Mutation of the Matrix Metalloproteinase At2-MMP Inhibits Growth and Causes Late Flowering and Early Senescence in Arabidopsis*

Dortje Goldack, Olga V. Popova, and Karl-Josef Dietz‡

From the Department of Physiology and Biochemistry of Plants, Faculty of Biology, University of Bielefeld, D-33615 Bielefeld, Germany

This study characterizes the expression and functional significance of the member of the matrix metalloproteinase (MMP) family At2-MMP from Arabidopsis. By transcript analysis, expression of At2-MMP was found in leaves and roots of juvenile Arabidopsis and leaves, roots, and inflorescences of mature flowering plants showing strong increase of transcript abundance with aging. Cell specificity of expression of At2-MMP was studied by in situ hybridizations in leaves and flowers of Arabidopsis. In leaves, the gene was expressed in the phloem, in developing xylem elements, epidermal cells, and neighboring mesophyll cell layers. In flowers, signals were localized in pistils, ovules, and receptacles. In an Arabidopsis mutant (at2-mmp-1) carrying a tDNA insertion in At2-MMP, neither germination nor development of plants was modified in comparison to the wild type in the juvenile rosette stage. Starting with the onset of shoots, growth of roots, leaves, and shoots was inhibited compared with the wild type, and the plants were characterized by late flowering. Besides the flowering, at2-mmp-1 plants showed fast degradation of chlorophyll in leaves and early senescence. These results demonstrate the involvement of At2-MMP in plant growth, morphogenesis, and development with particular relevance for flowering and senescence.

The family of zinc-dependent endopeptidases that has been particularly characterized in vertebrates is divided into four subfamilies based on structural and functional characteristics: matrix metalloproteinases (matrinins), adamalysins, serralysins, and astacins. All members of these zinc metalloproteinases have a similar structure with the conserved consensus sequence HE^XXXHE^XXXH in their catalytic site and a conserved methionine residue that forms the "Met-turn" structure (1). Members of the subfamily of matrix metalloproteinases are gelatinases, collagenases, and stromelysins (2). Matrix metalloproteinases are synthesized as prepro-enzymes with a signaling peptide targeting the enzyme to the extracellular space. The pre-domain is followed by a pro-peptide with a conserved PRCG(V/N)PD motif that contains the so-called "cysteine switch." This Cys residue ligates the catalytic zinc thus maintaining the latency of the inactive pro-enzymes (3). In vitro, pro-matrix-metalloproteinases can be activated proteolytically by proteinases as well as by mercurial compounds and reactive oxygen, for example, whereas in vivo activation by proteinases is most likely (3). The function of matrix metalloproteinases is the degradation and remodeling of the extracellular matrix (2). The enzymes play a role in development, embryogenesis, and organ morphogenesis but also in wound healing in vertebrates (4). They also participate in pathological processes such as cancer and arthritis (5). In vertebrates, the enzyme activity of matrix metalloproteinases is transcriptionally regulated as well by proteolytic activation of the mature enzyme from the inactive pro-enzyme and by co-secretion with endogenous inhibitors (4).

In addition to vertebrates, matrix metalloproteinases have also been identified from Caenorhabditis elegans (6), sea urchins (7), and from Chlamydomonas reinhardtii with the gamete lytic enzyme that digests the cell wall of gametes (8). The first matrix metalloproteinase (MMP)3 isolated from higher plants was SMEP1 from soybean (9) that was subsequently cloned (10). SMEP1 was shown to be expressed in adult leaves but could not be detected in other plant tissues or in young developing leaves (10). In cucumber the matrix metalloproteinase Cs1-MMP is expressed in leaves during senescence and may participate in programmed cell death (11).

In the Arabidopsis thaliana data base five genomic sequences homologous to matrix metalloproteinases were identified (12). Expression of the Arabidopsis enzymes was detected in roots, leaves, stems, and flowers. Proteolytic activity was demonstrated for one of these proteinases, At1-MMP. At1-MMP hydrolyzed both synthetic peptides and myelin basic protein but not gelatin or casein (12).

Here a detailed characterization of tissue specificity and age dependence of expression of the member of Arabidopsis matrix metalloproteinases At2-MMP is presented. For the first time cell specificity of expression of a plant matrix metalloproteinase was studied by in situ hybridizations and by promoter-reporter gene fusion. Finally, an Arabidopsis mutant carrying a tDNA insertion in the At2-MMP locus was identified and used to demonstrate the physiological role of the gene in plant growth and developmental processes, particularly in flowering and senescence.

EXPERIMENTAL PROCEDURES

Plant Material—Plants of A. thaliana (Columbia) were grown in a growth chamber with 10 h of light (240 μmol quanta m⁻² s⁻¹; 23 °C) and 14 h of darkness (18 °C) with 50% relative humidity. After germination in rock wool soaked with nutrition solution (1.25 mM KNO₃, 1.5 mM Ca(NO₃)₂, 0.75 mM MgSO₄, 0.5 mM (NH₄)₂HPO₄, 72 μM Fe-EDTA micronutrients (see Ref. 13) H and L) the 3-week-old plants were transferred to aerated 5-liter hydroponic tanks. For stress treatment, plants were transferred to nutrition solution containing 50 mM NaCl for 24 h, containing 150 μM CdCl₂ for 48 h, or were sprayed with 45 μM methyl jasmonate in 0.1% ethanol (14) on leaves and inflorescences and

* This work was supported by the Deutsche Forschungsgemeinschaft Sonderforschungsbereich 549. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed. Tel.: 49-521-106-5589; Fax: 49-521-106-6039; E-mail: karl-josef.dietz@biologie.uni-bielefeld.de.

The abbreviations used are: MMP, matrix metalloproteinase; RT, reverse transcriptase; RACE, rapid amplification of cDNA ends.
exposed for 48 h. Non-stressed plants were grown in parallel and harvested at the same time. Plants used for the experiments were in the rosette stage at the age of 4 weeks or were flowering at the age of 10 weeks. Plants were harvested 5 h after the onset of illumination.

The At2-mmp-1 mutant was identified from the seed pool 02_13 (N40069; insert number 02_15_06) obtained from the Nottingham Arabidopsis Center (UK).

RNA Extraction and RT-PCR—RNA was isolated by guanidinium isothiocyanate extraction from leaves, roots, and inflorescences from A. thaliana (15).

cDNA synthesis was performed with SuperScript II reverse transcriptase (Invitrogen) from each 3 μg of total RNA using oligo(dT) primers. The sequences of the forward and reverse primers used for amplifying the full-length sequences of At2-MMP (GenBank™ accession number AC002062) were 5'-CCACCATGAGTTTGTGTTTCCGCTT-3' (S1) and 5'-CTACGGTAAGAACACGACG-AATCC-3' (S2). For PCR, Advantage2 polymerase mix (CLONTECH) was used, and the sequence was cloned in the vector PCR2.1-TOPO (Invitrogen). For RT-PCR expression studies, the following forward and reverse primers from the coding region of At2-MMP were used: 5'-GGTGAAGCTTGAGGCCTTCA-3' and 5'-ATCTGCGTCTAGGTGGAA-3'. With this combination a 446-bp fragment was amplified. The fragments were cloned in PCR-TOPO II (Invitrogen). For expression studies with the non-coding region of the transcripts, the following forward primers were used: 5'-AAGGCAAGCACAGG-GAA-3' and 5'-CACACAGAACGCTGAA-3' that amplify a 482-bp fragment. The PCR settings were 1 cycle 94 °C for 1 min 30 s, 1 min 94 °C, 1 min 55 °C, 2 min 72 °C, and a final extension at 72 °C for 10 min. For the transcripts, cycle numbers in the linear range of amplification were determined and used for the expression analyses by RT-PCR. At2-MMP was amplified with 35 cycles from tissue of 4-week-old plants and with 30 cycles from 10-week-old plants. For direct comparison of expression in juvenile and flowering plants, 33 cycles were used. In stress experiments At2-MMP transcripts were amplified with 28 cycles from leaf and root tissue and with 30 cycles from inflorescences of 10-week-old Arabidopsis plants, whereas 34 cycles were used for 4-week-old plants. The PCR products from RT-PCR amplifications were separated on 1.7% (w/v) agarose gels and stained with ethidium bromide. Photographic documentation was performed with a gel documentation system (INTAS, Göttingen). Absence of genomic DNA from the cDNA preparations was verified as described previously (16).

For identification of the at2-mmp-1 mutant, the following tDNA-specific primers were used: 5'-GGTGCAGCAAAACCCACACTTTGTTTGGCCGACACTCCTTACC-3' and 5'-ATCTGCGTCTAGGTGGAA-3'. 3'-RACE amplifications, total RNA was isolated from rosette leaves of 10-week-old at2-mmp-1 plants as described above, and cDNA synthesis was performed with SuperScript II reverse transcriptase (Invitrogen) using a 3'-RACE anchor primer (SMART RACE, CLONTECH). PCR was performed with the primer dSm5 and the SMART RACE primer mix (CLONTECH). The DNA fragments amplified were cloned in PCR 2.1 (Invitrogen). Sequencing was performed at the sequencing facility of the University Bielefeld.

Extraction of Genomic DNA, Promoter Constructs, Plant Transformation, and Histochemical Detection of Reporter Gene Activity—Genomic DNA was extracted as described previously (17). A 1249-bp genomic DNA fragment upstream of the start methionine for translation was amplified by PCR with Advantage2 Polymerase mix (CLONTECH) with the following primer pair: 5'-CTTATTTGTCCTCCGCCCCACCTTACGTCGTC-3' and 5'-GGTGTTTTTGGATTGATGGATGGCAAC-3'. The amplified DNA fragment was cloned in the vector pBlue-TOPO (Invitrogen) upstream of the lacZ reporter gene.

10-Week-old Arabidopsis plants were transformed with the pAt2-MMP::lacZ constructs by particle bombardment (PDS1000, Bio-Rad). 0.25 μg of plasmid DNA was precipitated on 1.6-μm gold particles for each bombardment according to the protocol of the manufacturer. For the bombardments 1200 pounds/square inch rupture discs were used. After bombardment, the plants were allowed to recover under normal growth conditions for 24 h. Leaves from the bombarded plants were vacuum-infiltrated with 1% glutaraldehyde in Z buffer at room temperature for 1 h to inhibit endogenous esterase activity (18, 19). Tissues were cut into three times in Z buffer and then vacuum-infiltrated with staining solution as described previously (19) with 0.1% 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-gal) at room temperature for 1 h followed by incubation at 37 °C for 14 h. Finally, the leaves were treated with Z buffer and incubated with 70 and 100% ethanol to remove chlorophyll. Leaves from plants bombarded with the promoter-less vector pBlue-TOPO did not show β-galactosidase activity.

In Situ Hybridization and Microscopic Images—Tissue sections from inflorescences and leaves from 10-week-old Arabidopsis plants were fixed with formaldehyde-acetic acid, dehydrated, and embedded with Paraplast Plus (Fisher) as described previously (20). Sections of 10 μm were cut on a Leica microtome and stained with toluidine blue. The leaf material used for the tissue sections was taken from the middle of the leaf blades half-way between the main vein and the edge of the leaves.

RESULTS

Tissue-specific and Developmental Differences in At2-MMP Expression—The genomic sequence of the matrix metalloproteinase At2-MMP has recently been identified in the Arabidopsis data base by Maitland et al. (12) under the GenBank™ accession number AC002062 on chromosome 1. The genomic sequence of At2-MMP is intronless and contains an open reading frame of 378 amino acids. The predicted polypeptide shows the prepro-enzyme structure typical for zinc endopeptidases with a signal peptide, a pro-peptide, and a catalytic domain with the signature motif HEXXXHXXXHXH and the conserved methionine residue that forms the Met-turn structure (1). In a converse manner to At4-MMP, but similar to the other matrix metalloproteinase homologues identified in Arabidopsis, At2-MMP contains a non-cleavable C-terminal trans-membrane domain (compare with Ref. 12). Analysis using PSORT (psort.imb.u-tokyo.ac.jp) proposed localization of At2-MMP in the plasma membrane.

The expression of At2-MMP was studied by RT-PCR amplification in juvenile 4-week-old Arabidopsis plants in the rosette stage and in 10-week-old flowering Arabidopsis plants. A fragment from the coding region of the gene was amplified by gene-specific primers, and actin was amplified as a loading control. In 4-week-old Arabidopsis plants, transcripts of At2-MMP were detected in leaves and roots with a higher transcript abundance in the leaf tissue (Fig. 1A). In 10-week-old flowering A. thaliana plants, At2-MMP was expressed in leaves, roots, and inflorescences. The highest signal strength was obtained from root tissue, and in leaves and flowers a lower level of expression was detected (Fig. 1B).

To analyze developmental effects, the transcript abundance of At2-MMP was compared in leaves and roots of juvenile and flowering Arabidopsis. In both tissue types the expression of the metalloproteinase was strongly induced with aging of the plants (Fig. 1C).

Histochemical Analysis of Promoter Activity and in Situ Hybridization—The tissue specificity of At2-MMP expression was studied by histochemical detection of promoter activity in cauline leaves and rosette leaves of 10-week-old Arabidopsis plants. A genomic fragment 5′-upstream of the start methionine of the At2-MMP coding sequence was fused to the β-galactosidase reporter gene, and Arabidopsis plants were transformedbiologically with the promoter-reporter-gene construct. The plants were allowed to recover under normal growth conditions before β-galactosidase activity induced by the At2-MMP promoter was histochemically detected. Reporter gene expression could be found in both cauline and rosette leaves with signals of similar staining intensity. The signals were distributed on the leaf blade without preference of a particular leaf area (Fig. 2A).

For analysis of cell specificity of At2-MMP transcription abundance, in situ hybridizations were performed in leaf and flower
Matrix Metalloproteinase in A. thaliana

**Fig. 1.** Tissue specificity and age dependence of expression of At2-MMP in Arabidopsis. Transcript levels were quantitated in juvenile 4-week-old Arabidopsis plants and flowering 10-week-old Arabidopsis plants. A fragment of the coding region of At2-MMP was amplified by RT-PCR. Actin was amplified as a loading control. A, 4-week-old Arabidopsis plants. L, leaves; R, roots (35 PCR cycles). B, 10-week-old Arabidopsis plants. L, leaves; R, roots; F, inflorescences (30 PCR cycles). C, L4, leaves of 4-week-old plants; L10, leaves of 10-week-old plants; R4, roots of 4-week-old plants; R10, roots of 10-week-old plants (35 PCR cycles).

Cross-sections from 10-week-old Arabidopsis plants using a fragment from the coding region of the gene as a probe. In flowers, signals were detected in the receptacle in the two to three mesophyll layers neighboring the epidermis but was not detectable in sepals and petals. Strong expression of At2-MMP occurred in the gynoecium in the cell layers neighboring the epidermis with signal intensities increasing toward the styles. Furthermore, strong hybridization of antisense-At2-MMP RNA could be observed in ovules (Fig. 2, C–F). In leaves, expression of the matrix metalloproteinase was detected in phloem cells next to the metaxylem and in selected protoxytem cells. Moreover, epidermal cells and mesophyll cell layers toward the leaf surface were showing At2-MMP signals (Fig. 2, G and H).

**Expression of At2-MMP in Response to Methyl Jasmonate, Cadmium, and NaCl**—The regulation of At2-MMP transcription in response to methyl jasmonate, to the heavy metal cadmium, and to salt stress was studied by RT-PCR amplification. Methyl jasmonate is known to induce pathogenesis-related and wound responses in plants (21) and was applied to 4- and 10-week-old Arabidopsis plants by spraying of leaves, stems, and inflorescences as a 45 μM solution. The heavy metal cadmium is a highly toxic abiotic stressor that inhibits plant growth (22) was added at 150 μM to the nutrition solution for 48 h. For salt stress, the nutrition solution was supplemented with 50 mM NaCl, and the plants were exposed for 24 h.

In rosette leaves of 4-week-old Arabidopsis plants, both methyl jasmonate treatment and exposure to cadmium increased the transcript level of At2-MMP, whereas the signal strength did not change in response to NaCl. Conversely, in roots the transcription was stimulated by NaCl but was not modified by jasmonate and cadmium (Fig. 3). In 10-week-old plants, the At2-MMP transcript level in roots was not affected by the three stressors. In leaves and inflorescences the expression was not modified by jasmonate (Fig. 4) and NaCl (not shown) but was strongly inhibited by exposure to cadmium (Fig. 4).

**Characterization of a tDNA Knock-out Mutant for At2-MMP**—The tDNA insertion mutant at2-mmp-1 was identified from a seed pool that was obtained from the Nottingham Arabidopsis Center, UK (SLAT collection, insert number 02_13_06), by PCR using a combination of sequence-specific and tDNA-specific primers. The identified F1 at2-mmp-1 plant was self-pollinated, and from the F2 generation 12 plants homozygous for at2-mmp-1 were PCR-based identified for further characterization.

From genomic DNA of the F1 plant a PCR fragment was amplified with the At2-MMP sequence-specific primer S1 and the tDNA-specific primer T2. The amplified DNA fragment was
Matrix Metalloproteinase in A. thaliana

FIG. 3. Expression of At2-MMP in juvenile Arabidopsis in response to methyl jasmonate, cadmium, and NaCl. Transcript amounts were quantitated by RT-PCR using fragments of the coding region of the gene. Actin was amplified as a loading control. A, Lc, leaves from non-stressed control plants; LCd, leaves from plants treated with 150 μM CdCl₂ for 48 h; LJa, leaves from plants treated with 45 μM methyl jasmonate for 48 h; Rc, roots from non-stressed control plants; RCd, roots from plants treated with 150 μM CdCl₂ for 48 h; RJa, roots from plants treated with 45 μM methyl jasmonate for 48 h. B, Lc, leaves from non-stressed control plants; Ls, leaves from plants treated with 50 mM NaCl for 24 h; Rs, roots from plants treated with 50 mM NaCl for 24 h.

cloned, and sequence analysis revealed the integration of the tDNA into the At2-MMP locus at bp 415 of the open reading frame of At2-MMP. From genomic DNA isolated from the at2-mmp-1 mutant plants, the full-length coding DNA sequence could not be amplified using the S1-S2 primer combination. This difference in obtaining full-length At2-MMP PCR products from wild type genomic DNA but not from at2-mmp-1 plants allowed to distinguish clearly between the wild type and the mutant plants and indicated that the at2-mmp-1 plants analyzed were homozygous for the tDNA insertion without the mutant plants and indicated that the at2-mmp-1 plants analyzed were homozygous for the tDNA insertion without carrying the wild type gene (Fig. 5A). By using the tDNA-specific primer dSpm8, a single PCR product was obtained by 3'-RACE PCR amplifications from cDNA synthesized from RNA of at2-mmp-1 mutant plants (Fig. 5B). Sequencing of the PCR product revealed its identity as chimeric DNA composed of the 3’-end of the inserted tDNA as well as the 3’-end of the At2-MMP coding region and demonstrated integration of the cDNA up to bp 412 at the 3’-end of the gene (Fig. 6). The results from the 3’-RACE experiments show that At2-MMP carrying the tDNA insertion is expressed in at2-mmp-1 plants. Furthermore, obtaining only one PCR-product from the transcript population of at2-mmp-1 plants by using tDNA-specific primers suggests that the mutant plants carry a single tDNA insertion.

Phenotypic Effects of the Mutation of the At2-MMP Locus—There was no difference of germination time and germination rate observable between wild type plants and the at2-mmp-1 mutants. The development of the second leaf pair was slower in the mutant plants than in the wild type, whereas further development was not modified in comparison to the wild type plants during the juvenile rosette stage. Starting with the development of shoots, growth of at2-mmp-1 plants became significantly slower than that of the wild type (Fig. 7). At the age of 6 weeks root length of the mutant was about 60% of the wild type; leaf length was about 70%, and shoot length was about 15%. The development of flowers started 2–3 weeks later in the mutant than in the wild type under the growth conditions used in our experiments. The final length reached by at2-mmp-1 plants was about 80% of the wild type for roots and...
about 70% for leaves and shoots (Fig. 8). At the age of 10 weeks the mutant plants showed fast degradation of chlorophyll and pronounced senescence in both rosette and cauline flowers, whereas wild type plants grown in parallel showed initial signs of senescence in the oldest rosette leaves but no chlorophyll degradation in younger rosette and cauline leaves (Fig. 9).

Transverse sections from rosette leaves of 10-week-old wild type Arabidopsis plants and at2-mmp-1 revealed reduced cell size and thus indicated inhibition of elongation in at2-mmp-1 compared with wild type plants (Fig. 10).

**DISCUSSION**

Expression of At2-MMP Is Developmentally Regulated—In animals, matrix metalloproteinases are the major group of proteinases that mediate the turnover of components of the extracellular matrix (4). Degradation of the extracellular matrix has an important role for physiological processes as embryogenesis, organ morphogenesis, and bone remodeling, for example (5). Unregulated enzyme activity of matrix metalloproteinases is involved in the development of diseases including cancer, arthritis, and atherosclerosis (4). There are 20 soluble members of matrix metalloproteinases known that are secreted to the extracellular space, and furthermore, there have been 5 membrane-anchored matrix metalloproteinases described that have a predicted localization in the plasma membrane (see Refs. 5 and 23; psort.ims.u-tokyo.ac.jp).

In contrast to animals, little is known on the matrix metalloproteinase homologues in plants. In this study At2-MMP was chosen as one of the five matrix metalloproteinases that were recently identified in the Arabidopsis data base (12) for a detailed analysis of expression and physiological function.

Transcript analysis shows expression of At2-MMP in all tissues investigated, i.e. roots, leaves, and inflorescences. In both leaf and root tissue expression of At2-MMP was developmentally controlled with strong induction in mature flowering Arabidopsis. Maidment et al. (12) studied the expression of five Arabidopsis metalloproteinases in 2-week-old plants and found transcripts in leaves, stems, and flowers with different gene-specific transcript levels. At2-MMP was detected with stronger expression in roots than in flowers and leaves corresponding to the expression pattern that we found in flowering 10-week-old Arabidopsis plants in this study, whereas in juvenile 4-week-old plants At2-MMP transcript abundance was higher in leaves than in roots. In contrast to At2-MMP, Maidment et al. (12) found expression of At5-MMP to be strongest in stems and At3-MMP had higher transcript abundance in leaves and roots than in flowers and stems. In a converse manner, the matrix metalloproteinase SMEP1 from soybean was only detected in leaf tissue starting at the age of 10 days. Expression increased up to the age of 20 days and increased slightly thereafter. The enzyme was neither expressed in young leaves nor in other
for metalloproteinases but not gelatin and casein. Besides, the At1-MMP activity was inhibited by TIMP1 and TIMP2 as well as by the metalloproteinase hydroxamate inhibitor BB94 (12).

According to the ability of plant metalloproteinases to cleave similar substrates as the animal enzymes, a role of the plant proteases in defense against pathogens seemed plausible. Thus, metalloproteinases might be involved in proteolysis of foreign proteins in the plant extracellular matrix. In addition, members of the family of the plant subtilisin-like proteases were transcriptionally activated by pathogen attack in tomato (25, 26) and by treatment with methyl jasmonate that mediates wounding and pathogen responses in plants (21).

In this study we compared the expression of At2-MMP in response to methyl jasmonate, the highly toxic heavy metal cadmium, and NaCl in developing 4-week-old rosette plants and in mature 10-week-old flowering plants at the border to senescence. Our results demonstrated that expression of At2-MMP is not regulated as a general stress response in Arabidopsis but is tightly controlled in a tissue-responsive way with developmental differences. In 4-week-old plants leaf tissue but not roots showed transcriptional activation of the enzyme in response to methyl jasmonate and cadmium, whereas in root tissue expressional up-regulation was observed specifically in response to salt stress. In mature plants presenting the main developmental phase of At2-MMP expression, transcription of the enzyme was inhibited in inflorescences and leaves by cadmium treatment but was not modified by the metal in roots as well as in response to methyl jasmonate and salinity in all tissues tested. According to these data we conclude that At2-MMP plays a minor role in general stress responses in Arabidopsis and is particularly not of significant importance for adaptation to wounding stress or pathogenesis response.

**Physiological Characterization of an At2-MMP Mutant**—The extracellular matrix of plants consists by up to 10% of the dry weight of proteins including enzymes such as hydrolases and peroxidases, pathogenesis-related proteins, signal sensing, and structural proteins (27). Physiological roles of extracellular proteolytic processes and particularly of matrix metalloproteinases rarely have been investigated in plants but are well documented in animal systems.

In mice, knock-out mutants have been generated and characterized for several matrix metalloproteinases. Interestingly, in knock-out mice with defects of soluble MMPs some developmental phenotypes were detected (23). In contrast, knock-out mutants of the membrane-type MT1-MMP were characterized by dwarfism, arthritis, and fibrosis, for example (28). By overexpression of seven different soluble matrix metalloproteinases, morphogenesis was not affected in Madin-Darby canine kidney cells, whereas three membrane-type MMPs accelerated, disrupted, or modified branching tubulogenesis in these cells (29). These data demonstrated that in animal systems membrane-type MMPs have a key role in pericellular proteolysis of the extracellular matrix including its remodeling and are essential for development, cell invasion, and morphogenesis (28, 29).

In the present study, an Arabidopsis mutant of the membrane-type At2-MMP was identified carrying a tDNA insertion. The mutant phenotype showed the involvement of the enzyme in growth and development of maturing plants. Roots as well as leaves, shoots, and inflorescences exhibited growth inhibition and retarded development. Furthermore, the mutant plants were characterized by late onset and slow development of flowers, and their final size was reduced compared with wild type plants. Interestingly, aging of the plants was not slowed down in comparison to the wild type, but the mutation caused acceleration of senescence. Based on expressional studies that re-
revealed Cs1-MMP transcripts only in senescing cucumber leaves, Delorme et al. (11) suggested an involvement of Cs1-MMP in programmed cell death. The authors hypothesized that the enzyme may contribute to proteolysis of cell residues, for example (11). Our data demonstrate an earlier onset of senescence and cell death in at2-mmp-1 mutants than in wild type plants as well as growth inhibition of organs. Apparently, the activity of At2-MMP is related to a delay of senescence and programmed cell death and is necessary for regular plant growth and development.

In general, the physiological functions of extracellular proteolytic processes in plants may include the remodeling of the extracellular matrix during growth and development. Turnover of extracellular matrix proteins could be involved in the generation of secondary cell walls during plant maturation, formation of secondary plasmodesmata, of vascular xylem elements, and intercellular lytic spaces, as well as regulation of receptor interactions and signal transduction by receptor modification. Interestingly, At2-MMP was mainly expressed in maturing flowering plants, and phenotypic differences of the at2-mmp-1 mutant from wild type plants were particularly pronounced starting with the plant shooting and onset of flowers. Our data demonstrated that extracellular proteolytic processes mediated by At2-MMP are involved in growth and development during the developmental phase of flowering. However, it is likely that morphogenesis and growth of juvenile plants requires extracellular proteolytic processes as well. Members of other families of extracellular proteases may substitute for the function of matrix metalloproteinases in the juvenile developmental phase of plants. Candidate enzymes are members of extracellular subtilisin-like proteases, for example, that have been shown to be preferentially expressed in young developing plants (30). Future studies on the regulation of age-dependent expression, identification of signal transduction pathways, and in vivo studies will further clarify our understanding on the significance of extracellular proteases for plant morphogenesis and development.

Finally, the hypothesis of a more specific function of At2-MMP may be suggested which involves receptor shedding or activation and will have to be tested. Initiation of senescence and programmed cell death involves hormonal stimuli such as ethylene and salicylate (31, 32); other stimuli such as cytokinins counteract the development of senescence (33). The stimuli are sensed by receptors that in turn activate signaling cascades and trigger or silence the senescence program. Assuming the constitutive expression of such a receptor, and either its continuous degradation or specific activation by MMP, MMP-deficient tissue could be hypersensitized leading to inhibited growth and premature activation of the senescence program (34). The phenotype of the at2-mmp-1 mutant plants is in agreement with such a hypothesis.

REFERENCES

1. Stocker, W., and Bode, W. (1995) Curr. Opin. Struct. Biol. 5, 383–390
2. Borkakoti, N. (2000) J. Mol. Med. 78, 261–268
3. Nagase, H., and Woessner, J. F. (1999) J. Biol. Chem. 274, 21491–21494
4. Brew, K., Dinakarpandian, D., and Nagase, H. (2000) Biochim. Biophys. Acta 1477, 267–283
5. Wang, Y., Johnson, A. R., Ye, Q. Z., and Dyer, R. D. (1999) J. Biol. Chem. 274, 35045–35049
6. Wada, K., Sato, H., Kinoh, H., Kitaj, M., Yamamoto, H., and Seiki, M. (1998) Gene (Amst.) 211, 57–62
7. Lepage, T., and Gache, C. (1999) EMBO J. 9, 3003–3012
8. Kinoshita, T., Fukuzawa, H., Shimada, T., Saito, T., and Matsuda, Y. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4693–4697
9. Graham, J. S., Xiong, J., and Gillikin, J. W. (1991) Plant Physiol. 97, 786–792
10. Pak, J. H., Liu, C. Y., Huangpu, J., and Graham, J. S. (1997) FEBS Lett. 404, 283–288
11. Delorme, V. G., McCabe, P. F., Kim, D. J., and Leaver, C. J. (2000) Plant Physiol. 123, 917–927
12. Maidment, J. M., Moore, D., Murphy, G. P., Murphy, G., and Clark, I. M. (1999) J. Biol. Chem. 274, 34706–34710
13. Ostrem, J. A., Olson, S. W., Schmitt, J. M., and Bohnert, H. J. (1987) Plant Physiol. 84, 1270–1275
14. Penninekx, I. A. A. M., Thomma, B. P. H. J., Buchala, A., Metraux, J. P., and Broekaert, W. F. (1998) Plant Cell 10, 2103–2113
15. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
16. Goldack, D., and Dietz, K. J. (2001) Plant Physiol. 125, 1643–1654
17. Gustinich, S., Manfolleti, G., del Sal, G., and Schneider, C. (1991) BioTechniques 11, 298–302
18. Teeri, T. H., Lehvasliho, H., Franck, M., Uotila, J., Heino, P., Palva, E. T., Van Montagu, M., and Herrera-Estrella, L. (1989) EMBO J. 8, 343–350
19. Kononowicz, H., Wang, Y. E., Habeck, L. L., and Gelvin, S. B. (1992) Plant Cell 17, 27–27.
20. McKhann, H. I., and Hirsch, A. M. (1993) in Methods in Plant Molecular Biology and Biotechnology (Glick, B. R., and J. E. Thompson, J. E., eds) pp. 179–205, CRC Press, Inc., Boca Raton, FL
21. Creelman, R. A., and Mullet, J. E. (1997) Annu. Rev. Plant Physiol. 48, 355–381
22. Sandalio, L. M., Dalurzo, H. C., Gomez, M., Romero-Puertas, M. C., del Rio, I. A. (2001) J. Exp. Bot. 52, 2115–2126
23. Quaranta, V. (2000) J. Cell Biol. 149, 1167–1170
24. McGeehan, G., Burkhart, W., Anderegg, R., Beckerer, J. D., Gillikin, J. W., and Graham, J. S. (1992) Plant Physiol. 99, 1179–1183
25. Tornero, P., Caneviero, V., and Vera, P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6332–6337
26. Jordi, L., Cams, J., Caneviero, V., and Vera, P. (1999) J. Biol. Chem. 274, 2360–2365
27. Dietz, K. J. (2000) Prog. Bot. 62, 215–237
28. Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, A., Mankani, M., Robey, P. G., Poole, A. R., Pidoux, I., Ward, J. M., and Birkedal-Hansen, H. (1999) Cell 99, 81–89
29. Hotary, K., Allen, E., Puntarieri, A., Yana, I., and Weiss, S. J. (2000) J. Cell Biol. 149, 1309–1323
30. Messdaghi, D., and Dietz, K. J. (2000) Biochim. Biophys. Acta 1480, 107–116
31. Johnson, P. R., and Ecker, J. R. (1998) Annu. Rev. Genet. 32, 227–254
32. Heath, M. (2000) Plant Mol. Biol. 44, 321–334
33. Brault, M., and Maldiney, R. (1999) Plant Physiol. Biochem. 37, 403–412
34. Beers, E. P., Wolfenden, B. J., and Zhao, C. (2000) Plant Mol. Biol. 44, 399–415

---

2 D. Goldack, P. Vera, and K. J. Dietz, non-published results.