Functional and Developmental Impact of Cytosolic Protein N-Terminal Methionine Excision in Arabidopsis

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Protein N-terminal methionine (Met) excision (NME) is carried out by two types of Met aminopeptidases (MAPs), MAP1 and MAP2, in eukaryotes. Three enzymes, MAP1A, MAP2A, and MAP2B, have been identified in the cytoplasm of Arabidopsis (Arabidopsis thaliana). MAP transcript quantification revealed a predominance of MAP2B and developmental and organ-specific regulation of both MAP1A and MAP2s. By combining reverse genetics and reverse chemogenomics in transgenic plant lines, we have devised specific and reversible switches for the investigation of the role of cytoplasmic NME in Arabidopsis and of the respective contributions of the two types of cytoplasmic MAPs throughout development. dsRNA interference and knockout (KO) plant lines targeting either MAP1A alone or both MAP2s simultaneously were constructed and shown to display wild-type phenotypes. In the MAP1A KO context, modulating MAP2 activity by treatment with various concentrations of the specific drug fumagillin impaired plant development, with particularly strong effects on the root system. Reciprocally, complete MAP2 inhibition in various MAP1A knocked-down genetic backgrounds also generated a gradient of developmentally abnormal plants, but the effects on the root system were milder than in the KO context. In the absence of MAP2 activity, the severity of the phenotype in the MAP1A knocked-down lines was correlated to the extent of MAP1A mRNA accumulation. Complete cytoplasmic NME inactivation blocked development after plant germination. Thus, in plants, (1) cytoplasmic NME is essential; (2) MAP1A and MAP2s are functionally interchangeable, which is not the case in fungi and animals, as a complete block of either MAP-type activity does not cause any visible molecular or phenotypic effect; and (3) a minimal level of cytoplasmic MAP is required for normal development.

In living organisms, whatever the cell compartment, proteins are always synthesized with Met as the first residue even if a nonconventional initiation codon (i.e. non-AUG) is used (for review and references, see Giglione and Meinnel, 2001; Giglione et al., 2004). However, this first Met is specifically removed from most mature proteins (Meinnel et al., 1993; Bradshaw et al., 1998; Giglione and Meinnel, 2001). In the course of protein N-terminal Met excision (NME), the free amino-terminal Met is removed by Met aminopeptidase (MAP) cleavage. Unlike eubacteria, which possess only one type of MAP (MAP1), eukaryotes possess a second type of MAP, MAP2, which has similar substrate specificity to MAP1 (Kendall and Bradshaw, 1992; Chen et al., 2002). MAP2s and MAP1s play identical enzymatic roles and have very similar three-dimensional (3D) structures, but their amino acid sequences differ considerably. In contrast, levels of sequence identity are high for MAPs of the same type (Lowther and Matthews, 2002; Giglione et al., 2004). The recent discovery that cytoplasmic MAP2s are the specific cellular target of natural compounds like fumagillin, acting as antiangiogenic, antitumoral, or immunosuppressive agents, has revived interest in NME (Ingber et al., 1990; Griffith et al., 1997, 1998; Sin et al., 1997; Turk et al., 1999; Towbin et al., 2003). Several of these compounds have also been shown to have antiproliferative effects in unicellular eukaryotes, such as ameba (Killough et al., 1952), microsporidians (Weiss et al., 2001), trypanosomatida, and apicomplexa (Zhang et al., 2002), all of which cause severe parasitic diseases in humans. Significant progress has recently been made in the characterization of this process in higher eukaryotes (for review, see Giglione et al., 2004). NME has been shown to occur in all compartments in which de novo protein synthesis occurs: the cytoplasm, mitochondria, and plastids. Experimental data have shown that MAP1s are found in both the organelles and the cytoplasm (MAP1As) of all higher eukaryotes, including mammals and plants (Giglione et al., 2000; Serero et al., 2003). Unlike MAP1s, MAP2s are found specifically in the cytoplasm (Giglione et al., 2004). In higher plants, various organelle-targeted MAPs (MAP1B, MAP1C, and MAP1D; Giglione et al.,...
RESULTS

Quantification of MAP Transcripts Shows the Predominance of MAP2B RNA and the Developmental and Organ-Specific Regulation of Both MAP1A and MAP2s

As observed in other species, Arabidopsis contains two types of cytosolic MAPs, MAP1A and two MAP2s, MAP2A and MAP2B. These are the only cytosolic MAPs isolated (Table I; Giglione et al., 2000). The three corresponding genes are MAP1A, MAP2A, and MAP2B. MAP2A and MAP2B have identical intron-exon organizations, produce mRNAs of the same size, and their open reading frames are 86% identical overall (Giglione et al., 2000). MAP2A and MAP2B catalytic domains are more than 90% identical. The amino acid sequence of the MAP1A catalytic domain is approximately 50% identical to those of the 3 organellar MAP1s. Low levels of identity occur between MAP1s and MAP2s (Supplemental Fig. 1). The data concerning the MAP protein family from Arabidopsis are summarized in Table I. Except for MAP1A (this article), no specific antibody is yet available for the other MAPs, including both MAP2s.

Few data on cytoplasmic MAP expression and regulation in a whole higher eukaryote are available. We therefore investigated the expression profiles of all three genes in various organs and during plant development by means of real-time PCR experiments. A cytoplasmic MAP transcript was present in all tissues and at all developmental stages examined. MAP2 transcripts accumulated in all organs tested, but the amount depended on the organ considered (Fig. 1A). MAP2B transcripts were the most abundant of the two MAP2s. MAP1A transcripts were observed in all organs tested, except siliques, in which they were undetectable. MAP1A transcripts were strongly induced during the early stage of development, beginning at root development (Boisson et al., 2003). Using an integrated array of experimental approaches, we were able to show that (1) cytoplasmic NME is essential for plant development; (2) a minimal level of total cytoplasmic MAP is required for correct plant development; (3) low levels of MAP2 cannot compensate for the absence of or very low levels of MAP1A; and (4) the natural compound fumagillin specifically targets the activity of both MAP2A and MAP2B, but not that of MAP1A in plants, as previously demonstrated in animals and fungi.
emergence on day after imbibition (DAI) 2. Significant levels of induction were also observed for both MAP2 transcripts until DAI 4 (Fig. 1A).

**Strong Decrease in the Level of Expression of the Genes of the Two MAP Groups Reveals No Apparent Phenotype**

Functional analysis of cytoplasmic NME in Arabidopsis by insertional reverse genetics is difficult because three genes are involved in the process (Table I) and MAP2A (At2g44180) and MAP1A (At2g45240) are very close neighbors on chromosome 2, making it difficult to identify double mutants if KO plants were isolated for each gene. To investigate NME by reverse genetics, we used dsRNA interference (RNAi) to construct plant lines with knocked-down (KD) MAP expression. First, two RNAi vectors, one targeting MAP1A and the other targeting both MAP2A and MAP2B, were constructed. We selected 50 drug-resistant T1 plants at random for each construct and followed their development until seed production. All were shown to have the relevant construct target gene-inverted repeats, as shown by PCR analysis, and produce the RNAi transcript, as shown by northern blotting (data not shown). Various MAP1A and MAP2 KD T1 lines ($\Psi_{map1A}$, $\Psi_{map2}$) were selected with this method. The level of the relevant target MAP gene transcript was determined by semiquantitative reverse transcription (RT)-PCR in 10-DAI-old plantlets grown on normal medium (Fig. 1B). MAP1A, MAP2B, and MAP2A transcript levels were compared with those in wild-type plants, and progeny from one of the cT T1 lines were examined. The plants from each $\Psi_{map1A}$ line had much lower levels (8% ± 1% of wild type) of MAP1A than wild type (Fig. 1, A and B). However, we also isolated several $\Psi_{map1A}$ lines with various, smaller decreases in MAP1A transcripts (data not shown). In the $\Psi_{map2}$ progeny plant, MAP2B and MAP2A transcript levels were strongly reduced (7% ± 3% and 29% ± 3% of wild type, respectively). MAP transcript levels were unaffected in the progeny of the cT T1 line. No obvious abnormalities in morphology (growth rate and size) or rate of development (germination time, flowering time, and seed yield) were detected after systematic comparisons with wild-type plants. In line with this, no difference was observed when the protein patterns of plants from several $\Psi_{map1A}$ and $\Psi_{map2}$ lines were compared with those of the wild-type or cT line (data not shown).

Together, these data strongly suggested that both MAP types were functionally interchangeable. To exclude that the small remaining amount of functional MAP1 or MAP2 observed in either RNAi line could be sufficient to allow normal plant development, KO mutants for both MAPs were searched. We screened various Arabidopsis T-DNA mutant collections in vitro or in silico for insertions in MAP1A and MAP2 genes. No true KO lines have yet been obtained for MAP2A. In the putative MAP2B-inserted line, SALK_100162, the T-DNA insertion turned out to have occurred outside the open reading frame, in contrast to the sequence data available. We therefore excluded this line from the analysis. One putative mutant line was identified for MAP1A with a T-DNA insertion point within the first intron of the gene (Fig. 2A). Lines homozygous for a T-DNA insertion resulting in MAP1A inactivation (map1A-1) were obtained and purified. The T-DNA insertion in line map1A-1 is located downstream of the first Cys codon of the first exon. As this Cys, which is part of the double zinc finger motif that is crucial for in vivo MAP1A activity in yeast (Zuo et al., 1995), line map1A-1 was expected to correspond to a true MAP1A KO mutant. RT-PCR analysis confirmed the absence of any detectable full-length MAP1A transcript in line map1A-1 in contrast to wild type (Fig. 2B). Accordingly, western-blot experiments showed that line map1A-1 contained no detectable MAP1A and corresponded to a true MAP1A inactivation (Fig. 2C). Line map1A-1 behaved similarly to wild type and displayed no visible mutant phenotype under standard greenhouse growth conditions. We investigated the expression profiles of the MAP genes by real-time PCR and noticed that both MAP2 transcript levels were identical to those of wild type (Fig. 1A). To definitely exclude the occurrence of any subtle differences between line map1A-1 and wild type, comparison of their two-dimensional gel electrophoresis (2-DE) protein patterns were performed.

### Table I. Various MAP genes and corresponding proteins in Arabidopsis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>TAIR Entry</th>
<th>No. of Open Reading Frame Codons (Size)</th>
<th>Percent Identity with MAP1A(^d) (MAP2B/MAP2A)</th>
<th>Cellular Localization of the Protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP2A</td>
<td>At2g44180</td>
<td>440 (49)</td>
<td>&lt;20%* (100)</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>MAP2B</td>
<td>At3g59990</td>
<td>439 (49)</td>
<td>&lt;19%* (86)</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>MAP1A</td>
<td>At2g45240</td>
<td>398 (44)</td>
<td>100</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>MAP1B</td>
<td>At3g25740</td>
<td>369 (35)</td>
<td>50</td>
<td>Chloroplast</td>
</tr>
<tr>
<td>MAP1C</td>
<td>At1g13270</td>
<td>344 (31)</td>
<td>46</td>
<td>Chloroplast, mitochondrond</td>
</tr>
<tr>
<td>MAP1D</td>
<td>At4g37040</td>
<td>350 (33)</td>
<td>48</td>
<td>Chloroplast, mitochondrond</td>
</tr>
</tbody>
</table>

\*Experimental data are from Giglione et al. (2000). Note the nomenclature used in this article was updated recently (Giglione et al., 2004). \#TAIR, The Arabidopsis Information Resource (http://arabidopsis.org/). \$Deduced protein size is expressed in kilodaltons and corresponds to that of the fully processed protein, taking into account NME and signal peptide cleavage at http://www.cbs.dtu.dk/services/TargetP. \#< indicates that only local identity in the catalytic domain was obtained and that the value indicated corresponds to that of the highest value determined for 10 contiguous amino acids (for an alignment of MAP1 and MAP2, see Supplemental Fig. 1).
As no MAP2 (i.e. MAP2A + MAP2B) KO line is yet available, we investigated the role of plant cytoplasmic NME further using an inhibitor active against cytoplasmic MAPs. Few such inhibitors are known (Giglione et al., 2004). Recently, l-Met, the product of the reaction catalyzed by MAPs, was shown to inhibit both Homo sapiens MAPs (HsMAPs), but with different binding constants: 0.15 mM for MAP2 and 5 mM for MAP1A (Dummitt et al., 2003). The 3D structures of the complex of l-Met with MAP1 and MAP2 are available under protein data bank (PDB) entries 1C21 and 1KQ9. These structures show that l-Met binds to conserved residues in the active site of both MAP species (Fig. 3A; Li et al., 2004). We expected l-Met to bind to both AtMAP1A and AtMAP2s as the catalytic domains of these two enzymes displayed an extremely high level (>75%; see alignment in Giglione et al., 2000) of identity to HsMAP2 (Giglione et al., 2004). The 3D structure of Arabidopsis MAP2s was modeled based on the structure of HsMAP2 complexed with l-Met. The 3D structure of the active site of AtMAP1A was modeled from the 3D structure of Escherichia coli MAP (EcMAP) complexed with l-Met. The models generated showed overlapping 3D folds of the active site of both MAP types when shown in the same orientation (Fig. 3A). They also revealed that all the residues involved in l-Met recognition were conserved in both plant MAP types, with the exception of Cys-59, which was replaced by an Ala in AtMAP1A (Fig. 3A). This substitution does not interfere with substrate or l-Met analog recognition (Li et al., 2004).

l-Met impairs the growth of a map1A yeast line by inhibiting MAP2 in the millimolar range of concentration (Dummitt et al., 2003). Wild-type and map1A-1 Arabidopsis seedlings were grown in the presence of equivalent amounts of l-Met. At a concentration of 2 mM l-Met, map1A-1 KO plants displayed strongly impaired development (Fig. 3B). Increasing the concentration of l-Met blocked development completely. These data suggested that AtMAP2s could be inhibited by l-Met in vivo. Nevertheless, l-Met caused wild-type plants to grow more slowly (Fig. 3B). We concluded that the inhibition of wild-type seedling growth by l-Met may have resulted from either (1) nonselective inhibition of both cytoplasmic MAP types, suggesting that cytosolic NME is essential; or (2) the metabolism of l-Met to generate compounds that play a major role in the plant (Ravanel et al., 1998), for which changes in concentration could retard plant development. Hence, to delineate the role of cytosolic NME, an inhibitor with no effect on wild type was required.

Modulating MAP2 Activity with Fumagillin Affects Plant Development in the map1A-1 Context

Fumagillin is a natural compound produced by the fungus Aspergillus fumigatus (McCown et al., 1951). Fumagillin and its derivatives, such as TNP-470 (also known as AGM-1470; Ingber et al., 1990), fumagillone (compound 4 in Turk et al., 1998), or ovalicin (Corey et al., 1994), specifically and covalently bind and inactivate MAP2s, with low nanomolar binding con-

![Figure 1](image-url)
constants, but have no physiological effect on MAP1s (Griffith et al., 1997, 1998; Sin et al., 1997; Liu et al., 1998; Turk et al., 1998; Brdlik and Crews, 2004). The chemical structure of these four related compounds is shown in Figure 4A. As fumagillin has been shown to strongly inhibit all MAP2s tested, both in vitro and in vivo, and as we identified no particular phenotype with either map RNAi transgenic plant lines or the KO mutant line map1A-1, we thought to use this drug to investigate further the role of cytoplasmic NME in Arabidopsis in vivo. The 3D structure of Arabidopsis MAP2s with fumagillin was modeled from that of HsMAP2 complexed with fumagillin (Liu et al., 1998). This 3D model (Fig. 4B) revealed that all the residues involved in fumagillin recognition, including the crucial His residue (His-231; see Fig. 4B) responsible for covalent attachment of the drug, were conserved in both plant MAP2s. The only nonidentical residue was the Phe-366 of HsMAP2, which was replaced by a Cys in AtMAP2A. However, it was recently demonstrated

Figure 2. Characterization of a map1A-1 plant line. A, Schematic representation of MAP1A gene disruption in Arabidopsis line SALK_021985 (map1A-1). The exon-intron structure of the gene is shown. Translation initiation and termination codons are indicated. The T-DNA insertion is shown with the border sequences of the insert (LB and RB) labeled to indicate the orientation of the insertion. The location of T-DNA end insertion was checked by DNA sequencing, PCR amplification, and restriction fragment length analysis. LB, Left border; RB, right border. B, RT-PCR analysis showing the absence of the MAP1A transcript in line map1A-1. MAP1A expression analysis in map1A-1 and wild-type lines was performed by RT-PCR analysis using cDNA of either line as template. cDNA synthesis is described in “Materials and Methods.” The amount of cDNA to be used in each experiment (about 2 ng) was estimated by real-time PCR analysis to yield the same amplification level for EF1A. The MAP1A cDNA-specific primers used in this experiment were 5'-CAGATGCTACAGCAGCTGTACTCTTTCCTGTC and 5'-ATTCGGTTCACTTGTGGAGTCCCATC and were expected to yield a 382-bp amplification band. The DNA ladder (lane 1) corresponded to the 1-kb plus ladder (Bio-Rad). For each cDNA (wild type or map1A), the amplification resulting from three distinct cycle numbers (23, 26, and 30) is shown. C, Western-blot analysis of 10-DAI-old seedlings. WT, Wild type. We analyzed 80-μg aliquots of total protein by 10% denaturing PAGE, followed by electroblotting and probing of the resulting membrane with anti-MAP1A antibody. D, 2-DE patterns of wild type (left) and line map1A-1 (right). MW, Molecular weight (M). The lowest molecular mass visible in the second dimension was 21 kD. The image shown is the most representative of the various experiments performed knowing that quantitative differences for the high-Mr spots were observed in various analysis of the same protein extract.
that the Phe-366Cys substitution has no effect on the binding of fumagillin to HsMAP2 (Brdlik and Crews, 2004). The greater specificity of fumagillin for both AtMAP2s than for AtMAP1A was fully expected as AtMAP1A (1) has the Thr residue (Thr-202) involved in fumagillin selectivity between MAP1 (Ala-362) and MAP2 (Fig. 3A; Brdlik and Crews, 2004); and (2) possesses a Cys residue (Cys-70) rather than the crucial His-231 of MAP2s (Figs. 3A and 4B). These residues are conserved in all Arabidopsis MAP1s, including the three organellar isoforms (Giglione et al., 2000). Fumagillin, a commercially available compound, therefore appeared to be a useful chemical tool for the specific inhibition of both cytosolic MAP2 activities in plants.

Arabidopsis seeds were therefore sown in the presence of various concentrations, up to 15 μM, of fumagillin. No effects on wild-type plants (Fig. 4C) were observed, indicating that this drug did not impair plant development at the concentrations used and did not inhibit any essential target protein. When line map1A-1, which produced only MAP2A and MAP2B, the putative fumagillin-sensitive isoforms, was grown under similar conditions, it was found to be highly sensitive to the drug (Fig. 4C). Similar data were obtained when TNP-470, fumagillione, or ovalicin were used (Fig. 4, A and D). In contrast, actinonin, a specific inhibitor of organellar NME that blocks peptide deformylase activity (Giglione and Meinnel, 2001; Serero et al., 2001; Giglione et al., 2003), had no effect within the same range of concentration (data not shown). These results are consistent with fumagillin being highly specific for the two MAP2s and demonstrate that the effect observed is dependent on MAP2 inhibition only. At extremely low concentrations (0.01 μM) of fumagillin, line map1A-1 displayed a dwarf phenotype (Fig. 4E) with very few secondary roots. These plants continued to grow slowly in the presence of the drug but developed a bushy leaf appearance (data not shown). Increasing the concentration of fumagillin increased the severity of the mutant phenotype, together with the effects on the root system. Finally, a strong block in development after germination was observed at fumagillin concentrations exceeding 1 μM (Fig. 4, C and F). These plants failed to turn green at or develop beyond the two-cotyledon stage. We compared wild-type and line map1A-1 seeds sown in the presence of 1 μM fumagillin with a wild-type or a map1A-1 mature embryo sown in the absence of the drug in which the primary seedling structure was morphologically recognizable and identified no structural differences. All
Figure 4. Fumagillin specifically targets and inhibits MAP2. A, Chemical structures of fumagillin and various structural analogs showing potent inhibition of MAP2. B, In silico model of the active binding site of AtMAP2B bound to fumagillin. See details in Figure 3A. The structure of fumagillin is indicated with blue sticks. Links between the metal cations and protein side chains are shown as red lines. The orientation of the fumagillin molecule is upside-down compared to A. C, 2-DAI-old wild-type or map1A-1 line plants grown in the presence of the indicated (top) concentration of fumagillin (micromolar). D, 10-DAI-old map1A-1 line plant grown in the presence of 5 μM of the indicated drug (structure shown in A). E, 10-DAI-old map1A-1 line plant grown in the presence of the indicated (top) concentration of fumagillin (micromolar). F, Comparison of the size and morphology of a wild-type mature embryo stained with di-acetate fluorescein (top left inset) with a map1A-1 line seedling germinating in the presence of 1 μM fumagillin. The scale for both is shown below the figure. G, 1D PAGE (12%) analysis of protein extracts originating from Arabidopsis plant lines (wild type or map1A-1) grown in the presence of the indicated concentration of fumagillin (micromolar). The gel was silver stained (Amersham). Molecular mass ranges corresponding to the first lane are indicated on the left border of the gel (Biolabs).
major structures were detected, including root, hypocotyls, and cotyledons, in both plants (Fig. 4F), indicating that MAP2 activity alone was sufficient for embryo formation but that development could not progress much beyond the mature embryonic stage if this activity was also inhibited. This suggested that fumagillin impaired cell expansion and the few early cell divisions in line map1A-1.

Next, experiments were performed with protein samples extracted from the map1A-1 or wild-type lines grown in the absence or presence of 1 μM fumagillin (i.e. a concentration allowing complete inhibition of both MAP2 activities in fungi). When one-dimensional (1D) or 2-DE experiments were performed with a wild-type plant grown in the presence of 1 μM fumagillin, no discrepancy was observed compared to the control, and the patterns were similar to those shown in Figure 2D. This indicated that the absence of MAP2 activity was likely to be compensated by that of MAP1A. In contrast, the protein pattern of line map1A-1 grown in the presence of more than 0.01 μM fumagillin was significantly modified compared to the control, as expected for the inhibition of an essential process that involves a majority of the proteins of the cytoplasm (Fig. 4G).

Thus, fumagillin specifically targets and inhibits both MAP2A and MAP2B, but not MAP1A, in plants. Moreover, our data indicate that (1) cytoplasmic NME (i.e. MAP1A + MAP2A + MAP2B activity) is an essential process in plants; (2) the function of both cytosolic MAP2 can be compensated by that of MAP1A; and (3) in the absence of MAP1A, the intrinsic level of MAP2 activity is crucial for correct development. As plant lines grew normally and showed identical protein patterns in the absence of either cytosolic MAP-type activity (i.e. MAP1A or MAP2A/B), we concluded that the two MAPs were interchangeable in plants.

Complete Inhibition of MAP2 Causes a Range of Developmental Perturbations in Plant Lines Producing Various Amounts of MAP1A

Having evaluated the impact of MAP2 activity on Arabidopsis cytoplasmic NME in genetic backgrounds devoid of MAP1A proteins, we then investigated the contribution of MAP1A proteins to this process. We sowed all the ψmap1A and ψmap2 lines separately in a growth medium containing the concentration of fumagillin previously shown to yield the maximal effect in line map1A-1 (5.4 μM). We used concentrations of fumagillin up to that used in the experiments with map1A-1 lines that completely blocked plant development, suggesting that the activity of the two MAP2 enzymes was completely inhibited. Seeds from wild-type plants, ctl, and ψmap2 T1 lines germinated and developed normally on fumagillin (Fig. 5A), with normal dark-dependent skotomorphogenesis (data not shown). In contrast, developmentally abnormal plants were observed in the progeny of all the ψmap1A lines grown in the presence of the drug. The most extreme phenotype, which was observed at various frequencies in the progeny from all ψmap1A T1 lines, was a complete block of plant development after emergence from the seed (Fig. 5A). The plants failed to turn green or to develop beyond the stage indicated and closely resembled map1A-1 plants grown in the presence of high concentrations of the drug (see Fig. 4, C and E). Nevertheless, most of the progeny from the most affected ψmap1A T1 lines grown in the presence of fumagillin displayed a lesser effect on the roots than was observed in KO map1A-1 plants (Fig. 4C).

The progeny from some of the ψmap1A T1 lines showed a gradient of less severe phenotypes on fumagillin medium than that typified by the complete developmental arrest occurring before leaf emergence. Such less extreme phenotypes included discolored and malformed cotyledons and leaves (Fig. 5B; see also Fig. 6) and slow growth. Such plants never reached the flowering stage. Light is known to cause an inhibitory effect on hypocotyl elongation. Because light-grown map1A-1 and ψmap1A seedlings in the presence of fumagillin showed extremely reduced hypocotyl elongation, we examined whether the hypocotyl was able to elongate in the dark. Similarly to wild type, all ψmap1A seedlings had strong elongation of the hypocotyl when grown in the dark in the absence of the drug (Fig. 5C). However, the most severely affected ψmap1A-1 seedlings failed to exhibit hypocotyl elongation, even by 10 DAI in the dark (Fig. 5C), when grown in the presence of fumagillin. This indicated that ψmap1A was severely defective in hypocotyl cell expansion/elongation and not responsive to dark treatment.

We assessed the correlation of MAP1A transcripts with the mutant phenotype. We used semiquantitative RT-PCR to determine MAP1A transcript levels in progeny plants of the same age from a single T1, ψmap1A line sown on fumagillin, which expressed the RNAi construct and displayed a severe or intermediate mutant phenotype or appeared normal. The values obtained were compared with those for wild-type and ctl T1 progeny plants of equivalent age, producing RNAi transcripts, and sown on fumagillin. MAP1A transcript levels clearly decreased with increasing severity of the mutant phenotype (Fig. 5, B and D). These results are consistent with those reported for map1A-1 KO plants (Fig. 4) in that these data demonstrate the need for a minimal level of cytoplasmic MAP activity for correct plant development. However, the greater sensitivity of map1A-1 KO plant roots to fumagillin than of ψmap1A lines suggests that a small amount of MAP1A can still ensure root development.

The Fumagillin-Induced NME Inactivation Phenotype Can Be Mimicked in an RNAi Plant Line Targeting the Complete Set of Cytoplasmic MAPs

We thought it should be possible to obtain viable transgenic plant lines displaying the above-described gradient of developmental arrest phenotypes by simultaneously using RNAi-mediated targeting of all cytoplasmic MAP transcripts with a single construct.
Figure 5. A gradient of developmental mutant phenotypes induced by fumagillin in \( \Psi_{map1A} \) RNAi plant lines, according to \( MAP1A \) transcript levels. Various RNAi plant lines were grown in the presence or absence of the indicated concentration of fumagillin. WT, Wild type; \( ctl \), corresponds to the control line (see text). A, Three-DAI-old \( T_1 \) progeny. The most divergent phenotypes attainable in the \( T_1 \) \( \Psi_{map1A} \) progeny are shown. B, The \( T_2 \) progeny from a \( T_1 \) \( \Psi_{map1A} \) line was sown on fumagillin. (X) indicates various plants for which \( MAP1A \), \( MAP2B \), and \( MAP2A \) RNA levels were analyzed. C, \( \Psi_{map1A} \)\( T_1 \) progeny grown in the dark for 10 DAI in the absence (top) or in the presence (bottom) of 5.4 \( \mu \text{M} \) fumagillin. D, \( MAP1A/EF1A \) RNA levels of the plants selected in B, with wild-type values set to 100. E, Triple RNAi lines (\( \Psi_{map1A2} \)) were grown for 10 DAI in the presence or absence of 5.4 \( \mu \text{M} \) fumagillin.
In the absence of fumagillin, the progeny of 11 out of 22 randomly selected Finale-resistant T1 lines ($\Psi$map1A2) displayed the range of mutant phenotypes characteristic of $\Psi$map1A T1 lines sown on fumagillin (Fig. 5E), with a milder effect on roots than observed with the $map1A$-1 KO line (Fig. 4, C and E). However, fumagillin increased the number of mutants with the most severe phenotype. This was demonstrated when the progeny from four T1 $\Psi$map1A2 construct lines, together with wild-type plants and the progeny of $\Psi$map1A T1 plants previously grown on 5.4 μM fumagillin and with a mild phenotype (left) were transferred to fumagillin-depleted growth medium for 3 more weeks (middle). The seedlings were then planted in soil and photographed 2 weeks later (right).

**Figure 6.** Reversible and irreversible effects of fumagillin on Arabidopsis $\Psi$map1A and $map1A$-1 seedlings, respectively. Arabidopsis $\Psi$map1A or $map1A$-1 seeds were sown in a growth medium without fumagillin, synchronized in the dark at 4°C for 2 DAI, and then incubated in a growth cabinet. Representative seedlings were photographed before and after sowing in the presence of the indicated concentration of fumagillin. A, $\Psi$map1A seedlings grown for 15 DAI before the addition of fumagillin. B, $map1A$-1 seedlings grown for 3 DAI before the addition of fumagillin. C, Three-week-old $\Psi$map1A T1 plants previously grown on 5.4 μM fumagillin and with a mild phenotype (left) were transferred to fumagillin-depleted growth medium for 3 more weeks (middle). The seedlings were then planted in soil and photographed 2 weeks later (right).
a strong mutant phenotype (observed with fumagillin) \( \Psi \) map1A \( T_1 \) line as controls, were grown in the presence or absence of fumagillin. These data confirmed our aforementioned conclusions on the essential role of NME in plant development. When transferred to soil, the least severely affected \( \Psi \) map1A2 \( T_2 \) lines gave rise to plants with multiple emerging primary inflorescence of similar sizes (data not shown).

Irreversible and Reversible Effects of Postgermination NME Inactivation on Plant Development

We investigated whether the cytoplasmic NME machinery was crucial for plant development in \( \Psi \) map1A plants sown directly on fumagillin either because this machinery was inhibited from the beginning of plant growth or because it also plays a role in later developmental stages. Our demonstration that fumagillin specifically inhibited the two MAP2s and the construction of KD and KO \( map1A \) lines provided us with appropriate tools for specific cytoplasmic NME inactivation at any developmental stage. Two-week-old \( \Psi \) map1A lines previously grown in the absence of fumagillin were transferred to a medium containing 5.4 \( \mu M \) of this drug. Under these conditions, in plant lines with very low MAP1A transcript levels, the drug slowed down development and plants prematurely underwent senescence, leading to the formation of necrotic lesions on the leaves (Fig. 6A). No effect was observed in \( \Psi \) map1A lines containing larger amounts of this transcript. In \( map1A-1 \) KO lines, we observed an almost immediate block of development, accompanied by rapid senescence of the apical region (Fig. 6B).

We then investigated the effects of transferring the progeny of \( \Psi \) map1A \( T_1 \) lines to fumagillin-free medium to determine whether the effects of this drug were reversible. Three-week-old \( \Psi \) map1A \( T_1 \) lines previously grown on fumagillin medium were transferred to fumagillin-depleted growth medium. Those with the most severe mutant phenotypes, similar to line 4 in Figure 5B, did not recover on fumagillin-free medium (data not shown). In contrast, mutants that had continued to develop, but slowly, similar to line 2 in Figure 5B, developed a bushy leaf appearance on transfer to fumagillin-free medium (Fig. 6C, middle), with shoots unable to grow upward away from the medium. These less severely affected mutants produced a large number of axillary buds rather than a single apical bud. Consistent with these findings, multiple inflorescences emerged following transfer to soil (Fig. 6C, right), rather than the more usual single main inflorescence, as seen in wild-type plants. Similar, but more marked phenotypes were observed with \( map1A-1 \) lines: the higher the initial concentration of fumagillin, the longer the delay observed in the transition from the vegetative to the flowering stage. These results indicate that NME is crucial for development after the initial germination stages and that the plant can recover from an inhibited NME system, but with perturbed development.

DISCUSSION

The general mechanism of action of MAPs has been extensively studied in yeast and bacteria and the removal or retention of the initiator Met seems to be directed by the second residue (for review, see Giglione et al., 2004). MAPs cleave the N-terminal l-Met only if the second residue is small. Hence, by means of MAP substrate specificity, NME contributes to the diversity of the N-terminal amino acids occurring in mature proteins.

MAP2s Versus MAP1s: Overlapping or Redundant Functions in Higher Eukaryotic NME?

In the cytoplasm of eukaryotes, two different MAP types, MAP1 and MAP2, exist. The significance of the occurrence of two types of MAPs is unclear. In yeast, the only organism in which this issue has been addressed, MAP1 is the dominant isoform; inactivation of MAP1 results in a slow-growth phenotype, whereas MAP2 inactivation has no great effect. These data suggest that MAP2 is redundant, with MAP1 the chief enzyme in fungal NME (Dummitt et al., 2003). Recent data have suggested that the catalytic efficiencies of MAP1 and MAP2 might be slightly different in vivo for some substrates (Turk et al., 1999). For example, in yeast, MAP1 is the most efficient enzyme in NME, whereas MAP2 increases the efficiency of this process for some substrates only if overproduced (Chen et al., 2002). However, both MAPs were less efficient if the second residue was Val, and MAP2 was less efficient than MAP1 if the second residue was Cys, Gly, or Thr.

The importance of these MAPs in animal cell growth and tumor progression has been investigated, particularly since the identification of MAP2 as the molecular target for the antiangiogenic agent fumagillin and the discovery that this enzyme has POEP activity. Fumagillin and its derivatives specifically block the NME activity of MAP2, selectively inhibiting endothelial cell proliferation by arresting the cells in the G1 phase (Zhang et al., 2000). The mechanism underlying this selective inhibition remains unclear. Recent data suggest that MAP2 is tightly regulated throughout development and tumorigenesis. Regulation has been shown to occur at the transcriptional and post-transcriptional levels. MAP2 seems to accumulate in a cell- and tissue-specific manner. In insects, MAP2 transcripts are uniformly distributed during early embryonic development, but at later stages, they accumulate only in specific tissues (Cutforth and Gaul, 1999).

MAP2 seems to be strongly and selectively expressed in germinial center B cells and some neoplastic cells, such as B-cell lymphoma cells, hepatoma cells, and mesothelioma cells (Wang et al., 2000; Catalano et al., 2001).
What the Plant Genetic System Contributes to Our Understanding of the Function of Cytoplasmic NME

To understand the roles of the two types of MAP enzyme in cytoplasmic NME, we need to modulate the activity of each type of MAP separately in a whole-cell system or in a whole, developing organism. This study of the cytoplasmic NME and the roles of MAP1 and MAP2 in a higher eukaryote is uncommon in that it is essentially based on the availability of: (1) a higher eukaryotic system devoid of MAP2-borne POEP activity; (2) a KO for cytoplasmic MAP1 (line map1A-1); and (3) a dual-compatibility epigenetic control system combining RNAi-triggered KD lines and drug susceptibility, making it possible to induce a loss-of-function or gain-of-function of all cytoplasmic MAPs at various developmental stages. We were therefore able to modulate NME production and activity at any time point. Various MAP KD mutants (Ψmap1A, Ψmap2, and Ψmap1A2) were isolated. Lines Ψmap1A, Ψmap2, and map1A-1 displayed a wild-type phenotype, indicating that the two types of MAPs are fully interchangeable in plants. Our data show that plant MAP2s do not have POEP activity, as predicted from examination of their N-terminal domains. If these enzymes had POEP activity, line Ψmap2 would display an apoptotic phenotype. Our data, showing that all map1A-1 mutants were viable, and the observation of aberrant developmental phenotypes in the presence of 1-Met or fumagillin suggest that MAP2 plays an expanded role in higher eukaryotes, as previously suggested. Our data also showed that cytoplasmic NME (i.e. MAP1A + MAP2A + MAP2B activity) as a whole is essential for plant development and that each individual component is dispensable, provided that other components are present. Finally, our data suggest that a true triple MAP KO mutant would be embryo lethal. These findings were confirmed by the occurrence of extremely severe mutant phenotypes in the triple RNAi lines. Moreover, in the absence of MAP1A, MAP2 levels become especially important for developmental progress and root development in particular.

Recently, Kim et al. (2004) has proposed that human MAP2 is not the target for the antiangiogenic effect of fumagillin, suggesting that the drug could also target other important cellular molecules in animal epithelial cells. With the plant system, we found that fumagillin has no effect on wild-type and Ψmap2 KD lines. This implies that the drug has no other antiproliferative target in Arabidopsis, confirming the strong specificity of fumagillin for MAP2, as reported in fungi and animals (Griffith et al., 1997, 1998; Sin et al., 1997). To get more insight into the specificity of fumagillin, we used several other drugs known to be highly specific for MAP2 and obtained similar results (Fig. 4D). Our data show that, in line map1A-1, MAP2 inhibition strongly impairs plant cell elongation/expansion (Fig. 5D) and suggest that it also blocks cell division. This latter conclusion is in line with earlier reports describing the inhibitory effect of the fumagillin analog TNP-470 on animal cell cycle (Abe et al., 1994; Yeh et al., 2000; Zhang et al., 2000; Lien et al., 2004).

A gradient of developmentally abnormal plants was observed in the progeny of Ψmap1A lines grown in the presence of saturating concentrations of fumagillin, with a clear relationship between MAP1A content and phenotype severity. These mutants had a phenotype similar to that observed with map1A-1 plants grown in the presence of various concentrations of fumagillin. However, the roots of these plants were less severely affected than those of map1A-1 KO plants grown in similar conditions. The higher sensitivity of map1A-1 KO plant roots to fumagillin than the Ψmap1A line in the presence of the drug suggests that MAP2 level or activity is limiting in this organ as a whole or in specific cell types. The much later flowering of the map1A-1 KO line replaced in the absence of fumagillin, correlated with the initial concentration of fumagillin, is also consistent with these conclusions. Our results suggest that MAP1A plays a major role in root development and in the transition between the vegetative and reproductive stages. This effect is not due to
reduced MAP2 transcript levels in the roots. MAP2 transcript levels were found to be similar in different organs and these transcripts were the most abundant of the three MAP transcripts. The strong induction and accumulation of all three MAP transcripts, particularly those corresponding to MAP1A, at the beginning of plant development (Fig. 1A), together with the fumagillin-induced stimulation of senescence in map1A-1 KO adult plants and the most severely affected ψmap1A lines, suggest that adequate cytoplasmic NME in the leaves is essential for plant growth. Taking into account our results and those of cytoplasmic MAPs of other eukaryotes, we could speculate on various nonexclusive hypotheses. First, in specific tissues, MAP2 is somehow more strongly inhibited, for instance, by a local higher concentration of free Met, in keeping with our data showing Met-induced inhibition of NME (Fig. 3). Second, there are some specific or fastidious substrates whose accumulation is strictly required for defined plant development pathways, and for which NME can still be ensured by a low concentration of MAP1A but not of MAP2. This is in line with the data indicating that MAP1 and MAP2 exhibit different cleavage efficiencies against the same substrates in yeast (Dummitt et al., 2003). As a result, MAP2s will efficiently ensure the cleavage of these substrates if present at their physiological limiting concentration or if overproduced.

Our studies generate a model of the functions of the three components of cytoplasmic NME from a higher eukaryote. The demand for cytoplasmic NME increases during development, as revealed by the transcriptional induction of all three MAPs, especially MAP1A (Fig. 1A). Transcript levels are strictly controlled and kept constant by the induction of a given MAP in most organs, including those in which MAP1A transcription has been reduced or stopped, as in flowers and siliques. In siliques, to compensate for artificially reduced MAP levels by increasing the levels of other MAPs.

MATERIALS AND METHODS

Plants and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia seeds were surface sterilized and sown on 0.5% Murashige and Skoog (Sigma, St.- Quentin en Yvelines, France) medium supplemented with 0.8% agarose (Becton Dick- inson, Le Pont de Claix, France) and 1% Suc (Sigma). When a MAP2 inhibitor like fumagillin was used, all media, whatever the concentration of the drug, was prepared in 100% methanol as 10-mM solutions. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ozyme, Saint-Quentin en Yvelines, France). The buffers used were those indicated by the manufacturer. Oligonucleotides were synthesized by MWG-BIOTECH (Courtabeuf, France). Nucleotide sequences were determined by the big-dye terminator V3 method, with a 16-capillary ABI PRISM 3100 genetic analyzer (PE- Applied Biosystems, Courtabeuf, France), using DNA templates purified with the QuiAprep mini-prep or QuiAgel quick gel extraction kits (Qiagen, Courtabeuf, France). PCR was performed with Eurobiotaq II (Eurobio, Les Ulis, France), on an Omnis-E thermal cycler (Hybaid, Cogex, Paris), unless otherwise stated. All PCR-derived fragments for cloning were amplified with Pfu DNA polymerase (Stratagene, La Jolla, CA) and sequenced after cloning to check that they were identical to the original fragment. Southern blotting was performed as described elsewhere (Sambrook et al., 1989), using positive membranes (Qiogene, Illkirch, France). The Megaprim eDNA labeling system (Amersham Biosciences, Orsay, France) was used to label probes with [α-32P]-dCTP (Amersham), according to the manufac- turer’s instructions. Hybridization and posthybridization washes were systematically carried out under stringent conditions. Radioactive signals were detected and quantified on a Storm-840 phosphorimager (Amersham), using ImageQuant 5.2 software (Molecular Dynamics, Amersham).

Plant RNAi Constructs

A strategy similar to that previously described by Chuang and Meyerowitz (2000) was followed. First, three RNAi vectors, the first targeting MAP1A, the second targeting both MAP2A and MAP2B, and the third targeting the three cytosolic MAP genes, were constructed. Coding sequences were inserted as inverted repeats on either side of a β-glucuronidase (GUS) gene spacer, the whole sequence being transcribed under the control of a strong promoter (double-p35S) in the binary vector pMS101, facilitating selection on Finale (AgrEvo, Montvale, NJ), a phosphinotrichin herbicide. A 280-bp sequence originating from the 5′ sequence of the cDNA encoding the MAP1A-specific zinc-binding domain was used (nucleotides 206–485, GenBank AF250960) to prime the down-regulation of a nonellar MAP1D gene (see Table I). We used a 359-bp MAP2B DNA sequence (nucleotides 598–956, GenBank AF300880) to target both MAP2A and MAP2B transcripts. This nucleotide sequence displays a high level of identity to the MAP2A sequence, with a significant number of identical stretches of 25 to 30 bp. We therefore fully expected both MAP2s to be targeted via dicer nuclease activity. Second, a construct including inverted cfl sequences (326 bp; nucleotides 298–625, EMBL V085618) was produced to serve as a control. RNAi constructs were generated by a two-step procedure in which the target gene-specific sequences were first amplified and cloned as inverted repeats on either side of an EcoRI/EcoRV fragment containing the GUS gene sequence (nucleotides 2,252–3,317; GenBank M14461) derived from plasmid pPR76. The inverted repeats separated by the GUS spacer sequence were then inserted into a plant transformation and expression vector, pMS101. Gene- specific fragments were amplified, using MAP cDNA fragments (Giglione et al., 2000) or plasmid pPR98 for the neomycin phosphotransferase gene (nptII) for control purposes, as templates. Complete details of the constructions are available (see supplemental material).

Construcs were used for the electrotrotransformation of Agrobacterium strain EHA105 (Hood et al., 1993), which was then used to transform Arabidopsis Columbia plants by the floral-dip method (Clough and Bent, 1998). Transgenic plants (T0) were germinated and selected on sand contin- uously soaked with 10% Finale (AgrEvo) in the greenhouse. Resistant plants were transferred to soil at the two-leaf stage. The control line (cfl) corresponds to a line expressing the nptII inverted repeats instead of the given MAP constructs. Line cfl has no target for nptII inverted repeats.

Materials and General Molecular Biology Methods

All chemicals were purchased from Sigma except TNP-470, ovalicin, and fumagillin, which were kindly provided by J.O. Liu (Baltimore). Fumagillin and its analogs were dissolved in 100% methanol as 10-mM solutions. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ozyme, Saint-Quentin en Yvelines, France). The buffers used were those indicated by the manufacturer. Oligonucleotides were synthesized by MWG-BIOTECH (Courtabeuf, France). Nucleotide sequences were determined by the big-dye terminator V3 method, with a 16-capillary ABI PRISM 3100 genetic analyzer (PE- Applied Biosystems, Courtabeuf, France), using DNA templates purified with the QuiAprep mini-prep or QuiAgel quick gel extraction kits (Qiagen, Courtabeuf, France). PCR was performed with Eurobiotaq II (Eurobio, Les Ulis, France), on an Omnis-E thermal cycler (Hybaid, Cogex, Paris), unless otherwise stated. All PCR-derived fragments for cloning were amplified with Pfu DNA polymerase (Stratagene, La Jolla, CA) and sequenced after cloning to check that they were identical to the original fragment. Southern blotting was performed as described elsewhere (Sambrook et al., 1989), using positive membranes (Qiogene, Illkirch, France). The Megaprim eDNA labeling system (Amersham Biosciences, Orsay, France) was used to label probes with [α-32P]-dCTP (Amersham), according to the manufac- turer’s instructions. Hybridization and posthybridization washes were systematically carried out under stringent conditions. Radioactive signals were detected and quantified on a Storm-840 phosphorimager (Amersham), using ImageQuant 5.2 software (Molecular Dynamics, Amersham).
Semi-quantitative Real-Time RT-PCR

Total RNA was extracted from Arabidopsis 10-DAY-old plants as previously described (Kay et al., 1987). Poly(A) RNA was isolated on oligo(dT) 25 Dynabeads (Dynal, Compiegne, France). We then subjected 200 ng of mRNA to reverse transcription. The cDNA (about 2 ng) was then amplified by PCR, using the specific oligonucleotides. Real-time PCR was optimized with the LightCycler FastStart DNA Master SYBR Green kit (Roche, Meylan, France) for each primer pair (amplification efficiency was always >90%), using a standard cDNA. Each cDNA sample was quantified at least twice, with respect to a standard cDNA quantified in the same conditions. All experiments were performed with a LightCycler and the data were processed with LightCycler version 1.0 quantitative software (Roche). Full details and oligonucleotide sequences are available (see supplemental data).

3D Modeling of the Structures of Cytosolic MAPs from Arabidopsis

The amino acid sequences of AtMAP2A and AtMAP2B were aligned with that of HsMAP2 and the amino acid sequence of AtMAP1A was aligned with that of EcMAP using InsightII software (Accelrys, San Diego). The 3D structures of HsMAP2 bound to fumagillin (PDB entry 1BOA; Liu et al., 2003) and the amino acid sequence of AtMAP1A was aligned with the homology modeler module. In each case, the lowest energy imposed onto the structure of fumagillin or L-Met bound to HsMAP2 or AtMAP2 was selected. The structures of HsMAP2 bound to fumagillin, along with T2 plants from a single wild-type plant were selected. The amino acid sequences of AtMAP2A and AtMAP2B were aligned with that of HsMAP2 and the amino acid sequence of AtMAP1A was aligned with the homology modeler module. In each case, the lowest energy structures of HsMAP2 bound to fumagillin, along with T2 plants from a single wild-type plant were selected. The 3D structures of HsMAP2 bound to fumagillin (PDB entry 1BOA; Liu et al., 2003) and the amino acid sequence of AtMAP1A was aligned with the homology modeler module. In each case, the lowest energy structures of HsMAP2 bound to fumagillin, along with T2 plants from a single wild-type plant were selected. The 3D structures of HsMAP2 bound to fumagillin, along with T2 plants from a single wild-type plant were selected.
methionine aminopeptidases in *Saccharomyces cerevisiae*. Arch Biochem Biophys 398: 87–93


Li X, Chang YH (1995) Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. Proc Natl Acad Sci USA 92: 12357–12361


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Plant Cytosolic N-Terminal Methionine Excision Is Vital

Plant Physiol. Vol. 137, 2005

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