Role of *Arabidopsis thaliana* PAS/LOV Protein in salt and dehydration stress conditions

Sarah Sansom  
*Department of Plant Cellular and Molecular Biology, Somers’ Lab  
Ohio State University, Columbus OH 43210*

**Abstract**

Plant functioning and growth are strongly affected by dehydration and salt stress conditions. Many genes are involved in the response pathways that are activated to tolerate stress. It has been shown that Arabidopsis PAS/LOV protein (PLP) is transcriptionally upregulated under high salt and dehydration stress conditions. In order to investigate the role of PLP in stress response, ten homozygous knockout insertion lines were identified by PCR. Four weak overexpression lines were also identified using RT-PCR. PLP, which contains a LOV domain, did not exhibit an obvious circadian cycling phenotype at the transcriptional level. Germination success on high salt medium was impaired in the PLP knockout when compared to Wild Type. However, measurement of root growth on high salt medium did not reveal any additional phenotype for PLP knockout. Transcriptional expression of two known stress response genes, RD29A and DREB2A, showed differential expression when comparing Wild Type and PLP knockout plants under strong stress conditions. Under both high salt and dehydration stress conditions, RD29A and DREB2A exhibit reduced upregulation and shortened expression period when PLP is absent. These results indicate that PLP is likely involved in the response pathways for dehydration and salt stress.

**Introduction**

Plants have developed ways to tolerate stressful conditions as a necessity. As generally rooted and immobile organisms, plants must be able to adapt to local environmental pressures. Pests, disease, extremes in temperature, drought and high salinity commonly confront plants. The uses of plants that are resistant to abiotic and biotic stress conditions has been valuable in agricultural production, and will likely become even more important in the future. Worldwide human consumption has exceeded environmental capacity. To compound the problem, the current population of approximately seven billion people is expected to increase to nine billion by 2050 (Glenn and Gordon, 2006). Urban sprawl
will likely limit the availability of land for agricultural use. Irrigation practices, which increase the amount of land available for agriculture, will also increase soil salinity and make long-term agriculture in these areas difficult. Thus, these scenarios have a strong probability of resulting in a worldwide food crisis unless preventative measures are taken. These challenges highlight the need to develop more stress-tolerant agricultural crops. The first step in developing stress-tolerant plants is to characterize and understand stress response mechanisms. In doing so, valuable insight will be gained that will be applied to the engineering or selective breeding of new agricultural products.

Plants that are exposed to environmental stress respond in many ways to minimize the deleterious effects of such conditions. Physiological, molecular and developmental changes result from exposure to such environmental pressure (Yamaguchi-Shinozaki and Shinozaki, 1994; Yamaguchi-Shinozaki and Shinozaki, 2005; Ramachandra-Reddy et al, 2004). Salt and dehydration stress elicit similar physiological responses. Stomatal closure, decreased nutrient uptake, repression of growth and metabolic pathways, and inhibition of photosynthesis are a portion of the physiological mechanisms that plants use to cope with these types of stress. On the molecular level, many genes have been shown to respond transcriptionally to these stresses as part of signal transduction cascades (Benzel and Reuveni, 1994; Yamaguchi-Shinozaki and Shinozaki, 2005; Shinozaki et al, 2003). Accumulation of osmolytes, such as proline, help increase osmotic pressure and may play an important role in dehydration tolerance (Tsugane et al, 1999). Increased production of hydrophilic proteins, chaperone proteins, and oxygen-radical scavenging proteins also help the plant survive under dehydration stress (Ramachandra-Reddy et al, 2004).
Salt and dehydration stress induce the production of the phytohormone abscisic acid (ABA), which in turn influences the transcription of many stress-response genes. A portion of these genes can be induced by exogenous application of ABA. ABA-independent stress response has also been identified. In ABA-deficient (aba) and ABA-insensitive (abi) mutants of Arabidopsis several ABA-inducible genes are still strongly expressed in response to stress (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2005). A number of genes involved in high salinity, drought, and ABA response pathways are shared and have potential to interact positively or negatively (Xiong et al, 1999; Kreps et al, 2002).

The gene selected for investigation in this study is the Arabidopsis PAS/LOV protein (PLP) (At2g02710). PLP is a small protein that contains an N-terminal Per-ARNT-Sim (PAS) domain and a C-terminal light, oxygen or voltage (LOV) domain. Due to mRNA splicing, three separate proteins are produced: PLPA, PLPB, and PLPC. LOV domains likely involve light-dependent conformational changes that augment the binding affinity of the molecule. In the presence of blue light, flavin mononucleotide (FMN) covalently bonds to a conserved cysteine residue in the LOV domain (Salomon et al, 2001). Of the known Arabidopsis proteins containing LOV domains, all have been categorized according to function except PLP. Other A. thaliana genes known to contain LOV domains include: PHOT1, PHOT2, ADO1/LKP1/ZTL, ADO2/LKP2 and ADO3/FKF1. (Crosson et al, 2003). Both the PAS and LOV domains can be important in protein-protein interactions (Más et al, 2003; Taylor and Zhulin, 1999).

Recently, it has been shown that PLP is strongly transcriptionally upregulated under dehydration and high salt stress conditions (Ogura et al, 2008). The increase of
PLP mRNA suggests that PLP may play a role in stress response pathways. PLP mRNA was not greatly increased by treatment with ABA (Ogura et al., 2008), suggesting that PLP would likely act in an ABA-independent response pathway. Investigation of expression patterns of known ABA-independent stress response genes in the absence PLP may give insight into the functional role of PLP in stress response. Two genes were selected for investigation: RD29A and DREB2A.

RD29A is induced rapidly after exposure to dehydration stress, salt stress, cold stress and ABA application. Rapid induction was observed within 20 minutes, and strong expression was observed after several hours. Induction of RD29A mRNA has been shown to respond differently to different stress conditions (Yamaguchi-Shinozaki and Shinozaki, 1993). The RD29A promoter contains separate cis-acting elements which function in both ABA-dependent and ABA-independent responses. Rapid response of RD29A to dehydration and high salt stress is ABA-independent, and is induced by a 9 bp dehydration-responsive element (DRE), TACCGACAT, in its promoter. DRE can function in response to high salt, dehydration and cold stress response, but is not responsive to ABA. The delayed response of RD29A to these stress conditions is ABA-dependent, and is induced by an ABA response element (ABRE) that is commonly found in the promoter regions of ABA-inducible genes. Therefore, two separate pathways can control RD29A induction (Yamaguchi-Shinozaki and Shinozaki, 1994).

DREB2A also exhibits rapid response to stress conditions. Induced expression was observed within 10 minutes of high salt and dehydration stress application, and was strongly expressed after several hours. Expression of DREB2A is similar to that of RD29A under high salt and dehydration stress conditions. DREB2A is not induced in
response to cold stress or ABA application. It can bind to the DRE sequence and induce transcription of other genes, including RD29A (Liu et al., 1998). In dreb2a mutants, RD29A expression was reduced (Sakuma et al., 2006 a). The C-terminal region of DREB2A functions in transcriptional activation of many stress-inducible genes. DREB2A expression under normal conditions does not result in downstream gene induction, and may be posttranslationally modified before binding to DRE (Sakuma et al., 2006 b).

In this study, PLP knockout (plp-ko) and PLP weak overexpression (PLP-ox) mutants were identified. Germination was observed in plp-ko mutant plants under high salt stress conditions. No phenotype was observed in plp-ko plants for root growth under high salt stress conditions. PLP was not shown to exhibit an obvious circadian cycling phenotype at the mRNA level. Increase in PLP mRNA under salt and dehydration stress conditions was confirmed. Under both stress conditions, RD29A and DREB2A exhibit reduced mRNA accumulation and shortened expression period when PLP is absent. These results indicate that PLP is likely involved in the response pathways for dehydration and salt stress.

Methods and Materials

Plant materials and growth conditions

Arabidopsis thaliana Columbia ecotype was grown on 0.8% Murashige and Skoog (MS) medium with filter paper for 3 weeks under 12 h light and 12 h dark conditions at 22°C. For the high salt stress procedure, rosette plants were harvested from the plates and placed in 250mM NaCl solution with gentle shaking under the same light and
temperature conditions. For the dehydration stress procedure, rosette plants were
harvested from the plates and placed in an equilibrated 75% humidity chamber on filter
paper under dim light at 22°C. Plants were collected at time points 0, 1, 2, 5, 10, and 24 h
and frozen in liquid nitrogen. RNA was extracted and used for RT-PCR analysis.

**Identification of PLP knockout mutants**

Putative T-DNA insertion lines were obtained (GABI-Kat) and screened by antibiotic
selection. PCR and gel electrophoresis were used to score for the presence of
homozygous insertion lines. PLP-F2 (5’-TTAGTCTGCGAAAGCGGTA-3’) and PLP-
TDNA (5’-ATATTGACCATCATCATTGC-3’) primers were used to confirm the
presence of T-DNA insertion at the PLP locus. PLP-F2 and PLP-R (5’-
TGAGCTTCTAACCAGCAACTC-3’) primers were used to determine if the insertion was
homozygous.

**Identification of PLP overexpression mutants**

The PLP cDNA was cloned into an expression vector (35S promoter driven) with an HA
tag fused to the N-terminus. This 35S::HA-PLP cassette was cloned into the
Agrobacterium-compatible vector pZP200 (Basta-resistant) and transformed into
Arabidopsis. Transformants were selected on soil by spraying with Basta and F3 seed
were tested for HA-PLP expression by immunoblotting seedling extracts with anti-HA
antibody. PLP-ox seeds were screened by antibiotic selection. RT-PCR and gel
electrophoresis were used to investigate the relative expression levels. Actin was
amplified using primers ACT 2F short (5’-CCATCACCACCACCACCCTCCTC-3’) and
ACT 2R (5’-GTTGAACGGAAGGGATTGAGAGT-3’). PLP was amplified using primers RT-LOVPAS-F (5’-CAGTACTATACGAGATGAAG-3’) and RT-LOVPAS-R (5’-TGAGCTTCTAACCACACTC-3’).

**Germination under high salt stress**

WT and plp-ko seeds were plated directly on 0.8% MS medium that contained 0mM, 50mM, 100mM and 150mM NaCl concentration. Plants were observed for 28 days under 12 h light and 12 h dark at 22°C. Germination was defined as the production of green cotyledons.

**Root growth under high salt stress**

WT and plp-ko seeds were plated on 0.8% MS medium with filter paper and grown for 10 days under 12 h light and 12 h dark at 22°C. Seedlings were then placed directly on 0.8% MS plates that contained 0mM, 50mM, 100mM and 150mM NaCl concentration. Plants were grown vertically for 7 days under the same light and temperature conditions. Root growth during this period was measured using ImageJ software.

**Expression Analysis**

RNA was extracted from plant tissues collected under stress conditions at time points 1, 5, 9, 13, 17 and 21 h. Reverse transcriptase was used to synthesize cDNA samples for each time point. RT-PCR and gel electrophoresis were used to investigate the relative expression levels of each gene. Actin was amplified using primers ACT 2F short and
ACT 2R. PLP was amplified using primers RT-LOVPAS-F and RT-LOVPAS-R. RD29A was amplified using primers RD29A-RT-F1 (5’-ATCAGGAATAAGGTTTGTGAATT-3’) and RD29A-RT-R1 (5’-TTTCTCTAGGTTCCTGTGAATT-3’). DREB2A was amplified using primers DREB2A-F2 (5’-GATTCTGCTAAGCTTGCTG-3’) and DREB2A-R2 (5’-AGTACCAAGGCCAAGCTGCTG-3’).

**Results and Conclusions**

**Identification of plp-ko and PLP-ox**

PCR was used to identify homozygous T-DNA insertion lines at the PLP locus (Fig. 1). A total of ten homozygous insertion isolates were identified. RT-PCR was used to identify PLP overexpression mutants (Fig. 2). A total of four weak overexpression lines were identified. Overexpression lines were not used for phenotypic or expression analysis due to problematic seed germination. Knockout mutant lines were then used for subsequent phenotypic and expression analysis experiments.

**Figure 1.** PLP knockout mutant lines were identified by PCR scoring for the presence of homozygous T-DNA insertion at the PLP locus. A positive band for combination of PLP forward and TDNA reverse primers indicates the presence of a TDNA insertion at the locus. A negative band for combination of PLP forward and PLP reverse primers indicates that the insertion is homozygous. Denoted line numbers indicate homozygous insertion lines.
Figure 2. PLP overexpression lines were identified by RT-PCR analysis by selecting lines that exhibited increased mRNA levels due to the presence of the transgene. The top band is PLP, and the lower band is actin. Denoted line numbers indicate the strongest PLP transcript expression in comparison to actin levels.

**PLP Expression Under Normal Conditions**

Due to the presence of a LOV domain in PLP, it is possible that PLP mRNA may exhibit light-dependent circadian cycling. RT-PCR of WT samples showed no obvious circadian cycling phenotype of PLP at the mRNA level (Fig 5). Cycling of PLP transcript will not need to be taken into consideration when examining mRNA expression profiles under stress conditions.

Figure 5. PLP expression under normal 12 h light and 12 h dark conditions was assayed using RT-PCR at continuous time points. A representative RT-PCR gel electrophoresis sample is shown. Graph reflects the average of 2 biological trials. Error bars show the standard error of the means.

**Germination and Root Growth Under High Salt Stress**

Comparison of WT and plp-ko germination under high salt stress conditions revealed decreased germination of plp-ko seeds (Fig 3). Under no salt stress, WT and plp-ko seeds
had nearly identical germination rates of approximately 100% (Fig 3A). Germination at 50mM and 100mM NaCl concentrations yielded similar results for WT and plp-ko. However, addition of 150mM salt to the growth medium resulted in lower germination success for plp-ko seeds than for WT. Two biological trails were conducted, but results from one trial are not shown due to very low germination of WT and no germination of plp-ko seeds. WT seeds germinated under these conditions 17.5% after 28 days. Knockout seeds only germinated 2.5% (Fig 3B). Plants were also studied for root growth under high salt stress conditions. Comparison of WT and plp-ko root growth on several concentrations of salt after 7 days revealed no phenotypic difference (Fig 4). Root growth of WT and plp-ko was comparable at all salt concentrations tested.

**Figure 3.** Germination rates of WT vs. plp-ko plants under different stress conditions. Error bars show the standard error of the mean. (A) Germination on MS medium with no salt. Bar represents average of two biological trials, with 40 samples each. (B) Germination on 150mM NaCl MS medium. Only one biological trail is shown, with 40 samples.
**Figure 4.** Comparison of WT and plp-ko root growth on varying NaCl concentrations after 7 days. Bars represent average of two biological trials. Error bars show standard error of the mean.

**Gene Expression Under High Salt and Dehydration Conditions**

PLP transcriptional upregulation was confirmed by RT-PCR in both high salt and dehydration stress conditions in WT plants (Fig 6). Under high salt stress conditions, PLP was visibly increased after one hour, and expressed very strongly after two hours. Expression of PLP remained strong throughout the 24-hour collection period (Fig 6A). When dehydration stress was applied, PLP was not shown to be strongly upregulated until time point 24 (Fig 6B). The dehydration stress could be considered weak, at 75% humidity. It is possible that the plant dehydration stress was too weak to elucidate an immediate activation of stress response pathways, and may explain why PLP induction is delayed in comparison to high salt stress response. Little induction of PLP was observed in both of the control conditions. This data confirms that PLP induction was a specific response to the applied salt and dehydration stress conditions and not due to harm during removal from plates.
Investigation of the expression pattern of DREB2A under high salt conditions yielded conflicting results (Fig 7). Expression of DREB2A in WT showed induction within one hour of stress treatment, with a peak expression at 10 hours. Peak expression was then followed by a reduction in mRNA accumulation (Fig 7A). Two biological trials of high salt stress were conducted using the same conditions. The first biological trial yielded a striking reduction of DREB2A induction in plp-ko plants. Only an extremely faint signal was observed at time point 10 hours (Fig 7B). The second biological trial
revealed moderate induction of DREB2A at time points 2 and 5 hours. Time point 10 data is not available in this representative figure. Expression of DREB2A is reduced at time points 5 and 24 in plp-ko compared to WT data (Fig 7C). It is unclear why these biological trials yield significantly different results in plp-ko plants. Despite the differences in expression, however, it seems that DREB2A is consistently reduced in expression in plp-ko in comparison to WT.
Figure 7. RT-PCR is used to show DREB2A expression under high salt stress and control conditions at continuous time points. (A) Expression of DREB2A in WT. (B) Expression of DREB2A in plp-ko in the first biological trial. (C) Expression of DREB2A in plp-ko in the second biological trial. Graphs represent technical repeat averages of 2 biological trials with standard error of the means.

DREB2A expression under dehydration stress revealed different patterns in WT and plp-ko plants as well (Fig 8). DREB2A expression in WT looked very similar to that of PLP under the same conditions. A strong induction of DREB2A is not observed until time point 24 hours (Fig 8A). No DREB2A induction was observed in plp-ko at any time point (Fig 8B).
Figure 8. RT-PCR is used to show DREB2A expression under dehydration stress and control conditions at continuous time points. (A) Expression of DREB2A in WT. (B) Expression of DREB2A in plp-ko. Graphs represent technical repeat averages of 1 biological trial with standard error of the means.

Induction of RD29A was altered in plp-ko mutants compared to WT under high salt stress conditions (Fig 9). RD29A shows very strong induction within one hour of stress application in WT plants. Strong induction continues from time point 1 hour through 5 hours, and then decreases gradually (Fig 9A). In plp-ko mutants, RD29A is reduced at time point 1 compared to WT. Peak expression occurs at time point 2, and then gradually decreases (Fig 9B). RD29A expression in plp-ko is reduced after time point 2 when compared to WT.
Figure 9. RT-PCR is used to show RD29A expression under high salt stress and control conditions at continuous time points. (A) Expression of RD29A in WT. (B) Expression of RD29A in plp-ko. Graphs represent technical repeat averages of two biological trials with standard error of the means.

A change in expression pattern of RD29A was also observed under dehydration stress conditions when comparing WT and plp-ko mutants (Fig 10). In WT, rapid induction was observed within 1 hour. Two peaks of expression were observed, first at time point 2 hours and secondly at time point 24 hours (Fig 10A). Induction of RD29A in plp-ko showed peak expression at time point 5, and then gradually decreased (Fig 10B). Expression of RD29A in plp-ko is greatly reduced at all time points in plp-ko when compared to WT. It should be noticed that there is high expression of RD29A in both WT and plp-ko control treatments of 100% humidity. Background expression of RD29A is very comparable to stress treatment results in WT at all time points except 24 hours. Similarly, background expression of RD29A is nearly equivalent in plp-ko when comparing stress and control treatments. Despite the high background levels, there are striking differences in expression at time point 24 hours. RD29A induction in WT is greatly increased while induction in plp-ko remains low.
**Figure 10.** RT-PCR is used to show RD29A expression under dehydration stress and control conditions at continuous time points. (A) Expression of RD29A in WT. (B) Expression of RD29A in plp-ko. Graphs represent technical repeat averages from one biological trial with standard error of the means.

**Discussion**

PLP, which was shown previously to be strongly upregulated under high salt and dehydration stress at the transcriptional level (Ogura et al., 2008), was investigated for possible role in ABA-independent stress response pathway. PLP knockout mutants were useful in investigating phenotype and the changes in gene expression of known high salt and dehydration stress-response genes.

A reduced germination phenotype on high salt medium was identified for plp-ko, but not in root growth on high salt medium. The discovery of a phenotype under high salt stress indicates that PLP may play a role in stress response. Very young or germinating
plants may be more susceptible to high salt stress, and could therefore exhibit lower
germination success if the stress response pathway is impaired. The lack of phenotype for
root growth on high salt concentrations suggests that PLP does not play a role in root
growth regulation. It would be interesting to investigate if PLP induction under stress
conditions is organ-specific. It would seem likely that if PLP is acting in a light-
dependent manner due to the presence of a LOV domain, that PLP would play a more
important role in tissues regularly exposed to light, such as leaves. However, there is very
little research available that investigates light-dependent interactions acting in high salt
and dehydration stress response pathways. Further research may focus on germination
phenotypes on high salt medium under varying light conditions. If the blue-light-
dependent LOV domain in PLP is acting in an important capacity for stress response,
then germination may be even more greatly impaired under red light conditions than
white light. It should be investigated if the light-dependent interactions of the LOV
domain are important in high salt and drought stress response, or if it is acting in another
capacity.

PLP was not shown to exhibit a circadian cycling phenotype at the transcriptional
level. However, previous research has shown that the LOV domain of two splice variants,
PLPA and PLPB, is functional. Interaction with several proteins was diminished under
blue light in yeast. The PLP LOV domain is acting in a blue light-dependent manner, the
result of which is unclear (Ogura et al, 2008). Although PLP does not exhibit circadian
cycling at the transcriptional level, study at the protein level for oscillations may be of
interest. Previous research has revealed that one other LOV-containing protein,
ZEITLUPE (ZTL), is constitutively expressed but exhibits protein oscillation through
protein-protein interaction with GIGANTEA (GI) (Kim et al., 2007). Circadian cycling of PLP at the protein level may be controlled in a similar way. However, PLP has not been shown to interact with GI (unpublished data). Future experiments should investigate if PLP exhibits circadian cycling phenotypes at the protein level.

Analysis of PLP expression confirmed induction under high salt and dehydration stress conditions. It was also shown that in the absence of PLP, several known stress response genes, RD29A and DREB2A, were differentially expressed under stress conditions. These genes, which are part of the ABA-independent stress response pathway, consistently exhibited reduced upregulation and shortened expression period in plp-ko. Further biological repeats are necessary to confirm the expression pattern of PLP, DREB2A and RD29A under dehydration stress. Further biological repeats are also necessary to determine the true expression pattern of DREB2A under high salt stress conditions. These results suggest that PLP is acting in an upstream capacity in the ABA-independent stress response pathway. PLP is somehow influencing this pathway to induce and maintain expression of known stress response genes. Further research should focus on elucidating how PLP is influencing this pathway. This may be accomplished by selecting other known stress-response genes in this pathway to investigate expression profiles in the absence of PLP. It would also be useful to assay PLP protein-protein interaction partners. Several proteins have been previously identified which interact with PLP: VITAMIN C DEFECTIVE 2 (VTC2), VTC2-LIKE (VTC2L), AND BEL1-LIKE HOMEODOMAIN 10 (BLH10A AND BLH10B) (Ogura et al., 2008). VTC2 is an enzyme in vitamin C biosynthesis (Linster et al., 2007). VTC2L function has not been identified. BLH10 is known to act in ovule development (Hackbusch et al., 2005), but
physiological function remains to be identified. It is not yet known if the decreased interactions of these proteins with PLP under blue light are due to a direct LOV interaction, or if the LOV conformational change in blue light indirectly affects these interactions (Ogura et al., 2008). Identification of PLP protein-protein interactions with known stress response genes should be investigated to determine how PLP is affecting the high salt and dehydration stress response pathways.

**Literature Cited**


