Genetic analysis of methyltransferases involved in choline synthesis of *Arabidopsis thaliana*

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ABSTRACT

In plants, S-adenosyl-L-methionine-dependent phospho-base *N*-methyl transferases catalyze the three sequential methylations of phosphoethanolamine to phosphocholine, the precursor for choline and the major membrane phospholipid phosphatidylcholine. The enzyme phosphoethanolamine *N*-methyltransferase (PEAMT) catalyzes the first and committing step in choline synthesis, a step for which no known by-pass has been found. In *Arabidopsis thaliana* there are two loci annotated as encoding PEAMT and a putative PEAMT, At3g18000 (*NMT1*) and At1g73600 (*NMT3*), respectively. A related gene product that catalyzes the last two methylations is encoded by locus At1g48600 (*NMT2*). The objective of this study was to investigate the role of *NMT3* in *Arabidopsis*. Three SALK lines carrying independent T-DNA insertions in At1g73600 were used: SALK_062703, SALK_016929c and SALK_120703c.

Genomic DNA was used for PCR and sequence analysis of the products established the insertion of T-DNA in the protein coding region of At1g73600 for all three lines. Gene expression was analyzed by q-PCR. Primer design was particularly important for *NMT3* transcript quantification by q-PCR. In SALK_062703 *nmt3* mutants, the T-DNA is in exon 8 and in the SALK_120703c line it is in intron 6. In both cases, no *NMT3* transcripts were detected using primers that annealed to sites 3' to the position of the T-DNA in the gene. However, low levels of transcripts were detected using primers that annealed at positions 5' to the site of T-DNA insertion. In the SALK_016929c line the position of the T-DNA insertion was in exon 2 and primers annealing near the site of the T-DNA insertion showed no *NMT3* expression in this mutant but amplifying the mid portion of the gene showed WT levels of *NMT3* transcripts. Thus all the mutants produce truncated *NMT3* transcripts and by avoiding areas that overlap truncated transcript regions we could differentiate between *NMT3* knock-out or knock-down expression.

Wild-type (*NMT3*) and *nmt3* seedlings from the three lines grown on defined media plates showed no difference with respect to primary root length, number or density of lateral roots, and total root length. Exposing seedlings to salt (50 or 75 mM NaCl) led to reductions in root growth but again, roots of wild-type plants were indistinguishable

from the mutant seedlings. One anomaly relates to the *nmt3* SALK_120703c line which showed two root phenotypes. On saline media most of the seedlings had longer roots that resembled the wild-type and other mutant lines and about a third had shortened roots. Whether the seedlings had long or short roots on salt, they all lacked *NMT3* transcripts. This line is likely carrying another insertion that yields a salt-sensitive root phenotype. Mutant plants at four-weeks looked like wild-type plants and time of flowering was not reproducibly delayed or accelerated in mutant plants relative to wild-type.

In wild-type seedlings the relative expression level of the three *NMT* genes is similar at day or night with transcript abundance ranked in the order *NMT3* > *NMT2* > *NMT1*. *nmt3* seedlings harvested midday showed no detectable *NMT3* expression but the abundance of *NMT1* transcripts was 6.2-fold and 1.7-fold higher relative to wild-type in shoots and roots, respectively. At night, *NMT1* expression in shoots of *nmt3* seedlings decreased 4.8-fold relative to the level of *NMT1* expression at midday while transcripts detected in roots increased slightly (1.3-fold). Using SALK_036291 *nmt1* seedlings we found that *NMT3* expression in shoots and roots was modestly up-regulated in the absence of *NMT1* expression and the expression of *NMT3* is lower at night than during the day. Also, regardless of the genotype or time of day, *NMT2* expression remained constant even when *NMT1* and *NMT3* transcripts underwent several-fold changes in abundance. Interestingly, four-week old *nmt3* plants of the SALK_062703 line showed that *NMT3* expression is knocked-out in leaves but only knocked-down in roots.

NMT3 was the most highly expressed of the three *NMT* genes monitored by q-PCR. Nonetheless, three independent T-DNA insertion lines defective for *NMT3* expression were wild-type by appearance and as such, offer compelling evidence that NMT3 is not required by *Arabidopsis*. The increased expression of *NMT1* in *nmt3* plants and *NMT3* in *nmt1* plants strongly suggests that plants compensate for the loss of one gene by up-regulating, to varying extents, the expression of the remaining *NMT* gene. If this is the case, a reasonable prediction made for a cross between *nmt1* and *nmt3* plants is that it would be lethal unless plants have yet another way to circumvent the loss of an essential enzyme for this committing metabolic bottleneck in choline synthesis.

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ABBREVIATIONS

µg	Microgram(s)
µL	Microliter(s)
µM	Micromolar
µmol	Micromole
AG	Agamous transcription factor
BADH	Betaine aldehyde dehydrogenase
CDP/CTP	Cytidine di/tri phosphate
CK	Choline kinase
CMO	Choline monoxygenase
CTT	Cytidylyltransferase
C	Celsius
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter (s)
Cq	Quantification cycle
DEPC	Diethylpyrocarbonate
DEA	Dimethylethanolamine
DNA	Deoxyribonucleic acid
EA	Ethanolamine
EFM	Early-flowering mutant phenotype
GB	Glycine betaine

g	Gram(s)
h	Hours(s)
Hmz	Homozygous
Kg	Kilogram(s)
L	Liter(s)
M	Molar
m	Meter(s)
mM	Millimolar
min	Minute(s)
mL	Millilitre
mRNA	Messenger ribonucleic acid
MEA	Methylethanolamine
MS	Murashige & Skoog
NMT	*N*-methyltransferase
nm	Nanometre(s)
ORF	Open reading frame
Pi	Inorganic phosphate
pmol	Picomole
PA	Phosphatidic acid
P-Cho	Phosphocholine
P-DEA	Phosphodimethylethanolamine

P-EA	Phosphoethanolamine
P-EAMeT	Phosphoethanolamine-*N*-methyltransferase
PLMT	Phospholipid-*N*-methyltransferase
P-MEA	Phosphomethylethanolamine
Ptd-Cho	Phosphatidylcholine
Ptd-DEA	Phosphatidyldimethylethanolamine
Ptd-EA	Phosphatidylethanolamine
Ptd-MEA	Phosphatidylmethylethanolamine
PVP	Polyvinylpyrrolidone
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
s	Second(s)
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SE	Standard error of the mean
T-DNA	Transfer DNA
uORF	Upstream open reading frame
UTR	Untranslated region
V	Volt(s)
WT	Wild type

LITERATURE REVIEW

Plant environmental stress

Stress is a product of the negative interaction between an organism and its environment and its presence is often detected as a decrease in plant productivity, such as crop yield, relative to the productivity of the same plant in the absence of stress. Sources of stress are often classified as arising from biotic or abiotic sources. Biotic stress is a product of interactions between plants and other living organisms ranging from viruses and other plants to herbivores (Kluth et al., 2002). In contrast, abiotic stress results from the interaction between an organism and its physical environment such as drought, salinity, and extreme hot or cold temperatures (Bray et al., 2000, Vierling & Kimpel, 1992).

Biotic Stress

Plant pathogens cause biotic stress in plants and these include viruses, bacteria, or fungi. Frequently pathogens that cause biotic stress are transferred to plants by herbivores (Kluth et al., 2002), providing one indication that plant interactions with other organisms can be complex and dynamic. To reduce the impact of biotic stress plants have developed chemical defenses such as phytoalexins and these defenses can render plants more resistant to an invasive organisms (Liu et al., 1992). Despite the operation of a plant immune system to defend against infection by some microbial organisms, other pathogens such as *Pseudomonas syringae* can bypass immunity defenses to cause diseases (Nomura et al., 2006). What is critical to plants developing resistance to invasion by pathogens is having mechanisms that enable the plant to perceive and recognize the presence of a pathogen. Recognition between plants and their pathogens can be highly specific. This specificity is conferred by proteins encoded by plant resistance genes where the products of these genes translates perception into an adaptive or defense response (Jones et al., 2004).

Abiotic stress

Drought, salinity, and/or exposure to cold or high temperatures are unfavorable environmental conditions for most plants and plants experiencing these conditions seldom reach their maximum yield potential (Boyer, 1982).

Temperature stress

Plants grow in nearly every climate found on this planet but species and natural accessions of species differ with respect to the ideal temperature optima for their growth or thresholds that limit their survival. When temperatures for plants exceed their optimal range the plant becomes stressed (Iba, 2002).

The photosynthetic apparatus is recognized as one of the plant components that is sensitive to high temperature stress (Berry & Bjorkman, 1980). The inhibition of photosynthesis by high temperature stress limits plant growth and reproduction (Seemann et al., 1984a). Excessively high temperatures can result in stomatal closure thereby decreasing gas exchange required for photosynthesis (Seemann et al., 1984b). Prokaryotes and eukaryotes often produce a group of proteins upon exposure to elevated temperature known as heat shock proteins (Hsp). For example, Hsp100/ Clp (casein lytic protease), specifically ClpB, is a family of proteins serving as molecular chaperones that remodel proteins in various ways such as helping reassemble dissociated protein complexes or dismantling protein aggregates to restore functional complexes (Lee et al., 2007). ClpB proteins are found in diverse organisms including bacteria and some are cell compartment specific such as the cytosol of *Saccharomyces cerevisia*e or higher plants (Schirmer et al., 1996). Unravelling the role of ClpB family members has benefited from the use of mutants defective in their regulation. For example, one of the loci associated with a ClpB cytosolic protein in *Arabidopsis, HOT1*, encodes Hsp101, an Hsp that accumulates to a comparatively high abundance when plants are exposed to elevated temperatures (Queitsch et al., 2000). Use of the *hot1* mutant shows that the loss of Hsp101 does not alter the phenotype of plants under normal conditions and that Hsp101 accumulation is necessary for thermo tolerance (Hong & Vierling, 2000). More recently,

mutants in other ClpB family members show that some are targeted to the mitochondria or chloroplast but T-DNA insertion mutants of the genes encoding these products provide no evidence that they play a role in thermo tolerance as is the case for HSP101 (Lee et al., 2007).

Winter stresses include freezing cold temperatures, sudden frost, flooding as ice melts and general oxidative stress (Thomashow, 1999). Stressful low temperatures induces photo inhibition, a condition where damage to photosystems leads to the reduced capacity to convert electron flow into ATP and reduced carbon contributing to diminished plant vigor and survival (Fowler et al., 1999).

Morphological, physiological, and biochemical characteristics are changed when plants are exposed to low temperature with many changes leading to increased cold tolerance (Thomashow, 1999). However, deleterious changes also occur as plants are injured by exposure to freezing temperatures or when they are chilling sensitive to begin with. Membrane damage related to cold exposure includes phase transitions and decreased membrane fluidity that can cause ion leakage from cells and inactivate processes requiring fluid membrane lipids and intact membranes (Iba, 2002). To avoid these adverse effects, some plants increase their cold tolerance to subzero temperature by a period of exposure to low but non-freezing temperatures in a process call acclimation (Hüner et al., 2012). During acclimation plants may actively accumulate osmoprotectants such as sugars, soluble proteins, glycine betaine and proline to offset the effects of cellular dehydration (Yancey, 2005).

Osmotic Stress

Osmotic stress can be caused by several different environmental factors that reduce the availability of water to plants such as drought, salt and freezing temperatures. All of these factors contribute to reduced crop yields through the resulting impacts of osmotic stress on crop productivity. Although catastrophic losses of crops can happen due to severe drought, even mild water deficits at crucial points in plant development such as anthesis can decrease crop yields (Boyer, 1982, Bray et al., 2000). Conditions

accompanying osmotic stress include a decrease of xylem pH and conductivity and an increase in ABA content, the latter providing a hormone trigger that mediates many stress responses (Wilkinson & Davies, 1997, Bahrun et al., 2002). The increased production of reactive oxygen species (ROS) in mitochondria, chloroplasts, and peroxisomes are all possible by-products of altered rates of respiration and photosynthesis under water limiting conditions (Apel & Hirt, 2004). ROS can oxidize protein, DNA, lipid and other cellular components that impair cellular function (Mittler et al., 2004, Dat et al., 2000). The superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$) are some examples of ROS (Apel & Hirt, 2004).

Arabidopsis thaliana as a model organism for biological studies

Arabidopsis thaliana is a small crucifer in the family Brassicaceae that has become the genetic model for plant biology (Meinke et al., 1998b, McCourt & Benning, 2010). *Arabidopsis* has been a good model organism for plant research because it is compact, a prolific seed producer, and compared to many crop species it has a small, diploid genome. The 120-megabase genome of *Arabidopsis* is organized into five chromosomes and contains approximately 20,000 genes. T-DNA collections have become a powerful tool in elucidating the role of many gene products like that of Hsp101 discussed earlier (An et al., 1986; Bechtold & Pelletier, 1998; Lee et al., 2007). Thousands of T-DNA insertion lines have been created with insertions scattered throughout the genome and many of these lines are publicly available for a minor fee that contributes to maintenance and curation expenses. The Arabidopsis Biological Resource Center at Ohio State University is a popular source of seeds for plant research needs including insertion mutant lines (http://abrc.osu.edu/). In addition to public collections, other mutant lines are being produced by private companies interested in functional genomics (Sundaresan, 1996, Meinke et al., 1998).

With respect to form and development, *Arabidopsis* seeds are 0.5mm in length, vegetative plants have rosette leaves ranging from 2 to 10 cm in diameter and the transition to flowering is accompanied by the emergence of bolts 15 to 20 cm long from

the rosette (Meinke et al., 1998a). The entire life cycle (seed to seed) is completed in six to eight weeks. *Arabidopsis* can self-fertilize and through self or manual cross pollination thousands of seeds are produced from a single plant very easily (Weigel & Glazebrook, 2002). The fully sequenced genome has been publicly available since December 2000 and now multiple *Arabidopsis* relatives including natural accessions have been sequenced providing valuable resources for comparative genomics research. (Schneeberger et al., 2011, Cao et al., 2011).

Use of T-DNA insertions for mutagenesis

T-DNA insertion is an effective mutagen for genome-wide mutagenesis studies (Krysan et al., 1999). However, the expression of the mutated genes in homozygous insertion mutants must be evaluated to determine if the T-DNA insertion has knocked out or knocked down gene expression. The effectiveness of an insertion of T-DNA depends on its position in a gene so not all mutants will have altered expression of a mutagenized gene (Delatte et al., 2005, Pastuglia et al., 2006).

In some case, an insertion of T-DNA into an intron or exon in the protein coding region of a gene will effectively knock-out or knock-down its expression; however, this is not always the case. For example, when two T-DNA mutants in the *Arabidopsis annexin 2* (*AnnAt2*) gene were tested there was no transcript detected in *annAt2-1* but *annAt2-2* showed similar expression to wild type lines (Lee et al., 2004). Importantly, even though a truncated transcript may be expressed and used to produce a truncated protein, these products may not be functional (Noh & Amasino, 2003, Okushima et al., 2005, Gusmaroli et al., 2004). It is still unclear why some T-DNA insertion mutants show transcripts associated with mutagenized genes while other mutants do not even in cases where the independent T-DNA insertions are found in the same exon as is the case for the *PIE1* (*PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1*) gene. *pie1* plants are early flowering. However, while some *PIE1*-associated transcripts were detected in *pie1-2* and *pie1-4* plants, no *PIE1* transcripts were detected in *pie1-3*, even though all of the T-DNA insertions in these independent mutant lines map to the same exon. The authors

attributed the difference in transcript expression between the lines to the possibility that the truncated *PIE1* mRNA is more unstable when produced in *pie1-3* plants (Noh & Amasino, 2003). Moreover, there is evidence that in some cases alternative splice transcript variants of a single gene may show differential expression with T-DNA insertions. The aldehyde dehydrogenase (*ALDH*)-*3H1* locus of *Arabidopsis* encodes five alternative splice variants that normally show low levels of expression. However, a T-DNA insertion in exon 1 of this gene resulted in elevated expression of the transcript splice variants revealing an undiscovered promoter in intron 1 of the gene (Missihoun et al., 2012).

Wang (2008) reviewed some of the results of T-DNA insertions reported by different labs in order to determine whether some general properties could be identified based upon the location of the T-DNA relative to the remainder of the gene. He reported that insertion of T-DNA before the start codon or in the promoter region tends to produces more knock-down mutants than knock-out. T-DNA inserted in the promoter region resulted in no expression of the mutated genes in 41% of 17 lines compared and reduced expression in 53% of the cases (Wang, 2008). An insertion in the promoter region also can lead to mis-expression of the downstream region. A T-DNA insertion in the promoter region of *AGP17*, a gene that encodes an arabinogalactan protein widely distributed in plants, was associated with the presence of transcripts in roots but not leaves compared with wild type that showed expressed transcripts in leaves and roots (Gaspar et al., 2004). This result showed that the expression of the gene downstream of the promoter can vary within an individual plant if the promoter is disrupted (Wang, 2008). The least effective type of insertion is when T-DNA is inserted after the stop codon which often results in unaltered expression of the mutagenized gene (Wang, 2008). However, this is not always the case as inserts 260 bp after the stop codon of *CSLA9* (Gaspar et al., 2004) or 650-700 bp after the stop codon of *TFL1* (Ohshima et al., 1997) resulted in knock-outs.

Choline synthesis in plants

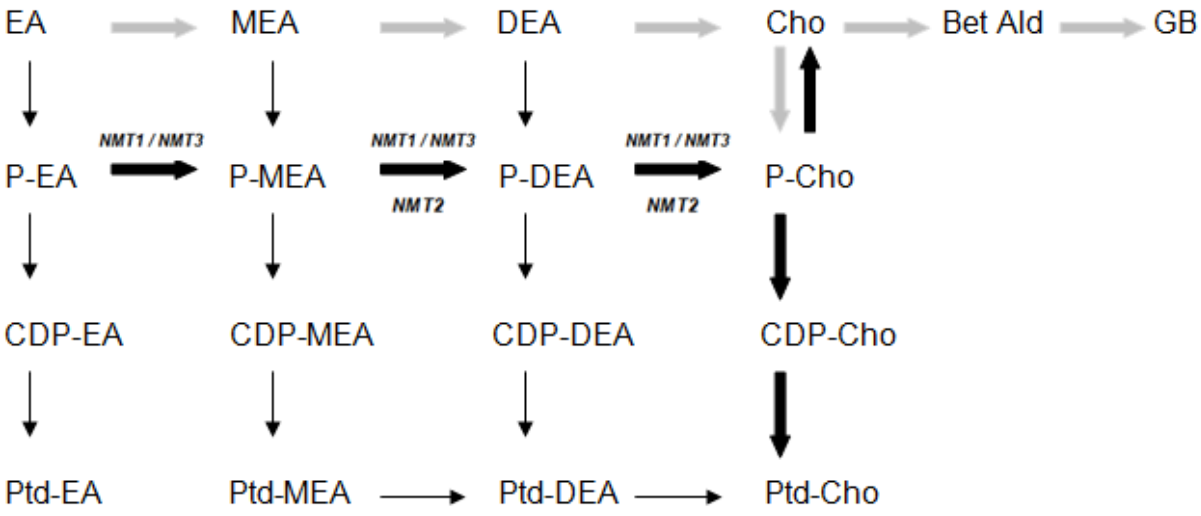
Choline can be produced by plants via free base, phosphobase or phosphatidylbase intermediates (Hanson & Rhodes, 1983, Datko & Mudd, 1988a, Prud'homme & Moore, 1992b) (Fig. 1). Dephosphorylation of phosphocholine (P-Cho), the polar head group of the abundant membrane lipid phosphatidylcholine, also can produce choline (Hitz et al., 1981). Choline synthesis begins when ethanolamine (EA) is derived from serine by decarboxylation (Rontein et al., 2001). Ethanolamine kinase then phosphorylates EA to produce phosphoethanolamine (P-EA) (Delwiche & Bregoff, 1958) and then P-Cho is synthesized by three sequential *N*-methylations of P-EA: phosphoethanolamine P-EA → phosphomethylethanolamine (P-MEA) → phosphodimethylethanolamine (P-DEA) → phosphocholine (P-Cho). These reactions are performed by a single enzyme called phosphoethanolamine-*N*-methyltransferase (PEAMT) (Nuccio et al., 2000b). The route of choline production has been demonstrated to be important for many plants including the eudicots *Arabidopsis* (Bolognese & McGraw, 2000), *Spinach oleracea* (spinach) and *Beta vulgaris* (sugar beet) (Hanson & Rhodes, 1983) and the moncot *Lemna pauscicostata* (Datko & Mudd, 1988b).

The phosphatidylbase (Ptd-) and free bases can also be sequentially methylated from Ptd-MEA (Ptd-MEA→Ptd-DEA→Ptd-Cho) (Datko & Mudd; 1988a) or EA (Prud'homme & Moore, 1992a). Datko and Mudd (1988a) showed that methylation occurs at the phosphatidyl pathway in soybean and carrot but not all steps occur through these intermediates. These authors reported that the committing step for the synthesis of choline in carrot or soybean is the methylation of P-EA by PEAMT and that subsequent steps occur at either the phosphatidyl or phosphobase level (Mudd & Datko, 1986, Datko & Mudd, 1988a). For carrot cell cultures the methylation reactions proceed at both the phosphobase and phosphatidylbase levels by converting P-EA to P-MEA and then P-MEA can be methylated directly or converted first to Ptd-MEA prior to methylation.(Datko & Mudd, 1988a). In contrast, for soybean tissue culture they found that the P-EA methylated to P-MEA by PEAMT was converted to Ptd-MEA but then all further methylations occur at only the level of Ptd bases (Ptd-MEA→Ptd-

Figure 1. Choline and phosphatidylcholine biosynthesis pathways in plants

The free base, phosphobase (P-base) and phosphatidylbase (Ptd-base) intermediates are shown. Three methylation steps and the enzymes responsible are designated by bold letters: NMT1, NMT2 and NMT3 where NMT signifies *N*-methyltransferase. The methylation of P-EA is a committing step in plant Ptd-Cho synthesis with subsequent methylations at the P-base (heavy arrows) or Ptd-base level (light arrows). *NMT1* and *NMT3* catalyze the methylation of all three P-bases leading to P-Cho synthesis whereas *NMT2* cannot use PEA as a substrate.

EA, ethanolamine; MEA, methylethanolamine; DEA, dimethylethanolamine; Cho, choline; Bet Ald, betaine aldehyde; GB, glycine betaine; P-EA, phosphoethanolamine; P-MEA, phosphomethylethanolamine; P-DEA, phosphodimethylethanolamine; P-Cho, phosphocholine; Ptd-EA, phosphatidylethanolamine; Ptd-MEA, phosphatidylmethylethanolamine, Ptd-DEA, phosphatidyldimethylethanolamine; Ptd-Cho, phosphatidylcholine. [Reproduced from (Begora, et al., 2010)].


EA
MEA
DEA
Cho
Bet Ald
GB
NMT1 / NMT3
NMT1 / NMT3
NMT1 / NMT3
P-EA
P-MEA
P-DEA
P-Cho
NMT2
NMT2
CDP-EA
CDP-MEA
CDP-DEA
CDP-Cho
Ptd-EA
Ptd-MEA
Ptd-DEA
Ptd-Cho

DEA→Ptd-Cho; Datko & Mudd 1986). Choline synthesis in other organisms does not necessarily follow the same route as plants. In mammals and yeast the phosphatidyl base is a dominant pathway (Greenberg et al., 1983) but in the malaria causing plasmodium (*Plasmodium falciparum)* the pathway followed resembles the phosphobase route of plants. Thus there is evidence of diversity in choline metabolism in different organisms and the enzymes responsible for the synthesis of this important metabolite.

The role of choline in plants

The universal role for choline in plants is as the polar head group in the membrane phospholipid Ptd-Cho (Moore, 1982). However, choline has other functions in plants. One important end product in plants and other organisms is in the production of the quaternary ammonium compound called glycine betaine (GB) (Hanson & Nelsen, 1978, Yancey, 2005). GB is a compatible solute and osmoprotectant in plants (Hanson & Nelsen, 1978, Hitz et al.,1981, Jones & Storey, 1978). Plant species such as sugar beet and spinach accumulate GB in response to water deficits and salinity (Coughlan & Jones, 1982, Hanson & Rhodes, 1983). Plant leaves are where the synthesis of GB mainly occurs (Hanson & Rhodes, 1983). In spinach the phosphobase methylation pathway operates in the cytosol (Weretilnyk et al., 1995) and the two-step oxidation of choline to GB takes place in the choloroplast (Weigel et al., 1986). The enzyme P-Cho phosphatase hydrolyzes P-Cho to produce free choline in spinach (Summers & Weretilnyk, 1993) that must then move across the chloroplast membrane to the site of GB synthesis. The movement of choline in plants may affect its use as a precursor for GB. Nuccio et al (2000a) used computer modeling combined with radiotracer analyses to show that choline transport into the chloroplast likely limits GB synthesis in tobacco. (Weretilnyk & Hanson, 1990). With exposure to salt the activities of several enzymes in this pathway (EA kinase and phospho-base methyltransferases) are up regulated (Summers & Weretilnyk, 1993).

Many plants including *Arabidopsis* and tobacco cannot accumulate GB because they lack the enzyme choline monoxygenase (CMO). CMO catalyzes the first step in a two-step pathway of GB synthesis and so its absence limits the ability of these plants to accumulate GB in spite of having a functional BADH (Nuccio et al., 1998). However, plants that constitutively express active spinach CMO in chloroplasts can produce GB but they do not accumulate significant amounts of GB (Nuccio et al., 2000a) and an insufficiency of choline was thought to be a likely problem. Over-expression of the spinach PEAMT in transgenic tobacco plants was performed to augment the endogenous tobacco PEAMT activity and increase the pool of free choline available for conversion to GB (McNeil et al., 2001). The expanded choline pool led to a 30-fold increase in free choline relative to the control plants but the levels of GB in these transgenic plants remained too low to be useful in osmotic stress protection compared to the plants such as spinach that naturally accumulated GB (Hitz & Hanson, 1980).

Arabidopsis has been transformed with the *codA* gene isolated from *Arthrobacter globiformis* (Sakamoto et al., 2000). This bacterial gene encodes the enzyme choline oxidase. Plants carrying this transgene also synthesize GB to a higher level relative to wild-type plants and improvements in cold stress tolerance and salt shock were observed in *Arabidopsis*, a species normally intolerant of these conditions. Despite this different approach, a common observation made is that one can engineer plants to successfully increase GB but transgenic plants do not accumulate GB to the same extent as plants like spinach that naturally accumulate this metabolite.

Enzymes involved in phosphocholine (P-Cho) synthesis

Methyltransferase enzymes

The transfer of a methyl group from one molecule to another is catalyzed by methyltransferases. These enzymes are involved in the methylation of many substrates including nucleic acids, proteins, and carbohydrates (Moffatt & Weretilnyk, 2001). The most common methyl donor molecule used by all kinds of methyltransferases is S-adenosyl-L-methionine (AdoMet) (Cheng & Roberts, 2001). In view of their

importance in plant metabolism, biosynthesis, detoxification, protein sorting and repair, signal transduction and nucleic acid processing, methyltransferases have been extensively studied (Joshi & Chiang, 1998, Martin & McMillan, 2002).

Phosphoethanolamine N-methyltransferase (PEAMT) and phosphomethylethanolamine N-methyltransferase (PMEAMT)

As discussed earlier, P-Cho is synthesized through a series of reactions catalyzed by methyltransferases with the first enzyme in choline synthesis, PEAMT, capable of catalyzing all three methyl transfer reactions (Fig. 1) (Weretilnyk et al., 1995, Nuccio et al., 2000b). PEAMT from a number of plants has been biochemically characterized including *Arabidopsis*, *spinach* and *wheat* (Bolognese & McGraw, 2000, Nuccio et al., 2000b, Charron et al., 2002). The gene corresponding to this enzyme in *Arabidopsis* is annotated as *AtNMT1* (or simply *NMT1*) and is found on chromosome 3 (Bolognese & McGraw 2000). The critical contribution of this enzyme towards plant development has been shown using mutant plants deficient in the activity of this enzyme. The presence of a T-DNA insertion in *Arabidopsis PEAMT* was associated with a phenotype of increased lateral roots and delayed flowering (Cruz-Ramírez et al., 2004).

A second methyltransferase in this pathway is phosphomethylethanolamine *N*-methyltransferase (PMEAMT) which shares 89% amino acid sequence identity with PEAMT. The activity of only two methyl transfer reactions, namely P-MEA→P-DEA→P-Cho, is associated with PMEAMT. The activity of PMEAMT was identified and its protein partially purified from spinach leaves (Smith et al., 2000, Weretilnyk et al., 1995). *Arabidopsis PMEAMT* has been cloned by functional complementation of a Ptd-Cho deficient yeast strain and the gene product studied *in vitro* by radio assay using extracts prepared from the transformed yeast as well as leaves of *Arabidopsis*. *Arabidopsis* PMEAMT, like the enzyme from spinach, can catalyze only the last two methylation reactions leading to P-Cho synthesis (BeGora et al., 2010). The gene encoding PMEAMT is located on chromosome 1 and is designated *AtNMT2* or simply *NMT2*.

A third, closely related gene is found on chromosome 1 in the *Arabidopsis* genome along with *NMT2* and is annotated as a third, putative *PEAMT* designated *AtNMT3* or *NMT3*. The predicted protein sequence shares 82% amino acid identity with *NMT1* (Macleod, 2010). Macleod (2010) cloned the gene encoding NMT3 and over-expressed the protein in *E. coli* and showed this enzyme to be functionally equivalent to PEAMT in its ability to perform all three methylations of P-EA to P-Cho. For ease of discussion, the nomenclature for *Arabidopsis* used in this report will refer to PEAMT encoded by the gene on chromosome 3 as NMT1 and the two genes on chromosome 1 encoding PMEAMT as NMT2 and the second PEAMT enzyme as NMT3.

Regulation of PEAMT

The activity and protein level of spinach PEAMT decreases in the dark (Drebenstedt, 2001). In the presence of salt and light, the activity of this enzyme is increased and this up-regulation is associated with increased protein levels (Weretilnyk et al., 1995). It is not clear how light regulates PEAMT activity. The activity of PMEAMT (corresponds to *Arabidopsis* NMT2) does not show light-responsive activity changes in contrast to PEAMT (Summers & Weretilnyk, 1993). This evidence served as an early indicator that PMEAMT is a different enzyme from PEAMT.

In plants studied to date, PEAMT (NMT1) is the enzyme that regulates flow through the pathways that produce choline and Ptd-Cho (Datko & Mudd, 1988a, Datko & Mudd, 1988b, Mou et al., 2002b). Not surprisingly, this enzyme is subject to regulation at various levels including the availability of light. One of the products of the methylation reaction is *S*-adenosyl-L-homocysteine (AdoHcy) which is also a potent inhibitor of PEAMT activity (Nuccio et al., 2000b; Datko & Mudd, 1988a; Datko & Mudd; 1988b, Smith et al., 2000). Another product of the phosphobase methylation pathway, P-Cho, also inhibits the NMT enzymes *in vitro*. The activity of 400-fold purified spinach PEAMT was dereased 71% in the presence of 1mM of P-Cho and 47 % in presence of 0.01 mM of AdoHcy compared to controls (Smith et al., 2000).

Importance of PEAMT/NMT *in planta*

The physiological role of NMT1 activity in *Arabidopsis* has been examined *in vivo*. Suppressed expression of *Arabidopsis NMT1* (*XPL1*) produces a short primary root, fewer root hairs, a high number of lateral roots, late flowering and reduced levels of P-Cho, Ptd-Cho and Cho in short epidermal cells in roots (Cruz-Ramírez et al., 2004). *xipol1* seedlings also have a reduction in the biosynthesis of Ptd-Cho in roots and leaves. The content of Ptd-Cho in leaves and roots is decreased by ~18 and 23%, respectively, compared with wild type plants. *xipol1* plants also showed a decrease of 5% in P-Cho levels and 7% in choline levels in leaves and roots compared with wild type plants. The results of RNA *in situ* hybridization indicate that the expression level of *NMT1* is higher in roots and less in leaves, inflorescences, and siliques of wild type plants (Cruz-Ramírez et al., 2004). Since NMT1 is believed to be the only enzyme responsible for the synthesis of P-Cho and choline in *Arabidopsis* it is not surprising that its reduced expression is associated with decreased root and shoot choline and P-Cho content. In the case of *xipol1*, exogenous additions of choline or P-Cho can rescue the mutant and restore the wild type root phenotype which indicates that a functional choline kinase enzyme exists in *Arabidopsis* and that choline is limiting in this mutant plant line (Cruz-Ramírez et al., 2004).

The role of the NMT family in *Arabidopsis* was also studied by the production of RNAi knockdown lines (Mou et al., 2002a). These lines showed the severe morphological phenotype of pale green leaves, early senescence, temperature-sensitive male sterility and siliques producing fewer seeds and it is likely that the use of RNAi led to reduced expression of all the *NMT* genes. The authors of this study reported that the total choline content was reduced by 64% compared to wild type levels. This plant line also showed a hypersensitivity to treatment with 200 mM NaCI relative to wild type plants indicating that silencing *NMT* results in hypersensitivity to high salinity.

Post transcriptional regulation of the *Arabidopsis NMT1* is mediated by an upstream open reading frame (uORF) (Alatorre-Cobos et al., 2012). The conserved

uORF is not found in *Arabidopsis NMT2* and *NMT3*. The spinach and corn *NMT* homologues also have the uORFs (Tabuchi et al., 2006). Tabuchi et al (2006) propose that the posttranscriptional regulation by *AtNMT1* uORF may play a role to prevent excessive choline production. When spinach is grown in 10mM choline chloride the PEAMT protein content and transcript levels of *SoPEAMT* were reduced in the roots providing evidence that choline levels likely play a vital regulatory role in the regulation of *PEAMT* expression (Tabuchi et al., 2006).

Phosphatidylcholine (Ptd-Cho) synthesis in plants

Ptd-Cho is the most abundant non-plastid phospholipid in plants and accounts for up to 60% of total membrane lipids (Moore, 1976; Datko & Mudd, 1988b). Ptd-Cho content can be modulated and the levels and fluidity of Ptd-Cho can be altered in response to abiotic stress in plants such as exposure to high salinity (Pical et al., 1999). The amount and degree of polyunsaturation of fatty acyl chains comprising Ptd-Cho in the plant membrane is associated with changes in the freezing tolerance of plants with a higher degree of unsaturation making membranes more fluid at lower temperatures (Uemura et al., 1995). Also, a greater amount of Ptd-Cho is produced in response to salt stress in *Arabidopsis* (Tasseva et al., 2004). In addition, Ptd-Cho is important for phosphatidic acid (PA) production. PA is a signalling molecule/secondary messenger that plays diverse roles in plant development (Li et al., 2009) and Ptd-Cho can be converted into PA and free choline by the enzyme phospholipase D (PLD) (Qin & Wang, 2002). Inhibition, overexpression, or silencing of PLD has an impact on plant development including altering root and root hair development of *Arabidopsis* (Ohashi et al., 2003).

There are two different pathways to produce Ptd-Cho in plants: the CDP-Cho pathway also known as the Kennedy pathway and the methylation pathway. In the CDP-Cho pathway, choline kinase phosphorylates choline and then P-Cho is converted to Ptd-Cho via cytidine-5'-diphosphate choline (CDP-choline) as an intermediate (Tasseva et al., 2004). The alternative route for Ptd-Cho synthesis is the sequential methylation of phosphatidylbase intermediates to produce Ptd-Cho (Datko & Mudd, 1988b). There is,

however, no known phosphatidylethanolamine (Ptd-EA) methyltransferase in plants and this ensures that PEA and not Ptd-EA serves as a precursor for Ptd-Cho synthesis. (Weretilnyk, & Summers, 1992)

The phosphatidylbase methylation pathway accounts for only10% of total Ptd-Cho in control plants and approximately 6%,1% and 9% under cold stress, salt stress and mannitol stress, respectively, in *Arabidopsis* plants (Tasseva et al., 2004). The majority of Ptd-Cho is produced by the CDP-choline (Kennedy) pathway. The CDP- choline pathway seems to be controlled by choline kinase (CK) and CDP- choline cytidylyltransferase (CTT) (Tasseva et al., 2004). Under cold and salinity stress conditions Ptd-Cho production is increased through the operation of the CDP-choline pathway (Inatsugi et al., 2002, Tasseva et al., 2004) while enzymes needed for the methylation of phosphobases and phosphatidylbases are down-regulated (Tasseva et al., 2004). There is additional genetic evidence that operation of these pathways is flexible and responsive to plant needs for choline and Ptd-Cho. Phospholipid methyltransferase (AtPLMT) catalyzes the methylation at the phosphatidyl base level in *Arabidopsis* (Keogh et al., 2009). Using a Ds-transposon insertion line of *Arabidopsis*, Keogh et al. (2009) showed that the loss of *AtPLMT* leads to a plant with no visible phenotype and the amount of Ptd-Cho produced is unchanged relative to wild type. These results indicate that flux through the phosphobase methylation and CDP-choline pathways can compensate for the loss of the direct methylation route in plants when AtPLMT activity is disrupted.

Quantitative real time polymerase chain reaction (q-PCR)

Reverse transcription (RT) followed by the polymerase chain reaction (RT-PCR) has proven to be a powerful technique to analyze gene expression in all types of organisms (Livak & Schmittgen, 2001; Winer et al., 1999). A more recent variation on this approach has been called Real Time quantitative PCR (q-PCR) and it offers advantages in that it is highly accurate, very sensitive and allows for precise quantification of not only rare transcripts but also small changes in gene expression

(Morrison et al., 1998). The technique has additional desirable features in that it is easy to perform, the equipment is frequently available in labs, and the assays are rapid and reproducible once assay conditions have been validated and standardized for a given application (Pfaffl, 2001).

There are two types of quantification in q-PCR. One is absolute quantification which requires an internal or external calibration curve generated using known concentrations of cDNA or RNA plotted against the PCR amplification fluorescence signal. The transcript abundance of a sample is then estimated from comparisons between the PCR signal of the target template to the calibration curve (Pfaffl, 2001). In order to use a calibration curve it is important to confirm that the target templates for both the sample and the standards are being amplified with identical PCR amplification efficiencies. The determination of amplification efficiency is part of the process called validation (Pfaffl, 2001). More frequently, however, the version of q-PCR used is not an absolute determination but a relative quantification. In this approach the expression of target genes is estimated in terms that are relative to the expression of reference genes (Livak & Schmittgen, 2001). Livak and Schmittgen (2001) derived the equation which is basis for the relative method of quantification: $2^{-\Delta\Delta Ct}$. In their equation, a Ct value is the quantification cycle where the PCR signal crosses the threshold of detection in a progressive series of successive PCR cycles. The Ct value for the target gene of interest is then be compared to the Ct values for several reference genes.

"Cq" is the standard name for the Ct value according to the recognized Real-time PCR Data Markup Language (RDML) guideline (Bustin et al., 2009). Using the "$2^{-\Delta\Delta Ct}$ method" (as this approach is referred to in the literature), an untreated control serves as the calibrator where $\Delta\Delta Ct$ equals zero and 2^0 equals one. Consequently, all other fold-changes in gene expression are then relative to the conrol value of 1 (Livak & Schmittgen, 2001). According to Pfaffl (2001), the reliable and accurate relative quantification of gene expression relies upon on reliable, standardized, reference genes. A reliable reference gene is presumed to have constant expression levels in different tissues, all developmental stages of an organism under study and regardless of the

experimental conditions or treatments a tissue or organism experiences. Therefore, some of the most frequently used reference genes for q-PCR in plants and animals include well studied genes: *18S rRNA*, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *polyubiquitin* (*UBQ*), *actin* (*ACT*), *elongation factor-1α* (*EF-1α*) and *α-tubulin* and *β-tubulin* (*TUA* and *TUB*) genes (Czechowski et al., 2005). In summary, because of its high through-put potential, high sensitivity, and superior accuracy, q-PCR has become a powerful gene expression analysis tool to study gene expression in plants.

MATERIALS AND METHODS

1. Chemicals and Reagent Preparation

Chemicals and enzymes were purchased from Sigma (Sigma-Aldrich Canada, Oakville, ON) unless otherwise stated. All solutions were prepared with de-ionized water purified by a Barnstead NANO pure 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/mobixlab/).

2. Plant material and growth conditions

2.1 Seeds

The T-DNA insertion plant lines used in this study were obtained from the Arabidopsis Biological Resource Center (ABRC) at the University of Ohio (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). Specifically, the lines used and the loci with the reported T-DNA insertions were: SALK_036291 (At3g18000), SALK_006037 (At1g48000), SALK_062703 (At1g73600), SALK_016929c (At1g73600), and SALK_120703c (At1g73600). Information regarding these lines and the primers used to validate genotypes is given in Table1. SALK_062703 *NMT3* and *nmt3* plants were identified through genotyping analysis and propagated in the lab. *Arabidopsis thaliana* ecotype Columbia (Col-0) was also obtained from ABRC for use in this study and references to wild-type (WT) in the text refer to this line.

2.2 Surface sterilization of seeds

About 40 to 50 seeds from each seed line were sterilized in 1.5 mL microcentrifuge tubes with 1mL of 70% v/v ethanol at room temperature for 2 min with gentle shaking. After the ethanol was removed, 5 mL of Sterilization Solution consisting of 30% v/v Javex Bleach™ (Clorox Company of Canada, Brampton, ON) and 0.1% v/v Triton X-100 (Cat. No. X100) in H_2O. The tubes were then incubated at room temperature for 10 min with periodic inversion. The seeds were washed with 1 mL of H2O until no bubbles were seen; seeds were suspended in sterile 1 mL 0.1% w/v

Table 1. Gene specific primers for genotyping

Primer ID	Locus	SALK Line	Forward Primers (5'-3')	Reverse Primers (5'-3')	Tm °C
NMT1-GT[a]-1	At3g18000	SALK-036291	TTCTTCAGGACAAACCAGCCTTG	AAACCAAGTAGCATAACACAAATGC	55
NMT2-GT-1	At1g48600	SALK-006037	AGGAACCAACCTACCACAACC	GATTCGATTTTTGTGCGATTC	61
NMT3-GT-1	At1g73600	SALK-062703	TCGAGCTCGAGCTTTCTATG	TATGCTGGATCTGAAACCTGG	60
NMT3-GT-2		SALK-120703c	TGCTGGAATTGGTCGTTTTAC	TCCACGAATTCCTTTGTTGTC	60.1
NMT3-GT-3		SALK-016929c	TCCCGGTTCCACTAAAGAATC	AGAACAAGCATGGACGTGAAC	60.1
LBP#1.3[b]			ATTTTGCCGATTTCGGAAC		55~61

[a] Gene specific primers used in genotyping (GT) the specific SALK mutant lines
[b] T-DNA left border primer for all SALK mutant lines

Phytagel™ (Cat. No. P8169) solution and then the tubes containing suspended seeds were capped and stored at 4°C overnight**.**

2.3 Seed germination on defined media plates

Surface-sterilized seeds were transferred to a 100 × 20 mm cell culture dish (Corning Inc., Corning, NY, Cat. No. 83.1802) containing Murashige and Skoog (MS) medium (Murashige & Skoog, 1962). The MS medium was prepared using 1.0 mL MS vitamins (Cat.No. M7150), 0.215 g Murashige and Skoog Salt Mixture (Cat. No. M5524), 1% w/v sucrose (BioShop Canada Inc. Cat.No. SUC507.2) and solidified with 0.8% Phytagel. The perimeter of the plate was sealed with Micropore™ surgical tape (Fisher Scientific, Ottawa, ON, Cat. No. 19-027-761) and placed in a germination chamber (Conviron, Controlled Environments Limited, Canada; Model No: CMP6010) set for a 24 h photoperiod with a light intensity of 50 µmol m^{-2} s^{-1} at 22°C for 7 days before seedlings were transplanted to plates containing defined nutrient media.

2.4 Preparation of culture plates for seedling studies

Media used for seedling production and salt stress studies were formulated using the components listed in Table 2. After combining the volumes of various stock solutions and water, the nutrient solution was made 2% (w/v) with respect to sucrose then the pH of the medium was checked using an Orion DUAL STAR pH Meter (Thermo Fisher Scientific Inc., Rockford, IL, Cat. No. 2115000) to ensure it was between 5.5 and 6. After verifying the pH, 0.8 % (w/v) Phytagel was added and then the solution containing agar was autoclaved. The sterile culture medium was allowed to cool slightly before being poured into 100 × 20 mm square plates (BD Biosciences, Mississauga, ON, Cat. No. 35112). The plates were left at room temperature to solidify overnight and then they were inverted and placed in a plastic sleeve for storage at 4°C until needed.

Table 2. Composition of media used to test the response of *Arabidopsis* seedlings to salt stress

Component	Stock	Final	Salt concentration (NaCI, mM)		
			0	50	75
Macronutrients	M	mM	Volume (mL)		
$CaCI_2$-$2H_2O$	0.5	0.5	1	1	1
$MgSO_4$	0.5	0.5	1	1	1
KNO_3	0.5	0.5	1	1	1
Na-PO_4	0.5	0.5	1	1	1
Micronutrients	mM	μM			
$FeSO_4.7H_2O$	10	100	10	10	10
H_3BO_3	100	100	1	1	1
$MnCI_2$	20	20	1	1	1
$CuSO_4$	1	1	1	1	1
$ZnSO_4.7H_2O$	3	3	1	1	1
Na_2MoO_4	1	0.4	0.4	0.4	0.4
$CoCI_2$	0.1	0.01	0.1	0.1	0.1
Additions	M				
NaCI	2		0	25	37.5
H_2O			981.5	956.5	944

2.5 Seedling transplant to culture media

Four days after germination, the small seedlings (at 4 leaf stage with 2 rosette leaves and 2 cotyledons) with root length averaging 1 mm were carefully transferred from the MS plates to culture plates (see Section 2.4). The plates with seedlings were oriented vertically in a Conviron growth chamber (Conviron, Controlled Environments Limited, Canada; Model No: PGC20) set at 22°C with a 12 h light/12 h dark photoperiod under a light intensity of 150 µmol m^{-2} s^{-1}.

Seedlings were photographed at the same time each day using a Nikon D90 camera (NIKON Corp., JAPAN; Model No: 5005520) fitted with a lens (AF-S NIKKOR 18-55 mm 1:3.5-5.6G; Model No: 51136801). The photographs were used to determine changes in primary root length and lateral root numbers and length over the experimental period using ImageJ 1.46r software (http://rsbweb.nih.gov/ij/). When the primary roots reached the bottom of the plate (approximately 5 to 6 days after transplant) the seedlings were harvested. For diurnal expression studies the seedlings were harvested at two times during the day: mid-way through the light cycle and mid-way through the dark cycle. The shoots and roots were harvested separately and after harvest, tissues were immediately flash-frozen in liquid nitrogen and stored at -80°C until use. This experiment was performed three times with each repeat including a minimum of 90 seedlings.

2.6 Seedling salt stress experiment

Seedlings produced on MS plates (see Sections 2.3 and 2.4) were transplanted at 4 days post-germination to defined medium culture plates modified to contain 50 or 75 mM NaCl (Table 2). As a control, seedlings were also transplanted to plates that did not contain added NaCl. After the seedlings were transplanted from MS media to defined culture media with or without added salt, the plates were positioned vertically in a chamber at 22°C with a 12 h light/12 h dark photoperiod under a light intensity of 150 µmol m^{-2} s^{-1}. Seedlings were photographed at the same time each day as described in Section 2.4. The plants were harvested at the same stage based upon similar root length for seedlings across the various treatments. The shoots and roots were harvested

separately and the photographs were used to determine changes in primary root length and lateral root numbers and length over the experimental period using ImageJ 1.46r. A sample (90 to 110 mg) of root and shoot tissue was flash-frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation. The entire experiment was repeated three times.

2.7 Plant Growth in Pots and Tissue Collection

Following surface-sterilization and overnight storage at 4°C (see Section 2.2), seeds were transferred to a soil mix prepared using 6 parts Promix BX soil (Premier Horticulture Inc., Quakertown, PA) and 1 part Turface (Plant Products, Brampton, ON). The trays with pots were transferred to a dark cold room for 3 days at 4°C to allow the seeds to stratify. After cold treatment, the trays were moved to a growth chamber set to at 22°C and a light intensity of 120 to 150 µmol m^{-2} s^{-1} at 23°C with a 9 h light/16 h dark or 12 h light/12 h dark photoperiod. Plants were watered as required and fertilized once every two weeks with 1g/L of 20-20-20 fertilizer (Plant Products Ltd. Brampton, ON). Unless otherwise specified, plant tissue was harvested from 4-week-old plants. For RNA extraction a mass of 90 to 110 mg of leaves and roots tissues were harvested, flash-frozen in liquid N_2 then stored at -80 °C. Plant tissue was usually harvested mid-way through the light period when phosphobase NMT activity is maximal (Weretilnyk et al. 1995).

3. Molecular Analyses

3.1 DNA Preparation

DNA was extracted from leaves of 2-week-old plants using FTA® Plant Saver Card (Whatman™, Cat. No. WB120065). Leaf tissue was crushed against the FTA paper using the end of a pestle and then fragments of the paper (approximately 2 mm diameter) were cut out using scissors and the segments put into a 1.5-mL microcentrifuge tube. To the contents of the PCR tube, 200 µL of FTA Purification Reagent (Whatman™, Cat. No. WB120204) was added and the tube was incubated for 5 min at room temperature then the solution was discarded. This washing step was repeated twice. After the second treatment, 200 µL of 1X TE Buffer (10 mM Tris-HCI, 0.1 mM Na_2-EDTA, pH 8.0) was

added to the PCR tube and the tube was incubated for 5 min at room temperature. After incubation the buffer was discarded and this washing step was repeated two times in total. After the final removal of liquid the paper fragments were dried by transferring the opened PCR tubes to a heating block set at 56°C for 10 to 20 min.

3.2 Analyzing T-DNA Insertion lines of *Arabidopsis thaliana*

Table 1 lists the primers used for PCR assays and sequence information for the left and right gene-specific primers used in genotyping specific SALK mutant lines. Left and right gene-specific primers and T-DNA specific left border primers were designed using the T-DNA primer design tool from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/tdnaprimers.html) (Alonso et al., 2003). The forward 5'-AGGAACCAACCTACCACAACC -3' and reverse 5'-GATTCGATTTTTGTGCGATTC-3' gene-specific primers were used to identify *nmt2* plants homozygous for a T-DNA insertion. To identify *nmt3* SALK_062703 line plants the gene-specific forward 5'-TCGAGCTCGAGCTTTTCTATG-3'and reverse 5'-TATGCTGGATCTGAAACCTGG-3' primers were used. To identify *nmt3* SALK_016929c line plants the gene-specific forward 5'-TCCCGGTTCCACTAAAGAATC-3' and reverse 5'-AGAACAAGCATGGACGTGAAC-3' primers were used. To identify *nmt3* SALK_120703c line plants the gene-specific forward 5'-TGCTGGAATTGGTCGTTTTAC-3' and reverse 5'- TCCACGAATTCCTTTGTTGTC-3' primers were used. PCR reactions to confirm *nmt1* plants used the forward 5'-TTCTTCAGGACAAACCAGCCTTG-3' and reverse 5'-AAACCAAGTAGCATAACACAAATGC-3' primers. All PCR reactions used to confirm the presence of the T-DNA insertion used the T-DNA specific left border primer 5'- TGGTTCACGTAGTGGGCCATCG-3'. PCR reactions (50 μL volume) contained 34.5 μL H_2O, 5 μL of 10 x PCR buffer (20 mM Tris-HCl, pH 8.8), 5 μL of 25 mM $MgCl_2$, 1 μL of each primer (10 pmol/ μL), 1μL of 10 mM dNTP mix, 1 μL DNA and 0.3 units of Taq DNA polymerase (Cat. No. D6677). An Eppendorf Mastercycler

Thermocycler (Eppendorf, Brinkmann Instrument.Inc, Germany; Cat. No. 5345-003558) was used to carry out the PCR reactions. PCR amplification began with a denaturation step for 3 min at 94°C followed by 30 cycles of amplification. Each cycle of amplification included 30 s denaturation at 94°C, 50 s primer annealing at the optimum primer-specific temperature (Table 1), 1 min extension at 72^{o}C with the last cycle using a 10 min extension at 72^{o}C. The products amplified by PCR were stored at -20^{o}C or analyzed immediately by agarose gel electrophoresis.

3.3 Agarose gel electrophoresis

The PCR products were separated by electrophoresis on 1.2 % (w/v) agarose gels. The gel was prepared using 800 μL 50 X TAE (0.4 M Tris-base pH 8.0, 10 mM Na_2-EDTA, 200 mM NaOAc), 39.2 mL H_2O and 0.48 g agarose (BioShop, Cat. No. AGA 001.100).The mixture was heated in a microwave oven stopping at 30 s intervals to swirl the mixture until the agarose had dissolved. The solution was allowed to cool slightly before adding 5 μL GelRed Nucleic Acid Stain (Biotium, Inc. Hayward, CA. Cat. No. 41003) and then the mixture was immediately poured into the gel tray and allowed to solidify at room temperature. The PCR reaction product (10 μL) was mixed with 2 μL 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 Na_2-EDTA, 60% (v/v) glycerol) and then loaded onto the gel. A lane containing 0.8 μg of 1 Kb Plus DNA Ladder (Fermentas Biosciences, Burlington, ON. Cat. No. SM0314) mixed with 2 μL 6X loading dye and 8 μL of H_2O was included on each gel. Electrophoresis was performed at 70 V for 1 h using a PowerPac 300 V supply (Biorad, Cat. No.165-5050). The amplicon and molecular mass DNA standards were visualized on an AlphaImager™ 2200 transilluminator with AlphaImager v.5.5 software (Fisher Scientific, Ottawa, ON) and images produced using a Mitsubishi CP700D printer/cutter (Mitsubishi Electric Canada Inc., Markham, ON).

3.4 RNA Preparation

All reagents used for RNA extraction were provided by the Sigma Spectrum Plant Total RNA kit (Cat. No. STRN50) and the RNA preparation was depleted of DNA using the Sigma On Column DNase1 kit (Cat. No. DNASE10-1SET).

Approximately 100 mg of previously frozen leaves or roots were ground to a fine powder in liquid nitrogen in a fume hood using a mortar and pestle. The powder was transferred to a 2.0 mL-microfuge tube (supplied in the kit) and then 500 μL of Lysis Solution was added before the powder thawed. The sample tube was incubated in a water bath at 56°C for 5 min then centrifuged in Centrifuge 5415D (Eppendorf, Hamburg, Germany; Cat. No. 0061966) at maximum speed for 1 min at room temperature. The supernatant was transferred to the filtration column and both were centrifuged at maximum speed for 3 min at room temperature. Next, 750 μL Binding Solution was added to the flow-through and the contents mixed by drawing the solution up and down a pipette tip using a pipettor. The solution was then loaded into a Binding Column over a collection tube and the column assembly centrifuged at maximum speed for 1 min. The flow-through was then discarded. A volume of 300 μL of Wash Solution 1 was added to the Binding Column and the column was centrifuged at maximum speed for 1 min. After the column assembly was centrifuged, a volume of 80 μL DNase 1 solution (Cat.No. DNASE10-1SET; 10 μL DNase1 and 70 μL of DNase1 Buffer) was added by pipette to the centre of the column and the column, with DNase 1, was incubated at room temperature for 15 min. After incubation, 500 μL Wash Solution 1 was added and the column was centrifuged at maximum speed for 1 min. After centrifugation, 500 μL Wash Solution 2 was added to the column and the column was centrifuged at maximum speed for 0.5 min. This wash step was performed twice. After centrifugation, the flow-through was discarded and the column centrifuged at maximum speed for 1 min to remove residual wash solution. Finally, a volume of 50 μL Elution Solution was added to the column, the column was incubated at room temperature for 4 min and then the column was centrifuged for 1 min at maximum speed to collect the eluted RNA.

A NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc. Model. 2000UV-Vis) was used to measure the absorbance of the RNA sample to determine concentration and quality. An absorbance ratio for A_{260}/ A_{230} between 1.8 and 2.0 indicates RNA lacks contaminating carbohydrates and other plant metabolites while an A_{260}/ A_{280} ratio between 1.8 and 2.0 is generally accepted as indicating that the RNA is free of protein. The RNA concentration and yield was calculated from the A_{260} value corrected for dilution then multiplied using the conversion of 1 OD_{260} equal to 0.04 µg RNA/µL. The RNA samples were immediately used for cDNA synthesis or stored at -80°C.

3.5 cDNA synthesis

Approximately 1µg/µL of total RNA was used to synthesize first-strand cDNA following the Sigma-Aldrich manufacture's recommendations. The first strand synthesis required 1µL RNA, 1µL 10 mM dNTP mix, 1µL Oligo(dT)18 primer and 7 µL nuclease-free H_2O and the reaction tubes were incubated at 70°C for 10 min. After incubation, 1 µL of M-MLV reverse transcriptase (200U/ µL; Cat. No. M1302), 2 µL M-MLV reverse transcriptase buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, and 15 mM $MgCI_2$), 0.5 µL RNase Out™ ribonuclease inhibitor (Invitrogen Canada, Inc., Burlington, ON, Cat. No.10777019) and 6.5 µL nuclease-free H_2O were added to yield a final, combined volume of 20 µL. The reaction was incubated at 37°C for 50 min and then heated between 80°C to 95°C for 10 min to deactivate the reverse transcriptase enzyme. The cDNA synthesis products were used immediately or stored at -20°C.

3.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

First-strand cDNA synthesized was used to analyze expression of *NMT1 and NMT3* in both wild type and mutant plants. Gene-specific primers were used to reverse transcribe the RNA by PCR (see Table 3). The expression of *ubiquitin 10* was used as an internal control to assess the quantity and quality of the cDNA. Primers used are listed in Table 3. Each RT-PCR reaction included 2 µL of 20 pmol of each forward and reverse

primer, 2.5 µL of 1X PCR buffer containing 20 mM Tris-HCL pH 8.8, 0.5 µL of 10 mM dNTP mix, 2.5 µL of 25 mM $MgCl_2$, 0.5 µL of 0.05 units/µL of Taq DNA polymerase, 2 µL (24 µg) cDNA in a total volume of 50 µL. For transcript analysis (SALK_062703,SALK_120703c and SALK_016929c lines) the initial denaturation step was carried out for 2 min at 94°C followed by 30 cycles with each cycle composed of 30 s denaturation at 94°C, 30 s annealing at 62°C, 1 min extension at 72°C followed by a final extension at 72°C for 10 min. For *NMT3 expression in* SALK_016929c mutants the initial denaturation step was followed by 40 cycles of amplification with each composed of 30 s denaturation at 94^{o}C, 30 s annealing at 67°C, and 1 min extension at 72°C followed by a final extension for 10 min at 72°C. The products amplified by PCR were stored at -20^{o}C or separated immediately by agarose gel electrophoresis.

3.7 Real-time quantitative polymerase chain reaction (q-PCR)

The q-PCR was performed in an optical 96-well clear plate (BioRad Laboratories, Inc, Cat. No. MLL9601) and the plate was covered with a Microseal®'B' Film plate (BioRad Laboratories, Inc, Cat. No. MSB100). To quantify the expression level of a target gene the expression patterns of four reference genes (*At5g12240, At5g60390, At3g62250* and *At5g08290*) were quantified along with each target gene (see Table 3 for primer sequences). q-PCR was performed in a 10 µL reaction containing 0.6 µL of water, 5 µL of 0.5X LuminoCt SYBR Green q-PCR Ready-Mix (Cat. No. L6544), 0.2 µL of 10 µM forward primers (200 nM), 0.2 µL of 10 µM reverse primers (200 nM) and 4 µL (24 µg) of template cDNA. The q-PCR reactions were performed using the BioRad C1000 Thermocycler equipped with the BioRad CFX96 Real Time Detection System. The primer annealing temperature was selected based on the optimum annealing temperature determined for each pair of primers (Table 3). The cycle conditions were: 1 cycle denaturation at 95°C for 3 min followed by 39 cycles of amplification (5 s denaturation at 95°C, 30 s annealing and extension at 63.4°C). After the amplification procedure was completed the product melting cycle was performed to determine whether a single or multiple products were generated during amplification. For this step the plate was held

Table 3. Primers for Amplification of cDNA

RT-PCR	Locus	SALK Line	Forward Primers (5'-3')	Reverse Primers (5'-3')	Tm °C
NMT2-RT[a]	At1g48600	SALK-006037	GATTGGATGGGTCAAGCCAG	GAATAGAGCTGGCTTGTCTTGG	67
NMT3-RT	At1g73600	SALK-062703	ACAACCCAACACACTACCGTG	TGCATCCAGTTCCCGTTTCA	62
NMT3-RT		SALK-120703c	ACAACCCAACACACTACCGTG	TGCATCCAGTTCCCGTTTCA	62
NMT3-RT		SALK-016929c	TCCAGACTCGCTATCGAAGG	AGCCAGCATATCTGGTTCTG	65
Ubiqutin10	At4g05320		GATCTTTGCCGGAAACA ATGGA GGATGGT	CGACTTGTCATTAGAAAGAAAGAGATAACAGG	62
q-PCR					
NMT1-7	At3g18000	SALK-036291	TGTGGACTTGTTGAGTGGTGT	AGACCAAACATAATTCGGTTTAC	63.3
NMT2-15	At1g48600	SALK-006037	ACCGTTCCTTTGGAGGACAA	GGCACAACGACTCGAGTAAGA	61.4
NMT3-3	At1g73600	SALK-062703	TTTCGAGGAGGTAATCGCGG	TGCATCCAGTTCCCGTTTCA	62.3
NMT3-5			GATGCTGGTTTCGAGGAGGT	ACTGCATCCAGTTCCCGTTT	62.3
NMT3-4			AGACGTTGGGTGCGGAATAG	CAATGCCCACAACATCCACG	60.4
NMT3-11			GCGAGGAGCGTGAAATCCAAAA	AGGACGTTCTTCTTTGTCGAGG	61
NMT3-6			TCCAAAGCTTCTGACCTCGACA	GCCGGCCTTCTGAGCTAATTC	61
NMT3-8			AGTGCTAGAGTTTGGTGCTGGA	GTCAACCGCAATGACCTGGC	61
NMT3-13			ATGGACAAAGGTTGGCGGGTAT	TAGGTTCACGGTAGTGTGTTGGG	61
At5g12240	At5g12240		TCCTCGACCTCTTCCCAGTCGT	GCAGCCGCTTTCAGATTTTCCCA	63.4
YL8S	At5g08290		TCTGCACTCCGGTTGGGCTGT	TCAGCAACAGACGCAAGCACC	63.4
EF1a2	At5g60390		ACGCCCCAGTTCTCGATTGCCACA	AGCAACGGTCTGCCTCATGTCCCT	61
UBQ5	At3g62250		TCGTCCTCCGTCTCCGTGGT	TCCCAGCTCCACAGGTTGCGT	61

[a] Gene-specific primers were used to reverse transcribe the RNA by PCR (RT)

at 52°C for 0.5 sec and then the temperature was increased by 0.5°C at 5 s increments until a final temperature of 95°C was reached. Three technical replicates were conducted for each biological replicate. The resulting q-PCR and melt curve data was analyzed based on CFX96 Manager Software versions 3.0 (BioRad Inc).

3.8 Primer validation

The gene-specific primers for q-PCR were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHomeAd), an NCBI program that uses the algorithm Primer3 (Rozen and Skaletsky 2000). The primer sequences were checked with the nucleic-acid folding software mfold for DNA (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) to predict secondary structure related problems associated with the primers. The primer pairs were then validated by q-PCR using cDNA derived from mRNA of *Arabidopsis* seedlings or mature plants.

The first step for validation of the primers involved determining the optimal annealing temperature for the primers. This determination was made by performing a temperature gradient spanning ± 5°C of the predicted optimal annealing temperature and changing the temperature in successive reactions by 0.5°C increments. The lowest quantification cycle (Cq) value is the point where the fluorescence crosses the threshold for detection (Heid, et.al., 1996) and this corresponds to the temperature considered to be the optimal annealing temperature (see Table 4).

When each amplification cycle leads to a doubling of product the spacing of the fluorescence curves is determined by the equation: 2^n = dilution factor, where n (Equation 1) is the number of cycles between curves for different primer dilutions at the fluorescence threshold (or the difference between the Cq values of the curves). A standard curve comprised of Cq values from eight dilutions of a given sample of cDNA was used to determine the reaction efficiency for a given primer pair (Livak & Schmittgen, 2001). The dilutions of template cDNA required for the eight-point standard curve are derived from Equation 1(Livak & Schmittgen, 2001) and each primer set was tested for the appropriate dilution necessary for q-PCR using this formula:

Table 4. Primer sequence validation for q-PCR primers

Primer ID	Efficiency Parameters					Validation Results		
	Tm *°C*	Cq	DF[a]	Points	Slope	Efficiency *%*	Melt Curve	Size *bp*
NMT1-7	63.3	21	3	6	0.997	108.9	Single peak	70
NMT2-15	61.4	20	4	6	0.998	105.9	Single peak	103
NMT3-3	62.3	15	5	7	0.999	109.8	Single peak	70
NMT3-5	62.3	15	6	6	1.000	106.6	Single peak	80
NMT3-4	60.4	15	6	5	0.999	98.6	Single peak	74
NMT3-11	61	21	3	6	0.999	100.9	Single peak	113
NMT3-6	61	21	3	6	0.996	104.4	Single peak	136
NMT3-8	61	21	3	6	0.998	102.4	Single peak	79
NMT3-13	61	21	3	6	0.995	107	Single peak	101
At5g12240	63.4	21	4	7	0.989	97.1	Single peak	200
YL8S	63.4	20	5	6	0.996	102	Single peak	150
EF1a2	61	19	7	7	0.999	92.9	Single peak	233
UBQ5	61	15	6	6	0.996	106.9	Single peak	183

[a] DF represented as Dilution Factor

$$n = (35\text{-}Cq)/8 \qquad \text{Equation 1.}$$

The correlation or regression coefficient (r or r^2 value) of a standard curve provides an estimate of how well the experimental data fit the regression line. An efficiency value between 90 to 110% with a regression value of 0.98 or higher at the optimal temperature is considered valid parameters for use in q-PCR (Bustin *et. al.* 2009). Validation ensures that the q-PCR analysis results are amenable to statistical analysis and the results can be interpreted with confidence.

During validation the efficiency of amplification is determined (as described above) as are properties of the amplicon generated. To ensure that a single product is amplified and that the product is of the predicted size the amplified products of q-PCR were analyzed by electrophoresis on a 2% (w/v) agarose gel (0.48 g agarose, 40 ml 1X TAE buffer, 4 µL GelRed nucleic acid stain) for 2.0 h at 50 V (see section 3.3 for details). The stained amplicons were visualized on a transilluminator to confirm that a single gene product was amplified. This final step of validation is evidence that the amplicon is a product of the target gene of interest.

4.0 Statistical Analysis

All data are presented as the mean +/- SE. Statistical analysis was performed by one way or two-way ANOVA followed by Tukey's post hoc analysis using R 2.13.1 (http://www.r-project.org/). The exception is Figure 12, 18 and 19 which was analyzed using GraphPad Prism 5.0 (http://www.graphpad.com/scientific-software/prism/). Figures of the data were prepared using GraphPad Prism 5.0. Differences were considered statistically significant at $P<0.05$. Information on replication is provided in the figure legends. ANOVA tables are reported in Appendices B and C.

Results

5. Analyzing T-DNA insertion lines by polymerase chain reaction (PCR)

5.1 Genotyping the SALK_062703 line

The *Arabidopsis* SALK-062703 T-DNA insertion line was genotyped to confirm the presence of the T-DNA insertion in individual plants using primers specific to *NMT3*. Figure 2A showed the gene structure map of *NMT3* (locus At1g73600) with the position of primers for genotyping relative to the reported position of the T-DNA in exon 8.

The SALK-062703 line included wild type and mutant plants. Figure 2B shows typical PCR products used to distinguish plants as homozygous for the T-DNA or *nmt3* (single 540 bp amplicon; Lanes 2 to 4) from WT or *NMT3* (single 1167 bp amplicon; Lanes 5 and 6) plants. Individual *nmt3* plants heterozygous for the T-DNA insert were detected by the presence of both bands following PCR amplification of genomic DNA (not shown) but these plants were not used in this study. The products amplified by PCR were extracted from the gels and sequenced then this information was submitted to the Basic Local Alignment Search Tool (BLAST; Altshul et al., 1990) program which confirmed the amplicon identity as a product originating from the *Arabidopsis NMT3*. The sequence information is given in Appendix A.

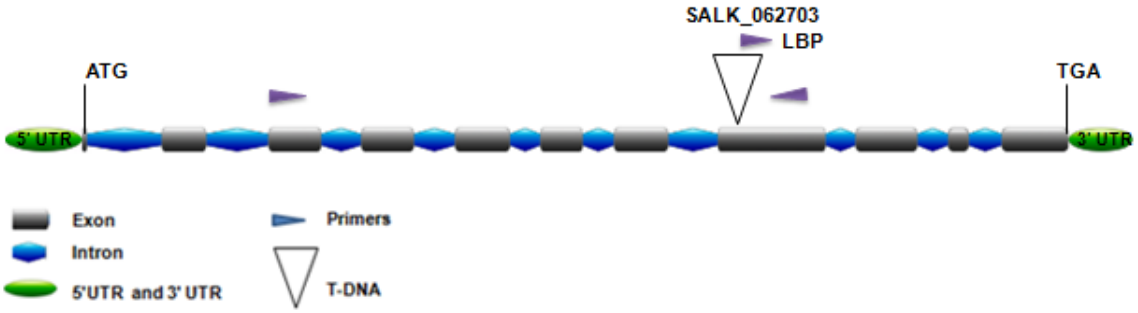
5.2 Genotyping the SALK_016929c and SALK_120703c lines

Genomic DNA extracted from individual plants of the SALK_016929c and SALK_120703c T-DNA insertion lines was used to confirm the presence of the T-DNA insertion. Figures 3A and 3B showed the gene structure maps proposed for *NMT3* in SALK_120703c and SALK_016929c lines, respectively, along with the relative positions of the primers used for genotyping. PCR products were amplified consistent with the predicted locations of T-DNA present in intron 6 of *NMT3* in the SALK_120703c (Fig. 3A) and exon 2 of the same gene in the SALK_016929c line (Fig. 3B). *nmt3* plants that were identified as homozygous for the T-DNA insertion were identified by PCR amplification of genomic DNA and PCR products compared to those of Col-0 WT plants. Figure 3C shows the products of genotyping reactions. Two different RP and LP gene-

Figure 2. *NMT3* gene structure map for the SALK_062703 line with genotyping results verifying presence of T-DNA

A. Map of *NMT3* showing the position of T-DNA insertion site in SALK_062703 line with non-coding UTRs in green, exons in grey and introns in blue. Positions of left (LP) and right (RP) gene-specific primers relative to the T-DNA insertion are shown. **B**. PCR reaction products using genomic DNA isolated from leaves analyzed by electrophoresis on agarose gels. PCR products amplified using gene-specific and T-DNA left-border primers (LBP) to test for the presence of a T-DNA insertion. Lane 1: Molecular size markers; Lanes 2 to 4: 540 bp amplicon amplified with the RP and LBP primers identifying plants homozygous (Hmz) for the T-DNA insertion (*nmt3*) and Lanes 5 and 6: single product of 1167 bp amplified with RP and LP gene-specific primers identifying plants as wild-type (*NMT3*). Primer sequences are given in Table 1. PCR and electrophoresis conditions are given in the Materials and Methods section.

A


SALK_062703
LBP
ATG
TGA
5' UTR
3' UTR
Exon
Intron
5'UTR and 3' UTR
Primers
T-DNA

B

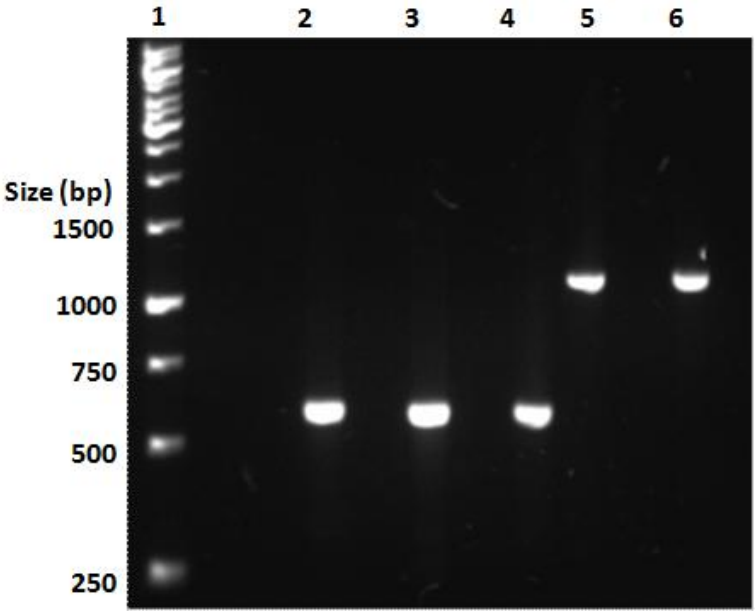
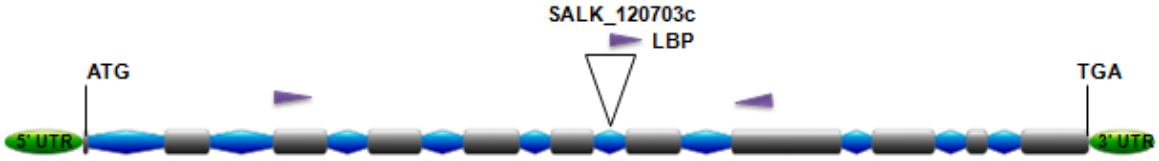

1
2
3
4
5
6
Size (bp)
1500
1000
750
500
250

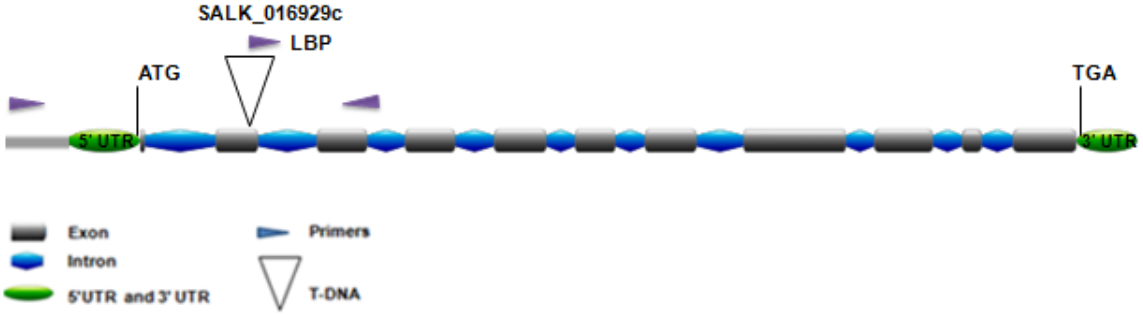
Figure 3. *NMT3* gene structure map for the SALK_016929c and SALK_120703c lines with genotyping results verifying presence of T-DNA insertions

A, B: Maps showing the position of T-DNA insertion sites in *NMT3* for SALK_120703c and SALK-016929c lines, respectively. Non-coding UTRs are shown in green, exons in grey and introns in blue. The upstream region 5' *NMT3* for SALK_016929c is shown as a thickened, light grey line. Positions of left (LP) and right (RP) gene-specific primers relative to the T-DNA insertion are shown for both lines. **C.** PCR reaction products using genomic DNA isolated from leaves of the SALK lines analyzed by electrophoresis on agarose gels. PCR products were amplified using gene-specific and T-DNA left-border primers (LBP) to test for the presence of a T-DNA insertion. For comparison, genomic DNA was isolated from an *Arabidopsis* Columbia wild type (Col-0) plant and tested using LP and RP primers customized for the mutant lines. Lane 1: Molecular size markers; Lanes 2 and 3: SALK_016929c 500 bp amplicon produced using the RP and LBP primers for two plants homozygous (Hmz) for T-DNA insertion (*nmt3*) and Lane 4: the 1042 bp product of Col-0 WT produced using the same primer set. Lanes 5 and 6: SALK_120703c 520 bp amplicon produced using the RP and LBP primers for two plants homozygous (Hmz) for the T-DNA insertion (*nmt3*) and Lane 7: the 1146 bp product of Col-0 WT produced using the same primer set. Primer sequences are given in Table 1. PCR and electrophoresis conditions are given in the Materials and Methods section.

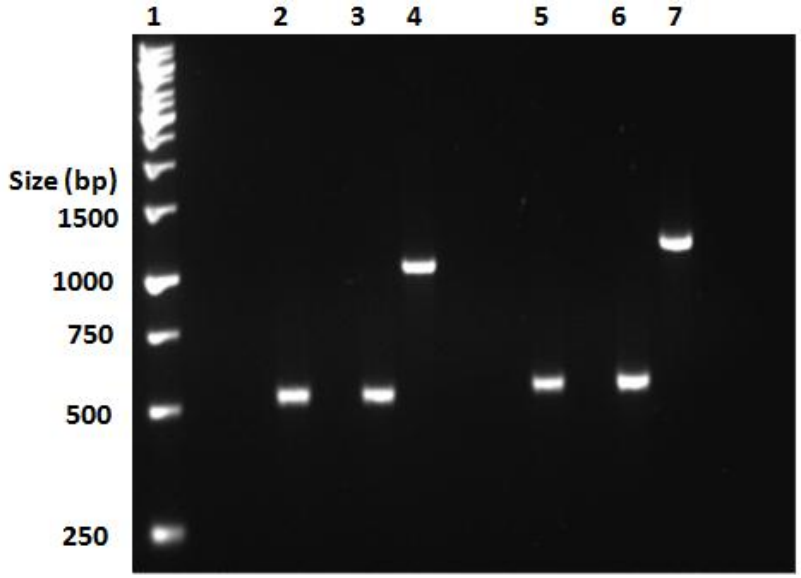
A


SALK_120703c
LBP
ATG
TGA
5' UTR
3' UTR

B


SALK_016929c
LBP
ATG
TGA
5' UTR
3' UTR
Exon
Intron
5'UTR and 3'UTR
Primers
T-DNA

C


1
2
3
4
5
6
7
Size (bp)
1500
1000
750
500
250

specific primer sets amplified either a 1042 or 1146 bp product from Col-0 WT genomic DNA. The gene-specific RP and T-DNA LBP amplified 500 bp and 520 bp products corresponding to *nmt3* plants of the SALK_016929c and SALK_120703c, respectively. Both the PCR amplified products were extracted from gels and sequenced and a search using the BLAST program matched their sequences to locus At1g73600 (*NMT3*).

Figure 3C shows representative results of the genotyping analyses of plants with a 500 bp PCR amplicon product for (Lanes 2 and 3) corresponding to *nmt3* SALK_016929c plants carrying a T-DNA insertion and a 520 bp PCR amplicon product (Lanes 5 and 6) identifying *nmt3* SALK_120703c plants carrying a T-DNA insertion. For comparison, using Col-0 WT DNA as a source of template for PCR genotyping, primers designed to identify SALK_016929c and SALK_120703c *nmt3* plants generated 1042 bp (Fig. 3C, Lane 4) and 1146 bp (Fig. 3C, Lane 7) products, respectively.

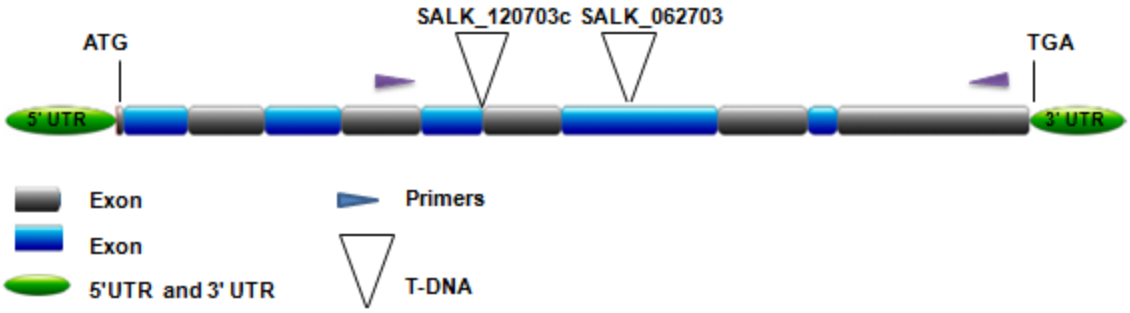
5.3 Transcript analysis of SALK_062703 and SALK_120703c nmt3 plants by RT-PCR

In order to use q-PCR to quantify transcripts associated with *NMT3* the suitability of the cDNA was tested first using RT-PCR. cDNA was prepared from isolated RNA from leaf and root tissue from Col-0 WT plants and *nmt3* plants representing the SALK-062703 and SALK-120703c plant lines. The expression of the gene corresponding to *ubiquitin10* (locus At4g05320) was used as a reference standard. For the SALK_062703 and SALK_120703c plants, gene specific primers were designed to anneal on exon 5 (LP) and exon 10 (RP) to amplify a product of 824 bp (Fig. 4A). cDNA prepared from SALK_062703 and SALK_120703c plants found to be homozygous for the presence of a T-DNA insertion (*nmt3*) had no detectable transcripts in leaves or roots (Fig. 4B, Lanes 2 to 5). In contrast, the expected 824 bp amplicon was produced from cDNA extracted from leaves and roots of Col-0 using the same NMT3 primers (Fig. 4B, Lane 6 and 7). The absence of RT-PCR product cannot be explained by poor RNA or cDNA from the mutant plants as *ubiquitin10* shows no difference in expression between the leaves and roots of the T-DNA lines and Col-0 (Fig. 4B, Lanes 8 to 13).

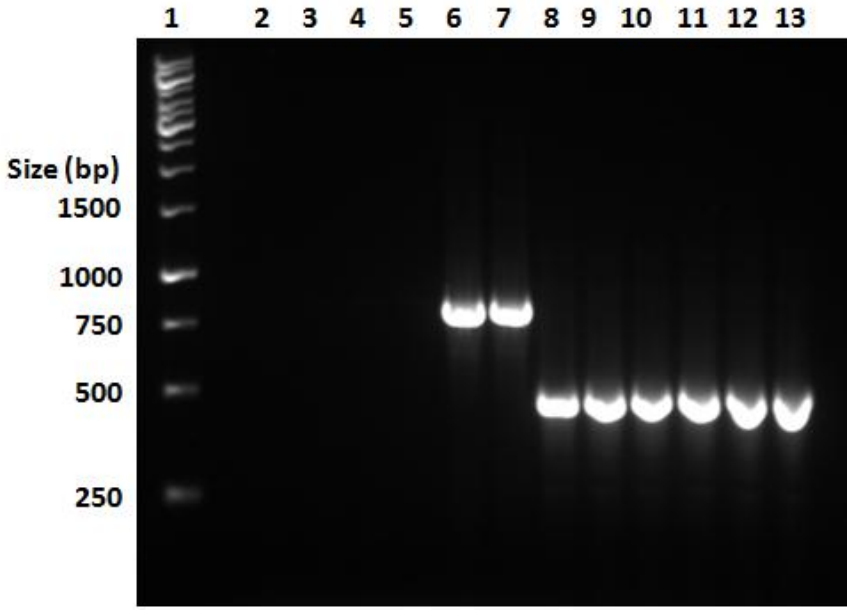
Figure 4. RT-PCR analysis of transcripts associated with *NMT3* from leaves and roots of representative SALK_062703 and SALK_120703c mutant plants

A. Gene structure map of *NMT3* from SALK_062703 and SALK_120703c lines showing the position of the T-DNA insertion site relative to *NMT3* primers used for RT-PCR reactions. The map shows non-coding UTR (green), exons (grey and blue).
B. Analysis of PCR products resolved by electrophoresis on agarose gels. Lane 1: Molecular size markers followed by cDNA amplification of *NMT3* from various sources: Lane 2: *nmt3* SALK_062703 leaves; Lane 3: *nmt3* SALK_062703 roots; Lane 4: *nmt3* SALK_120703c leaves; Lane 5: *nmt3* SALK_120703c roots; Lane 6: Columbia WT (Col-0) leaves; Lane 7: Columbia WT (Col-0) roots and cDNA amplification of *ubiquitin10*: Lane 8: *nmt3* SALK_062703 leaves; Lane 9: *nmt3* SALK_062703 roots; Lane 10: *nmt3* SALK_120703c leaves; Lane 11: *nmt3* SALK_120703c roots; Lane 12: Columbia WT (Col-0) leaves; Lane 13: Columbia WT (Col-0) roots. Primer sequences are given in Table 3. cDNA preparation, RT-PCR and electrophoresis conditions are given in the Materials and Methods section.

A


ATG
SALK_120703c
SALK_062703
TGA
5' UTR
3' UTR
Exon
Exon
5'UTR and 3' UTR
Primers
T-DNA

B


1 2 3 4 5 6 7 8 9 10 11 12 13
Size (bp)
1500
1000
750
500
250

5.4 Transcript analysis of SALK_016929c nmt3 plants by RT-PCR

cDNA was prepared from isolated RNA of the *nmt3* SALK_016929c plant line to test the suitability of the cDNA for q-PCR measurements of *NMT3* transcript abundance. For reference, the expression of genes corresponding to *ubiquitin10* was used. For the SALK_016929c line gene specific primers were designed to anneal within the non-coding 5'UTR (LP) and exon 6 (RP) to produce an expected 819 bp product (Fig. 5A). Figure 5B shows that no transcripts associated with *NMT3* are detected using cDNA prepared from leaves (Lanes 2 and 3) or roots (Lane 4) of the *nmt3* SALK_016929c. The predicted 819 bp *NMT3*-derived product was amplified from the cDNA template prepared from leaf and root tissue of Col-0 plants using the same primers showing that the primers are suitable for detecting *NMT3* expression (Fig. 5B, Lanes 5 and 6). All sources of *NMT3* template showed comparable amplification of *ubiquitin10* showing that the *nmt3* plants of the SALK_016929c lack *NMT3* transcripts but this absence is not due to poor RNA or cDNA (Fig. 5B, Lanes 7 to 11).

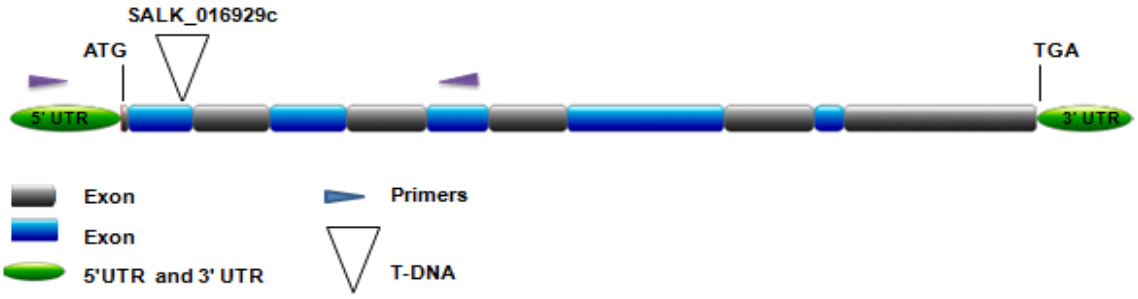
6. Primer selection for *NMT* q-PCR analysis for different SALK mutant lines

q-PCR was used to estimate the transcript level of *NMT3* in plants representing the three T-DNA insertion lines: SALK_062703, SALK_120703c and SALK_016929c. A gene map showing the positions of the primers used is found in Figure 6A as are the map positions of the T-DNA insertions. *NMT3* expression in plants of the SALK_062703 line was tested using primers (*NMT3*-3) designed to anneal on exon 10 and exon 11 near the 3'untranslated region (3'-UTR). For the SALK_120703c line, the effect of the T-DNA insertion in intron 6 was tested using two different pairs of primers (*NMT3-4* and *NMT3-3*) with both amplifying regions closer to the 3' region of the gene (Fig. 6A). In the SALK_016929c line the T-DNA insertion resides in exon 2 and primers that anneal near the 5'UTR region (after the start codon) (*NMT3-11*) were designed for use in q-PCR. The resulting products generated by q-PCR were analyzed by agarose gel electrophoresis to confirm the size of the product and determine whether it matched the

Figure 5. RT-PCR analysis of transcripts associated with *NMT3* from leaves and roots of representative SALK_016929c mutant plants

A. Gene structure map of *NMT3* of the SALK_016929c line showing the position of T-DNA insertion relative to *NMT3* primers used for RT-PCR reactions. The map shows non-coding UTR (green), exons (grey and blue). **B.** Analysis of RT-RCR products resolved by electrophoresis on agarose gels. Lane 1: Molecular size markers followed by cDNA amplification of *NMT3* from various sources: Lanes 2 and 3: *nmt3* SALK_016929c leaves; Lane 4: *nmt3* SALK_016929c roots; Lane 5: Columbia WT (Col-0) leaves; Lane 6: Columbia WT (Col-0) roots and cDNA amplification of *ubiquitin10*: Lane 7 and 8: *nmt3* SALK-016929c leaves; Lane 9: *nmt3* SALK_016929c roots; Lane 10: Columbia WT (Col-0) leaves; Lane 11: Columbia WT (Col-0) roots. Primer sequences are given in Table 3. cDNA preparation, RT-PCR and electrophoresis conditions are given in the Materials and Methods section.

A


SALK_016929c
ATG
TGA
5' UTR
3' UTR
Exon
Exon
5'UTR and 3' UTR
Primers
T-DNA

B

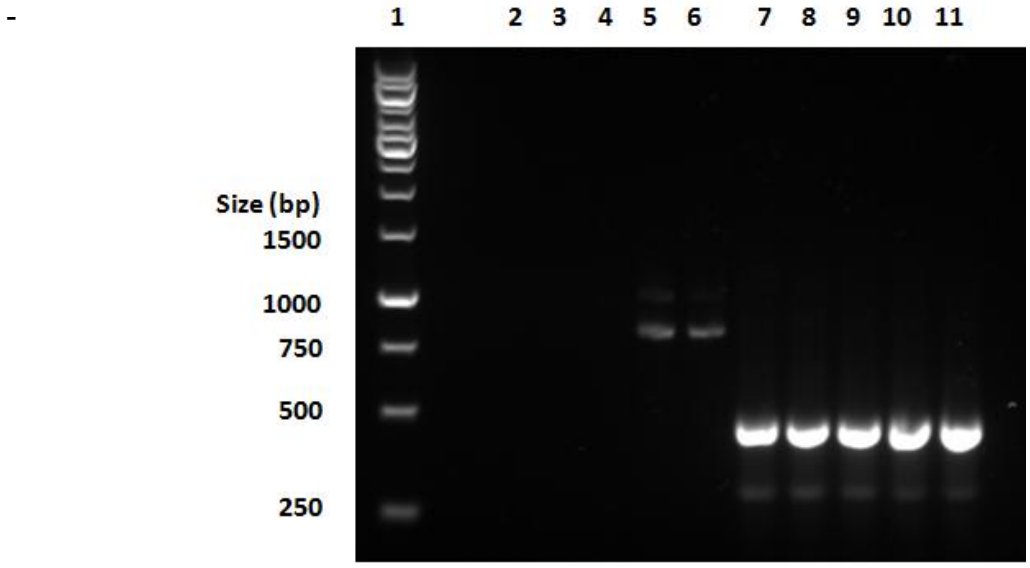
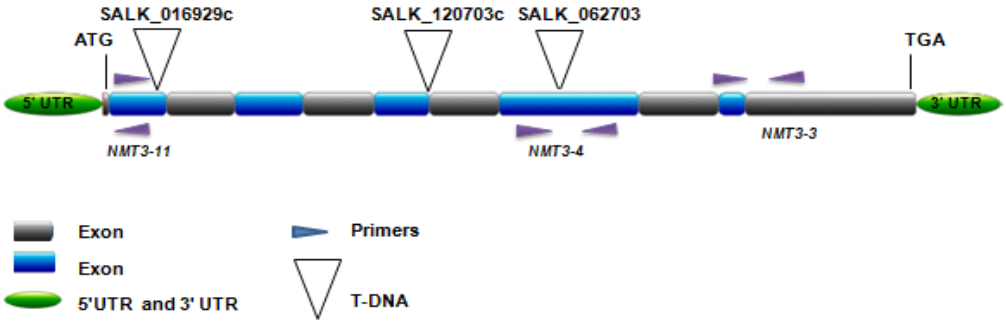

1 2 3 4 5 6 7 8 9 10 11
Size (bp)
1500
1000
750
500
250

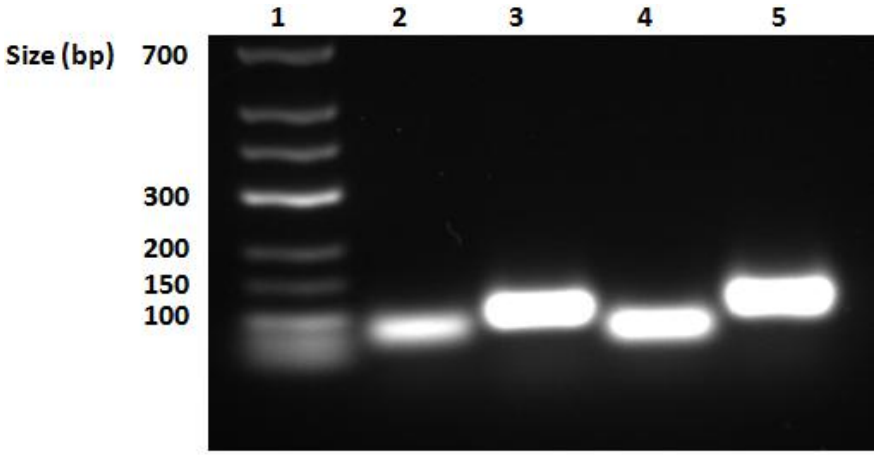
Figure 6. Primer selection for *NMT* q-PCR analysis of SALK mutants

A. Gene structure maps of *NMT3* in SALK_062703, SALK_120703c and SALK_016929c lines. The map shows exons (grey and blue) and non-coding UTRs (green) with primer positions for amplification of cDNA by q-PCR denoted by arrows. In SALK-062703 the T-DNA is associated with exon 8 of *NMT3* and RP and LR gene-specific primers (*NMT3-3*) were used to amplify DNA between exons 10 and 11. In SALK_120703c the T-DNA associated with *NMT3* maps to intron 6 and *NMT3-4* and *NMT3-3* primer pairs were used to amplify a fragment of exon 8 and a region between exons 10 and 11, respectively. In SALK_016929c the T-DNA insertion maps to exon 2 of *NMT3* and the *NMT3-11* primer pair used for q-PCR amplified a section of exon 2. cDNA prepared from Col-0 *Arabidopsis* (WT) leaves was used for q-PCR following conditions described in the Materials and Methods section. The PCR products were resolved by electrophoresis on a 2% agarose gel for 1.5 hours at 60 volts. **B.** Lane 1: Molecular size markers; Lane 2: *NMT3-3* (70 bp); Lane 3: *NMT2-15* (103 bp); Lane 4: *NMT1-7* (70 bp); Lane 5: *NMT3-11* (113 bp); and **C.** Lane 6: *At5g12240* (200 bp); Lane 7: *At5g08290* (*YL8S*, 150 bp); Lane 8: *At5g60390* (*EF1α- 2*; 233 bp); and Lane 9: *At3g62250* (*UBIQ5*; 183 bp). Primer sequences are given in Table 3.

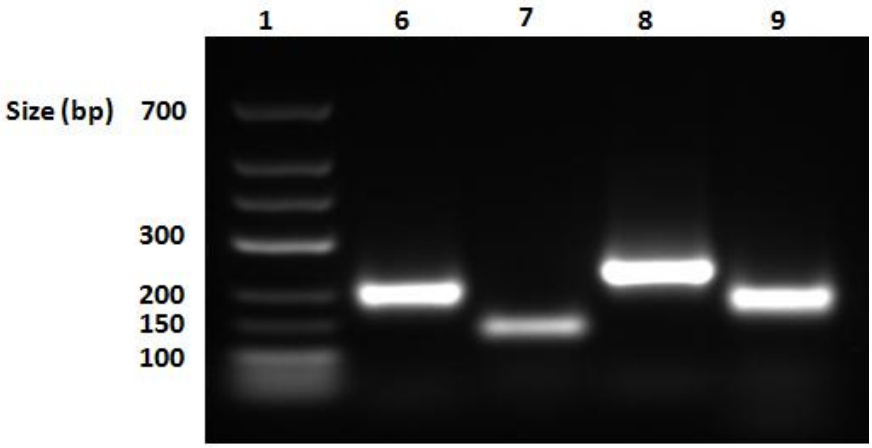
A


SALK_016929c
SALK_120703c
SALK_062703
ATG
TGA
5' UTR
3' UTR
NMT3-11
NMT3-4
NMT3-3
Exon
Exon
5'UTR and 3' UTR
Primers
T-DNA

B


1
2
3
4
5
Size (bp)
700
300
200
150
100

C


1
6
7
8
9
Size (bp)
700
300
200
150
100

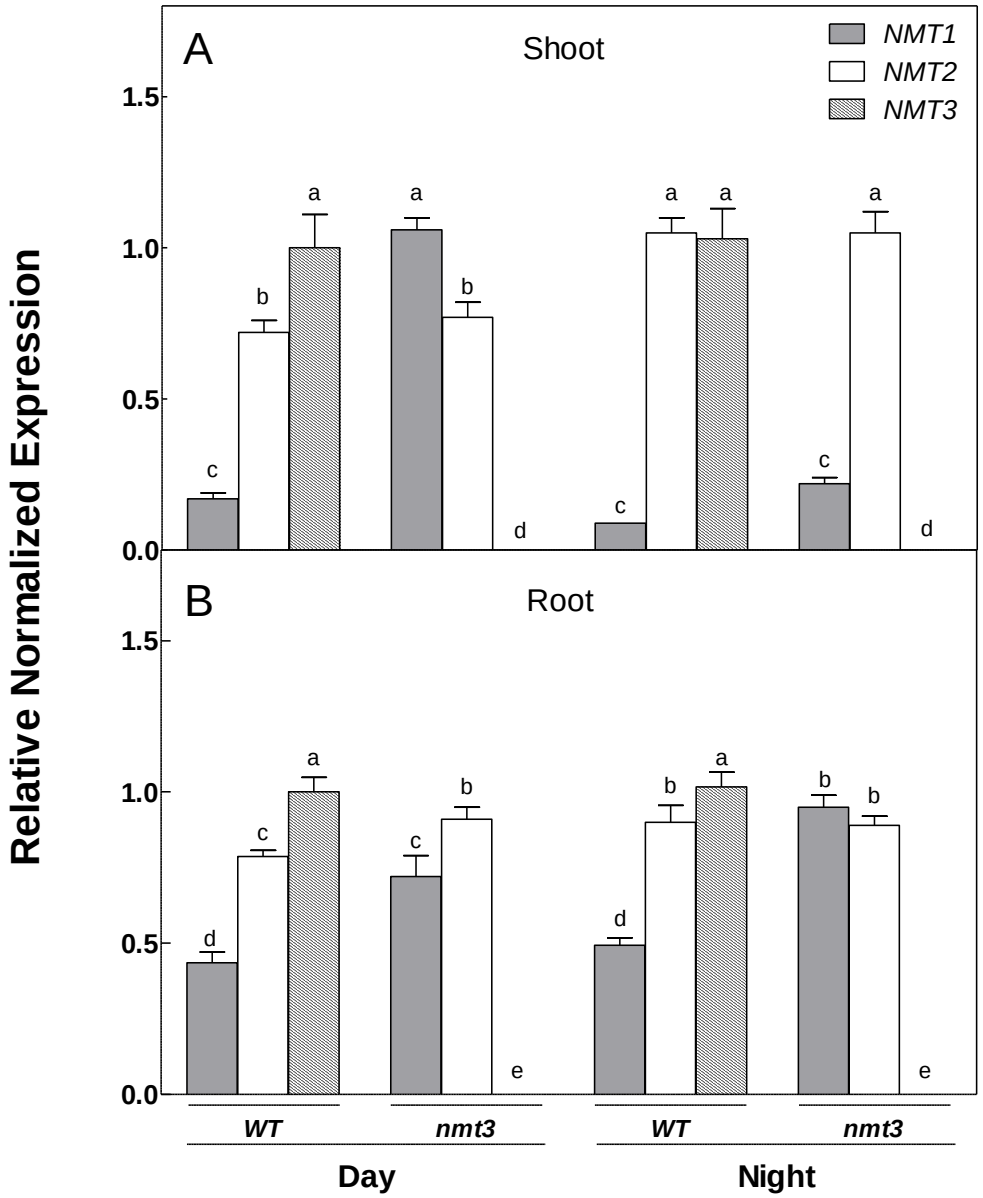
predicted size (Fig. 6B and C). The product size of target genes are shown in Figure 6B and are as follows: primer pair *NMT3-3*, 70bp (Lane 2); primer pair *NMT2-15*, 103 bp (Lane 3); primer pair *NMT1-7*, 70 bp (Lane 4); and primer pair *NMT3-11*, 113 bp (Lane 5). For the reference genes the products are shown in Figure 6C and the sizes were as follows: primer pairs for *At5g12240*, 200 bp (Lane 6); primer pair for *At5g08290* (*YL8S*, 150 bp (Lane 7); primer pair for *At5g60390* (*EF1α- 2*), 233 bp (Lane 8); and primer pair for *At3g62250* (*UBIQ 5*), 183 bp (Lane 9). For both the target gene and reference primers the expected product size based upon their annealing location and the actual amplicon size obtained after PCR are in good agreement and in all cases only a single product was observed on gels.

6.1 *Testing harvest conditions: are there diurnal changes in NMT expression in shoots and roots of nmt3 and NMT3 SALK_062703 (WT) Arabidopsis seedlings?*

Transcript abundance for many genes can fluctuate diurnally through the influence of circadian regulation and this can influence the time of day selected for harvesting (Strayer et al., 2000). For this study *Arabidopsis* seedlings were grown on nutrient plates until the primary root reached the bottom of the plate and the shoot and root tissues were harvested separately at midpoints of the 12 h light (day) and 12 h dark (night) period of the daily cycle. The expression of *NMT1, NMT2* and *NMT3* were assayed by q-PCR and the data was normalized to the expression of four references genes for shoots (Fig. 7A) and roots (Fig. 7B) harvested under light or dark conditions. Figure 7 shows that transcripts associated with all three genes were detected following q-PCR analysis of cDNA prepared from WT shoots and roots harvested during mid-day or at night. In contrast, *nmt3* seedlings showed no detectable *NMT3* transcripts in either tissue at midday or in the dark. Interestingly, there is also a different pattern of gene expression between WT and *nmt3* seedlings with respect to *NMT1* and *NMT2* in light and dark conditions. Specifically, in WT shoots *NMT1* transcript abundance is significantly lower by 4.2-fold and 5.8-fold than that of *NMT2* or *NMT3* transcripts, respectively. In the absence of *NMT3* expression, shoots of the *nmt3* seedlings show significantly higher expression of

Figure 7. Expression of *NMT1, NMT2* and *NMT3* in *NMT3* SALK_062703 WT and *nmt 3* SALK_062703 *Arabidopsis* seedlings determined during the light and dark phases of the photoperiod.

A. Shoots and **B.** Roots of five-day-old seedlings were used to prepare cDNA. Tissue was harvested at midpoint of the 12 h light (day) period and midpoint of the 12 h dark (night) period of the daily cycle. Expression of *NMT1, NMT2* and *NMT3* was assayed by q-PCR and transcript abundance normalized to the expression associated with four references genes (*At5g12240, YL8S, EF1α-2* and *UBQ5*) and reported as relative to the expression level of *NMT3* SALK_062703 WT (arbitrarily set as 1). Data are the mean ± SE for three independent biological replicates with a minimum of 54 seedlings analyzed for each line per replicate. ANOVA showed that "genes", "lines" "time", "lines × genes" "lines × time", "genes × time", "lines × genes × time" were significant ($P < 0.001$). Means of "line × time of day" identified by the same letter are not significantly different as determined by Tukey post hoc testing.


A
Shoot
B
Root
NMT1
NMT2
NMT3
Relative Normalized Expression
1.5
1.0
0.5
0.0
WT
nmt3
WT
nmt3
Day
Night

NMT1 (6.2-fold), but no change in *NMT2* transcript abundance. In contrast, in the dark the only difference in shoot *NMT* gene expression is the loss of *NMT3* associated transcripts in the *nmt3* mutant, the relative abundance of transcripts associated with *NMT1* and *NMT2* is unchanged between shoots of WT and *nmt3* seedlings. *NMT* expression in roots of WT *Arabidopsis* seedlings resembles the shoot expression pattern for the three genes with transcript abundance in the order of *NMT3* > *NMT2*> *NMT1*. That order of gene expression is unchanged in the dark. Again, the roots of *nmt3* seedlings lack transcripts associated with *NMT3* and the expression of *NMT1* and *NMT2* have increased 1.7-fold and 1.2-fold, respectively, compared to WT roots harvested during the day. In root tissues harvested in the dark the pattern of *NMT* gene expression in WT and *nmt3* roots is similar although again there is a 1.9-fold increase in transcripts associated with *NMT1* in the roots of *nmt3* seedlings. The results shown in Figure 7 indicate that while the relative expression patterns differ between shoots and roots, transcripts associated with all three genes can be easily detected in tissue collected during the day or night. Thus the absence of *NMT3* associated transcripts in *nmt3* seedlings cannot be explained by circadian regulation of this gene. Given the ease of collecting tissue during the day, the remaining studies were done using tissue harvested midway through the light period. Also, the relative expression patterns for WT plants showed lowest transcript abundance for *NMT1* and highest transcript abundance for *NMT3* with the expression of *NMT2* in between and relatively constant. However, in shoots or roots of *nmt3* seedlings harvested in the light or in the dark, the relative abundance of *NMT1* shows statistically significant up-regulation relative to its expression in WT seedlings. The presence (or absence) of light did not contribute to this change in relative expression between the three *NMT* genes.

6.2 Differential expression of NMT genes in the shoots and roots of nmt3 and NMT3 SALK_062703 (WT) Arabidopsis plants at rosette plant stage

Figure 8 shows that the three *NMT* genes were differentially expressed in the shoots (Fig. 8A) and roots (Fig. 8B) of SALK_062703 *nmt3* and *NMT3* WT plants. As was found for the seedlings of this line, no *NMT3* transcripts were detected in the shoots (Fig. 8A) and again, increased (1.9-fold) expression of *NMT1* was found in *nmt3* plants while *NMT2* expression was unchanged. However, *NMT3* transcripts were detected in the roots of *nmt3* plants albeit at a very low level relative to the other tissues tested (Fig. 8B). This result indicates that the *NMT3* expression may be knocked out in the shoots but is only knocked down in the roots of more mature plants. Moreover, *NMT1* and *NMT2* were differently expressed in the *nmt3* plants with *NMT1* expression significantly higher in the absence of WT levels of *NMT3* expression and highest when *NMT2* expression in roots of *nmt3* plants is lower relative to WT levels. This suggests that *NMT1* expression may play a compensatory role to alleviate the lack or lower expression of *NMT2* and/or *NMT3*.

6.3 Testing for diurnal changes in expression of NMT genes in the shoots and roots of nmt1 SALK_036291and SALK_062703 NMT3 (WT) Arabidopsis seedlings.

The light and dark period expression of *NMT1, NMT2* and *NMT3* was determined in *nmt1* seedlings (carrying *NMT3*) and in seedlings from the SALK_062703 line carrying both *NMT1* and *NMT3* serving as the WT for comparison. For this study *Arabidopsis* seedlings were grown on nutrient plates until the primary root reached the bottom of the plate at which point the shoot and root tissues were harvested midway through the 12 h light (day) or 12 h dark (night) period of the daily cycle. The expression of *NMT1, NMT2* and *NMT3* in leaves (Fig. 9A) and roots (Fig. 9B) was assayed by q-PCR and their transcript abundance was normalized to the expression of four references genes. The expression of *NMT1* in shoots was low relative to the other two *NMT* genes but surprisingly, it was not significantly different between WT and *nmt1* seedlings

Figure 8. Expression of the *NMT1, NMT2* and *NMT3* in *NMT3* SALK_062703 WT and *nmt3* SALK_062703 *Arabidopsis* mature plants during light cycle of the photoperiod.

A. Shoots and **B.** Roots of four-week-old plants were used to prepare cDNA. Tissue was harvested at the midpoint of the 9 h light (day) period of the daily cycle. *NMT1, NMT2* and *NMT3* expression was assayed by q-PCR and results were normalized to the expression of four references genes (*At5g12240, YL8S, EF1α-2* and *UBQ5*) and reported relative to the expression of *NMT3* SALK_062703 WT arbitrarily set as 1. Data are the mean ± SE for three independent biological replicates with a minimum of three plants analyzed for each line per replicate. ANOVA showed that "genes", "lines" and "genes × lines" were significant ($P < 0.05$). Means of "genes × lines" identified by the same letter are not significantly different as determined by Tukey post hoc testing.

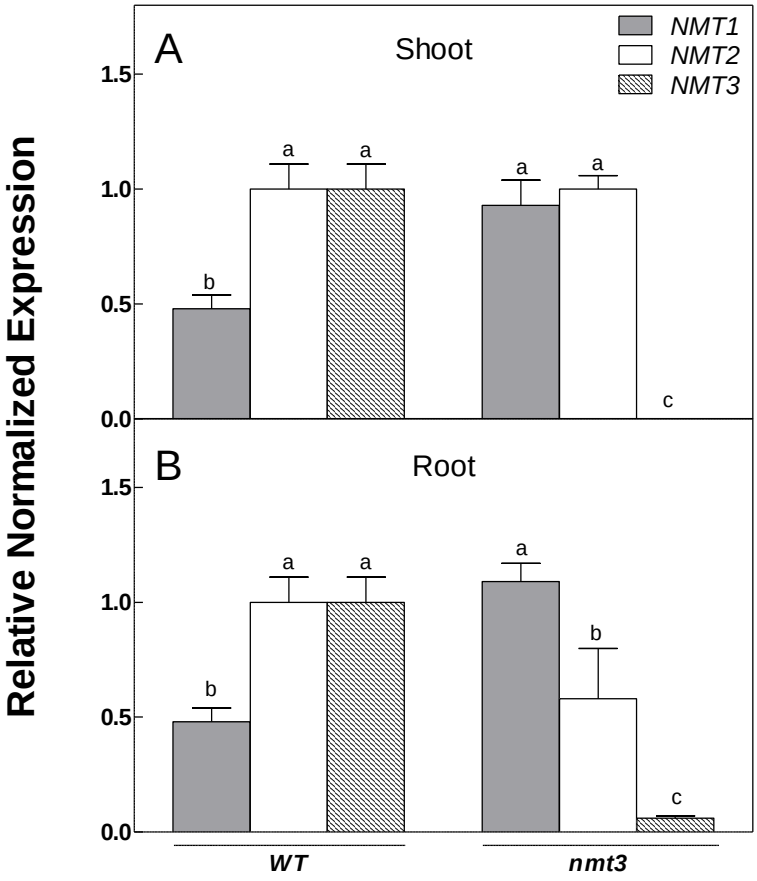
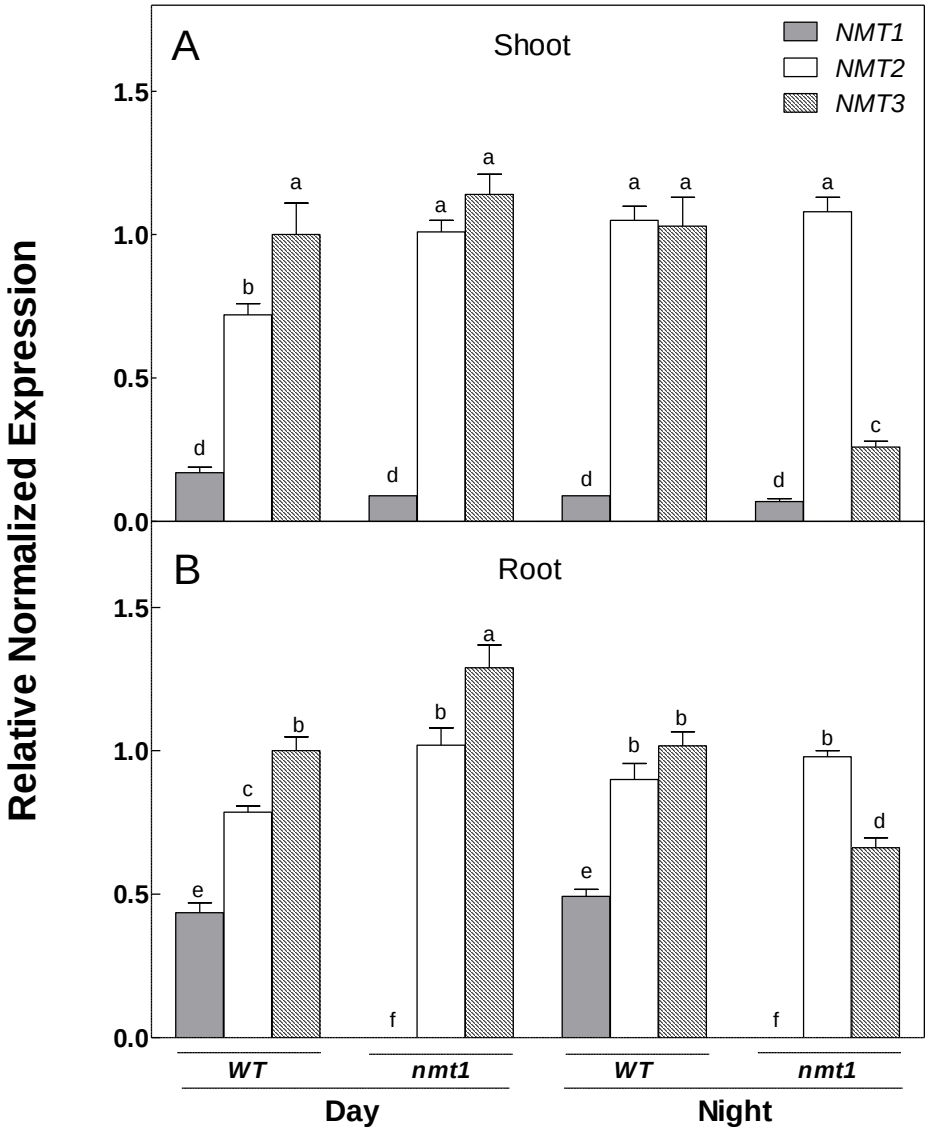

A
Shoot
B
Root
NMT1
NMT2
NMT3
Relative Normalized Expression
1.5
1.0
0.5
0.0
a
b
c
WT
nmt3

Figure 9. Expression of *NMT1, NMT2* and *NMT3* in SALK_036291, *NMT3* SALK_062703 WT and *nmt 1* SALK_036291 *Arabidopsis* seedlings determined during light and dark phases of the photoperiod.

A. Shoots and **B.** Roots of five-day-old seedlings were used to prepare cDNA. Tissue was harvested at the midpoint of the 12 h light (day) period and midpoint of the 12 h dark (night) period of the daily cycle. Expression of *NMT1, NMT2* and *NMT3* was assayed by q-PCR and transcript abundance normalized to the expression associated with four references genes (*At5g12240, YL8S, EF1α-2* and *UBQ5*) and reported was relative to the expression of *NMT3* SALK_062703 of WT arbitrarily set as 1. Data are the mean ± SE for three independent biological replicates with a minimum of 54 seedlings analyzed for each line per replicate. ANOVA showed that "genes", "lines", "time", "lines × genes", "lines × time", "genes × time", and "lines × genes × time" were significant ($P < 0.001$). Means of "lines × time of day" identified by the same letter are not significantly different as determined by Tukey post hoc testing.


A
Shoot
B
Root
NMT1
NMT2
NMT3
Relative Normalized Expression
1.5
1.0
0.5
0.0
WT
nmt1
WT
nmt1
Day
Night

(Fig. 9A). However, *NMT1* expression was not detected in roots of the *nmt1* plants (Fig. 9 B).

NMT2 expression was stable and high relative to the expression of *NMT1* for shoots or roots in either light or dark conditions. *NMT3* expression was also the same or higher than *NMT2* except in shoots of *nmt1* seedlings under dark conditions where *NMT3* expression was reduced 4.4-fold relative to the expression of this gene in *nmt1* seedlings exposed to light (Fig. 9A). This reduced expression of *NMT3* relative to *NMT2* also holds true as significant for roots in the dark but the decrease is less marked.

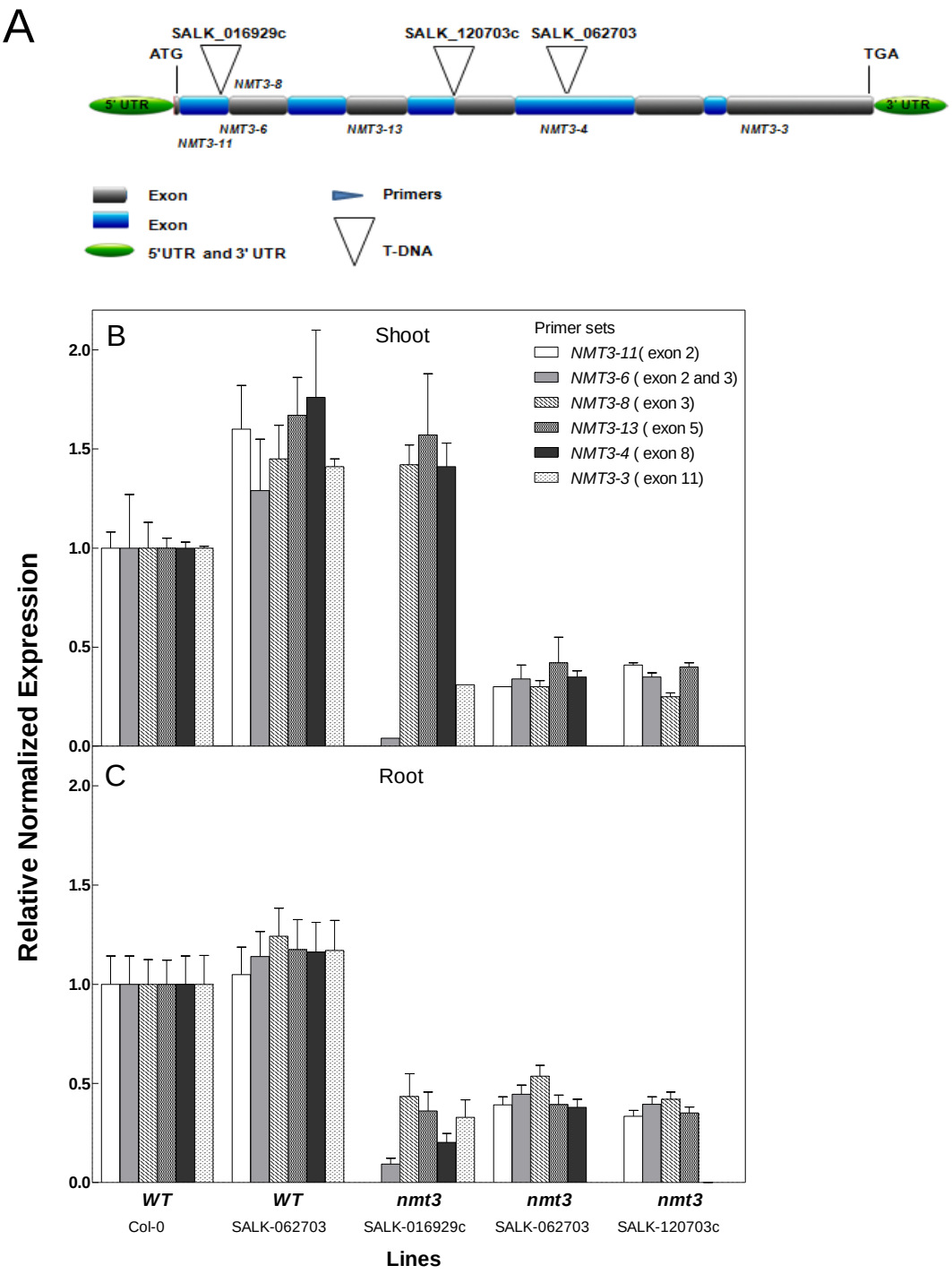
6.4. Primer design influences estimates of NMT3 primer abundance by q-PCR.

Insertion of a T-DNA element into an exon or intron in the protein-coding region of a gene can partially or completely knock-out or knock-down the expression of a target gene (Wang, 2008). Therefore, in order to analyze *NMT* gene regulation in the mutant plants attention must be given to the selection and design of primers as well as the conditions used for amplification. Figure 10 shows gene structure maps of *NMT3* along with the position of the T-DNA insertion for the three mutant lines used in this project: SALK_062703, SALK_20703c and SALK_016929c. q-PCR was used to assay the expression of *NMT3* in seedling shoots and roots from these lines including WT SALK_062703 and Col-0 plants as a reference. Information on the properties of these primers is summarized in Table 4. All of the primer sets used passed the validation criteria necessary for use in q-PCR including a high efficiency and production of a single, amplified product. In Figure 10, Panels B and C the gene expression data was normalized to the expression of four reference genes (*At5g12240, YL8S, EF1α-2* and *UBQ5*) and reported relative to the q-PCR data for the Col-0 seedlings arbitrarily set as 1).

Figure 10 (Panels B and C) show that all of the primer sets used are suitable for detecting the abundance of *NMT3* transcripts and that the estimates show no significant difference within the two lines carrying WT versions of this gene (Col-0 and SALK_062703) whether looking at shoot or root-specific expression. The same outcome is not true for the mutant lines. For example, the insertion of T-DNA in *nmt3*

Figure 10. Genotype and oligonucleotide primer design influences estimates of *NMT3* primer abundance by q-PCR.

Five-day-old *Arabidopsis* seedlings grown on defined media plates were used for RNA extraction and cDNA preparation. **A**. The gene structure map for *NMT3* showing positions of the forward and reverse primer pairs used for q-PCR. The line-specific T-DNA insertion sites are shown as are the exons (grey and blue). **B.** Shoots and **C**. Roots of *nmt3* SALK_062703, SALK_120703c, SALK_016929c, *NMT3* SALK_062703 and Col-0 wild-type were used for q-PCR. Primer pairs were designed to anneal and amplify DNA fragments associated with different exons of *NMT3*. The gene expression data is reported relative to the q-PCR data for Col-0 wild-type seedlings arbitrarily set as 1. All data was normalized to the expression of four reference genes: *At5g12240, YL8S, EF1α-2* and *UBQ5*. The expression data shown is the mean +/- SE for three technical replicates with a minimum of 18 seedlings per line. Information about the primer pairs used for q-PCR is given in Table 4 of Materials and Methods.


A
SALK_016929c
SALK_120703c
SALK_062703
ATG
TGA
NMT3-8
5' UTR
3' UTR
NMT3-6
NMT3-11
NMT3-13
NMT3-4
NMT3-3
Exon
Exon
5'UTR and 3' UTR
Primers
T-DNA
B
Shoot
C
Root
Primer sets
NMT3-11(exon 2)
NMT3-6 (exon 2 and 3)
NMT3-8 (exon 3)
NMT3-13 (exon 5)
NMT3-4 (exon 8)
NMT3-3 (exon 11)
Relative Normalized Expression
2.0
1.5
1.0
0.5
0.0
WT
Col-0
WT
SALK-062703
nmt3
SALK-016929c
nmt3
SALK-062703
nmt3
SALK-120703c
Lines

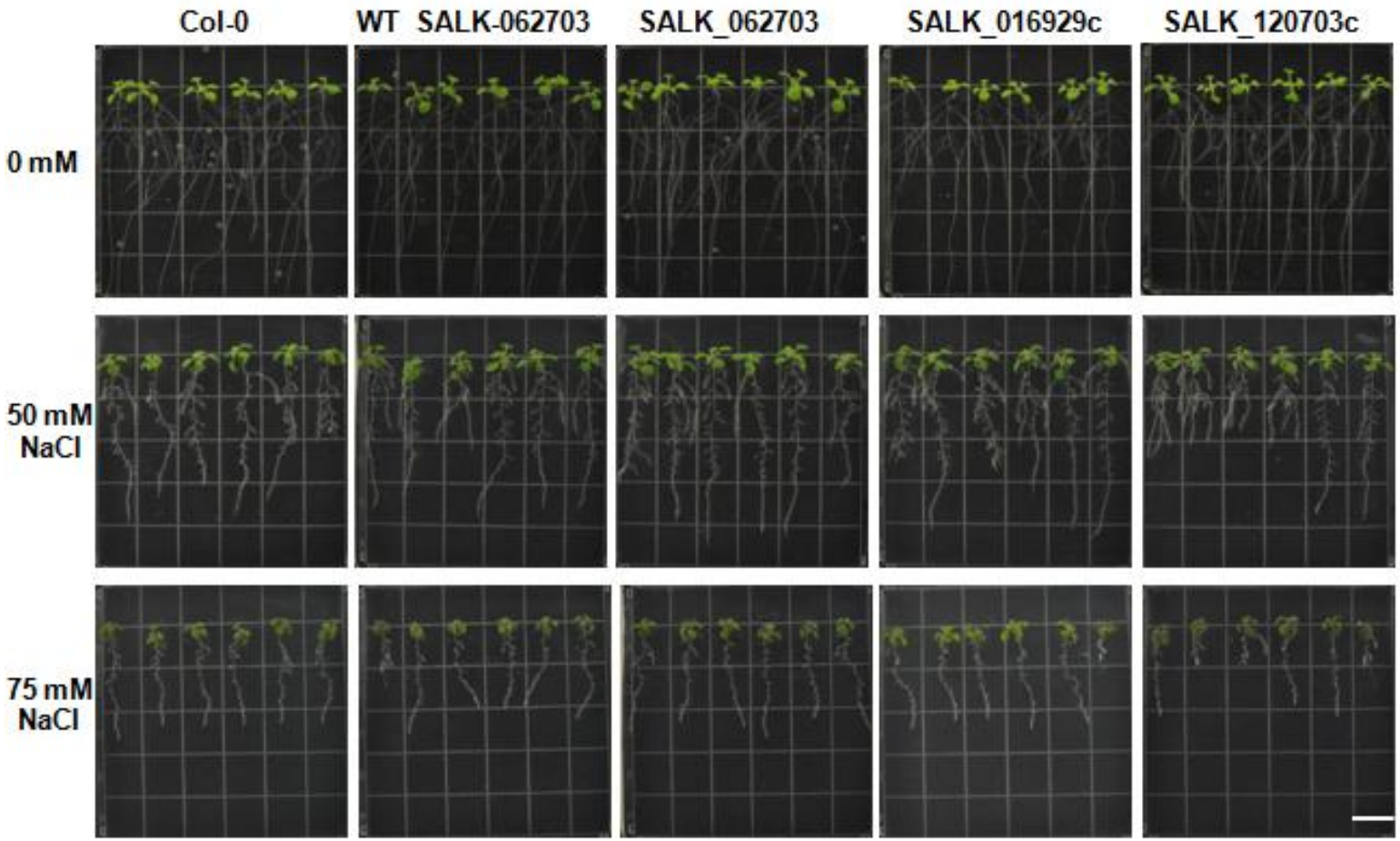
SALK_016929c is in exon 2 near the non-coding 5'UTR region of *NMT3*. Seedlings carrying this insertion show no expression of this gene in either shoots or roots when the *NMT3-11* primer set was used (Fig. 10B and C). However, three primers sets annealing 3' relative to the site of T-DNA insertion (*NMT3-4, NMT3-8,* or *NMT3-13*) all but *NMT3-6* showed the presence of transcripts associated with *NMT3* and the level of expression to be as high as WT plants of the SALK_062703 line and higher than the Col-0 line. Using primers annealing to exon 11 (*NMT3-3*), however, showed a lower level of gene expression and one that was more consistent with *NMT3* transcript levels detected in the SALK_062703 and SALK_120703c lines. In contrast, the same primer sets used for root-specific *NMT3* expression provided a similar pattern of abundance although again, the lowest estimates of none or negligible expression came from the *NMT3-11* primer set. The T-DNA insertions in *nmt3* SALK_062703 and SALK_120703c plants are in exon 8 and intron 6, respectively (Fig. 10A). No transcripts associated with *NMT3* were detected in shoots or roots of *nmt3* SALK_062703 with the primer set that annealed after exon 8 (*NMT3-3*) but every other primer set showed a level of gene expression approximately 3-fold and 2.7-fold lower than the level of transcripts found in Col-0 shoots or roots, respectively. Seedlings of the SALK_120703c line provided a similar outcome. No *NMT3* transcripts were detected in shoots or roots when primer sets that annealed after exon 6 of *NMT3* were used (*NMT3-4* or *NMT3-3*; Fig. 10B and C). However, transcripts were detected in both shoots and roots when using the remaining primer sets. This level of expression was not significantly different from the levels detected in the *nmt3* SALK_062703 seedlings.

7. Representative *Arabidopsis* seedlings grown on defined media in the presence and absence of NaCl

To determine if the reduced *NMT3* expression has an effect on seedling response to salinity we subjected seedlings to media containing different concentrations of NaCl. Seedlings were grown for 5 d on nutrient media lacking added salt (0 mM NaCl) or for 7 d on media supplemented with 50 or 75 mM NaCl (Fig. 11). A longer time was needed

Figure 11. **Representative *Arabidopsis* seedlings grown on defined media in the presence and absence of NaCl.**

Seedlings were grown for 5 d on nutrient media lacking added salt (0 mM) or for 7 d on media supplemented with 50 or 75 mM NaCl. The lines used for this comparison were: Columbia wild-type (Col-0), *NMT3* SALK_062703 wild type (WT) and three *nmt3* lines: SALK_062703, SALK_016929c and SALK_120703c. In the case of SALK_120703c all seedlings had long roots on media lacking salt but on 50 or 75mM NaCl almost half of the seedlings (28 out of 60 seedlings) showed a shortened primary root relative to other seedlings on the plate revealing a salt-responsive root length phenotype in this line. Scale bar is 13.5 mm.

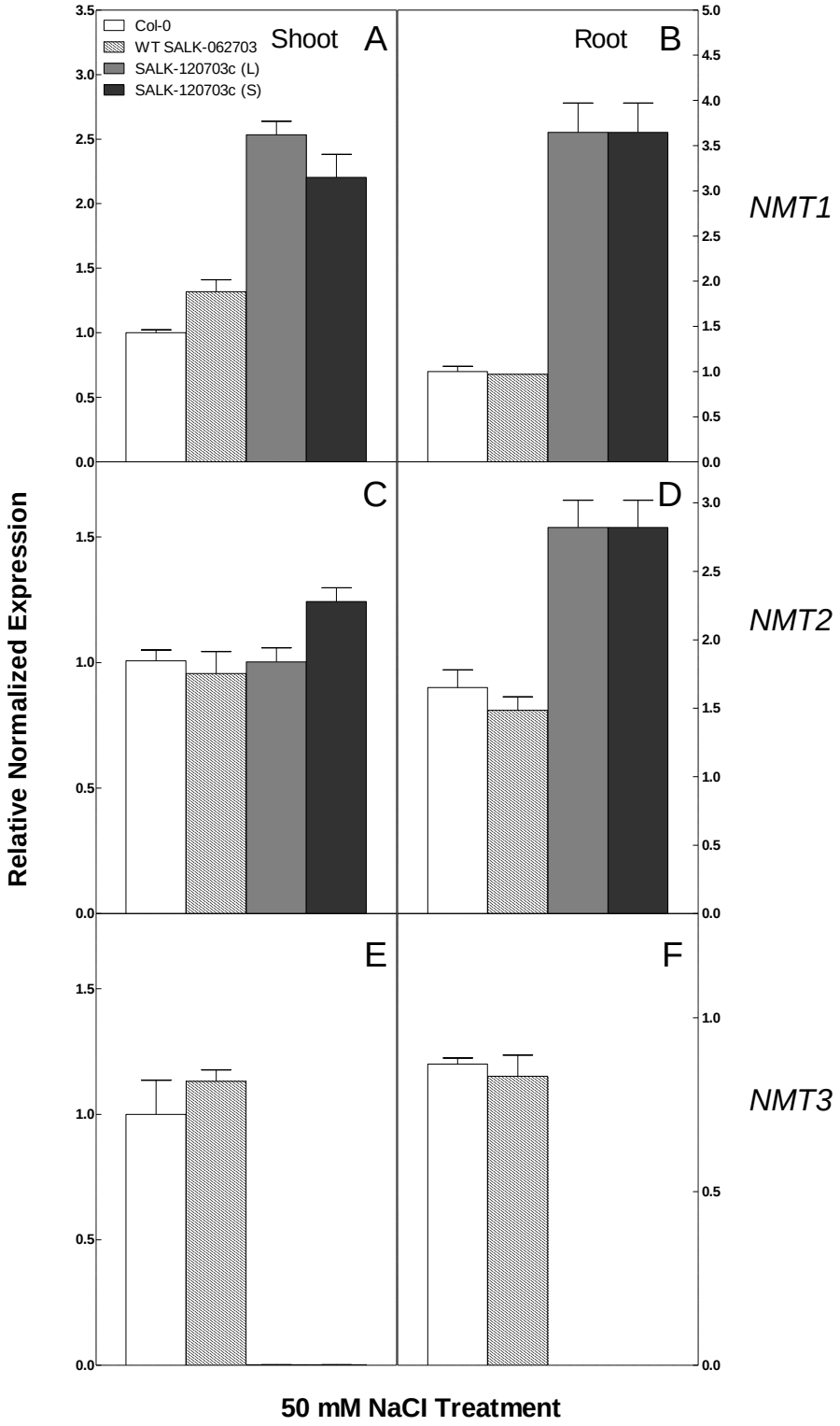

Col-0
WT SALK-062703
SALK_062703
SALK_016929c
SALK_120703c
0 mM
50 mM NaCl
75 mM NaCl

for the plants on salt to grow to a size that would yield enough tissue to process for RNA isolation and manipulate easily for biomass measurements. The lines used for this comparison were: Columbia wild-type (Col-0), *NMT3* SALK_062703 (WT) and three *nmt3* lines: SALK_062703, SALK_016929c and SALK_120703c.

The appearance of representative seedlings from all lines and treatments at the day of harvest is shown in Figure 11. A visual comparison between *NMT3* SALK_062703 WT and Col-0 WT seedlings show that they all have long roots and green shoots when grown without NaCl. When NaCl is added to the medium, the roots are shorter even at 7 days and the leaves of the shoots are not as fully expanded. The same outcome is true for the *nmt3* SALK_062703 and SALK_016929c seedlings. In the absence of salt the seedlings resemble WT plants in having long roots and green shoots and showing no deformities and in the presence of NaCl in the medium the roots are shorter and shoots are smaller. An unexpected observation related to the SALK_120703c line in that all of the *nmt3* seedlings looked alike when NaCl was absent from the medium but for this line the addition of either 50 or 75mM NaCl led to dramatically reduced root elongation for about half of the seedlings (28 out of 60 seedlings) revealing a salt-responsive root-length phenotype among the plants in this line. This raises the question whether this line carries a second, unrelated T-DNA insertion that is responsible for this salt-responsive short-root phenotype. To address this question, q-PCR was used to determine whether the transcript levels associated with *NMT* for *nmt3* SALK_120703c were the same for seedlings showing long or short root development when grown on salt. The results of this analysis are given in Figure 12 and shows that *NMT1* and *NMT2* transcripts are detected and not significantly different with respect to their abundance in shoots or roots of the SALK_120703c seedlings (Panels A, B, C and D) but *NMT3* transcripts were not detected in either shoots or roots of seedlings displaying the salt-responsive root length phenotype (Fig. 12E and F). As a result, we conclude that the shortening of some seedling roots with salt is likely due to the presence of a second mutation that segregates independently of *NMT3* in the SALK_120703c line. All of the analyses of root growth and *NMT* expression from this point were made on plants that did not show the excessively

Figure 12. **Relative expression of *NMT* genes in shoots and roots of SALK_120703c *Arabidopsis* seedlings grown on saline media.**

Seedlings were grown on media containing 50 mM NaCl for 7 d and shoots and roots were harvested separately, pooled, and used for RNA extraction followed by cDNA synthesis for q-PCR analysis. **A, C, E**. Shoots and **B, D, F**. Roots. **A, B.** *NMT1* gene expression. **C, D.** *NMT2* gene expression. **E, F.** *NMT3* gene expression. q-PCR was used to assay the expression of two different phenotypes of *nmt3* SALK_120703c: long root (L) and SALK_120703c: short root (S). For comparison, *NMT3* SALK_062703 wild-type and Columbia wild-type (Col-0) plants are included. Details of seed germination, transplant and seedling growth are given in the Materials and Methods. The gene expression data is reported relative to the q-PCR data for the Col-0 line arbitrarily set as 1. The data for *NMT* expression was normalized to the expression of four reference genes: *At5g12240, YL8S, EF1α-2* and *UBQ5*. The data is for one biological replicate with a minimum of 18 seedlings per line. Standard error bars are for three technical repeats.


Col-0
WT SALK-062703
SALK-120703c (L)
SALK-120703c (S)
Shoot
Root
A
B
C
D
E
F
NMT1
NMT2
NMT3
Relative Normalized Expression
50 mM NaCl Treatment

shortened roots on saline media. By necessity, however, due to the salt-responsive nature of the phenotype some of the plants carrying this second mutation are included in measurements taken for plants on growth media without NaCl (0 mM).

7.1 Root measurements for mutant and wild type lines under various salt treatments

The primary root length of the three *nmt3* lines and two WT lines were comparable on the day of transplant and all reached about 60 mm on the day of harvest (Fig. 13A). The 50 mM NaCl treatment resulted in slower growth in all of the lines and the primary root length of the plants was about 33 mm on the day of harvest (Day 7) indicating growth inhibition by salt stress (Fig. 13B). As expected from the visual appearance of the seedlings, both WT and *nmt3* mutant lines showed little primary root growth with the 75 mM NaCl treatment (Fig. 13C). In summary, the ANOVA analysis showed that "days", "salt concentrations" and "salt concentrations × days" were all significant ($P \leq 0.01$) but there was no statistical significance for "lines", "salt concentrations × lines", "lines × days" and "salt concentrations × days × lines" interactions. Thus the statistics confirms the impression that there was no difference in how salt affected the different lines tested (mutant and WT) and that exposure to salt has a significant effect on time required by roots to grow.

The lateral root density and the number of lateral roots of seedlings were parameters that were also documented and measured from photographs. The lateral root density among seedlings from the three *nmt3* lines and two WT lines on 0 mM NaCl was similar (Fig. 14A). The lateral root density for all of the lines was not significantly different at 7 d when the plants on the NaCl-containing plates were harvested (Fig. 14B, C). Indeed the ANOVA showed no statistical significance for "lines", "salt concentrations × lines", "lines × days" ,"salt concentrations × days" and, "salt concentrations × days × lines", whereas "days" and "salt concentrations" was significant at $P \leq 0.01$. Thus there was no difference in seedling lateral root density as a result of the *nmt3* genotype. The number of lateral roots of seedlings from all of the lines increased with time and there was no significant difference between them with respect to the number of lateral roots on the

Figure 13. Primary root growth of *Arabidopsis* seedlings grown on defined media in the presence and absence of NaCl.

Seedlings were grown on nutrient media **A**. lacking added salt (0 mM) or on media supplemented with **B.** 50 mM NaCl or **C.** 75 mM NaCl. Root measurements were made using photographs taken at the day of transplant (Day 1), Day 3 following transplant and the day of harvest (Day 5 for seedlings grown without added NaCl or Day 7 for seedlings exposed to 50 or 75 mM NaCl). The lines used for this comparison were: Columbia wild-type (Col-0), *NMT3* SALK_062703 (WT) and three *nmt3* lines: SALK_062703, SALK_016929c and SALK_120703c. The data is the mean +/- SE for two independent biological replicates. Each replicate had a minimum of 18 seedlings per line. ANOVA showed that "days", "salt concentrations" and "salt concentrations × days" were significant at $P \leq 0.01$ whereas there was no statistical significance for "lines", "lines × salt", "lines × days", and "lines × salt concentrations × days" interactions.

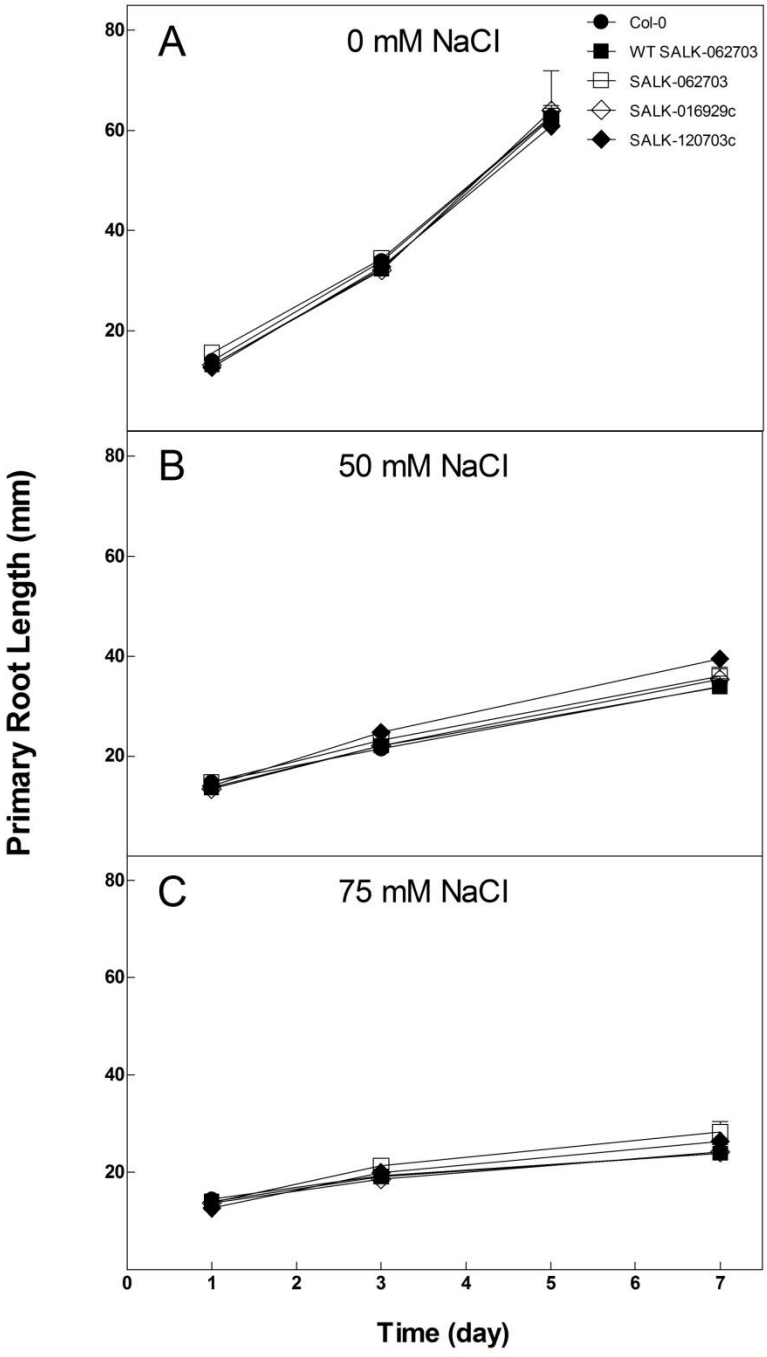
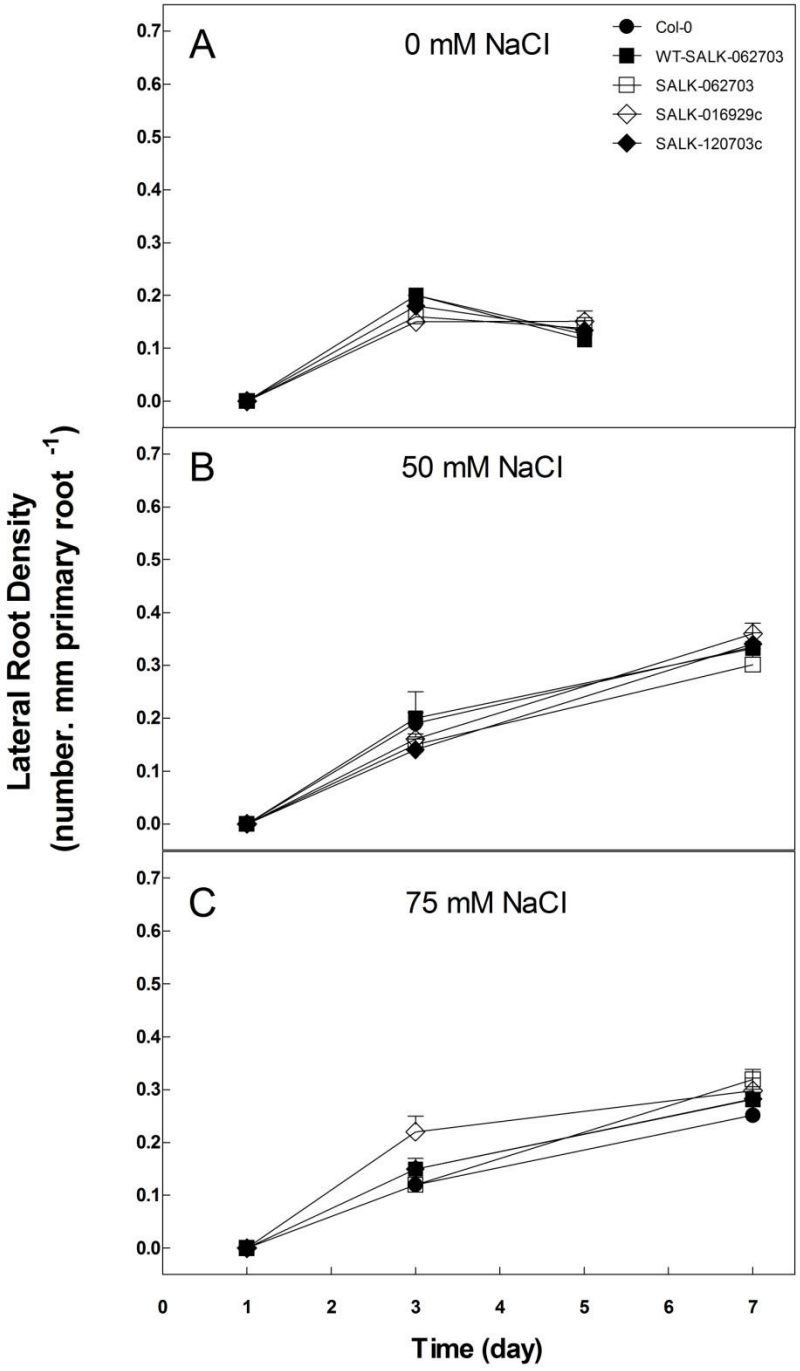

A
0 mM NaCl
B
50 mM NaCl
C
75 mM NaCl
Col-0
WT SALK-062703
SALK-062703
SALK-016929c
SALK-120703c
Primary Root Length (mm)
Time (day)
0
1
2
3
4
5
6
7
20
40
60
80

Figure 14. Lateral root density of *Arabidopsis* seedlings grown on defined media in the presence and absence of NaCl.

Seedlings were grown on nutrient media **A.** lacking added salt (0 mM) or on media supplemented with **B.** 50 mM NaCl or **C.** 75 mM NaCl. Root measurements were made from photographs taken on day of transplant (Day 1), Day 3 following transplant, and the day of harvest (Day 5 for seedlings grown without added NaCl or Day 7 for seedlings exposed to 50 or 75 mM NaCl). The lines used for this comparison were: Columbia wild-type (Col-0*), NMT3* SALK_062703 (WT) and three *nmt3* lines: SALK_062703, SALK_016929c and SALK_120703c. Data are the mean ± standard error (SE) for two independent biological replicates. Each replicate had a minimum of 18 seedlings per line. ANOVA showed no statistical significance for "lines", "lines × salt concentrations", "lines × days", "salt concentrations × days" and, "lines × salt concentrations × days", whereas "days" and "salt concentrations" were significant at $P \leq 0.01$.


A
0 mM NaCl
B
50 mM NaCl
C
75 mM NaCl
Col-0
WT-SALK-062703
SALK-062703
SALK-016929c
SALK-120703c
Lateral Root Density
(number. mm primary root -1)
Time (day)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
3
4
5
6
7

0 mM NaCl treatment (Fig. 15A). While the number of lateral roots of all lines was higher on 50 mM NaCl medium (Fig. 15B) compared to 0 or 75 mM NaCl (Fig. 15A and C), the effect was not significant. Statistical analysis by ANOVA showed that 'days", "salt concentrations" and "salt concentrations × days" were significant at $P \leq 0.01$ whereas there was no statistical significance for "lines", "salt concentrations × lines", "lines × days" and, "salt concentrations × days × lines" interactions. Again, the *nmt3* and WT seedlings all showed the same number of lateral roots when exposed to the same treatment.

The total root lengths of seedlings (primary root length plus length of lateral roots) calculated for the day of transplant (Day 1), growth Day 3 following transplant, and the day of harvest (Day 5 for the seedlings grown without added NaCl medium or Day 7 for the seedlings exposed to 50 or 75 mM NaCl) (Fig. 16A). The baseline values of the total root length for seedlings of three *nmt3* and WT lines were similar on 0 mM NaCl medium and they all reached about 160 mm on the day of harvest. There was no salt-responsive phenotype observed for the *nmt3* lines that distinguished them from the two WT lines (Fig. 16 B and C). Again, the analysis by ANOVA showed that "days", "salt concentrations" and "salt concentrations × days" were significant at $P \leq 0.01$ whereas there was no statistical significance for "lines", "salt concentrations × lines", "lines × days" and, "salt concentrations × days × lines" interactions.

In summary, the *nmt3* genotype is not associated with a change in root morphology and any changes in root growth in response to salt in the medium are shared equally by WT (*NMT3*) seedlings.

7.2 Relative expression of NMT genes in shoots of Arabidopsis seedlings in response to NaCl in the growing medium

q-PCR was used to assay the expression of the three *NMT* genes in the shoots of SALK_062703 (WT), Columbia (Col-0), *nmt3* SALK_062703, SALK_12703c, and SALK_016929c seedlings. Figure 17 shows the relative normalized expression levels

Figure 15. **Lateral root number for *Arabidopsis* seedlings grown on defined media in the presence and absence of NaCl.**

Seedlings were grown on nutrient media **A.** lacking added salt (0 mM) or on media supplemented with **B.** 50 mM NaCl or **C.** 75 mM NaCl. Counts of lateral root numbers were made using photographs taken at the day of transplant (Day 1), Day 3 following transplant, and the day of harvest (Day 5 for seedlings grown without added NaCl or Day 7 for seedlings exposed to 50 or 75 mM NaCl). The lines used for this comparison were: Columbia wild-type (Col-0), *NMT3* SALK_062703 (WT) and three *nmt3* lines: SALK_062703, SALK_016929c and SALK_120703c. Data are the mean ± standard error (SE) for two independent biological replicates. Each replicate had a minimum of 18 seedlings per line. ANOVA showed that 'days", "salt concentrations" and "salt concentrations × days" were significant at $P \leq 0.01$ whereas there was no statistical significance for "lines", "lines × salt concentrations", "lines × days", and "lines × salt concentrations × days" interactions.

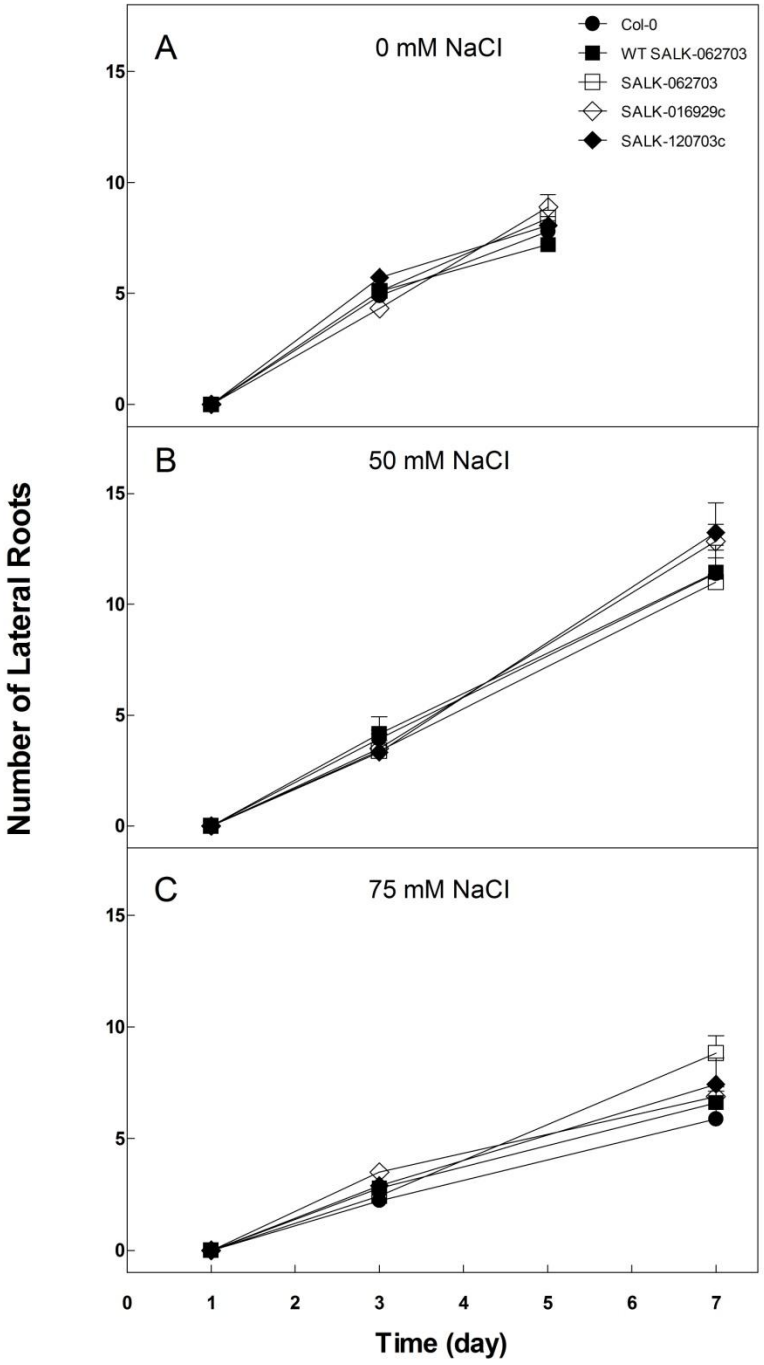

A
0 mM NaCl
B
50 mM NaCl
C
75 mM NaCl
Col-0
WT SALK-062703
SALK-062703
SALK-016929c
SALK-120703c
Number of Lateral Roots
Time (day)
0
5
10
15
0
1
2
3
4
5
6
7

Figure 16. **Total root length of *Arabidopsis* seedlings grown on defined media in the presence and absence of NaCl.**

Seedlings were grown on nutrient media **A.** lacking added salt (0 mM) or on media supplemented with **B.** 50 mM NaCl or **C.** 75 mM NaCl. Root measurements (primary root length plus length of lateral roots) were made from photographs taken on the day of transplant (Day 1), Day 3 following transplant and the day of harvest (Day 5 for seedlings grown without added NaCl or Day 7 for seedlings exposed to 50 or 75 mM NaCl). The lines used for this comparison were: Columbia wild-type (Col-0), *NMT3* SALK_062703 (WT) and three *nmt3* lines: SALK_062703, SALK_016929c and SALK_120703c. Data are the mean ± standard error SE for two independent biological replicates. Each replicate had a minimum of 18 seedlings per line. ANOVA showed that "days", "salt concentrations" and "salt concentrations × days" were significant at $P \leq 0.01$ whereas there was no statistical significance for "lines", "lines × salt concentrations ", "lines × days", and "lines × salt concentrations × days " interactions.

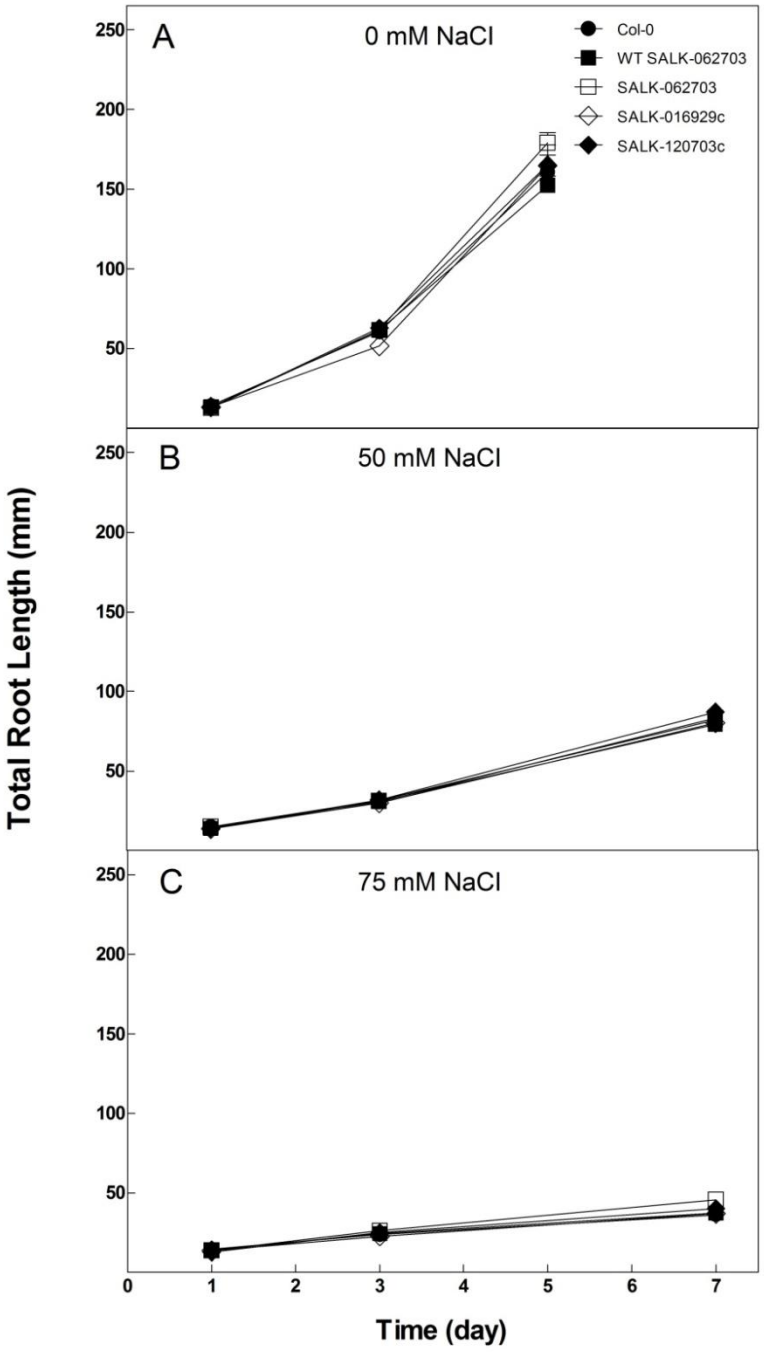
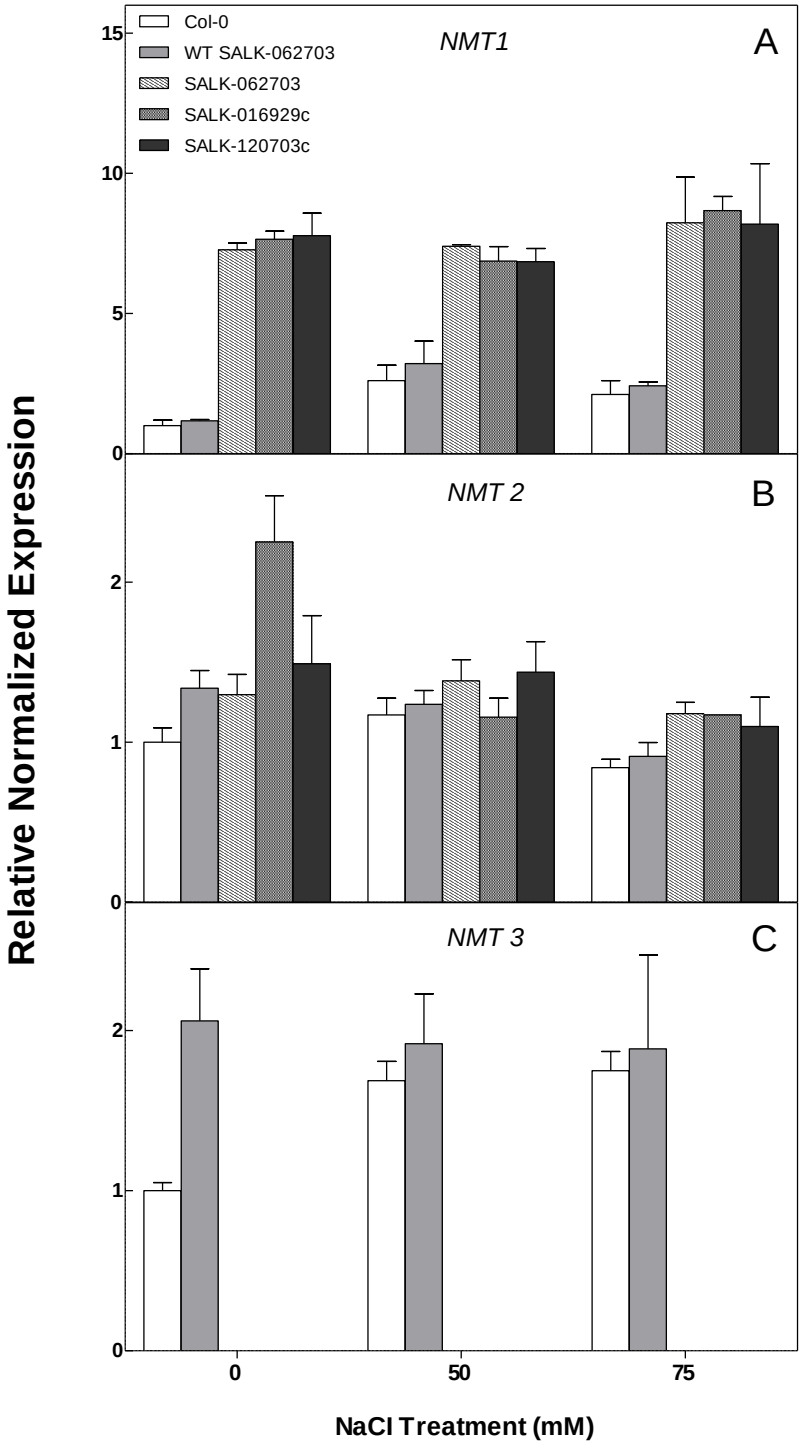

A
0 mM NaCl
B
50 mM NaCl
C
75 mM NaCl
Col-0
WT SALK-062703
SALK-062703
SALK-016929c
SALK-120703c
Total Root Length (mm)
Time (day)
250
200
150
100
50
0
1
2
3
4
5
6
7

Figure 17. **Relative expression of *NMT* genes in shoots of *Arabidopsis* seedlings grown on saline media.**

Seedlings were grown on media containing no salt for 5 d (0 mM) or on 50 or 75 mM NaCI for 7 d and shoots were harvested, pooled, and used for RNA extraction followed by cDNA synthesis for q-PCR analysis. **A.** *NMT1* gene expression. **B.** *NMT2* gene expression and **C.** *NMT3* gene expression. q-PCR was used to assay the expression of *NMT3* in *nmt3* SALK_062703, SALK_120703c, SALK_016929c, *NMT3* SALK_062703 (WT) and Columbia wild-type (Col-0). Details of seed germination, transplant and seedling growth are given in the Materials and Methods. The gene expression data is reported relative to the q-PCR data for the Col-0 line arbitrarily set as 1. The data for *NMT* expression was normalized to the expression of four reference genes: *At5g12240, YL8S, EF1α-2* and *UBQ5*. The data is the mean +/- SE for two independent biological replicate experiments each had a minimum of 18 seedlings per line. ANOVA showed "lines" to be significant ($P \leq 0.01$) but not "salt concentrations" or "lines × salt concentrations" interaction.


Col-0
WT SALK-062703
SALK-062703
SALK-016929c
SALK-120703c
NMT1
A
NMT 2
B
NMT 3
C
Relative Normalized Expression
NaCl Treatment (mM)
0
50
75
0
5
10
15
0
1
2
0
1
2

for *NMT*s in the shoot tissue of the lines as a function of NaCI treatment. In the absence of added NaCl, the normalized fold-expression of *NMT1* in three *nmt3* lines was 7.6 fold higher than the expression of the same gene in WT and Col-0 seedlings ($P \leq 0.01$) (Fig. 17A). The same expression pattern also appeared in 50 and 75 mM concentration. Under 50 mM NaCI conditions the three *nmt3* lines also showed 2.3 fold higher level of transcript abundance relative to the two WT lines (Fig. 17A). When supplied with 75 mM NaCI media, the expression of *NMT1* in the shoots of *nmt3* lines showed a 3.9-fold higher expression level than the level of transcripts detected in the two WT lines (Fig. 17A). However, ANOVA showed no significant interaction between lines and treatment (salt) or treatments (salt) alone but there was a significant difference in the gene expression patterns related to lines ($P \leq 0.01$) (Fig. 17A). Thus there was no significant difference in transcript abundance between the mutant lines or the WT lines under different concentration of NaCI but the WT and mutant lines showed differences in *NMT1* expression regardless of salt exposure or not.

The salt content of the media had no significant effect on gene expression level of *NMT2* in the lines tested (Fig. 17B). One minor but not significant difference was for the *nmt3* SALK_016929c line where seedlings on 0 mM NaCI showed a 1.7-fold higher expression levels than plants of the other lines (Fig. 17B). As expected, the transcripts associated with *NMT3* were not detected in the three *nmt3* lines and that did not change with NaCl treatment (Fig. 17C).

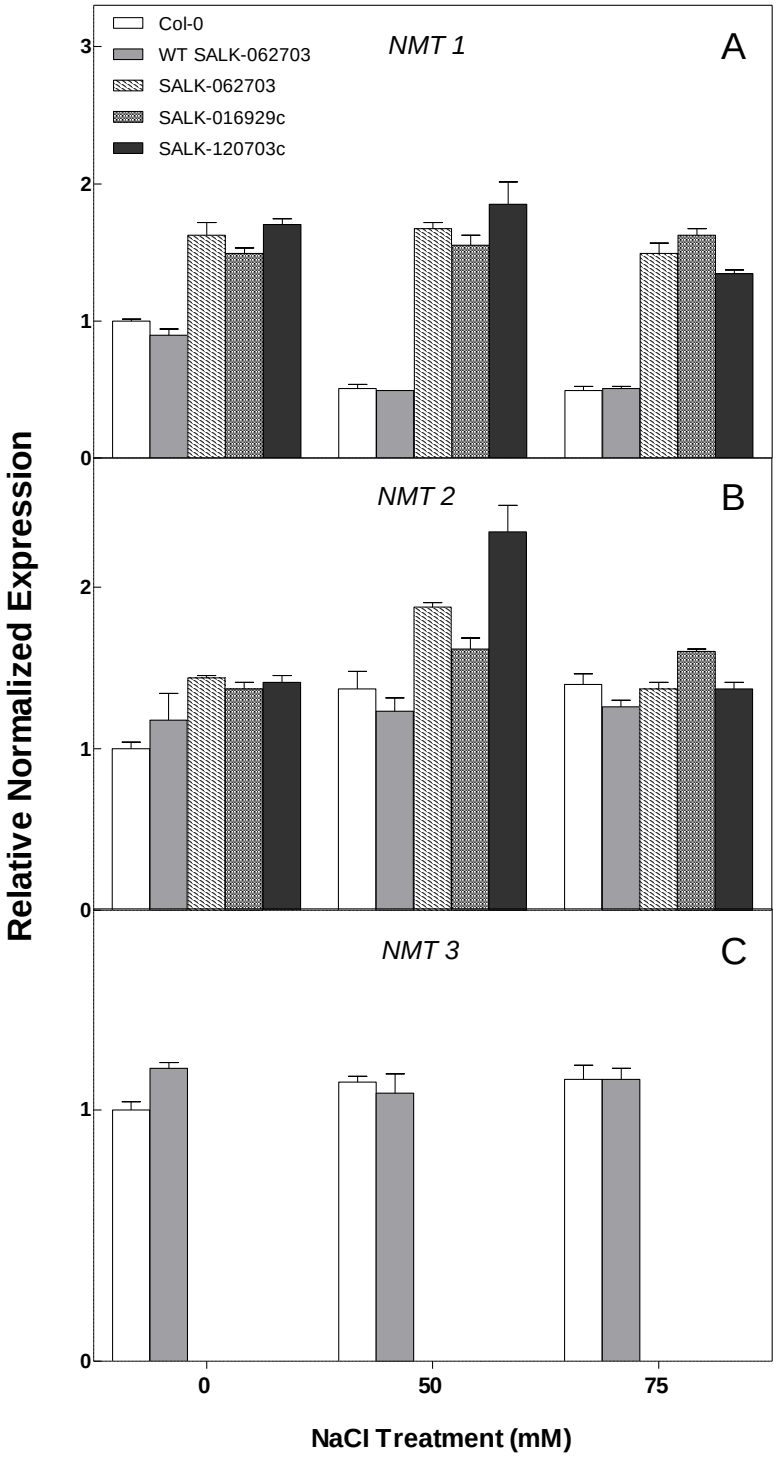
7.3 Relative expression of NMT genes in roots of Arabidopsis seedlings in response to NaCl

q-PCR was used to assay the expression of the three *NMT* genes in the roots of SALK_062703 (WT), Columbia (Col-0), *nmt3* SALK_062703, SALK_12703c, and SALK_016929c seedlings. On the 0 mM NaCl medium *NMT1* expression in the three *nmt3* lines was significantly (1.6-fold) higher than the expression level detected in WT lines (Fig. 18A). The relative expression of *NMT1* in roots of *nmt3* lines grown on media supplemented with 50 or 75 mM NaCI was the same as the seedlings of the same lines

grown on 0 mM NaCl but the expression of *NMT1* in WT seedlings was only half the level for WT seedling roots on 0 mM NaCl (Fig. 18A). There were no significant differences between the level of *NMT2* transcript abundance for the three *nmt3* mutant lines on 0 mM NaCl or 75 mM NaCl media. *NMT2* transcripts were slightly elevated in roots of *nmt3* seedlings grown on 50 mM NaCl relative to the WT lines but there is also greater variability in the data for this treatment leaving the significance of this increase in question (Fig. 18B). The reduced biomass of root tissue for seedlings on salt medium likely contributes to the variability because several biological replicates need to be combined to obtain enough root tissue for q-PCR analysis. No transcripts associated with *NMT3* were detected in roots of the three *nmt3* lines grown in the presence or absence of salt (Fig. 18C). The expression level of *NMT3* in WT seedling roots was the same for roots all treatments (Fig. 18C).

Figure 18. **Relative expression of *NMT* genes in roots of *Arabidopsis* seedlings grown on saline media.**

Seedlings were grown on media containing no salt for 5 d (0 mM) or on 50 or 75 mM NaCI for 7 d and roots were harvested, pooled, and used for RNA extraction followed by cDNA synthesis for q-PCR analysis. **A.** *NMT1* gene expression. **B.** *NMT2* gene expression and **C.** *NMT3* gene expression. q-PCR was used to assay the expression of *NMT3* in *nmt3* SALK_062703, SALK_120703c, SALK_016929c, *NMT3* SALK_062703 (WT) and Columbia wild-type (Col-0). Details of seed germination, transplant and seedling growth are given in the Materials and Methods. The gene expression data are reported relative to the q-PCR data for the Col-0 line arbitrarily set as 1. The data for *NMT* expression was normalized to the expression of four reference genes: *At5g12240, YL8S, EF1α-2* and *UBQ5*. The data is for one biological replicate with a minimum of 18 seedlings per line. Standard error bars are for q-PCR three technical repeats.


Col-0
WT SALK-062703
SALK-062703
SALK-016929c
SALK-120703c
NMT 1
A
NMT 2
B
NMT 3
C
Relative Normalized Expression
NaCl Treatment (mM)
0
50
75
0
1
2
3

DISCUSSION AND CONCLUSIONS

In *Arabidopsis*, choline is synthesized *via* methylation of the free base phosphoethanolamine (PEA) catalyzed by the enzyme PEAMT (Fig. 1) (Bolognese & McGraw, 2000). There are three loci associated with PEAMT: one encoding PEAMT (At3g18000; *NMT1*), one encoding PMEAMT (At1g48600; *NMT2*) and a third encoding a putative PEAMT (At1g73600; *NMT3*). Previously, our laboratory reported that PMEAMT (NMT2) cannot methylate PEA but can methylate PMEA and PDEA (BeGora et al., 2010) and disruption of the activity of PMEAMT was not associated with a discernible morphological phenotype. This finding was evidence that the initial and committing step in the P-Cho synthesis pathway is carried out by NMT1 and /or NMT3 making one or both of these enzymes essential for choline synthesis in *Arabidopsis*.

Recently, Alatorre-Cobos et al (2012) reported that NMT1 must be the more critical of the two enzymes because disrupting its activity leads to a severe change in root development with a reduction to primary root length and proliferation of lateral roots. These authors noted that the shoots of the mutant resembled WT although there was delayed flowering. Macleod (2010) studied a single *nmt3* line of *Arabidopsis* (SALK_062703) that lacks *NMT3* expression. He observed that mutant plants do not show a discernible phenotype, an outcome that would support the proposal of Alatorre-Cobos et al (2012) on the relative importance of these two enzymes. Alternatively, the lack of phenotype for *nmt3* plants could be explained by compensatory changes in *NMT1* expression and NMT1 activity and not simply a reflection of the relative importance of *NMT1* and its gene product to a WT plant. With this background in mind, the present study was aimed to investigate the following: A) validate three SALK insertion lines as *nmt3,* B) use q-PCR to monitor the expression of the *NMT* genes in *nmt3* and *nmt1* plants to discern the patterns of expression for all three genes, and C) test if exposure to osmotic stress imposes a condition that alters the expression levels of these genes as has been the case for glycine-betaine accumulating species.

Early in this work, seeds corresponding to the three SALK insertion lines (SALK_062703 with its associated WT, SALK_120703c and SALK_016929c) and Col-0 WT were planted and the plants produced were observed. The various *nmt3* plants were visually indistinguishable from WT plants at the seedling stage (Fig. 11) and at the rosette stage of four-week old plants (Fig. 19). Macleod (2010) reported an accelerated time of flowering phenotype for the *nmt3* SALK_062703 line. This early flowering phenotype was also observed for the same line in this study at a frequency of ranging from 25 to 75% among mutant plants grown at different times. In other respects (bolt size, silique formation, etc.) the mutant plants undergoing flowering are visually indistinct from flowering WT plants. Since flowering is widely affected by stress and it was a variable and even ephemeral trait with respect to frequency in the study by Macleod (2010), this association was noted but not explored further in this project. In the absence of a strongly altered morphological or developmental phenotype, the first steps were directed towards ensuring that the SALK lines used were authenticated as having a T-DNA insertion in *NMT3* and that *NMT3* expression was altered before assessing the expression of *NMT3* (and the related *NMT* genes) in these lines.

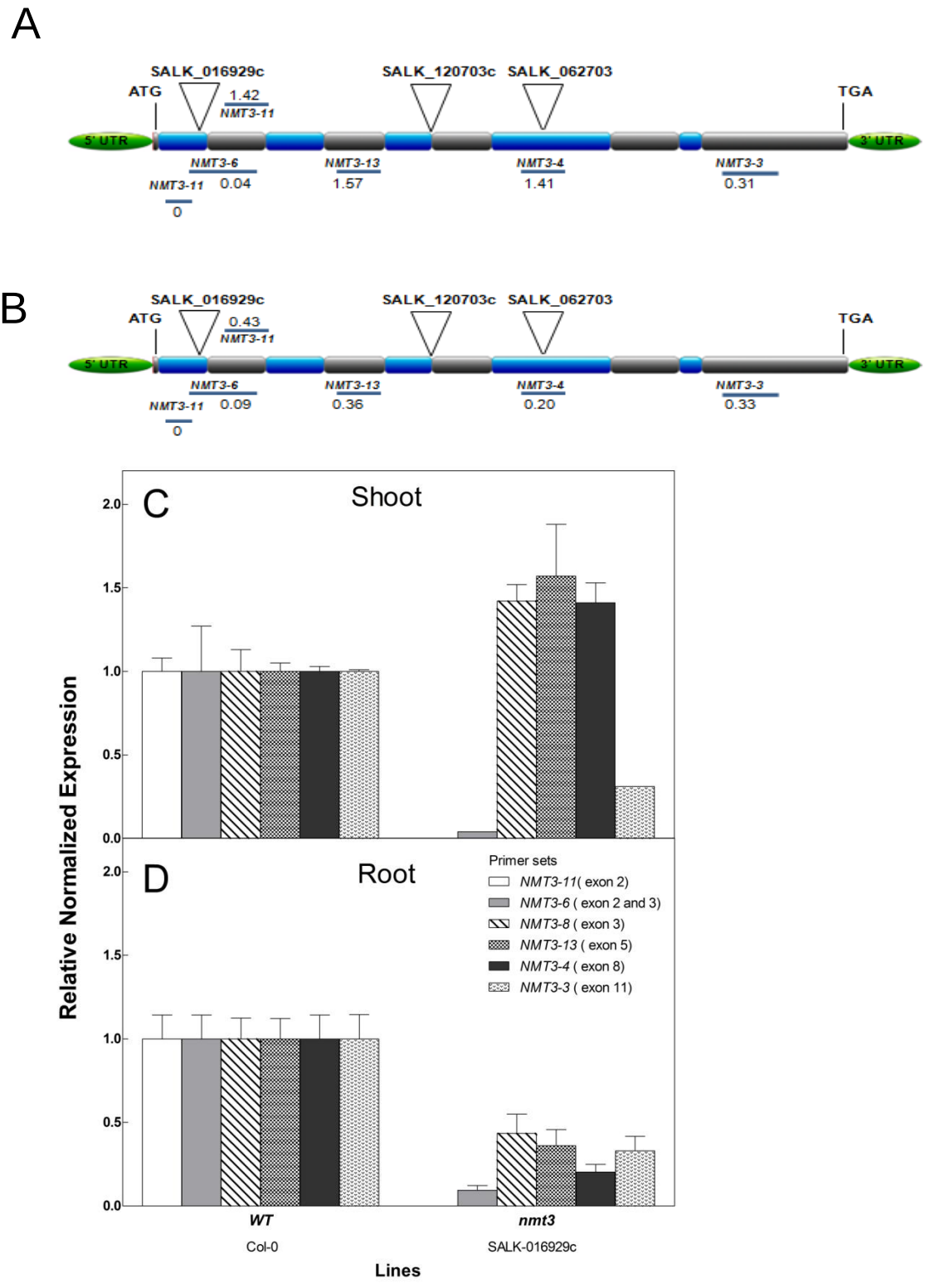
Selection of suitable primers for q-PCR proved to be a challenge. The first difficulty is the high degree of sequence overlap for the three *NMT* genes making gene-specific primer selection for amplification difficult. Longer products from RT-PCR were sequenced showing that appropriate selection of gene-specific primers is possible despite the high sequence identity for the three genes. However, q-PCR products are too short to sequence so for this application melt curve analysis provided evidence that only a single product was being generated during q-PCR amplification (Table 4). In early analyses we expected that a single set of primers should suffice for use in all of the lines being tested. However, test runs of RT-PCR and q-PCR showed that truncated transcripts associated with *NMT3* were being produced by plants in the lines reported to have T-DNA insertions in locus At1g73600 (see Fig. 10). The most dramatic example was found for the SALK_016929c which has a T-DNA insertion associated with exon 2 of *NMT3* (data from Fig. 10 is summarized in Fig. 20). The use of primer pairs annealing to the cDNA

Figure 19. **Representative 4-week-old *Arabidopsis* wild type and *nmt3* mutant plants** Top Row (left to right): WT (*NMT3*) SALK_062703 and two Col-0 wild type plants. Bottom Row (left to right): SALK_062703, SALK_016929c and SALK_120703c *nmt3* plants. Scale bar is 4 cm.


SALK-062703 WT
Col-0 WT
Col-0 WT
SALK-062703
SALK-016929c
SALK-120703c

Figure 20. Genotype and oligonucleotide primer design influences estimates of *NMT3* primer abundance by q-PCR.

Five-day-old *Arabidopsis* seedlings grown on defined media plates were used for RNA extraction and cDNA preparation. The gene structure maps for *NMT3* showing positions of the forward and reverse primer pairs used with relative values for q-PCR amplification for **A.** Shoots of SALK_016929c and **B.** Roots of SALK_016929c. The line-specific T-DNA insertion site is shown as are the exons (grey and blue). **C.** Shoots and **D**. Roots of *nmt3* SALK_016929c and Col-0 wild-type were used for q-PCR. Primer pairs were designed to amplify DNA fragments associated with different exons of *NMT3*. The gene expression data is reported relative to the q-PCR data for Col-0 wild-type seedlings arbitrarily set as 1. All data was normalized to the expression of four reference genes: *At5g12240, YL8S, EF1α-2* and *UBQ5*. The expression data shown is the mean +/- SE for three technical replicates with a minimum of 18 seedlings per line. Information about the primer pairs used for q-PCR is given in Table 4 of results.


A
SALK_016929c
SALK_120703c
SALK_062703
ATG
TGA
1.42
NMT3-11
5' UTR
3' UTR
NMT3-6
NMT3-13
NMT3-4
NMT3-3
NMT3-11
0.04
1.57
1.41
0.31
0
B
SALK_016929c
SALK_120703c
SALK_062703
ATG
TGA
0.43
NMT3-11
5' UTR
3' UTR
NMT3-6
NMT3-13
NMT3-4
NMT3-3
NMT3-11
0.09
0.36
0.20
0.33
0
C
Shoot
D
Root
Relative Normalized Expression
2.0
1.5
1.0
0.5
0.0
Primer sets
NMT3-11(exon 2)
NMT3-6 (exon 2 and 3)
NMT3-8 (exon 3)
NMT3-13 (exon 5)
NMT3-4 (exon 8)
NMT3-3 (exon 11)
WT
Col-0
nmt3
SALK-016929c
Lines

template at a position downstream of the insertion (even as close as exon 3) shows WT levels of *NMT3* transcripts while no detectable product is observed with a primer set that anneals near the site of the T-DNA insertion in exon 2. Evidence that the lack of amplification by these primers is not due to faulty primer design is shown by the readily detectable levels of *NMT3* transcripts for WT sources of template cDNA following q-PCR.

T-DNA insertion into the exon or intron of protein-coding genes may result in knocking-out or knocking-down the expression of a target gene (Wang, 2008). We had both types of insertions available: two in exons and one in an intron. In our study, the insertion of T-DNA in *nmt3* SALK_062703 was in exon 8. The primer pairs used to test gene expression for this insertion line were located on exon 11 (RP) and exon 10 (LP) (Fig 6A), which were designed to anneal towards the 3'UTR of *NMT3*. As assessed by RT-PCR (Fig 4B) and q-PCR (Fig. 10B and C), no transcripts were detected in the shoots and roots of *nmt3* plants of the SALK_062703 line. However, when the primers to amplify the same gene in the same line were designed to anneal upstream of the T-DNA insertion we found low, but detectable levels of transcripts in shoots and roots (Fig.10B and C). Thus in this line, there is evidence that truncated *NMT3* transcripts are present. In contrast to the exon position of the T-DNA in the SALK_062703, the T-DNA insertion of the *nmt3* SALK_120703c line was in an intron (No. 6). The two primer pairs designed to anneal downstream (3') of the T-DNA insertion site in the *nmt3* SALK_120703c line showed that there was no expression of the *NMT3* gene in this line (Fig. 10B and C). However, primers annealing 5' to the site of insertion (ie, *NMT3-13* directed to exon 5) shows *NMT3* to have an almost identical, albeit low expression level in the shoots and roots of *nmt3* SALK_120703c and SALK_062703 plants. These findings indicate that knock-downs of gene expression are possible with T-DNA insertions in either an exon or intron of *NMT3* but that neither insertion yielded a knock-out mutant (Fig.10B and C). It is important to consider, however, that even when a truncated transcript is expressed and translated to produce a truncated protein, the latter may still not be functional (Noh & Amasino, 2003, Okushima et al., 2005, Gusmaroli et al., 2004). As discussed earlier,

even the T-DNA inserted the exon 2 of *nmt3* SALK_016929c line which places the T-DNA near the 5'end of the gene is not a knock-out mutant as *NMT3* transcripts that correspond to the middle of the gene, presumably truncated versions, are readily detected by q-PCR. In each case discussed above, whether truncated transcripts are translated into active or inactive NMT3 protein is not known. A comparison of enzyme activity measurements from plant tissues would help address this uncertainty.

In advance of carrying out a gene expression profile of *NMT* genes in *Arabidopsis*, it was important to establish that the absence of transcripts could not be ascribed to trivial explanations including our use of an inappropriate time of harvest. Light and salt exposure increase the level of steady state transcripts encoding spinach *PEAMT* with low levels of transcripts during the day and higher levels at night when both spinach PEAMT activity and protein was lowest (Weretilnyk et al., 1995, Smith et al., 2000, Drebenstedt, 2001). This study shows that under light conditions, transcripts associated with *NMT3* are significantly higher than those related to *NMT1* in WT plants and that holds for both roots and shoots of seedlings harvested under light or dark conditions (Fig. 7). Thus *NMT3* expression does not appear to be light-regulated in WT plants (Fig. 7A). The relatively high and stable level of *NMT3* expression in roots of WT plants harvested during the dark cycle versus day cycle also suggests this gene is not light-responsive. However, in shoots of *nmt3* plants during the day, *NMT1* expression is found to be equal to the level of *NMT3* transcripts in WT seedlings and this level of transcript abundance drops for shoots (but not roots) harvested in the dark. These observations suggest that 1) increased *NMT1* expression appears to compensate for the lack of *NMT3* expression in *nmt3* seedlings and 2) in the *nmt3* background *NMT1* gene expression is diurnally regulated.

The same experiment was performed using *nmt1* plants of the SALK_036291 line (Fig. 9). Interestingly, *NMT1* transcripts were detected, albeit at relatively low levels, in shoots of all seedlings whether under light or dark conditions (Fig. 9A). In contrast, the expression of *NMT1* was not detected in roots, the location that is reportedly most severely compromised by the loss of this gene (Cruz-Ramírez et al., 2004). Also in

shoots and roots, the expression of *NMT3* is up-regulated relative to WT levels in *nmt1* seedlings harvested under day conditions but not under night conditions where *NMT3* transcripts are significantly lower relative to the day time-point. Thus in *nmt1* plants showing no detectable *NMT1* transcripts in roots we find: 1) *NMT3* expression in higher than *NMT1* expression at midday in both shoots and roots, 2) with respect to compensatory changes there is only a modest increase in *NMT3* expression in *nmt1* plants and 3) in the *nmt3* background there is evidence of light-regulation in *NMT3* expression with *NMT3* transcript abundance decreasing at night with the greatest change seen in shoots of *nmt1* plants where this reduction is 4.4-fold (Fig. 9A).

The observed diurnal behavior of *NMT1* and *NMT3* expression, particularly in WT plants is not consistent with light being the trigger for gene expression. However, with the observed changes in the expression pattern of these genes in the *nmt1* and *nmt3* mutants at midday and midway through the dark period, light-regulation could be inferred. However, with these contrasting patterns in mind, the involvement of light is not likely direct but more consistent with a response or condition within the plant that is responsive or regulated by light and their effect, indirectly, serves to regulate expression of these genes. Alatorre-Cobos et al. (2012) proposed that choline and P-Cho regulate the translation of the *Arabidopsis* gene they designate as *XIPOTL1* (aka *NMT1*) via interaction with a conserved, upstream uORF. The translational efficiency of this gene is regulated through the uORF so that transcript abundance is not linked to enzyme activities. Thus it would be valuable to have enzyme activities from these sources to see if transcript abundance correlates at all to enzyme activities in *nmt3* plants.

BeGora *et al* (2010) proposed that the role of NMT2 (aka PMEAMT) was to ensure that all of the P-EA that was converted to P-MEA was then fully methylated to produce P-Cho. This would prevent the build-up of Ptd-MEA or Ptd-DEA in phospholipids of membranes, a potentially deleterious condition. This important role for NMT2 could explain the lack of diurnal changes in spinach PMEAMT activity and its presence in both roots and leaves (Weretilnyk et al., 1995). Figures 7A and B show that *NMT2* expression is consistently higher than *NMT1* but usually lower than the expression

level of *NMT3* in WT plants for both shoots and roots of *Arabidopsis* seedlings. In the absence of *NMT3* expression in *nmt3* plants where *NMT1* expression has increased, *NMT2* transcript levels remain high, an observation consistent with this gene behaving more like a house-keeping gene with respect to showing stable expression in seedlings or plants harvested in the light or dark period of the day (Fig. 7).

With the Cruz-Ramirez et al., (2004) report that *nmt1* seedlings show significant alterations to roots, we examined *nmt3* seedlings for any signs of a root-related phenotype. However, there was no statistically significant difference in primary root length, density or number of lateral roots, total root length or any visible properties of the shoot when WT and *nmt3* seedlings are grown on tissue culture medium (Fig 13, 14,15,16). This was not surprising given our earlier observations with 4-week old rosettes or flowering *nmt3* plants where the plants were not distinguishable from WT plants. In many plants, the requirement for choline is increased in response to stress (Hanson & Rhodes, 1983). When *Arabidopsis* seedlings were salinized, root development was delayed for all of the genotypes in the comparison so the absence of detectable *NMT3* transcripts did not seem to have any detrimental impact on root or shoot development over that uniformly imposed on all of the lines by the presence of salt.

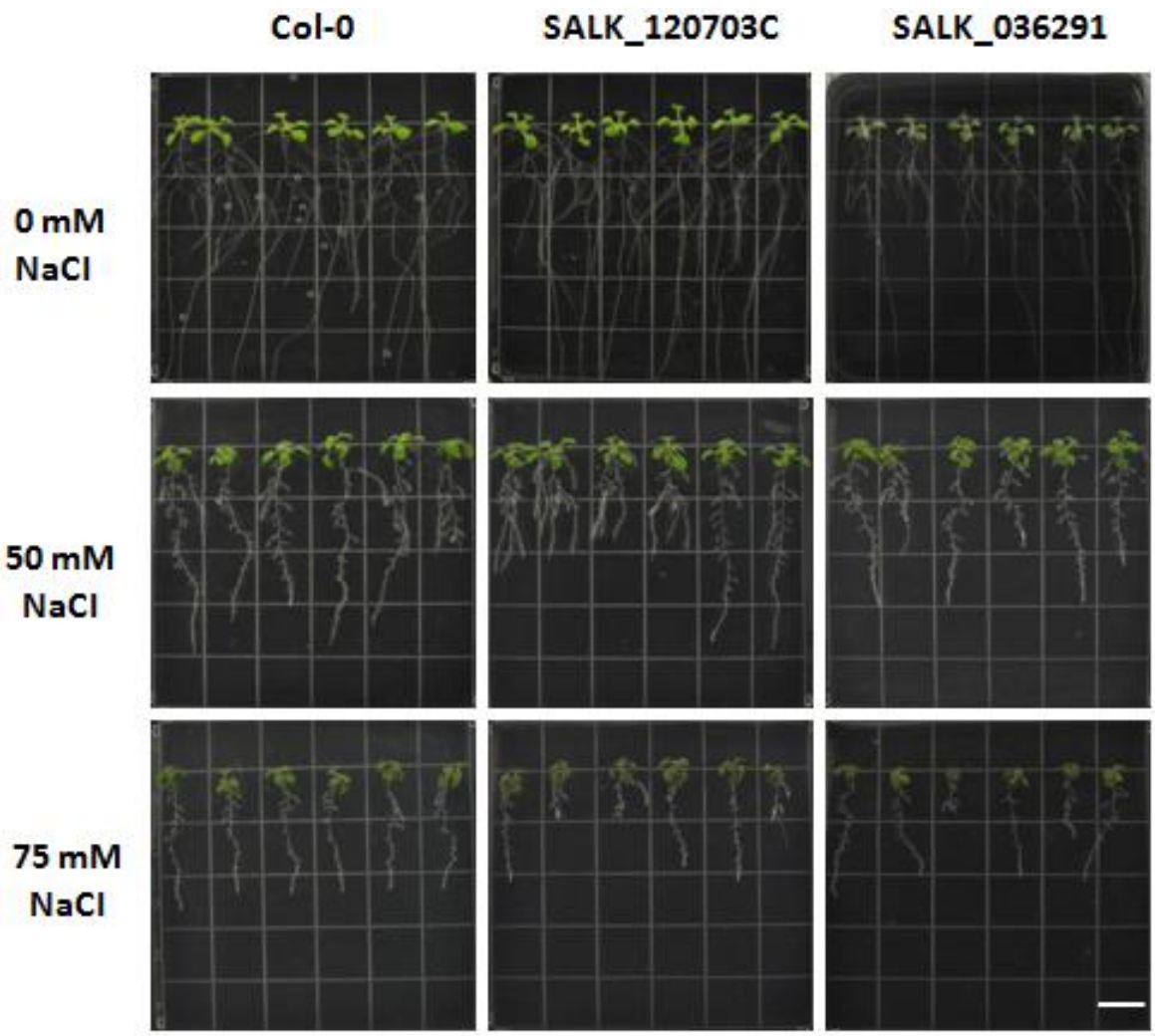
The study reported by Cruz-Ramirez et al. (2004) showed that the *Arabidopsis* line (*xpl*) lacked *NMT1* transcripts and that all of the plants had an aberrant root phenotype but the aerial part of the plants resembled WT plants. Our laboratory also reported that there was no morphological abnormality in terms of flowering and reproduction when the *atpmeamt* mutants plants were shown to be deficient in *NMT2* related transcripts (BeGora et al., 2010). The three *nmt3* lines used in this study also show no noteworthy developmental or morphological phenotype, in contrast to the *xpl* plants. Taken together, these comparisons suggests that *NMT1* and *NMT3* do not have overlapping functions, particularly where the development of roots is concerned and that *NMT1* likely has a dominant role in choline metabolism because its defective expression leads to the only phenotypic alteration. This brings us back to the one root-related phenotype we did observe and that was for the SALK-120703c line where about 47% of

the seedlings carrying a T-DNA insertion in At1g73600 (and anomalous expression of *NMT3*) developed shortened roots but only while on plates containing saline media (Fig. 11). This root-related phenotype was not detected in the other lines where *NMT3* transcript levels were also not detected. We questioned how *nmt1* seedlings would perform under our conditions. Figure 21 shows a comparison of WT, *nmt3* (SALK_120703c) and *nmt1* (SALK_036291) seedlings grown in the absence and presence of NaCl added to the medium. In the results section, we attributed the inconsistent, altered salt-related root phenotype for SALK_120703c *nmt3* plants as likely being due to the presence of a second, unrelated mutation in that line. For comparison, we grew SALK_036291 *nmt1* seedlings (parent plants genotyped as carrying the T-DNA insertion in *NMT1*) on plates and found, in contrast to the report by Cruz-Ramirez (2004), that some plants in this line also have long roots and that their growth is compromised with salt just as is the case for WT plants. Occasionally short roots show up among the WT plants, but in the *nmt1* line short roots appear among all roots with a frequency of 35 % which is above that observed for WT plants at 15%. There are at least two explanations to account for this observation and this apparent contradiction to the report of Cruz-Ramirez (2004). It may be that the SALK_036291 *nmt1* line also harbors a second mutation and that this mutation leads to an aberrant root phenotype even in the absence of salt stress (Fig. 21, 0 mM NaCl). Inadvertent selection for the altered roots may now have led to lines selected for the presence of at least two T-DNA insertions, one associated with short roots and the other for a knock-out of *NMT1*. Alternatively, it may be that the stress associated with decreased performance in the PEA methylation pathway as a consequence of the *nmt* genotype produces a condition that exerts its greatest impact on root development and that the extent of this stress and/or when it happens during plant development is unpredictable. In the absence of additional information we cannot exclude either possibility.

In conclusion, our findings show that the loss of *NMT3* expression in three, independent *nmt3* lines has negligible impact on seedling root development and no indications that it has a detrimental effect on plant development or reproduction. It is

Figure 21. **Representative *Arabidopsis* seedlings grown on defined media in the presence and absence of NaCl.**

Seedlings were grown for 5 d on nutrient media lacking added salt (0 mM) or for 7 d on media supplemented with 50 or 75 mM NaCl. The lines used for this comparison were: Columbia wild-type (Col-0), SALK_120703c *nmt3* and SALK_036291 *nmt1*. In the case of SALK_120703c all seedlings had long roots on media lacking salt but on 50 and 75 mM NaCl almost half of the seedlings (28 out of 60 seedlings) exposed to salt showed a shortened primary root relative to other seedlings revealing a salt-responsive root length phenotype in this line. SALK_036291 seedlings tested in parallel also had long and short primary roots but this distinguishing phenotype was found on media lacking salt 0 mM NaCl (113 out of 316), containing salt 50 mM (10 out of 22) and 75 mM NaCl (13 out of 24). Scale bar is 13.5 mm.


Col-0
SALK_120703C
SALK_036291
0 mM NaCl
50 mM NaCl
75 mM NaCl

possible that exposure to adverse environments, including cold or hot temperatures or water deficits, may reveal a phenotype associated with this genotype but the compensatory changes in the expression of *NMT1* would suggest otherwise. Rather, it appears that *Arabidopsis* has a significant capacity to compensate for the loss of gene product (NMT3) that catalyzes a reaction that is regarded as essential for plant metabolism without, apparently, deleterious consequences. How this compensation is performed is not adequately explained by transcript abundance measurements.

Future studies should associate enzyme activities and, if possible, protein levels for the three gene products with changes in transcript abundance. The latter may prove to be problematic given the high identity in amino acid sequence of the three products. Nonetheless, enzyme activities would shed light on the contribution of the three methyltransferases and a useful comparison with the transcript abundance estimates reported in this thesis. Moreover, given the proposed role of P-Cho and choline in phosphobase methylation as regulating the translation of *nmt1* (Alatorre-Cobos, 2012), measurements of these metabolites could provide additional insight into the mechanisms regulating choline synthesis in *nmt3 Arabidopsis* plants.

Appendix A- Sequence Alignment

The products amplified by PCR were extracted from the gels and sequenced. The DNA sequence information was submitted to the Basic Local Alignment Search Tool (BLAST) program and the best match for each amplicon is given. The analysis confirmed the amplicon identity as a product originating from the *Arabidopsis NMT3* (locus At1g73600). The amplicons were produced by PCR using the following primer pairs: Left Border Primer (LBP#1.3) for all *nmt3* lines and the right primers customized for each the mutant lines: NMT3-GT-1 (RP) for SALK_062703 plants, NMT3-GT-2 (RP) for SALK_120703c plants and NMT3-GT-3 (RP) for SALK_016929c plants.
"Query" represents *nmt3* sequence (from MOBIX) and "Subject" is the genomic DNA sequence from NCBI for locus At1g73600 (Gene ID: 843694)

SALK_062703
Right Primer: (NMT3-GT-1) - TATGCTGGATCTGAAACCTGG
LBP#1.3 ATTTTGCCGATTTCGGAAC

```
Query  194       CAATGCTGGCTTGTCCTGAGAGTAAGACCAGGGATTACAAAATGGATACCAAATTAAAGT  253
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672859  CAATGCTGGCTTGTCCTGAGAGTAAGACCAGGGATTACAAAATGGATACCAAATTAAAGT  27672800

Query  254       CATAAGGGAAGAAGAAATAGCGGAAGCTTATACTTGGATATGTAGAATGGTGTCTCTGCT  313
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672799  CATAAGGGAAGAAGAAATAGCGGAAGCTTATACTTGGATATGTAGAATGGTGTCTCTGCT  27672740

Query  314       ATAAATAACATCAAAGGTGTTATCAGGATACTCCTTCTTGGTGCAATCAGCTACTTCGAA  373
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672739  ATAAATAACATCAAAGGTGTTATCAGGATACTCCTTCTTGGTGCAATCAGCTACTTCGAA  27672680

Query  374       TTCTACAGAGCATTTGAGTCCTATTGCGTGTTCAAGCGCAAAAGAGATCATGTTTACAGA  433
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672679  TTCTACAGAGCATTTGAGTCCTATTGCGTGTTCAAGCGCAAAAGAGATCATGTTTACAGA  27672620

Query  434       TAGATCAATGCCCACAACATCCACGTCAAAGTTCTCAGCCATGTAGAAGTCCCCTCCTCC  493
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672619  TAGATCAATGCCCACAACATCCACGTCAAAGTTCTCAGCCATGTAGAAGTCCCCTCCTCC  27672560

Query  494       TATTCCGCACCCAACGTCTAGAACTTTTTGGCCAGGTTTCAGATCC-GCATA  544
                 |||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  27672559  TATTCCGCACCCAACGTCTAGAACTTTTTGGCCAGGTTTCAGATCCAGCATA  27672508
```

SALK_120703c
Right Primer: (NMT3-GT-2) - TCCACGAATTCCTTTGTTGTC
LBP#1.3 ATTTTGCCGATTTCGGAAC

```
Query  196       TTATAATGTTTGGACTCATATTTTTCGAAAATTTCAGATATGCTGGCTTTGGCAGAAAGT  255
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672177  TTATAATGTTTGGACTCATATTTTTCGAAAATTTCAGATATGCTGGCTTTGGCAGAAAGT  27672236

Query  256       CAGTTCGGATAATGATAGGGGCTTCCAACGCTTCTTGGACAATGTCCAGTATAAGTCTAG  315
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672237  CAGTTCGGATAATGATAGGGGCTTCCAACGCTTCTTGGACAATGTCCAGTATAAGTCTAG  27672296

Query  316       TGGTATCTTACGCTATGAGCGTGTCTTTGGAGAAGGGTTTGTTAGCACAGGGGGACTCGG  375
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672297  TGGTATCTTACGCTATGAGCGTGTCTTTGGAGAAGGGTTTGTTAGCACAGGGGGACTCGG  27672356

Query  376       TATGCTCTTTAGTCATCGAGTTTGTATCAACTTTGCAGTATTGATGGTTCTGTTTTTGAA  435
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672357  TATGCTCTTTAGTCATCGAGTTTGTATCAACTTTGCAGTATTGATGGTTCTGTTTTTGAA  27672416

Query  436       TTTAAGTTATACAGAACCATTAAGTGAAGCTATCTTTTCTTCTTATTATACTTGTTTCTT  495
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27672417  TTTAAGTTATACAGAACCATTAAGTGAAGCTATCTTTTCTTCTTATTATACTTGTTTCTT  27672476

Query  496       TTGATATAGAGACAACAAAGGAATTCGTGGA  526
                 ||||||||||||||||||||||||||||||
Sbjct 27672477    TTGATATAGAGACAACAAAGGAATTCGTGGA 27672507
```

SALK_016929c
Right Primer: (NMT3-GT-3) - AGAACAAGCATGGACGTGAAC
LBP#1.3 ATTTTGCCGATTTCGGAAC

```
Query  159       AGAACGTCCTGAGGTTTATTTCGACTCACCCACTATAAAGTTATCATCTTTGCTATATAT  218
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27671136  AGAACGTCCTGAGGTTTATTTCGACTCACCCACTATAAAGTTATCATCTTTGCTATATAT  27671195

Query  219       AGATCATTTTGAGATATGTATGCCTTGATGAGTTGAATCTTTGCTGTCCTTTATTGTGTT  278
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27671196  AGATCATTTTGAGATATGTATGCCTTGATGAGTTGAATCTTTGCTGTCCTTTATTGTGTT  27671255

Query  279       ATGGACTTATGAAGGAAAGCTTCTAAAGGCTTAAATTATGTGTGTGTTTGTGTCAGATAC  338
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27671256  ATGGACTTATGAAGGAAAGCTTCTAAAGGCTTAAATTATGTGTGTGTTTGTGTCAGATAC  27671315

Query  339       TTGCGTTTCTTCCACCTATTGAAGGGACAACAGTGCTAGAGTTTGGTGCTGGAATTGGTC  398
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27671316  TTGCGTTTCTTCCACCTATTGAAGGGACAACAGTGCTAGAGTTTGGTGCTGGAATTGGTC  27671375

Query  399       GTTTTACTACTGAATTAGCTCAGAAGGCCGGCCAGGTCATTGCGGTTGACTTCATTGAAA  458
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27671376  GTTTTACTACTGAATTAGCTCAGAAGGCCGGCCAGGTCATTGCGGTTGACTTCATTGAAA  27671435

Query  459       GTGTTATCAAAAAGGTTGTTAGTTCACGTCCATGCTTGTTCT  500
                  |||||||||||||||||||||||||||||||||||||||||
Sbjct  27671436  GTGTTATCAAAAAGGTTGTTAGTTCACGTCCATGCTTGTTCT  27671477
```

Appendix B- Statistical analysis of gene expression by q-PCR: Seedling data

SALK_062703 Shoot	Degrees of Freedom	Sum Sq	F-value	P-value
Lines	1	0.6993	24.988	***
Gene	2	5.2988	94.672	***
Time	1	0.0569	2.034	.
Lines: Gene	2	11.1290	198.839	***
Line: Time	1	0.5363	19.163	***
Gene: Time	2	2.6893	48.049	***
Lines: Gene: Time	2	0.7833	13.994	***

SALK_062703 Root	Degrees of Freedom	Sum Sq	F-value	P-value
Lines	1	0.9631	72.1403	***
Gene	2	2.3469	87.8992	***
Time	1	0.1131	8.4683	**
Lines: Gene	2	8.9182	334.0168	***
Lines: Time	1	0.0003	0.0190	
Gene: Time	2	0.0795	2.9757	***
Lines: Gene: Time	2	0.1002	3.7510	***

Significant codes: '***' 0.001, '**' 0.01, '*' 0.05, '.' 0.1, '' 1

SALK_036291 Shoot	Degrees of Freedom	Sum Sq	F-value	P-value
Lines	1	0.4261	48.361	***
Gene	2	21.0240	1193.191	***
Time	1	0.4450	50.511	***
Lines: Gene	2	0.5859	33.249	***
Lines: Time	1	0.3066	34.799	***
Gene: Time	2	0.7745	43.956	***
Lines: Gene: Time	2	0.7870	44.668	***

SALK_036291 Root	Degrees of Freedom	Sum Sq	F-value	P-value
Lines	1	0.4261	48.361	***
Gene	2	21.0240	1193.191	***
Time	1	0.4450	50.511	***
Lines: Gene	2	0.5859	33.249	***
Lines: Time	1	0.3066	34.799	***
Gene: Time	2	0.7745	43.956	***
Lines: Gene: Time	2	0.7870	44.668	***

Significant codes: '***' 0.001, '**' 0.01, '*' 0.05, '.' 0.1, '' 1

Appendix C- Statistical analysis of root measurements

Primary Root Length	Degrees of Freedom	Sum Sq	F-value	P-value
Lines	4	0.1654	4.0262	**
Salt concentration	2	4.2853	208.6684	***
Days	1	19.1241	1862.4511	***
Lines: Salt concentrations	8	0.1079	1.3132	
Lines: Days	4	0.2991	7.2832	***
Salt concentrations: Days	2	8.0446	391.7241	***
Lines: Salt concentrations: Days	8	0.0695	0.8464	

Lateral Root Density	Degrees of Freedom	Sum Sq	F-value	P-value
Lines	4	0.0888	1.0401	
Salt concentrations	2	1.6988	39.7958	***
Days	1	30.3966	1424.0953	***
Lines: Salt concentrations	8	0.1935	1.1331	
Lines: Days	4	0.0384	0.4501	
Salt concentrations: Days	2	0.0757	1.7725	
Lines: Salt concentrations: Days	8	0.0588	0.3442	

Significant codes: '***' 0.001, '**' 0.01, '*' 0.05, '.' 0.1, '' 1

Lateral Root Number	Degrees of Freedom	Sum Sq	F-value	P-value
Lines	4	0.016	0.0910	
Salt concentrations	2	0.752	8.7485	***
Days	1	102.996	2396.7879	***
Lines: Salt concentrations	8	0.205	0.5950	
Lines: Days	4	0.168	0.9795	
Salt concentrations: Days	2	5.241	60.9862	***
Lines: Salt concentrations: Days	8	0.146	0.4250	

Total Root Length	Degrees of Freedom	Sum Sq	F-value	P-value
Lines	4	0.095	1.8644	
Salt concentrations	2	2.740	108.1168	***
Days	1	74.090	5846.8282	***
Lines: Salt concentrations	8	0.052	0.5174	
Lines: Days	4	0.055	1.0915	
Salt concentrations: Days	2	19.936	786.6035	***
Lines: Salt concentrations: Days	8	0.081	0.8031	

Significant codes: '***' 0.001, '**' 0.01, '*' 0.05, '.' 0.1, ' ' 1

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