Thesis

Recombinant wheat ALMT1 protein expression in *Pichia pastoris*

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Abstract

Gamma aminobutyric acid (GABA), is a non-protein amino acid, found in all kingdoms of life (archaea, bacteria and eukaryotes) (Bouché, Lacombe, *et al.* 2003). In mammalian brains, binding of GABA to the $GABA_A$ receptor activates chloride channels allowing chloride influx to the cells and regulates neuronal firing during stress (Palacios *et al.* 1981). In plants, GABA has been shown to be a metabolite that changed under abiotic and biotic stresses (MacGregor *et al.* 2003; Mekonnen *et al.* 2016; Perez-Alfocea *et al.* 1994; Ramputh & Bown 1996; Rhodes *et al.* 1986). However, recent discovery of a putative GABA binding site on a family of anion channels (Aluminium Activate Malate Transporters – ALMT) involved in aluminium stress tolerance suggests that GABA regulates these channels and may act as a stress signalling molecule (Ramesh *et al.* 2015).

The discovery of a putative binding site (12 amino acid stretch) on the wheat ALMT (TaALMT1) as a transducer of GABA signalling has broadened the area of research into the plant signalling pathways (Ramesh *et al.* 2015). However, the structure or topology of TaALMT1 or other ALMTs is not known and the molecular determinants of GABA regulation of anion channels is not yet completely understood (Fromm 2020). Thus, it is difficult to identify the exact physiological role of GABA and its role in stress signalling and tolerance in plants.

In this project TaALMT1 was expressed in the yeast *Pichia pastoris* with the aim of purifying the recombinant protein for determining its tertiary or 3D structure via Cryo-EM studies and bioinformatics was used to identify other plant proteins with putative GABA binding sites to understand the GABA regulation.

Amplified ALMT1 was cloned into pPICZB with *Xho*I and *Xba*I restriction sites followed by transformation into *E. coli* competent cell DH5α. Plasmid DNA was extracted, sequenced to

confirm the DNA sequence, and then transformed into *Pichia pastoris.* In the process of colony PCR to confirm integration, Thermopol Taq polymerase (NEB) was found to be more suitable than Go Taq Polymerase (Promega) and X33 strain of *Pichia* was better suited for expression of protein than SMD1168 strain. 24-48 hours of induction with methanol was sufficient for protein induction. Following transformation into *Pichia*, protein was induced by addition of methanol over a period of 24-120 h. The cells of the *Pichia* were disrupted using glass beads in a bead beater and induction of protein expression confirmed using anti-His antibody via dot blot. However, protein expression could not be confirmed via western blots. Future work is to optimise protein induction followed by affinity purification of the recombinant protein for tertiary structure determination.

Further to identify the other proteins that might have a putative GABA binding site, a consensus sequence DVFXXXXWXXEXL, based on different plant ALMTs and mammalian GABA receptors was used in a BLAST search. The search identified plant ALMTs as well other proteins. However, 20 different proteins from both monocot and dicot plants were with the putative GABA binding sites selected for this study. highly conserved amino acids Valine, Phenylalanine and Tryptophan which have been shown to be important for GABA regulation. These proteins need to be functionally characterized in the future to determine if they are regulated by GABA.

1. Introduction/Literature Review

Gamma aminobutyric acid (GABA), is a non-protein amino acid, found in all kingdoms of life (archaea, bacteria and eukaryotes) (Bouché, Lacombe, *et al.* 2003). More than seven decades ago, GABA was discovered in potato tubers, followed by its discovery in mammalian brains as an inhibitory neurotransmitter (Steward 1949) (Awapara *et al.* 1950) Research progressed

in animals with the discovery of GABA receptors ($GABA_A$, $GABA_B$ and GABA rho) and drugs and inhibitors to study these in detail. In mammalian brains, binding of GABA to the $GABA_A$ receptor activates a chloride conductance that allows chloride to move into the cell. This shifts the membrane potential towards the chloride equilibrium potential and therefore regulates neuronal firing during stress (Palacios *et al.* 1981). In plants, GABA was thought of as a metabolite that changed under abiotic and biotic stresses such as heat, cold, hypoxia, dehydration, acidosis, salinity, virus infection and during defence against herbivory (Perez-Alfocea *et al.* 1994; Ramputh & Bown 1996; Rhodes *et al.* 1986). GABA is synthesised in the cytoplasm via decarboxylation of glutamate in the GABA shunt (Figure 1) and it is suggested that under stress, increases in activity of the GABA shunt provide GABA to the tricarboxylic acid cycle (TCA) to maintain energy production (Bown & Shelp 1997). Though there was speculation that GABA might be a signalling molecule in plants, no receptor was identified until recently. In 2015, a putative GABA binding site was discovered in a family of anion channels (Aluminium (Al)- Activated Malate Transporters – ALMT) in plants, providing evidence that GABA might act as a stress signal (Ramesh *et al.* 2015).

Members of the ALMT family are widely distributed in plant genomes and are involved in a variety of functions including Al tolerance, seed development, microbe interactions, stomatal regulation, fruit acidity and mineral nutrition (Bai *et al.* 2012; Meyer *et al.* 2011; Piñeros *et al.* 2008; Rudrappa *et al.* 2008; Sasaki *et al.* 2004; Xu, M *et al.* 2015). The discovery of a putative GABA binding motif (12 amino acid stretch) on the wheat *ALMT (TaALMT1*) as a transducer of GABA signalling has broadened the area of research into the stress signalling pathways in plants (Ravi *et al.* 2018).

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Figure 1. GABA Shunt. *GABA is synthesised via the metabolic pathway known as GABA shunt and involves decarboxylation of glutamate to GABA by the action of glutamate decarboxylase. GABA shunt is upregulated during stress and increased GABA feeds into the Krebs cycle to maintain energy production in plants (Bown & Shelp 1997; Shelp et al. 2017). GDH-L-glutamate dehydrogenase, GAD-glutamate decarboxylase, GABA-T-GABA transaminase, SSADH-succinic-semialdehyde dehydrogenase,* CO_2*-carbon dioxide,* NH_3*-ammonia.*

Research has shown that the putative GABA binding motif in ALMTs show similarity to the motif for GABA binding from mammalian (rat) $GABA_A$ receptors, and GABA gated anion channels are important in plant stress tolerance (Ramesh *et al.* 2015; Ravi *et al.* 2018). Modulation of GABA binding to its receptor and manipulation of GABA levels *in planta* could be carried out to improve plant stress tolerance, however, the structure or topology of the *TaALMT1* or other *ALMTs* in which the putative GABA site was identified, is not yet known (Fromm 2020). Thus, it is difficult to identify the exact physiological/signalling role of GABA, its role in stress signalling and tolerance in plants.

In this project, TaALMT1 will be cloned and transformed into yeast *Pichia pastoris* with the aim of purifying the recombinant protein for determining its structure or 3D shape via Cryo-EM studies. Identification of the structure will enable us to study how this protein is regulated

by GABA, the conformational changes that might occur when GABA binds to the ALMTs and the changes that might occur to the topology of the protein in the membrane when subjected to site directed mutagenesis. Elucidation of the structure will help us understand better the role of GABA in stress tolerance and how informed modulation of the GABA regulation of this anion channel could improve stress tolerance in plants.

Gamma aminobutyric acid (GABA)

GABA is produced in the cytosol by the decarboxylation of the Glutamate by an enzyme Glutamate decarboxylase (GAD) and is metabolised through GABA transaminase (GABA-T) and in the mitochondria through succinic semialdehyde dehydrogenase (SSADH). The final product of this cycle is succinate which feeds into the Krebs cycle and this pathway is called the GABA shunt (Figure 1) (Tuin & Shelp 1994). Under stress, GABA shunt is upregulated to provide anaplerotic succinate to the Kreb's cycle and maintains energy production. The enzymes that regulate the GABA shunt pathway have been reported in different organisms in different subcellular locations, for instant, SSADH in yeast, is found in the cytosol (Coleman *et al.* 2001), similarly, in Arabidopsis, GABA-T is localized to the mitochondria (Cao, J *et al.* 2013).

GABA in mammals

Several studies have shown that GABA and L-glutamate are the most abundant neurotransmitters in mammalian brain; while GABA is the major inhibitory neurotransmitter, L-glutamate is an excitatory neurotransmitter (Curtis & Watkins 1963; McCormick 1989). Both are key mediators of synaptic plasticity and neuroendocrine function (Lynex *et al.* 2004). Initially, GABA was assumed to act as an inhibitory neurotransmitter in brain and research progressed with discovery of its location, (nerve terminals) and its receptor $GABA_A$

(ionotropic) and $GABA_B$ (metabotropic) in mammalian brains (Palacios *et al.* 1981). GABA effects the inhibitory processes throughout the nervous system through binding to its receptors, ligand gated $GABA_A$ and $GABA_C$ and G-protein-coupled receptors $GABA_B$, (Jones, KA *et al.* 2000). In mature neurons, binding of GABA to the $GABA_A$ receptor activates a chloride conductance allowing for passive chloride influx into the cytoplasm causing membrane hyperpolarization which eventually leads to the inhibitory action of the GABA (Watanabe & Fukuda 2015); this regulates neuronal firing during stress and hence function as a calming factor (Cooper & Selman 1974). In animals, effects of GABA have been widely studied focusing on the nervous system, where its receptors regulate the function of brain and early development of the central nervous system (Ganguly *et al.* 2001; Lin *et al.* 1994). Studies in 2000, revealed that the GABA receptors are also expressed in different human tissues such as liver, heart, lungs, and ovary. Thus, these findings suggests that GABA could be a signalling molecules in other parts of the body and not only the brain (Calver *et al.* 2000). Disturbances in the homeostasis of GABA in the brain are responsible for the development of epilepsy, depression, Parkinson's disease, Huntington's chorea, Alzheimer's disease, and Stiff-Man syndrome (Lynex *et al.* 2004).

GABA in Plants

Presence of GABA in several parts of the plants including the roots, shoots, fruits, nodule, xylem, phloem, flowers, embryo, and cotyledon suggests that it has important roles to play in growth and development (Hijaz & Killiny 2020; Kinnersley & Turano 2000). In plants, higher content of GABA and lower content of glutamate causes abnormal growth and development for instance transgenic tobacco expressing a mutant petunia GAD lacking calmodulin (CaM)

binding domain showed impaired development with short stems having short cortex parenchyma that lead to variation in the levels of the critical metabolites (Baum *et al.* 1996).

The concentration of GABA varies across plant species, and across different organs, tissues, and compartments. In plants, GABA was thought of as a metabolite that changed under abiotic and biotic stresses (Perez-Alfocea *et al.* 1994; Ramputh & Bown 1996; Rhodes *et al.* 1986). In cytosol, these stresses stimulate the increase in levels of calcium (Ca^{2+}) which form complexes with CaM and in the physiological pH range, Ca^{2+}/CaM complex activates GAD leading to GABA synthesis. This enzyme is also activated due to increase in cytosolic acidification which can result from the mechanical damage that rupture the vacuolar membranes and release organic acids (Roberts *et al.* 1984). In 2003, GABA was reported in the reproductive part i.e. stigma, style and pollen tubes of *Arabidopsis* and shown to have both direct and indirect effects; stimulate growth of pollen tube, inhibit the elongation of pollen tube at high concentrations and act as signal that guides the pollen tube navigation (Palanivelu *et al.* 2003).

Many previous studies have demonstrated GABA as a metabolite that changes in response to stress [Table I] (Bouché, Lacombe, *et al.* 2003; Liu & Zhou 2018; MacGregor *et al.* 2003; Shelp *et al.* 1999). Among all the stresses, salt induced GABA accumulation has been broadly studied in different types of plants like rice, barley, tobacco, *Arabidopsis, alfalfa,* soybeans, *Populus x canescens* (Fougere *et al.* 1991; Renault *et al.* 2010; Xing *et al.* 2007) (Dluzniewska *et al.* 2006; Patterson *et al.* 2009; Zhang, Jingtao *et al.* 2011). Under drought stress, GABA was found to be increased in soybean, Arabidopsis, bean, sesame and turnips (Bor *et al.* 2009; Mekonnen *et al.* 2016; Raggi 1994; Serraj *et al.* 1998) and other studies show that GABA also accumulates under hypoxic stress in *Prunus persica* (Salvatierra *et al.* 2016). Interestingly, some studies found that GABA does not always accumulate under stress (Kishinami 1988). GABA was found to accumulate in undifferentiated meristematic cells such as cultured rice

cells and root tips during ammonium assimilation when auxin was applied exogenously (Kishinami 1988).

GABA has been shown to accumulate in herbivory and pathogen infection as a defence responses (Scholz *et al.* 2015; Solomon & Oliver 2001). The GABA levels were found to increase by 5-fold within 10 min when the leaf was being crawled upon by the tobacco budworm and in soybean it increased by 11-fold when crawled upon by *Choristoneura rosaceana* (Scholz *et al.* 2015; Solomon & Oliver 2001). These results suggested that GABA might be a signalling molecule in plants, but the mechanism was unknown.

After the discovery of GABA as a signalling molecule in animals and bacteria (Chevrot *et al.* 2006), it was speculated to be a signalling molecule in plants as well. GABA has been shown to control the level of Reactive Oxygen Species (ROS) intermediates to prevent cell death either by limiting NADH or Ca^{2+} signalling (Bouché, Fait, *et al.* 2003); to function in plant-insect interactions i.e., tobacco plants with higher content of GABA had significantly lower number of eggs of northern root-knot nematodes (McLean *et al.* 2003) and plant-bacterial interactions by interfering with the diffusion of plant pathogen *Agrobacterium tumefaciens* due to variation in GABA levels in the plant tumours (Lang *et al.* 2016). It has also been recently shown to be involved in stomatal regulation in plants to reduce the loss of water through transpiration, evaporation and osmosis during drought (Mekonnen *et al.* 2016; Xu, B *et al.* 2021) providing evidence towards it being a signalling molecule.

For a molecule to be involved in signalling, it requires the following characteristics:

(1) alter expression of genes and/or activate enzymes.

(2) alter metabolism and/or growth.

(3) have receptor or sensor molecules (Grill & Himmelbach 1998; Iten *et al.* 1999; Sheen *et al.* 1999; Solano & Ecker 1998).

Table I. Stress-Related Kinetics of GABA Accumulation in Plants (Kinnersley & Turano 2000).

Plant	Stress	GABA % of Control	Time	Reference
Asparagus cells	Acidosis	300	15 s	(Crawford *et al.* 1994)
Soybean leaves	Mechanical damage	1800	1 min	(Ramputh & Bown 1996)
Radish leaves	Anoxia	10,000	4 h	(Streeter & Thompson 1972)
Cowpea cells	Heat	1,800	24 h	(Mayer *et al.* 1990)
Bean leaves	Drought	200	3 d	(Raggi 1994)
Tomato root	Salt	200	4 d	(Bolarin *et al.* 1995)
Tomato leaves	Viral	130	13 d	(Cooper & Selman 1974)

GABA regulation of anion channels

In mammalian brains, GABA regulates ion flow through its receptors $GABA_A$ (Chloride channels) and $GABA_B$ (G-protein coupled receptors), across cell membranes (Barnard *et al.* 1998). The $GABA_A$ receptor is a macromolecular protein that functions as a chloride channel with a binding site for GABA and other drugs like barbiturates, picrotoxin and benzodiazepines (Macdonald & Twyman 1991; Pinal & Tobin 1998). The discovery of a putative GABA binding motif with similarity to the mammalian $GABA_A$ receptors in the ALMT proteins that are a family of anion channels in plants provided the first evidence for its signalling role and implications in modulating stress resilience *in planta* (Ramesh *et al.* 2015).

In acidic soils, aluminium (Al^{3+}) becomes more soluble and readily available to plant roots causing toxicity and is detrimental to root growth (Foy 1984). To reduce this stress, plant release anions (malate) from the root tips which bind to Al^{3+} ions and prevent damage to the root cells. In 1995, studies showed that in acidic soils where Al^{3+} toxicity is prevalent, plants that exuded more malic acid from their root tips were more tolerant than plants with less malic acid efflux (Figure 2) (Delhaize & Ryan 1995). The research progressed and in 2004, the gene

that regulated the malic acid efflux in wheat was functionally characterised and named as TaALMT1 (Sasaki *et al.* 2004). The ALMTs are localised to the plasma membrane of the cells. Since then, several ALMTs have been characterised in different plants (Bai *et al.* 2012; Meyer *et al.* 2011; Piñeros *et al.* 2008; Xu, M *et al.* 2015). The ALMT family is multigenic family; in Arabidopsis 14 ALMTs have been identified, in soybean 34, grapevine 13, and 9 in rice have been characterised (Hoekenga *et al.* 2006; Liu 2016; Peng *et al.* 2018). All members of the ALMT family are not involved in conferring Al tolerance (Sasaki *et al.* 2004); some are involved in diverse functions such as seed development (Xu, M *et al.* 2015), microbe interactions (Rudrappa *et al.* 2008), stomatal regulation (Meyer *et al.* 2011; Xu, B *et al.* 2021) fruit acidity and mineral nutrition (Bai *et al.* 2012; Piñeros *et al.* 2008). All these functions are regulated through the anion channels, ALMTs that are present in different parts of the plants.

GABA binding motif in plants

There is a putative GABA binding motif in ALMT proteins; a 12 amino acid stretch (TVFLFPVWAGEDV, Figure 3) that shows similarity to the motif for GABA binding from mammalian $GABA_A$ receptors and this motif is important in modulating plant stress tolerance (Ramesh *et al.* 2015). The figure shows a sequence alignment for the putative GABA binding motif generated by a bioinformatic analysis with the sequences of different plant ALMTs including wheat, barley, grapevine, Arabidopsis, and rice along with the $GABA_A$ receptor from rat.

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Figure 2. Roots exposed to Al in acidic soil with low pH. *Root growth in near isogenic lines of wheat ET8 and ES8 exposed to 50 µM Al in the soil. ET8 is Al tolerant and exudes more malic acid from the root tips while ES8 exudes less malic and is sensitive to Al toxicity. The exudation of malic acid in the near isogenic lines differ in expression of TaALMT1 with ES8 having lower expression of TaALMT1 than ET8 (Delhaize & Ryan 1995).*

The ALMTs shown in the sequence alignment were further characterised for GABA sensitivity (defined as inhibition of anion stimulated inward currents by GABA) by expression in *Xenopus laevis* oocytes and tobacco BY2 suspension cells; the EC_{50} values ranged from 0.6 to 6.8 µM for the various ALMTs which were interestingly lower than that determined for mammalian GABA receptor (Ramesh *et al.* 2015). There are three aromatic amino acids, shown in the boxed region (Figure 3) that are important for the GABA binding in the mammalian $GABA_A$ receptor. These amino acids were identified through the mutational analysis, point mutations on $Phe\text{-}Arg^{64\text{-}66}$ (Boileau *et al.* 1999) and reduced the sensitivity of both agonist and antagonist of $GABA_A$ from rat (Sigel *et al.* 1992). Similarly, in plants, site directed mutagenesis of Phe^{213} and Phe^{215} in the TaALMT1 were performed to probe the putative GABA binding site and the results suggested that these residues are important for binding GABA in the wheat TaALMT1 (Ramesh *et al.* 2015). However, mutations (Phe^{213} and Phe^{215}) in this site do not completely reduce or abolish the sensitivity of the TaALMT1 for GABA (Ramesh *et al.* 2015) which suggests that there could be other regions on this protein that might affect the GABA sensitivity (Fromm 2020; Ramesh *et al.* 2017).

Furthermore, in 2018 research revealed that some ALMTs in addition to being regulated by GABA, could also transport GABA (Ramesh *et al.* 2018) and that cytosolic GABA inhibits anion transport through wheat ALMT1 (Long *et al.* 2020).

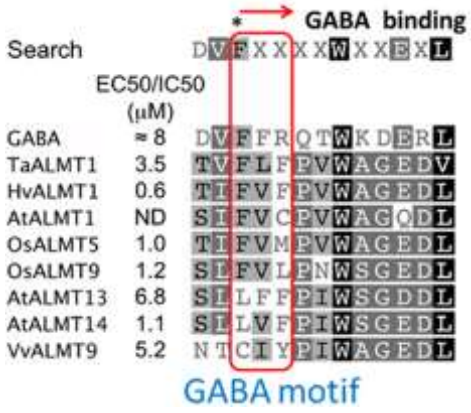


Figure 3. ***Sequence alignment*** *of GABA$_A$ receptor from rat and ALMTs from different plants such as wheat (TaALMT1), barley (HvALMT1), Arabidopsis (AtALMT1, 13 and 14), rice (OsALMT 5 and 9), and grapevine (VvALMT9) showing the putative GABA motif (Ramesh et al. 2015). Boxed region shows amino acids important for GABA binding. Asterisk indicates the aromatic amino acid important for GABA binding.*

Emerging evidence indicates that GABA is a stress signal and variation in its endogenous levels lead to some plants being more stress tolerant, and some less stress tolerant. However, the molecular determinants of this regulation are not yet understood (Gilliham & Tyerman 2016; Ramesh *et al.* 2018; Ramesh *et al.* 2015).

The recent discovery of the GABA regulated ion channels i.e., ALMTs in plants meets the criteria for GABA to be a potential signalling molecule as exogenous GABA negatively regulates root growth, alters ALMT and GAD expression and ALMTs have a putative GABA binding site (Ramesh *et al.* 2015). These results open new pathways for the research in plant biology.

Structure of GABA receptors

$GABA_A$ receptors from mammals have been well characterised (Cully *et al.* 1994; Hilf & Dutzler 2009; Miller & Aricescu 2014; Wong *et al.* 2015; Yamasaki *et al.* 2017). They are the members of Cys loop of the pentameric ligand-gated ion channels (pLGICs) and are found in eukaryotic animals and in some prokaryotes but were thought to be completely absent in multicellular plants and fungi (Schofield *et al.* 1987). The $GABA_A$ receptor is made of three subunits, α, β, γ that associate in a defined ratio to form a heteromeric functional receptor and both the α, and β subunits are important for GABA binding. In comparison, the structure of putative GABA binding motif on ALMTs is poorly understood. It is still unknown whether the ALMT channels are monomeric or multimeric with either homomeric or heteromeric combinations (Ramesh *et al.* 2017). Several studies have been conducted to predict the topology of the TaALMT 1, (Dreyer *et al.* 2012; Motoda *et al.* 2007) but they contradict with each other; one suggests that TaALMT1 consists of six transmembrane domain (TMDs) and both the N-terminus and C-terminus face the extracellular space (Motoda *et al.* 2007; Zhang, Jingbo *et al.* 2013) whereas the other suggests that it consists of eight TMDs and its N-terminus and C-terminus are located inside and outside of cells, respectively (Dreyer *et al.* 2012).

Absence of a defined topology and structure for the ALMTs limits the ability to predict the location of the amino acid residues in the putative GABA binding motif, to understand the conformational changes that occur upon GABA binding to its site and upon site directed mutagenesis. This impairs our ability to manipulate these proteins and understand their role in stress tolerance in plants.

Gaps in research and Biotechnological Significance

The review of literature suggests that the plants with the higher levels of GABA are more stress tolerant and that TaALMT1 is the first protein discovered that contains a putative GABA

binding site (Ramesh *et al.* 2015). GABA plays a role in tolerance to many stresses such as salinity, drought, hypoxia and in plant defence (Perez-Alfocea *et al.* 1994; Ramputh & Bown 1996; Rhodes *et al.* 1986), but ALMT proteins are not involved in providing tolerance to all these stresses. This suggests that there *could be other proteins with GABA binding sites* and that GABA might be regulating the activity of these proteins either directly or indirectly to confer stress tolerance. Furthermore, *the lack of clearly defined topology or 3D structure of the ALMTs* is a major limitation to modulate the activity of these proteins to manipulate for improved stress tolerance.

Aims and Hypotheses

The hypotheses are:

There are plant proteins other than ALMTs that may be regulated by GABA and recombinant ALMT protein could be an ideal tool for the better understanding of GABA regulation of proteins.

The aims are:

1. To identify other plant proteins with putative GABA binding sites.
2. To make progress towards determining the structure of the ALMT and the GABA binding site.

2. Identification of novel proteins with putative GABA binding sites

Gamma aminobutyric acid (GABA), a non-protein, amino acid, was thought to be a metabolite in plants as its concentrations changed rapidly under both biotic and abiotic stresses such as pathogen or viral infections, viruses heat, cold, hypoxia, salinity and acidity (Perez-Alfocea *et al.* 1994; Ramputh & Bown 1996; Rhodes *et al.* 1986). In response to various stresses, GABA is synthesised in the cytosol and moved to the mitochondria to provide anaplerotic succinate to

the Tricarboxylic acid cycle (TCA) to maintain energy production (Shelp *et al.* 1999). As GABA is a major inhibitory neurotransmitter with signalling roles in mammalian brains, a signalling role for GABA in plants was hypothesised. It was not until 2015, that a putative GABA binding site was identified in a family of anion channels -Aluminium Activated Malate Transporter (ALMT) from wheat and this provided evidence towards GABA being a signalling molecule in plants (Ramesh *et al.* 2015). To date, wheat protein TaALMT1 is the only one protein characterised that is regulated by GABA. As GABA has been shown to accumulate rapidly when plants are faced with adverse environmental conditions, it is highly likely that there are other plants proteins that have putative GABA binding sites and be regulated by GABA. Further, ALMT proteins play a role in aluminium toxicity tolerance but no other abiotic stresses; thus, there could be other proteins with the GABA binding motifs that are regulated by GABA and enable plants to tolerate stress (Perez-Alfocea *et al.* 1994; Ramputh & Bown 1996; Rhodes *et al.* 1986).

The aim of this study is therefore to identify proteins other than the ALMTs that have GABA binding site/s. To identify proteins having putative GABA binding site/s, bioinformatic analysis was performed with a consensus sequence (DVFXXXXWXXEXL) based on the ALMT sequences from plants (Ramesh *et al.* 2015) using BLAST search. The sequence was used to probe plant genome databases via the BLAST program in NCBI (https://www.ncbi.nlm.nih.gov/). BLAST searches all the databases in plants that have similar sequences until the cluster of residues in the consensus are fully or partially aligned. It can be performed on either amino acid or nucleotide sequences. The results from this sequence alignment help to predict the function of proteins either individual, families or even the entire protein complement of a newly sequenced organism (Alberts *et al.* 2002). Only proteins that have a high e-value and shows 80% are more of query covered were selected as potential candidates.

3. Expression of TaALMT1 in *Pichia pastoris*

Structural study of different proteins is carried out using heterologous expression in *S. cerevisiae*, *Pichia pastoris* or insect cells and involves various molecular techniques. The structural studies of GPCRs were carried out in insect cell based system but it is more expensive and time-consuming than *P. pastoris* (Maeda & Schertler 2013). *P. pastoris* showed better expression of fungus high affinity phosphate transporter PiPT compared to *Saccharomyces cerevisiae and* seems to be an ideal expression system for membrane protein expression for structural studies (Pedersen et al. 2013).

Molecular techniques for protein expression consist of various steps, from cloning into vector, transformation into expression system to purification of the proteins. In this study, wheat gene TaALMT1 was expressed in the eukaryotic expression system *P. pastoris* which is widely used for the biopharmaceuticals and complex protein production due to its ability to perform post-transcriptional modifications (Spadiut & Herwig 2014). It can be grown on either methanol or glucose and under fermentation conditions, high cell densities can be maintained. Initially *P. pastoris* was used to isolate single-cell proteins (Wegner 1983). Later in 1988, it was genetically modified to get high yield of industrially and medically important proteins (Cregg & Madden 1988). This system can be used to produce large quantities of the required protein at very low cost, with easy harvesting and storage (Lueking et al. 2000). In *P. pastoris,* medium density cultures can be achieved which reduces proteolysis and cellular stress related with high density cultures (Jahic *et al.* 2003; Mattanovich *et al.* 2004). Furthermore, when human Aquaporin 1 (hAQP1) was expressed in *P. pastoris* two times more proteins was produced and it was correctly localised to the membrane (Byrne 2015). Therefore, *P. pastoris* strain is more suitable for the expression of membrane proteins. Hence, it is widely used in molecular biology for the study of expressed foreign proteins.

4. Methods

4.1 Bioinformatic analysis

The consensus sequence DVFXXXXWXXEXL generated from sequence alignments of ALMTs with mammalian $GABA_A$ receptor was used in a BLAST program in NCBI (https://www.ncbi.nlm.nih.gov/) to search twenty different plant genome databases from both monocot and dicot plants such as wheat, barley, pea, chickpeas, and parsley. BLAST searches all the databases in plants that have similar sequences until the cluster of residues in the consensus are fully or partially aligned. The full-length sequences that showed high e values 190 and the proteins other than ALMTs were aligned with Clustal X using the program Geneious Prime and a consensus of putative amino acids (13 residues) was extracted from these sequences. Sequences that showed at least 80% similarity to the consensus sequence were selected for further analysis.

The extracted sequences were then aligned using the program Geneious Prime to generate a sequence alignment that shows the highly conserved amino acids

4.2.1 Primer design and Polymerase Chain Reaction (PCR)

The primers were designed to the nucleotide sequence of wheat TaALMT1. Both the forward and reverse primers were designed to amplify the full-length wheat specific gene starting from ATG to end of the sequence, and the expected size of the product was 1380 bp. To design the primers, primer 3 (https://bioinfo.ut.ee/primer3-0.4.0/) a web-based program was used with the primer size 33-35 bases and <50% GC content; the restriction sites *Xho*I and *Xba*I were added to the forward and reverse primers respectively and the primers were synthesised by Sigma. For the amplification of the gene, the melting temperature of the primer was checked using NEB Tm Calculator. Both the primers were mixed and was diluted 1/10 from 100 µM to make 10 µM and was stored at -20°C until further use. For the amplification of the required gene,

proof-reading Taq polymerase (Phusion, NEB) and the gene specific forward and reverse primers were used. Master mix was prepared for a total PCR reaction volume of 50 µl where 5µl of 5 X buffer Phusion, 1µl of TaALMT1 template DNA or TaALMT1 F213C template DNA (DNA sequences in the Appendix, page 68), 1µl each of forward and reverse primers and dNTPs, 0.5µl of Phusion HiFi Taq and 41.5µl of Milli Q water were mixed. Touch down (TC) PCR reaction was run at 98°C for 2 minutes for initial denaturation followed by another cycle of 98°C for 30s (denaturation), 58-68°C for 30s (annealing), 72°C for 30 seconds (extension); followed by 1 cycle of 72°C for 5 minutes for final extension with the total of 30 cycles and hold at 4°C. The amplified PCR product was stored at -20°C. For the analysis of the PCR, gel electrophoresis was performed.

4.2.2 Gel electrophoresis

Gel electrophoresis was used to analyse the PCR products. 0.8 % i.e., 0.8 g of agarose was used to make 100 ml of gel in 1X TAE with 2.5µl of Gel Red (Biotium) as a DNA binding and Fluorescing agent. An aliquot of 5µl of PCR products were loaded onto the gel and electrophoresed in 1X TAE as running buffer. The gel was run for 45 minutes at 85 V followed by visualisation and imaging using Gel Doc EZ imager (BioRad). Amplified PCR products were gel purified using MinElute reaction clean up protocol (Qiagen) or the Wizard Gel and PCR clean-up system (Promega).

4.2.3 Cloning

The vector pPICZB was used for the cloning of the amplified PCR product into the multiple cloning sites *Xho*I and *Xba*I. The Figures 4 and 5 show the schematic representation of the cloning strategy used in this study.

After the analysis of the PCR product by gel electrophoresis, restriction digestion was performed for both PCR product and vector (pPICZB) with the same restriction enzymes *Xho*I

and *Xba*I. For this process, 10µl of cut smart buffer was used with 1.5µl of each restriction digestion enzyme, 25µl of insert (PCR product) or vector (pPICZB) and 62µl of Milli Q water to make the total volume of 100µl. After mixing all the ingredients, it was incubated at 37° C for 3 hours then heat inactivated at 65° C for 20 minutes.

To concentrate the product for better results, either reaction purification or gel purification of the products were performed by MinElute reaction clean up protocol or the Wizard Gel and PCR clean-up system. The products were quantitated using a nanodrop and the concentrations ng/µl and purity were recorded (260/280 and 260/230 ratios).

4.2.4 Ligation and Transformation

For ligation, both the purified PCR product and vector having high concentrations were chosen. Ligation calculator, Insilico http://www.insilico.uni-duesseldorf.de/Lig_Input.html was used for the calculation of the vector and insert in the ratio 3:1 and the ligations were set up as detailed in the Appendix with appropriate controls. For the transformation, competent cells of *E. coli* strain, DH5α were used which were stored at -80°C. 5µl of the ligated product was mixed with the competent cells and left on the ice for 20-30 minutes, followed by heat shock at 42° C for 45 seconds and kept back on ice for 2 minutes. Then, 400µl of low salt LB liquid (Appendix page 70) was added to the tube and mixed by flicking and incubated in a shaking incubator at 37° C for 1 hour. The transformation mix (10 and 100µl was then plated on low salt LB plates with 25µg/ml of selective antibiotic zeocin and the plates were sealed and incubated at 37° C, overnight.

4.2.5 Analysis of transformants

After the overnight incubation, the colonies on the plates were counted and compared with negative control. Only single and large colonies were selected for the Colony PCR to identify recombinant plasmids with the AOX forward and reverse primers. Prior to colony PCR, the

colonies were re-streaked on to low salt LB plates with 25µg/ml of selective antibiotic zeocin and grown at 37°C overnight for increasing the inoculum or cell numbers.

Thirty colonies were dropped into tubes containing 20µl of milli Q water each and heated to 95°C. A master mix was prepared with final volume of PCR reaction being 25µl and consisted of 5µl of 5 X buffer Phusion, 2.5µl of treated colonies, 1µl of forward and reverse primers and dNTPs each, 0.125µl Taq, 1.25µl of magnesium chloride ($MgCl_2$) and 14.4µl of Milli Q water. The PCR cycle was 98°C for 30s for initial denaturation followed by 37 cycles of 98°C for 30s (denaturation), 55°C for 30s (annealing), 72°C for 30 seconds (extension); followed by 1 cycle

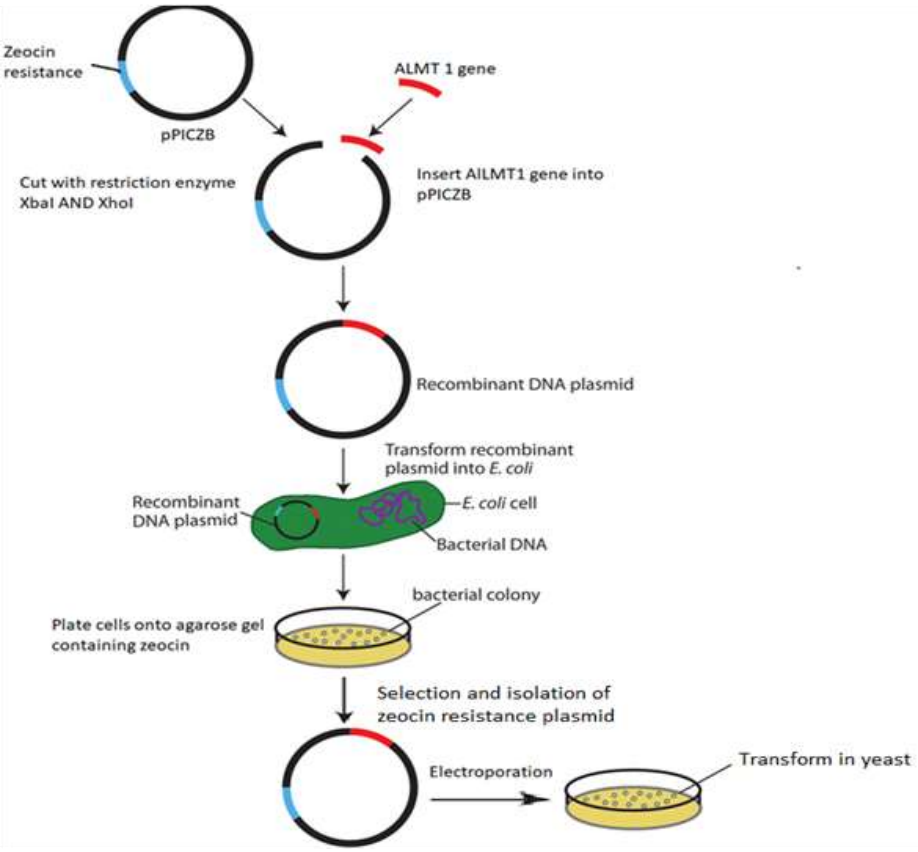


Figure 4. *Overall strategy for cloning and expression of TaALMT1 protein in Pichia pastoris.*

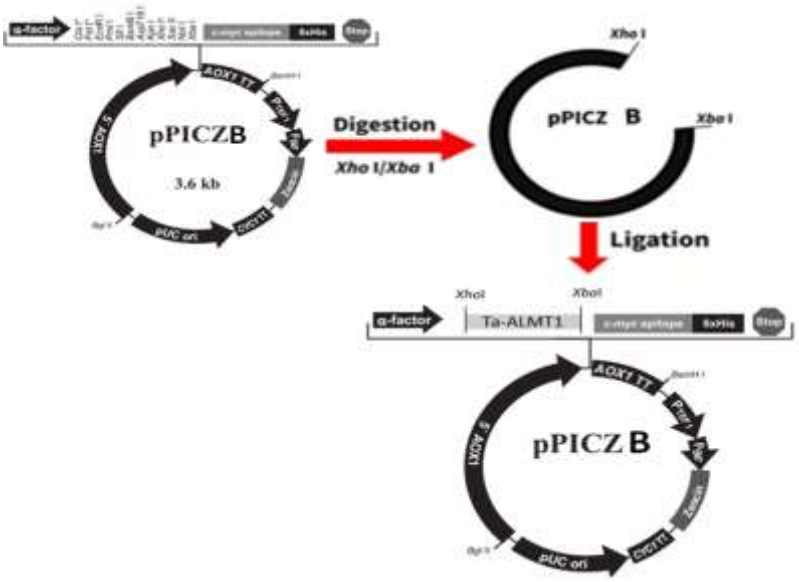


Figure 5. *Integration of TaALMT1 into the vector pPICZB after ligation.*

of 72°C for 10 minutes for final extension and 4°C hold. The PCR product was analysed via gel electrophoresis as described above (section 4.2.2). The transformants with the correct size amplified products were selected for further analysis.

A small amount of the re-streaked colony was picked with a toothpick and dropped into 5 ml of low salt LB with 25µg/ml of zeocin and grown overnight at 37°C in a shaking incubator. The liquid cultures were used to extract plasmid DNA using GenElute Plasmid Miniprep kit (Sigma) as per the manufacturer's instructions. Plasmid DNA was quantitated using nanodrop and 1 µg each of DNA with M13 F and R (Universal primers) was sent for Sanger sequencing (sequencing results in the Appendix, pages 70-71) to Australian Genome Research Facility (AGRF) to confirm integration of TaALMT1 into pPICZB.

4.2.6 Electroporation of TaALMT1 into *P. pastoris*

Plasmid DNA confirmed by sequencing was used to integrate TaALMT1 into P. pastoris for recombinant protein expression. 12µg of the plasmid DNA was dried down in heating block at 50°C to concentrate, followed by resuspension in 20µl of water. The DNA sample was

linearised using *Pme*I restriction enzyme by incubation at 37°C overnight, heat inactivated at 65°C for 20 minutes and run on a 0.8% agarose gel to confirm linearisation.

4.2.7 Ethanol precipitation

Linearised plasmid DNA was subjected to ethanol precipitation for purification. Briefly, 0.1 volume of 3M Sodium acetate (pH 5.2) and 2.2-2.5 volumes of ice-cold 100% ethanol was added to the linearised product, mixed using vortex and kept at -80°C overnight for precipitation. The samples were then centrifuged at full speed at 4°C for 25-30 minutes, supernatant was carefully pipetted off and 50-100ul of 70% ethanol was added and tubes were centrifuged for 10 min. The ethanol was removed carefully, tubes were air dried at 37°C for 15 minutes, resuspended in 15µl of water and stored at -20°C until further use.

4.2.8 Growth of *P. pastoris*

A single colony of *P. pastoris* strains (X33 and SMD1168) were grown in 5 ml YPD in a 50 ml sterile falcon tube at 30°C overnight. An aliquot of 0.1– 0.5 ml of the overnight culture was added to 500 ml of YPD in a 1 litre flask and grown overnight at 30 °C to an OD_{600} = 1.3–1.5. The cells were centrifuged at 1,500 × g for 5 minutes at 4°C and the pellets were resuspended with 500 ml of ice-cold, sterile water and centrifuged. Centrifugation process was repeated 2 more times followed by resuspension with 250 ml of ice-cold, sterile water, 20 ml of ice-cold, sorbitol (1M). The cells were resuspended in 1 ml of ice-cold 1 M sorbitol to a final volume of approximately 1.5 ml was added to the pellets and the cells were kept on ice and used on the same day.

4.2.9 Electroporation

80µl of the prepared cells were mixed with 10µg of linearized DNA (in 5–10µl sterile water) and transferred to an ice-cold 0.2 cm electroporation cuvette (BioRad). The cuvettes were

incubated with the cells on ice for 5 minutes, then the electroporation was carried out using a BioRad Micropulser with a charging voltage of 1.8 KV. Immediately 1 ml of ice-cold 1 M sorbitol was added to the cuvettes. The cuvette contents were transferred to sterile 15-ml tubes and incubated at 30°C without shaking for 2 hours. After 2 hours, the 50, 100, and 200µl each of the cells were plated on separate, labelled YPD plus 1 Molar (M) sorbitol (YPDS) plates containing 100µg/ml Zeocin for X33 strain, for SMD1168, YPDS + 1 % glycerol, MDH, MDH + 1 % glycerol MMH and MMH + 1 % glycerol and the plates were incubated for 3–10 days at 30°C until colonies / transformants were visible. Details of the plates in the Appendix, pages 71-73.

4.2.10 Screening of transformants

After 3 days of incubation, 40 colonies from each plate were taken and further streaked on to plates containing the respective media with 500µg /ml Zeocin and further incubated for another 3 days at 30°C. Then, a small amount cells were picked with a toothpick and re-suspended in 20µl of 20 mM NaOH and boiled at 95°C for 10 minutes. The tubes were centrifuged and 2.5µl of supernatant was used for the colony PCR with 0.125µl of Go Taq polymerase (Promega), 1µl of forward and reverse AOX primers, 5µl of buffer, 0.5µl of dNTPs and remaining water making the final volume to 25µl in each reaction. The PCR cycle was set-up with initial heating at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 30 second, annealing at 55°C for 30 second and extension at 72°C for 30 second with the final extension at 72°C for 5 minutes. After PCR, 5µl of the samples were run on 0.8 % agarose gel with 3µl of loading dye and 2µl of 1 kb DNA ladder. Due to problems with Go Taq polymerase (Promega), Thermopol Taq polymerase (NEB) was used in later screen of transformants via colony PCR (detailed in the results section 5.3.5).

4.2.11 Induction of protein expression

After confirming integration of TaALMT1 into *Pichia*, 5 colonies were selected for each of the strains; 5 ml liquid cultures with 100 µg/ml of zeocin were set up using BMGY for X33 strain, and MGHY for the SMD1168 strain of the *Pichia* and grown overnight at 30°C. These liquid cultures were used to spike 50 ml of culture medium as described above for overnight culture in 250ml flasks. The 50 ml overnight cultures were centrifuged at 3000g for 5 minutes and resuspended in 50 ml of BMMY and MMH with 100µg/ml of zeocin and transferred to 250 ml baffle flasks for the induction of protein. Methanol (0.5%) was added to the culture flasks every 24 hours for 5 days. An aliquot 10 ml of each liquid culture was sampled from the 50 ml culture for 5 days i.e., 24, 48, 72, 96, and 120 hours to check for induction of protein expression. The aliquoted 10 ml samples were centrifuged at 3000g for 5 minutes and the pellets were stored at -80°C until further use.

4.2.12 Breaking of *Pichia* cells

For the breaking of the cells, acid washed, 425-600 microns glass beads were used. Approximately the same weight of glass beads as the pellets were added to 2 ml Precellys tubes specially designed for use with Precellys 24 homogenizer. Frozen Pichia cell pellets were thawed on ice, resuspended in buffer + protease inhibitor (details in Appendix, page 74) in approximate volume equal to the weight of pellet. The cells were transferred to the 2 ml tubes with glass beads. Then tubes were placed in the homogenizer and set to shake at 5000 g for 30s at 4°C, to break open the cells. This process was repeated 3 times. After that, centrifugation was carried out at 3000rpm for 5 minutes. The supernatant was used to estimate the amount of protein in the samples using Pierce Protein assay kit. Briefly, 150µl of Protein assay reagent was added to 1:10 dilution of the samples; BSA was used at different concentrations i.e., 2000, 1000, 500, 250, 125, 62.5µg/ml to generate a standard curve and for negative control Protein

assay reagent was used and the absorbances were measured at 660 nm in a BMG Omega microplate reader.

4.2.13 Dot blot

5 µl of undiluted supernatants from each sample were placed on a nitrocellulose membrane (Amersham™ Protran ® Premium 0.45 µm Nitrocellulose membrane, 300 mm X 4m) having 0.4µm permeability and the samples left to air dry at room temperature for 30 minutes. After 30 minutes, the membrane was placed in 1X TBS tween for couple of minutes to wet, then blocking buffer (5% skim milk +1X TBS tween) was added and kept for shaking in a shaker for 1 hour at room temperature. After 1 hour, the blocking buffer was discarded and primary antibody i.e., Anti-His Rabbit antibody (dilution 1:1000, Cell signalling Technology) was added and placed at 4°C on a shaker, overnight.

After overnight incubation, the membrane was washed with 1X TBS 3 times for 10 minutes each time at room temperature. Then, secondary antibody i.e., Goat anti-Rabbit (dilution 1:5000, Cell signalling Technology) was added and kept for 1 hour on a shaker at room temperature. After that it was washed with 1X TBS tween 3 times for 10 minutes each, dried and visualised in ODESSEY CLx (LI-COR).

4.2.14 Coomassie stain and Western Blot

After dot blot, the samples with the higher expression of the protein were chosen for Western blot. 20 µg of the total proteins from each sample were mixed with 10µl of Laemmli sample buffer (BioRad) in the first attempt but no results were obtained (result section 5.3.8 and Figure 17). Therefore, amount of protein loaded was increased to 40 µg for each sample and mixed with 10µl Laemmli sample buffer. The mixture was heated at 100°C for 10 minutes. After 10 minutes the samples were loaded into the wells of 12 % Tris-SDS gel along with the protein ladder and purified human AQP1 protein as the positive control. The samples were allowed to

run for an hour at 170 V. The gel for Coomassie stain was stained with the Coomassie (40 % Methanol, 10 % Acetic acid and 0.1 % Brilliant Blue) whereas the gel for Western Blot was placed onto a nitrocellulose membrane subjected to a wet transfer for 90 minutes at 60 V at 4°C. After 90 minutes, the nitrocellulose membrane was blocked with 20 ml of blocking buffer (5% skim milk + 1X TBS tween) for an hour at room temperature. The Anti-His Rabbit antibody (dilution 1:1000) was added and the membrane placed on a shaker overnight at 4°C. After an overnight incubation, the membrane was washed 3 times with the blocking buffer and incubated with Goat anti-rabbit secondary antibody for an hour with at room temperature.

5. Results and Discussion

5.1 Aim 1: To identify other plant proteins with putative GABA binding sites.

From the 13 amino acids identified, it was observed that Valine, Phenylalanine and Tryptophan were highly conserved in all the plants (Figure 6). Previous studies have shown that the aromatic amino acid Phenylalanine (F) at position 3 in the alignment is important for GABA regulation in the wheat protein TaALMT1 (Ramesh et al., 2015). Site directed mutagenesis of this amino acid to cysteine has been shown to impair GABA regulation of TaALMT1 and grapevine ALMT – VvALMT9. Similar results have been shown in mammalian $GABA_A$ receptor wherein site directed mutation of phenylalanine to cysteine results in GABA sensitivity i.e., abolish protein function (Boileau *et al.* 1999; Sigel *et al.* 1992). In the review published in 2017 (Ramesh *et al.* 2017), sequence alignment shown consists of plant proteins mainly ALMTs and kinases from Arabidopsis with putative GABA binding site whereas in this research, putative GABA binding site was identified in plants proteins other than ALMTs from both monocot and dicots. But, without functional characterization of these proteins, it is not possible to categorise these proteins as regulated by GABA.

The proteins identified in this study include a diverse array such as ACT-like proteins Serine/Threonine kinases, Inorganic phosphate transporter, Cycloartenol, cytokine oxidase/dehydrogenase, NADH dehydrogenase, tyrosine-protein kinase, high affinity sulphate transporter, Zinc finger like, potassium channel and NADH-plastoquinone oxidoreductases (Table II). Among them, Serine/threonine like proteins were the top hits in most of the plants. The diversity of proteins identified with putative GABA binding sites suggests that GABA may directly or indirectly regulate the activity of these proteins under certain environmental conditions.

The proteins identified from barley, rice, chickpeas, pumpkin, cucumber, and sunflower shown (Figure 6 and Table II) are different isoforms of Serine/Threonine protein kinases. In the cells, protein kinases phosphorylate or dephosphorylate proteins for the regulation of the cellular functions. Protein kinases in the living organisms are abundant and participate in cell signalling mechanisms (Akamine *et al.* 2002). The MAPK (Mitogen-Activated Protein Kinase i.e., serine/threonine protein kinase) cascades are the major pathways for the intracellular responses stimulated by the extracellular stimuli such as osmotic shock, growth factor, cytokines, ultraviolet irradiation (UV), toxin, and so on in eukaryotes (Herskowitz 1995; Ip & Davis 1998). In various plant species, MAPK homologues have been isolated and are found to be involved in responses to environmental stimuli such as osmotic and UV irradiation (Mizoguchi *et al.* 1997).

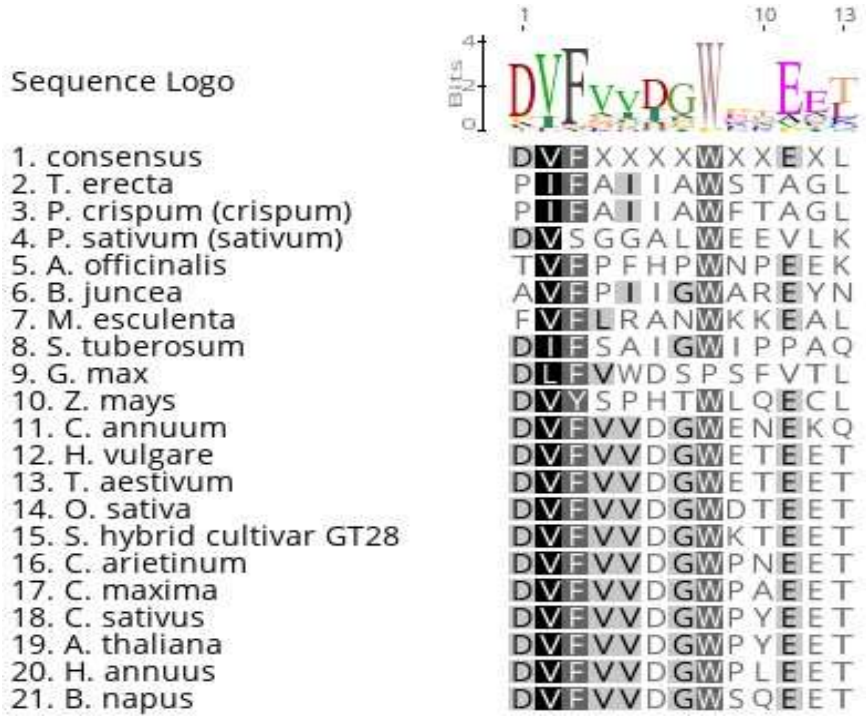


Figure 6. *Sequence alignment of putative GABA binding sites extracted from full length amino acid sequences after a BLAST search against 20 different plant genomes. Shown here are extracted sequences from both monocot and dicot plants.*

In tobacco plants, the activation of tobacco MAPK is involved in defence against the multiple pathogen invasion (Yang, K-Y *et al.* 2001). Recent research in Arabidopsis with the isoform of Serine/Threonine protein kinase STY46 showed that it was induced by abiotic stresses such as salinity and osmotic stress, by prolonged darkness to induce a sugar deficit during a 48hours study period with additional 24hours darkness after 12/12hours day/night photoperiod and exogenous abscisic acid treatments, and under reduced carbon availability (Dong et al., 2020). The results suggest that this kinase could be the part of the salinity stress response pathway that utilises starch (that act as a sugar source) during early plant growth (Dong *et al.* 2020).

Table II: 20 different plant proteins with putative GABA motifs identified via Blast search.

Name	Type of protein	Full Form	ACCESSION NUMBER
H. vulgare	Serine/threonine-protein kinase HT1	*Hordeum vulgare* (Barley)	KAE8777147.1
C. arietinum	Serine/threonine-protein kinase STY46	*Cicer arietinum* (Chickpea)	XP_004497215.1
C. maxima	Serine/threonine-protein kinase STY46	*Cucurbita maxima* (Pumpkin)	XP_022990296.1
C. sativus	Serine/threonine-protein kinase STY46	*Cucumis sativus* (Cucumber)	XP_004144866.1
H. annuus	serine/threonine-protein kinase STY46	*Helianthus annuus* (Common sunflower)	XP_035846319.1
O. sativa	Serine/threonine-protein kinase STY8	*Oryza sativa* (Japanese rice)	XP_015636462.1
Z. mays	Cycloartenol Synthase	*Zea mays* (Maize)	PWZ43462.1
T. aestivum	unnamed protein product	*Triticum aestivum* (Wheat)	SPT21144.1
P. sativum	cytokinin oxidase/dehydrogenase 1	*Pisum sativum* (Pea)	ABK32520.1
M. esculenta	protein DETOXIFICATION 33-like	*Manihot esculenta* (Cassava)	**XP_0215993431**
S. hybrid cultivar GT28	constitutive triple response 1 protein	*Saccharum hybrid* cultivar	QGQ60456.1
T. erecta	NADH dehydrogenase	*Tagetes erecta* (African marigold)	AAC37463.1
B. napus	tyrosine-protein kinase	*Brassica napus* (Rape seed)	NP_001303099
B. juncea	high-affinity sulphate transporter 1;1	*Brassica juncea* (Mustard)	AFX60922.1
G. max	zinc finger protein CONSTANS-LIKE 13-like	*Glycine max* (Soybean)	NP_001278943.1
A. thaliana	protein kinase like protein	*Arabidopsis thaliana*	CAB37503.1
C. annuum	putative hippocampus abundant transcript-like protein 1-like	*Capsicum annuum* (Capsicum)	KAF3660808.1
S. tuberosum	inorganic phosphate transporter 1	*Solanum tuberosum* (Potato)	CAA67395.1
A. officinalis	potassium channel KOR1	*Asparagus officinalis* (Asparagus)	XP_020269274.1
P. crispum	NADH-plastoquinone oxidoreductase subunit 5	*Petroselinum crispum* (Parsley)	YP_004733890.1

The results suggest that this kinase could be the part of the salinity stress response pathway that utilises starch (that act as a sugar source) during early plant growth (Dong *et al.* 2020). Similarly, the HT1 (High Leaf Temperature) isoform of the serine/threonine plays a main role in abscisic acid (ABA) independent CO_2-induced stomatal regulation (Hashimoto *et al.* 2006; Hõrak *et al.* 2016). Thus, the Serine/Threonine protein kinases seem to be important for the stress tolerance in plants. It is of interest that under stresses such as salinity, there is rapid increase in GABA concentrations along with induction of other tolerance pathways (Perez-Alfocea *et al.* 1994). Thus, it is probable that if there are proteins such as STY46 that have a have a role in salinity tolerance and show putative GABA binding motifs, they may be regulated directly or indirectly by increasing GABA levels.

Stresses in plants can be both biotic and abiotic such as heat, cold, hypoxia, salinity, drought, virus infection and so on (Perez-Alfocea *et al.* 1994; Ramputh & Bown 1996; Rhodes *et al.* 1986). To cope with these stresses, plant adopt various mechanism or signalling pathways that are mutually interconnected molecular cascades and networks that allow plants to maintain their growth and development (Mulligan *et al.* 1997). An essential signalling cascade i.e., multistep phosphorelay (MSP) in plants, helps to incorporates a huge range of both exogenous and endogenous stimuli that could be both hormone dependent and independent and helps to stimulate essential plants hormones for the balanced growth and development of the plants and its adoption towards different stresses (Pekárová *et al.* 2016). Different types of the plant hormones (cytokinin, ethylene and abscisic acid) were found to be regulating the MSP however, cytokinin was observed to be the key regulator of the MSP activity (Pekárová *et al.* 2016). Studies have shown that stress induced accumulation of GABA may be involved in cross talk with cytokinin, abscisic acid, ethylene, auxin, and other plants hormones (Baum *et al.* 1996; Kathiresan *et al.* 1997; Lugan *et al.* 2010; Pospíšilová *et al.* 2016).

Abscisic acid (ABA),is essential plant hormone which is required for growth and development of plants under normal condition whereas in abiotic stresses it plays a vital role as a endogenous messenger to cope with the stress (Lalk & Dörffling 1985). Under stress conditions, ABA induces the defence mechanisms by regulating the stomatal apertures which activates the Ca^{2+} influx and increases concentration of the cytosolic Ca^{2+} (McAinsh *et al.* 1990). Increased level of Ca^{2+} accelerate the dimerization of C-terminal domains induced by Ca^{2+}-CaM, that activate Glutamate decarboxylase (GAD) (Snedden *et al.* 1996). GAD is an enzyme that helps to convert glutamate (Glu) into GABA. The protein from *T. erecta* and *P. crispum* (Table 1) ACT-LIKE Nicotinamide adenine dinucleotide hydrogen (NADH) dehydrogenase negatively regulates the enzyme succinic-semialdehyde dehydrogenase (SSADH) involved in GABA catabolism in the mitochondria (Bouché, Fait, *et al.* 2003; Busch *et al.* 2000). NADH is a complex molecule involve in oxidative metabolism to produce ATP in mitochondria and it is 6a component of NADH-ubiquinone oxidoreductase (Complex I) (Hatefi & Stempel 1969). In the process of the energy production, NADH is oxidised to NAD^+ by NADH dehydrogenase which is used to transfer proton to the intermembrane space from the mitochondrial matrix (Weiss *et al.* 1991). In peas, NADH dehydrogenase complex located in thylakoids has been shown to be involved in protection from oxidative stress and the oxidation of NADH to reduce plastoquinone was found to be mediated by Ndh complex (Sazanov *et al.* 1998). In Arabidopsis, NADPH oxidase is involved in the formation of the reactive oxygen species (ROS), which activates Ca^{2+} channels and regulates plant cell expansion (Foreman *et al.* 2003).

Several studies have shown that ABA, auxin, phytotoxins, gravity and many more stimuli can induce ROS production (Bais *et al.* 2003; Joo *et al.* 2001; Pei *et al.* 2000). In Arabidopsis guard cells, production of H_2O_2 is induced by ABA and if the production of H_2O_2 is blocked then the stomatal closure is inhibited (Pei *et al.* 2000). Production of the ROS in both mammalian and

plants, boosts the phosphorylation and activation of several proteins including MAPK which mediate specific defence mechanisms (Kovtun *et al.* 2000; Sundaresan *et al.* 1995).

Stomatal closure of the plants also depends on the uptake of the potassium via the potassium channels which regulate the turgor of guard cells (Zeiger *et al.* 1987). ABA regulated stomatal closure results from decrease in guard cell turgor due to loss of anions and potassium from the guard cells (MacRobbie 1992). Potassium channels are a diverse group of membrane protein family that are distributed in almost all cell types, help in generation of electrical signals by allowing the potassium ions to flow selectively and rapidly throughout the cell membrane and control large number of cellular functions (Jan 1999). In plants potassium, plays a vital role in maintenance of osmotic processes, cytosolic pH, elongation of cells, translocation of photosynthates and it is also a very important factor for the tolerance of various stresses such as salinity, fungal diseases, and drought (Amtmann *et al.* 2008; Baker & Weatherley 1969; Perrenoud 1977). In mammalian brain, $GABA_B$ receptors function through G-proteins by modulating Ca^{2+} and K^+ channels and are involved in various physiological processes such as gating and desensitization, inwardly rectifying K^+ currents in neurons and transient receptor potential channel expression (Gao *et al.* 2007; Lainez *et al.* 2010) whereas in plants not much is known about the GABA regulation of K^+ channels.

In Arabidopsis, ABA signalling is interfered by Zinc Finger Protein 3 during seed germination and plant development by negatively regulating the expression of several ABA-induced genes (Joseph *et al.* 2014). Zinc is an essential element that helps in the correct folding of proteins and is also required for the recognition of specific DNA as the distinct classes of zinc finger (GATA-type, Gal4-type) acts as nucleic acid binding modules and binds with the double stranded DNA (Lowry *et al.* 2009; Sabbah *et al.* 1987). The zinc is either coordinated by four Cys ligands or two Cys and two His ligands (Evans & Hollenbergt 1988). In the eukaryotic genomes, Zinc finger proteins are the one of the most abundant proteins that have

extraordinarily diverse functions which includes RNA packaging, DNA recognition, regulation of apoptosis, transcriptional activation, lipid binding, protein folding and assembly (Calver *et al.* 2000; Choo & Klug 1993; De Guzman *et al.* 2000). In plants such as Arabidopsis, rice and wheat, Zinc Finger proteins have been shown to improve tolerance to salt stress either by regulation of downstream ion-transport-related genes or by increasing the excretion of sodium ions (Na^+) and reducing stomatal apertures (Huang *et al.* 2000; Ma *et al.* 2016; Sakamoto *et al.* 2004). Zinc finger proteins are also found to be involved in other stresses such as osmotic stress, cold, drought, oxidative stress and high light stress in the research mainly carried out in Arabidopsis and plants such as wheat, potatoes, rice and soybeans (Davletova *et al.* 2005; Kim *et al.* 2016; Mittler *et al.* 2006; Tang *et al.* 2013; Xu, D *et al.* 2008). In this study (Table II), soybean ACT- like Zinc finger protein has been identified to have a putative GABA binding site but limited information is available for the connection between GABA and Zn in plants whereas in animals, Zn^{2+} is suggested to have important modulatory role in epilepsy and negatively regulates GABA by inhibiting GABA receptor at mossy fiber synaptic varicosities, (Carver *et al.* 2016; Coulter 2000; Ruiz *et al.* 2004). Several studies have shown the interconnection between the homeostasis of Zn and phosphate transporters in many crop species like tomato, maize, wheat, and Arabidopsis and shown that under Zn deficient conditions, plants over accumulate Pi in the shoot and vice-versa (Huang *et al.* 2000; Loneragan *et al.* 1982; Reed 1946). However detailed information on the molecular basis of the interactions between Pi and Zn is still poorly understood (Huang *et al.* 2000; Watts-Williams *et al.* 2013). Phosphate is a very essential nutrient required for plant growth and phosphate deficiency limits the crop yields (Tasaki *et al.* 2002). Phosphorous availability tightly controls the expression of PHT 1 a high-affinity phosphate transporter gene at the transcriptional level (Muchhal & Raghothama 1999). The family of inorganic phosphate transporters in Arabidopsis are involved in Pi uptake and its distribution from roots to shoots

especially under low phosphorous (Remy et al. 2012). In acidic soils with low pH, phosphorous is found in highly stable complexes that limit utilisation by plants and affects root architecture. Interestingly GABA concentrations increase with decrease in pH and thus it is possible that changing GABA concentrations may regulate the activity of phosphate transporters leading to increased mobilisation of Pi (Din *et al.* 2021; Sasaki *et al.* 2004; Wang *et al.* 2015).

In another study with Arabidopsis it was observed that there was a significant co-relation between the sulphate and ABA metabolism as the sulphate acts as primary source to promote the anti-transpiration effect of ABA in leaves that helps plants cope with water and salt stress (Cao, MJ *et al.* 2014; Ernst *et al.* 2010). In plants, sulphate is the main source for the synthesis of the amino acids containing sulphur that can be used in the assimilatory pathway. In nature, sulphur is usually available in the reduced form, therefore sulphate may become limiting for the growth of plants. In most of the higher plants, high affinity sulphate transporters help in the acquisition of sulphate into roots (Hawkesford 2003; Smith *et al.* 1997; Takahashi *et al.* 2000) and sulphate transporters initiate the defence mechanism against strong oxidative stress by mediating sulphate transport (Zuber *et al.* 2010). In rice, high affinity sulphate transporter was shown to be involved in tolerance against various heavy metal stress (Kumar *et al.* 2019). Sulphur is involved in the detoxification of the heavy metals and ROS and its metabolism is directly connected to ethylene and polyamine related stress responses by Methionine salvage cycle (Bürstenbinder *et al.* 2007; Xiang & Oliver 1998). Furthermore, in studies with elevated arsenic (As) on carbon, nitrogen and sulphur metabolism and GABA shunt activity showed that the level of the GABA determines the stress tolerance; maintains the supply of succinate and fumarate for Tri-Carboxylic acid (TCA) cycle; allows proper nitrogen and amino acid metabolism and modulation of sulphur metabolism for the detoxification (Pathare *et al.* 2013). These results show some link between sulphate and GABA, but further studies are necessary for better understanding if GABA is involved in regulating the sulphate metabolism. Under

abiotic stress, cytokinin (CK) levels in the plants decrease which leads to the senescence of the leaves and shoot growth inhibition (Yang, J *et al.* 2002). Treatment with cytokinin in tobacco has shown to delay leaf senescence (Van Staden *et al.* 1988). Chemically cytokinin, are N6 substituted purine derivatives. These are the types of plants hormones which help in the regulation of the huge number of developmental functions like root and shoot branching, development of leaves and also helps in chloroplast development (Mok 1994) whereas cytokinin oxidase/dehydrogenase play an important role in regulating the cytokinin levels in different plants likes barley (Zalewski *et al.* 2014), maize (Brugière *et al.* 2008), wheat (Ogonowska *et al.* 2019) and rice (Ashikari *et al.* 2005). Cytokinin oxidase/dehydrogenase in Arabidopsis and tobacco exhibit an important role to tolerate the excessive stress created by drought and salinity (Werner *et al.* 2010). Tobacco plants with zinc tolerance gene i.e., isopentyl transferase (*ipt)* with SAG12 promoter show elevated CK production under drought and salinity, both stresses in which GABA levels increase. This suggests that there may be a crosstalk between GABA and CK to regulate stress tolerance in plants (Pavlíková *et al.* 2014). Under various stress conditions, ABA is accumulated as a result of modification of xylem differentiation mediated by T-Spm (Thermo spermine) which also modulates auxin signalling (Yoshimoto *et al.* 2016). The research conducted in 2017 suggests that polyamine (PA) oxidase enzyme is involved in tightly controlled interplay between CKs and auxin which is necessary for the proper differentiation of xylem (Alabdallah *et al.* 2017). Interactions between hormone production, GABA accumulation and polyamine metabolism is likely to change various plant physiological processes and it requires further research to explore the cross talk between all these (Podlešáková *et al.* 2019).

Like ABA and CKs, Ethylene (ET) also accumulates in response to the same biotic and abiotic stresses in which GABA levels also increase (Review (Podlešáková *et al.* 2019) . ET is produced from the activated form of methionine i.e., S-adenosyl-methionine (SAM), a

precursor also for the synthesis of polyamines (PA). Several studies were conducted to study the connection between GABA and ET; in sunflower application of exogenous GABA enhanced 1-aminocyclopropane-1-carboxylic acid (ACC) transcript levels and resulted in a 14-fold increase in ET production (Kathiresan *et al.* 1997). In the same study 6-8 days old seedlings were excised and treated with different chemicals such as l-glutamate, L-Alanine, and GABA, it was observed that the time for the accumulation of GABA post treatment was only 30s whereas level of the ET increased after 20 min, (Kathiresan *et al.* 1997). Similarly in another study with pathogen infection with *Pseudomonas syringae* and *Alternaria brassicicola* in *Arabidopsis* revealed that GAD4 was strongly upregulated along with jasmonates (JA), Salicylic acid (SA) and ET after first 12- and 24-hour infection (De Vos *et al.* 2005).

5.2 Conclusion and Future work

From the above discussion, it is evident that the proteins identified by BLAST search (Figure 6 and Table II), have been shown to be involved in stress tolerance in various studies in plants. Interestingly GABA accumulation occurs under many of the same stresses, so it is not surprising that many of these proteins show putative GABA motif or binding sites. There is already some evidence of cross talk between ethylene and GABA, but little evidence exists of GABA regulation of other identified proteins. The sequence alignment (Figure 6) shows conservation of key amino acids responsible for GABA binding, however no conclusions can be drawn until these proteins are heterologously expressed and characterised to study if GABA can regulate their activity. If GABA regulation is established, these proteins can be further studied with site directed mutagenesis and T-DNA knock out plants to confirm their role in stress tolerance.

5.3.1 Amplification of ALMT1 and cloning into vector pPICZB (Aim 2)

The TaALMT1 gene from wheat was amplified using the forward and reverse primers containing restriction site *XbaI* and *XhoI* and cloned into the yeast expression cloning vector pPICZB. The cloning vector consists of Myc, and His-tags in the C-terminus and the His-tag can be used in the purification of the protein. A DNA sequence of 1380 bp, for wheat ALMT, was obtained after touch-down Polymerase Chain Reaction (PCR) using ALMT F / ALMT R (TaALMT1 original) or the ALMT F1 / ALMT R1 (TaALMT1 F213C mutant) primers (Figure 7). Both the vector and PCR products were digested with the *XhoI and XbaI* as shown in the figures 9 and 10. After restriction digestion, the PCR products were ligated into the vector pPICZB.

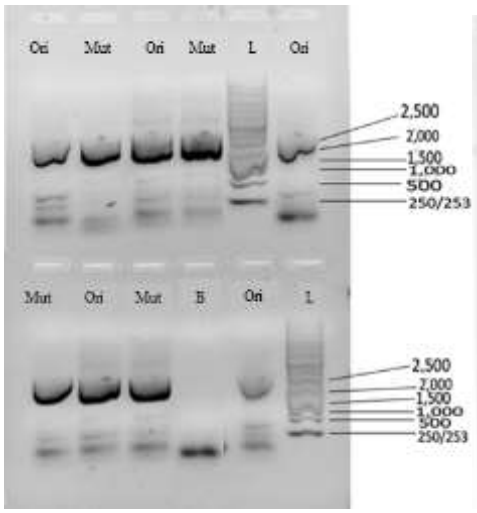


Figure 7

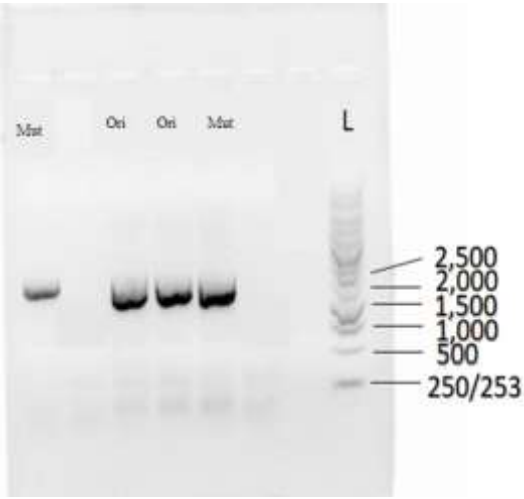
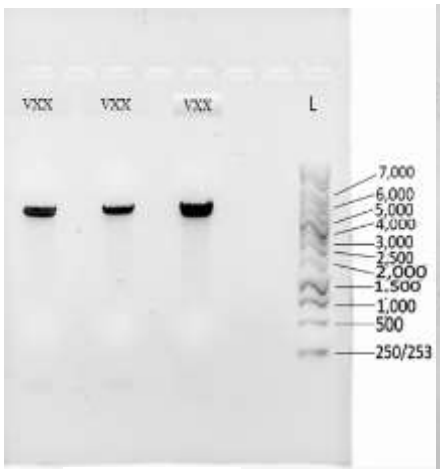


Figure 8

Figures 7 and 8*. **(7)** Gel electrophoresis after touchdown PCR showing the amplification of 1380 bp product for TaALMT1 original (Ori) and TaALMT1 F213C mutant (Mut). L is 1 Kb ladder (Promega) and the lane before the ladder is negative control i.e., water only.* ***(8)*** *Gel purified PCR products.*



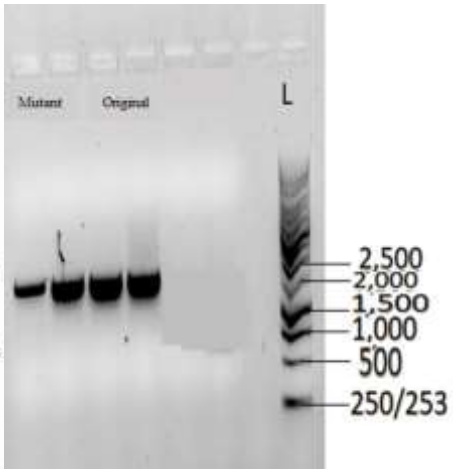


Figure 9 **Figure 10**

Figures 9 and 10. *Gel purified products after restriction digestion with XhoI and XbaI.* ***(9)*** *pPICZB.* ***(10)*** *Amplified PCR products.*

5.3.2 Transformation into *E. coli*

The TaALMT1 original and site directed mutant gene ligated into the vector pPICZB were transformed into the *E. coli* competent cell DH5α. The bacterial cells were grown on low salt LB at 37°C for overnight. After overnight incubation, some colonies were seen on the plates (Table III).

Table III*: Total number of the colonies grown in the low salt LB plates for each sample after transformation in E. coli.*

Sample	Volume plated (ul)	Number of colonies
Ta-Ori pPICZB	50	55
Ta-Ori pPICZB	100	189
Ta-Mut pPICZB	50	39
Ta-Mut pPICZB	100	120

The plasmid DNA was extracted from selected transformants and subjected to PCR analysis to confirm amplification of the correct sized insert as shown in Figure 11.

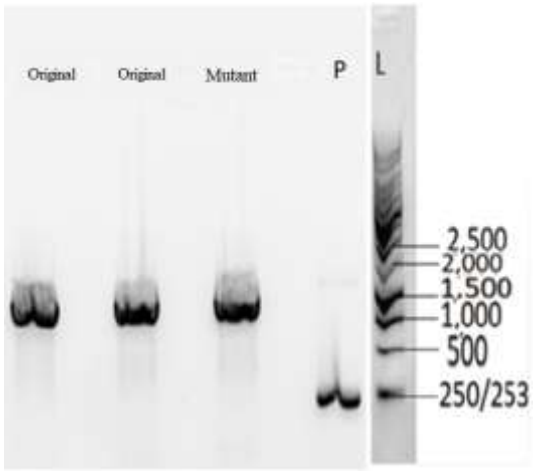


Figure 11*: Gel showing the correct sized (~1380) amplified products from colony PCR of the transformants.*

5.3.3 Sequencing

The recombinant pPICZB-ALMT1 transformants were confirmed by Sanger sequencing (details in the Appendix, pages 70-71).

5.3.4 Linearisation and electroporation into *P. pastoris*

The plasmid DNA (12µg each) from the transformants confirmed after sequencing were linearized using the restriction enzyme *Pme*I (Figure 12) and ethanol precipitated to concentrate the pDNA. Approximately 7-8 µg of pDNA could be obtained after purification from 12µg; quantitation of plasmid DNA (pDNA) is shown in Table IV.

Purified pDNA was then electroporated into competent *P. pastoris* X33 and SMD1168 strains, grown in different media with zeocin (100 µg/ml) at 28-30° C for 3 days. In comparison to X33, SMD1168 strain of *Pichia* showed very tiny and large numbers of colonies. SMD1168 strain of *Pichia,* is histidine deficient therefore, minimal media with histidine MDH + 1% glycerol, MMH + 1% glycerol and YPDS + 1 % glycerol were used. The reason to use different media was because there is less information about SMD1168 strain of *Pichia* and in some papers YPDS was used while in others MDH was used.

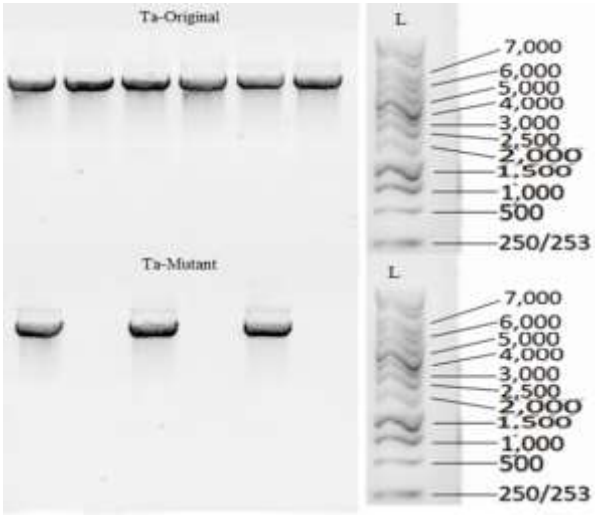


Figure 12: *Gel showing linearised pDNA after digestion with PmeI. Lanes in the top gel=Ta-ori pPICZB; lanes in the bottom part of the gel = Ta mutation TaALMT1 F213C; L = 1Kb ladder (Promega).*

Electroporation was performed several times, initially, low concentration of zeocin 25µg/ml instead of 100µg/ml of zeocin was used therefore, large number of colonies were observed and negative result in colony PCR. On the 4th attempt, 50-100 colonies were obtained in X33 strain of *Pichia pastoris* (Table V). Twenty to thirty colonies were selected and restreaked onto 500µg/ml zeocin but integration of pDNA could not be confirmed via colony PCR.

Even though colony PCR was unsuccessful, a few colonies were selected, grown in either BMMY or MMH for 24 hours and a dot blot was performed to check for protein expression. The results showed very faint expression of protein as shown in the Figure 13. Human Aquaporin 1(hAQP1) purified protein obtained from co-supervisor Dr Henderson, University of Adelaide was used as positive control for the dot blot and total protein extracted from untransformed X33 *Pichia* strain was used as the negative control.

Table IV: *Concentration of pDNA after linearisation and purification by ethanol precipitaion.*

Sample	Concentration (ng/µl)	260/280	Yield (µg)
Ta-Ori pPICZB 1	501.5	1.73	7522.5
Ta-Ori pPICZB 2	517.7	1.72	7765.5
Ta-Ori pPICZB 3	530.6	1.67	7959
Ta-Ori pPICZ B 4	445.3	1.72	6679.5
Ta-Ori pPICZB 5	446.3	1.75	6694.5
Ta-Ori pPICZB 6	453.5	1.74	6802.5
Ta-Mut pPICZB 1	475.1	1.70	7126.5
Ta-Mut pPICZB 2	519.1	1.64	7786.5
Ta-Mut pPICZB 3	439.9	1.71	6598.5

***Table V**. Number colonies obtained after electroporation into Pichia pastoris and plating on low salt LB agar plates with zeocin.*

Constructs	Volume of transformation plated (µl)	No. of colonies
Ta-Ori pPICZB	50	> 100
Ta-Ori pPICZB	100	> 300
Ta-Mut pPICZB	50	> 100
Ta-Mut pPICZB	100	> 250

5.3.5 Trouble shooting confirmation of *Pichia* integration

As the integration of gene of interest could not be confirmed with colony PCR, it was assumed that either the electroporation of the genes into *Pichia* strain X33 was not working or there was a problem with the colony PCR. Therefore, it was decided to repeat electroporation with another Pichia strain SMD1168 in addition to X33 and include positive control (BACP-8 His pDNA, obtained from A/Prof Peter Anderson, Flinders University for the electroporation and colony PCR). The positive control BACP-8 His showed the expected size product in the colony PCR, but no results were obtained for the TaALMT1 transformants.

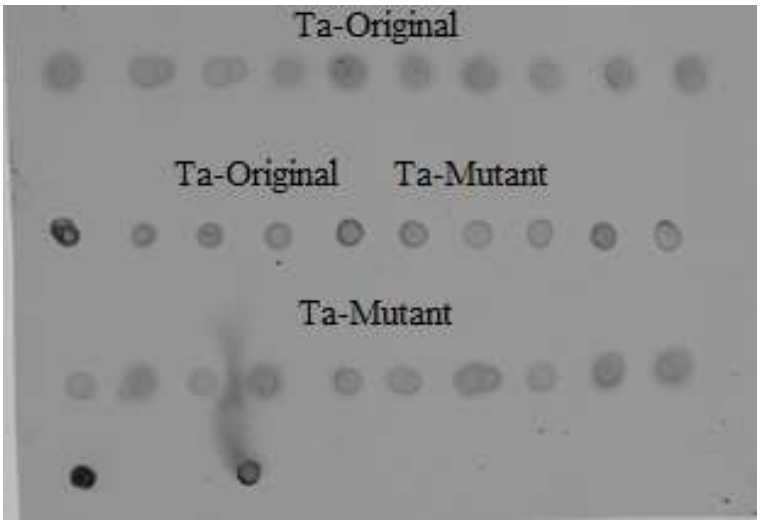


***Figure 13**. Dot Blot to check the induction of protein after 24 hours. 5ul each of total protein was used in the dot blot. Half of the samples were of Ta-Original pPICZB and other half samples were of Ta-Mutant pPICZB with aquaporin protein as a positive control and protein from X33 untransformed Pichia strain as negative control.*

A different strain of *Pichia,* SMD1168 was used for electroporation that showed tiny but large number of colonies. Single colonies were picked carefully from plates with 100µg/ml zeocin and streaked onto 500µg/ml of zeocin. Colony PCR was performed with 15 colonies from each construct which were treated as detailed in the methods section 4.2.10, positive control pDNA (TaALMT 1 pPICZB and BACP-8 His), empty plasmid pPICZB and negative control water. The PCR products were once again only amplified in the positive control. These results suggested that the problem was not with electroporation but with the colony PCR as colonies that were treated with 20 mM NaOH did not amplify any bands but the positive control that were untreated showed correct sized bands.

Thus, it was decided that the PCR would be performed with NEB Thermopol Taq polymerase, in addition to GoTaq polymerase from Promega. Two colonies from each construct and two colonies of BCAP-8 His were lysed with 20mM NaOH and one colony of each was heated with water to 95° C for lysis and were mixed with the master mixes containing two different Taq polymerases, GO Taq polymerase and Thermopol Taq polymerase. The colony PCR with GO Taq polymerase amplified no bands whereas the colony PCR with Thermopol Taq polymerase amplified bands not only in the positive controls but also in the transformants. This result showed that there was an integration, but the GO Taq polymerase was not amplifying the products as the NaOH treatment for the lysis of the cells prior to the PCR was inactivating the GoTaq polymerase (Figures 14 and 15).

5.3.6 Recombinant protein induction

Colonies that grew in 500µg/ml zeocin and confirmed to show integration of the genes of interest into *Pichia* were chosen for further analysis. From X33 strain, a total of 13 colonies showed correct size bands on the gel whereas from SMD1168, more than 30 colonies showed correct size bands for Ta-ori pPICZB, and Ta-mut pPICZB. For the induction, 5 colonies of

Ta-ori pPICZB and 1 colony of Ta-mut pPICZB in X33 were used and 5 colonies of Ta-ori pPICZB and 4 colonies of Ta-mut pPICZB in SMD1168 strain were used. Protein samples collected after methanol induction at time points ranging from 24-120 hours were used in the dot blot. Protein extracted from the untransformed X33 and SMD1168 cells were used as negative controls.

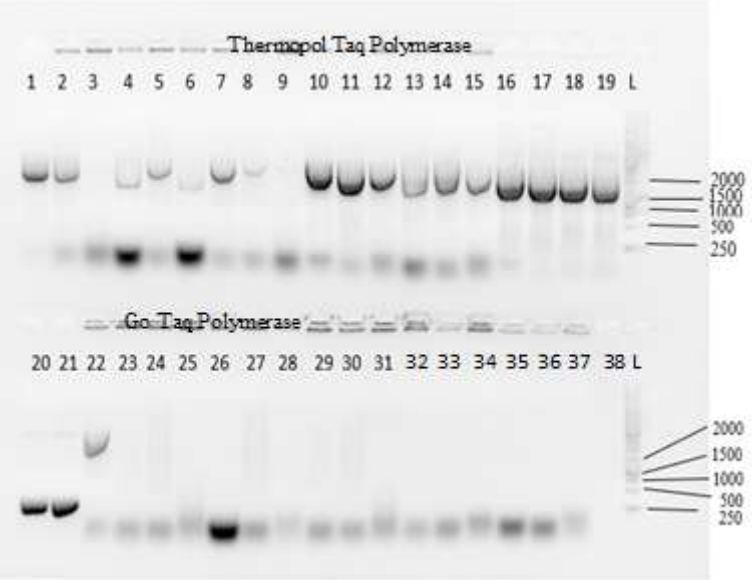


Figure 14. *Gel showing the results of colony PCR. The PCR for samples 1-22 were performed with Thermopol Taq polymerase while the PCR for samples 22-38 were performed with Go Taq polymerase. Ta-original colonies 1, 2, 4, 5, 10, 11,16, 23, 24, 26, 27, 32, 33 were treated with 20 mM NaOH while the colonies 3, 6, 12, 17, 25,28, 34 were treated with water. Ta-mutant colonies 7, 8, 29, 30 were treated with 20 mM NaOH while the colonies 9 and 31 were treated with water. Colonies 13,14, 35 and 36 BCAP-8 were treated with 20 mM NaOH while the colonies 15 and 37 BCAP-8 were treated with water. Plasmid DNA for BCAP-8 treated with either NaOH or water were in lanes 18 and 19, respectively. The negative controls were the plasmid DNA from the empty vector pPICZB in lanes 21 and 22 and were treated with NaOH or water, respectively.*

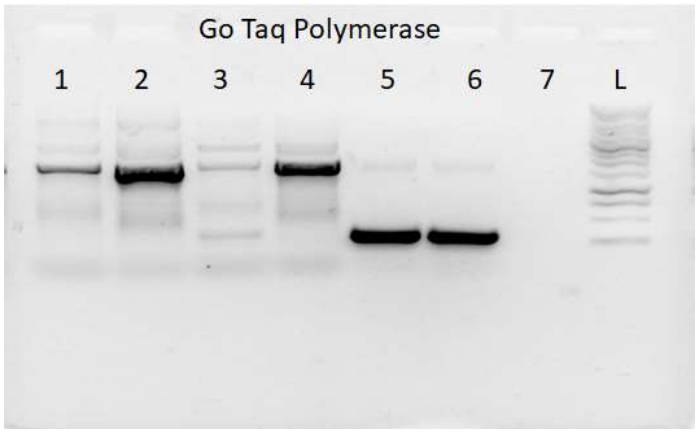


Figure 15. *One selected transformant from either the NaOH treated, or water treated was subjected to colony PCR with NEB Thermopol Taq polymerase to confirm the results. Lane 1, Ta- original in NaOH; 2, Ta- original in water; Lane 3, BCAP-8 in NaOH; lane 4 BCAP-8 in water; Lane 5, empty vector pPICZB in NaOH and Lane 6, empty vector pPICZB in water.*

5.3.7 Dot Blot

The result from the dot blot (Figure 16) shows that the induction of protein is optimal at 24 and 48 hours for the pDNA integrated into X33 whereas for SMD1168, no optimal induction time was identified as indicated by very faint signals at all time points.

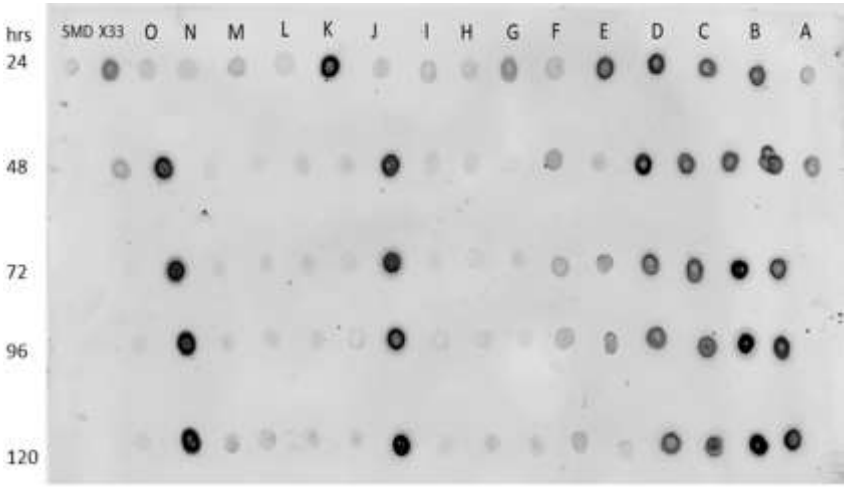


Figure 16. *Dot Blot to check the induction of protein at different time points (24-120 hours). 5 ul each of total protein was used in the dot blot. Lanes A-E, Ta-Ori pPICZB (X33); lanes F-J, Ta-Ori pPICZB (SMD); lane K, Ta-Mut pPICZB in X33; lanes L-O, Ta-Mut pPICZB in SMD 1168; followed by X33 and SMD 1168 untransformed strains.*

5.3.8 Coomassie Stain and Western blot

Following the results of the dot blot, 20µg of the protein from each sample, negative control (X33) and positive control (1µl of purified hAQP1 protien) were loaded on to a 12 % Tris-SDS gel and stained with coomassie blue (Figure 17). Bands were detected only in two lanes; in the negative control X33 and a sample integrated in X33 strain of the *Pichia.* The protein samples were requantified using Pierce Protein assay (Thermofisher) to confirm equal loading and the gel was rerun (Figure 18). This gel showed bands for all the protein samples used.

The results in Figure 16 were used as a guide to perform Western blots as detailed in the methods section 4.2.14, however no signals could be detected in either the samples or the positive control. It is highly likely that using an old secondary antibody may have resulted in the lack of signals. This is particularly true for the positive control hAQP1 that has been confirmed to be expressed via western blots and patch clamping by my co-supervisor Dr. Henderson. Delay in obtaining the new secondary antibody (ordered in April) and limitation of time has prevented me from working to resolve this issue with the western blots.

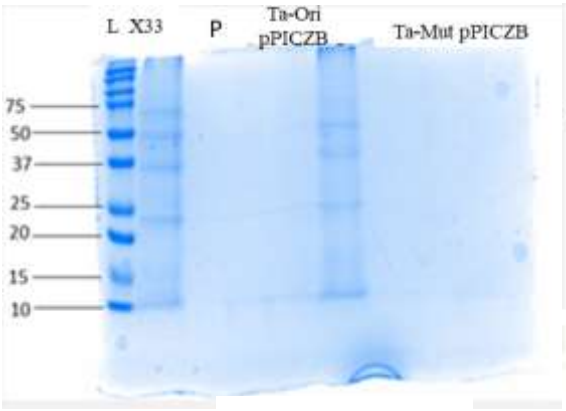


Figure 17

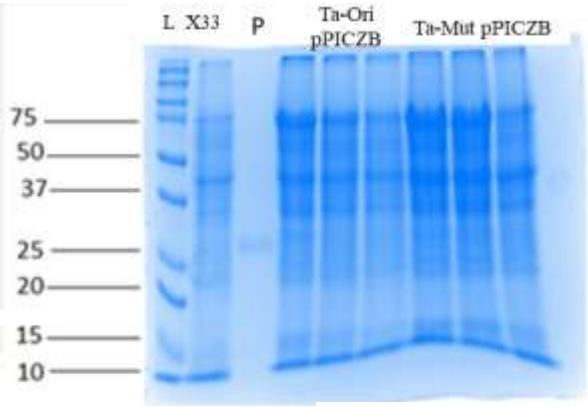


Figure 18

Figures 17 and 18: *Coomassie stained gels. Lane 1 ladder, lane 2 X33 untransformed; lane 3 hAQP1; lanes 4-9 total proteins from Ta-original and Ta-mutant at 24, 48 and 96 hours respectively.*

5.3.9 Limitations and Future work

Due to problems with colony PCR required to confirm the integration of the genes into *Pichia*, more than three months of valuable time was lost. The use of an old secondary antibody due to delays in delivery of the newly ordered antibody meant that there was no time left to optimise the Western blots. Further, process of functional characterisation of the His-tagged TaALMT1 and its mutant as well as purification of the recombinant protein was not attemped due to limited timeframe. These experiments will be carried out in the future to investigate the structure of the *ALMT 1* using Cryo EM.

5.4 Discussion

The aim was to integrate the TaALMT1 and the mutant pDNA into *Pichia* for recombinant protein expression and purification to generate a Cryo-EM structure. This study shows the successful expression of the wheat gene TaALMT1, starting from the cloning into the vector pPICZB and expressing in different heterologous expression systems such as *E. coli* and *Pichia.* Gene specific primers i.e., TaALMT forward and reverse primers were used for the amplification of the gene by Touch-down (TD) PCR. TD-PCR is the method of PCR which is used to enhance the specificity and product formation. It can also be incorporate to solve the problem related to high annealing temperature and high % of G+C content (Korbie & Mattick 2008). *Pichia pastoris* strains were chosen as host due to various advantages such as easy handling, ability to retain post-transcriptional modification and its direct relation to production of recombinant protein through *AOX1* promoter. Linearised, purified pDNA was electroporated into two different strains of *Pichia,* X33 and SMD1168.

Dot blot was used to check the level of expression of the protein which showed that the expression level of the protein in X33 strain was better than the SMD1168 which suggests that the X33 strain is more suitable for the integration of TALMT1.

ALMT is an anion channel regulated by GABA under acidic stress that elute malate to reduce aluminium stress in palnts. These ALMT channels are located at the plasma membrane of the cells and one of the reason for less information about the strucutre of the ALMT 1 protein is because it very hard to crystallize due to its hydrophobic nature and the general techiniques like NMR and X-Ray Crystallography used for the identification of the structure of the proteins cannot be used (Carter 1979; Cherezov & Caffrey 2007; Ostermeier & Michel 1997). Inspite of ALMTs being an anion channel and having a GABA binding motif similar to $GABA_A$ receptor in animals, they share a little sequence homology with the mammalian GABA receptors. In fact the only similarity is in a 12 amino acid stretch towards the end of the 6^{th} transmembrane domain (Ramesh *et al.* 2015). Further the lack of crystal structures of proteins similar to ALMTs means that *in silico* analysis of the ALMT protein via homology modelling cannot be carried out. Several studies were conducted for the prediction of the topology of TaALMT 1 using computer generated models using a bacterial protein that showed less than 30 % homology to the ALMT protein but the results from these studies contradict functional data (unpublished) and further, it is still unclear what the exact topology of the ALMTs proteins is in the membrane (Dreyer *et al.* 2012; Motoda *et al.* 2007). Thus it is difficult to predict the location of the 12 amino acid putative GABA binding motif slowing down our understanding of the structure-function of ALMT-mediated transportation processes (Ligaba *et al.* 2013).

Moreover, ALMT anion channel elute malic acid that chelates Al^{+3} alone with GABA and also helps to release phosphorous from its stable complex form (Din *et al.* 2021). However, there is lack of crystal structure of the *ALMT* that prevents the modelling of the ALMT transporters and hence has slow down the understanding of the structural function of ALMT-mediated transportation processes (Ligaba *et al.* 2013).

Structural study of the protein can solve many unknown facts of the proteins such as the functional sites or residues, sizeable cavities or clefts to bind substrate in the protein structure,

hydrophobicity of the protein surface that are involved in protein-protein interactions and also help to predict other features such as whether the proteins bear the similar protein fold to understand the evolution of the protein (Jones, S & Thornton 1997; Laskowski *et al.* 1996; Lesk 1998; Orengo *et al.* 1999; Petock *et al.* 2003). In comparison to animals, research into the plant GABA binding motif is in its initial phase. In plants it is still unknown which side of the ALMT1 binds to GABA, kinetics of GABA binding, whether other regions in ALMT proteins are involved in the regulation by GABA. As such the structural study of the ALMT1 can play a vital role to understand the role of GABA and its involvement in the stress tolerance in plants.

Plant feel stressed in each biotic and abiotic stresses which not only affects physiological role; seed germination, plant growth, fruit yield and many more but also the cellular processes such as respiration, photosynthesis and membrane premeability in plants. Intensive study related stresses in plant is very necessary to deal with the increasing frequencies of adverse environmental conditions. With the variation in the geographical features all over the world and with the increasing harsh environmental conditions, very few plants can tolerate stress and remain productive to produce food which is sufficient to the feed the growing global population. To feed the all the people all over the world, crops need to be more stress resilient and the study of the proteins that regulate stress tolerance in plants could guide us towards solving this problem or provide options to choose the best way to engineer plants to be more stress tolerant and thrive with increased productivity.

5.5 Conclusion

Research in GABA has a very long history of more than six decades. In animals, a greater number of studies have been conducted than in plants. However, the recent discovery of plant ALMT proteins with GABA binding site has opened new pathways for the research into GABA to make plants more stress tolerant to cope with changing environment and global warming.

To exploit the properties of GABA to improve plant stress resilience, a knowledge of the structure of ALMT would be very important.

6. Future perspectives

Further study of the 20 novel proteins identified can be carried out to explore GABA regulation. For the identification of putative GABA binding site, site directed mutagenesis can be carried out and further characterisation can be completed by characterising these proteins via electrophysiology in *Xenopus* oocytes, tobacco BY2 cells and yeast. The wheat recombinant proteins expressed in *Pichia* can be purified by subjecting them to affinity purification and the purified proteins can be sent for Cryo-EM studies to determine the 3D structure of the ALMT protein.

7. References

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8. Appendix

Primers for cloning

TaPichia_F1 CAGCTCGAGATGGATATTGATCACGGCAGAGAG

TaPichia_R1 CAGTCTAGATACAAAATAACCACGTCAGGCAAAGG

Polymerase chain reaction (PCR)

Master Mix:	3 X Master Mix
5 X buffer Phusion = 5 µl	15 µl
Template DNA =1 µl	_
Primer F/R (10 µM) = 1 µl	3 µl
dNTP (10mM) = 1 µl	3 µl
Phusion HiFi Taq = 0.5 µl	1.5 µl
Milli Q water = 41.5 µl	124.5 µl
Total = 50 µl	49 µl in each tube

Restriction Digestion

Cut smart buffer = 10µl

Restriction digestion (*Xho* I) = 1.5 µl

Restriction digestion (*Xba* I) = 1.5 µl

Insert (either PCR product or Vector) = 25 µl

Milli Q water = 62 µl

= 100 µl

Ligation

If vector =1 µl having 7.4 ng/ µl concentration,

Then required insert = 9.206 ng/µl

And if the insert concentration (x) = 31.3 ng/µl

Then, x µl = 9.206 x (1/31.3)

:. x = 0.294 µl i.e., required insert

If vector =1 µl having 7.4 ng/ µl concentration,

Then required insert = 9.206 ng/µl

And if the insert concentration (x) = 31.3 ng/µl

Then, x µl = 9.206 x (1/31.3)

:. x = 0.294 µl i.e., required insert

Even the other component increases or decreases, the insert and the vector remain the same (should be used as per calculation)

Recipe:

Vector = 1 µl

Insert =0.294 µl

Buffer = 2 µl

Water = 13.06 µl

Ligase = 1 µl

Total = 20 µl

Colony PCR

Master Mix

Milli Q water = 14.4 µl

5 x buffer = 5 µl

dNTPs = 0.5 µl

Taq = 0.125 µl

AOX F/R = 2 µl

$MgCl_2$ =1.25 µl

Colony lysate = 2.5 µl

Total = 25 µl

Low Salt LB liquid/ Agar

For 1 litre
dissolve the following in 950 ml deionized water,
Add 10 g tryptone,
5 g yeast extract
5 g NaCl 2.
For agar, add 15 g/litre agar before autoclaving.
Adjust the pH of the solution to 7.5 with 1 N NaOH and bring the volume up to 1 litre.
Autoclave for 20 minutes at 15 lb/sq. in and 121°C. Let cool to ~55°C and add desired antibiotics at this point.
For Low Salt LB medium with Zeocin™, add Zeocin™ to 25µg/ml final concentration. Store at 4°C. If you have added Zeocin™, store medium in the dark.

Primers for sequencing

*TaALMT1*F ATGGATATTGATCACGGCAGAG

*TaALMT1*R TTACAAAATAACCACGTCAGGCAAAGG

Sanger sequencing results

TaALMT1 original

```
   1 ATGGATATTG ATCACGGCAG AGAGAGCGAC GGCGAGATGG TGGGCACCAT CGCCAGCTGC
  61 GGGCTGCTGC TCCACTCGCT TCTCGCCGGG CTCGGGCGTC GCGCCGCCGG GTTCGCCCGG
 121 AAGGTGGGCG GCGCCGCGCG GGAGGACCCG AGGCGGGTGG CGCACTCGCT CAAAGTCGGC
 181 CTGGCGCTCG CGCTGGTGTC CGTCGTCTAC TTCGTCACGC CGCTCTTCAA CGGCCTCGGG
 241 GTGTCCGCGA TATGGGCCGT GCTCACCGTC GTCGTCGTCA TGGAGTACAC CGTCGGTGCC
 301 ACGCTGAGTA AAGGCTTGAA CAGAGCCTTG GCGACGTTGG TGGCTGGCTG CATCGCCGTC
 361 GGAGCTCATC AGTTAGCTGA ATTAGCTGAA CGCTGTGGTG ATCAGGGAGA GCCCATAATG
 421 CTTACCGTGC TCGTCTTCTT CGTAGCGTCA GCGGCGACGT TCTTGCGCTT CATCCCGGAG
 481 ATCAAGGCCA AGTACGACTA CGGCGTGACC ATCTTCATAC TGACCTTCGG TCTGGTGGCC
 541 GTGTCGAGCT ACAGAGTGGA GGAGCTCATC CAGCTCGCGC ACCAGCGGTT CTACACCATA
 601 GCCGTCGGCG TCTTCATCTG CCTCTGCACC ACCGTCTTCC TCTTCCCCGT CTGGGCCGGA
 661 GAGGACGTCC ACAAGCTCGC CTCCGGCAAC CTCGACAAAC TCGCTCAGTT CATTGAAGGA
 721 ATGGAATTCA ACTGCTTTGG CGAAAACAGT GTTGCAAATA ATTTTGGGGG AAAAGATTTC
 781 CCCCAAATGC ACAAGAGCGT CCTTAATTCG AAGGCCACTG AGGACTCTTT GTGCACCTTT
 841 GCCAAATGGG AGCCTCGTCA TGGCCAGTTC AGATTTCGAC ACCCATGGAG TCAATACCAG
 901 AAGCTGGGAA CTCTTTGTCG CCAATGTGCG TCTTCTATGG AGGCTCTTGC TTCATATGTC
 961 ATCACAACCT CAAAAACCCA GTGCCCTGCT GCAGCCAACC CTGAGCTATC ATGTAAGGTT
1021 CGAAAAACAT GTGGCGAAAT GAGCTTGCAT TCCTCCAAGG TGCTTAGGGA TCTCGCAATG
1081 GCAACTCGAA CAATGACTGT GCCGTCTCCA GTGAATATCA CCATGGCTAC AGCCGTGAAA
1141 GCAGCGGAAA GCCTCAGAAG CGAGCTTGCA GAGAACACGG CTCTGTTGCA AGTGATGCAT
1201 GTGGCCGTCA CCGCAACACT TCTTGCGGAC TTGGTTGATA GGGTGAAGGA AATCGCGGAA
1261 TGTGTTGATG TCCTAGCAAG ACTGGCGCAC TTTAAGAACC CCGAGGACAC AAAAAATGTC
1321 GTTGTTAGTA CCGTGAGTCG AGGGATAGAC GAACCTTTGC CTGACGTGGT TATTTTGTAA
```

Amino acid sequence

MDIDHGRESDGEMVGTIASCGLLLHSLLAGLGRRAAGFARKVGGAAREDPRRVAHSLKVG
LALALVSVVYFVTPLFNGLGVSAIWAVLTVVVVMEYTVGATLSKGLNRALATLVAGCIAV
GAHQLAELAERCGDQGEPIMLTVLVFFVASAATFLRFIPEIKAKYDYGVTIFILTFGLVA
VSSYRVEELIQLAHQRFYTIAVGVFICLCTTVFLFPVWAGEDVHKLASGNLDKLAQFIEG
MEFNCFGENSVANNFGGKDFPQMHKSVLNSKATEDSLCTFAKWEPRHGQFRFRHPWSQYQ
KLGTLCRQCASSMEALASYVITTSKTQCPAAANPELSCKVRKTCGEMSLHSSKVLRDLAM
ATRTMTVPSPVNITMATAVKAAESLRSELAENTALLQVMHVAVTATLLADLVDRVKEIAE
CVDVLARLAHFKNPEDTKNVVVSTVSRGIDEPLPDVVIL*

TaALMT1 F213C mutant

```
   1 ATGGATATTG ATCACGGCAG AGAGAGCGAC GGCGAGATGG TGGGCACCAT CGCCAGCTGC
  61 GGGCTGCTGC TCCACTCGCT TCTCGCCGGG CTCGGGCGTC GCGCCGCCGG GTTCGCCCGG
 121 AAGGTGGGCG GCGCCGCGCG GGAGGACCCG AGGCGGGTGG CGCACTCGCT CAAAGTCGGC
 181 CTGGCGCTCG CGCTGGTGTC CGTCGTCTAC TTCGTCACGC CGCTCTTCAA CGGCCTCGGG
 241 GTGTCCGCGA TATGGGCCGT GCTCACCGTC GTCGTCGTCA TGGAGTACAC CGTCGGTGCC
 301 ACGCTGAGTA AAGGCTTGAA CAGAGCCTTG GCGACGTTGG TGGCTGGCTG CATCGCCGTC
 361 GGAGCTCATC AGTTAGCTGA ATTAGCTGAA CGCTGTGGTG ATCAGGGAGA GCCCATAATG
 421 CTTACCGTGC TCGTCTTCTT CGTAGCGTCA GCGGCGACGT TCTTGCGCTT CATCCCGGAG
 481 ATCAAGGCCA AGTACGACTA CGGCGTGACC ATCTTCATAC TGACCTTCGG TCTGGTGGCC
 541 GTGTCGAGCT ACAGAGTGGA GGAGCTCATC CAGCTCGCGC ACCAGCGGTT CTACACCATA
 601 GCCGTCGGCG TCTTCATCTG CCTCTGCACC ACCGTCTGCC TCTTCCCCGT CTGGGCCGGA
 661 GAGGACGTCC ACAAGCTCGC CTCCGGCAAC CTCGACAAAC TCGCTCAGTT CATTGAAGGA
 721 ATGGAATTCA ACTGCTTTGG CGAAAACAGT GTTGCAAATA ATTTTGGGGG AAAAGATTTC
 781 CCCCAAATGC ACAAGAGCGT CCTTAATTCG AAGGCCACTG AGGACTCTTT GTGCACCTTT
 841 GCCAAATGGG AGCCTCGTCA TGGCCAGTTC AGATTTCGAC ACCCATGGAG TCAATACCAG
 901 AAGCTGGGAA CTCTTTGTCG CCAATGTGCG TCTTCTATGG AGGCTCTTGC TTCATATGTC
 961 ATCACAACCT CAAAAACCCA GTGCCCTGCT GCAGCCAACC CTGAGCTATC ATGTAAGGTT
1021 CGAAAAACAT GTGGCGAAAT GAGCTTGCAT TCCTCCAAGG TGCTTAGGGA TCTCGCAATG
1081 GCAACTCGAA CAATGACTGT GCCGTCTCCA GTGAATATCA CCATGGCTAC AGCCGTGAAA
1141 GCAGCGGAAA GCCTCAGAAG CGAGCTTGCA GAGAACACGG CTCTGTTGCA AGTGATGCAT
1201 GTGGCCGTCA CCGCAACACT TCTTGCGGAC TTGGTTGATA GGGTGAAGGA AATCGCGGAA
1261 TGTGTTGATG TCCTAGCAAG ACTGGCGCAC TTTAAGAACC CCGAGGACAC AAAAAATGTC
1321 GTTGTTAGTA CCGTGAGTCG AGGGATAGAC GAACCTTTGC CTGACGTGGT TATTTTGTAA
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Amino acid sequence

MDIDHGRESDGEMVGTIASCGLLLHSLLAGLGRRAAGFARKVGGAAREDPRRVAHSLKVG
LALALVSVVYFVTPLFNGLGVSAIWAVLTVVVVMEYTVGATLSKGLNRALATLVAGCIAV
GAHQLAELAERCGDQGEPIMLTVLVFFVASAATFLRFIPEIKAKYDYGVTIFILTFGLVA
VSSYRVEELIQLAHQRFYTIAVGVFICLCTTVCLFPVWAGEDVHKLASGNLDKLAQFIEG
MEFNCFGENSVANNFGGKDFPQMHKSVLNSKATEDSLCTFAKWEPRHGQFRFRHPWSQYQ
KLGTLCRQCASSMEALASYVITTSKTQCPAAANPELSCKVRKTCGEMSLHSSKVLRDLAM
ATRTMTVPSPVNITMATAVKAAESLRSELAENTALLQVMHVAVTATLLADLVDRVKEIAE
CVDVLARLAHFKNPEDTKNVVVSTVSRGIDEPLPDVVIL*

Stock Solutions

10X YNB

Add 34 g of YNB without ammonium sulphate and amino acids and 100 g of ammonium sulphate in 1000 ml of water and filter sterilize. Heat the solution to dissolve YNB completely in water. Store at 4°C. The shelf life of this solution is approximately one year.

500X B (0.02% Biotin)

Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at 4°C. The shelf life of this solution is approximately one year.

100X H (0.4% Histidine)

Dissolve 400 mg of L-histidine in 100 ml of water. Heat the solution at 50°C to dissolve. Filters sterilize and store at 4°C. The shelf life of this solution is approximately one year.

10X M (5% Methanol)

Mix 5 ml of methanol with 95 ml of water. Filters sterilize and store at 4°C. The shelf life of this solution is approximately two months.

10X GY (10% Glycerol)

Mix 100 ml of glycerol with 900 ml of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is greater than one year.

1 M potassium phosphate buffer, pH 6.0:

Combine 132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 and adjust the pH = 6.0 ± 0.1 with phosphoric acid or KOH. Sterilize by autoclaving and store at room temperature. The shelf life of this solution is greater than one year.

Yeast Extract Peptone Dextrose Medium (1 litre)

Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water.
Add 20 g of agar if making YPD agar.
Autoclave for 20 minutes on liquid cycle. Cool to ~60°C.
Add 100 ml of 10X D.
The liquid medium is stored at room temperature. Store YPD agar at 4°C.

Minimal Glycerol Medium + Histidine (1 litre)

Combine aseptically 800 ml autoclaved water with 100 ml of 10X YNB,
2 ml of 500X B, and
100 ml of 10X GY
For growth of his4 strains in this medium, a version can be made that contains histidine (called MGYH) by adding 10 ml of 100X H stock solution. Store at 4°C. The shelf life of this solution is approximately two months.

Minimal Dextrose Histidine (1 litre)

For medium, autoclave 800 ml of water for 20 minutes on liquid cycle.
For agar media, add 15 g of agar with 800 ml of water and autoclave.
Cool to about 60°C and then add:
100 ml of 10X YNB
2 ml of 500X B
100 ml of 10X D
add 10 ml of 100X H stock solution.
Mix and store at 4°C.

Minimal Methanol Histidine (1 litre)

For medium, autoclave 800 ml of water for 20 minutes on liquid cycle 2

For agar, add 15 g of agar + 800ml of water and autoclave.
Cool autoclaved water to 60°C and add:
100 ml of 10X YNB
2 ml of 500X B
100 ml of 10X M
add 10 ml of 100X H stock solution.
Mix and store at 4°C

Buffered Minimal Glycerol Buffered Minimal Methanol (1 litre)

Autoclave 690 ml water for 20 minutes on liquid cycle.
Cool to room temperature, then add the following and mix well:
100 ml 1 M potassium phosphate buffer, pH 6.0 100 ml
10X YNB
2 ml 500X B
100 ml 10X GY
BMMH, add 100 ml 10X M instead of glycerol.
To add histidine, add 10 ml of 100X H stock solution. Mix and store at 4°C.
Store media at 4°C. The shelf life of this solution is approximately two months.

Buffered Glycerol-complex Medium Buffered Methanol-complex Medium.

Dissolve 10 g of yeast extract, 20 g peptone in 700 ml water.
Autoclave 20 minutes on liquid cycle.
Cool to room temperature, then add the following and mix well:
100 ml 1 M potassium phosphate buffer, pH 6.0
100 ml 10X YNB
2 ml 500X B
100 ml 10 X GY 4
add 100 ml 10X M.

Store media at 4°C. The shelf life of this solution is approximately two months.

Breaking Buffer

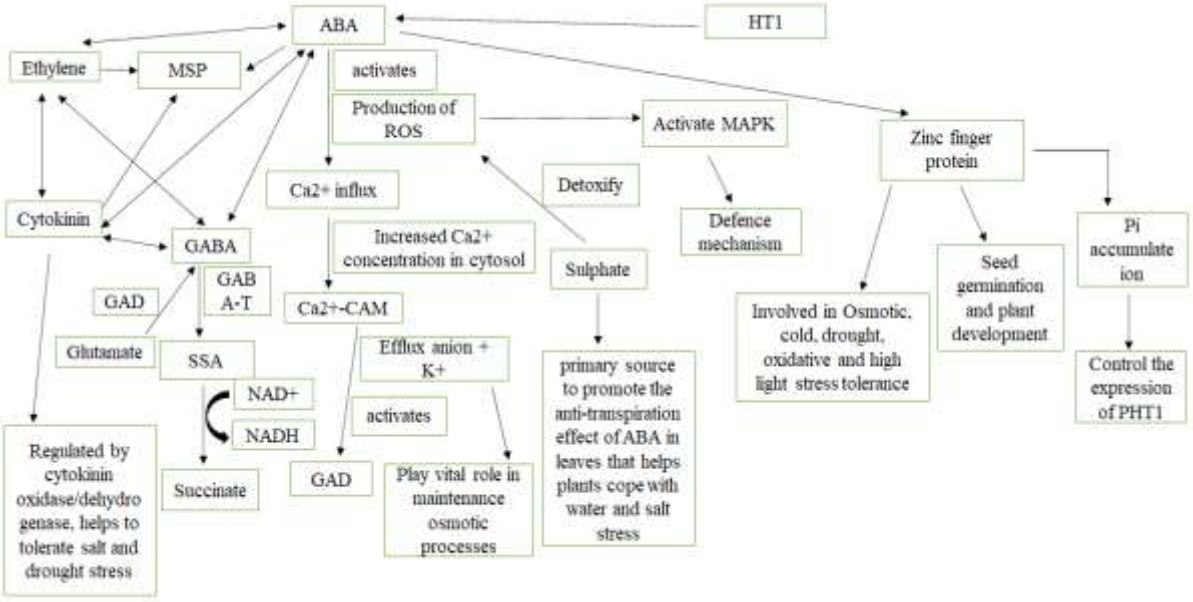
50 mM sodium phosphate, pH 7.4 1 mM PMSF (phenylmethylsulphonyl fluoride or other protease inhibitors) 1 mM EDTA 5% glycerol
For 1 litre, dissolve the following in 900 ml deionized water:
6 g sodium phosphate (monobasic) 372 mg EDTA 50 ml glycerol.
Use NaOH to adjust pH and bring up the volume to 1 litre. Store at 4°C.
Right before use, add the protease inhibitors.

1 X TBS Tween

Add 5 ml of 20 TBS Tween in 95 ml of water.

Blocking Buffer/ Blocking Buffer with antibody

Use 5% of skim milk in 15 ml of 1 X TBS Tween. Add antibiotic 1: 1000 dilution for the primary antibody (Anti Rabbit) and 1:5000 dilution for Goat Anti Rabbit.


ABA
HT1
Ethylene
MSP
activates
Production of ROS
Activate MAPK
Zinc finger protein
Ca2+ influx
Detoxify
Cytokinin
GABA
Increased Ca2+ concentration in cytosol
Defence mechanism
Pi accumulate ion
Sulphate
GAD
GAB A-T
Ca2+-CAM
Seed germination and plant development
Glutamate
SSA
Efflux anion + K+
Involved in Osmotic, cold, drought, oxidative and high light stress tolerance
NAD+
NADH
activates
primary source to promote the anti-transpiration effect of ABA in leaves that helps plants cope with water and salt stress
Control the expression of PHT1
Regulated by cytokinin oxidase/dehydro genase, helps to tolerate salt and drought stress
Succinate
GAD
Play vital role in maintenance osmotic processes