2009

Functional study of Disproportionating Enzyme 2 in the Arabidopsis cold response

Sunita Patil
San Jose State University

FUNCTIONAL STUDY OF DISPROPORTIONATING ENZYME 2 IN THE *ARABIDOPSIS* COLD RESPONSE

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Sunita Patil

May 2009

SAN JOSE STATE UNIVERSITY

The Undersigned Thesis Committee Approves the Thesis Titled

FUNCTIONAL STUDY OF DISPROPORTIONATING ENZYME 2 IN THE *ARABIDOPSIS* COLD RESPONSE

by
Sunita Patil

APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

Dr. Robert Fowler,	Department of Biological Sciences	5/22/09 Date
Dr. David Bruck,	Department of Biological Sciences	5/21/09 Date
Dr. Zhiyong Wang,	Department of Plant Biology Carnegie Institution of Washington	5-18-2009 Date

APPROVED FOR THE UNIVERSITY

Associate Vice President	Office of Graduate Studies and Research	5/29/09 Date

ABSTRACT

FUNCTIONAL STUDY OF DISPROPORTIONATING ENZYME 2 IN THE *ARABIDOPSIS* COLD RESPONSE

by Sunita Patil

Disproportionating Enzyme 2 (DPE2) is a cytosolic protein found in *Arabidopsis thaliana*. When exposed to cold temperature, the protein has been shown by 2D difference gel electrophoresis to be enriched in a microsomal compartment. The first aim of this study was to test whether DPE2 translocates to a microsomal compartment in response to cold by both a biochemical approach (western blot) and a cellular approach (green fluorescent protein tagging and confocal microscopy). The second aim was to determine if the *dpe2* knockout mutants were more freeze tolerant than the wild type plants. Western blot analysis indicated that the protein translocated to a microsomal compartment in response to cold. These findings could not be confirmed by confocal microscopy due to co-suppression of transgene expression. Whole plant freeze tests and electrolyte leakage analysis showed that knocking out the *DPE2* gene did not make *Arabidopsis* more freeze tolerant.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Zhiyong Wang, head of the Wang Lab at Carnegie Institution of Washington in Stanford, for welcoming me into his lab and for providing valuable guidance throughout my research. I thank Dr. Robert Fowler for kindly agreeing to be my graduate advisor when Dr. Matthes resigned from SJSU. He was always there to answer any questions that I had during my thesis work. I am grateful to Dr. David Bruck and Dr. Fowler for taking the time to help me edit my thesis report. I wish to thank Dr. David Matthes for guiding me with my coursework and inspiring me to do thesis research. I am indebted to Dr. Zhiping Deng from the Wang lab at Carnegie for guiding me with my experiments and training me in plant biology techniques. I also wish to thank all my friends at the Wang lab for their support and for providing a stimulating environment in which to learn. I wish to thank my parents for always loving and encouraging me. Lastly, and most importantly I thank my husband, Rohit, for constantly supporting and motivating me to achieve my goals.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Chilling stress has a major impact on the geographical distribution, survival and agricultural productivity of plants. Plants from temperate regions are tolerant to cold but only above freezing temperatures. When plants are exposed to freezing temperatures, ice forms in intercellular spaces and leads to cellular dehydration as well as membrane damage.

Exposure to low, non-freezing temperatures increases tolerance to freezing by a process called cold acclimation, which involves several physiological and molecular changes such as activation or repression of gene expression, accumulation of protective metabolites (Zhu et al., 2007), and changes in membrane lipid composition and soluble and membrane protein composition (Yano et al., 2005). Freeze tolerance is also enhanced by sugar accumulation in leaves (Yano et al., 2005).

Knowledge of the localization of a protein or a change in its localization provides information about the functional role of the protein. Translocation of a protein, for example, from the cytoplasmic compartment to the plasma membrane or an internal membrane-limited compartment, is essential for signaling events (O'Rourke et al., 2005).

Transitory starch in leaf chloroplasts is broken down in the dark to maltose and glucose (Lu and Sharkey, 2004). The maltose produced in chloroplasts is exported to the cytoplasm by a maltose transporter, MEX1 (Niittylä et al., 2004), and metabolized by Disproportionating enzyme 2 (Chia et al., 2004; Lu and Sharkey, 2004).

Disproportionating enzyme 2 (DPE2) is an amylomaltase (4-alpha-d-glucanotransferase). It transfers a glycosyl unit of maltose to glycogen or amylopectin, ultimately leading to the conversion of maltose to sucrose and the release of another glycosyl unit as free glucose (Chia et al., 2004; Lu and Sharkey, 2004). The enzyme is localized to the cytoplasm in *Arabidopsis thaliana* leaves (Chia et al., 2004). The enzyme is also found in a small but diverse group of plants like *Solanum tuberosum* (potato), *Oryza sativa* (Asian rice), *Vitis vinifera* (common grape vine), and *Triticum aestivum* (common wheat). Two-dimensional difference gel electrophoresis has shown that DPE2 is an early cold-response protein (Z. Deng, unpublished observation). When plants of the Columbia ecotype of *A. thaliana* were exposed to 2°C conditions for 30 minutes, DPE2 was found to be enriched in a microsomal compartment.

dpe2 knockout mutants show increased maltose accumulation in leaves. The amount of maltose is 20-90 times higher in the mutant plants (Lu and Sharkey, 2004). Temperature is one of the regulating factors for maltose metabolism (Lu and Sharkey, 2006). Maltose has compatible solute (organic molecules of low molecular weight that are not toxic to cells at high concentrations) properties (Kaplan and Guy, 2004). Compatible solutes increase cell osmotic potential during cold stress and maintain the structural integrity of proteins and membranes. Maltose protects the photosynthetic electron transport chain in chloroplasts during freezing stress (Kaplan and Guy, 2004). Being a soluble sugar, maltose may be involved in cold signaling and regulation of cold-inducible gene expression (Rolland et al., 2006).

The above findings point to the involvement of DPE2 in the *Arabidopsis* cold response, suggesting a negative correlation between level of *DPE2* expression and cold tolerance.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild type *A. thaliana* (ecotype Columbia) and *dpe2* knockout seedlings were grown on separate halves of plates containing one-half Murashige-Skoog salts (MS), 1% (w/v) sucrose and 0.7% (w/v) Phytoblend (Caisson Laboratories, Inc., North Logan, UT) at pH 5.7. Before plating, seeds of each of these varieties were sterilized in 70% (v/v) ethanol and 0.1% (v/v) TritonX-100 for 15 min followed by a brief rinse in 90% (v/v) ethanol. For western blotting, wild type seedlings were grown from seeds grown in liquid medium containing one-half MS salts and 1% (w/v) sucrose on a platform shaker under continuous light. For growth in liquid culture, the seeds were sterilized with 80% (v/v) bleach.

Time Course Western Blotting

Microsomal, Soluble, and Total Protein Extraction

The protocol described by Tang et al. (2008) was followed with modifications. Wild type *A. thaliana* var Columbia seeds were germinated in liquid medium (one-half MS salts, 1% sucrose, pH 5.7). Ten-day-old seedlings were transferred to liquid media pre-cooled to 1°C. After 5, 10, 30, or 60 min of cold treatment, tissue from approximately 40 seedlings per time point was harvested. The seedlings were surface dried with tissue paper and frozen and ground in liquid nitrogen with a mortar and pestle. The tissue was weighed and then vortexed with three volumes of microsomal extraction buffer (25 mM HEPES, 0.33 M sucrose, 10% (v/v) glycerol, 0.6% (w/v) PVP, 5 mM EDTA, 1 mM NaF, 5 mM DTT, 1 μM Bestatin, 1 μM Pepstatin, 2 μM Leupeptin and 1 mM PMSF, pH 7.5).

To remove tissue debris, the samples were centrifuged twice at 10,000 rpm for 10 min. After saving some of the homogenate as the total protein fraction, microsomal membranes were pelleted by spinning the rest of the homogenate at 55,000 rpm at 4°C for 1 h. The supernatant was retained as the soluble fraction and the pellet was resuspended in 100 µl of extraction buffer by slowly pipeting up and down and stored at -80°C. The total, soluble, and microsomal proteins were quantified by a Bradford assay kit (Bio-Rad, Hercules, CA).

Western Blot Analysis

Microsomal, soluble, and total proteins extracted from ten-day-old Columbia seedlings, as described above, were subjected to western blot analysis. The samples were prepared by adding 2X SDS sample buffer (160 mM Tris, 4% (w/v) SDS, pH 6.8, 20% (v/v) glycerol, 0.04% (v/v) bromophenol blue, 2% (v/v) 2-mercaptoethanol) to each fraction and heating at 95°C for 3 min. The proteins were separated by polyacrylamide gel electrophoresis on a 7.5% gel and transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4°C with 5% (w/v) non-fat dry milk in 1X PBS and 0.1% (v/v) Tween-20 (PBST). It was then incubated with anti-DPE2 primary antibody (1:5000 dilution) for 1 h at room temperature. The membrane was rinsed four times for 10 min each with PBST and incubated with horseradish peroxidase enzyme-conjugated secondary antibody (1:2000 dilution) for 1 h at room temperature. After rinsing the membrane with PBST as described earlier, Supersignal® west dura extended duration substrate (Pierce Scientific, Rockford, IL) was added. After incubation for 2 min the excess supersignal solution was drained and the chemiluminescence detected with a

cooled CCD camera. Band intensities were quantified by the image analysis software, ImageQuant (GE Healthcare, Livingston, NJ).

Creating Transgenic Plants

CaMV35S:*DPE2-GFP* and CaMV35S:*YFP-DPE2* Constructs

N-terminal *YFP* and C-terminal *GFP*-tagged *DPE2* constructs, driven by the Cauliflower mosaic virus constitutive promoter (CaMV-35S), were made using the Gateway® Technology from Invitrogen (Carlsbad, CA). *DPE2* cDNA in a pUNI vector obtained from the Salk Institute Genomic Analysis Laboratory was PCR amplified using gene-specific primers (forward: 5′CACCATGATGAATCTAGGATCTCTTTCG 3′ and reverse: 5′ TGGGTTTGGCTTAGTCGAGCCATTG 3′) and Pfu DNA polymerase (Promega, Madison, Wisconsin). The four-base sequence, CACC, was added at the 5′ end of the forward primer to allow directional cloning. The PCR product was run on a 0.8% agarose gel and was cut and purified using the QIAquick Gel Extraction Kit from Qiagen (Gaithersburg, MD). The purified PCR product was directionally cloned into the pENTR/D/SD TOPO entry vector by means of the pENTR™ Directional TOPO cloning kit (Invitrogen, Carlsbad) to create an entry clone. The 6 µl TOPO cloning reaction was set up by incubating the 4 µl (16 ng/µl) of purified PCR product with 0.5 µl of TOPO vector (15-20 ng/µl linearized purified plasmid). The inserts from positive clones were sequenced (Sequetech Corporation, Mountain View, CA) to check for insertion in the correct orientation in the entry vector and for mutations. The plasmid preparation from a positive clone was digested with *Mlu*I restriction enzyme to remove the replication origin of the entry clone because both the entry clone and the destination vector contained a

kanamycin selection marker. The digested products were run on a 0.8% agarose gel to separate the band consisting of the replication origin from the rest of the entry clone. The larger band consisting of the entry clone minus the replication origin was transferred into pMDC84 and pEarleyGate 104 destination vectors with the Gateway Technology from Invitrogen (Carlsbad, CA) involving the recombination enzyme, LR Clonase™. The resulting expression vectors were sequenced (Sequetech Corp, Mountain View, CA) to check if the *DPE2* gene was in frame with the Yellow Fluorescent Protein (*YFP*) and the Green Fluorescent Protein (*GFP*) genes, and the constructs were transformed separately into *Agrobacterium tumefaciens* by electroporation.

Transformation by Floral Dip Method

dpe2 knockout mutants in the Columbia background and wild type Columbia were transformed with the *YFP* or *GFP*-tagged constructs by *Agrobacterium*-mediated floral infiltration (Bent, 2006). The agrobacteria transformed with the constructs were cultured at 28°C in 500 ml liquid Luria-Bertani (LB) media supplemented with the antibiotics, kanamycin, for selection of the constructs and gentamycin for selection of agrobacteria. The cultures were spun down in a Sorval centrifuge at 4000 rpm for 5 min and the bacterial pellet was resuspended in infiltration medium (0.5% (w/v) MS salts, 2.5% (w/v) sucrose, 0.05% (v/v) Silwet L-77, and benzamino purine). Inflorescences of healthy *Arabidopsis* plants were immersed in the bacterial suspension for 10 min and the pots containing the inoculated plants were individually wrapped in plastic wrap for 24 h. Seeds of the transformed plants were collected after 6 weeks.

Selection and Analysis of Transformed Plants

T1 seeds collected from the transformed plants were sterilized with 70% (v/v) ethyl alcohol and 0.1% (v/v) TritonX-100 as described earlier and plated on one-half MS plates containing 20 μg/ml hygromycin to select for positive transformants and 30 μg/ml carbenecillin to prevent *Agrobacterium* contamination. The seedlings were grown for three days in the dark and transferred to light. T1 plants transformed with the N-terminal YFP construct were selected by spraying young seedlings with Basta. The T1 seedlings were observed under a confocal microscope for expression of the transgene.

The tissue from the T1 Columbia plants transformed with the *DPE2-GFP* construct were subjected to western blot analysis for expression of the transgene. The tissues were ground in liquid nitrogen and weighed. The samples were prepared by adding two volumes of 2X SDS sample buffer (4% SDS, 160 mM Tris, pH 6.8, 20% glycerol, 0.04% bromophenol blue, and 2% 2-mercaptoethanol), vortexing and heating at 95°C for 3 min. The samples were vortexed again and spun down and then equal volumes of supernatant were loaded on a 7.5% polyacrylamide gel. Western blot analysis was conducted as described above.

Seeds from T1 plants with high levels of gene expression were grown on one-half MS plates with 20 μg/ml hygromycin and were observed under a confocal microscope. The seedlings were then placed on ice for 10 min and observed again under a confocal microscope. The constructs were also injected separately into young tobacco leaves for

transient expression of the fusion proteins. Confocal imaging was performed 48 h after leaf infiltration.

Whole Plant Freeze Test

The protocol described by Agarwal et al. (2006) was followed with some modifications. Plant survival upon exposure to -6°C was tested. Plates containing two-week-old unacclimated wild type and *dpe2* knockout seedlings were floated for 16 h in a circulating freezing water bath (VWR scientific, San Francisco, CA) set at -1°C. Ice chips were sprinkled on the plates to initiate ice nucleation, and the temperature was lowered to -6°C at the rate of 1°C/h. The plates were taken out of the water bath after incubating them at the desired temperature for 2 h. The plates were thawed for 12 h at 4°C in the dark and then transferred to room temperature in a growth chamber under continuous light. Differences in the number of surviving seedlings were determined after four days.

Electrolyte Leakage Measurement

The protocols described by Kaplan and Guy (2004) and Agarwal et al. (2006) were followed with modifications. One leaf per plant was excised from four-week-old unacclimated wild type plants and *dpe2* knockout mutant plants grown at room temperature and under continuous light. The leaves were individually wrapped in water-saturated Kimwipes and placed in separate microfuge tubes. The tubes were submersed at 0°C for 30 min in a circulating, freezing water bath (VWR Scientific, San Francisco, CA). Chips of ice were placed in contact with the Kimwipes to initiate ice nucleation. The temperature was lowered at the rate of 1°C/h. When -6°C was reached, the tubes were removed from the water bath and placed immediately on ice, and the tissue was allowed to thaw gradually in this 4°C environment. The leaves were then transferred into 10 ml deionized water and shaken overnight at room temperature. On the following day, 5 ml of water from each tube was removed to a separate tube to be used as a pre-boil sample. The leaves in the remaining deionized water, to be used as post-boil samples, were autoclaved at 15 psi pressure for 15 min. Electrolyte levels of the pre-boil and post-boil samples were measured with an ion conductivity meter, and percentage ion leakage was calculated for each sample as the ratio of ion concentration before boiling to ion concentration after boiling multiplied by 100.

RESULTS

Time Course Western Blot

Total, soluble, and microsomal protein fractions from cold-treated wild type Columbia seedlings were analyzed by western blotting to study the translocation kinetics of DPE2 in response to cold treatment. Following 5-10 min of cold treatment at 1°C, DPE2 was found to be enriched in the microsomal fraction (Fig. 1, Table I). The total (Fig. 2, Table II) and soluble fractions (Fig. 3, Table III) showed no change in DPE2 levels.

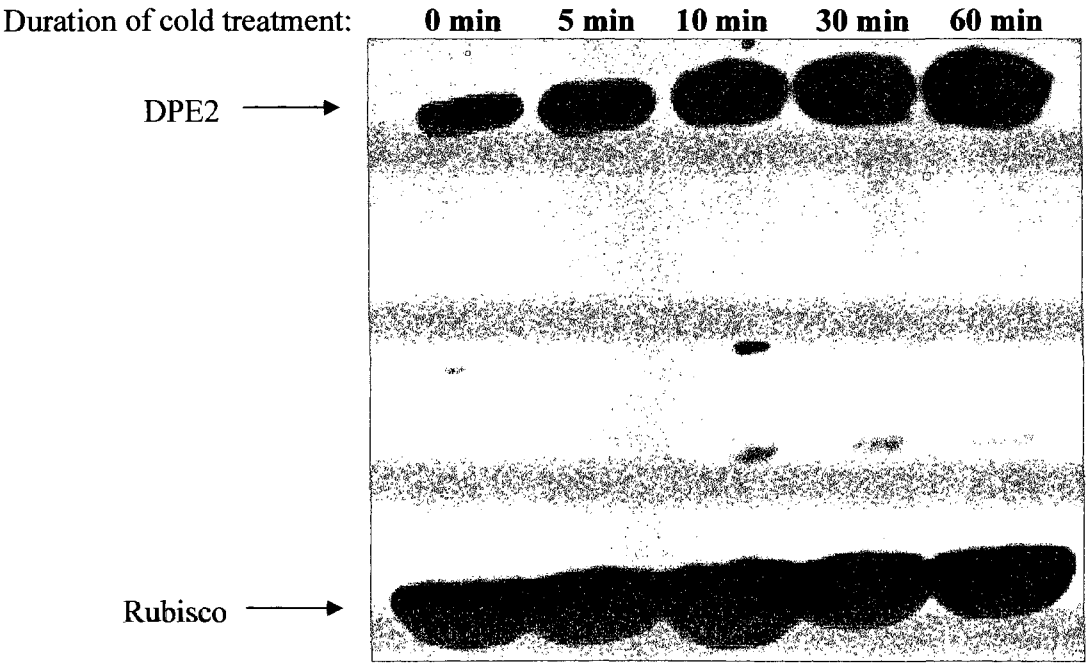

Figure 1. Western blot of microsomal protein fractions of cold-treated Columbia seedlings. Bands at the top of the gel correspond to DPE2 protein (110 kDa). The lower nonspecific, bright bands are bands of Rubisco (ribulose-1,5-bisphosphate carboxylase), used as loading controls.

Table I. *ImageQuant analysis of western blot of microsomal protein fractions*

The microsomal protein fractions isolated from cold-treated Columbia seedlings were subjected to western blot analysis. Ratio of DPE2 band intensity to Rubisco band intensity was used to compare DPE2 levels between samples.

Duration of Cold Treatment (min)	Ratio of DPE2 Band to Rubisco Band Intensity
0	1
5	1.7
10	1.94
30	2.33
60	2.26

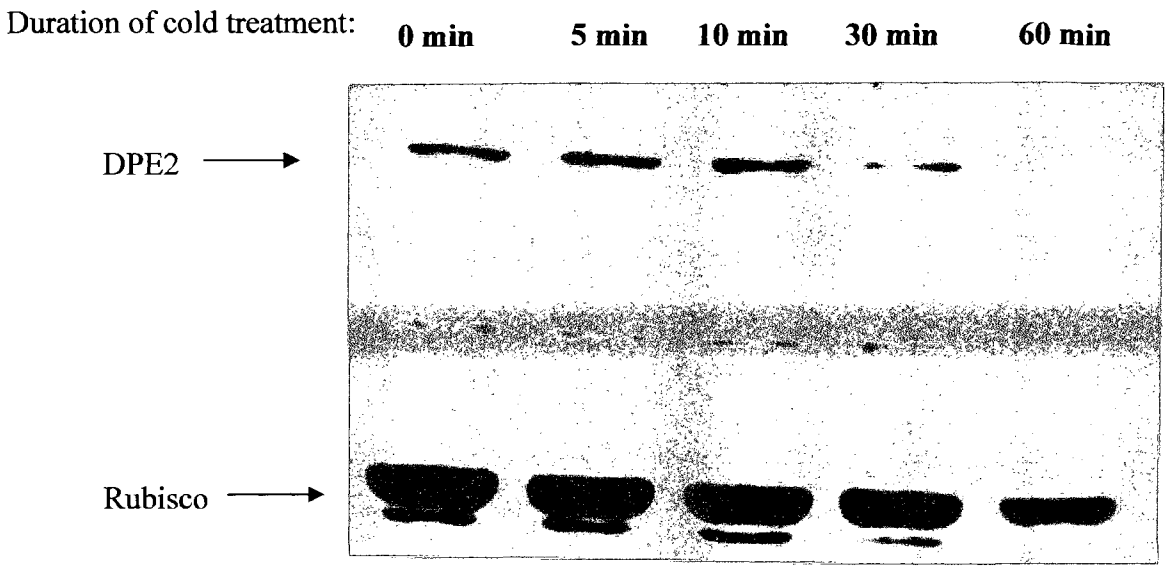

Figure 2. Western blot of total protein isolated from cold-treated Columbia seedlings. Bands at the top of the gel correspond to DPE2 protein (110 kDa). The lower nonspecific bright bands are bands of Rubisco used as loading controls.

Table II. *ImageQuant analysis of western blot of total protein*

Total protein isolated from cold-treated Columbia seedlings were subjected to western blot analysis. Ratio of DPE2 band intensity to Rubisco band intensity was used to compare DPE2 levels between samples.

Duration of Cold Treatment (min)	Ratio of DPE2 Band to Rubisco Band Intensity
0	1
5	1.14
10	1.04
30	0.74
60	0.56

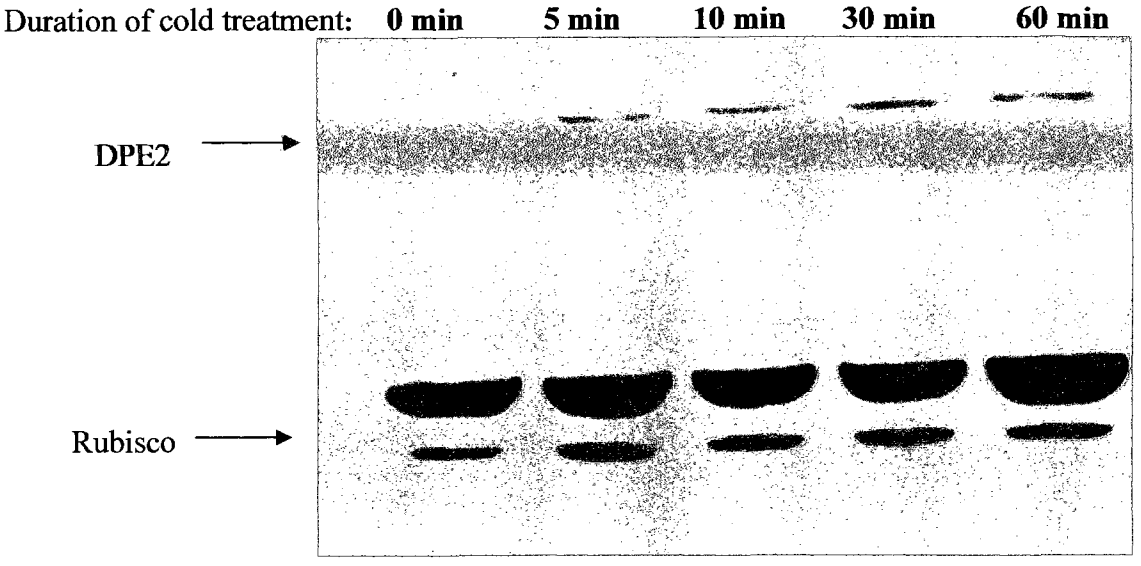

Figure 3. Western blot of soluble protein fractions of cold-treated Columbia seedlings. Bands at the top of gel correspond to the DPE2 protein (110 kDa). The lower nonspecific bright bands are bands of Rubisco, used as loading controls.

.

Table III. *ImageQuant analysis of western blot of soluble protein fractions*

The soluble protein fractions isolated from cold-treated Columbia seedlings were subjected to western blot analysis. Ratio of DPE2 band intensity to Rubisco band intensity was used to compare DPE2 levels between samples.

Duration of Cold Treatment (min)	Ratio of DPE2 Band to Rubisco Band Intensity
0	1
5	1
10	1.01
30	1.05
60	0.74

Creating Transgenic Plants

Making *GFP* and *YFP*-tagged *DPE2* Constructs

The PCR amplification of *DPE2* cDNA in a pUNI vector with Pfu polymerase led to a 2869 bp product. The product was run on a 0.8% agarose gel (Fig. 4), cut out of the gel, and purified to separate it from the plasmid vector.

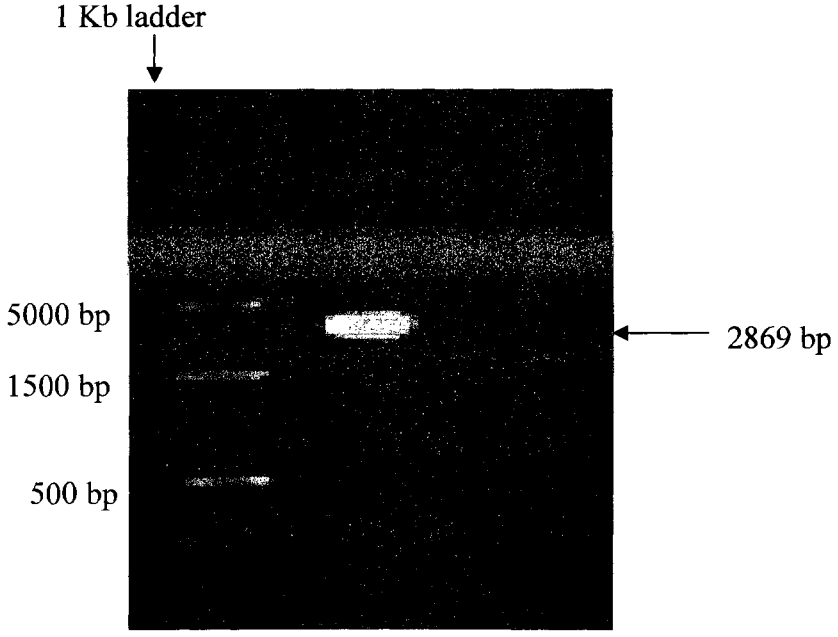

Figure 4. Agarose gel electrophoresis of the PCR product of the *DPE2* cDNA insert in pUNI vector. The product was run on a 0.8% agarose gel and it migrated to a position consistent with its expected size of 2869 bp.

The product of the TOPO cloning reaction was transformed into chemically competent *E. coli* TOP10 cells. The colonies produced were analyzed by PCR for the presence of the insert in the correct orientation (Fig. 5). The plasmid preps from the positive clones were sequenced and digested with *Mlu*I restriction enzyme to remove the pUC origin of replication (Fig. 6), as both the entry clone and destination vector had the same kanamycin selection marker.

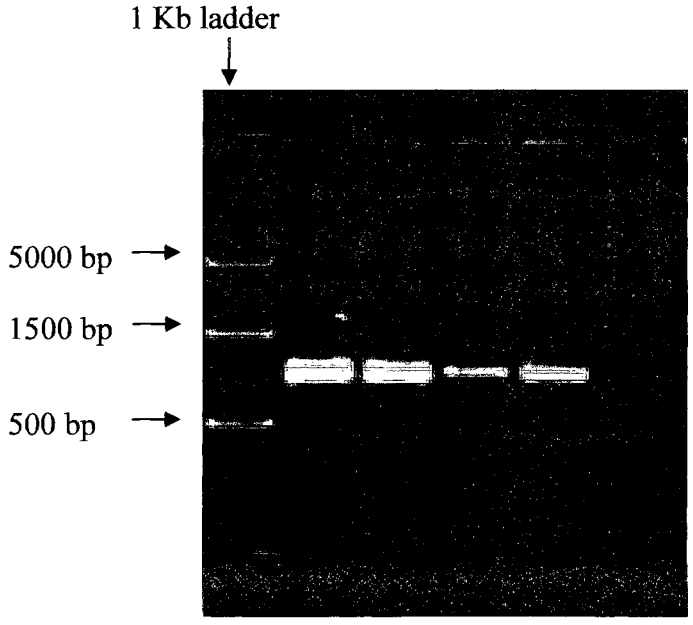

Figure 5. Colony PCR. TOP10 *E. coli* colonies transformed with the entry clone were analyzed by PCR for the presence of insert in the correct orientation. The positive clones showed an approximate 1000 bp band.

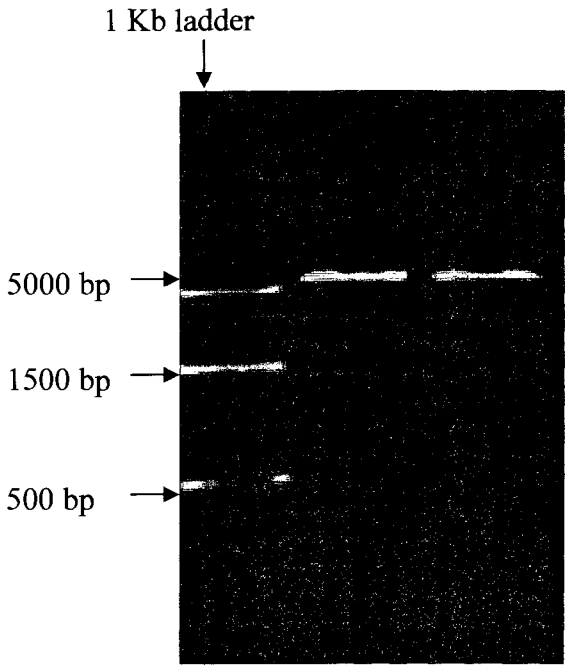

Figure 6. *Mlu*I digestion of entry clone. The entry clone created by the insertion of *DPE2* cDNA in a pENTR/D/SD TOPO entry vector was digested with *Mlu*I restriction enzyme. The bright upper band is the entry clone without the replication origin. The replication origin ran as the lower band.

Selection of Transformants and Analysis

When T1 seedlings (Col/*DPE2-GFP*) were grown in the dark, hygromycin-resistant positive transformants could be distinguished from sensitive seedlings by their long hypocotyls and green leaves (Fig. 7). Rosette leaf tissues from four-week-old T1 seedlings were analyzed by western blot (Fig. 8). The level of transgene expression varied among different lines with some showing high levels and others none.

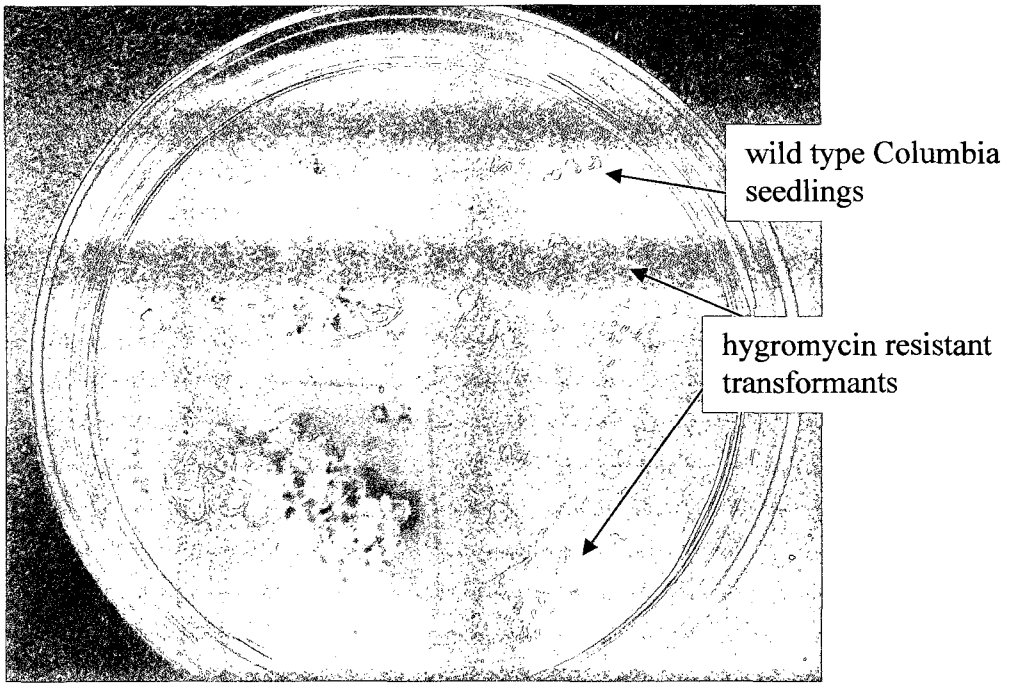

Figure 7. Hygromycin selection of T1 seedlings. Columbia seedlings transformed with the *DPE2-GFP* construct were grown in the dark on one-half MS plates containing hygromycin. Positive transformants had long hypocotyls and green leaves.

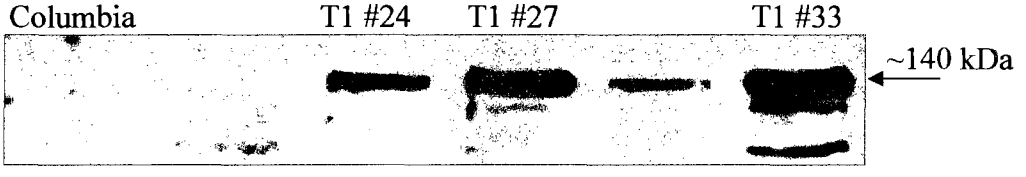

Figure 8. Western blot of Col/*DPE2-GFP* transgenic plants with anti-GFP antibody. Wild type Columbia tissue was used as a negative control. The size of the DPE2-GFP fusion protein is approximately 140 kDa. T1 #27 and T1 #33 are transgenic lines that showed high levels of protein expression.

Both N and C terminal, *YFP* and *GFP*-tagged *DPE2* constructs were separately transformed into *Arabidopsis* plants because it was not known whether the N and/or the C terminal of DPE2 is important for its subcellular localization. The constructs were also transiently transformed into tobacco leaves to check for expression of the fusion proteins and to compare the localization of the proteins in the two species. The YFP-DPE2 fusion protein localized to the nucleus and cytoplasm in both tobacco (Fig. 9) and *Arabidopsis* plants (Fig. 10). The DPE2-GFP fusion protein showed cytoplasmic localization and low expression levels in both types of plants (Fig. 11, Fig. 12). As co-suppression can occur in different tissues and at different times in the photoperiod in *Arabidopsis* and lead to reduced transgene expression, different plant parts such as leaves, hypocotyls and roots were visualized at different time points in the photoperiod. In addition one-week-old *Arabidopsis* seedling leaves and three-week-old rosette leaves were visualized by confocal microscopy, as co-suppression can occur at different developmental stages. The GFP signal was low under all the above conditions (data not shown).

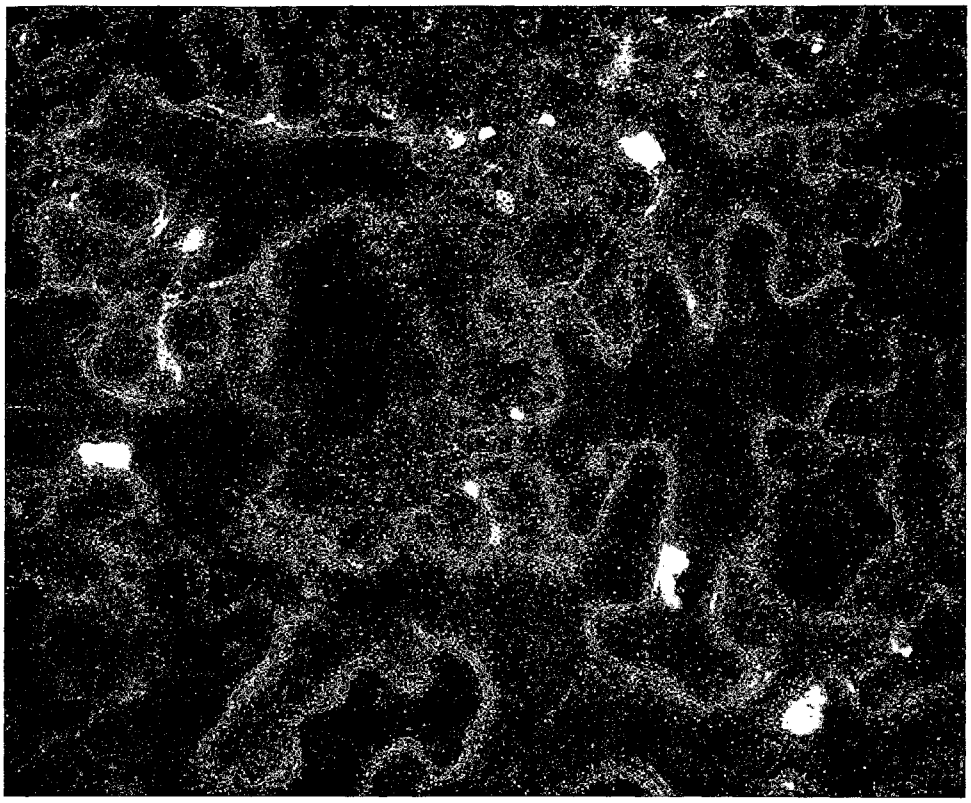

Figure 9. Confocal micrograph of abaxial epidermal surface of a tobacco leaf showing the subcellular localization of the YFP-DPE2 fusion protein (appearing green in micrograph). The construct was transiently transformed into tobacco by leaf infiltration. YFP-DPE2 localized to both nucleus and cytoplasm. The red fluorescing organelles are auto-fluorescing chloroplasts.

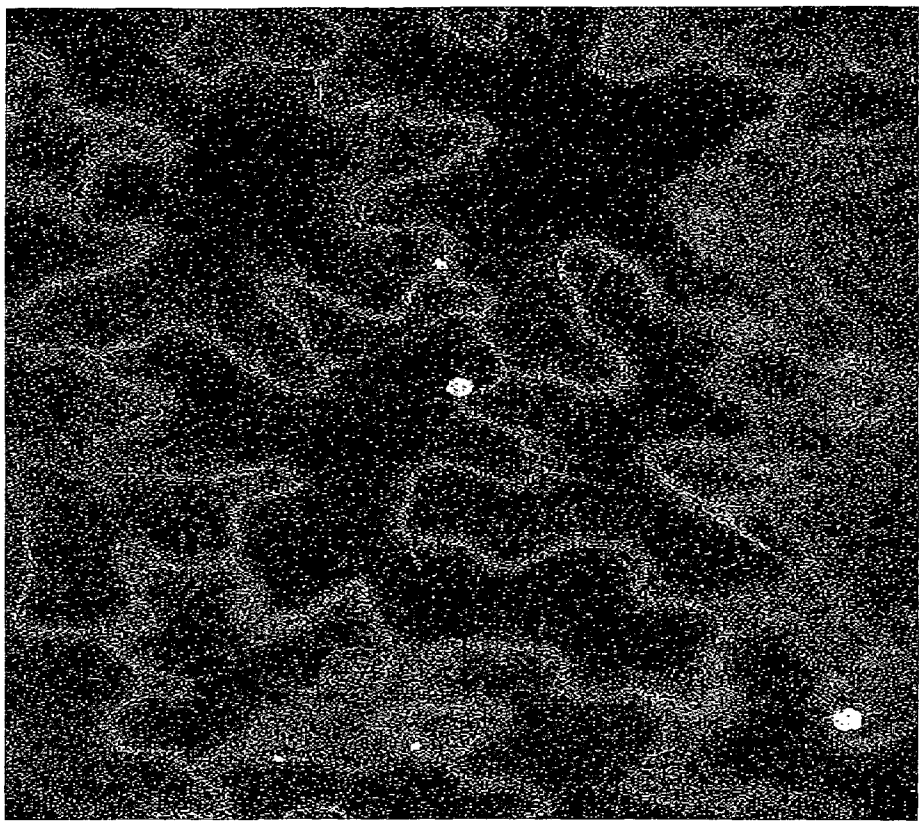

Figure 10. Confocal micrograph of abaxial leaf epidermal surface of *Arabidopsis* showing the subcellular localization of the YFP-DPE2 fusion protein. The construct was stably transformed into *Arabidopsis* by the floral dip method and showed both nuclear and cytoplasmic localization.

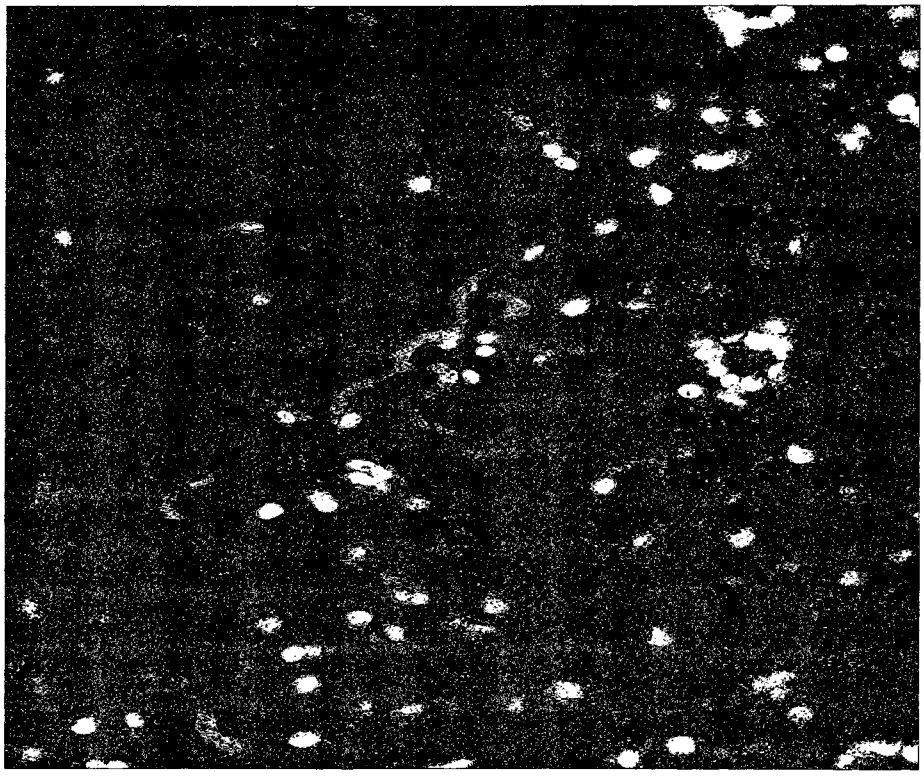

Figure 11. Confocal micrograph of abaxial leaf surface of tobacco showing the subcellular localization of the DPE2-GFP fusion protein. The construct was transiently transformed into tobacco by leaf infiltration. It showed low levels of expression that localized to minimal portions of cytoplasm. The red fluorescing organelles are chloroplasts.

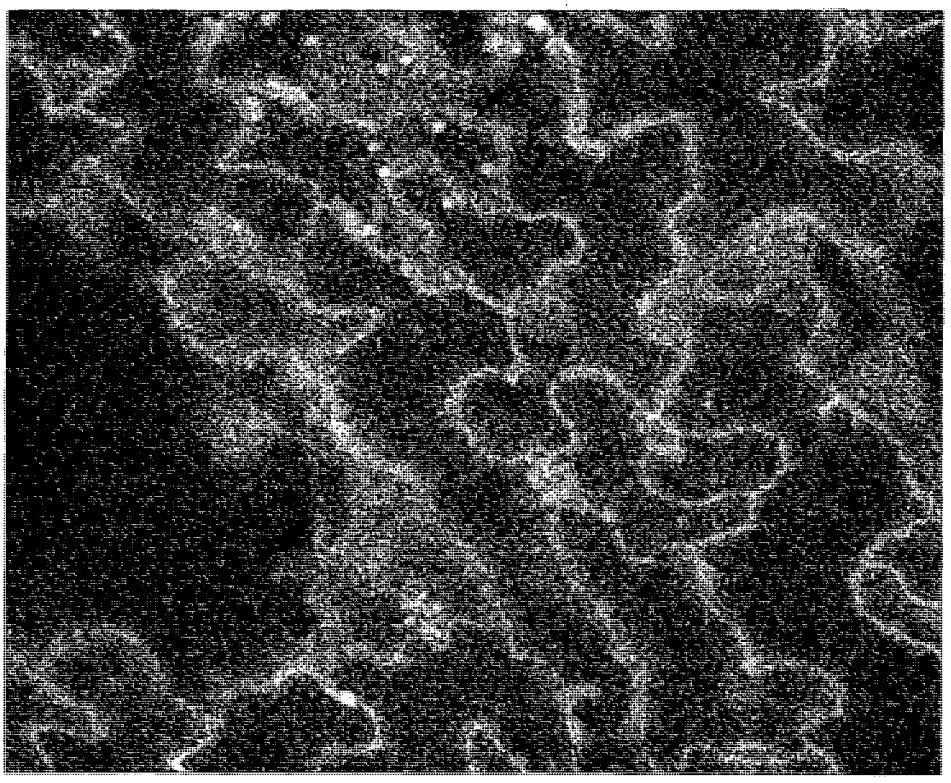

Figure 12. Confocal micrograph of abaxial leaf surface of *Arabidopsis* showing the subcellular localization of the DPE2-GFP fusion protein. The protein was expressed at low levels and localized to the cytoplasm. The red and yellow organelles are chloroplasts.

Whole Plant Freeze Test

Columbia wild type and *dpe2* knockout seedlings were subjected to freezing temperatures to look for differences in freeze tolerance. The seedlings maintained at room temperature were used as negative controls (Fig. 13). There was no significant difference in survival of the two types of seedlings (Fig. 14).

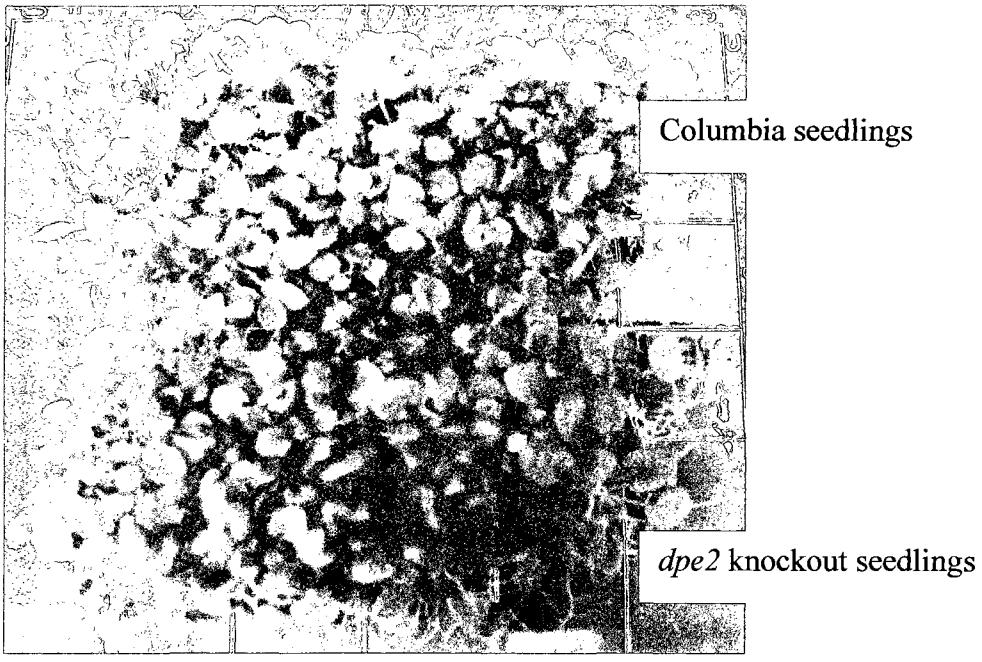

Figure 13. Room temperature control for whole plant freeze test. Unacclimated wild type Columbia and *dpe2* knockout seedlings were grown under continuous light conditions and room temperature on separate halves of one-half MS plates.

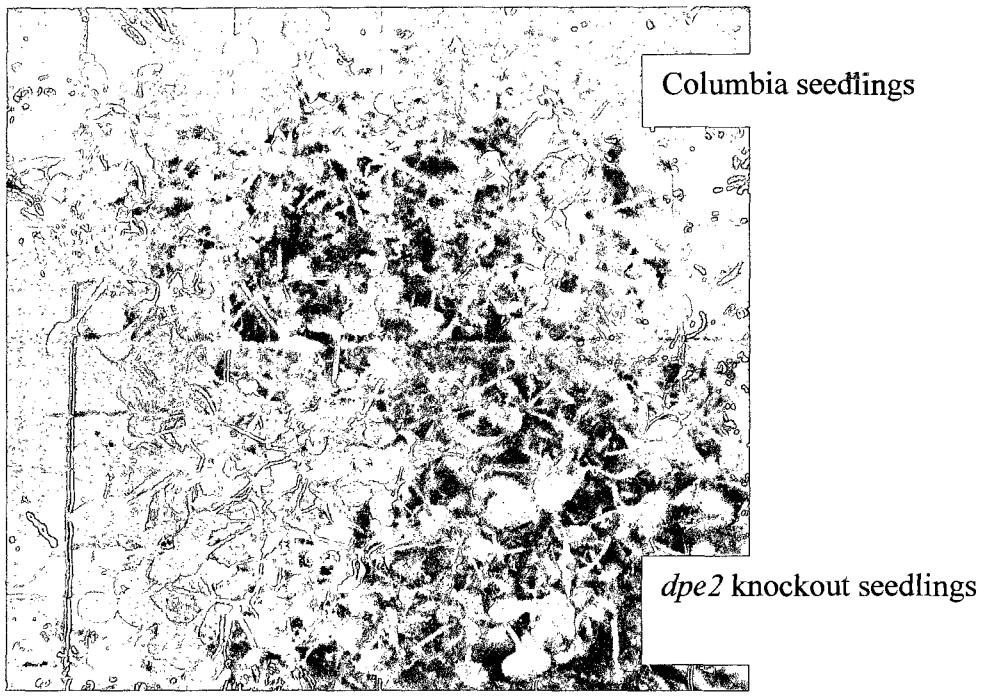

Figure 14. Whole plant freeze test. Unacclimated wild type Columbia and *dpe2* knockout seedlings were grown on opposite halves of one-half MS plates and exposed to -6°C for 2 h.

Electrolyte Leakage Analysis

The plasma membrane integrity in the rosette leaf cells of unacclimated Columbia and *dpe2* knockout mutants when freeze stressed was compared by measuring electrolyte leakage. There was no significant difference in electrolyte leakage between the two plant types when their leaves were exposed to -6°C (Fig. 15).

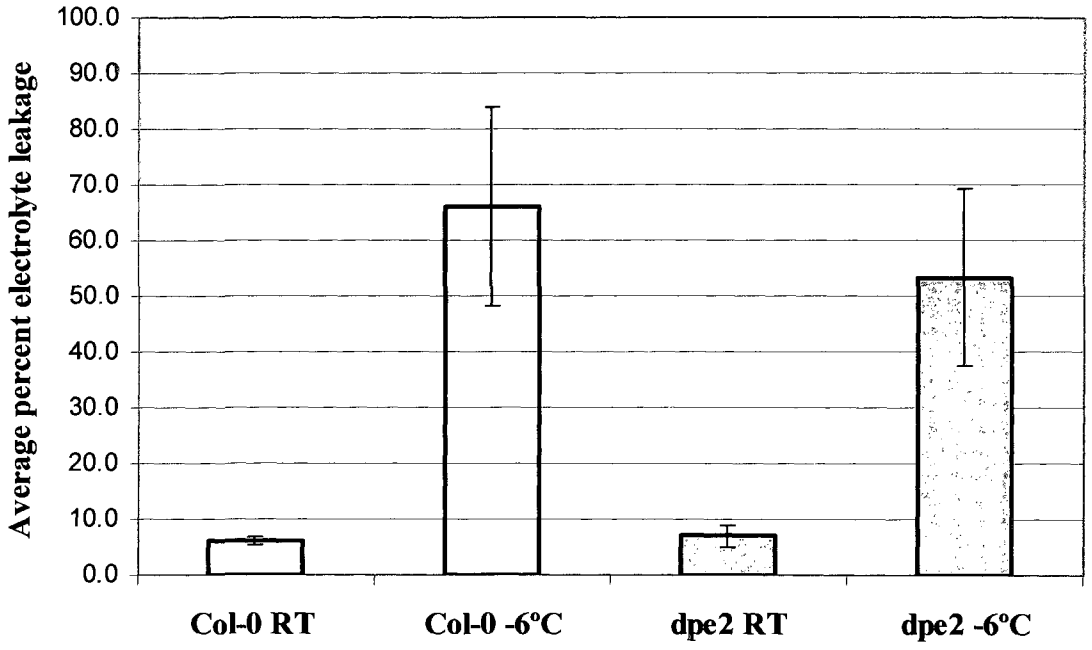

Figure 15. Electrolyte leakage analysis in rosette leaves of four-week-old wild type and *dpe2* knockout plants exposed to -6°C. Error bars indicate +/- SD of three experimental replications.

DISCUSSION

Time Course Western Blot Analysis

The enrichment of the microsomal compartment with DPE2 following cold treatment was consistent with the results from 2D difference gel electrophoresis (Z. Deng, unpublished data). Although these data suggest protein translocation from a cytoplasmic to a microsomal compartment, they need to be confirmed by GFP tagging and subsequent microscopic imaging. The change in the DPE2 level of the microsomal compartment could be due to aggregates that form during extraction and pellet during ultracentrifugation.

Transgenic Plants and Confocal Imaging

Confocal imaging was done to complement the results of the western blot. The YFP-DPE2 fusion protein showed both nuclear and cytoplasmic localization, which was contrary to the wild type protein showing only cytoplasmic localization. This pattern could have occurred because the N-terminus of the protein is involved in localization. Therefore, the above-mentioned construct was not used for studying protein translocation in response to cold stress. The 2X 35S:*DPE2-GFP* construct showed low levels of expression and localization to the cytoplasmic compartment in both *Arabidopsis* and tobacco leaves. The GFP signal was too weak to discern the change in location of DPE2 following cold treatment.

The level of transgene expression could have been low because of co-suppression. Co-suppression is a form of post-transcriptional gene silencing in plants where the levels

of mRNA in the cytoplasm are reduced without affecting the rate of transcription initiation. Several hypotheses have been suggested to explain co-suppression (Elmayan et al., 1998). One hypothesis is that co-suppression results from high levels of transcription due to a strong promoter. Another hypothesis is that interaction between the transgene mRNA and homologous gene mRNA leads to the formation of aberrant RNAs that cause sequence-specific RNA degradation. A third hypothesis is that a shorter aberrant poly(A)- RNA pairs with the transgene mRNA leading to its cleavage. The vector pMDC84 that was used for making the *DPE2-GFP* construct has the 2X 35S strong promoter. Using the pEarleyGate series of vectors with the 1X 35S promoter or a native promoter for making *GFP* constructs with *DPE2* may prevent co-suppression.

Whole Plant Freeze Test

The survival of wild type and *dpe2* mutant plants when exposed to -6°C was compared, as -4°C is the LT_{50} of wild type Columbia plants (temperature at which only half the plants survive). At -6°C, any differences in survival should be discernable. The freeze tolerance in unacclimated plants was studied, as maltose levels increase in response to cold and *dpe2* knockout plants have increased maltose levels even in the absence of cold. Therefore, any differences in freeze tolerance between wild type and mutant plants should be manifested in the absence of acclimation. This study showed that knocking out *DPE2* does not make *Arabidopsis* plants more freeze tolerant.

Electrolyte Leakage Analysis

The percent ion leakage in leaves of unacclimated wild type and *dpe2* knockout mutants when exposed to -6°C freezing temperatures was compared. The lack of a significant difference in response of the two types of plants suggests that maltose accumulated in the leaf cell cytoplasm does not contribute to the integrity of plasma membrane during freeze stress.

The change in localization of DPE2 in response to cold treatment suggests a functional role of the protein in cold tolerance. The activity of the protein localized to the cytoplasm should be compared to that localized to the microsomal compartment. It is possible that the change in localization leads to reduced activity of the protein and in turn the increased accumulation of maltose.

LITERATURE CITED

Agarwal M, Hao Y, Kapoor A, Dong C, Fuji H, Zheng X, Zhu J (2006) A R23 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. J Biol Chem **281**: 37636-37645

Bent A (2006) *Arabidopsis* thaliana floral dip transformation method. Methods Mol Biol **343**: 87-103

Chia T, Thorneycroft D, Chapple A, Messerli G, Chen J, Zeeman SC, Smith S, Smith A (2004) A cytosolic glucosyltransferase is required for conversion of starch to sucrose in *Arabidopsis* leaves at night. Plant J **37**: 853-863

Elmayan T, Balzergue S, Beon F, Bourdon V, Daubrenmet J, Guenet Y, Mourrain P, Palauqui J, Vernhettes S, Vialle T, Wostrikoff K, Vaucheret H (1998) *Arabidopsis* mutants impaired in cosuppression. Plant Cell **10**: 1747-58

Kaplan F, Guy CL (2004) Beta-amylase induction and the protective role of maltose during temperature shock. Plant Physiol **135**: 1674-1684

Kaplan F, Guy CL (2004) RNA interference of *Arabidopsis* beta-maylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. Plant J **44**: 730-743

Lu Y, Sharkey TD (2004) The role of amylomaltase in maltose metabolism in the cytosol of photosynthetic cells. Planta **218**: 466-73

Lu Y, Sharkey TD (2006) The importance of maltose in transitory starch breakdown. Plant Cell Environ **29**: 353-366

Niittylä T, Messerli G, Trevisan M, Chen J, Smith AM, Zeeman SC (2004) A previously unknown maltose transporter essential for starch degradation in leaves. Science **303**: 87-9

O'Rourke N, Meyer T, Chandy G (2005) Protein localization studies in the age of 'Omics'. Curr Opin Chem Biol **9**: 82-87

Rolland F, Baena-Gonzales E, Sheen J (2006) Sugar sensing and signaling in plants: conserved novel mechanisms. Annu Rev Plant Biol **57**: 675-709

Tang W, Deng Z, Oses-Prieto J, Suzuki N, Zhu S, Zhang X, Burlingame, Wang Z (2008) Proteomics studies of brassinosteroid signal transduction using prefractionation and two-dimensional DIGE. Mol Cell Proteomics **7**: 728-38

Yano R, Nakamura M, Yoneyama T, Nishida I (2005) Starch-related ∝-glucan/water dikanse is involved in the cold induced development of freezing tolerance in *Arabidopsis*. Plant Physiol **138**: 837-846

Zhu J, Dong CH, Zhu JK (2007) Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Curr Opin Plant Biol **10**: 290-5