be attributed to changes in stomatal conductance, electron transport rate, leaf water potential, chlorophyll fluorescence, osmotic potential and leaf ion concentration (Medrano *et al*. 2002; Patakas and Noitsakis 1999; Schultz and Matthews 1988; Walker *et al*. 1981).

2.2.2 Effect of salinity on fruit set and yield

There are several studies determining the loss in yield in different ways due to salt stress. The yield loss in grapevines is observed in terms of fruit set, coulure and millerandage. Fruit set is determined by total number of flowers turned into berries (Live green ovaries or LGOs, seeded and seedless berries). Coulure is defined as an excessive shedding of ovaries or very young berries, which results in relatively few numbers of true berries. Millerandage which is yet another abnormal condition in which excessive numbers of small berries are mixed with sparse numbers of full sized berries (Dry *et al*. 2010). Hawker and Walker (1978) demonstrated the reduction in berry set and development by application of NaCl to rooted

cuttings of Cabernet Sauvignon under glasshouse conditions. While Stevens *et al*. (1999) studied the effect of salinity over six seasons in the field and found an effect of salt in sixth season which was a yield loss due to a reduction in berry weight.

2.3 Effect of salinity on pollen grains and pollen tube

In angiosperms the male gametophyte (the pollen grain) germinates on the stigmatic surface and elongates through the style tissue to reach the ovule to make fertilization successful. Pollen viability is one of the most important determinants for normal fruit set in all fruit crops including grapevines (Bouquet and Danglot 1996; Dantas *et al*. 2005; Kelen and Demirtas 2003; Mullins *et al*. 1992). Since the discovery of a pollen germination media by Brewbaker and Kwack (1963) *in vitro* pollen germination assays have become an important tool to study the physiology and biochemistry of germinating pollen grains and pollen tubes. A lot of such experiments have been performed to check the effect of salinity on the pollen viability, PG and PTG to check the pollen performance under saline conditions (Dadashi 2008; Dhingra and Varghese 1985; El Sayed *et al*. 1991; Reddy and Goss 1971). Reddy and Goss (1971) observed a decrease in PG by 21% from the control treatment in Petunia under saline conditions. Dhingra and Varghese (1985) observed that increasing salinity treatment from 0<80<120<160 meq l^{-1} results in decrease in pollen viability and germination in maize plants; at 160 meq l^{-1} salinity, the viability (PG) was reduced by 29.7% from control treatment. Similar research was done by El Sayed *et al*. (1991) showing that increase in salinity was detrimental to pollen viability and PG; incorporation of hydrogen polymer in the sand in which the plants were grown helped in reducing the salinity by absorbing excess salts which were responsible for the lower pollen viability. There are previous studies showing *in vivo* PTG contributing to reduced fruit set in grapevine (Bouquet and Danglot 1996; Ebadi *et al*. 1995a; Ebadi *et al*. 1995b; Mullins *et al*. 1992). One of the research groups also used *in vitro* studies as an important tool to screen the best grapevine varieties for breeding and vineyard establishment (Sharafi and Bahmani 2011). Hence, pollen tube growth is a crucial contributing factor towards fertilization and during salinity its growth is affected, so is a likely factor that reduces the fruit set.

2.3.1 Different ion and ion channels are involved in pollen tube growth

As biological membranes are not permeable to charged or large molecules, ion channel and transporter proteins are required to transport these solute ions into and out of cells (Hedrich 2012). There are different sets of ion transporters in different cell types that co-ordinate the physiology of the plant. There is also a complex network of signaling molecules that regulate the activity of these ion channels with their activity changing during the course of the day,

during development and stress events (Hedrich 2012). PTG is one such physiological process that requires the co-ordinated activity of ion channels and transporters in different cell types that co-ordinate the physiology of the cell (Hedrich 2012).

2.3.2 Anion channels are essential for PTG

The role of various ions in PG and PTG has been discovered including Ca^{2+}, protons (H^{+}), potassium (K^{+}) and chloride (Cl^{-}) (Fig. 1, A) (Brewbaker and Kwack 1963; Breygina *et al*. 2009; Malhó *et al*. 2000; Schiøtt *et al*. 2004; Steer 1989; Steinhorst and Kudla 2013).

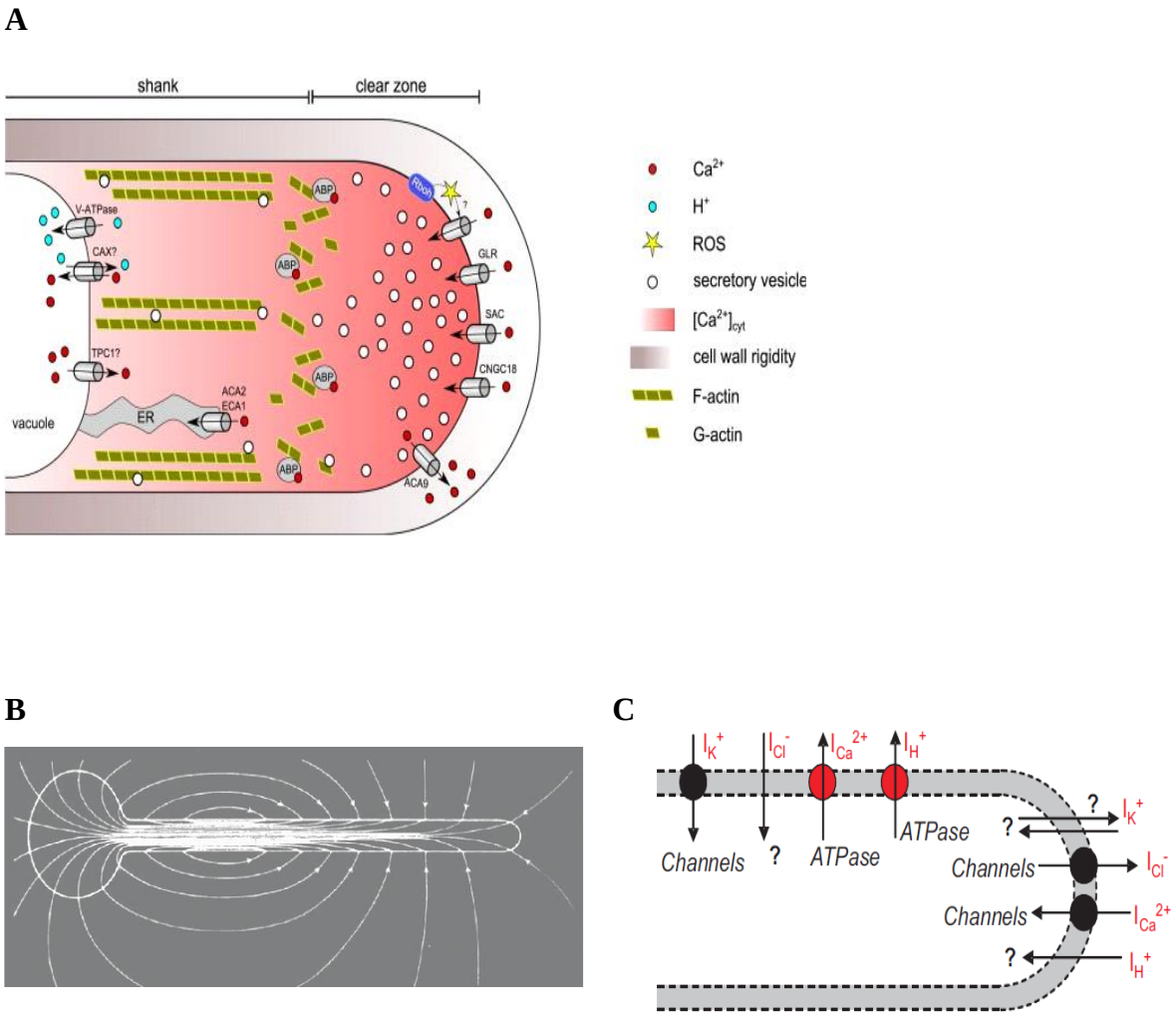


Figure 1. Current model for ion fluxes around pollen tube. **(A)** Ca^{2+} regulated processes and changes in Ca^{2+} concentration in a growing pollen tube. This schema illustrates the diverse Ca^{2+} fluxes and concentration as well as their impact on actin organization in the pollen tube (Steinhorst and Kudla 2012). **(B)** Electrical model for lily (*Lilium longiflorum*) pollen tube, deduced from extracellular electric currents as determined by original voltage- vibrating probe. A net current of positive charges enters the tip, leaking by the tube and grain (Adapted from Weisenseel *et al.* 1995). **(C)** Inorganic ion fluxes around the pollen tube of Tobacco (*Nicotiana tabaccum*) as recorded using the ion- specific vibrating probe technique (Michard *et al.* 2009).

The activity of ion channels and hence the fluxes across the pollen tube plasma membrane are linked through the influence of the membrane potential on the electrochemical gradient for ion movement (Fig. 1, B and C) (Michard *et al*. 2009). Affecting the activity of one class of ion transport protein will impinge upon the activity of other classes of transporter and affect the flux of all ions across the plasma membrane, and will affect PTG. Signaling molecules that affect chloride fluxes across the PT will therefore affect PTG. Recently, it has been hypothesized that GABA may be a signal that regulates chloride flux across PT membranes, and it is already established that GABA affects pollen tube growth (Palanivelu *et al*. 2003).

Transcriptomic analysis of *Arabidopsis thaliana* has shown that there are at least 459 possible transporter genes expressed during PG and PTG. Of these 459 genes at least 8 were found to be pollen specific (Pina *et al*. 2005; Wang *et al*. 2008). Several gene families responsible for encoding putative cation channels were shown to be expressed during PG and PTG (Becker *et al*. 2003; Feijó *et al*. 1999; Mouline *et al*. 2002; Schiøtt *et al*. 2004; Song *et al*. 2009; Sze *et al*. 2004). There are different gene families for different ions e.g. for Ca^{2+}[*CNGC* (Cyclic Nucleotide Gated ion Channels), *GLR* (Glutamate receptor Channels) (Mouline *et al*. 2002), *CaCA* (Ca^{2+} Cation Antiporter), *ACA* (Schiøtt *et al*. 2004)**,** *CHX* (Cation- Hydrogen Exchanger) (Sze *et al*. 2004)], for H^+ [AHA(H^+-Pumps), H^+-ATPase (Feijó *et al*. 1999)], for K^+ [SPIK (shaker family) (Mouline *et al*. 2002), transporters from CHX or AtKT/ AtKUP/HAK (Becker *et al*. 2003; Song *et al*. 2009)] but the identity of Cl^- channels remains to be identified.

Zonia *et al*. (2002) discovered a marked efflux of Cl^- from the apex of Tobacco pollen tubes (Fig. 2, A and B). They confirmed that the efflux of Cl^- was necessary for the PTG through the application inositol 3, 4, 5, 6- tetra*kis*phosphate which induced cell volume increase and disrupted Cl^- efflux. This observation is sufficient to prove that along with Ca^{2+}, H^+ and K^+ this anion also plays a pivotal role in pollen tube growth where it could be presumable to play a role in turgor regulation. Later studies (Breygina *et al*. 2009) further supported the previous studies by the application of 5-nitro-2-(3-phenylpropylamine)-bezoic acid (NPPB) and niflumic acid (NA) the blockers of Cl^- channel activity to show the complete inhibition of PG and significantly reduced the Cl^- efflux (Matveyeva *et al*. 2003) and also results in depolarization of plasma membrane and disruption of pollen tube apical compartmentalization (Breygina *et al*. 2009). Even now the actual identity of the Cl^- channels in pollen tube have not been discovered. Anion permeable channels have been identified in plants belonging to the slowly activating anion channel family (SLACs/SLAH) and the Aluminum (Al^{3+})-activated malate transporters/Quickly activating anion channel family (ALMTs/QUAC)

(Hedrich *et al*. 1990; Linder and Raschke 1992; Schroeder and Keller 1992). Plant cells require a complex mixture of diverse anions including chloride (Cl^-), nitrate (NO_3^-), sulphate (SO_4^-), phosphates (PO_4^-) and organic anions such as citrate, malate and oxalate in varying proportions (Barbier and Buygoo *et al*. 2000), and these two protein families can transport these anions with various degrees of specificity. It is possible that members of either of these families encode the proteins responsible for anion fluxes across pollen tube membranes.

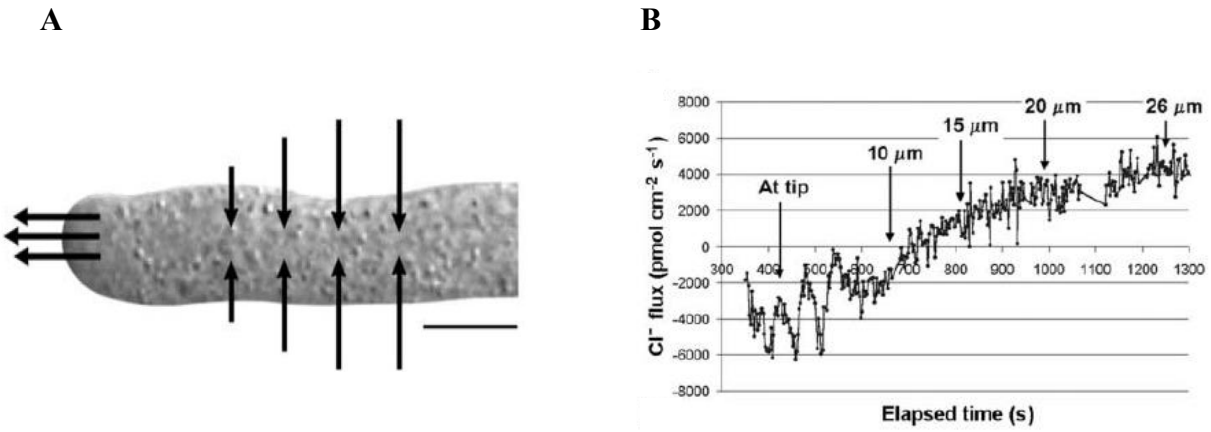


Figure 2. (A) Profile of Cl^- flux starting at the apex and continuing to 26µm distal to the tip. There is an inversion of oscillatory efflux to net influx at ~12µm distal to the tip. **(B)** A Graphic representation of the vectorial flux of Cl^- into the pollen tube and out from the apex. Bar = 10µm (Zonia *et al.* (2002).

2.4 What is GABA?

γ –Aminobutyric acid, is a four carbon non-protein amino acid. It was first discovered in the year 1949 in potato tuber tissue (Steward *et al*. 1949). Later it was also discovered in animals as a major inhibitory neurotransmitter in the central nervous system (CNS). In both plants and animals, GABA is metabolized *via* a common pathway known as the GABA shunt. The GABA shunt is composed of three enzymes; namely, Glutamate decarboxylase (GAD) a cytosolic enzyme, GABA transaminase (GABA-T) and Succinic Semialdehyde Dehydrogenase (SSADH) the mitochondrial enzymes (Fig. 3), and it has recently been proposed to act as a signaling molecule in plants as well as an essential intermediate in C/N signaling (Bouche and Fromm 2004; Fait *et al*. 2008).

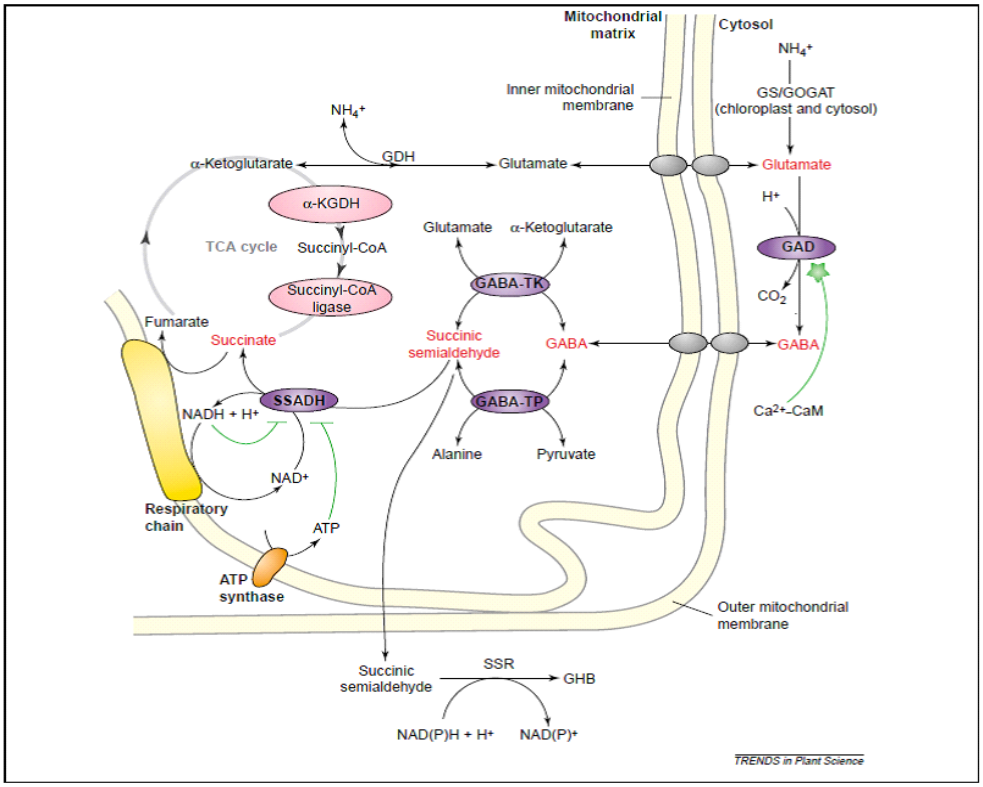


Figure 3. GABA shunt, the γ- Aminobutyric acid pathway and its regulation in plants (Bouch and Fromm, 2004).

2.4.1 Different enzymes of GABA- shunt

2.4.1.1 GAD:- Glutamate decarboxylase (EC 4.1.1.15) is the Ca^{2+}-dependent calmodulin (CaM) binding protein. It catalyzes the first step in the GABA shunt that is the irreversible decarboxylation of glutamate to GABA. This enzyme is located in the cytosol and exhibits an acidic pH optimum of ~ 5.8 (Breitkreuz *et al*. 1999) (Fig. 3).

2.4.1.2 GABA-T:- GABA Transaminase (EC 2.6.1.19) is located in the mitochondria (Shelp *et al*. 1995; Zhang and Bown 1997). After GABA is transported into the mitochondria, it is converted to succinic semialdehyde (SSA). GABA-T has two types depending upon the type of amino acid acceptors. It could either use α- ketoglutarate (GABA-TK) or pyruvate (GABA-TP) as amino acid acceptors, producing glutamate and alanine respectively. This enzyme exhibits alkaline pH of ~ 8 to 10 (Shelp *et al*. 1995). SSA then enters the TCA cycle after the reduction with SSADH or instead could it be reduced to γ- hydroxybutyric acid (GHB) via a succinic semialdehyde reductase (SSR), which is located in the cytosol in animal cells and possibly in plant cells as well. In animals it has the auto inhibitory role while in plants its role is still unknown (Breitkreuz *et al*. 1999) (Fig. 3).

2.4.1.3 SSADH:- Succinic semialdehyde dehydrogenase (EC 1.2.1.16) is also located in the mitochondria and further reduces the SSA to succinate (Bown and Shelp 1997). This enzyme

exhibits alkaline pH of ~ 9. It is an irreversible reaction requiring 1 NAD and 1 ATP molecule. Both NAD and ATP can inhibit the activity of SSADH through feedback inhibition (Fig. 3).

2.4.2 Role of GABA Shunt:-

The GABA shunt is associated with various physiological responses, including regulation of pH, contributing to the C:N balance, defense against insects, protection against oxidative stress, osmoregulation and signaling (Bouche and Fromm 2004).

2.4.2.1 Regulation of pH:- In the first step of the GABA shunt, GAD uses cytosolic protons (H^+) to convert glutamate to GABA *via* a decarboxylation reaction. In doing so it contributes to the regulation of pH in the cytoplasm. As observed in *Asparagus* exposed to cell-permanent weak acids the cytosolic pH decreased by 0.6 with half time of 2 s, and GABA levels increase by 200-300% within 15 s (Crawford *et al*. 1994). Acidic pH is one of the crucial factors controlling the activity of GAD other than Ca^{2+}- CAM. In bacteria the GABA shunt plays a role in acid resistance (Becker *et al*. 2003; Castanie-Cornet *et al*. 1999). When *Escherichia coli* is exposed to such conditions GAD is activated and uses the protons by catalyzing the decarboxylation of glutamate to GABA.

2.4.2.2 Contributing to the C:N balance:- Levels of GABA is observed to be very high in certain tissues. It can contribute up to 50% of total free amino acids in cherry tomato fruits (Beuve *et al*. 2004; Mazzucotelli *et al*. 2006; Rolin *et al*. 2000). In addition sometimes GABA can also acts as a sole nutrient source as was observed in *Arabidopsis* (Breitkreuz *et al*. 1999). GABA shunt is also a way of assimilating carbon from glutamate and to generate C:N fluxes that enter the TCA cycle.

2.4.2.3 Protection against oxidative stresses:- During various stresses the production of Reactive Oxygen Species (ROS) is enhanced in organelles such as the chloroplast and mitochondria by interfering with electron transport chain (ETC) (Mittler 2002). In *Arabidopsis*, the disruption of the *SSADH* gene results in plants undergoing necrotic cell death when exposed to environmental stresses, owing to accumulation of ROS (Bouché *et al*. 2003). They used *ssadh1* mutant to study the role of the GABA shunt in H_2O_2 regulation. The *ssadh1* mutant accumulated more H_2O_2 as compared to the wild type plants. Another group (Cramer *et al*. 2007) observed in grapevines (cv. Cabernet Sauvignon) a marked increase in the transcript of GABA-T and hence showing the role of GABA shunt in limiting the ROS under water deficit and salt stress (Bouché *et al*. 2003).

2.4.2.4 Osmoregulation:-In response to environmental stress (drought, heat, salt and heavy metal stress) GABA levels increase in a similar way to other osmolytes, and accumulation during stress is shown in many studies (Bolarín *et al*. 1995; Mayer *et al*. 1990; Raggi 1994; Thomson *et al*. 1996). Although GAT1 (GABA transporter) is present as reported in Arabidopsis (AtGAT1) but proline transporters from *Arabidopsis* (AtProT2) (Breitkreuz *et al*. 1999) and tomato (LeProT1) (Breitkreuz *et al*. 1999) also transport GABA along with proline. AtProT2 is strongly induced during water or salt stress conditions (Rentsch *et al*. 1996) hence suggesting its role in mitigating stress levels as the other osmolytes (Shelp *et al*. 1999). Heber *et al*. (1971) also reported that GABA can protect biological membrane from inactivation during freezing just as the other osmoprotectants do.

2.4.2.5 GABA as a signalling molecule:- Evidence that GABA could also act as a signal similar to its role in animals (as a neurotransmitter) came from the studies on *Arabidopsis* GABA transaminase mutant ***pop2*** (*Pollen–pistil 2*) which revealed that a GABA concentration gradient in the style is required to guide pollen tubes to the ovary (Palanivelu *et al*. 2003). Also (Lancien and Roberts 2006) showed from the studies on *Arabidopsis* that in the presence of high external Ca^{2+} concentrations, GABA strongly down- regulates seven of the nine 14-3-3 genes expressed in *Arabidopsis* seedlings, along with ethylene and Abscisic acid. Immediate increases in cytosolic Ca^{2+} occur in response to stress (Knight *et al*. 1992; Malho and Trewavas 1996). Increased cytosolic Ca^{2+} forms complexes with CaM and Ca^{2+}/ CaM activates GAD that increases GABA biosynthesis (Aurisano *et al*. 1995; Cholewa *et al*. 1997).

2.4.3 Main pathways of GABA formation in plants

2.4.3.1 GABA shunt

As mentioned above GABA is formed from glutamate via a decarboxylation reaction catalyzed by GAD. This is the major pathway contributing to the total GABA formation in plants during normal and stress conditions (Breitkreuz *et al*. 1999).

2.4.3.2 Polyamines (PAs) catabolism

GABA is also proposed to be formed from PAs which are aliphatic, N_2 containing, low molecular weight polycationic molecules. They are widespread in all types of living organisms. The major PAs are the diamine Putrescine (Put), the triamine Spermidine (Spd) and the tetramine Spermine (Spm). During the catabolism of PAs, Spd and Put {catabolised by PAO (Polyamine Oxidase) and DAO (Diamine Oxidase) respectively} (Cona *et al*. 2006) further get converted into GABA *via* a Δ^1 – pyrroline intermediate formation (Bhatnagar *et al*.

2001; Bouchereau *et al*. 1999; Turano *et al*. 1997). Xing *et al*. (2007), demonstrated that the GABA in 2- week old soybean [*Glycine max* (L.) Merr.] seedlings increased significantly, and was concomitant with decrease in PAs level and increase in DAO activity. They applied AG (Aminoguanidine) a specific inhibitor of DAO which resulted in accumulation of free PAs due to strong inhibition of DAO activity but decreased GABA levels (by 39%) thus suggesting that raised GABA reported earlier was due to the PA oxidation catalyzed by DAO. Similarly Bai *et al*. (2013) also reported the GABA accumulation in germinating fava bean during hypoxia. In contrast Shelp *et al*. (2012) reported that during abiotic stresses accompanied by changes (less) in oxygen levels the activity of DAO is affected as they require oxygen for their reaction. Hence the main pathway of GABA formation is through the regulation of GAD instead of Put catabolism through DAO.

A lot of work has been done to see the effect of exogenous application of certain PAs. It was found to improve fruit set in a variety of plants including mango (Singh and Janes 1999), apricot (Alburquerque *et al*. 2006), apple (Costa *et al*. 1985) and olive (Rugini and Mencuccini 1985). Interestingly exogenous application of GABA also results in improvement of the yield, as reported in variety of plant types including *Stellaria longipes* (Kathiresan *et al*. 1998), potato (Narayan and Nair 1990), onions (Drost 1999). In all these cases AuxiGro was used which is a commercial product containing both glutamic acid and GABA (1:1). But it is still speculative that these two mitigation processes work in accordance or independently, it needs further research.

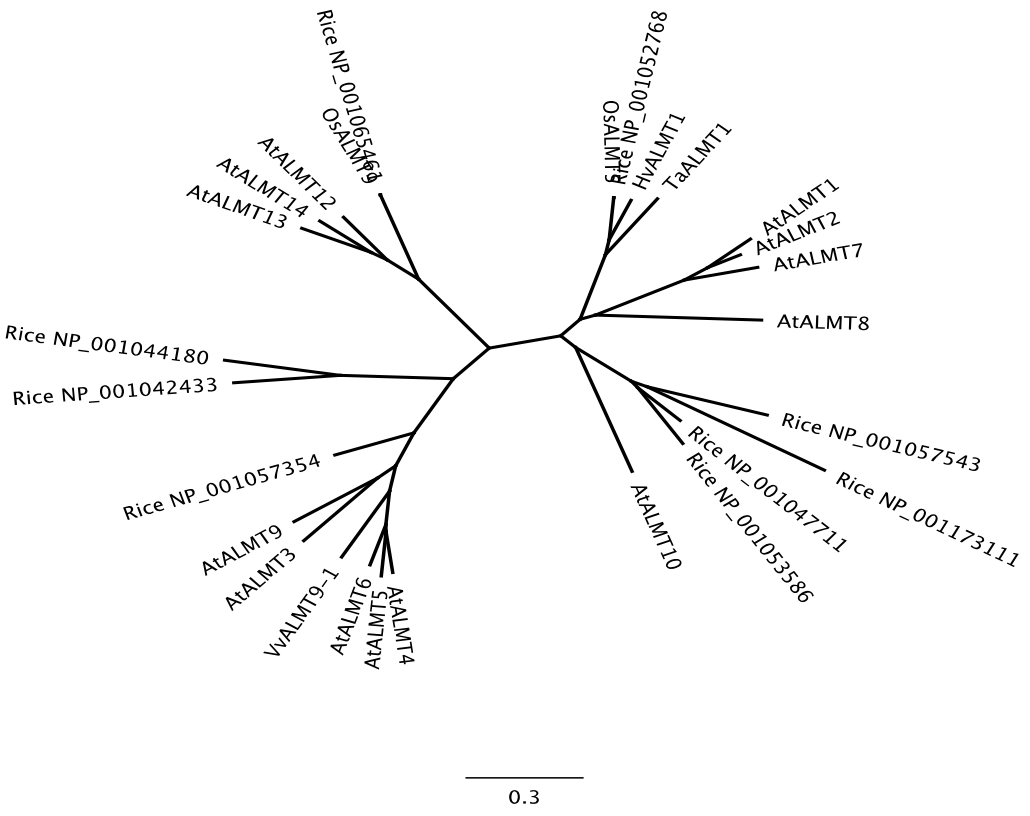
2.5 What are ALMTs?

The Al^{3+}- activated Malate transporters gene family (ALMTs) have recently been proposed to encode for some Rapid (R)-type anion channel activity in plant cells (Meyer *et al*. 2010). R-type channels are so named as they rapidly activate/deactivate within milliseconds, they are also voltage-dependent. ALMTs were first described in plasma membrane of root cells using the patch clamp technique (Kollmeier *et al*. 2001; Piñeros and Kochian 2001) and were genetically identified in *Triticum aestivum* (TaALMT1) (Sasaki *et al*. 2004) and *Arabidopsis thaliana* (AtALMT1) (Hoekenga and Maron). Both homologues TaALMT1 and AtALMT1 has been described to be involved in aluminum (Al^{3+}) resistance by releasing malate from the root tip, thereby chelating Al^{3+} in the rhizosphere (Meyer *et al*. 2010). Although the name suggests the ALMTs to be Al^{3+} activated but most of the ALMTs are not Al^{3+} activated. As was observed in maize where ZmALMT1 is Al^{3+} independent and transports inorganic anions such like, Cl^{-}, NO^{3-}, and SO^{4-} rather than malate (Piñeros and Kochian 2001).

2.6 Relationship between ALMTs and GABA

In animals, GABA mediates pre-synaptic inhibition of primary afferent fibers in the motor neuron system. It regulates brain excitability via $GABA_A$ receptors. $GABA_A$ receptors are macromolecular proteins composed of a chloride ion- selective channel with binding sites for picrotoxin, barbiturates, benzodiazepines and GABA (Macdonald and Twyman 1991). GABA binds with $GABA_A$ receptors to regulate (gating) opening and closing of Cl^- ions and produces a flow Cl^- ions into neurons (Macdonald and Twyman 1991). Recently it was reported that ALMTs have putative GABA binding motif that has strong homology with binding motif of animal $GABA_A$ receptor (Ramesh *et al*., 2015) (Fig. 4, B).

A



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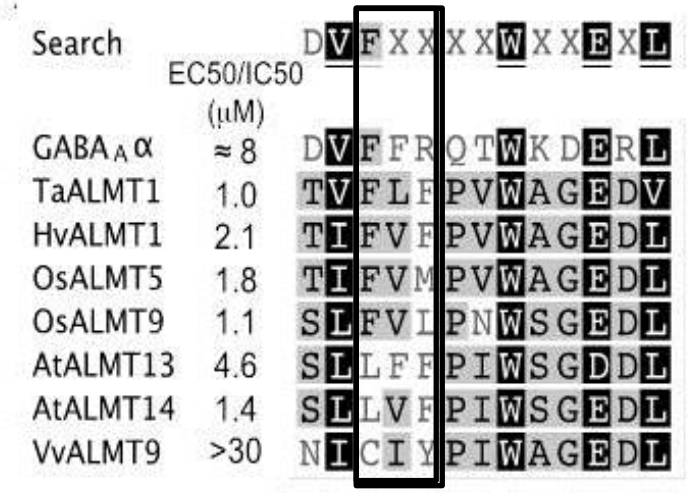

Figure 4. (A) A Family tree of ALMTs. **(B)** Comparison of the GABA binding sites in the Mammals (Rat) and plants (*Triticum aestivum*, *Hordeum vulgare*, *Oryza sativa*, *Arabidopsis thaliana* and *Vitis vinifera*. (Ramesh *et al*., 2015).

Interestingly, the anion flux through the plant ALMTs can be regulated by GABA (Ramesh *et al*., 2015). ALMTs are unique in plants, and thought to be present in all the plant types and they are strongly suggested to play the discrete roles depending upon their localization in different cell types (Fig. 4, A). As an example, they have confirmed roles in aluminium tolerance (Hoekenga and Maron 2006), stomatal aperture control (AtALMT12) (Meyer *et al*. 2010; Sasaki *et al*. 2010) in vacuolar malate accumulation (AtALMT9, 6) (De Angeli *et al*. 2013; Kovermann *et al*. 2007; Michard *et al*. 2011) and in mineral nutrition (ZmALMT1) (Piñeros *et al*. 2008) and are speculated to play even, as few of the family have been characterized. At first ALMTs were identified in only stomatal guard cells and roots but now they have been identified and characterized in other cell types as well, like seed endosperm (AtALMT11), in pollen tubes (AtALMT12) and in pericycle and hypocotyls (AtALMT10), and in pericycle and seed coat (AtALMT7). There are 14 ALMTs identified in Arabidopsis, 13 in Grapevine, 33 in Soybean and 9 in Rice (Delhaize *et al*. 2007).

As GABA binding sites have been detected on pollen tube (Hoekenga and Maron 2006), and ALMT are present in pollen tubes it could be speculated that ALMT proteins are the sites of GABA binding. Also the role of ALMT in plants may parallel the role of $GABA_A$ receptors in animals by regulating the membrane potential of cells, and in so doing could regulate ion flux across pollen tube membranes (and other cell types).

2.7 Conclusion and aims of research

In grapevines salinity is one of the major environmental constrains limiting the yield and productivity. There has been a considerable work done to find that there is a decrease in yield due to salt stress, but the factors responsible for this reduced yield are still obscure. Pollen tube growth is essential in fertilization but how pollen tube growth is regulated during grapevine fruit set is still poorly explored. Although there are various factors affecting the fertility and ions play a crucial role. Different ions (Ca^{2+}, H^{+}, K^{+} and Cl^{-}) are important for the growth of pollen tube and failure of any one of these ion transporters result in slow or stunted PTG. There are several reports showing the transporters for Ca^{2+}, H^{+}, K^{+} but there are still no reports for Cl^{-} channels as earlier reported CLC transporters were later found to be NO_3 transporters. Some of the ALMTs were found to transport Cl^{-} and are best candidates for Cl^{-} transporters. Recently, GABA was found to guide the pollen tubes but the actual mechanism underlying this guidance is still not known.

The present research will be carried out with the following aims:

1) to explore if GABA concentration in flowers increases during salt stress
2) to explore if salt stress regulates the expression of gene/s responsible for GABA accumulation.
3) to find if GABA has any effect on Pollen germination (PG) and pollen tube growth (PTG) in grapevine?
4) to explore which *ALMT* are expressed in grapevine pollen tubes and may be responsible for the GABA effect upon pollen tube growth?

Chapter 3.

Optimizing an *in vitro* pollen germination and pollen tube growth assay and assessing the effect of GABA

Introduction

In order for angiosperm fertilisation to occur the pollen grain, which contains a male gametophyte, must be transferred to female tissue for germination and growth of the pollen tube through the stylar region to the ovule. Since the discovery of an *in vitro* pollen germination assay by Brewbaker and Kwack (1963), there has been a significant body of work done to explore the role of various factors that influence PG and PTG.

The *in vitro* pollen germination assay media recipe, type of media, temperature and pH play a crucial role in this assay and if any one of these conditions is not properly optimized it will lead to a reduction in PG and PTG. Standard pollen germination media consists of sucrose, boric acid, and other various ions (i.e. H^+, K^+, Ca^{2+}, Cl^-, NO_3^-, Mg^{2+} and SO_4^{2-}) each component has its own role. To start with sucrose acts as a nutrient source and as an osmoticum (Brewbaker and Kwack 1963) to prevent the pollen bursting when hydrated in pollen germination media however the proper sucrose concentration also plays an important role to act as an osmoticum (Messerli and Robinson 2003). Boric acid plays an important role in growth of the pollen tube by binding to the phosphoinositides, glycoproteins and glycolipids of membrane hence contributing to the membrane stability (Blevins and Lukaszewski 1998). Apart from these ions transport processes are also crucial for pollen tube growth with Ca^{2+}, H^+, K^+ and Cl^- fluxes across the pollen tube plasma membrane all being essential for PTG. Among ions Ca^{2+} plays a major role as a second messenger in regulation of cytoskeleton and vesicle trafficking by maintaining endomembrane trafficking and controlling actin dynamics needed for tip focused growth (Steinhorst and Kudla 2013). External Ca^{2+} source help to maintain the internal Ca^{2+} gradient in order to maintain the PTG. In stigma and style too external Ca^{2+} guides the elongating pollen tubes towards the ovary to help reaching ovules for fertilization procedure (Bednarska 1989; Tirlapur *et al*. 1993). H^+ ions are important in maintaining the acidic pH at the tip of the growing pollen tube required for proper binding of Actin Depolymerizing Factor (ADF)/cofilin (Actin binding proteins) to bind F-actin to maintain continuous growth (Bamburg 1999; Feijó *et al*. 1999). K^+ and Cl^- ion has a role in maintaining the turgor pressure (Messerli et al. 1999; Zonia et al. 2002a). Mg^{2+} is added to the media because Mg^{2+} acts as a co- factor for ATPases (George *et al*. 2008; Palmgren 2001) and helps in maintenance of ionic concentration in pollen cytoplasm. NO_3^- acts as a nitrogen source in the media and SO_4^{2-} is used by the plants in lipid synthesis and in regulating the structure of proteins through the formation of S-S bridges (George *et al*. 2008).

The media used for *in vitro* assays could be made solid, semisolid and liquid depending upon the type of studies to be conducted. In addition to media recipe and type the temperature and pH also play an important role determining the pollen germination percentage and pollen tube growth (Boavida and McCormick 2007; Holdaway-Clarke and Hepler 2003). The most suitable incubation temperature to observe pollen tube growth *in vitro* was found to be 25°C for grapevine (Sharafi and Bahmani 2011) and other plant types like Arabidopsis (*Arabidopsis thaliana*), Cherimoya (*Annona cherimola* Mill.), Feijoa (*Acca sellowiana* (berg) Burret) and Cotton (*Gossypium hirsutum* L.) (Franzon *et al*. 2005; Liu *et al*. 2006; Pickert 1988; Rosell *et al*. 1999). Also slightly an acidic pH in the range of 5-6 was found to be the most favourable for better PG and PTG (Holdaway-Clarke and Hepler 2003) which is very important to maintain an acidic pH at the tip of the pollen tube for its continuous growth (Holdaway-Clarke and Hepler 2003).

In the present study we optimized the suitable way to grow the grapevine pollen tubes to ensure maximum PG percentage and appropriate PTG along with the ideal way of administering exogenous GABA treatment to pollen tubes.

Materials and method

Plant material and growth conditions

Vines were prepared from cuttings and grown following the method of Baby *et al*. (2014). Briefly, Shiraz (clone BVRC17) cuttings were taken from the University of Adelaide Coombe vineyard, Waite Campus, Urrbrae, South Australia (34°9’ S, 138°6’ E). Cuttings with 5-6 nodes were cut at the basal end and treated with 1.5g/L of rooting hormone Indole Butyric Acid (IBA) (Clonex, Growth technology, Perth, Australia) and planted for pre-rooting, in washed coarse river sand in a heat-bed maintained at 25˚C, in a dark cold room maintained at 4˚C for 5 weeks. After 5 weeks the cuttings were planted in pots of 2 L capacity filled with perlite: vermiculite (1:1) (Smoult Mobile Horticulture Suppliers, Australia) and transferred to CER (controlled environment room) (Fig. 1, A). Growth conditions were 27˚C during the day and 22˚C at night with a 16 h photoperiod. Humidity in the growth room was maintained at 35% using a dehumidifier (Secco Ultra, Applied Climate Control Pty Ltd, Australia) and light intensity was 400 µmol photons/m^2/s. Evaporation from the pots was minimised by covering the pots with aluminium foil wrapped in cling wrap. When the vines reached the flowering stage (50% caps fall) (Fig. 1, B and C) after 4-5 weeks at EL stage 23 (Coombe 1995) the flowers were harvested using different methods and stored for later use for doing *in vitro* assay.

Storage of pollen grains

As flowers last for just one week, pollen grains were stored for later use. To discover the most suitable method of pollen storage that assures more germination capacity different methods were tried. Two temperatures were selected for storage depending upon the literature -20°C (Connor and Towill 1993) and Liquid Nitrogen (LN) (Crisp and Grout 1984; Parfitt and Almehdi 1983). For -20°C storage pollen were stored in two ways one directly after harvesting and the second by drying the pollen grains in a desiccator containing silica gel granules for 1 day (24 hrs) and stored at -20°C. For storage at -80°C the whole bunch of flowers were first snap frozen in LN in big falcon tubes (50 mL) and then stored at -80°C. For the pollen germination assay the pollen stored at -20°C were directly used while for -80°C they were first thawed in a water bath at 37°C for 1 minute and used for the assay within one hour. Twenty-five flowers (5 flowers each from 5 different inflorescences from different plants) were taken for each treatment.

In vitro pollen germination assay

For germinating pollen grains two types of media were used one solid and one liquid. The modified Brewbaker and Kwack (1963) pollen germination media (PGM) was used. The PGM contained 15% Sucrose, 1 mM Boric acid, 1 mM $CaNO_3$, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 5 mM MES with pH adjusted to 5.5 with 1M TRIS. It was made solid by adding 1% agar (Agarose, BIOLINE Cat. No. BIO- 41025) besides above salts. For GABA treatments liquid media was used and agar is skipped. Liquid media is not autoclaved after adding GABA instead it is made fresh every time using the stock solutions.

For solid media petri plates were used (30 mm) and the hot (40-50°C) media is poured using glass pipet. About 2 ml of media is poured in each petri plate to make the fine even thin layer of media to ensure proper PTG through it. Pollen were sprinkled on the settled agar plates by using a fine brush (not demonstrated in figures). While for liquid media double cavity slides are used instead of petri plates. For this the flower is held upside down and dipped in the media to avoid shedding it to the slide area without media ensure that all the pollen grains are within the drop so that at the end the non- germinated (NG) pollen which actually got deprived of media do not interfere with results. The small petri plates for solid media and double cavity slides for liquid media were kept in round petri dish containing moistened filter paper to maintain a humid environment (Fig. 2).

Incubation at different temperatures

The petri plates were kept at two different temperatures at 20°C (on the lab bench) and 25°C (incubator) for 6 hours.

Measurement of pollen tubes

After 6 hours the germinated pollen grains were compared in terms of PG and PTG. A photomicrograph was taken using a TOUPCAM camera attached to a microscope (Zeiss, Axiophot, West Germany) at different magnifications 2.5X and 10X. Later pollen tubes were measured using Image J software (http://rsb.info.nih.gov/ij/). Data was analysed using a software Graph Pad Prism (6.0).

GABA treatment

GABA was added directly to the solid media by pouring about 2 ml of GABA solution over the agar surface to make a thin layer of GABA solution covering the agar layer while added directly to the liquid media along with other salts as mentioned above in the media recipe and the pH is adjusted to 5.5 with 1M TRIS. For GABA treatment the $CaNO_3$ was dropped and $CaCl_2$ was increased to 2 mM (Palanivelu *et al*. 2003).

Results

Storage of pollen grains

Stored pollen grains were germinated and compared with fresh pollen grains germinated to see the difference after storage. The pollen grains stored directly at -20°C showed freezing injury and were unable to germinate (Fig. 4, A) as compared to other two methods (storage at -20°C after drying in the desiccator and snap freezing in LN and storage at -80°C). There was a significant difference observed for PG and PTL between fresh pollen and the ones stored at -20°C but comparable with pollen grains stored at -80°C (Fig. 4, B). The PG in fresh pollen was observed to be 74.4% as compared to 41% in pollen grains stored at -20°C showing 33.4% reduction but was similar to pollen grains stored at -80°C. While the average PTL in fresh pollen and pollen grains stored at -80°C was found to be 81% longer as compared to PTL of pollen grains stored at -20°C after drying (Fig. 4, C). PG and PTL was found to be exactly same in fresh pollen and pollen stored at -80°C.

Solid vs liquid media for in vitro pollen germination assay

The pollen grains germinated equally well on both solid and liquid media with similar percentage (Fig. 3, A) but the pollen tubes in the liquid media grew 87.34% more as compared to pollen tubes grown on solid media (Fig. 3, B)

Incubation at different temperatures

Temperature plays an important role for incubation. The pollen grains stored at -80˚C were incubated at two different temperatures 20°C and 25°C to find the suitable one. The PG was found to be similar with no significant difference but the PTL was found to be 62.48% more for incubation at 25°C as compared to 20°C (Fig. 4, B and C).

GABA treatment

GABA was reported to play an important role in pollen tube guidance along the stylar region (Palanivelu et al. 2003). Out of two ways of administering GABA liquid media was selected as in solid media the GABA changes the pH and the agar is dislodged. When observed under the microscope the PTL was less after 10 mM GABA but that was due to change in a pH rather than a change due to GABA effect. While for liquid media the pH was adjusted after adding GABA and considered the ideal way as compared to the solid media. While for liquid media the pH was adjusted after adding GABA to avoid this situation (Table. 1).

Table.1 Addition of GABA changes pH with increasing trend with more GABA.

GABA concentration	Initial amount of MES	Initial pH after adding GABA	Amount of MES/TRIS added to adjust pH5.5	Final pH
Control	5 mM	3.8	0.75 mM TRIS	5.5
1 mM	5 mM	4.8	0.75 mM TRIS	5.5
2 mM	5 mM	5.0	0.75 mM TRIS	5.5
5 mM	5 mM	5.1	0.60 mM TRIS	5.5
10 mM	5 mM	5.2	0.35 mM TRIS	5.5
20 mM	5 mM	5.4	0.05 mM TRIS	5.5
50 mM	5 mM	5.6	extra 2.5 mM MES	5.5
100 mM	5 mM	5.8	extra 10 mM MES	5.5

A

B

C

Figure. 1. (A), Maintenance of Grapevine cuttings in the CER. **(B)**, Grapevine plant at flowering stage and **(C)**, 50% caps fall stage for flowers storage at -20°C and LN.

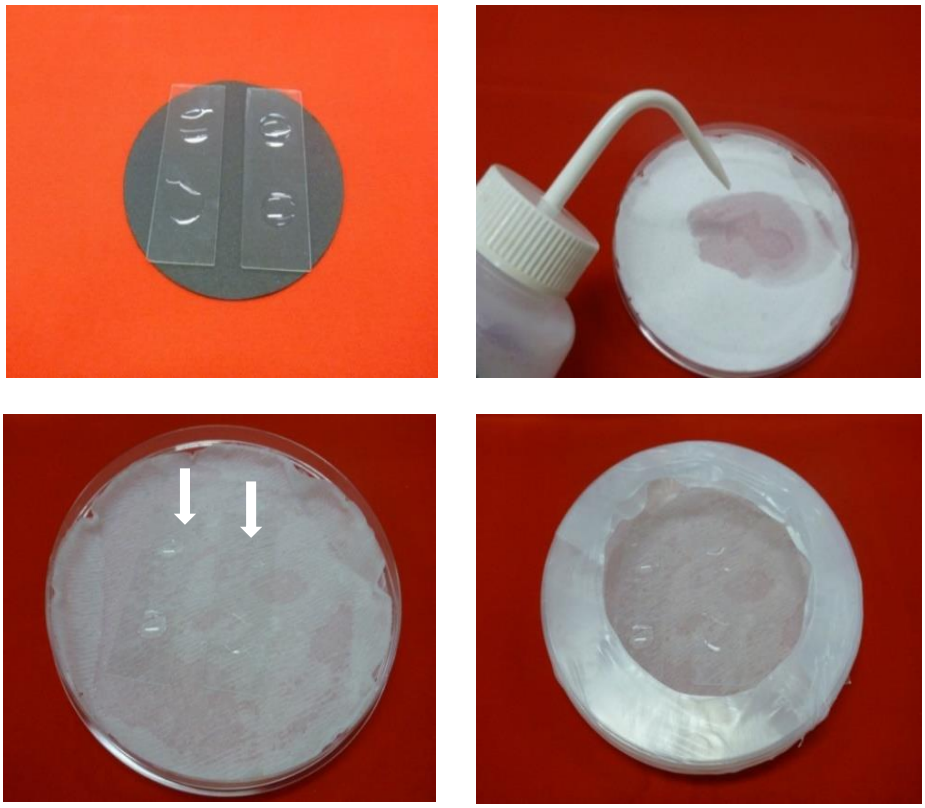

Figure. 2. *In vitro* pollen germination assay demonstration in pictures. Anti-clockwise demonstration of preparation of slides (indicated by arrows) for *in vitro* assay and keeping them in bigger petri plates (180 mm) with moistened filter paper covered and secured with paraffin tape after keeping slides inside.

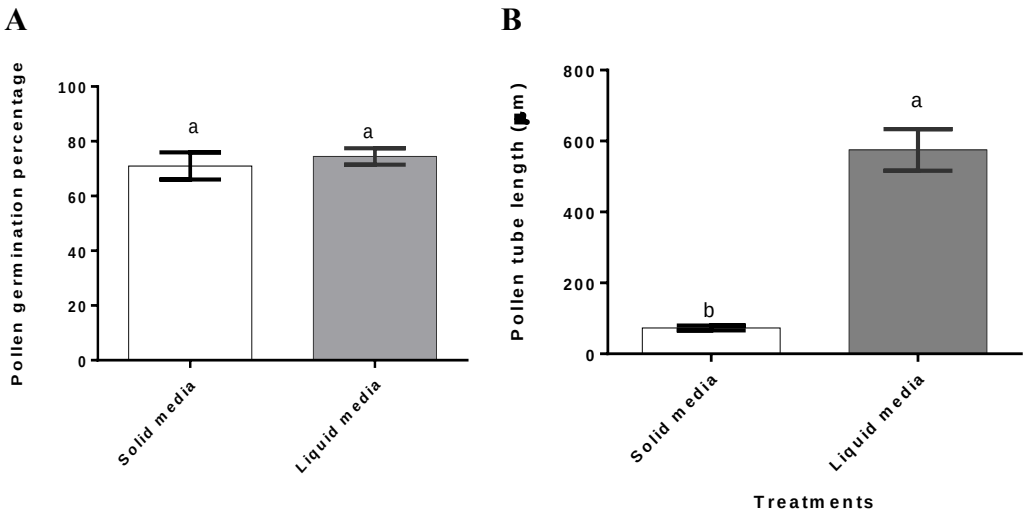

Figure. 3. Comparison of *In vitro* pollen germination assay experiment using solid and liquid media in terms of PG and PTL in an incubator (25°C). N=50 (SEM). **(A)**, There was no significant difference observed for PG between solid and liquid media. **(B)** But a significant difference was observed for PTG between different incubation temperature 20 and 25° C (unpaired t test, P value < 0.0001).

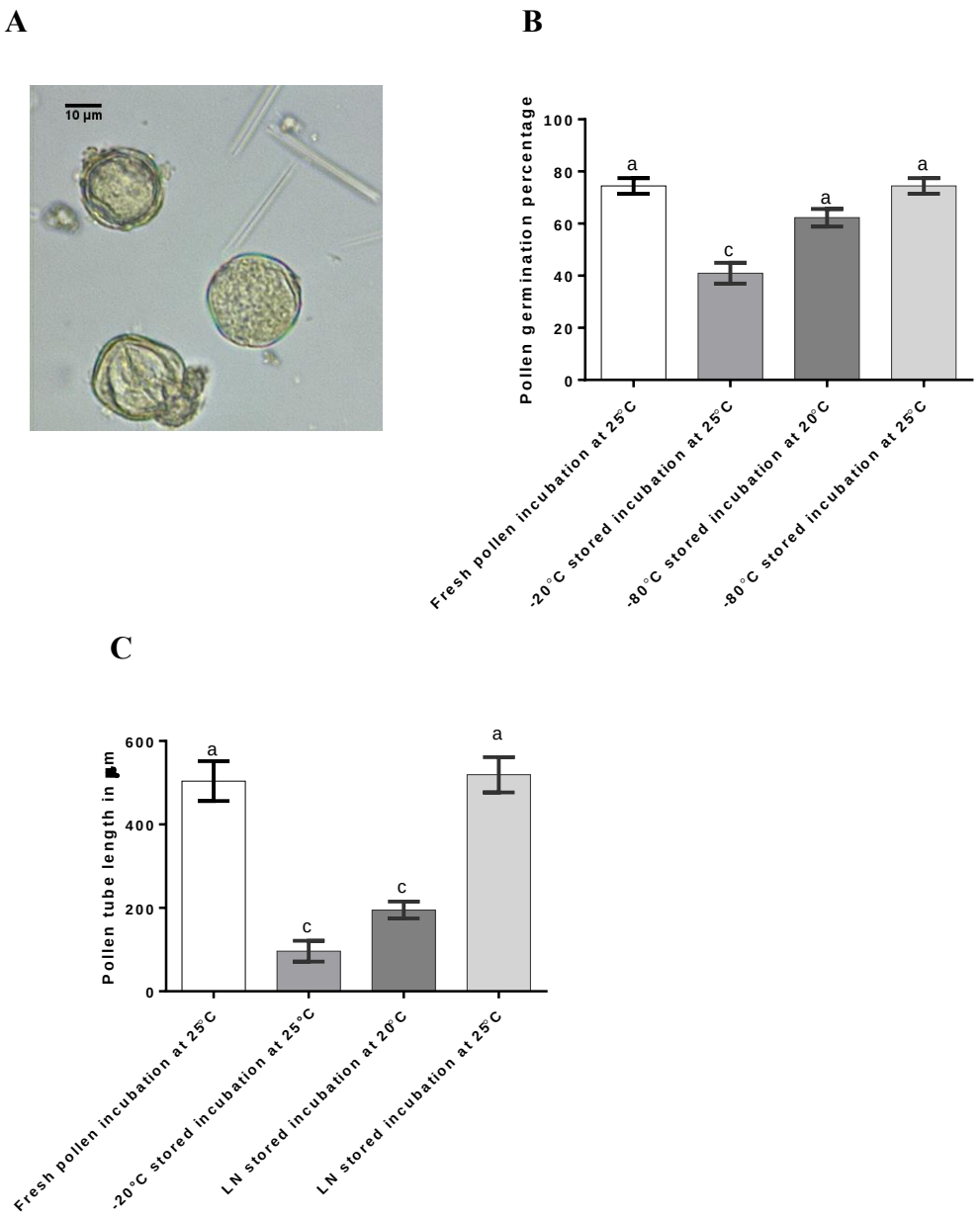

Figure. 4. **(A),** Ruptured pollen grains leading to leakage when rehydrated due to freezing injury (63X). Comparison of PG **(A)** and PTL **(B)** between fresh pollen grains and pollen grains stored at different temperatures (-20°C and -80°C) and incubation at different temperatures (20°C and 25°C). N=50 (SEM). **(B)**, A significant difference was observed between fresh and -20°C stored pollen grains for PG (One way ANOVA, P value < 0.0001). **(C),** Also a significant difference was observed for PTL between fresh and -20°C and -80°C stored and incubated at 25°C (One way ANOVA, P value < 0.0001. Magnification 63X, scale bar 10 µm.

Discussion

In vitro pollen germination assay is an important tool used to understand the physiology and biochemistry behind its growth since it was first discovered (Brewbaker and Kwack 1963a). But for that we need to ensure that the material (pollen grains) we are using are either fresh or has same viability as fresh pollen grains. For this we compared different ways of storage. Pollen grains were stored at two different temperatures at -20°C and -80°C. For -20°C they were stored in two ways one directly without drying and the second by drying them in the desiccator with $CuSO_4$ flakes followed by storage at -20°C. Pollen grains storage directly at -20°C without drying resulted in imbibition damage (Fig.4, A) as observed earlier (Crowe *et al*. 1989). When the pollen grains are dried first and then stored at -20°C they remained in a preserved form and germinated normally when rehydrated. While the directly stored pollen grains get ruptured due to initial moisture content and resulted in imbibition damage (Fig. 4, A). Previous reports indicate that drying the pollen grains before storage helps in long term storage (Yates *et al*. 1991). Also dehydration of pollen grains at maturation and dispersal is a natural phenomenon which gets rehydrated when it reaches stigmatic surface (Heslop-Harrison 1979). Instead if they are stored without drying it results in rupturing of pollen walls leading to the leakage of vital contents of the pollen grains to the media resulting in the failure of germination. Comparatively pollen grains dried and stored at -20°C germinated normally but still showed significantly less PG and PTG as compared to pollen grains snap frozen and stored at -80°C (Fig. 4 B and C). Out of the three ways of storage snap freezing and then storage at -80°C gave maximum PG and PTG which was found to be similar to the fresh pollen grains (Fig. 4, A, B and C). There are previous reports where snap freezing (-196°C) and storage at -80°C was found to be the best one for long term storage for grapevine pollen and similar to fresh pollen grains (Olmo 1942; Parfitt and Almehdi 1983). Before using these pollen grains in the *in vitro* assay they were thawed at 37°C to bring the pollen grain walls back to liquid crystalline layer to avoid imbibition damage (Crowe *et al*. 1989). Liquid media grew better as compared to solid media (Fig. 3, A-D) hence it was chosen for final experiments. In the present study two different temperatures 20°C and 25°C were tested for incubation and 25°C is selected over 20°C for better PTG (Fig. 4, B and C). There are previous reports where this temperature (25°C) is used for incubation in *in vitro* assays for better PG and PTG in a variety of species like Arabidopsis (*Arabidopsis thaliana*), Cherimoya (*Annona cherimola* Mill.), Feijoa (*Acca sellowiana* (berg) Burret) and Cotton (*Gossypium hirsutum* L.) (Franzon *et al*. 2005; Liu *et al*. 2006; Pickert 1988; Rosell *et al*. 1999). In grapevine too this temperature was used for incubation (Sharafi and Bahmani 2011). In the present study GABA was administered in two ways either by pouring it on the solidified agar

layer or by dissolving it directly in the media by adjusting the pH later. It was found that if we add GABA after the agar plates are set it liquefies the set agar by changing its pH. To avoid this situation liquid media was chosen and GABA was added before adjusting the pH (Table. 1). The main purpose of using liquid media was to ensure that the GABA is available to the pollen grains/tubes at the buffered pH 5.5 so that the pollen performance is just due to GABA and not due to pH change as it happened in solid media.

Conclusion

Present studies demonstrated an ideal way to store, grow and administer exogenous GABA to ensure maximum PG and PTG ensuring the effect seen in terms of PG and PTG after GABA application is not due to pH change but just due to exogenous GABA.

Chapter 4. Do GABA-gated ion channels have a role in regulating fruit set?

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Do GABA-gated ion channels have a role in regulating fruit set?

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Abstract

Pollen tubes are integral to fertilization during sexual reproduction; any kind of impairment in their growth leads to reduced, or to no fruit set. Salinity reduces grapevine fruit set. In other plant species salinity reduces pollen germination (PG) and pollen tube growth (PTG) but this has not studied for detail in grapevine. Another factor affecting the growth of pollen tubes is gamma-aminobutyric acid (GABA) concentration; GABA concentration can increase in plant tissue under salinity stress. In the present study, the role of GABA in determining fruit set in grapevine under salinity stress was examined. Shiraz vines (Clone BVRC17) were grown under controlled conditions with a treatment of 35 mM NaCl applied to half the vines. Salt treated vines had lower fruit set, as did field grown vines treated with 10 or 20 mM GABA. Pollen from salt treated plants germinated less and had a slower growth rate than from control plants. Endogenous GABA concentrations were found to be about 2 fold more in salt treated flowers. GABA was found to stimulate PTG and PG *in vitro* at low concentrations (1–5 mM), whereas it was inhibitory at higher concentration (100 mM), consistent with the observations in other species. The effect of pharmacological agents such as muscimol (10 µM), as an analog of GABA, and bicuculline (100 µM) as an allosteric inhibitor for GABA binding supported the hypothesis of GABA regulating PTG through modulating the activity of pollen localised Aluminium-activated malate transporter (ALMT) proteins. Expression of *VvALMT 9- like* and *VvALMT 10* in pollen tubes gives further evidence of a putative role of a GABA-ALMT interaction in controlling PTG.

Key words**:** Grapevines, **γ-** aminobutyric acid (GABA), Aluminum activated Malate Transporters (ALMTs), Pollen tube growth, Pollen germination, Muscimol, Bicuculline.

Introduction

In angiosperms pollen tubes play an important role in sexual reproduction by transporting male gametes, otherwise known as sperm cells, to the female embryo sac tissue making fertilization possible. Pollen tube growth in grapevine flowers is an important determinant for successful fertilization and fruit set (Bouquet and Danglot 1996; Dantas *et al*. 2005; Ebadi *et al*. 1995a; Kelen and Demirtas 2003; Mullins *et al*. 1992). Poor fruit set can lead to higher levels of coulure and millerandage indices and hence poor yields (Dry *et al*. 2010), which can decrease wine quality (Walker *et al*. 2010). The growth of the pollen tube in the style relies on various processes including cytoskeletal rearrangements, vesicle trafficking, signaling and signal transduction pathways (Steinhorst and Kudla 2013). Ion transport processes are also

crucial for pollen tube growth with Ca^{2+}, H^+, K^+ and Cl^- fluxes across the pollen tube plasma membrane all being essential, with roles in maintaining a tip focused Ca^{2+} gradient, an acidic pH within the cell wall and turgor pressure (Holdaway-Clarke and Hepler 2003; Song *et al*. 2009). These ions are transported through membrane proteins which can be influenced by signals from the pistil and hence contribute to pollen pistil interactions. Pollen-pistil interactions are influenced by many endogenous chemical factors such as serine, polyamines, and GABA (Gentile *et al*. 2012; Michard *et al*. 2011; Palanivelu *et al*. 2003). Variation in any of these factors can lead to altered pollen tube growth and in many cases a failure in fertilization.

γ- aminobutyric acid (GABA) gradients guide pollen tubes to the micropylar end of the embryo sac leading them to the ovary (Palanivelu *et al*. 2003). The *pop 2* (*Pollen–pistil 2)* gene, also known as *GABA-transaminase* (*GABA-T*), is responsible for degradation of GABA and hence influencing the GABA concentrations in wild type flowers. In mutant flowers, lacking *GABA-T* the GABA concentration is high and the gradient is abolished which restricts the pollen tube growth in the style via an undetermined mechanism. Interestingly, stress events, including salinity, increase GABA concentrations in plant tissues (Kinnersley and Turano 2000). Salinity is a major environmental factor that constrains optimal fruit set in the grapevines (Hawker and Walker 1978). As such GABA is potentially a key regulator of grapevine fruit set under salinity and other stresses.

As stated earlier ion transport processes are known to be essential in pollen tube growth but a few limited number of transporters have been identified, and mostly for Ca^{2+}, H^+ and K^+ (Brewbaker and Kwack 1963; Ge *et al*. 2009; Holdaway‐Clarke and Hepler 2003; Malhó *et al*. 2000; Schiøtt *et al*. 2004; Song *et al*. 2009; Steer 1989; Steinhorst and Kudla 2013). Information is more limited regarding anion transport. The slowly activating anion channel homolog 3 (*SLAH3*) and quickly activating anion channels QUAC (*ALMT 12*) are transcribed in *Arabidopsis thaliana* pollen tubes (Gutermuth *et al*. 2013; Pina *et al*. 2005) in other tissues these are associated with NO_3^- and Cl^- transport (Meyer *et al*. 2010)). Recently, it has been suggested that GABA regulates anion transport activity through ALMT proteins and this in turn can regulate plant tissue growth (Ramesh *et al*., 2015). This may provide a mechanism by which GABA acts to inhibit pollen tube growth (Palanivelu *et al*. 2003). Recently the same research group co- related the effect of exogenous GABA on Ca^{2+} permeable channels resulting in more Ca^{2+} influx which leads to activation of Ca^{2+}/CAM that binds to GAD leading to GABA synthesis. But higher concentration of exogenous GABA leads to down stream regulation of Ca^{2+}/CAM and feedback control of Ca^{2+} permeable channels by GAD

limiting the PTG (Yu *et al*. 2014). Previous reports have shown R-type anion channels activated by intracellular Ca^{2+} (Colcombet *et al*. 2001; Hedrich *et al*. 1990; Zimmermann *et al*. 1998). It is likely that ALMTs are activated by intracellular Ca^{2+} at lower GABA concentrations but when exogenous GABA concentrations are higher it binds to ALMT channel proteins making them close and possibly affecting the efflux of Cl^- crucial for PTG. The present study investigated whether GABA influenced pollen tube growth and fruitset in grapevine and, whether GABA-regulation of ALMT activity constituted a plausible explanation for regulating pollen tube growth and fruitset in grapevine, especially under saline conditions.

Materials and Methods

Plant materials and growth conditions (study in the growth room)

Vines were prepared from cuttings and grown following the method of Baby *et al*. (2014) with the following modifications. Control plants were watered with modified half strength Hoagland's solution and 35 mM NaCl was added to each of the salt treated vines. Only one axillary inflorescence and five leaves were kept for each plant as shown in Chapter 3 Fig. 1, B. After 4-5 weeks, when plants had reached the flowering stage (50% caps fall; EL stage 23, (Coombe 1995) flowers (the whole inflorescence) were harvested into a 50 mL Falcon tube, snap frozen in Liquid Nitrogen (LN) and stored at -80˚C. These flowers were then used for various experiments such as *in vitro* pollen germination assays, the GABase assay and gene expression studies.

Effect of Bicuculline 100 µM on fruit set

In order to examine the effect of Bicuculline (100 µM) on fruit set it was applied exogenously on to inflorescences just before anthesis (EL stage 18). The pH was adjusted to 6.0 with 5 mM MES/TRIS for Bicuculline sprays and spray control (MQ water). Each inflorescence was sprayed daily for three consecutive days and the material was collected on the third day after the last application.

Effect of GABA on fruit set (Field trial)

Shiraz vines (Clone BVRC12, on rootstock Ruggeri 140) in the University of Adelaide 'Coombe' vineyard, Waite Campus, Urrbrae, South Australia (34°9' S, 138°6' E) were marked for the GABA study. Vines were planted in 1992 at a vine and row spacing of 2.7m X 3.0m. Vines are trained to a single wire, bilateral cordon and vertical shoot positioning and spur pruned to between 22-26 nodes. Based on the results from the *in vitro* pollen germination assay experiment three GABA concentrations were selected for exogenous application to

assess their effect on fruit set (10, 20 and 50 mM). The pH was adjusted to 6.0 with 5mM MES/ TRIS for spray and spray control at EL stage 18 (Coombe, 1995). Spraying was done on alternate days one week before the onset of flowering three times.

Reproductive performance studies

Reproductive performance was measured in all three of the above mentioned experiments by enclosing inflorescences in a mesh bag, these were secured with cable ties just before flowering stage (EL 17, Coombe, 1995) and removed after the completion of flowering stage (EL 26, Coombe, 1995). Reproductive measures - bunch weight, berry weight, berry number (seeded, seedless and LGO's), Millerandage Index (MI) and Coulure Index (CI) were determined (Dry *et al*. 2010) (Table 1,5 and 6).

In vitro pollen germination assay

Twenty-five flowers (5 flowers each from 5 different inflorescences from different plants) for each treatment were taken and thawed at 37˚C for one minute in a water bath and used within one hour. Pollen tubes were germinated in liquid media that was optimised in pilot experiments (see chapter 3). The PGM contained 15% Sucrose, 1 mM boric acid, 1 mM $MgSO_4$, 1mM $CaCl_2$, 1 mM $CaNO_3$ and 5 mM MES for basic media and 1 mM boric acid, 1 mM $MgSO_4$, 2 mM $CaCl_2$ and 5 mM MES for GABA treatments (pH adjusted to 5.5 with 1 M TRIS) (Palanivelu *et al.*, 2003). The pollen grains were germinated in cavity slides (Sky Double cavity slides, 1.2-1.5 mm Interleaved, Livingstone, International Pty Ltd.) kept within a 80 mm round petri dish containing moistened filter paper to maintain a humid environment, these were kept in an incubator maintained at 25°C for 4 and 6 hrs. After 4 and 6 hrs a photomicrograph was taken using a TOUPCAM camera attached to a microscope (Zeiss, Axiophot, West Germany) at different magnifications 2.5X and 10X. Later pollen tubes were measured using Image J software (http://rsb.info.nih.gov/ij/). A minimum of 50 pollen tubes were measured per treatment and total numbers of pollen were allocated into different categories depending upon the lengths of pollen tubes ranging between non-germinated to pollen tubes in excess of 2000 µm in length.

GABase assay

Endogenous levels of GABA were estimated according to (Zhang and Bown 1997). For this 100 mg of ground fresh plant material was taken and added to methanol to deactivate the GAD enzyme. A 90.3 µL sample was added to the assay mix containing 0.5 M potassium pyrophosphate (pH 8.6), 5 mM 2-β, Mercaptoethanol, 4 mM β-$NADP^+$, 20 mM α-ketoglutarate and 2 units of *Pseudomonas fluorescens* GABase in a final volume of 200 µl.

Absorbance was measured at OD_{340nm} in a BMG Labtech FLUOstar Omega plate reading spectrophotometer. A standard curve of known GABA concentrations was constructed and used to interpolate sample GABA concentrations in µmoles/g FW (Fig. 1 in supplementary figures).

RNA extraction semi quantitative PCR (semi q PCR)

Frozen flowers, obtained from inflorescences stored as described for the pollen germination assay were ground to a fine powder using a pestle and mortar that was already cooled with LN. Total RNA was extracted from ground frozen flowers (-80°C) using the Spectrum™ total RNA kit (Sigma, Cat. # STRN 50-1KT) and cDNA was synthesized from 2 µg of total RNA by using Superscript III (Invitrogen, Cat. # 18080-044, 10000 U) and primers were designed using (BLAST and Primer3plus) and listed in (Table 3 and 4). For semi q PCR, *Taq* DNA polymerase (Cat # M0267S) was used for *ALMT* gene expression studies (*ALMT1, 2, 3, 4, 6, 7, 8, 8_like, 9_like, 9-like_2, 10, 11, 12* and *13*) and Phusion® High fidelity DNA polymerase (BioLabs, Cat # M0530S) was used for the GABA shunt enzymes Glutamate decarboxylase (EC 4.1.1.15) (*GAD1, GAD2 and GAD3*) and GABA Transaminase (EC 2.6.1.19) (*GABA-T*). Amplification of cDNA was carried out using denaturation at 95˚C for 30 seconds followed by 35 cycles at 95˚C for 10 seconds, 55˚C for 10 seconds, 68˚C for 10 seconds and 68˚C for 10 minutes for *ALMT* and denaturation 98˚C for 30 seconds followed by 35 cycles at 98˚C for 15 seconds, 49-64˚C (for different primers) for 30 seconds, 72˚C for 30 seconds and 72˚C for 10 minutes for the GABA shunt enzymes. *VvActin 1* and *VvUbiquitine-L40* was used as a house keeping gene for normalising the expression. Gel electrophoresis was performed on a 2% agarose gel run at 100 V for 45 minutes. The expression of the genes was visualized against the 100 bp DNA ladder (BioLabs, Cat. # N3231S).

Real time qRT-PCR

The semi quantitative PCR product for each gene was purified using a PCR purifying Combo kit (Life Technologies, K220001) and sequenced by AGRF (http://www.agrf.org.au/). The sequenced PCR product was used as a template to make standards with a serial dilutions ($1/8^{th}$) up to 6 points in triplicates. qRT- PCR reaction mixture consists of 10 µM forward and reverse primers, 2X KAPA® SYBR FAST qPCR master mix (KAPA, ABI Prism, Biosystems Product code- KR0390) and 1 µl of cDNA. qRT- PCR was carried out using QuantStudio 12K Flex RT-PCR system (Applied Biosystems, Cat # 4471087) and QuantStudio™ 12K Flex software v 1.22 and specific primer pairs (Table, 4) using manufacturer's instructions.. Reactions were performed in triplicates following the 3 step PCR conditions for each gene consisting of 40 cycles with following: 95°C for 1 seconds, 49-64°C (for different primers)

for 20 seconds, 68°C for 5 seconds. Relative gene expression ratios were calculated using primer pair efficiency by Pfaffl method (Pfaffl 2001). *VvActin 1* was used as a reference gene for normalisation.

RNA extraction from pollen tubes

For extraction of RNA from pollen tubes the pollen grains were germinated on the liquid media described above for about 6 hrs. Germinated pollen grains were separated from non-germinated ones by filtering it through 50 µm mesh falcon strainers (Easy strainer ™ 40 µm Cat.-no. 542 040) and RNeasy Plant mini kit (Qiagen, Cat no. 74903) was used to extract the total RNA as per manufacturer's instructions. cDNA was synthesized from 150 ng of total RNA using Superscript III (Invitrogen, Cat. no.18080-044, 10000 U). The presence of *ALMT* in pollen tubes was detected by running all the primers (Table. 3) both on pollen and pollen tube cDNAs. For PCR *Taq* DNA polymerase (BioLabs, Cat # M0267S) was used for gene expression studies on *ALMT* (1, 2, 3, 4, 6, 7, 8, 8_like, 9_like, 9-like_2, 10, 11, 12 and 13). Semi-quantitative PCR was also performed to visualise expression of *ALMT* in pollen grains and pollen tubes. Amplification of cDNA was carried out at denaturation 95˚C for 30 seconds followed by 35 cycles at 95˚C for 10 seconds, 55˚C for 10 seconds, 68˚C for 10 seconds and 68˚C for 10 minutes for *ALMT*. *Vv Elongation-factor-1-α* (*Vv EF 1-α*) was used as the house keeping gene for normalisation. Gel electrophoresis was done on 1.2% agarose and run at 100 V for 45 minutes. The expression of the genes was visualized against a 100 bp DNA ladder (BioLabs, Cat.no. N3231S).

Results

Effect of salinity on fruit set

Salt treated vines were compared to control vines. Salt treatment showed visual difference on leaf lamina and bunches as compared to control conditions (Fig. 1, A and B). Salt treated vines had lower percent fruit set, bunch weight, berry weight, total number of berries, seeded berries/bunch, LGOs/bunch and higher CI and MI than the control treatments (Table 1). Percent fruit set in salt stressed bunches was reduced by 44% from the control treatment. CI increased by 21% and MI was increased by 59% in salt treated plants. Bunch weight was 46% lower and total berry weight 53% lower in salt treated vines as compared to control ones. There was no significant difference observed for number of seedless berries and LGOs but seeded berries were 46% lower in salt stressed vines (Table. 1).

Effect of salinity on pollen tube growth and pollen germination percentage

The pollen grains were germinated using PGM as described above and 50 pollen tubes were measured at two time points 4 and 6 hrs after germination as a pilot experiment to see the difference in PTL with time (Fig. 2, A). There was a significant difference observed for PTL between control and salt treated (54% lower) conditions at 4 hours while no significant difference was observed after 6 hours. Similarly the growth rate for the whole pollen population was observed to be significantly higher for control pollen as compared to pollen harvested from salt treated flowers. It was observed to be 0.825 µm/minute as compared to 0.17 µm/minute in salt treated condition (Fig. 2, B).

To further investigate all pollen grains (200-300) were counted and sorted into different categories depending upon their viability (germinated or non-germinated) and pollen tube lengths. This provided a clear picture of the whole population variability in terms of their lengths at two time points (4 hrs and 6 hrs) (Fig. 3, A and B). At 4 hours the number of non-germinated (NG) pollen were significantly more in salt treated (64%) as compared to control conditions (30%). All other categories of pollen tubes between 1-100 µm and 100-500 µm were significantly higher in control (45% and 22% respectively) samples compared to salt treated (25% and 9.7% respectively) ones (Fig. 3, A). After 6 hours the PG was found to be similar in control and salt treatment and pollen tubes ranging between 1-100 µm were still higher in control (52%) as compared to salt treated one (32%). But pollen tubes between 100-500 µm were found to be higher in salt treated (22%) as compared to control one (9%) (Fig. 3, B).

Effect of GABA on PTG and PG percentage

GABA had a biphasic response both at 4 and 6 hours showing first an increase and then a gradual decrease in PTL (Fig. 4, A and B). The effect of GABA was more prominent at 4 hours of incubation compared to 6 hours. At the end (6 hours) the lower concentrations (1-5 mM) were observed to stimulate PTG while the higher concentrations were inhibitory, especially 100 mM (Fig. 4, B). However there was not much difference observed in the growth rate of the whole population among different GABA concentrations except 100 mM at 4 hours (Fig. 4, C).

Total number of pollen grains (200-300) were counted and categorized based upon their viability (germinated or non-germinated) and PTL (Table. 2). The amount of NG pollen increased gradually with increasing GABA concentration however the difference was more significant at 100 mM GABA concentration. 35% NG pollen grains were observed in the

control, which was significantly less when compared to 100 mM GABA where 74% of pollen were NG. All other categories were found to be similar in all the treatments (Table. 2).

GABase assay

Endogenous GABA levels were significantly greater (1.8 fold) in salt stressed flowers at 3.8 µ moles/g FW compared to control flowers that was 2.12 µ moles/g FW (Fig. 5).

Pharmacological approach to show GABA sensitivity for ALMT channel

After incubating pollen tubes for 6 hours muscimol (10 µM) was observed to inhibit while bicuculline (100 µM) was found to accelerate the growth of pollen tube when added to the PGM. Muscimol decreased the PTL by 45% while bicuculline increased it by 29%. When applied in combination bicuculline increased the PTL by 36% (Fig. 6).

Effect of GABA on Grapevine fruit set

Percentage fruit set was significantly reduced with an exogenous spray of 10 and 20 mM GABA but improved with the higher concentration of 50 mM GABA (Table. 5). Consistent with this, CI increased significantly at lower GABA concentrations and decreased with higher GABA concentration. However, MI remained the same in all the GABA treated bunches. Bunch weight, berry weight, total number of berries, seeded berries/bunch and LGOs/bunch remained the same among different treatments.

Effect of Bicuculline on fruit set

There was no significant difference observed for any parameter of reproductive performance between spray control and Bicuculline (Table. 6).

RNA extraction, Semi quantitative PCR (semi q PCR) and Real time q RT-PCR

During salt stress conditions there was no significant difference observed for the relative gene expression of *GAD1, GAD2, GAD3* and *GABA-T* (Fig. 9).

ALMTs in grapevine flowers and pollen tubes

Thirteen *ALMT* were identified from the Grapevine (*Vitis vinifera* L.) genome. The expression of all *ALMT* gene family members (Table. 3 and Fig. 7) identified in grapevine was tested, five *ALMT* were expressed in flowers including *ALMT 7, 9-like, 9-like_2, 10* and *13* (Fig. 8, A). Total RNA was also extracted from pollen grains and pollen tubes. Out of the 13 grapevine *ALMT* only two *ALMT* gene members were detected in pollen tubes (*Vv ALMT 9-like* and *Vv ALMT 10*) and only one in pollen grains (*Vv ALMT 10*) (Fig. 8, B).

Discussion

Pollen tubes play a vital role in fertilization; any kind of impairment in their growth leads to reduced, or no fertilization, which leads to a reduction in fruit set. Salinity is one of the major abiotic stresses that reduce fruit set in different plants including grapevine (Hawker and Walker 1978; Walker *et al*. 2010; Walker *et al*. 2002). In the present study the pollen harvested from salt treated vines showed a significant difference in pollen tube length compared to control pollen. When the total pollen grains were categorized into different groups depending upon germination capacity and PTL after 4 hours growth the number of NG pollen were significantly greater in salt treated sample as compared to the control treatment also there were fewer pollen tubes from 1-1000 µm in salt treated one (Fig. 3, A). This is in accordance with previous studies that have shown that salt treatment reduces PG and PTG (Dadashi 2008; Dhingra and Varghese 1985; El Sayed *et al*. 1991; Reddy and Goss 1971) suggesting that pollen grains harvested from salt treated vines germinating slowly in the initial hours of growth (Fig. 2 A, B and 3 A, B). In addition to this under *in vivo* conditions it is likely that elevated GABA levels might be acting as an additional constraint for growing pollen tubes inside the style, which cannot be explored in *in vitro* studies.

GABA was recently found to be a crucial factor in guiding pollen tubes to the ovary (Palanivelu *et al*. 2003). GABA has been observed to increase in concentration in plant tissues under saline conditions (Akçay *et al*. 2012; Bai *et al*. 2013; Baki *et al*. 2000; Renault *et al*. 2010; Xing *et al*. 2007) and also regulates *Arabidopsis* PTG (Palanivelu *et al*. 2003). We investigated whether GABA concentration was altered in grapevine flowers during salinity treatment and whether application of GABA to pollen tubes *in vitro* could replicate the PTG inhibition observed in salt treated plants. Firstly, we found that the endogenous GABA concentrations in salt treated flowers were indeed significantly higher compared to control flowers; under the saline conditions (35 mM NaCl) accumulation of GABA was almost 2 fold greater than in control flowers (Fig. 5). Secondly, when GABA was applied to the pollen grains harvested from control plants it had a biphasic effect by stimulating PTL at lower concentrations but inhibiting it at higher concentrations (Fig.4, A and B). When the total population (200-300 pollen grains) was separated into different categories the NG pollen grains were found to be significantly higher in 100 mM GABA treatment (Table. 2). This result is in accordance with the previous research done by Palanivelu *et al*. (2003) where they showed that in *Arabidopsis* GABA at lower concentrations stimulates growth as observed in wild type but when present at a higher concentration as observed in mutant flowers (*pop2*), lacking the *GABA-T* gene, growth is inhibited. They hypothesise that a gradient in GABA

from 20 µM at stigma, 60 µM in the style and 110 µM in the ovary walls guides the pollen tubes to ovules for successful fertilization. In *pop 2* mutant flowers this GABA gradient increasing towards the ovary relative to stigma gets altered due to absence of *GABA-T* leading to misguidance of the pollen tubes. Further to support their hypothesis *in vitro* studies were performed and demonstrated that exogenous GABA concentrations from 1-10 mM were stimulatory for PTL while higher concentrations were inhibitory. Similar studies were reported in a conifer *Picea wilsonii* where the GABA concentrations above 500 mM were found to be inhibitory for PTG and PG (Ling *et al*. 2013) and Tobacco (*Nicotiana tabaccum*) where the PTG was stimulated with lower GABA concentration (1 mM) and inhibited by higher concentrations (10mM-100mM) (Yu *et al*. 2014). This suggests that the involvement of GABA in guiding the pollen tubes is conserved among the taxa (angiosperms and gymnosperms) in the plant kingdom.

As a pharmacological approach muscimol (10 µM) as an analogue and bicuculline (100 µM) as an allosteric inhibitor for GABA receptor were applied to detect the presence of GABA binding sites on pollen tube membranes taking reference from similar studies conducted in animals (Andrews and Johnston 1979; Kerr and Ong 1992; Krogsgaard-Larsen and Christensen 1980; Olsen and Sieghart 2008). Interestingly muscimol (10 µM) inhibited while bicuculline (100 µM) stimulated PTG suggesting the presence of GABA binding sites on pollen tubes (Fig. 6).

After finding that exogenous GABA application had an effect on PG and PTL an experiment was designed to assess three GABA concentrations (10, 20 and 50 mM) on PG and PTG to determine its effect on fruit set. Exogenous GABA sprays in Tomato (*Solanum lycopersicum* L.) and Bitter Gourd (*Momordica charantia*) (Ashrafuzzaman *et al*. 2010; Kinnersley 1998) were found to increase fruit set. In the present study the percent fruit set in GABA sprayed vines was found to decrease with lower concentrations (10 and 20 mM) and increase when a higher concentration (50 mM) of GABA was applied (Table. 5). The decrease in percent fruit set with 10 and 20 mM GABA concentrations was consistent with the results from *in vitro* pollen germination assays where the lower concentrations of GABA (1-5 mM) stimulated while the higher concentration (100 mM) inhibited the PTG (Fig. 4, B). A decrease in fruit set was observed to be due to excessive shedding of flowers leading to a higher CI in treatments with lower fruit set. However with 50 mM GABA there was no reduction in fruit set observed. This may be due to the anomalously low flower number at the start of the treatment which may have led to a bogus result. This experiment would have to be repeated to be sure whether high GABA concentrations increase or decrease fruit set.

Bicuculline spray was also applied exogenously to see its effect on fruit set but there was no significant difference observed in terms of reproductive performance (Table. 6). It was reported earlier that bicuculline is unstable at 24°C if the stock solution is adjusted between pH 5.5 to 7 while stable even at 37°C if the stock solution is adjusted to pH 3 (Olsen *et al*. 1975; Pong and Graham 1973). In the present experiment the vines were maintained according to the method described in Baby *et al*. (2014) under 27/22°C as day/night temperature regimes. It is likely that at this temperature the bicuculline spray could not maintain stability until it is absorbed into the tissue which could be the reason for its failure to influence PTG and hence fruit set.

GABA is mainly generated in the GABA shunt pathway by an irreversible decarboxylation reaction of glutamate to GABA through an enzyme GAD (EC 4.1.1.15). It is further degraded by GABA -T (EC 2.6.1.19) to succinic semi aldehyde (SSA) (EC 1.2.1.16). During various types of stresses either greater activity of GAD or underactivity of GABA-T leads to more accumulation of GABA. Other than this major pathway, GABA is also reported to be generated as a bi product from the breakdown of polyamines (PAs); spermidine (Spd) and putrescine (Put) *via* DAO (Diamine Oxidase) and PAO (Polyamine Oxidase) respectively (Bhatnagar *et al*. 2001; Bouchereau *et al*. 1999; Cona *et al*. 2006; Turano *et al*. 1997). In the present study, endogenous GABA levels were nearly 2 fold higher in salt treated flowers compared to control ones (Fig. 5) which may have been generated by either of the pathways responsible for GABA production. In order to determine the enzyme or gene responsible for the GABA accumulation, gene expression studies were done using RT-PCR of GABA shunt enzymes (*GAD1, GAD2, GAD3* and *GABA-T*). But no significant difference was observed between control and salt treated vines for the relative gene expression of these GABA shunt enzymes (Fig. 9). Our reports are in accordance with previous reports in tomato fruits (*Solanum lycopersicum* L.) where they demonstrated that during salt stress GABA levels were high and it was attributed to GAD protein levels rather than transcript abundance (Yin *et al*. 2010). In the present study we have analysed the transcript level but did not see the protein abundance. Moreover GAD is an enzyme that is modified post translationally to increase GABA production and therefore transcription does not necessarily have an influence over GABA concentrations in the short term (Bown *et al*. 2006; Shelp *et al*. 1999). As stated earlier there is an alternative pathway where GABA is generated as a breakdown product from PAs (spd and put). Further investigation is required to determine the role of GAD protein or any other pathway in GABA generation and accumulation in grapevine during saline conditions (35 mM NaCl).

Recently in Tobacco GABA binding sites have been found on the protoplast membrane of pollen (Yu *et al*. 2006) but the identity of these GABA binding sites is still unknown. Interestingly, ALMTs have recently been shown to bind GABA (Ramesh *et al*., 2015) and members of the ALMT family have been detected in *Arabidopsis* and tobacco pollen tubes (Gutermuth *et al.*, 2013; Pina *et al.*, 2005). Therefore, ALMTs are strong candidates for being GABA binding sites. In the present study out of 13 *ALMT* gene family members found in grapevine five (*Vv ALMT 7, 9-like, 9-like_2, 10 and 13*) were detected in grapevine flowers (Fig. 8, A) with two of them (*Vv ALMT 9-like and 10*) present in pollen tubes and only one in pollen grains (*Vv ALMT 10*) (Fig. 8, B). Recently, ALMT 9 is reported to be a malate activated Cl^- channel located in the tonoplast (De Angeli *et al*. 2013) and found to be GABA sensitive (Ramesh *et al*. 2015) as described in the dosage response curve for inward malate current where wild type Vv ALMT 9 to GABA with an IC50 of 5.38 µM. When the Tyrosine residue at position 237 is mutated to Cysteine the GABA sensitivity is abolished increasing the IC50 to 697 µM (Fig. 10). There are previous reports about Cl^- transport across pollen tube membranes to be essential to maintain the turgor pressure necessary for PTG maintenance (Zonia *et al*. 2002). While ALMT 10 has not been characterized but was found to be expressed in elongating cell/tissues (pollen tubes/ roots) in the present study suggesting its role in elongation procedure. Interestingly *Vv ALMT 9-like* was found to be expressed in both pollen grains and pollen tubes while *Vv ALMT 10* was present in pollen tubes further supporting its role in elongation. This research suggests that Vv ALMT 9-like or 10 could constitute a GABA binding site in pollen and they are candidates for anion transport and the mechanism by which GABA effects on pollen are transduced.

Conclusion

In the present study the role of salinity in affecting the pollen and fruit set is studied in detail. There was difference in the pollen performance between pollen grains harvested from control and salt treated (35 mM NaCl) in the initial hours (up to 4 hours) but later there was no significant difference. Endogenous GABA levels were found to be significantly higher in salt treated flowers. However, no significant difference was observed for transcript levels of GABA shunt enzyme encoding genes (*GAD1, GAD2, GAD3 and GABA-T*) between control and salt treatment. Further study in terms of finding protein abundance is required to understand the role of GAD or GABA-T in GABA generation if any. At the same time any other possible pathway also needs an exploration to understand this mechanism equally. When pollen grains harvested from control plants were germinated on PGM supplemented

with GABA showed the similar effect as reported earlier (Ling *et al*. 2013; Palanivelu *et al*. 2003). Pharmacological studies were performed using muscimol as an analogue and bicuculline an allosteric inhibitor of ALMT to gather evidence to support ALMT-GABA interaction in controlling PTG. It further supported the role of GABA in PTG regulation. Although when sprayed exogenously on flower bunches bicuculline could not show any effect on reproductive performance. In summary the pollen tubes are inhibited by the higher GABA concentrations and GABA levels go up during salt conditions suggesting its possible role in determination of fruit set in grapevine during saline conditions.

In the present study two ALMT gene family members *Vv ALMT 9-like* and *Vv ALMT 10* were shown to be expressed in pollen tubes which are possible candidates of GABA binding sites reported earlier. We studied the effect of GABA on grapevine pollen tubes and expressed ALMTs in pollen grains and tubes finding clues for the involvement of GABA-gated anion channels (ALMTs) in PTG regulation. Although we found ALMTs in pollen tubes but still they are not localised in the pollen grains and tubes. Further study is required to find the exact location for the expression of these gene/s through *in situ* PCR studies. Moreover as there are no reports available for ALMT 10 regarding the localisation and GABA sensitivity further studies for characterising this gene member will help in understanding its role in PTG regulation.

Table 1. Effect of salinity on reproductive performance due to salt treatment (35 mM). N= 10 ±SEM.

Parameters	Treatments		P value	Significance
	Control	Salt		
Flower caps/bunch	265	229	0.2021	ns
Seeded berries/bunch	106	57	0.0004	***
Seedless berries/ bunch	1.6	0.3	0.1059	ns
LGOs	8.4	10	0.4306	ns
Total bunch weight	80	39	<0.0001	****
Total berry weight	75	40	<0.0001	****
Total number of berries	108	58	0.0004	***
% Fruit set	42	25	0.0015	**
Coulure Index	5.405	6.876	0.0108	*
Millerandage Index	1.615	3.961	0.0008	***

Unpaired t test was performed to see the significance among the treatments.

Table 2. Comparison of the complete pollen population between control and GABA concentrations (1-100 mM). Total pollen grains were separated into different groups in terms of germination (percent NG pollen grains) and pollen tube lengths (1-100, 100-500, 500-1000 and 1000-2000 µm)

Type of pollen tube (in %)	Treatments								P value	Signifi -cance
		GABA concentrations								
	Control	1mM	2 mM	5 mM	10 mM	20 mM	50 mM	100 mM		
NG	35a	39a	27a	39a	26a	39a	49ab	74b	0.0096	**
1-100 µm	36a	38a	40a	44a	48a	36a	34a	14a	0.5559	ns
100- 500 µm	16a	17a	25a	14a	21a	21a	13a	8.6a	0.4157	ns
500- 1000 µm	4.5a	0b	6.9a	2.1a	3.6a	3.5a	3.8a	2.5a	0.011	*
1000- 2000 µm	0.4a	0a	0.18a	0.18a	0.18a	0a	0a	0.2a	0.4418	ns

One way ANOVA and Unpaired t test was performed to see the significance among the treatments. Different letters indicate significant difference among different treatments.

Table 3. List of all the grapevine (*Vitis vinifera* L.) ALMT gene members and primers tested in semi quantitative PCR studies.

List of Grapevine (*Vitis vinifera* L.) ALMT gene members	Forward and Reverse Primer
Vv ALMT 1	AGG TTT GAA TAG GGG GTTGG GAC GGT TGA GAG CCT TTG TT
Vv ALMT 2	ATC TGG TGG GAT AGG TGA GC CAT ACA GGG CAA ACG ACA AT
Vv ALMT 3	GCG ATA CGA TTA TGG GTT GC TGA GGA TGG TTG ATA GCC TTT T
Vv ALMT 4	GCT ACC GAG ATG AAG ACG CA CAC CAA CCC ATA CCG GAC AA
Vv ALMT 6	CAG GAT TTG CTC ATA GAA GTG C CCG AGG TCA AGG ACT GTT TC
Vv ALMT 7	GGC TGT TTG GGG GAA TAC TT TCA GGG TTG ATG AGG GTT GT
Vv ALMT 8	CCA AGA CGA CCC AAG AAA AA CGA GAC CCC AAA ACT GTC AT
Vv ALMT 8 like	TGA TAC TGG GTT TCT TCG TCT TC CCC TCA TCT CGG TAA GCA CA
Vv ALMT 9	TCT CCT ATG ACC TCT CAG ACC A TGG TAA GAC TCT GCC GTC ACT
Vv ALMT 9 like	CTT GGG TGC TGG GGT ATG TT CCC CGC ATA AAG TGG ATC GT
Vv ALMT 9 like 2	GCT ACT CTG TTG GGG TTT GC TCC AGG GCT TAG TTT TTC CA
Vv ALMT 10	GAA ATG CTA TGT GGG GAG TCA TCA CGA AGG GCT CAA GTT TT
Vv ALMT 11	TAA CAG AAC AGC GGC AAC TTT ACT GAT GGA ACA AAC CGA GAA
Vv ALMT 12	CAG GGC AGC AGT ATG TGA AA CTG AAT GCG GGG TCT GAA T
Vv ALMT 13	GGC TTG CTG TCC TAC TTG TG GCT CTG TTG AAT CCC CTG TT

Table 4. List of forward and reverse primers for GABA shunt genes used for semi q and RT- PCR studies.

GABA Shunt genes	Forward Primer	Reverse Primer
GAD 1	GGG ATA CCG AAA TGT GAT GG	GAA GCA TGT CTG CAA CCT CA
GAD 2	CGT GAG GAG ATT GCC AAT GTA	AGA AGA CGC ATT TGG AGG TG
GAD3	CAA GGT CCA ACA GAG CAA CA	CCA CAA ACT CAA CCG CTC TT
GABA-T	CAG AGT GCA AGA AGC ATG GA	TGA GTT CCT TCA CCC TCT CTT C

Table 5. Effect of different concentrations of GABA (10, 20 and 50 mM) on reproductive performance. N= 10 ±SEM.

Parameters	Treatments			P value	Significance
	Spray control	10 Mm	20 mM	50 mM	
Flower caps/bunch	308a	327a	411a	269a	0.0133	ns
Seeded berries/bunch	233a	176a	226a	198a	0.1099	ns
Seedless berries/ bunch	4.5a	4.7a	0.7a	1.6a	0.4075	ns
LGOs	42a	34a	40a	34a	0.8501	ns
Total bunch weight	214a	175a	170a	165a	0.0417	ns
Total berry weight	204a	166ab	160b	158b	0.033	*
Total number of berries	247a	173b	216ab	196ab	0.0298	*
% Fruit set	81a	58b	59b	74a	0.0002	***
Coulure Index	1.02a	3.08b	3.19b	1.35a	<0.0001	****
Millerandage Index	1.32a	1.92a	1.64a	1.5a	0.6356	ns

One way ANOVA was performed to see the significance among the treatments. Different letters indicate significant difference among different treatments.

Table 6. Effect of Bicuculline (100 µM) on reproductive performance measures. N= 7 ±SEM.

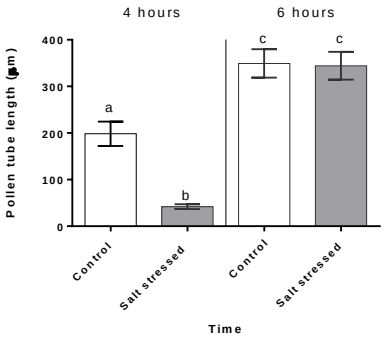
Parameters	Treatments		P value	Significance
	Spray control	Bicuculline 100 µM		
Flower caps/bunch	215	204	0.7456	ns
Seeded berries/bunch	97	88	0.5002	ns
Seedless berries/ bunch	0.14	0.42	0.2707	ns
LGOs	1.85	4.28	0.1492	ns
Total bunch weight	50	50	0.9414	ns
Total berry weight	45	47	0.9215	ns
Total number of berries	97	88	0.7532	ns
% Fruit set	47	45	0.7007	ns
Coulure Index	5.32	5.88	0.4061	ns
Millerandage Index	0.43	0.48	0.8011	ns

Unpaired t test was performed to see the significance among the treatments.

(A) **(B)**

Figure 1. (A), Effect of salinity (35mM) on the leaf with margins showing Cl^- toxicity similar with previous reports (Prior *et al*. 1992: Walker *et al*. 1996). **(B)**, Comparison of bunches between control and salt stressed treatment showing lesser number of berries resulting in reduced fruit set due to MI and CI. Scale bar, 10mm.

(A)



(B)

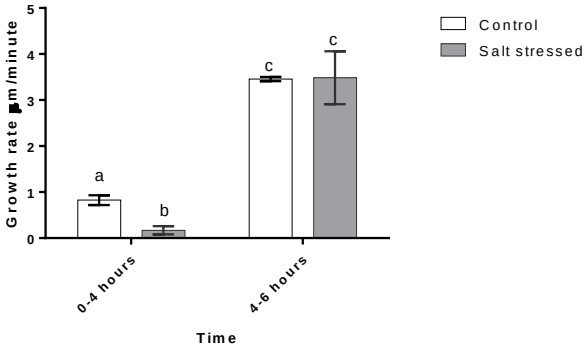
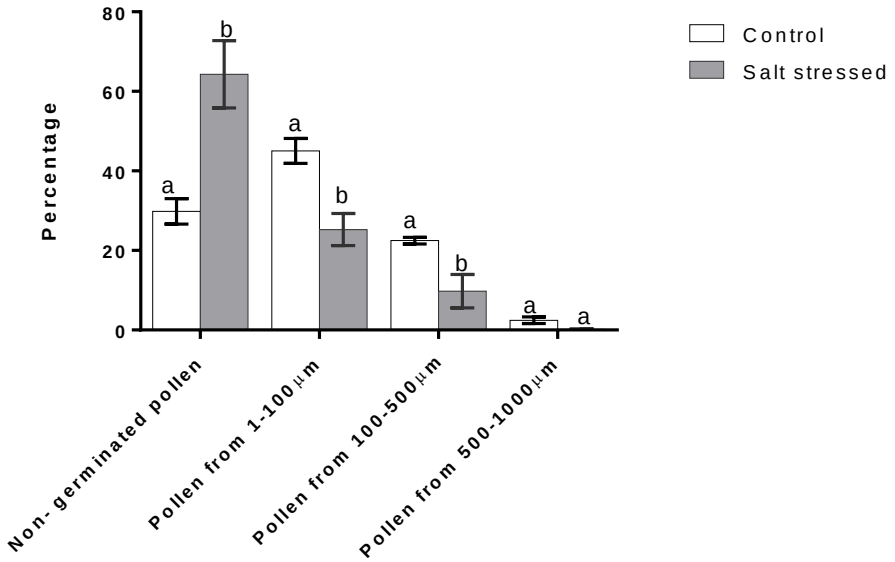


Figure 2. The effect of salinity stress on pollen tube length at 4 and 6 hours **(A)** and Growth rate of whole pollen population at 4 and 6 hours **(B)**. Pollen tubes were grown *in vitro* using liquid pollen germination media and incubated at 25°C. A significant difference in PTL (Unpaired t test, P value <0.0001) and growth rate (Unpaired t test, P value 0.0209) was observed at 4 hours However at 6 hours there was no significant difference. Values are means of two different experiments with 50 different replicates ± SEM. Different letters indicate significant difference among different treatments.

(A)



(B)

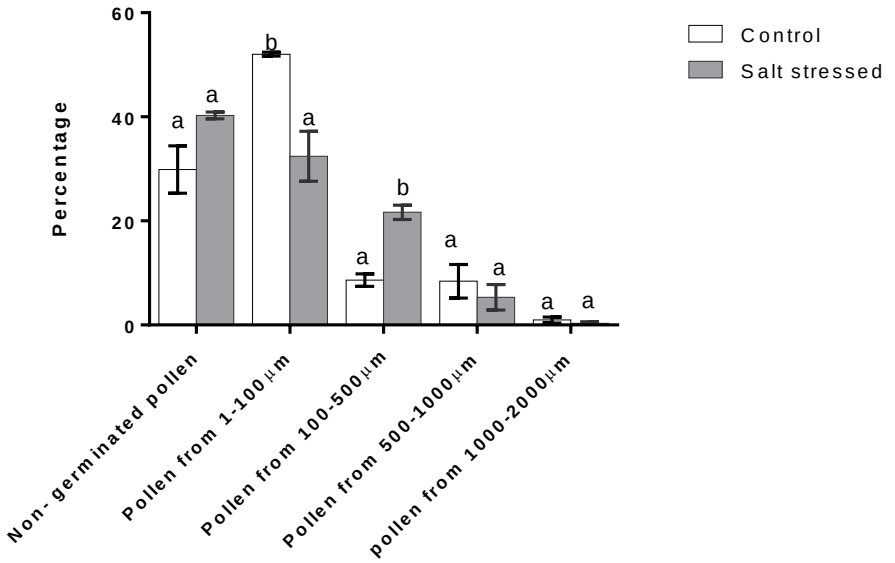
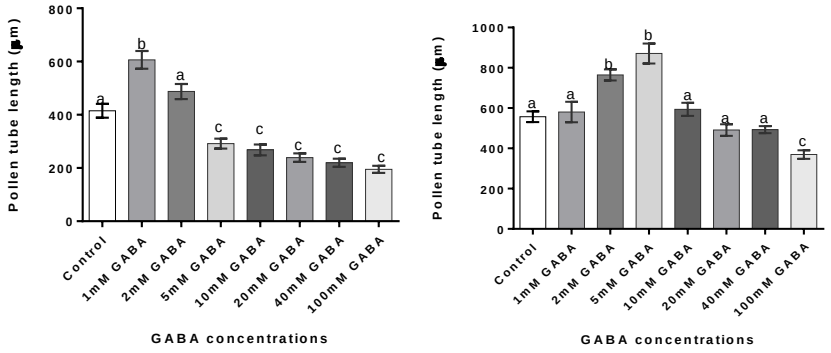


Figure 3. Comparison of complete pollen grains profile among control and salt stress conditions at 4 **(A)** and 6 hours **(B)** after germinating pollen grains at 25°C. N=200-300 (± SEM). At 4 hours **(A)** a significant difference was observed for NG (t test, P value 0.0314), pollen tubes between 1-100 µm (Unpaired t test, P value 0.0304) and 100-500 µm (Unpaired t test, P value 0.0481). However at 6 hours **(B)** no significant difference was observed for NG while a significant difference was observed for pollen tubes ranging between 1-100 µm (Unpaired t test, P value 0.0095) and 100-500 µm (Unpaired t test, P value 0.0276) with longer pollen tubes in salt treatment. Different letters indicate significant difference among different treatments.

(A) **(B)**



(C)

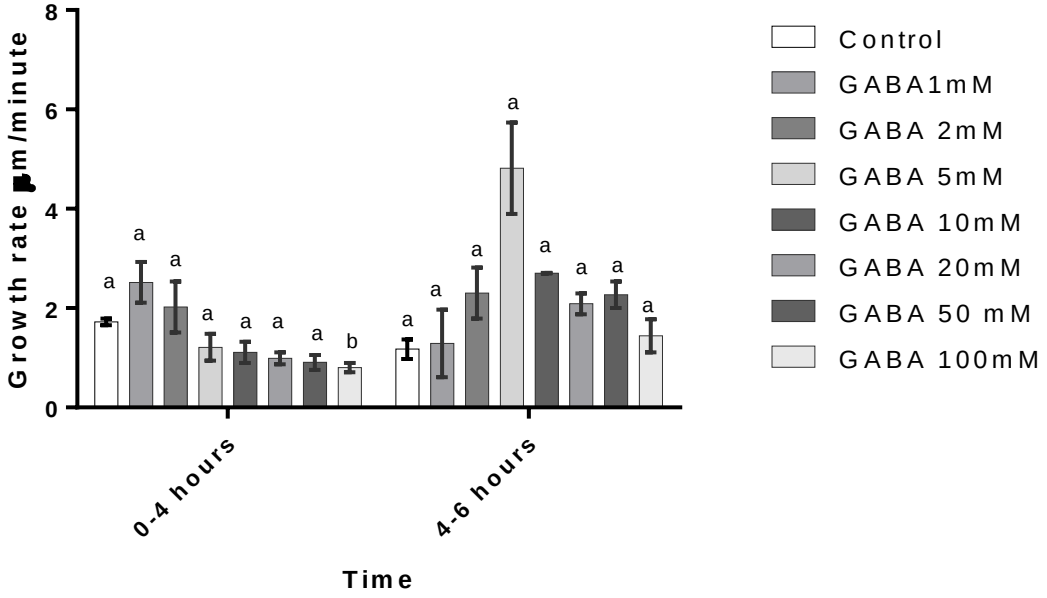


Figure 4. The effect of GABA on pollen tube length through *in vitro* pollen germination assay experiment at 4 and 6 hours of incubation at 25°C (A) and growth rate of the whole population (B). **(A and B)**, A biphasic GABA effect was seen at both 4 and 6 hrs. 2mM and 5 mM GABA concentrations were found to be stimulatory (one way ANOVA, P value <0.0001). While the 100 mM GABA were inhibitory for PTG (one way ANOVA, P value 0.0007) at the end. **(C)**, For growth rate a significant difference was observed between control and 100 mM GABA (one way ANOVA, P value 0.01) at 4 hours but no significant difference was observed among any treatment at 6 hours. Values are means of 50 different replicates ± SEM. Different letters indicate significant difference among different treatments.

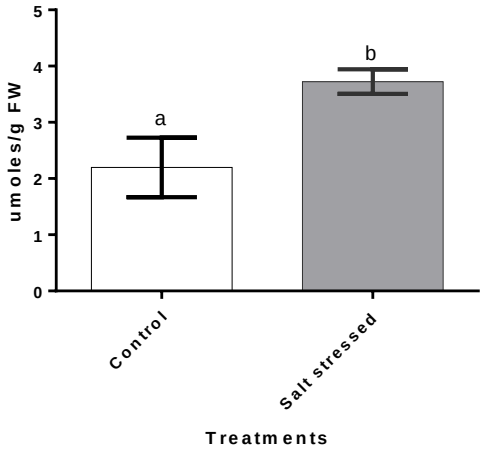


Figure 5. Endogenous GABA levels in µ moles/g FW values for control and salt stressed flowers. A significant difference was observed between control and salt treated flowers (Unpaired t test, P value 0.0377). Values are means of 5 different replicates ± SEM. Different letters indicate significant difference among different treatments.

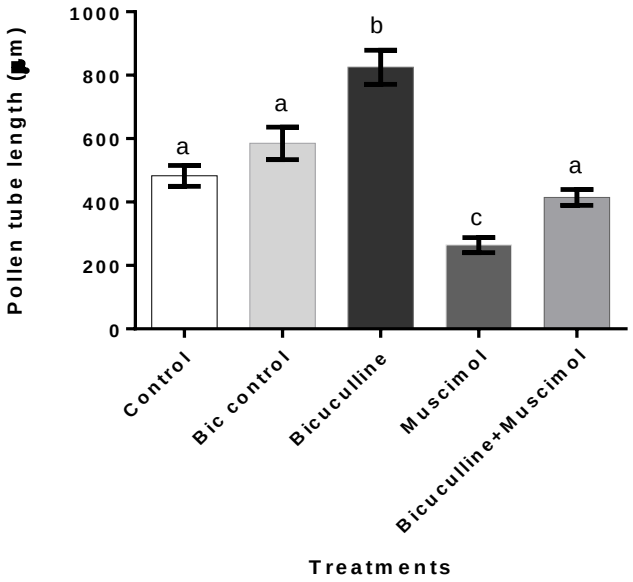


Figure 6. Effect of Bicuculline (100 µM), Muscimol (10 µM) and Bic-Muscimol on pollen tube length after 6 hours**.** Values are means of 200-300 replicates ± SEM in the second graph (one way ANOVA, P value < 0.0001). Bicuculline stimulated the PTG by 29% while muscimol inhibited it by 45%. When applied together the effect of muscimol is abolished by bicuculline resulting in 36% increase than muscimol. Different letters indicate significant difference among different treatments. This figure is included in one of the paper Ramesh *et al*. (2015).

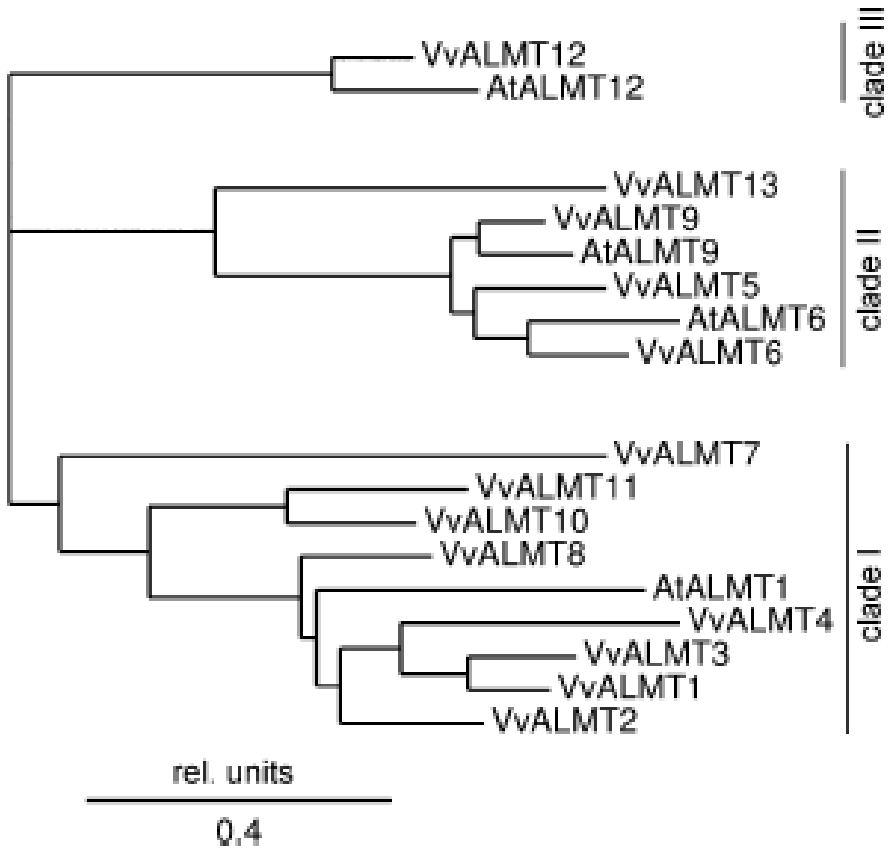
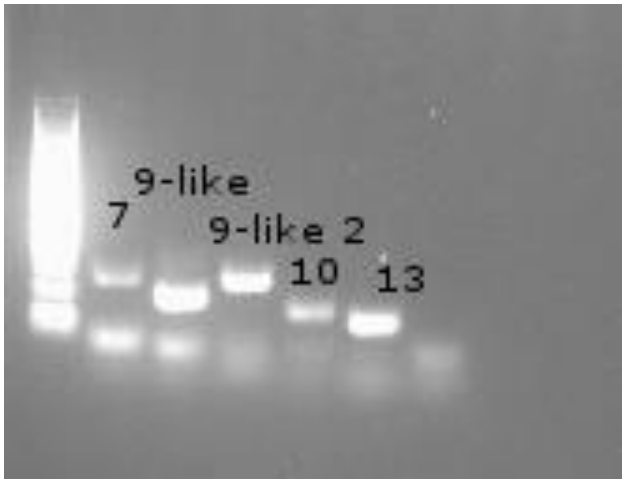


Figure 7. Adapted from De Angeli *et al*. (2013). Dendrogram of ALMT protein family in *Vitis vinifera*. Based on multiple amino acid sequence alignments using ClustalW (Thompson et al 1994) the 13 members of ALMT protein family were classified into three main clades as in *A. thaliana*. Clade I member: Vv ALMT 1, Vv ALMT 2, Vv ALMT 3, Vv ALMT 4, Vv ALMT 7, Vv ALMT 10, Vv ALMT 11 (GSVIVT01036162001, GSVIVT0103750001, GSVIVT01037569001, GSVIVT01036157001, GSVIVT01011122001, GSVIVT01019627001, GSVIVT01027186001). Clade II member: Vv ALMT 5, Vv ALMT 6, Vv ALMT 9, Vv ALMT 13 (GSVIVT01011922001, GSVIVT01011922001b, GSVIVT0100827001, GSVIVT01019447001). Clade III member: Vv ALMT 12 (GSVIVT01013184001).

(A)



(B)

ALMT 9-like **ALMT 10** **EF**

Figure 8. The semi-quantitative PCR to show the expression of *ALMT 7*, *9-like*, *9-like_2*, *10* and *13* in flowers **(A)** and expression of *ALMT 9-like* and *10* in pollen tubes relative to *Vv Elongation Factor-1-α* (*Vv EF1-α*) as a HK gene **(B)**.

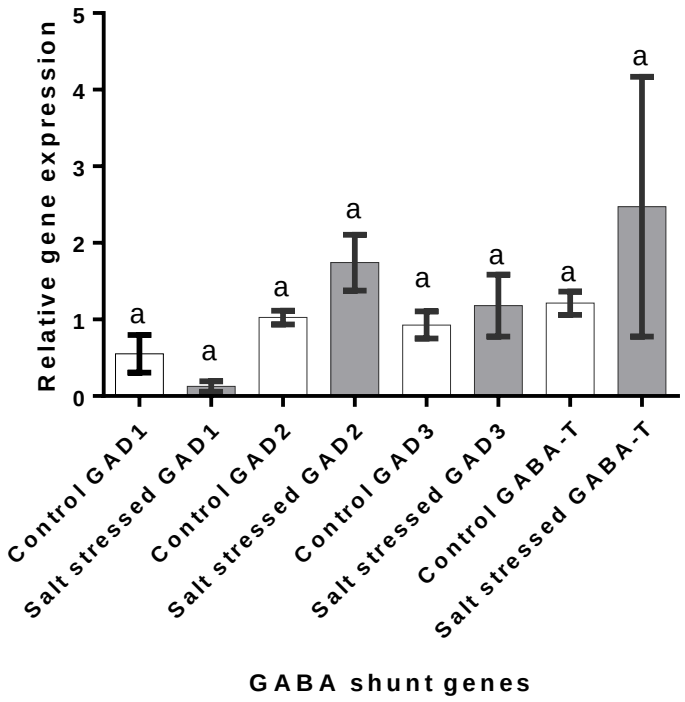


Figure 9. Relative gene expression levels of *GAD1, GAD 2, GAD 3 and GABA-T* to *Vv Actin 1(Vv Act1*) in control and salt stressed flowers. No significant difference was observed for any of these genes between control and salt treated flowers (Unpaired t-test, P value 0.3387).

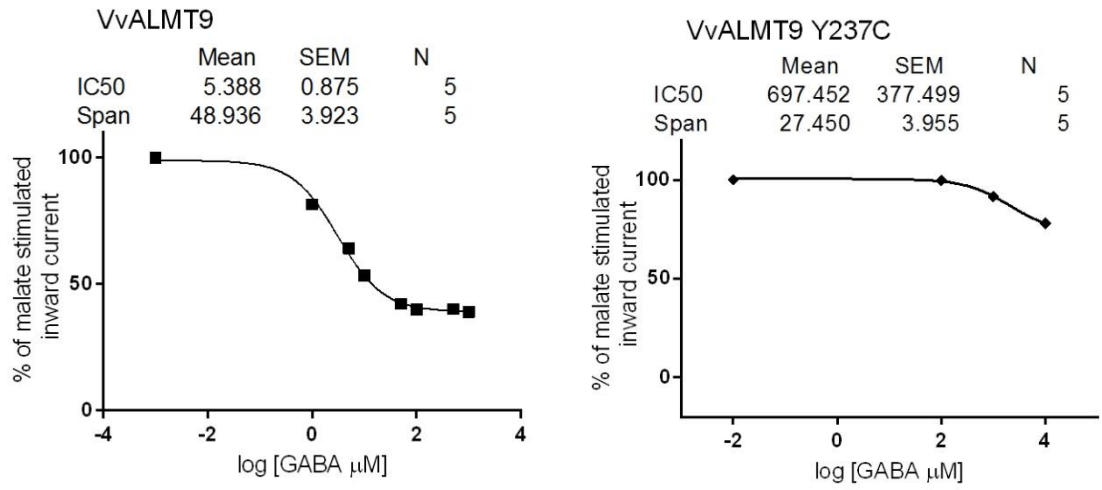


Figure 10. Dosage response curve of wild type Vv ALMT 9 to GABA with an IC50 of 5.38 µM **(A)** when Mutated with Tyrosine residue at position 237 to cysteine reduces (abolishes) GABA sensitivity and increases IC50 to 697.452 µM **(B)**. This suggests that Tyrosine plays an important role in GABA sensitivity in Vv ALMT 9. Ramesh *et al*. (2015).

Supplementary figures

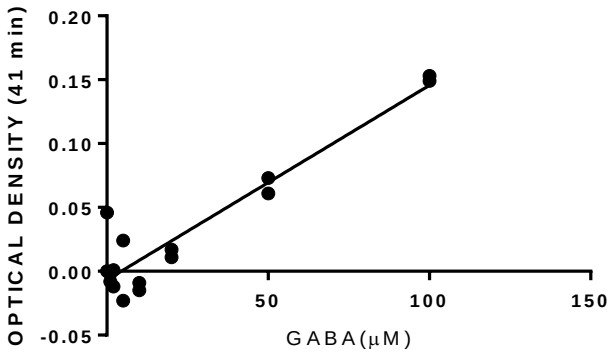
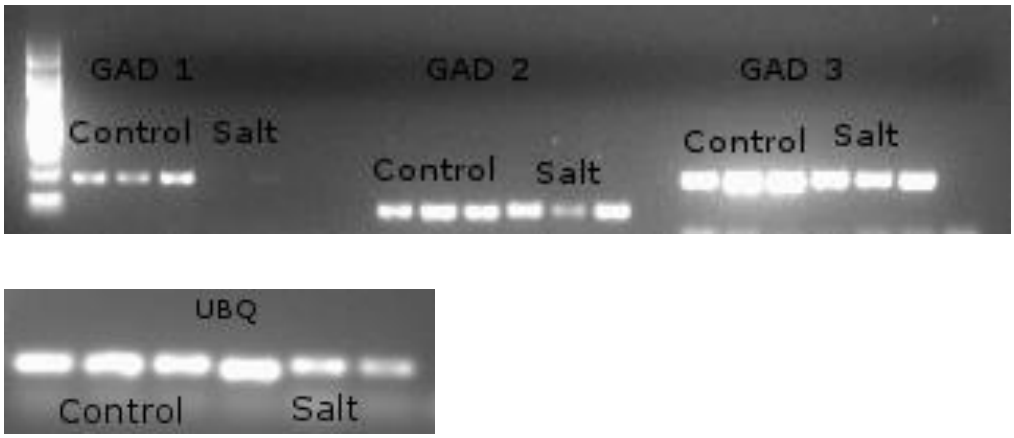


Figure 1. Base corrected data of GABase test for estimation of endogenous GABA levels in control and salt treated samples. Graph shows the optical density values for the GABA standard 1-200 µM and interpolated values of samples (control and salt treated) tested.

(A)



(B)

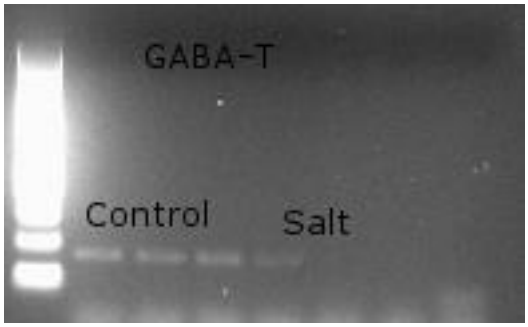


Figure 2. Semi- quantitative PCR of GABA-shunt enzyme encoding genes (*GAD1, GAD2, GAD3 and GABA-T*) relative to *Vv Ubiquitine-L40* (*Vv UBQ*) as a HK gene.

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Chapter 5.

General discussions and Future Directions

Chapter 5 General discussions and future directions

Pollen tubes play a vital role in fertilization; any kind of impairment in their growth leads to reduced, or no fertilization, which leads to a reduction in fruit set. Salinity is one of the major abiotic stresses that reduce the fruit set in different plants including grapevine (Hawker and Walker 1978; Walker *et al*. 2010; Walker *et al*. 2002). In the present study, the *in vitro* pollen germination method was optimized to ensure proper PG and PTG of grapevine pollen, and an accurate and suitable way to administer GABA or other drugs (bicuculline or muscimol) to see their effect on PTG regulation. In Chapter 4 various *in vitro* studies were conducted to understand the performance of pollen grains harvested from different conditions (control and salt treated plants), to analyse the effect of exogenous GABA, Muscimol and Bicuculline application and to grow the pollen tubes for extraction of RNA for gene expression studies.

Chapter 3 describes the optimization study to find suitable conditions for grapevine PG and PTG by trialling different types of storage conditions, media, and incubation temperatures. For pollen grain storage -80˚C was selected because it ensured the pollen were stored without any perturbation in germination rate over fresh pollen (Olmo 1942; Parfitt and Almehdi 1983). Liquid media was selected to carry out further studies because it is easy to administer the exogenous GABA or any other drugs that were not heat stable, and allowed the easy control of external pH. Out of two temperatures (20˚C and 25˚C), 25˚C was selected based on the greater PG number and PT lengths achieved; this was previously reported to be optimal in many other species besidesgrapevine such as Arabidopsis (*Arabidopsis thaliana*), Cherimoya (*Annona cherimola* Mill.), Feijoa (*Acca sellowiana* (berg) Burret) and Cotton (*Gossypium hirsutum* L.) (Franzon *et al*. 2005; Liu *et al*. 2006; Pickert 1988; Rosell *et al*. 1999; Sharafi and Bahmani 2011) .

Chapter 4 comprises the major part of this present study where potential factors that affect the pollen grain/tube and fruit set following salinity treatment is studied in detail. The performance of salt treated pollen grains in terms of germination, PTL and growth rate was checked to find any difference in its potential from the control and were all found to be less in the initial hours of germination, when germinated *in vitro* (Chapter 4, Fig. 2, A, B and Fig. 3, A). A major aim of this study was to identify whether GABA increased in grapevine floral tissue upon salinity stress. Here, we found that GABA concentrations were found to increase significantly, about 2-fold in salt treated flowers, over control flowers. This meant that the next aim of the study was valid, to test whether GABA could act as a potential signal in floral

tissue to regulate PG and PTG and therefore be a factor in reducing fruit set under salinity stress. When pollen grains were germinated on media supplemented with GABA the PTL was found to be stimulated with lower concentrations but inhibited by the higher concentrations (Chapter 4, Fig. 4, B) suggesting its possible role in regulating PTG inside the style; this shows that grapevine pollen has similar intrinsic responses to GABA as previously observed in Arabidopsis (*Arabidopsis thaliana*) and Spruce (*Picea wilsonii*) and Tobacco (*Nicotiana tabaccum*) (Ling *et al*. 2013; Palanivelu *et al*. 2003; Yu *et al*. 2014). Difference in the pollen grain potential for the first 4 hours between control and salt treatment could be due to an abnormal development of microspores inside the anther lobe (microsporangium), which needs further exploration. Furthermore, in the present study the pollen grain performance in terms of PG, PTL and growth rate (whole population) was studied but to further explore the growth of pollen tube in salt treatment further study is needed where the growth of individual pollen tubes is tracked over time and to study whether there is any difference in rate of exocytosis and eventually the cell wall synthesis following salt or GABA treatment (Onelli and Moscatelli 2013). If we could find any difference in the growth pattern it will give us further clue for the presence of some endogenous factors if any in addition to exogenous ones (GABA). Also we compared the pollen grains harvested from salt treated vines on the basic media not the one supplemented with GABA, such an approach may provide a further clue to see if salt treated pollen grains are more susceptible to increased GABA levels; this has been observed in Arabidopsis *pop2* tissues which have elevated GABA concentrations due to abolition of the GABA-T enzyme (Renault *et al*. 2010).

GABA was also sprayed on flower bunches to see if it had any effect on fruit set and interestingly the fruit set decreased with 10 and 20 mM GABA concentrations (Chapter 4, Table. 5); this was due to the increased CI indicating excessive shedding of young ovaries due to lack of fertilization. Although this effect was not consistent with higher concentration (50 mM) these data could be due to the low flower number in these samples compared to the spray control, which would artificially inflate fruit set. These data need repetition with uniform number of flowers with spray control in future.

Pharmacological studies were performed using muscimol as an analogue and bicuculline an allosteric inhibitor of ALMT to gather evidence to support ALMT-GABA interaction in controlling PTG (Chapter 4, Fig. 6). Our data further supported the role of GABA in PTG regulation as muscimol, an analogue of GABA reduced the PTG, and bicuculline, an allosteric inhibitor of GABA binding to mammalian $GABA_A$ receptors increased the PTG (Chapter 4, Fig. 6). However, when sprayed exogenously on flower bunches, bicuculline

could not show any effect on reproductive performance. Bicuculline is unstable at high temperature and pH so it is likely its effects could not be ascertained over the long term. We found promising results with the bicuculline treatment in the *in vitro* pollen germination assay studies leading to 29% longer pollen tubes (Chapter 4, Fig. 6) a better management of conditions for the spray could give a boom in grapevine fruit set. Alternatively we can increase the number of sprays to achieve better fruit set.

In order to find the genes responsible for GABA generation and accumulation gene expression studies were done using qRT-PCR on floral tissue to see the difference in the expression level of GABA shunt enzymes, the main pathway for GABA generation and degradation, but no significant difference was observed for transcript levels of these genes between control and salt treatment (Chapter 4, Fig. 9). Further study in terms of assaying protein abundance and activity is required to understand the role of GAD or GABA-T in GABA generation in floral tissues of grapevine. At the same time there are several other possible pathways that needs exploration to understand if they are involved in the elevation in GABA seen under salinity such as degradation of PAs by DAO and PAO (Cona *et al*. 2006; Xing *et al*. 2007).

In the present study two ALMT gene family members *VvALMT 9-like* and *VvALMT* 10 were shown to be expressed in pollen tubes (Chapter 4, Fig. 8, B), which are possible candidates of GABA binding sites reported in the PT of other species (Hoekenga and Maron 2006). A fluorescent muscimol conjugate could be used to confirm whether there are GABA binding sites on grapevine pollen (Allen *et al*. 2008; Wang *et al*. 2000). Although we found *ALMTs* expressed in pollen tubes we still do not know their localisation in the pollen grains and tubes. Further study is required to find the exact location for the expression of these gene/s through *in situ* PCR studies (Przybecki *et al*. 2006), these could be combined with the muscimol Bodipy or ALMT-fluorescent protein fusions to see if expression patterns, protein localisation and muscimol-BODIPY fluorescence overlap. Moreover, as there are no reports available of ALMT10 in grapevine regarding the localisation and GABA sensitivity, further studies for characterising this gene member will help in understanding its role in PTG regulation. For instance, some electrophysiological studies using two- microelectrode voltage clamp techniques for heterologous gene expression studies with Xenopus oocytes to assess the ion selectivity of the ALMTs (Dascal 1987) will further help in understanding its contribution towards PTG. VvALMT9 was found to be GABA sensitive in such an assay, so may be the transporter that transduces the GABA signal in pollen.

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Appendix

Table 1. Effect of 20 mM GABA spray on reproductive performance. N= 10 ±SEM.

Parameters	Treatments		P value	Significance
	Spray control	GABA 20 mM		
Flower caps/bunch	286	254	0.3525	ns
Seeded berries/bunch	113	109	0.7114	ns
Seedless berries/ bunch	6.9	0.3	0.0366	*
LGOs	11	12	0.9226	ns
Total bunch weight	72	75	0.4406	ns
Total berry weight	66	70	0.3554	ns
Total number of berries	113	109	0.7114	ns
% Fruit set	42	45	0.4977	ns
Coulure Index	5.2	5	0.57	ns
Millerandage Index	1.2	1	0.6516	ns

Unpaired t test was performed to see the significance among the treatments

Table. 2. Effect of 100 mM GABA spray on reproductive performance. N=7 ±SEM.

Parameters	Treatments		P value	Significance
	Spray control	GABA 100 mM		
Flower caps/bunch	215	122	0.001	***
Seeded berries/bunch	97	93	0.7812	ns
Seedless berries/ bunch	0.28	0.14	0.6627	ns
LGOs	1.8	1.42	0.6542	ns
Total bunch weight	50	51	0.6788	ns
Total berry weight	45	46	0.8179	ns
Total number of berries	97	94	0.8381	ns
% Fruit set	47	77	<0.0001	****
Coulure Index	5.3	2.2	<0.0001	****
Millerandage Index	0.43	0.2	0.1873	ns

Unpaired t test was performed to see the significance among the treatments