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Recycling of methylthioadenosine is essential for normal vascular development and reproduction in *Arabidopsis thaliana*

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ABSTRACT

5'-methylthioadenosine (MTA) is the common by-product of polyamine (PA), nicotianamine (NA) and ethylene biosynthesis. The methylthiol moiety of MTA is salvaged by 5'-methylthioadenosine nucleosidase (MTN) in a reaction producing methylthioribose (MTR) and adenine. The MTN double mutant, mtn1-1mtn2-1, retains ~14% of the MTN enzyme activity present in the wild type, and displays a pleiotropic phenotype that includes altered vasculature and impaired fertility. These abnormal traits were associated with increased MTA levels, altered PA profiles and reduced NA content. Exogenous feeding of PAs partially recovered fertility whereas NA supplementation improved fertility and also reversed interveinal chlorosis. The analysis of PA synthase crystal structures containing bound MTA suggests that the corresponding enzyme activities are sensitive to available MTA. Mutant plants that expressed either MTN or human methylthioadenosine phosphorylase (which metabolizes MTA without producing MTR) appeared wild type, proving that the abnormal traits of the mutant are due to MTA accumulation rather than reduced MTR. Based on our results, we propose that the key targets affected by increased MTA content are thermospermine synthase activity and spermidine-dependent post-translational modification of eukaryotic initiation factor 5A.
INTRODUCTION

The Met recycling pathway (or Yang cycle) is present in all types of organisms where its primary role is to recycle sulfur-containing metabolites (Albers, 2009). In most plants, bacteria and protozoa, 5'-methylthioadenosine (MTA) nucleosidase (MTN; EC 3.2.2.16) irreversibly hydrolyses MTA to methylthioribose (MTR) and adenine (Fig. 1); the resulting MTR is phosphorylated to 5'-methylthioribose 1-phosphate (MTR-1P) by MTR kinase (MTK; EC 2.7.1.100). Recently, two novel enzymes that catalyze intermediate steps in generating Met from MTR-1P were identified and characterized: 5'-methylthioribose-1-phosphate isomerase and dehydratase-enolase-phosphate-complex (Pommerrenig et al., 2011).

Several of the enzyme activities involved in Met recycling in eukaryotes such as humans, differ from those in plants. For example, MTA is converted to MTR-1P by methylthioadenosine phosphorylase (MTAP; EC 2.4.2.28). Continuous MTA metabolism is extremely important in mammals as loss of MTAP activity is associated with cancer (Bertino et al., 2011), and accumulation of MTA has been linked to tumor progression (Stevens et al., 2009).

MTA metabolism may be equally important to plants, as it is the by-product of polyamine (PA), nicotianamine (NA) and ethylene biosynthesis (Fig. 1). PAs such as putrescine (Put), spermidine (Spd), spermine (Spm) and thermospermine (Tspm) are cationic organic molecules that are essential for plant development and stress responses. Some of the documented roles of PAs include vascular differentiation, embryogenesis, cell division and responses to abiotic stresses such as salt, osmotic, drought and wounding (Vera-Sirera et al., 2010; Takahashi and Kakehi, 2010). For example, recent evidence suggests roles for both Tspm and H$_2$O$_2$ produced from PA catabolism in the control of xylem differentiation (Muñiz et al., 2009; Tisi et al., 2011). PAs have also been implicated in RNA processing, chromatin remodeling, membrane fluidity and protein activation.
(Baron and Stasolla, 2008, Takahashi and Kakehi, 2010). NA, a product of another MTA generating reaction, is a metal chelator involved in long distance transport of ions. NA ion complexes ultimately support gametogenesis and embryo development (Curie et al., 2009 and Lan et al., 2011). Finally, ethylene is a phytohormone that impacts numerous processes, including seed germination, seedling growth, fruit ripening, flower development and abscission (Yang and Hoffman, 1984).

The Arabidopsis genome has two MTN-encoding genes (At4g38800 and At4g34840) that are annotated as AtMTN1 (MTN1) and AtMTN2 (MTN2), respectively (Rzewuski et al., 2007). Using northern blot analysis Oh et al. (2008) showed that MTN1 is expressed in roots, stems, flowers and cauline and rosette leaves. Public microarray data reveal that MTN2 transcripts are about 10 times less abundant than those of MTN1 in these organs (Winter et al., 2007). When compared on a tissue level MTN1, MTN1 is expressed preferentially in the cortex of roots and xylem of stems. On the other hand, MTN2 transcripts are most abundant in apical-basal cells of embryos, in developing pollen, stigma, ovules, and leaf guard cells (Winter et al., 2007). Recently it has also been shown that both MTNs are abundant in the phloem tissue (Pommerrrenig et al., 2011). Although MTN1 and MTN2 polypeptides share 64% amino acid sequence identity they have distinct substrate specificities and pH optima (Siu et al., 2008). Thus, it was initially inferred that these two enzymes may have distinct roles in plant metabolism: in vitro MTN1 accepts only MTA as a substrate while MTN2 can also accept S-adenosylhomocysteine to a limited extent (Siu et al., 2008). More recent crystallography and protein dynamic analyses revealed that MTN1 binds to S-adenosylhomocysteine but is incapable of hydrolysing it (Siu et al., 2011).

Recently we described the physiology of several single T-DNA insertion mutants in MTN1 and MTN2: mtn1-1 (T-DNA insertion in the third intron), mtn1-2 (T-DNA insertion in the sixth exon), mtn2-1 (T-DNA insertion in the fourth exon) and
mtn2-2 (T-DNA insertion in the fourth exon) (Bürstenbinder et al., 2010). Based on biochemical analyses, we established that MTN1 is responsible for 80% of the MTN activity in crude extracts of four-day-old seedlings and rosette leaves of three-week-old plants grown on media (Bürstenbinder et al., 2010). When grown on soil or germinated on sulfur sufficient-media containing 500μM MgSO₄, seedlings of single mutants were phenotypically indistinguishable from the wild type (WT). However, when the sulfur source is 500 μM MTA, both mtn1-1 and mtn1-2 mutants have impaired seedling and root growth (Bürstenbinder et al., 2010). These MTN-deficient seedlings also have altered PA profiles with increased Put and Spm compared to seedlings grown on media containing 500μM MgSO₄. Interestingly, no significant changes in either NA content or ethylene production were detected in either mtn1-1 or mtn2-1. In an attempt to lower MTN levels further, the two homozygous mutant lines were crossed and a double mutant was isolated. The resulting mtn1-1mtn2-1 plants exhibited a pleiotropic phenotype with developmental abnormalities (Bürstenbinder et al., 2010). The details and physiological basis for these pleiotropic traits, in terms of MTN’s enzyme activity and MTA regulation was unclear.

Here, we report on the effect of MTN deficiency based on a comprehensive examination of the development and physiology of the homozygous mtn1-1mtn2-1 double mutant. In addition to measuring its residual MTN enzyme activity and profiling relevant metabolites, we analyzed the effect of MTA accumulation based on predicted structures of Spd and Tsp synthases co-crystallized with this ligand. The effect of exogenous supplementation of relevant compounds on the mutant phenotype was tested to reveal abnormalities in the cellular metabolism of the MTN-deficient plants. The results of these analyses indicate the critical importance of MTA metabolism in maintaining the biosynthesis of NA and PAs that are essential for normal plant vascular development and reproduction.
RESULTS

MTN activity in mtn1-1mtn2-1 mutants reflects decreased transcript and protein abundance

RT-PCR was performed on cDNA isolated from the buds and rosette leaves of WT and mtn1-1mtn2-1 plants. In both organs, MTN1 transcript levels were higher than MTN2 in the WT background (Fig. 2A). A comparison of transcript abundance in mtn1-1mtn2-1 revealed substantially decreased MTN1 and undetectable levels of MTN2 in both leaves and buds. Immunoblot analysis (Fig. 2B) showed a similar abundance of MTN1 protein in WT leaves and buds but reduced levels in mtn1-1mtn2-1. Interestingly, MTN-deficient buds had higher amounts of MTN1 in their buds than in their leaves. As documented previously by Bürstenbinder et al. (2010), MTN2 protein cannot not be detected in either WT or mtn1-1mtn2-1 tissues by immunoblotting with a MTN2-specific polyclonal antibody (Bürstenbinder et al. 2010).

WT and mutant bud extracts were assayed for MTN enzyme activity to determine whether or not this correlated with MTN protein abundance and phenotype. Floral buds were chosen for the assay based on preliminary studies indicating that this organ had the highest MTN specific activity (Fig.S1). Compared to the 7 - 8% retained MTN activity observed in mtn1-1 seedlings grown on MTA (Bürstenbinder et al., 2010), mtn1-1mtn2-1 buds had 14% residual MTN activity (1.21 ± 0.27 nmol mg⁻¹ protein min⁻¹ in WT; Fig. 2C). Thus our results show that mtn1-1mtn2-1 is a knock-down mutant, with MTN1 providing the residual MTN activity. Attempts to create a mutant completely lacking MTN activity by crossing homozygous knock-out lines, mtn1-2 and mtn2-2, failed due to embryo lethality.

MTN deficiency affects both vegetative and reproductive development

The mtn1-1mtn2-1 mutants developed no obvious defects until the formation of the first true leaves. Subsequently an array of abnormalities developed upon activation of the floral meristem. Here we focus on the changes in vascular development and reproduction.
Vegetative phase phenotypes

Ten days after germination (DAG), the first true leaf of WT seedlings is greater than 1 mm in length (stage 1.02 described in Boyes et al., 2001; Fig. 3A). In comparison to WT, mtn1-1mtn2-1 seedlings did not reach this stage until 12 days after sowing. In addition to their delayed development, the first true leaves of mtn1-1mtn2-1 exhibited interveinal chlorosis (Fig. 3B): a condition where the veins of the leaf remain green while the area between the veins becomes yellow in color. This chlorosis was most notable in emerging young leaves when seedlings were grown on ½ strength Murashige and Skoog (MS; Murashige and Skoog, 1962) medium (as opposed to soil). Upon maturation of the leaves or transfer of the plants to soil, the interveinal chlorosis gradually disappeared.

As the mtn1-1mtn2-1 mutants matured, abnormalities of the rosette leaves became apparent. Clearing and observation of the 6th leaf revealed an increase in venation, particularly adjacent to the leaf margins (Fig. S2A, B). Leaves of mtn1-1mtn2-1 leaves also had thicker mid-veins (Fig. 3C, D). Further examination of embedded leaf sections showed that the thicker veins in mtn1-1mtn2-1 resulted from an increase in xylem, phloem and cambial cells (Fig. 3E, F). Irrespective of the larger leaves, the leaf vasculature of mtn1-1mtn2-1 was similar to that of the Tspm synthase mutant, thick vein (tkv, Clay and Nelson 2005), which is allelic to acaulis5 (acl5; Hanazawa et al., 1997; Kakehi et al., 2008). ACL5 is specifically expressed in xylem vessel elements and both acl5 and tkv have an over-proliferation of xylem vessels in their stems. This increased xylem is proposed to result from reduced polar auxin transport (PAT; Clay and Nelson 2005). Thus based on similarities to tkv and acl5, we assayed auxin transport and followed vascular patterning in the stems of mtn1-1mtn2-1.

Auxin transport was measured in WT and mutant inflorescence stem segments using 14C-indole acetic acid (IAA). The basipetal transport of 14C-IAA in the mtn1-1mtn2-1 mutant was 46% of that detected in WT stems while the acropetal transport in the stem segments was normal. IAA transport in tkv, measured as a
control, was 64% that of WT (Fig.4A) which is consistent with the initial description of this mutant by Clay and Nelson (2005).

We next examined stem cross-sections of the MTN double mutant in more detail. WT stems contain 6-8 symmetrically arranged vascular bundles ~2-3 cm above the base of primary inflorescence (Fig. 4B) whereas corresponding sections of \textit{mtn1-1mtn2-1} stems usually had ten or more vascular bundles. These mutants also have increased number of small cells in all stem tissues (Fig. 4C). Furthermore, the symmetrical arrangement of the vascular bundles in the WT was disrupted in the \textit{mtn1-1mtn2-1} mutant, leading it to have an irregular stem circumference.

Auxin maxima resulting from differences in auxin distribution in the shoot apical meristem determine the position of leaf primordia and the formation of leaf traces (Dengler 2006). The leaf traces arise from vascular bundles or vascular sympodia in the stem (Dengler 2006). Thus we considered that the increased number of vascular bundles in \textit{mtn1-1mtn2-1} mutants might reflect changes in auxin distribution. To examine this we monitored GFP expression from a synthetic auxin-responsive promoter \textit{DR5rev::GFP} (Friml et al., 2003) in this mutant background. The resulting images showed that \textit{mtn1-1mtn2-1} stem cross sections always had a greater number of auxin maxima than WT, corresponding to the mutant’s increased number of vascular bundles (Fig.4D-G).

\textit{Reproductive phase phenotypes}

The \textit{mtn1-1mtn2-1} siliques arrested when they were 2-3 mm (stage 16 of the developmental milestones described by Smyth et al., [1990]) and very few siliques produced viable seeds. To determine the basis for this observed sterility, reciprocal crosses were performed: WT pollen was applied to \textit{mtn1-1mtn2-1} pistils and \textit{mtn1-1mtn2-1} pollen grains were transferred to stigma of the male sterile mutant \textit{apt1-3}. The resulting fertilization was documented by recording the number of crosses producing viable seeds (Table 1). Both \textit{mtn1-1mtn2-1} and
apt1-3 pistils were receptive to WT pollen, and mtn1-1mtn2-1 pollen was able to germinate on WT and apt1-3 pistils resulting in successful seed production in all cases. Thus, both mtn1-1mtn2-1 male and female gametes were capable of producing viable heterozygous progeny although with reduced efficiency relative to WT.

Further examination revealed several factors that contribute to decreased fertility in mtn1-1mtn2-1. Anthers of stage 14 mtn1-1mtn2-1 flowers were indehiscent (Fig. 5A). In order to observe pollen, anthers of mtn1-1mtn2-1 needed to be opened manually (Fig. 5D). These anthers contained abnormally formed pollen grains (Fig. 5E, F). Specifically, most of the mtn1-1mtn2-1 grains were round in shape and lacked the groove present in WT pollen (Fig. 5B, C). To assess the viability of the abnormal pollen grains, anthers of stage 14 flowers were stained with fluorescein diacetate, a stain that is taken up only by viable cells. Almost all of the WT pollen was able to take up the fluorescein diacetate (N = 45), whereas mtn1-1mtn2-1 had more unstained than stained pollen: 37% of MTN-deficient anthers had no viable pollen, 20% had less than five viable pollen grains, almost half (40%) had five to ten and only 3% had an excess of ten viable pollen grains (N= 65).

Next, in vivo pollen tube formation was assessed; pistils were fixed 24 h after pollination and visualized with aniline blue (Fig.S2C to H). Reciprocal crosses performed between mtn1-1mtn2-1 and WT pollen proved the mutant stigma to be receptive to pollen, with 90% of the successfully pollinated pistils containing pollen tubes. Attempts to manually pollinate apt1-3 or mtn1-1mtn2-1 with mtn1-1mtn2-1 pollen were not successful as no pollen tubes were observed for either cross 24 h later. Collection of stage 14 mtn1-1mtn2-1 flowers and staining them with aniline blue showed that 22% had self-pollinated and of those pollinated, 48% had pollen tubes. Thus, mtn1-1mtn2-1 pollen are capable of forming pollen tubes in vivo but apparently need longer than 24 h to extend.
Carpel and ovule development was also aberrant in stage 14 \textit{mtn1-1mtn2-1} flowers; carpels had wide or duplicated stigmas, while ovules of the pistils had un-extended integuments when compared to WT (Fig. 5G, H). About 10\% of \textit{mtn1-1mtn2-1} flowers examined contained one to three fully developed ovules that appeared to WT.

The \textit{mtn1-1mtn2-1} phenotype complemented by MTN1 over-expression

In the Yang cycle, MTA is converted to MTR-1P via two steps: first, MTA is hydrolysed by MTN to form MTR and adenine, and the resulting MTR is subsequently metabolized by MTK. Unlike \textit{mtn1-1mtn2-1} seedlings, MTK-deficient seedlings are not impaired in their growth and development when provided with sulfur-sufficient conditions (Sauter et al., 2004) suggesting MTN is the more crucial of the two enzyme activities. In order to verify that the complex phenotype of \textit{mtn1-1mtn2-1} was solely due to MTN deficiency, the MTN1 gene was re-introduced to the double mutant. The pleiotropic phenotype of \textit{mtn1-1mtn2-1} was fully complemented by a 2.4 Kb genomic fragment that contained the full length coding sequence of \textit{MTN1} along with 356bp of the upstream region (Fig. 6A). These results were further confirmed by the ectopic expression of \textit{MTN1} cDNA under the control of the constitutive polyubiquitin10 (UBQ10) promoter in the \textit{mtn1-1mtn2-1} background. We therefore concluded that the pleiotropic phenotype of \textit{mtn1-1mtn2-1} was the result of MTN-deficiency, and that replenishing MTN1 alone was sufficient to compensate for the lack of MTN2.

The basis of \textit{mtn1-1mtn2-1} phenotype is accumulation of MTA, not loss of MTR

Although the observed vascular and reproductive defects of \textit{mtn1-1mtn2-1} were restored by MTN transgene expression, we reasoned that these abnormal traits could be due to either a lack of MTA metabolism or decreased MTR. Targeted profiling of Met metabolites in inflorescences and rosette leaves, revealed statistically significant increases of MTA in \textit{mtn1-1mtn2-1} (p< 0.05). MTA was
elevated two-fold in rosette leaves (Fig. 6B) and five-fold in the mutant inflorescences, leading us to conclude that MTN deficiency leads to increased MTA content. However, based on these data, it could not be excluded that the lack of MTR might also contribute to the complex phenotype of the mutant. To test this, we introduced a cDNA encoding human MTAP activity (UBQ10::hMTAP) into the mutant. This enzyme phosphorylates MTA directly into MTR-1P without producing MTR. The phenotype of the double mutant was fully complemented by constitutive expression of hMTAP (Fig. 6C).

Modeling of Spd synthase and Tspm synthase complexes with MTA

MTA is a potent inhibitor of both human Spm synthase (approximate $K_i = 0.3 \mu M$) and Spd synthase ($K_i = 2-10 \mu M$, Wu et al., 2007). Analysis of crystal structures containing bound ligands of these enzymes with MTA explains the difference in affinity: the hydrophobic binding site of Spm synthase is larger allowing it to have more extensive interactions with the adenine residue of MTA than does Spd synthase. The conserved structural folds of the human and plant PA enzymes allowed us to model MTA binding to these targets. 3-D models were developed for Arabidopsis Spd synthase1 and Tspm synthase and then their MTA complexes were visualized as for human Spd synthase1 and Spm synthase (Wu et al., 2008). Analysis of these 3-D models revealed that the MTA-binding pockets of Spd synthase1 and Tspm synthase are very similar (Fig. 7A,B): the CH$_3$S moiety of MTA makes van der Waals contact with L90, L92 and V156 of Spd synthase1, or L76, I78 and V142 of Tspm synthase. The ribose ring of MTA is predicted to establish hydrogen bonds with the side chains of Q76 and E151 of Spd synthase 1 or the side chains of Q62 and E137 of Tspm synthase. N-6 of MTA adenine forms hydrogen bonds with the side chain of D182 of Spd synthase1 or D168 of Tspm synthase. The adenine ring of MTA is placed between I137 on one side and L187 and L199 on the other side in the Tspm synthase-MTA complex. In the Spd synthase1-MTA complex, the adenine ring of MTA is sandwiched between I151 and L212. The residues I151 and L212 of Spd synthase1 and the residues I137 and L199 of Tspm synthase are structurally
conserved, while the L187 residue of Tspm synthase is substituted by serine (S202) in Spd synthase1 (Hanazawa et al., 2000). Therefore, the hydrophobic surfaces involved in interaction with the adenine ring of MTA are predicted to be very different. The hydrophobic surface (in yellow) made by I152 in Spd synthase1 is similar to the corresponding one in Tspm synthase (I138) (Fig. 7C, D). On the other side of the adenine ring of MTA, the hydrophobic surface (in magenta) of Spd synthase1 made by L212 is shifted and partially covers the adenine ring of MTA, whereas in the Tspm synthase, the hydrophobic surfaces made by L187 and L199 are large and positioned to cover the adenine ring completely. Although MTA binds to all three PA synthases, these modeling experiments predict that similar to human Spm synthase, Tspm synthase may be more sensitive to inhibition by MTA than Spd synthase1 due to extensive hydrophobic interactions between Tspm synthase and the adenine moiety of MTA.

*mtn1-1mtn2-1* plants exhibit altered PA profiles
We next investigated whether increased MTA abundance in the mutant was associated with changes in free PA content. To do this, we monitored both leaves and inflorescences for Put, Spd, and Spm using HPLC analysis. Unfortunately Tspm abundance was so low that it was not readily detected in WT samples (Naka et al., 2010) and as a result, could not be measured. For those PAs that could be measured, differences were observed in both *mtn1-1mtn2-1* leaves and inflorescences: Put levels increased in *mtn1-1mtn2-1* whereas Spd and Spm levels decreased (Fig. 8A, B). Of these, a statistically significant difference was only observed in the Put content of inflorescences.

We reasoned that if these changes were physiologically relevant, reactions dependent on PAs would be affected. Spd is a cofactor for the post-translational activation of the eukaryotic translation initiation factor eIF5A. The butylamine moiety that is provided solely by Spd is transferred to eIF5A in a two-step reaction causing it to become hypusinated on a specific lysine residue (Pegg and
Casero, 2011). Thus, we tested the degree of hypusination of eIF5A in *mtn1-1mtn2-1* leaf extracts by 2-D gel electrophoresis followed by immunoblotting with eIF5A-specific antibodies as an indicator of intracellular Spd availability. The amount of hypusinated eIF5A was two-fold lower in *mtn1-1mtn2-1* indicating a clear reduction in hypusination of eIF5A in comparison to WT leaf extracts (Fig. 8C, D).

**MTN-deficiency results in altered NA levels**

Upon recognizing that the free PA profiles changed in response to increased MTA abundance, we next analyzed NA levels of *mtn1-1mtn2-1*. We determined that NA was significantly reduced in inflorescences and mature rosette leaves of *mtn1-1mtn2-1* compared to WT (p < 0.05; Fig. 9A). In WT plants, the highest NA content (268 ± 58 pmol mg FW⁻¹) was detected in inflorescences with leaves having 60% less NA (106 ± 50 pmol mg FW⁻¹) whereas the NA content of *mtn1-1mtn2-1* inflorescences was 34% lower than WT and undetectable in rosette leaves.

Since NA is known to play a key role in ion homeostasis and long distance transport, we investigated whether the ion content (Cu, Fe, Zn, and Mn) of *mtn1-1mtn2-1* was different from WT, using inductively coupled plasma atomic emission spectroscopy (Gadapati and Macfie, 2006). In the mutant inflorescences, contents of Cu, Fe and Zn were significantly lower, with Fe and Zn showing the greatest reduction compared to WT (p< 0.05; Fig.9B). The ion profiles of mutant and WT rosette leaves were similar with reduced Zn and Cu abundance, except the Mn content in the mutant was significantly higher than in the WT (p< 0.05; Fig.9C). Interestingly, *mtn1-1mtn2-1* had significantly decreased Fe levels in the inflorescences compared to WT while these levels were not significantly different in rosette leaves (p< 0.05). Confirming these results, interveinal chlorosis was absent in the reproductive leaves of mature *mtn1-1mtn2-1* plants.

**Exogenous feeding of PA and NA partially restored fertility of mtn1-1mtn2-1**
Chemical rescue of fertility

Since both free PA and NA levels were affected in the mtn1-1mtn2-1 mutant, we postulated that MTA-producing reactions were being product inhibited. We then hypothesized that the exogenous application of target compounds would reverse abnormal phenotypes. To test this, we chemically complemented mtn1-1mtn2-1 mutants with exogenous application of Spd, Spm, Tspm or NA (NA was extracted from NA over-producing transgenic Arabidopsis plants [Pianelli et al., 2005]).

To treat MTN-deficient plants, F2 seeds of MTN1-1/mtn1-1mtn2-1/mtn2-1 plants were grown on media supplemented with Spm, Spd, Tspm or NA. After 2-3 weeks on media, seedlings were transplanted to soil and genotyped via PCR. With the exception of Tspm, drops of the corresponding compound were then applied daily to shoot apices of mtn1-1mtn2-1 plants until the plants were eight-weeks-old. Examination of the treated plants showed that mtn1-1mtn2-1 fertility was partially recovered using any of three compounds (Spm, Spd or NA): partial recovery of fertility was indicated by the presence of extended siliques (Fig. 10A) with fertile seed (Fig. 10B to E) on individual branches. Surprisingly partial fertility was also recovered in a similar manner when the seeds were germinated on the indicated compounds for 14 or more days and transferred to soil without being provided with further supplement (Fig. 10F).

Generally, siliques with unfertilized ovules fail to extend beyond 3.5 mm, while siliques containing 60 seeds reach a maximal length of 15 mm (Meinke, 1994). Thus the fertility of the plants without further supplement was scored by categorizing the siliques based on their length in to three groups: (1) 5-7 mm, (2) 8-10 mm and (3) more than 10 mm (Fig. 10A). Most of the siliques produced were in the first two categories while only Spd and Tspm gave rise to siliques that were >10 mm long. Of the four treatments, the most effective was 100 μM Spd (78 ± 5 siliques / plant) and the least effective was 100 μM Spm (Fig. 10F). Both NA and Tspm treatments produced 25-35 siliques/ plant.
The number of seeds per silique in each group of the Spd experiment was further determined: 5-7cm group had 1 ± 0.4 seeds per silique, while the 8-10 mm and >10 mm group had 6 ± 1, and 14 ± 4 seeds per silique, respectively. Some seeds in the 5-7 mm and 8-10 mm category showed abnormal shape and color (Fig. 10B) when compared to WT (Fig. 10C). To determine the viability of these abnormal seeds approximately 100 seeds from 6 siliques were germinated on media and scored: 8-10 mm and >10 mm siliques had 33 ± 10% and 66 ± 20% germination, respectively (Fig. 10G).

**Chemical rescue of interveinal chlorosis by NA**

In addition to testing for recovered fertility, rescue of interveinal chlorosis with the application of exogenous PAs (Put, Spd, Spm or Tspm) or NA was also investigated. Of the compounds tested, only media supplemented with NA completely eliminated the interveinal chlorosis normally observed at 10DAG in \textit{mtn1-1mtn2-1} seedlings. Mutant seedlings that exhibited interveinal chlorosis on \( \frac{1}{2} \text{MS} \) developed green leaves two days after being transferred to \( \frac{1}{2} \text{MS} \) media supplemented with NA. Moreover, hydroponically grown \textit{mtn1-1mtn2-1} supplemented with 20 \( \mu \text{M} \) NA lacked interveinal chlorosis (\( N=8 \), Fig. S3).

**DISCUSSION**

Our detailed characterization of the \textit{mtn1-1mtn2-1} mutant has established a correlation between MTN deficiency, abnormal vasculature and low reproductive efficiency. A similar phenotype has been recovered in \textit{mtn1-1} plants expressing an artificial microRNA specific for \textit{MTN2}, further substantiating the link between MTN deficiency and these traits (data not shown). The first distinct phenotype of \textit{mtn1-1mtn2-1} coincides with the development of true leaves. As recently reported, in Arabidopsis and \textit{Plantago major} the Yang cycle (i.e. MTA recycling) mainly occurs in leaf veins (Pommerrenig et al., 2011). Thus, it is likely that the flux of MTA synthesis first increases substantially in leaves as vein development proceeds.
Molecular characterization of the \textit{mtn1-1mtn2-1} mutant showed a reduction in MTN transcript levels consistent with the presence of a T-DNA insertion within the transcribed region of each gene. Protein abundance and enzyme activity reflected transcript levels, with the \textit{mtn1-1mtn2-1} mutant having \~14\% residual MTN activity, indicating its substantially reduced capacity for MTA hydrolysis. The MTN assay results were consistent with our previous analysis of \textit{mtn1-1} and \textit{mtn1-2} single mutant seedlings grown on sulfur-deficient medium supplemented with MTA (Bürstenbinder et al., 2010). These seedlings have less than 20\% of the MTN activity of WT, and display severe growth retardation. Interestingly, they also have increased MTN activity when grown in the presence of MTA. Since MTN transcript levels did not change substantially under these conditions, the activation of MTN must occur at the protein level (Bürstenbinder et al., 2010). While the molecular basis of this activation remains unknown, we assume that the increased MTA content in the double mutant activates the residual MTN enzyme, leading to cellular variations in enzyme activity.

**MTA accumulation is the basis for \textit{mtn1-1mtn2-1} phenotype**

While our data pointed to the lack of MTA hydrolysis as being the cause of the abnormalities in \textit{mtn1-1mtn2-1} plants the contribution of reduced MTR content was also considered. This concept arose from comparing the completely normal phenotype of MTR kinase mutants (Sauter et al., 2004) with the complex phenotype of MTN-deficient plants. Both mutants are unable to salvage Met, yet MTR kinase mutants have MTR and are normal whereas the MTN-deficient plants lack MTR and are abnormal. Thus we tested the involvement of MTR deficiency in the phenotype of \textit{mtn1-1mtn2-1} by introducing constitutive expression of \textit{MTN1} and h\textit{MTAP} transgenes into \textit{mtn1-1mtn2-1} plants. MTAP metabolizes MTA to MTR-1P without producing MTR. Complementation of the mutant phenotype by either transgene confirms that the abnormal development of MTN-deficient plants is due to their increased MTA content rather than a lack of MTR or Met salvage itself.
MTA accumulates in cells of *mtn1-1mtn2-1* that are actively synthesizing PA or NA; this MTA accumulation is obviously both tissue-specific and developmentally dependent. When *mtn1-1* seedlings are grown on sulfur-deficient media supplemented with MTA, presumably all their cells are exposed to MTA resulting in retarded shoot and root growth (Bürstenbinder et al., 2010). We propose that these phenotypes are also due to MTA inhibition of NA and PA synthases based on the similarity of their metabolite profiles with those of *mtn1-1mtn2-1* plants.

The MTA abundance in the leaves of *mtn1-1mtn2-1* plants was two-fold higher than in WT: 1.7 ± 0.2 pmol mgFW⁻¹ as compared to 0.8 ± 0.1 pmol mg FW⁻¹ in WT. This change was in the same range as that of *mtn1-1* grown on MTA: a two-fold increase in four-day-old seedlings and 1.5-fold increase in three-week-old plants (Bürstenbinder et al., 2010). Interestingly, the MTA accumulation in the double mutant was greater in flowers than in leaves. The MTN level of the *mtn1-1mtn2-1* inflorescences was 24 ± 2 pmol mg FW⁻¹ compared to 4.6 ± 0.2 pmol mg FW⁻¹ in WT. Thus, in *mtn1-1mtn2-1* plants, the degree of MTA accumulation is tissue specific. In accordance with our MTA abundance values, Tassoni et al. (2000) and Naka et al. (2010) found that all PAs (Put, Spd, Spm and Tspm) are higher in WT flowers compared to mature leaves. Thus flowers must have an increased requirement for MTN activity to hydrolyse the MTA produced during PA biosynthesis.

**MTA binds NA synthase, SPD synthase, SPM synthase and TSPM synthase**

MTA is known to inhibit PA synthetic enzyme activities, while increasing SAMDC activity (Albers, 2009). For example, mammalian Spm synthase and Spd synthase activities are MTA sensitive (Pegg, 1981; Wu et al., 2008). Our *in silico* modelling leads us to propose that MTA inhibits Arabidopsis Spd synthase and Tspm synthase with the latter being the most sensitive. This inhibition is likely quite dynamic since the stimulation of SAMDC by MTA theoretically increases flux to PA synthesis and further enhances MTA production.
Barley NA synthase is product inhibited by MTA ($K_i = 5 \mu M$; Herbik, 1997). We suspect that the Arabidopsis enzyme is similarly affected given its amino acid sequence identity (46%) and similar domains (Herbik et al., 1999). Moreover, the mutant plants have chlorotic leaves that are reversed by NA supplementation. Thus, we conclude that MTA inhibits PA and NA synthases in a spatially and temporally dependent manner reflecting the flux through the Yang cycle and the cellular abundance of the target enzyme activities.

Molecular basis of the altered vascular development of *mtn1-1mtn2-1*

The increased venation of *mtn1-1mtn2-1* rosette leaves likely contributes to the variation in their free PA content as PA biosynthesis predominantly occurs in leaf vasculature (Pommerrenig et al., 2011). Increased vein thickness of the MTN mutant rosette leaves is similar to that reported for *tkv* (Clay and Nelson 2005). Since our modeling predicted that Tspm synthase activity is inhibited by MTA, and Tspm is required for normal xylem differentiation (Muñiz et al., 2008), it was of interest to examine Tspm content. Unfortunately, Tspm quantification is a challenge due to its low abundance; Tspm was not detected in our system. Despite this observation, our results lead us to believe that *mtn1-1mtn2-1* plants have reduced Tspm.

Aside from Tspm effects, PA catabolism may also contribute to the changes in vascular development in *mtn1-1mtn2-1*. The altered PA content of the mutant (e.g. increased Put) may lead to increased PA oxidase activity and $H_2O_2$, a documented signal for increased vascular tissue differentiation (Tisi et al., 2011). However, we did not detect increased $H_2O_2$ in 14-day-old *mtn1-1mtn2-1* seedlings using a histochemical stain (Figure S4). Similarly, semi-quantitative RT-PCR of mature leaves of *mtn1-1mtn2-1* did not show significant changes in the transcript levels of three representative PA oxidases (PAO1, PAO2, PAO5) compared to WT (Figure S4). However, more detailed analysis is necessary to
determine if PAO activities change in the mature mutant plants, or as a result of Spd treatments.

The vascular abnormalities of mtn1-1mtn2-1 appear to be a direct effect of changes in PAT. We base this conclusion on the similarities between the mtn1-1mtn2-1 vasculature and the effects of reducing PAT by naphthylphthalamic acid treatment (Mattsson et al., 1999); both sets of plants have thick veins in their rosette leaves, and an increased number of vascular bundles in their stems. Ibañes et al. (2009) created a computational model for vascular bundle development based on DR5::GUS expression of naphthylphthalamic acid-treated plants and anatomical analysis of brassinosteroid (BR) signalling mutants. Their model predicts PAT-related auxin maxima determine the spacing between vascular bundles while BR controls bundle number. If this is the case, then our results suggest that BR signalling changes may also contribute to the vascular phenotype of mtn1-1mtn2-1.

The question arises then, why do acl5 and tkv that also have reduced PAT (Clay and Nelson, 2005; Vera-Sirera et al., 2010) lack the increase in vascular bundles characteristic of mtn1-1mtn2-1 plants? We propose that the differing phenotypes of these Tspm-deficient plants reflect the degree to which PAT is inhibited. mtn1-1mtn2-1 plants have less residual auxin transport as compared with tkv (46% versus 64%, respectively). The greater reduction in PAT in mtn1-1mtn2-1 plants may lead to an increase in their BR content or sensitivity, causing them to have an increased number of bundles. The modest PAT changes in tkv (and acl5) may not be sufficient to trigger the crosstalk with BR. This reasoning is consistent with the proposal that the PAT changes in acl5 are secondary to its vascular defects (Vera-Sirera et al., 2010). Direct investigation of BR content and signaling in Tspm-deficient mutants is needed to clarify BR involvement in these phenotypes.

**NA deficiency of mtn1-1mtn2-1 causes altered ion homeostasis leading to interveinal chlorosis and reproductive abnormalities**
In addition to free PAs, NA levels were significantly affected in mtn1-1mtn2-1 tissues: NA was undetectable in double mutant rosette leaves, while inflorescences exhibited a 3.7-fold reduction relative to WT. This NA deficiency is similar to that of the chloronerva tomato mutant (Bohme and Scholz, 1960), quadruple NA synthase Arabidopsis mutants (nas4x-2 and nas4x-1; Klatte et al., 2009), and transgenic naat tobacco lines over-expressing NA aminotransferase (Takahashi et al., 2003). All these plants have interveinal chlorosis and reduced fertility along with reduced NA content. In all cases, this deficiency is associated with altered profiles of Fe, Zn, Mn and Cu. Similar to the previously published reports describing mutants with decreased NA, mtn1-1mtn2-1 plants had lower levels of all these ions in both inflorescences and rosette leaves, with the exception of Mn which increased in leaves.

**Restoration of seed set in mtn1-1 mtn2-1**

The reduced presence of double mutants in the segregating progeny of MTN1/mtn1-1mtn2-1mtn2-1 plants is indicative of a reproduction problem. The double mutant was commonly recovered from these segregating populations at a frequency of 1/7th to 1/10th, in contrast to the expected 1/4. At least two factors may contribute to the reduction of the double mutant in these F2 populations. 1) mtn1-1mtn2-1 embryos may have insufficient Spd to support their development. This hypothesis is based on the observation that the Spd content of mtn1-1mtn2-1 inflorescences is lower than in the WT. If the MTN-deficient embryos are similarly affected this would cause them to delay or arrest their development as reported for Spds synthase double mutants spds1-1spds2-1 that lack Spd (Imai et al., 2004). The biochemical basis for the embryo lethality of Spd mutants is not known but may relate to the Spd-dependent modification of eIF5a; deoxyhypusine synthase mutants that lack the rate-limiting enzyme activity required for eIF5a hypusination arrest during embryo sac development (Pagnussat et al., 2005). 2) Alternatively the abnormal growth of mtn1-mtn2-1 pollen tubes may contribute to the reduced recovery of the homozygous double mutant seed. Although we do not know the basis of the pollen tube defect in
mtn1-1mtn2-1 this may be associated with their altered polyamine metabolism since mutants with reduced PAO3 activity also have abnormal pollen tube growth (Wu et al., 2010). Exogenous supply of 20 µM NA, 100 µM Spd, 100 µM Spm or 50 µM Tspm improved the fertility of mtn1-1mtn2-1 to a limited extent. When each of these chemicals is supplied exogenously, the plants presumably have a reduced need to synthesize these chemicals. As a result the amount of MTA generated in vivo by these biosynthetic pathways is reduced and thus the product inhibition of PA or NA synthases is alleviated, thereby improving fertility. Among the compounds tested, 100 µM Spd was the most effective; Spm was the least and Tspm and NA had intermediate effects. Application of 100 µM Put, 10 or 100 µM of IAA, or 5 µM of ACC did not produce observable differences. The simplest explanation of these results is that exogenous Spd supplies a limiting compound that is required for fertility or proper embryo development. For example the Spd supplement may provide an increased precursor pool for Tspm synthesis. The Tspm may induce more normal xylem differentiation and restore sufficient transport of compounds essential for seed development including phytohormones and metal cofactors. Interestingly, a recent report by Tisi et al. (2011) shows that increased xylem differentiation and secondary wall deposition is induced in maize roots by exogenous Spd feeding. This is apparently induced by H2O2 arising from PA catabolism. Similar H2O2 signaling may contribute to the Spd effect on mtn1-1mtn2-1 plants reported here. It is also possible that the Spd acts by another route aside from simply being the precursor for Tspm or affecting PA catabolism, such as via eIF5A modification. A recent study of Cucurbita maxima (pumpkin) phloem has shown that eIF5A modification occurs in phloem sap (Ma et al., 2010) consistent with the localization of MTN enzymes in companion cells (Pommerrenig et al., 2011). A comprehensive developmental and metabolite analysis of Spd-treated mtn1-1mtn2-1 plants will be required to elucidate the molecular mechanism underlying the effects of both MTN deficiency and Spd feeding. A deeper understanding of this process will provide important insight of the long recognized link between PA and plant development.
CONCLUSION
Although MTN-deficient plants have a complex phenotype, each abnormal trait can be traced back to an effect of MTA accumulation. Given the different enzyme activities inhibited by MTA and their broad range of contributions to plant metabolism, increases in MTA content have a broad impact on both a cellular and developmental scale.

METHODS
Plant material and growth conditions
Seeds for mtn1-1 (SALK_085385) and mtn2-1 (SALK_071127) single T-DNA insertion lines used to generate mtn1-1mtn2-1 were obtained from the Arabidopsis Biological Resource Center. The male sterile mutant, apt1-3 (Moffatt and Somerville, 1998), was used for reciprocal crosses. All Arabidopsis seeds were surface sterilized with chlorine gas before sowing on plates with half strength MS medium solidified with 0.8% (w/v) agarose (Murashige and Skoog, 1962); these plates were incubated in the dark at 4°C for 48 h for seed stratification. On the third day after sowing, plates were transferred to a tissue culture chamber (TC7, Conviron, Winnipeg, Canada) under continuous light: 24h at 21°C with 80µmol m⁻²s⁻¹ photosynthetically active radiation for 10 days. Seedlings were then transplanted to individual pots (25 cm³) containing a 1:1 soil mixture of Sunshine LC1 mix and Sunshine LG3 germination mix (SunGro Horticultural Inc, Washington, USA) and maintained in a growth chamber (Conviron, Winnipeg, Canada) under long day conditions: 16h at 22°C with 150 µmol m⁻²s⁻¹ photosynthetically active radiation. The plants were watered every two days and fertilized with 20:20:20 (N: P: K) fertilizer mix (Plant products Co Ltd, Brampton, Canada) once a week until maturity. Hydroponic experiments were set up as described in Pierre et al. (2003) and maintained in the same growth chambers.
To document the first obvious developmental defect of \textit{mtn1-1mtn2-1} compared to WT, plate-based-growth and soil-based-growth assays were conducted as detailed in Boyes et al. (2001). In the plate-based assay the time taken to develop the first true leaves \textgreater 1mm along with daily lengths of roots were measured over duration of 11 days. The reproductive defects were evaluated in the stage 14 flowers as described by Smyth et al. (1990).

Buds and rosette leaf samples were obtained from WT and \textit{mtn1-1mtn2-1} plants two weeks after bolting unless otherwise stated. When sampled, WT plants were five-weeks-old whereas \textit{mtn1-1mtn2-1} plants were approximately seven-weeks-old in order to similar developmental stages to be evaluated. Samples were collected before noon for all the experiments.

**Microscopy**

\textit{Bright- light microscopy}

Developmentally similar (i.e., having the same number of first order veins arising from the mid vein) WT and \textit{mtn1-1mtn2-1} rosette leaves and stage 14 pistils (Smyth et al., 1990) were first incubated in clearing solution (3:1, glacial acetic acid:ethanol [EtOH]) for 2 h. Upon clearing, the tissue was rehydrated by moving it from 70\% EtOH to water in a gradient series of 15 min intervals each, and softened by incubating overnight in 8 M NaOH. For visualization, leaf samples were then stained with 0.1\% (w/v) Toluidine Blue O (TBO; pH 6.8) for 15s and observed with a Zeiss Axiovert 200M microscope (Carl Zeiss Inc, Toronto, Canada). The ovules of the cleared pistils were observed with Differential Interference Contrast (DIC) optics of a Zeiss Axiovert 200M microscope.

The same stage leaves of both genotypes were fixed in solution (50 mM sodium cacodylate, 2\% paraformaldehyde [w/v, pH 7], 0.1\% glutaraldehyde) for 1 h under vacuum at room temperature (RT). The samples were then transferred to fresh fixative and incubated overnight at 4°C. After the appropriate amount of fixation, the samples were dehydrated in a series of EtOH under vacuum at RT,
going from 10 to 70% EtOH in 15% intervals for 1 h each. The samples were left in 70% EtOH and 0.2% eosin overnight at 4°C. Once fully dehydrated, the tissue was slowly infiltrated with LR White plastic resin (hard grade, Canemco, Mississauga, Canada), in a series of EtOH to resin volumes: 3:1, 1:1, 1:3 for 2 h each, then 100% LR white for 1 h, then in fresh LR overnight. Once the samples were fully infiltrated with resin, the LR white was refreshed and the samples were polymerized in a 60-65°C oven for 2-24 h. Once the resin had hardened, tissue was sectioned at 1 µm thickness using an ultramicrotome (UltracutE, Reichert, New York, USA) and stained with TBO for observation with Zeiss Axiovert 200M microscope.

**Scanning Electron Microscopy**

Anthers of stage 14 flowers from WT and *mtn1-1mtn2-1* were placed in fixative (4% [w/v] paraformaldehyde [pH 7], 2% [w/v] glutaraldehyde dissolved in potassium buffered saline for 1 h under vacuum at RT. Upon replacing the fixative solution, the samples were left to incubate overnight at 4°C. The tissue was then dehydrated in a 10% ethanol gradient (50% - 95%) at 30 min intervals under vacuum. Upon reaching 95% EtOH concentration, samples were left overnight at 4°C. The samples were then moved to HPLC-grade acetone for 2 h at RT. The samples were dried using a mass critical point dryer, mounted on 2” stubs and sputter coated with gold particles. The tissue was visualized using a Hitachi S570 scanning electron microscope.

**Laser Scanning Confocal Microscopy**

The expression patterns of *DR5rev::GFP* stem sections of both WT and *mtn1-1mtn2-1* backgrounds were observed using a Carl Zeiss LSM 510 confocal microscope (Carl Zeiss Inc. Jena, Germany). The GFP signals were excited with an argon ion laser at 488 nm, and emissions were captured with a 505/530 nm band pass filter. Chlorophyll autofluorescence was also excited at 488 nm and was captured with 650 nm long pass filter.

**Chemical complementation experiments**
Seeds were placed on ½ MS media supplemented with one of the following chemicals: 20 μM of NA (crude KI extract, see below), 20 μM of WT extract, 100 μM Spd, 100 μM Spm, and 50 μM Tspm. After two days of cold incubation these plates were transferred to continuous light for 21 days. The seedlings were then transplanted to soil or hydroponic media and grown under long day conditions. In the case of soil-grown plants, chemicals were supplied daily as drops (~20μl) in ½ MS to each vegetative and reproductive meristems over a period of 3-4 weeks. However, Tspm was not applied as daily drops due to its limited availability.

**Extraction of NA from K1 plants**

NA-overproducing Arabidopsis transgenic lines (K1, Pianelli et al., 2005) were grown until the full rosette stage. NA was extracted from the rosette leaves ground in 80°C dH₂O; corresponding extracts from WT leaves were used as controls. The K1 extract contained 50 pmol mgFW⁻¹ NA compared to WT and neither KI nor WT extracts contained detectable PAs, indicating that the K1 is a source of NA but not PAs.

**Complementation of mtn1-1mtn2-1**

For complementation, pMTN1::MTN1, UBQ10::MTN1 coding sequences (CDS) and UBQ10::hMTAP CDS were introduced to mtn1-1mtn2-1 mutants. The UBQ10 (AT4G05320) promoter (693bp) was amplified using UBQ10pF and UBQp blunt R primers (Table S1) and cloned in to pSAT5.nosP.RNAi.nosT (EUO49865) with Agel and HinfI to generate pSAT5.UB10p.nosT. This vector was then used as a recipient for MTN1 and hMTAP CDSs. Binary vectors pGreenII 0229 (www.pgreen.ac.uk) and pPZP-RCS2-BAR (CD3-1057, Arabidopsis Biological Resource Centre) were used for Agrobacterium–mediated transformation.

Genomic MTN1 was amplified using the following primers: gMTN1-F and gMTN1-R (Table S1). The 1.9 kb genomic MTN1 fragment with 162 bp of promoter was then digested with Spe1 and BamHI and cloned directly into binary
vector pGreenII 0229. The MTN1 cDNA was amplified with the following primers: MTN1CDS-F and MTN1CDS-R (Table S1). The resulting 0.8kb fragment was digested with XmaI and BamHI and cloned first into pSAT5.UB10p.nosT. After sequencing, the UB10p::CDS cassette was transferred top PZP-RCS2-BAR. Similarly, the 0.8 kb hMTAP cDNA fragment was amplified from hMTAP cDNA with hMTAP-F and hMTAP-R primers (Table S1), cloned into the SmaI site of pSAT5-UBQ10p-nosT and the resulting UBQ10::hMTAP cassette inserted into pPZP-RCS2-BAR.

MTN1 constructs were introduced into Agrobacterium strain GV3101Soup, while UBQ10::MTN1CDS and UBQ10::hMTAP were placed in Agrobacterium strain GV3101. Homozygous mtn1-1 plants were transformed with their respective Agrobacterium strains using the floral dipping method (Bechtold et al, 1993). Transgenic T1 individuals were selected for by spraying 10 day-old soil grown seedlings with 40 mg/ L Basta (glufosinate ammonium, Wilson laboratories inc. Dundas, Canada). The expression of MTN1 and hMTAP in the surviving T1 plants was confirmed by immunoblot, and successful transformants were crossed with mtn1-1/mtn1-1MTN2-1/mtn2-1 plants. The resulting F1 progeny was germinated on media containing Basta and genotyped by PCR to identify mtn1-1mtn2-1 F2 individuals. F2 seeds of three lines were selected and screened on Basta supplemented MS media and genotyped to identify mtn1-1mtn2-1 seedlings.

RT-PCR

Total RNA was extracted from leaves and buds of WT and mtn1-1mtn2-1 plants using Tri Pure Isolation Reagent (Roche). Two micrograms of total RNA was used for first strand synthesis with SuperScript™ reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. PCR reactions were performed as described in Bürstenbinder et al., 2007 using AtMTN1-F3, AtMTN1-R3, AtMTN2-F1, AtMTN2-R1 and Actin 8F and Actin 8R primers (Table S1) to
determine the transcript abundance of *MTN1* (851bp), *MTN2* (306bp) and actin (700bp).
**MTN enzyme assay**
Bud protein extracts were prepared from developmentally matched flowering plants in 50mM potassium phosphate buffer [pH 7, Bürstenbinder et al., 2010]. Twenty micrograms of soluble protein from buds was used to determine MTN specific activity using a xanthine oxidase-coupled enzyme assay (Lee et al., 2005). Absorbance was measured every 10 min over 1 h with a DU530 spectrophotometer (Beckman Coulter, Krefeld, Germany) at 470 nm modified from the protocol outlined in Bürstenbinder et al. (2010).

**Polar auxin transport assay**
Polar auxin transport in primary inflorescence stem segments of WT, mtn1-1mtn2-1, and tkv was measured using a modification of procedure provided by Okada et al. (1991) method as described in Clay and Nelson (2005). The inflorescence stem segments were immersed in 14C labeled IAA (Sigma) in either an acropetal or basipetal orientation. After 19h of incubation, the uppermost 5mm of the stem segments were removed and incubated overnight in liquid scintillation cocktail (Cytoscint, MP Biomedicals, and Toronto, Canada). Released radioactivity was counted using a scintillation counter (Model LS 1701, Beckman, Munich, Germany).

**Immunoblot analysis**
Ten micrograms of total soluble protein was extracted as described above, separated on a 12.5% SDS polyacrylamide gel (Laemmli, 1970) and transferred onto a polyvinyldenedifluoride membrane using a semi-dry electro-blotting system (Bio-Rad laboratories, Mississauga, Canada). The membrane blot was cut near the 34 kDa marker so the bottom portion could be incubated for 2 h with anti-MTN1 (1: 2500) antibody, while the top portion was incubated for the same time with monoclonal antibody for actin (MP Biomedicals, Toronto, Canada; 1: 3000). Bound antibodies were detected following incubation with horseradish
peroxidase-conjugated secondary antibodies, using the ECL Plus Western Blotting Detection System (GE Healthcare, Mississauga, Canada) and Amersham Hyperfilm™ ECL.

2-D electrophoresis
Protein was extracted using phenol followed by ammonium acetate-methanol precipitation as described by Zheng et al., (2007). Protein was quantified using RC/DC protein assay kit (Bio-Rad laboratories, Mississauga, Canada). These proteins were then separated based on isoelectric focusing on a Multiphore II system (GE Healthcare, Mississauga, Canada) using immobiline dry strip gels with non-linear pH gradients (pH 4-7NL). These strips were placed on SDS polyacrylamide (12.5%) gels as detailed in Zheng et al., (2007). The separated proteins were then blotted as described for immunoblot analysis above. The membrane blot was then incubated overnight at 4°C, with eIF5A specific primary antibody (1: 3000). The blots were detected as described above. Digital images of these films were analysed using Image Quant TL analysis software (GE Healthcare, Mississauga, Canada).

Determination of PA content
Rosette leaves and inflorescences of WT and mtn1-1mtn2-1 were mixed with 5% (v/v) perchloric acid at a ratio of 1:4 (w/v) and frozen at –20°C. Samples were further processed and Put, Spd and Spm were quantified by high performance liquid chromatography as described by Minocha et al., (1990, 1994).

Determination of NA content
Nicotianamine was extracted from leaves and inflorescences of the WT and mtn1-1mtn2-1 plants and quantified after labeling with 9-fluorenyl methyl chloroformate as described in Supplementary material S1 of Klatte et al., 2009.
Determination of MTA content
Leaves and inflorescences of the WT and mtn1-1mtn2-1 plants (0.1 g) were ground in liquid nitrogen to a fine powder, which was extracted with 1 ml of 0.1 M HCl for 15 min at 4°C. Cell debris were removed by centrifugation twice at 28,000 g for 15 min at 4°C. MTA was determined as described in Rzewuski et al. (2007) using the same HPLC system.

Detection of metal contents
Samples were dried for constant weight at 60°C, and subjected to acid digestion with Omni-Trace1 nitric acid (EMI Scientific), and then heated to 85 – 90°C. Nitric acid blanks were processed to ensure that metals were not added during sample preparation. The standards Tomato (NIST Standard #1573a) and Corn (NIST Standard # 1573a) leaves were also processed without sample to quantify the recovery of Zn, Cu, Mn and Fe during the extraction procedure. Filtered samples were prepared and analyzed for metals as described by Gadapati and Macfie (2005) by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Modeling of MTA inhibition for Tspm synthase and Spd synthase 1
A 3-D model of ACL5 was built by Swiss-Model suite (Arnold et al., 2006) using the crystal structure of PA aminopropyl transferase from Thermus thermophilus (PDB ID 1UIR; Ohnuma et al., 2011) as a template (39% identity). The crystal structure of Arabidopsis Spd synthase1 is available in PDB (PDB ID 1XJ5). To obtain Tspm synthase-MTA and Spd synthase1-MTA complexes, MTA was docked using DOCK 4.0 (Ewing et al., 2001). Programs COOT (Emsley and Cowtan,2004) and PyMOL (DeLano Scientific, San Carlos, Canada) were used for structure visualization and figure preparation.

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SUPPLEMENTAL MATERIAL

**Figure S1.** MTN activity of various organs harvested from 6-week-old WT plants

**Figure S2.** Leaf vein pattern and pollen tube growth of *mtn1-1mtn2-1* compared to WT.

**Figure S3.** Rescue of Interveinal chlorosis.

**Figure S4.** Gene expression of polyamine oxidases and H$_2$O$_2$ production.

**Table S1.** Primer sequences.
LITERATURE CITED

Albers E (2009) Metabolic characteristics and importance of the universal methionine salvage pathway recycling methionine from 5′-methylthioadenosine. IUBMB Life 61: 1132-1142


Figure Legends

Figure 1. Overview of enzymatic reactions that generate 5′-methylthioadenosine (MTA)

MTA is generated as a by-product of polyamine (PA), nicotianamine (NA) and ethylene biosynthesis. 5′-methylthioadenosine nucleosidase (MTN, EC 3.2.2.16) hydrolyses MTA to 5′-methylthioribose (MTR) and adenine. The MTR is converted to Met through several steps, the first of which, MTR kinase (EC 2.7.1.100), catalyzes the production of MTR-1P. Methionine adenosyltransferase (EC 2.5.1.6) condenses Met and ATP to form S-adenosylmethionine (SAM). SAM decarboxylase (SAMDC, EC 4.1.1.50) removes carboxyl group from SAM to generate decarboxylated SAM (DCSAM). An aminopropyl group is transferred from DCSAM when putrescine (Put) is converted to spermidine (Spd) via Spd synthase (SPDS, EC 2.5.1.16). Similarly, another aminopropyl group is transferred from DCSAM when Spd is converted to spermine (Spm) or its isomer thermospermine (Tspm) via Spm synthase (SPMS, EC 2.5.1.22) or Tspm synthase (ACL5, EC 2.5.1.79) respectively. NA is produced from the condensation of three SAM molecules by NA synthase (NAS, EC 2.5.1.43). 1 aminocyclopropane-1-carboxylic acid (ACC) is also derived from SAM by ACC synthase (ACS, EC 4.4.1.14) in the rate-limiting step in the biosynthesis of ethylene. The dotted line indicates the additional steps involved in converting MTR into Met. The compounds that are in bold letters are the key compounds of interest in the present study.

Figure 2. MTN transcript and protein abundance with corresponding enzyme activity.

Results are averages (± SD) of 3-10 independent biological replicates.

(A) RT-PCR showed that MTN1 and MTN2 transcript abundance was reduced in mtn1-1mtn2-1. Actin was used as the reference gene to ensure equal loading of the cDNA. N=3.
(B) Immunoblot analysis indicated that MTN1 was present in buds and leaves of WT. In mtn1-1mtn2-1 MTN1 was considerably lower in buds and undetectable in the leaves. Detection of actin protein confirmed equal protein loading. N=3.

(C) Specific enzyme activity of MTN1 was measured in buds harvested from plants two-weeks after bolting. Statistically significant variables are indicated by different letters (P < 0.05; N = 10).

Figure 3. Vegetative phenotypes of mtn1-1mtn2-1 mutants compared to WT.

(A, B) WT and mtn1-1mtn2-1 seedlings at the 1.02 development stage when first true leaves are > 1mm. WT seedlings had green true leaves (A) compared to mutant seedlings that exhibited interveinal chlorosis (B).

(C, D) Developmentally matched adult leaves (equal number of secondary veins arising from the mid veins) of WT (C) and mtn1-1mtn2-1 (D), were cleared and stained with Toluidine Blue O to reveal the increased vein thickness in mtn1-1mtn2-1. Trichomes of the mutant were larger and the leaves tended to acquire more stain compared to the WT. Scale bars = 1.5 mm.

(E, F) Transverse sections through the mid veins of developmentally matched adult leaves of WT (E) and mtn1-1mtn2-1 (F) plants. The mtn1-1mtn2-1 exhibited an increase number of xylem (xy), phloem (ph) and cambial (ca) cells. Scale bars = 15 µm.

Figure 4. Polar auxin transport and stem vascular arrangement

(A) Reduced polar auxin transport of ¹⁴C-IAA in excised inflorescence stem segments of mtn1-1mtn2-1 mutants placed basipetally (inverted orientation) in ¹⁴C-IAA. Three independent experiments were conducted using segments of 32 plants for each genotype. Error bars indicate SE. Statistically significant values (P < 0.05) are indicated in different letters.

(B-C) Free hand cross sections of mtn1-1mtn2-1 and WT taken at the basal end of the primary inflorescence stem. Composite images of confocal laser scanning image and pseudo DIC image. Pseudo DIC image shows the increased number
of vascular bundles of the \textit{mtn1-1mtn2-1} (C) compared to WT (B). Confocal laser image shows the lignified inter and intra fascicular fibers stained with berberine. Scale bars: B, C = 50 µm.

(D-G) Auxin maxima (*) visualized using DR5rev::GFP mostly corresponded to number of vascular bundles in cross-sections taken from the basal end of the primary inflorescence stem (D, E) of WT (D, F) and \textit{mtn1-1mtn2-1} backgrounds (E, G). Higher magnification images reveal auxin maxima primarily localized to the xylem (xy) tissue of the vascular bundles (F, G). Note that the green fluorescence observed on lignified xylem of the inter-fascicular and intra-fascicular (IF) was autofluorescence. Scale bars: D, E = 50 µm; F, G = 150 µm.

Figure 5. Reproductive abnormalities of \textit{mtn1-1mtn2-1}

(A-F) Scanning electron micrographs of WT (A) and manually opened (D) anther sacs showed abnormal pollen development in the mutant. The \textit{mtn1-1mtn2-1} pollen grains were irregularly shaped (F), and lacked the characteristic furrow (indicated by the arrow) seen in the WT (B, C). At times the mutant pollen appeared clumped with each other (E) compared to WT (B). Scale bars = A, B = 45 µm; B, C E, F = 5 µm.

(G-H) Differential interference contrast (DIC) images of stage 14 flower pistils. WT had mature ovules post-fertilization stage (G) while the \textit{mtn1-1mtn2-1} ovules (H) corresponded to those normally observed in stage 12 WT flowers: outer integuments were not fully extended over the nucellus. Scale bars = 25 µm.

Figure 6. Complementation of \textit{mtn1-1mtn2-1} phenotype by MTN1 expression, MTA content and complementation of \textit{mtn1-1mtn2-1} phenotype by hMTAP.

(A) Complementation of \textit{mtn1-1mtn2-1} with MTN1::MTN1 3 weeks after bolting.

(B) Steady state levels MTA of inflorescences and rosette leaves. Mean values of five replicates were plotted with error bars indicating the SD. Statistically significant values (P < 0.05) are indicated in different letters.

(C) Complementation of \textit{mtn1-1mtn2-1} with UBQ10::hMTAP shown at bolting.
Figure 7. Structural basis for inhibition of Tspm synthase and Spd synthase1 enzymes by MTA.
(A, B) Close-up views of the MTA-binding pocket for Spd synthase 1 and Tspm synthase, respectively. MTA and the residues interacting with MTA in both structures are shown in stick-and-ball representation, while carbon atoms for residues are in the same color as the corresponding structure, whereas carbon atoms of MTA are in gray.
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Figure 8. PA profiles and two dimensional gels showing eukaryotic initiation factor 5A (eIF5A).
(A, B) Altered PA profiles of inflorescences (A) and rosette leaves (B) of mtn1-1mtn2-1 and WT (N = 4). Error bars represent SD. Statistically significant values (P < 0.05) are indicated in different letters.
(C, D) The hypusinated (H) and non-hypusinated (NH) eIF5A in rosette leaves of mtn1-1mtn2-1 (D) compared to WT (C). Images are representations of three replicates. The Rubisco large sub unit polypeptide was used as the reference for quantification (*). N=3. Molecular mass standards (in KDa) are indicated to the left in C, D.

Figure 9. Nicotianamine content and metal profiles of leaves and buds
Nicotianamine could not be detected in the rosette leaves (A; N = 5) compared to WT and was lower in mtn1-1mtn2-1 inflorescences (N = 3). In addition to nicotianamine, Fe, Mn, Zn and Cu levels were also reduced in mtn1-1mtn2-1 inflorescences (B) and leaves (C; N = 5) compared to WT. Each n is a pool of three biological replicates. Error bars indicate the standard error of the mean; FW, fresh weight; DW, dry weight. Statistically significant values (P< 0.05) are indicated in different letters while insignificant values indicated in same letters.
Figure 10. Exogenous NA and PA restored the fertility phenotype of \textit{mtn1-1mtn2-1}.

(A-E) Siliques and seed morphology of restored plants. The restored seeds of \textit{mtn1-1mtn2-1} were categorized in groups based on their lengths; most of the seeds of > 10 mm siliques has normal WT-looking seeds (B) while the former two categories had seeds with abnormal color and shape (C). Closer observation of these abnormal seeds showed (D) that they had developed seed coats similar to WT (E). Scale bars: A = 1.5 mm, B, C= 300 µm, D, E 100 µm.

(F) Number of siliques that developed when \textit{mtn1-1mtn2-1} was grown in ½ MS media supplemented with 20 µM NA, 100 µM spermine (Spm), spermidine (Spd) or 50 µM thermospermine (Tspm). The restored siliques were of various lengths they were categorized into three groups based on their lengths (N = > 20 siliques per treatment). Statistically significant values (P< 0.05) are indicated in different letters.

(G) Number of seeds/ silique and percent seed germinated from \textit{mtn1-1mtn2-1} plants grown on ½ MS supplemented with 100 µM Spd. Seed counts were average of 60 siliques from 6 plants for 5–7 mm and 8-10 mm categories while 36 siliques from 6 plants for >10mm category. Percent germination was assessed only on seeds collected for latter 2 categories (N = 6 siliques). Bars represent the standard deviation (SD). Statistically significant values (P< 0.05) are indicated in different letters while insignificant values indicated in same letters.

\textbf{Table 1}. Viable seeds obtained from reciprocal crosses between WT and mutants (\textit{apt1-3} and \textit{mtn1-1mtn2-1})
Figure 1: Overview of enzymatic reactions that generate 5'-methylthioadenosine (MTA)

MTA is generated as a by-product of polyamine (PA), nicotianamine (NA) and ethylene biosynthesis. 5'-methylthioadenosine nucleosidase (MTN, EC 3.2.2.16) hydrolyses MTA to 5'-methylthioribose (MTR) and adenine. The MTR is converted to Met through several steps, the first of which, MTR kinase (EC 2.7.1.100), catalyzes the production of MTR-1P. Methionine adenosyltransferase (EC 2.5.1.6) condenses Met and ATP to form S-adenosylmethionine (SAM). SAM decarboxylase (SAMDC, EC 4.1.1.50) removes carboxyl group from SAM to generate decarboxylated SAM (dcSAM). An aminopropyl group is transferred from dcSAM when putrescine (Put) is converted to spermidine (Spd) via Spd synthase (SPDS, EC 2.5.1.16). Similarly, another aminopropyl group is transferred from dcSAM when Spd is converted to spermine (Spm) or its isomer thermospermine (Tspm) via Spm synthase (SPMS, EC 2.5.1.22) or Tspm synthase (ACL5, EC 2.5.1.79) respectively. Polyamine oxidase (PAO; EC 1.5.3.11) back convert Spd/Tspm to Spd and to Put and produce H₂O₂. Other H₂O₂ producing reactions that oxidise PA are not shown. NA is produced from the condensation of three SAM molecules by NA synthase (NAS, EC 2.5.1.43). 1-aminocyclopropane-1-carboxylic acid (ACC) is also derived from SAM by ACC synthase (ACS, EC 4.4.1.14) in the rate-limiting step in the biosynthesis of ethylene. The dotted line indicates that there are many steps involved in converting MTR into Met. Highlighted compounds are the key compounds of interest in the present study.
Figure 2. *MTN* transcript and protein abundance with corresponding enzyme activity. Results are averages (± SD) of 3-10 independent biological replicates.

(A) RT-PCR showed that *MTN1* and *MTN2* transcript abundance was reduced in *mtn1-1mtn2-1*. Actin was used as the reference gene to ensure equal loading of the cDNA. N=3.

(B) Immunoblot analysis indicated that MTN1 was present in buds and leaves of WT. In *mtn1-1mtn2-1* MTN1 was considerably lower in buds and undetectable in the. Detection of actin protein confirmed equal protein loading. N=3.

(C) Specific enzyme activity of MTN1 was measured in buds harvested from plants two-weeks after bolting. N = 10.
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(A) Reduced polar auxin transport of \(^{14}\text{C}\)-IAA in excised inflorescence stem segments of \textit{mtn1-1mtn2-1} mutants placed basipetally (inverted orientation) in \(^{14}\text{C}\)-IAA. Three independent experiments were conducted using segments of 32 plants for each genotype. Error bars indicate SE. Statistically significant values (P < 0.05) are indicated in different letters while insignificant values indicated in same letters.

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Exogenous compound

+ NA + Spm + Spd + Tspm

Number of siliques (counts)

0 20 40 60 80

5-7 mm 8-10 mm >10 mm

Number of seeds / silique

0 20 40 60 80 100

8-10 mm >10 mm

Percent germination

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