IDENTIFICATION OF PUTATIVE-PEAMT T-DNA MUTANTS IN ARABIDOPSIS

IDENTIFICATION OF PUTATIVE-*S*-ADENOSYL-L-METHIONINE: PHOSPHOETHANOLAMINE-*N*-METHYLTRANSFERASE T-DNA MUTANTS IN ARABIDOPSIS

By

AMBER GLEASON, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2008)

(Biology) McMaster University

Hamilton, ON

TITLE: IDENTIFICATION OF PUTATIVE-*S*-ADENOSYL-L-METHIONINE: PHOSPHOETHANOLAMINE-*N*-METHYLTRANSFERASE T-DNA MUTANTS IN ARABIDOPSIS

AUTHOR: Amber Gleason, B.Sc. (University of Guelph)

SUPERVISOR: Dr. Elizabeth A. Weretilnyk

NUMBER OF PAGES: xi, 108

ABSTRACT

Some plants such as spinach, sugar beet, and wheat accumulate the quaternary ammonium compound glycine betaine when exposed to stresses in their environment. Environmental stress can be in the form of an excess or deficiency of water, high salt content, and/or exposure to excessively low or high temperatures and many if not all of these stresses are associated with cell dehydration.

Glycine betaine is an organic solute that is believed to help restore the osmotic potential of a cell undergoing dehydration by reducing water loss and preventing damage to the structure and function of macromolecules. However, many plants such as Arabidopsis, tobacco, and rice do not accumulate glycine betaine. Given the perceived benefits of glycine betaine production by plants under stress, studies have been carried out to identify factors regulating its production.

Glycine betaine is synthesized by the two-step oxidation of choline. The capacity to synthesize phosphocholine for choline production has been found to limit the production of glycine betaine in non-accumulating plants such as tobacco. As such, genetic engineering has been used to enhance the production of choline to up-regulate the synthesis of glycine betaine. This strategy has required knowledge of the enzyme(s) catalyzing the three *N*-methylation steps of the phosphocholine biosynthetic pathway.

This study focused on a gene product identified as putative-phosphoethanolamine *N*-methyltransferase (putative PEAMT) based upon its similarity to a spinach *N*-methyltransferase known to convert phosphoethanolamine to phosphocholine. This gene

is located at the locus At1g73600 on chromosome I of Arabidopsis and its predicted amino acid sequence has high similarity to two other genes encoding *N*-methylating enzymes located at At3g18000 (a biochemically confirmed PEAMT) and At1g48600 (annotated as a putative PEAMT).

In this study, publicly available microarray data was examined to identify an expression profile of transcripts associated with the At1g73600 gene in organs and tissues of Arabidopsis at various developmental stages. A summary of the microarray data shows the highest abundance of transcripts for At1g73600 to be in the rosette leaves of Arabidopsis at 18.0 – 20.9 days of growth.

Arabidopsis plants grown from seeds from four SALK lines reported to have a T-DNA insert in the At1g73600 gene were screened for the presence of a T-DNA tag using a three primer PCR design strategy. Individual plants from two of the lines were found to have a T-DNA insert present. RT-PCR was then used to analyze the expression of transcripts associated with the At1g73600 gene in these mutant lines. Transcripts were not detected among the amplified products from cDNA produced from the SALK line designated 062703 but they were found at reduced levels in cDNA of SALK line 016929c

.

In future studies the two T-DNA mutant lines identified in this study can be used to assign a biological role for the product of the At1g73600 gene by examining the phenotype of these mutant plants relative to that of wild-type plants under normal and/or stressed conditions. The line found with no expression associated with the At1g73600

gene will be useful in crosses with T-DNA knock-out mutants of genes at loci At3g18000 and At1g48600. Systematic knock-outs for each of the genes in isolation and in combination will help discern whether there is functional redundancy in their biological roles or if their individual expression contributes uniquely towards the development of a plant or its stress response. Given the associated role for PEAMT in phosphatidylcholine metabolism, lipidomics could be used to determine if the composition of the plant membranes is altered relative to wild-type when the At1g73600 gene is knocked-out.

ACKNOWLEDGEMENTS:

I would like to thank Dr. Elizabeth Weretilnyk and Dr. Peter Summers for all their support and guidance throughout my Masters.

A special thanks to Rick Gierczak for all his support and aid in screening for SALK_016929c mutant plants and RT-PCR work on SALK_062703 homozygous plants reported in Figures 8 and 10, respectively.

I would also like to thank Michael BeGora, Jeff Dedrick, Mitch Macleod, and Ashley Tattersall for all their help, encouragement, and contribution to making the lab a very fun and enjoyable environment in which to work.

Table of contents:

List of Tables:

List of Figures:

List of Abbreviations:

bp	Base pairs
ddH_2O	Double distilled water
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Gly Bet	Glycine Betaine
MOBIX	Institute for Molecular Biology and Biotechnology, McMaster University
PCho	Phosphocholine
PCR	Polymerase chain reaction
PEA	Phosphoethanolamine
PDEA	Phosphodimethylethanolamine
PEAMT	Phosphoethanolamine *N*-methyltransferase
PMEA	Phosphomethylethanolamine
PMEAMT	Phosphomethylethanolamine *N*-methyltransferase
PtdCho	phosphatidylcholine
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
TAIR	The Arabidopsis Information Resource

INTRODUCTION

ENVIRONMENTAL STRESS

Environmental stress for an organism can be defined as an "adverse force or influence that tends to inhibit normal systems from functioning" (Hopkins, 1995). These stresses can be categorized into two distinct groupings describing their origins: abiotic or biotic.

Biotic environmental stress

The sources of biotic stresses are living entities such as insects and pathogens and are the consequences of their interactions with other organisms in the environment (Maffei et al., 2006). These interactions can be detrimental to normal plant processes, as is the case when competition for limiting resources such as water and nutrients arise between a plant and another living organism(s) (Nilsen and Orcutt, 1996). In this regard, competition removes or critically limits resources required by the plant for growth and sustainability (Nilsen and Orcutt, 1996). Alternatively, adverse biotic interactions can be more direct as in the case of grazing by herbivores or penetration of plant organs by disease-causing plant pathogens (Maffei et al., 2006).

Abiotic environmental stresses

Abiotic stresses are consequences of non-biological interactions between a plant and its environment and can include factors such as salinity, water stress (drought and

flooding), temperature extremes (chilling, freezing), chemical toxicity, and intense light (Chen and Murata, 2002). This study focused on better understanding an enzyme in a pathway that has been implicated in increasing the capacity of plants to tolerate exposure to abiotic stresses (Yancey et al., 1982). As such, abiotic stresses will be discussed in greater detail in this review.

WATER STRESS

Water stress can originate from either an excess (flooding) or deficiency of water (drought) (Baruch, 1994). Environments with poor drainage and that experience heavy rainfall and/or frequent overflow from rivers or streams are particularly prone to flooding (Kozlowski, 1997). By way of contrast, drought-prone areas are typically in arid regions that receive little to no rainfall (Price et al., 1998).

Deficiency of water - Drought stress

A plant exposed to a water-limited environment will experience several physiological changes as the water deficit causes the cellular protoplasm to shrink, solutes to be accumulated to higher than normal levels, and photosynthesis to be hindered by increasing stomatal closure (Kramer, 1950). At the cellular level, dehydration-associated changes can affect the structure of proteins and lipids and these perturbations eventually disrupt the normal functioning of metabolic processes (Bray, 1993).

The effects of water deficiency are also reported to cause disturbances to normal plant processes on a "secondary level" (Parida and Das, 2005). In this respect, a water

deficiency in cells leads to the increased production of potentially damaging reactive oxygen species such as superoxide (O_2^-) and hydroxyl radicals (OH^-) that mainly disrupt cellular processes through the oxidation of nucleic acids, proteins and lipids (Fridovich, 1986). Under non-stressed conditions, a balance is maintained between the amount of damaging reactive oxygen species synthesized in a cell and their removal by antioxidants (Apel and Hirt, 2004). However, under stressed conditions such as water deficit, the capacity to generate sufficient antioxidants to counteract or inhibit the oxidative activities of the reactive oxygen species can be exceeded and thus the maintenance of cellular balance would be hindered (Harper and Harvey, 1978).

Excess of water - Flooding

An initial response to flooding by plants is often a decrease in the water content of leaves and wilting. This seemingly paradoxical outcome is due to decreased root aeration caused by an increased amount of water present in the surrounding soil. The decrease in leaf water content is caused by an inability of roots to take up water. Water uptake is passive but it responds to a water potential gradient that is established by the uptake of ions from the soil, a process requiring energy (Cosgrove, 1993).

A consequence of too much water is a lack of oxygen in the soil referred to as hypoxia (Smit, 1989; Peng et al., 2005). Hypoxia inhibits aerobic respiration leading to a decreased concentration of adenylates present in the cell which impairs normal ion uptake and transport (Huang et al., 2003). The reduced uptake of ions from the soil into the plant contributes to the development of deficiencies of valuable minerals such as nitrogen (Yan

et al., 1996; Alaoui-Sosse et al., 2005). Hypoxia causes a greater amount of ACC (1-aminocyclopropane 1-carboxylate) to be synthesized (Grichko and Glick 2001) and ACC is the precursor to the hormone ethylene. As a consequence of ACC content increasing, the amount of ethylene in the plant also increases (Grichko and Glick 2001a). An increased amount of ethylene production in leaves may also cause leaf chlorosis, a decrease in the amount of fruit produced, or leaf necrosis (Grichko and Glick 2001b). Eventually leaves can show epinasty as ethylene causes the petioles of leaves to turn downwards (English et al., 1995; Grichko and Glick 2001b). Epinasty in conjunction with the enhanced closure of stomatal openings causes the rate of transpiration in the plant to be slowed down (Jackson 1997). Also, an increase in the amount of ethylene being synthesized in the roots due to hypoxia causes decreased root elongation and an increase in the number of adventitious roots (Chen and Murata, 2002; Bragina et al., 2003).

TEMPERATURE STRESS

High temperatures

The precise temperature(s) at which plants are injured by heat varies between plant species and even among specific ecotypes of a plant species (Hansen et al., 1994). The primary injuries initiated by high temperatures center around lipid phase transitions that cause the stability of the membrane to deteriorate (Pike et al., 1979). Lipid

transitions alter the normal molecular order of the membranes and hence the normal properties of those structures (Hansen et al., 1994). The transition of lipids due to high temperature exposure occurs when the hydrocarbon chains of the phospholipids of the membrane melt (Steim et al., 1969). In addition to membrane destabilization, cellular metabolism is also affected (Hansen et al., 1994). For example, in tomato, detrimental effects on metabolic activity and membrane composition due to lipid transitions occurred when temperatures reached 30°C or greater (Hansen et al., 1994).

Frequently water deficits arise in conjunction with excessive heat (Hamerlynck et al., 2000). As a result, the stomatal openings of the leaves will close to reduce water loss and this, in turn, will limit the photosynthetic capabilities of the plant through the resultant reduction of available CO_2 (Mata and Lamattina, 2001). Limited photosynthetic capacity for a plant causes an overall inhibition to growth as the supply of photosynthate needed for growth is decreased (Lal et al., 1996).

Chilling temperatures

For most chilling intolerant plants, the temperature range capable of causing cellular damage capable of leading to death is between 1-12°C (Somerville, 1995). The specific temperature at which maximal damage occurs may vary with the species or even among different genetic lines within a given species (Hodges et al., 1997). For example, the temperature identified as constituting chilling for maize has been reported to occur at 11°C (Hodges et al., 1997). When maize experiences chilling temperatures it risks incurring severe cellular damage through the accumulation of reactive oxygen species

that are produced by the plant in greater than normal abundance in response to low temperature exposure (Hodges et al., 1997). Reactive oxygen species are defined as a highly reactive oxygen by-product (Salin, 1988) capable of causing oxidative damage to nucleic acids, proteins and lipids (Fridovich, 1986). Examples of these compounds include H_2O_2 (hydrogen peroxide), O_2^- (superoxide), HO^- (hydroxyl radical) (Halliwell and Gutteridge, 1985), and $^1O_2$ (singlet oxygen) (Elstner, 1987). Reactive oxygen species accumulate during chilling temperatures due to the decrease in photosynthesis accompanying exposure to chilling which limits CO_2 fixation (Allen 1995). As a result, CO_2 fixation is unable to keep up with the increased reduction of Photosystem I which leads to oxygen competing for electrons from Photosystem I (Allen 1995). Oxygen is consequently prematurely reduced to produce reactive oxygen species (Allen 1995).

The severity of cellular damage due to chilling is largely dependent upon the length of exposure of the plant to these temperatures (Hodges et al., 1997). Minimal damage was reported when "chilling sensitive" plants such as sunflower and tomato were exposed to chilling conditions for less than three days (Hodges et al., 1997). However, exposure for more than three days led to the inhibition of starch synthesis (Hodges et al., 1997). By way of contrast, some plants like wheat and Arabidopsis are "chilling-tolerant plants" and are able to withstand low but non-freezing temperatures without experiencing damage (Somerville, 1995).

Freezing temperatures

Just as plants display varying capabilities to tolerate chilling temperatures, there is considerable variation among plants with respect to tolerance towards freezing temperatures (Thomashow, 1998). While Arabidopsis is regarded as chilling-tolerant, adverse effects of exposure to freezing temperatures are reported to occur between -2°C to -8°C (Welti et al., 2002). However, Wanner and Junttila (1999) showed that Arabidopsis plants exposed to 1°C for one day were able to withstand freezing temperatures of at least -8°C and when kept at 1°C for two to three days the plants were able to withstand temperatures of at least -12°C (Wanner and Junttila, 1999). This improved capacity to withstand freezing following exposure to low but non-injurious temperatures is called acclimation and Arabidopsis is an example of a plant species able to acclimate. Acclimation is accompanied by the accumulation of starch, soluble sugars and proline and it allows Arabidopsis to develop a greater tolerance to withstand more severe freezing temperatures than an un-acclimated plant (Wanner and Junttila, 1999). Not all plants require acclimation to become freezing tolerant. Non-acclimated winter rye can withstand exposure to temperatures below - 20°C, a much lower temperature range than Arabidopsis (Krol et al., 1984).

Exposure to freezing temperatures can result in changes to the normal membrane composition of plant cells (Thomashow, 1998). The damage to membranes at freezing temperatures occurs when cells undergo dehydration as ice forms extracellularly (Thomashow, 1998). It is believed that the normal membrane lipid composition of plant

cells is adversely affected by the enhanced hydrolysis of lipids under these conditions (Welti et al., 2002) and from lesions formed on the membrane surface (Steponkus et al., 1993). Lipids are hydrolyzed during freezing stress due to the increased activation of phospholipases, such as phospsolipase D which hydrolyzes PtdCho to phosphatidic acid (Welti et al., 2002).

A common consequence of environmental stress associated with freezing temperatures is ice encasement (Hetherington et al., 1978). Ice encasement can be detrimental to a plant as it severely hinders gas exchange and this leads to the accumulation of anaerobic metabolites in plant cells that can interfere with the normal functioning of basic metabolic processes (Andrews and Pomeroy, 1979). A study on wheat seedlings undergoing ice encasement shows that ice encasement decreases the capacity of seedlings to tolerate cold temperatures and, with time, can lead to plant death (Andrews and Pomeroy, 1975). Ice encasement contributes to an accumulation of ethanol that can become toxic to the plant and higher than normal levels of CO_2 (Andrews, 1977).

SALINITY

Many areas across the globe experience highly saline conditions as either a natural feature of a given environment or as an outcome of human interference (Kozlowski, 1997). Highly saline environments are often found in arid to semi-arid regions

throughout the world (Neumann, 1997) and areas prone to tidal flooding (Kozlowski, 1997). Repeated application of water containing salt combined with the concentration of salts through evaporative water loss can eventually produce salt deposits, including salt encrustations, in environments not naturally prone to saline conditions. Human activities that increase salinity include the use of salt in de-icing roads leading to saline run-off entering roadsides (Kozlowski, 1997) or irrigating land with salt-containing groundwater (Flowers and Yeo, 1995).

Various approaches have been used to experimentally produce saline conditions under laboratory conditions. High environmental saline conditions have been re-created in controlled laboratory experiments by exposing plants to 300 mM NaCl for 10 days (Gagneul et al., 2007). In many experiments, there is no period of time allowed between no-salt and high salt exposure (Taji et al., 2004; Lee et al., 2003; Spicket et al., 1993). However, some researchers advocate incrementally raising the level of salt exposure to plants to allow plants to acclimate to an increasing osmotic challenge (Summers and Weretilynk, 1993; Ishitani et al., 1995; Gaxiola et al., 2001). For example, a more modest mimic of salt stress has been achieved by growing plants in vermiculite and irrigating pots with nutrient solutions or water containing 50 mM NaCl initially and then increasing the concentration of salt step-wise every three days to final concentrations up to 300 mM (Summers and Weretilnyk, 1993).

Salt stress negatively affects plants in at least two important ways, with both corresponding to a disruption of the homeostasis of previously maintained ionic and water

potential balances (Hagemann and Erdmann, 1997). An initial consequence of high saline soils is the development of a water deficit in the plant and this deficit causes a loss of turgor pressure (Rausch et al., 1996). Turgor loss is experienced at the level of individual cells when water loss exceeds uptake either as a consequence of low water availability or high external salt in the soil (Rausch et al., 1996). The maintenance of sufficient turgor pressure is required for cell elongation and is also crucial to the function of specific cells such as the guard cells in regulating stomatal aperture (Cutler et al., 1977). The ionic homeostasis is disrupted when high Na^+ and Cl^- content in the soil leads to the decreased uptake of important ions such as K^+ and Ca^{2+} required for the biochemical and metabolic maintenance of the plant (Niu et al., 1995).

IMPACT OF ENVIRONMENTAL STRESS

Exposure of crop plants to abiotic stress has been reported to decrease the productivity of important crops by at least 50% (Bray et al., 2000). Certain environmental and societal changes are predicted to occur in the future that will increase the adverse impact of these stresses (Kozlowski, 1997). For example, global climate changes are expected to alter precipitation patterns and increase the incidence of environments experiencing drought conditions along with population increases taxing already finite supplies of water (Chaves et al., 2003). Also, the requirement for irrigation in agriculture is increasing and an expected loss of 50% of arable land to excessive salinity has been predicted by the year 2050 (Wang et al., 2003). These problems will

require the development of more crop species with a stronger capacity to tolerate highly saline environments (Flowers and Yeo, 1995; Wang et al., 2003).

Fortunately, there is evidence that plants living under extreme environmental conditions can acquire stress tolerance traits leading to improved productivity and a greater capacity to adapt favourably to these conditions (Yancey et al., 1982). While local adaptation is one such mechanism contributing to improved tolerance traits, genetic engineering attempts geared to increase plant tolerance to stress has had some success in recent years (Hayashi et al., 1997).

STRATEGIES LEADING TO INCREASED ABIOTIC STRESS TOLERANCE

Plants have evolved different response mechanisms to cope with the detrimental effects caused by stress in the environment. For example, these responses, or adaptive strategies, can be initiated by exposure to insufficient or excess water in plants found in drought, saline, or flood prone environments. These responses are often categorized as either escape / avoidance, or osmotic adjustment strategies (Price et al., 1998).

An example of avoidance is drought escape. In this case plants avoid drought by completing their entire life cycle before the onset of severe water deficits in their environment (Price et al., 1998). Many short-lived desert plants use this strategy to cope with the perpetual lack of water in their native environment (Hopkins et al., 1995).

An example of a non-avoidance tolerance strategy involves use of a Na^+ / H^+ antiporter to compartmentalize potentially perturbing ions away from salt-sensitive biochemical processes (Apse et al., 1999). The increase of Na^+ ions in the vacuoles through the action of the Na^+ / H^+ antiporter causes the water potential of the cell to decrease. A developing reduction in root water potential allows for the formation of a water potential gradient that can drive water uptake by plant roots despite the low soil water potential typical of drying or saline soil (Kant et al., 2006). At the cellular level, in order to maintain plant cellular functions and prevent excessive water movement from the cytosol to the vacuole, the water potential of the cytosol must be in near equilibrium with that of the vacuole. This required balance is achieved through a process called osmotic adjustment (Yancey et al., 1982).

Osmotic adjustment

Osmotic adjustment offers protection against excessive water loss and alterations to the normal functioning of metabolic pathways (Nelson and Bohnert, 1998). This protection is achieved by increasing the capacity of cells to take up water and re-establish the homeostatic balance of ions (Nelson and Bohnert, 1998). Osmotic adjustment can proceed by either an energy "expensive" or "inexpensive" route depending on the severity / type of stress(es) and the availability of specific ions in the environment (Gagneul et al., 2007).

In the energy inexpensive route, osmotic adjustment is established through the uptake of ions such as NO_3^-, Na^+ and K^+ from the surrounding soil when, and if, readily

available sources of these ions are present in the immediate environment (Gagneul et al., 2007). However, if these ions are not appropriately compartmentalized in vacuoles they can disrupt the pre-existing cytoplasmic ionic homeostasis (Gagneul et al., 2007). This route is considered less expensive in terms of energy since there are no requirements for the production of carbon skeletons and so is more suited towards osmotic adjustment under low stress conditions (Gagneul et al., 2007). In contrast, the energy expensive route involves the accumulation of low molecular weight organic solutes (Gagneul et al., 2007). Under non-stressed environmental conditions these solutes or their precursors may serve in important metabolic processes in the plant but are redirected from these roles under stress (Gagneul et al., 2007).

OSMOPROTECTIVE SOLUTES

Organic solutes such as sugar alcohols and proline often accumulate in stress-tolerant plants when the plants are subjected to harsh environmental conditions such as high salt, desiccation, and extreme temperature variation (Rathinasabapathi, 2000). These organic solutes have been called "osmoprotectants" and are believed to aid the plant in tolerating osmotic stresses primarily through the prevention of turgor pressure loss from the cells and the restoration of the ionic balance (Weretilnyk et al., 1990).

Compartmentalization of organic, osmoprotective solutes in the cytoplasm of cells is believed to counter-balance the accumulation of perturbing ions in vacuoles

(Weigel et al., 1986). Ideally, organic ions must not perturb metabolism in the cytoplasm even at concentrations high enough to balance the high osmotic pressure in vacuoles and so have been called "compatible" osmotically active solutes (or osmolytes) as a consequence (Weigel et al.,1986). For example, when spinach was exposed to stress, the concentration of glycine betaine in the chloroplast was elevated from a modest concentration of 20 mM to a much higher concentration of 300 mM (Brouquisse et al., 1989). In spinach, exposure to 200 mM NaC led to a four to six-fold increase in glycine betaine content over that of non-salinized plants (Brouquisse et al., 1989).

Compatible organic solutes may serve to maintain the structure and therefore the inherent function of macromolecules necessary for normal cellular processes (Sakamoto and Murata, 2002). In addition, some osmoprotective compounds are believed to protect plant cells from the damaging effects of harmful reactive oxygen species that can be produced in greater amounts in plants experiencing stressful conditions (Bohnert and Shen, 1999).

Compatibility requires that the accumulation of organic solutes should not perturb plant metabolism (Galinski, 1993). The compatibility of these compounds is attributed to specific characteristics of these compounds such as high solubility and polarity in addition to the absence of charge at physiological pH (Galinski, 1993). Many compatible solutes are amino acid derivatives of *N*-acetylated amino acids, *N*-derivatized carboxamides of glutamine, ecoines, and betaines (Galinski, 1993). Other examples of common osmoprotectants synthesized and accumulated by plants under stressful

environmental conditions is the amino acid proline and carbohydrates including the polyol pinitol and disaccharide trehalose (Garcia et al, 1997).

Studies have found that very high concentrations of these solutes may be required to aid in the protection of a plant experiencing environmental stress (Bohnert and Shen, 1999). A reported concentration of 500 mM has been suggested as necessary for any protective effects to be seen but this is a concentration which is rarely reached by any osmolyte under in vivo conditions (Bohnert and Shen, 1999). However, it has been suggested that some osmolytes may protect particular membranes and proteins at concentrations other than the 500 mM estimated minimum and it is possible that the local concentration of solutes around specific surfaces is functionally more important for protective effects to be realized (Bohnert and Shen, 1999).

While compatible solutes can accumulate to high concentrations during periods of exposure to stress, many have important roles in plants aside from the stress tolerance response (Jolivet et al., 1982). For example, the amino acid proline is present in proteins and is particularly abundant in the proteins comprising the cell wall (Molinar et al., 2007).

GLYCINE BETAINE

The quaternary ammonium compound glycine betaine is an important organic solute accumulated by many plants when exposed to adverse environmental conditions (Rhodes and Hanson, 1993; Bray et al, 2000). This osmolyte is also found in animals,

micro-organisms and prokaryotes (Sakamoto and Murata, 2002). In organisms capable of accumulating glycine betaine the content of this compound can become very high (Sakamoto and Murata, 2002). For example, in plants that accumulate glycine betaine the content of this metabolite can range between 40 to 400 μmol ·g DW^{-1} (Rhodes and Hanson, 1993; Sakamoto and Murata, 2002). In plants that do not accumulate glycine betaine the content of this metabolite is typically $1/1000^{th}$ the level of an accumulating species (Rhodes and Hanson, 1993).

Typical of compatible organic solutes, glycine betaine carries a positively charged, fully substituted N group and a negatively charged carboxyl group (Fig. 1). This property makes glycine betaine a zwitterion where the differential charges can influence its ability to associate with the hydrophilic and hydrophobic portions of macromolecules such as proteins or membrane lipids (Ohnishia and Murata, 2006). In particular, one study reported that glycine betaine acts to maintain protein structure by allowing the formation of a hydration shell around a protein and avoiding direct contact between glycine betaine with the protein surface itself (Sakamoto and Murata, 2002). However, alternate theories on how glycine betaine protects protein structure suggest that under minimal water situations, water is freed by glycine betaine binding directly to the protein. As a consequence of this binding, the protein is protected from denaturation by allowing water to interact with the glycine betaine-bound protein domains (Sakamoto and Murata, 2002).

METABOLISM OF GLYCINE BETAINE

Glycine betaine synthesis has been well studied in several plants including spinach (Weigel et al., 1986), sugar beet (Russell et al., 1998), barley (Kishitani et al., 1994), and wheat (Allard et al., 1998). These plants are examples of glycine betaine accumulating species (Rhodes and Hanson, 1993; Waditee et al., 2007), although glycine betaine appears to present more broadly among plants albeit at much lower levels (Weretilnyk and Hanson, 1990). Examples of plants that do not accumulate glycine betaine are tobacco, Arabidopsis, and rice (Smith et al., 2000).

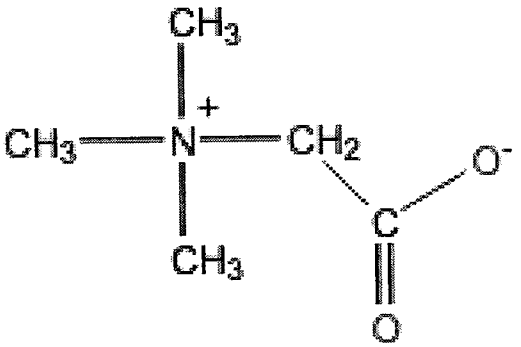
Glycine betaine synthesis proceeds by a two-step oxidation of choline and both reactions have been shown to reside in the chloroplasts of sugar beet and spinach (Russell et al., 1998; Weigel et al., 1986). In the first step, choline forms betaine aldehyde through the action of the enzyme choline monooxygenase (Rathinasabapathi et al., 1997). This enzyme has only been found in plants (Rathinasabapathi, 2000). The second step, producing glycine betaine, is catalyzed by the enzyme betaine aldehyde dehydrogenase (Weretilnyk and Hanson, 1989). Previous studies have shown that both enzymes show salt-responsive increases in activity when a plant is exposed to saline environmental conditions (Rathinasabapathi et al, 1993; Hanson et al., 1985; Weigel et al., 1986; Brouquisse et al., 1989). The rate of glycine betaine production through this pathway is also up-regulated when glycine betaine-accumulating plants are exposed to cold temperatures, drought, or saline environmental conditions (Hanson and Rhodes, 1983; Allard et al, 1998; Mou et al., 2002). However, Arabidopsis is an example of a plant that

does not accumulate glycine betaine and appears to lack genes encoding the enzymes required for the two-step biosynthetic pathway for this compound (Mou et al., 2002).

FIGURE 1: Chemical structure of glycine betaine

This figure was reproduced from the London South Bank University Applied Science website (http://www.lsbu.ac.uk/water/kosmos1.html).

Glycine betaine is a compatible organic solute accumulated by many organisms when subjected to water and salinity stresses in their environment . As shown in the figure, glycine betaine contains a positively charged, fully substituted N and a negatively charged carboxyl group, producing an electrically neutral zwitterion.


CH3
+
CH3
N
CH2
O-
C
CH3
O

CHOLINE SYNTHESIS IN PLANTS

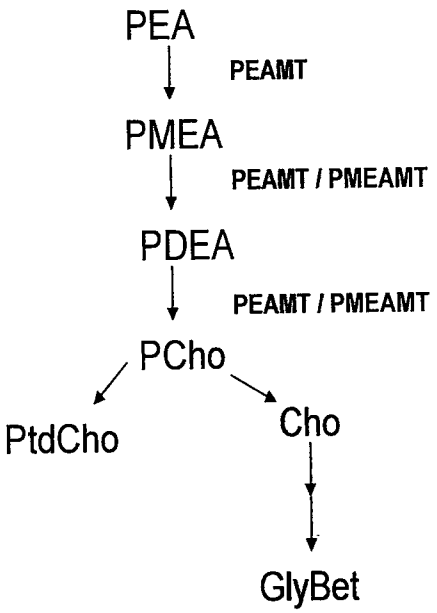
In plants, choline is synthesized by different routes depending on the particular plant species (reviewed by Nuccio et al., 2000). In spinach and sugar beet, choline is synthesized through what is termed the phospho-base pathway while in plants such as soybean, choline is synthesized through a phosphatidyl-base pathway. As such, reactions in spinach involve water-soluble intermediates while reactions in soybean involve phospholipid intermediates.

In spinach, the synthesis of choline involves three, sequential *N*-methylation reactions to produce phosphocholine (PCho) (Weretilnyk et al., 1995). PCho is then hydrolyzed to produce choline (Cho) that can then be oxidized to glycine betaine (Weretilnyk et al., 1995). The first reaction for Cho synthesis by the phospho-base pathway in spinach involves the *N*-methylation of phosphoethanolamine (PEA) to phosphomethylethanolamine (PMEA) by the enzyme phosphoethanolamine *N*-methyltransferase (PEAMT) using *S*-adenosyl- L -methionine as methyl donor (Fig. 2; Weretilnyk et al., 1995). The next two methylation reactions involve the conversion of PMEA to phosphodimethylethanolamine (PDEA) and PDEA to PCho (Weretilnyk et al., 1995). These are catalyzed by either PEAMT or phosphomethylethanolamine *N*-methyltransferase (PMEAMT) (Weretilnyk et al., 1995).

FIGURE 2: Choline synthesis pathway in *Spinacia oleracea* L.

In spinach, choline synthesis proceeds via a phospho-base pathway involving three sequential *N*-methylation reactions (figure adapted from Summers and Weretilnyk 1993). Arrows are incorporated as representing only foward reactions for the purposes of this review, but do not preclude possible reverse reactions.

Abbreviations used: Cho, choline; PEA, phosphoethanolamine; PMEA, phosphomethylethanolamine; PDEA, phosphodimethylethanolamine; PCho, phosphocholine; PtdCho, phosphatidylcholine; GlyBet, glycine betaine; PEAMT, PEA-*N*-methyltransferase; PMEAMT, PMEA-*N*-methyltransferase


PEA
PEAMT
PMEA
PEAMT / PMEAMT
PDEA
PEAMT / PMEAMT
PCho
PtdCho
Cho
GlyBet

PRIMARY ROLE FOR CHOLINE IN GLYCINE BETAINE NON-ACCUMULATING PLANTS

Choline is a ubiquitous and essential metabolite in plants. As PCho, choline can be used as a precursor for phospholipid phosphatidylcholine (PtdCho) (Smith et al., 2000). PCho can be used by the enzyme CTP:P-choline cytidylytransferase to form PtdCho, an important component of the phospholipids comprising the cellular membranes of a plant (Smith et al., 2000). In fact, PtdCho is the most prevalent phospholipid incorporated into plant cell membranes, contributing as much as 60% of all lipids in the membrane structure (Cruz-Ramirez et al, 2004). In addition, PtdCho has an important role in cellular signalling processes through the formation of phosphatidic acid. Phosphatidic acid is an important signalling component involved in processes such as root hair formation (Cruz-Ramirez et al., 2004).

N-METHYLATING ENZYMES OF THE PHOSPHO-BASE CHOLINE SYNTHESIS PATHWAY

The enzyme reported to control the flux rate of the choline synthesis pathway in Arabidopsis is PEAMT, and so this enzyme is considered to be the most important regulatory enzyme of this pathway (Mou et al., 2002). Choline synthesis and the activity of this enzyme have been shown to be feedback inhibited by PCho present in the plant (Hanson and Rhodes, 1983; Smith et al., 2000; Nuccio et al., 2000). Original work on choline metabolism showed that exogenous applications of choline to cell cultures or

tissue cultured plants would also down-regulate choline synthesis in the system under study (Mudd and Datko, 1989a; Mudd and Dakto, 1989b; Nuccio et al., 2000). The essential nature of this pathway was demonstrated in transgenic plants where PEAMT was silenced and those plants showed increased sensitivity to salinity and temperature-sensitive male sterility (Cruz-Ramirez et al., 2004).

The gene that encodes a product that converts PEA to PCho (PEAMT) is found on chromosome 3 in Arabidopsis at locus At3g18000 (Bolognese and McGraw, 2000). There are two more genes in the Arabidopsis genome whose products are annotated as encoding putative PEAMT products. These are both found on chromosome 1 (Cruz-Ramirez et al., 2004; At1g73600: AC079676; At1g48600: AY133811). The confirmed biochemical activities of these two gene products have not been reported. The gene at locus At1g48600 has been cloned and its biochemical activity is under study (BeGora, unpublished). The second putative-PEAMT gene is found at locus At1g73600 but its capacity to use PEA as a substrate has not been determined. An alignment of the two predicted protein products of genes on chromosome 1 with the protein encoded by At3g18000 show that the product of At1g73600 shares 85.1% and that of At1g48600 86.6% amino acid sequence similarity to Arabidopsis PEAMT (BeGora, unpublished). The high amino acid sequence similarity between these proteins encoded by the three genes is shown in Figure 3 and is suggestive of similar if not identical enzymatic activities for the three protein products.

FIGURE 3: Alignment of sequences encoding the three nuclear genes of Arabidopsis associated with loci At3g18000, At1g48600 and At1g73600

These three genes are annotated as being involved in the methylation of phosphoethanolamine and hence, implicated as playing a role in choline synthesis. Of these genes, only the product encoded by At3g18000 has been shown to encode phosphoethanolamine *N*-methyltransferase (PEAMT) by in vitro assays (Bolognese and McGraw, 2000) and catalyses all the three sequential *N*-methylations of PEA to PCho. The two genes encoding similar gene products are identified as At1g48600 and At1g73600 but the biochemical activities associated with their products has not yet been published. The product of At1g73600 shares 85.1% and that of At1g48600 86.6% amino acid sequence similarity to PEAMT (BeGora, unpublished).

```
At3g18000       ------------MAASYEEERDIQKNYWIEHSADLTVEAMMLDSRASDLDKEERPEVLS
At1g48600       ----------------------------MEHSSDLTVEAMMLDSKASDLDKEERPEVLS
At1g73600       MAHSHTNGAISPSFSKDLCEEREIQKNYWKEHSVGLSVEAMMLDSKASDLDKEERPEILA
                                             *** .*:********:***********:*:

At3g18000       LLPPYEGKSVLELGAGIGRFTGELAQKAGELIALDFIDNVIKKNESINGHYKNVKFMCAD
At1g48600       LIPPYEGKSVLELGAGIGRFTGELAQKAGEVIALDFIESAIQKNESVNGHYKNIKFMCAD
At1g73600       FLPPIEGTTVLEFGAGIGRFTTELAQKAGQVIAVDFIESVIKKNENINGHYKNVKFLCAD
                ::** **.:***:******** *******::**:***:..*:***.:*******:**:***

At3g18000       VTSPDLKITDGSLDLIFSNWLLMYLSDKEVELLAERMVGWIKVGGYIFFRESCFHQSGDS
At1g48600       VTSPDLKIKDGSIDLIFSNWLLMYLSDKEVELMAERMIGWVKPGGYIFFRESCFHQSGDS
At1g73600       VTSPNMNFPNESMDLIFSNWLLMYLSDQEVEDLAKKMLQWTKVGGYIFFRESCFHQSGDN
                ****:::: : *:***************:*** :*::*: * * ****************.

At3g18000       KRKSNPTHYREPRFYSKVFQECQTRDAAGNSFELSMIGCKCIGAYVKNKKNQNQICWIWQ
At1g48600       KRKSNPTHYREPRFYTKVFQECQTRDASGNSFELSMVGCKCIGAYVKNKKNQNQICWIWQ
At1g73600       KRKYNPTHYREPKFYTKLFKECHMNDEDGNSYELSLVSCKCIGAYVRNKKNQNQICWLWQ
                *** ********:**:*:*:**: .*  ***:***::.********:**********:**

At3g18000       KVSSENDRGFQRFLDNVQYKSSGILRYERVFGQGFVSTGGLETTKEFVEKMNLKPGQKVL
At1g48600       KVSVENDKDFQRFLDNVQYKSSGILRYERVFGEGYVSTGGFETTKEFVAKMDLKPGQKVL
At1g73600       KVSSDNDRGFQRFLDNVQYKSSGILRYERVFGEGFVSTGGLETTKEFVDMLDLKPGQKVL
                *** :**:.**********************:*:*****:*******  ::*********

At3g18000       DVGCGIGGGDFYMAEKFDVHVVGIDLSVNMISFALERAIGLSCSVEFEVADCTTKHYPDN
At1g48600       DVGCGIGGGDFYMAENFDVHVVGIDLSVNMISFALERAIGLKCSVEFEVADCTTKTYPDN
At1g73600       DVGCGIGGGDFYMAENFDVDVVGIDLSVNMISFALEHAIGLKCSVEFEVADCTKKEYPDN
                ****************:***.*****************:****.***********.* ****

At3g18000       SFDVIYSRDTILHIQDKPALFRTFFKWLKPGGKVLISDYCRSPKTPSAEFSEYIKQRGYD
At1g48600       SFDVIYSRDTILHIQDKPALFRTFFKWLKPGGKVLITDYCRSAETPSPEFAEYIKQRGYD
At1g73600       TFDVIYSRDTILHIQV----------------------------------------------
                :**************                                              -

At3g18000       LHDVQAYGQMLKDAGFTDVIAEDRTDQFMQVLKRELDRVEKEKEKFISDFSKEDYDDIVG
At1g48600       LHDVQAYGQMLKDAGFDDVIAEDRTDQFVQVLRRELEKVEKEKEEFISDFSEEDYNDIVG
At1g73600       ------------------------------------------------------------


At3g18000       GWKSKLERCASDEQKWGLFIANKN
At1g48600       GWSAKLERTASGEQKWGLFIADKK
At1g73600       -------------------------
```

A study by Cruz-Ramirez et al (2004) proposed redundant functions between members of the PEAMT gene family encoded at the At1g48600, At1g73600, and At3g18000 loci. In this study, mutant Arabidopsis plants were identified with a T-DNA insertion in the At3g18000 gene encoding a PEAMT product (Cruz-Ramirez et al., 2004). A decrease in the biosynthesis of PCho was observed in these mutants as well as severe disruption to the normal development of the roots (Cruz-Ramirez et al., 2004). It was the severity of the damaging effects to root structure in the At3g18000 mutants that raised questions regarding the products of members of the PEAMT gene family possibly contributing to PCho synthesis differently in areas of the plant where they are expressed at different levels. Support for this hypothesis has been found in microarray data showing the expression of the transcripts associated with At1g73600 and At1g48600 to be higher in leaves than At3g18000 whose expression was, in relative terms, higher in roots (Cruz-Ramirez et al., 2004). There is also microarray data available on the expression of the At1g73600 gene under conditions of stress such as cold, wounding, UV-B and chemical treatment (http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression).

GENETIC ENGINEERING TO INCREASE GLYCINE BETAINE PRODUCTION IN ARABIDOPSIS

Any attempt to increase the capacity of a plant to respond positively to environmental stress requires a thorough knowledge of all the pathways involved in the plants tolerance mechanisms and, as such, complete knowledge of all the enzymes involved in those pathways (Nelson and Bohnert, 1998),. Numerous genetic engineering attempts have been conducted and these studies have achieved some success in that the accumulation of certain compounds such as trehalose, fructans and ononitol are shown to produce a modest increase in the tolerance of transgenic plants with respect to abiotic environmental stresses (Nelson and Bohnert, 1998).

A study involving the transfer of a single gene encoding an enzyme involved in glycine betaine production was conducted to determine if its expression had any impact on the accumulation of glycine betaine in plants that do not usually accumulate this compound (Hayashi et al., 1997). This approach involved transforming Arabidopsis with the gene encoding the enzyme choline oxidase (*codA* from the soil bacterium *Arthrobacter globiformis*) responsible for converting choline to glycine betaine and led to a modest increase in the tolerance of the plants exposed to environmental stress (Hayashi et al., 1997). However, in a comparable approach, a modest increase in the capacity of transgenic tobacco plants to withstand abiotic stress was correlated with a slight increase in the content of glycine betaine (Nuccio et al., 1998). In this latter study glycine betaine accumulation was shown to be limited by an insufficient supply of choline.

RESEARCH USING T-DNA INSERTIONAL MUTANTS

T-DNA insertional mutants have been used to study the function of various genes in Arabidopsis (Alonso et al., 2003; Krysan et al., 1996). T-DNA insertion has become a valuable strategy for knocking out or knocking down the functions of genes. The use of T-DNA in Arabidopsis serves to both disrupt the expression of a specific gene and the presence of the T-DNA inserted in a gene allows for more easy identification of the disrupted gene through simple PCR reactions (Krysan et al., 1996). The known DNA sequence of the inserted T-DNA and the availability of a fully sequenced plant genome allows for primers to be designed that are specific to a target gene of interest and to the T-DNA itself (Krysan et al., 1996).

The use of T-DNA as an insertional mutagen has been applied successfully in studies designed to reveal the biological function of a specific gene (Ruegger et al., 1998; Winkler et al., 1998; Meissner et al., 1999). For example, the disruption of genes encoding products involved in the response pathway of ethylene was used to assess the utility of T-DNA insertion approach for the determination of gene function (Alonso et al., 2003). Screening for insensitivity to ethylene helped identify four of the genes in the ethylene response pathway. Therefore, through characterization of T-DNA mutant plants the role of genes that were not previously associated with ethylene responses were found to have a significant role in this signal transduction pathway.

T-DNA SALK LINES

The Arabidopsis Biological Resource Center at the University of Ohio (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm) offers seeds for purchase corresponding to many plant lines in which T-DNA insertions have been found in various genes. Multiple plant lines are available that have T-DNA insertions specifically associated with the At1g73600 gene. For example, the SALK lines designated: SALK_016929c, SALK_016929, SALK_062703, SALK_028641, SALK_017793, SALK_017121, SALK_128987 are all lines reported to have a T-DNA tag within the Atg73600 gene (http://signal.salk.edu/cgi-bin/tdnaexpress). The designation of "c" on the identification number of some lines refers to seeds for plants reported to have confirmed homozygosity for the presence of a T-DNA insertion.

MATERIALS AND METHODS

All chemicals used were purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON) unless stated otherwise. All solutions were prepared with de-ionized water purified by a Barnstead NANOpure II water purification system (SYBRON / Barnstead, Dubuque, IA). All primers were synthesized by The Institute for Molecular Biology and Biotechnology (MOBIX) (http://www.science.mcmaster.ca/mobix/).

PLANT MATERIAL

Seeds

Seeds for the Arabidopsis thaliana wild-type CS 6000 and SALK lines were purchased from the Arabidopsis Biological Resource Center at the University of Ohio (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). The four Arabidopsis T-DNA SALK lines used in this study (SALK_016929c, SALK_016929, SALK_062703, SALK_028641) are shown in Table I.

TABLE I: Four Arabidopsis SALK lines obtained from ABRC were selected to screen for presence of a T-DNA insertion associated with locus At1g73600

SALK line identification number	**Parent line**	**Homozygosity for T-DNA insertion reported upon seed purchase confirmed/not confirmed**
016929c	CS_60000	confirmed
016929	CS_60000	not confirmed
028641	CS_60000	not confirmed
062703	CS_60000	not confirmed

Seed Surface Sterilization

Approximately 15 seeds were placed in 1.5 mL microfuge tubes for surface sterilization. To each tube 1 mL of 70% v/v ethanol was added. The tubes were inverted periodically for 2 min. The ethanol was poured out of the tubes, and 1 mL of seed sterilization solution consisting of 30% v/v commercial Javex 5 bleach (Clorox Company of Canada, Brampton, ON) and 0.1 % v/v Tween 20 (Sigma, Lot. No. 74H101015) was added. Each tube was inverted periodically for 10 min. The sterilization solution was poured off, and seeds rinsed five times with 1 mL of sterile H_2O discarding the water each time. A 1 mL volume of 0.1 % w/v sterile phytablend (Caisson Laboratories, Cat. PTC001, Lot. No. 10601505, Rexburg, ID) was added to suspend the seeds. The seeds were subsequently incubated overnight at 4°C.

Seed Germination

Surface sterilized seeds were transferred to Murashige and Skoog (MS) plates (Murashige and Skoog, 1962) containing 1.0 % w/v sucrose (Fisher Scientific, Lot. No. 044832, Fair Lawn, NJ), 1.0 mL MS vitamins (Sigma, Lot. No. 14K2353), and 0.215 g Murashige and Skoog Salt Mixture (Sigma, Lot. No. 027K23061). The outside of the plates were sealed with micropore surgical tape and placed inside an incubation chamber (Percival 50036, Boone, IA) set for a 24 hour photoperiod at 22°C for 7 to 10 days.

Seedling Growth Conditions

Seedlings from the MS plates were then transplanted to sterile Promix BX soil (Premier Horticulture Inc., Quakertown, PA) in black, rectangular 7.7 cm x 4.4 cm plastic pots (The Lerio Corp, Mobile, AL), and placed in a Conviron CMP 3244 (Conviron, Winnipeg, MA) controlled environment chamber. The chamber was maintained at 22°C with a 12 h photoperiod and a light intensity of 120 μmol m^{-2} s^{-1}. Once in the chamber, plants were misted daily with sterilized H_2O for one week after which the soil was watered as needed until leaves of the plant were selectively harvested. To avoid possible cross pollination between the different lines, mutants of different genotypes and wild-type plants were separated in the growth chamber (Kliebenstein, 2004; Schierup et al., 2001).

Tissue Harvesting and Seed Collection

Leaf material was usually harvested from plants four weeks after transplant. Tissue was removed using sterile tweezers and then placed into sterile 1.5 mL microfuge tubes. The tubes were closed and then the contents flash-frozen by immersing the tubes in liquid nitrogen. The tubes were then placed into a -80°C freezer for storage. Seeds were collected from plants when the siliques were brown, approximately 8 weeks after transplant. Seeds were stored at room temperature in 5 X 3 in. envelopes.

EXTRACTION AND ANALYSIS OF NUCLEIC ACIDS

DNA Preparation

One leaf per plant, approximately 2.5 cm long and 0.5 cm wide (between 25 – 50 mg) was harvested and dropped directly into liquid nitrogen. Leaves were then ground by hand with disposable pestles (VWR International, Mississauga, ON) at room temperature for approximately 10 s in 400 µL of extraction buffer containing 200 mM Tris-HCL (Tris; BioShop, Lot. No. 764661) pH 7.5, 250 mM NaCl (BioShop, Lot. No. 4F1801, Burlington, ON), 25 mM EDTA (Ethylenedinitrilotetraacetic acid; EMD Chemicals Inc., Lot. No. 45082519, Darmstadt, FRG), and 0.5% w/v SDS (sodium dodecyl sulfate, BDH Inc., Lot. No. 107318/28914). The macerated tissue was vortexed for 5 s then the tubes were incubated at room temperature for 60 min. Following incubation, tubes were centrifuged for 1 min at 16100 g in a 5415 D Centrifuge (Eppendorf, Cat. No. 0061966, Hamburg, FRG) and then 300 µL of supernatant was transferred to a new sterile 1.5 mL microfuge tube. An equal volume of isopropanol (Burdick and Jackson, Lot. No. COO44, Muskegon, MI) was added to the supernatant, the contents mixed by inversion and then incubated at room temperature for 2 min. The mixture containing precipitated DNA was centrifuged for 6 min at room temperature, the supernatant discarded, and the DNA pellet dissolved using 25 µL of H_2O (Edwards, et al., 1991). The solution containing DNA was stored at -20°C until it was used.

RNA Preparation

Unless otherwise stated, all steps were carried out at room temperature. RNA was extracted from Arabidopsis leaf tissue using the Qiagen RNeasy Mini Kit (50) (Cat. No.74104, Lot. No. 41525549) following the protocol described by the manufacturer. Two leaves, each approximately 2.5 cm long and 0.5 cm wide (between 25-50 mg), were harvested and flash frozen immediately in liquid nitrogen. The leaves were ground by hand with a disposable pestle in 450 µL of extraction buffer containing guanidinium thiocyanate and 4.5 µL of 14.3M β-mercaptoethanol (Sigma, Lot. No. 39F-060715). After vigorous vortexing, the lysate was pipetted unto a QIAshredder spin column and centrifuged at 16100 g for 2 min in a 5415 D Centrifuge. The supernatant was transferred to a sterile 1.5-mL microfuge tube, 225 µL of absolute ethanol (Commercial Alcohols Inc, Brampton, ON) was added and the contents mixed thoroughly by pipetting. From this sample, 650 µL was transferred to the membrane of a RNeasy Mini Column and then they were centrifuged together for 15 s at 9300 g. The column flow-through was discarded and then 700 µL of "Buffer RW1" containing ethanol and guanidinium thiocyanate was dispensed onto the membrane of the spin column and centrifuged as before at 9300 g for 15 s. The flow-through was again discarded and then 500 µL of "Buffer RPE" containing ethanol was dispensed by pipette onto the membrane. The entire column containing solution was centrifuged for 15 s at 9300 g. The flow-through fraction was discarded and another 500 µL volume of "Buffer RPE" was added to the membrane and centrifuged at 16100 g for 1 min. After the flow-through was discarded

the RNeasy column was transferred to a sterile, 1.5 mL collection tube and RNA was eluted from the membrane using 30 μL of RNase-free H_2O. The H_2O was dispensed directly onto the membrane and the column with contents were centrifuged for 1 min at 9300 g. The tubes containing column eluate were closed and then flash-frozen in liquid nitrogen and stored in a -80°C freezer prior to use.

Screening for the T-DNA insertion by Polymerase Chain Reaction (PCR)

Left and right gene-specific primers and T-DNA-specific left border primers were designed using information provided by the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/tdnaprimers.html; Alonso et al. 2003). The basic strategy for screening for the presence of a T-DNA insertion by PCR is shown in Figure 4 (http://signal.salk.edu/tdnaprimers.2.html).

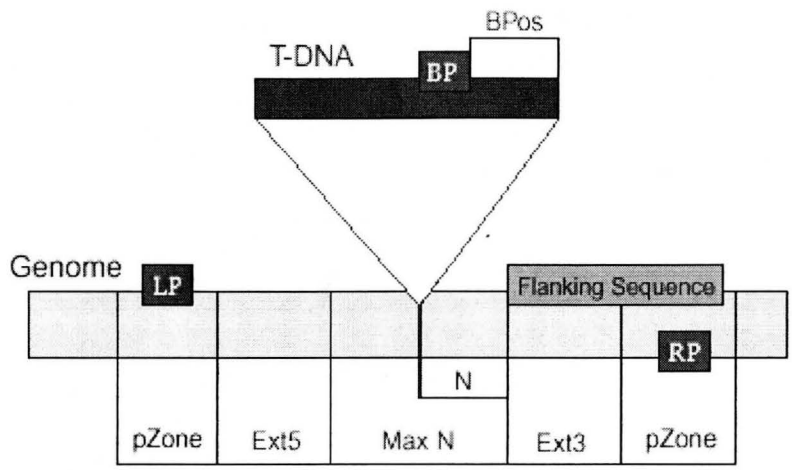
FIGURE 4: Three-primer design to identify T-DNA insertion mutants associated with At1g73600

Panel A is a figure taken from the Salk Institute Genomic Analysis Laboratory website (http://signalsalk.edu/tdnaprimers.2.html) and it shows how the three-primer design is used for detection of T-DNA insertion in alleles of At1g73600 Panel B is a figure taken from the Salk Institute Genomic Analysis Laboratory website (http://signalsalk.edu/tdnaprimers.2.html) and it shows the banding patterns produced by PCR amplification of DNA using left and right gene-specific primers and a left border primer specific to the T-DNA insert.

Explanation of Terms and Abbreviations used in Panel A:

pZone: 100 bp regions of genome used to pick up left and right genomic primers; **N**: 0-300 bp region of genome between T-DNA insertion site and 5'end of gene flanking sequence; **MaxN**: Maximum region of genome between T-DNA insertion site and flanking sequence of gene, set to default value of 300 bp; **Ext3, Ext5**: Genomic regions between the MaxN and pZones not used to pick up left and right genomic primers; **BPos**: Length of T-DNA insert from 3' end of left T-DNA-specific border primer to T-DNA insertion site.

A

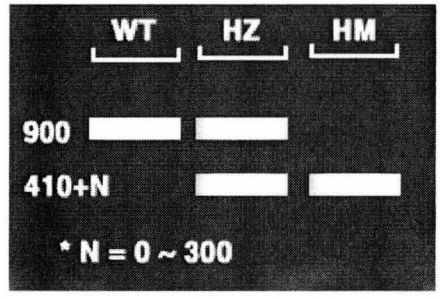


BP- Left T-DNA border primer

LP- Left gene-specific primer

RP –Right gene-specific primer

B



WT – Wild-type – LP + RP
HZ – Heterozygote – LP + RP + BP
HM – Homozygote – BP + RP

The left and right gene-specific primers were designed to amplify a product of 900-1000 bp with the exact size dependent upon the specific SALK line as seen in Figure 4B. The left border primer specific to the T-DNA was selected to amplify a smaller product of about 450-800 bp, also seen in 4B. Identification and basic information on the left and right primers specific to each SALK line and the left border primers used to screen for T-DNA insertion in the alleles of At1g73600 are reported in Table II. The left and right gene-specific and left border T-DNA primers were synthesized by MOBIX (http://www.science.mcmaster.ca/mobix/), and diluted to 25 pmol / μL for use.

Calculations for primer dilutions were performed using an Excel Spreadsheet program and optical density value provided by The Institute for Molecular Biology and Biotechnology (MOBIX, http://www.science.mcmaster.ca/mobix/). Information provided by Oligo calculator was used to determine the optimal melting temperature of each primer for PCR reactions (http://www.pitt.edu/~rsup/OligoCalc.html). The initial melting temperature used for each primer was determined by inputting the sequence into Oligo Calculator and decreasing the calculated temperature by 5°C. If multiple products were obtained, the stringency of the primers was empirically tested by increasing the melting temperature by increments of 2°C until one product was obtained.

The PCR reaction designed to screen for the presence of a T-DNA insertion at At1g73600 contained final concentrations of 25 pmol / μL each of left and right gene- specific primers and the left border-specific T-DNA primer, 10 mM dNTP mix, 25 mM $MgCl_2$, 10 x PCR buffer containing 20 mM Tris-HC pH 8.8, and 1 μL of Taq DNA polymerase

(Fermentas, Burlington, ON). An Eppendorf Mastercycler thermocycler (Eppendorf, Lot. No. 5345 003558) was used to carry out the PCR reactions. The initial denaturation step was carried out at 94°C for 3 min and was followed by 40 cycles of denaturation at 94°C for 30 s, annealing at the optimum primer-specific temperature for 50 s, and extension at 72°C for 1 min ending with final extension at 72°C for 10 min. The annealing temperatures usually fell between 52°C-62°C for the primers used in this research (see Table II). DNA products amplified by PCR were stored at -20°C, or resolved immediately by agarose gel electrophoresis.

TABLE II: Primers used in PCR reactions used to screen SALK Arabidopsis lines for presence of T-DNA at locus At1g73600

SALK Line or T-DNA	Position	MOBIX Primer ID(all stocks)	Sequence	Optimal Tm
016929c (016929)	Left	ML 07-1100 ML 07_17514 ML 07_19980 ML 07_10399 ML 07_250	5'-TCCCGGTTCCACTAAAGAATC-3'	52° C
	Right	ML 07-1101 ML 07_17515 ML 07_19981 ML 07_10398 ML 07_251	5'-AGAACAAGCATGGACGTGAAC-3'	52° C
028641	Left	ML 07-19976	5'-TTAAAGGAACCTCACAGCCAC-3'	50° C
	Right	ML 07-19977	5'-TATGCTGGATCTGAAACCTGG-3'	52° C
062703	Left	ML 07-19974	5'-ATGTAAACCGAAATCGGAAGC-3'	52° C
	Right	ML 07-19975	5'-GAAAGAACAAGCATGGACGTG-3'	52° C
T-DNA (all lines)	Left border #1	ML 07-249 ML 07_10400 ML 07_18283 ML 07_19860 ML 07_20572	5'-GCGTGGACCGCTTGCTGCAAC T-3'	52°C
	Left border #2	ML 07-19276	5'-TGGTTCACGTAGTGGGCCATC G-3'	58°C

cDNA Synthesis and Analysis

RNA prepared using the RNeasy kit was used for cDNA Synthesis. cDNA copies were produced using Superscript II Reverse Transcriptase RNase H (Invitrogen, Cat No. 18064-014 Lot No. 1297384, Carlsbad, CA). The first strand synthesis required final concentrations of 25 pmol /μL of forward and reverse primers (see Table III), 10 mM dNTP mix, 5x First-Strand Buffer (Invitrogen; 250 mM Tris-HCL pH 8.3, 375 mM KCL, and 15 mM $MgCl_2$), 0.1 M DTT (dithiothreitol; Invitrogen), and 1 μL of RNaseOUT Ribonuclease Inhibitor (40 units / μL; Invitrogen, Cat. No. 10777-019).

The cDNA was amplified by PCR using left and right specific primers to the Arabidopsis actin gene on chromosome 2 associated with locus At2g37620. The left primer specific to the actin gene was designed to anneal to an exon sequence that straddled either side of an intron. This design was used to ensure that RNA and not DNA was amplified during the cDNA synthesis and subsequent PCR reaction step.

TABLE III: Information regarding the actin and At1g73600 gene-specific primers used in RT-PCR reactions

	Actin		**At1g73600**	
	Left primer	**Right primer**	**Left primer**	**Right primer**
MOBIX Primer ID	ML 07-2774	ML 07-2775	ML 07-1332	ML 07-1333
Sequence	5'-CGTCTTGATCTTGCTGGTCGTGAC C-3'	5'-CGTCTTGATCTTGCTGGTCGTGAC C-3'	5'-CTGAATTAGCTCAGAAGGCC-3'	5'-CAGAACCAGTATGCTGGT-3'
Optimal Tm	62° C	62° C	60° C	60° C
Stock concentration	728 pmol /µL	822 pmol /µL	836 pmol /µL	852 pmol /µL

Gene-specific primers were designed to anneal to sites flanking an intron of At1g73600 using information obtained from The Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/isect.2.html). As for the actin, this strategy would ensure that cDNA from RNA and not trace contamination from genomic DNA would be amplified in the RT-PCR reaction, as the RNA should have introns spliced. Figure 5 shows how the design of gene-specific primers allowed for discrimination between cDNA and DNA. The RT-PCR procedure with actin primers was used as an internal control to assess the quality of the RNA isolated from the leaf tissue. Any difference in the intensity of the bands associated with the actin product is used to help interpret a variation in the intensity of the bands associated with the gene-specific primers. In this way, any absence of product amplified using gene-specific primers in the presence of a strong actin product from the same RNA sample would not be interpreted as being the consequence of degraded mRNA but more likely due to the absence of transcripts associated with this gene in the original tissue.

FIGURE 5: Primer design for actin gene-specific (locus At2g37620) left and right primers

The sequence was developed using information provided by The Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/isect.2.html). The right primer was designed to correspond to the exon sequence that spanned the intron. This was done to reduce the likehood of amplifying genomic DNA encoding actin. Lower case letters correspond to nucleotides associated with introns and upper case letters correspond to nucleotides associated with exons.

ACGTCCTGAGgtttatttcgactcacccactataaagttatcatctttgctatatataga
tcattttgagatatgtatgccttgatgagttgaatctttgctgtcctttattgtgttatg
gacttatgaaggaaagcttctaaaggcttaaattatgtgtgtgtttgtgtcagATACTTG
CGTTTCTTCCACCTATTGAAGGGACAACAGTGCTAGAGTTTGGTGCTGGAATTGGTCGTT
TTACTACTGAATTAGCTCAGAAGGCCGGCCAGGTCATTGCGGTTGACTTCATTGAAAGTG
TTATCAAAAAGgttgttagttcacgtccatgcttgttctttctagctacttataagactt
gtatttttctcgagttttgtttgatttttttctctaatgagagtttcatctccagAATGA
GAACATTAACGGTCACTACAAGAACGTCAAATTTCTGTGCGCTGATGTCACATCACCAAA
TATGAACTTTCCAAATGAGTCTATGGATCTGATATTCTCCAACTGGCTGCTAATGTATCT
CTCTGATCAAGAGgtagccatattatttattactgagggaaccacttttaaactgtacac
Acttagactaatcgcttatatcccttggacaaattttcatgtcttttcatcaaattgcag
GTTGAAGATTTGGCGAAAAAGATGTTACAATGGACAAAGGTTGGCGGGTATATTTTCTTT
CGGGAGTCTTGTTTCCATCAGTCTGGTGATAACAAGCGGAAGTACAACCCAACACACTAC
CGTGAACCTAAATTTTACACAAAGgtgtgctacctataaatccctccactcttctgtttc
tagacgacaattatttgaactctggtacatgaattcctctcagCTTTTCAAAGAATGCCA
TATGAATGACGAAGATGGGAATTCGTATGAACTCTCTTTGGTTAGCTGTAAATGCATTGG
AGCTTATGTGAGAAACAAAAAGAACCAGAACCAGgtaattggcctcttcatcttttagcc
acccttttgatggtatctcttataatgtttggactcatatttttcgaaaatttcagATATG
CTGGCTTTGGCAGAAAGTCAGTTCGGATAATGATAGGGGCTTCCAACGCTTCTTGGACAA
TGTCCAGTATAAGTCTAGTGGTATCTTACGCTATGAGCGTGTCTTTGGAGAAGGGTTTGT
TAGCACAGGGGGACTCGgtatgctctttagtcatcgagtttgtatcaactttgcagtatt
gatggttctgtttttgaatttaagttatacagaaccattaagtgaagctatcttttcttc
ttattatacttgtttctttgatatagAGACAACAAAGGAATTCGTGGATATGCTGGATC
TGAAACCTGGCCAAAAAGTTCTAGACGTTGGGTGCGGAATAGGAGGAGGGGACTTCTACA
TGGCTGAGAACTTTGACGTGGATGTTGTGGGCATTGATCTATCTGTAAACATGATCTCTT
TTGCGCTTGAACACGCAATAGGACTCAAATGCTCTGTAGAATTCGAAGTAGCTGATTGCA
CCAAGAAGGAGTATCCTGATAACACCTTTGATGTTATTTATAGCAGAGACACCATTCTAC

The PCR amplification of cDNA involved final concentrations of 25 pmol / uL of each forward and reverse primer, 10X PCR buffer containing 20 mM Tris-HCL pH 8.8, 10 mM dNTP mix, 25 mM $MgCl_2$, and 1.0 µL Taq DNA polymerase (Fermentas, Burlington, ON). The first denaturation step was carried out at 94°C for 3 min and it was followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 50 s at 60°C-62°C (depending on the primers used, see Table III), and extension for 1 min at 72°C. The final extension was carried out for 10 min at 72°C. Preparation and dilution of primer stocks to 25 pmol/uL and selection of suitable annealing temperatures was described earlier. RT-PCR products were analyzed by gel electrophoresis on 1.5% w/v agarose gels in TAE buffer.

Gel Electrophoresis of PCR and RT-PCR products

DNA products were separated by electrophoresis using a 1.5% (w/v) agarose (BioShop, Lot No. 6G1437) gel. A Bio-Rad Mini-Sub Cell GT (BioRad Laboratories, Mississauga, ON) gel apparatus was used for the procedure. The gel was prepared using 800 µL of 50X TAE buffer stock (0.4 M Tris-base pH 8.0, 0.01 M Na_2EDTA, 0.2 M sodium acetate (Caledon Laboratories Ltd., Lot. No. 61362, Georgertown, ON) diluted with 39.2 mL H_20 to a final volume of 40 mL, to which 0.40g of agarose (BioShop, Lot. No. AGA001.100) was added. The solution was heated in the microwave for 3 x 15 s, with the flask being swirled after each 15 s. To the heated and slightly cooled solution containing molten agarose, 2 µL of 10 µg / µL ethidium bromide stock solution (Boehringer Mannheim, Lot. No. 12499420-19, Darmstadt, FRG). A volume of 8 uL of

PCR product was mixed with 2 uL of 6X loading dye (10 mM Tris-HCL pH 7.6, 0.03 % w/v bromophenol blue, 0.03 % w/v xylene cyanol FF, 60 mM EDTA, 60 % v/v glycerol). To determine the size of the amplified products a lane containing 0.50 µL of 1 ug / µL 1 Kb Plus DNA ladder (Invitrogen, Cat. No. 10787-018) was combined with 2 µL of 6X loading dye and 8 µL of H_20 was run on each gel. Electrophoresis was performed at a constant voltage of 60 V for 1.5 h on a PowerPac 300 V power supply (Biorad, Cat No. 165-5050). DNA fragments were visualized on a transilluminator (ImageMaster VDS, Pharmacia BioTech), and photos taken using a Fujifilm Thermal imaging FTI-500 camera (Fuji, Japan).

RESULTS

Analysis of expression pattern associated with the At1g73600 gene product

To determine whether the At1g73600 gene is transcribed in Arabidopsis, microarray data from The Arabidopsis Information Resource (TAIR) microarray database (http://www.arabidopsis.org) was analyzed. The signal given on this website is a measure of the relative level of gene expression of the transcript determined under specific conditions and this signal correlates directly to the strength of hybridization between the abundance of message and its hybridization to oligos associated with the At1g73600 gene product on an Affymetrix Gene Chip (http://www.arabidopsis.org). An equal to or greater than 1000 signal intensity was taken as significant for the purpose of this analysis. The only significant expression of the At1g73600 gene in wild-type Arabidopsis plants was found to be in the rosette leaves where a signal intensity of 3954 was recorded and in the shoots (defined as comprising the stem, leaves, and flowers) with a signal of 1618 (Table IV).

Additional microarray data provided by Genevestigator (https://www.genevestigator.ethz.ch/) on relative At1g73600 gene expression at different developmental stages of wild-type Arabidopsis shows that the highest expression associated with the At1g73600 locus in rosette leaves to occur following 18.0-20.9 days

TABLE IV: Transcript abundance associated with At1g73600 in different organs of Arabidopsis compiled using data reported on the Arabidopsis Information Resource (TAIR) microarray database (http://www.arabidopsis.org) (Site accessed January 11, 2006). Plants were grown under long days at a light intensity of 125 μmol $m^{-2}s^{-1}$ and at day temperature of 25°C and a night temperature of 20°C.

Organ	**Developmental Stage**	**Signal Intensity[a]**
Rosette leaf	15	3954
Flower	29	407
Stem	29	634
Shoot (stem, leaves, and flowers of plant)	18	1618
Root	18	381
Sepal (modified leaf of flower)	21	298
Petal	21	24
Stamen (male organ of flower)	21	434
Carpel (female organ of flower)	21	244

[a] A signal intensity equal to or greater than 1000 was taken as significant for the purpose of this analysis

of growth followed by a second peak of transcript abundance at 29.0 – 35.9 days of growth. These growth stages are shown in the cartoon of Figure 6.

FIGURE 6: Standardized age and development comparisons for Arabidopsis

This figure was adapted from one available at the Genevestigator website (http:www.genevestigator.ethz.ch/at/index.php)

Growth stages in Arabidopsis development are standardized and correspond to the days following germination. Available microarray data shows transcripts associated with At1g73600 to be highest during leaf development in rosettes (http:www.genevestigator.ethz.ch/at/index.php).

	Developmental Stage	Age (Days)
	Seed germination	1.0 – 5.9
	Leaf development – cotyledons open 2 rosette leaves	6.0 – 13.9
	Leaf development – 4 rosette leaves	14.0 – 17.9
	Leaf development – 8 rosette leaves	18.0 – 20.9
	Leaf development – 13 rosette leaves	21.0 – 24.9
	Inflorescence emergence	25.0 – 28.9
	Flower production – first flower open	29.0 – 35.9
	Flower production – 50% of flower buds to be produced open	36.0 – 44.9
	Silique shattered	45.0 – 50.0

Screening for the presence of a T-DNA insertion

Leaf material from SALK_016929c, SALK_016929, SALK_028641, and SALK_062703 T-DNA SALK and wild-type Arabidopsis lines was harvested for DNA extraction. PCR reactions using left and right At1g73600 gene-specific primers and a T-DNA-specific left border primer were then used to identify the presence of the T-DNA insert in individual plants. The size of the products formed by these three primers was used to distinguish plants that have no T-DNA insert from plants heterozygous or homozygous for the presence of T-DNA (Fig. 4B).

The presence of a band in the 900-1000 bp molecular size range among the PCR products would indicate that the plant has a wild-type genotype in that there is no T-DNA insert in either allele of the gene of interest (Fig. 4B). The presence of a single PCR-amplified DNA product in the 450-850 bp size range would signify the presence of a T-DNA insert in both alleles of the gene and hence a plant homozygous for the mutation (Fig.4B). The presence of two bands among the products, one of ca 1000 bp and a second approximately half the size would indicate a plant heterozygous for the T-DNA insert (Fig. 4B). Additional PCR reactions confirmed which two primers from the original three-primer PCR screening reaction were responsible for the product visualized on a gel. For example, in a plant homozygous for the T-DNA insertion no PCR product would be expected if only the gene-specific primers on either side of the T-DNA insertion were used for amplification due to the large size of the insert. The size of the insert was 12841 bp (http://signal.salk.edu/pBIN-pROK2.txt-new). Likewise, a wild-type plant should not

yield a PCR product if only the left border primer of the T-DNA and the right gene-specific primers are used. This approach also confirmed that the smaller product of ca 300 bp arose due to the presence of a T-DNA insert. The predicted size range for each PCR product specific SALK line is provided by the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/tdnaprimers.2.html) (Table V).

A product of ca 450 bp was consistently found amplified from wild-type and all SALK line DNA when the three primers were used. This band was found to be an artefact in that it was produced when the left border primer #1 was used alone but was not found when only the left gene-specific, right gene-specific, or left border #2 primers were used on the same DNA extracts under identical PCR conditions.

Table VI outlines the Arabidopsis plants from each T-DNA SALK line that were either homozygous or heterozygous for T-DNA insertion or wild-type with respect to the At1g73600 gene.

TABLE V: Four SALK lines were screened for the presence of T-DNA insertions in At1g76300 based on the presence or absence of PCR-amplified DNA products of predicted sizes given below (http://signal.salk.edu/tdnaprimers.2.html). The SALK line-specific primers used for PCR reactions are reported in Table II.

Arabidopsis SALK line identification	**Estimated PCR-amplified product sizes** ***bp***		
	Wild-type	**Heterozygous for T-DNA**	**Homozygous for T-DNA**
016929c[a]	1042	1042 451 - 751	451 - 751
016929	1042	1042 451 - 751	451 - 751
062703	1009	1009 458 - 758	458 - 758
028641	981	981 431 - 731	431 - 731

[a] c designates confirmed line

TABLE VI: Screening by PCR identified a number of Arabidopsis plants from each T-DNA SALK line that were either homozygous or heterozygous for T-DNA insertion or wild-type with respect to the At1g73600 gene

SALK line identification	Homozygous	Heterozygous	Wild-type
016929c	7	0	0
016929	0	0	2
028641	0	2	9
062703	5	7	3

Plants from the SALK_062703 line were screened for the presence of T-DNA (Fig. 7). Two PCR reactions were carried out. In the first reaction, PCR #1, a single product estimated to be 1009 bp was amplified when DNA extracted from wild-type plants was used. No bands were detected with any plants of the SALK_062703 line. This reaction used At1g73600 gene-specific right and left primers (ML 07_19975 and ML 07_19974; Table II). In PCR #2, the T-DNA specific left border primer #2 (ML 07_19276:Table II) was used with the At1g73600 gene-specific right primer yielding no band when DNA from wild-type plants was used and a single band of 711 bp in each of the SALK,_062703 plants tested. This was consistent with the expected 458 bp to 758 bp range predicted if a T-DNA insertion in the SALK_062703 line was present based upon information provided by the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/tdnaprimers.2.html; Table V).

In both cases, the size of the bands produced by PCR were determined using the equation of the line given by the Rf values of the 1 Kb plus DNA ladder standards shown in the first lane of PCR#1 and #2 in Figure 7.

Since PCR #1 was carried out without the T-DNA-specific left border primer, any product corresponding to ca 1009 bp (Table V) would indicate a wild-type or heterozygous genotype for individual plants of the SALK_062703 line. This product was only amplified from the Col-O wild-type and was absent from PCR products of the SALK_062703 plants indicating that the six plants screened from this line are homozygous for the presence of T-DNA (Fig. 7).

FIGURE 7: Confirmation of SALK_062703 plants homozygous for the presence of T-DNA associated with the At1g76300 locus.

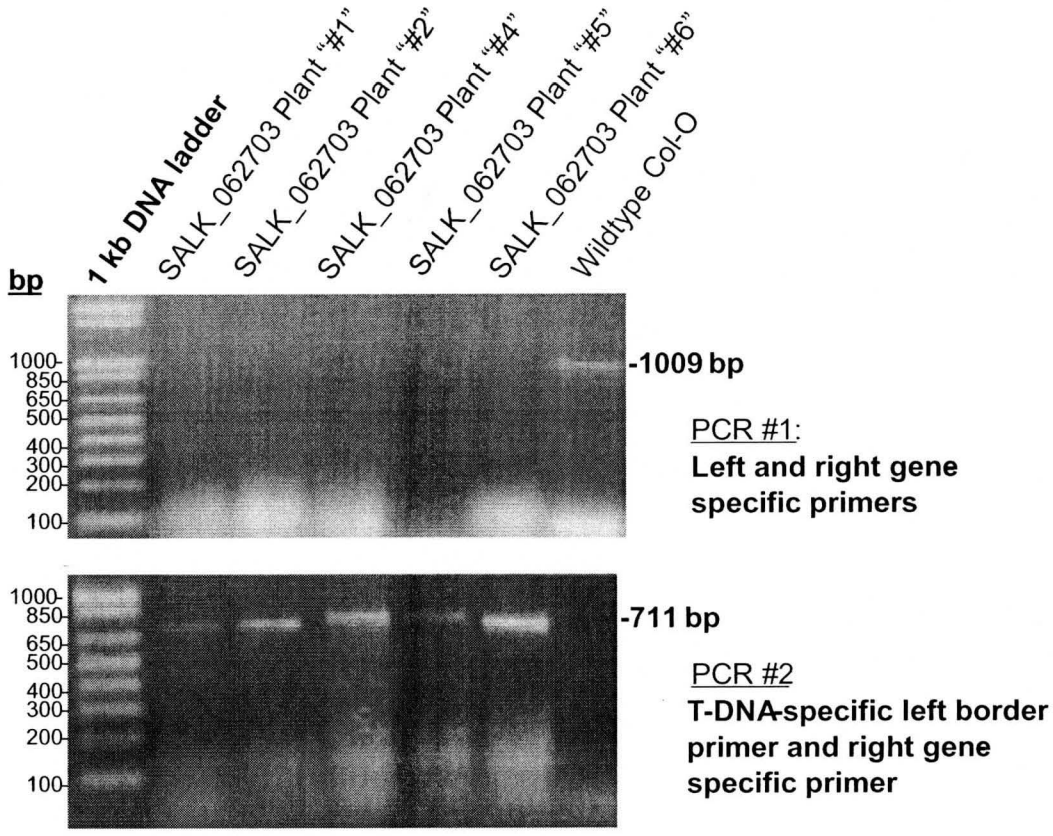
Six plants transplanted May 01 / 07 were used to screen for the presence of a T-DNA insertion by PCR. Reactions included DNA from a wild-type Col O and five SALK_062703 individuals (designated 1, 2, 4 through 6; #3 did not survive transplant). were screened by PCR for the presence of a T-DNA insertion. Information on primer design was obtained from http://signal.salk.edu/tdnaprimers.2.html.

PCR reaction #1:

Left and right At1g73600 gene-specific primers designed specific to the SALK_062703 line were used (ML 07_19974 and ML 07_19975; Table II) In this reaction, a plant homozygous for the T-DNA insertion would show no amplified product and plants bearing wild-type or heterozygous for this allele show a product of ca 1009 bp (Table V).

PCR reaction #2:

The right At1g73600 gene-specific primer (ML 07_19975) and the T- DNA-specific left border primer #2 (ML 07_19276) (see Table II). In this PCR reaction, wild-type plants would show no amplified product whereas plants homozygous or heterozygous for the T-DNA insertion would yield a product of approximately 458-758 bp (Table V).

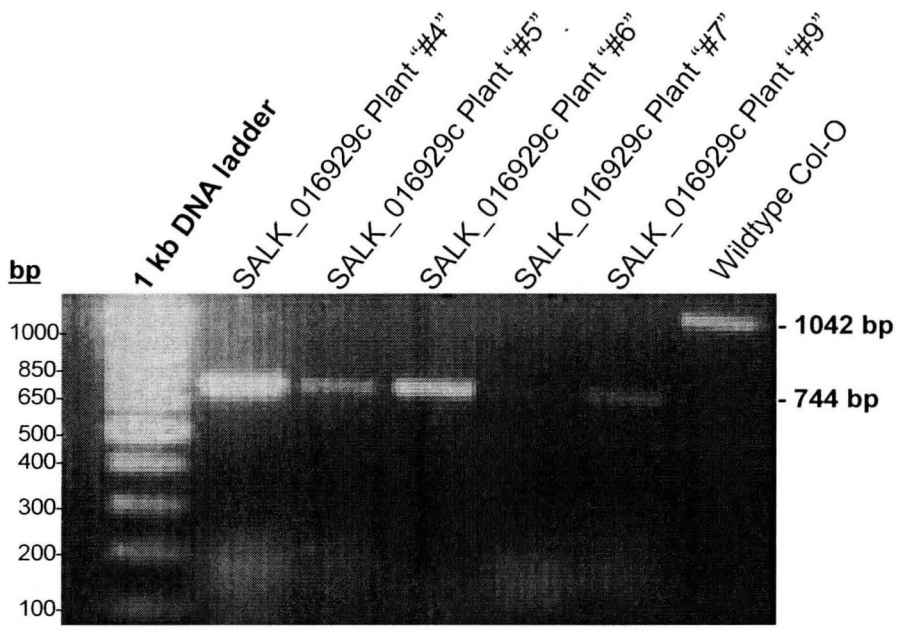

1 kb DNA ladder
SALK_062703 Plant "#1"
SALK_062703 Plant "#2"
SALK_062703 Plant "#4"
SALK_062703 Plant "#5"
SALK_062703 Plant "#6"
Wildtype Col-O
bp
1000
850
650
500
400
300
200
100
-1009 bp
PCR #1:
Left and right gene specific primers
-711 bp
PCR #2
T-DNA-specific left border primer and right gene specific primer

The confirmation that SALK_016929c plants are indeed homozygous for the presence of T-DNA insertions at the At1g73600 locus is shown in Figure 8. One band is PCR-amplified from each sample of DNA extracted from a SALK_016929c plant following reactions that included the left and right gene-specific primers for the SALK_016929c line (ML 07_1100 and ML 07_1101; Table II) and a T-DNA specific left border primer #2 (ML 07_19276; Table II). This band was amplified from several SALK_016929c plants and was consistent with the 451 bp to 751 bp range expected for plants with a T-DNA insertion in the At1g73600 of this line. Also, no comparable product was amplified from the DNA of a Col-O wild-type plant where a band of 1042 bp corresponding to the predicted product size for an undisrupted At1g73600 gene was produced under the same conditions.

FIGURE 8: Confirmation of Arabidopsis SALK_016929c mutants homozygous for the presence of T-DNA

DNA isolated from the SALK line 016929c and Arabidopsis Col-O wild-type plants was used in PCR amplification reactions using left and right At1g73600 gene-specific primers (ML 07_1100 and ML 07_1101; Table II) for the SALK_016929c line and T-DNA-specific left border primer #2 (ML_19276; Table II). DNA was extracted from leaves of individual SALK_016929c plants designated plant"#4", plant "#5", plant "#6" and plant "#9"; transplanted April 01 / 07 and wild-type plants transplanted April 07 / 07.

This PCR reaction design was used to identify any plants from the SALK_016929c line that were homozygous for T-DNA insertion in the At1g73600 gene. Wild-type plants with the At1g73600 gene undisturbed would produce a product of 1042 bp under this reaction design. Homozygous plants would produce a product in the range of 451-751 bp, as predicted by the information available at the Salk Institute Genomic Analysis Laboratory website (http://signal.salk.edu/tdnaprimers.2.html).

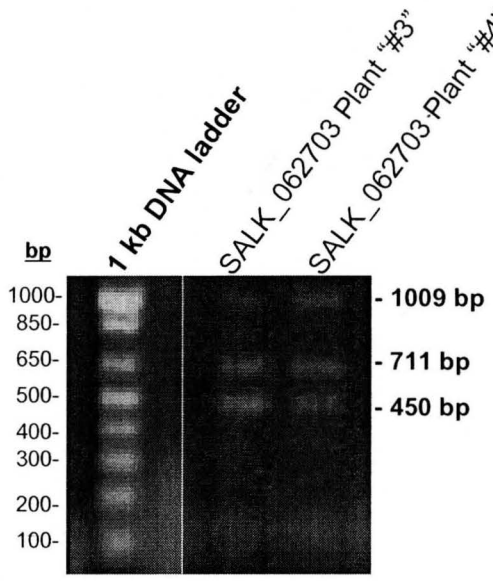

bp
1 kb DNA ladder
SALK_016929c Plant "#4"
SALK_016929c Plant "#5"
SALK_016929c Plant "#6"
SALK_016929c Plant "#7"
SALK_016929c Plant "#9"
Wildtype Col-O
1000
850
650
500
400
300
200
100
- 1042 bp
- 744 bp

An example of a PCR reaction used to identify a T-DNA insertion for plants of the SALK_062703 line is shown in Figure 9. The plants shown are heterozygous for the presence of T-DNA. Heterozygote plants were allowed to self but only 20 plants were screened for T-DNA insertion and no homozygous plants were identified, a larger amount of progeny from selfed heterozygotes need to be screened to identified homozygous plants.

FIGURE 9: Identification of SALK_062703 plants heterozygous for the presence of T-DNA insertion associated with At1g73600.

PCR amplification of DNA extracted from the rosette leaves of plants from the SALK_062703 line using the left and right At1g73600 gene-specific primers (ML 07_19976 and ML 07_19977; Table II) and a T-DNA-specific left border primer #1 (ML 07_941;Table II). These PCR reactions identified two plants as heterozygous for the T-DNA insertion.

DNA was extracted from two SALK_062703 plants designated "#3" and "#4" (transplanted March 06 / 07) and PCR reactions generated several distinct bands. For the SALK_062703 line a PCR product associated with the undisrupted At1g73600 gene is predicted to be 1009 bp (Salk Institute Genomic Analysis Laboratory; http://signal.salk.edu/tdnaprimers.2.html; Table V). If the T-DNA insertion is only in one allele a second product in size range of 458-758 bp should also be present. Since both plants produced products consistent with these sizes they are heterozygous for the presence of T-DNA. The third band at 450 bp was determined to be an artefact generated by the T-DNA left border-specific primer #1 as use of this primer alone gave the same band in mutant and wild-type samples (see Materials and Methods).


bp
1 kb DNA ladder
SALK_062703 Plant "#3"
SALK_062703 Plant "#4"
1000-
850-
650-
500-
400-
300-
200-
100-
- 1009 bp
- 711 bp
- 450 bp

Estimation of transcript abundance in SALK mutants by Reverse Transcription-PCR (RT-PCR)

RT-PCR has proven to be an efficient method for the detection of mRNA transcripts of various genes (Freeman et al., 1999). The rationale for performing RT-PCR was to determine if wild-type plants expressed transcripts corresponding to At1g76300 and whether this expression was disrupted in the mutants carrying T-DNA insertions. RNA was extracted from leaf tissue of Columbia (Col-O) wild-type Arabidopsis plants and from plants identified to have T-DNA insertion in both alleles of the At1g73600 gene from the SALK_016929c and SALK_062703 lines. RNA was also extracted from wild-type plants specific to these lines. As described earlier, the cDNA synthesized from the extracted RNA was initially amplified with primers specific to the actin (At2g37620) gene and subsequently with primers specific to the transcribed product of the At1g73600 gene.

SALK_062703 line:

Using the actin-specific primers (ML 2341 and ML 2514; Table III)) a product of predicted size 511 bp is expected to be amplified by RT-PCR. The lower panel of Figure 10 shows this to be the case for all of the plants tested and little difference was seen with respect to the intensity of this product between the individual plants tested although

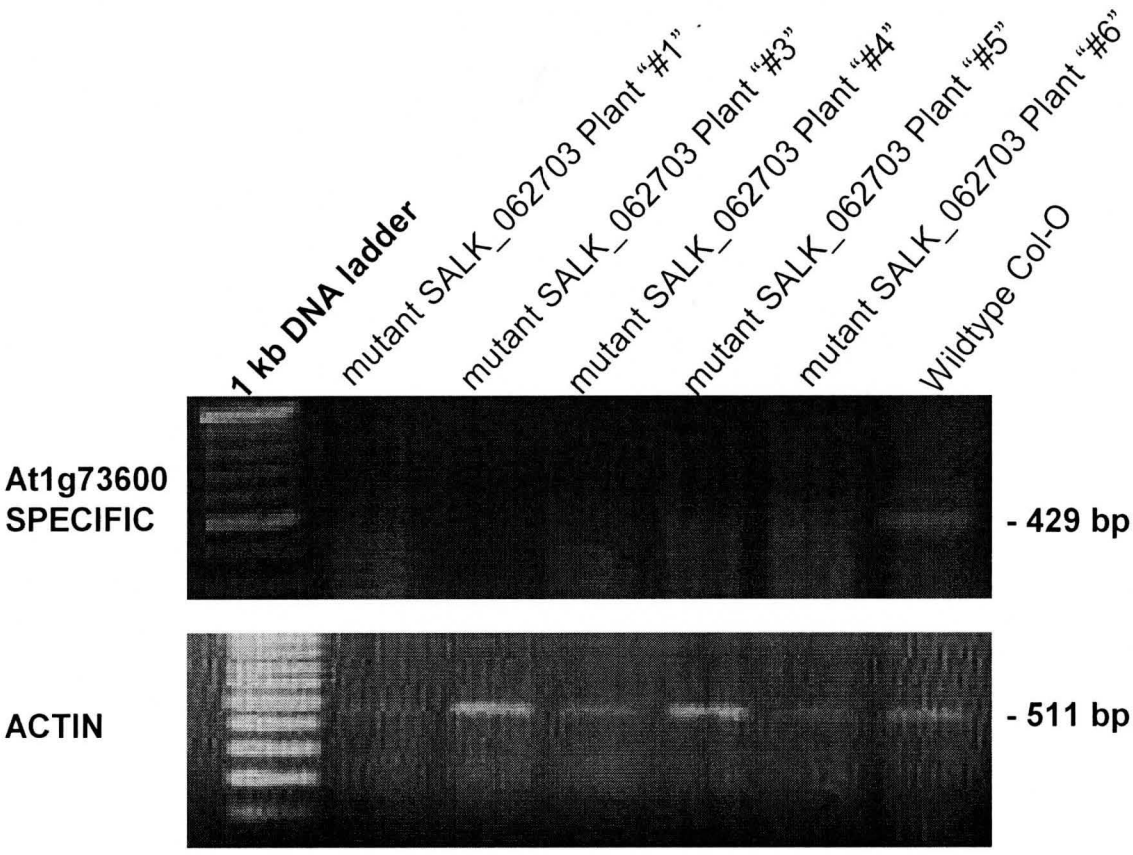
SALK_062703 plants #3 and #5 yielded slightly more intense actin-related product. Therefore, the quality and amount of mRNA extracted from each of these samples was considered to be relatively consistent with respect to quality.

Use of the At1g73600-specific primers (ML 1332 and ML 1333; Table III) gave a single RT-PCR product of 429 bp; a size consistent with one expected to be amplified from cDNA for this gene by these primers. Only the RNA extracted from a Col-O wild-type plant led to an amplified product of this size. This outcome is evidence that transcripts associated with the At1g73600 were not present in samples from plants of the SALK_062703 line and an indication that the At1g73600 gene is not being expressed in these plants.

FIGURE 10: Estimation of transcript abundance for products of the At1g73600 gene in SALK_062703 homozygous mutant plants

The abundance of transcripts associated with At1g73600 was estimated by RT-PCR for individuals of the SALK_062703 line found to be homozygous for the presence of T-DNA insertion through PCR screening. At1g73600-associated transcript abundance was also estimated for Arabidopsis Col-O wild-type plants.

For each reaction 2µl cDNA was used and transcripts associated with actin served as an internal control for cDNA quality. RNA was extracted from SALK_062703 mutants designated #1, #3, #4, #5 and #6; transplanted May 01 / 07 (see Fig. 7).. Actin left and right gene-specific primers (ML 07_ 2514 and ML 07_2341 and At1g73600 gene-specific left and right primers (ML 07_1332 and ML 07_1333); Table III) were used. The number of cycles used in this PCR was 35.


1 kb DNA ladder
mutant SALK_062703 Plant "#1"
mutant SALK_062703 Plant "#3"
mutant SALK_062703 Plant "#4"
mutant SALK_062703 Plant "#5"
mutant SALK_062703 Plant "#6"
Wildtype Col-O
At1g73600 SPECIFIC
- 429 bp
ACTIN
- 511 bp

SALK_016929c line:

The expected size of product amplified by the actin-specific primers is 511 bp. All RNA extracts prepared from plants of this line produced an actin-associated product of this size (Fig. 11). In this experiment, two different PCR reactions were performed and the results are shown as separate panels. Using gene specific primers, PCR reaction showed the SALK_016929c plants designated #1 and #4 which was judged to be homozygous for the presence of T-DNA produced products similar in size and releative intensity to that amplified from RNA prepared from the Col-O wild-type plant. However, the RT-PCR amplified product from the homozygous T-DNA mutant SALK_016929c plant designated "#2" (transplanted May 1st 2007) is slightly less intense relative to the wild-type control but the low intensity of the actin product for this sample raises some doubt as to whether the low At1g73600gene-specific band intensity is meaningful. As such, more plants were screened.

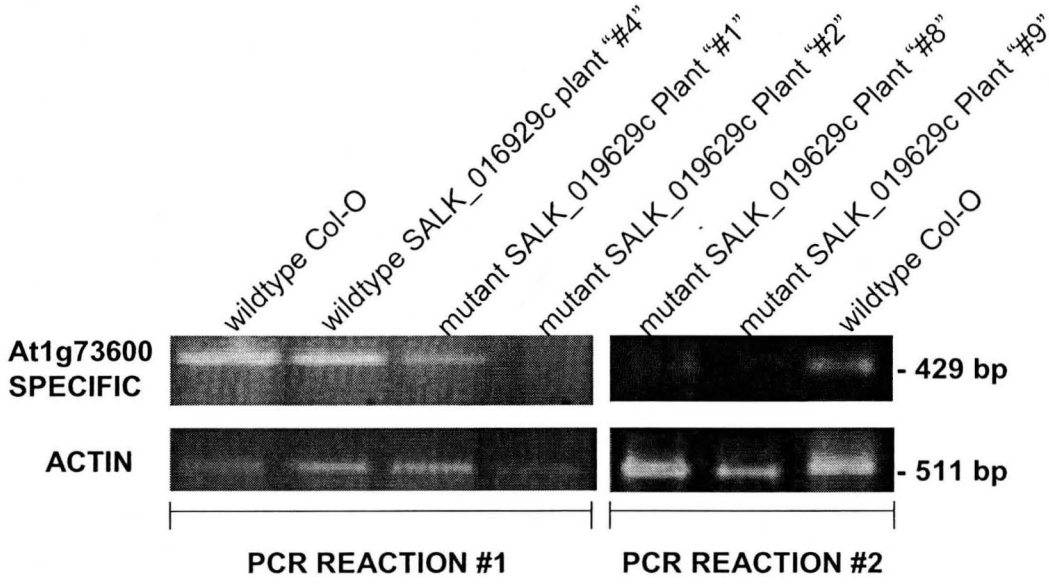
In PCR reaction #2 the intensities of the bands amplified by the actin-specific primers were comparable for the T-DNA mutant plants "#8" and "#9" (transplanted May 01 / 07) and the Col-O Arabidopsis wild-type (Fig. 11). Use of the At1g73600-specific primers (ML 1332 and ML 1333; Table III) with these RNA samples yielded a product of 429 bp in the wild-type, a faint band in mutant #8 and no detectable band in mutant #9. A product of 429 bp is expected if there are transcripts associated with At1g73600 present. RNA from the Col-O wild-type plant produced an intense 429 bp band indicating that the At1g73600 gene was expressed. In contrast, plants #8 and #9 provided RNA yielding a

strong actin product but transcripts associated with the At1g73600 gene are in relatively low abundance in plant #8 and likely absent from plant #9. Thus the T-DNA insertion does not knock-out but may knock-down the expression of At1g76300 in the SAL_016929c plants.

FIGURE 11: Estimation of At1g73600-associated transcript abundance in SALK_016929c homozygous mutant plants and an Arabidopsis Col-O wild-type plant by RT-PCR.

Products of two RT-PCR reactions are shown. The reactions were performed under the same conditions with the same primers but used RNA originating from different plants as the source of cDNA.

Reactions used 2 µL of cDNA prepared from RNA isolated from SALK_016929c mutants designated plants #1, #2, #8, and #9 all transplanted May 01 / 07 and SALK_016929c plant #4 transplanted January 16 / 07. Parallel reactions used actin left and right gene-specific primers (ML 07_ 2514 and ML 07_2341) to assess RNA template quality and At1g73600 gene-specific left and right primers (ML 07_1332 and ML 07_1333). See Table III for primer information. The number of cycles used in this PCR was 35.


wildtype Col-O
wildtype SALK_016929c plant "#4"
mutant SALK_019629c Plant "#1"
mutant SALK_019629c Plant "#2"
mutant SALK_019629c Plant "#8"
mutant SALK_019629c Plant "#9"
wildtype Col-O
At1g73600 SPECIFIC
- 429 bp
ACTIN
- 511 bp
PCR REACTION #1
PCR REACTION #2

DISCUSSION

At1g73600 gene expression

Publicly available microarray data is useful to help determine whether a gene is expressed and can provide insight into the function(s) of a particular gene product of interest (Bedhomme et al., 2005; Seki et al., 2002). Currently it is possible to use available microarray data to detail the expression patterns of particular genes of interest in specific organs or tissues of Arabidopsis during developmental stages or under various treatments and/or conditions such as drought, high salt and intense light (Seki et al., 2002). This data can be used as a basis and/or accompaniment to other gene expression determination experiments such as RT-PCR (Birnbaum et al., 2003).

In this study, data from The Arabidopsis Information Resource (TAIR) microarray database (http://www.arabidopsis.org) describing the expression pattern for At1g73600 gene in the organs and tissues of Col-O Arabidopsis wild-type plants showed that expression of this gene was found to occur primarily in rosette leaves (Table IV). Genevestigator (https://www.genevestigator.ethz.ch/) was also used to determine at which stage of development the At1g73600 gene was expressed and it was determined that "developed" rosettes at 18.0 – 20.9 days of growth had the highest level of expression of the At1g73600 gene in the rosette leaves compared to all other developmental stages. Second highest At1g73600 gene expression occurred at 29.0 – 35.9 days of growth.

This information was very useful in that it told us that the gene is indeed expressed in plants as some genes are silent. Also, with future RT-PCR studies in mind, this information directed us towards using rosette leaves for RNA extraction in order to maximize our likelihood of detecting At1g76300-associated transcripts in plants potentially disrupted with respect to the expression of this gene.

Identifying SALK lines displaying disruption of At1g76300 gene expression

The overall objective of this study was to obtain mutants that abolish or reduce At1g76300 gene function. Having mutants with reduced expression of this gene would enable us to compare phenotypes between these and wild-type plants in order to identify a biological role for this gene. The biological function of many other genes in Arabidopsis has been discovered using this powerful approach and successes include ascribing roles to members of the P450 gene family (Winkler et al., 1998), genes encoding MYB transcription factors (Meissner et al., 1999), and the AtRAD51 gene (Li et al., 2004).

The phenotype of a mutant often provides important insights into the action(s) of a gene and its product. For example, a previously uncharacterized transport inhibitor response 1 (TIR1) gene was disrupted and then it was found that the function of this gene is correlated to auxin response reactions and not, as suspected, in auxin transport processes (Ruegger et al., 1998). A clue to its role was revealed when mutant plants

showed elongation of the hypocotyls and proliferation of cells at the root tip; a phenotype not observed in wild-type plants.

There may also be no easily identifiable phenotype for knock-out mutants (Meissner et al., 1999). This can be due to the difficulties of detecting subtle changes in the mutant phenotype or the complicating impact of functional redundancy between members of gene families that can mask the action of a similar gene (Vision et al., 2000; Simillion et al., 2002). In some cases, the lack of a recognizable phenotypic characteristic of a knock-out mutant may be due to the environmental conditions that the plants are grown in necessitating the use of a wide variety of conditions for phenotypic analyses (Meissner et al., 1999). It may well be that a change in the wild-type phenotype of a mutant may only be provoked under highly specific environmental conditions or treatment combinations such as high salinity or drought that might suggest the function of the gene in question to be related to the stress response of the plant (Meissner et al., 1999). However, before any phenotypic analysis, mutant plants must be available and ideally with the expression of the gene of interest knocked out.

Insertional mutagenesis involving T-DNA has been widely used in Arabidopsis (Feldmann 1991, Meissner et al., 1999; Krysan et al., 1999). The use of T-DNA as an insertional mutagen is primarily chosen due to the stability of the insertion through several generations and the relative ease of detecting the mutation through simple PCR screening reactions (Jeon et al., 2000). However, the use of T-DNA as an insertional mutagen does have its drawbacks. Integration of T-DNA into the plant genome can cause

re-arrangements at the insertion site and the T-DNA element itself (Gheysen et al., 1987) in the form of duplications, deletions, and inversions (Mayerhofer et al., 1991). Due to the random distribution of T-DNA integration into the plant genome (Chyi et al., 1986; Wallroth et al., 1986) many mutagenized plant lines are needed to find an insertion in any particular gene of interest (Galbiati et al., 2000). The use of T-DNA as an insertional mutagen is hindered by the frequent incidence of other T-DNA insert(s) integrating into other areas of the genome unlinked to the gene of interest (Alonso et al., 2003). For example, in a plant line bearing a T-DNA insert in a gene of interest Li et al (2004) found a second T-DNA insert in the same plant line. Since the second mutation may affect the phenotype of the mutant plant, outcrossing may be required to separate the two sites of T-DNA integration (Li et al., 2004). Having multiple mutant lines to establish a phenotype can address some of these concerns in that an overlapping phenotype shared by independent mutants is more likely to be the outcome of no expression of the mutant gene in common.

In this study, seeds from four Arabidopsis T-DNA SALK lines: SALK_016929c, SALK_016929, SALK_062703 and SALK_028641 were obtained from the Arabidopsis Biological Resource Center at the University of Ohio (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). Three of these SALK lines were reported to have a T-DNA insert at a different location within the At1g73600 gene. SALK_016929 (and its confirmed stock of seeds SALK_016929c) and SALK_062703 were reported to have T-DNA inserted in an exon of the At1g73600 gene, and

SALK_028641 in an intron (http://signal.salk.edu/cgi_bin/tdnaexpress?JOB=TEXT$TYPE=GENE&QUERY=At1g73600). The SALK_016929c line was confirmed for the presences of a homozygous T-DNA insertion in the At1g73600 gene through PCR reactions (http://www.arabidopsis.org) Despite the assurance that this line was confirmed, we subjected all four lines to screening by PCR to identify and verify the genotype of plants found to be homozygous for T-DNA insertion in both alleles of the At1g73600 gene.

Out of the four SALK T-DNA lines screened, plants homozygous for T-DNA were only identified from the SALK_062703 and SALK_016929c lines. As shown in Table VI, five homozygous mutant plants were identified in the SALK_062703 line, and seven in the SALK_016929c line. No homozygotes with respect to T-DNA insertions were identified from the unconfirmed SALK_016929 seed stock and the SALK_028641 line. However, seven plants were identified as heterozygous for T-DNA insertion among individuals of the SALK_062703 line, five as homozygous and three as wild-type in the same line. From the SALK_028641 line two heterozygous plants were identified and nine wild-type individuals.

The lack of homozygous plants in two of the SALK lines screened, particularly SAL_028641, may be a true reflection of the absence of such individuals in the population of seeds received or it may be related to the screening process itself. However, it is possible that the PCR screening failed to identify an appropriate mutant even if it is present. The nature of the T-DNA element and its integration into the plant

genome can cause difficulties in easily identifying inserts by the simple PCR screening strategy used in this study.

In general, each PCR screening should include T-DNA border primers from both the left and right ends, due to the frequent occurrence of rearrangements and truncations at the T-DNA ends (Krysan et al., 1996). For example, in the isolation of T-DNA insertions in particular genes of interest by Meissner and co-workers (1999), one of the populations screened was found to have disruptions in the left and right borders of the T-DNA. In that population, a non-disrupted T-DNA right border was found in only 25% of the lines, and a non-disrupted T-DNA left border was found in only 50% of the lines screened. Additionally, it was reported that another problem with using T-DNA as an insertional mutagen was due to the frequent occurrence of loci with numerous T-DNA elements in tandom (Meissner et al., 1999). As such, in lines where an insertion in a gene of interest could not be found by PCR screening, filter hybridization to probe for the presence of a T-DNA insertion has been successful (Meissner et al., 1999). Also, in some cases PCR screening may not work because the T-DNA can be integrated in a rearranged configuration, allowing for amplification of product by only one border primer specific to the T-DNA element (Krysan et al., 1996). In the case of SALK_016929 where no homozygotes were found it is more likely that the prevalence of a homozygous genotype was very low among the seedlings screened and this was corrected in the confirmed line SALK_0162929c which was previously screened for the presence of a T-

DNA insert by PCR reactions by the Arabidopsis Biological Resource Center before seed purchase.

RT-PCR

RT-PCR complements microarray data in estimating the relative amount of a particular mRNA in a biological sample (Birnbaum et al., 2003). In studies of T-DNA insertion mutants the absence of mRNA transcripts corresponding to a gene of interest in a mutant compared to its wild-type plant would strongly suggest the disruption of a given gene has created a plant completely deficient for its product or a T-DNA "knock-out" mutant. As discussed below, not all T-DNA insertions lead to a disruption of gene expression. As such, RT-PCR is an important strategy to carry out before launching further investigations to elucidate the role of a particular gene.

Isolated mRNA serves as a template for cDNA and the amplification of mutant and wild-type plant cDNA by primers specific to actin serves as an internal control to ensure that the quality and relative quantity of mRNA extracted from mutants and wild-type plant tissues are comparable between samples. Comparisons made between the intensity of PCR product related to the actin gene amplification between mutant and wild-type plants can be used to interpret the abundance of product associated with

amplification of the At1g73600 gene transcripts. With this in mind, any difference in the intensity of bands visualized on gels between homozygous and wild-type cDNA amplified by actin or At1g73600 gene-specific primers could be attributed to differing levels of gene expression in the two plants and not due to a sample source made up of highly degraded RNA.

cDNA was amplified from mRNA extracted from SALK lines where we recovered mutants homozygous for the T-DNA insertion in At1g73600. As such, plants from SALK_016929c and SALK_062703 lines were used in combination with their wild-type siblings for RT-PCR analyses. Both were reported to have disruptions in the exon of the At1g76300 gene and since plants that were homozygous for the presence of T-DNA were identified, these lines are of particular interest for future phenotypic analyses (Table VI). Only heterozygotes were identified for plants of the SALK_028641 line and T-DNA was reported to be associated with an intron so these plants were not studied further.

cDNA from SALK_062703 plants identified as homozygous for the presence of T-DNA did not amplify any product when included in a 35 cycle PCR reaction with the At1g73600 gene-specific primers (Fig. 10). However, in that same RT-PCR analysis, the wild-type cDNA did amplify product at the expected size of 429 bp. The amplification of a readily detectable product with actin-specific primers shows that the RNA was of suitable quality for amplification of a gene product had At1g73600-associated transcripts been present. Therefore, this result is consistent with the conclusion that SALK_062703 line homozygous T-DNA mutant plants have no detectable transcripts for the At1g73600

gene. This outcome would make these plants very useful in future phenotype studies. However, it would be useful as well to address an anomaly seen in the wild-type cDNA amplified At1g73600 gene-specific product. A faint double band was obtained for the wild-type cDNA amplification by the gene-specific primers, suggesting possible DNA contamination. The At1g73600 gene-specific primers were designed to span an intron to minimize DNA amplification as well as the primers for the actin gene (Fig. 5), as explained in the RT-PCR section of the Materials and Methods. Therefore, in the future DNase could be used to further reduce the likelihood of DNA contamination and exclude this as a possible source of the second band.

A more complicated outcome was seen when cDNA synthesized from four mutant SALK_016929c, wild-type SALK_016929c, and Col-O wild-type RNA was amplified with both the actin and At1g73600 gene-specific primers (Fig. 11). PCR-amplified cDNA prepared from mutant plants "#1" and "#8" (Fig. 11) show an At1g73600-related band as a product. Relative to wild-type transcript levels this product is less abundant but transcripts associated with this gene appear to be expressed in these plants nonetheless. Plants #2 and #9 for this line showed no transcripts associated with this gene even when the number of PCR cycles was increased from 30 to 35 cycles. Mutant phenotype screens have included mutant lines with reduced expression as well as on lines with no gene expression at all (Meissner et al., 1996). Therefore, the mutant SALK_016929c plants identified in this study could still be useful in phenotype studies to identify the function of the At1g73600 gene.

Comparable expression results as that obtained for the SALK_016929c line mutants were found in a study that identified T-DNA insertion in several genes of a transcription factor gene family in Arabidopsis where reduced, no expression, or wild-type level expression was identified by RT-PCR analysis of individual mutants for 15 MYB transcription factor genes (Meissner et al., 1999). In this study, RT-PCR analysis of T-DNA insertion mutants for 8 of these genes, showed significantly decreased levels of transcripts where no expression had been expected (Meissner et al., 1999).

Another reason for detecting transcripts in suspected mutant plants is redundancy (Meissner et al., 1996). Due to the large degree of gene duplication in the genome of Arabidopsis many closely related members of multigene families exist (Vision et al., 2000; Simillion et al., 2002; Blanc et al., 2000). The close sequence similarity of the genes in these families makes it highly likely that they have similar, or redundant functions and the presence of transcripts in an expected knock-out could be due to the contributions of mRNA associated with closely related genes (Meissner et al., 1999). Functional redundancy has been reported for many Arabidopsis genes, such as the AB12 and ABI1 genes (Leunge et al., 1997), phytochromes B, D, and E (Franklin et al., 2003) and MADs-box gene family (Pinyopich et al., 2003). The At1g73600 gene is closely related in amino acid sequence to the At3g18000 and At1g48600 genes (BeGora, unpublished) so primer selection could be very important in distinguishing individual members of these genes in sections where DNA sequence may also be similar. Therefore, the primers were aligned to the other two members of the PEAMT gene family using

Gene Runner (http://www.generunner.net/). Although very similar in sequence, it is possible for members of closely related gene families to have very different functions, and display distinct phenotypes (Pelaz et al., 2000; Liljegren et al., 2000). Therefore, to study the function of one gene of family with closely related members, mutants bearing T-DNA inserts in each of the members can be identified by PCR and combined in one line through selective crossing (Meissner et al., 1996; Krysan et al., 1996). Phenotypic comparisons of plants with different genotypes with respect to gene family members can then prove helpful in ascribing biological roles to the products of related genes.

It has been reported previously that insertion in either an intron or exon of a gene will very likely cause a mutated gene to have no expression (Azpiroz-Leehan and Feldmann, 1997). However, the apparent expression of transcripts associated with At1g76300 in SALK_016929c plants need not be due to the presence of related transcripts. The effect or ability of the T-DNA element to completely knock-out the expression of a gene can be due to where an element integrates into a given gene (Winkler et al., 1998; Azpiroz-Leehan and Feldmann 1997; Oppenheimer et al., 1991; Klucher et al., 1996). When *Agrobacterium tumefaciens* is used to transform Arabidopsis with T-DNA, the T-DNA will insert randomly into the plants genome (Krysan et al., 1996). Therefore, the T-DNA element can insert into various locations of a gene such as exons and introns (Azpiroz-Leehan and Feldmann, 1997), 5' and` 3' untranslated regions (Winkler et al., 1998), or 1 kb to 2 kb 5' or 3' of the transcribed region (Oppenheimer et al., 19991; Klucher et al., 1996). For T-DNA insertion in the 1 kb to 2kb 5' or 3' of the

transcribed region of the gene of interest, detectable changes from the wild-type phenotype and significant changes in the gene transcript levels has been reported (Oppenheimer et al., 1991; Klucher et al., 1996). Several studies have reported that the extent to which the expression of a gene with a T-DNA insert is altered is affected greatly by the site in the gene where the T-DNA integrates (Winkler et al., 1998; Azpiroz and Feldmann, 1997; Oppenheimer et al., 1991; Klucher et al., 1996; Meissner et al., 1999). Also, the transcription of genes in Arabidopsis can be controlled by circadian clocks (Harmer et al., 2000). The level of RNA being transcribed at a particular point can be higher or lower than normal levels depending on the time of day, therefore timing of tissue harvesting could affect the level of gene expression determined by RT-PCR (Kreps and Simon, 1997). Tissue for mutant and wild-type plants was collected together between 11:00 a.m. – 12:00 p.m.

Future work

Beyond the scope of this thesis, the following studies would be useful in elucidating the biological role for the gene product encoded by At1g73600.

Cloning of the At1g73600 gene and assaying for biochemical activity

Overexpressing the At1g73600 gene product and assaying for biochemical activity using the phospho-bases PEA, PMEA or PDEA would determine if any of these chemicals were suitable substrates and products could be verified by TLC analysis. An

analogous strategy was used successfully to identify the biochemical activity associated with the closely related At1g48600 gene product (BeGora, unpublished). In this manner it would be possible to determine if the At1g73600 gene product is involved in choline biosynthesis, and whether this gene catalyzes all three methylation reactions involved in PtdCho biosynthesis as is the case for the product encoded by At3g1800.

It is interesting to note that genes encoding products at At1g48600 and At3g1800 were both cloned by functionally complementing a *Saccharomyces cerevisiae* yeast mutant (CPBY19) (Bolognese and McGraw, 2000; BeGora, unpublished). The CPBY19 strain contains mutations in the opi3 and cho2 genes which normally function in PtdCho synthesis, therefore this strain of yeast requires choline to survive. In both cases, cloning of genes involved in choline biosynthesis began with an Arabidopsis cDNA library in the pFL61 vector being used to transform the mutated yeast Any cDNA found to complement the mutation allows the yeast to survive on selective medium and this screen did not yield a cDNA corresponding to the At1g73600 gene (BeGora, unpublished). This result may have been due to the low abundance of the At1g73600 gene in the cDNA library used or possibly the At1g73600 gene does not function in the choline biosynthetic pathway. These previous transformations that found clones with cDNAs corresponding to the At3g1800 and At1g48600 genes used a Landsberg erecta ecotype whole seedling library (BeGora, unpublished) and it may be that the transcripts associated with the At1g73600 gene are too low in abundance for this approach to be successful.

Phenotypic characterization of At1g73600 mutant plants

Initial phenotypic screens can be carried out under normal environmental conditions (22°C, 12 h photoperiod, light intensity of 120 μmol $m^{-2}s^{-1}$) by examining the At1g73600 SALK_062703 homozygous mutant plants and recording their characteristics compared to that of wild-type plants grown under the same conditions. Visible phenotypic alterations from wild-type plants such as an increase in the number of lateral roots and the development of short epidermal cells were seen in T-DNA tagged At3g18000 mutant plants grown at normal environmental conditions, and due to the high sequence similarity and possible redundant functions of the At3g1800 and At1g73600 gene products, visible phenotypic changes may be readily identified in At1g73600 mutant plants without exposure to stress treatments (Cruz-Ramirez et al., 2004).

All aspects of the plants should be examined such as the plant as a whole (biomass, plant height) including separate measurments for roots (root length, number of root hairs, number of lateral roots) and shoots (leaf color, size, shape) (Nameth, 2007; http://signal.salk.edu/index.htm/; Cruz-Ramirez et al., 2004; Mou et al., 2002). Other features of normal plant functioning could be assessed in the mutant plants such as time to flowering and fertility (Mou et al., 2002).

The high sequence similarity existing between the At1g73600 and At3g18000 genes is suggestive of similar if not identical biological and biochemical function(s) (BeGora, unpublished). However, these two genes are expressed at very different levels in the roots and leaves (Cruz-Ramirez et al., 2004). Microarray data from Genevestigator (http://www.genevestigator.ethz.ch/gv/index.jsp) shows the At3g1800 gene to be

expressed at much higher levels in the roots than the At1g73600 gene which, by comparison, is expressed at much greater levels in the leaves. Additionally, the third member of the PEAMT gene family At1g48600 is most highly expressed in the roots similar to the expression of At3g18000 (http://www.genevestigator.ethz.ch/gv/index.jsp).

When mutant plants with significantly reduced levels of PEAMT encoded by the At3g1800 gene were analyzed, it was reported that the only significant phenotypic changes relative to wild-type plants related to the structure and growth of the roots where the At3g1800 gene is most highly expressed (Cruz-Ramirez et al., 2004). Therefore, it is anticipated that any significant phenotypic changes associated with a disrupted expression of At1g73600 would be found in leaves where that gene is most highly expressed and so phenotypic characterizations should directed at revealing its function in this tissue.

The differential expression of the three members of the PEAMT gene family does not necessarily mean they encode products with dissimilar roles in synthesizing PtdCho. It may, however, suggest that these genes contribute differentially towards PtdCho in various plant organs where they are expressed at different levels. This possibility is supported by At3g1800 T-DNA mutant plants that were found to produce less PtdCho during root development, and showed severe changes to the root structure and growth compared to wild-type plants (Cruz-Ramirez et al., 2004).

In addition to phenotypic analysis carried out under normal conditions, both mutant and wild-type plants should be grown and characterized under varying environmental conditions and stresses such as high temperatures and high salt content to

determine if expression of At1g73600 is stress- responsive. It may be that the product of this gene only functions under specific conditions and/or stresses as is the case when PEAMT is suppressed and plants are male sterile at high temperatures (Mou et al., 2002). Due to the high sequence similarity and possible redundancy between the At3g1800 and At1g73600 gene products a phenotype for At1g73600 mutants may be revealed at high temperatures as well. In the study examining silenced PEAMT plants, the effect of temperature was studied by growing plants at 20°C, 23°C, and 26°C and that may be useful for mutants of At1g76300 as well (Mou et al., 2002).

The effect of salinity on mutant plants should be examined as it has been previously reported that variations in the quantity of PtdCho is linked to salt stress responses (Kinney et al., 1987; Pical et al., 1999; Mou et al., 2002). This link was shown when mutants whose endogenous PEAMT activity was silenced were found to be hypersensitive to salt stress (Mou et al., 2002). The effects of salinity can be tested by growing seeds on agar plates with and without NaCl added and examining the phenotypes of seedlings on those plates versus wild-type controls. For example, in the study examining PEAMT mutants, salt stress was created by adding 100mM and 200 mM NaCl to the media of plates (Mou et al., 2002). In this study mutant plants hypersensitive to salinity were shown to have significantly reduced growth relative to wild-type plants when both were grown on media supplemented with 200 mM salt. (Mou et al., 2002).

Analysis of lipid composition of At1g73600 mutant plants

PtdC comprises 40-60% of membrane lipids (Moore, 1990). The importance of PtdC synthesis in plants like Arabidopsis that lack the enzymes required for glycine betaine production may be seen by examining the composition of membranes. Therefore, leaves from homozygous At1g73600 mutant plants could be harvested and analyzed by gas chromatography/mass spectrometry to determine if the absence of product associated with the At1g73600 gene has any effect on the composition of membranes.

Crossing independent mutant lines to produce double mutants for phenotypic characterization

Since the At1g73600 gene is a member of a closely related gene family with the possibility of redundant functions existing between the three members of this family, creating a combined knock-out line deficient for the At1g73600, At3g18000, and At1g48600 gene products would be valuable in determining the specific function of the At1g73600 gene. The generation of double knock-out lines can be achieved through crossing independent mutant lines (Meissner et al., 1999; Li et al., 2004).

An insertional mutant line for the At3g18000 gene has been previously identified and phenotypically characterized, as well as another mutant line available from the SALK T-DNA insertion collection that has been verified in the same study and shown to display phenotypic differences to that of wild-type plants (Cruz-Ramirez et al., 2004). This line is identified as SALK_036291 and is reported to have a T-DNA insertion in an exon at the At3g18000 locus and seeds are available (http://www.arabidopsis.org/index.jsp).

Homozygous T-DNA mutant plants have been identified for the At1g48600 gene with T-DNA tagged lines reported to have elements integrated in the promoter region of the At1g48600 gene (Nameth, 2007; http://signal.salk.edu/index.htm/). However, transcripts of this gene were still detected by RT-PCR analysis, and no line was confirmed with At1g48600 gene expression completely disrupted (Nameth, 2007; http://signal.salk.edu/index.htm/). The crossing of knock-out mutants of members of the PEAMT gene family would be better accomplished when a line is found with T-DNA integrated into the protein coding region of the At1g48600 gene where no transcripts are detected. However, the line with reduced expression of At1g48600 transcripts could still be used for phenotypic analysis or crosses.

An ideal approach would be to cross the At3g1800 knock-out plant with one from an At1g48600 knock-out line. This would provide a way of knowing if the At1g73600 gene is involved in PCho or PtdCho biosynthesis by measuring choline content in this line. The choline content of any plant can be measured in the roots or leaves by an assay used by Nuccio et al. (1998) and others (Cruz-Ramirez et al., 2004). Mutant plants deficient for PEAMT activity were found to have 64% less choline than wild-type plants by this assay (Cruz-Ramirez et al., 2004). However, these authors used RNAi to suppress PEAMT activity and it is possible that the other two related members were also at least partially silenced leading to significantly reduced choline content in these plants (Cruz-Ramirez et al., 2004). Having a mutant specifically deficient in the action of the At1g73600 gene product would address this concern. It may also reveal whether this

gene product is a more significant contributor to PtdCho synthesis in leaves than roots as would be suggested from its pattern of expression.

REFERENCES

Alaoui-Sosse B, Gerard B, Binet P, Toussaint ML, Badot PM (2005) Influence of flooding on growth, nitrogen availability in soil, and nitrate reduction of young oak seedlings (*Quercus robur* L.). Ann. For. Sci. **62:**593-600

Allard F, Houde M, Krol M, Ivanov A, Huner NPA, Sarhan F (1998) Betaine improves freezing tolerance in wheat. Plant and Cell Physiology. **39**: 1194-1202

Allen RD (1995) Dissection of oxidative stress tolerance using transgenic plants. Plant Physiology. **107:** 1049-1054

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Sterenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker P, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Horn E, Karnes M, Mulholland C, Ndubaku R, Schmid I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-Wide Insertional Mutagenesis of *Arabidopsis thaliana*. Science. **301**: 653-657

Andrews CJ, Pomeroy MK (1975) Survival and cold hardiness of winter wheats during partial and total ice immersion. Crop Science. **15:** 561-566

Andrews CJ (1977) The accumulation of ethanol in ice encased winter cereals. Crop Science. **17:** 157-161

Andrews CJ, Pomeroy MK (1979) Toxicity of anaerobic metabolites accumulating in winter wheat seedlings during ice encasement. Plant Physiology. **64:** 120-125

Apel K, Hirt H (2004) ROS: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology. **55**: 373-399

Apse MP, Aharon GS, Snedden WS, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. Science. **285:**1256–1258

Azpiroz-Leehan R, Feldmann KA (1997) T-DNA insertion mutagenesis in Arabidopsis: going back and forth. Trends in Genetics **13:**152-156

Baruch Z (1994) Responses to drought and flooding in tropical forage grasses. Plant and Soil. **164:** 87-96

Bedhomme M, Hoffmann M, McCarthy EA, Gambonnet B, Moran RG, Rébeillé F, Ravanel S (2005) Folate Metabolism in Plants. Journal of Biological Chemistry. **280:** 34823-34831

Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW (2003) A gene expression map of the *Arabidopsis* root. Science. **302:** 1956-1960

Blanc G, Barakat A, Guyot, Cooke R, Delseny M (2000) Extensive duplication and reshuffling in the Arabidopsis genome. The Plant Cell. **12:** 1093-1102

Bolognese CP and McGraw P (2000) The isolation and characterization in yeast of a gene for *Arabidopsis* S-Adenosylmethionine: Phospho-ethanolamine *N*-methyltransferase. Plant Physiology. **124:**1800-1813

Bohnert HJ, Shen B (1999) Transformation and compatible solutes. Scientific Horticulture. **78:** 237-260

Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Gorlach J (2001) Growth stage-based phenotypic analysis of *Arabidopsis*: A model for high throughput functional genomics in plants. The Plant Cell. **13:** 1499-1510

Bragina TV, Rodionova NA, Grinieva GM (2003) Ethylene production and activation of hydrolytic enzymes during acclimation of maize seedlings to partial flooding. Russian Journal of Plant Physiology. **50:**794-798

Bray EA (1993) Molecular responses to water deficit. Plant Physiology. **103:** 1035-1040

Bray EA, Bailey-Serres J, Weretilnyk EA (2000) Responses to abiotic stresses. *In* Gruissem W, Buchnnan B, Jones R, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp 1158-1249

Brouquisee R, Weigel P, Rhodes D, Yocum CF, Hanson AD (1989) Evidence for a ferredoxin-dependent choline monooxygenase from spinach chloroplast stroma. Plant Physiology. **90:** 322-329

Chen THH, Muarata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solute. Current Opinion on Plant Biology. **5**: 250-257

Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to drought-from genes to the whole plant. Functional Plant Biology. **30:** 239-264

Cosgrove D (1993) Water uptake by growing cells-An assessment of the controlling roles of wall relaxation, solute uptake, and hydraulic conductance. Journal of Plant Science. **154:** 10-21

Chyi YS, Jorgensen RA, Goldstein D, Tanksley SD, Loaiza-Figueroa F (1986) Locations and stability of *Agrobacterium*-mediated T-DNA insertions in the *Lycopersicon* genome. Molecular and General Genetics. **204:**64-69

Cruz-Ramirez A, Lopez-Bucio J, Ramirez-Pimental G, Zurita-Silva A, Sanchez-Calderon L, Ramirez-Chavez E, Gonzalez-Ortega, Herrera-Estrella L (2004) The xipotl mutant of *Arabidopsis* reveals a critical role for phospholipid metabolism in root system development and epidermal cell integrity. The Plant Cell. **16:** 2020-2034

Cutler JM, Rains DW, Loomis RS (1977) The importance of cell size in the water relations of plants. Physiologia Plantarum. **40:** 255-260

Desprez T, Amselem J, Caboche M, Hofte H (1998) Differential gene expression in *Arabidopsis* monitored using cDNA arrays. The Plant Journal. **14:** 643-652

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Research. **19:** 1349

Elstner EF (1987) Metabolism of activated oxygen species *In* DD Davies, ed, The Biochemistry of Plants. Biochemistry of Metabolism, Academic Press, San Diego, CA. **2:** 252–315

English PJ, Lycett GW, Roberts JA and Jackson MB (1995) Increased 1-aminocyclopropane-1-carboxylic acid oxidase activity in shoots of flooded tomato plants raise ethylene production to physiologically active levels. Plant Physiology. **109:** 1435-1440

Feldmann KA (1991) T-DNA insertion mutagenesis in *Arabidopsis*: Mutational spectrum. The Plant Journal. **1:** 71-82

Flowers JJ, Yeo AR (1995) Breeding for salinity resistance in crop plants: Where next? Australian Journal of Plant Physiology. **22:** 875-884

Franklin KA, Praekelt U, Stoddart WM, Billirgham OE, Halliday KJ, Whitelam GC (2003) Phytochromes B, D, and E Act Redundantly to Control Multiple Physiological Responses in Arabidopsis. Plant Physiology. **131**:1-7

Freeman WM, Walker SJ, Vrana KE (1999) Quantitative RT-PCR analysis: Pitfalls and potential. Biological Techniques. **26:** 112-125

Fridovich I (1986) Biological effects of the superoxide radical. Archives of Biochemistry Biophysics. **247**: 1–11

Gagneul D, Ainouche A, Duhaze C, Lugan R, Larher FR, Bouchereau A (2007) A reassessment of the function of so-called compatible solutes in the halophytic Plumbaginaceae *Limonium latifolium*. Plant Physiology. **144:** 1598-1611

Galbiati M, Moreno MA, Nadzan G, Zouyrelidou M, Dellaporta SL (2000) Largescale T-DNA mutagenesis in *Arabidopsis* for functional genomic analysis. Functional and Integrative Genomics **1:** 25–34

Galinski EA (1993) Compatible solutes of halophilic eubacteria: Molecular principles, water-solute interaction, stress production. Cellular and Molecular Life Sciences. **49:** 487-496

Garcia AB, Engler JA, Iyer S, Gerats T, Montagu MV, Caplan AB (1997) Effects of osmoprotectants upon NaCl stress in rice. Plant Physiology. **115:** 159-169

Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought and salt tolerant plants result from overexpression of the AVP1 H^+ pump. Proceedings of the National Academy of Sciences USA. **98:**11444-11449

Gaymard F, Pilot G, Lacombe B, Bouchez D, Bruneau D, Boucherez J, Michaux-Ferriere N, Thilbaud JB, and Sentenac H (1998) Identification and disruption of a plant shaker-like outward channel involved in K1 release into the xylem sap. Cell **94:** 647–655

Grichko VP, Glick BR (2001a) Amelioration of flooding stress by ACC deaminase-containingplant growth-promoting bacteria. Plant Physiology and Biochemistry. **39:** 11-17

Grichko VP, Glick BR (2001b) Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35***S***, ***rolD*** or PRB-1***b*** promoter. Plant Physiology and Biochemistry. **39:**19-25

Hagemann M and Erdmann N (1997) Environmental stresses. *In* Rai AK ed., Cyanobacterial Nitrogen Metabolism and Environmental Biotechnology. Springer, Heidelberg, Narosa Publishing House, New Delhi, India. pp.156–221

Halliwell B, Gutteridge JMC (1985) Free Radicals in Biology and Medicine, Clarendon Press, Oxford University Press, New York

Hamerlynck EP, Huxman TE, Loik ME, Smith SD (2000) Effects of extreme high temperature, drought and elevated CO_2 on photosynthesis of the Mojare desert evergreen shrub, Larrea tridentate. Plant Ecology. **148:** 183-193

Hanson AD, Rhodes D (1983) ^{14}C tracer evidence for synthesis of choline and betaine via phosphoryl base intermediates in salinized sugarbeet leaves. Plant Physiology. **71:** 692-700

Hanson AD, May AM, Grumet R, Bode J, Jamieson GC, Rhodes D (1985) Betaine synthesis in chenopods: Localization in chloroplasts. Proceedings of the National Academy of Sciences USA **82:** 3678-3682

Hansen LD, Afzal M, Breidenbach RW, Criddle RS (1994) High and low temperature limits to growth of tomato cells. Planta. **195**: 1-9

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science. **290:** 2110-2113

Harper DB, Harvey BMR (1978) Mechanism of paraquat tolerance in perennial ryegrass. II. Role of superoxide dismutase, catalase and peroxidase. Plant, Cell and Environment. **1:** 211-215

Hayashi H, Alia, Mustardy L, Deshnium P, Ida M, Murata N (1997) Transformation of *Arabidopsis thaliana* with the codA gene for choline oxidase: Accumulation of glycine betaine and enhanced tolerance to salt and cold stress. The Plant Journal. **12:** 133-142

Hetherington PR, Broughten HL, McKersie BD (1978) Ice encasement injury to microsomal membranes from winter wheat crowns. I. Comparison of membrane properties after lethal ice-encasement and during a post-thaw period. Plant Physiology. **85:** 1068-1072

Hong Z, Ueguchi-Tanaka M, Umemura K, Uozu S, Fujioka S, Takatsuto S, Yoshida S, Ashikari M, Kitano H, Matsuoka M (2003) A rice Brassinosteroid-deficient mutant, ebisu dwarf (d2), is caused by a loss of function of a new member of cytochrome P450. The Plant Cell. **15:** 2900-2910

Hodges DM, Andrews CJ, Johnson DA, Hamilton RI (1997) Antioxidant enzyme responses to chilling stress in differentially sensitive unbred maize lines. Journal of Experimental Botany. **48:** 1105-1113

Hopkins WG (1995) Introduction to Plant Physiology. John Wiley and Sons, New York

Huang S, Greenway H, Colmer TD (2003) Responses by coleoptiles of intact rice seedlings to anoxia: K+ net uptake from the external solution and translocation from the Caryopses. Annals of Botany. **91:** 271-278

Ishitani M, Nakamura T, Han SY, Takabe T (1995) Expression of the betaine aldehyde dehydrogenase gene in barely in response to osmotic stress and abscisic acid. Plant Molecular Biology. **27:** 307-315

Jackson M (1997) Hormones from roots as signals for the shoots of stressed plants. Trends in Plant Science. **2:** 22-28

Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kin C, Jung S, Lee S, Yang K, Nam J, An K, Han MJ, Sung RJ, Choi HS, Yu JM, Choi JH, Cho SY, Cha SS, Kin SI, An G (2000) T-DNA Insertional mutagenesis for functional genomics in rice. The Plant Journal. **22:** 561-570

Jolivet Y, Larher F, Hanelin J (1982) Osmoregulation in halophytic higher plants: The protective effect of glycine betaine against the heat destabilization of membranes. Plant Science Letters. **25:** 193-201

Kant S, Kant P, Raveh E, Barak S (2006) Evidence that differential gene expression between the halophyte, Thellungiella halophila, and Arabidopsis thaliana is responsible for higher levels of the compatible osmolyte proline and tight control of Na^+ uptake in T. halophila. Plant, Cell and Environment. **29:** 1220-1234

Kinney AJ, Clarkson DT, Loughman BC (1987) The regulation of phosphatidylcholine biosynthesis in rye (*Secale cereale*). Biochemical Journal **242:** 755-759

Kishitani S, Watanabe K, Yasvda S, Arakawa K, Takabe T (1994) Accumulation of glycine betaine during cold acclimation and freezing tolerance in leaves of winter and spring barley plants. Plant, Cell and Environment. **17:** 89-95

Kliebenstein DJ (2004) Secondary metabolites and plant environmental interactions: A view through Arabidopsis thaliana tinged glasses. Plant, Cell and Environment. **27:** 675-684

Klucher KM, Chow H, Reiser L, Fischer RL (1996) The AINTEGUMENTA gene of Arabidopsis required for ovule and female gametophyte development is related to the floral homeotic gene APETALA2. Plant Cell. **8:** 137-153

Kozlowski TT (1997) Responses of Woody Plants to Flooding and Salinity. Tree Physiology Monograph no. 1. Heron Publishing (Victoria BC, Canada)

Kramer PJ (1950) Effects of wilting on the subsequent intake of water by plants. American Journal of Botany. **37:** 280-284

Kreps JA and Simon AE (1997) Environmental and genetic effects on circadian clock-regulated gene expression in *Arabidopsis*. The Plant Cell. **9:** 297-304

Krol M, Griffith M, Huner HPA (1984) An appropriate physiological control for environmental temperature studies: Comparative growth kinetics of winter rye. Canadian Journal of Botany. **62:** 1062-1068

Krysan PJ, Young JC, Tax F, Sussman MR (1996) Identification of transferred DNA insertions with *Arabidopsis* genes involved in signal transduction and ion transport. Proceedings of the National Academy of Sciences USA **93**: 8145-8150

Krysan PJ, Young JC, Sussman MR (1999) T-DNA an insertional mutagen in Arabidopsis. The Plant Cell. **11:** 2283-2290

Lal A, Ku MSB, Edwards GE (1996) Analysis of inhibition of photosynthesis due to water stress in the C_3 species Hordeum vulgare and Vicia faba: Electron transport, CO_2 fixation and carboxylation capacity. Photosynthesis Research. **49:** 57-69

Lee JT, Prasad V, Yang PT, Wu JF, Ho THD, Chang YY, Chan MT (2003) Expression of *Arabidopsis* CBF1 regulated by an ABA/stress inducible promoter in transgenic tomato confers stress tolerance without affecting yield. Plant, Cell and Environment. **26:**1181-1190

Leung J, Merlot S, Giraudat J (1997) The Arabidopsis ABSCISIC ACID INSENSITIVE2 (AB12) and ABIf Genes Encode Homologous Protein Phosphatases 2C Involved in Abscisic Acid Signal Transduction. The Plant Cell. **9:** 759-771

Li W, Chen C, Markmann-Mulisch U, Timofejeva L, Schmelzer E, Ma H, Reiss B (2004) The Arabidopsis $AtRAD_{51}$ gene is dispensible for vegetative development but required for meiosis. Proceedings of the National Academy of Science USA. **101**: 10596-10601

Liljegren SJ, Ditta GS, Eshed Y, Savidge B, Bowman JL, Yanofsky MF (2000) SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. Nature. **404:** 766-770

Maffei M, Bossis S (2006) Electrophysiology and Plant Responses to Biotic Stress. Plant Electrophysiology. Springer. Berlin. pp. 461-481

Mata CG, Lamattina L (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiology. **126:** 1196-1204

Mayerhofer R, Koncz-Kalman Z, Nawrath C, Bakkeren G, Crameri A, Angelisk, Redei GP, Schell J, Hohn B, Koncz C (1991) T-DNA integration: a mode of illegitimate recombination in plants. The EMBO Journal. **10:** 697–704

Meissner RC, Jin H, Cominelli E, Denekamp M, Fuertes A, Greco R, Kranz HD, Penfield S, Petroni K, Urzainqui A, Martin C, Paz-Ares J, Smeekens S, Tonelli C, Weisshaar B, Baumann E, Klimyuk V, Marillonnet S, Patel K, Speulman K, Tissier AF, Bouchez D, Jones JJD, Pereira A, Wisman E, Bevan M (1999) Function Search in a Large Transcription Factor Gene Family in Arabidopsis: Assessing the Potential of Reverse Genetics to Identify Insertional Mutations in R2R3 *MYB* Genes. The Plant Cell. **11:**1827–1840

Molinar HBC, Marur CJ, Daros E, Freitas de Campos MK, Portela de Carvalho JFR, Filho JCB, pereira LFP, Vieira LGE (2007) Evaluation of the stress-inducible production of proline in transgenic sugercane (*Saccharum* spp.): Osmotic adjustment, chlorophyll fluorescence and oxidative stress. Physiologia Plantarum. **130:** 218-229

Moore TS (1990) Biosynthesis of phosphatidylinositol. Inositol metabolism. Plant Physiology. **9:** 107–112

Mou Z, Wang X, Fu X, Dai Y, Han C, Ouyang J, Bao F, Hu Y, Jiayang L (2002) Silencing of phosphoethanolamine *N*-methyltransferase results in temperature-sensitive male sterility and salt hypersensitivity in *Arabidopsis*. The Plant Cell. **14:** 2031-2043

Murashige T, Skoog F (1962) A revised medium for rapid plant growth and bioassays with tobacco tissue culture. Plant Physiology **15:** 473-497

Mudd SH, Datko AH (1989a) Synthesis of methylated EA moieties: regulation by Cho in *Lemna.* Plant Physiology. **90**: 296–305

Mudd SH, Datko AH (1989b) Synthesis of methylated EA moieties: regulation by Cho in soybean and carrot. Plant Physiology. **90**: 306–310

Nelson DE, Bohnert HJ (1998) Regulation of cell-specific inositol metabolism and transport in plant salinity tolerance. The Plant Cell. **10:** 753-764

Nilsen ET, Orcutt DM (1996) The Physiology of Plants Under Stress. John Wiley and Sons. New York

Niu X, Bressan RA, Hasegawa PM, Pardo JM (1995) Ion homestasis in NaCl stress environments. Plant Physiology. **109:** 735-742

Neuman P (1997) Salinity resistance and plant growth revisited. Plant, Cell and Environment. **20:** 1193-1198

Nuccio ML, Russell BL, Nolte KD, Rathinasabapathi B, Gage DA, Hanson AD (1998) The endogenous choline supply limits glycine betaine synthesis in transgenic tobacco expressing choline monooxygenase. The Plant Journal. **16:** 487-496

Nuccio ML, Ziemak MJ, Henry SA, Weretilnyk EA, Hanson AD (2000) cDNA cloning of phosphoethanolamine *N*-methyltransferase from spinach by complementation in Schizosaccharomyces pombe and characterization of the recombinant enzyme. Journal of Biological Chemistry. **275:** 14095-14101

Ohnishi N, Murata N (2006) Glycine betaine counteracts the inhibitory effects of salt stress on the degradation and synthesis of D1 protein during photoinhibition in *Synechococcus* sp. PCC 7942. Plant Physiology. **141:**758-765

Oppenheimer DG, Herman PL, Sivakumaran S, Esch J, Marks MD (1991) A myb gene required for leaf trichome differentiation in Arabidopsis is expressed in stipules. Cell. **67:** 483-493

Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: A review. Ecotoxicology and Environmental Safety. **60:** 324-349

Pelaz S, Ditta GS, Baumann E, Wisman E, Yanofsky MF (2000) B and C oral organ identity functions require SEPALLATA MADS-box genes. Nature. **405:** 200-203

Peng HP, Lin TY, Wang NN, Shih MC (2005) Differential expression of genes encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis* during hypoxia. Plant Molecular Biology. **58**:15-25

Pical C, Westergren T, Dove SK, Larsson C, Sommarin M (1999) Salinity and hyperosmotic stress include rapid increases in phophatodylinositol 4,5-bisphosphate, diacylglycerol pyrophosphate, and phosphatidylcholine in Arabidopsis. Journal of Biological Chemistry. **274:** 38232-38240

Pike CS, Berry JA, Raison JK (1979) Fluorescence polarization studies of membrane phospholipids phase separations in warm and cool season plants. *In* Lyons JM, Graham D, Raison JK, eds. Low Temperature Stress in Crop Plants: the Role of the Membrane. Academic Press, New York. pp.305-318

Pinyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, Wisman E, Yanofsky MF (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature. **424:** 85-88

Price AH, Cairns JE, Horton P, Jones GH, Griffiths H (1998) Linking drought-resistance mechanism to drought avoidance in upland rice using a QTL approach: progress and new opportunities to integrate stomatal and mesophyll responses. Journal of Experimental Botany. **5:** 989-1004

Rausch T, Kirsch M, Low R, Lehr A, Viereck R, Zhigang A (1996) Salt stress responses of higher plants: The role of proton pumps and Na+/H+ antiporters. Journal of Plant Physiology. **148:** 425-433

Rathinasabapathi B, Burnet M, Russell BL, Gage DA, Liao PC, Nye GJ, Scott P, Golbeck JH, Hanson AD (1997) Choline monooxygenase, an unusual iron-sulfur enzyme catalyzing the first step of glycine betaine synthesis in plants: Prosthetic group characterization and cDNA cloning. Proceedings of the National Academy of Sciences USA. **94:** 3454-3458

Rathinasabapathi B (2000) Metabolic engineering for stress tolerance: Installing osmoprotectant synthesis pathways. Annals of Botany. **86:** 709-716

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulphonium compounds in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology. **44**: 357–384

Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle (1998) The TIR1 protein of Arabidopsis functions in auxin response and is related to human SkP2 and yeast Grr1p. The Plant Cell. **12:** 198-207

Russell BL, Rathinasabapapathi B, Hanson AD (1998) Osmotic stress induces expression of choline monoxygenase in sugar beet and Amaranth. Plant Physiology. **166:** 859-865

Sakamoto A, Murata N (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant, Cell and Environment. **25:**163-171

Salin ML (1988) Toxic oxygen species and protective systems of the chloroplast. Physiologia Plantarum. **72:** 681–689

Schierup MH, Mable BK, Awadalla P, Charlesworth D (2001) Identification and Characterization of a Polymorphic Receptor Kinase Gene Linked to the Self-Incompatibility Locus of *Arabidopsis lyrat.* Genetics. **158:** 387-399

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono V, Karmiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression

profiles of 7000 Arabidopsis genes under drought, cold and high salinity stresses using a full-length cDNA microarray. The Plant Journal. **31:** 279-292

Simillion C, Vandepoele K, Van Montagu MCE, Zabeau M, Van der Peer Y (2002) The hidden duplication past of Arabidopsis thaliana. Proc Natl Acad Sci USA. **99:** 13627-13632

Smit B, Stachowiak M, Volkenburgh EV (1989) Cellular processes limiting leaf growth in plants under hypoxic root stress. Journal of Experimental Botany. **40:** 89-94

Smith DD, Summers PS, Weretilnyk EA (2000) Phosphocholine synthesis in spinach: characterization of phosphoethanolamine *N*-methyltransferase. Physiologia Plantarum. **108:** 286-294

Somerville C (1995) Direct tests of the role of membrane lipid composition in low-temperature-induced photoinhibition and chilling sensitivity in plants and cyanobacteria. Proceedings of the National Academy of Sciences USA. **92:** 6215-6218

Spicket CM, Smirnoff N, Ratcliffe RG (1993) An in vivo nuclear magnetic resonance investigation of ion transport in maize (Zea mays) and Spartina anglica roots during exposure to high salt concentrations. Plant Physiology. **102:** 629-638

Steponkus PL, Uemura M, Webb MS (1993) A contrast of the cryostability of the plasma membrane of winter rye and spring oat. *In* Steponkus PL, ed. Advances in Low Temperature Biology. Vol. **2**. London: JAI Press, pp. 211-304

Stein JM, Tourellotte ME, Reinert JC, McElhaney RN, Rader RL (1969) Calorimetric evidence for the liquid-crystalline state of lipids in a biomembrane. Proceedings of the National Academy of Science USA. **63:** 104-109

Summers PS, Weretilnyk EA (1993) Choline synthesis in spinach in relation to salt stress. Plant Physiology. **103:** 1269-1276

Taji T, Seki M, Satou M, Saburai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, Shinozaki K (2004) Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis*-related halophyte salt cress using *Arabidopsis* microarray. Plant Physiology. **135:** 1697-1709

Thomashow MF (1998) Role of Cold-Responsive Genes in Plant Freezing Tolerance. Plant Physiology **118:** 1–8

Vision TJ, Brown DG, Tanksley SD (2000) The Origins of Genomic Duplications in *Arabidopsis*. Science. **290:** 2114-2117

Waditee R, Bhuiyan NH, Hirata E, Hibino T, Tanaka Y, Shikata M, Takabe T (2007) Metabolic engineering for betaine accumulation in microbes and plants. Journal of Biological Chemistry. **282:** 34185-34193

Waterhouse PM, Graham MW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proceedings of the National Academy of Sciences USA. **95:** 13959-13964

Walbot V (1992) Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. Annual Review of Plant Physiology and Plant Molecular Biology. **43:** 49-78

Wallroth M, Gerats AGM, Rogers SG, Fraley RT, Horsch RB (1986) Chromosomal localization of foreign genes in *Petunia hybrida*. Mol. Gen. Genet. **202:** 6-15

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. **218:** 1432-2048

Wanner LA, Junttila O (1999) Cold-induced freezing tolerance in Arabidopsis. Plant Physiology. **120:** 391-399

Weigel P, Weretilnyk EA, Hanson AD (1986) Betaine aldehyde oxidation by spinach chloroplasts. Plant Physiology. **82:** 753-759

Welti R, Li W, Li M, Sung Y, Zhou HE, Rajashekar CB, Williams TD, Wang X (2002) Profiling membrane lipids in plants stress responses. The Journal of Biological Chemistry **277:** 31994-32002

Weretilnyk EA, Bednarek S, McCue KF, Rhodes D, Hanson AD (1989) Comparative Biochemical and immunological studies of the glycine betaine synthesis pathway in diverse families of dicotyledons. Planta **178**: 342-352

Weretilnyk EA, Hanson AD (1990) Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. Proceedings of the National Academy of Sciences USA. **87:** 2745-2749

Weretilnyk EA, Smith DD, Wilch GA, Summers PS (1995) Enzymes of choline synthesis in spinach: response of phospho-base *N*-methyltransferase activities to light and salinity. Plant Physiology. **109:**1085-1091

Weretilnyk EA, Moffat BA, Griffith M (2005) Expressed sequence tags from the Yukon ecotype of Thellungiella reveal that gene expression in response to cold, drought, and salinity shows little overlap. Plant Molecular Biology. **58:** 561-574

Winkler RG, Frank MR, Galbraith DW, Feyereisen R, Feldmann KA (1998) Systematic reverse genetics in the Cytochrome P450 Gene Superfamily. Plant Physiology. **118:** 743-750

Winzeler EA, Shoemaker DD, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly C, Davis K, Dietrich F, Dow SW, Bakkoury ME, Foury F, Friend SH, Gentalen E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H, Liebundguth N, Lockhart DJ, Danila AL, Lussier M, M'Rabet N, Menard P, Mittmann M, Pai C, Rebischung C, Revuelta JL, Riles L, Roberts CJ, Ross-MacDonald P, Scherens B, Snyder M, Sookhai-Mahadeo S, Storms RK, Véronneau S, Voet M, Volckaert G, Ward TR, Wysocki R, Yen GS, Yu K, Zimmermann K, Philippsen P, Johnston M, Davis W (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science. **285:** 901-906

Wong CE, Li Y, Whitty BR, Diaz-Camino C, Akhter SR, Brandle JE, Golding GB, Weretilnyk EA, Moffatt BA, Griffith M (2005) Expressed sequence tags from the Yukon ecotype of Thellungiella salsuginea reveal that gene expression in response to cold, drought, and salinity shows little overlap. Plant Molecular Biology **58**: 561-574

Yan B, Dai Q, Liu X, Huang S, Wang Z (1996) Flooding-induced membrane damage, lipid oxidation and activated oxygen generation in corn leaves. Plant and Soil.**179:** 261-268

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science. **217:** 1214-1222

Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. Development, **130:** 4859–4869