GENETIC APPROACHES TO IMPROVE DROUGHT TOLERANCE
OF TOMATO AND TOBACCO

DISSERTATION

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* * * * *

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Plant survival relies on resisting episodes of adverse environmental stresses. Water stress is one of the most severe constraints in crop production. Plants reduce water loss by decreasing stomatal aperture when water supply is limited. The stress hormone ABA activates a signal transduction pathway culminating in stomatal closure. Numerous studies have been conducted to understand in-depth mechanisms involved in ABA signaling and stomatal functions, and numerous components including bzip transcription factors such as ABFs/AREBs and phosphoinositides such as IP$_3$ have been identified. However, most of progress has been from model plant Arabidopsis, and still the biochemical, cellular, and molecular mechanisms underlying plant drought response are not completely understood. The objective of this study was to increase the resistance of crops (tobacco and tomato) to water deficit stress. To achieve this goal transgenic plants were generated using two genes, a putative tomato type I inositol 5 polyphosphatase (5PTase) that terminates inositol 1,4,5 trisphosphate (IP$_3$) signaling and ABRE binding factor $ABF4$ derived from Arabidopsis.

Inositol 1,4,5 triphosphate (IP$_3$), as one of phosphoinositides, is known to transduce a stress signal by changing its level in response to water deficit, osmotic stress, and low temperature in plants. Recently it was shown that type I inositol 5 phosphatases (5PTases), At5PTase1 and AtIP5PII/At5PTase2, regulate IP$_3$ level in plant like in animals and that
up-regulation of these genes decreases IP₃ levels which leads to a reduction in the expression of ABA-/drought-responsive genes in Arabidopsis. On the basis of sequence similarity to Arabidopsis 5PTases, four tomato cDNAs (Le5PT1-4) that encode putative tomato type I 5PTase proteins were identified. Predicted protein sequences of identified Le5PTs had conserved catalytic domains that are required for 5PTase enzyme activity. Two clones, Le5PT1 and Le5PT2 were similar to AtIP5P1/At5PTase2 and At5PTase1, respectively. The expression of Le5PT1 was down-regulated in early time point under dehydration, NaCl, and exogenous ABA treatment, indicating that Le5PT1 may play a negative role in stress signaling. Transgenic tobacco plants with 35S:Le5PT1 did exhibit weak expression of the drought inducible gene, NtERD10B, but did not show correlation with resistance to water deficit stress. Transgenic tobacco and tomato plants, however, exhibited retarded growth, suggesting that Le5PT1 may have a relation to plant development.

AtABF4, a bzip transcription factor, is known to induce the expression of ABA-responsive genes. The expression of Arabidopsis ABF4/AREB2 gene under the control of guard cell specific KST1 promoter was shown to significantly increase drought tolerance in tobacco and tomato plants. The transgenic plants exhibit significantly lower water loss per unit leaf area compared to wild type plants. ABF4-mGFP protein strongly accumulated in guard cell nuclei. The possibility that ABF4 protein can activate 4XKST1-rd29B:mGFP-GUS fusion construct in a water stress dependent manner was tested. The results show that ABF4 itself may not be sufficient to induce GUS expression even under water stress. These results demonstrate that drought-inducible transcription factors may be an agronomically useful tool to alter crop drought tolerance.
TO MY PARENTS, BROTHER AND SISTERS
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I am happy to take this graceful opportunity to express many thanks to lead for me to reach at this moment.

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ABSTRACTS AND PRESENTATIONS

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CHAPTER 1

REVIEW OF LITERATURE

1.1 WATER STRESS AND PLANT BREEDING

Plants continuously experience episodes of environmental stresses, such as water deficit. Water deficit is one of the main factors for the yield loss in plants. Therefore, it is important to understand how plants adapt to water deficit. Drought tolerance is the ability of plants to survive water deficit stress and to maintain plant growth under water deficit conditions. There are different ways plant species to adapt to this adverse condition. Some plants can 1) finish their life cycle during optimum conditions, 2) conserve water by reducing water loss for example, by reducing leaf size or reducing stomatal pore, 3) maintain growth even during water deficit by retaining water content, or 4) increase water use efficiency of limited available water (Bressan et al., 2002). These mechanisms can be utilized as indicators in breeding strategy to improve drought-tolerant crops.

Water deficit is a serious and imminent problem worldwide. Therefore, where irrigation is not economical or difficult because of water scarcity, breeding strategies have focused on drought tolerance. Although conventional breeding has drawbacks such as backcross to get rid of undesirable traits, it has been successful.

During the last two decades, however, tremendous advances in plant biotechnology have enabled scientists to add traits of interest into crops by recombinant DNA technology. Through gene transfer techniques, it is possible to introduce target genes
even from other organisms, which is impossible through traditional hybridization (Grover, 2000). It is still difficult to introduce a trait regulated by multiple genes, but some unique genes have been successfully integrated into some plant species to improve plant tolerance to environmental stresses including drought tolerance (Table 1.1).

Since the majority of water loss in plants is via stomata, an avenue with potential to improve drought tolerance is by modifying guard cell behavior. This study was initiated to generate plants that have the ability to conserve more water by modifying stomatal behavior using a biotechnological approach.
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Table 1.1 Transgenic crops tolerant for water and salt stress
1.2 THE EFFECTS OF WATER STRESS IN PLANTS

Water deficit is defined as the water content of plant that is lower than a species-specific level, which results in various physiological and molecular changes (Bressan et al., 2002). In the early stages of water deficit, plants reduce their leaf size via reduction of leaf growth and leaf number. These changes are helpful to minimize excessive water loss and to conserve available water, which can be used later. Reduction in leaf size is the consequence of water deficit because cell expansion relies on turgor pressure that is dependent on water content. In contrast, roots can use more energy for their growth into deeper moist soil, which is enabled by re-allocation of energy from the reduction of consumption in upper part of plant. These changes in leaf size and root growth occur under slow developing water deficit conditions. When dehydration is rapid, however, plants use different mechanism to reduce water loss through transpiration by closing numerous small pores, called stomates. Stomatal pores are closed by a decrease in turgor pressure in the guard cells that can be induced by direct water loss to air or by dehydration of other plant organs. Since the stomatal pore is the passage for plant to uptake CO₂ required for assimilation, prolonged stomatal closure ultimately reduces photosynthesis. Interestingly, however, photosynthesis does not decrease as a result of water stress until it is severe. This is important for plant growth. In addition, water deficit increases wax deposition in cuticle that decreases water loss. Although water loss through the epidermis is relatively small, under severe water stress wax accumulation probably is beneficial to reduce water loss (Bressan et al., 2002).

Plants can only uptake water when their water potential is lower than soil, but as available water decreases it becomes impossible to absorb water from soil with lower
water potential than plants. However, some plants such as sugar beet maintain higher stomatal conductance and turgor at lower water potential. Lower water potential can result from the accumulation of compatible solutes such as proline, sugar alcohols, glycine betain, and ions, and this process is called osmotic adjustment. This change requires the expression of various genes that are involved in synthesis of compatible solutes such as proline and glycine betaine (Bressan et al., 2002). In addition, LEA proteins and heat shock proteins HSPs (Wehmeyer et al., 1996) are produced during water deficit, and they are believed to have roles in protein protection. Group III LEA proteins are especially thought to have important role in drought tolerance. Accumulation of barley HVA1, a member of group III LEA family of proteins, increases drought tolerance. Along with water loss from living cell, the driving force for cellular organization is diminished and the membranes undergo structural changes due to protein denaturation. The decrease in cellular volume due to lower water levels can cause crowding of cytoplasmic components, and the cytoplasm becomes increasingly more viscous, increasing the chance for undesirable molecular interactions that can cause protein degradation and membrane fusion (Hoekstra et al., 2001). Therefore, desiccation resistance is correlated with the presence of considerable quantities of compatible solutes and specific proteins, which can reduce damage during water deficit stress. These compounds facilitate living cells to maintain cellular structure as well as the function of proteins (Hoekstra et al, 2001).

Nevertheless, the control of water loss by stomatal closure has, in particular, received attention since plants lose more than 90% of the water through the stomatal pore. For this reason, many scientists are trying to understand the mechanism underlying in stomatal
function, which consequently can be used to improve plant drought tolerance by modulating stomatal aperture. Many studies revealed the pivotal role of the phytohormone abscisic acid (ABA) in stomatal functions, suggesting a possible application in molecular breeding since stomatal closure is induced as effectively by ABA as by water stress. In this respect, ABA biosynthetic or signaling components have been successfully used for the last decade and can be used in the future to alter stomatal activities, possibly resulting in improved plant drought tolerance (Pei et al, 1998; Lemichez et al, 2001; Iuchi et al, 2001).

1.3. MECHANISMS OF STOMATAL OPENING AND CLOSING

Plant water status not only relies on available water in the soil, but the leaf itself also has an important role in the regulation of water content. Stomata are small pores that are surrounded by a pair of guard cells, that are located largely in the leaf epidermis in plants. Although the structure of guard cells is slightly different between monocot and dicot plants, the underlying mechanisms of operation do not differ. The opening and closing of stomata are induced by volume changes caused by turgor pressure in guard cells in response to various signals. Stomatal pores form the connection between the interior of the leaf and the air, and are crucial for the uptake of CO₂ that ultimately is integrated into various plant components by photosynthesis. Inevitably plants lose water via stomata as the consequence of CO₂ influx, but transpiration has a positive role in lowering body temperature thereby reducing damage from heat stress. Stomatal apertures are regulated by various stimuli, such as water status, light, temperature, CO₂ concentration, and hormones (Schroeder et al., 2001a; Bressan et al., 2002).

1.3.1 Stomatal opening and closing
Numerous studies have shown that a network of signaling mechanisms exists in guard cells. Stomatal opening occurs when guard cells swell and increase their volume. The volume change is driven by the difference of water potential across the plasma membrane. The change in water potential is initiated by H\(^+\)-efflux across plasma membrane by a proton pump (H\(^+\)-ATPases) that is activated by various stimuli, such as blue light. The H\(^+\)-efflux is believed to cause plasma membrane hyperpolarization resulting in the influx of potassium ions (K\(^+\)) into guard cells via inward K\(^+\)-channels. Elevation of K\(^+\) increases osmotic pressure that ultimately promotes water uptake. At the same time, Cl\(^-\) known as a counterpart for K\(^+\) also increases in guard cells. K\(^+\) is a major solute that supports stomatal opening in the beginning (Schroeder et al., 2001a; Bressan et al., 2002).

Figure 1.1 Simplified hypothetic model for ABA-induced stomatal opening and closure.
In contrast, stomata close with reduction in volume of guard cells. Stomatal closure is induced by abscisic acid (ABA), darkness, and high internal leaf CO₂ concentration. However, it is not well understood how guard cells recognize these stimuli (Schroeder et al., 2001b). Although various stimuli induce stomatal closing, ABA-induced stomatal closure is widely used to understand underlying mechanisms of stomatal closing. Stomatal opening is driven by ion influx, whereas stomatal closing requires ion efflux. ABA induces cytosolic Ca²⁺ accumulation. This high Ca²⁺ accumulation inhibits the proton pump and K⁺ inward channels, both of which are required for stomatal opening. In addition, Ca²⁺ concentrations promote anion (Cl⁻ and malate) efflux by activating anion channels in plasma membrane. These combined actions cause depolarization across the plasma membrane and activates outward K⁺ channels. ABA also increases cytoplasmic pH in the guard cells that activates outward K⁺ channels. Ultimately, turgor pressure decreases with reduction in osmotic pressure due to loss of solutes.

1.4 ABSCISIC ACID METABOLISM

Abscisic acid (ABA) is a phytohormone that has many important roles in vegetative growth (Arenas-Huertero et al., 2000; Barrero et al., 2005), seed germination and dormancy, stomatal closure, as well as plant defense to abiotic and biotic stresses in plant life cycle (Audenaert et al., 2002; Mauch-Mani and Mauch, 2005). In addition, recent studies revealed that ABA interacts with other hormones (Mauch-Mani and Mauch, 2005) such as ethylene, salicylic acid, and jasmonic acid, and probably with glucose since high concentration of glucose increases ABA levels in germinating seeds (Gazzarrini and McCourt, 2001).
Nevertheless, ABA is best known as a stress hormone because of its involvement in plant responses to environmental stresses. The ABA signal pathway is the most well established hormonal signaling pathway in plants. Upon various stresses ABA initiates adaptive responses via modulating gene expression required for adaptation and stress signaling pathways (Seo and Koshiba, 2002; Taylor et al., 2000). The level of ABA increases in response to various environmental stresses, especially upon water deficit stress (Taylor et al., 2000), and the endogenous level of ABA is correlated with various plant responses to stresses (Seo and Koshiba, 2002). In contrast to rapid increases in ABA levels upon being subjected to water deficit, water potential and relative water content decrease due to limited water availability in soil.

One of most important aspects, however, is ABA-induced stomatal closure that is crucial for plant response to water deficit stress, and this ultimately culminates in preventing water loss from plants. With studies to unravel stomatal functions, many new components that may have important roles in these actions have been found, for example inositol triphosphate (IP3), phospholipase C (PLC) and D (PLD), transcription factors involved in ABA signal transduction pathway, Ca^{2+}, and cyclic ADP-ribose (Sanchez et al., 2004, Finkelstein et al, 2002). Despite the findings of numerous components related to ABA-mediated stomatal response, it is still not well understood how these components are connected.

1.4.1 ABA biosynthetic pathway in higher plants

Abscisic acid (ABA) is a metabolite of the isoprenoid pathway, and it contains 15 carbon atoms (C_{15}) derived from cleavage of C_{40} carotenoids rather than from the C_{15} precursor, farnesyl diphosphate that plants have. A C_{15} ABA precursor, xanthoxin, is
cleaved from 9'-cis-neoxanthin and 9'-cis-violaxanthin catalyzed by 9-cis-epoxycarotenoid dioxygenases in chloroplasts. The last two conversion steps occur in cytoplasm: xanthoxin to abscisic aldehyde by a short-chain alcohol dehydrogenase (ABA2), abscisic aldehyde to ABA via the oxidation of abscisic aldehyde by abscisic aldehyde oxidase (AAO3) (Fig. 1.2).

On the other hand, ABA can be catabolized by two different reactions, hydroxylation and conjugation. Three hydroxylation pathways exist and generate 7', 8', and 9'-hydroxy ABA via oxidation of one of the three methyl groups in the ring structure. However, the formation of 8'-hydroxy ABA, which is catalyzed by a cytochrome P450 monooxygenase (P450), is genetically accepted as the major ABA catabolic pathway. The 8'-hydroxy ABA is spontaneously changed to (-)-phaseic acid (PA) and is further catalyzed into (-)-dihydrophaseic acid (DPA). In addition to catabolic hydroxylation, the carboxyl and hydroxyl groups of ABA and the oxidative catabolites can be inactivated by conjugation with glucose that generates ABA glucosyl ester and ABA glucosides, respectively (Nambara and Marion-Poll, 2005).

Recent studies have shown that the accumulation of ABA via expressing NCED genes increases drought tolerance in Arabidopsis and tobacco plants (Iuchi et al., 2001; Qin and Zeevaart, 2002).

1.4.2 Elucidation of Abscisic acid biosynthesis in plants

Current knowledge of the biosynthetic pathway and functions of ABA has been tremendously enhanced through the characterization of ABA-deficient mutants including ataba1, ataba2, ataba3, vp14 in maize, notabilis in tomato, atnced3, ataaao3, and sitiens in tomato. These mutants have been identified by distinct phenotypes, such
as wilting symptoms, precocious germination, and insensitivity of ABA-deficient mutants to high concentrations of sugar. To date, numerous ABA mutants have been identified on the basis of various screening methods. The characterization of these mutants revealed that NCED is a committed enzyme for ABA biosynthesis from an carotenoid precursor (Schwartz et al., 2003) and that AAO3 is involved in the last step of ABA biosynthesis. Interestingly, however, the aao3 mutant did not affect precocious germination, which was shown for the maize vp14 ABA deficient mutant, suggesting the possibility that another AAO gene may be responsible for precocious germination. In addition, biochemical assays demonstrated that tomato ABA biosynthetic mutants (flacca and sitiens) still produce some ABA. These findings indicate that more than one pathway in ABA biosynthesis exists (Rock and Zeevaart, 1991).
Figure 1.2. Simplified ABA biosynthetic pathway and mutants identified in plants.
1.4.2.1 Abscisic acid biosynthetic genes in plants

NCED: Some ABA biosynthetic mutants exhibit a wilting phenotype even without water deficit stress or precocious germination. *notabilis* in tomato and *vp14* in maize have been identified because of their phenotype of wilting and precocious germination. Characterization of these mutants revealed that NCED enzymes cleave *cis*-violaxanthin and neoxanthin to xanthoxin. NCED genes have been identified and characterized in several plant species. *PvNCED1, LeNCED1,* and *AtNCED3* genes are induced by water stress. Overexpression of *AtNCED3* increased ABA levels and drought tolerance in Arabidops (Iuchi et al., 2001), as does the overexpression of its homolog, *pvNECD1,* in *Nicotiana plumbaginifolia* (Qin and Zeevaart, 2002). However, tomato *notabilis* and maize *vp14* do not exhibit a severe ABA-deficient phenotype, suggesting that gene redundancy may reduce the effect of these mutations to an extent since *NCED* is a multi-gene family. In addition, some *NCED* genes might be involved in different processes via temporal and spatial expression since they have different expression patterns in Arabidopsis (Tan et al., 2003; Iuchi et al., 2004) and avocado (Chernys and Zeevaart, 2000). In tomato, interestingly, the expression of both *LeNCED1* gene and *LeZEP1* exhibit a circadian rhythm (Thompson et al., 2000) even though they have different patterns, providing a possibility that ABA biosynthesis may somehow relate to light/dark cycles. However, there is no further information available whether these diurnal fluctuations are correlated with water deficit and ABA level in plants.

AAO3: Abscisic aldehyde oxidase (AAO) is involved in the final step of ABA biosynthesis. There are four *AAO* genes in Arabidopsis; however, only AAO3 has been shown to have substrate specificity to abscisic aldehyde. Further characterization of the
*aa03* mutant has shown that AAO3 is the main enzyme responsible for ABA synthesis in vegetative tissues (Seo et al., 2000a; Seo et al., 2000b) and in seeds (Gonzalez-Guzman et al., 2004; Seo et al., 2004). Arabidopsis *aa03* mutant exhibited a wilting phenotype as shown in AAO mutants of tomato *flacca* and *sitien* mutants. However, the rest of the *AAO* genes have not been characterized so far, and it is of interest whether these AAOs also have the same role like AAO3.

1.5 MECHANISM OF ABA ACTION

1.5.1 Transcriptional regulation of ABA-responsive genes in plant

ABA transduces its signal via ABA-dependent signaling transduction pathway (Fig. 1.2). Numerous ABA-responsive genes involved in this signaling pathway have been identified, but still it is controversial how and where plants perceive the ABA signal. The search for receptors, however, has not been successful so far. Therefore, most progress has been limited to down-stream events after ABA perception, such as transcriptional regulation that activates ABA-responsive genes.

A major landmark in ABA signaling research was the identification of genes responsive to ABA. The expression of ABA-responsive genes is correlated with plant responses to environmental stresses, in particular to water deficit stress (Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong et al., 2002; Finkelstein et al., 2002). ABA-responsive genes have been used in many studies to identify regulatory genes, transcription factors. Transcriptional levels of ABA-responsive genes are highly abundant following the imposition of water deficit stress, but it is difficult to separate ABA response from salt or cold stress since many stress-induced genes respond to various stresses. Nevertheless, through promoter dissection analyses of ABA-responsive genes,
four major ABA-responsive cis-acting regulatory elements have been identified and are called ABA-responsive elements (ABREs). Several studies revealed that these cis-acting elements are sufficient to lead gene expression upon ABA treatment (Yamaguchi-Shinozaki and Shinozaki, 1994; Uno et al., 2000; Choi et al., 2000; Narusaka et al., 2003). Many genes inducible by ABA have consensus cis-elements (C/T)ACGTGGC. These ABREs have a core ACGT sequence that is part of a G-box (CACGTC). Some ABREs have coupling sequences CE3 (ACGCACGTCGTCCTC) or motif III (GCCGCACGTCGTCCTC), and these elements share CGCGT as a consensus sequence. The CE3 and motif III elements are equivalent to ABREs containing ACGT, and are crucial for the induction of gene expression by ABA (Hobo et al., 1999) although one base pair of the core sequences differs from ABREs with ACGT (G instead of A in their core ACGT sequences). More than one copy of an ABRE or combination with other cis-acting elements is probably required for sufficient ABA induction (Choi et al., 2000). ABRE or the drought-responsive element [DRE/C-repeat (CRT)] alone is not sufficient to induce the expression of the reporter gene, but both together show synergistic effects in response to dehydration (Narusaka et al., 2003). This suggests that there is cross-association between ABRE elements and DRE elements, and these elements together have a synergistic effect that leads to the expression of drought-responsive genes.

Using binding assays, various transcription factors that bind and regulate ABA-responsive genes which have ABA cis-acting element in their promoter regions have been identified. To date, it is estimated that more than 81 bZip transcription factors exist in Arabidopsis (Riechmann et al., 2000), and they are divided into ten groups based on similarity (common domains). Among them only one group of bZip subfamily, group A,
is believed genetically to be involved in ABA response (Riechmann et al., 2000). ABI5 (ABA insensitive 5 found in seed germination) and its homologs, ABRE binding factors including (ABFs and AREBs), is included into this subfamily (Choi et al., 2000; Uno et al., 2000; Finkelstein et al., 2002).

1.5.2 Secondary messengers

1.5.2.1 Ca\(^{2+}\) in stomatal closure

Numerous studies have revealed the importance of Ca\(^{2+}\) as a secondary messenger involved in ABA-induced stomatal closing. ABA-induced cytosolic Ca\(^{2+}\) increases result from the release of Ca\(^{2+}\) in intracellular organelles, as well as influx from extracellular areas through Ca\(^{2+}\) channels located on the plasma membrane. The stomatal response to water stress is related to the activities of several ion channels localized at the plasmalemma and the tonoplast. ABA induces depolarization of the plasma membrane potential that results in a massive redistribution of ions and solutes from the vacuole to the apoplast. Along with this redistribution, cytosolic Ca\(^{2+}\) concentration and pH increase. Elevation of Ca\(^{2+}\) concentration inhibits the inward rectifying K\(^{+}\) channel, which is required for stomatal opening (Grill and Himmelbach, 1998). Ca\(^{2+}\) activates the plasmalemma slow-type (S) anion channel, which releases Cl\(^{-}\) and malate into apoplast. The depolarization of the guard cells by ABA activates influx of Ca\(^{2+}\) from extracellular areas. These sequential processes drive stomatal closure by causing loss of turgor pressure of the guard cell.
1.5.2.2 Phospholipase D (PLD) and Phosphatidic acid (PA) as secondary messengers in ABA signal transduction

Phosphatidylcholine is known as a major substrate for phospholipase D (PLD), which generates phosphatidic acid (PA) as a byproduct. PLD can be activated by environmental stresses, such as water deficit stress and osmotic stress, and by exogenous ABA. ABA treatment increases the activity of PLD, which in turn induces stomatal closing (Jacob et al., 1999) and the expression of ABA-responsive genes in rice (Gampala et al., 2001). To date, several PLD genes have been found in different species and revealed that PLDs are involved in multiple plant responses (Wang et al., 2000; Zhang et al., 2004). A knockout mutant of the PLD enzyme, PLDα1, is sensitive to water deficit stress and loses more water than wild type Arabidopsis. Phosphatidic acid (PA) produced by PLDα1 binds and inactivates ABI4, resulting in transducing ABA signaling (Zhang et al., 2004). In an earlier study, however, it was reported that ABA did not increase AtPLDδ (another Arabidopsis PLD gene) expression, but dehydration did (Katagiri et al., 2001). This indicates that AtPLDδ may be involved in ABA-independent manner in response to dehydration. In addition, PA is not only involved in the response to dehydration, but it is also involved in seed germination (Katagiri et al., 2005), indicating that PLD and its byproduct, PA, have diverse functions in plant development. Additionally Zhang et al. (2004) reported that phosphatidic acid (PA) is also involved in ABA-induced stomatal closure by interacting with ABI1 protein phosphatase 2C enzyme (PP2C) (Zhang et al., 2004) that have positive role in ABA-induced stomatal closure.
1.5.2.3 ROS in ABA-induced stomatal closure

Plants inevitably produce several reactive oxygen species (ROS) as byproducts of photosynthesis, such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. In addition, although ROS can cause detrimental oxidative damage, plants generate and use ROS as signaling molecules. Recent studies provide that ABA increases reactive oxygen species (ROS) in guard cells (Pei et al., 2000; Zhang et al., 2001; Suhita et al., 2004). ROS, as a second messenger, promotes ABA-induced stomatal closure (Apel and Hirt, 2004). Abscisic acid-insensitive mutants, abi1-1 and abi2-1, exhibit a pleiotropic phenotype. They are insensitive to exogenous ABA in germination assays, impaired in stomatal function, and have reduced ABA-responsive gene expression in vegetative tissues (Allen et al., 1999; Chak et al., 2000; Wu et al., 2003). ABI1 and ABI2 encode type 2C protein phosphatases (PP2C) that share 86% similarity in amino acid sequences and structure.

Opening of ABA-induced Ca$^{2+}$ channels is disrupted in the ABA-insensitive mutant, abi1-1, but H$_2$O$_2$-induced stomatal closure is not impaired. In the ABA-insensitive mutant, abi2-1, however, H$_2$O$_2$ induced stomatal closing is blocked. H$_2$O$_2$-induced stomatal closure also is lost in the ABA insensitive gca2 mutant, as well in open stomata 1 (ost1) mutant that loses the ability to control transpiration under drought conditions (Mustilli et al., 2002). Based on these results, it was proposed that ABI1 and OST1 regulate ROS-mediated ABA signaling in upstream of ABI2 (Mustilli et al., 2002; Suhita et al., 2004). These findings provide that H$_2$O$_2$ is an important component in ABA-induced stomatal closure (Murata et al., 2001).
1.5.3 ABA signal transduction

Numerous studies have been conducted to reveal underlying mechanisms in ABA signaling, but no ABA receptors have been identified so far. It is believed that both extracellular and intracellular receptors may exist in plants. In contrast, many signaling intermediates have been identified. For example, many components, such as ABI (ABA insensitive) genes, involved in seed development have been discovered from analysis of ABA sensitivity using seed germination assays. Transcriptional regulators such as ABFs/AREBs, MYB transcription factors have been identified through protein binding assays using promoters of ABA-responsive genes such as RD29A and RD29B. In addition, ABA signaling involves GTP-binding proteins, phospholipases, and phosphatases, and secondary messengers, such as inositol 1,4,5 triphosphate (IP$_3$), PA, PLC, PLD, protein kinases. These components are involved in various ABA signaling such as seed germination, seedling development, ABA-induced stomatal closure, and other stress responses (Finkelstein and Rock, 2002). However, it is still largely unknown how these components are connected in ABA signaling pathway.

1.5.3.1 Phospholipase C in ABA signaling

Phospholipase C (PLC) is an enzyme involved in IP$_3$ release from phosphatidylinositol 4,5 bisphosphate by cleaving phosphodiester bonds between inositol and diacylglycerol. In animals, PLCs have an N-terminal pleckstrin homology (PH) domains, rooted from two repeated repeats in the protein, pleckstrin (platelet and leukocyte C kinase substrate), that are involved in membrane binding and activation other protein, and EF-hand motifs. In addition, C2 domain in PLCs has role for interactions between Ca$^{2+}$ and lipids/proteins. Although they lack a PH domain and have incomplete
EF motifs, all plant PLCs can hydrolize inositol 1,4,5 triphosphates (IP$_3$) from phosphatidylinositol 4,5 bisphosphate in a Ca$^{2+}$-dependent manner. Diacylglycerol (DAG) is produced following IP$_3$ cleavage from phosphatidyl 4,5 bisphosphate (PIP$_2$) by PLC, and is rapidly converted into phosphatidic acid, another second messenger. To date, nine putative PLC genes have been found in Arabidopsis and AtPLC1 is shown to be involved in ABA signaling. Expression of AtPLC1 is necessary for IP$_3$ release (Sanchez and Chua, 2001).

1.5.3.2 The involvement of phosphoinositides in ABA signal pathway

Phosphoinositides include phosphatidylinositol and its derivatives (Fig. 1.3). In mammals, phosphoinositides have been known for decades for the importance of their dual functions as precursors of second messengers and signaling molecules by themselves. As signaling molecules, phosphoinositides are involved in various signaling pathways as well as development, such as actin cytoskeleton assembly, and membrane trafficking (Martin, 1998). These diverse functions are possible because of multiple phosphorylation in the inositol head of phosphatidylinositol including free phosphoinositols, such as inositol 1,4,5 triphosphate (IP$_3$) and inositol hexakisphosphate (IP$_6$). To date, various enzymes involved in phosphoinositide signaling and biosynthesis have been identified and characterized in animals (Martin, 1998; De Matteis et al., 2002).

Compared to animals the role of phosphoinositides is still poorly understood in plants. During last decade, however, new information has emerged and shows that phosphoinositides are involved in various plant processes, such as calcium mobilization, signal transduction, cytoskeletal rearrangements, and membrane trafficking (Wang, 2004) and defense against pathogen attack (Laxalt and Munnik, 2002).
Figure 1.3. The phosphoinositides in plants. Abbreviations: InsP₆, inositol 1,2,3,4,5,6 hexaphosphate; Ins(1,4,5)P₃, inositol 1,4,5 trisphosphate; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol-3-phosphate; PtdIns(3,5)P₂, phosphatidylinositol (3,5) bisphosphate; PtdIns(3,4)P₂, phosphatidylinositol (3,4) bisphosphate; PtdIns(4,5)P₂, phosphatidylinositol (4,5) bisphosphate; PtdIns (4,5)P₃, phosphatidylinositol (4,5) trisphosphate; PtdIns (4,5)P₄, phosphatidylinositol (4,5) tetrakisphosphate.
1.5.3.2.1 Phosphatidylinositol 4,5 bisphosphate

In eukaryotic cells phosphatidylinositol (PtdIns) is the basic building block of the intracellular inositol lipids consisting of D-myo-inositol-1-phosphate (Ins1P) linked via its phosphate group to diacylglycerol. The inositol head group of PtdIns has five free hydroxyl groups except for postions 2, and 6. Phosphatidylinositol-4-phosphate (PI4P) is synthesized by phosphatidylinositol-4-kinases. PIP4 subsequently is used for most PtdIns(4,5)P2 synthesis via phosphatidylinositol-4-phosphate 5-kinase. PtdIns(4,5)P2 is important for membrane biogenesis and vesicle trafficking from the ER to the Golgi and plasma membrane. In plant cells, PtdIns 4 kinase and PtdInsP 5 kinase activities are associated with the plasma membrane, nucleus, endomembranes, cytosol and the cytoskeleton (Munnik et al., 1998).

PtdIns (4,5) P2 serves not only as a precursor of the two secondary messengers, IP3 and diacylglycerol, but also acts as a pivotal signaling molecule itself in many cellular processes. For example, PtdIns (4,5) P2 is important for the regulation of cytoskeletal dynamics (Hwang and Lee, 2001), vesicle trafficking and ion transport in plants (Stewenson et al., 2000). In addition PtdIns (4,5) P2 is accumulated under different salt stress treatments, which coincides with IP3 elevation as well (DeWald et al., 2001).

PtdIns (4,5) P2 is ubiquitous throughout the membranes and establishes a network of initiation sites for generation of rapid, transient increases in IP3 in response to stimuli. Under water deficit stress ABA activates phospholipase C, which cleaves IP3 from PtdIns (4,5) P2 (Munnik et al., 1998; Stewenson et al., 2000). Hydrolyzed IP3 is involved in Ca2+ oscillation in the cell (Reddy, 2001).
1.5.3.2.2 Inositol 1,4,5 trisphosphate (IP₃)

Plant water status is precisely controlled by ABA by inducing stomatal closure in response to water deficit stress (Allen et al., 1999; Schroeder et al., 2001b; Lemichez et al., 2001; Iuchi et al., 2001). Stomatal closure is associated with cytosolic Ca²⁺ concentrations, which is in part regulated by inositol 1,4,5 trisphosphate (IP₃) that increases upon environmental stresses and ABA treatment (Gilroy et al., 1998). The vacuole is believed to be a major reserve for Ca²⁺ release into cytoplasm in plants. IP₃ acts as a secondary messenger that can activate Ca²⁺ channel in the vacuole, ER, and other organelles in plant cell. IP₃-triggered Ca²⁺ release is suggested to contribute to ABA-induced stomatal closure (Macrobbie, 2000). IP₃ activates IP₃ receptors on the endoplasmic reticulum and the tonoplast, and the signal triggers the release of Ca²⁺ from organelles in the cell. Increased concentration of Ca²⁺ induces K⁺ and Cl⁻ efflux and thereby resulting in stomatal closure.

1.5.3.2.3 Inositol hexakisphosphate (IP₆)

In addition, IP₆ also is considered to be involved in the ABA inhibition of stomatal opening by inactivating the plasma membrane influx K⁺ channels (Lemtiri-Chlieh et al., 2003). The precursor of IP₆ is presumed to be IP₃ and it is the most abundant inositol phosphate in cells, but its roles are poorly understood. IP₆ elevation has been shown to occur in guard cells in response to ABA treatment (Yang et al., 2001; Lemtiri-Chlieh et al., 2003), suggesting IP₆ also has a role in ABA-induced stomatal closure.
1.5.3.3 Inositol polyphosphate 5 phosphatases

Diverse inositol phosphatases have been found and characterized in animals. There are different types of phosphatases, such as inositol polyphosphate 1 phosphatases, - 4 phosphatases, and - 5 phosphatases (Majerus et al., 1999). Inositol polyphosphate 5 phosphatases among known inositol phosphatases are well characterized in animals.

1.5.3.3.1 Inositol polyphosphate 5 phosphatases (5PTase) in animals

Since the first inositol polyphosphate 5 phosphatases (5PTase) had been identified in human platelets, 5PTases have since been found in other mammalian cells, yeast, and in plants (Stolz et al., 1998; Majerus et al., 1999). These enzymes are Mg\(^{2+}\)-dependent phosphomonoesterases. Based on their substrate specificity these enzymes have been classified into four groups (Majerus et al., 1999) so far and further analysis may include more subfamilies in the future. Four well known substrates for 5PTases are Ins-1,4,5-P\(_3\) (IP\(_3\)), Ins-1,3,4,5-P\(_4\), phosphatidyl (4,5) bisphosphate (PIP\(_2\)), phosphatidyl (3,4,5) triphosphate (PtdIns-3,4,5-P\(_3\)), and probably IP\(_6\). These enzymes have two distinct motifs required for enzyme activity: (F/I)WXGDXN(F/Y)R and (R/N)XP(S/A)(W/Y)(C/T)DR(I/V)(L/I) (Majerus et al., 1999).

The major function of inositol polyphosphate phosphatases is the termination of phosphoinositide signals that are produced by various phosphoinositide kinases in response to stimuli (Kisseleva et al., 2002). There are four classes of 5PTases based on their substrate specificity as follows:

**Type I 5PTase:** Group I 5PTases remove 5’phosphate from only free water-soluble substrates, such as Ins-1,4,5-P\(_3\) (IP\(_3\)), Ins-1,3,4,5-P\(_4\). They are believed to function as a terminator of calcium signaling. Human type I 5PTase is involved in the control of Ca\(^{2+}\)
oscillations (De Smedt et al., 1997). Transfected cells with an antisense 5PTase I cDNA results in elevation of IP₃ as well as cytoplasmic Ca²⁺ levels.

**Group II 5PTases:** Type II phosphatases, OCRL-1, and Synaptojanin 1 belong to group II enzymes that target all phosphoinositides having 5’phosphate. Mutation of OCRL-1 causes Lowe's oculocerebrorenal syndrome (OCRL). Synaptojanin 1 (involved in synaptic vesicle trafficking) is known to inhibit phospholipase D (PLD) activity, and synaptojanin-1 5PTase-deficient mice have neurological defects and early death (Cremona et al., 1999).

**Group III 5PTases:** Group III enzymes hydrolyze only substrates having a 3’ phosphate such as Ins-1,3,4,5-P₄ and PtdIns-3,4,5-P₃. These phosphatases, such as SHIP and SHIP2 5PTases, have SHIP domains (SH₂-containing inositol-5-phosphatase) that are important for interaction with other proteins (Damen et al., 1996). SHIP 5PTases are suggested to be involved in cell proliferation through cytokine signaling, and malfunction of this 5PTase causes early death and myeloproliferative syndrome (Helgason et al., 1998).

**Group IV 5PTases:** These enzymes are poorly understood to date; type IV enzymes can hydrolyze PtdIns-3,4,5-P₃ as well as PtdIns-4,5-P₃ (Kesseleva et al., 2000).

1.5.3.3.2 Inositol 1,4,5 trisphosphate 5 phosphatase (5PTase) in plants

In mammals, various 5PTases have been identified and characterized. Malfuction of those 5PTases causes serious problems in growth, Ca²⁺ signaling, and cancer, suggesting that these enzymes have critical roles in mammals (De Smedt et al., 1997; Helgason et al., 1998; Cremona et al., 1999).
While the critical roles of 5PTases in animals are well documented, much less is known about their functions in plants. An inositol-1-monophosphatase (IMP) has been identified in tomato (Gillaspy et al., 1995), but until recently, few inositol 5 phosphates have been found and analyzed (Table 1.2). To date, all of these genes have been characterized in Arabidopsis (Zhong and Ye, 2004; Berdy et al., 2001; Sanchez and Chua, 2001; Carland and Nelson, 2004; Ercetin and Gillaspy, 2004), but there are no reports from other plant species.

Two 5PTases genes (At5PTase1 or AtIP5PII/At5PTase2) have been characterized and are likely involved in ABA signaling. At5PTase1 or AtIP5PII overexpression causes reduction in IP3 levels (Berdy et al., 2001), and exogenous ABA does not change IP3 levels in plants over-expressing At5PTase2 (Sanchez and Chua, 2001). When ABA was applied to these plants, the expression of ABA-regulated genes was reduced compared to wild type plants. In addition, AtIP5PII plants were less sensitive to ABA than wild type in seed germination, indicating that 5PTase has an important role in seed development (Sanchez and Chua, 2001).
<table>
<thead>
<tr>
<th>Species</th>
<th>Genes</th>
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<th>Characteristics (s)</th>
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<td>Type II/PI(4,5)P, PI(3,4,5)P₃, Ins(1,4,5)P₃</td>
<td>Secondary wall development</td>
<td>Zhong et al., 2004</td>
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</table>

Table 1.2 Inositol 5 phosphatase genes characterized in plants

27
1.6 ABA-INDEPENDENT DROUGHT STRESS RESPONSES IN PLANTS

Sessile plants experience frequent episodes of water deficit stress. Their survival strongly relies on how fast and effectively they respond to these adverse stresses and minimize the resultant damage. Water stress changes various biochemical and physiological aspects in plants. Since plant cell growth and shape are largely dependent on turgor pressure, water loss often results in wilting or even in plasmolysis under the severe dehydration. Numerous studies have shown that various genes are upregulated in response to water deficit stress in plant (Shinozaki and Yamaguchi-Shinozaki, 1997). Although many drought-inducible genes are also inducible by ABA, some drought-responsive genes are up-regulated in an ABA-independent manner. These findings raise the possibility that there may be an ABA-independent drought response pathway in plants (Fig. 1.4). Recent studies have revealed that ABA-independent drought stress pathways exist in plant under drought, salt, or cold stresses (Shinozaki and Yamaguchi-Shinozaki, 1997).

ABA-independent drought stress response is proposed on the basis of genetic studies using ABA-deficient (aba) or ABA-insensitive mutants (abi). Several genes, such as \( RD29A \), \( KIN1 \), \( KIN2 \), and \( COR47 \), are induced by drought, salt, or cold, but not by ABA in these mutants. These results indicate these genes are involved in a drought response in an ABA-independent manner (Shinozaki and Yamaguchi-Shinozaki, 1997). The Arabidopsis \( RD29A \) gene is one of them, and has been extensively investigated. From promoter deletion analysis, Yamaguchi-Shinozaki and Shinozaki (1994) found 9bp conserved cis-acting element, DRE motif TACCGACAT (dehydration responsive element containing the C-repeat element, CRT underlined CCGAC), and this motif is critical to
lead gene expression under drought or cold stresses as well as salt stress. However, exogenous ABA did not change reporter GUS expression under the control of an artificial promoter containing the DRE motif. These results provide strong evidence that an unknown ABA-independent signaling pathway exists. DRE binding proteins, DREBs that activate drought-responsive genes upon binding DRE motif have been identified from a cDNA library screen using the yeast two hybrid system. Deduced amino acid sequences of DREB genes reveal that these proteins have a conserved EREBP/AP2 DNA binding domain, which are also involved in ethylene responses and flower morphogenesis. Except for this motif, these genes are not only very different in their amino acid sequences, but do exhibit different expression patterns under various stress conditions. DREB1A is strongly induced by cold stress, but does not respond to drought, salt, or ABA treatment. DREB2A and DREB2B gene expression can be induced by drought. These results suggest that these genes are involved in separate signal pathways, cold stress or water stress in ABA-independent manner (Liu et al., 1998; Chinnusamy et al., 2004). There appear to be additional genes that are also involved in water stress. C-repeat binding factors (CBF1, 2, 3) also can be induced by low temperature and bind CRT elements (CCGAC) within DRE motif (TACCGACAT) (Gilmour et al., 1998). Sucrose synthase 1 (SUS1) is strongly induced by cold stress in wild type Arabidopsis plants, as well as the aba-1 and aba-2 mutants, suggesting that SUS1 may sense osmotic changes in plants (Dejardin et al., 1999). Based on previous studies there is only one ABA-independent drought stress response pathway via DREB2 (Fig. 1.4).
Dehydration and salt stress

Figure 1.4. Simplified signaling cascades in plant response to water deficit stress.
1.7 OBJECTIVES

Numerous studies have been conducted to understand the in-depth mechanisms involved in plant drought responses, such as ABA and stomatal function. These studies provided considerable invaluable information, such as the existence of stress-related transcription factors and the central role of phosphoinositides. Although the biochemical, cellular, and molecular mechanisms underlying plant drought response are still not completely understood, immense progress has been obtained from model plant Arabidopsis. Outcome from this progress not only is useful for unraveling mechanisms related to drought response, but also provides important information that can be applied to other plant species, such as crops, to improve drought tolerance.

The objective of this study was to increase the resistance of two crops (tobacco and tomato) to water deficit stress. To achieve this goal I attempted to generate transgenic plants using two genes, putative type I inositol 5 polyphosphatase (5PTase) from tomato and ABRE binding factor ABF4 from Arabidopsis (Choi et al., 2000).

Type I 5PTases, as a negative regulator, are known to terminate inositol 1,4,5 trisphosphate (IP$_3$) signaling by hydrolyzing 5 position phosphate (Berdy et al., 2001). Since the accumulation of IP$_3$ is suggested as an early step in ABA signal pathway triggered by drought in plant, the expression level of type I 5PTase may be crucial in plant response to drought, providing a unique opportunity for genetic manipulation of plant drought response. Therefore, it is hypothesized that plant drought response may be modulated by an altered 5PTase level. To test this hypothesis, four putative tomato 5PTase genes (Le5PT1-4) have been cloned. Le5PT1 is used since the expression of Le5PT1 is dramatically suppressed by dehydration.
In addition, many transcription factors involved in ABA signaling pathway have been identified and characterized in model plant *Arabidopsis*, but most of them have not been applied to crop plants. One of bzip transcription factors, ABA responsive element binding factor 4 (*AtABF4*) has been shown to dramatically improve drought tolerance in Arabidopsis (Kang et al., 2002). In this study, the goal is to specifically express the *AtABF4* gene in guard cell and to test whether *AtABF4* expression in guard cell also can improve plant drought tolerance like 35S:*AtABF4* Arabidopsis. To test this hypothesis, *AtABF4* gene under control of guard cell specific *KST1* (Plesch et al., 2001) or strong tobacco mosaic virus *CaMV 35S* promoter is introduced into tobacco and tomato plants.
CHAPTER 2

GUARD CELL SPECIFIC EXPRESSION OF ARABIDOPSIS ABF4 ALTERS DROUGHT TOLERANCE IN TOMATO AND TOBACCO

2.1 ABSTRACT

Plant survival relies on resisting episodes of adverse environmental stresses. Water stress is one of the most severe constraints in crop production. Plants reduce water loss by reducing stomatal aperture when water supply is limited. The stress hormone ABA activates a signal transduction pathway culminating in stomatal closure. Many transcription factors have been shown to be involved in ABA responses in Arabidopsis, but rarely applied to crops. Here, it is shown that the expression of Arabidopsis ABF4/AREB2 gene under the control of guard cell specific KST1 promoter significantly increases drought tolerance in tobacco and tomato plants. The transgenic plants exhibit significantly lower water loss per unit leaf area compared to wild type plants. The ABF4-mGFP protein strongly accumulated in guard cell nuclei. The possibility that the ABF4 protein can activate 4XKST1-rd29B:mGFP-GUS fusion construct in response to water deficit stress was examined. Interestingly, however, ABF4 itself was apparently insufficient to induce GUS expression even under water stress. Nevertheless, these results provide a tool that drought-inducible transcription factors can be used to alter crop drought tolerance via expressing in guard cells.
2.2 INTRODUCTION

During their life cycle, plants are subjected to multiple environmental stresses such as water deficit, cold, or salt stresses that commonly occur in natural environments. Therefore, the capability of plants to adjust to those adverse conditions in a timely fashion is critical for their survival. As a stress hormone, abscisic acid (ABA) is a pivotal player in adaptive responses to water stress by reducing water loss through inducing stomatal closure as well as changing the expression of stress-responsive genes. Since it is known that plants lose more than 95% of the water taken up by the roots through the stomates, ABA-induced stomatal closure is necessary for survival under water deficit conditions. Many studies using ABA deficient mutants have shown the importance of ABA in the response of plants to water stress (Burbidge et al., 1999; Seo and Koshiba, 2002; Thompson et al., 2004). In addition, numerous genes have been shown to be up-regulated under water deficit, such as \textit{RD29A} and \textit{B} (Yamaguchi-Shinozaki and Shinozaki, 1994), group 2 \textit{LEA} genes known as dehydrins (Kasuga et al., 2004), that have a role like osmolytes in protecting proteins from denaturation during water deficit stress.

Many ABA-inducible genes have a consensus \textit{cis}-acting element known as ABA-responsive elements (ABREs) in their promoters (Guiltinan et al., 1990, Yamaguchi-Shinozaki and Shinozaki, 1994). Most abundant ABREs elements contain C/TACGTGGC sequences (Kang et al., 2002). These ABA-inducible genes containing ABREs are up-regulated by binding of bZip transcription factors, such as ABRE element binding proteins (\textit{ABFs/AREBs}) (Choi et al., 2000; Uno et al., 2000). \textit{ABF2/AREB1} and \textit{ABF4/AREB2} are upregulated by dehydration and exogenous ABA. However, ABA is required for appropriate functions of \textit{ABF2/AREB1} and \textit{ABF4/AREB2} proteins in a
transactivation study using RD29B promoter-GUS fusion gene in Arabidopsis protoplasts (Uno et al., 2000). Transgenic Arabidopsis plants expressing ABF3 and ABF4 are significantly more tolerant to water deficit stress than wild type plants (Kang et al., 2002; Oh et al., 2005) and seeds of these transgenic plants are hypersensitive to ABA in germination assays (Kang et al., 2002). The expression of ABA-responsive genes such as RD29B and RAB18 also are up-regulated in these transgenic plants (Kang et al., 2002). Both transgenic plants exhibit reduced stomatal apertures compared with wild type Arabidopsis (Kang et al., 2002). This is evidence that drought tolerance of these transgenic plants is due to reduced water loss via stomatal closure. Interestingly, a microarray study demonstrated that ABF3 was up-regulated in guard cells treated with ABA, and various possible target genes by ABRE binding factors have been identified (Leonhardt et al., 2004), indicating that the expression of ABA responsive genes via ABRE binding proteins may be functionally well conserved in guard cells.

A dwarf phenotype is common in plants with altered levels of stress-inducible genes. As exogenous ABA inhibits plant growth, the dwarf phenotype in 35S:ABF3 and 35S:ABF4 transgenic plants is probably due to ABA-hypersensitive response caused by the transgene (Kang et al., 2002). A similar phenotype was also observed in plants over-expressing stress-inducible transcription factors such as LeCBF1, AtCBF1, and AtCBF3 (Gilmour et al., 2000; Hsieh et al., 2002; Zhang et al., 2004). AtCBF1 and AtCBF3 activate drought-responsive genes with DRE/CRT cis-acting element (core motif: G/ACCGAC) in their promoter regions, which is similar to ABRE elements. Interestingly, AtCBFs were also up-regulated by exogenous ABA (Knight et al., 2004), and a recent finding from analysis using DRE and ABRE elements in rd29A promoter indicates that a
possible network exists between drought and ABA signaling pathways (Narusaka et al., 2003). However, dwarf phenotypes were not observed when \textit{AtCBF3} and \textit{ABF3} were expressed under the control of \textit{ubiquitin1} promoter instead of strong cauliflower mosaic virus \textit{CaMV 35S} promoter (Oh et al., 2005). Both transgenic rice plants still exhibited drought tolerance without retarded growth (Oh et al., 2005). A normal growth phenotype was also recovered when \textit{AtDREB1A/AtCBF3} was expressed under the control of ABA-/drought-responsive \textit{RD29A} promoter in tobacco (Kasuga et al., 2004).

In this study, I investigated whether guard cell specific expression of an ABRE binding protein, \textit{ABF4/AREB2} (Choi et al., 2000; Uno et al., 2000), also could improve drought tolerance in tobacco and tomato plants while minimizing the dwarf phenotype. I found that the expression of \textit{ABF4/AREB2} under the control of guard cell specific \textit{KST1} promoter that was shown to lead gene expression specifically in the guard cells of potato (Plesch et al., 2001) was sufficient to increase drought tolerance in both crop plants by reducing transpiration. Moreover the growth of these transgenic plants was much less affected by transgene expression compared to Arabidopsis plants transformed with \textit{35S:ABF4} or \textit{35S:ABF3} (Kang et al., 2002). In addition, since the \textit{ABF4/AREB2} protein was shown to activate the \textit{RD29B-GUS} fusion gene in transient experiment using Arabidopsis mesophyll protoplasts (Uno et al., 2000), it was of interest to test whether the \textit{ABF4} protein also can transactivate a reporter gene specifically in guard cells. To accomplish this, a heterologous fusion construct (\textit{4XKST-\textit{rd29B-mGFP:GUS}}) consisting of four tandem repeats of 82bp fragment conferring guard cell specificity in the \textit{KST1} promoter (Plesch et al., 2001), \text{–}369bp \textit{RD29B} promoter (Yamaguchi-Shinozaki and Shinozaki, 1994), and \textit{mGFP-GUS} as reporter gene was generated. I tested whether a
heterologous 4XKST-rd29B-mGFP:GUS fusion gene, a guard cell specific and drought inducible reporter gene, could be activated by Arabidopsis ABF4 protein in tobacco plants.

2.3 MATERIALS AND METHODS

2.3.1 Plant Materials

Seeds of tomato (Lycopersicon esculentum cv. Ohio 8245) or tobacco (Nicotiana tabacum cv. Wisconsin 38) were surface sterilized in 50% sodium hypochlorite solution for 10 min, followed by 4 to 5 rinses with sterile water, and placed in Magenta boxes containing Murashige and Skoog medium (Sigma, St. Louis, MO, USA) supplemented with 30 g L⁻¹ sucrose, adjusted at pH 5.7, and solidified by 8 g L⁻¹ select agar (Sigma). Seedlings were grown for 3 to 4 weeks under 50 μmol m⁻² sec⁻¹ of constant fluorescent light at 25±3°C.

2.3.2 DNA Constructs

The Cloning of Arabidopsis ABF4

A 1.4kb full-length cDNA for ABF4 gene was isolated from 3-week-old Arabidopsis thaliana (Columbia) leaves using RT-PCR with one-step RNA PCR kit (Takara, Madison, WI, USA). The primer pairs used were as follows:

forward primer (5'- ACGCGTCGAC ATGGGAACTCACATCAATTTC-3', Sal 1),
reverse primer (5'- ACAGTCATGAACCATGGTCCGGTTAATGTC –3’, BspH 1). The amplified fragment was cloned into pGEM®-T Easy vector (Promega, Madison, WI) following the procedures in the manual supplied by the manufacturer. To produce the KST1:ABF4-mGFP construct, the Sal1 and BspH1 ABF4 fragment was ligated into Sal1/Nco 1 sites of a modified pCambia 1302 binary vector (Center for the Application of
Molecular Biology of International Agriculture, Black Mountain, Australia) containing the *KST1* promoter. To generate *CaMV 35S:ABF4-mGFP*, the *KST1* promoter was replaced with the *CaMV 35S* promoter.

**The Cloning of *KST1* Promoter**

A 0.3 kb *KST1* promoter having a guard cell-specific element (Plesch et al., 2001) was cloned from potato genomic DNA by PCR using EX-taq DNA polymerase (Takara). Primer pairs were as follow:

forward primers 5’- CGCGGATCCATCTGCGTACAGTCTACCT –3’, and reverse primer 5’-CATGCCATGGGTCGACTATTATATATTGCTGCTTCTTT-3’.

The amplified PCR fragment was digested with BamH 1 and Nco 1 and used to replace *CaMV 35S* promoter in the pUC18 vector. A 0.3 kb Hind III/Nco1 *KST1* fragment was used to replace the *CaMV 35S* promoter in the pCambia 1302 vector, which further was used to clone *AtABF4* cDNA in upstream of mGFP.

![Figure 2.1](image)

**Figure 2.1.** Constructs used for tobacco and tomato transformation. Arabidopsis *ABF4* gene was placed under the control of constitutive *CaMV 35S* or potato *KST1* guard cell specific promoter.
2.3.3 DNA Sequencing

The sequencing of plasmid DNA was performed using an Applied Biosystems 3730 DNA Analyzer at the Ohio State University Plant-Microbe Genomic facility.

2.3.4 Plant Transformation

Two-week-old tomato cotyledons or 1-month-old tobacco leaves were used for transformation. Tomato cotyledons and tobacco leaves were cross-cut into pieces and pre-cultured the adaxial side facing down in MS medium supplemented with 2% sucrose at pH 5.7 for 2 days. The pCambia vector 1302, containing KST1:ABF4-mGFP was transferred into Agrobacterium tumefaciens strain GV3101 by electroporation. Explants were co-cultured with overnight culture of A. tumefaciens GV3101 on the basis of procedure described in leaf disc transformation method (Horsch et al., 1998). After co-cultivation for 2 days, the explants were washed thoroughly with sterile distilled H2O containing 300 mg L⁻¹ cefotaxim and dried briefly on sterile filter paper. For tobacco regeneration, the explants were transferred into MS medium containing hygromycin B 30 mgL⁻¹ as described by Horsch et al. (1998). For tomato regeneration, however, after 7-day culture the explants were transferred into new MS medium supplemented with 2% sucrose, zeatin 2mg L⁻¹ and IAA 0.1mg L⁻¹, hygromycin B 30mgL⁻¹ at pH 5.7 (Park et al., 2003).

Putative transformants were rooted on MS medium containing 2% sucrose and 50 mgL⁻¹ hygromycin. The hygromycin-resistant plantlets were transferred to 2 L pots with soilless media (Metro-Mix 360, The Scotts-Sierra Co., Marysville, OH, USA) and grown in a greenhouse with a 16-h photoperiod. The plants were watered daily, and fertilized as needed.
2.3.5 The Expression Analysis for Arabidopsis *ABF4* in transgenic plants

**Tobacco Plants**

Total RNA was isolated from 3-week old seedlings in MS medium containing 2% sucrose using an RNeasy® Plant Mini Kit (Qiagen USA, Valencia, CA). RT-PCR was conducted using the one-step RNA PCR kit by following the manufacturer's protocol (Takara). The primer pairs used for RT-PCR of the expression of *ABF4* are as follows:

Forward primer 5’-ACGCGTCGACATG GAACTCACATCAATTTC-3’,
Reverse primer 5’-ACAGTCATGAACCATGGTCCGGTTAATGTC-3’.

A 25µL RT-PCR mixture containing total RNA (1.0µg) was reverse-transcribed using the Takara One-Step RNA PCR kit as described by protocol provided by the manufacturer. After reverse transcription, the mixture was used for PCR as following conditions: 20 to 25 cycles of at 94 °C for 30 sec, 55 °C for 40 sec, and 72 °C for 1 min 30 sec. Then, 10 µL of the PCR products were loaded onto 1% agarose gels, electrophoresed, and stained with 0.5 mg/ml ethidium bromide.

**Tomato Plants**

PCR was used to identify positive transgenic lines using genomic DNAs from segregating T₂ tomato plants with the same primer sets used in tobacco plants. Total RNAs were isolated from 1 or 2 month old PCR positive plants, and transgene expression was analyzed by RT-PCR as described previously for tobacco.

2.3.6 Analysis of Transgenic Plants under Water Deficit Conditions

Roughly 100 seeds from wild type and two transgenic tobacco plants were directly sowed into 2 L pots with soilless media (Metro-Mix 360), whereas wild type and transgenic tomato lines were sowed individually into 0.2 L pots filled with Metro-Mix
Plants were grown in greenhouse under 16hr photoperiod for 1 month. To test water deficit resistance, water was withheld from KST1:ABF4-mGFP transgenic tomato T2 and wild type plants until plants began to wilt. For tobacco, water was withheld for 20 days, irrigated, and the ability to recover examined.

2.3.7 Plant Water Usage

Two-week-old seedlings in MS (Sigma) medium were transferred into 2 L pots filled with Metro-Mix 360 and grown in the greenhouse for two months. Prior to recording daily water loss, 6 tobacco plants from 2 KST1:ABF4-mGFP transgenic lines (NK3 and NK27) and wild type tobacco plants were harvested to measure leaf area, height, leaf number, dry and fresh weight. Daily evapotranspiration was determined by lysimetry. Twelve pots containing one plant from each line were weighed using a top loading balance between at 11:00HR and 12:30HR each day for 10 days. The plants were irrigated each day at 11:30HR. Daily evapotranspiration was determined by calculating the difference in the weights of the pot at 12:30HR and the weight at 11:00HR the following day. Transpiration was deduced by subtracting the average amount of evaporation for the same period from three 2 L pots that contained soilless media but no plants. In addition, transpiration per unit leaf area was determined by dividing the amount each plants transpired during the previous 24h by the total leaf area. Water use efficiency (WUE) and transpiration ratio (TR) of tobacco plants were calculated as follows:

- WUE = dry weight increase / total water loss, during 10 day-period
- TR = total water loss / dry weight increase

For tomato, daily transpiration rates from T2 heterozygous KST1(35S):ABF4-mGFP plants and WT plants were compared as described for tobacco.
2.3.8 Confocal Laser Microscopy

The intercellular localization of the ABF4-mGFP fusion protein was determined using a PCM2000 confocal laser scanning microscope (Nikon) with argon and green HeNe lasers to detect GFP fluorescence.

2.3.9 Protein extraction and Western analysis

Total protein was obtained from one-month old leaf tissues (approximately 100 mg) of wild type or transgenic tobacco plants. To extract protein, 500μL of SDS extraction buffer (0.125 M tris-HCl, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8) was added and ground using a mortar and pestle. The slurry was transferred into a 2 mL Eppendorf tube and centrifuged for 3 min at 10,000 rpm at 4 °C. The supernatant was transferred to a new tube and mixed with 1.5 mL of 100% acetone. After 30 min incubation at 4°C, the samples were centrifuged for 3 min at 10,000 rpm at 4 °C and the pellet dissolved in 2X SDS protein loading buffer. Then, the equal amount of samples was separated on 8% SDS-PAGE gel. After electrophoresis, SDS-PAGE gel was stained by coomassie blue staining buffer, and protein levels were determined based on intensity. Protein was transferred to poly (vinylidene difluoride) membrane (Bio-Rad) by standard techniques (Sambrook et al., 1989). The membrane was blocked in 1X TBS (10 mM Tris HCl, pH 8.0/150 mM NaCl) containing 1% (wt/vol) nonfat dry milk at room temperature for 1 h, incubated with anti-GFP rabbit polyclonal antibody (Molecular Probes, Eugene, OR, USA), and developed using peroxidase-conjugated secondary antibody (dilution ratio of 1:5,000) by enhanced chemiluminescence (Amersham Pharmacia).
2.3.10 Transactivation Experiment with Transgenic Plants

Although various genes have been shown to be expressed in guard cells, there are no reports of a drought-responsive promoter that controls gene expression specifically in guard cells. In this regard, a heterologous promoter was generated to test whether a fusion promoter can be used to express target gene in guard cell under water deficit stress. *KST1* promoter from potato (*Solanum tuberosum*) contains two copies of guard cell specific elements (TAAAG) in –236 to –154bp (82bp) regions (Plesch et al., 2001). Four repeats of 82bp fragment of *KST1* promoter were ligated to upstream of –344bp *RD29B* promoter that is drought responsive. Next, this fusion construct was used to replace the *CaMV 35S* promoter in pCambia 1304 binary vector. Final reporter construct 4X*KST-rd29B:mGFP-GUS* in pCambia 1304 was used to transform tobacco plants by *A. tumefaciens*-mediated transformation procedure as described before. To generate F1 tobacco plants homozygous *KST1:ABF4-mGFP* transgenic plants (NK27) were crossed with wild type tobacco plants or with homozygous 4X*KST-rd29B:mGFP-GUS* transgenic plants. F1 transgenic plants were used in a transactivation test. Briefly, one-month-old F1 plants were watered or dehydrated by stopping irrigation, and then leaf tissues were excised and examined for GUS accumulation. GUS transcripts were analyzed by RT-PCR with primer pairs (Forward 5’ – TGGGAAAGCGCGTTACAAGAAAGC - 3’ Reverse primers 5’ - TGTAGAGCATTACGCTGCGATGGA - 3’) by following the same conditions as described before.

2.3.11 Histochemical GUS Assay

Two-week-old seedlings grown in MS (Sigma) medium supplemented with 2 % sucrose were transferred and grown in 200-cell square plug flat (16 cm³ for each cell)
with Metro-Mix 360 in the greenhouse for 2 more weeks. After that, eight plants from each transgenic tobacco line were used for GUS staining. Prior to the GUS histochemical assay, 4 plants of each transgenic line were covered with polyethylene film to reduce water loss, and 4 plants from each line remained uncovered. When the uncovered plants were severely wilted, leaves were harvested and used in the GUS assay. For the histochemical GUS assay (Campisi et al., 1999), leaf tissues were incubated in a solution consisting of 50 mM NaHPO4 (pH 7.2), 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), 0.5% Triton X-100 at 37°C for 48h. The staining solution was replaced with 70% ethanol and placed in a hot water bath at 70 °C to remove the chlorophyll.

2.3.12 Measurement of water potential and relative water contents

For tobacco, two KST1:ABF4-mGFP transgenic (NK3 and NK27) and wild type plants were grown in 2 L pots in greenhouse for 7 weeks. After that, each 3 plants from three genotypes were used to measure water potential using WP4-T Dewpoint Potentiometer (Decagon, Pullman, WA, USA). To determine solute and pressure potentials, leaf tissue was placed in a –70 °C freezer immediately after measuring the water potential. After 24h, the tissue was thawed, and the water potential measured again. This second water potential measurement was used as the solute potential. The pressure potential was calculated by subtracting the solute potential from the leaf water potential. To determine the relative water content (RWC), leaves of similar size were harvested at 13:00HR, and used to measure fresh weight (FW), turgid weight (TW). For TW, the tissue was fully hydrated by placing it in a 15 mL polyethylene cup with 3-5 mL of distilled water and placed in a refrigerator at 4 °C for 1 day, and the weight was re-measured. The tissue was then dried in an oven at 60 °C for 1 day and the dry weight
(DW) was determined. RWC was calculated using following equation:

\[ \text{RWC} = \frac{(\text{FW}-\text{DW})}{(\text{TW}-\text{DW})} \]

The water potential of 2-month old greenhouse tomato plants was measured using a Scholander-type pressure chamber (the Model 1000 pressure chamber, PMS, Albany, OR, USA). Three to five plants from T2 heterozygous \textit{KST1}(35S):\textit{ABF4} and wild type plants were used for measurement of water potential at 13:00HR (normal conditions) or 17:00HR (water-stressed). At the same time, leaf tissues were collected from each plant to measure RWC using the same method as described above in RWC measurement in tobacco plants.

2.4 RESULTS

2.4.1 Generation of Transgenic Tobacco and Tomato Plants with Arabidopsis \textit{ABF4}

To investigate whether guard cell-specific expression of Arabidopsis \textit{ABF4} can change the resistance to water deficit stress in crops, an \textit{ABF4-mGFP} fusion gene construct was placed under the control of guard cell specific 0.3kb \textit{KST1} promoter from potato (Mueller-Roeber et al., 1995; Plesch et. al., 2001). For tobacco, 11 \textit{KST1:ABF4-mGFP} transgenic lines were obtained from hygromycin selection and grown in the greenhouse to harvest seeds. However, no transgenic tobacco plants were obtained that contained \textit{35S:ABF4-mGFP} construct. Homozygous lines containing 1 copy of the transgene were selected based on Mendelian inheritance patterns. Two homozygous T3 tobacco lines (Fig. 2.2A, NK3, NK27; NK- \textit{Nicotiana tabacum} and \textit{KST1:ABF4-mGFP}) were used for further study. For tomato, three \textit{KST1:ABF4-mGFP} and two \textit{35S:ABF4-mGFP} transgenic lines were obtained from hygromycin selection. Two \textit{KST1:ABF4-mGFP} (Fig. 2.2B, lanes 4 and 5; LK-\textit{Lycopersicon esculentum} and \textit{KST1:ABF4-mGFP})
and one 35S:ABF4-mGFP (Fig. 2.2B, lanes 6 and 7; LK- *Lycopersicon esculentum* and 35S:ABF4-mGFP) segregating T2 transgenic lines were used for further analysis. The expression of Arabidopsis *ABF4* in the transgenic plants was only detected in the putative transgenic tobacco or tomato plants (Fig. 2.2A and B).

**Figure 2.2.** Analysis of transgene expression in tobacco and tomato plants. A, Expression level of *ABF4* in wild type (WT) and two *KST1:ABF4* homozygous transgenic tobacco lines (NK3, NK27) was examined by RT-PCR. RNA levels of two T3 transgenic lines and wild type tobacco were examined by RT-PCR using total RNA from a month old tobacco plants. B, Expression level of *ABF4* were examined by RT-PCR using total RNAs from wild type (WT) and two individual tomato plants from each lines of two *KST1:ABF4* T2 transgenic lines (LK21 and LK34) and one 35S:ABF4 T2 transgenic plants (LS41).
Figure 2.3. Expression and localization of ABF4-mGFP fusion proteins. A, ABF4-mGFP image was superimposed with bright field image from transgenic tobacco plant (NK27). B-C, ABF4-mGFP expression in NK3 transgenic tobacco plant. Expression of fusion protein in transgenic tobacco plant (NK3) was determined by anti-GFP antiserum and ABF4-mGFP image was visualized in tobacco guard cell nuclei under confocal microscope. D, Bright field image of C.
2.4.2 ABF4 accumulates strongly in nuclei of guard cells in tobacco plants

When expressed constitutively Arabidopsis ABF3 and ABF4 improved plant drought tolerance (Kang et al., 2002), resulting from the induction of ABA-responsive genes via the activation of ABRE elements in their promoters. Even though it was shown that ABF4 binds ABRE elements in in-vitro assays (Choi et al., 2000; Uno et al., 2000), there are no reports on whether ABF4 protein is localized in nuclei. To visualize the cellular localization of ABF4 in plants, ABF4 was fused to a mGFP reporter gene (Fig. 2.1). Heterologous ABF4-mGFP fusion protein accumulated in nuclei of tobacco plants. Although some the ABF4-mGFP protein was detected in the nuclei of epidermal cells (Fig. 2.3B and C), the strongest accumulation was found in nuclei of guard cells (Fig. 2.3A-C). The expression of ABF4-mGFP was analyzed by Western blot analysis, and the expected 75kD fusion protein was detected only in transgenic plant (Fig. 2.3B, NK3).

2.4.3 Guard cell specific ABF4 Expression Improves Drought Tolerance

To examine whether ABF4 expression under the control of the guard cell specific promoter KST1 affects drought tolerance like Arabidopsis constitutively expressing ABF4, KST1:ABF4-mGFP transgenic lines and wild-type plants were grown in pots in the greenhouse for one month with regular irrigation.

For tobacco, both transgenic plants exhibited minor wilting symptoms when water was withheld for 18 days, whereas wild type plants were severely wilted (Fig. 2.4A). After 20 days, however, all lines were severely wilted (Fig. 2.4A). To test the ability to recover from the 20 days drought treatment, the plants were irrigated and pictures taken 5 days later (Fig. 2.4A). The 2 transgenic lines recovered within 5 days after irrigation, whereas most wild-type plants were dead or did not fully recover (Fig. 2.4A).
For tomato, the same result was observed in \textit{KST1:ABF4-mGFP} and \textit{35S:ABF4-mGFP} transgenic tomato plants without irrigation in just one day (Fig. 2.5A). This may be because tomato loses nearly twice as much water as tobacco plants per unit leaf area (Fig. 2.7B).

These results suggest that the expression of \textit{ABF4} under the control of guard cell specific promoter \textit{KST1} apparently improved plant drought tolerance. However, the extent to which the low level of expression observed in epidermal cells (Fig. 2.3) contributes to the increase in drought tolerance is unknown.
Figure 2.4. *KST1:ABF4-mGFP* transgenic tobacco plants were more tolerant to water deficit stress than wild-type plants. A, One-month-old wild type (WT) and two T<sub>3</sub> transgenic plants (NK3 and NK27) in greenhouse were withheld from water for 20 days. After 18-day water withdrawal, wild type tobacco plants showed more severe wilting than both transgenic plants. Both wild type and transgenic plants were watered on day 20<sup>th</sup> when severe symptom of water stress developed. Transgenic plants recovered in 5 days after irrigation, in contrast most of wild type tobacco plants died or could not recover (n = ±100). B, Tolerance of two-month old transgenic plants to water deficit stress was observed just in one-day water withdrawal. Unlike Arabidopsis plants with *35S:ABF3* or *35S:ABF4*, no severe growth retardation was observed in *KST1:ABF4-mGFP* transgenic tobacco plants.
Figure 2.5. *KST1/35S:* ABF4-mGFP transgenic tomato segregating T₂ plants were more tolerant to water deficit stress than wild type. A, Wild type and transgenic plants (4-5 plants from each lines) were grown in greenhouse for one month and transgenic plants did not show obvious growth retardation at this stage. These plants were deprived of water for one day when wild type tomato plants exhibited wilting symptoms earlier than transgenic tomato plants (LK21 and LK34 from *KST1:* ABF4-mGFP or LS41 from 35S:* ABF4-mGFP). B, Three-month old 35S:* ABF4-mGFP transgenic plants exhibited severe growth retardation compared to KST1:* ABF4-mGFP transgenic tomato plants. C, T₂ segregating 35S:* ABF4-mGFP and KST1:* ABF4-mGFP transgenic and wild type tomato plants (10 seeds per each line) were directly sowed in pots and treated with repetitive water stress for 1 month by withholding irrigation until they show severe wilting each time. 35S:* ABF4-mGFP lines exhibited significant reduction in growth compared with wild type and KST1:* ABF4-mGFP (LK34) transgenic plants.
Figure 2.6. Daily water loss of KST/35S:ABF4-mGFP transgenic tobacco and tomato plants was much less than daily water loss of wild type plants. A, To measure daily water loss of wild type and two transgenic tobacco plants, each 12 plants from three genotypes (wild type: WT; two transgenic tobacco plants: NK3 and NK27) were grown for two month in greenhouse. Pots with plant were weighed at every 11:00HR, watered immediately after first measurement, and reweighed at 12:00HR to calculate water loss during 23h. Each value was average water loss of 12 plants from wild type tobacco plants (WT), and two KST1:ABF4-mGFP transgenic tobacco plants (NK3 and NK27). Compared to daily water loss of wild type tobacco plants, transgenic plants lost significantly less water. B, To examine daily water loss of wild type tomato plants, two independent segregating T2 lines with KST1:ABF4-mGFP (n ≥ 4 for LK21 and LK34), or one 35S:ABF4-mGFP segregating T2 line (n = 4 for LS41) were grown for two months in greenhouse and examined for transpiration. Plants were measured as the same way as (A) except for weighing them at every 16:00HR and 17:00HR. C and D, Cumulative daily water loss of A and B, respectively. Linear regression equations in graph C were as follows: y = 224x + 5 for WT, y = 179x + 7 for NK3, y = 180x - 7 for NK27. R² in graph D were as follows: R² = 0.98 for WT, 0.98 for LK21, 0.98 for LK34, 0.97 for LS41. Vertical bars denote SD of the mean of numbers of plants indicated.
Figure 2.7. Transpiration per unit leaf area of transgenic plants was significantly lower compared to wild type. Three-month old transgenic or wild type plants from three tobacco genotypes (WT, NK3, and NK27) or 4-5 individual plants from four tomato genotypes were used for these experiments. A and B, Time-course transpiration of transgenic plants per unit leaf area was examined by measuring water loss in pots with plants at every 30min for tobacco plants (A) or at every 2h for tomato plants (B) during the day. As shown in graphs, transpiration was significantly lower in transgenic tobacco and tomato plants, and 35S:ABF4-mGFP transgenic plants (LS41) exhibits lower water loss than KST1:ABF4-mGFP tomato plants (LK21 and LK34). C and D, The amount of transpiration per 100cm² leaf area of wild type and transgenic tobacco lines (C), and tomato plants (D) for one day (=23h) was consistent with the results from time-course transpiration. Vertical bars denote SD of the mean (n = 4 for A; n ≥ 4 for B and D; n ≥ 11 for C). In graph C and D, different letters indicate significant differences within three genotypes (P <0.05) for Tukey pairwise multiple comparison.
2.4.4 *ABF4* transgenic plants conserve water by reducing transpiration

Transgenic tobacco and tomato plants with *KST1:*ABF4-mGFP exhibited more drought tolerance than wild type plants. In order to investigate whether drought tolerance of *KST1:*ABF4-mGFP transgenic plants is correlated with stomatal activity as was found in *Arabidopsis* (Kang et al., 2002), daily water loss was measured by weighing pots with 2-month-old *KST1:*ABF4-mGFP transgenic or wild type plants. Daily water use pattern of *KST1*(35S):ABF4-mGFP transgenic tobacco and tomato plants were significantly lower than their wild type counterparts (Fig. 2.6, A and C).

The RWC of the transgenic tobacco plants was higher than wild type even under well-watered conditions (Fig. 2.8A). Interestingly, RWC of transgenic tomato plants were not different from wild type under non-stress conditions during 12:00 to 14:00HR, but it was higher than wild type during 16:00 to 18:00HR (Fig. 2.9A) when wild type exhibited minor wilting.

Water potential of transgenic tobacco and tomato plants under well-watered conditions was not different (Fig. 2.8B, 2.9B), but it was significantly higher in leaf samples taken from transgenic tomato plants at the end of the day (Fig. 2.9B) when wild type plants exhibited minor wilting symptoms. These results indicate that *KST1:*ABF4-mGFP transgenic tobacco and tomato plants conserve water by reducing transpiration. Although *KST1:*ABF4-mGFP transgenic tobacco plants did reduce transpiration and maintain higher RWC, water use efficiency (WUE) did not differ between wild type and transgenic tobacco plants (data not shown).
Figure 2.8. Measurement of water potential and relative water content in \textit{KST1:ABF4-mGFP} transgenic tobacco plants. A, Water potential of 45-day old \textit{KST1:ABF4-mGFP} transgenic tobacco plants was not different under normal conditions. B, However, relative water content was significantly lower in transgenic tobacco plants than in wild type. Vertical bars denote SD of the mean (n = 3 plants in triplicates for water potential; n = 6 individual plants for relative water content). Different letters indicate significant differences within three genotypes (P < 0.05) for Tukey pairwise multiple comparison.

Figure 2.9. \textit{ABF4} transgenic tomato plants have a higher water potential and relative water content under water deficit stress compared to wild type. To examine whether transgenic tomato plants show different parameters related to water status, water potential (A) and relative water content (B) were measured during 12:00 to 14:00HR (non-stress conditions) and 16:00 to 18:00HR (water stress conditions). As shown, water potential and relative water content of transgenic tomato plants were higher under water stress. Experiments were repeated for five or three times for water potential in non-stress or in water stress conditions, respectively. Relative water content was measured three times for water stress or four times for non-stress conditions at the same time as water potential experiments using similar size of leaf discs. Each value is the mean ± SD (n = 3 plants in triplicates for water potential except for 4 plants from LK34; n = 6 plants for relative water content as two repeats).
2.4.5 Guard Cell Specific Expression of \textit{ABF4} Resulted in Mild Pleiotropic Growth Effects

Although a growth retardation was not observed in early stages of development (Fig. 2.4, A and B; Fig. 2.5A) unlike Arabidopsis with 35S:ABF4 (Kang et al., 2002), \textit{KST1:ABF4-mGFP} transgenic tobacco and tomato plants exhibited growth retardation at later stages of development (Table 2.1 and 2.2; Fig. 4 and 5). The growth parameters in the transgenic tobacco plants were reduced in leaf area by 8%, fresh weight by 9%, dry weight by 16%, and height by 18% compared to wild type tobacco plants (Table 2.1).

Compared to wild type and \textit{KST1:ABF4-mGFP} plants, 35S:ABF4-mGFP tomato plants exhibited significant reduction in growth after repeated water deficit stress for one month (Fig. 2.5C, bottom left).

Recently studies have shown the potential of engineering for stress tolerance in crops using transcription factors, such as \textit{CBF1, CBF3, ABF3} and \textit{ABF4}. Overexpression of these transcription factors significantly improved stress tolerance, but the associated severe growth inhibition is a drawback when especially dealing with crops (Kang et al., 2002; Gilmour et al., 2000; Hsieh et al., 2002; Zhang et al., 2004). Although guard cell specific \textit{ABF4} expression reduced plant growth, the severity of the growth reduction was much less than that observed in Arabidopsis using a constitutive promoter in which \textit{ABF4} was expressed.
Table 2.1. Summary of growth parameters in 3-month-old KSTI:ABF4 transgenic tobacco plants. The means±standard deviations are shown; n=12. Different letters within column denote significant difference within three genotypes (P<0.05) for Tukey pairwise multiple comparison.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Leaf no.</th>
<th>Leaf area (cm²)</th>
<th>Fresh weight (g)</th>
<th>Final dry weight (g)</th>
<th>Height (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>14.3±0.5</td>
<td>3237±158a</td>
<td>166±9.9a</td>
<td>17.4±1.1a</td>
<td>41±1.7a</td>
</tr>
<tr>
<td>NK3</td>
<td>14.2±0.4</td>
<td>2977±114b</td>
<td>151±7.5b</td>
<td>14.7±0.7b</td>
<td>33±1.4b</td>
</tr>
<tr>
<td>NK27</td>
<td>14.2±0.6</td>
<td>3001±144b</td>
<td>150±8.0b</td>
<td>14.5±1.0c</td>
<td>34±2.9b</td>
</tr>
</tbody>
</table>

Table 2.2. Summary of plant growth during 10-day period. The means±standard deviations are shown; n=12. Different letters within column denote significant difference within three genotypes (P<0.05) for Tukey pairwise multiple comparison.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Leaf no. Increase</th>
<th>Leaf area Increase (cm²)</th>
<th>Fresh weight increase (g)</th>
<th>Dry weight increase (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.7±0.5a</td>
<td>1703±158a</td>
<td>56±3.8a</td>
<td>99±9.9a</td>
</tr>
<tr>
<td>NK3</td>
<td>5.3±0.4ab</td>
<td>1515±114b</td>
<td>51±2.7b</td>
<td>84±7.5b</td>
</tr>
<tr>
<td>NK27</td>
<td>5.1±0.6b</td>
<td>1550±144b</td>
<td>50±4.8b</td>
<td>85±8.0b</td>
</tr>
</tbody>
</table>
2.4.6 Stress-Inducible 4XKST1-RD29B Fusion Promoter Was Up-regulated by ABF4 Under Water Deficit Stress

*ABF4* overexpression activates ABA-responsive genes under non-stress conditions (Kang et al., 2002). Therefore, it is of interest whether activation of ABA-responsive gene by ABF4 proteins also occurs in guard cells of tobacco plants. Although various genes that are specifically expressed in guard cells have been identified, it is difficult to find an appropriate promoter that is inducible by ABA or water deficit stress. Therefore, a fusion promoter construct was generated consisting of 4 repeats of an 82 bp fragment of the *KST1* promoter, which contains guard cell specific elements (Plesch et al., 2001), and –344 bp *RD29B* promoter containing 2 copies of ABRE elements (Yamaguchi-Shinozaki and Shinozaki, 1994) as described before. The reporter, mGFP-GUS, was driven by this promoter. GUS expression was higher in guard cells of water-stressed plants containing the vector control (4XKST1:RD29B-mGFP-GUS) compared to counterparts of non-stressed vector control plants (Fig. 2.10B). F1 progeny resulting a cross of homozygous 4XKST1-RD29B:mGFP-GUS and KST1:ABF4-mGFP transgenic tobacco plants were assayed for GUS expression. Heterozygous F1 progeny resulting from a cross between 4XKST1-RD29B:mGFP-GUS plant and wild type tobacco plants were used as a control. Indeed, GUS expression of F1 plants subjected to the water deficit stress showed higher GUS accumulation in guard cells compared to non-stressed F1 plants with ABF4 and control plants (Fig. 2.10C).

A similar result was reported from *ubi1:ABF3* rice plant, in which ABF3 was not sufficient to transactivate *Wis18:GUS* (ABA-inducible promoter linked to GUS reporter gene) compared to the transactivation by exogenous ABA (Oh et al., 2005).
Figure 2.10. Transactivation of 4XKST-RD29B:mGFP-GUS fusion construct by ABF4. A, The constructs used for transactivation test. KST1-ABF4-mGFP was used as effector construct, and 4XKST1-rd29B:mGFP-GUS was reporter construct. B, Transgenic tobacco plants with 4XKST1-rd29B:mGFP-GUS were subjected to well-watered conditions as control or withheld from water for two days to give water deficit stress. GUS accumulation was higher under water stress (right) compared with non-stress conditions (left). T₃ transgenic tobacco plants were crossed with KST1:ABF4-mGFP T₃ transgenic tobacco plants (NK27) to generate heterozygous F₁ plants or wild type tobacco as a vector control. C, The 4XKST1-RD29B:mGFP-GUS fusion was activated by ABF4 in tobacco guard cells only under water deficit stress. One-month-old F₁ plants were withheld from water for a week and examined for GUS accumulation. Consider wild type plants with vector, transgenic plants exhibited higher GUS expression in guard cells. D. GUS expression in wild type tobacco (V, vector control) and F₁ transgenic plants (1 and 2, two lines from crossing 4XKST1-rd29B:mGFP-GUS with KST1:ABF4-mGFP) under dehydration and normal condition.
2.5. DISCUSSION

Numerous studies have been conducted to reveal guard cell signaling pathway related to stomatal closure (Mäser et al., 2003; Fan et al., 2004). In this study, transgenic plants were generated that possess the Arabidopsis \textit{ABF4}/\textit{AREB2} gene (Choi et al., 2000; Uno et al., 2000; Kang et al., 2002) under the control of guard cell specific promoter \textit{KST1} (Fig. 2.1, A and C) (Mueller-Roeber et al., 1995; Plesch et al., 2001). As expected, the reporter mGFP protein accumulated in the nuclei of tobacco guard cells (Fig. 2.3). In contrast to transient expression assays (data not shown), however, weak expression in nuclei of epidermal cells was observed (Fig. 3B and C). Prior to generating \textit{ABF4} transgenic plants, a transient expression assay of \textit{KST1} promoter using tobacco leaf was performed and found that 0.3 kb \textit{KST1} promoter leads the expression of the reporter gene, \textit{GFP} (S65T) (Sheen et al., 1995) in guard cell (data not shown). This indicates that the result from the transient expression systems may be not completely identical with those from the transgenic plants. In addition, even though tobacco and potato are closely related species, gene regulation may be different in the two species.

Transgenic tobacco and tomato plants were used to examine whether \textit{ABF4} expression in guard cells effectively can improve plant drought tolerance. Guard cell specific \textit{ABF4} expression in tobacco plants significantly enhanced plant drought tolerance (Fig. 2.4). In particular, most transgenic tobacco plants recovered from severe water deficit stress, compared to wild type (Fig. 2.4A). Similarly, both 35\textit{S}:\textit{ABF4-mGFP} and \textit{KST1}:\textit{ABF4-mGFP} transgenic tomato plants were more tolerant to water deficit stress (Fig. 2.5A). These results are consistent with those from previous studies, in which both Arabidopsis with 35\textit{S}:\textit{ABF4} or \textit{ABF3} and rice plants with \textit{ubi1}: \textit{ABF3} exhibited
more tolerance to water deficit stress (Kang et al., 2002; Oh et al., 2005). Interestingly, Arabidopsis CBF1 and CBF3, well known as components in cold/drought stress signaling via activating genes containing drought responsive element, DRE/CRT cis-element, also increased drought tolerance including cold tolerance in other plant species, such as tomato and Arabidopsis (Hsieh et al., 2002; Kasuga et al., 2004; Oh et al., 2005). This suggests that ABRE binding transcription factors, such as ABFs/AREBs are probably well conserved in other plant species, and that up-regulation of these genes may increase the tolerance to various stresses as shown in CBF3/DREB1Aa and CBF1 expression in Arabidopsis (Kasuga et al, 1999; Hsieh et al., 2002).

It was demonstrated that guard cell specific ABF4 expression conferred drought tolerance in tobacco and tomato plants. In order to examine whether drought tolerance is due to reduced transpiration, the daily and time course of water loss was observed. Indeed, daily water loss was significantly lower in KST1:ABF4-mGFP transgenic plants (Fig. 2.6). In addition, transpiration rate per unit leaf area supports that reduced water loss is due to lower transpiration of transgenic plants than that of wild type plants in two species (Fig. 2.7). The expression of ABF3 or ABF4 in Arabidopsis (Kang et al., 2002) and ABF3 in rice (Oh et al., 2005) conferred drought tolerance and 35S:ABF3 or 35S:ABF4 showed reduced stomatal aperture (Kang et al., 2002), which suggests that KST1:ABF4-mGFP plants may reduce water transpiration rate via reduction in stomatal aperture.

No obvious growth retardation was observed in early stage in the KST1:ABF4-mGFP plants (Fig. 2.4 and 2.5A), but older plants did exhibit (Table 2.1 and 2.2; Fig. 2.5, B and C). However, compared to transgenic Arabidopsis plants with 35S:ABF3 or 35S:ABF4 (Kang et al., 2002; Kim et al., 2004) the growth retardation of the
KST1:ABF4-mGFP transgenic plants was much less severe (Fig. 2.4B). Similar results were reported from rice plants with ubi1:ABF3 (Oh et al., 2005). Surprisingly, RD29A:DREB1A/CBF3 Arabidopsis (Pei et al., 1999; Kasuga et al., 1999) or ubi1:CBF3 rice plants like ubi1:ABF3 (Oh et al., 2005) also showed much less or no obvious dwarf phenotype, compared to Arabidopsis with 35S: CBF3 or tomato with 35S:CBF1 (Kasuga et al., 1999; Gilmour et al., 2000; Hsieh et al., 2002) including Arabidopsis with 35S:ABF3 (Kang et al., 2002). These phenotypes are believed to be due to the constitutive expression of stress-inducible genes (Kasuga et al., 1999; Kang et al., 2002). In tomato plants with 35S:CBF1, plant height was significantly reduced due to shorter internodes, and this was recovered by GA₃ application (Hsieh et al., 2002). This is similar to KST:ABF4-mGFP transgenic plants since although height was shorter in transgenic plant, leaf number was not different from WT.

Recent studies have revealed that ABF or AREB transcription factors activate ABRE elements (Choi et al., 2000; Uno et al., 2000; Narusaka et al., 2003; Oh et al., 2005). In order to test whether KST1:ABF4/AREB2 also can activate ABRE elements in tobacco plants, transgenic tobacco plants were generated containing a fusion construct consisting of a tetramer of 82bp KST1, -236 to -154 region of KST1 promoter which contains guard cell specific elements (Plesch et al., 2001), and the 344bp RD29B promoter, -344 to -1 region of RD29B promoter containing ABRE elements (Yamaguchi-Shinozaki and Shinozaki, 1994). F₁ tobacco plants were obtained from crossing 4XKST1-RD29B:mGFP-GUS transgenic plants with KST1:ABF4-mGFP transgenic or wild type plants, and used for this experiment. Since under non-stress conditions ABF3 or ABF4 expression elevated ABA-responsive genes, such as RD29B and RAB18 in Arabidopsis...
with 35SABF3 or 35SABF4 (Kang et al., 2002), similar up-regulation of GUS expression was expected in F₁ plants. Interestingly, GUS accumulation in both F₁ plants was weak or undetectable without water deficit stress, but was higher in plants having ABF4 than F₁ plants with reporter alone after water deficit stress (Fig. 2.10C, right). A similar result was reported recently from ubi:ABF3 trangenic rice and 35S/RD29A: DREB1A transgenic tobacco plants, in which the expression of wis18 or NtERD10A-D, drought/ABA inducible genes, was much stronger in transgenic plants after water stress compared to the expression without water deficit stress (Kasuga et al., 2004; Oh et al., 2005). Uno et al. (2000) suggested that phosphorylation may be required for maximum ABF4 function in activating ABA-responsive genes such as RD29B. Although transgenic tobacco with Arabidopsis ABF4 exhibited increased drought tolerance, maximum function of ABF4 may be still required for water deficit stress or possibly phosphorylation as shown in rice TRAB1 (Kagaya et al., 2002). As ABRE binding proteins, TRAB1 and ABF2 were properly activated via phosphorylation or interaction with additional proteins such as ARIA and VP1, respectively (Hobo et al., 1999; Kim et al., 2004). Therefore, it also may be that the ABF4 alone is insufficient for the activation of ABA-responsive genes. Another explanation is that a heterologous promoter consisting of a guard cell-specific element (4XKST1) and RD29B may be not a combination that results in strong gene expression in guard cells under drought stress. Consistent with this is the observation that the combination of DRE and ABRE elements is critical for ABA-responsive gene expression in Arabidopsis (Narusaka et al., 2003).
CHAPTER 3
POSSIBLE INVOLVEMENT OF PUTATIVE TOMATO INOSITOL
POLYPHOSPHATASE, Le5PT1, IN SEEDLING DEVELOPMENT
AND STRESS RESPONSE

3.1 ABSTRACT

Plants respond to environmental stresses through signaling cascades that lead to change in the expression of stress-responsive genes, which are important for adaptation and survival under adverse conditions. As one of phosphoinositides, inositol 1,4,5 triphosphate (IP₃) is known to transduce stress signal by changing its level in response to water deficit, osmotic stress, and low temperature in plants. Recently, it is shown that type I inositol 5 phosphatases (5PTases), At5PTase1 and AtIP5PII/At5PTase2, regulate IP₃ level in plant like in animal and that up-regulation of these genes decreases IP₃ level including the expression of ABA-/drought-responsive genes in Arabidopsis plants. On the basis of sequence similarity to Arabidopsis 5PTases, four tomato cDNAs (Le5PT1-4) that encode putative 5PTase proteins were identified. The predicted protein sequences of the Le5PTs had conserved catalytic domains required for 5PTase enzyme activity. Two clones, Le5PT1 and Le5PT2 were similar to AtIP5PII/At5PTase2 and At5PTase1, respectively. The expression of Le5PT1 was down-regulated early during a period of dehydration, NaCl, and exogenous ABA treatment, raising that Le5PT1 may have negative role in response to water deficit stress. Transgenic tobacco plants with
35S:Le5PT1-mGFP did exhibit weak expression of a drought-inducible gene, NtERD10B, which were not correlation with resistance to water deficit stress. Transgenic tobacco and tomato plants, however, exhibited retarded growth, suggesting that Le5PT1 may have a role to plant development.

3.2 INTRODUCTION

Under natural conditions plants encounter various adverse stresses including water deficit, salt stress, and cold stress. In response to these stresses, plants initiate signaling cascades that result in the development of defense response. Recent research has shown phosphoinositides (PIs) play an important role in development including stress signaling. PIs include membrane-bound phosphatidylinositol and its derivatives in which the inositol hydroxyl group can be phosphorylated or dephosphorylated. PIs are involved in various mechanisms in plant, such as membrane traffic (Kim et al., 2001), actin cytoskeleton organization (Zhong et al., 2004; Zhong et al., 2005), several stresses (Meijer et al., 2001; Xiong et al., 2001) or growth (Williams et al., 2005), or vascular patterning (Carland and Nelson, 2004). As phosphoinositides are involved in various signaling and development, many kinases and PI phosphatases are involved in generating (Jung et al., 2002; Park et al., 2003; Joo et al., 2005; Stevenson-Paulik et al., 2003; Perera et al., 2005) or degrading PIs in plants (Berdy et al. 2001; Sanchez and Chua, 2001; Burnette et al., 2003; Ercetin and Gillaspy, 2004; Carland and Nelson 2004; Zhong et al., 2004; Zhong and Ye 2004).

Inositol 1,4,5 triphosphate (IP₃) is generated via hydrolysis of phosphatidylinositol (4, 5) bisphosphate (PIP₂) by phosphatidylinositol-specific phospholipase C (PI-PLC) in plant (reviewed in Mueller-Roeber and Pical, 2002). As Arabidopsis phospholipase C,
AtPLC1, is up-regulated by environmental stresses (Hirayama et al., 1995), the level of IP3 increases in response to water deficit stress, osmotic stress, or cold stress (Takahashi et al., 2001; DeWald et al., 2001; Sanchez and Chua, 2001; Ruelland et al., 2002; Xiong et al., 2002). IP3 is involved in Ca^{2+} signaling, which has various roles in plant stress as well as development. IP3 injection into guard cell elevated Ca^{2+} (Blatt et al., 1990; Gilroy et al., 1990; Schroeder et al., 2001b). Since elevated Ca^{2+} mediates ABA-induced stomatal closure, it is possible that altered levels of IP3 might affect drought resistance in plants (Schroeder et al., 2001b). In addition, there are reports that IP3 is involved in plant growth, such as gravitropic growth and pollen growth. IP3 levels rapidly changed in response to gravistimulation in maize pulvini (Perera et al., 1999), which is followed by gravitropical growth. Pollen tube growth was also observed after the release of caged IP3 in Agapanthus umbellatus (Monteiro et al., 2005).

The level of IP3 is known to be regulated by dephosphorylation by type I 5PTases in animal (Majerus et al., 1999), but in plant it can be hydrolyzed by both 5PTases (Berdy et al., 2001; Sanchez and Chua, 2001) as well as 1PTase (Xiong et al., 2001). Therefore, both 1PTase and 5PTases deactivate IP3 by removing the 1' phosphate (Xiong et al., 2001) or the 5' phosphate (Berdy et al., 2001; Sanchez and Chua, 2001) to terminate IP3-related signaling. However, most identified inositol polyphosphatases belong to 5PTases (Berdy et al. 2001; Sanchez and Chua, 2001; Burnette et al., 2003; Ercetin and Gillaspy, 2004; Carland and Nelson 2004; Zhong et al., 2004; Zhong and Ye 2004) and only one 1PTase have been identified in plants (Xiong et al., 2001).

To date, at least four putative type I 5PTases have been identified in Arabidopsis (Berdy et al., 2001; Sanchez and Chua, 2001; Ruiqin Zhong and Ye, 2004). At5PTase1
and AtIP5PII/At5PTase2 (Berdy et al., 2001; Sanchez and Chua, 2001) are probably counterparts of human type I 5PTases with substrate specificity toward soluble phosphoinositides (Majerus et al., 1999). IP3 level decreased in Arabidopsis plants overexpressing At5PTase 1 or AtIP5PII/At5PTase 2 (Berdy et al., 2001; Sanchez and Chua, 2001). In transgenic Arabidopsis plants transformed with 35S:At5PTase 1 or 6XUAS-46:AtIP5PII/At5PTase 2, the induction of ABA/drought-responsive genes by exogenous ABA was lower than counterparts of wild type plants (Berdy et al., 2001; Sanchez and Chua, 2001). In addition, seed germination of AtIP5PII/At5PTase 2 transgenic Arabidopsis was not sensitive to ABA in germination assays, but ABA sensitivity was unaffected in antisense transgenic plants (Sanchez and Chua, 2001). Based on these findings, at least type I 5PTases affected ABA response via changing IP3 levels, indicating a possible connection to plant stress tolerance. However, there is a little evidence so far whether any of plant 5PTases directly affects water deficit resistance in physiological level. In addition, there are not many 5PTases that have been studied, and no 5PTases has been identified from other plant species except for Arabidopsis.

The objective of this study was to test whether the altered expression of 5PTase could affect plant drought tolerance via changing IP3 level. To test this hypothesis, four putative 5PTase homologs were identified from tomato plants on the basis of the similarity of amino acid sequences to Arabidopsis 5PTases. Prior to introducing into plants, their expression under water deficit stress was determined. Only one of them, Le5PT1, was down-regulated by dehydration, suggesting that Le5PT1 might be a good candidate to test this hypothesis. In this respect, Le5PT1 was used to introduced into tomato and tobacco plants for further characterization.
3.3 MATERIALS AND METHODS

3.3.1 Growth conditions

Seeds of tobacco and tomato (Nicotiana tabacum cv. Wisconsin 38, Lycopersicon esculentum cv. Ohio 8245, respectively) were surface-sterilized for 10 minutes using 50% Clorox, rinsed with sterile water four times, and germinated in a Magenta™ box (Sigma) containing Murashige and Skoog (MS) medium (Sigma, St. Louis, MO, USA) supplemented with 2% sucrose, adjusted to pH 5.8, and solidified with 0.7% agar (Sigma). For gene expression or transformation, seedlings were grown for 3 to 4 weeks under a continuous fluorescent illumination of 50 μmol m−2 sec−1 at 23±3°C. For whole plant physiological experiments, 2 to 3 week-old seedlings were transferred into soilless media (Metro-Mix 360, Scotts-Sierra, Marysvilles, OH) and grown for 2 month in the greenhouse at 25±3°C. Germination kinetics were investigated by measuring the time of radicle emergence on MS medium containing NaCl, ABA, or polyethylene glycol.

3.3.2 Gene Cloning and Sequence Analysis

Tomato EST clones were identified from a key-word search at tomato EST database provided by TIGR database or from BLAST search using At5PTase1 and At5PTase2 protein sequence (Berdy et. al., 2001; Sanchez and Chua, 2001) in the TIGR tomato database. Homologous ESTs were obtained from Clemson University Genomics Institute (Clemson, SC, USA) and confirmed by sequence analysis using Applied Biosystems 3730 DNA Analyzer at Ohio State University Plant-Microbe Genomic facility. Sequence information was used to clone tomato inositol poly-5-phosphatases. In order to get full-length cDNAs 5’ RACE had been performed using Smart RACE cDNA amplification kit (BD Biosciences Clontech, CA). Finally four putative tomato inositol poly-5-
phosphatases were identified and named Le5PT1 to Le5PT4.

3.3.3 5' RACE and Cloning of full-length potential 5 Phosphatase cDNA from tomato

The 5' RACE PCR was performed with total RNA extracted from one-month old tomato leaves using the RNeasy plant kit (Qiagen, CA) and following the protocol supplied with the Smart RACE cDNA amplification kit (BD Biosciences Clontech, CA). 5' RACE products were produced using four gene specific primers (Le5PT1: 5’-GCAAGAAGATTGTCTACGGTACCAAA-3’; Le5PT2: 5’-AGATCAAAGCATTCTTTCTCTATATC-3’; Le5PT3: 5’-AAAGTCAGCATTCCACTCGGCAATT-3’; Le5PT4: 5’-ATTCCCCTCGCTAAATTGTCTTCCCAA-3’), cloned into pGEM®-T Easy Vector (Promega, Madison, WI), and sequenced. The coding regions of these four genes were amplified by RT-PCR (Takara, Madison, WI) using four gene specific primer sets (Le5PT1 Forward: 5’-CCGCTCGAGGTCGACATGCAAATGAAAACAAGAAGG-3’, Reverse: 5’-GGACTAGTCCATGGACATGAGAGAGCTCAAGGCAAC-3’, Le5PT2 Forward: 5’-CGGGGTACCGTCGACATGAGAGAGCTCAAGGCAAC-3’, Reverse: 5’-TGCTCTAGACCATGGACGGAGCATGAGGCAAC-3’, Le5PT3 Forward: 5’-CCGCTCGAGGTCGACATGAGGGATTCTTCGATGAGGAG-3’, Reverse: 5’-CGCGGATCCGCTAGCCCATGGAAAAGAAGCATAGTTCGGTATAAC-3’, Le5PT4 Forward: 5’-CCGCTCGAGCCTAGGTCATGAGGGAATTTCGGAGGGGACTG-3’).

The Sal1/Nco1 fragment of Le5PT1 was ligated into Sal1/Nco1 sites of pUC18 vector,
and Le5PT2-4 cDNA fragments were cloned into pGEM®-T Easy Vector.

3.3.4 Stress treatments

Various stress treatments were imposed on 2-week old tomato seedlings that were germinated on filter paper in Magenta boxes containing liquid Murashige and Skoog (MS) medium (Sigma) supplemented with 1% sucrose, and adjusted to pH 5.8. The seedlings were rinsed with distilled water, replaced with fresh liquid MS medium without sucrose. After one day the MS medium was replaced with MS medium containing 20% polyethylene glycol (MW 8,000; Sigma), 50\(\mu\)M ABA, 250mM NaCl, or 2% sucrose. For cold and dark treatments, Magenta™ boxes containing the seedlings were placed at 4°C refrigerator or at room temperature without light to determine that light affect the expression of the Le5PTs. Plant samples were harvested at varying times after stress treatments and frozen in liquid nitrogen and stored at –70 °C for further analysis.

3.3.5 Analysis of Le5PTs expression patterns

Total RNA was isolated from tomato and tobacco plants using Rneasy Plant Mini kit (Qiagen, CA, USA). The RNA (10 \(\mu\)g) was electrophoresed in a 1.2% MOPS/EDTA agarose gel and transferred to a nylon filter. \(P_\text{32}\)-labeled probes were prepared from plasmid containing cDNA for Le5PT1 and Le5PT2 by PCR using the primer sets (Le5PT1: Forward 5’-AGATCATCTCAGGCCTTTTGG-3’,
Reverse 5’-CATACAAAGCCACTCATCAA-3’; Le5PT2:
Forward 5’-TTACAGTGCTGACCCTGAC-3’,
Reverse 5’- TTTCGCGAATGATGTCCTC-3’). Gene expression of Le5PT3 and Le5PT4 were analyzed by RT-PCR using One-step RNA PCR kit (Takara, Medison, WI, USA) with the primer sets (Le5PT3: Forward 5’-
ATCAACCAGGTGTATGGAG-3’, Reverse 5’-
CGACGATGAGAGAAGTATG-3’; Le5PT4: Forward
5’-TGGAGCAGAAGATCCAACAG-3’, Reverse 5’-ATGGACATGCTCACAGAGAC-
3’).

3.3.6 GFP fusion and Plant Transformation

The 2.2kb HindIII/Nco1 fragment (CaMV 35S:Le5PT1) from pUC18 vector was
ligated into the same sites in pCambia 1302 binary vector to produce CaMV 35S:Le5PT1-
mGFP. The resulting plasmid pCambia 1302, which contains the Le5PT1-mGFP coding
sequence under the control of the CaMV 35S promoter, was transformed into
Agrobacterium tumefaciens strain GV3101 by electroporation. Subsequently this plasmid
was transferred into tobacco and tomato plants by Agrobacterium-mediated
transformation. Three- to four-week-old tobacco leaf or tomato cotyledon explants were
pre-cultured upside down in MS medium supplemented with 2% sucrose at pH 5.8 for 2
days. Explants were inoculated by immersence in an overnight culture of A. tumefaciens
GV3101 for 10 min and dried by blotting on sterile filter paper. After 2 days, explants
were washed thoroughly using sterile distilled H2O containing 300 mg/L cefotaxim. For
tobacco plants, explants were regenerated as described by Horsch et al. (1988), and
tomato explants were regenerated by method described as Park et al. (2003).
Transformants were selected on MS medium containing 50 mgL\(^{-1}\) hygromycin B, and
transferred to soil, and grown in a greenhouse with a 16-h photoperiod at 25±3°C. Two
35S:Le5PT1-mGFP transgenic tobacco lines were selected for further study.
3.3.7 Water usage

Two-month old tobacco plants were used to measure daily transpiration rate and water use efficiency. On the initiation date of experiment, 6 plants from each of wild type and two transgenic lines were harvested to measure leaf area, fresh and dry weight, plant height, and leaf number, and these data were used as reference. Daily evapotranspiration of 12 plants from both 2 transgenic lines and wild type tobacco plants was recorded by measuring pot weight using a top balance between at 11:00HR and 12:30HR each day for 10 days. The plants were irrigated each day at 11:30HR. Daily evapotranspiration was determined by calculating the difference in the weight of the pot at 12:30HR and the weight at 11:00HR the following day. Transpiration was deduced by subtracting the average amount of evaporation for the same period from three 2 L pots that contained only soilless media. After 10 days plants were harvested as described for the reference plants.

3.3.8 Measurement of water potential and relative water content

Two 35S:Le5PT1-mGFP transgenic (NS11 and NS30) and wild type tobacco plants were grown in 2 L pots in greenhouse for 7 weeks. After that, each 3 plants from three genotypes were used to measure water potential using WP4-T Dewpoint Potentiometer (Decagon, Pullman, WA, USA). First, leaf water potential was measured using the same size of leaf tissues collected from young leaves at similar height, and leaf tissues were placed in a –70°C freezer. After one day, leaf tissues were used to measure leaf solute potential. Turgor pressure was calculated by subtracting solute potential from leaf water potential. To determine relative water content (RWC) during non-water stress conditions, leaves of similar size were harvested at 13:00HR and used to measure fresh weight (FW),
turgid weight (TW) after hydration for one day, and dry weight (DW). RWC was calculated using following equation:

\[
\text{RWC} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}}
\]

### 3.3.9 Protein extraction and Western analysis

Total protein was obtained from one-month old leaf tissues (approximately 100 mg) of wild type or transgenic tobacco plants. To extract protein, 500\(\mu\)L of SDS extraction buffer (0.125 M tris-HCl, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8) was added and ground using a mortar and pestle. The slurry was transferred into a 1.5mL Eppendorf tube and centrifuged for 3 to 4 min at 10,000 rpm at 4°C. The supernatant was transferred to new tube and mixed with 100% acetone. After 30 min incubation at 4°C, the samples were centrifuged for 3 min at 10,000 rpm at 4°C and the pellet dissolved in SDS sample buffer. Then, the equal amount of samples was separated on 8% SDS-PAGE gel. After electrophoresis, SDS-PAGE gel was stained by coomassie blue staining buffer, and protein levels were determined based on intensity. Protein was transferred to poly(vinylidene difluoride) membrane (Bio-Rad, Hercules, CA, USA) by standard techniques (Sambrook et al., 1993). The membrane was blocked in 1X TBS (10 mM Tris HCl, pH 8.0/150 mM NaCl) containing 1% (wt/vol) nonfat dry milk at room temperature for 1 h, incubated with anti-GFP rabbit polyclonal antibody (Molecular Probes, Eugene, OR, USA), and developed using peroxidase-conjugated secondary antibody (dilution ratio of 1:5,000) by enhanced chemiluminescence (Amersham Pharmacia).
3.4 RESULTS

3.4.1 Cloning of Tomato 5PTase Genes

In this study, potential tomato 5PTase homologs were identified from searching tomato expressed sequence tags (ESTs) in TIGR databases. This search resulted in four potential 5PTase homologs: Le5PT1 from EST clone, cLED2N23; Le5PT2, cTOD18D18; Le5PT3, cTOC18B10; Le5PT4, cLEX10B15. These EST sequences were similar to protein sequences of known Arabidopsis At5PTase1 and At5PTase2 (Berdy et. al., 2001; Sanchez and Chua, 2001). cDNA sequences were used to clone full-length tomato 5PTase genes. On the basis of sequence information, gene specific primers were designed and used to obtain four tomato 5PTases that were named Le5PT1 – 4. Sequence alignment of these predicted protein sequences revealed the higher degree of similarity with Arabidopsis 5PTase1 and 2 in the conserved catalytic domains (Fig. 3.1A) (Berdy et. al., 2001; Carland and Nelson 2004; Zhong et al., 2004). Using the blockmaker program, seven conserved regions were found in four putative tomato 5PTases Le5PT1-4, and the organization of these motifs are very similar to At5PTase1 and 2 (Fig. 3.1B). Sequence alignment and phylogenetic analysis showed that Le5PT1 is 54% identical to At5PTase2 in overall amino acid sequences and that Le5PT2 is 54% identical to At5PTase1 (Fig. 3.2). In previous studies both At5PTase1 and At5PTase2 showed strong substrate specificity to inositol 1,4,5 triphosphate (IP₃) (Berdy et. al., 2001; Sanchez and Chua, 2001), indicating that tomato Le5PT1 and Le5PT2 may have the same substrate affinity to IP₃.
Figure 3.1. Characteristics of putative tomato 5PTases, Le5PT1-4. A. Alignment of catalytic domains with known Arabidopsis 5PTases. Two conserved catalytic domains were found in all identified Le5PT1-4. B. Three Arabidopsis 5PTases and tomato 5PTases were used to generate blocks. Seven highly conserved regions were found by block maker software in Blocks WWW Server (http://blocks.fhcrc.org/make_blocks.html). C, Sequence alignment of Le5PTs amino acid sequences and Arabidopsis At5PTase1, At5PTase2, and CVP2.
Figure 3.2. Amino acid sequences of fourteen Arabidopsis 5PTases with accession ID and four tomato Le5PT1-4 were aligned to produce unrooted phylogenetic tree with ClustalW (http://clustalw.genome.jp/). Phylogenetic analysis showed similarity between identified tomato 5PTases and Arabidopsis 5PTases, Le5PT1 and AtPTase 2, Le5PT2 and AtPTase 1, and Le5PT3 and CVP2, respectively.
3.4.2 Analysis of *Le5PTs* Expression

To investigate the spatial and temporal expression patterns of *Le5PT1-4* in tomato, Northern blot and RT-PCR analysis were carried out using total RNAs from various tissues (Fig. 3.3B) and stress treatments (Fig. 3.3A). *Le5PT1* expression was reduced by dehydration and 20% polyethylene glycol (PEG), indicating that *Le5PT1* might be involved in response to water deficit stress. In addition, the expression of *Le5PT1* also was slightly down-regulated by 50\(\mu\)M ABA and 200mM NaCl treatments at early time points, but it fluctuated 3h after incubation of the treatments. In contrast, *Le5PT2* and *Le5PT3* expression increased along with time after dehydration, PEG, NaCl, ABA treatments (Fig. 3.3A). Interestingly, overall expression patterns of *Le5PT2* and *Le5PT3* showed similar patterns by stress treatments, indicating that these two genes may have similar roles. It is also possible that both genes are required for the response to stress.

Unlike other treatments, the expression of *Le5PT1* was strongly up-regulated 1h after cold treatment at 4\(^\circ\)C (Fig. 3.3A), and decreased in 24h. Since cold stress was carried out without light, the level of *Le5PT1* transcripts at 1h after by cold stress (4\(^\circ\)C) or dark treatment at room temperature was compared. Although the transcripts of *Le5PT1* also increased by dark treatment, it was lower than cold treatment, indicating that the expression of *Le5PT1* is likely responsive to cold stress as well as by light.

To examine *Le5PTs* expression patterns in different tissues, RT-PCR analysis was performed using total RNAs of old and young leaf, stem, and flower from 3-month-old tomato plants in the greenhouse. The expression patterns of the four putative tomato 5PTases were very different (Fig. 3.3B), suggesting that these genes are differentially regulated throughout development.
Figure 3.3. Expression of tomato Le5PTs in different tissues or in response to various stress treatments. A. One-month in-vitro grown tomato seedlings were dehydrated, or incubated in liquid MS media with ABA (50μM), NaCl (200mM), PEG (20%), sucrose (2%), in cold (4°C) without light or in the dark at RT. RNAs were isolated in 0.5, 1, 3, and 24h after treatments and used for Northern blot or RT-PCR analysis. B. Expression patterns of Le5PTs in different tissues. RT-PCR analysis was performed using RNAs extracted from old leaf (O), young leaf (Y), stem (S), and flower (F) from 3-month old plants grown in greenhouse.
3.4.3 Ectopic Expression of *Le5PT1* in Tobacco and Tomato

The expression of tomato *Le5PT1* was down-regulated by water deficit stress (Fig. 3.1A), suggesting that *Le5PT1* may have a role in response to water stress. To examine the physiological role of *Le5PT1*, transgenic plants with 35S:*Le5PT1*-mGFP construct (Fig. 3.4A) were examined for the expression of transgene by RT-PCR (Fig. 3.4. B, D). Two transgenic tobacco lines (NS11 and NS 30) that exhibited higher expression of *Le5PT1*-mGFP in RT-PCR analysis were selected for further study (Fig. 3.4B). Western blot analysis showed NS30 line had higher expression of fusion proteins (Fig. 3.4B). GFP expression was not observed, however, probably because the fusion protein is unstable or associated with substrates that impair GFP detection.
Figure 3.4. Generation of 35S:Le5PT1-mGFP transgenic plants. A, Constructs used for tobacco and tomato transformation. Tomato Le5PT1 gene was placed between strong promoter tobacco mosaic virus CaMV 35S promoter and mGFP in pCambia 1304 binary vector. B, Screening of 35S:Le5PT1-mGFP transgenic tobacco (underlined) by RT-PCR. C, The transcript levels of Le5PT1 in 1-month-old wild type (WT) and two homozygous transgenic tobacco lines (NS11, NS30) was examined by RT-PCR. D, Expression level in four T2 segregating transgenic tomato (LS5-6, 8-1-1, 9-1, and 55-5) and wild type plants were analyzed by RT-PCR.
3.4.4 Transgenic Tobacco Plants Carrying 35S:Le5PT1-mGFP Does Not Show ABA Insensitivity in Seed germination

In addition to promoting seed dormancy in plants, ABA inhibits seed germination. Various ABA-insensitive mutants, however, are insensitive to exogenous ABA. Arabidopsis plants overexpressing At5PTase2 exhibited insensitivity to exogenous ABA (Sanchez and Chua, 2000). In contrast, Arabidopsis plants with antisense At5PTase2 (Sanchez and Chua, 2001) or a 5PTase mutant, cvp2 (Carland and Nelson, 2004), exhibited hypersensitivity to exogenous ABA in seed germination assays. To examine whether 35S:Le5PT1-mGFP transgenic tobacco plants display altered seed germination, germination assays were performed in presence of 0.5 and 1μM ABA, or in presence of 50 and 75mM NaCl since fry1 (inositol 1PTase), a mutant with elevated IP3 levels, exhibited hypersensitivity to salt stress (Xiong et al., 2001). Although Le5PT1 is similar in predicted amino acid sequences to At5PTase2, the germination of seeds from 35S:Le5PT1-mGFP transgenic lines was not different from wild type tobacco plants in presence of 0.5 μM ABA (Fig. 3.5). One of the transgenic lines, NS11, exhibited slightly lower germination frequency than wild type tobacco seeds in 0.75 μM ABA, and interestingly, this is similar to the seed germination kinetics observed in 35S:At5PTase1 Arabidopsis plants (Burnette et al., 2003) rather than Arabidopsis plants with sense At5Ptase2 (Sanchez and Chua, 2001). In addition, there was no difference in germination kinetics in presence of NaCl compared to wild type.
Figure 3.5. Germination analysis of 35S:Le5PT1-mGFP transgenic tobacco plants. Seeds from WT and two transgenic tobacco plants were assayed for germination in MS media or containing sucrose, ABA, or NaCl as shown. Germination frequencies did not show statistical difference in all treatments. Each data point represents the mean ± SD values of triplicate experiments (n = 50 each).
3.4.5 Transgenic Tobacco with Le5PT1 Showed Retarded Growth

Although the germination kinetics of seeds from transgenic tobacco plants over-expressing Le5PT1 were not altered, seedling growth was slower than wild type. Cotyledon, hypocotyls, and root length were measured from 10-day-old seedlings grown in MS medium alone or containing 2% sucrose, 50mM, or 75mM NaCl. Cotyledon length of transgenic plants was slightly inhibited by NaCl treatment compared to wild type tobacco plants. However, hypocotyls and root growth were not much affected (Fig. 3.6, B-D). In older plant, growth retardation was observed in the transgenic plants for all measured growth parameters (Fig. 3.8; Table 3.1 and 3.2;).

<table>
<thead>
<tr>
<th>Lines</th>
<th>Leaf no.</th>
<th>Leaf area (cm²)</th>
<th>Final fresh weight (g)</th>
<th>Final dry weight (g)</th>
<th>WUE¹</th>
<th>WU²</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>14.3±0.5a</td>
<td>3237±159a</td>
<td>166±9.9a</td>
<td>17.4±1.1a</td>
<td>5.3±0.4a</td>
<td>2246±125a</td>
<td>41±1.7a</td>
</tr>
<tr>
<td>NS11</td>
<td>14.2±0.6a</td>
<td>3114±180a</td>
<td>156±10.5b</td>
<td>15.8±0.7b</td>
<td>5.0±0.2ab</td>
<td>2019±118b</td>
<td>35±1.8b</td>
</tr>
<tr>
<td>NS30</td>
<td>16.5±0.5b</td>
<td>2952±166b</td>
<td>135±10.3c</td>
<td>14.3±0.9c</td>
<td>4.8±0.2b</td>
<td>2028±116b</td>
<td>32±2.0c</td>
</tr>
</tbody>
</table>

Table 3.1. Summary of growth parameters in 2-month-old 35S:Le5PT1-mGFP transgenic tobacco plants. The means±standard deviations are shown; n=12. Different letters within column denote significant difference within three genotypes (P<0.05) for Tukey pairwise multiple comparison.

1 Water use efficiency: Dry weight (g)/Water loss (kg).
2 Water usage during 10-day period.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Leaf no.</th>
<th>Leaf area (cm²)</th>
<th>Fresh weight increase (g)</th>
<th>Dry weight increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Leaf</td>
<td>Stem</td>
</tr>
<tr>
<td>WT</td>
<td>5.7±0.5a</td>
<td>1703±159</td>
<td>43±3.8a</td>
<td>99±9.9a</td>
</tr>
<tr>
<td>NS11</td>
<td>5.5±0.6a</td>
<td>1574±180</td>
<td>35±3.5b</td>
<td>86±10.5b</td>
</tr>
<tr>
<td>NS30</td>
<td>7.2±0.5b</td>
<td>1669±166</td>
<td>32±4.4b</td>
<td>81±10.3b</td>
</tr>
</tbody>
</table>

Table 3.2. The plant growth during 10-day test period. The means±standard deviations are shown; n=12. Different letters within column denote significant difference within three genotypes (P<0.05) for Tukey pairwise multiple comparison.
Figure 3.6. *Le5PT1* transgenic tobacco plants exhibited growth retardation. A, Two-week-old *Le5PT1* transgenic tobacco seedlings grown in MS medium or 50mM NaCl. B, Cotyledon length. C, Hypocotyl length. D, Root length. Each 15 seedlings were used to measure cotyledon length, hypocotyl length, and root length. Vertical bars denote SD of the mean (n = 15). Different letters indicate significant differences within three genotypes (P <0.05) for Student-Newman-Keuls pairwise multiple comparison.
3.4.6 *Le5PT1* expression may not have a role in plant water stress

Recent evidence has shown that inositol 1,4,5 triphosphate (IP₃) may have a positive role in ABA-mediated stomatal closure (Schroeder et al., 2001b). Since it is known that IP₃ levels are regulated by type I 5PTases (Berdy et al., 2001, Sanchez and Chua, 2001), whether the altered expression tomato *Le5PT1* in tobacco has any effects on plant water status was examined. Although daily whole plant water usage, transpiration per unit leaf area, and water potential were not different between wild type and the two transgenic lines (Fig. 3.7A-C), water use efficiency was lower in transgenic plants (Table 3.1), indicating that to an extent the expression of Le5PT1 affect plant water status.
Figure 3.7. The expression of *Le5PT1* did not show any change related to water deficit stress in transgenic tobacco plants. A, Daily water loss was measured twice a day for 10 days by measuring pot weight with *Le5PT1* transgenic tobacco or wild type plants. In addition, pots only with soil were weighed to adjust water evaporation. B, Cumulative water loss. C, Transpiration per 100 cm² leaf area was calculated by dividing water loss per day by total leaf area. D, *Le5PT1* transgenic plants did not show significant difference in water potential, but both transgenic lines showed altered solute potential. Vertical bars denote SD of the mean (n = 12 for A-C; n = 3 for D).
Figure 3.8. Growth retardation was observed during overall growth period in Le5PT1 transgenic tobacco plants showed growth retardation in later stage. A, Picture of three-month-old plants grown in greenhouse. B, Leaf area. C, Fresh weight. D, Dry weight. In graph B-D, vertical bars denote SD of the mean (n = 12). Different letters indicate significant differences within three genotypes (P <0.05) for Tukey pairwise multiple comparison.

Figure 3.9. Drought inducible NtERD10B was down-regulated in Le5PT1 transgenic tobacco plants. The expression of three LEA group 2 gene, NtERD10B-D, was analyzed by RT-PCR using RNAs extracted from transgenic plants treated with ABA (50μM) or PEG (20%) for a day.
3.4.7 The expression of LEA gene was down-regulated in 35S:Le5PT1-mGFP transgenic tobacco plants

LEA group 2 proteins are known as dehydrins, and are suggested to minimize protein degradation that occurs when plants are subjected to water deficit stress (Goyal, et al., 2005). In tobacco, 4 LEA group 2 genes have been identified (Rizhsky et al., 2002; Kasuga et al., 2004), and they are up-regulated by water deficit stress (Kasuga et al., 2004). Therefore, if Le5PT1 has a role in the response to water deficit stress, it is possible that it may affect the expression of LEA genes. To test the effect of the overexpression of Le5PT1 on the regulation of three LEA genes (NtERD10A, C, and D), total RNAs were isolated from two independent transgenic lines (NS11 and NS30) and wild type tobacco plants treated with MS medium alone or with 50 μM ABA or 20 % PEG for 1 day at room temperature under continuous light. The expression was analyzed by RT-PCR. The level of NtERD10B transcripts in transgenic line, NS30, treated with 20% PEG was significantly lower than wild type plants, but there was no difference in plants treated with ABA (Fig. 3.9), indicating that Le5PT1 may be involved in drought response in ABA independent manner.

3.5. DISCUSSION

To date, several 5PTases have been characterized only in Arabidopsis (Berdy et al. 2001; Sanchez and Chua, 2001; Burnette et al., 2003; Ercetin and Gillaspy, 2004; Carland and Nelson 2004; Zhong et al., 2004; Zhong and Ye 2004), but no 5PTase has been identified from other plant species. Studies of Arabidopsis 5PTases revealed important roles in plant development, such as cotyledon vascular patterns (Carland and Nelson 2004); secondary cell wall synthesis (Zhong et al., 2004). In addition, in-vitro
studies revealed a possible involvement of IP$_3$ in stomatal closure (Schroeder et al., 2001b), and it is known that IP$_3$ level is regulated by type I 5PTases in animal system (Mejerus et al., 2001) and in Arabidopsis (Berdy et al. 2001; Sanchez and Chua, 2001).

In the present study, four tomato 5PTase homologs were identified based on similarity to Arabidopsis 5PTases (Fig. 3.1 and 3.2). All of them contain two conserved catalytic domains (Fig. 3.1A). Moreover, phylogenetic analysis revealed that _Le5PT1_, _Le5PT2_, and _Le5PT3_ are similar to Arabidopsis _At5PTase2_ (Sanchez and Chua, 2001), _At5PTase1_ (Berdy et al. 2001), and _CVP2_ (Carland and Nelson 2004), respectively (Fig. 3.2). Gene expression in young or old leaf exhibited different expression patterns (Fig. 3.3B) that suggest these 5PTases may have different roles during different stages of plant development.

In Arabidopsis, study of _AtIP5PII/At5PTase2_ showed that transgenic plants with _6XUAS-46:AtIP5PII/At5PTase2_ caused ABA insensitivity in seed germination and seedlings growth in the presence of ABA. To investigate whether transgenic tobacco plants with _35S:Le5PT1-mGFP_ exhibit similar characteristics since _Le5PT1_ showed the greatest similarity to _AtIP5PII/At5PTase2_ in predicted amino acid sequences among identified _Le5PT1-4_ genes, germination frequencies in presence of ABA were examined. Unlike Arabidopsis transformed with _AtIP5PII/At5PTase2_ no difference in the germination frequencies between wild type and two transgenic tobacco lines were observed. Perhaps combined with _Le5PT1_ expression under dehydration in wild type tomato (Fig. 3.3A) and the _NtERD10B_ expression under PEG treatment in transgenic tobacco plants (Fig. 3.9) with _35S:Le5PT1-mGFP_, it may be that _Le5PT1_ is involved in ABA independent drought response, and these results perhaps explain why seed
germination did not respond to ABA.

The 35S:Le5PT1-mGFP transgenic tobacco plants exhibited retarded growth, and the degree of phenotype was consistent with the level of Le5PT1 expression (Fig. 3.4B). In addition, in contrast to the seedling growth of Arabidopsis with 6XUAS-46:AtIP5PII in presence of ABA (Sanchez and Chua, 2001), 35S:Le5PT1-mGFP transgenic plants did not exhibit altered sensitivity to ABA. To determine whether Le5PT1 indeed has 5PTase enzyme activity and is involved in plant development, however, substrate specificity of the Le5PT1 protein as well as comparison of IP3 levels in wild type and transgenic plants is needed.

Arabidopsis 5PTases exhibited the different expression patterns in responses to phytohormones or environmental stresses (Ercetin and Gillaspy, 2004; Lin et al., 2004), suggesting that 5PTases may have various roles in hormonal and stress responses via phosphoinositides in plants. Therefore, it was of interest to examine whether any phytohormones change dwarf phenotype of transgenic tobacco plants with 35S:Le5PT1-mGFP. In this regard, seed germination of transgenic plants were carried out using several hormones including an inhibitor of ethylene biosynthesis: GA3, ABA, IAA, brassinosteroid, an ethylene biosynthesis inhibitor (AVG: aminoethoxyvinylglycine), and ethylene precursor (ACPC: 1-aminocyclopropane carboxylic acid), but none of them affected growth at least in the seedling stage (data not shown).

Several studies suggest that inositol 1,4,5 triphosphate has a role under water deficit stress, osmotic stress, or cold stress (Takahashi et al., 2001; DeWald et al., 2001; Ruelland et al., 2002; Sanchez and Chua, 2001; Xiong et al., 2002). In-vitro injection of
IP₃ promotes calcium release from organelles in guard cells (Schroeder et al., 2001b), which is an important for the action of ABA-induced stomatal closure that can ultimately reduce water loss under water deficit. Although the level of transcripts of Le5PT1 was down-regulated by dehydration (Fig. 3.3A), 35S:Le5PT1-mGFP transgenic tobacco plants did not exhibit any changes related to water deficit stress (Fig. 4.6, A-D) or salt stress with 300 or 500mM NaCl treatment (data not shown). In addition, Arabidopsis plants with At5PTase1 or AtIP5PII/At5PTase2 showed altered expression of stress responsive genes (Sanchez and Chua, 2001; Berdy et al., 2001). Interestingly, the tobacco LEA gene, NtERD10B, exhibited reduced level of transcripts by dehydration, indicating that there is still a connection between Le5PT1 and drought response.
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Rock CD, Zeevaart JA. (1991) The aba mutant of Arabidopsis thaliana is impaired in


