THE ROLE OF LRR RECEPTOR-LIKE KINASES IN ABA DEPENDENT TPK1 CHANNEL REGULATION

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Abstract

The increasing world population demands increased food production, but several abiotic factors, for example, drought is hampering increased food production. The problem of drought is increasing and further limiting the productivity of many crops. There could be a range of potential strategies that may help plants to tolerate and avoid drought conditions, for example, to produce stress tolerant plants, to understand and utilize signalling mechanisms in plants to adapt them to drought, to minimize yield loss in dryland areas and to reduce the water needs.

ABA (abscisic acid) is produced during drought stress causing the closure of the stomatal pore to prevent water loss. K^+ (potassium) release from the vacuole through K^+ transporters localised to the tonoplast, during these conditions this is one of the important steps. However, the mechanism of coupling of ABA to the tonoplast is not known yet. The AtTPK1 channel (*Arabidopsis thaliana* two pore potassium channel) is localised to the tonoplast and has been shown to have a role in the vacuolar K^+ release and stomatal closure. AtTPK1 is activated by phosphorylation and binding of 14-3-3 proteins. The surface of the plasma membrane of plants contains receptor-like kinases (RLKs) that are known to be involved in the early steps of osmotic-stress signalling. Binding of a ligand to the extracellular domain of the RLK activates the intracellular kinase domain, resulting in bringing extracellular environment signals into the intracellular targets. LRRs (Leucine-rich repeat) are a possible mechanism to link external ABA with TPK1 and because LRRs had been found to impact in TPK1 current, for example, two LRR receptor kinase candidates, KINASE1 and KINASE2 (*At3g02880* and *At4g21410*) were shown by patch clamp studies to affect TPK1 current stimulation. The BiFC (Bimolecular fluorescence complementation) studies also showed interaction of these kinases with TPK1 when they were treated with ABA. Therefore, it was hypothesized that they are involved in the activation of TPK1 (Isner et al., unpublished data). The kinase mutant lines were selected for further characterization in comparison to *tpk1* and WT (wild type) in different media conditions.

All the knockout lines showed shorter root lengths and lower fresh weights as compared to the wild type in K^+-deficient, higher K^+ and osmotic stress conditions. Lower fresh weights for the KO (knock out) lines as compared to the wild type were also observed in soil in control and moderate stress conditions. The lower growth of the *tpk1* and kinase KO lines as compared to the wild type may be because of the lack of TPK1 activity. The lack of kinase proteins may lead to the inactivation of TPK1 channels and thus it leads to the comparable results between the kinase KO lines and *tpk1* KO line. These results suggest a link between these kinases and TPK1 channel activity. These lines were also tested for the stomatal conductance under various ABA treatments applied to the excised leaves. The kinase KO lines and *tpk1* KO led to a delayed response in stomatal closure after exposure to different concentration of ABA (1 µM, 10 µM and 100 µM).

The similarities in phenotype between the kinases KO and channel KO mutants suggest there may be a relation between these kinases and the TPK1 channel. Combined with other, as yet unpublished data, the data from this report support the idea of the involvement of these kinases in ABA dependent regulation of TPK1.

Table of Contents

List of Figures:

List of Tables

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Author’s declaration

I hereby declare that I am the sole author of this thesis and this thesis is a presentation of original work. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Chapter 1

1 Introduction

1.1 Drought

Drought is a natural disaster of water-deficit stress condition. Generally drought is known as a long period of surface or underground water deficiency or unusually dry weather that persists long enough (for months or even years) to cause environmental, social and economic problems. This generally happens when a region receives continuously below average precipitation.

Drought occurs due to changes in many environmental factors such as temperatures, low relative humidity, high winds, timing and characteristics of rain or snow, poor water managements by humans or increased water usage.

Both surface and groundwater resources are affected by drought, resulting in reduced water supply, less nutrient availability, low crop production or harvest failure and a reduction in product varieties (Riebsame et al., 1994). Drought not only affects the productivity of the crops but also the vegetative parts by lowering the growth and photosynthesis. Drought causes long-term impacts on perennial crops (crops that live for more than two years). Almost all major crops are affected by drought depending upon the degree of desiccation. More than 50% of the Earth's surface areas, including agricultural lands are exposed to drought (Kogan, 1997). According to the UNCCD (United Nations Convention to Combat Desertification) classification system, 40 percent of the world's total land is considered as dry lands.

Plants require water for many physiological and biochemical processes, for example, photosynthesis, nutrient uptake and translocation, as well as for cooling (Farooq et al., 2009). Nowadays the crucial issues for agriculture are drought, salinity and low temperature. These affect the physical, physiological and even genetical behavior of the plants. Drought stress results in the disruption of the cellular membrane bilayer making it unusually porous (Mahajan and Tuteja, 2005) and difficult for the transport of materials in to and out of the cell. Upon dehydration organelles and cytosolic proteins may decrease their function or even may completely denature (Mahajan and Tuteja,

2005). Cell division may be reduced resulting in reduced plant growth. Water stress can result in reducing photosynthesis rate, ion uptake and respiration and ultimately it can result in plant death (Jaleel et al., 2008). Drought causes water stress, but can also often lead to salinity stress. Drought stress is a common detrimental factor in the world economy than salinity. Salinity is the high concentration of ions of salts such as Na^+, Ca^{2+}, Cl^- and HCO_3^- in the soil. Drought can also affect human activities. Drought leads to direct and indirect loss to farmers and consumers respectively.

By 2050, it is expected that the world population will increase by two to three billion. At the same time water shortages are anticipated to get more severe due to climate change, potentially resulting in reduced food production. Yet, by 2050, a 70 percent increase in food demand is expected, particularly in developing countries (Hugh Turral, FAO consultant). To increase food production a number of strategies can be employed:

- Improving farming practices and traditional plant breeding, to conserve water, such as making dams and other constructions that help to store water.
- Genetic engineering, to increase plant tolerance to drought stress.
- Understanding and exploiting signalling mechanisms in plants to adapt them to drought.

1.1.1 Different mechanisms of plants for tolerating drought stress

Plants adopt different mechanisms for drought tolerance (Osakabe et al., 2013). For example, xerophytes have different morphological and physiological adaptations to water deficient conditions such as the members of family Cactaceae use stems as water reservoirs. Other xerophytes have comparatively thick waxy layers or smaller leaf surfaces that help in reducing the water loss. Cotton plants respond to drought by accelerating senescence and by shedding of the old leaves. Some plants have extensive root systems either going deep into the earth or spreading on the surface of the earth (Henry et al., 2012) to explore and absorb distant water sources. Other plants like rice, wheat and barley do not have such mechanisms and therefore they are sensitive to drought.

Figure 1.1: Different plants adapt differently to stress.

Figure 1.1A: Cactaceae family having round stems and spined leaves. Figure 1.1B: Examples of crop plants; rice, wheat and barley.

One of the most important mechanisms to tolerate drought is the ability of plants to regulate the size of the stomatal opening. This ability is especially important during drought stress, when loss of water can cause serious damage to plants. The discovery and development of stress tolerant crops to avoid yield loss during water stress is therefore very important.

1.1.2 The phytohormone abscisic acid (ABA)

Abscisic acid (ABA) is produced in plants during drought stress. It is a naturally occurring plant hormone that was identified in the 1960s. It is important for the regulation of abiotic stress responses such as drought, salinity, seed development and dormancy, germination, stomatal closure and biotic stress (pathogen attack) (Cutler et al., 2010). It is synthesized from oxygenated carotenoids called xanthophylls, for example, neoxanthin, violaxanthin and zeaxanthin, (Li and Walton, 1990). ABA is applied either to intact leaves or isolated epidermis for closing the stomatal aperture (Jones and Mansfield, 1970). Wright and Hiron, (1972) showed that the level of ABA increases in the leaves that are osmotically stressed. The response of mesophyll and guard cells is different to ABA, this difference in response is important for the control of transpiration. Mesophyll cells work efficiently during stomatal closure because of the difference in response to ABA (Jones and Mansfield, 1970). Thus, understanding the

function of ABA is essential for the development of crops with better stress responses (Wang et al., 2011).

1.1.3 ABA receptors and ABA signalling pathway

During drought stress, ABA is produced and accumulated in the vascular tissues (xylem, phloem) of plants (Iuchi et al., 2001 and Behnam et al., 2013). ABA is delivered by membrane localised ATP-binding cassette (ABC) transporters into the guard cells (Kang et al., 2010). The sensing of ABA by the plant cell has recently been revealed (Pei et al., 1997). Several receptors are responsible for sensing ABA such as PYR (pyrabactin resistance) /PYL (pyrabactin resistance-like). The binding of ABA to a PYR /PYL family of ABA receptor-like proteins initiates the early steps of ABA signalling pathway (Ma et al., 2009; Park et al., 2009 and Cutler et al., 2010). The formation of ABA during drought stress brings about activation of chains of reactions that activate the production of reactive oxygen species (ROS) such as H_2O_2, which results in the transport of Ca^{2+} through both plasma membrane and tonoplast into the cytosol. The plasma membrane proton pumps H^+-ATPases that hyperpolarise the membrane are inhibited in the presence of ABA to induce stomatal closure (Merlot et al., 2007). The *Arabidopsis* genome contains 13 PYR1-like genes named Pyl1 to Pyl13. The 2C protein phosphatases (PP2Cs), (ABI1 and ABI2 (ABA INSENSITIVE 1 and 2)) are central regulators of ABA responses (Koornneef et al., 1984 and Merlot et al., 2001). In this pathway, the type 2C protein phosphatases (PP2Cs) and Sucrose non-fermentation kinase subfamily 2 (SnRK2s) act as negative and positive regulators of downstream signalling (Ma et al., 2009 and Park et al., 2009). In figure 1.2, during normal growth conditions when cellular ABA levels are low, ABI type protein phosphatases 2Cs are active and bind to a serine/threonine protein kinase SnRK2 such as Open Stomata 1 (OST1) and dephosphorylate to keep the SnRK2s in an inactive state. While during stress condition, the cellular ABA level rises and binds to PYR/PYL, (Ma et al., 2009 and Yin et al., 2009) which in turn inhibits and creates a binding surface for PP2Cs. SnRK2s are then released from PP2Cs and self-activated via auto-phosphorylation, in the nucleus the SnRK2s further phosphorylates ABRE-binding factors (ABF). The phosphorylated ABFs bind to ABA-responsive elements (ABREs) at the promoter of the ABA-responsive genes and lead to the transcription of ABA-responsive genes resulting in stomatal closure. While in the cytoplasm the activated

SnRK2s cause the activation of S-type anion channel such as SLAC1 a slow-activating sustained (S-type), (Schroeder and Hagiwara, 1989) and a rapid-transient (R-type), that helps in the removal of anions. The anion efflux through the K^+ efflux channel, GORK (guard cell outward-rectifying K^+), results in plasma membrane depolarization and in decreasing the activity of stomatal opening channels, such as KAT1/KAT2.

G protein coupled receptor has also been shown to be a ABA receptor (Liu et al., 2007). ABA responses in *Arabidopsis* are mediated by G protein-coupled receptors (GPCRs) by interacting with G protein alpha subunit (Liu et al.,2007). G-proteins act as molecular switches to couple the activation of GPCRs (Jin-Gui Chin, 2008). The GPCRs plays an important role in a range of signalling pathways. The GPCRs sense many extracellular signals and activates the hetromeric G proteins, which further transduce signals intracellular to appropriate downstream effectors (Narendra Tuteja 2009) to switch on cellular responses. G-proteins were found to regulate stomatal movements in *Vicia faba* using electrophysiological and pharmacological methods, (Huajian Zhang 2012). All these findings have greatly helped in understanding the functions of ABA but there is still a great ignorance about the coupling of ABA signalling to the vacuole.

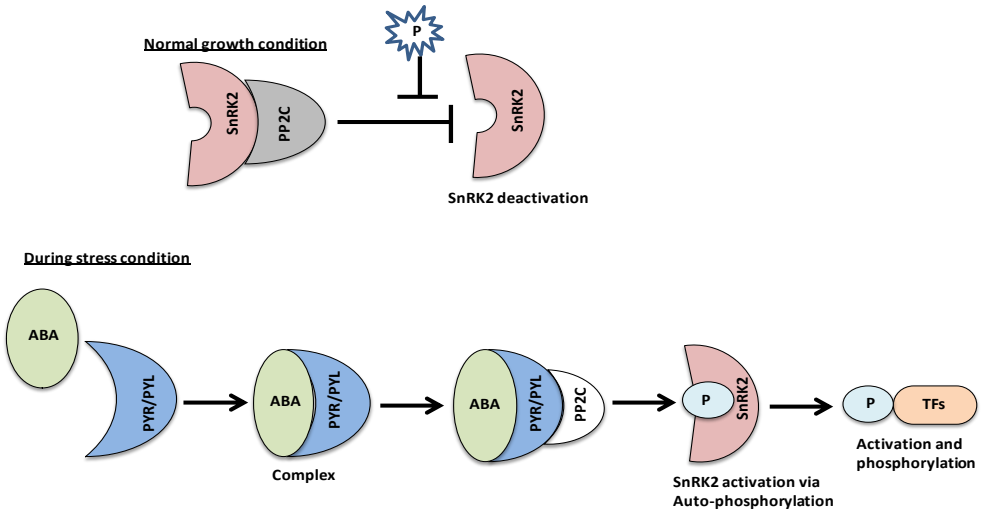

Figure 1.2: Signalling model of ABA.

In the normal growth conditions, PP2C phosphatase (2C protein phosphatases) attaches to SnRK2 kinases (Sucrose non-fermentation kinase subfamily 2) and deactivates their auto-phosphorylation. During stress conditions, ABA attaches to ABA receptors PYR/PYL (pyrabactin resistance/pyrabactin resistance-like) forming a complex resulting in the deactivation of PP2C phosphatases, resulting in the auto-phosphorylation of SnRK2 kinases that further results in the phosphorylation of transcription factors that helps in the transcription of ABA-responsive genes.

1.2 Stomata

1.2.1 Stomatal structure

Plants need to have a control over the exchange of water and gases into the plants and in their outside environment. This is important to adjust to their continuously changing environment. Therefore, the epidermis of aerial plant parts contain small pores called stoma (stomata: plural) that are used for the exchange of gasses and for the transpiration of water in and out of the leaf by controlling the size of the stomatal pores. In the terrestrial plants, stomata were first found over 400 million years ago (Edwards et al., 1998). On the basis of morphology stomata are divided into two shapes; linear dumbbell-shape in grasses and kidney shape in trees and shrubs. The size of one stoma ranges from 10—80 µm depending on the environmental condition and the type of the species (Willmer and Fricker, 1996). These pores are surrounded by a pair of specialized epidermal cells called the guard cells having cell wall, nucleus, cytoplasm, chloroplasts, vacuoles, radially arranged microfibrils and other cellular organelles. The guard cells are further surrounded by epidermal cell, spongy mesophylls, palisade, upper epidermis cells and cutical respectively. The inner wall of the guard cell is much thicker compared to the outer wall. When the guard cells are turgid the gap between the two cells is more and when the cell is flaccid the gap is less. Depending on the size, shape, structure, location and their behaviours stomata respond to a variety of signals which result in changes in environment from the cellular to the global level (Hetherington and Woodward, 2003). Stomatal pores occupy about 5% of the total leaf area (Willmer and Fricker, 1996). The internal structure of the leaf is shown below in figure 3 and stomata in figure 4 showing location of different cells, the shape and position of stomata and different other cells.

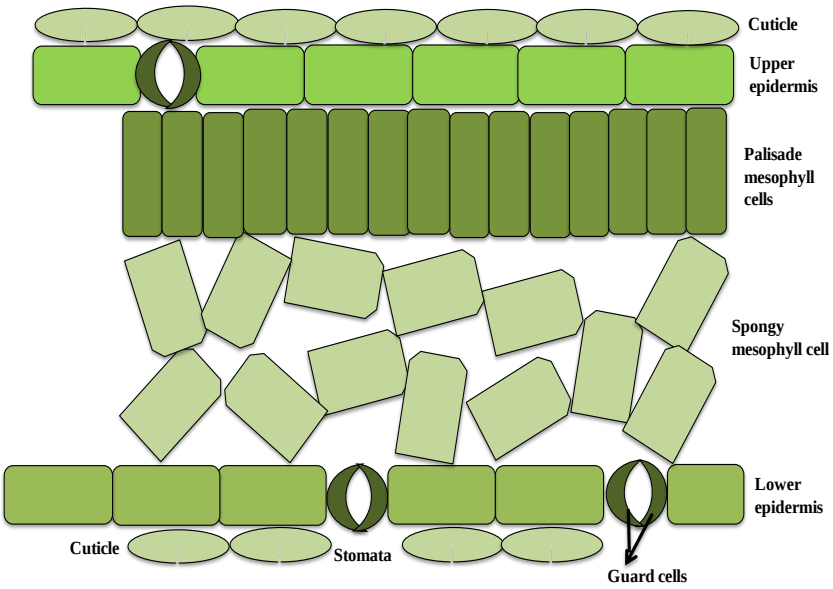

Figure 1.3: Internal structure of leaf.

The internal structure of a leaf showing the location of different cells, i.e., cuticle, upper epidermis, palisade mesophyll cells, spongy mesophyll cells, lower epidermis and guard cells.

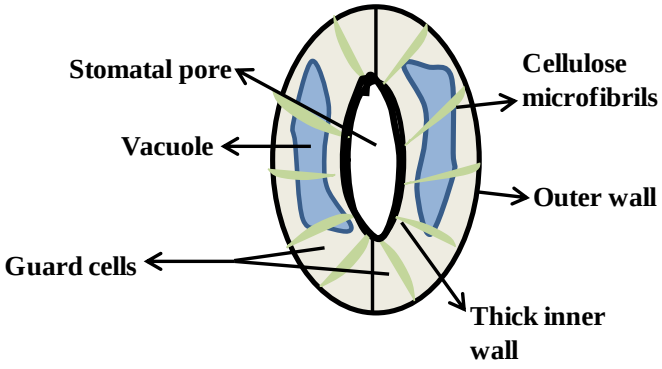

Figure 1.4: Structure of stomata.

The stoma is surrounded by a pair of guard cells, containing vacuoles, cellulose microfibrils and inner thick and outer thin walls.

1.2.2 Stomatal function

Stomata are involved in the maintenance of plant homeostasis. They regulate gas exchange between the leaves and the atmosphere. The transpiration of water from the leaves drives the water uptake by the roots and transport through the xylem allowing the uptake of CO_2 for photosynthesis and exit of oxygen as a by-product (Takemiya and Shimazaki, 2010) through stomata. The stomatal opening helps the plants to cool. Some large trees, for example deciduous trees, can transpire about 400 litres of water per day through their stomata (Raven et al, 1987). The annual maximum rate of water loss through stomata is 32 X 10^{15} kg in the uniform and warm forested areas (Alistair and

Woodward, 2003). Plants close their stomata during drought stress and therefore take-up very little CO_2 and the transpiration rate is also lowered (Arve, L. E. et al, 2011).

Guard cells respond to environmental stimuli such as blue light, red light, temperature, CO_2, humidity and plant hormones such as auxins, ethylene and abscisic acid to prevent excessive water loss by transpiration and to optimize plant growth (Vavasseur and Raghavendra, 2005). The more the turgor pressure in the guard cells, the greater is the opening of the stomatal aperture and vice versa. When the turgor pressure is more in the epithelial cells (subsidiary cells) the stomatal aperture is reduced due to the force applied by the epithelial cells on the guard cells (Buckley, 2005). During photosynthesis, CO_2 is consumed a lot, causing a decline in the concentration of CO_2, for compensating this, stomata open and let in more CO_2 to the cell from the atmosphere to continue cellular activities. On the other hand, during the absence of photosynthesis, high concentration of CO_2 is available and therefore, very little CO_2 is taken up. Thus it helps in the lower water loss through transpiration as the stomata are closed (Dietrich et al., 2001). During water stress conditions, plants can keep their stomata closed to reduce the water loss and sacrificing CO_2 uptake.

Guard cell also contains proteinecious cortical microtubules that are believed to be involved in the cell shape and development of stomata (Mitchison and Kirschner, 1984). The microtubules radiate from the inside of the stomatal pore toward outside of the guard cell (McDonald et al., 1993). These microtubules are involved in the deposition of cellulose microfibrils in distinctive orientation and distribution in characteristic regions of the guard cell wall that is important for the stomatal movement in response to the guard cell volume (Palevitz and Hepler, 1976).

Like other cells, guard cells also have cellular organelles such as vacuoles. The vacuole provides turgor, ion homeostasis, protein degradation, pH regulation, Ca^{2+} signaling and is a depository for both harmful and useful compounds. When plants absorb nutrients in excess, these are stored in the vacuoles. This stored food helps the plants to survive during periods of less food availability, when minerals such as NO_3^- and K^+ are released from the vacuole to maintain cytosolic homeostasis (Walker et al., 1996 and Orsel et al., 2002). As a storage organelle of the cell, vacuoles also store K^+ and K^+ accumulation develops turgor pressure within the cell which generates opening and closing of stomata and other tropisms.

1.2.3 Guard cell vacuolar K^+ and its role in stomatal conductance

Potassium is a very important cation present in about ~100 mM in almost all organisms. It provides a suitable ionic environment for metabolic processes in the cytosol**.** K^+ is used by cells for maintaining the regulatory processes in development, growth, reproduction, solute transport and osmotic balance for regulation of water in plants. Potassium is required for many physiological processes such as in the activation of many enzymes, photosynthesis and protein synthesis. A deficiency of potassium can lead to many growth related problems to the plants. Mainly, these could be a slow or arrested growth, chlorosis of leaves which is due to the low level of chlorophyll due to K^+ deficient conditions, reduced ability to fight against diseases, pests and bugs, unhealthy roots, early shedding of leaves before the time (Defoliation), formation of less ATP, resulting in the breakdown of transport of sugar and no or poor resistance to drought and high temperature. All these can result in lower crop quality and yield.

K^+ in the guard cells helps in the opening of stomata (Allen and Sanders, 1995). Accumulation of K^+ results in the reduction of water potential causing the increase of water uptake. The water causes turgidity of the guard cells, which results in the opening of stomata. The cell wall of guard cells contains asymmetrical arranged microfibrils that help in opening and closing of stomata (Shimazaki et al., 2007). The stomatal conductance also depends on the accumulation of the sugars (Talbott and Zeiger, 1998).

1.2.4 Potassium channels and transporters

The plasma membrane, endomembranes and tonoplast of plants contains membrane proteins for the transport of potassium (Maathuis, 2007). There are two main types of K^+ channels in the plasma membrane, i.e. inward rectifying K^+ (K^+_{in}), i.e. AKT1, and outward rectifying K^+ (K^+_{out}) channels, e.g. GORK (Guard cell outward rectifying K^+ channel).

The vacuoles of the cells also contain channels and carrier transporters. The *Arabidopsis* genome contains 15 genes coding for proteins that form K^+ channels (Szczerba et al., 2009). There are three types of vacuolar K^+ channels, i.e., fast activating K^+ channels (FV), slow activating K^+ channels (SV) and vacuolar channels (VK) (Hedrich and Neher, 1987; lvashikina and Hedrick, 2005 and Pottosinet et al., 2004). Tandem-pore potassium (TPK) channels belong to voltage independent K^+ channels. There are five isoforms of tandem-pore potassium (TPK) channels, i.e., TPK1, TPK2, TPK3, TPK 4

and TPK5 in *Arabidopsis*. They are localized to the tonoplast of the vacuole except TPK4 which is localized to the plasma membrane (Becker et al., 2004). There are two TPK isoforms in rice (Oryza sativa) that are known as TPKa and TPKb. Shin Hamamoto et al., (2008) characterised NtTPK1 (Nicotiana tabacum) as a novel tonoplast potassium channel in tobacco. NtTPK1 was found in flowers, leaves and roots.

1.3 TPK1 a Vacuolar potassium channel

1.3.1 TPK1 structure and localization

Using approaches such as electrophysiology, reverse genetics, and homologous gene expression, the TPK1 gene was shown to have a role in K^+ homeostasis, stomatal functioning and seed germination (Gobert et al., 2007). TPK1 is the VK channel and the most abundantly expressed isoform of the vacuolar potassium channel family. TPK1 is considered to be the most prominent member of the TPK family (Zimmermann et al., 2004). It is expressed at the tonoplast. Schonknecht et al., (2002) and Czempinski et al., (2002) showed TPK1 channel to be expressed in the vacuolar membrane of *Arabidopsis* and tobacco. TPKs have four trans-membrane domains (4-TMD), a two pore structure and both pores contain the GYGD motif (Voelker et al., 2006), responsible for K^+ selectivity (Maathuis, 2007) as shown in figure 1. Most of the TPK channels have two clear 'EF hands' localized in the C-terminal domain where Ca^{2+} binds and a 14–3–3 protein (GRF6) binding site, Ca^{2+} is involved in TPK1 regulation (Maathuis, 2007). The phosphorylation of a serine residue (S42) in the N-terminus of the TPK1 channel, by calcium-dependent protein kinases (CDPKs) (Latz et al., 2013) helps in binding of 14-3-3 proteins to the TPK1 channel. This 14-3-3 protein further helps in the activation of the TPK1 channel (Latz et al., 2007). TPKs have been identified in roots, leaves and flowers and have been found in the tonoplast membrane. The proposed structure of TPK1 is shown below.

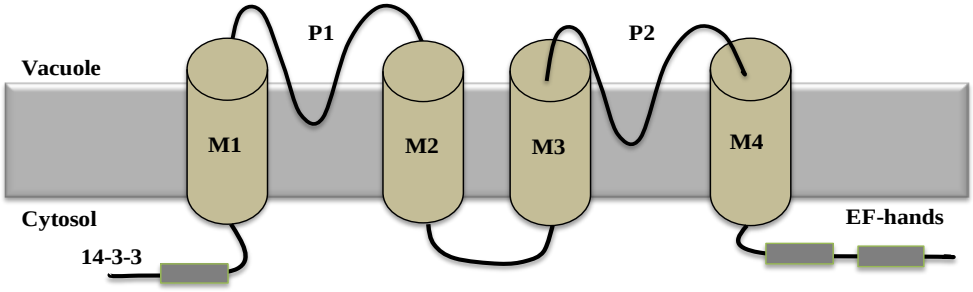

Figure 1.5: Proposed topology of the two pore K^+ channel.

Proposed topology of the TPK1, showing four transmembrane domains (M1, M2, M3, and M4), two pore regions (P1 and P2), 14-3-3 binding site in the amino terminus and a two carboxy terminal Ca^{2+} binding EF-hands.

1.3.2 Function

At the tonoplast TPK1 mediates K^+-selective currents between cytoplasmic and vacuolar compartments. TPK1 activity is independent of vacuolar membrane voltage, but is regulated by cytoplasmic Ca^{2+} and cytoplasmic pH (Gobert et al., 2007). Gobert et al., (2007) showed the absence of current in the vacuolar membrane in the TPK1 loss of function mutants. TPK1 was also reported by Gobert et al., (2007) to be involved in the translocation of intracellular K^+. TPK1 is also involved in the redistribution of K^+ between the different tissues of the plants.

In stomatal guard cells, TPK1 is involved in the removal of Potassium from the guard cell vacuole. Lower concentration of potassium in the guard cells helps in the closure of stomatal pore for preventing or decreasing the rate of transpiration during periods of less water availability. When the TPK1 gene is knocked out, K^+ release in the presence of ABA is delayed, causing slower stomatal closure which can reduce plant growth, but removal of TPK1 has no effect on K^+ uptake during stomatal opening (Gobert et al., 2007). This suggests that during stomatal closure TPK1 could be a possible pathway for vacuolar K^+ release in the presence of ABA.

TPK1 is activated under different environmental factors such as the concentration of potassium, elevated Ca^{2+} concentration in the cell, pH of the cell, S42 phosphorylation and 14-3-3 proteins binding to an N-terminal domain (Latz et. al 2007) and stress hormones such as ABA.

1.3.3 Regulation of TPK1

There are many potential factors that are involved in TPK1 regulation, such as cytoplasmic calcium, pH (Gobert et al., 2007), 14-3-3 proteins (Latz et al., 2007), K^+ concentration and phosphorylation. The 14–3–3 binding motif in TPK1 is phosphorylated and activated by the calcium-dependent protein kinases (CDPKs) (Latz et al., 2013). 14-3-3 protein further helps in the enhanced activation of the TPK1 channel (Latz et al., 2007). Thus, ABA may lead to the activation of TPK1 via phosphorylation of the 14-3-3 domain.

1.3.4 Previous work

The reduction of water loss during abiotic stress such as drought stress can improve growth and quality of the plants. Investigating the pathways of water conservation by plants may help improving water use efficiency in plants. Potassium channels such as TPK1 are involved in the movement of stomata by removing K^+ from the guard cell vacuole thereby resulting in the closure of stomatal pore and resulting in the prevention of water loss. Thus understanding of the activation of TPK1 channel is important. Isner et al., (unpublished data) showed the increased activity of TPK1 in *Arabidopsis* guard cell vacuoles, through patch clamp studies when ATP and 14-3-3 were added to the bath solution. To see the effect of phosphorylation, the vacuoles were further washed with 100 mM NaCO3, no effect on the phosphorylation was observed, which suggested that the kinase responsible for TPK1 phosphorylation and 14-3-3 dependent activation is associated to the tonoplast. Thus, for the identification of that kinase bioinformatics analysis was performed by Isner et al., using SUBA data base. For 17 kinase isoforms (KINASE1 to KINASE17) loss of function mutants were obtained and their activities were observed in the presence of ATP+14-3-3. Out of 17 kinase mutants, two candidate kinase knockout lines, i.e. *kinase1_1* of *At3g02880* and *kinase2* of *At4g21410* were shown to affect TPK1 current stimulation. The current stimulation by these kinases was lower. BiFC approach was further used for analysing the interaction of the kinases to the TPK1 channel. Fluorescence was observed in the guard cell protoplasts that were co-transformed i.e, TPK1-YFP_{Ct} with KINASE1_1-YFP_{Nt} or with KINASE2-$YFP_{Nt.}$ The interaction for the KINASE1_1 was found at the tonoplast while for the KINASE2 the interaction was found at the endoplasmic reticulum. According to the *Arabidopsis* Information Resource (TAIR) both kinases are members of the leucine LRR-RLK

family and are located to the plasma membrane and are expressed ubiquitously in plants. Preliminary data suggest that KINASE1_1, can move from the plasma membrane to the tonoplast through endocytosis (Isner et al., unpublished data). Cysteine-rich receptor-like kinases (CRKs) are shown to be regulated by stress or hormones (Zhang et al, 2013). Nemhauser et al., (2006) showed CRK29 (*At4g21410*) to be significantly down-regulated by ABA. Chen et al., (2010) suggested that the LRR kinase (*AT3G02880*.1) phosphorylation state increases at a C-terminal position after an hour treatment with ABA. The above discussed work showed that this may be the case that the kinases are involved in the activation of TPK1.

1.4 Kinases

MacRobbie (2006) suggested that after ABA application, K^+ is released from the vacuole within minutes. This rapid ABA induced K^+ release may involve a quick posttranslational change and protein-protein interaction. Isner et al., (unpublished data) showed kinases to be involved in the regulation of TPK1. The potential role of kinases in ABA dependent activation of TPK1 were studied here. Kinases are the key regulators of cell functions. Kinases are involved in the transfer of a phosphate (PO_4^{3-}) group from ATP to a substrate (the process called phosphorylation). The substrates may be protein, lipids, carbohydrates, amino acids and nucleic acids. The phosphate group is covalently attached to a specific substrate. Phosphorylation is an important process for the function and activity of many proteins. It activates and deactivates many protein enzymes. Kinases also help in determining the location of many proteins. Specific kinases are often named after their substrates for example, protein kinases (Cyclin dependent kinases, Mitogen-activated protein kinases), lipid kinases (Phosphatidylinositol kinases, Sphingosine kinases) and carbohydrate kinases (Hexokinase, Phosphofructo kinase). A brief description of the functions of these kinases are below:

Protein kinases are involved in the signal transduction (Hideji Karbiet and Setsuko Komatsu, 1995) of various stimuli such as biotic and abiotic stresses. Protein kinases attach phosphate groups to a specific amino acids for modifying protein function. Some protein kinases have been found to be Ca^{2+} dependent (D. M. Roberts and A. C. Harmon 1982) known as CDPKs (calcium dependent protein kinases). Another class of protein kinases, Cyclin dependent kinases (CDKs) belongs to serine/threonine protein kinases. During different stages of cell division the CDKs make sure that the cells

progress in an organized manner (Francis D, 2007). Mitogen activated protein kinase (MAPK) are involved in response to abiotic stress (Alok Krishna Sinha, 2011). In *Arabidopsis* the expression of AtMEKK1 (*Arabidopsis thaliana* MAP kinase kinase) and AtMPK3 (*Arabidopsis thaliana* MAP kinase kinase kinases) could be induced during drought stress (Mizoguchi T, 1996). Lipid kinases attach phosphate group to lipids in the cell and helps in changing the reactivity and localization of the lipids. For example, Phosphatidylinositol kinases phosphorylates lipids. The phosphorylated lipids (phosphoinositides) help in cell signalling, lipid signalling and membrane trafficking (Munnik T, 2011). Sphingosine kinases are lipid kinases that catalyzes the formation of lipid second messenger (Olivera A 2001) such as sphingosine-1-phosphate from the precursor sphingolipid sphingosine. Hexokinase a carbohydrate kinase phosphorylates six carbon sugars (hexoses). While phospofructo kinase phosphorylates Fructo 6-phosphate.

While Phosphatases are involved in the removal of PO_4^{3-} group from a substrate through hydrolysis (the process called dephosphorylation) that is opposite to the function of kinases. The phosphatases are named after their substrates for example, phosphotyrosine, (Tyrosine-specific phosphatases), phosphoserine/-threonine (serine-/threonine-specific phosphatases).

1.4.1 Receptor-like kinases (RLKs)

Plant cell responds to different environmental stress conditions (stimuli), for example biotic or abiotic stress condition, through receptors present on the cell surfaces. The plant response to these stimuli could be in various forms, for example, division, expansion or elongation of the cells, cell death or abscission of leaves, synthesis of chemical compounds and preparing themselves to fight against the infections and pathogens (Morris and Walker 2003). Receptor like kinases (RLKs) are transmembrane receptor proteins that assist communication from cell to cell and between the cell and its outside environment (Shiu and Bleecker, 2001). RLK is the largest family of such receptors. In *Arabidopsis* there are more than 400 identified membrane-associated RLKs. The first plant RLK was identified in 1990 in maize by Zan et al., in 2013. RLKs have also been shown to play a role in light responses and have been suggested to be present throughout the plant (Shiu et al., 2004).

1.4.2 Leucine-rich repeat RLKs (LRR–RLKs)

Leucine rich repeat-RLKs are the largest group of receptor-kinases in *Arabidopsis* (Diévart and Clark, 2003). In *Arabidopsis* there are 120 genes of (LRR)-RLKs. Osakabe et al., (2013) suggested that the surface of plasma membranes of some plants contains RLKs that are known to be involved in the early steps of osmotic-stress signalling. LRR-RLKs have tandem repeats of about 24 amino acids with conserved leucines (Colette et al., 2011). It has an extracellular membrane for sensing stimuli, the extracellular LRR domain that are localized at the plasma membrane function as a receptor and is involved in protein-protein interactions (Walker, 1994) in response to various environmental and developmental signals (Dievart and Clark, 2004). A C-terminal cytoplasmic serine/threonine kinase domain involved in protein phosphorylation. The (LRR)-RLKs are known to be involved in the plant growth, development, differentiation, stress response (Zan et al., 2013) and disease resistance (Song et al., 1995).

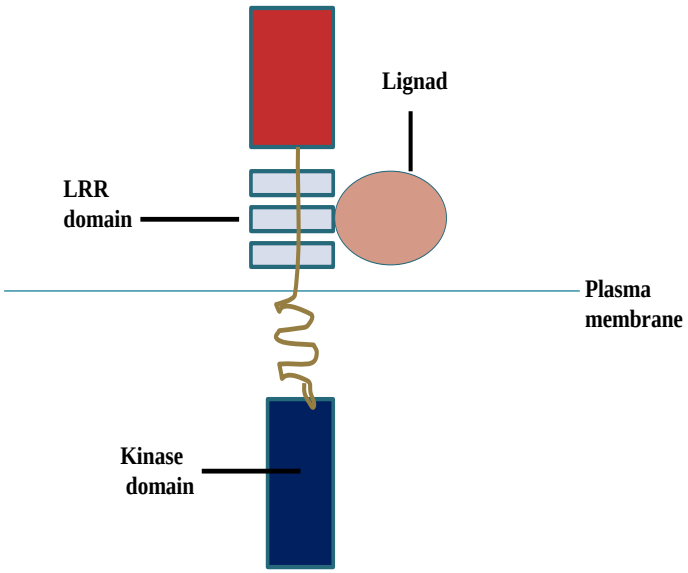

Figure 1.6: Structure of leucine-rich repeat receptor-like kinase.

LRR-RLK has an extracellular leucine rich repeat domain and a cytoplasmic kinase domain.

1.4.3 Objectives

Isner et al., (unpublished data) showed increased activity of TPK1 in *Arabidopsis* guard cell vacuoles. The kinases responsible for TPK1 phosphorylation and 14-3-3 dependent activation was shown to be associated to the tonoplast. Thus, for the identification of that kinase bioinformatics analysis was performed and candidates tested using a loss of function approach. Two candidate kinases i.e. KINASE1 (gene code *At3g02880)* and KINASE2 (*At4g21410)* were shown to affect TPK1 current stimulation. The current stimulation by these kinases was lower. BiFC approach was further used for analysing the interaction of the kinases with the TPK1 channel. Fluorescence was observed in the guard cell protoplasts that were co-transformed. The interaction for the KINASE1_1 was found at the tonoplast while for the KINASE2 the interaction was found at the endoplasmic reticulum.

Therefore the objectives of the thesis were:

To investigate the role of the TPK1 channel, KINASE1 and KINASE2 in the growth and stomatal conductance of *Arabidopsis*. To achieve this, wild type and loss of function mutants were compared in growth experiments and stomatal conductance measurements and the results are discussed.

Chapter 2

2 Materials and Methods

2.1 Plant material

The seeds of different lines of *Arabidopsis* were obtained from Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/cgi-bin/tdnaexpress). Ecotype Colombia was used as wild type. Two T-DNA insertion lines (Salk_019840 and Gabi_047F11) of LRR-RLK *at3g02880*, and one T-DNA insertion line (Sail_447_FO6) of LRR-RLK *at4g21410* were used. As documented in the Signal Salk database, T-DNA is inserted in the first exon of *at3g02880* in Salk_019840 mutant line, while insertion is in the 300 UTR in case of Gabi_047F11mutant line of the same gene.The T-DNA is inserted in the first exon of *at4g21410* in Sail_447_FO6. These lines were named formally as *kinase1_1* (Salk_019840), *kinase1_2* (Gabi_047F11) and *kinase2* (Sail_447_FO6). These lines were used to study the effect of these genes on growth and stomatal conductance in *Arabidopsis* under different conditions.

2.2 Growth medium and growth conditions

2.2.1 Seed sterilization

The *Arabidopsis* seeds were sterilized using 70% ethanol for 1 minute, then using 70% ethanol with a drop of tween-20 for 15 minutes and followed by five times washing with sterilized distilled water.

2.2.2 Growth on ½ MS plates

Arabidopsis seeds were grown in different media conditions to study plant growth. Seeds were sown on ½ MS (Murashige and Skoog medium) control plates containing macronutrients (10.30 mM NH_4NO_3, 1.49 mM $CaCl_2.2H_2O$, 0.750 mM $MgSO_4.7H_2O$, 0.625 mM KH_2PO_4, and 9.39 mM KNO_3) and micronutrients (0.050 μM H_3BO_3, 0.052 μM $CoCl_2.6H_2O$, 0.05 μM $CuSO_4.5H_2O$, 0.049 μM $FeSO_4.7H2O$, 0.0499 μM $MnSO_4.4H_2O$, 0.516 μM $Na_2Mo4O_4.2H_2O$, 14.9 μM $ZnSO_4.7H_2O$). The control medium was supplemented with additional KCl to increase the concentration of K^+ up to 50 mM in the medium for the salt stress treatment. While for the osmotic stress

treatment the control medium was supplemented with sorbitol solution to 50 mM (w/v) and 80 mM (w/v) sorbitol. KH_2SO_4 and KNO_3 in the control medium were replaced with the equimolar concentrations of Na^+ salts, i.e., NaH_2PO_4 and $NaNO_3$ for the zero K^+ condition.

2.2.3 Growth in soil

Seeds were sown with the help of sterile toothpicks on P40 trays containing compost from Sinclair named Levingtons F2 + sand. Each pot contained one seed. The trays were kept in the cold room for 2—3 days, and then were transferred to the growth room. The trays were regularly watered by the horticulture technicians for 3 to 4 weeks.

2.2.4 Growth environment

The plants were kept in the growth room under 20-23 C^0 at 16hrs day length.

2.3 Chemicals and consumables

The chemicals used in this study were purchased from different companies including Sigma (UK), Fischer Scientific (UK) and the consumables were purchased from Star Lab (UK), Eppendorf (UK). The RNA extraction kits, gel purification and protein assay kits were purchased from Qiagen (UK) and Fermentas (UK) Thermo scientific.

2.4 DNA extraction from *Arabidopsis* plants

DNA was extracted from individual *Arabidopsis* plants according to the CTAB method. The leaves were ground to fine powder in liquid nitrogen and powder was quickly mixed with 500 µl pre-warmed CTAB (Hexadecyl trimethyl-ammonium bromide) buffer and incubated at 65 °C for 60 minutes. After vortexing the mixture, 300 µl of chloroform: isoamylalcohol solution (24:1v/v) was added. The mixture was vigorously shaken and centrifuged for 7 min in a microfuge. The top aqueous layer was transferred to clean, sterilized, Eppendorf tubes and DNA was precipitated by adding 2 volumes of 96 % ethanol and 4% 3 M NaAc (pH 5.2). The mixture was vortexed and left at room temperature for 30 minutes to precipitate the DNA. The mixture was then centrifuged for 10 min at 13000 RPM to obtain the DNA pellet. Finally, the pellet was rinsed in 70 % ethanol. It was then dried for 10 min and resuspended in 100 µl sterilized water.

2.5 Total RNA isolation from *Arabidopsis* plants

Total RNA was extracted from leaf tissues of individual homozygous *Arabidopsis* plants, using an RNase easy KIT (Qiagen, UK). Approximately 100 mg leaves were ground to fine powder in liquid nitrogen with the help of a grinder, while kept frozen. The tissue was transferred to RNase-free 2 ml tubes, allowing the liquid nitrogen to evaporate, but not letting the tissue to thaw. 450 µl of buffer RLT (a lysis buffer) was added and vortexed vigorously. The lysate was added to a Q1Ashredder spin column placed in a 2 ml tube and was centrifuged for 2 min on full speed. The supernatant was transferred to a clean 1.5 ml Eppendorf tube. 0.5 ml of 96% ethanol was added to the lysate and was mixed immediately by pipette. The sample was then transferred to an RNeasy spin column and then centrifuged for 30 Sec at 10,000 RPM. The follow-through was discarded. To wash the RNeasy column 350 µl of buffer RW1 (a washing buffer) was added and centrifuged for 30 Sec. 10 µl of the DNase I stock solution was added to 70 µl of buffer RDD and was mixed by inverting the tube several times. 80 µl of DNAse I incubation mixture was directly poured over the RNeasy spin column membrane and left on the bench for 15 minutes. To wash the RNeasy column, 350 µl of buffer RW1 was added and centrifuged for 30 Sec and the flow-through was discarded. To wash the RNeasy spin column 500 µl of buffer RPE (a wash buffer) was added and then centrifuged for 30 Sec at 10,000 RPM. 500 µl of buffer RPE was again added and then centrifuged for 2 minutes at 10,000 RPM. For avoiding any carryover of buffers and contaminations the RNeasy spin column was placed in a new 2 ml collection tube and was centrifuged for 1 min at 10,000 RPM. The RNeasy spin column was removed and placed in a 1.5 ml Eppendorf tube. For eluting the RNA 50 µl of RNase free water was added to the RNeasy spin column and then centrifuged for 1 minute at 1000 RPM.

2.6 cDNA synthesis

Two micrograms of the RNA and 0.5 µg of oligo-dT primer per microgram of RNA were mixed and water was added to make the total volume up to 15 µl. For melting the secondary structure in the template, the tube was heated to 70 ^{o}C for five minutes. The tube was then cooled on ice to prevent the secondary structure from reformation. The tube was then briefly spun for collecting the solution. M-MLV 5X reaction buffer (5 µl), dNTP mix (1.25 µl), and a recombinant Rnasin ribonuclease inhibitor (0.5 µl) were added to the tube. The enzyme used for the synthesis of the first strand of cDNA was

the Moloney murine leukemia virus Reverse transcriptase (M-MLV RT), an RNA-dependent DNA polymerase reverse transcriptase. M-MLV RT (1 µl) was added and the final volume was adjusted to 25 µl. The sample was mixed gently and was incubated at 42^{o}C for 60 minutes.

2.7 Screening of the putative mutant lines using PCR

The putative T-DNA insertion lines were screened by PCR technique. Different sets of primers, i.e., gene specific (forward and reverse) and T-DNA specific primers were used. DNA obtained from the leaf tissues of the *kinase1_1*, *kinase1_2* and *kinase2* was used as a template for the PCR.

PCR mix was prepared in 2 ml Eppendorf tube by adding and mixing 5 µl Go-Taq Flexi buffer, 2 µl of $MgCl_2$, 1 µl of dNTP, 1 µl of each primer, 0.1 µl of Go-Taq polymerase, 2-3 µl of the template DNA and the final volume was made to 25 µl (per reaction) by adding the required amount of water. PCR conditions varied according to the requirement of the primer pairs (Table 2.1) and the size of the amplicon. The PCR products were resolved by electrophoresis in a 1% agarose gel (w/v).

2.8 Identification of homozygous lines and RT-PCR analyses

RT-PCR was performed for identifying the homozygous lines of *kinase1_1*, *kinase1_2* and *kinase2*. The cDNA from individual homozygous plants was prepared and used as a template for the RT-PCR to analyse the transcript level of the respective genes. PCR mix was prepared in 2 ml Eppendorf tube by adding and mixing 5 µl Go-Taq Flexi buffer, 2 µl of $MgCl_{2,}$ 1 µl of dNTP (10 mM of each deoxyribonucleotide triphosphates), 1 µl of each primer, 0.1 µl of Go-Taq polymerase (5U/µl), 2-3 µl of the template cDNA and the final volume was made to 25 µl (per reaction) by adding the required amount of water. PCR was performed on a Mastercycler machine. PCR conditions varied according to the requirement of the primer pairs (Table 2.1) and the size of the amplicon. The PCR products were resolved by electrophoresis in a 1% agarose gel.

Table 2.1 Primers used to screen the knockout mutant lines.

Gene ID	Primer Sequence	Product Size in bp (base pairs)
Salk_ 019840 F	TATTCCGAGTTCGTTGTCGTC	998 bp
Salk_ 019840 R	ATGAGAAGTTGTCCCATCACG	998 bp
Sail_447_F06 F	AAATACAGCAGGGATGTG	1184 bp
Sail_447_F06 R	TCTCAGCATCACAACAACTCG	1184 bp
RP_Gabi_047F11	CAGCAGTTGAATGAAGAAGGC	1289 bp
LP_Gabi_047F11	TTGCTTCCACGGTTTAAAAAC	1289 bp
T-DNA Lb	ATATTGACCATCATACTCATTGC	580 bp
Gabi_047F11 F	CGTGATGGGACAACTTC	643 bp
Gabi_047F11 R	ACTTGACCCCAAACAATATTCAAATC	643 bp
Lb2-Sail	GCTTCCTATTATATCTTCCCAAATTAC	650 bp
Lba1	TTTTTCGCCCTTTGACGTTGTTGGAGT	1000 bp
Actin F	ACGAGCAGGAGATGGAAAC	510 bp
Actin R	ACCCCAGCTTTTTAAGCCTT	510 bp

2.9 Growth analysis

2.9.1 Root length analyses on ½ MS plates

Plants were grown in different media conditions on ½ MS plates to analyse their root length. One week old plants were transferred from the control ½ MS plates to different media conditions (10 mM K^+ [control], 0 mM K^+, 50 mM K^+, 50 mM sorbitiol, 80 mM sorbitol) and were analysed for root length after two weeks. Three plants of each genotype were plated on a single plate. Three plates of each media condition were used in one replicate. The experiment was replicated three times. The root lengths were

marked at the beginning and at the end of the experiment as initial and final root length respectively. Plates were scanned and the root lengths were measured using Image J software (http://imagej.en.softonic.com/). The percent increase in root length per day was determined using the equation, $[\{(L_f-L_i)/L_i\}/\text{No of days}]*100$ where L_f is the final root length, L_i is the initial root length, No of days is the total duration plants exposed to different treatments.

2.9.2 Fresh weight analyses on ½ MS plates

The plants were grown on ½ MS plates as mentioned in the section 2.2.2. The total weight of three plants of each genotype growing on one plate was taken. Each replicate of the experiment contained three plates for each media conditions. The Experiment was replicated three times. Percent gain of fresh weight was calculated using the equation, (total fresh weight/No of days) *100.

2.9.3 Fresh weight analyses in soil

The plants were grown on soil as mentioned in the section 2.2.3. The fresh weight of the soil grown plants was analysed in stress conditions. Three weeks old plants were exposed to control, moderate stress and severe drought stress condition for two weeks. The plants in control conditions were watered with 220 ml of water while the moderate stressed with 110 ml and severe stressed with 55 ml of water per tray. Each tray contained six plants. Each replicate of the experiment contained three plants of each genotype in each tested condition. The experiment was replicated three times. After two week treatment, plants were removed from the soil, roots were washed with water and dried with blotting paper and then fresh weights of the plants were taken using a balance. The percent gain of the fresh weight was calculated using the equation as mentioned in section 2.9.2.

2.10 Whole leaf stomatal conductance measurements:

To see if stomatal conductance of the kinase KO lines (*kinase1_1*, *kinase1_2*, *kinase2*), *tpk1* and wild type leaves show different responses to ABA, similar size leaves were exposed to 1 µM, 10 µM and 100 µM of ABA for 30 minutes. The leaves of 3 to 4 weeks old plants were used in the experiment. In each replicate of the experiment, eight leaves of each KO line were analysed in comparison with wild type. The excised leaves

were kept in stomatal opening buffer (consisting of 10 mM KCl and 10 mM Mes-KOH (pH 6.15) with their abaxial surface up for one hour. As a control value, stomatal conductance of eight leaves of each genotype per treatment was measured with an infrared gas analyser (Li-Cor 6400 (LI-COR, Cambridge, UK) portable photosynthesis system). Half of the leaves of the KO and wild type were then transferred to control and ABA treatment for 30 minutes. After 30 minutes leaves were removed from the solutions and their stomatal conductance was measured. The average of stomatal conductance of the leaves at control condition was subtracted from the ABA treated condition and the values were then calculated in percentage.

2.11 Statistical treatment of data

The data for the growth and stomatal conductance experiments were obtained from three individual replicates. The significance was analysed using unpaired two-tailed Student t-tests using MS excel program. Significance levels were at $p<5\%$ unless indicated otherwise. The error bars in the figures represent standard errors.

Chapter 3

3 Results

3.1 Screening of *Arabidopsis* thaliana kinase mutant lines

Two putative mutant lines (*kinase1_1* and *kinase1_2***)** of the LRR-receptor like kinase (gene code) *At3g02880* and one putative mutant line (*kinase2*) of the LRR-RLK (gene code) *At4g21410* were identified through bioinformatics analyses and electrophysiological experiments and were thought to be involved in the activation of TPK1. For further phenotypic characterisation of these lines in different conditions, these lines were tested by using PCR and RT-PCR at both DNA and cDNA level.

Gene specific primers (forward and reverse) and LBA (left border primer) and SAIL were used for the screening (Table 2.1). The PCR results showed amplification when a gene specific and a T-DNA primer was used for all the three mutant lines which indicates the insertion of T-DNA in the genes (figure 3.1 A, B and 3.3 B). The results also showed absence of amplification with gene specific forward and reverse primers for all the three putative mutant lines which suggests that these lines are homozygous for T-DNA insertion (figure 3.2 A, B and 3.3 A).

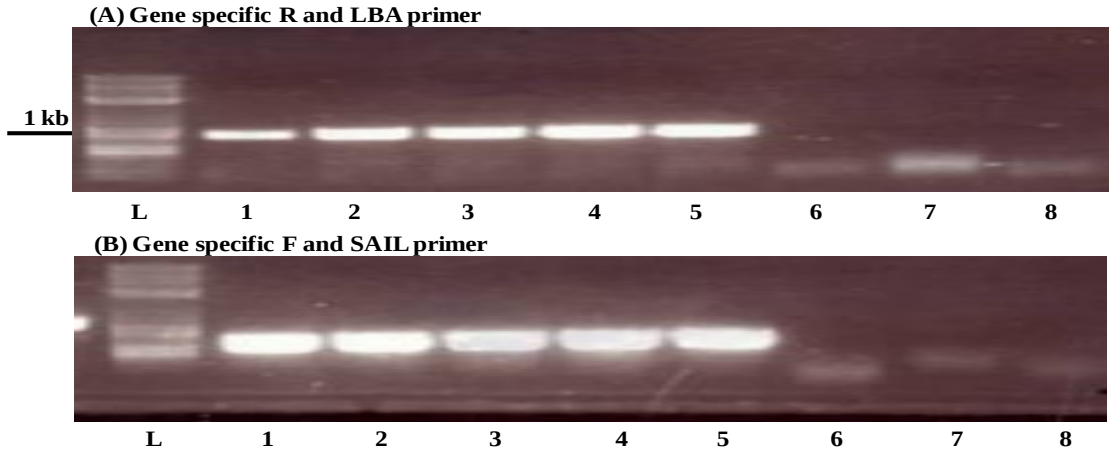

Figure 3.1: PCR for screening the *kinase1_1* and *kinase2* knockout mutant lines.

Figure 3.1A: PCR for screening of the *kinase1_1* knocout mutant line with gene specific reverse and left border primer. Lane 1-5: *kinase1-1* mutant line DNA, lane 6: water (-ive control); lane 7 and 8: gDNA wild type (+ive control). Figure 3.1B: Screening of the *kinase2* knocout mutant line with gene specific forward and T-DNA primer; Lane 1-5: gDNA from the *kinase2* mutant line, lane 6: water (-ive control), lane 7 and 8: gDNA wild type (+ive control), L: is the ladder in both A and B.

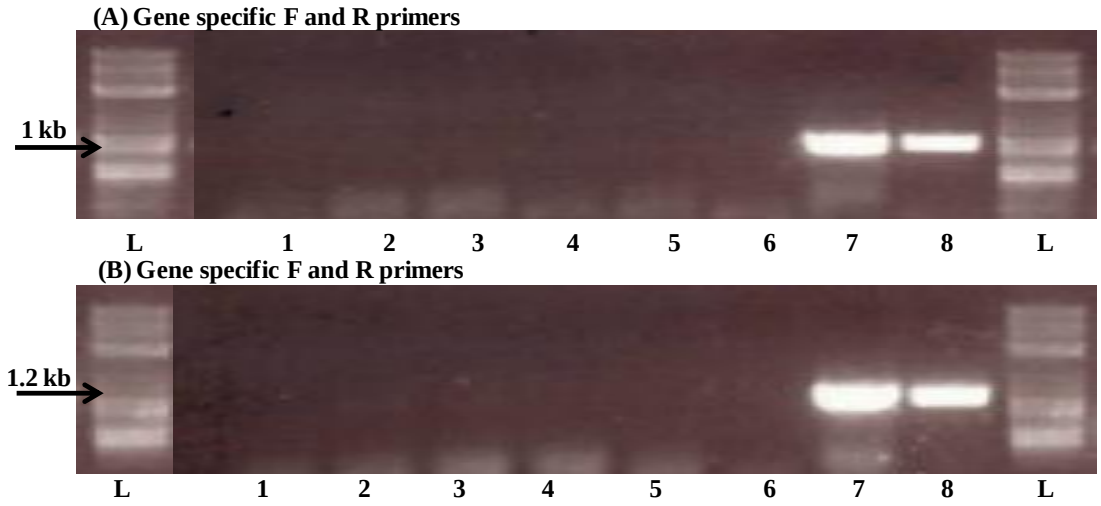

Figure 3.2: PCR for screening the *kinase1_1* and *kinase2* knockout mutant lines.

Figure 3.2A: PCR for screening of the *kinase1_1* knockout mutant line with gene specific forward and reverse primers. Lane 1-5: gDNA from mutant line, lane 6: water (-ive control); lane 7 and 8: gDNA wild type (+ive control); Figure 3.2B: Screening of the *kinase2* knockout mutant line with gene specific forward and reverse primers. ; Lane 1-5: gDNA from the kinase mutant line, lane 6: water (-ive control), lane 7 and 8: gDNA wild type (+ive control), L: is the ladder in both A and B.

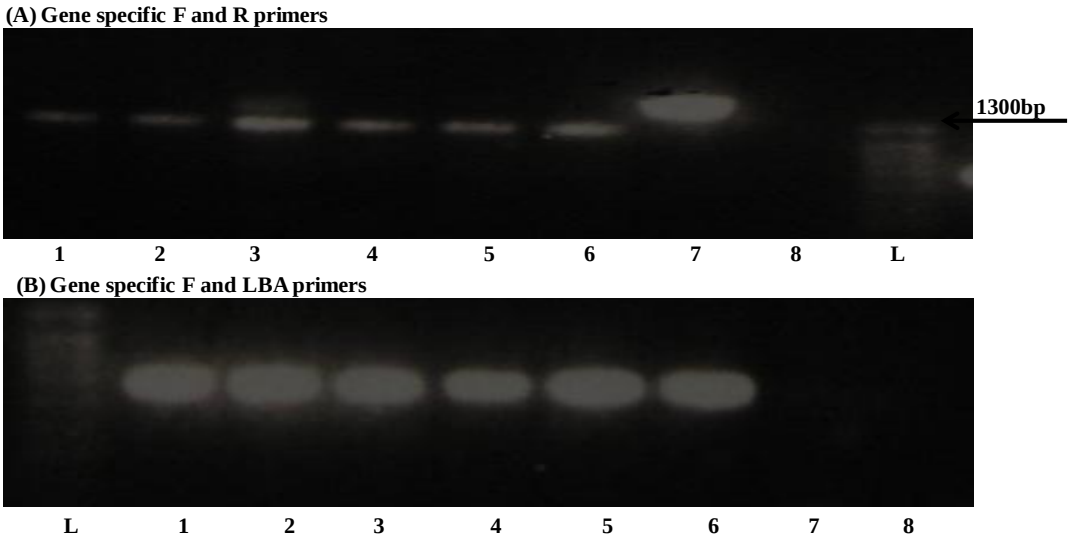

Figure 3.3: PCR for screening the *kinase1_2* knockout mutant lines.

Figure 3.3A: PCR for screening of the *kinase1_2* knockout mutant line with gene specific forward and reverse primers. Lane 1-6: gDNA from mutant line, lane 7 gDNA wild type (+ive control); Lane 8: water (-ive control).Figure 3.3B: Screening of the *kinase1_2* knockout mutant line with gene specific forward and left border primer. Lane 1-6: gDNA from the kinase mutant line, lane 7 gDNA wild type (+ive control); lane 8 water L is the hyper ladder iv.

The above mentioned mutant lines were further tested by RT-PCR to check the transcript level of these genes. The results showed a lack of transcript for the lines *kinase1_1* (figure 3.4) and *kinase2* (figure 3.5) with gene specific and actin forward and

reverse primers. However, we observed the presence of a transcript for the *kinase1_2* when gene specific forward and reverse primers were used (figure 3.6).

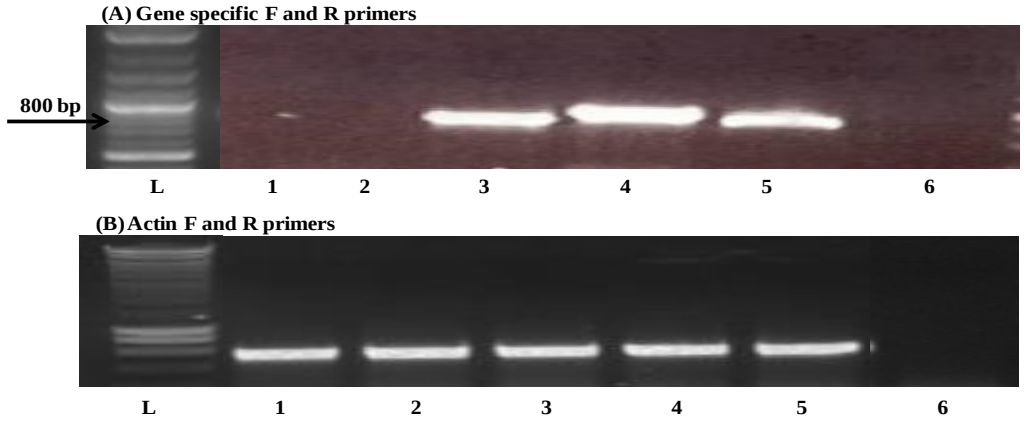

Figure 3.4: RT-PCR for *kinase1_1* knockout mutant line.

Figure 3.4A: RT-PCR for screening of *kinase1_1* knockout mutant line with gene specific forward and reverse primers. Figure 3.4B: RT-PCR screening of *kinase1_1knockout* mutant line with actin primers, Lane 1 and 2: cDNA from mutant line *kinase1_1* lane 3: cDNA from wild type, lane 4: gDNA from WT (+ control), lane 5: cDNA from another kinase mutant line (+ive control), lane 6: water (-ive control), L: is the ladder.

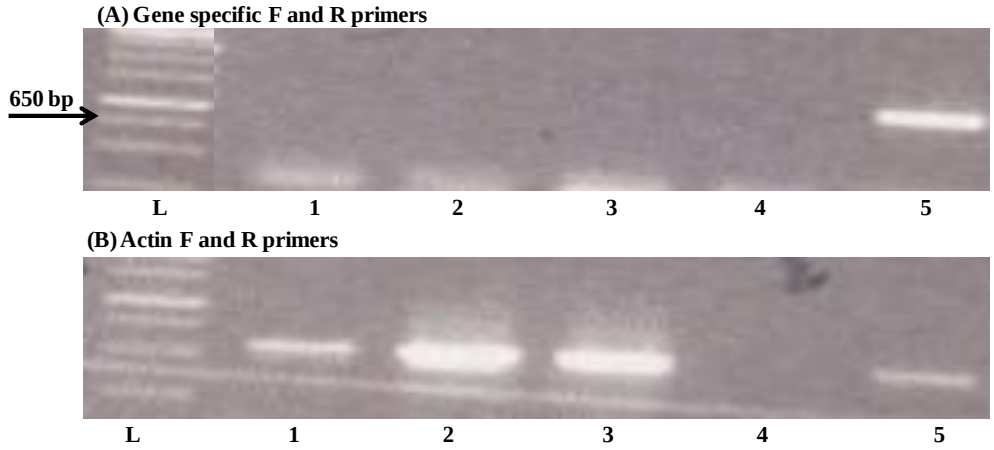

Figure 3.5: RT-PCR for *kinase2* knockout mutant line.

Figure 3.5A: RT-PCR for screening of *kinase2* knockout mutant line with gene specific forward and reverse primers. Figure 3.5B: RT-PCR screening of *kinase2* knockout mutant line with actin primers, Lane 1, 2 and 3: cDNA from mutant line *kinase2*, lane 4: water, lane 5 cDNA from WT (+control), L: is the ladder.

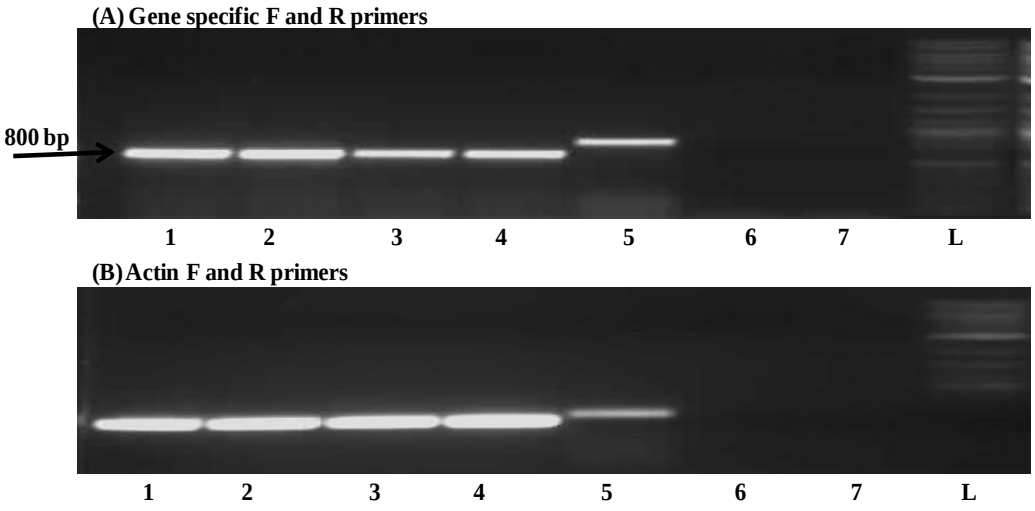

Figure 3.6: RT-PCR for *kinase1_2* knockout mutant line.

Figure 3.6A: RT-PCR for screening of *kinase1_2* knockout mutant line with gene specific forward and reverse primers. Figure 3.6B: RT-PCR screening of *kinase1_2* knockout mutant line with actin primers. Lane 1 and 2: cDNA from mutant line *kinase1_2*, lane 3 and 4: cDNA from wild type (+ive control), lane 5 gDNA from wild type (+ive control): lane 6 and 7 water (-ive control), L is the ladder.

3.2 Characterization of wild type, *tpk1* and kinase mutant lines

3.2.1 Analyses of root length

To see whether the loss of function in the kinase and TPK1 genes had any effect on the root length and fresh weights of the plants, root length of the wild type and knockout lines was analysed in several conditions. The results showed that there was no significant difference in root growth between any of the tested genotypes in control and 80 mM sorbitol conditions. However, all the three knockout lines showed less root length per day as compared with the wild type plants at 0 mM K^+, 50 mM K^+ and 50 mM sorbitol (figure 3.7) indicating a strong connection between the kinases and TPK1 channel.

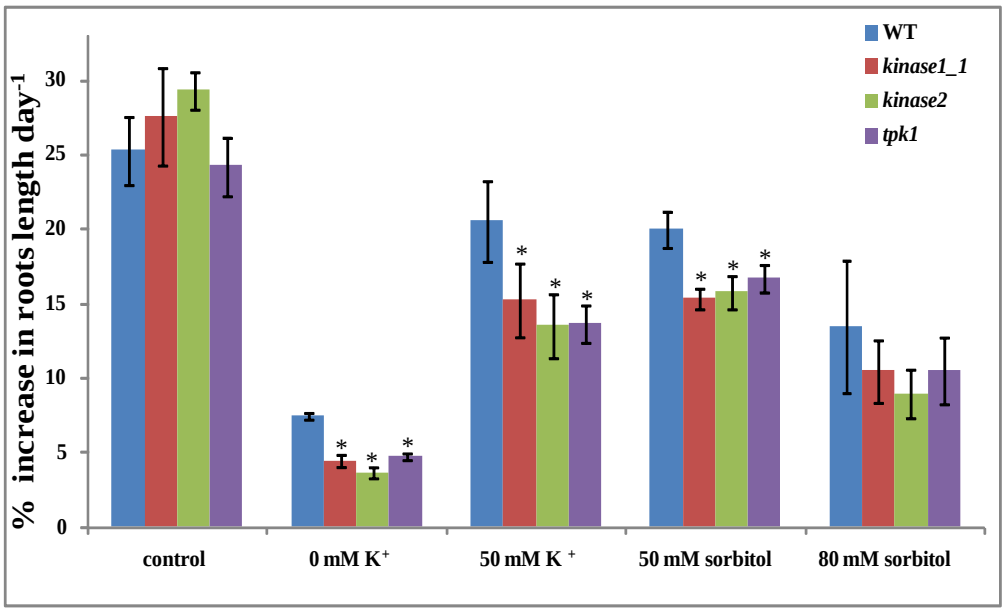

Figure 3.7: Percent increase in roots length of wild type and transgenic lines exposed to different media conditions on ½ MS plates.

Figure 3.7: Percent increase in root length per day of the wild type and three mutant lines as mentioned in the figure. The plants were grown for two weeks under different conditions (10 mM K^+ [control], 0 mM K^+, 50 mM K^+, 50 mM sorbitol and 80 mM sorbitol) and root lengths were measured. Data are from three independent experiments, and the bars in the figure represent the standard errors. * denotes a significant difference by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines.

3.2.2 Analyses of fresh weight on plates

Fresh weights of the wild type and knockout lines was analysed (figure 3.8) in several conditions to observe whether the loss of function in the kinase and TPK1 genes had any effect on the fresh weights of the plants. The results showed no difference in the fresh weight of all the tested genotypes in control, 50 mM K^+ and 50 mM sorbitol conditions. However, all the knockout lines showed less fresh weight as compared with the wild type plants in 0 mM K^+ and 80 mM sorbitol conditions (figure 3.8). The results again pointing towards a relation between the kinases and TPK1 channel.

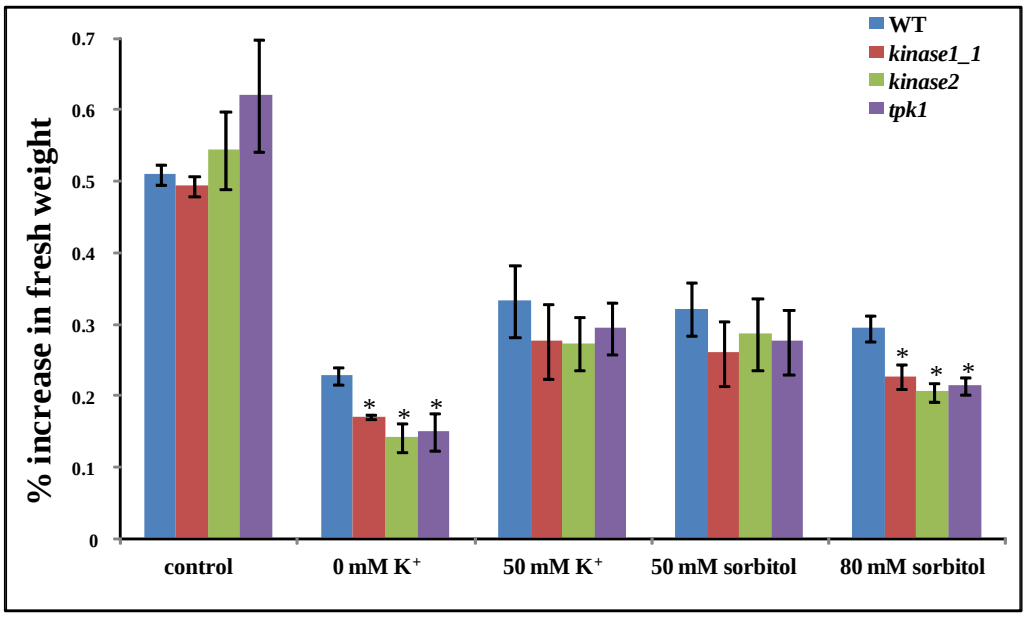

Figure 3.8: Percent increase in fresh weights of wild type and transgenic lines exposed to different media conditions on ½ MS plates.

Figure 3.8: Percent increase in fresh weights of the wild type and three mutant lines as mentioned in the figure. The plants were grown for two weeks under different conditions (10 mM K^+ [control], 0 mM K^+, 50 mM K^+, 50 mM sorbitol and 80 mM sorbitol) and fresh weight of each plant was recorded. Data are from three independent experiments, and the bars in the figure represent the standard errors. * denotes a significant difference by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines.

3.2.3 Analyses of fresh weight in soil

To see whether loss of function in the kinase and TPK1 genes has any effect on the fresh weights of plants, six soil grown plants were kept per tray and then each tray was exposed to control (220 ml water), moderate (110 ml water) and severe drought (55 ml water) stress conditions. The fresh weight of the wild type and knockout lines was then analysed. The results showed less fresh weight for all the knockout lines as compared with the wild type plants in control and moderate stress conditions. No difference was observed for all the genotypes at severe drought stress in soil (figure 3.9).

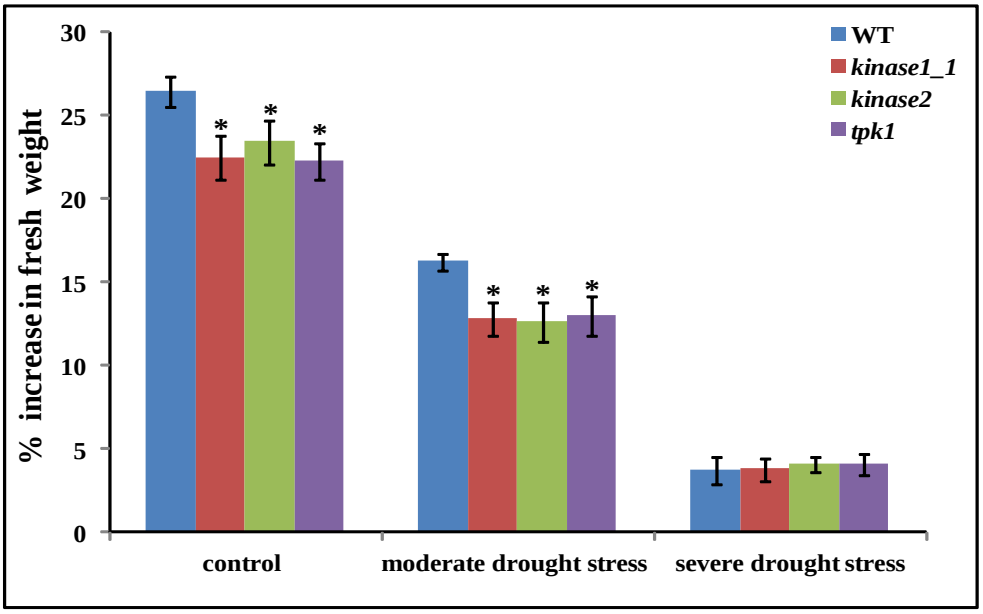

Figure 3.9: Percent increase in fresh weights of wild type and transgenic lines exposed to different conditions in soil.

Figure 3.9: Percent increase in fresh weights of the wild type and three mutant lines as mentioned in the figure. The plants were grown in soil in control conditions and then were exposed to experimental conditions (control, moderate drought and severe drought) for two weeks and fresh weight of each plant was recorded. Data are from three independent experiments, and the bars in the figure represent the standard errors. * denotes a significant difference by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines.

3.3 Stomatal conductance

Isner et al., (unpublished data), showed LRR kinases to be involved in the phosphorylation of AtTPK1 in ABA dependent manner. Therefore, further to see any phenotypic relation among, the knockout lines of LRR kinases and *Attpk1* stomatal conductance in comparison with the wild type plants were tested (figure 3.10). In general, the data showed less reduction in stomatal conductance in response to ABA for all the tested KO lines as compared to the wild type. The similarities in the stomatal conductance phenotype between the kinase knockouts and *tpk1* KO mutants suggest a relation between these kinases and the TPK1 channel. Combined with other, as yet unpublished data, the data from this report support the idea of the involvement of these kinases in ABA dependent regulation of TPK1.

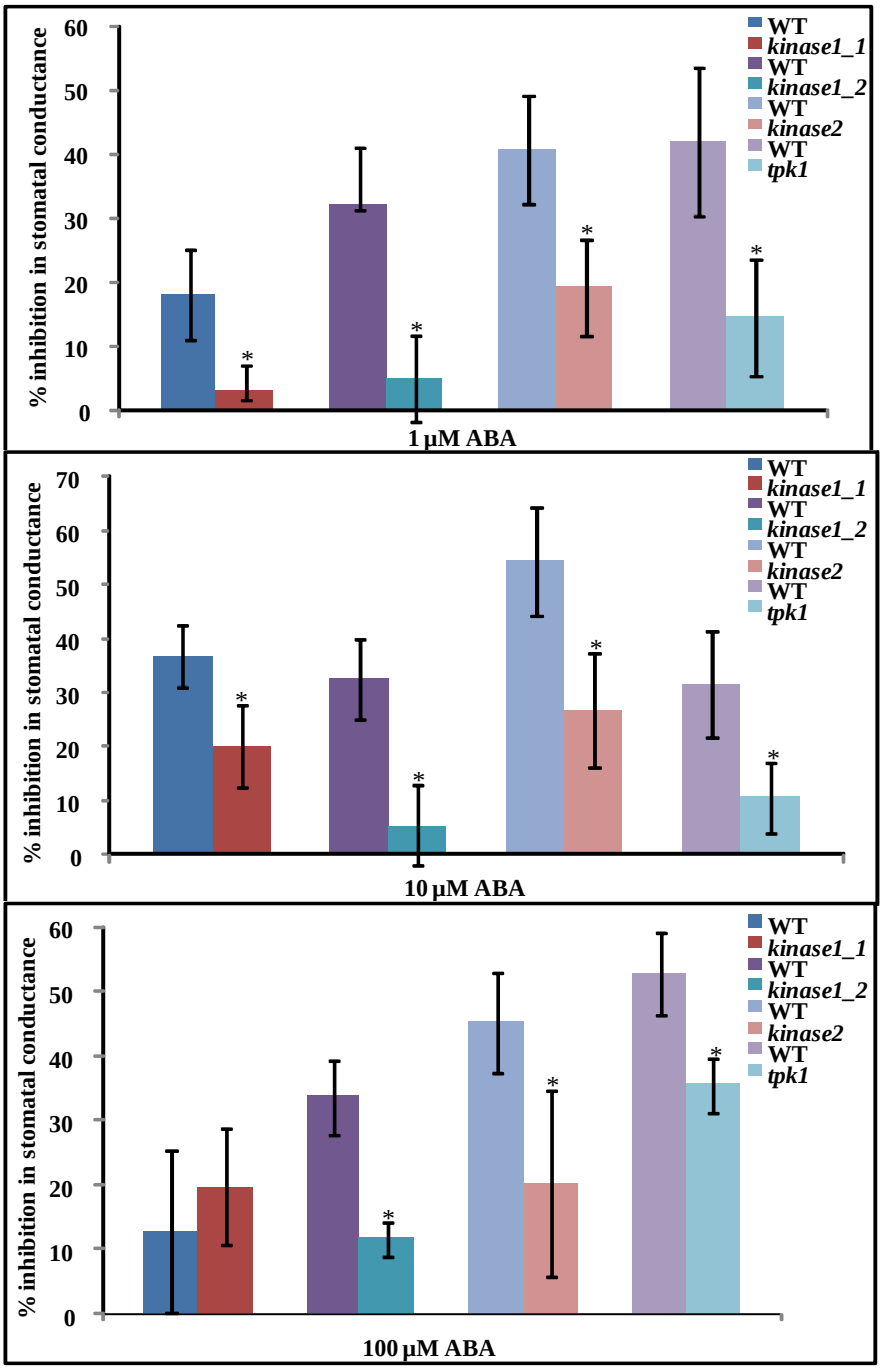

Figure 3.10: Analyses of the stomatal conductance of wild type and mutant lines at different ABA concentrations, i.e., 1 µM ABA (figure 3.10A), 10 µM ABA (figure 3.10B) or 100 µM ABA (figure 3.10C).

The stomatal conductance of various genotypes (as mentioned in figure 3.10) was analysed under different concentrations of ABA. The leaves were incubated in stomatal opening buffer for one hour and then their initial stomatal conductance was measured with an infrared gas analyser. The leaves were then kept in two conditions, i.e., control, which has opening buffer, 1 µM ABA (figure 3.10A), 10 µM ABA (figure 3.10B) or 100 µM ABA (figure 3.10C) for 30 minutes. The final conductance was expressed as a percentage of the initial conductance measured in wild type leaves which was set at 100%. Data show averages ± SEM for one independent experiment by using 8 leaves for each genotype. The error bars represent the standard error. * denotes a significant difference by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines. The average of control experiments was subtracted from the average of the ABA treated experiments.

CHAPTER 4

4 Discussion and conclusions

The vacuolar potassium channel AtTPK1 has been shown to have a major role in ABA-dependent stomatal closure (Gobert et al., 2007). The activated TPK1 effluxes K^+ from the guard cell vacuole resulting in the closure of the stomata and then prevents the loss of water from the plant. How the coupling of ABA to AtTPK1 at the tonoplast takes place is not yet known.

There is a possible way that kinases may bring external ABA and attaches it with AtTPK1. As discussed in the previous work section, *kinase1_1* and *kinase2* knockout lines were shown by patch clamp study to affect TPK1 current (Isner et al., unpublished data). Therefore, these kinases were further studied in relation to their interaction with TPK1. Growth experiments and stomatal conductance were performed for studying the involvement of these kinases in TPK1 activation. Kinase knockout lines (*kinase1_1*, *kinase1_2*, and *kinase2*), *tpk1* and WT were used and their roles were studied and compared.

4.1 Kinases have role in TPK1 channel regulation

Growth experiments were done to see whether the loss of function in the kinase and TPK1 genes had any effect on the root length and fresh weights of the plants. Root length and fresh weights of the wild type and knockout lines was analysed in several conditions. The data obtained for the growth experiments show that there was no significant difference found in the root lengths (figure 3.7) and fresh weights (figure 3.8) among all the tested genotypes in control conditions. These results are similar to those of Gobert et al., (2007) who showed there was no growth phenotype for *Attpk1* in control conditions. This suggests a limited role of TPK1 under control growth conditions. The data showed shorter root lengths (figure 3.7) and less fresh weights (figure 3.8) for all the KO lines of kinases and of *tpk1* as compared with the wild type at 0 mM K^+ conditions. Gobert et al., (2007), showed the same growth phenotype for the *Attpk1* knockout lines at low K^+ conditions while AtTPK1 overexpressor plants were shown to grow better in K^+-deficient condition. Ahmad et al., (unpublished data) also showed a better growth in low K^+ conditions for rice that overexpresses TPKs. The

lower growth of all the KO lines as compared to the wild type may be because of the impaired K^+ distribution within the plant body as it has been suggested that TPK1 is involved in the redistribution and translocation of K^+ in the plant (Gobert et al., 2007). As hypothesized earlier, the TPK1 current is stimulated by the two mentioned kinases, therefore, loss of function of these genes may lead to lack of TPK1 activation. This lack of TPK1 may, therefore, be responsible for the lack of K^+ distribution and translocation in the kinase KO lines. Efficient distribution of nutrients could lead to better growth of the plants. Thus the efficient release of K^+ from the root vacuoles through TPK1 channels may help loading of more K^+ into the xylem and this will help in the K^+ distribution from root to shoot. On the other hand, higher K^+ loading to the xylem, may increase K^+ absorption through K^+ transporters localised in the plasma membrane at the root soil boundary. Ahmad et al., (unpublished data) showed higher K^+ content in the rice TPK overexpressor plants in the K^+ deficient conditions which supports the idea that TPK activity may affect root K^+ absorption. The absence of TPK1 activity in *tpk1* and the kinase knockouts may lead to reduced K^+ release from the guard cell vacuole and therefore may keep the stomata open, as seen in the stomatal conductance experiment. Indeed, the *tpk1* and the kinase knockouts showed less reduction in stomatal conductance in the presence of ABA. The resulting increased water loss could be a reason for the lower fresh weight of the KO lines. As shown from the stomatal conductance data all the knockout lines showed a slower response to ABA. The kinase KO lines phenocopies the *Attpk1* knockout, suggesting a role of the two kinases in the regulation of TPK1.

At 50 mM K^+ conditions, all the KO lines showed shorter root length as compared to the WT. Gobert et al., (2007) showed less growth for the *tpk1* knockouts than the wild type at 80 mM K^+. These results suggest that TPK1 has a role in the high K^+ condition and plays a role in maintaining cell turgor and for controlling the concentration of K^+ in the cell. They did not find any difference in the overall tissue K^+ content of the TPK1 overexpressor and knockout line in comparison with the wild type. This suggested that the difference in the seedling growth of these lines may be attributed to differential K^+ distribution within the plant. The difference in the K^+ distribution of different genotypes may be because of the difference in TPK1 expression.

At osmotic stress conditions (50 mM and 80 mM sorbitol), all the KO lines showed shorter root lengths and less fresh weights as compared to the WT (figure 3.7 and 3.8). These results were further analysed in the soil under different water regimes and we observed less fresh weights for all the KO lines as compared to the wild type at control and moderate stress conditions (figure 3.9). Our results are also comparable to overexpressing lines of rice TPKb which showed better growth in the osmotic stress conditions (Ahmad et al., unpublished data). There could be many reasons for the observed lower fresh weights; i.e. (a) the loss of TPK1 may affect the distribution of K^+ and therefore may damage the growth of the knockout lines, (b) there may be higher water loss in the KO lines as the loss of TPK1 function may delay the closing of stomata, (c) the KO lines may have comparatively less K^+ content due to the lack of TPK1 activity which could be a reason for the lower growth of the KO lines. Ahmad et al., (unpublished data) found a higher K^+ content in rice TPK overexpressor lines and suggested that increased TPK activity might be a reason for improved K^+ nutrition under osmotic stress conditions. (d) The lower weights of the kinase knockouts could be because of the impaired activity of the receptor-like kinases, as it is known that RLKs are involved in the growth and development of plants and that they respond to many environmental stress conditions such as salt and drought. For example Ouyang et al., (2010) showed the sensitivity of RLK OsSIK1 gene knockout mutants, i.e. sik1-1 (Oryza sativa stress-induced protein kinase gene 1) and sik1-2 (Oryza sativa stress-induced protein kinase gene) plants under drought treatment.

4.2 LRR-RLK knockouts and TPK1 knockout shows similar response to ABA

Delay in stomatal closure may lead to higher water loss which will result in the plant weight loss. An increase of 200-300% was found in the phosphorylation of TPK1 after treating the intact cells with ABA (Isner et al., unpublished data). The binding of 14-3-3 proteins to the N-terminus of the TPK1 depends on the phosphorylation of the serine risidue in the 14-3-3 binding motif of the TPK1 (Latz et al., 2007). The 14–3–3 binding motif in TPK1 is phosphorylated and activated by the calcium-dependent protein kinases (CDPKs) (Latz et al., 2013) 14-3-3 protein further helps in the enhanced activation of the TPK1 channel (Latz et al., 2007). RLKs have been shown to be essential for a quick drought stress response. Alex et al., (2012) showed the up-regulation of RLK genes in root and shoot in the presence of 1 hour drought treatment.

Here we showed that in the presence of light, different concentrations (1 µM, 10 µM and 100 µM) of ABA induced a lower decrease in the stomatal conductance of all the KO lines as compared to the WT. Osakabe et al., (2005) also showed that the RPK1 (a

leucine-Rich-Repeat Receptor-Like kinase 1) KO lines (rpk1-1 and rpk1-2) in *Arabidopsis* showed decreased sensitivity to ABA during stomatal closure and suggested that that RPK1 gene is involved in the early steps in the ABA signalling pathway and early ABA perception.

The data for the stomatal conductance experiment (figure 3.10) showed less reduction in stomatal conductance for all the KO lines compared to the wild type, when they were exposed to different concentrations of ABA. The less reduction might be a reason for the greater water loss and less fresh weights. These results are also comparable to the results of Gobert et al., (2007) where in light conditions, at 10 μM the stress hormone ABA induced a rapid decrease in WT leaf conductance but the response to ABA was slower in the *tpk1* KO lines and slightly faster in the TPK1ox. The difference between the controls in the three panels might be because the experiments were done in different stages of the plants growth, or it might be because the experiments were performed at different times of the day.

In the presence of ABA, guard cell vacuolar K^+ is released through TPK1 helping in the closure of stomata. The stomatal conductance data obtained for the LRR-RLK knockouts phenocopied *tpk1* which provides good evidence for the link between kinases and TPK1 channel. Binding of a ligand to the extracellular domain of the LRR-RLKs activates the intracellular kinase domain, resulting in bringing extracellular environment signals into the intracellular targets. As there is no such activity in the LRR-RLKS knockouts, this likely disrupts the response to ABA and signals to TPK1 which in turn causes the lower reduction in the stomatal conductance.

4.3 The presence of a strong stomatal conductance phenotype in spite of high levels of transcript in the *kinase1_2* mutant is puzzling

The *kinase1_2* line was found to be homozygous for the T-DNA insertion (figure 3.3). However, at the transcript level, overexpression of the gene was found (figure 3.6) which clearly suggests that this is not a null mutant. Interestingly, the phenotype for this line was the same as that for the *kinase1_1* mutant. There could be many reasons for this anomaly i.e. For example, the inserted T-DNA could have caused a mutation. Another possibility could be the presence of another T-DNA insertion somewhere else in the genome which affects the stomatal conductance of this line.

4.4 Model of activation of TPK1 in the presence of ABA

Based on results presented here from growth and stomatal conductance experiments and unpublished data from Isner et al, the following model can be composed. In the presence of ABA, the LRR-RLK KINASE1_1, that is present on the plasma membrane internalizes to the tonoplast and then attaches to the TPK1 causing the phosphorylation of the N-terminal domain of TPK1. Phosphorylation allows binding of the 14-3-3 proteins, causing the activation of the TPK1 channel. Activation of TPK1 causes efflux of potassium from the guard cell vacuole to the cytoplasm. In the next phase guard cell outward rectifying K^+ channel (GORK) then remove K^+ from the cell. The removal of anions and K^+ from guard cells results in a reduction of guard cell turgor and leads to stomatal closure (Gobert et al., 2007; Schroeder and Hagiwara, 1989).

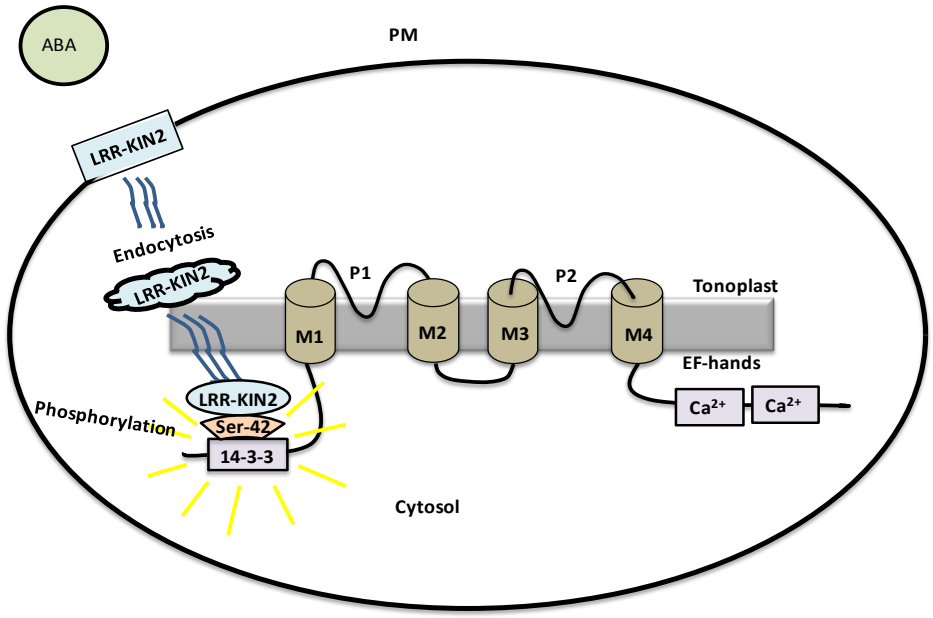

Figure 4.1: Model of activation of TPK1 in the presence of ABA.

In the presence of ABA, RLK *kinase1_1* internalise to the tonoplast through endocytosis where it causes the phosphorylation of the N-terminal domain of TPK1, resulting in the attachment of the 14-3-3 proteins and then causing the activation of the TPK1 channel.

Through BiFC study the interaction between the KINASE1_1 and TPK1 was shown by Isner et al, and our comparable results for the physiological role of the LRR knockouts *kinase1_1* and *kinase1_2* and *tpk1* knockouts from growth and stomatal conductance data suggests the involvement of this kinase in the activation of TPK1 in the presence of ABA. From growth and stomatal conductance data no significant difference between

kinase1_1 and *kinase2* was observed. While from Isner et al., (unpublished data) the expression of the *kinase1_1* was observed in the tonoplast and in the endoplasmic reticulum for the *kinase2* which they suggest a possible role of *kinase2* in the upstream of *kinase1_1*.

4.5 Further studies

- Another *at3g02880* null mutant allele should be tested to ensure that the observed stomatal conductance phenotype is due to a mutation in this gene.
- TPK1 phosphorylation was shown by Isner et al., (unpublished data) to be ABA-dependent. To see if TPK1 phosphorylation is dependent on KINASE1_1, ABA-dependent TPK1 phosphorylation should be tested in the kinase KO line.
- The *tpk1* and kinase knockout line may be analysed for K^+ content to see the effect of these proteins in K^+ nutrition.
- The KINASE1 and KINASE2 were found to be involved in the phosphorylation of TPK1. It would be interesting to identify the phosphatase that dephosphorylates TPK1To further characterise the role of KINASE1 and KINASE2, these proteins could be overexpressed in Arabidopsis and plants could be analysed for phenotypes

Appendices

Roots length from ½ MS plates

CONTROL	WT	*kinase1_1*	*kinase2*	*tpk1*
REPLICATE 1	25.2815788	33.79620766	31.31889177	30.52216214
REPLICATE 2	14.75261621	11.81640322	22.88073002	15.69213448
REPLICATE 3	26.69774119	33.47181185	31.77812175	23.73841913
REPLICATE 4	32.82025819	31.14506028	29.20069525	26.82674968
REPLICATE 5	28.75159363	32.35219366	29.39295884	27.39176288
REPLICATE 6	23.47344392	22.46095652	31.52671446	21.268277
T-TEST		0.31127542	0.09174635	0.377117393
0 mM K^+				
REPLICATE 1	7.03425	4.310952105	3.220141151	4.582947684
REPLICATE 2	8.049986469	4.590777069	4.129398532	4.511523731
REPLICATE 3	7.026988636	5.043321918	5.206883675	5.076416753
REPLICATE 4	7.203425	5.3011642	2.920141151	5.229476838
REPLICATE 5	7.094998647	4.80691	3.15367679	5.115237314
REPLICATE 6	8.326983344	2.50691	3.409068837	3.776416753
T-TEST		0.004078329	0.000300221	0.001547539
50 mM K^+				
REPLICATE 1	15.55815716	10.54660881	14.18131432	13.8623885
REPLICATE 2	25.54673847	14.61230259	12.6063021	17.49548833
REPLICATE 3	21.57729522	19.55755771	11.93940648	12.25115768
REPLICATE 4	28.05048379	25.42482502	15.52122843	15.92650499
REPLICATE 5	10.08572162	9.234324665	5.28059578	8.480200228
REPLICATE 6	22.59357074	12.00615129	21.79531511	13.99774417
T-TEST		0.031464637	0.025061847	0.011075609
50 mM sorbitol				
REPLICATE 1	17.45064818	15.5631077	16.74934171	16.14077485
REPLICATE 2	20.39063971	13.39609969	14.73296747	16.15731819
REPLICATE 3	16.99702521	13.96171674	12.10665349	15.85886133
REPLICATE 4	25.37688051	16.75029888	20.13410187	20.38158355
REPLICATE 5	19.81824844	17.95930303	14.53845967	13.85449516
REPLICATE 6	19.67987038	14.47147079	16.60309464	18.08746653
T-TEST		0.010286716	0.003199197	0.013785755
80 mMsorbitol				
REPLICATE 1	5.758991172	8.495870731	9.663905216	12.74054899
REPLICATE 2	3.948157059	4.250292267	5.015791575	5.363323447
REPLICATE 3	15.9466174	11.86588674	11.4562756	19.68809016
REPLICATE 4	30.76866049	17.85178355	12.18003851	11.13112755
REPLICATE 5	20.18653544	14.3699439	12.28418621	9.313344261
REPLICATE 6	4.320043775	5.956060742	3.119152573	4.51682426
T-TEST		0.263329497	0.225248131	0.496940577

Fresh weight of plants from ½ MS plates

CONTROL	WT	*kinase1_1*	*kinase2*	*tpk1*
REPLICATE 1	0.5365	0.48675	0.5805	0.5675
REPLICATE 2	0.49566667	0.47366667	0.612	0.518
REPLICATE 3	0.4945	0.52	0.438	0.775
T-TEST		0.55553367	0.56116563	0.31916115
0 mM K^+				
REPLICATE 1	0.215	0.165	0.15	0.142
REPLICATE 2	0.25266667	0.17533333	0.17166666	0.198
REPLICATE 3	0.216	0.171	0.102	0.11
T-TEST		0.02926331	0.02660629	0.03522842
50 mM K^+				
REPLICATE 1	0.274	0.251	0.2375	0.25
REPLICATE 2	0.43166667	0.37966667	0.34733333	0.365
REPLICATE 3	0.293	0.235	0.237	0.269
T-TEST		0.05459527	0.05127039	0.11504648
50 mM SORBITOL				
REPLICATE 1	0.25	0.173	0.19	0.185
REPLICATE 2	0.372	0.32666667	0.35793333	0.33666667
REPLICATE 3	0.342	0.31	0.309	0.31
T-TEST		0.0611802	0.11574359	0.05215497
80 mM SORBITOL				
REPLICATE 1	0.28488889	0.201	0.17933333	0.19566667
REPLICATE 2	0.32866667	0.25866667	0.219	0.236
REPLICATE 3	0.269	0.22	0.218	0.211
T-TEST		0.02175393	0.04252256	0.01848902

Fresh weights of Soil grown plants

CONTROL	WT	*kinase1_1*	*kinase2*	*tpk1*
REPLICATE 1	24.7333333	19.9666667	21.9466667	20.1033333
REPLICATE 2	27.9666667	22.9666667	26	23.2966667
REPLICATE 3	26.6666667	24.5166667	22.3	23.4933333
T-TEST		0.04903984	0.04970711	0.0137347
MODERATE	WT	*kinase1_1*	*kinase2*	*tpk1*
REPLICATE 1	16.6666667	11.96	12	12.8
REPLICATE 2	16.8333333	14.7666667	14.8666667	15.0666667
REPLICATE 3	15.2	11.6333333	11.0333333	11.0333333
T-TEST		0.04583796	0.04918672	0.04948397
SEVERE	WT	*kinase1_1*	*kinase2*	*tpk1*
REPLICATE 1	2.93333333	3.6	5	2.93333333
REPLICATE 2	5.4	2.7	3.93333333	4.36666667
REPLICATE 3	2.8	5	3.36666667	4.93333333
T-TEST		0.97286413	0.74061262	0.73207895

Leaf stomatal conductance at 100 µM ABA

	CONTROL						ABA							
	WT			*kinase1_1*			WT			*kinase1_1*			WT	*kinase1_1*
	Initial	final	change in aperture	Initial	Final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	ABA-con	ABA-con
REP 1	1.16	1.11	0.043	0.9	0.76	0.157	1.68	1.1	0.35	1.21	0.57	0.53	0.3	0.37
REP 2	1.17	1.05	0.102	0.99	0.9	0.089	1.01	0.74	0.27	1.3	0.71	0.45	0.17	0.36
REP 3	0.764	0.6	0.214	0.92	0.73	0.203	0.86	1	-0.2	0.581	0.39	0.33	-0.38	0.13
REP 4	0.754	0.88	-0.16	0.5	0.5	-0.00	0.6	0.52	0.13	0.496	0.32	0.36	0.29	0.37
REP 5	0.624	0.42	0.326	0.46	0.39	0.142	0.54	0.47	0.14	0.601	0.58	0.03	-0.19	-0.1
REP 6	0.612	0.44	0.284	0.49	0.39	0.201	0.42	0.2	0.52	0.792	0.75	0.05	0.23	-0.2
REP 7	0.551	0.42	0.235	0.64	0.6	0.062	0.87	0.68	0.22	0.52	0.29	0.45	-0.01	0.39
REP 8	0.646	0.6	0.066	0.47	0.4	0.135	0.65	0.22	0.67	0.443	0.29	0.36	0.6	0.22
T-TEST	0.306													

	CONTROL						ABA							
	WT			*kinase1_2*			WT			*kinase1_2*			WT	*kinase1_2*
	Initiatl	final	change in aperture	Initiatl	Final	change in aperture	Initiatl	final	change in aperture	Initiatl	final	change in aperture	ABA-con	ABA-con
REP1	1.37	1.2	0.146	1.09	0.8	0.241	0.58	0.3	0.42	0.63	0.4	0.4	0.274	0.16
REP2	0.99	0.6	0.3939	0.79	0.6	0.253	0.8	0.4	0.52	0.94	0.6	0.4	0.127	0.14
REP3	0.91	0.9	0.0372	1.02	0.7	0.305	1.09	0.4	0.6	0.95	0.6	0.4	0.566	0.06
REP4	1.02	0.9	0.1176	1.11	0.9	0.205	1.11	0.6	0.5	0.87	0.6	0.3	0.378	0.14
REP5	0.99	0.8	0.2222	0.98	0.8	0.205	0.76	0.4	0.53	0.97	0.8	0.2	0.309	0.02
REP6	1.2	1	0.1667	0.8	0.6	0.249	0.98	0.5	0.53	0.85	0.5	0.4	0.359	0.17
T-TEST	0.003													

	CONTROL						ABA							
	WT			*kinase2*			WT			*kinase2*			WT	*kinase2*
	Initial	final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	ABA-con	ABA-con
REP1	0.89	1	-0.165	1.47	1.3	0.102	1.14	0.6	0.4	1.1	0.9	0.2	0.611	0.124
REP2	0.89	1	-0.15	0.98	0.9	0.058	1.31	0.6	0.5	0.91	0.8	0.9	0.671	0.847
REP3	1.88	1.7	0.0851	1.44	1.4	0.0208	1.97	0.8	0.6	1.51	0.9	0.6	0.509	0.608
REP4	1.28	1.1	0.1719	1.88	1.7	0.1223	1.58	0.9	0.4	1.37	0.3	0.2	0.264	0.127
REP5	1.17	1.1	0.0256	1.15	0.8	0.3426	1.01	0.6	0.4	0.94	0.4	0.4	0.413	0.054
REP6	0.92	0.8	0.1277	1.47	1	0.3429	0.79	0.2	0.7	0.88	0.3	0.3	0.606	-0.04
REP7	2.59	1.5	0.4093	1.19	1	0.1748	2.6	1.3	0.5	1.47	0.4	0.3	0.11	0.111
REP8	2.23	1.9	0.13	1.13	0.5	0.5575	1.71	0.8	0.6	1.63	0.5	0.3	0.428	-0.22
T-TEST	0.05													

	CONTROL						ABA							
	WT			*tpk1*			WT			*tpk1*			WT	*tpk1*
	Initial	final	change in aperture	Initial	Final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	ABA-con	ABA-con
REPL1	0.9	0.9	-0.004	1.47	1.6	-0.088	0.78	0.5	0.32	1.47	1.2	0.2	0.327	0.3
REPL2	1.04	1.1	-0.01	1.11	1	0.081	1.11	0.5	0.53	1.08	0.8	0.3	0.542	0.18
REPL3	1.02	1.1	-0.108	1.15	1	0.147	1.29	0.4	0.69	1.01	0.4	0.6	0.796	0.43
REPL4	1.66	1.4	0.1747	1.28	1	0.254	0.87	0.3	0.67	0.91	0.4	0.6	0.498	0.34
REPL5	1.56	1.5	0.0449	1.63	1.3	0.215	1.46	0.6	0.61	1.01	0.4	0.6	0.561	0.4
REPL6	2.24	3	-0.344	1.51	1.3	0.146	1.02	0.9	0.09	0.92	0.4	0.6	0.434	0.47
T-TEST	0.02													

Leaf stomatal conductance at 10 µM ABA

	CONTROL							ABA						
	WT			*kinase1_1*			WT			*kinase1_1*			WT	*kinase1_1*
	Initial	final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	ABA-con	ABA-con
REP1	1.04	0.7	0.3154	0.8	0.57	0.2943	1.09	0.5	0.6	0.8	0.4	0.6	0.26	0.3
REP2	1.43	1	0.2727	1.09	0.68	0.3752	0.67	0.4	0.5	0.55	0.3	0.4	0.19	0.1
REP3	1.07	0.8	0.2402	0.99	0.61	0.3843	0.54	0.2	0.6	0.61	0.3	0.5	0.35	0.1
REP4	1.09	1	0.055	1.46	1.03	0.2945	1.36	0.5	0.6	1.11	0.4	0.6	0.57	0.3
REP5	1.31	0.9	0.2763	1.33	0.87	0.3451	1	0.3	0.7	0.78	0.5	0.4	0.41	0
REP6	0.98	1.2	-0.26	0.6	0.76	-0.25	1.08	1.1	0	0.56	0.4	0.3	0.27	0.6
REP7	0.48	0.5	0.008	0.65	0.6	0.073	0.63	0.3	0.5	0.54	0.5	0.1	0.54	0.1
T-TEST	0.04													

	CONTROL						ABA							
	WT			*kinase1_2*			WT			*kinase1_2*			WT	*kinase1_2*
	Initial	final	change in aperture	Initial	final	change in aperture	Initatl	final	change in aperture	Initatl	final	change in aperture	ABA-con	ABA-con
REP1	1.24	0.9	0.2597	1.29	1	0.209	1.31	0.7	0.46	1.11	0.7	0.4	0.199	0.17
REP2	1.68	1.2	0.2857	1.05	0.5	0.527	1.28	0.4	0.66	1.49	1.2	0.2	0.371	-0.31
REP3	1.2	0.6	0.4833	1.41	1.3	0.05	0.96	0.5	0.52	1.18	0.9	0.2	0.032	0.16
REP4	1.61	1.5	0.0683	2.59	2.1	0.189	1.25	0.5	0.57	1.79	1.4	0.2	0.5	0.05
REP5	1.32	1	0.2348	1.78	1	0.427	1.25	0.5	0.59	2.08	1	0.5	0.352	0.09
REP6	0.97	0.8	0.1533	1.53	0.9	0.397	1.2	0.4	0.66	1.13	0.5	0.6	0.507	0.17
T-TEST	0.01													

	WT			*kinase2*			WT			*kinase2*			WT	*kinase 2*
	Initial	final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	Initiatl	final	change in aperture	ABA-con	ABA-con
REP1	1.04	0.7	0.3154	0.87	0.7	0.2314	1.09	0.3	0.8	1.02	0.5	0.5	0.441	0.297
REP2	1.44	1	0.3188	1.16	0.7	0.4043	0.64	0.2	0.7	1.47	0.7	0.5	0.356	0.092
REP3	1.02	0.7	0.3039	1.08	0.5	0.5657	0.94	0.3	0.7	1.03	0.6	0.4	0.382	-0.14
REP4	0.95	1	-0.054	0.74	0.8	-0.013	0.97	0.3	0.7	1.06	0.4	0.6	0.758	0.599
REP5	1.24	1.1	0.0968	1.19	1	0.1513	1.54	0.8	0.5	0.81	0.3	0.6	0.384	0.438
REP6	0.61	0.9	-0.516	0.72	0.6	0.1	0.66	0.4	0.4	0.68	0.4	0.4	0.936	0.311
T-TEST	0.04													

	CONTROL						ABA							
	WT			*tpk1*			WT			*tpk1*			WT	*tpk1*
	Initial	final	change in aperture	Initial	Final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	ABA-con	ABA-con
REP1	1.04	0.7	0.3154	1.33	0.9	0.311	1.09	0.5	0.57	1.45	0.8	0.5	0.257	0.15
REP2	1.43	1	0.2727	1.38	0.9	0.378	0.67	0.4	0.46	0.65	0.5	0.2	0.188	-0.15
REP3	1.07	0.8	0.2402	1.21	0.8	0.32	0.54	0.2	0.59	0.52	0.3	0.4	0.349	0.1
REP4	1.06	0.8	0.2538	1.21	0.9	0.252	0.64	0.4	0.33	0.92	0.6	0.3	0.073	0.05
REP5	1.26	0.8	0.3778	1.21	0.9	0.249	0.79	0.3	0.59	0.84	0.4	0.5	0.208	0.22
REP6	0.99	1.1	-0.085	1.16	0.9	0.244	1.02	0.3	0.74	0.93	0.4	0.6	0.822	0.36
REP7	0.96	0.6	0.3725	1.05	0.9	0.179	0.99	0.3	0.68	1.17	1	0.2	0.31	0.01
T-TEST	0.04													

Leaf stomatal conductance at 1 µM ABA

	WT			*kinase1_1*			WT			*kinase1_1*			WT	*kinase1_1*
	Initial	final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	ABA-con	ABA-con
REP1	1.83	1.4	0.2514	1.51	1.13	0.2517	1.78	1.4	0.2	1.55	0.9	0.4	-0	0.1
REP2	1.29	1	0.238	1.25	0.93	0.2552	1.23	0.8	0.3	1.61	1.3	0.2	0.09	-0
REP3	0.84	0.6	0.3111	1.13	0.68	0.4009	1.96	0.5	0.8	0.88	0.6	0.3	0.46	-0.1
REP4	1.05	0.9	0.1429	0.97	0.55	0.4269	1.64	0.9	0.5	0.83	0.5	0.4	0.31	-0
REP5	0.94	0.7	0.2639	1.49	1.06	0.2886	1.64	0.9	0.4	0.93	0.7	0.3	0.18	-0
REP6	1.23	0.9	0.274	1.11	0.76	0.318	1.5	1	0.3	1.24	0.6	0.5	0.06	0.2
T-TEST	0.05													
	CONTROL						ABA							
	WT			kinase1_2			WT			kinase1_2			WT	kinase1_2
	Initial	final	change in aperture	Initiatl	final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	ABA-con	ABA-con
REPL1	0.33	0.3	0.003	0.6	0.6	0.068	0.7	0.7	0.06	0.85	0.7	0.2	0.058	0.12
REPL2	0.47	0.5	-0.161	0.47	0.4	0.18	0.52	0.5	0.09	0.52	0.5	-0	0.249	-0.2
REPL3	0.43	0.5	-0.155	0.76	0.7	0.109	0.71	0.6	0.1	0.45	0.3	0.3	0.251	0.19
REPL4	1.52	1.4	0.0789	2.36	2	0.174	1.18	0.5	0.56	1.13	0.9	0.2	0.485	0.06
REPL5	1.67	1.5	0.1198	2.77	2.7	0.011	2.46	0.5	0.8	0.96	1.1	-0.1	0.677	-0.11
REPL6	1.66	1.5	0.0843	1.75	1.9	-0.08	0.93	0.7	0.29	1.09	0.9	0.1	0.202	0.22
T-TEST	0.02													

	CONTROL						ABA							
	WT			*kinase2*			WT			*kinase2*			WT	*kinase2*
	Initial	final	change in aperture	Initial	Final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	ABA-con	ABA-con
REP1	1.15	1	0.0957	1.44	1.1	0.2569	1.79	0.8	0.6	1.06	0.7	0.3	0.469	0.075
REP2	1.31	0.9	0.3321	1.18	0.9	0.2076	2.02	1	0.5	1.39	1	0.3	0.178	0.073
REP3	1.55	1.4	0.071	1.64	1.3	0.2256	1.28	0.7	0.4	1.72	0.6	0.6	0.36	0.412
REP4	1.01	0.9	0.1337	1.66	1.4	0.1807	0.96	0.4	0.6	1.14	0.9	0.2	0.42	0.042
REP5	0.63	0.8	-0.319	1.25	1.1	0.136	1.42	0.8	0.4	1.35	1	0.2	0.764	0.108
REP6	1.36	1.2	0.125	1.63	1.4	0.1166	1.41	0.9	0.4	1.38	0.6	0.6	0.246	0.435
T-TEST	0.04													

	CONTROL						ABA							
	WT			*tpk1*			WT			*tpk1*			WT	*tpk1*
	Initial	final	change in aperture	Initial	Final	change in aperture	Initial	final	change in aperture	Initial	final	change in aperture	ABA-con	ABA-con
REP1	1.25	1.3	-0.072	1.56	1.2	0.244	1.05	0.4	0.64	1.24	0.5	0.6	0.708	0.35
REP2	1.41	1	0.2695	1.71	1.4	0.158	1.19	0.6	0.52	1.44	0.7	0.5	0.247	0.33
REP3	1.11	0.9	0.1991	1.78	1.4	0.225	1.36	1.2	0.15	1.43	0.9	0.4	-0.05	0.16
REP4	0.96	1.2	-0.216	2.81	2.8	0.004	0.73	0.6	0.21	1.41	1.1	0.2	0.427	0.19
REP5	0.53	0.6	-0.093	1.31	1	0.272	1.06	0.6	0.45	1.47	1.5	0	0.538	-0.26
REP6	0.52	0.5	-0.008	1.26	1	0.228	0.95	0.3	0.63	0.93	0.6	0.3	0.64	0.1
T-TEST	0.05													

List of Abbreviations

ABA:	Abscisic acid
ABF:	Downstream transcription factors
ABI1 and ABI2:	ABA Insensitive 1 and ABA Insensitive 2
ABREs:	ABA-responsive promoter elements
ATP:	Adenosine Tri phosphate
BiFC:	Bimolecular fluorescence complementation
Bp:	Base pair
cDNA:	Complementary DNA
CDPKs:	Calcium-dependent protein kinases
DNA:	Deoxyribonucleic acid
dNTP:	Deoxyribonucleotide triphosphates
GORK:	Guard cell outward rectifying potassium channel
GRF6:	General regulatory factor
K^+_{in}:	Inward rectifying K^+
K^+_{out}:	Outward rectifying K^+
KO:	Knockouts
LRR–RLKs:	Leucine-rich-repeat Receptor-like protein kinases
OST1:	Open Stomata 1
PCR:	Polymerase chain reaction
PP2Cs:	Protein phosphatases
PYL:	Pyrabactin resistance-like
PYR:	Pyrabactin resistance

qRT-PCR:	Quantitative reverse transcriptase PCR
RLKs:	Receptor-like protein kinases
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
Sik:	Stress-induced protein kinase
SLAC:	Slow anion channel
SnRK2s:	Sucrose non-fermentation kinase subfamily 2
SV:	Slow activating K^+ channels
TAIR:	The *Arabidopsis* Information Resource
T-DNA:	Transferred DNA
TMD:	Trans-membrane domains
TPK:	Tandem Pore Potassium channel
UNCCD:	United Nations Convention to Combat Desertification
VK:	Vacuolar channels
WT:	wild type

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