Arabidopsis thaliana matrix metalloproteinases (MMPs) in plant defense against pathogens

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List of Abbreviations

ABA	Abscisic acid
aba2	ABA-deficient2
abi2	ABA-insensitive 2
Arabidopsis	*Arabidopsis thaliana*
ATS	*Arabidopsis thaliana* medium with sucrose
BAK1	BRI1-associated kinase 1
BRI1	Brassinosteroid insensitive 1
Ca^{2+}	Calcium ions
CaMV 35S	Cauliflower mosaic virus promoter 35S
Cb	Carbenicilli
cDNA	Complementary DNA
CDs	Coding sequence
cfu	Colony forming unit
CM	Complex medium
Col-0	*Arabidopsis thaliana* ecotype Columbia-0
Coin1	Coronatine-insensitive 1
C-terminal	Carboxy terminal
d	Day(s)
DAMP	Damage-associated molecular patterns
dai	Day after inoculation
DEPC	Diethylpolycarbonate
DMSO	Dmethyl sulfoxide
DM	double mutant
dNTPs	Desoxyribonucleotides
ECM	Extracellular matrix
EDS1	Enhanced disease susceptibility 1
EDTA	Ethylenediaminetetraacetic acid
EFR	Elongation factor Tu receptor
EF-Tu	Elongation factor thermo-unstable
ein2	Ethylene insensitive2
ERF	Ethylene-responsive factor
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
EV	Empty vector
Flg22	A 22-amino-acid-long peptide derived from flagellin
FLS2	Flagellin Sensitive 2
g	gram(s)
GFP	Green fluorescent protein
GPI	glycosylphosphatidylinositol
h	Hour (s)
hai	Hour(s) after inoculation
HR	Hypersensitive response
H_2O_2	Hydrogen peroxide

Hpx	Hemopexin
HR	Hypersensitive response
ics1	Isochorismate synthase 1
IPTG	Isopropyl-β-D-thiogalactopyranoside
IWF	Intercellular washing fluid
JA	Jasmonic acid
jar1	Jasmonic acid resistant 1
jin1	Jasmonate-insensitive 1
kDa	Kilodalton(s)
l (L)	Litre(s)
LB	Left border primer
LB medium	Luria-Bertani medium
log	Decimal logarithm
LP	Left primer
LPS	Lipopolysaccharides
LRR	Leucine-rich repeat
m	Milli
M	Molar (mol/l)
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
min	Minute(s)
MMP	Matrix metalloproteinase
MPI	Matrix metalloproteinase inhibitor
mRNA	Messenger RNA
MS medium	Murashige and Skoog medium
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NahG	Bacterial salicylate hydroxylase gene
NASC	Nottingham *Arabidopsis* stock centre
NB	Nucleotide binding
NCBI	National center for biotechnology information
NPR1	Non-expressor of pathogenesis-related genes 1
N-terminal	Amino terminal
PAD3	Phytoalexin deficient 3
PAD4	Phytoalexin deficient 4
PAMPs	Pathogen associated molecular patterns
PAGE	Polyacrylamide gel-electrophoresis
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDB	Protein data bank
½ PDB	½ Potato dextrose broth
PDF	Pathogen-defense genes
pH	Negative decimal logarithm of the H+ concentration
PR1	Pathogenesis related protein 1

PRRs	Pattern recognition receptors
Pst	*Pseudomonas syringae* pv. *tomato* DC3000
Psta	*Psuedomonas syringae* pv. *tabaci*
PsT1	*P. syringae* pv. *tomato* T1
PTI	PAMP-triggered immunity
pv.	Pathovar
Q-PCR	Quantitative polymerase chain reaction
R-gene	Resistance gene
RNA	Ribonucleic acid
Rif	Rifampicin
ROS	Reactive oxygen species
RP	Right primer
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SA	Salicylic acid
SDS	Sodium dodecyl sulfate
SMEP1	Soybean metalloendoproteinase 1
SOC	Super optimal broth medium with catabolite repression
Spc	Spectinomycin
T-DNA	transfer DNA
TIMP	Tissue inhibitor of matrix metalloproteinases
tir1	Transport inhibitor response 1
TM	transmembrane
TLR	Toll-like receptor
TTSS	Type III secretion system
UBQ5	Ubiquitin5
U	(enzymatic) Unit
Zn^{2+}	Zinc ion
°C	Degree Celsius
5’	upstream region of gene or sequence
3’	downstream region of gene or sequence
::	fused to gene fusion constructs
%(v/v)	volume percent
%(w/v)	weight/volume percent
µ	Micro

1. Introduction

1.1 Matrix metalloproteinases (MMPs) in mammals

Matrix metalloproteinases (MMPs) are a group of highly conserved proteolytic enzymes containing zinc ion in the active site, which was first described in 1962 (Gross & Lapiere, 1962). They are classified as the matrixin subfamily of zinc metalloprotease family M10 according to MEROPS database. There are 23 MMPs found in human (Nagase *et al.*, 2006). MMPs are secreted or attached to the cell surface, thereby largely restricting their activity on the modulation of membrane proteins or extracellular proteins in the secretory pathway. They are key players in the remodelling of the extracellular matrix, associated with a number of physiological and pathological processes (Birkedal-Hansen *et al.*, 1993; Stamenkovic, 2003; Vu & Werb, 2000; Parks *et al.*, 2004). Non-matrix substrates were also found for MMPs and indicated more sophisticated roles for MMPs in regulation of cellular behaviour and cell-cell communication (McCawley & Matrisian, 2001).

1.1.1 Structure of MMPs in mammals

A typical MMP consists of a signal peptide, a propeptide, a catalytic domain, a linker peptide and a hemopexin (Hpx) domain (Nagase *et al*., 2006). Exceptions are MMP-7, MMP-26 and MMP-23 which lack the linker peptide and Hpx domain. The "cysteine switch" motif PRCGXPD in the propeptide and the zinc binding motif HEXXHXXGXXH in the catalytic domain are shared structural signatures for all MMPs (Nagase & Woessner, 1999). The conserved cysteine in the propetide coordinate with the catalytic zinc ion and this Cys-Zn^{2+} coordination maintains the latency of proMMPs (Van Wart & Birkedal-Hansen, 1990). The three histidines in the zinc binding motif are responsible for coordinating the catalytic zinc ions (Visse & Nagase, 2003). The catalytic domain also contains a conserved methionine, forming a ''Met-turn'' eight residues after the zinc binding motif, which forms a hydrophobic basis for the zinc ion and the three liganding histidine residues (Bode *et al.*, 1993). The C-terminal Hpx domain has a function in modulating substrate specificity and binding to tissue inhibitors of metalloproteinases (TIMPs) (Parks *et al*., 2004).

Based on the differences of domain compositions, MMPs have been subdivided into different groups as shown in Fig.1 – 1. For instance, those MMPs that are secreted and bound to the plasma membrane are distinct by the intrinsic motifs, such as a transmembrane domain, a glycosylphosphatidylinositol (GPI) anchor or an N-terminal signal peptide. The transmembrane domains and GPI anchors are connected to the hemopexin domain with a

short linker. However, there are few agreements about how these subdivisions should be assigned in the field (Parks *et al*., 2004).

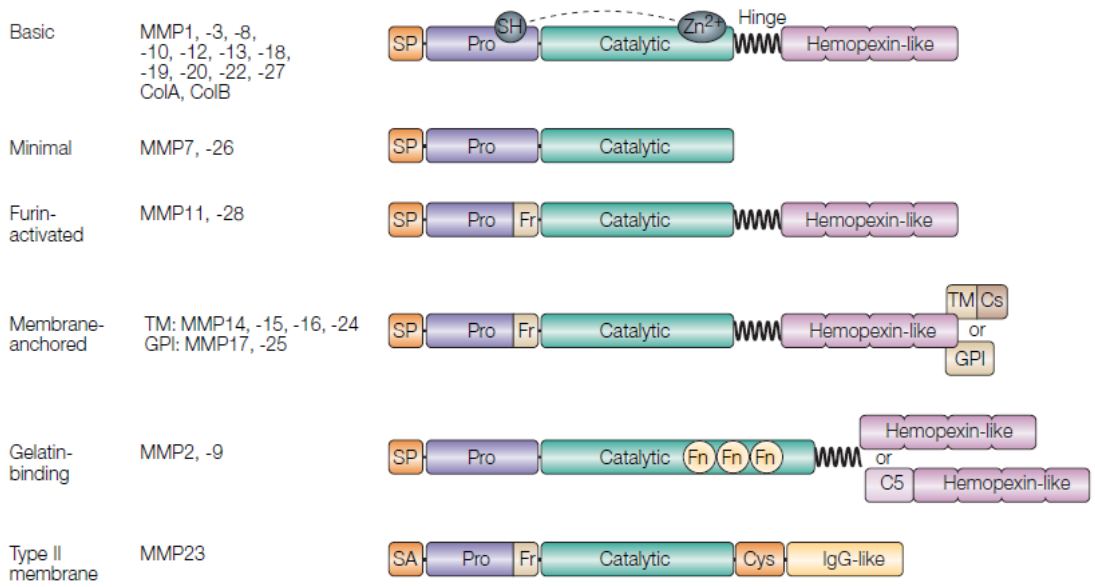


Fig.1 - 1. Domain structure of the mammalian MMP family. C5, type-V-collagen-like domain; Col, collagenase-like protein; Cs, cytosolic; Cys, cysteine array; Fn, fibronectin repeat; Fr, furin-cleavage site; Pro, pro-domain; SH, thiol group; SP, signal peptide; Zn, zinc (Parks *et al*., 2004).

1.1.2 Regulation of MMPs activities in mammals

Like other secreted proteinases, the MMP activities are regulated at four points: gene expression, compartmentalization, pro-enzyme activation and enzyme inactivation (Parks *et al*., 2004). In general, MMPs are not expressed in normally healthy tissues but detected in activated cells such as cells in remodelling process, diseased tissues or cultured cells. The transcriptional regulation of MMPs is tightly controlled by internal/external signals in a temporal and spatial manner. Once MMPs are synthesized, they are secreted as inactive proteolysis enzymes (Nagase & Woessner, 1999; Woessner, 1991). The activation of ProMMPs requires the disruption of the Cys-Zn^{2+} interaction and removal of the propeptide, which is achieved by proteinases or by non-proteolytic agents (Visse & Nagase, 2003). Several MMPs contain one RXKR or RRKR sequence linking the pro- and catalytic domains. Such a short sequence is known as a furin cleavage site and acts as a target site for pro-protein convertases or furins (Fig.1 - 1). Compartmentalization discriminately places the MMPs in distinct pericellular spaces and results in local enrichment of the enzyme targeting their specific substrates (Nagase *et al*., 2006). The localization of MMPs under physiological conditions often dictates their biological functions.

There are four endogenous TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) in the tissues which were shown to inhibit the MMP activity by binding to the catalytic sites (Brew & Nagase, 2010; Visse & Nagase, 2003). Homologues of the TIMPs are distributed widely among both invertebrate and vertebrate animals. TIMPs are secreted proteins of approximately 20-29 kDa and have distinct N-terminal domain and C-terminal domain (Parks *et al*., 2004; Sternlicht & Werb, 2001, Visse & Nagase, 2003). The N-terminal domain was fully active to inhibit MMPs. N-TIMPs have been widely used in characterizing the biochemical and biophysical properties of TIMPs and for investigating structure-function relationships. TIMPs differ in their affinity for specific inhibitor-protease pairs and their interaction does not necessarily lead to inhibition (Brew & Nagase, 2010). Among the four TIMPs, TIMP-3 has the broadest inhibition spectrum and TIMP-1 has the most restricted inhibitory range (Brew & Nagase, 2010). Due to the high expression level of MMPs in diseased cells (i.e., tumor), the concept of MMP inhibitors as targets for anticancer therapy has received extensive attention in the clinic field.

1.1.3 Biological function of MMPs in mammals

The main function of MMPs was thought to be the breakdown of extracellular matrix (ECM) molecules in the tissue. However, the ECM acts as an extracellular scaffold as well as a reservoir of biologically active molecules. Degradation or removal of components in ECM or at the cell surface may alter cell-matrix and cell-cell interactions. In addition, some non-ECM molecules are also possible substrates for MMPs. Thus, MMPs function much more beyond ECM modification (Nagase *et al*., 2006). On one hand, many MMPs degrade the physical barriers. On the other hand, some MMPs also act on multiple singling pathways during many physiological processes and in disease.

MMPs play central roles in various physiological processes including morphogenesis, wound healing, tissue repair and remodelling (Nagase *et al*., 2006; Nagase & Woessner, 1999; Page-McCaw *et al.*, 2007), in the regulation of inflammation and immunity (Parks *et al*., 2004), and in progression of diseases such as cancer (Egeblad & Werb, 2002).

In most cases, MMP deficient mice exhibited no or minor altered phenotypes under unchallenged condition. One exception is MMP14-deficient mice, which have severe bone deformations (Holmbeck *et al.*, 1999; Zhou *et al.*, 2000). Similarly, loss of MMP9 was found to be associated with growth plate defect (Page-McCaw *et al.*, 2007). However, MMP-deficient mice revealed various phenotypes under challenged conditions, such as injury, cancer, inflammation or infection (Parks *et al*., 2004). Numerous findings indicate that MMPs have specific and essential roles in tissue repair, angiogenesis, host defense,

tumor progression and inflammation. They may have evolved to respond to environmental pressures.

Several MMPs were expressed at injury sites and were important for the wound closure (Parks, 1999). For instance, the catalytic activities of MMP1, MMP7 and MMP9 are implicated in wound repairing (Dunsmore *et al.*, 1998; McGuire *et al.*, 2003; Pilcher *et al.*, 1997). Some MMPs are strongly induced during bacterial infections (Burke, 2004; Quiding-Järbrink *et al.*, 2001,). MMP3 was suggested to be important in defense against bacteria because MMP3-deficient mice showed impaired immunity to intestinal bacterial infection (Li *et al.*, 2004). Likewise, MMP7 has been shown to activate antimicrobial peptides α-defensins (cryptdins), in Paneth cells of mouse small intestine (Wilson *et al.*, 1999). MMP7 knockout mice are more susceptible to infection with *Salmonella typhimurium*. Recently, the protective roles of MMP2 and MMP9 were demonstrated in the early host immune response against *Streptococcus pneumoniae* infection (Hong *et al*., 2011).

Apart from the secretion of antimicrobial peptides, some MMPs may also kill bacteria, directly modulate chemokine activity and establish chemokine gradients (Parks *et al*., 2004). A direct antimicrobial function has been elucidated for the hemopexin domain of MMP-12 (Houghton *et al.*, 2009). MMP-12 deficient mice exhibited impaired bacterial clearance and increased mortality when infected with gram-negative and gram-positive bacteria. The antimicrobial properties of MMP-12 were mainly attributed to a unique four amino acid sequence within the hemopexin-like domain and did not require catalytic activity of the enzyme. Together with other reports, these findings highlighted the significance of nonproteolytic functions for MMPs (Kessenbrock *et al.*, 2010).

MMPs have been implicated in cancer formation for more than 40 years, and it is well known that MMP-mediated breakup of ECM resulted in cancer cell invasion and metastasis (Liotta *et al.*, 1980, Bourboulia & Stetler-Stevenson, 2010). In addition to invasion, MMPs contribute to multiple steps of tumor progression, including tumor promotion, angiogenesis and metastasis. They are up-regulated in virtually all human and animal tumors and the stage of tumor progression is positively correlated with the expression of MMP family members (MMP-1, 2, 3, 7, 9, 11 and 14) (Sun, 2010). Based on the notion that MMPs are important contributors for tumor progression, a number of efforts have been put in the use of MMP inhibitors for suppression of cancer in the last decades (Gialeli *et al.*, 2011). However, this strategy is largely unsuccessful in the clinical trial for treating patients (Kessenbrock *et al.*, 2010). Recently, more experimental evidence indicated that several MMPs also exhibit tumor-suppressing effects. These MMPs should be regarded as the anti-targets in cancer (Decock *et al.*, 2011; Overall & Kleifeld, 2006a; Overall & Kleifeld, 2006b). Specific

inhibitors for certain MMPs should be considered in the future therapeutic strategies (Gialeli *et al*., 2011). The fact that MMPs affect physiology and pathology in different and even opposite ways indicated the complexity of MMP functions (Rivera *et al.*, 2010).

1.2 MMPs in plants

MMPs in mammals have been extensively studied during the last fifty years. Such studies in the mammal system set a solid basis for plant researchers. Despite a widespread presence of MMPs in the plant kingdom, information about their functions is only accumulating very recently in this emerging field. Characterization of MMPs have been described in several plant species including soybean (Cho *et al.*, 2009; Graham *et al.*, 1991; Liu *et al.*, 2001; Ragster & Chrispeels, 1979; McGeehan *et al.*, 1992) *Arabidopsis* (Golldack *et al.*, 2002; Lenger *et al.*, 2011; Maidment *et al.*, 1999), cucumber (Delorme *et al.*, 2000), tobacco (Kang *et al.*, 2010; Schiermeyer *et al.*, 2009), *Medicago truncatula* (Combier *et al.,* 2007) and Loblolly pine (Ratnaparkhe *et al.*, 2009). Suggested functions of plant MMPs covered many aspects of physiological processes such as leaf expansion (Graham *et al*., 1991), flowering (Golldack *et al*., 2002), seed development and germination (Ratnaparkhe *et al*., 2009), senescence (Golldack *et al*., 2002), PCD (Delorme *et al*., 2000) and abiotic stresses (Cho *et al*., 2009). Moreover, some plant MMPs have been shown to participate in the interaction with pathogenic and beneficial microbes (Combier *et al.*, 2007; Kang *et al*., 2010; Liu *et al*., 2001; Schiermeyer *et al*., 2009)

1.2.1 Structure of plant MMPs

In the *Arabidopsis thaliana* genome, the presence of five MMPs has been described (Maidment *et al*., 1999). Distinct from mammalian MMPs, all five AtMMPs are intronless genes and this feature was also indicated in MMPs from other plants such as soybean, cucumber and *Medicago truncatula* (Combier *et al*., 2007; Delorme *et al*., 2000; Liu *et al*., 2001; Pak *et al*., 1997). Like the animal cognates, all the plants MMPs possess signal peptide, propeptide domain and catalytic domain (Fig.1 - 2). However, the predominant C-terminal hemopexin-like domain of mammal MMPs is lacking in plant MMPs. In the propeptide domain, plant MMPs contain a conserved cysteine switch PRCGXXD motif. The catalytic domain is characteristic with the zinc-binding motif (HEIGHXLGLXH) followed by the conserved methionine residue of the Met turn. Variant sequences for the zinc-binding motif were found with an E to Q substitution for some MMPs in legume plants (Cho *et al*., 2009; Combier *et al.*, 2007). The residue E is essential for mammalian MMP activity, such a

mutation in the active site may lead to low or no protease activity of the MMPs (Rowsell *et al.*, 2002). To date, no crystal structure for plant MMPs is available.

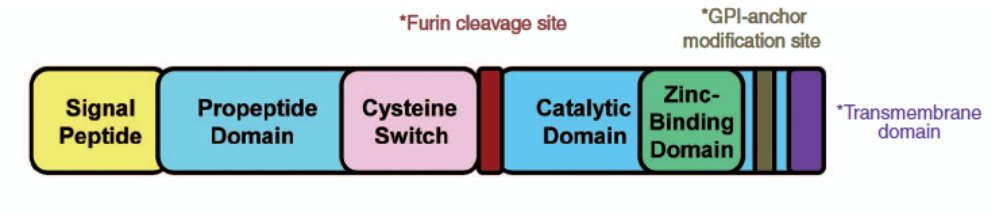


Fig.1 - 2. General structure of plant MMPs. Relevant domains identified are color-coded. Positions of putative furin cleavage sites, GPI-anchor modification sites and C-terminal transmembrane domains, when predicted, are indicated. The graph is taken from Flinn (2008) without modification.

1.2.2 Activation of the plant MMPs activities

For most of the mammalian MMPs, they gain activities by disruption of the Cys-Zn^{2+} interaction between the conserved cysteine residue and the active site Zinc (Parks *et al.*, 2004; Sternlicht *et al.*, 2001). In general, plants MMPs were secreted as enzymes in an inactive form. They also require a further activation step to achieve the proteolytic activity by cleaving off the cysteine switch (Flinn, 2008). In soybean, mature GmMMP2 without the propeptide showed higher activity than pro-GmMMP2 (Liu *et al.*, 2001). The *Arabidopsis* At1-MMP can be activated through the use of MMP activator 4-aminophenyl mercuric acetate (APMA) to cleave the propeptide domain (Maidment *et al.*, 1999). In contrast, the processing of recombinant *Pta1-MMP* occurs spontaneously without the presence of APMA (Ratnaparkhe *et al.*, 2009). Cucumber Cs1-MMP appeared to process itself through autocatalytic activation (Delorme *et al.*, 2000).The NtMMP1 in tobacco was found to undergo autocatalytic processing within a sequence motif similar to MMP-3 and MMP-10 (Birkedal-Hansen *et al.*, 1993; Mandal *et al.*, 2010)

1.2.3 Substrates and inhibitors of plant MMPs

Artificial substrate like azocoll and myelin basic protein (MBP) have often been used as substrate in activity assay for plant MMPs (Liu *et al.*, 2001; Maidment *et al.*, 1999; Ragster & Chrispeels, 1979; Ratnaparkhe *et al.*, 2009; Schiermeyer *et al.*, 2009). The native SMEP1 in soybean leaves displayed an Azocollase. A activity which can be inhibited by EDTA (Graham *et al.*, 1991; Ragster & Chrispeels, 1979). The protease activity of recombinant At1-MMP protein was confirmed by the degradation of MBP but not gelatine or casein (Maidment *et al.*, 1999). In addition, the activity of At1-MMP protein was inhibited by human tissue inhibitors of metalloproteinases (TIMPs). Recombinant *Pta1-MMP* showed the

same proteolytic activity against MBP which was inhibited by EDTA and the active site-binding hydroxamate inhibitor GM6001 (Ratnaparkhe *et al.,* 2009). Furthermore, *GmMMP2* was also found to be able to degrade MBP and azocoll and EDTA could inhibit the activity of *GmMMP2*.

In spite of the progresses made in the analysis of plant MMPs, the physiological substrates are almost unknown. For mammalian MMPs, the components in extracellular matrix and a variety of cell surface proteins are identified as MMP substrates (Cauwe *et al.*, 2007). Whether or not plant MMPs might act on similar types of proteins existing in the extracellular space and the plasma membrane remains to be investigated. In addition, the presence of endogenous MMP inhibitors in plants is still an open question awaiting answers. Efforts in finding natural products for inhibition of mammalian MMPs in medicine plants might provide insights into potential candidates for endogenous inhibitors of plant MMPs (Longatti *et al.*, 2011; Seo *et al.*, 2005)

1.2.4 Subcellular localization of plant MMPs

Plant MMPs were predicted to be secreted or attached to the cell surface through a GPI-anchor (Flinn, 2008). The localization may differ depending on the presence of glycosylphosphatidylinositol (GPI) anchor linkage and C-terminal transmembrane domain. Studies on SMEP1 suggested that the enzyme was extracellular and a portion of the mature form was tightly bound to the cell wall (Pak *et al*., 1997). Two reports using GFP reporter fusion constructs have demonstrated the membrane localization of NtMMP1 and *Slti114* (Cho *et al*., 2009; Schiermeyer *et al*., 2009). In the case of *Arabidopsis* MMPs, all but At4-MMP contain a N-terminal signal peptide and a C-terminal transmembrane domain (Maidment *et al*., 1999). In addition, At2-, At4- and At5-MMP contain a putative GPI modification site. Their subcellular localizations remain to be addressed experimentally.

1.2.5 Function of plant MMPs

1.2.5.1 Tissue remodeling

Over three decades ago, the first plant metalloproteinase activity was detected in soybean leaves (Ragster & Chrispeels, 1979). This protein was shown to posess an Azocollase-A activity in soybean leaf extracts and was sensitive to EDTA. Later on, this proteinase was purified and designated SMEP1 (**S**oybean **me**talloendo**p**roteinase 1) (Graham *et al*., 1991, McGeehan *et al*., 1992). SMEP1 started to accumulate ten to fourteen days after leaf emergence. Older leaves contain larger amounts of SMEP1 compared with younger leaves. The protein accumulation occurred in parallel with the mRNA transcript. Thus, the

expression of SMEP1 in soybean leaves is tightly controlled in a temporal manner and has been speculated to have a role in remodelling of the extracellular matrix during leaf expansion (Pak *et al.*, 1997).

1.2.5.2 Senescence and programmed cell death (PCD)

In cucumber (*Cucumis sativus* L. cv Marketmore), northern-blot analysis showed that Cs1-MMP was expressed at the boundary of senescence and cell death in the cotyledon development (Delorme *et al.,* 2000). Although the physiological substrates remain unclear, the expression of Cs1-MMP in late senescence and early programmed cell death (PCD) suggested its involvement in triggering or regulating PCD in cucumber cotyledon. At2-MMP from *Arabidopsis thaliana* is another example linking plant MMPs with senescence. An earlier onset of senescence and cell death were observed in *at2-mmp-1* mutants (Golldack *et al*., 2002). This finding supported a positive role of At2-MMP in the delay of senescence and programmed cell death. In soybean (*Glycin max*), the matrix metalloproteinase *Slti114* was recently cloned and its expression in cotyledon exhibited an age-dependent manner (Cho *et al.*, 2009). Its expression was increasing after germination and most abundant on the ninth day. Afterwards, the transcript level drastically declined and became undetectable in the yellowish cotyledons. Thus, *Slti114* may act as a signal inducer in cotyledon senescence (Cho *et al*., 2009).

1.2.5.3 Seed development and germination

Publicly available data from Genevestigator indicated that At1-MMP and At4-MMP had the maximal or near maximal expression in mature siliques/seeds. In addition, At4-MMP was also highly expressed during seed germination (Flinn, 2008). These expression data implicated a potential role of plant MMPs in seed germination and development. Following this concept, Ratnaparkhe and colleagues (2009) initiated studies on Loblolly pine (*Pinus taeda*) and analysed the MMP expression during embryogenesis and seed germination. They found that expression of *Pta1-MMP* increased in embryo and megagametophyte from proembryo to early cotyledonary stage and decreased during late embryogenesis and maturation drying (Ratnaparkhe *et al.,* 2009). In addition, *Pta1-MMP* may participate in the completion of seed germination based on MMP inhibitor studies.

1.2.5.4 Flowering

There is a single report correlating a plant MMP with flowering. The *at2-mmp-1* mutant showed late flowering comparing with wild-type plants (Golldack *et al*., 2002). Interestingly,

the expression of At2-MMP was most abundant in both leaf and root tissue in mature flowering *Arabidopsis*. This implicated that extracellular proteolytic processes mediated by At2-MMP are involved in growth and development during the developmental phase of flowering.

1.2.5.5 Abiotic stresses

Several expression studies indicated the possible involvement of plant MMPs in the response to abiotic stresses. In four-week-old *Arabidopsis* plants, At2-MMP expression was induced by NaCl in root and stimulated by cadmium treatment in leaves (Golldack *et al*., 2002). Likewise, *GmMMP2* in soybean was induced by wounding and dehydration (Liu *et al*., 2001). In addition, *GmMMP2*-related *Slti114* in soybean was induced by low temperature and wounding (Cho *et al*., 2009). These expression data suggested potential roles of plant MMPs in the adaptation to abiotic stresses, however, direct evidences are required to validate their functions and elucidate the molecular mechanisms.

1.2.5.6 Plant-microbe interaction

Proteases play an important role in plant defense through perception of pathogens, regulation of signaling cascades or direct attacking of invading pathogens (van der Hoorn & Jones, 2004). Apart from their involvement in development, senescence, PCD and abiotic stresses, plant MMPs have been demonstrated as regulators in host-microbe interactions as well.

The SMEP1 in soybean was assumed to contribute to leaf defenses against pathogen attack (Pak *et al*., 1997). Soybean *GmMMP2* was responsive to the infection of the oomycete pathogen *Phytophthora sojae* in both compatible and incompatible interactions as well as the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* (Liu *et al*., 2001). It may act via the activation of antimicrobial peptide to combat invading pathogens. In cassava, a gene similar to *GmMMP2* was identified as a differentially expressed gene during hypersensitive response caused by *Psudomonas syringae* pv. *Tomato (Pst)* (Kemp *et al*., 2005). Up-regulated by incompatible *Pst* infection, this cassava MMP was speculated to participate in PCD or target invading pathogens in cells undergoing HR. *Arabidopsis At3-MMP* was rapidly induced by the flg22 peptide in seedlings (Zipfel *et al.*, 2004). In tobacco, the membrane-bound NtMMP1 was induced by bacterial pathogens and may possess a role in pathogen defenses at the cell periphery (Schiermeyer *et al*., 2009). Very recently, Kang *et al*., (2010) suggested a positive role of *Nicotiana benthamiana* NMMP1 in the defense against bacterial infection. This is the first report that pinpoints a role of plant MMPs in pathogen defenses using RNAi and overexpression methods. MtMMPL1 from the legume *Medicago*

truncatula was found as a novel specific marker and regulator of Rhizobium infection (Combier *et al*., 2007). During the symbiotic association between *Medicago* and *Sinorhizobium*, the constitutive MtMMPL1 overexpression lines showed reduced nodule number compared with wild type. In contrast, the RNAi lines exhibited similar nodule numbers but with enlarged infection threads and more bacteria. Mounting evidence support the notion that plant MMPs are essential players in plant-microbe interaction. The exact role of specific MMP in pathogen response and the underlying mechanisms, however, are yet to be further elucidated.

1.3 Plant innate immunity

The arms race between the plants and pathogens never stopped. Unlike animals, plants are growing in a fixed niche and have to face all kinds of abiotic and biotic stresses. For biotic stresses, plants need weapons to combat invading bacteria, fungi, oomycetes, nematodes and a variety of herbivores. The surviving strategy of plants in such a challenging environment relies largely on the plant innate immunity, which evolved to perceive and fight against harmful pathogens. The plant innate immunity comprises of two interconnected branches termed as PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl, 2006).

1.3.1 PAMP-triggered immunity (PTI)

PAMP-triggered immunity (PTI) constitutes the first layer of plant innate immunity relying on the recognition of pathogen-associated or microbe-associated molecular patterns (PAMPs/MAMPs) by membrane-localized pattern recognition receptors (PRRs) (Boller & Felix, 2009; Jones & Dangl, 2006). PTI, formerly called basal or horizontal resistance, is thought to be an ancient form of innate immunity and shows an obvious similarity to innate immunity in animals (Boller & Felix, 2009; Chisholm *et al.*, 2006; Jones & Dangl, 2006; Smith *et al.*, 2003). For example, the flagellin of bacteria was perceived through the leucine-rich-repeat (LRR) domain of the membrane receptor FLS2 (flagellin-sensitive 2) (Chinchilla *et al.*, 2006; Gómez-Gómez & Boller, 2000). Similarly, Toll-like receptor TLR5 in mammals could also perceive bacterial flagellin using its LRR domain though the detected site of flagellin is structurally distinct from that of FLS2 (Hayashi *et al.*, 2001; Smith *et al*., 2003). Another good example of plant PTI is the activation of immune responses upon the recognition of bacterial EF-Tu (elongation factor Tu) by the receptor kinase EFR (elongation factor Tu receptor) (Kunze *et al.*, 2004; Zipfel *et al*., 2006). Other well characterized PAMP/PRR pairs include chitin/CERK1 in *Arabidopsis* (Miya *et al.*, 2007), chitin/CEBiP in

rice (Kaku *et al.*, 2006), xylanase/LeEIX1/2 (ethylene-inducing-Xylanase) in tomato (Ron & Avni, 2004) and Ax21/XA21 in rice (Lee *et al.*, 2009). The activation of PTI is normally accompanied with a wide range of intracellular responses including rapid ion influx, generation of reactive oxygen species (ROS), activation of MAP kinase cascades, induction of marker genes and cell wall reinforcement (Zipfel, 2008).

1.3.2 Effector-triggered immunity (ETI)

Efficient PTI can trigger resistance to subsequent infection with pathogen, however, successful pathogens evolved strategies to interfere with PTI and achieve virulence. In many cases, suppression of PTI is achieved by secreted virulence effectors (Abramovitch *et al.*, 2006; Block *et al.,* 2008; Block & Alfano, 2011; Chisholm *et al*., 2006; Jones & Dangl, 2006). During the long-term co-evolution between plants and pathogens, some plants evolved resistance proteins (R proteins) to directly or indirectly recognise such effectors and usually undergo a local cell death known as the hypersensitive responses (HR) at the infection site to restrict pathogen proliferation. This event is termed effector-triggered immunity (ETI) and serves as the second layer in plant immune responses (Boller & He, 2009). ETI is regarded as a faster and stronger version of PTI (Tao *et al.*, 2003; Truman *et al.*, 2006). However, ETI is dependent on the recognition between specific effector and the plant resistance gene and restricted to a narrow range of plant-pathogen interactions. A four phased zigzag model was proposed and well accepted as a current concept of the plant immune system (Fig. 1 - 3)

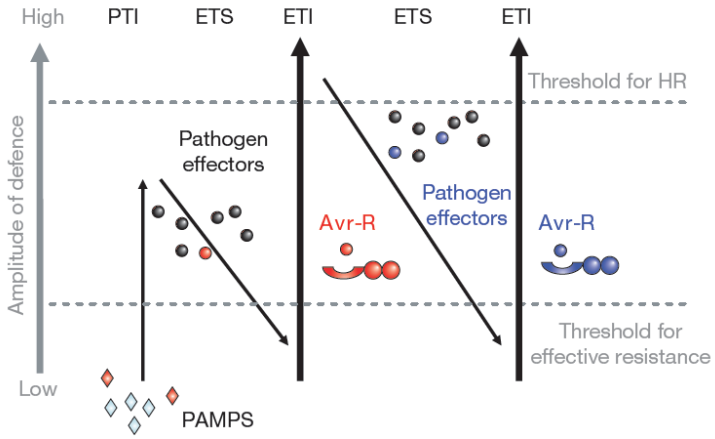


Fig.1 - 3. Zigzag model of the plant immune system (Jones & Dangl, 2006). The proposed model illustrates the quantitative output of the plant immune system and the evolutionary relationship between PTI and ETI. In phase I, plant PRRs recognize PAMPs and activates PTI that prevent pathogen colonization. In phase 2, successful pathogens suppress PTI using secreted effectors and results in effector-triggered susceptibility (ETS). In phase 3, Specific recognition of an effector by the cognate plant R proteins results in ETI, which leads to

strong disease resistance. ETI is regarded as a stronger and amplified version of PTI and often accompanied with an induction of an HR at the infection site. In phase 4, natural selection drives pathogens to evade ETI by loss of the read effectors, or by gain of new effectors (in blue) that suppress ETI. Subsequently, natural selection results in new R proteins to recognize the newly acquired effectors and triggers ETI again.

Most plant R genes encode NB-LRR (nucleotide binding-leucine rich repeat) proteins and around 125 NB-LRR proteins are present in the *Arabidopsis* genome. The signalling events required for the R gene-mediated ETI is largely unclear. R proteins are likely folded in a signal competent state with the help of cytosolic heat shock protein 90 and other receptor co-chaperones (Holt *et al.*, 2005; Schulze-Lefert, 2004). NB-LRR activation requires intra-and intermolecular conformational changes and inappropriate NB activation seems to be tightly controlled by the autoinhibition of LRR domains (Takken *et al.*, 2006). On the other hand, the C-terminus of LRR domains provides pathogen recognition specificity and is required for full host defense (Takken & Tameling, 2009).

1.3.3 Reactive oxygen species (ROS) in plant-pathogen interaction

As one of the earliest events during plant-pathogen interaction, reactive oxygen species (ROS) burst is of crucial importance in regards to the outcome of the interactions. ROS production may contribute to plant disease resistance either directly via its antimicrobial activity or indirectly through induction of defense related genes, cell wall strengthening and orchestration of cell death (Boller & Felix, 2009; Bolwell, 1999; Lamb & Dixon, 1997; Levine *et al.*, 1994; Torres *et al.*, 2006; Zurbriggen *et al.*, 2009). ROS production is largely dependent on the activity of membrane-localized NADPH oxidases (respiratory burst oxidase homologs, Rboh) (Kobayashi *et al.*, 2006; Torres *et al.*, 2006), with AtRbohD being the most important for PAMP-triggered oxidative burst (Nühse *et al.*, 2007; Zhang *et al.*, 2007). Rboh genes have been identified as relatives to mammalian gp91*phox* (Torres & Dangl, 2005; Yoshioka *et al.*, 2003). Genetic studies using double mutants of *Arabidopsis rbohD* and *rbohF* confirmed their essential function in pathogen-induced oxidative burst (Torres *et al.*, 2002).

Due to the different infection strategies of biotrophic and necrotrophic pathogens, they seem to have distinct responses to ROS generated in the host plants (Heller & Tudzynski, 2011). Successful infection of biotrophic and hemibiotrophic fungi depend on the prevention of a strong oxidative burst and the hypersensitive response of their host, by suppression of PTI responses or by scavenging the host-derived ROS during the early infection phase (Molina & Kahmann, 2007; Shetty *et al.*, 2007). Thus, the oxidative burst generated in the host plant is an effective process to combat biotrophic pathogens. However, necrotrophic pathogens may

exploit host or endogenous ROS burst to facilitate infection and colonization (Asai & Yoshioka, 2009; Govrin & Levine, 2000; Segmüler *et al.*, 2008; Temme & Tudzynski, 2009).

1.4 Objectives

Understanding the molecular basis of plant immune responses is of central significance to sustainable agriculture and food security. The model plant *Arabidopsis thaliana* has been used extensively to uncover the fundamental mechanisms of plant resistance towards diverse pathogens (Nishimura & Dangl, 2010). As evolutionary conserved proteinases, the crucial roles of mammal MMPs in pathological processes implicated similar functions for plant MMPs. Apart from a few studies, details about the functions and mode of actions for plant MMPs are largely unclear. This is partly due to the potential difficulties of the identification of their physiological substrate and endogenous inhibitors. The aim of the present work is to analyse the possible involvement of the *Arabidopsis* MMP family in plant defenses against different pathogens. To this end, *Arabidopsis* T-DNA insertion mutants for the At-MMP genes were identified and characterized for their role in pathogen responses. In addition, the expression profiles of At-MMPs under pathogenic conditions were verified to select the best candidate genes and provide the first evidences for their involvement in defense responses. Constitutive overexpression of At-MMPs were be used as a tool to further confirm the findings from the mutant analysis. Altered responses to pathogen in the overexpression plants were examined together with the comparison of gene expression and monitoring of PAMP/DAMP-mediated production of reactive oxygen species (ROS) production. Another focus of this project was to characterize the At2-MMP proteins in respect of the subcellular localization and its protease activity. Transient transformation of a reporter fusion construct was employed to address the question of localization and studies on recombinant proteins demonstrated its features as a typical MMP. The final goal of this study would be a substantial understanding of the functions of MMP in model pathosystem and transfer of the knowledge from model plants to crop plants.

2. Materials and methods

2.1 Plants growth condition

Arabidopsis plants were grown in growth chamber under 8h light/16h darkness regime and 22℃ at day/18℃ in night and 60% humidity. *Arabidopsis* seeds were sown in soil (soil: sand = 3:1 (v/v)) and kept at 4℃ for 2 days in the dark before being placed in growth chamber. Covers were removed after the seeds sprouted and the first true leaves were emerging. The transplanting was performed two weeks later. 5-6 weeks old plants were used for pathogen inoculation.

For gene expression during the interaction with *Piriformospora indica*, the surface sterilized Col-0 seeds were grown in petri dish containing ATS medium. T_0 surface sterilize the seeds, Col-0 seeds were washed with ddH_2O for 2 min to remove the inflorescence and clean the seeds surface then rinsed in 70% ethanol for 1 min. Afterwards, seeds were surface sterilized with 3% NaOCl for 10 min under shaking. The seeds were then rinsed 5 times with ddH_2O, 4 min for each time and sown on ATS medium. ATS medium consists of 0.45% gelrite, 1% sucrose, 5 mM KNO_3, 2.5 mM KPO_4, 3 mM $MgSO_4$, 3 mM $Ca(NO_3)_2$, 50 µM Fe-EDTA, 70 µM H_3BO_3, 14 µM $MnCl_2$, 0.5 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.2 µM Na_2MoO_4, 10 µM NaCl and 0.01 µM $CoCl_2$. To enhance germination, seeds in Petri plates were first placed in the dark at 4℃ for 2 days.

2.2 Pathogen inoculation and quantification

2.2.1 Fungal and bacterial strains

In this study, *Botrytis cinerea B05.10*, *Golovinomyces orontii*, *Pseudomonas syringae* pv. *tomato* DC3000*,* and *Piriformospora indica* were used for gene expression and pathogen inoculation.

2.2.2 *Botrytis cinerea* inoculation

Botrytis cinerea strain B05.10 was grown on HA agar (1% malt extract, 0.4 % yeast extract, 0.4 % glucose, 1.5% agar, pH 5.5) as described previously (Doehlemann *et al.*, 2006). Rosette leaves from 6-week-old soil-grown *Arabidopsis* plants were detached and placed in Petri dishes containing 0.5% agar, with the petiole embedded in the medium. To infect plants, conidia were collected from 14-day-old culture, and the spore density was adjusted in 12 g L^{-1} potato dextrose broth (PDB, Duchefa Biochemie, Haarlem, The Netherlands) to $2x10^5$ conidiospores mL^{-1} for gene expression and $5x10^4$ conidiospores mL^{-1} for pathogen resistance

assay. Inoculation was performed by placing 3ul spore suspension on the both side of leaf or 5 µl of spore suspension in the leaf centre. Half-strength PDB was used as mock inoculation. Inoculated plants were maintained at high humidity with a transparent cover in a growth chamber. Depending on the symptom development, two to six days after infection, the leaf samples were first photographed with a ruler as a scale followed with lesion size measurement using ImageJ. For gene expression study, the inoculated plants were placed in a transparent box tightly covered to keep high humidity. The boxes were incubated in a growth chamber under short day condition. Leaf samples were harvested at 0h, 8h, 16h, 24h, 48h and 72h after inoculation and immediately frozen in liquid nitrogen then stored at -80℃ prior to use.

2.2.3 Powdery mildew (*Golovinomyces .orontii, G. orontii*) inoculation

For *G. orontii* inoculation, leaves from heavily infected plants were detached to collect the conidia spores with Tween H_2O (1:20,000). The spore suspension with a density of 50,000 conidia mL^{-1} was immediately (within 15 min) sprayed on 5-week-old healthy plants. Mock treatment was done by spraying Tween H_2O (1:20,000). After inoculation, plants were moved to a growth chamber under an 8 h/16 h light/dark regime at 22℃. For gene expression, leaves were harvested at 0h, 6h, 12h, 18h, 24h, 72h and 120h and frozen in liquid nitrogen then stored at -80℃ prior to use. For quantification of the fungal growth, visible disease symptoms were photographed at least 6 days after inoculation (check every day). When clear symptom appeared, the infected plants were harvested, weighed for fresh weight and rinsed with Tween H_2O to collect the conidia spores. The number of conidia per mg fresh weight was determined to quantify the fungal growth.

2.2.4 *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) inoculation

Pst DC3000 was grown at 28℃ on King's B medium (1% protease peptone, 0.15% anhydrous k_2HPO_4, 1.5% glycerol, 1% Agar, pH 7.0) containing 50mg/L Rif for selection. After incubation at 28°C for two days, the bacterial were scrapped off with sterile 10mM $MgCl_2$ using a glass spatula and the concentration was adjusted to OD600 = 0.3 for gene expression. The bacterial suspension was pressure-infiltrated into leaves with a needleless syringe on half leaves. The non-injected other half leaves were harvested for gene expression minimizing the wounding effect.

For bacterial resistance assessment, *Pst* DC3000 suspension was diluted to OD = 0.01with 10mM $MgCl_2$. The inoculation was done by pressure infiltration. Leaf disks (0.5 cm^2) were

harvested at 0 and 48 h after inoculation and ground in 10 mM $MgCl_2$. After grinding of the tissue, the samples were thoroughly vortex-mixed and diluted 1:10 serially (10^{-1} to 10^{-5}). Samples were finally plated on King's B medium supplemented with 50mg/L rifampicin. Plates were placed at 28℃ for 2 days and the colony-forming units were counted.

2.2.5 *Piriformospora indica* (*P. indica*) inoculation

Piriformospora indica culture was maintained at 22℃ on complex medium (CM) containing 0.5% 20 × Salt solutions, 2% Glucose, 0.2% Pepton, 0.1% Yeast extract, 0.1% Casamino acid, 1ml Microelement, 1.5% Agar-agar. Chlamydospores were collected from three-week-old *P. indica* plates with sterilized Tween20-H_2O (1:5000). The spore concentration was adjusted to 5×10^5 chlamydospores mL^{-1}. For each square Petri plate, 1 mL spore suspension was loaded on three-week-old *Arabidopsis* roots followed with 30 seconds gentle shaking to ensure uniform distribution of the spores. The mock treatment was done with Tween20-H_2O (1:5000). The mock and *P. indica* inoculated roots were harvested at the time points 0 day, 1day, 3day and 7 day and flash frozen in liquid nitrogen. All root samples were stored at -80℃ prior to RNA extraction.

2.3 Extraction of DNA/RNA and PCR

2.3.1 Extraction of DNA and PCR

Genomic DNA extraction was performed to identify T-DNA insertion mutants and transgenic plants. For identification of single mutants, the REDExtract-N-Amp plant PCR kit (Sigma-Aldrich, St. Louis, MO, USA) was used for DNA extraction and PCR amplification according to the manufacturer's instructions. Briefly, 0.5-0.7 cm leaf disks were cut from each plant with scissors and transferred with forceps into a 1.5ml microcentrifuge tube containing 60 µL of Extraction Solution. The leaf disk was mashed several times with pipette to be covered with Extraction solution. Scissors and forceps were rinsed with 70% ethanol and dried between samples. Tubes were vortexed briefly to cover the leaf disk with Extraction Solution, and then heated at 95℃ in a heating block for 10 min. After the incubation, 60 µL of Dilution Solution was added with brief vortex. Two microliters of diluted extract after short centrifuge was used for PCR and the remaining extract was stored at 4°C (with leaf disk inside the tube). Each PCR reaction contained 5 µL REDExtract-N-Amp PCR reaction mix, 0.5 µM forward and reverse primer, 2 µL DNA extract in a final volume of 10 µL. PCR conditions were: 94℃for 5 min, then 45 cycles of 94℃ for 30 s, at optimum annealing temperature for 30 s, and 72℃ for 40-90 s (calculated for 1 kb per min).

The reaction was completed with a final extension at 72℃ for 5 min. PCR products were loaded directly onto a 1.5% agarose gel for electrophoresis.

For double mutant identification, a quick and dirty protocol was used for DNA extraction. The DNA was extracted in a simplified way with compromised purity but suitable for PCR amplification. Two pieces of small leaves in the size of 1 cm^2 were harvested from each plant in 2 mL collection tube. Samples were frozen in liquid nitrogen and leaf disks were crushed using a pre-cooled plastic stick. 500 µL DNA extraction buffer (200mM Tris-HCl pH 7.5, 250mM NaCl, 25mM EDTA and 0.5% SDS) was added to the collection tubes with brief vortex. After incubation at room temperature for 10 min, 500 µL chloroform was added and vortex vigorously. Samples were then centrifuged at 13, 000 rpm for 10 min. The supernatant (500 µL) was transferred to a new eppendorf tube and mixed with 500 µL isopropanol by inverting. The mixture was incubated at room temperature for 2 min then centrifuged at 13,000 rpm for 10 min. The pellet was kept and washed with 500 µL 70% ethanol by vigorous vortex. Samples were then centrifuged again at 13,000 rpm for 5 min. The pellet was air dried under laminar flow for 10 min and dissolved with 100 µL MiliQ H_2O. The dissolved DNA was used for PCR reaction (25 uL) which contains 0.2 mM dNTPs, 1x PCR Buffer, 1.5 mM $MgCl_2$, 0.4 µM forward and reverse primer, 2 µL DNA and 0.75 U DNA polymerase. PCR reactions are initiated with 94℃ for 5 min and then amplified for 35 cycles including 94℃ for 30s, at optimum annealing temperature for 30 s, and 72℃ for 40-90 s (calculated for 1 kb per min) with a final extension at 72℃ for 5 min.

2.3.2 Extraction of RNA

For RNA extraction, five to six weeks old *Arabidopsis* leaves were harvested and immediately frozen in liquid nitrogen. Leaf samples were crushed to fine powder in liquid nitrogen with mortars and pestles. The 2 ml microcentrifuge tubes were filled with leaf powder to one third of the volume. One mL RNA extraction solution (38% Phenol, 0.8 M Guanidine Thiocyanate, 0.4M Ammonium Thiocyanate, 0.1M Natrium Acetate pH 5, 5% Glycerol) was added to each sample. After vigorous vortext till the powder was well suspended, 200 µL chloroform was added and vortexed again. Samples were placed on ice during the proceeding of the rest samples. All samples were vortexed for 15 seconds and centrifuged at 4℃ and 13,500rpm for 15 min. The supernatant was transferred in a new microcentrifuge tube (2 mL) with 850 µL chloroform and vortexed briefly. Samples were centrifuged at 4°C and 13,500rpm for 15 min. The supernatant was transferred to a new tube with 1mL 5 M LiCl and mixed by brief vortex followed with overnight incubation at -20℃

to increase the RNA yield. Centrifuge was performed at 4℃ and 13,500rpm for 20 min. The supernatant was discarded and the pellet was washed with 70% ethanol before centrifuge at 4℃ and 13,500rpm for 10min. The washing step was repeated and then the pellet was air dried under clean bench. H_2O_{DEPC} was added to dissolve the pellet. RNA concentration was determined by NanoDrop ND-1000 Spectrophotometer (peqLab Biotechnologie GmbH, Erlangen, Germany). The RNA integrity was verified on denaturing 1.5% agarose-gel containing 5% formaldehyde in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). The gel was visualized with a UV transilluminator.

2.4 RT-PCR

2.4.1 cDNA synthesis

RT-PCR was preformed with Fermentas reagents. RNA extraction was performed as described above. Three microgram RNA from each sample was treated with DNase I and RNase Inhibitior. Each sample was added with a mixture containing 1μL DNase I (1U/μL, Fermantas, Germany), 1μL 10×Dnase I buffer and 0.25 μL Rnase I inhibitor (40 U/μL). DEPC-treated MilliQ H_2O was added to each sample to reach 10 μL final volume. After 30 min incubation at 37℃, 2 μL EDTA 25mM was added to each sample and incubated for 10 min at 70℃. The RNA concentration was measured again after DNase I treatment. One microgram RNA was used for cDNA synthesis with 1 μL oligo$(dT)_{18}$ primer (100 μM) and 1 μL Random hexmaer primer(100 μM). The reactions were first filled up with DEPC H_2O to 12.5 μL. After 5 min incubation at 70℃, samples were placed on ice for 2 min. A second mixture containing 4 μL 5×M-MulvRT buffer and 0.5 μL RNase Inhibitor (40 U/μL) was added to each sample. After 5 min incubation at 37℃, 1 μL Revert Aid ^{TM} –M-Mulv Reverse Transcriptase was add in each reaction. The reactions were incubated in a TProfessional thermocycler (Biometra GmbH, Germany) following the program of 25℃ for 10 min, 42℃ for 60 min and 70℃ for 10min. Eventually, 80 μL MilliQ H_2O was added to each sample. Two aliquots were made from the cDNA samples and stored at -20℃ prior to use.

2.4.2 Reverse transcription polymerase chain reaction (RT-PCR)

For RT-PCR, cDNA synthesized above was used as template to amplify the target genes. In all cases, the *Arabidopsis* housekeeping gene ubiquitin 5 (UBQ5, AGI: AT3G62250) was used as an internal control for equal loading. The expression of *Arabidopsis* matrix metalloproteinase gene (At-MMPs), PR1, PDF1.2 and ERF1 were examined.

2.5 Identification of T-DNA insertion mutants

2.5.1 Identification of single mutants

The T-DNA insertion *at2-mmp* mutant HM-280 (GABI_416E03, NASC code N348998) was generated from GABI-Kat population (Rosso *et al*., 2003). The *at2-mmp* mutant HM-257 (Salk_082450, NASC code N582450) was identified from the Salk *Arabidopsis* T-DNA insertion population (Alonso *et al*., 2003). Both mutant lines are in the background of columbia (Col-0) ecotype. T-DNA insertions were confirmed by PCR using a combination of a border primer of T-DNA and a gene-specific primer. Another PCR was performed to identify plants homozygous for the insertions using the above gene-specific primers and respective reverse primer. RT-PCR was performed to examine the At2-MMP transcript level in the mutants using the primers. Same procedure was followed for identification of *at3-mmp*, *at4-mmp* and *at5-mmp* mutants.

2.5.2 Production and identification of double mutants

2.5.2.1 Crossing of *Arabidopsis* T-DNA mutants

Double mutants were generated from crossing of two single mutants. *at2-mmp* and *at3-mmp* single mutants were grown in soil under short day condition. After 6weeks, the plants were transferred to long day condition to promote flowering. For crossing (about 10 weeks old), mature siliques as well as open flowers and buds from mother plants were removed with fine forceps. The meristems with too small buds were also removed and 3-5 flower buds with suitable size were remained on the mother plants. Anthers from these flower buds were carefully removed with forceps. After emasculation of the mother plants, pollen from open and mature flower on father plants were tapped on the stigma of mother plants. The crosses were documented including mother plant, father plant, date and number of pollinated flowers. Between different crosses, forceps were cleaned with 96% ethanol then with H_2O and dried with tissue paper. Three to four weeks later, siliques with the hybrid seeds were mature and harvested by cutting them into a paper bags.

2.5.2.2 Identification of double mutants

For each crossing combination, at least 40 plants were grown to ensure the presence of homozygous double mutants. DNA extraction was performed as described above. Double mutants were identified by PCR with specific primer from parent lines. In brief, four serial PCR were applied for identification of homozygous double mutants. For the first PCR, LP and RP primer form male parent were used. The sample which gives no product in PCR 1 were selected for further test in PCR 2, using LP and RP primer from female parent.

Likewise, the sample which gives no product in PCR 2 was remained for PCR 3, in which the plants were confirmed with the LB and RP primer from male parent. As a final proof, the double mutant should give a product with LB and RP primer from female parent in PCR 4.

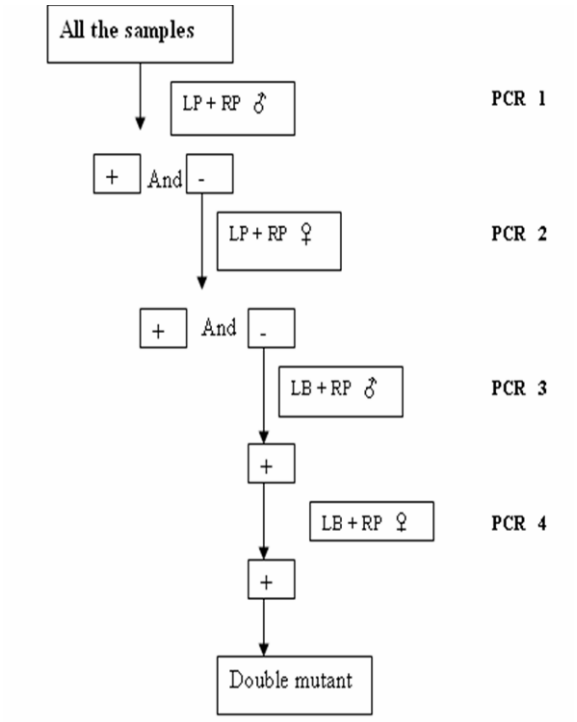


Fig.2 - 1. Identification of homozygous T-DNA insertion double knockout mutants. Four PCR reactions were performed with LP, RP and LB primers. PCR1 was preformed with LP and RP primer of male parent. PCR1 negative plants were tested by PCR2 with LP and RP primer of female parent. Both PCR1 and PCR2 negative plants were tested by PCR3 with LB and RP primer of male parent. PCR3 positive plants were then verified by PCR4 with LB and RP primer of female parent. LP: Left primer. RP: Right primer. LB: Left border primer

2.6 Overexpression of *At2-MMP* and *At3-MMP*

2.6.1 Cloning and construction of transformation vectors

To generate the *35S:MMP2* and *35S:MMP3* constructs, the genomic DNA of the *Arabidopsis* was used to amplify the full length sequence by PCR. The restriction sites *Bam*HI and *Hind*III were introduced in the gene specific full-length primers. Amplification was achieved using the Phusion™ high-fidelity DNA polymerase (New England Biolabs, UK). Primer pairs At1g70170_MMP2_F (#449)/ At1g70170_MMP2_R (450) and At1g24140_MMP3_F (#447)/At1g24140_MMP3_R (448) were used for amplification of *At2-MMP* and *At3-MMP*, respectively. The full length fragment was first cloned into pGEM-T easy vector (Promega, Madison, USA*)* and sequenced by LGC Genomics (Berlin, Germany). The fragment were then released through *Bam*HI/*Hind*III digestion, ligated into the cloning plasmid p35S-BM and subcloned into the *Sfi*I restriction sites of the *Agrobacterium* transformation vector pLH6000 in the sense orientation behind the *CaMV*

35S promoter. The empty vector cassette from p35S-BM including the 35S promoter and NOS terminator was also subcloned into the pLH6000 vector at *Sfi*I sites.

The pLH6000-p35S-*MMP2*, pLH6000-p35S-*MMP3* and pLH6000-p35S were transformed into the *Agrobacterium* strain AGL1 by electroporation using an *E. coli* Pulser™ transformation appratus (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instruction. Briefly, the 0.2 cm *E.coli* Pulser electroporation cuvette was chilled on ice. One μL aliquots of plasmid DNA (about 100 ng) was mixed with 50 μL of elctro-competent AGL1 cells by pipetting up and down. After 1 min incubation on ice, the *E. coli* Pulser™ apparatus (Bio-Rad) was set to a voltage of 2.5 KV for transformation. Within 10 seconds of the pulse, 1mL SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.0) was added to the cuvette and cultured at 28℃ for 1 h. The cells was then plated on YEB medium containing 25 μg/mL Rif, 25 μg/mL Cb and 50μg/mL Spc. Two days later, positive colonies were confirmed by PCR and selected for liquid culture. Minprep extraction of the plasmid was performed using NucleoSpin® Plasmid DNA purification kit (MACHEREY-NAGEL GmbH, Düren, Germany). Plasmids were confirmed again using *Sfi*I digestion.

2.6.2 Stable transformation of *Arabidopsis* plants

Agrobacterium-mediated transformation of *A. thaliana* was carried out by means of the floral dip method (Clough & Bent, 1998). Col-0 plants were grown in soil under short-day conditions for 5 weeks and moved to long-day conditions afterwards. First flower stem were cut to encourage proliferation of more secondary flower stems. Plants were used for transformation one week after the first clipping.

YEB liquid medium (1%Bacto-Peptone, 1%Yeast extract, 0.5% NaCl) was used for *Agrobacterium* culture. An initial liquid culture of *Agrobacterium* harbouring pLH6000-p35S-BM, pLH6000-p35S-*MMP2* and pLH6000-p35S-*MMP3* plasmids were started from a single colony in 100 ml medium (YEB +25 μg/mL Rif +25 μg/mL Cb +50μg/mL Spc) and grown at 28℃ for 2 days. One day before the infiltration, 10 ml of the pre-culture was used to inoculate 200 mL YEB medium containing appropriate antibiotics. After 6 h incubation at 28℃, *Agrobacterium* cells (OD = 2.0) were harvested by centrifugation at 5,000 rpm for 10min under room temperature and resuspended in 200 mL infiltration medium (1/2 MS salts including vitamins, 5% sucrose, pH 5.7) supplemented with 44 nM 6-benzylaminopurine (BAP, 10 ul L^{-1} of a 1 mg mL^{-1} stock in dimethyl sulfoxide [DMSO]) and 0.005% Silwet L-77.

A glass bell jar connected via a condensation trap to a Leybold Trivac oil pump (type S 8B/AF 4-8) was used for vaccum infiltration. A glass tray filled with 200 mL of the *Agrobacterium* suspension was placed in the jar. Pots with plants were inverted, to allow the submersion of inflorescence shoots in the suspension under a pressure of about 16 mbar for 5 min. After the 5 min treatment the vacuum was immediately released and the infiltration step was repeated. The 200 mL bacterial suspension was re-used for three pots. After the infiltration treatment, the plants were covered with a transparent cover and in darkness for 2 days before transfer to a long-day growth chamber. Mature seeds were collected in paper bags after about 5 weeks.

Seeds from T_0 plants were sowing in ½ MS containing 30mg/L hygromycin and incubated in a 4℃ refrigerator for 2 days before being transferred to a short day condition growth chamber. Transformants were selected by their hygromycin resistance. After two weeks, green seedlings with fully expanded cotyledons, the first pair of true leaves and elongated roots were considered as transformants. The plants which had yellow leaves and short roots were autoclaved. The putative transformants with green leaves and long roots was transformed to soil culture. Two weeks later, these plants were tested by PCR using the REDExtract-N-Amp plant PCR kit.

2.6.3 Extraction of intercellular washing fluid (IWF)

The intercellur washing fluid was extracted from 6 weeks old *Arabidopsis* plants using an infiltration-centrifugation method. About 30 fully-expanded rosette leaves were detached from 10 plants, mounted in a metal tea filter and immersed in a beaker containing pre-cooled (4℃) phosphate buffer (0.2 M K_2HPO_4, 0.2M KH_2PO_4, pH 7.4). The beaker was placed in a vacuum chamber and subjected to several consective rounds of vacuum treatment for 2 min followed by abrupt release of vacuum. The infiltrated leaves were blotted dry with filter paper and gently placed in a 15 ml falcon tube on a grid separated from the tube bottom. The IWF was collected from the bottom of the tube after centrifugation at 300 g for 2 min at 4℃. IWF extracted from transgenic and control platns were fractionated on 12 % SDS-PAGE and their proteolytic activities were evaluated against MBP.

2.7 Recombinant protein

2.7.1 Construction of pET32a-MatMMP2

For construction of the expression constructs, the catalytic domain (mature MMP2) or full length CDs without signal peptide (ProMMP2) were cloned in frame into pET32a(+)

(Novagen, Madison, WI) containing a His-tag at the N terminus of the expressed protein and transformed into *Escherichia coli* strain BL21(DE3) pLysS. The primer pairs ProMMP2_Fwd (#555) and ProMMP2_Rev (#556) for ProMMP2, MatMMP2_BamHI (#633) and MatMMP2_HindIII (#634) for MatMMP2 were used in the amplification respectively. At3-MMP full length CDs without signal peptide was cloned in frame into pET32a (+) containing the His-tag at N-terminal and expressed in BL21 (DE3). As the control, empty vector pET32a (+) was expressed in BL21 (DE3) and BL21 (DE3) pLysS.

2.7.2 Induction and purification of recombinant protein

Single colony from fresh plate was used to inoculate 5 mL Luria-Bertani (LB) medium containing 100 mg/L ampicillin and allowed to grow overnight at 37℃ on a shaker. The overnight culture was added to fresh LB + AMP at the ratio 1:100. The bacteria were grown at 37℃ with shaking until the OD600 reach 0.6-0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and further incubated for 4 hours. Then, bacteria were harvested by centrifugation at 9,500 rpm for 20 min at 4℃. The bacterial pellet was dissolved in 20 mL lysis buffer (10 mM Tris-Hcl pH 8.0, 1u/ml DNaseI, 1 mg/ml lysozyme, 0.1mM PMSF) and cell was disrupted by sonication (Bendelin UW 2070). The sonication was performed by 8 cycles each with 15seconds sonication and 15 seconds on ice. Then the samples were centrifuged at 9,500 rpm for 15 min at 4℃. The pellet was resuspended in lysis buffer again and repeated with the sonication two times. Subsequently, 10ml buffer B (100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, 8 M urea, the pH was adjusted to 8.0 using NaOH) was added to pellet and incubated at room temperature for 1 h with gentle shaking. Thereafter, the cell debris was precipitated from the lysate solution by centrifugation at 9,500 rpm for 30 min (Sorvall SS34 Centrifuge). The supernatant was collected, mixed with 2ml 50% Ni-NTA slurry (Qiagen, Hilden, Germany) and incubated at room temperature for 1h. The lysate-resin mixture was carefully loaded into an empty column with the bottom cap still attached. The cap was open until the lysate-resin mixture fall down and the flow through fraction was collected. The column was washed with 10 mL washing buffer C (100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, 8 M urea, pH adjusted to 6.3 using HCl) then eluted three times with buffer D (100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, 8 M urea, pH adjusted to 5.9 using HCl) and labeled as E1.1, E1.2 and E1.3. After the first elution, the column was eluted three times with elution buffer (100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, 8 M urea, pH adjusted to 4.5 using HCl) and labeled as E2.1, E2.2 and E2.3. 10 µL protein from each

fraction was loaded on 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, gel was fixed by fixation solution (40% Ethanol, 10% Acetic acid) for 30 min and stained with staining solution (20% Methanol, 60% H_2O, and 20% colloidal Coomassie blue Roti ®-Blue, Roth, Karlsruhe, Germany) over night followed with destaining for 1h in destaining solution (40% methanol, 10% glacial acid, 50% water). The gel was scanned.

The collected proteins were desalted and concentrated using an ultra-filtrate column (VIVASPIN 15 ml concentrator) with a cut-off molecular weight of 30 kDa (Vivascience, Lincoln, UK) and refolded in refolding buffer (20mM Tris-H_2SO_4, pH 7.5, 5mM $CaSO_4$, 100mM $NaSO_4$, 1uM $ZnSO_4$, 10% glycerol, 0.05% Brij35, 0.02% NaN_3) using a dilution method with a final concentration of 10-50 μg mL^{-1}. The refolded protein was incubated at 4°C overnight. After refolding, the protein was concentrated using VIVASPIN concentrator. Protein quantity was estimated by A280 using NanoDrop ND-1000 spectrophotometer. Purity and integrity of pET32a-MatMMP recombinant protein was determined by separating protein aliquots using SDS-PAGE. The concentrated protein was aliquoted, frozen in liquid nitrogen and stored at -80℃.

2.7.3 Proteolytic activity assay

Enzymatic analysis was performed as previously described (Maidment *et al.*, 1999, Ratnaparkhe *et al.*, 2009). Degradation of myelin basic protein (MBP) was assessed using bovine MBP (Sigma) at a final concentration of 0.25 mg/mL in 100 mM Tris–HCl pH 7.5, 5 mM $CaCl_2$, 0.05% Brij 35, 0.02% NaN_3. A total of 3 μg of recombinant protein was incubated with MBP in a 15 μL reaction volume for the indicated time (30 min, 4 hours and 30 hours) at 37℃, and products were analyzed by 20% SDS-PAGE to visualize MBP degradation. The gels were stained with coomassie blue overnight and then destained for 1 hour. Inhibition of MMP activity was determined by the addition of EDTA at a concentration ranging from 100 nM to 5 mM.

Thermolysin (Sigma, 88303) at the concentrations of 5 μg/mL was used with 0.25 μg/mL MBP in a 15 μL reaction volume for 5min, 15min, 30min, 1h, 2h at 37℃. The products were analyzed by SDS-PAGE to visualize MBP degradation using 20% separating gel. After electrophoresis, gel was fixed by fixation solution for 30 min. Then the gel was stained with staining solution over night. Destaining was performed with destaining solution for 1h.

2.7.4 *B. cinerea* germination assay

The antifungal activities of the recombinant protein were evaluated against *B. cinerea* using spore germination assay. Fungal conidia (2 x 10^4 conidia mL^{-1}) were incubated with 60 µg/mL recombinant protein from At2-MMP and empty vector in microtiter plate at room temperature. After incubation for 10 h, the spore germination was monitored microscopically and photographed.

2.7.5 Western blot

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane (Roti®-PVDF, pore size 0.4um, ROTH, Germany) with semi-dry electrophoretic transfer cell (Bio-Rad) at 15 V for 1 h. The PVDF membrane was balanced with 1× Towbin buffer (25 mM Tris, 192 mM glycine and 20 % [v/v] methanol) for 15 min. After protein transfer, the membrane was washed three times with PBST buffer (0.1 % [v/v] Tween 20,137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 , pH 7.4) and 10 min for each time. Non-specific binding sites were blocked using 5 % (w/v) milkpowder (ROTH, Germany) in PBST buffer at room temperature for 2 h. After three times washing with PBST buffer, the N-terminal and the C-terminal antibody from tobacco (N-11.5.1.5 or C-6.3.2) were uses as primary antibody respectively with overnight incubation at 4℃ on a shaker (Mandal *et al.*, 2010). The membrane was washed three times for 5 min in PBST buffer and incubated with anti-mouse lgG peroxidase conjugate (sigama-9044) at the dilution 1:5000 in PBST buffer for 2 h at room temperature. After three times washing, the blot was developed using Chmiluminescent substrate (SuperSignal® WestPico).

2.8 Subcellular localization of At2-MMP

The full length cDNA of At2-MMP was amplified using the primer pair At1g70170_MMP2_F (5'-ATGGATCCAATCCGAAAACCACCATGAG-3', #449) and At2-MMP *Xho*I (5'-CCGCTCGAGCGAAATCAAACATAGGTATAGGACA-3', #635) (restriction sites underlined) and cloned downstream of CaMV 35S promoter in p35S-Nos-BM (DNA Cloning Service, Hamburg) to result in *35S::MMP2*. The GFP CDS without stop codon was amplified from *35S::GFP* using GFP-*Sal*I (5' CGCGTCGACCATGGTGAGCAAGGGCGAGGA-3', #631, *Sal*I site underlined) and GFP-*Xho*I (5'-CCGCTCGAGGTCTTGTACAGCTCGTCCATGC-3', #632, *Xho* I site underlined) and cloned in frame to At2-MMP at its Sal I site, which is between the peptidase domain and the putative GPI anchor. For transient transformation, detached leaves from 5-

week-old, short-day grown Col-0 plants were cobombarded with the construct *35S::MMP2-GFP* and the control plasmid *35S::mCherry*. 24 hours after bombardment, the subcellular localization of MMP2-GFP was analyzed by confocal laser scanning microscopy. Plasmolysis was performed with 50% glycerol for 5 min.

2.9 Oxidative burst assay

Pre-clearing of chitin was performed as described with modifications (Petutschnig *et al.*, 2010). In brief, crab shell-derived chitin (Sigma) was ground with mortar and pestle to a very fine powder and the 10 mg/ml stock slurry was prepared with water. The extract was vortexted vigorously for 15 min. Then after 30s heat with microwave, the extract was treated with 5 min socication. The supernatant was removed by centrifugation at 4,000 rpm and 4℃ for 10 min. ROS detection using leaf disks from soil grow plants was performed as described previously (Keppler *et al.*, 1989, Goméz-Goméz *et al.*, 1999). The leaf disks 5mm in diameter were cut from 7-week-old healthy plants with sharp puncher and were floated overnight in 200 µL H_2O in 96-well plates to minimize wounding effect. For ROS measurement, the water was replaced with 200 µL mastermix aqueous containing 30 µg/mL luminol (Sigma, 15mg/ml stock in DMSO) and 20 µg/mL horseradish peroxidase (10mg/ml in water, Sigma, P6782) under low-light condition. Elicitors used to stimulate ROS generation include three PAMPs (100 nM flg22, 100 nM elf18, and 1 mg/mL chitin) and one DAMP (100 nM Pep1). 20 µL of the elicitors (10x concentrated) was injected by TECAN Infinite®F200 microplate reader (TECAN, Switzerland) to trigger ROS production. Luminescence was measured for 70 cycles, 60 seconds per cycle.

2.10 Phylogenetic reconstruction

The MMP protein sequences from fourteen monocot and dicot plant species were retrieved from NCBI based on blastp and tblastn search using the sequences from At1-MMP and At5-MMP as queries. The GenBank accession numbers of proteins or cDNA used for the translation are listed as follows: At1-MMP (NP_193397), At2-MMP (NP_177174), At3-MMP (NP_173824), At4-MMP (NP_182030), At5-MMP (NP_176205), BoMMP1 (ACB59207), ChrMMP1 (BAA01400), ChrMMP2 (BAB68383), ChrMMP3 (XP_001694591), Cs1-MMP (CAB76364), SMEP-1 (AAB26959, Gm), GmMMP2 (AAL27029), ACU24527 (ACU24527, Gm), *Slti114*(ABW96008, Gm), HvMMP1 (BAJ94792), HvMMP2 (BAJ93963), HvMMP3 (BAJ94176), HvMMP4 (BAJ90264), MtMMPL1 (CAA77093), ACJ84310 (ACJ84310, Mt, translation), NMMP1(ADD21635, Nb), NtMMP1 (ABF58910), OsMMP1 (NP_001048075), OsMMP2 (NP_001057259),

OsMMP3 (NP_001065361), PtMMP1 (XP_002318762), PtMMP2 (XP_002322292), PtMMP3 (XP_002326645), PtMMP6 (XP_002338425), PtMMP7(XP_002325947), PtMMP8 (XP_002311050), Pta1-MMP (ACJ24812), LeMMP1 (AK322919, translation), SlMMP2 (AK328733, translation), VvMMP1 (XP_002267298), VvMMP2 (CBI32079), VvMMP3 (XP_002274477), VvMMP4 (XP_002267238), VvMMP5 (CAN59960), VvMMP6 (XP_002275556), VvMMP7 (XP_002280833), VvMMP11 (XP_002275671), ZmMMP1 (NP_001151749), ZmMMP2 (NP_001142095). Five human MMPs are selected including HsMMP1 (NP_002412), HsMMP7 (NP_002414), HsMMP8 (AAZ38714), HsMMP13 (AAH67523) and HsMMP20 (AAT70722).

The peptide sequences of the domains spanning the cysteine switch and Met-turn motif of the MMP protein were aligned by Muscle package within MEGA5 program (Tamura *et al.*, 2011). The multiple alignments were adjusted with gaps manually inserted for optimal alignment based on the conserved features of the cysteine switch and the zinc-binding domains. The Neighbor-Joining method implemented in MEGA5 for amino acid sequences were used for phylogenetic tree reconstruction. The bootstrap consensus tree inferred from 10,000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown above the branches.

3. Results

3.1 Phylogenetic anaylsis of the MMP family in plants

MMP sequences for human, single-celled green algae *Chlamydomonas reinhardtii* and 14 selected monocotyledonous and dicotyledonous plant species were retrieved from NCBI database (section 2.10). All previously characterized plant MMPs were included in the phylogenetic reconstruction. Based on the alignment of conserved region spanning the cysteine switch and Met-turn motif of the MMP protein sequences (Fig. S1), a non-rooted phylogenetic tree was constructed using Neighbor-Joining method (Tamura *et al*., 2011). Basically, the selected MMPs in higher plants can be classified into four subgroups, with some features linked to certain plant species or specified functions (Fig.3 - 1). For group I and II, each can be clearly separated into two subclades representing MMP branches from dicots and monocots. At2-MMP, At3-MMP together with At5-MMP is assigned in the group I, containing LeMMP1, NtMMP1 and NMMP1 which are known to be pathogen responsive (Frick and Schaller, 2002; Kang *et al*., 2010; Schiermeyer *et al*., 2009). At1-MMP and At4-MMP belong to group II in which some members have been proposed to be involved in development (Flinn, 2008; Ratnaparkhe *et al*., 2009). No monocot MMP were identified in group III and thus it appears to be a dicot-specific group. Two members from group III, SMEP-1 and Cs1-MMP have a suggested function in cell death and leaf expansion, respectively (Delorme *et al*., 2000; Graham *et al*., 1991). Group IV members are till now exclusively found in the legume plants *Glycine max* and *Medicago truncatula* and thus seem to be legume-specific MMPs.

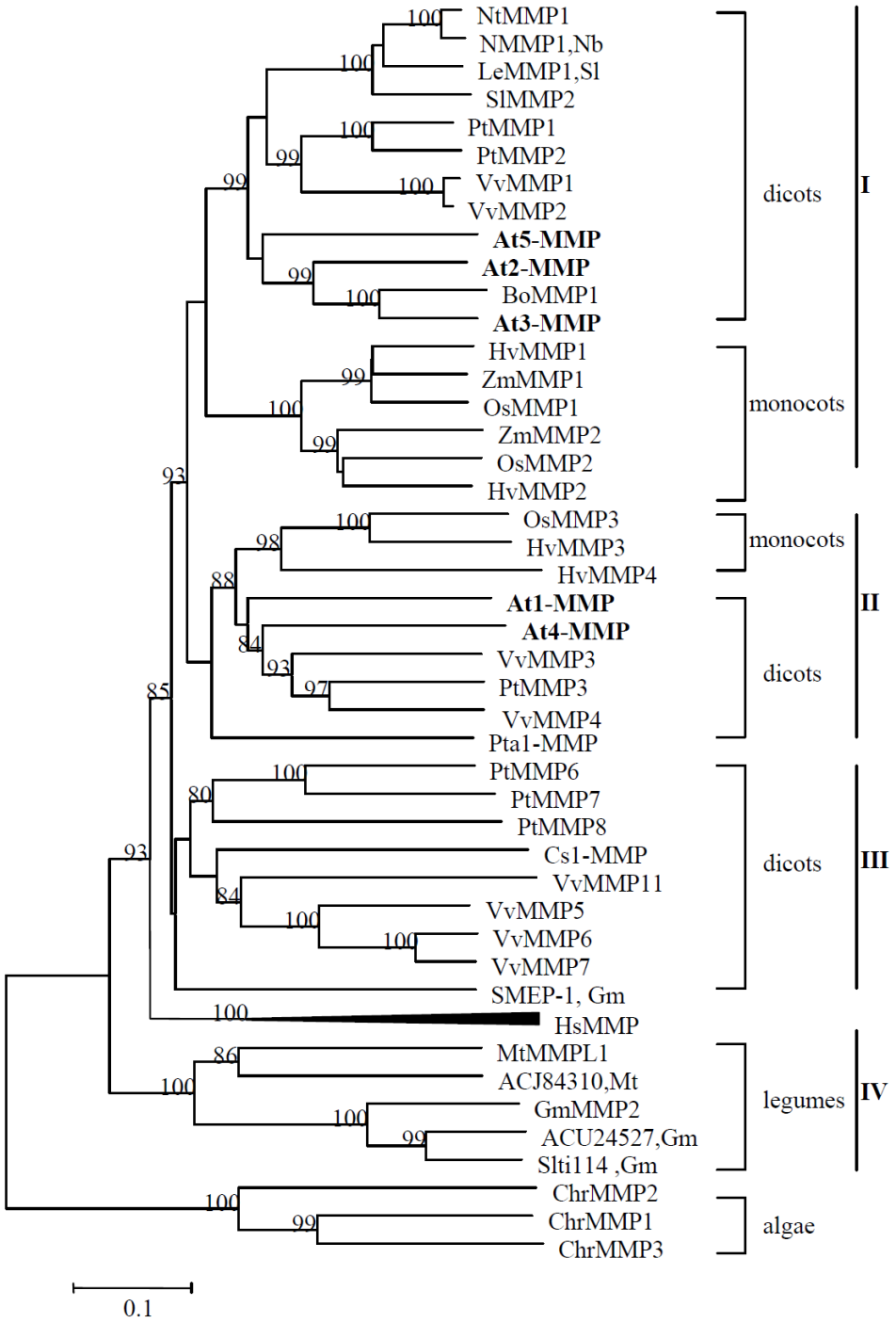


Fig.3 - 1. Molecular phylogenetic anaylsis of the MMP family in plants. The evolutionary history was inferred by Neighbor-Joining method based on the p-distance model using a conserved region spanning the cysteine switch and Met-turn motif of 49 MMP protein sequences from human and 15 plant species. At (*Arabidopsis thaliana*), Bo (*Brassica oleracea*), Chr (*Chlamydomonas reinhardtii*), Cs (*Cucumis sativus*), Gm (*Glycine max*), Hs (*Homo sapiens*), Hv (*Hordeum vulgare*), Mt (*Medicago truncatula*), N (*Nicotiana benthamiana*), Nt (*Nicotiana tabacum*), Os (*Oryza sativa*), Pt (*Populus trichocarpa*), Pta (*Pinus taeda*), Sl (*Solanum lycopersycum*), Vv (*Vitis vinifera*) and Zm (*Zea mays*). Genebank accession numbers for proteins or the corresponding cDNA are listed in section 2.10. Bar represents the number of amino acid differences per site (Tamura *et al*., 2011).

3.2 At-MMPs gene expression during the interaction with various pathogens

3.2.1 At-MMPs expression during the infection of *Botrytis cinerea*

In *Arabidopsis*, there are five homologues in the matrix metalloproteinses family and are named *At1-MMP* to *At5-MMP* (Maidment *et al*., 1999). From the Genevestigator database (www.genevestigator.com), some members of AtMMPs showed differential expression pattern after pathogen infection. To investigate the expression pattern of the five MMP genes, gene-specific primers were designed (Table. S1). The 5-week-old plants were inoculated with necrotrophic fungus *B. cinerea* and the infected leaves were harvest at different time points (0h, 8h, 16h, 24h, 48h and 72h) (section 2.2.2).

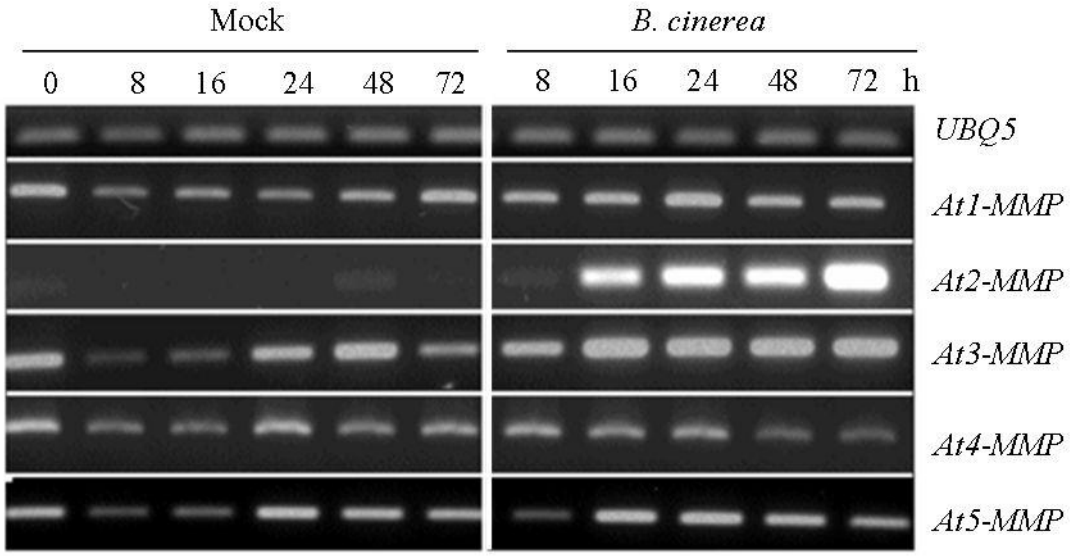


Fig.3 - 2. Expression profile of *At-MMPs* in Col-0 leaves after *Botrytis cinerea* infection Five-week-old *Arabidopsis* Col-0 plants were inoculated with *B. cinerea* by placing 5 ul spore suspension ($2x10^5$ spores/mL in 1/2 potato dextrose broth (PDB)) in the centre of the rosette leaves. Mock treatment was performed with 1/2 PDB. Total RNA was extracted from leaves at the indicated time points after *B. cinerea* infection and used for RT-PCR. 28 cycles were used for *UBQ5*, which served as the internal control for normalization, and 35 cycles for *At-MMPs*.The experiments were repeated three times with similar results. h: hours

After *B. cinerea* infection, *At1-MMP* was slightly up-regulated from 8 h to 48 h. At 24 h infection, *At1-MMP* showed the highest expression level among all the time points (Fig.3 - 2). For *At2-MMP*, the basal expression level was quite low and almost non-detectable in the mock treatment. However, *At2-MMP* showed a strong induction in response to *B. cinerea* infection (Fig.3 - 2). The up-regulation of At2-MMP was visible 8 h after infection and was increasing from 16 h to 72h. At 72 h post inoculation, *At2-MMP* had the highest expression level. For *At3-MMP*, its induction pattern is very similar but earlier than *At2-MMP*. At 8 h after *B. cinerea* infection, *At3-MMP* already showed clear induction. Notably, *At3-MMP* expression in mock treatment is associated with photoperiod rhythm. Its expression level appeared high in light (0 h, 24 h, 48 h and 72 h) and low in dark (8h and 16 h). There was

little difference for *At4-MMP* expression between mock treatment and *B. cinerea* treatment. For *At5-MMP*, it had similar expression pattern as *At3-MMP* in mock treatment. *B. cinerea* appeared to upregulate *At5-MMP* expression at 16 hpi but had little influence in later time points through the infection.

3.2.2 At-MMPs expression during the infection of *Golovinomyces orontii*

To examine the gene expression of At-MMPs after biotrophic pathogen infection, plants were inoculated with powdery mildew fungus *Golovinomyces orontii*. A spore suspension of 50,000 conidia mL^{-1} was prepared and sprayed immediately on 6-week-old healthy Col-0 plants (section 2.2.3). *At1-MMP* had similar expression between the mock treatment and *G. orontii* inoculation. For *At2-MMP*, it was clearly up-regulated at very late time point 120 hpi. *At3-MMP* had same expression level in the mock treatment and *G. orontii* inoculated treatment except that there was a slight up-regulation at 12h. *At4-MMP* was down-regulated after *G. orontii* inoculation starting from 24h to 120h. For *At5-MMP*, no clear differences in expression could be observed.

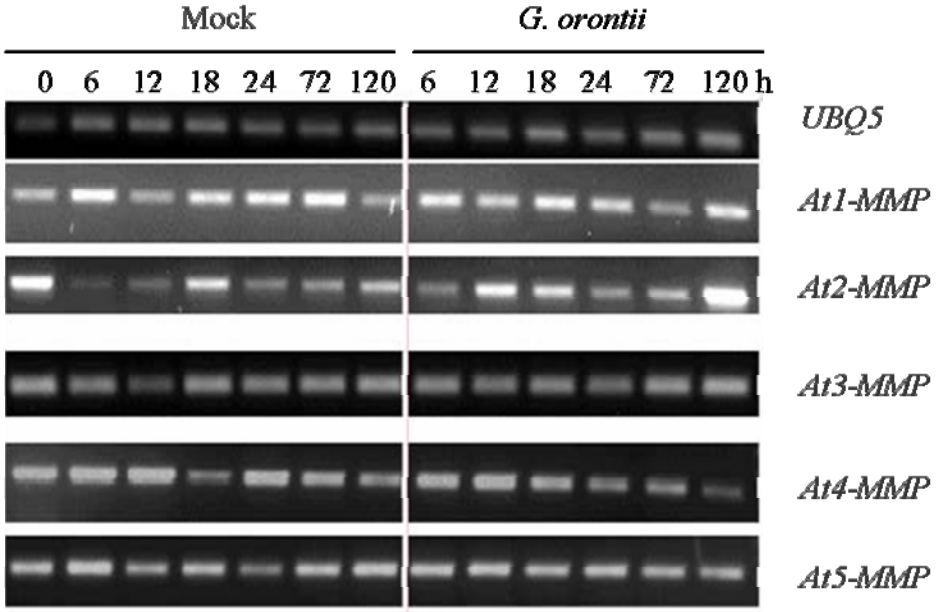


Fig.3 - 3. Expression profile of *At-MMPs* in Col-0 leaves after *Golovinomyces orontii* infection. Five-week-old plants were inoculated with *G. orontii* by spraying spore suspension (50,000 conidia ml^{-1} in 1: 20,000 Tween20/water). Mock treatment was performed with Tween20 water. Leaves were harvested at the indicated time points after *G. orontii* infection and used for total RNA extraction. RT-PCR was performed using UBQ5 as an internal control. The experiments were repeated three times with similar results. h: hours

3.2.3 At-MMP expression during the infectin of *Pseudomonas syringae*

The *Pseudomonas syringae* inoculation was performed by infiltration with *Pseudomonas syringae pv tomato* DC3000 (*Pst*DC3000) suspension (OD_{600} = 0.1 in 10 mM $MgCl_2$) on 6-week-old *Arabidopsis* Col-0 plants (section 2.2.2). The injection was done following a half-leaf method shown in Fig.3 - 4. The results revealed that *At1-MMP*, *At4-MMP* and *At5-MMP* showed no clear systemic changes during *P. syringae* infection. *At2-MMP* was slightly up-regulated starting from 8 h and was obviously up-regulated at 24h. *At3-MMP* was up-regulated from very early time point 4 h after injection.

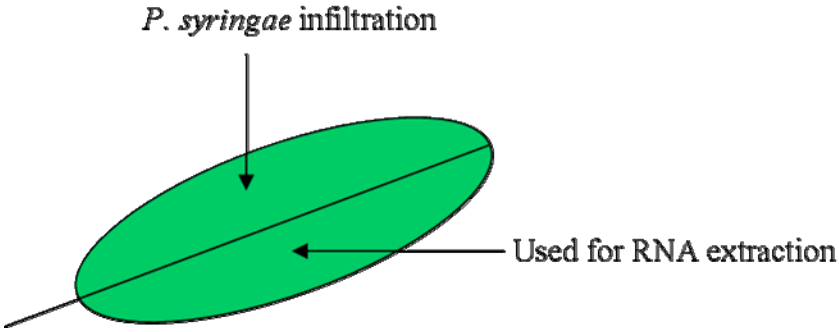


Fig.3 - 4. Half-leaf method in the infection of *Pseudomonas syringae* pv *tomato* DC3000. Schematic representation of the procedure is shown. Half leaf was infiltrated with *P. syringae* suspension using a needleless syringe and the other half leaf was harvested for At-MMPs gene expression assay.

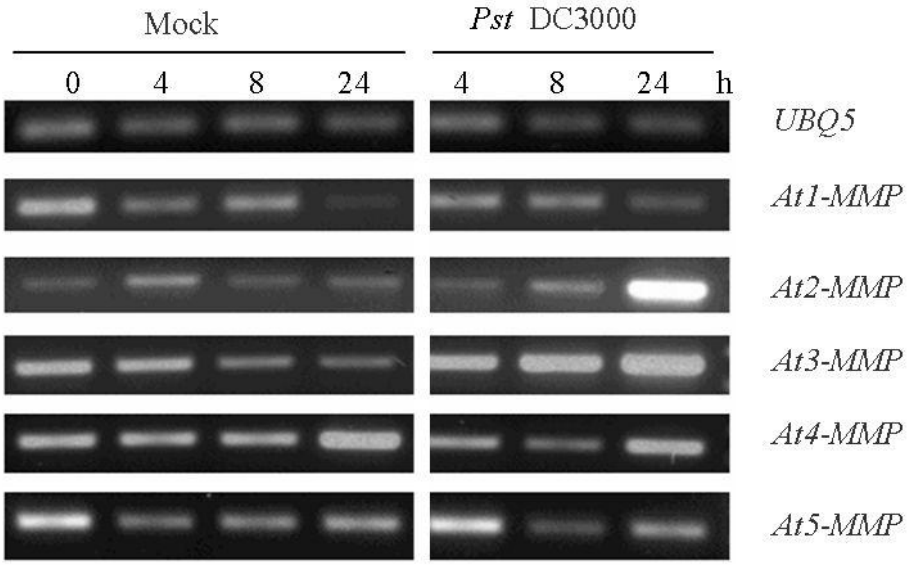


Fig.3 - 5. Expression profile of *At-MMPs* in Col-0 leaves after infection with bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000. *At2-MMP* and *At3-MMP* are induced by *Pst* infection. Six-week-old *Arabidopsis* Col-0 plants were infiltration with *Pst* DC3000 suspension with OD600= 0.1. The mock treatment was performed by infiltration with 10 mM $MgCl_2$. Leaves were harvested at the indicated time points after *Pst* DC3000

infection and used for total RNA extraction. RT-PCR was performed using UBQ5 as an internal control. The experiments were repeated three times with similar results. h: hours

3.2.4 At-MMPs gene expression in roots in the interaction with *Piriformospora indica*

Seeds of wild type Col-0 plants were sterilized and sowed on ATS medium in vertical Petri dishes. 3-week-old plants were used for *P. indica* inoculation with a spore concentration of 5×10^5 spores/mL (section 2.2.5). Based on the RT-PCR result, I observed that *At1-MMP* appeared to be slightly down-regulated starting from 1 day after inoculation. *At2-MMP* had much higher expression levels in roots but not altered due to *P. indica* treatment. For *At3-MMP*, it was up-regulated at 1day after inoculation and down-regulated from 3 days, then it was strongly down-regulated 7 days after inoculation. *At4-MMP* and At5-MMP appeared to have no altered expression after *P. indica* inoculation.

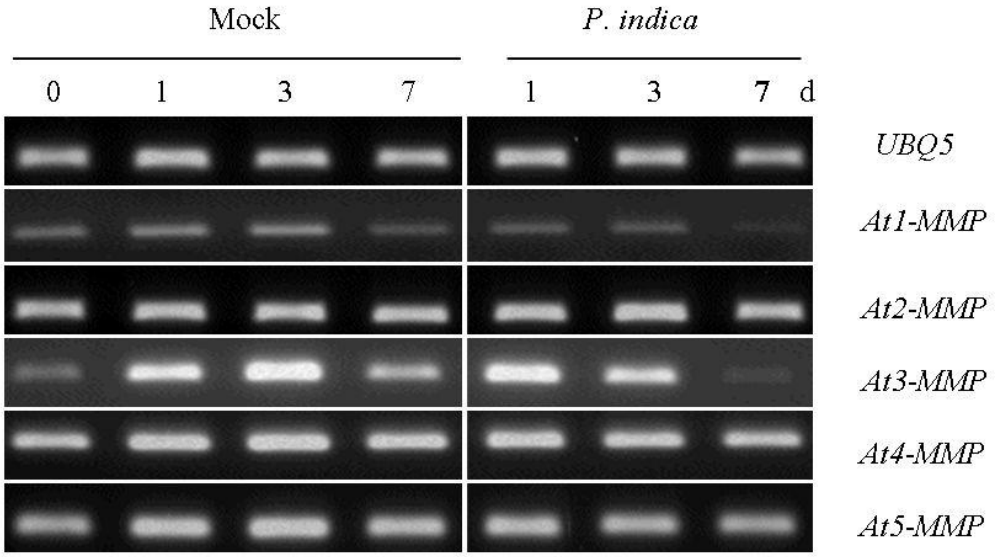


Fig.3 - 6. Expression profile of *At-MMPs* in Col-0 roots after colonization with the mutualistic fungus *Piriformospora indica*. The roots of 3 weeks old plants grown on ATS medium were inoculated with *P. indica* spores (5×10^5 spores/mL). The mock treatment was done with Tween20-water. The roots were harvested at the indicated time points after *P. indica* infection and used for total RNA extraction. RT-PCR was performed using UBQ5 as an internal control. The experiments were repeated two times with similar results. d: days.

3.2.5 Expression of *At2-MMP* and *At3-MMP* in signaling mutants

As described above, *At2-MMP* and *At3-MMP* were obviously induced upon *B. cinerea* infection. To determine which signal is required for the regulation of *At2-MMP* and *At3-MMP* expression, *Arabidopsis* mutant plants with compromised signaling pathways, namely salicylic acid (SA; NahG, *ics1, npr1-1, pad3-1*), jasmonic acid (JA; *jar1, jin1*) and ethylene (*ein2-1*) pathway mutants were tested for the *At2-MMP* and *At3-MMP* expression after *B. cinerea* infection. Representative disease symptoms of the *B. cinerea*-infected mutants are shown in Fig.3 - 7. The lesion sizes of all mutants were bigger than Col-0 plants thus more

susceptible to *B. cinerea*. Regarding *At2-MMP* expression, all the mutants showed similar expression pattern as Col-0 plants (Fig.3 - 8). Therefore, *Botrytis* induced expression of *At2-MMP* is likely independent of SA, JA and ethylene signaling pathway, induced by multiple pathways or upstream of the tested genes.

In all the mutants, *At3-MMP* had similar expression level and pattern with Col-0 plants (Fig.3 - 8). Hence, *B. cinerea* induced expression of *At3-MMP* is also independent of SA, JA and ethylene signaling pathway, or upstream of the tested genes.

Fig.3 - 7. Disease symptoms of the SA, JA or ET signaling mutants after *Botrytis cinerea* infection. The 5-week-old plants were inoculated with *B. cinerea* by placing 5 μL spore suspension ($2x10^5$ spores/mL in 1/2 potato dextrose broth (PDB)) in the centre of the rosette leaves. The photographs were taken 3 days after inoculation.

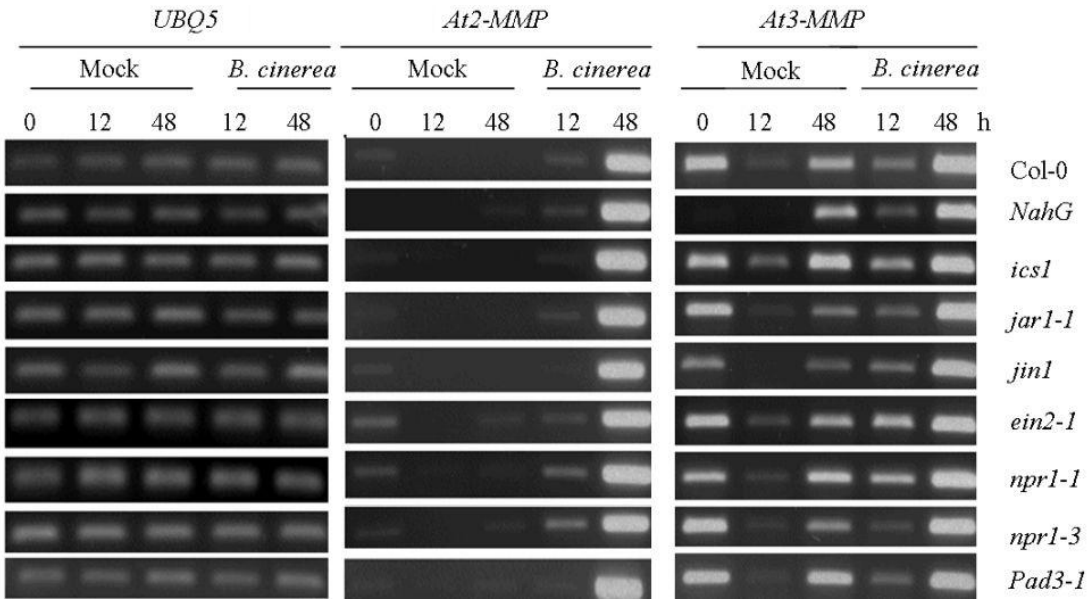


Fig.3 - 8. *B. cinerea* induced *At2-MMP* and *At3-MMP* expression is independent of SA, JA or ET signaling. Leaves from Col-0 and different signaling defective mutants were harvested at the indicated time points after *B. cinerea* infection and used for total RNA extraction. RT-PCR was performed using UBQ5 as an internal control. h: hours

To gain further information about the signaling pathway of *At2-MMP* and *At3-MMP*, more mutants were included in the expression analysis. Abcidic acid (ABA; *abi2, aba2*), gibberellic acid (GA; *della pentuple*), cytokinin (*ahp1,2,3,4,5-1*), JA (*coi1-16*), auxin (*tir1-1*), brassinosteroids (*bri1-116*) mutants, and a phytoalexin deficient plant (*pad4*) with corresponding wild type plants Ler and Col-0 were inoculated with *B. cinerea*. The infected leaves of 5 weeks old plants were harvested at 12 h and 48 h. After RNA extraction and cDNA synthesis, *At2-MMP* and *At3-MMP* expression was examined. Regarding the lesion size, *abi2* and *della pentuple* were significantly more susceptible than the wild-type Ler plants. Likewise, the mutant lines *bri1-116, aba2, coi1-16, tir1-1, pad4* and *ahp1, 2, 3, 4, 5-1* were also more susceptible than their background Col-0 (Fig.3 - 9). The mutant lines *coi1-16* and ABA mutants (*aba2* and *abi2*) were hypersusceptible to *B. cinerea* as evidenced by the complete rot and overgrowth with fungal mycelium (Fig.3 - 9).

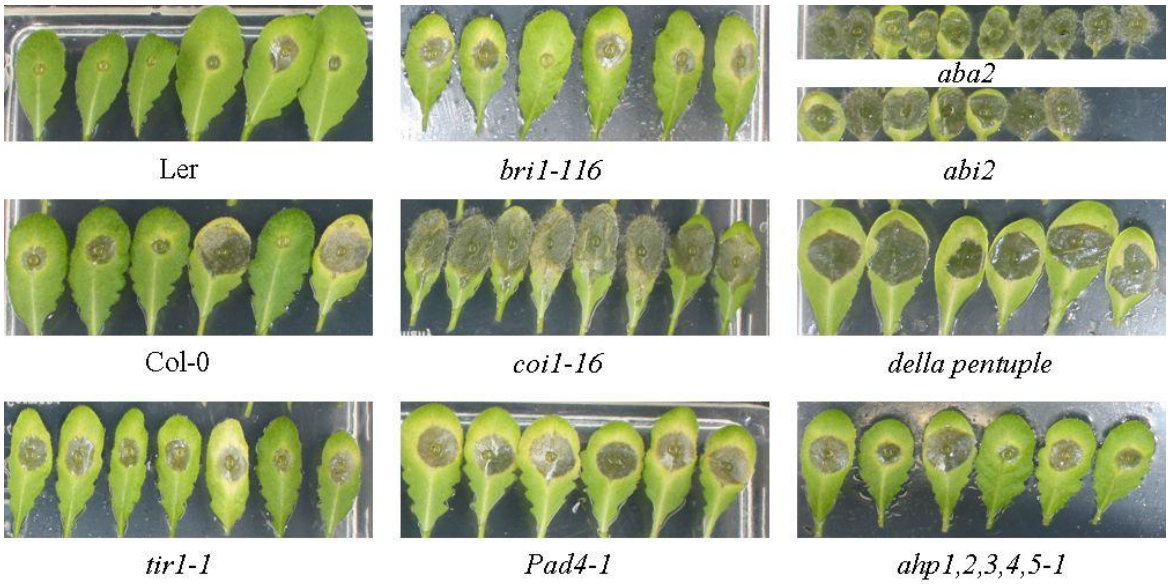


Fig.3 - 9. Disease symptoms of the signaling mutants after *B. cinerea* infection. The detached rosette leaves from 5-week-old plants were inoculated by placing 5 µl of *B.cinerea* spore suspension ($2x10^5$ spores/mL in 1/2 potato dextrose broth (PDB)) in the centre of the leaves. The photograph was taken 3 days after inoculation. *abi2* and *della pentuple* were in Ler background. The rest mutants *bri1-116, aba2, coi1-16, tir1-1, pad4* and *ahp1, 2, 3, 4, 5-1* was in Col-0 background.

In *aba2* mutants, which are defective in ABA synthesis, *At2-MMP* was expressed at similar level in mock treated and *B. cinerea* infected plants. The *B. cinerea*-induction of *At2-MMP* is not as clear as in the other mutants (Fig.3 - 10). This abolished induction of *At2-MMP* by *B. cinerea* in *aba2* mutant might imply the requirement for ABA but has to be confirmed in independent experiments. Similarly, the expression of *At2-MMP* in *B. cinerea*-infected *pad4-1* mutants was reduced, indicating a role of PAD4 in the regulation of *At2-MMP* expression. In other mutants lines such as *della pentuple, ahp1, 2, 3, 4, 5-1, tir1-1, bri1-116* and *coil1-16,* they had very similar expression pattern as Col-0. These results suggested that *B. cinerea*-induced *At2-MMP* expression might require direct or indirect regulation from ABA and PAD4. For *At3-MMP*, the induction by *B. cinerea* in the mutants appeared to have similar pattern as in the wild-type plants (Fig.3 - 10, right).

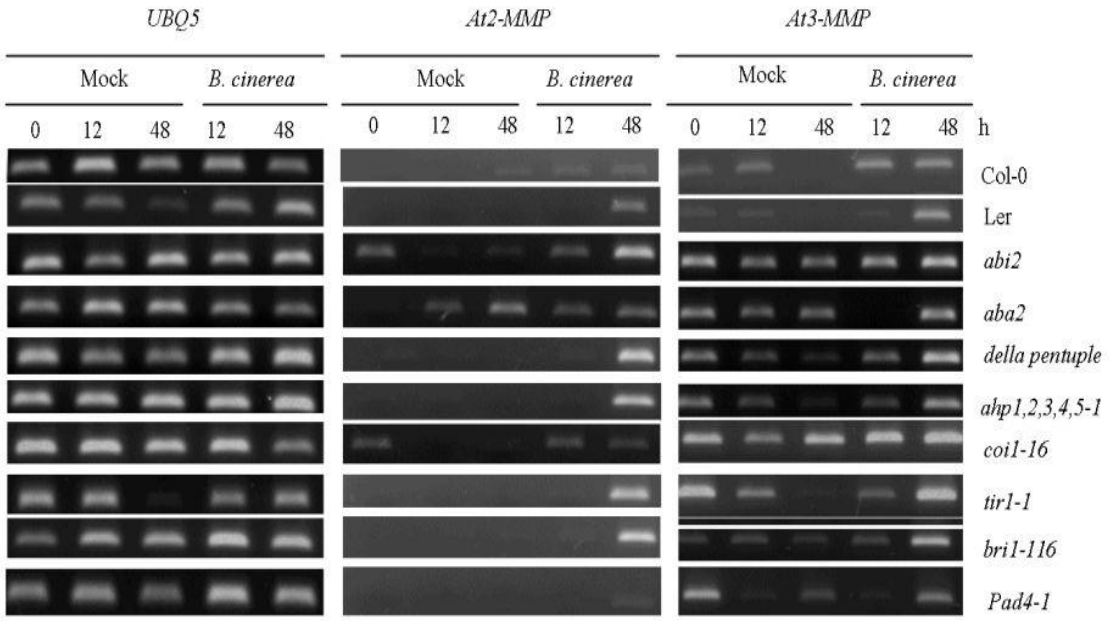


Fig.3 - 10. *B. cinerea* induced *At2-MMP* and *At3-MMP* expression in other signaling mutants. Leaves from Col-0 and different signaling defective mutants were harvested at the indicated time points after *B. cinerea* infection and used for total RNA extraction. RT-PCR was performed using UBQ5 as an internal control. *abi2* and *della pentuple* are in Ler background. The other mutants *bri1-116, aba2, coi1-16, tir1-1, pad4* and *ahp1, 2, 3, 4, 5-1* are in Col-0 background.

3.2.6 At2/3-MMP expression in *mpk3*, *mpk6* and *eds1* mutants

In *Arabidopsis*, the mitogen-acitivated protein kinases (MAPKs) MPK3 and MPK6 are well-known regulators of a wide array of defense responses (Asai *et al*., 2002; Ren *et al*., 2008). To elucidate the possible regulation of *At2/3-MMP* by MAP kinases, *mpk3* and *mpk6* mutant were included with *B. cinerea*. *At2-MMP* showed almost the same expression level and pattern in the *mpk3* and *mpk6* mutants as in wild type Col-0. In both mutants, *At2-MMP* was slightly up-regulated at 12 h and was significant induced at 48 h. *At3-MMP* was up-regulated at 48h after *B. cinerea* in *mpk3*, *mpk6* and Col-0 plants. There is some difference between the *mpk3*, *mpk6* mutant and Col-0. In *mpk3* mutant, *At3-MMP* had higher expression level at 0 h and lower expression at 48 h in the mock compared with Col-0. After 12 h *B. cinerea* infection, *At3-MMP* expression in *mpk3* mutant was higher than that in Col-0 plants, which might imply a negative regulation of *At3-MMP* by MPK3 in the early phase of infection (Fig.3 - 11). In *mpk6* mutant, *At3-MMP* had lower expression at 12 h and 48 h in the mock than in Col-0 plants. Despite these slight differences, the induction of *At2-MMP* and *At3-MMP* by *B. cinerea* appeared not impaired in *mpk3* or *mpk6* mutants.

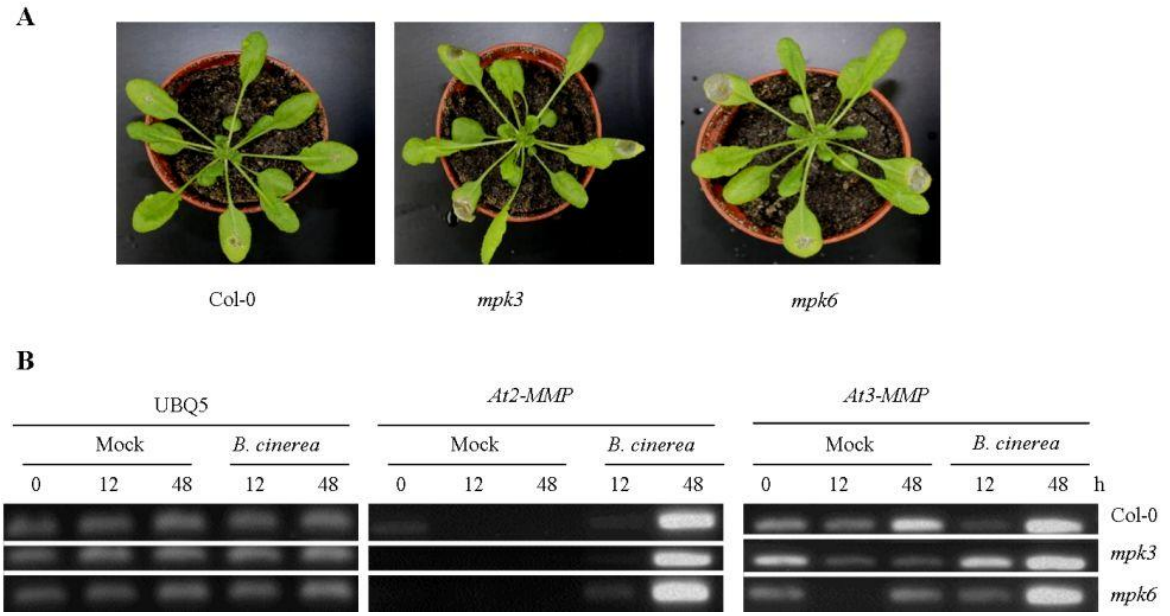


Fig.3 - 11. Expression profile of *At2-MMP* and *At3-MMP* in *mpk3* and *mpk6* leaves after *B. cinerea* infection. Five-week-old *Arabidopsis* Col-0 plants were inoculated with *B. cinerea* by placing 5 µL of spore suspension ($2x10^5$ spores/mL in 1/2 potato dextrose broth (PDB)) in the centre of the rosette leaves. Mock treatment was performed with 1/2 PDB. Leaves were harvested at the indicated time points after *B. cinerea* infection and used for total RNA extraction. RT-PCR was performed using UBQ5 as an internal control. **A**, Disease symptoms of the *mpk3* and *mpk6* mutants after *B. cinerea* infection. The pictures were taken at 3 days after inoculation. **B**, *B. cinerea* induced *At2-MMP* and *At3-MMP* expression is independent of *MPK3* and *MPK6*.

As described previously (Torres *et al*., 2003), MMP2-induction by *Pst* DC3000 infection was compromised in *eds1-2* mutant. To elucidate the possible correlation between EDS1 and *At-MMP* expression, I used *eds1-2* mutant for *B. cinerea* inoculation. In *eds1-2* mutant, *At2/3-MMP* showed same expression pattern and same expression level as Col-0 plants. This result suggested that, unlike in the case of *P. syringae*, EDS1 does not influence the *At2-MMP* expression during *B. cinerea* infection.

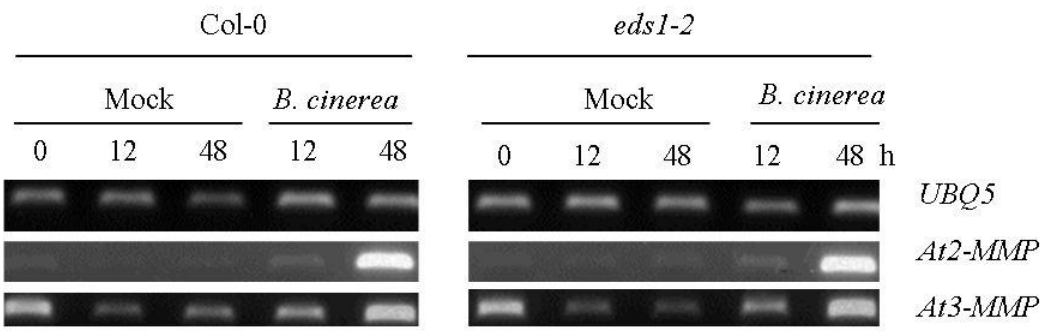


Fig.3 - 12. *B. cinerea* induced *At2-MMP* and *At3-MMP* expression is independent of *EDS1*. Five-week-old *Arabidopsis* Col-0 plants were inoculated with *B. cinerea* by placing 5 µL of

spore suspension ($2x10^5$ spores/mL in 1/2 potato dextrose broth (PDB)) in the centre of the rosette leaves. Mock treatment was performed with 1/2 PDB. Leaves were harvested at the indicated time points after *B. cinerea* infection and used for total RNA extraction. RT-PCR was performed using UBQ5 as an internal control.

3.3 Identification of AtMMP T-DNA insertion mutants

3.3.1 Identification of single mutants

The availability of large-scale T-DNA insertion mutants for *Arabidopsis thaliana* has greatly facilitated functional genomics studies. To verify the potential functions of At-MMPs, I ordered T-DNA insertion lines for At2-MMP to At5-MMP from NASC (European *Arabidopsis* stock centre). No lines were available for At1-MMP at the time the project was initiated. These mutant lines were segregating and thus required identification of homozygous individuals. The homozygous mutants were identified by PCR (Fig.S2 - S6).

Table.3 - 1. Identification of homozygous T-DNA insertion mutants

At-MMP	NASC ID	Number of plants tested	Number of homozygous plants
At2-MMP	N582450	8	2
At2-MMP	N348998	10	4
At3-MMP	N115923	17	4
At3-MMP	N103532	6	1
At4-MMP	N327098	10	5
At4-MMP	N532466	10	4
At5-MMP	N619909	10	5
At5-MMP	N593137	9	5
At5-MMP	N656052 HM	10	10
At5-MMP	N660426 HM	10	10

Table.3 - 2. List of mutants lines used in this study

MMP	gene	Accession number	NASC ID	Name	Source	Homozygous plant
At1-MMP	AT4G16640	Z97341	not available	NO	NO	no
At2-MMP	AT1G70170	AC002062	N582450	SALK_082450 (AV) (BF)	SALK	HM-257,HM-259
At2-MMP	AT1G70170	AC002062	N348998	GK-416E03.01	GABI	HM-279,HM-280,HM-281,HM-282
At3-MMP	AT1G24140	AC002396	N103532	SM_3.15171	JIC	HM-411
At3-MMP	AT1G24140	AC002396	N115923	SM_3.28404	JIC	HM-402,HM-403,HM-406,HM-418
At4-MMP	AT2G45040	AF062640	N327098	GK-075C07.01	GABI	HM-268,HM-269,HM-270,HM-276,HM-277
At4-MMP	AT2G45040	AF062640	N532466	SALK_032466 (E) (AE)	SALK	HM-357,HM-362,HM-364,HM-365
At5-MMP	AT1G59970	AC005966	N619909	SALK_119909(AV)(CH)	SALK	HM230,HM-233,HM-234,HM-237,HM-260
At5-MMP	AT1G59970	AC005966	N593137	SALK_093137(AV)(BR)	SALK	HM-264,HM-265,HM-267,HM-246,HM-247
At5-MMP	AT1G59970	AC005966	N656052HM	SALK_147513C	SALK	HM-366,HM-367,HM-368,HM-369,HM-370 HM-371,HM-372,HM-373,HM-374,HM-375
At5-MMP	AT1G59970	AC005966	N660426HM	SALK_052676C	SALK	HM-376,HM-377,HM-378,HM-379,HM-380 HM-381,HM-382,HM-383,HM-384,HM-385

3.3.2 Production of double mutants

Double mutants were generated by crossing the homozygous single-mutant lines. In this study, double mutants were produced from crossings *at2-mmp* x *at4-mmp* or *at2-mmp* x *at3-mmp*. F1 hybrids were self-crossed and at least 40 F2 plants from each combination were used for selection of homozygous double mutants. The workflow for identification of homozygous double mutants is shown in material and method (Fig. 2 - 1). Homozygous plants don't produce amplification with LP/RP primers but with LB/RP primers (Fig.S2 - S6). In principle, four PCR reactions were therefore required to identify the individuals homozygous for both T-DNA insertions in the crossing combination.

at4-mmp mutant HM-268 (N327098) and *at2-mmp* HM-280 (N348998), *at2-mmp* HM-257 (N582450) were both used as father parent and mother parent for the double mutant. After two generations of PCR selection, the homozygous double mutants were identified as listed in Table.3 - 3.

Table.3 - 3. List of identified *at2-mmp at4-mmp* double knockout mutants

Combinations	Number of tested F1 plants	True Hybrid in F1 (Line used for F2 generation is underlined)	Number of tested F2 plants	Homozygous double mutant
♂ HM-268 (*at-4mmp*, N327098) ♀ HM-280 (*at2-mmp*, N348998)	10	6 F1-451, F1-452, F1-453, F1-454, F1-459, F1-460	46	F2-7 F2-20 F2-26
♂ HM-280 (*at-2mmp*, N348998) ♀ HM-268 (*at4-mmp*, N327098)	10	5 F1-461, F1-462, F1-465, F1-466, F1-470	40	F2-57, F2-68
♂ HM-268 (*at4-mmp*, N327098) ♀ HM-257 (*at2-mmp*, N582450)	10	8 F1-471, F1-472, F1-473, F1-476, F1-477, F1-478, F1-479, F1-480	42	F2-99, F2-103, F2-105, F2-115, F2-126
♂ HM-257 (*at2-mmp*, N582450) ♀ HM-268 (*at4-mmp*, N327098)	10	10 F1-481, F1-482, F1-483, F1-484, F1-485, F1-486, F1-487, F1-488, F1-489, F1-490	40	F2-155, F2-161

at2-mmp HM-280 (N348998) and two *at3-mmp* mutant HM-411 (N103532) and HM-402 (N115923) were used as father parent and mother parent for the double mutant to have different combination. After two generations of PCR selection, the homozygous double mutants were identified as listed in Table.3 - 4.

Table.3 - 4. List of identified *at2-mmp at3-mmp* double knockout mutants

Combinations	Number of tested F1 plants	True Hybrid in F1 (Line used for F2 generation is underlined)	Number of tested F2 plants	Homozygous double mutant
♂ HM-280 (*at2-mmp*, N348998) ♀ HM-411 (*at3-mmp*, N103532)	8	8 F1-51, F1-52, <u>F1-53</u>, F1-54, F1-55, F1-56, F1-57, F1-58	40	F2-140, F2-142 F2-148, F2-153 F2-164, F2-168
♂ HM-411 (*at3-mmp*, N103532) ♀ HM-280 (*at2-mmp*, N348998)	13	13 F1-38, F1-39, <u>F1-40</u>, F1-41, F1-42, F1-43, F1-44, F1-45, F1-46, F1-47, F1-48, F1-49, F1-50	42	F2-103, F2-112 F2-113, F2-127
♂ HM-280 (*at2-mmp*, N348998) ♀ HM-402 (*at3-mmp*,N115923)	10	10 F1-28, F1-29, F1-30, F1-31, F1-32, F1-33, <u>F1-34</u>, F1-35, F1-36, F1-37	60	F2-II-11
♂ HM-402 (*at3-mmp*, N115923) ♀ HM-280 (*at2-mmp*, N348998)	6	6 F1-22, F1-23, F1-24, <u>F1-25</u>, F1-26, F1-27	50	F2-II-7

3.4 Pathogen responses of At-MMP mutants

3.4.1 Responses of At-MMP mutants *to Golovinomyces orontii*

As mentioned above, some At-MMPs showed altered expression after pathogen infection. To investigate the functions of At-MMPs in pathogen resistance, I inoculated all the MMP mutants with biotrophic fungus *Golovinomyces orontii* (section 2.2.3). The 5-week-old healthy plants were sprayed with 50,000 conidia mL^{-1} spore suspension. After 11 days of infection, conidia were rinsed from leaves and the number of conidia/mg fresh weight was determined microscopically (Fig.3 – 13, A). All the mutants tested showed no significant difference in comparison with Col-0 plants. The disease symptoms 11 days after powdery mildew infection are shown in Fig.3 - 13, B.

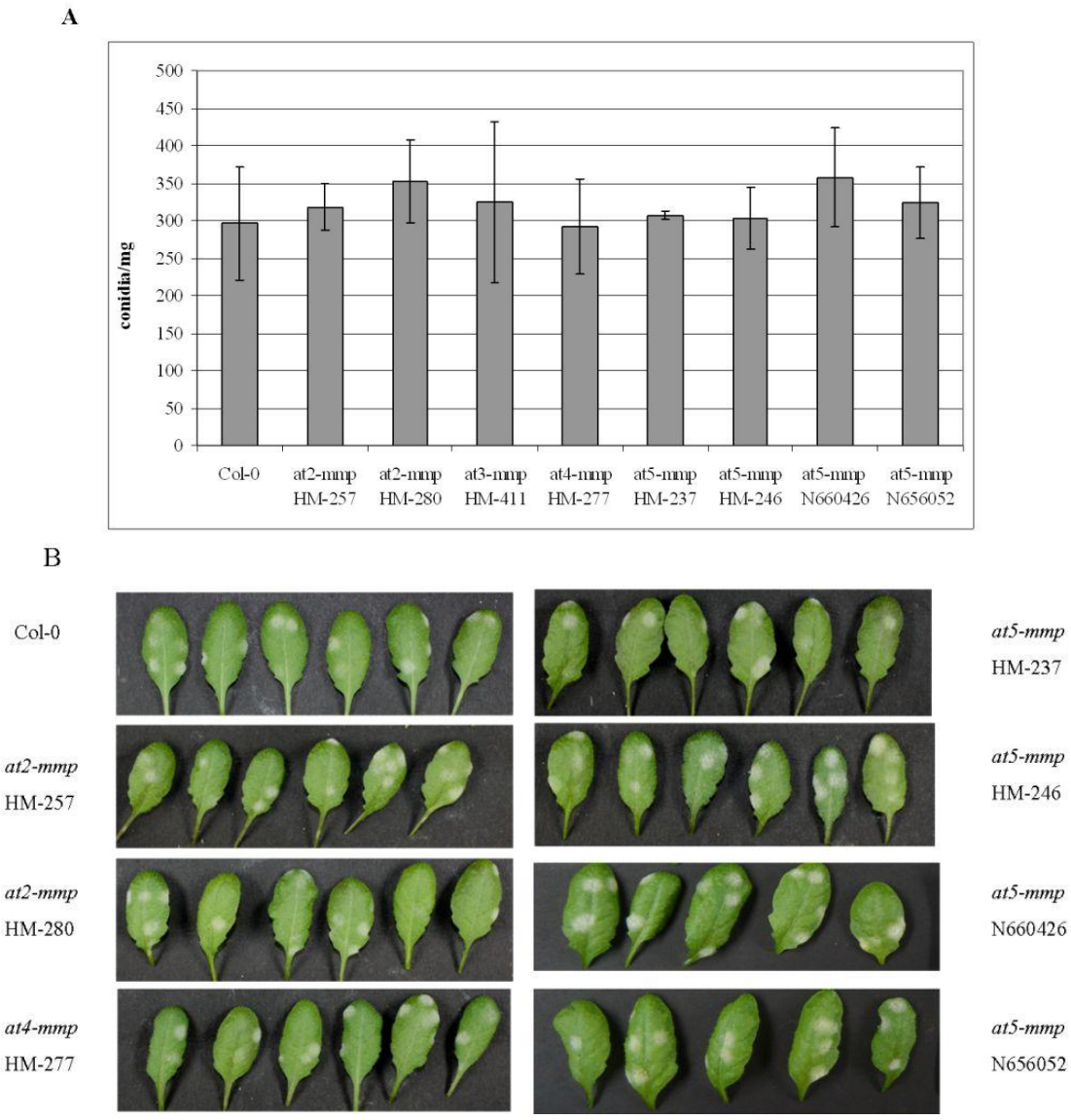


Fig.3 - 13. Disease signs of *at-mmp* mutants and Col-0 plants after powdery mildew inoculation. Six-week-old plants were sprayed with 50,000 conidia/ mL spore suspension of the powdery mildew fungus *G. orontii*. Eleven days after inoculation, the leaves were detached and the amount of conidia per mg of leaf fresh weight was determined for at least five individually treated plants. The error bars indicate the standard error. **A**, Conidia per mg leaf fresh weight for *at-mmp* mutants and Col-0 plants. **B**, Disease symptoms of the *at-mmp* mutants and Col-0 plants. Images were taken 11 days after inoculation.

3.4.2 Responses of At-MMP mutants to *Pseudomonas syringae*

Based on the At-MMPs gene expression profile during compatible interaction with biotrophic bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 (Fig.3 - 5), *At2-MMP* and *At3-MMP* were induced due to infection. To investigate the involvement of *At-MMPs* during *Pst* infection, the mutant lines and Col-0 plants were infiltrated with virulent strain *Pst* DC3000 (OD600=0.01 in 10mM $MgCl_2$). Leaf disks of 0.5cm diameter were harvested at two time points (0 h and 48 h after inoculation). Serial dilutions from 10^{-1}

to 10^{-5} were prepared from the corresponding leaf extracts and spread on agar plates. After 2 days incubation at 28°C, the colony number was counted and calculated as cfu/cm^2 to determine the growth of the bacteria (section 2.2.4). Both *at2-mmp* mutants (HM-257 and HM-280) and *at5-mmp* mutants (HM-237 and HM-246) had similar level of bacterial growth with Col-0 plants. The *at4-mmp* mutant line (HM-277) showed a significant difference with Col-0 in the preliminary trial (Fig. 3-14).

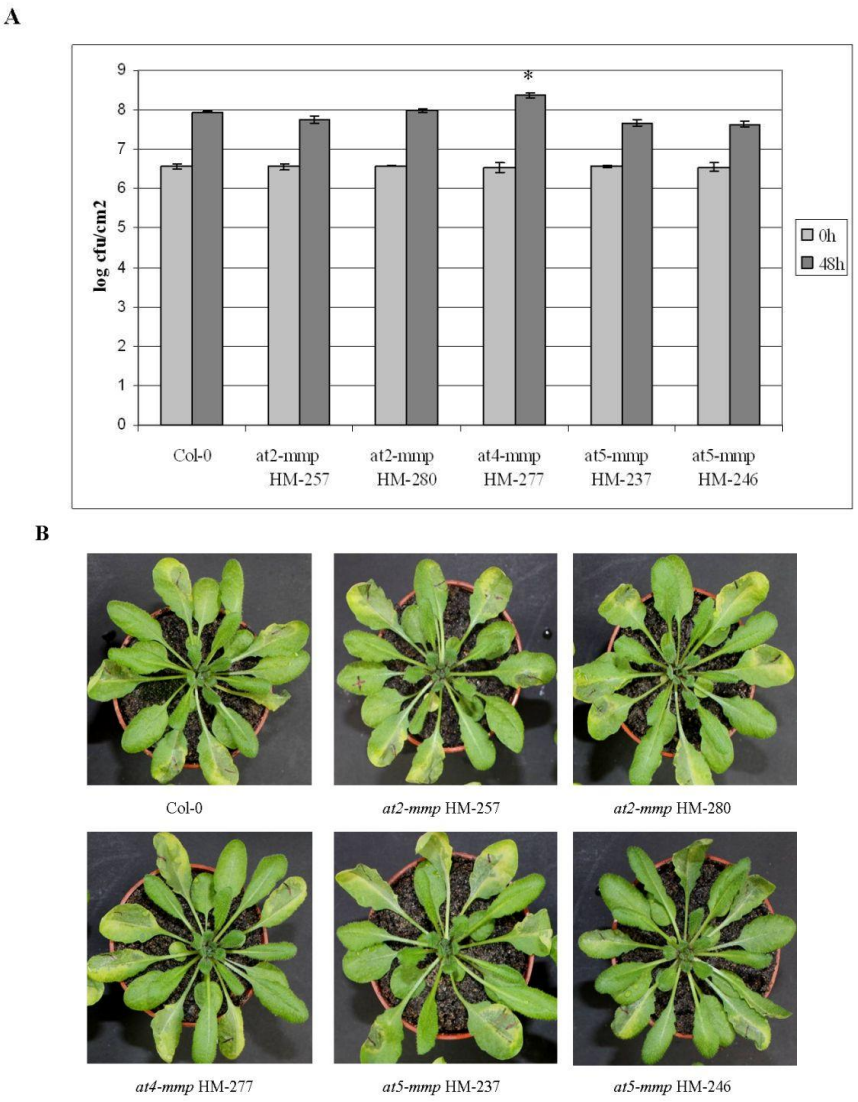


Fig.3 - 14. Responses of *at-mmp* mutants to *Pst DC3000*. **A**, The *at-mmp* mutants and Col-0 plants were inoculated through injection with a suspension of *Pst DC3000* (OD_{600}=0.01 in 10mM $MgCl_2$). Leaf disks of 0.5 cm diameter were harvested at 0 h and 48 h after inoculation. Columns and error bars represent mean and standard error from 10 plants. *at4-mmp* mutant HM-277 showed significant difference from the control plants ($p \leq 0.05$) according to Student's t-test. **B,** Pictures of representative inoculated plants were taken 3days after infection. The injected leaves were marked as shown.

3.4.3 Responses of At-MMP mutants to *Botrytis cinerea*

In wild type Col-0 plants, expression of *At2-MMP* and *At3-MMP* is induced by *B. cinerea* infection (Fig.3 - 2). To study the At-MMPs function during the *B. cinerea* infection, the *at-mmp* mutants were examined for the altered responses after *B. cinerea* inoculation (section 2.2.2). The detached leaf was cut from 6 weeks old plants and put into the plastic box with 0.5% H_2O-Agar. The lid was closed to keep the higher humidity. The pictures were taken at 6 days after inoculation. The lesion size was measured with ImageJ. Two independent *at2-mmp* mutants (HM-257 and HM-280) were more susceptible than wild type plants (Fig.3 - 15) and HM-280 show significantly susceptible. Two independent *at3-mmp* mutants (HM-402 and HM-411) were also more susceptible than Col-0 plant (Fig.3 - 15) and HM-411 showed the significantly susceptible to the Col-0 plants. *at4-mmp* mutant (HM-277) showed non-significantly smaller lesion size than Col-0 (Fig.3 - 15). Surprisingly, the two independent *at5-mmp* mutants HM-237 and HM-246 exhibited opposite phenotype (Fig.3 - 15). For line HM-237, the lesion size is slightly bigger than that in Col-0. However, the line HM-246 displayed significantly increased resistance to *B. cinerea* in comparison with Col-0 (Fig.3 - 15).

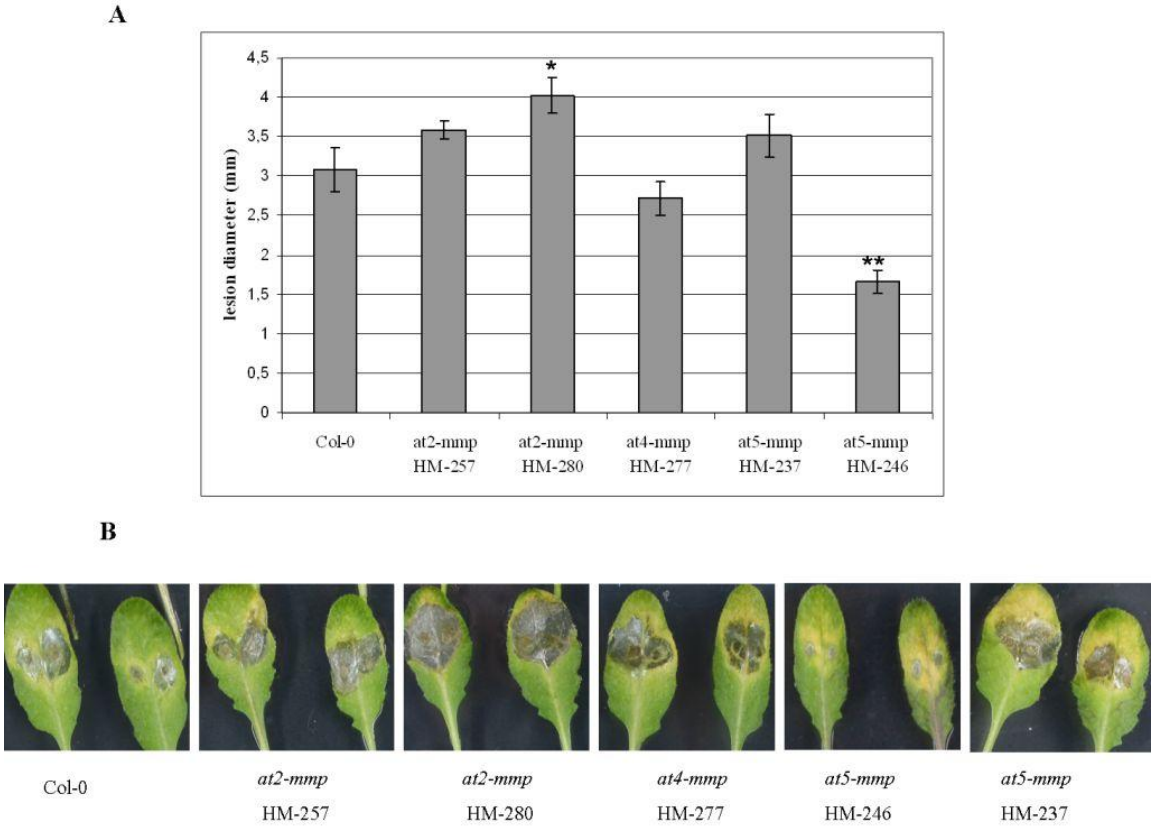


Fig.3 - 15. *at2-mmp* and *at3-mmp* mutants showed enhanced susceptibility to the necrotrophic pathogen *B. cinerea.* Leaves were cut from 6-week-old plants and placed in plastic boxes containing 0.5% H_2O-Agar. The detached leaves were inoculated by placing 3 µL spore suspension (5×10^4 conidiospores/mL) on both sides of the middle vein. The lesion

size was measured by Image J. **A**, Diameter of lesions on leaves of Col-0 and *at-mmp* mutants 6 days after inoculation with *B. cinerea.* Data represent average ± SE of at least 20 lesions. Bars represent the standard error. Asterisks indicate significant difference to control on the basis of Students's t-test (*, P≤0.05; **, P≤ 0.01). **B**, Disease symptoms on Col-0 and *at-mmp* mutant leaves. The photograph was taken 6 days after inoculation.

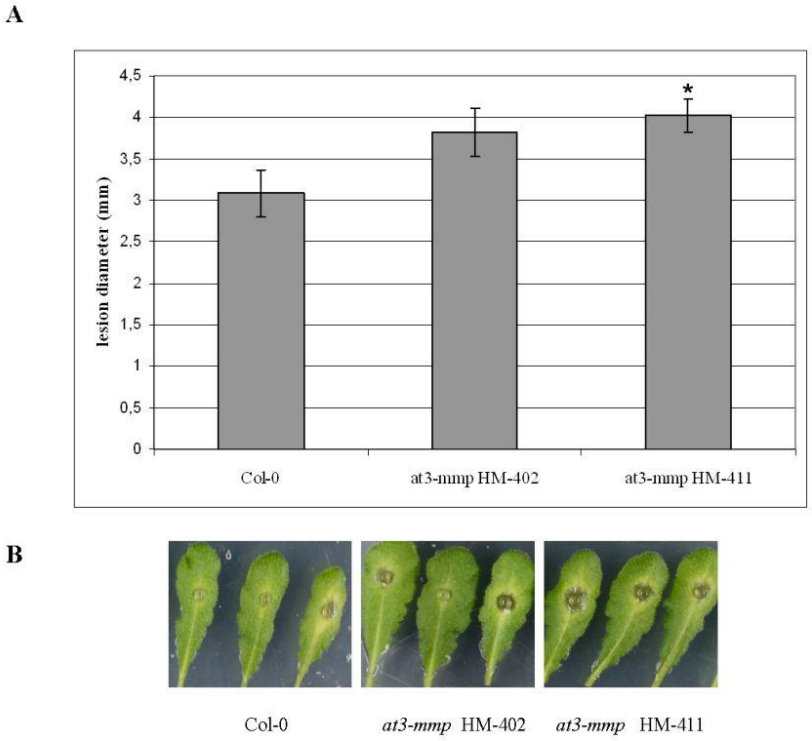


Fig.3 - 16. *at3-mmp* mutant (HM-402 and HM-411) showed increased susceptibility to the necrotrophic pathogen *B. cinerea.* The rosette healthy leaf was cut from the 6 weeks old plants and placed in plastic boxes containing 0.5% H_2O-Agar. The detached leaves were inoculated by placing 5 µL spore suspension (5×10^4 conidiospores/mL) on the middle vein. The lesion size was measured by Image J. **A**, Size of lesion formed in leaves of Col-0 and *at3-mmp* mutant (HM-402 and HM-411) 2days after inoculation with *B. cinerea.* Data represent average ± SE of at least 10 lesions. Bars indicate the standard error. *at3-mmp* (HM-402) increased the lesion size but not significantly. *at3-mmp* (HM-411) were significant different with Col-0 plants. Bars indicate the standard error. The asterisk indicats data set significantly different according to Students's t-test (P≤ 0.05). **B**, Symptoms of Col-0 and *at3-mmp* mutants (HM-402 and HM-411) leaves infected with *B. cinerea*. The photograph was taken 2 days after inoculation.

Double mutant *at2-mmp at3-mmp* produced using two different At3-MMP mutant lines were also tested regarding their resistance towards *B. cinerea* (Fig.3 - 17). The leaves were cut from 6 weeks old plants and were put into the plastic box with 0.5% H_2O-Agar. The detached leaves were inoculated by placing 5 µl spore suspension (5×10^4 conidiospores/mL) on the middle vein. The lesion size was measured by ImageJ (section 2.2.2). The double mutant lines showed bigger lesion size compared with the parental single mutant or the wild

type Col-0 plants. Both line DM F_3-II-7 and F_3-II-11 were highly significant susceptible compare to Col-0 and were more susceptible to parental line *at2-mmp* HM-280 and *at3-mmp* HM-411. Most double mutants of *at2-mmp* HM-280 and *at3-mmp* HM-411 showed significant susceptible to Col-0 Plants and were susceptible to parental line (Fig.S9). This indicates an additive effect of the mutations in At2-MMP and At3-MMP.

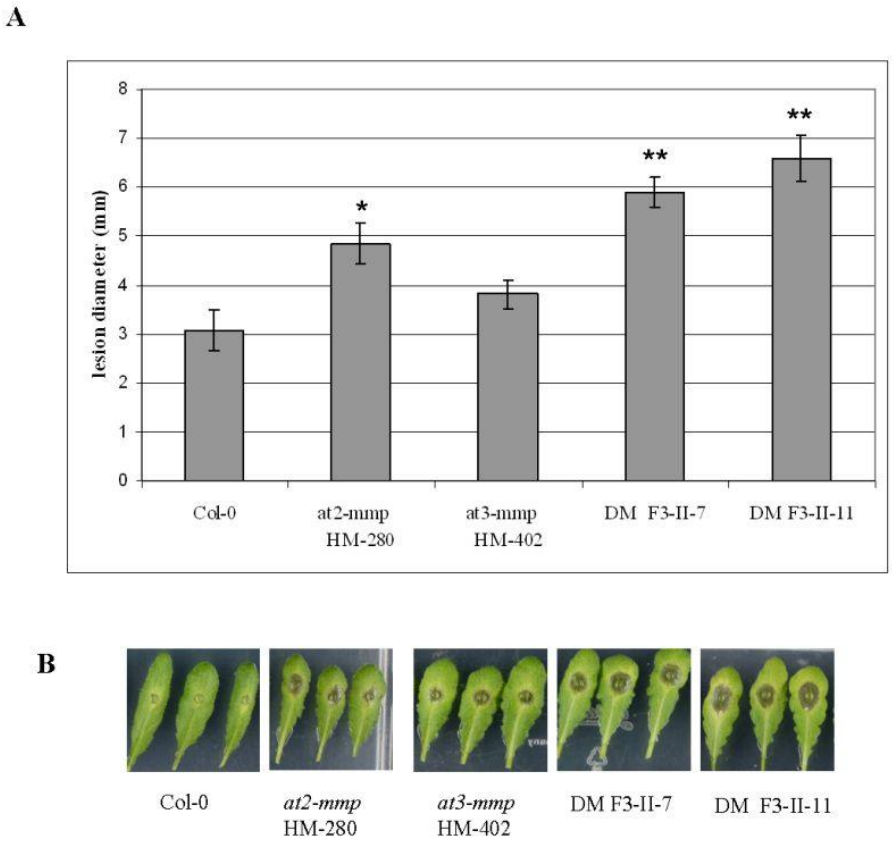


Fig.3 - 17. Double mutants of *at2-mmp* and *at3-mmp* F_3-II-11 and F_3-II-7 showed enhanced susceptibility to the necrotrophic pathogen *B. cinerea.* The rosette leaves were detached from 6-week-old plants and placed in plastic boxes containing 0.5% H_2O-Agar. The detached leaves were inoculated by placing 5 µL spore suspension (5×10^4 conidiospores/mL) on the middle vein. The lesion size was measured by ImageJ. **A**, Size of lesions formed in leaves of homozygous double mutants F_3-II-11 and F_3-II-7, parental lines *at2-mmp* (HM-280) and *at3-mmp* (HM-402) and wild type Col-0 two days after inoculation with *B. cinerea.* Data represent average ± SE of at least 20 lesions. Bars show the standard error. Asterisks indicate significant difference to control on the basis of Students's t-test (*, P≤0.05; **, P≤ 0.01). **B**, Disease symptoms on detached leaves of the double mutants F_3-II-11, F_3-II-7, single mutants *at2-mmp* (HM-280) and *at3-mmp* (HM-402) and Col-0 plants two days after infection. DM: double mutant.

3.5 Generation and characterization of *At2-MMP* and *At3-MMP* overexpression plants

3.5.1 Generation of *At2-MMP* and *At3-MMP* overexpression lines

Gene expression profiles and analysis of mutatns pointed At2-MMP and At3-MMP as promising candidate in plant innate immunity. In order to further verify the At2-MMP and At3-MMP gene function during plant defense, At2-MMP and At3-MMP overexpression plants were generated. The coding sequence of At2-MMP and At3-MMP was amplified by PCR using specific primers with flanking restriction sites for *Bam*HI and *Hind*III. The full length fragment was cloned into pGEM-T-easy vector, verified by sequencing and then cut out through BamHI/HindIII digestion, ligated into the cloning plasmid p35S-BM and subcloned into the SfiI sites of the *Agrobacterium* transformation vector pLH6000 in sense orientation under control of *CaMV 35S* promoter (Fig.3 - 18, A). As a control, the empty vector (EV) construct containing only the *CaMV 35S* promoter and nos terminator was used for transformation. The transgenic seeds were harvested from T_0 plants and were sown on ½ MS medium containing 30mg/L hygromycin. The transgenic seedlings were efficiently and reliably selected based on the presence of elongated roots and expanding true leaves (Fig.3 - 18, B).

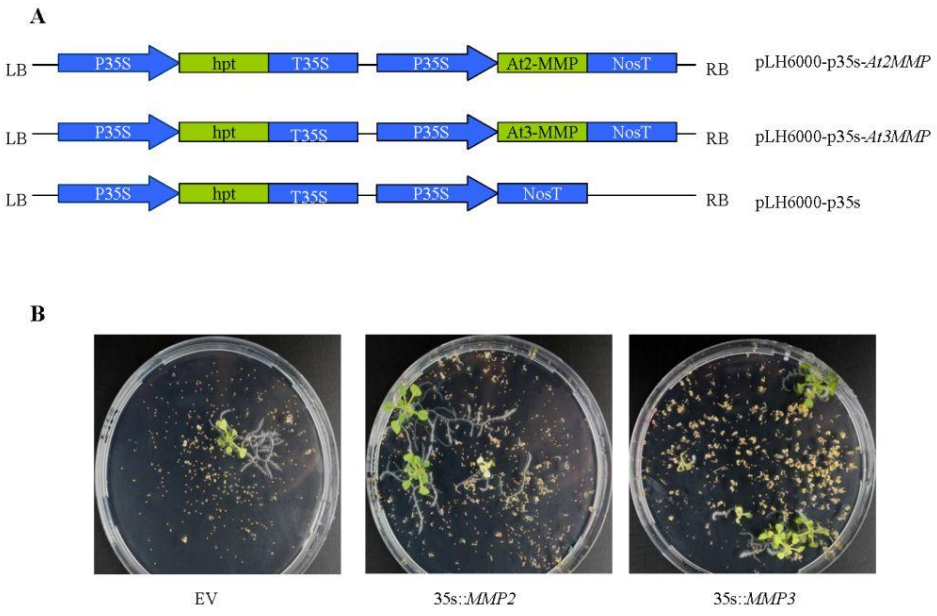


Fig.3 - 18. **A**, Schematic diagram of the constructs *35S::MMP2*, *35S::MMP3* and control construct (empty vector). **B**, Seeds from *Agrobacterium*-infiltrated F_0 plants were selected in ½ MS medium containing 30mg/L hygromycin. The putative transformants were able to grow on the selection medium.

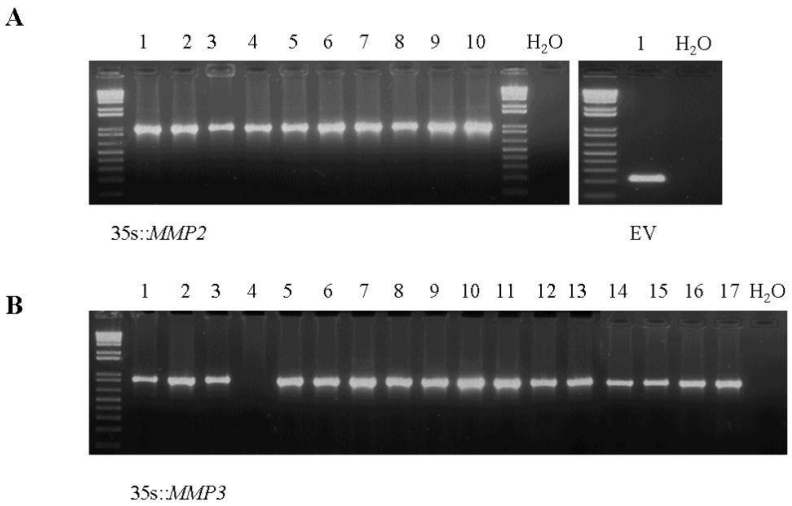


Fig.3 - 19. PCR identification of T_1 transgenic plants *35S::MMP2*, *35S::MMP3* and EV (empty vector) control plants. The REDExtract-N-Amp PCR kit was used for DNA extraction and PCR amplification following the manufacturer's instructions. **A**, Identification of *35S::MMP2* plants and EV transgenic plants. The *At2-MMP* forward primer (#207) and NOS terminator primer (#516, supplemental, Table S1) were used for *35S::MMP2*. Primers for 35S promoter (#517) and NOS terminator (#516) were used for EV transgenic plants. **B**, Identification of *35S::MMP3* plants. The *At3-MMP* forward primer (#211) and NOS terminator primer (#516) were used for PCR.

The putative transformants from *35S::MMP2*, *35S::MMP3* and EV were confirmed by PCR using a vector-specific primer and a gene-specific primer (Fig.3 - 19). In addition, the elevated expression level of *At2-MMP* in *35S::MMP2* and *At3-MMP* in *35S::MMP3* was analyzed by quantitative real-time PCR (Fig.3 - 20). The *35S::MMP2* lines L6 and L7, which had the highest and lowest transcript levels, were selected for further studies. The *35S::MMP3* lines L11 and L18 were selected for the further studies, with L18 had the highest expression level and L11 had a lower expression level (Fig. 3 -20, B) (Only a few seeds were harvested form L22 line of *35S::MMP3*)

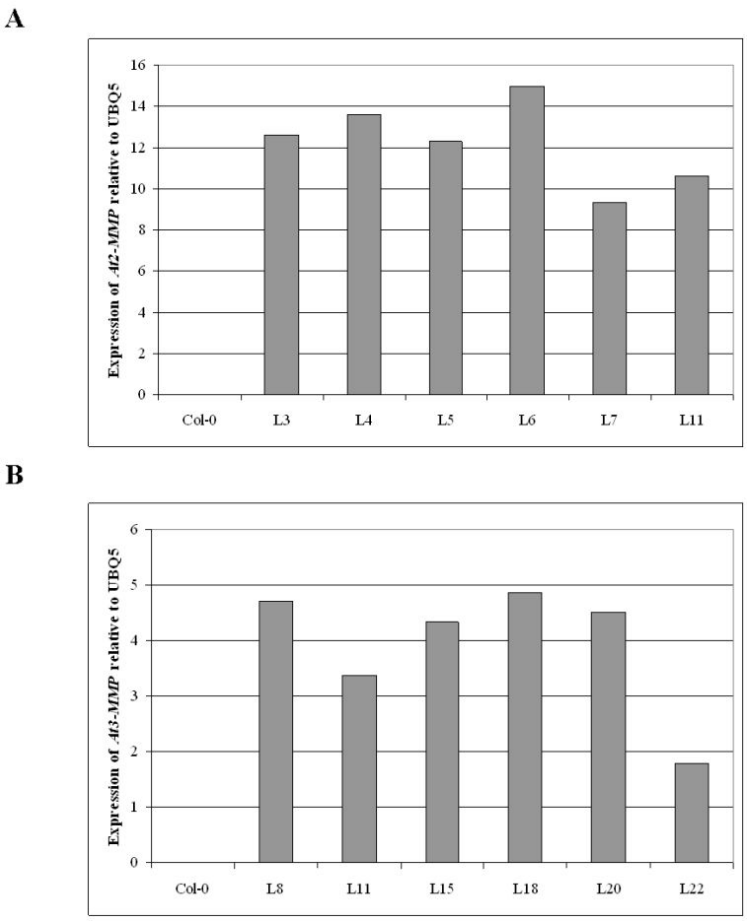


Fig.3 - 20. Expression level of *At2-MMP* and *At3-MMP* in the corresponding overexpression plants. Total RNA was extracted from leaves of six independent lines for both overexpression lines. UBQ5 was used as the reference gene in the quantitative RT-PCR. **A**, *At2-MMP* expression in *35S::MMP2* transgenic plants. At2-MMP specific primer pair was used for amplification. **B**, *At3-MMP* expression in *35S::MMP3* transgenic plants. At3-MMP specific primer pair was used for amplification.

3.5.2 Early flowering in *35S::MMP2* lines

The Col-0, EV controls and *35S::MMP2* transgenic plants (L6 and L7) were grown under short day conditions for eight weeks. The *35S::MMP2* plants revealed no difference in growth or morphology as compared with wild-type Col-0 plants in the vegetative stage. However, occasional flowering occurred much earlier compared to wild-type and EV control (Fig.3 - 21). This is consistent with a previous study, in which mutation of *At2-MMP* led to early senescence and late flowering (Golldack *et al*., 2002). No altered senescence was observed in *At2-MMP* overexpression plants. No difference was observed in the At3-MMP overexpression plants.

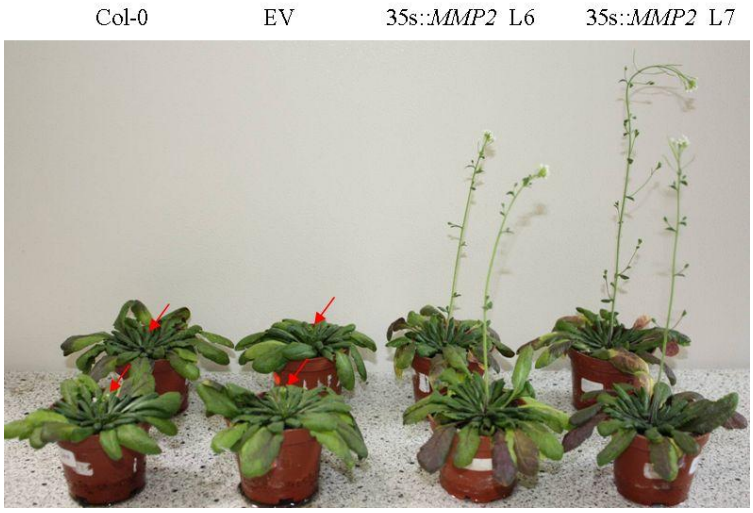


Fig.3 - 21. Early flowering phenotype of *35S::MMP2* transgenic plants. The plants were grown under short-day condition (8/16 h of light/dark regime). Image was taken 9 weeks after germination.

3.5.3 Salt responses in *At2-MMP* and *At3-MMP* overexpression lines

From the Genevestigator microarray data (Zimmermann *et al*., 2004), *At2-MMP* and *At3-MMP* were induced by NaCl treatment. To investigate the functions of *At2-MMP* and *At3-MMP* regarding salt stress response, the surface sterilized seeds were grown on ½ MS medium under short day conditions for 10days. Thereafter, seedlings from Col-0, EV, *35S::MMP2* (L6 and L7), and *35S::MMP3* (L11and L18) were transferred to ½ MS medium containing various NaCl concentrations (0, 50, 100 and 200mM). After two weeks, root lengths and sizes of L6 and L7 plants were similar to Col-0 and EV plants in ½ MS medium without salt. Under treatment with 50 mM NaCl, the growth of Col-0 plants was dramatically reduced showing smaller leaves and shorter roots. The in parallel transformed control EV plants and *35S::MMP2* were still growing similar to pants in 0 mM NaCl. Under 100 mM salt stress, the Col-0 plants were almost dying whereas the growth of EV plants and *35S::MMP2* plants (L6 and L7) was only slightly reduced. No clear difference was observed between EV and *35S::MMP2* plants under 50 mM and 100 mM NaCl. In the presence of 200 mM NaCl, all plants finally died but overexpression line L6 and L7 first produced longer roots than control (Fig.3 - 22.).

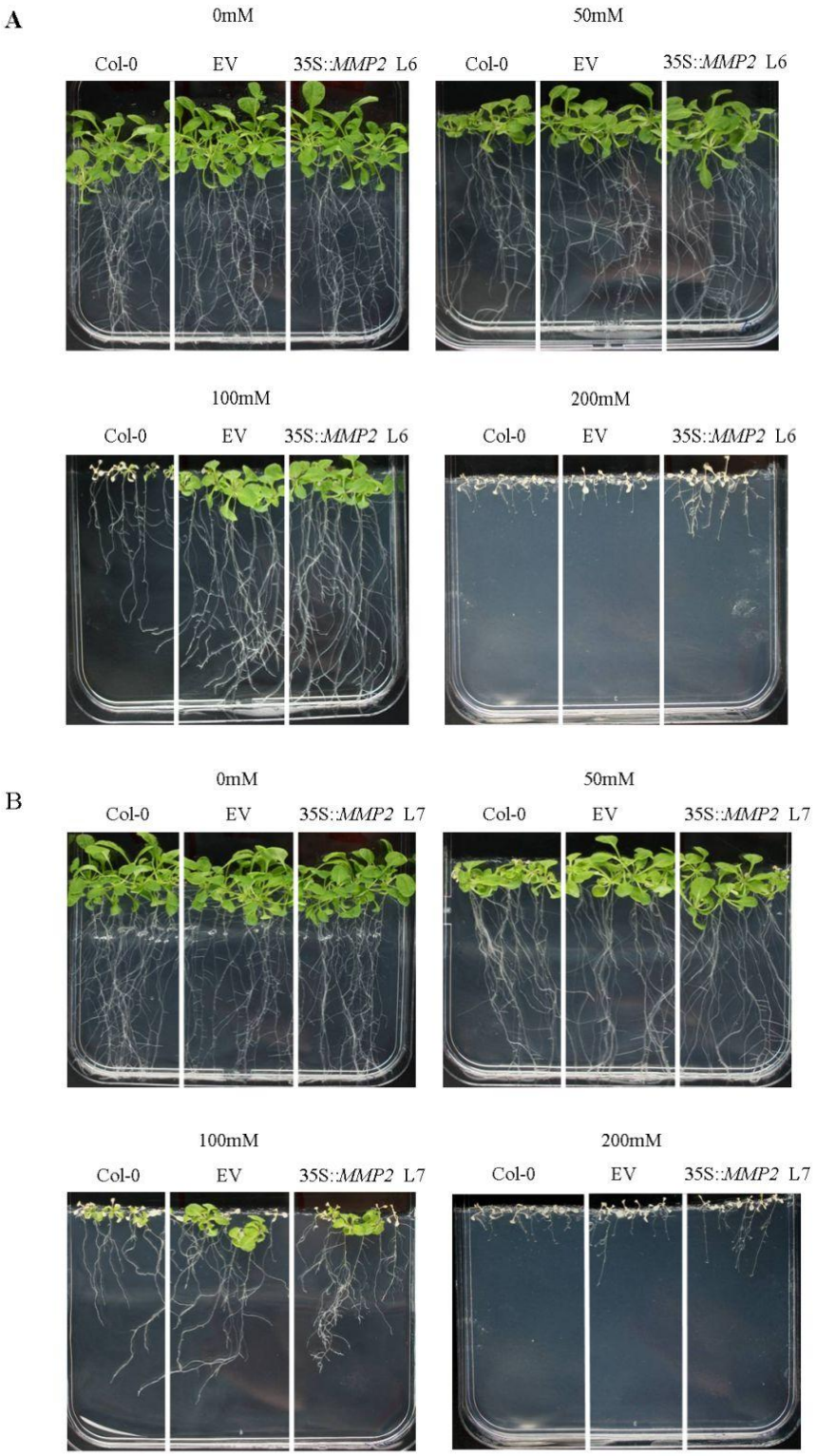


Fig.3 - 22. Responses of *35S::MMP2* plants (L6 and L7) to NaCl stress. The surface sterilized seeds were grown on ½ MS medium for 10 days and then seedlings were transferred to Petri dishes with ½ MS medium supplemented with 0, 50, 100 and 200 mM NaCl. Photographs were taken two weeks after transfer. **A**, Response of L6 to NaCl stress. **B**, Response of L7 to NaCl stress.

The *35S::MMP3* plants (L11 and L18) were also tested for their salt tolerance. Through all the NaCl concentration tested (0, 50, 100 and 200 mM), *35S::MMP3* and EV plants were

growing better than Col-0 plants concerning shoot size and root length (Fig.3 - 23). However, no difference was observed between *35S::MMP3* and EV plants at all NaCl concentrations. Hence, I conclude that *35S::MMP3* overexpression did not influence the NaCl tolerance.

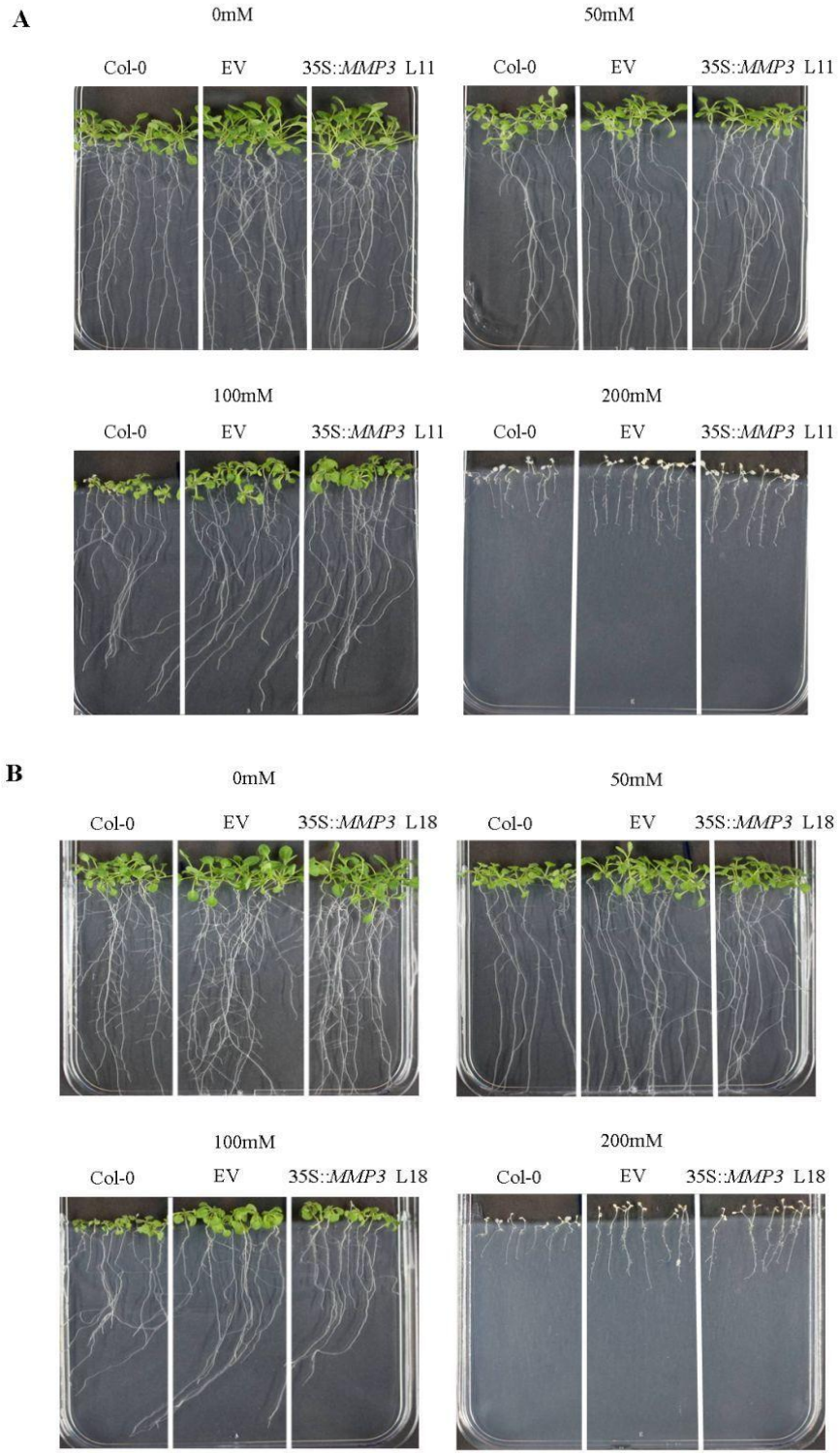


Fig.3 - 23. Responses of *35S::MMP3* plants (L11and L18) under NaCl stresses. The surface sterilized seeds were grown on ½ MS medium for 10 days and the seedlings were then transferred to petri dishes with ½ MS medium supplemented with 0, 50, 100 and 200mM NaCl. Photographs were taken two weeks after transfer. **A**, Response of L11 to NaCl stress. **B**, Responses of L18 to NaCl stress.

3.5.4 Pathogen responses in *At2-MMP* and *At3-MMP* overexpression plants

Both *At2-MMP* and *At3-MMP* overexpression plants were tested for the responses to *B. cinerea* infection. Detached leaves from 6-week-old *35S::MMP2* (line L6 and L7) and *35S::MMP3* plants (line L11and L18) were inoculated with *B. cinerea* spores. Control Col-0 and EV plants had similar lesion sizes with the latter slightly bigger diameters. Both L6 and L7 *35S::MMP2* lines showed significantly ($p \leq 0.05$) reduced lesion size in response *B. cinerea* compared to Col-0 and EV plants (Fig.3 - 24). Therefore, overexpression of *At2-MMP* enhanced the resistance to *B. cinerea*.

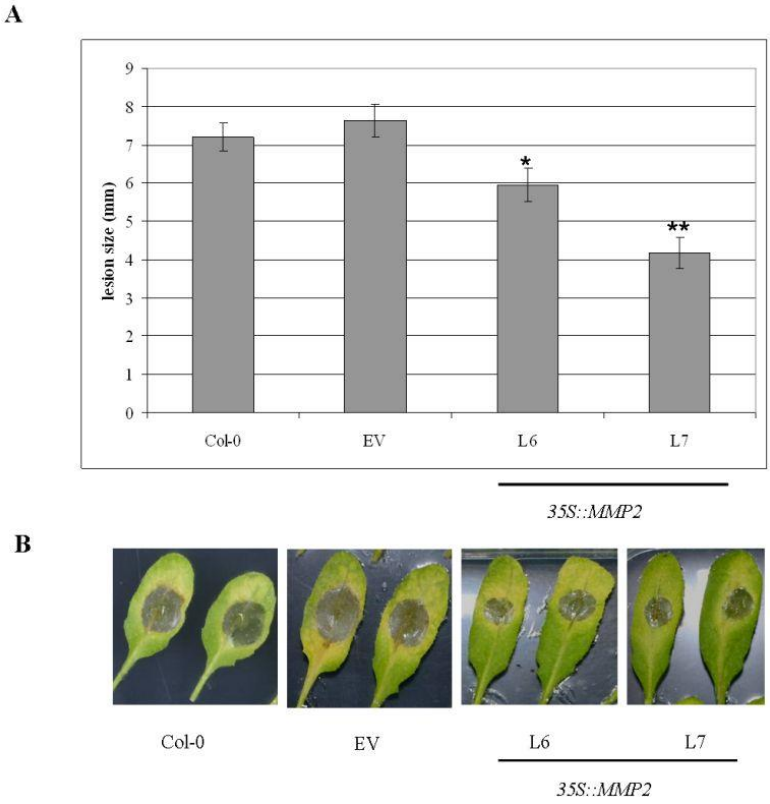


Fig.3 - 24. *35s::MMP2* plants (L6 and L7) showed increased resistance to the necrotrophic pathogen *B. cinerea.* The rosette leaves were detached from 6-week-old plants and placed in closed transparent plastic boxes containing 0.5% H_2O-Agar. The detached leaves were inoculated by placing 5ul spore suspension (5×10^4 conidiospores/mL) on the middle vein. The lesion size was measured by Image J. **A**, Size of lesions formed in leaves of Col-0, EV and *35S::MMP2* L6 and L7 plants after inoculation with *B. cinerea.* Data represent average ± SE of at least of 20 lesions. Bars indicate the standard error. The asterisks indicates significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$) according to Student's t-test. **B**, Disease symptoms on Col-0, EV, L6 and L7 of *35s::MMP2* leaves. The photographs were taken 4 days after inoculation.

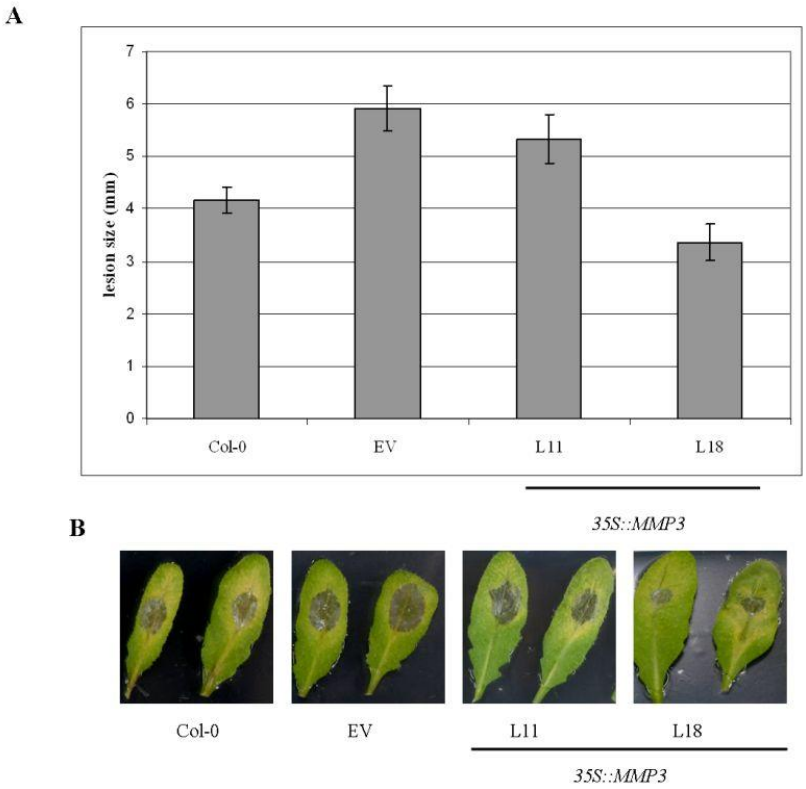


Fig.3 - 25. *35S::MMP3* plants (L11 and L18) showed increased resistance to the necrotrophic pathogen *B. cinerea.* The rosette healthy leaves were cut from 6-week-old plants and placed in plastic boxes containing 0.5% H_2O-Agar. The detached leaves were inoculated by placing 5 µL spore suspension (5×10^4 conidiospores/mL) on the middle vein. The lesion size was measured by Image J. **A**, Size of lesions formed in leaves of Col-0, EV and *35S::MMP3* L11 and L18 plants after inoculation with *B. cinerea.* **B**, The leaves of inoculated Col-0, EV, L11 and L18 of *35S::MMP3* plants 4 days after infection.

Similar to *35S::MMP2* plants, *35S::MMP3* plants (L11 and L18) also displayed reduced lesion size in comparison with EV. The disease symptoms were shown in Fig.3 - 25. This result indicates a similar role of *At3-MMP* as *At2-MMP* in *B. cinerea* resistance.

3.5.5 PAMP/DAMP-mediated ROS production

To gain insight into the mode of actions of matrix metalloproteaes in plant immune responses, I investigated in At-MMP mutants and overexpression plants the PAMP/DAMP-mediated production of reactive oxygen species (ROS) which are essential components of stress and defense signaling. I measured the oxidative burst (section 2.9) in *Arabidopsis* leaf disks after treatment with four elicitors including three PAMPs (100 nM flg22 peptide, 100 nM elf18 peptide and 1 mg/mL chitin) (Gómez-Gómez *et al*., 1999; Kunze *et al*., 2004; Miya *et al*., 2007) and one DAMP (100 nM Pep1 peptide (Krol *et al*., 2010). The oxidative burst was recorded by H_2O_2–dependent luminescence of luminol. Four independent At2-MMP overexpression lines, independent *at2-mmp* and *at3-mmp* single mutants, and double

mutants were included for comparison with the wild-type Col-0 and empty-vector (EV) transformed plants.

In both the Col-0 and EV plants, the PAMP flg22 was able to induce a strong transient oxidative burst within a few minutes (Fig.3 - 26, A). Intriguingly, a dramatic lower H_2O_2 level was observed in all the four independent *At2-MMP* overexpression lines compared with the Col-0 and EV plants in all the elicitor treatments. This impaired ROS production in the overexpression plants was most severe in the lines L7 and L11. They showed completely abolished oxidative burst upon flg22 treatment similar as that of flg22-insensitive mutant *fls2-17* (Fig.3 - 26*,* A). *at2-mmp* mutants (HM-257 and HM-280) showed wild-type level of ROS production after flg22 treatment (Fig.3 - 26, B). A significantly enhanced ROS production was observed in *at3-mmp* mutants (HM-402 and HM-411) and similarly increased ROS concentrations were found in *at2-mmp at3-mmp* double mutants (Fig.3 - 26, C and D).

Chitin is considered to be the predominant component of fungal cell wall and a potent PAMP (Miya *et al*., 2007). To gain insight into the responses to fungus-derived PAMP in At-MMP mutants and overexpression lines, I measured the chitin-mediated ROS production. Similarly, the *35S::MMP2* plants showed almost abolished ROS production after chitin treatment compared with Col-0 and EV (Fig.3 – 27, A). *at2-mmp* mutants (HM-280 and HM-257) showed ROS production very similar to Col-0 (Fig.3 – 27, B). In *at3-mmp* mutants (HM-402 and HM-411), the chitin-induced ROS was higher than that of Col-0 (Fig.3 – 27, C). Three out of the four double mutants lines showed higher ROS than Col-0 but lower than *at3-mmp* mutants in response to chitin treatment (Fig.3 – 27, D).

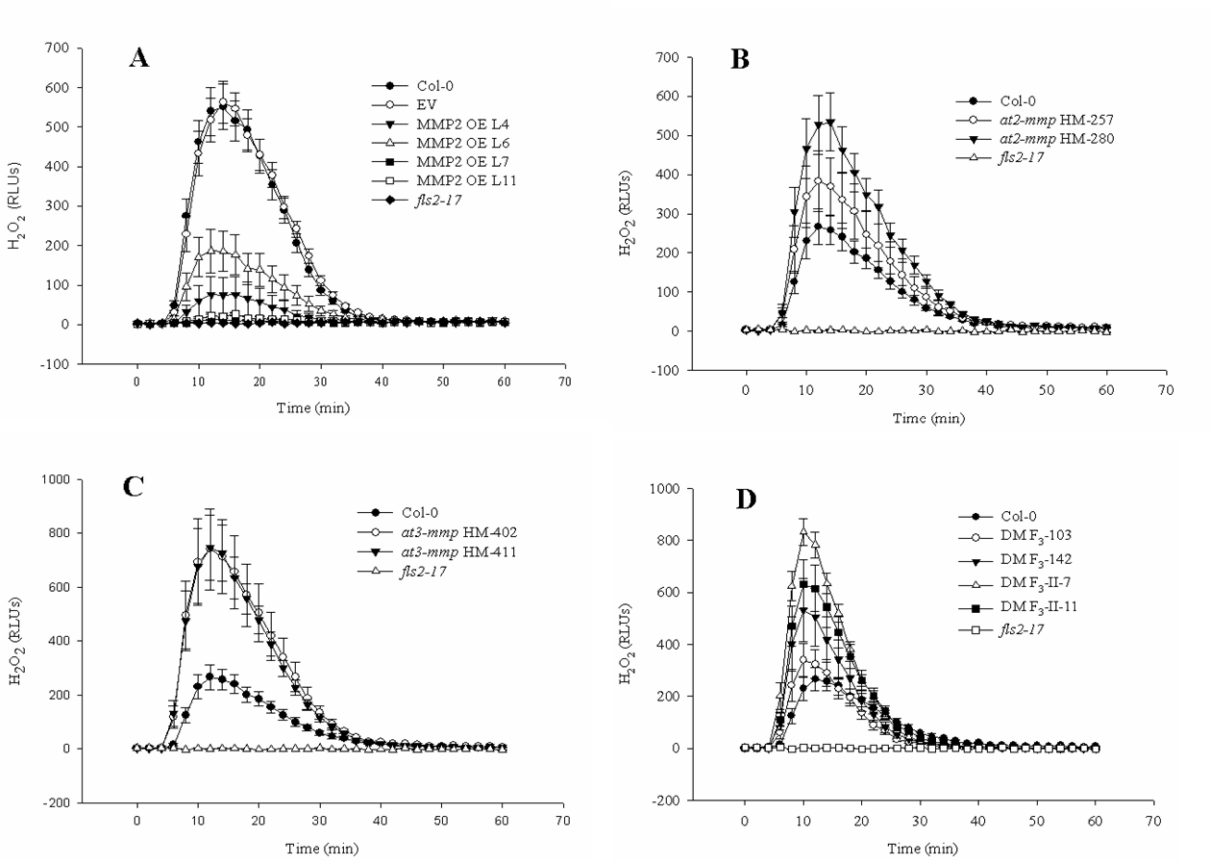


Fig.3 - 26. ROS production in response to 100 nM flg22. Leaf disks were cut from the soil-grown seven-week-old plants grown under short day condition. Oxidative burst was induced by 100 nM flg22 and measured in relative light units (RLU). Results are mean ± SE (n = 8)**.** **A**, Oxidative burst in leaf discs of wild-type Col-0, empty-vector transformants (EV), four independent At2-MMP overexpression lines (L4, L6, L7 and L11), and the receptor mutant *fls2-17*. **B**, Oxidative burst in leaf discs of *at2-mmp* mutants (HM-257 and HM-280), Col-0 and *fls2-17*. **C**, Oxidative burst in leaf discs of *at3-mmp* mutants (HM-402 and HM-411), Col-0 and *fls2-17*. **D**, Oxidative burst in leaf discs of *at2-mmp at3-mmp* double mutants, Col-0 and *fls2-17*. DM: double mutant.

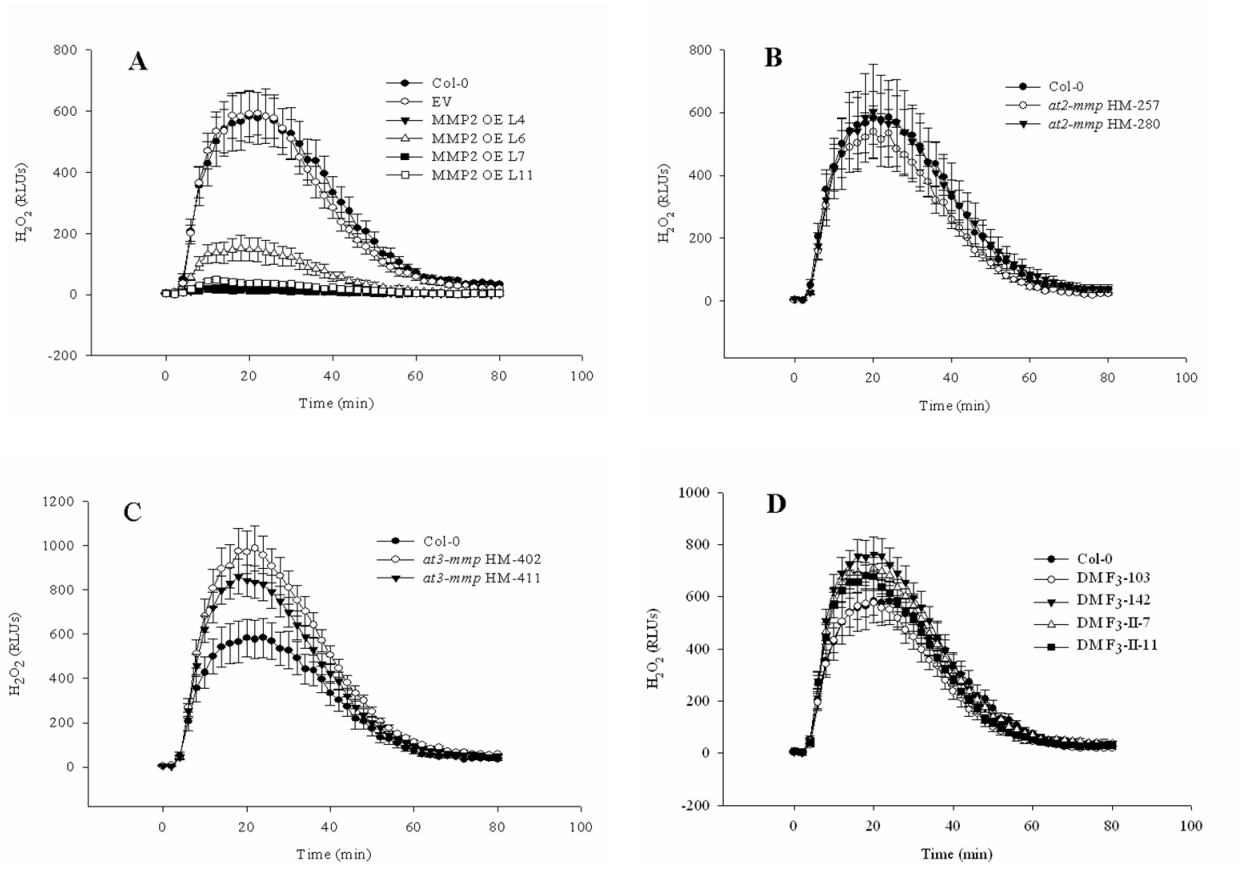


Fig.3 - 27. ROS production in response to 1mg/mL chitin. Leaf disks were cut from the soil-grown seven-week-old plants grown under short day condition. Oxidative burst was induced by 1 mg/mL chitin and measured in relative light units (RLU). Results are mean ± SE (n = 8)**.** **A**, Oxidative burst in leaf discs of wild-type Col-0, empty-vector transformants (EV) and four independent At2-MMP overexpression lines (L4, L6, L7 and L11). **B**, Oxidative burst in leaf discs of *at2-mmp* mutants (HM-257 and HM-280) and Col-0. **C**, Oxidative burst in leaf discs of *at3-mmp* mutants (HM-402 and HM-411) and Col-0. **D**, Oxidative burst in leaf discs of *at2-mmp at3-mmp* double mutants and Col-0. DM: double mutant.

The peptide elf18 is a conserved fragment from bacterial PAMP EF-Tu and able to induce plant immune responses upon the recognition by the membrane-bound receptor EFR (EF-Tu Receptor) (Kunze *et al*., 2004). To gain insight into the responses to elf18 in At-MMP mutants and overexpression lines, I measured the elf18-mediated ROS production. The elf18 insensitive mutant efr-1 was used as negative control. Similar to flg22 and chitin treatment, the *35S::MMP2* plants showed much lower ROS production after elf18 treatment compared with Col-0 and EV (Fig.3 - 28, A). *at2-mmp* mutants (HM-280 and HM-257) showed same ROS production as Col-0 (Fig.3 - 28, B). In *at3-mmp* mutants (HM-402 and HM-411), the level of elf18-induced ROS is close to that in Col-0 but the peak appeared earlier (Fig.3 - 28, C). Double mutant *at2-mmp/at3-mmp* showed higher ROS in response to elf18 than Col-0 (Fig.3 - 28, D) and the single mutants (Fig.3 - 28, B and C).

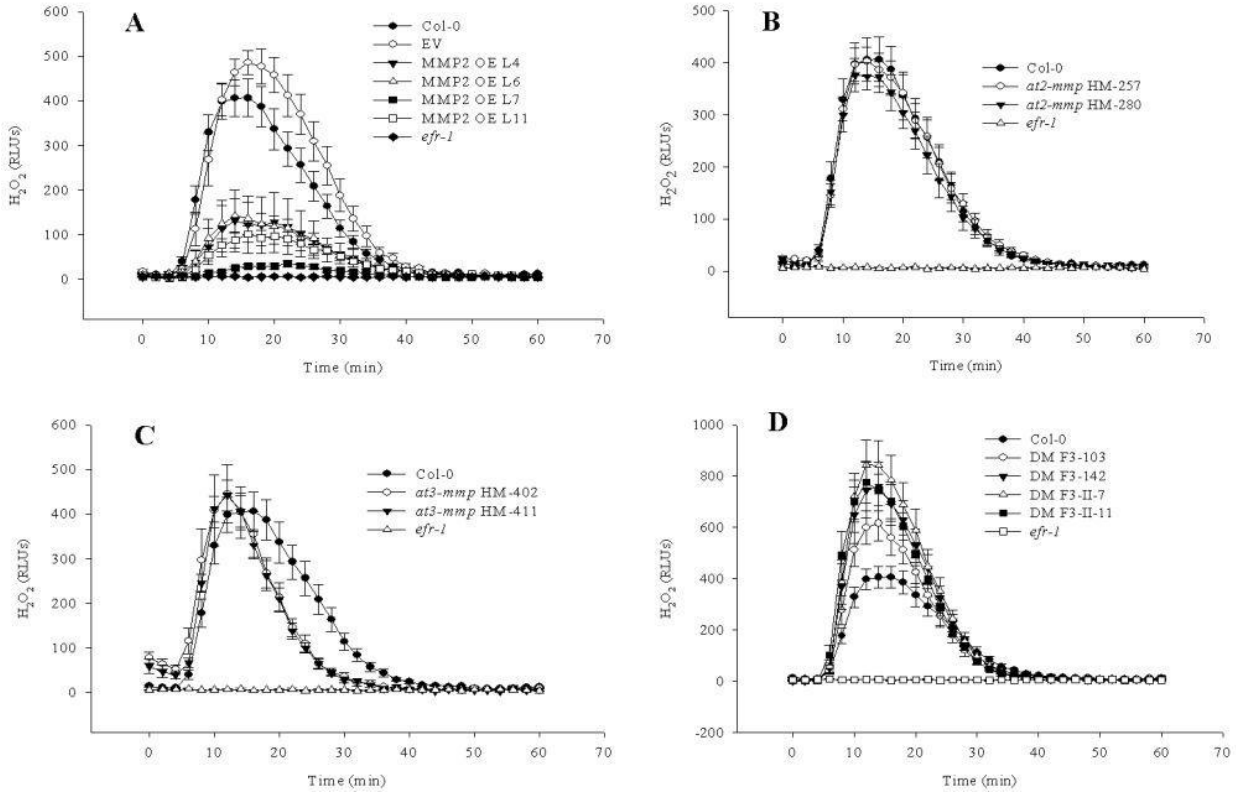


Fig.3 - 28. ROS production in response to 100 nM elf18. Leaf disks were cut from the soil-grown seven-week-old plants grown under short day condition. Oxidative burst was induced by 100 nM elf18 and measured in relative light units (RLU). Results are mean ± SE (n = 8). **A**, Oxidative burst in leaf discs of wild-type Col-0, empty-vector transformants (EV), four independent At2-MMP overexpression lines (L4, L6, L7 and L11) and the elf18 receptor mutant *efr-1* plants. **B**, Oxidative burst in leaf discs of *at2-mmp* mutants (HM-257 and HM-280), Col-0 and *efr-1*. **C**, Oxidative burst in leaf discs of *at3-mmp* mutants (HM-402 and HM-411), Col-0 and *efr-1*. **D**, Oxidative burst in leaf discs of *at2-mmp at3-mmp* double mutants, Col-0 and *efr-1*. DM: double mutant.

The *Arabidopsis*-derived DAMP Pep1 is known to induce plant defense signaling. To study the potential involvement of At-MMP in the Pep1-mediated signaling, I measured the Pep1-induced ROS production in *At-MMP* mutant and At2-MMP overexpression lines. For *35S::MMP2* plants, the Pep1-mediated oxidative burst is much lower than in control plants (Fig.3 - 29, A). No difference in Pep1-triggered ROS production was observed between *at2-mmp* mutant and Col-0. Interestingly, the Pep1-induced ROS in *at3-mmp* and *at2-mmp/at3-mmp* double mutants were substantially lower than in Col-0 (Fig.3 - 29, C and D). These data imply a requirement of At3-MMP in Pep1-mediated ROS production.

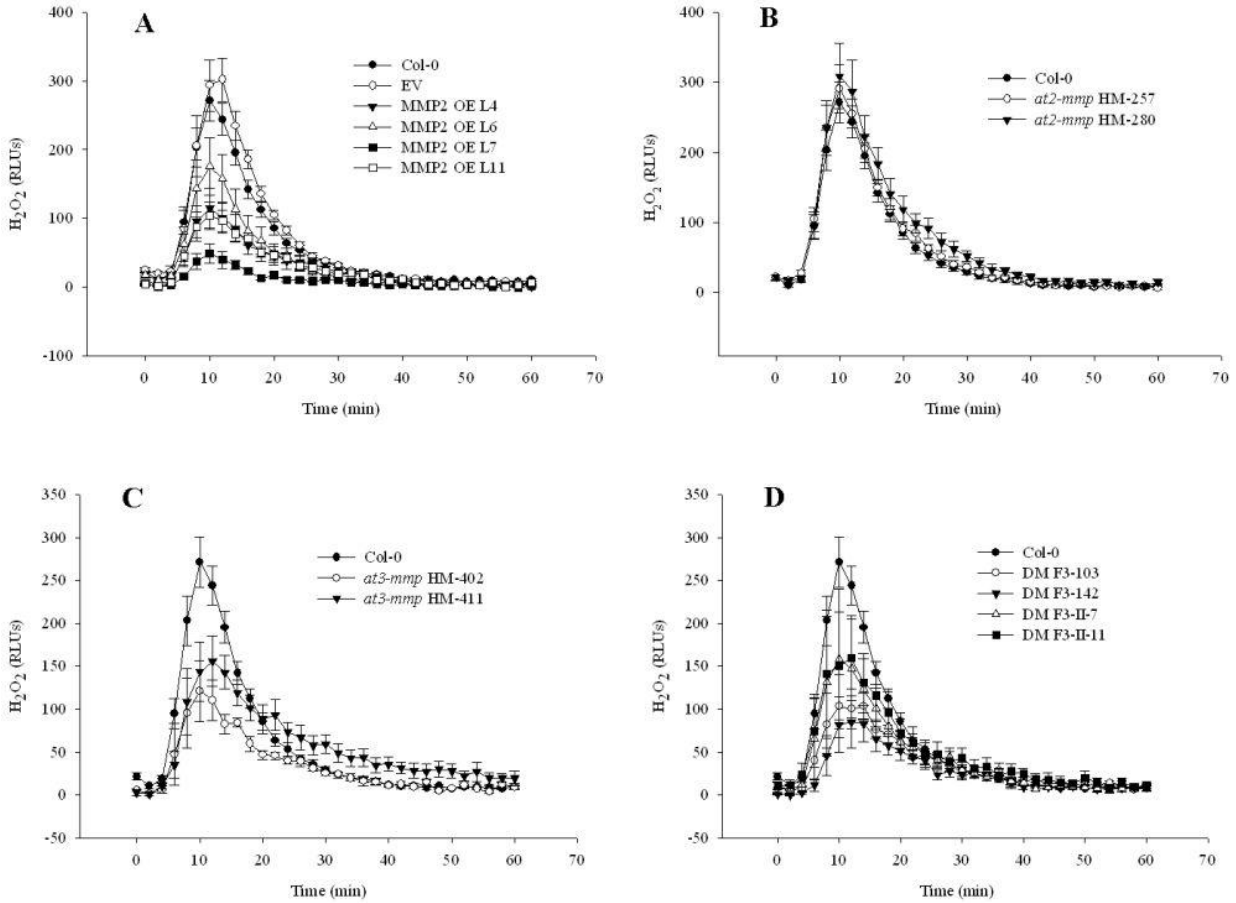


Fig.3 - 29. ROS production in response to 100 nM Pep1. Leaf disks were cut from the soil-grown seven-week-old plants grown under short day condition. Oxidative burst was induced by 100 nM Pep1 and measured in relative light units (RLU). Results are mean ± SE (n = 8)**.** **A**, Oxidative burst in leaf discs of wild-type Col-0, empty-vector transformants (EV) and four independent At2-MMP overexpression lines (L4, L6, L7 and L11). **B**, Oxidative burst in leaf discs of *at2-mmp* mutants (HM-257 and HM-280) and Col-0. **C**, Oxidative burst in leaf discs of *at3-mmp* mutants (HM-402 and HM-411) and Col-0. **D**, Oxidative burst in leaf discs of *at2-mmp at3-mmp* double mutants and Col-0. DM: double mutant.

In addition, I used the commercially available thermolysin to further investigate the involvement of metalloprotease activities in ROS production. Thermolysin is a neutral metalloproteinase enzyme produced by the gram-positive bacteria *Bacillus thermoproteolyticus* (Endo, 1962). Thermolysin was first examined for its proteolytic activity using MBP (myelin basic protein) as substrate (section 2.7.3). The thermolysin concentration of 5µg/mL was selected in the activity test according to a previous study (Altincicek *et al*., 2007). Degradation of MBP by thermolysin within five minutes confirmed its strong activity (Fig.3 - 31).

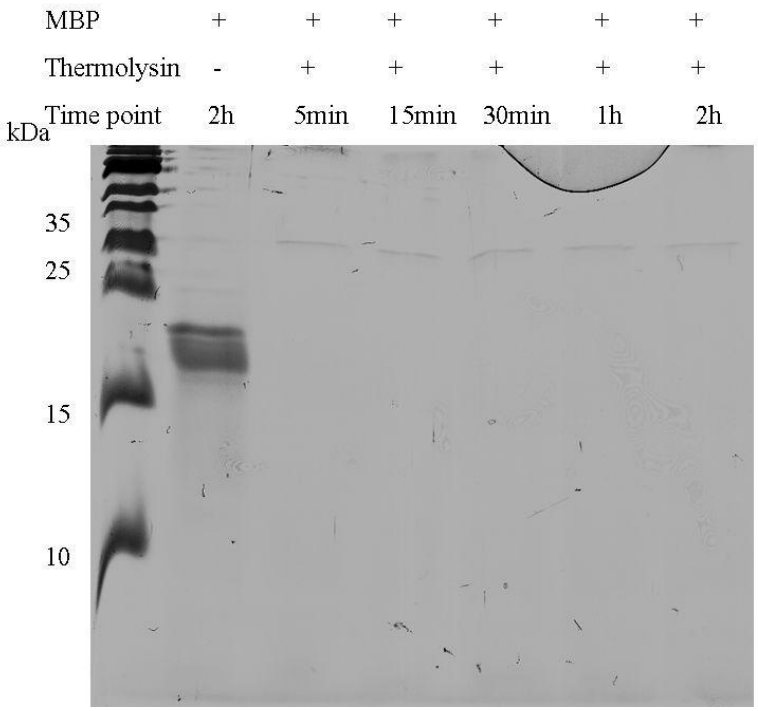


Fig.3 - 30. Determination of activity of bacterial metalloproteinase thermolysin by MBP degradation assay. MBP was incubated at 37 ^{o}C for in the presence of thermolysin (5 µg/mL) for the indicated time points. MBP incubation alone for 2 h was used as control. The products were loaded on SDS-PAGE gel.

To check plant responses to thermolysin, leaf disks were cut from Col-0 plants and measured for ROS production (section 2.9) in the presence of thermolysin (12.5µg/mL). Flg22 (100 nM) was used as the positive control in ROS assay (Gómez-Gómez *et al*., 1999). As expected, flg22 triggered a strong oxidative burst but thermolysin did not induce any ROS generation in the Col-0 leaf disks (Fig.3 - 31, A). Interestingly, a mixture of flg22 and thermolysin was not able to induce oxidative burst anymore in Col-0 plants (Fig.3 - 31, B). The abolished flg22-mediated oxidative burst by thermolysin provides a possible link between metalloproteinase and ROS generation.

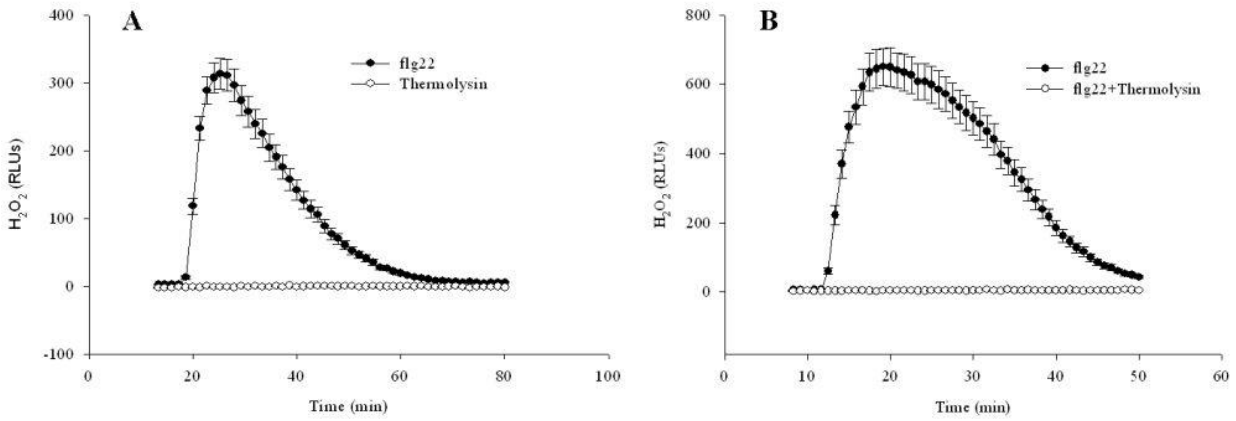


Fig.3 - 31. flg22-triggered oxidative burst was abolished by thermolysin. The leaf disks of Col-0 were cut from seven weeks old plants which were grown in soil under short day conditions. Oxidative burst was measured in relative light units (RLU) after treatment with 100 nM flg22, 12.5 µg/mL thermolysin or a combination of both. Results are mean ± SE (n = 24). **A**, Oxidative burst in Col-0 leaf disks after flg22 or thermolysin treatment. **B**, Oxidative burst triggered in Col-0 leaf disks triggered by flg22 with or without thermolysin.

3.5.6 Expression analysis of marker genes during *Botrytis cinerea* infection

To gain insight into the molecular basis of the At-MMP-related signaling cascade, the expression of three *Arabidopsis* marker genes PDF1.2 (plant defensin), PR1 (pathogenesis-related) and ERF1 (ethylene-responsive factor) during *B. cinerea* infection was compared between Col-0, *at2-mmp* and *35S::MMP2*. PDF1.2, PR1 and ERF1 are crucial component in the JA, SA and Ethylene signaling (Berrocal-Lobo *et al*., 2002; Delaney *et al*., 1994; Manners *et al*., 1998). Ubiquitin 5 was used as control for normalization. The expression profiles of PDF1.2, PR1 and ERF1 are shown in Fig.3 - 32.

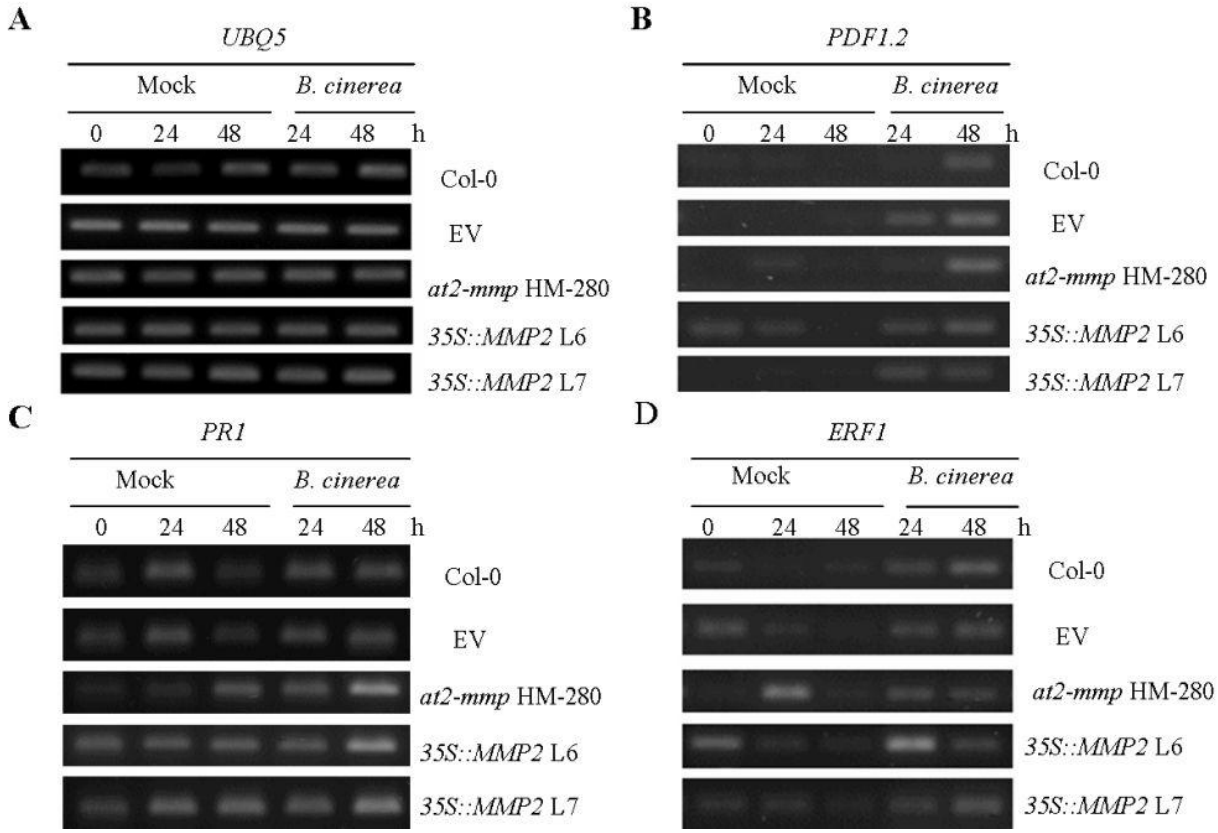
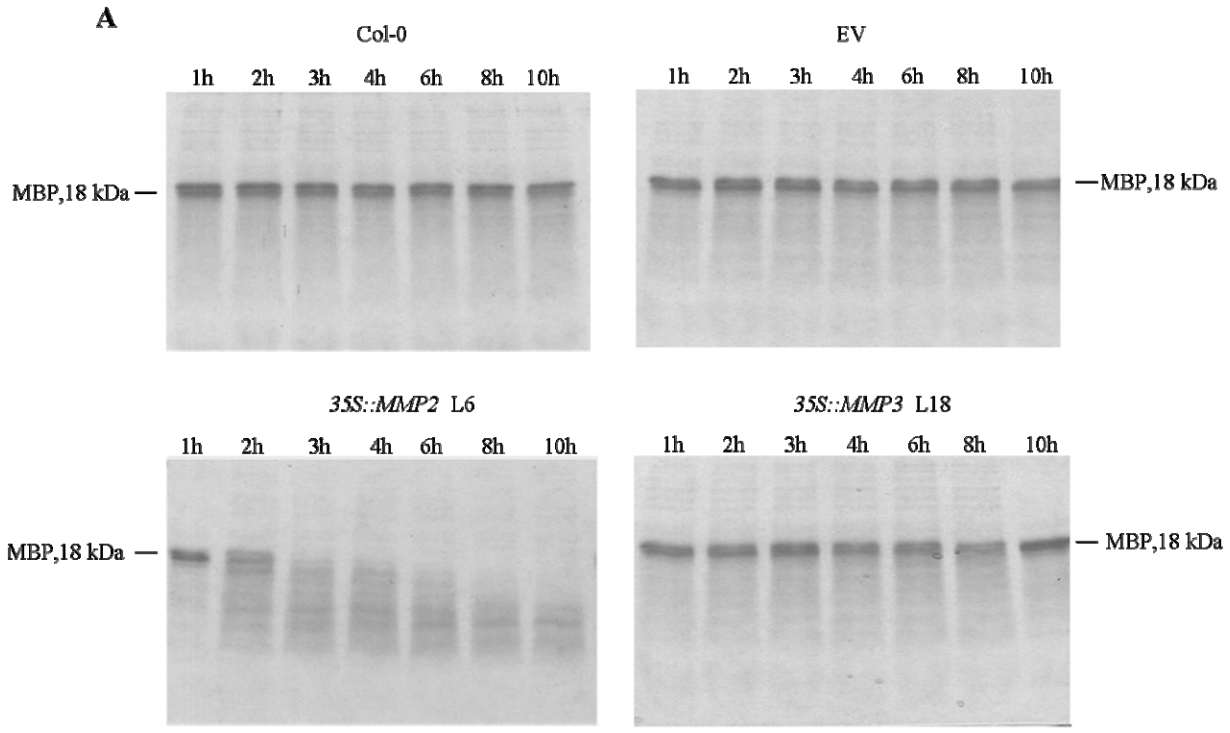


Fig.3 - 32. Expression profile of *PDF1.2*, *PR1* and *ERF1*. Leaves from Col-0, empty control (EV) transformant, *at2-mmp* and 35S::MMP2 (L6 and L7) 6-week-old plants were harvested at the indicated time points after *B. cinerea* infection and used for total RNA extraction. RT-PCR was performed using UBQ5 as an internal control. h: hours.

On the basis of RT-PCR results, the expression of PDF1.2, PR1 and ERF1 were induced after *B. cinerea* inoculation compared with the mock treatment in *at2-mmp* mutant, *35S::MMP2* plants and the control plants (Fig.3 - 32). However, no differences were observed for the expression of the three marker genes between these lines. The At2-MMP conferred resistance is unlikely through acting on *PDF1.2*, *PR1* or *ERF1*.

3.5.7 Intercellular washing fluid from *35S::MMP2* and *35S::MMP3* plants

To verify the proteolytic activity of At2-MMP and At3-MMP in the overexpression lines, intercellular washing fluid (IWF) was extracted form Col-0, EV, *35S::MMP2* plants (L6) and *35S::MMP3* plants (L18). Myelin basic protein (MBP) was used as substrate to determine the proteolytic activity of different IWFs. The IWF from different plants was incubated with MBP for 10 h. The final product was separated by 20% SDS-PAGE (Fig.3 - 33, A). After incubation, only the IWF from L6 plants of *35S::MMP2* could degrade the substrate MBP. The IWF from *35S::MMP3* (L18) plant was not able to degrade MBP (Fig. 3-33, A). The degradation of MBP by IWF from L6 was inhibited by EDTA. With the increase of EDTA concentration, the level of MBP degradation is decreased (Fig.3 - 33, B).



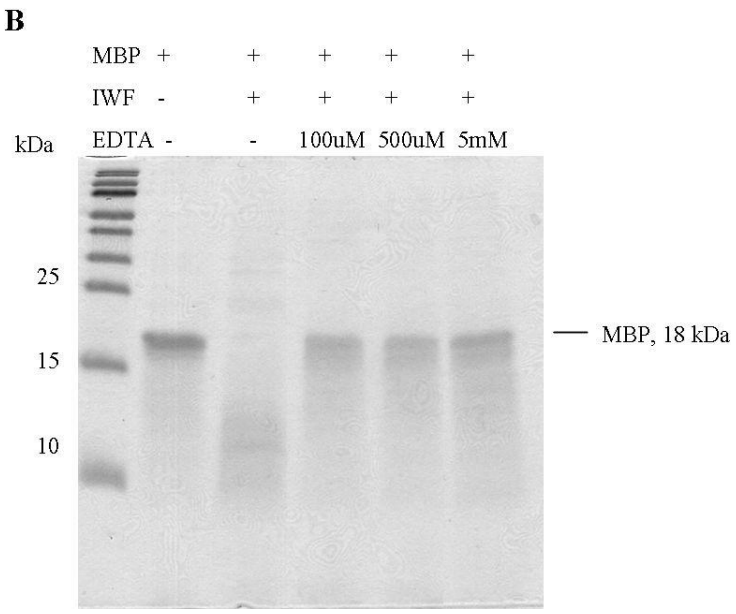


Fig.3 - 33. Proteolytic activity of the IWF from *35S::MMP2* and *35S::MMP3* plants. **A**, IWF activity was determined by degradation of MBP. IWF from *35S::MMP2* (L6) plant displayed activity against the substrate MBP. **B**, Activity of the IWF from 35S::*MMP2* (L6) was inhibited by EDTA. As the rising of EDTA concentration, the degradation of MBP decreases.

3.6 Characterization of At2-MMP and At3-MMP recombinant proteins

To verify the enzymatic activity of At2- and At3-MMP, the coding sequences of At2- and At3-MMP excluding N-terminal signal peptide were amplified by PCR. These fragments were cloned in frame to the C-terminal Thioredoxin-His-S (THS) tag in pET32a (+) vector. As the cloned sequences contain both the propeptide domain and catalytic domain, the encoded proteins were termed Pro-MMP2 and Pro-MMP3. The expression constructs used in the production of recombinant protein are shown in Fig.3 - 34, A, where pET32a vector was included to express the 20 kDa THS tag as a control.

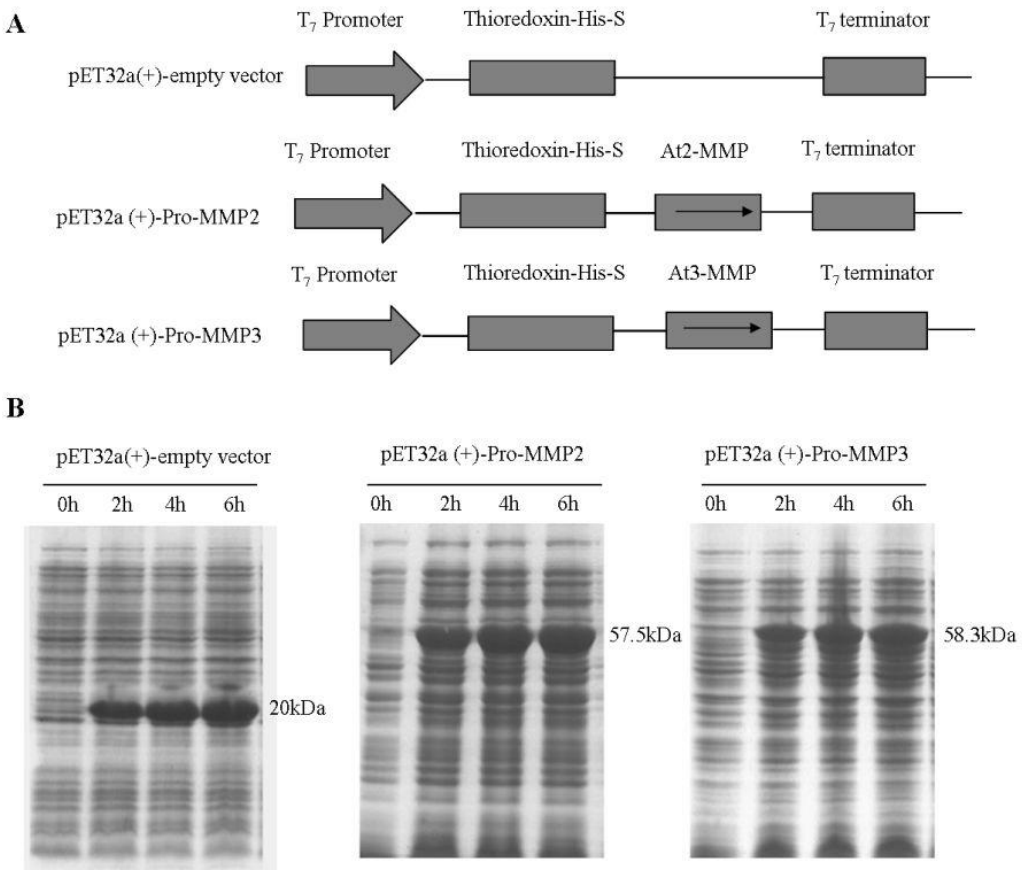


Fig.3 - 34. Expression of Pro-MMP2 and Pro-MMP3 in *E. coli*. **A**, The schematic diagram of the expression constructs pET32a (+) empty vector, pET32a (+)-Pro-MMP2 and pET32a (+)-Pro-MMP3. Coding sequences of *At2-MMP* and *At3-MMP* were cloned in frame to the C-terminal of Thioredoxin-His-S (THS) tag in pET32a (+) vector. The calculated protein size is shown on the right side. **B**, IPTG induction of THS tag, THS-tagged Pro-MMP2 and THS-tagged Pro-MMP3 in *E. coli*. Bacterial cells were harvested at indicated time points (0, 2, 4 and 6 h) after IPTG addition.

Initially, all the three constructs were transformed in the *E.coli* strain BL21 (DE3). Protein induction was performed by adding 1 mM IPTG. The induction was observed strong for THS tag and Pro-MMP3 in BL21 (DE3) strain (Fig.3 - 34, B), but very weak for Pro-MMP2 (data not shown). Thus, pET32a-Pro-MMP2 was transformed in the strain BL21 (DE3) pLysS and the expression level of Pro-MMP2 was quite strong after IPTG induction (Fig.3 - 34, B). Based on the induction dynamics of Pro-MMP2 and Pro-MMP3 (Fig.3 - 34, B), 4 h IPTG induction was used in the subsequent large-scale culture. To purifiy the Pro-MMP2 recombinant protein, *E.coli* cells transformed with pET32a-Pro-MMP2 or pET32a were harvested, lysed by lysozyme and sonicated for three times. After centrifugation, both the supernatant and pellet were loaded on SDS-PAGE gel to verify the solubility of Pro-MMP2. THS tag was found in the supernatant, but most of the Pro-MMP2 protein was present in pellet (data not shown). The recombinant Pro-MMP2 protein was therefore purified under

denaturing condition (section 2.7.2). In the presence of 8 M urea, the dissolved Pro-MMP2 was loaded on Ni-NTA column which has high binding affinity to the His-tagged protein. As a result, the THS-tagged Pro-MMP2 appeared to bind poorly to the Ni-NTA column and a small portion of the protein was purified (Fig.3 - 35).

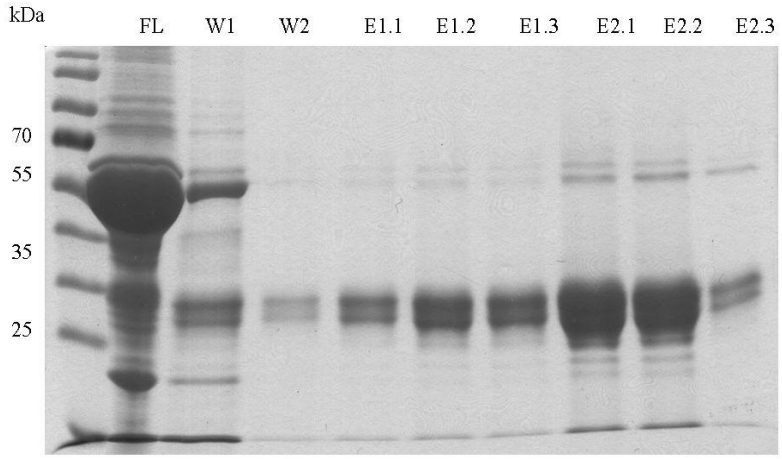


Fig.3 - 35. Purification of the Pro-MMP2 recombinant protein. Purification of Pro-MMP2 protein was performed under denaturing condition. FL: flow through. W: wash. E: elution.

In the final elution, there is one band close to 55 kDa, which is in agreement with the predicted size of 57.5 kDa. Other two bands are around 30 and 35 kDa, which may indicate a cleavage within the propeptide (Fig.3 - 35). The protein was subsequently refolded with dilution method as described previously (Maidment *et al*., 1999). After the refolding, the three dominant bands are still present.

The activity of Pro-MMP2 recombinant protein was determined by the myelin basic protein (MBP) degradation assay (section 2.7.3). With the presence of Pro-MMP2 protein, MBP was degraded after 30 h incubation at 37 ^{o}C (Fig.3 - 36, A). The activity of Pro-MMP2 was inhibited by EDTA, a chelator of metal ions required for MMP activity (Fig.3 - 36, B).

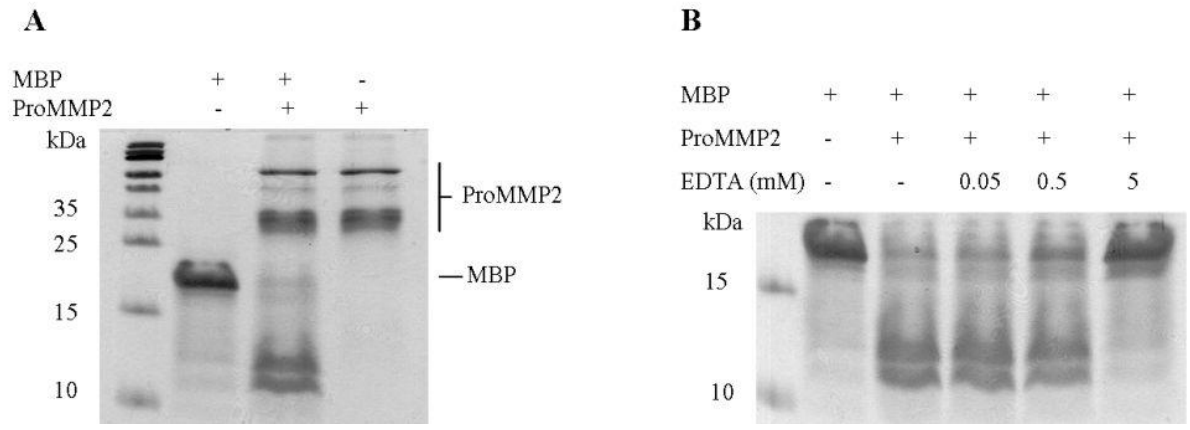


Fig.3 - 36. Activity determination of Pro-MMP2 recombinant protein by MBP degradation assay. **A**, Degradation of myelin basic protein (MBP, 18kDa) by Pro-MMP2 recombinant

protein. MBP was incubated with ProMMP2 at 37 ℃ for 30 hours. **B**, Inhibition of the Pro-MMP2 activity activity by EDTA. Incubation was performed at 37 ℃ for 30 hours.

Because the Pro-MMP2 mainly exists in the inclusion body, the purification must work under the denaturing condition and need refolding after purification. These possible reasons caused the low activity of recombinant protein. In addition, most of the recombinant protein was not binding to the Ni-NTA column and lost in the flow throw. To improve the purification and activity, a shorter fragment containing only the catalytic domain of At2-MMP was cloned in pET32a (+) vector. The fusions construct starts from the amino acid D in the cysteine switch motif PRCGNPD and ends before the C-terminal transmembrane domain. The expected protein was designated Mat-MMP2 (mature MMP2) and the THS-tagged Mat-MMP2 has a predicted size of 45 kDa (Fig.3 - 37, A).

Both the THS tag and Mat-MMP2 were expressed in *E.coli* strain BL21 (DE3) pLysS. High induction of the protein production was confirmed by SDS-PAGE (Fig.3 - 37, B). Though majority of Mat-MMP2 protein still existed as inclusion body after three times sonication (data not shown), a small portion of Mat-MMP2 was found in the supernatant after the third and fourth sonication (Fig.3 – 38).

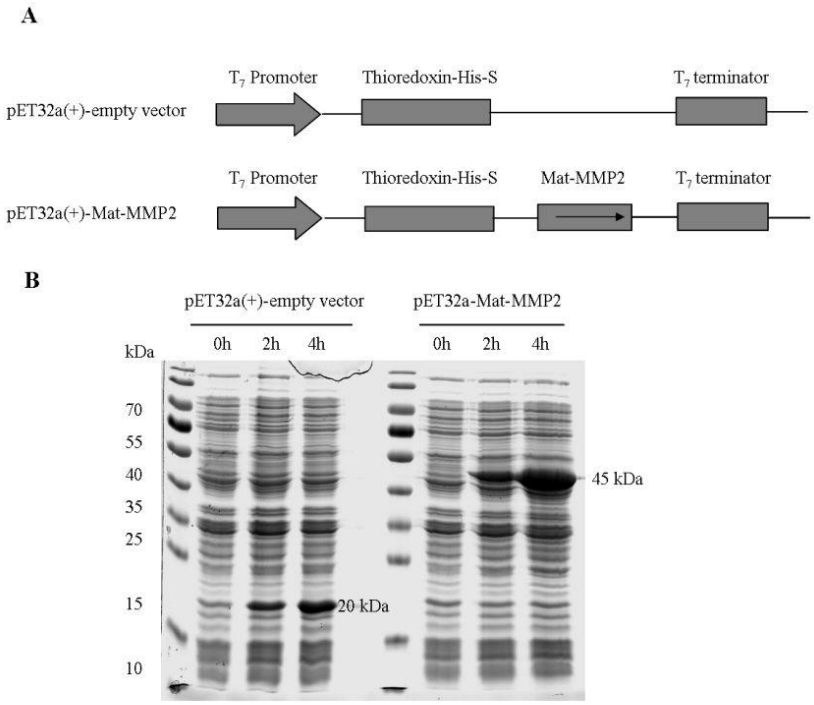


Fig.3 - 37. Expression of Mat-MMP2 in *E. coli*. **A**, The schematic diagram of the expression constructs pET32a (+) empty vector and pET32a (+)-Mat-MMP2. Catalytic domain of *At2-MMP* was cloned in frame to the C-terminal of Thioredoxin-His-S (THS) tag in pET32a (+) vector. The calculated protein size is shown on the right side. **B**, IPTG induction of THS tag and THS-tagged Mat-MMP2 in *E. coli*. Bacterial cells were harvested at indicated time points (0, 2 and 4 h) after addition of 1 mM IPTG and loaded on 12% SDS-PAGE.

The weak bands present in the supernatant from the third and fourth sonication (S3 and S4) have the same size as the predicted THS-tagged Mat-MMP2 (Fig.3 – 38, A). They were collected, centrifuged and tested for their enzymatic activity against MBP (section 2.7.3). The activity of the native Mat-MMP2 recombinant protein was tested regarding the degradation of MBP. MBP was incubated with Mat-MMP2 at 37 ℃ for 0min and 30min. It was observed that MBP was efficiently degraded within 30 min (Fig.3 -38, B). In contrast, MBP was not degraded in the empty vector control reaction.

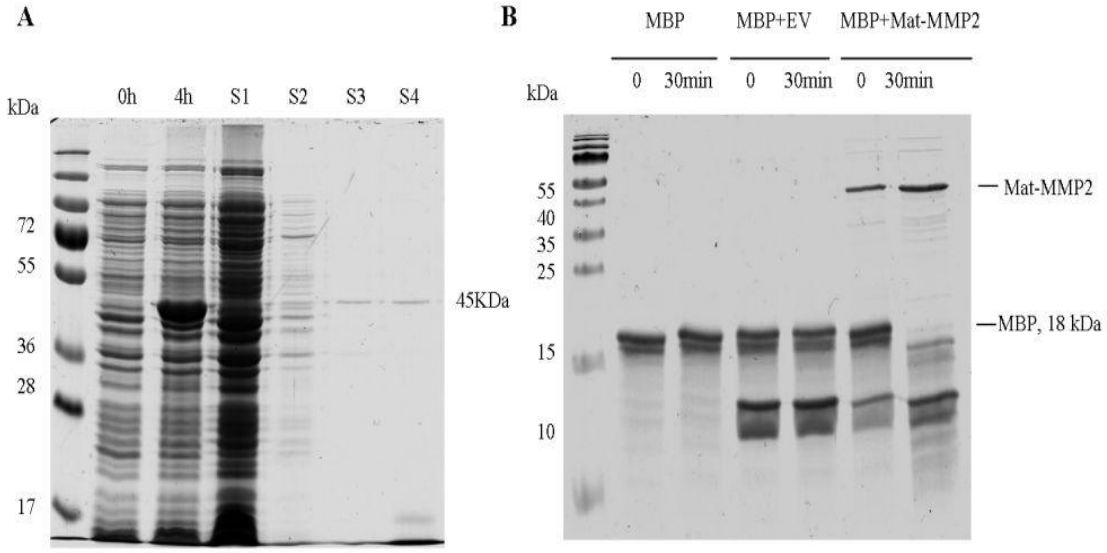


Fig.3 - 38. Native form of Mat-MMP2 and its enzymatic activity. **A**, Presence of native Mat-MMP2 protein in the supernatant after multiple sonications. 0h: pellet harvested before the addition of IPTG; 4 h: pellet harvested 4h after IPTG induction; S1: the supernatant after first sonication. S2: the supernatant after twice sonication. S3: the supernatant after three times sonication. S4, the supernatant after four times sonication. Samples were separated by 12% SDS-PAGE. **B**, Degradation of MBP by native Mat-MMP2. Both Mat-MMP2 or proteins harvested from empty vector (EV) transformed cells were harvested from the supernatant after three times sonication. MBP was indubated with or without the presence of Mat-MMP2 at 37 ^{o}C for 0 min and 30 min. Samples were separated by 20% SDS-PAGE.

Overexpression of At2-MMP increased the resistance to *B. cinerea* in transgenic *Arabidopsis thaliana* plants (Fig.3 - 24). To address the question whether At2-MMP has a direct anti-fungal activity, *B. cinerea* spores were incubated together with recombinant – Mat-MMP2. Ten hour after incubation, the germination of *B. cinerea* was examined by microscopy. I found that the spores of *B. cinerea* germinated and grew similar to the "empty vector" recombinant tag peptide, Tris-HCl buffer and H_2O control. Therefore, Mat-MMP2 had no direct inhibitory effect on the *B. cinerea* germination (Fig.3 - 39). The observed

enhanced resistance in *At2-MMP* overexpression lines therefore is more likely due to an indirect way.

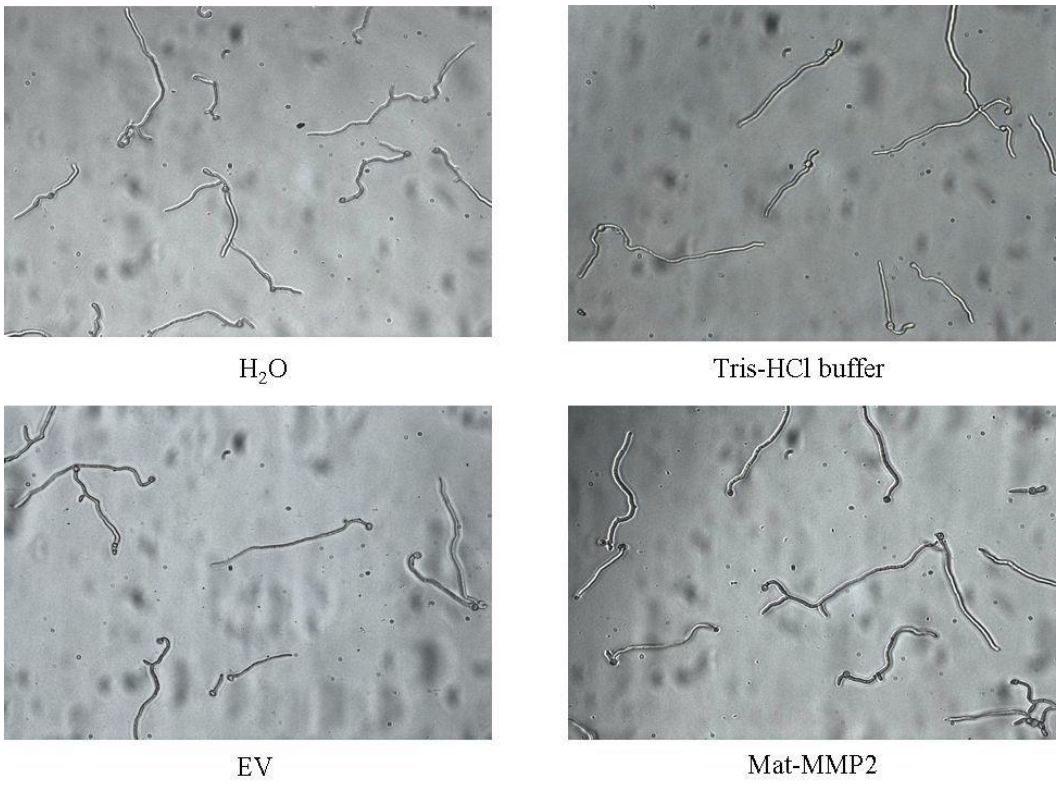


Fig.3 - 39. At2-MMP protein exhibits no direct inhibition on the spore germination of *B. cinerea*. The *B. cinerea* spores were incubated with proteins produced from *E. coli* cells transformed with EV (pET32a (+)-empty vector) or pET32a (+)-Mat-MMP2 for 10 hours at room temperature in darkness. The pictures were taken 10 h after incubation.

3.7 Structure and subcellular localization of At2-MMP

3.7.1 3D modeling of At2-MMP and At3-MMP

To gain insight into the structure of At2-MMP and At3-MMP, 3D modeling was performed with PyMOL using the amino acid sequences of both peptides (performed by Cathleen, Zocher, Nutritional Biochemistry, JLU). Using the X-ray structure of human MMP1 (PDB-ID: 1SU3) (Jozic *et al.,* 2005) and human MMP3 (PDB-ID: 1SLM) (Chen *et al*., 1999) as template for At2-MMP and At3-MMP respectively, the 3D structures can be well-modelled in the conserved domain such as cysteine switch motif and the catalytic domains (Fig.3 - 40, Fig.3 - 41). Overlapping of the catalytic domains indicated highly similar structure, with three invariable histidines and active Zinc site matching each other. For At2-MMP, the C-terminus modeling failed completely (Fig.3 - 40, A). This is due to the distinct C-terminal structures between At2-MMP and human MMP1. The C-terminal hemopexin domain in human MMP1 is not present in At2-MMP. In stead, At2-MMP has a specific C-terminal transmembrane domain.

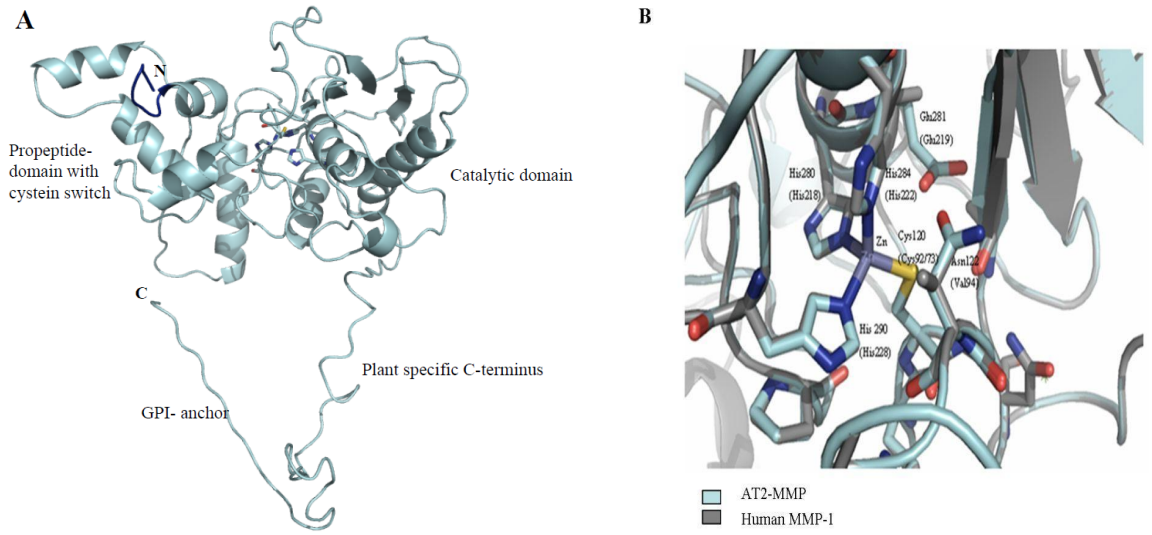


Fig.3 - 40. The 3D modeling of At2-MMP protein. The 3D structure was predicted by Cathleen Zocher using PyMOL. **A**, 3D modeling of At2-MMP. The propeptide domain, catalytic domain and C-terminus were indicated. **B**, Overlap of the catalytic domain from At2-MMP and human MMP1. The three conserved histidine residues in the catalytic domain are shown as aromatic rings. The active site Zn atom was indicated.

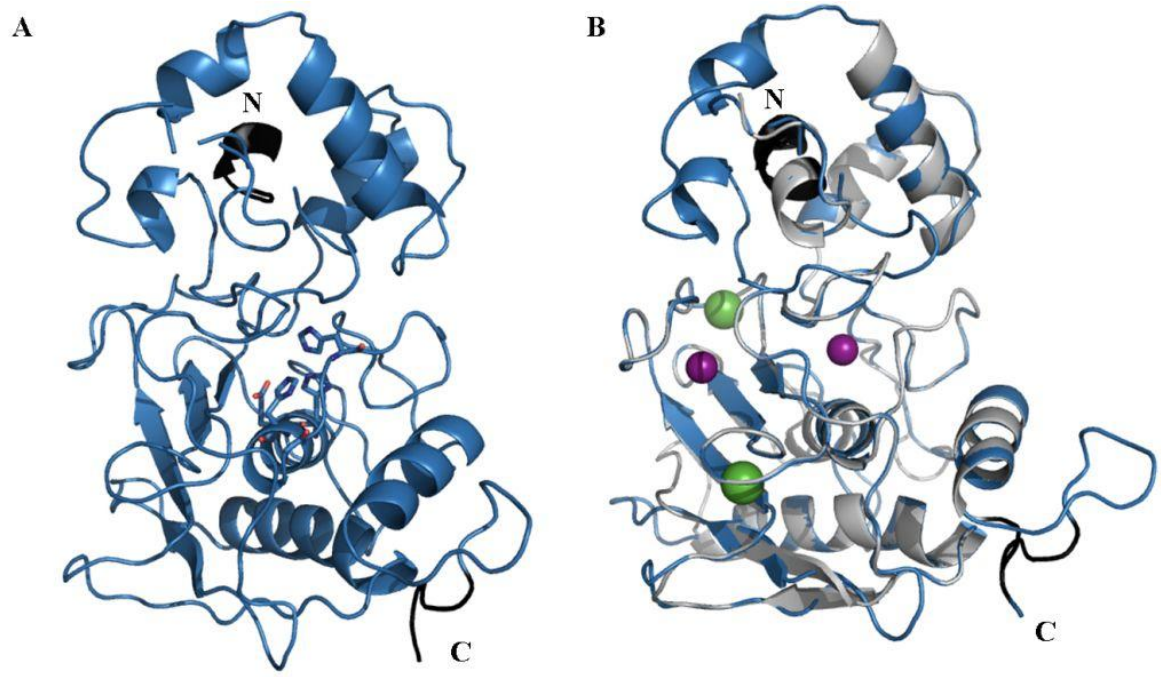


Fig.3 - 41. 3D modeling of At3-MMP protein. The 3D structure was predicted by Cathleen Zocher using PyMOL. **A**, 3D modeling of At3-MMP. N-terminal and C-terminal of the protein are indicated. Three histidine residues in the catalytic domain are shown as aromatic rings. **B**, Overlap of At3-MMP and human MMP3. The three conserved histidine residues in the catalytic domain are shown as aromatic ring. The zinc (purple) and calcium (green) atoms in the active site are shown.

3.7.2 Immunodetection of At2-MMP

In tobacco, the characterized NtMMP1 was showing 65.6%, similarity to At2-MMP at the amino acid level (Schiermeyer *et al*., 2009). Both the N-terminal and C-terminal-specific antibody is available for NtMMP1. To test the feasibility of immunodetection of At2-MMP using NtMMP1 antibody, western blotting was performed using N-terminal antibody and C-terminal antibody as described (Mandal *et al*., 2010) (section 2.7.5). Preliminary trial showed no specific detection of At2-MMP protein using NtMMP1 antibody (Fig.3 - 42). The immunodetection of At2-MMP using NtMMP1 antibody seems to be questionable and need further optimization.

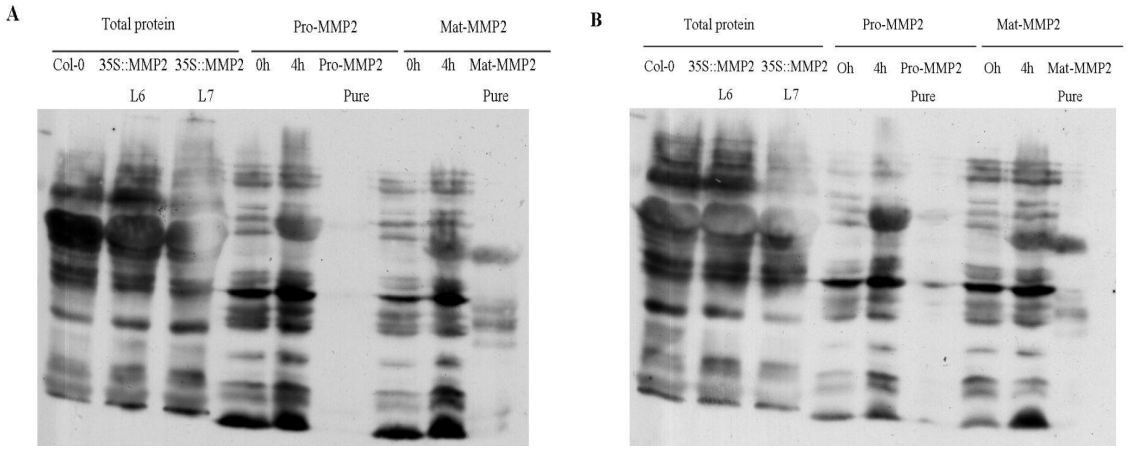


Fig.3 - 42. Immunodetection of At2-MMP protein using NtMMP1 antibody. Total protein extacted from *Arabidopsis* plants (Col-0, *35S::MMP2* L6/L7) or At2-MMP protein expressed in *E. coli* were loaded on 12% SDS-PAGE. 0h, pellet harvested before adding IPTG; 4h, pellet harvested 4h after IPTG induction; pure Pro-MMP2: recombinant protein Pro-MMP2 after Ni-NTA purification; Pure Mat-MMP2: recombinant protein Mat-MMP2 after Ni-NTA purification. **A**, Western blot with N-terminus antibody (N-11.5.1.5). **B**, Western blot of At2-MMP with C-terminus antibody (C-6.3.2).

3.7.3 Subcellular localization of At2-MMP

At2-MMP has a predicted signal peptide (aa 1-20) in the N-terminus and a putative GPI-anchor modification site and a transmembrane domain at the C-terminus (Fig.3 - 43, A). It was predicted to be located in the extracellular space by SubLoc (http://www.bioinfo.tsinghua.edu.cn/SubLoc/) and TargetP program (http://www.cbs.dtu.dk/services/TargetP/). However, WoLF PSORT (http://wolfpsort.org/) predicted that At2-MMP is bound to the plasma membrane. These conflicting predictions on the subcellular localization of At2-MMP needed to be verified experimentally.

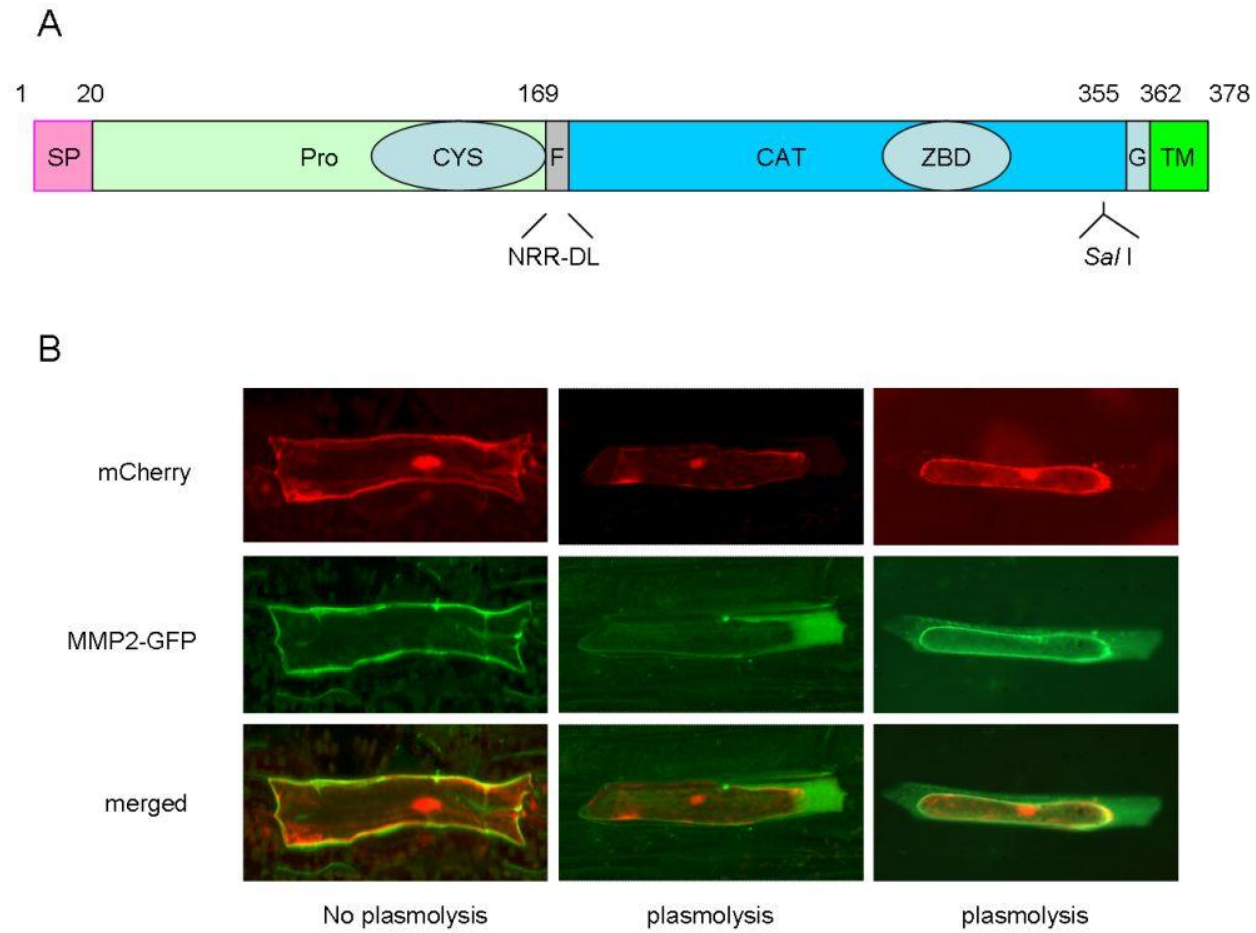


Fig.3 - 43. Structure and subcellular localization of At2-MMP. **A**, Schematic presentation of domain structure for At2-MMP-GFP fusion construct. The numbers on the top indicate the positions of the amino acid residues flanking various domains. SP: signal peptide; Pro: propeptide domain; CYS: cystein switch; F: predicted furin-cleavage site; CAT: catalytic domain; ZBD: zinc-binding domain; G: putative GPI-anchor modification site; TM: transmembrane domain. *Sal* I site (GTCGAC, aa 337-338) shown on the bottom is downstream of the zinc-binding domain and upstream of the GPI-anchor modification site. GFP CDS was cloned in frame at the *Sal* I site of At2-MMP to yield MMP2-GFP. **B**, Cell surface-localization of At2-MMP. The plasmid *35S::MMP2-GFP* was co-bombarded into *Arabidopsis* leaves with the *35S::mCherry* construct (section 2.8). Left panel: Without plasmolysis treatment, GFP fluorescence was observed at the cell wall-plasma membrane interface. Middle and right panel: after plasmolysis induction with 50% glycerol for 5 min, the GFP fluorescence was mainly detected in the apoplast (middle) or on the plasma membrane (right).

To prove the subcellular localization of At2-MMP, we constructed a GFP protein fusion of At2-MMP (section 2.8). The fusion construct was driven by the CaMV 35S promoter and contained the GFP in frame at the *Sal* I site of At2-MMP (Fig.3 - 43, A), which is between the zinc-binding domain and the putative GPI-anchor modification site. The construct *35S::MMP2-GFP* was delivered into *Arabidopsis* leaves through particle bombardment. As shown in Fig.3 - 43 (B, middle left), green fluorescence indicative for the transiently expressed MMP2-GFP fusion protein was exclusively detected on the cell surface

surrounding the interface of cell wall and plasma membrane. By contrast, red mCherry signal from a co-bombarded *35S::mCherry* plasmid was found through the cell periphery, cytoplasm and nucleus (Fig.3 - 43, B, left top). To verify whether the MMP2-GFP was bound to the membrane or secreted to the extracellular space, we performed plasmolysis with 50% glycerol. After induction of plasmolysis, the GFP signal was mainly observed in the space between plasma membrane and cell wall (Fig.3 - 43, B, middle column) or on the plasma membrane (Fig.3 - 43 B, right column). However, the mCherry signal remained within the cytoplasm (Fig.3 - 43, B). Therefore, we concluded that At2-MMP was present both in the apoplast and plasma membrane.

4. Discussion

4.1 Phylogenetic analysis of plant MMP family

As in vertebrates and invertebrates, MMPs have a widespread presence in plant kingdom from single-celled green algae *Chlamydomonas reinhardtii* to flowering plants with 5 members in *Arabidopsis thaliana*. I performed a phylogenetic reconstruction of 44 plant MMPs including all the till now characterized MMPs from 14 plants. This resulted in the classification of plant MMPs into four groups (Fig. 3 – 1) which were tightly linked to the taxonomy status and evolutionary relationships of the plants. *Arabidopsis* At2-MMP, At3-MMP and At5-MMP belong to group I also containing tomato and tobacco MMPs such as LeMMP1, NtMMP1 and NMMP1 which were known to be responsive to fungus elicitor treatment or bacterial pathogen infection (Frick and Schaller, 2002; Kang *et al.,* 2010; Schiermeyer *et al.,* 2009). Other members were implicated by their pathogen induction pattern as regulators in pathogen responses according to genevestigator expression profile. Monocots also possess group I MMP members, which might exhibit similar functions in stress and pathogen responses but waits to be functionally validated. The present study confirmed that expression of *At2-MMP* and *At3-MMP* was induced by pathogen infection. In group II, At1-MMP and At4-MMP had highest gene expression level during the seed development (Flinn, 2008). It was also reported that Pta1-MMP from Loblolly pine (*Pinus taeda*) was expressed during seed development, germination completion and early seedling establishment (Ratnaparkhe *et al.,* 2009). Thus, group II MMPs in plants may be associated with developmental processes. In group III, there are only MMP members from dicots identified in this group indicating that this group may have evolved in some dicot plants and may have specific functions. The functions of group III members are yet largely unknown. One of the group III members, the cucumber MMP Cs1-MMP was suggested to be involved in programmed cell death during the establishment and aging of cotyledons (Delorme *et al.,* 2000). Another member, SMEP-1 GM from soybean, might function in leaf expansion (Graham *et al.,* 1991). Hence, I speculate that MMP in group III might be involved in diverse processes including cell death and leaf expansion. Group IV members were exclusively found in the legume plants *Glycine max* and *Medicago truncatula* thus might be legume-specific MMPs. Their presence is of great interest as they may be essential regulators in the association with rhizobia in legume plants. One example is MtMMPL1 from *Medicago truncatula,* belonging to the group IV, was previously identified as a marker for root and nodule infection by *Sinorhizobium meliloti* (Combier *et al*., 2007). Overexpression and RNA interference approach supported a negative role of MtMMPL1 in

the regulation of *Rhizobium* infection. The MMPs in the unicellular green algae *Chlamydomonas reinhardtii* was the most basal and assumed ancient MMP among these MMPs and was in a distinct group (Fig. 3 – 1).

An invariant DLESV sequence was previously suggested to be a plant-specific motif with unknown functions on the N-terminal side of the zinc-binding region HEXGHXXGXXH in MMP proteins (Maidment *et al.,* 1999). According to the multiple alignments of MMPs from human, insects and plants, I could confirm that the DLESV motif or its variants were exclusively found in plants. In human MMP and insect MMPs, the corresponding region appeared to have a distinct consensus sequence of NLFLV. Interestingly, the DLESV motif was not present in the single-celled green algae *Chlamydomonas reinhardtii*. In the evolutionary process, this motif might have evolved as adaptation of land plants and represent one diversification point from ancient MMPs to modern MMPs. In addition, the plant MMPs are small families compared with mammalian MMPs. The expansion of MMPs in mammals might implicate the requirement of diverse functions in a more sophisticated extracellular matrix in mammals.

Interestingly, the fact that none of the plant MMP members contain introns whereas the animal MMPs do may implicate that they have evolved due to retro-position. Most human MMPs contain a C-terminal hemopexin domain which is absent in plant MMPs. An exception is human MMP7 (matrilysin) which also lacks the hemopexin domain (Maidment *et al*., 1999) and shows together with MMP20 (enamelysin), highest similarity to At2- and At3-MMP.

4.2 Subcellular localization of plant MMPs

4.2.1 Stability of At2-MMP in other plant species

Transient transformation of onion epidermal cells with reporter gene fusion constructs is a well-established system in plant cell biology (Scott *et al.,* 1999). Due to its high transformation efficiency and convenience, GFP imaging in living onion epidermal cells has been widely used in the determination of subcellular localizations by plant researchers. For instance, the nuclear localization of WRKY proteins from *Arabidopsis* and grape vine (*Vitis vinefera*) were experimentally verified in onion epidermal cells through particle bombardment (Kim *et al.,* 2006; Liu *et al.,* 2011; Xu *et al.,* 2006). In the present study, I performed co-bombardment of a *35S::MMP2-GFP* fusion construct with the *35S::mcherry* control plasmid in onion epidermal cells. As a result, large number of cells expressing mcherry was observed indicating high efficiency of transformation after the bombardment. However, the GFP signal was hardly detectable (data not shown). The same results were

obtained when I used barley epidermal cells for transformation, another system routinely used in our laboratory. But as the *35S::MMP2-GFP* fusion construct was expressed properly in *Arabidopsis* leaves, I concluded that the exogenously-introduced At2-MMP was unstable in onion and barley tissues, which may be caused by wrong folding or degraded as "non-self" protein by proteases in these plants. Another possible reason for the instability of GFP signal is improper pH range in the apoplast, because GFP fluorescence is pH-dependent and unstable at low pH (Sansebastiano *et al*., 1998). The lack of GFP fluorescence in onion cell walls was observed previously and attributed to the low pH in the cell wall (Scott *et al*., 1999). Treatment of the cells by soaking in neutral buffer (pH 7.0) may help to observe GFP signals in the apoplast of onion cells.

4.2.2 Co-presence of At2-MMP in the apoplast and plasma membrane

Most of MMPs in mammals are secreted to the extracellular space or attached to the cell surface (Parks *et al*., 2004). Plant MMPs are also expected to be extracellular or bound to plasma membrane (Flinn, 2008). Two plant MMPs, *Slti114* from soybean and NtMMP1 from tobacco, have been experimentally demonstrated as membrane-localized metalloproteinases (Cho *et al.,* 2009; Schiermeyer *et al.,* 2009). For NtMMP1, the C-terminal hydrophobic domain was suggested to mediate the membrane integration Due to the presense of a cleavable signal sequence at the N terminus and a predicted transmembrane domain at the C terminus, At2-MMP was also suggested to be located in the plasma membrane via GPI-anchor linkage (Maidment *et al.,* 1999). Indeed, I observed a membrane-localized GFP signal after transient transformation using *35S::MMP2-GFP* fusion construct (Fig.3 – 43). In addition, presence of the GFP was also detected in the apoplast after induction of plasmolysis by 50% glycerol (Fig.3 – 43, B, right column). As glycerol is a mild agent that thought to be harmless to plasma membrane, the release of MMP2 into the extracellular space is therefore more likely active. Many cell surface proteins are integrated into plasma membranes via covalent GPI anchors that are post-translationally linked to the C-terminus of the protein. Simpson *et al*. (2009) suggested that leaching of the GFP signal from membrane to apoplast is due to stress-induced phospholipase C activity, which is known to release GPI-anchored proteins (Low, 1987; Svetek *et al.*, 1999).

4.3 Expression analysis of At-MMPs

4.3.1 Pathogen-induction of At-MMP expression

In mammals and insects, some MMPs are strongly induced during bacterial infections (Burke, 2004; Lo´pez-Boado *et al*., 2001; Quiding-Järbrink *et al.*, 2001), wounding (Hieta *et*

al., 2003; Lohi *et al*., 2001) or treatment with MAMPs such as bacterial lipopolysaccharide (LPS) (Altincicek and Vilcinskas, 2008). As evolutionary conserved enzymes, MMPs in plants may also contribute in plant immunity. To determine whether At-MMPs are involved in immune responses, I started with the expression profiling of At1-MMP to At5-MMP during interaction with various plant pathogenic or mutualistic microbes including *B. cinerea*, *G. orontii*, *Pst* DC3000 and *P. indica*. From my observation, *At2-MMP* and *At3-MMP* exhibit very similar expression pattern in the interaction with *B. cinerea* and *Pst DC3000*. Both of them were up-regulated by the necrotrophic fungal pathogen *B. cinerea* (Fig.3 – 2) and the hemibiotrophic bacterial pathogen *P. syringae* (Fig. 3 – 5). After inoculation with biotrophic *G.* orontii, *At2-MMP* expression was up-regulated at very late time point (120 hai) (Fig. 3 – 3). *At3-MMP* was also induced by flg22 (Fig. S 8). Only a few other analyses demonstrated also pathogen-induced expression of plant genes encoding MMP proteins (Kang *et al.,* 2010; Liu *et al.,* 2001; Schiermeyer *et al.,* 2009; Torres *et al*., 2003; Zipfel *et al.,* 2004). Expression of NMMP1 in *Nicotiana benthamiana was* induced by a compatible pathogen *Pseudomonas syringae* pv. *tabaci* (*Psta*) and an incompatible pathogen *P. syringae* pv. *tomato* T1 (*Ps*T1) as well as by defense signaling molecules SA and ET (Kang *et al.,* 2010). In the incompatible PsT1 infection, NMMP1 expression was significantly increased 3 h to 12 h after treatment but decreased after 24 h. Such an induction pattern seems to be associated with hypersensitive response (HR) (Kang *et al*., 2010). Similarly, the expression of *GmMMP2* from soybean was shown to be highly up-regulated during compatible and incompatible interaction with oomycete pathogen *Phytophthora sojae* (Liu *et al.,* 2001). Moreover, increase of the *GmMMP2* transcript was in coincidence with an increased matrix metalloproteinase activity during the infection of *Pseudomonas syringae* pv. *glycinea*.

These biotrophic and necrotrophic pathogens have evolved distinct strategies to establish association with the host plants. Due to the capability in killing the host cells through secretion of toxins and tissue degrading enzymes, the grey mould fungus *B. cinerea* is considered as a necrotrophic pathogen. In contrast, the powdery mildew fungus *G. orontii* is a biotrophic pathogen that relies on living host cells for growth and proliferation. The hemibiotrphic bacteria *Pst* DC3000 starts with a biotrophic phase in the infection and subsequently the host cells die at a later stage. It appeared that induction of *At2-MMP* and *At3-MMP* was associated with the process of tissue damage caused by pathogens. The expression of *At2-MMP* and *At3-MMP* was increasing from early infection to late stage, during which the leaf necrosis and lesions became visible (Fig.3 – 2; Fig. 3 – 5). *At3-MMP* was induced by flg22 treatment from 15 min but *At2-MMP* was not clearly induced during

the early time points (Fig. S 8). *At3-MMP* plays a role in the early signaling event during the plant-microbe interactions. *At2-MMP* and *At3-MMP* expression may be components in the host alarming signaling responses upon pathogen-mediated cell destruction. The secreted and activated At2-MMP and At3-MMP might act on the yet unidentified substrate to generate danger signals. Recent findings from the lepidopteran model *Galleria mellonella* support such a danger model of immune activation (Altincicek and Vilcinskas, 2008). The first MMP identified in *G. mellenella* (Gm1-MMP), was found to degrade collagen-IV, which in turn activated the innate immunity.

After inoculation with root-colonizing mutualistic fungus *P. indica*, *At2-MMP* almost showed no change in the transcript level and *At3-MMP* was significantly down-regulated at late stage (Fig.3 – 6). It was recently reported that *P. indica* has evolved an extraordinary capacity in broad-spectrum suppression of host innate immunity during the colonization of *Arabidopsis* roots (Jacobs *et al.,* 2011). Induced expression of *At3-MMP* may be a typical part of the effective innate immune system that *P. indica* is confronted with during the establishment of mutualistic association. Thus, the observed downregulation of At3-MMP may be a result of active suppression of innate immunity.

In the *P. indica* colonization study, *At2-MMP* had very high expression in both mock and *P. indica* treated roots. In contrast, it showed very low basal expression in leaves. This is in agreement with the previous description that *At2-MMP* had higher expression in root than in leaves (Maidment *et al.,* 1999). In my study, *At1-MMP* had higher expression in leaves than in roots. However, Maidment *et al*., (1999) found that *At1-MMP* expression in roots was higher than in leaves. These contrasting results might reflect age-associated altered expression of *At1-MMP*. In this study, leaf samples were harvested from soil-grown five-week-old plants and the roots were harvested from three-week-old plants growing in ATS medium. The investigation by Maidment and colleagues was performed on 14-day-old *Arabidopsis* seedlings (Maidment *et al*., 1999)

4.3.2 Analysis of signaling pathways for At-MMP expression

Many defense-related genes are activated via a SA-dependent or JA-dependent pathway (Glazebrook, 1999; Glazebrook, 2001). The connection between the gene expression and defense signaling molecules has been demonstrated for some plant MMPs. For example NMMP1, the first MMP identified from *Nicotiana benthamiana*, was highly induced by ET but not by methyl jasmonate after 3 h of treatment (Kang *et al.,* 2010). The transcripts of soybean *GmMMP2* were up-regulated by pathogens and yeast extracts but not stimulated by

SA or JA treatment (Liu *et al.,* 2001). The *GmMMP2* expression seemed to follow a novel signaling pathway where SA and JA are not the key components.

To determine which pathway is involved in the regulation of *At2-MMP* and *At3-MMP* expression, *Arabidopsis* mutant plants with compromised signaling pathways including SA (NahG, *ics1*, *npr1-1*, *pad3*), JA (*jar1-1*, *jin1*) and ethylene (*ein2-1*) mutants were tested for the *At2-MMP* and *At3-MMP* expression after *B. cinerea* infection (Fig.3 - 8). Based on the similar induction pattern of At2-MMP and *At3-MMP* between wild-type plants and the SA, JA or ET signaling mutants, I demonstrated that the *B. cinerea*-induction of *At2-MMP* and *At3-MMP* was likely to be independent of SA, JA or ET signaling. The other possibility is that *At2-MMP* and *At3-MMP* induction act upstream of all the studied central signaling nodes. In addition, it's notable that methyl jasmonate treatment could stimulate At2-MMP expression in rosette leaves of 4-week-old plants but not 10-week-old plants (Golldack *et al.,* 2002). Therefore, the regulating role of JA in At2-MMP expression is ambiguous. The possibility is not ruled out that the *B. cinerea*-induced At2-MMP expression is partially modulated by JA signaling.

In the further analysis, ABA (*abi2* and *aba2*), GA (*della pentuple*), cytokinin (*ahp1,2,3,4,5-1*), JA(*coi1-16*), auxin (*tir1-1*), brassinosteroids (*bri1-116*), phytoalexin deficient (*pad4*) and wild type plants Ler and Col-0 plants were inoculated with *B. cinerea*. As shown in Fig. 3 – 10, most mutants showed very similar expression patterns as the wild type plants Ler and Col-0. Interestingly, *aba2* (ABA deficient 2) mutant showed a similar expression pattern after mock treatment and *B. cinerea* inoculation (Fig. 3 – 10). *B. cinerea*-induction of At2-MMP was not observed in *aba2* mutant. *At2-MMP* was likely to be regulated by ABA signaling. However, further investigations are required to confirm this hypothesis. Moreover, At2-MMP was previously suggested as a component of R gene-mediated disease resistance in *Arabidopsis thaliana* (Torres *et al*., 2003). Expression of *At2-MMP* was much stronger in RPM1-dependent incompatible interactions, but its expression in *eds1-2* mutants after *Pst DC3000* infection was compromised in comparison to the wild-type plants. I expected the expression of *At-MMP* differs between the *eds1-2* and wild type plants after *B. cinerea* inoculation. As a result, At2-MMP and At3-MMP had similar expression in the *eds1* mutant as in wild-type plants (Fig. 3 – 12). It indicated that *B. cinerea*-induced expression of *At2-MMP* and *At3-MMP* was independent of *eds1*. In *Arabidopsis*, two mitogen-activated protein kinases MPK3 and MPK6 are rapidly activated after pathogen infection (Ren *et al.,* 2008). In the present study, *mpk3* and *mpk6* mutants were inoculated with *B. cinerea* for expression analysis (Fig. 3 – 11). The expression profiling of *At2-MMP* and *At3-MMP* during the *B. cinerea* infection in the *mpk3* and *mpk6* mutants and Col-0 plant was examined. For *At2-*

MMP, expression in both *mpk3* and *mpk6* mutants was similar to Col-0. For *At3-MMP*, the expression in *mpk3* mutant at early time points (0h and 12 h) was higher than that of Col-0. This might indicate a role of MPK3 in the negative regulation of *At3-MMP*.

4.4 Functions of plant MMPs

4.4.1 Plant MMPs in development

Golldack *et al.,* (2002) showed that mutation of *At2-MMP* resulted in late flowering and early senescence. Upon the onset of shoots, growth of roots, leaves, and shoots was inhibited in the *at2-mmp-1* mutant compared with the wild type. Consistent with the previous finding, I observed a delayed flowering in *at2-mmp* mutants under short day conditions. Moreover, constitutive overexpression of *At2-MMP* resulted in early flowering (Fig. 3 – 21). Therefore, *At2-MMP* plays dual roles in pathogen defense and in shoot induction. Apart from the pathogen response, the *At3-MMP* might be involved in development as well on the basis of its microarray expression profile (data not shown). Its transcript level was increasing from seed germination to bolting and reached the peak value during formation of young flowers. Similar findings from insect models support such a notion that some MMPs function as coordinators linking development and immunity (Altincicek and Vilcinskas, 2008; Knorr *et al*., 2009). Studies on the lepidopteran model *Galleria mellonella* revealed that *Gm1-MMP* playes a role in both metamorphosis and innate immunity (Altincicek and Vilcinskas, 2008). In addition, results from the model insect *Tribolium castaneum* indicated that RNAi-based knowck-down of MMP-1 caused dual effects including defective embryogenesis, abnormal intestines and higher susceptibility to entomopathogenic fungus *Beauveria bassiana (*Knorr *et al*., 2009*)*. Dual role in pathogen resistance and development was recently demonstrated for ZMP-2, one MMP in the nemotode model species *Caenorhabditis elegans* (Altincicek *et al*., 2010). Thereby, my finding provides additional evidence revealing the evolutionarily conserved roles of MMPs between plants and animals.

4.4.2 Plant MMPs in abiotic stress responses

Expression data suggested potential roles of plant MMPs in the adaptation to abiotic stresses. NaCl treatment (50 mM) was shown to stimulate *At2-MMP* expression in roots but not in leaves (Golldack *et al.,* 2002). At2-MMP expression was also stimulated by cadmium treatment in leaves (Golldack *et al*., 2002). From Genevestigator data, *At3-MMP* expression was up-regulated by NaCl stress. To verify their role in salt stress responses, I used the homozygous *35S::MMP2* and *35S::MMP3* overexpression plants to test the salt tolerance with NaCl at different concentrations. *35S::MMP3* plants did not show any difference in the

salt sensitivity compared to the control plants (Fig. 3 – 23). This indicates that some salt-induced genes may not play important roles in salt tolerance (Xiong and Zhu, 2002). For *35S::MMP2* (L6 and L7) plants, the roots length was longer than that of Col-0 and EV plants in the presence of 200mM NaCl (Fig. 3 – 22). The results support a positive role of At2-MMP in the adaptation to NaCl stress. It is notable that also the empty vector transformants were showing enhanced salt tolerance in comparison with the wild-type Col-0 plants under all the studied NaCl concentrations. Reports from soybean support the involvement of MMPs in abiotic stress responses such as wounding, dehydration and low temperature (Cho *et al*., 2009; Liu *et al*., 2001).

4.4.3 Plant MMPs in immune responses

Growing evidence from expression studies implicates the involvement of plant matrix metalloproteinases in pathogen defenses (Liu *et al.,* 2001; Schiermeyer *et al.,* 2009). Recently, NMMP1-mediated bacterial resistance was revealed using transient overexpression and silencing assay in tobacco (Kang *et al.,* 2010). Strong evidence that in *Arabidopsis* the pathogen-inducible At2-MMP functions as a crucial regulator of defense against *B. cinerea* comes from analysis of both transgenic overexpression lines and independent T-DNA insertion mutants. Constitutive overexpression of *At2-MMP* enhanced the disease resistance (Fig. 3 – 24), whereas the *at2-mmp* mutants displayed increased susceptibility to *B. cinerea* (Fig. 3 – 15). At3-MMP acts in a very similar way as At2-MMP in the resistance towards *B. cinerea*. The additive function of At2-MMP and At3-MMP was evidenced by the more severe disease symptoms in *at2-mmp/at3-mmp* double mutants than the parental single mutants (Fig. 3 – 17).

I addressed the question whether *At2-MMP* act directly on the pathogens or not. Therefore I performed germination assays using recombinant At2-MMP protein which did not support any direct antimicrobial activity of At2-MMP on *B. cinerea* spores (Fig. 3 – 39). Hence, the action of At2-MMP in defense against pathogens is likely through an indirect way. In addition to the *B. cinerea*-induction of At2-MMP independent of the classical defense signaling SA, JA or ET, the 35S::MMP2 plants did not constitutively show enhanced expression of the defense marker genes *PR1*, *PDF1.2* or *ERF* (Fig. 3 – 32). Thus I proposed that At2-MMP mediates immunity via a yet uncharacterized novel pathway.

In mammalian system, the matrilysin MMP7 mediated antibacterial activity through the activation of α-defensin (Wilson *et al.,* 1999). A similar mechanism was proposed for GmMMP2 in soybean, which might mediate the activities of plant-derived enzymes in the degradation of fungal cell wall and aid in the release of antimicrobial substances (Liu *et al.,*

2001). At2-MMP may be subject to a similar mode of action in the defense against microbes. Nevertheless, the direct targets and the endogenous substrates of At2-MMP in the extracellular matix are unknown. Identification of its natural substrates is an urgent task and might help to understand the mechanisms underlying the functioning and signaling of At2-MMP during plant immune responses.

4.4 4 At-MMPs and MAMP/DAMP-mediated oxidative burst

Based on the oxidative burst assay, I observed significant impaired production of reactive oxygen species (ROS) after MAMP and DAMP treatment in At2-MMP overexpression lines. The reduced oxidative burst is unlikely due to T-DNA insertion-mediated destruction of MAMP/DAMP receptors because several independent elicitors were used. ROS production is largely dependent on the activity of membrane-localized NADPH oxidases (respiratory burst oxidase homologs, Rboh) (Torres *et al.,* 2006), with AtRbohD being the most important for MAMP-triggered oxidative burst (Nuhse *et al.,* 2007; Zhang *et al.,* 2007). At2-MMP, which localizes in both plasma membrane and apoplast, might target the membrane-bound NADPH oxidases thereby reducing ROS generation. It needs to be further verified whether the MAMP/DAMP-mediated activation of NADPH oxidase is blocked due to over-presence of At2-MMP proteinase, which may help to elucidate the mechanisms underlying the impaired ROS in At2-MMP overexpression plants.

As one of the earliest events during plant-pathogen interaction, reactive oxygen species (ROS) burst is of crucial importance in regards to the outcome of the interaction. ROS production may contribute to plant disease resistance either directly via its antimicrobial activity or indirectly through induction of defense related genes, cell wall strengthening and orchestration of cell death (Bolwell, 1999; Lamb and Dixon 1997; Levine *et al.,* 1994; Torres *et al.,* 2006; Zurbriggen *et al.,* 2009). Due to the different infection strategies of biotrophic and necrotrophic pathogens, they seem to have distinct responses to ROS generated in the host plants (Heller and Tudzynski, 2011). Successful infection of biotrophic and hemibiotrophic fungi depend on the prevention of a strong oxidative burst and the hypersensitive response of their host, by suppression of PTI responses or by scavenging the host-derived ROS during the early infection phase (Molina and Kahmann, 2007; Shetty *et al.,* 2007). Thus, from the host plants' side, the oxidative burst is an effective process to combat biotrophic pathogens. However, necrotrophic pathogens may exploit host or endogenous ROS burst to facilitate infection and colonization (Govrin and Levine, 2000; Segmüller *et al.,* 2008). It was suggested that *B. cinerea* does not face oxidative stress in planta, at least not via H_2O_2 (Temme and Tudzynski, 2009). Similar finding was also presented for another

necrotrophic fungus, *Leptosphaeria maculans,* which infects *Brassica napus* (Li *et al.,* 2008). Under our experimental conditions, the At2-MMP overexpression lines demonstrated a compromised H_2O_2 production and enhanced resistance to *B. cinerea*. This is in agreement with a previous finding that H_2O_2 levels during HR correlated positively with *B. cinerea* growth (Govrin and Levine, 2000). Moreover, our observation is supported by recent findings that ROS functioned negatively in resistance or positively in expansion of disease lesions during *B.cinerea-N.benthamiana* interaction (Asai and Yoshioka, 2009; Asai *et al.,* 2010). However, studies from Galletti *et al.,* (2008) revealed that the elicitor-triggered host H_2O_2 accumulation appeared to be independent of effective resistance against *B. cinerea*. Thus, the mechanism underlying the At2-MMP mediated resistance to *B. cinerea* and how ROS signaling participates negatively in defense responses against *B. cinerea* awaits further investigation.

The Pep1-induced ROS production was compromised in *at3-mmp* mutants (Fig. 3 – 29, C). This indicates that At3-MMP is required for the Pep1-mediated ROS production. In contrast, an enhanced ROS production after flg22 and chitin was observed in *at3-mmp* mutants in comparison with Col-0. Thus, At3-MMP appeared to negatively regulate flg22 or chitin-dependent ROS generation. In elf18 treatment, the ROS level is similar in *at3-mmp* mutants and Col-0 plants (Fig. 3 – 28, C). However, the peak of oxidative burst appeared to be earlier in *at3-mmp*. Taken together, these results provide evidences that At3-MMP is a crucial regulator in MAMP/DAMP-mediated ROS production. The negative role of At3-MMP in flg22/chitin-induced ROS and the positive role in Pep1-induced ROS signaling might imply antagonism between Pep1 and the other two MAMP signaling. The interplay between different MAMP and DAMP signaling therefore forms the basis of a sophisticated network including positive and negative feedback mechanisms. Such antagonism between different MAMP/DAMP sensing was recently described (Nicaise *et al*., 2009).

In my studies, thermolysin, a metalloproteinase derived from the gram-positive bacteria *Bacillus thermoproteolyticus* had high proteolytic activity which was proven by the degradation of MBP within 5 min (Fig. 3 – 30). In a previous study, artificially injected thermolysin mediated resistance to invading pathogens due to activation of innate immune responses in lepidopteran insect *Galleria mellonella* (Altincicek *et al.,* 2007). I observed that thermolysin could not induce oxidative burst in *Arabidopsis*. Moreover, the mixture of flg22 and thermolysin was not able to induce ROS production (Fig. 3 – 31, B). Thermolysin appeared to abolish flg22-mediated ROS production in *Arabidopsis*. Flg22-triggered ROS production in plants is requires the recognition of active form of flg22 by the receptor-like kinase FLS in the plasma membrane (Chinchilla *et al*., 2006). Therefore, thermolysin might

act either directly on the flg22 peptide, or indirectly acting on the flg22 receptor in the plasma membrane.

In the present study, the ROS production was also monitored after treatment with the recombinant Mat-MMP2 protein. Interestingly, recombinant Mat-MMP2 was able to induce oxidative burst in Col-0 plants while the At2-MMP OE plants are compromised in PAMP-triggered ROS. This oxidative burst seems to require the proteolytic activity because heat-inactivated Mat-MMP2 almost lost the capability to induce ROS production. Moreover, the Mat-MMP2-mediated ROS was determined in the *fls2* and *bak1* mutants. In *fls2* mutants, the ROS production by Mat-MMP2 is similar as in Col-0 plants. However, Mat-MMP2 did not induce any oxidative burst in *bak1* mutants (Fig. S 11, B). These results are preliminary data and require further verification, but they provide an exciting hypothesis that At2-MMP protein might behave in the processing of endogenous substrate that activate subsequent defense responses via functional BAK1.

4.5 Activity of At-MMP proteins

The 3D modelling of At2-MMP and At3-MMP was predicted by PyMOL software using the crystal structure of human MMP1 and human MMP3 as template respectively. The C-terminal of At2-MMP seems to be distinct from human MMP1 and thus difficult to be modelled. Despite that, the N-terminal part of At2-MMP was well-matched in the modeling due to the presence of conserved structures including the cystein switch and catalytic domain. The catalytic domains are in good agreement with each other according to the overlapping of the images.

MMPs are synthesized as prepro-enzymes and secreted as inactive Pro-MMPs in most cases (Nagase and Woessner, 1999). The activation of MMPs requires the disruption of the Cys-Zn (cysteine switch) interaction and the removal of the propeptide domain containing the cysteine switch achieved by proteinases or non-proteolytic agents. A few MMPs (human MMP-11 and MMP-14) can be activated intracellularly by furin (Pei & Weiss, 1995, Pei & Weiss, 1996). After purification of Pro-MMP2 recombinant protein, always three bands showed up on the SDS-PAGE (Fig. 3 – 35). The biggest band was about 57 kDa and other two small bands were about 35 kDa and 30 kDa. A furin cleavage site was predicted between the propeptide domain and catalytic domain of Pro-MMP2. The calculated fragment sizes after putative furin of furin-like cleavage were 35 kDa and 30 kDa respectively. The three bands might therefore indicate that At2-MMP underwent an autolytic activation through removing of the propeptide domain. The exact cleavage site still awaits experimental verification.

The thioredoxin-His-S-tagged Pro-MMP2 was first expressed in *E. coli* strain BL21(DE3) but with very low expression level compared to Pro-MMP3 and an empty vector (data not shown). Therefore, *E. coli* strain BL21(DE3) pLysS was used to express the Pro-MMP2 and showed very high expression level. In general, the BL21(DE3) competent cells is an all purpose strain featured with high-level protein expression and easy induction. The BL21(DE3)pLysS competent cells provide tighter control of protein expression for the purpose of toxic proteins and are resistant to chloramphenicol. In this regard, Pro-MMP2 might be toxic to BL21(DE3) cells and tightly controlled on the transcription level by the BL21(DE3) cells.

In the present study, proteolytic acitivity of At2-MMP was confirmed by degradation of MBP and this proteolytic process was inhibited in the presence of EDTA. Moreover, the activity of intercellular washing fluid (IWF) from *35S::MMP2* (L6) plants against MBP was also inhibited by EDTA (Fig. 3 – 33, B). Matrix metalloproteases in general contain essential zinc and calcium ions in the catalytic domain (Vu and Werb, 2000). As a chelator of metal ions, EDTA competed with the catalytic domain for Zn^{2+} and Ca^{2+} and subsequently disarmed their proteolytic activity. On the other hand, metal ions could stimulate the activity of MMPs. For instance, the Loblolly pine (*Pinus taeda*) Pta1-MMP recombinant protein showed a Zn^{2+}–dependent protease activity against MBP (Ratnaparkhe *et al.,* 2009). In soybean, the proteolytic activity of SMEP1 was shown susceptible to metal chelating agents and the inactivated enzyme can be restored by the addition of $ZnC1_2$ (Graham *et al.,* 1991). The activity of tomato MMPs *Sl*MMP1 and *Sl*MMP2 recombinant proteins could be stimulated by Ca^{2+} ions (Pasule, 2010). In this study, $ZnCl_2$ also increased the activity of Pro-MMP2 but not significantly (data not shown). In the future work, significance of calcium ions in the activity stimulation of At2-MMP should also be verified.

In the study, all the proteolytic activities of recombinant proteins were determined by MBP degradation experiments. Compared to the Pro-MMP2, the native Mat-MMP2 showed higher activity and was able to degrade MBP within 30 min. Mat-MMP2 protein contains only the catalytic domain which avoids any negative interaction with the cysteine switch in the zinc-binding site. Likewise, the mature protein of soybean (*Glycine max*) GmMMP2 was shown to be more efficient than pro- GmMMP2 with regard to the degradation of MBP (Liu *et al.,* 2001). Though with substantially increased activity, Mat-MMP2 appeared to be unstable under my conditions. After short storage of Mat-MMP2 at 4 ℃, the prominent band disappeared and showed a laddering pattern on SDS-PAGE. Therefore, the propeptide might contribute to maintain the stability of At2-MMP protein.

Two antibodies for N-and C-terminal partpf tobacco NtMMP1 were produced (Mandal *et al.,* 2010). For an immunoblot detection of At2-MMP, these two antibodies were tested in western blot withtotal protein from leaf extract, Pro-MMP2 and Mat-MMP2 recombinant proteins. However, no specific detection of At2-MMP protein was achieved in a preliminary test. An optimized western blot protocol might help to improve the detection of At2-MMP using the NtMMP1 antibody. Or in further investigation, At2-MMP specific antibody should be produced to achieve the *in planta* characterization of At2-MMP dynamics and biochemical properties. For instance, the stimulated expression and accumulation of At2-MMP in response to different biotic stimuli and membrane/apoplast-enrichment of At2-MMP protein could be verified using such an antibody.

4.6 Future perspectives

The role of plant MMPs in plant-microbe interaction is largely unknown. The present work has set up a starting point to better understand the functions and mechanisms of plant MMPs in innate immunity. Evidences of At2-MMP and At3-MMP in immunity are based on pathogen-induced expression, altered resistance in mutants and overexpression lines and altered ROS production in response to various elicitors. However, a number of questions remain unsolved.

First of all, the role of At2-MMP and At3-MMP in *P. syringae* infection is an interesting question and requires further evidences. At3-MMP showed a high induction within 30 min after flg22 treatment (Zipfel *et al.,* 2004). Our data showed that At3-MMP was induced by flg22 both in root and leaf at 15min (Fig. S 8). The flg22-mediated ROS was compromised in At2-MMP overexpression plants and enhanced in *at3-mmp* plants. Thus, I expect an altered MAMP-triggered immunity (MTI) in these plants. Virulent and avirulent strains from *P. syringae* should be tested for their growth and proliferation in such plants.

Secondly, mechanism underlying the impaired MAMP/DAMP-mediated ROS production in *35S::MMP2* is yet unknown. At2-MMP as a proteinase can act either upstream or downstream in the process of ROS production. To test whether At2-MMP acts on the upstream pattern recognition receptors (PRRs), growth retardation assay and immunoblot detection of PRRs in *35S::MMP2* plants should be considered. On the other hand, to correlate At2-MMP with the H_2O_2-generating NADPH oxidases, experiments should be designed to test the potential regulatory role of At2-MMP on the activators/inhibitors of NADPH oxidases or its direct regulation of NADPH oxidase.

An urgent and challenging task for plant MMP research is the identification of endogenous substrates under normal growth conditions and pathogenic conditions. As such, the exact

roles and mechanisms of plant MMP will be more clearly understood on a biochemical level. Furthermore, studies on matrix metalloproteinase inhibitors (MPI) have received extensive attention in medical field due to its potential use for cancer suppression (Coussens *et al.,* 2002; Gialeli *et al*., 2001; Vanlaere and Libert, 2009; Vihinen *et al*., 2005). Some of the natural products from plants were found to inhibit mammalian MMP activities (Bodet *et al*., 2007; Longatti *et al.*, 2011; Seo *et al.*, 2005). Tissue inhibitor of metalloproteinases (TIMP) was found in insects which share no sequence similarities with vertebrate MPIs (Vilcinskas and Wedde, 2002). MPIs have not yet been identified in plants and the presence of MPI in plants is still questionable. Homology-based approach in the searching for TIMP in plants failed to identify any candidate, however, non-TIMP-like inhibitors might be present in plants. The identification of endogenous MMP inhibitors may help to fully understand the nature of plant MMP activity.

5. Summary

Matrix metalloproteinases (MMPs) are evolutionary conserved metal-dependent endopeptidases widely present in animals and plants. Mammalian MMPs are well known as central regulators in a number of physiological and pathological processes such as tissue remodeling and cancer progression. Little is known about the detailed function and molecular mechanisms of MMPs in plants. Main focus of the present study was to analyze the potential involvement of *Arabidopsis* MMPs family in plant immune responses.

Phylogenetic analysis using 44 MMPs from fourteen higher plants resulted in a clear classification of four subgroups, which are associated with certain plant species or functions. Group I MMPs appear to be pathogen-responsive whereas group II members seem to be involved in development. Group III was found to be a dicot-specific group and group IV is solely present in legume plants.

The expression profiles of five At-MMPs were examined in Col-0 plants during the interaction with distinct microbes, including the biotrophic fungus *Golovinomyces orontii*, necrotrophic fungus *Botrytis cinerea*, hemibiotrophic bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and the symbiotic mycorrhizal-like fungus *Piriformospora indica*. *At2-MMP* and *At3-MMP* were clearly up-regulated after infection with *Pst* DC3000 as well as *B. cinerea*. This indicates a potential involvement of MMPs in pathogen resistance.

Arabidopsis T-DNA insertion mutant lines were tested for altered resistance. Mutation of At4-MMP and At5-MMP did not exhibit clear changes in pathogen resistance. *At2-MMP* mutants were identified to be more susceptible to *B. cinerea* infection. In contrast, At2-MMP overexpression lines *35S::MMP2* showed enhanced resistance to *B. cinerea*. Moreover, *35S::MMP2* exhibited early flowering compared with control plants. At3-MMP showed similar altered responses as At2-MMP based on mutants and overexpression analysis. Double mutants *at2-mmp/at3-mmp* were produced and showed significantly enhanced susceptibility to *B. cinerea*. These results confirmed the essential role of At2- and At3-MMP in the resistance towards *B. cinerea*.

Bombardment-mediated transient transformation was used to clarify the subcellular localization of At2-MMP. Plasmolysis experiments demonstrated that a *MMP2-GFP* fusion protein was co-present in the plasma membrane and apoplastic space. In addition,

recombinant proteins for Pro-MMP2 and mature MMP2 (Mat-MMP2) were produced and tested for proteolytic activity. The Pro-MMP2 contains the auto-inhibitory propeptide domain and catalytic domain but lacking the N-terminal signal peptide, whereas the Mat-MMPs contains only the catalytic domain. As a result, Mat-MMP2 exhibited substantially higher activity than Pro-MMP2 against the substrate myelin basic protein (MBP). Activities of MMP2 can be inhibited by the metal chelator EDTA. Like most mammalian MMPs tested, the recombinant At2-MMP protein showed no direct antimicrobial activity towards *B. cinerea* conidia spore germination and hyphae growth.

Expression pattern of *At2-MMP* and *At3-MMP* in Col-0 was compared in mutants which are compromised in SA (NahG, *ics1, npr1-1, PAD3*), JA (*jar1, jin1*) or ET (*ein2-1*) signalling after *B. cinerea* infection. The similar induction of At2-MMP and At3-MMP in all the mutants suggested that *B. cinerea*-induced expression of At2/3-MMP was likely independent of SA, JA and ET signalling. At2- and At3-MMP expression was also found to be independent of MPK3 and MPK6. Further analysis in the mutants with impaired signalling of ABA (*abi2, aba2*), GA (*della pentuple*), cytokinine (*ahp1,2,3,4,5-1*), JA (*coi1-16*), auxin (*tir1-1*), brassinosteroids (*bri1-116*) and phytoalexin (*pad4*) indicated that ABA and PAD4 may be required for full *B. cinerea*-induced *At2-MMP* expression.

Reactive oxygen species (ROS) are early host responses to pathogen infection. To decipher the mode of actions for At2- and At3-MMP mediated immune responses, the ROS production in *35S::MMP2*, *at2-mmp* and *at3-mmp* mutants was monitored after treatment of pathogen-associated molecular pattern molecules (PAMPs, flg22, elf18 and chitin) and a danger- associated molecular pattern peptide (DAMPs, Pep1). Intriguingly, the PAMP/DAMP-triggered ROS production was largely impaired in *35S:MMP2* plants. Wild-type level of ROS generation was observed in *at2-mmp* mutants. In contrast, flg22 and chitin-induced ROS generation was enhanced in *at3-mmp* mutants. Preliminary data also indicated that recombinant At2-MMP protein was capable of inducing ROS production in Col-0 plants.

Taken together, At2-MMP and At3-MMP are playing essential roles in *Arabidopsis* immune responses likely through the modulation of ROS production. Future studies are suggested to focus on their physiological substrates, mode of actions and roles in resistance to other pathogens such as bacterial pathogens.

6. Zusammenfassung

Matrix-Metalloproteinasen (MMPs) sind evolutionär konservierte metall-abhängige Endopeptidasen, die in tierischen und pflanzlichen Organismen weit verbreitet sind. Säugetier MMPs sind zentrale Regulatoren in einer Reihe von physiologischen und pathologischen Prozessen, wie Auf- und Abbau von Gewebe und Tumorwachstum. Über die genauen Funktionen und molekularen Mechanismen pflanzlicher MMPs ist dagegen wenig bekannt. Ziel der folgenden Arbeit war daher die Analyse der potentiellen Rolle der Arabidopsis MMP Proteinfamilie bei der pflanzlichen Abwehr.

Phylogenetische Analysen mit 44 MMPs aus 14 Pflanzenarten ergaben eine klare Einteilung der Proteine in vier Untergruppen, die jeweils mit spezifischen Beschaffenheiten oder Funktionen korreliert werden können. Gruppe I MMPs sind responsiv gegenüber Pathogenen, wogegen Mitglieder der Gruppe II in Entwicklungsprozessen involviert zu sein scheinen. MMPs der Gruppe III kommen ausschließlich in dikotylen Pflanzen vor und Gruppe IV ist auf Fabaceen beschränkt.

Von den fünf Arabidopsis At-MMPs wurden die Expressionsprofile während der Interaktion von Col-0 Pflanzen mit ausgewählten Mikroorganismen untersucht. Hierzu wurden der biotrophe Pilz *Golovinomyces orontii*, der nekrotrophe Pilz *Botrytis cinerea*, das hemibiotrophe Bakterium *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 und der symbiotische, Mykorrhiza-ähnliche Pilz *Piriformospora indica* verwendet. Bei *At2-MMP* und *At3-MMP* wurde nach Infektion mit *Pst* DC3000 und *B. cinerea* eine deutlich gesteigerte Expression beobachtet. Dies weist auf eine mögliche Rolle der MMPs in der Pathogenabwehr hin.

Mehrere Arabidopsis T-DNA Insertionslinien wurden daraufhin auf eine veränderte Resistenz gegnüber *B. cinerea* getestet. Eine Mutation von At4-MMP und At5-MMP zeigte keine signifikanten Änderungen in der Pathogenabwehr. *At2-MMP* Mutanten waren dagegen suszeptibler gegenüber dem Pilz. Im Gegensatz dazu wiesen At2-MMP Überexpressionslinien (*35S::MMP2*) eine erhöhte Resistenz gegen *B. cinerea* auf. Zusätzlich wurde bei diesen Pflanzen eine frühere Blütenbildung als bei Kontrollpflanzen beobachtet. In Mutations- und Überexpressionlinien von At3-MMP wurden ähnliche Phänotypen wie für At2-MMP festgestellt. Deshalb wurden *at2-mmp/at3-mmp* Doppelmutanten generiert. Diese

wiesen eine stark erhöhte Suszeptibilität gegenüber *B. cinerea* auf. Die Ergebnisse bestätigen eine essentielle Rolle von At2- und At3-MMP in der Resistenz gegen *B. cinerea*.

Transiente Expression mittels *Particle-Bombardment* wurde verwendet um die subzelluläre Lokalisation von At2-MMP zu studieren. Plasmolyse Experimente ergaben eine duale Lokalisation des Proteins an der Plasmamembran und dem apoplasmatischen Raum. Zusätzlich wurde rekombinantes Proenzym Pro-MMP2 und prozessiertes MMP2 (Mat-MMP2) hergestellt und auf proteolytische Aktivität getestet. Pro-MMP2 enthält die auto-inhibitorische propeptid Domäne und die katalytische Domäne, aber kein N-terminales Signalpeptid. Dagegen weist Mat-MMP2 nur die katalytische Domäne auf. Mat-MMP2 zeigte eine substantiell höhere Aktivität gegenüber dem Substrat Myelin-Basisches Protein (MBP), als Pro-MMP2. Die Aktivität von MMP2 ist durch den Metallchelator EDTA inhibierbar. Wie die meisten getesteten Säugetier MMPs wies rekombinantes At-MMP2 keine direkte antimikrobielle Wirkung gegenüber Sporenkeimung und Hyphenwachstum von *B. cinerea* auf.

Die Expressionsmuster von At2-MMP und At3-MMP in Col-0 Pflanzen nach Infektion mit *B. cinerea* wurden mit denen in Mutantenlinien verglichen, die eine gestörte SA (NahG, *ics1*, *npr1-1*, *pad3*), JA *(jar1*, *jin1*) oder ET (*ein2-1*) Signalweiterleitung aufweisen. Die mit dem Wildtyp vergleichbare Induktion von At-MMP2 und At3-MMP in allen Mutanten weist darauf hin, dass die *B. cinerea* induzierte Expression dieser Proteine wahrscheinlich unabhängig von SA, JA und ET Signalwegen erfolgt. Zusätzlich wurde die Expressionsinduktion als unabhängig von den MAP Kinasen MPK3 und MPK6 gefunden. Eine weitere Analyse von Mutanten mit gestörten Signalwegen für ABA (*abi2*, *aba2*), GA (*della pentuple*), Cytokinin (*ahp1,2,3,4,5-1*), JA (*coi1-16*), Auxin (*tir1-1*), Brassinosteroiden (*bri1-116*) und Phytoalexinen (*pad4*) weist darauf hin, dass ABA und PAD4 für eine vollständige *B. cinerea* induzierte Expression von *At2-MMP* notwendig zu sein scheinen.

Die Produktion von reaktiven Sauerstoffspezies (ROS) gehört zu den frühen Abwehrreaktionen von Pflanzen. Ziel war es, die Rolle von At2-MMP und At3-MMP bei der pflanzlichen Abwehr genauer zu untersuchen. Hierzu wurde die ROS Produktion in *35S:MMP2*, *at2-mmp* und *at3-mmp* Pflanzen nach Behandlung mit *microbe-associated molecular patterns* (MAMPs, flg22, elf18 und chitin) und *danger-associated molecular patterns* (DAMPs, Pep1) untersucht. Interessanterweise zeigten *35S:MMP2* Pflanzen eine deutlich verringerte ROS Produktion als Antwort auf die MAMP/DAMP Behandlung. Kein

Unterschied zum Wildtyp konnte in *at2-mmp* Mutanten beobachtet werden. Im Gegensatz dazu zeigten *at3-mmp* Pflanzen eine erhöhte Produktion von ROS als Reaktion auf flg22 und Chitin. Vorläufige Daten deuten zusätzlich darauf hin, dass rekombinantes At2-MMP Protein selbst zur Produktion von ROS in Col-0 Pflanzen führt.

Zusammengefasst spielen die Matrix-Metalloproteasen At2-MMP und At3-MMP wichtige Rollen bei Immunreaktionen in Arabidopsis, wahrscheinlich durch die Modulation der ROS Produktion. Zukünftige Studien sollten auf die Substrate der At-MMPs, ihre genaue Funktionsweise und ihre Beteiligung an Reaktionen gegenüber anderen Pathogenklassen, wie zum Beispiel Bakterien, abzielen.

7. References

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8. Supplementary

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                          10        20        30        40        50        60
                           |         |         |         |         |         |
ACU24527_Gm          -NIKGLSVVKDYLSEYGYIE--------SSRPFNNSFDQE-TMSAIKTYQKFSNLPVTGV
Slti114_Gm           -NITGLYIVKDYLSDYGYIE--------SSGPFNDSFDQE-IISAIKTYQNFSNLQVTGG
GmMMP2               --APPVSLIKDYLSNYGYIE--------SSGPLSNSMDQETIISAIKTYQQYYCLQPTGK
ACJ84310_Mt          -KYKGLDQIKQYLQNFGYLE--------QSGPFNNTLDQE-TVLALKTYQRYFNIYAGQD
MtMMPL1              --IQGLSKIKQHLYHFKYLQGL------YLVGFDDYLDNK-TISAIKAYQQFFNLQVTGH
NtMMP1               -KVDGLAKIKKYFYNFGYIP--------SLSNFTDDFDDA-LESALKTYQQNFNLNTTGV
NMMP1_Nb             -KVDGLAKIKKYFYNFGYIS--------SLSNFTDDFDDA-LESALKTYQQNFNLNTTGV
LeMMP1_Sl            -TVDGLAKIKKYFHYFGYINN-------SSTNFTDDFDDT-LESALKTYQLNFNLNTTGV
SlMMP2               -KVDGIAKIKKYFQHFGYINN-L-----TSFNFTDEFDDT-LESALKTYQRNFNLKATGV
PtMMP1               -KYDGLAKLKSYFQYFGYIPNS-------LSNFTDDFDDS-LESALRTYQQNFNLNITGQ
PtMMP2               -KYDGLAKLKHYFQYFGYIPNS-------LSNFTDDFDDY-LESALRTYQQNFNLNVTGE
VvMMP1               -KADGLAKLKEYFHYFGYIHN---------SNYTDDFDDA-FEQALKTYQLNFNLNTTGQ
VvMMP2               -KADGLAKLKEYFHYFGYIHN---------SNYTDDFDDA-FEQALKTYQLNFNLNTTGQ
BoMMP1               -KVDGLYKIKQYFQHFGYIPQT------LPGNFSDDFDDI-LKNAVEMYQRNFKLNITGE
At3-MMP              -KYDGLYMLKQYFQHFGYITETN-----LSGNFTDDFDDI-LKNAVEMYQRNFQLNVTGV
At2-MMP              -NVDGLYRIKKYFQRFGYIPET------FSGNFTDDFDDI-LKAAVELYQTNFNLNVTGE
At5-MMP              -NINGLSKLKQYFRRFGYITTT---------GNCTDDFDDV-LQSAINTYQKNFNLKVTGK
HvMMP1               -ERDGLARLKDYLSHFGYLSEAP-----TSSPFSDAFDAE-LEAAVATYQRNFGLNATGV
ZmMMP1               -ERQGLAGLKDYLSHFGYLPPPP-----PSSPFSDAFDQN-LEAAIATYQRNFGLNATGA
OsMMP1               -EREGLGRLKDYLSHFGYLPPPP-----SSSPYSDAFDDS-LEAAIAAYQRNFGLNATGE
OsMMP2               -ERQGLGKLKDYLWHFGYLSYPSSSS--LSPSFNDLFDAD-MELAIKMYQGNFGLDVTGD
HvMMP2               -ERQGLARLKDYLSRFGYLPAPP-----AK--FNDMFDAD-METAIRTYQHNFGLEATGQ
ZmMMP2               -QQQGLAKVKDYLSRFGYLPPES-----SG-SFNDVFDAD-LEEAIKVYQRNFGLGITGV
Pta1-MMP             -RMQGLPDLKRYFRRFGYLSAQN------N--VTEDFDEA-VESAVRTYQKNFGLNVTGV
PtMMP3               -QVSGMSELKEYFNRFGYLPIPDE------NNFTDIFDKQ-FESAVIAYQTNLGLPVTGK
VvMMP4               -QVSGMSELKKYFQRFGYLPVPN-------TNFTDVFDSR-FETAVIMYQTKLGLPVSGK
VvMMP3               -RISGMAELKKYFNRFGYLGFQD-------GNVTDVFDTR-LESAVAAYQAKLGLPVTGR
At4-MMP              PDVS-IPEIKRHLQQYGYLPQNK---------E--SDDVS-FEQALVRYQKNLGLPITGK
At1-MMP              -HVSGVSELKRYLHRFGYVNDGS-------EIFSDVFDGP-LESAISLYQENLGLPITGR
OsMMP3               -HVTGLAELKRYLARFGYMAKP-------GRDTTDAFDEH-LEVAVRRYQTRFSLPVTGR
HvMMP3               -RVTGLGDLKRYLATFGYMPKPAGAGAEHGGGPMDAFDEH-LEAAVKRYQSRLSLPVTGR
HvMMP4               -NGSVTDGLRRYLARFGYASSA-------------PDDAD-GRVVVSLYQSTLGLPVTGR
SMEP-1               -NYKGLSNVKNYFHHLGYIPNAP--------HFDDNFDDT-LVSAIKTYQKNYNLNVTGK
PtMMP6               -TVEGLVELKQYLKRFGYYPS-------DVNLMTSDFDDL-LESALKTYQNYFHLNVTGI
PtMMP7               -TVEGLIELKQYLKKFGYYPS-------DITLTSSDFDDH-LELALKTYQEYFHLNVTGN
PtMMP8               -SVKGLHELKRYLEKFGYLKYGHQGKKGHNHANDDEFDDL-LESAIKAYQQNHHLNVTGS
VvMMP6               -KVEGIHKLKKYLGQFGYLSYSHS-KYHTHAN-DDDFDDL-VESAIKTYQTNYHLNATGS
VvMMP7               -EVNGIQKLKKYLEQFGYLSYSRS-KNQTHAD-DDDFDDL-LESAIKTYQANYHLEATGD
VvMMP5               -KVKDIHKLKKYLQQFGYLSYSHS-EHQTHAD-NDDFDDL-LEFAIKTYQTNYYLKASGN
VvMMP11              -KVEGIHKVKKYLQHFGYLGSTHS-QTETQVDSEDHFDDA-LESAIKAFQTYYHLKPTGI
Cs1-MMP              -TKQGIHQIKKYLQRFGYITTNIQ-KHSNPIF-DDTFDHI-LESALKTYQTNHNLAPSGI
HsMMP20              TWRNNYRLAQAYLDKYYTNKEGH-----QIGEMVARGSNS-MIRKIKELQAFFGLQVTGK
HsMMP13              -SEEDLQFAERYLRSYYHPTN-------LAGILKENAASS-MTERLREMQSFFGLEVTGK
HsMMP1               -QEQDVDLVQKYLEKYYNLKN-D-----GRQVEKRRNSGP-VVEKLKQMQEFFGLKVTGK
HsMMP8               -KEKNTKIVQDYLEKFYQLPS-N-----QYQSTRKNGTNV-IVEKLKEMQRFFGLNVTGK
HsMMP7               -----------YLKRFYLYDS------------ETKNANS-LEAKLKEMQKFFGLPITGM
GLE_ChrMMP1          -TKSAFRWIRPPP---ARPPPFR--------RPPPAQTP----YVHKVEYTELQILCPQT
XP_001694591_Chr3    -LKSAFRFVRPPPN--HRPPPFR--------KPPPAASPS-PAFTHEVEYNELQILCPQE
BAB68383_ChrMMP2     YMEGAQAEVRHPPPLRRRPPPTRG------RQQSPPGSGG-AVLDTVTTPPQLQLVCPQI
                                                                           :
Prim.cons.           3KVDGLAKLKKYLQRFGYLPNP2S3KH2SSGNFTDDFDDSTLESAIKTYQ2NFGLNVTGK

                          70        80        90       100       110       120
                           |         |         |         |         |         |
ACU24527_Gm          PNKQLIQQMLSLRCGVPDVN-------------------------FDY-----------
Slti114_Gm           LNKELIQQMLSIRCGVPDVN-------------------------FDY-----------
GmMMP2               LNNETLQQMSFLRCGVPDIN-------------------------IDY-----------
ACJ84310_Mt          SLRKILQHIALPRCGVPDMN-------------------------FTY-----------
MtMMPL1              LDTETLQQIMLPRCGVPDIN-----------------------PDI-----------
NtMMP1               LDAPTIQHLIRPRCGNADVV----NGTSTMNSGKPPAG---SQN----------------
NMMP1_Nb             LDAPTIEHLIRPRCGNADVV----NGTSTMNSGKPSAG---SQN----------------
LeMMP1_Sl            LDANTIQHLIKPRCGNADVV----NGTSTMNSGKPPAG---SPT----------------
SlMMP2               LDAPTIQHLIKPRCGNADLV----NGTSTMNAGKP-------------------------
PtMMP1               LDDQTVNHIVRPRCGNPDIV----NGSSSMNSGKTHNT---SSS----H-----------
PtMMP2               LDDQTVNHVVRPRCGNPDII----NGSTSMNSGKTNNT---SSSH---H-----------
VvMMP1               LDEATLNQIVSPRCGNADIE----NGSSSMNSGKSTPS---TSG----H-----------
VvMMP2               LDEATLNQIVSPRCGNADIE----NGSSSMNSGKSTP------------------------
BoMMP1               LDELTLQHVVIPRCGVPDVV----NGTSTMLNGGRRRTYEVSFSGRSQR-----------
At3-MMP              LDELTLKHVVIPRCGNPDVV----NGTSTMHSG--RKTFEVSFAGRGQR-----------
At2-MMP              LDALTIQHIVIPRCGNPDVV----NGTSLMHGG-RRKTFEVNFS--RTH-----------
At5-MMP              LDSSTLRQIVKPRCGNPDLI----DGVSEMNGG--------------KI-----------
HvMMP1               LDPPTVSQMVAPRCGVADVI----NGTSTMDR---NAS---AAG----------------
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ZmMMP1              LDPSTVSQMVAPRCGVADVI----NGTSTMAR---SSS---ADA----------------
OsMMP1              LDTDTVDQMVAPRCGVADVI----NGTSTMDR---NSS---AAA----------------
OsMMP2              LDAATVSQMMAPRCGVADVV----NGTSTMGGG---------GG----------------
HvMMP2              LDAATVAKMMSPRCGVADII----NGTSSMG-----------KT----------------
ZmMMP2              MDASTVAQMMAPRCGVADII----NGTSTMGGGSASAS---AAH----------------
Pta1-MMP            LDEATISQLMVPRCGREDII----NGSSAMR----------------------------
PtMMP3              LDSDTISMMVSPRCGVSDTK-------T-H-G---------------TT----------
VvMMP4              LDSKTITAIVSPRCGVSDT--------T-P-L---------------ED----------
VvMMP3              LDSETLSQMMSPRCGMRDA------------M---------------QP----------
At4-MMP             PDSDTLSQILLPRCGFPDDV-------EPK-T---------------AP----------
At1-MMP             LDTSTVTLMSLPRCGVSDTH-------MTINN---------------DF----------
OsMMP3              LDNATLDQIMSPRCGVGDDDVERPVSVALSPG----------------AQ----------
HvMMP3              LDVVTLDQMMSPRCGVQDD-----HGASVTP-----------------EH----------
HvMMP4              LDAPTLDLLATPRCGVPDL-----QHSSQAN-----------------AT----------
SMEP-1              FDINTLKQIMTPRCGVPDIIIN----TNKTTS----------------------------
PtMMP6              LDDSTIKQMMIPRCGMHDIT-----------PNN---------TKSNYTK----------
PtMMP7              LDSSTIQQMMIPRCGMPDIIN----TPSAKPNS---------TKSKHKK----------
PtMMP8              LDNSTVHEMMQPRCGVPDVVN---------GTK---------HYHTHKS----------
VvMMP6              LDSETVSQMVKPRCGAADIIN----GTNWMRSGKK------GHHHGHGS----------
VvMMP7              LDSETVSEMVKPRCGVADIIN----GTNWMLSGKKRQY--HGHGHGHGS----------
VvMMP5              LDSETVSVMVKPRCGVADIIN----GTSRMRSGSR------SYPHGYGS----------
VvMMP11             LDAPTATQMSRTRCGVPDNPP----VTNNINS----------HGHSH----------
Cs1-MMP             LDSNTIAQIAMPRCGVQDVIK----NKKTKKRNQN-----FTN-NGHTH----------
HsMMP20             LDQTTMNVIKKPRCGVPDV------------------------------------------
HsMMP13             LDDNTLDVMKKPRCGVPDV------------------------------------------
HsMMP1              PDAETLKVMKQPRCGVPDV------------------------------------------
HsMMP8              PNEETLDMMEKPRCGVPDS------------------------------------------
HsMMP7              LNSRVIEIMQKPRCGVPDV------------------------------------------
GLE_ChrMMP1         IDSVTGYPMDDPRCNVPRATVAAGEEALTIRNEFELLNGDVLNVTLEEVDTPENPSRRRL
XP_001694591_Chr3   VDSSTNYTRDDPQCAVPRGSVAVGDEAEAVRAEFRLLSGDLVNVTLEEVDESEPGSRRRM
BAB68383_ChrMMP2    IDIVTGYPMDDPSCYVPRVVLAQGEAAEQMRNENGVSSGDIMNVTLDVS-----ANG---
                                 *
Prim.cons.          LDSETLQQMV2PRCGVPDVINA4GNGTSTMNSGK2RASG2VSN2HGH2HD22E23SRRR2

                                130       140       150       160       170       180
                                  |         |         |         |         |         |
ACU24527_Gm         -------------------------NFTDDNTSYPKAGHRW-----FPN-------RNL
Slti114_Gm          -------------------------NSTDDNISYPKAGHRW-----FPN-------RNL
GmMMP2              -------------------------NFTDDNMSYPKAGHRW-----FPH-------TNL
ACJ84310_Mt         -------------------------DSTND-ISYPK-GNQW-----FPKGT-----KNL
MtMMPL1             -------------------------NPDFG-FARAQ-GNKW-----FPKGT-----KEL
NtMMP1              -------------------------MHTVAHFSFFPGRPRW-----PDSKTD------L
NMMP1_Nb            -------------------------IHTVAHFSFFPGRPRW-----PESNRD------L
LeMMP1_Sl           -------------------------MHTVAHYSFFPGSPRW-----PANKRD------L
SlMMP2              -------------------------HTVAHYSFFPGRPKW-----PEGKTD------L
PtMMP1              -------------------------VHTVSHYSFFTGMPRW-----R--KQA------L
PtMMP2              -------------------------LHTVSHYSFFTGQPRW-----R--KQA------L
VvMMP1              -------------------------FHTVGHYSFFDGKPVW-----PESKRN------L
VvMMP2              ------------------------------------------------------------
BoMMP1              -------------------------FHAVKRYSFFPGEPRW-----PERRRN------L
At3-MMP             -------------------------FHAVKHYSFFPGEPRW-----PRNRRD------L
At2-MMP             -------------------------LHAVKRYTLFPGEPRW-----PRNRRD------L
At5-MMP             -------------------------LRTTEKYSFFPGKPRW-----PKRKRD------L
HvMMP1              -------------------------H-GRHLYTYFPGGPMW-----PPFRRE------L
ZmMMP1              -------------------------H-GRHLYAYFPGGPTW-----PPFRRD------L
OsMMP1              -------------------------LRGRHLYSYFPGGPMW-----PPFRRN------L
OsMMP2              -------------------------VRGRGLYSYFPGSPRW-----PRSRTT------L
HvMMP2              -------------------------VHGRNLYSYFPGSPSW-----PRSKKS------L
ZmMMP2              -------------------------ARGRNLFTYFPGSPSW-----PRSRKS------L
Pta1-MMP            ----------------------------GRGLFPFFPGSPRW-----GPDKRV------L
PtMMP3              -------------------------FQATKHFSYFYGKPRW-----GRQAPV-----IL
VvMMP4              -------------------------VHETRHFAYFYGKPRW-----ARVPPM-----TL
VvMMP3              -------------------------MHAAMHYVYFPGKPRW-----ARPIPM-----TL
At4-MMP             -------------------------FHTGKKYVYFPGRPRW-----TRDVPL-----KL
At1-MMP             -------------------------LHTTAHYTYFNGKPKW-----NRDT--------L
OsMMP3              -------------------------GGVVSRFTFFKGEPRW-----TR-SDP---PIVL
HvMMP3              -------------------------GGAVSRFTFFKGKPRW-----TRRSDPDPDPVSL
HvMMP4              -------------------------ATATARFAFFDGQPRW-----AR--APG--HFLL
SMEP-1              -------------------------FGMISDYTFFKDMPRW-----QAGTTQ------L
PtMMP6              -------------------------FHMVMHYTFFNGMPKW-----RPSKY------HL
PtMMP7              -------------------------VHVVAHYAF--GAQKW-----PPSKY------AL
PtMMP8              -------------------------IHTLAHYNFIPENPRW-----TKR--------QL
VvMMP6              -------------------------LRTVAHYSFFSGSPRW-----PPSKT------YL
VvMMP7              -------------------------LRTVAHYSFFPGSPRW-----PPSKT------YL
VvMMP5              -------------------------FHTVAHYSFLAGSPRW-----PPSKT------HL
VvMMP11             -------------------------LNIGTHYAFFPNKPRW-----PAGKR------HL
Cs1-MMP             -------------------------FHKVSHFTFFEGNLKW-----PSSKL------HL
HsMMP20             ----------------------------ANYRLFPGEPKW-----KKNT--------L
HsMMP13             ----------------------------GEYNVFPRTLKW-----SKMN--------L
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HsMMP1                  ------------------------------AQFVLTEGNPRW-----EQTH--------L
HsMMP8                  ------------------------------GGFMLTPGNPKW-----ERTN--------L
HsMMP7                  ------------------------------AEYSLFPNSPKW-----TSKV--------V
GLE_ChrMMP1             LSIIREEQRTGRVLLAT-SAELPTPTFRLKSLKSILKGSQKE----IYAGKPIDLRTIVY
XP_001694591_Chr3       LSQLREELATGRRLLNTDTAPLPGRSFRLKSLRPVRQTEQKE----IYTGTPIDLRTVVF
BAB68383_ChrMMP2        -------------------------SFAVTSLKTVMLGSQKGGCKCIYSGTPADDRVVMY

Prim.cons.              LS22REE22TGR2LL2TD2A2LP22SFHTVAHYSFFPGSPRWGCKCIP2SKRDDLR2V2L

                                190       200       210       220       230       240
                                  |         |         |         |         |         |
ACU24527_Gm             TYGFLP--E-----N--QIPDNMTKVFRD--------SFARWAQASG--------TLSLTE
Slti114_Gm              TYGFLP--E-----N--QIPDNMTKVFRD--------SFARWAQASG--------TLSLTE
GmMMP2                  TYGFLP--E-----N--QIPANMTKVFRD--------SFARWAQASG-------VLNLTE
ACJ84310_Mt             TYGFAP--K-----N--EIPLNVTNVFRK--------ALTRWSQTTR-------VLNFTE
MtMMPL1                 TYGFLP--E-----S--KISIDKVNVFRN--------AFTRWSQTTR-------VLKFSE
NtMMP1                  TYAFL----P---QN-GLT-DNIKSVFSR--------AFDRWSEVT--------PLSFTE
NMMP1_Nb                TYAFL----P---QN-GLT-DNIKSVFSR--------AFDRWSEVT--------PLTFTE
LeMMP1_Sl               TYAFA----P---QN-GLT-DDIKIVFTR--------AFDRWSEVT--------PLTFTE
SlMMP2                  TYAFL----P---AN-NLT-DDIKSVFSR--------AFDRWSEVT--------PLSFTE
PtMMP1                  TYAFL----P---GN-QLT-DEVKTVFSR--------AFDRWSTVI--------PLTFTQ
PtMMP2                  TYVFS----P---EN-QLS-DEVKAVFSR--------AFDRWSTVI--------PLNFSQ
VvMMP1                  TYGFL----P---DN-QLS-DTVKAVFTS--------AFERWAAVT--------PLTFTE
VvMMP2                  ---------------------------------------AFERWAAVT--------PLTFTE
BoMMP1                  TYAFE----P---QN-NLA-EEVKSVFSR--------AFVRWAEVI--------PLTFRR
At3-MMP                 TYAFD----P---RN-ALT-EEVKSVFSR--------AFTRWEEVT--------PLTFTR
At2-MMP                 TYAFD----P---KN-PLT-EEVKSVFSR--------AFGRWSDVT--------ALNFTL
At5-MMP                 TYAFA----P---QN-NLT-DEVKRVFSR--------AFTRWAEVT--------PLNFTR
HvMMP1                  RYAIT--ATA---AT-SIDRATLGAVFAR--------AFARWSDAT--------TLRFAE
ZmMMP1                  KYAIT--ATS---AA-SIDRSTLSDVFAR--------AFSRWAAAT--------NLRFAE
OsMMP1                  RYAIT--ATS---AT-SIDRATLSAVFAR--------AFSRWAAAT--------RLQFTE
OsMMP2                  RYAIT--ATS---QT-SIDRATLSKVFAS--------AFARWSAAT--------TLNFTE
HvMMP2                  RYAIT--AAT---ET-TIDRATLSRVFAS--------AFARWSAAT--------TLNFTE
ZmMMP2                  TYAVT--QTS---LT-SIDRATLSQVLAR--------AFARWSAAT--------TLTFTE
Pta1-MMP                SYAFSPDHEV---LS-EISLAELSTVVGR--------AFKRWADVI--------PITFTE
PtMMP3                  TYAFSQNNMI---D--YISLKDIKTVFKR--------AFSRWAQVI--------PVSFME
VvMMP4                  TYSFSRENMI---E--SLNSSEMKSVFER--------AFSRWASVI--------PVNFTE
VvMMP3                  TYAFSPENLV---G--YLSLEDIRGAFKL--------AFARWASVI--------PVSFSE
At4-MMP                 TYAFSQENLT---P--YLAPTDIRRVFRR--------AFGKWASVI--------PVSFIE
At1-MMP                 TYAISKTHKL---D--YLTSEDVKTVFRR--------AFSQWSSVI--------PVSFEE
OsMMP3                  SYAVSPTATV---G--YLPPAAVRAVFQR--------AFARWARTI--------PVGFVE
HvMMP3                  TYAVSPTATV---G--YLPADDVRAVFRR--------AFERWARVI--------PVAFVE
HvMMP4                  TYAVLSTPPY---QPLPLPRKAVRGAFRA--------AFARWARVI--------PARFRE
SMEP-1                  TYAFS----P---EP-RLD-DTFKSAIAR--------AFSKWTPVV--------NIAFQE
PtMMP6                  TYTFGSD-GV---QV--VDMDTLRSVCSD--------AFKKWSDVS--------PLTFQE
PtMMP7                  TYRFGS--GV---QV--VGSDTLRSVCSK--------AFQTWAKVS--------KFTFRE
PtMMP8                  TYKFRS--SV---QV--PAAQNIRSICAK--------AFQRWAQVT--------EFTFQE
VvMMP6                  TYAFLP-----------GTPSWAMSPVSR--------AFGQWDSAT--------HFTFGS
VvMMP7                  TYAFLP-----------GTPNWAMSPVSR--------AYGRWDSAT--------HFTFGW
VvMMP5                  TYAFLS-----------GTPSTTMSAVTR--------AFGQWASAT--------NFSFAE
VvMMP11                 LYSLDS-----------ASHPEAANAVAN--------AFGAWAGVT--------NFTFER
Cs1-MMP                 SYGFLP-----------NYPIDAIKPVSR--------AFSKWSLNT--------HFKFSH
HsMMP20                 TYRISK-------YTPSMSSVEVDKAVEM--------ALQAWSSAV--------PLSFVR
HsMMP13                 TYRIVN-------YTPDMTHSEVEKAFKK--------AFKVWSDVT--------PLNFTR
HsMMP1                  TYRIEN-------YTPDLPRADVDHAIEK--------AFQLWSNVT--------PLTFTK
HsMMP8                  TYRIRN-------YTPQLSEAEVERAIKD--------AFELWSVAS--------PLIFTR
HsMMP7                  TYRIVS-------YTRDLPHITVDRLVSK--------ALNMWGKEI--------PLHFRK
GLE_ChrMMP1             IMDFSSCKLSGWSAPATLTPEKVTSDMLR-GASAPTNNLANYYGACSYEKTLFNPDNFLV
XP_001694591_Chr3       IMDFSDCRMP--QAPA-VTKERVTRDMLK-TSASPANNLAGYYSTCSYGKTIFNPDNFIV
BAB68383_ChrMMP2        LLDFSSCQGYGMAAPAALTPEVAWRLLSQDGSSAPGSSFKAYQETCSYGRRLLNPDNVVV
                                                                  :         .
Prim.cons.              TYAF2PTATPG23QNPSLTPD2VKSVFSRDGSSAP3NAFARWASVT2YGKTLFNPLTFTE

                                250       260       270       280       290       300
                                  |         |         |         |         |         |
ACU24527_Gm             TT--------------------------------------YDNADIQVGFYNFTD----
Slti114_Gm              TT--------------------------------------YDIADIQVGFYNFTA----
GmMMP2                  TT--------------------------------------YDNADIQVGFYNFTY----
ACJ84310_Mt             TTS-------------------------------------YDDADIKIVFNNMN-----
MtMMPL1                 ATS-------------------------------------YDDADIKIGFYNISY----
NtMMP1                  TAS-------------------------------------FQSADIKIGFFAGDH----
NMMP1_Nb                IAS-------------------------------------FQSADIKIGFFAGDH----
LeMMP1_Sl               IAS-------------------------------------YQSADIKIGFFSGDH----
SlMMP2                  IPS-------------------------------------FQSADIKIGFLTGDH----
PtMMP1                  ADS-------------------------------------INAADIGIGFYSGDH----
PtMMP2                  TDS-------------------------------------IYTADIRIAFFSGDH----
VvMMP1                  SDS-------------------------------------YYSADLRIAFYTGDH----
VvMMP2                  SDS-------------------------------------YYSADLRIAFYTGDH----
BoMMP1                  VES-------------------------------------FSTSDISIGFYTGEH----
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At3-MMP             VER-----------------------------------FSTSDISIGFYSGEH----
At2-MMP             SES-----------------------------------FSTSDITIGFYTGDH----
At5-MMP             SES-----------------------------------ILRADIVIGFFSGEH----
HvMMP1              AAS-----------------------------------ESDADITIGFYAGSH----
ZmMMP1              TAS-----------------------------------ESDADITIGFYSGSH----
OsMMP1              VSS-----------------------------------ASNADITIGFYSGDH----
OsMMP2              AAS-----------------------------------AADADITIGFYGGDH----
HvMMP2              TAS-----------------------------------ASDADITIGFHSGDH----
ZmMMP2              TAS-----------------------------------ERDADITIGFYAGDH----
Pta1-MMP            SSD-----------------------------------YSSADIKVGFYSGDH----
PtMMP3              IED-----------------------------------YPSADIRIGFYYRDH----
VvMMP4              TED-----------------------------------FGSADIKIGFYSGDH----
VvMMP3              TDT-----------------------------------YSFADIKIGFYRGDH----
At4-MMP             TED-----------------------------------YVIADIKIGFFNGDH----
At1-MMP             VDD-----------------------------------FTTADLKIGFYAGDH----
OsMMP3              TDD-----------------------------------YEAADIKVGFYAGNH----
HvMMP3              TDD-----------------------------------YDKADIKVGFYEGSH----
HvMMP4              TRD-----------------------------------YNTADVRVGFLAGDH----
SMEP-1              TTS-----------------------------------YETANIKILFASKNH----
PtMMP6              ASD-----------------------------------GASANIVIAFYRGDH----
PtMMP7              ATG-----------------------------------GASADIVIEFFSGDH----
PtMMP8              VSV-----------------------------------SSPADIVIGFHRRDH----
VvMMP6              IQD-----------------------------------HTSADMTISFHRLDH----
VvMMP7              IQD-----------------------------------YTSADMTISFHRLDH----
VvMMP5              TQD-----------------------------------YTNADMKIGFQRGDH----
VvMMP11             TSD-----------------------------------PKIANLYISFKVRDH----
Cs1-MMP             VAD-----------------------------------YRKADIKISFERGEH----
HsMMP20             INS-----------------------------------GEADIMISFENGDH----
HsMMP13             LHD-----------------------------------GIADIMISFGIKEH----
HsMMP1              VSE-----------------------------------GQADIMISFVRGDH----
HsMMP8              ISQ-----------------------------------GEADINIAFYQRDH----
HsMMP7              VVW-----------------------------------GTADIMIGFARGAH----
GLE_ChrMMP1         LGPVPVPCIGGVTPPPRPPR---PPRPPPRAGSTISS---LSRRNDTYDDWWDLSKYCTA
XP_001694591_Chr3   VGPVPMKCAGNIPAIPRPPRPAPPPRPPPRTISTKPAPPPLSRRNATYEDWWDLSRFCSA
BAB68383_ChrMMP2    VG--PCRCP-ALAPSPTSGR----ARTLVGISPAAAN---ALRMNSTHIGWWDLSRSCTP
                                                                :
Prim.cons.          TASVP33C3G333P3PRPPRPAPPPRPPPR33ST333PPPLYSSADIKIGFYSGDH3CTA

                                  310       320       330       340       350       360
                                    |         |         |         |         |         |
ACU24527_Gm         LSIKMEV-YGGSLIF-LQPDSSKKGVVLLDG-NMGW------------------------
Slti114_Gm          LGIEVEV-YGGSLIF-LQPDSSKKGVVLMDG-NIGW------------------------
GmMMP2              LGIDIEV-YGGSLIF-LQPDSTKKGVILLDGTNKLW------------------------
ACJ84310_Mt         ------------------------------------------------------------
MtMMPL1             NSKEVID-VVVSDFF-IN----------LRS----F------------------------
NtMMP1              NDGEPFDGPMGTLAHAFSPP---GGHFHLDG-DENW------------------------
NMMP1_Nb            NDGEPFDGPMGTLAHAFSPP---GGHFHLDG-DENW------------------------
LeMMP1_Sl           NDGEPFDGPMGTLAHAFSPP---AGHFHLDG-EENW------------------------
SlMMP2              NDGEPFDGPMGTLAHAFSPP---AGHFHLDG-EENW------------------------
PtMMP1              GDGEPFDGVLGTLAHSFSPP---SGQFHLDG-DENW------------------------
PtMMP2              GDGEPFDGVLGTLAHAFSPQ---NGRLHLDD-DEHW------------------------
VvMMP1              GDGEAFDGVLGTLAHAFSPT---NGRFHLDG-EENW------------------------
VvMMP2              GDGEAFD---G----------------------ENW------------------------
BoMMP1              GDREPFDGFMGTLAHAFSPP---TGHFHLDG-AENW------------------------
At3-MMP             GDGEPFDGPMRTLAHAFSPP---TGHFHLDG-EENW------------------------
At2-MMP             GDGEPFDGVLGTLAHAFSPP---SGKFHLDA-DENW------------------------
At5-MMP             GDGEPFDGAMGTLAHASSPP---TGMLHLDG-DEDW------------------------
HvMMP1              GDGEAFDGPLGTLAHAFSPT---DGRFHLDA-AEAW------------------------
ZmMMP1              GDGEPFDGPLGTLAHAFSPT---DGRFHLDA-AEAW------------------------
OsMMP1              GDGEAFDGPLGTLAHAFSPT---DGRFHLDA-AEAW------------------------
OsMMP2              GDGEAFDGPLGTLAHAFSPT---NGRLHLDA-SEAW------------------------
HvMMP2              GDGEAFDGPLGTLAHAFSPT---DGRFHLDA-SEAW------------------------
ZmMMP2              GDGEAFDGPLGTLAHAFSPT---DGRFHLDA-AEAW------------------------
Pta1-MMP            GDGHPFDGPLGTLAHSFSPP---DGRFHLDA-AESW------------------------
PtMMP3              GDGQPFDGVLGVLAHAFSPE---NGRFHLDA-SETW------------------------
VvMMP4              GDGEPFDGVLGVLAHAFSPQ---NGRFHFDR-AETW------------------------
VvMMP3              GDGEPFDGVLGVLAHAFSPE---SGKFHLDA-AETW------------------------
At4-MMP             GDGEPFDGVLGVLAHTFSPE---NGRLHLDK-AETW------------------------
At1-MMP             GDGLPFDGVLGTLAHAFAPE---NGRLHLDA-AETW------------------------
OsMMP3              GDGVPFDGPLGILGHAFSPK---NGRLHLDA-SEHW------------------------
HvMMP3              GDGVPFDGPLGVLGHAFSPK---NGRLHLDA-AERW------------------------
HvMMP4              GDGEPFDGPLGVLGHAFSPP---SGQLHLDA-AERW------------------------
SMEP-1              GDPYPFDGPGGILGHAFAPT---DGRCHFDA-DEYW------------------------
PtMMP6              GDGYPFDGPGKILAHAFSPE---NGRFHYDA-DEKW------------------------
PtMMP7              GDQSPFDGPGNQLAHAFYPQ---DGRLHYDA-DENW------------------------
PtMMP8              NDGSAFDGPGGTLAHATPPVR--NAMFHFDA-DENW------------------------
VvMMP6              GDGYPFDGPGGTIAHAFAPT---NGRFHYDG-DETW------------------------
VvMMP7              GDGSPFDGPGGTIAHAFAPT---NGRFHYDG-DETW------------------------
VvMMP5              GDGHPFDGPGGTIAHSFPPT---DGRLHFDG-DESW------------------------
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VvMMP11             GDGRPFDGRGGILAHAFAPT---DGRFHFDG-DETW------------------------
Cs1-MMP             GDNAPFDGVGGVLAHAYAPT---DGRLHFDG-DDAW------------------------
HsMMP20             GDSYPFDGPRGTLAHAFAPGEGLGGDTHFDN-AEKW------------------------
HsMMP13             GDFYPFDGPSGLLAHAFPPGPNYGGDAHFDD-DETW------------------------
HsMMP1              RDNSPFDGPGGNLAHAFQPGPGIGGDAHFDE-DERW------------------------
HsMMP8              GDNSPFDGPNGILAHAFQPGQGIGGDAHFDA-EETW------------------------
HsMMP7              GDSYPFDGPGNTLAHAFAPGTGLGGDAHFDE-DERW------------------------
GLE_ChrMMP1         SEQQAWERAAEAYAQAIVAQDPNSATGKKLQGILQWRERRRNIYILPPGVKCS--WSGYA
XP_001694591_Chr3   SEQQAWERAAEAFAQAEVAKNPNDPEMQKLARLLQWRTRRRNLFILPSNVRCS--WAGYA
BAB68383_ChrMMP2    AEMSAVERAAEAFAQQIVAKDPNSADGRKLQAILQWRERRRNIYVLPPGSSCAHSWPAIA

Prim.cons.          GDGEPFDGPLGTLAHAFSPTSG2NGRFHLDA4DENWRERRRNIYILPPGV3CSHSW3GYA

                                   370       380       390       400       410       420
                                    |         |         |         |         |         |
ACU24527_Gm         -LLPSENASLS---K-----DDGVLDLETAAMHQIGHLLGLDHSHKE--DSVMYPYILSS
Slti114_Gm          -LLPSENATLS---K-----DDRVLDLETVAMHQIGHLLGLEHSPKE--DSVMYPYILSS
GmMMP2              -ALPSENGRLS---W-----EEGVLDLESAAMHEIGHLLGLDHSNKE--DSVMYPCILPS
ACJ84310_Mt         -TEK--------------------LDLETAAMHQIGHLLGLEHSSDS--KSIMYPTILPS
MtMMPL1             -TIRLEAS--------------KVWDLETVAMHQIGHLLGLDHSSDV--ESIMYPTIVPL
NtMMP1              -VIDGVPIVEGNFFS-----ILSAVDLESVAVHEIGHLLGLGHSSVE--DSIMFPSLAA-
NMMP1_Nb            -VIDGAPIVEGNFFS-----ILSAVDLESVAVHEIGHLLGLGHSSVE--DSIMFPSLAA-
LeMMP1_Sl           -VIDGAPIVDGNFFS-----ILSAVDLESVAVHEIGHLLGLGHSSVE--DAIMYPTLGA-
SlMMP2              -VVDGVPVNEGNFFS-----ILSAVDLESVAVHEIGHLLGLGHSSVE--DSIMYPSLES-
PtMMP1              -VVTGDVRTS----S-----LTTAVDLESVAVHEIGHLLGLGHSSVE--ESIMYPTISS-
PtMMP2              -VVTDDVRTS----T-----LTSAVDLESVAVHEIGHLLGLGHSSVE--ESIMYPSISS-
VvMMP1              -VATGDVTTS----S-----ISSAIDLESVAVHEIGHLLGLGHSSVE--EAIMYPTITS-
VvMMP2              -VATGDVTTS----S-----ISSAIDLESVAVHEIGHLLGLGHSSVE--EAIMYPTITS-
BoMMP1              -IVS-GEGGDG-FLT-----ERAAVDLESVAVHEIGHLLGLGHSSVQ--DSIMFPTIST-
At3-MMP             -IVS-GEGGDG-FIS-----VSEAVDLESVAVHEIGHLLGLGHSSVE--GSIMYPTIRT-
At2-MMP             -VVS-GD-LDS-FLS-----VTAAVDLESVAVHEIGHLLGLGHSSVE--ESIMYPTITT-
At5-MMP             -LISNGEISRR-ILP-----VTTVVDLESVAVHEIGHLLGLGHSSVE--DAIMFPAISG-
HvMMP1              -VA--DGQGGD---A-----PG-AVDLESVAVHEIGHLLGLGHSSVQ--GAIMYPTIRT-
ZmMMP1              -VAGSDVSRSS---A-----TG-AVDLESVAVHEIGHLLGLGHSSVP--DAIMYPTIRT-
OsMMP1              -VASGDVSTSS---S-----FGTAVDLESVAVHEIGHLLGLGHSSVP--DSIMYPTIRT-
OsMMP2              -VAGGDVTRAS---S-----NA-AVDLESVAVHEIGHILGLGHSSAA--DSIMFPTLTS-
HvMMP2              -VAGGDVSRAS---L-----DA-AVDLESVAVHEIGHLLGLGHSSVE--GAIMYPTITS-
ZmMMP2              -DASGDVARAA---S-----DV-AVDLESVAVHEIGHLLGLGHSAEP--AAIMFPTITS-
Pta1-MMP            -TV--DLSSDS---A-----AT-AIDLESIATHEIGHLLGLGHTTEK--AAVMYPSIAP-
PtMMP3              -ALDFETVKSR----------V-AVDLESVATHEIGHILGLAHSSVK--EAVMYPSLSP-
VvMMP4              -TVDFESEKSR----------V-AVDLESVATHEIGHILGLAHSSVK--EAVMYPSLSP-
VvMMP3              -AVDLESEKST----------V-AIDLESVATHEIGHLLGLAHSPVK--ESVMYPSLKP-
At4-MMP             -AVDFDEEKSS----------V-AVDLESVAVHEIGHVLGLGHSSVK--DAAMYPTLKP-
At1-MMP             -IVDDDLKGSSE---------V-AVDLESVATHEIGHLLGLGHSSQE--SAVMYPSLRP-
OsMMP3              -AVDFDVDATAS----------AIDLESVATHEIGHVLGLGHSASP--RAVMYPSIKP-
HvMMP3              -ALDFAGETKAS---------A-AIDLESVATHEIGHVLGLGHSTSP--QAVMYPSIKP-
HvMMP4              -AVG-DLDAAGA---------G-AVDLESVATHEIGHVLGLAHSSAP--DAVMYPSLKP-
SMEP-1              -VASGDVTKSP---------VTSAFDLESVAVHEIGHLLGLGHSSDL--RAIMYPSIPP-
PtMMP6              -ST--NPAMD----------QIDLESVAVHEIGHLLGLAHSSDS--NAVMYPSIAA-
PtMMP7              -ST--DPAMD----------QIDLETVTVHEIGHLLGLYHSKDHP-EAIMYPTTQR-
PtMMP8              -SE--NPGPN----------QMDLESVAVHEIGHLLGLDHNDDPNADAIMSSGIPS-
VvMMP6              -SI--GAVPN----------AMDLETVALHEIGHLLGLGHSSVQ--NAIMFPSISS-
VvMMP7              -SI--GAVPN----------AMDLETVALHEIGHLLGLGHSSVQ--NAIMFPSIST-
VvMMP5              -VV--GAVAG----------AFDVETVALHEIGHLLGLGHSSVE--GAIMFPTIAY-
VvMMP11             -VI--GAVAN----------SMDLQTVARHEIGHLLGLAHTPVQ--EAIMYAIISP-
Cs1-MMP             -SV--GAISG----------YFDVETVALHEIGHILGLQHSTIE--EAIMFPSIPE-
HsMMP20             -TM----------------GTNGFNLFTVAAHEFGHALGLAHSTDP--SALMYPTYKYK
HsMMP13             -TS----------------SSKGYNLFLVAAHEFGHSLGLDHSKDP--GALMFPIYTYT
HsMMP1              -TN----------------NFREYNLHRVAAHELGHSLGLSHSTDI--GALMYPSYTF-
HsMMP8              -TN----------------TSANYNLFLVAAHEFGHSLGLAHSSDP--GALMYPNYAFR
HsMMP7              -TDG---------------SSLGINFLYAATHELGHSLGMGHSSDP--NAVMYPTYGNG
GLE_ChrMMP1         DVTCTSATCSAYVRG------YSDTNAMQVIMHEAMHNYGLEHAGRGTLEYGDATDVMGD
XP_001694591_Chr3   DVTCTSATCSAYVRG------YGSATTPHVVIHEVMHNYGLEHAGRGFVEYGDQTDVMGD
BAB68383_ChrMMP2    ESLCTSATCGVFVDSSTLLPNAAAPRPPHILMHEMLHVLGLTHAGRGLKEAGDPTDIMGT
                                                    *:  *  *: *        .
Prim.cons.          DVVDGDVTTSSNFFSSTLLPITSAVDLESVAVHEIGHLLGLGHSSVE542AIMYPTISPS

                                   430       440       450
                                    |         |         |
ACU24527_Gm         QSQQRKVQLSNSDKANIHLQFAKHDSDLTSPN
Slti114_Gm          Q--QRKVKLSNSDKANIHLEFAKHDS------
GmMMP2              H--QRKVQLSKSDKTNVQHQF------------
ACJ84310_Mt         Q--QKKVQITDSDNLAIQKLY------------
MtMMPL1             H--QKKVQITVSDNQAIQQLY------------
NtMMP1              G--TRRVELANDDIQGVQVLYGSNPN------
NMMP1_Nb            G--TRRVELANDDIQGVQVLYGSNPN------
LeMMP1_Sl           G--TRRVELRNDDILGVQELYGSNPN------
SlMMP2              G--IRRVELVEDDIKGVQELYGSNPN------
PtMMP1              R--TKKVELADDDIQGIQVLYGSNPN------
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PtMMP2              R--TRKVELATDDIEGIQTLYGSNPN------
VvMMP1              R--TKKVELASDDIQGIQELYGSNPN------
VvMMP2              R--TKKVELASDDIQGIQELYGSNPN------
BoMMP1              G--RRKVDLHSDDVEGVQYLYGSNPN------
At3-MMP             G--RRKVDLTTDDVEGVQYLYGANPN------
At2-MMP             G--KRKVDLTNDDVEGIQYLYGANPN------
At5-MMP             G--DRKVELAKDDIEGIQHLYGGNP-------
HvMMP1              G--TRKVDLESDDVQGIQSLYGTNPN------
ZmMMP1              G--TRKVELEADDVQGIQSLYGSNPN------
OsMMP1              G--TRKVDLESDDVLGIQSLYGTNPN------
OsMMP2              R--TKKVNLATDDVAGIQGLYGNNPN------
HvMMP2              R--TQKVELANDDVVGIQSLYGGNPN------
ZmMMP2              R--TSKVDLAQDDVAGIQNLYGGNPN------
Pta1-MMP            R--TRKVDLVLDDVDGVQYVYGANPN------
PtMMP3              R--SKKVDLKIDDVNGVQALYGSNPN------
VvMMP4              R--RKKVDLKRDDVEGVQALYGPNPN------
VvMMP3              R--AKKADLKLDDIEAVQALYGSNPN------
At4-MMP             R--SKKVNLNMDDVVGVQSLYGTNPN------
At1-MMP             R--TKKVDLTVDDVAGVLKLYGPNPK------
OsMMP3              R--EKKVRLTVDDVEGVQALYGSNPQ------
HvMMP3              L--EKKADLTVDDVEGVQLLYGSNPD------
HvMMP4              R--TRKAELTLDDVRGVQALYGSNPR------
SMEP-1              R--TRKVNLAQDDIDGIRKLYGINP-------
PtMMP6              G--TKKRNLAQDDIDGIHALYGN---------
PtMMP7              G--SKKRDLAQDDIDGIHALYSN---------
PtMMP8              G--IAKRDLRADDVQGVRALYGFAN-------
VvMMP6              G--VTKG-LHEDDIQGISALYNR---------
VvMMP7              G--VTKG-LHEDDIQGIRALYNR---------
VvMMP5              G--VTKG-LNEDDIQGIQALY-----------
VvMMP11             G--VTKG-LNQDDIDGIRALYTG---------
Cs1-MMP             G--VTKG-LHGDDIAGIKALYRV---------
HsMMP20             N--PYGFHLPKDDVKGIQALYGPRKVFLGKPT
HsMMP13             G--KSHFMLPDDDVQGIQSLYGPGDE---DPN
HsMMP1              ---SGDVQLAQDDIDGIQAIYGRSQN----PV
HsMMP8              E--TSNYSLPQDDIDGIQAIYGLSSN----PI
HsMMP7              D--PQNFKLSQDDIKGIQKLYGKRSN------
GLE_ChrMMP1         FNKAGKGLLCPNAPNMYRIGWAKPINEPG---
XP_001694591_Chr3   YSKASSGLLCPNAPNMYRIGWAKPLNEPG---
BAB68383_ChrMMP2    FGGAGRGLLCPNAPNMYRIGWAKPINEPG---
                          :  .       :
Prim.cons.          GSKTRKVDLAQDDIQGIQALYGSNPNEPG3PN
```

Fig.S 1. Multiple alignment of 49 selected MMPs from human, algae and 14 flowering plants spanning the cysteine switch and Met turn. Alignment was performed with ClustalW. Accession numbers are listed in section 2.10.

Table. S 1. List of primers used in this study. Primer names, sequences and internal ID number are included in the table. Introduced restriction sites are shown in bold italics.

Primers	**Sequence 5'--3'**	**IPAZ ID**
At1-MMP Fwd	CGATTCGGTTACGTCAATGAT	#215
At1-MMP Rev	CACGGTTCCATTTCGGTTTA	#216
At2-MMP Fwd	CAACGGTACTTCGTTGATGC	#207
At2-MMP Rev	GAAGTTTAACGCGGTGACG	#208
At3-MMP Fwd	GCGGTAGGAAAACCTTCGAG	#211
At3-MMP Rev	TGATGTCAGAGGTCGAGA ACC	#212
At4-MMP Fwd	CGGTGATCCCAGTATCCTTC	#213
At4-MMP Rev	GCCACAGATTCCAAA TCGAC	#214
At5-MMP Fwd	GCC GAA ACG GAA ACG AGA	#209
At5-MMP Rev	CATGCGCTA ATGTTCCCA TA	#210
N582450LP	AGAGTGAAGTTTAACGCGGTG	#217
N582450RP	TGAATTCAATAACTCCAAATTTATAAAG	#218
GABI_416E03LP	TTTCCATTGGAATCATTCACC	#226
GABI_416E03RP	TTACGTTTCCCTGTCGTGATC	#227
N103532_LP	TGAGTTGAGAGATCAAACCCG	#390
N103532_RP	CGACGATATTCTCAAGAACGC	#391
N115923_LP	GAACCTTCTACCGAGGAATGG	#392
N115923_RP	TTTAACCGGTCCTTTACCACC	#393
GABI_075C07LP	GTCGAATCCAAATTTGGTGTG	#224
GABI_075C07RP	CATCGGCTAGATTTGTCGAAG	#225
S032466.54LP	CTGCCTAATGGGCTATGTCAG	#292
S032466.54RP	TTGGAGGTGACGTTTGATTTC	#293
S032466.55LP	AGCCTTTTGGAAAACGATAGC	#294
S032466.55RP	ATCTCCCGTAGCTTCTTCTCG	#295
N619909 LP	CGCCGCAGAATAATTTAACAG	#219
N619909 RP	CCTAAGAAACTACCATTAATGATGCTC	#221

N593137 LP	CGCCGCAGAATAATTTAACAG	#219
N593137 RP	TGTTGTATGTGATGACTTCGTAAGTC	#220
N656052LP	AAA TCG TCA GTG CAA TTA CCG	#288
N656052RP	TCG AAC TAA GCC GAG TGA AAC	#289
N660426LP	ATGTTCACCGGAGAAAAATCC	#290
N660426RP	TCAGAAAATGGTTTCTCTCGG	#291
Spm32	TACGAATAAGAGCGTCCATTTTAGAGTGA	#443
LBb1	GCGTGGACCGCTTGCTGCAACT	LBb1
GABI_LB	CCCATTTGGACGTGAATGTAGACAC	#222
GABI_RB	CCAAAGATGGACCCCCACCCAC	#223
At1g24140_MMP3_F	AG***G GAT CC***T TGG ATG GTG AGG ATT TGT G	#447
At1g24140_MMP3_R	GC***A AGC TT***G CAT GAA TGA AGA ACC AAT CT	#448
At1g70170_MMP2_F	AT***G GAT CC***A ATC CGA AAA CCA CCA TGA G	#449
At1g70170_MMP2_R	CG***A AGC TT***C GAA ATC AAA CAT AGG TAT AGG ACA	#450
MMP2FL_Fwd	AAT CCG AAA ACC ACC ATG AG	#511
MMP2FL_Rev	CGA AAT CAA ACA TAG GTA TAG GAC A	#512
MMP3FL_Fwd	TCT TCG CTC CTT CTC CAG TC	#509
MMP3FL_Rev	TGC ATG AAT GAA GAA CCA ATC T	#510
Kan1	AGT TCC TCT TCG GGC TTT TC	#300
Kan2	ACC GCT TAA AAG ATA CG	#301
Pro MMP2_fwd	AT***G GAT CC***T GGT TCT TCC CTA ACT CCA C	#555
Pro MMP2_rev	CG***A AGC TT***C TAC GGT AAG AAC CAC AAG A	#556
MMP3PRO_fwd	AG***G GAT C***CG GTT TCT ATA CAA ACT CCT C	#557
MMP3PRO_rev	GC***A AGC TT***C TAC ACT AAA TAC AAA AAT AAT C	#558
MPK6_LP	TCA TCT TCA TCT CCC AAA TGC	#559
MPK6_RP	TTA TCC GAA GAA CAT TGC CAG	#560
MPK3_LP	ATT TTT GTC AAC AAT GGC CTG	#561

MPK3_RP	TCT GCC TTT TCA CGG AAT ATG	#562
GFP-*Sal*I	CGC ***GTC GAC*** CAT GGT GAG CAA GGG CGA GGA	#631
GFP-Xho3	CCG ***CTC GAG*** GTC TTG TAC AGC TCG TCC ATG C	#632
At2-MMP-Xho3	CCG ***CTC GAG*** CGA AAT CAA ACA TAG GTA TAG GAC A	#635
MatMMP2_BamHI	CG***G GAT CC***G ACG TGG TCA ACG GTA CTT CG	#633
MatMMP2_HindIII	CCC ***AAG CTT*** GAC CGT CGA TTC TCC AGG CGG C	#634
HM280 Fwd	AAC GAT CAC GAC AGG GAA AC	#646
HM280 Rev	CGA CGG TTG ACA ACA AGA GA	#647

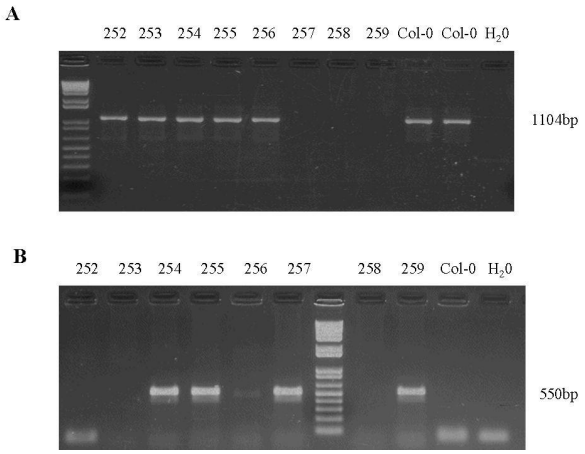


Fig.S 2. Identification of homozygous T-DNA insertion line N582450 (*at2-mmp,* HM-257). Genomic DNA was extracted and amplified using REDExatract-N-Amp PCR kit. Size of the PCR products is shown at the right side. **A**, PCR amplification using the gene specific primer LP (#217) and RP(#218). **B**, PCR confirmation of T-DNA insertion using a combination of the border primer LBb1 and gene specific primer RP (#218). LP, left primer; RP, right primer; LB, Left border.

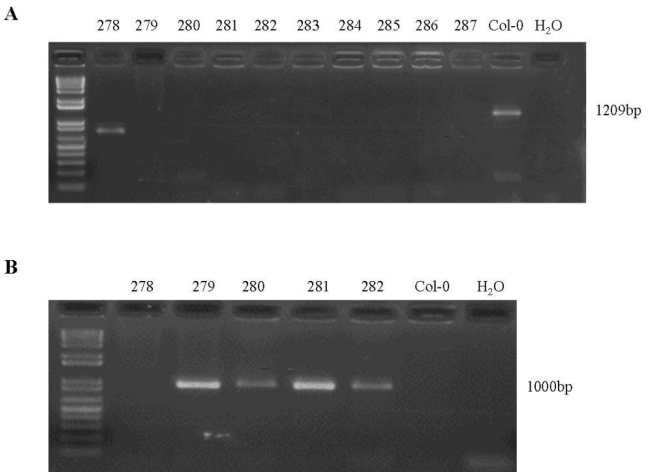


Fig.S 3. Identification of homozygous T-DNA insertion line N348998 (*at2-mmp,* HM-280). Genomic DNA was extracted and amplified using REDExatract-N-Amp PCR kit. Size of the PCR products was shown at the right side. **A**, PCR amplification using the gene specific primer LP (#226) and RP (#227). **B**, PCR confirmation of T-DNA insertion using a combination of the border primer LB (#222) and gene specific primer RP (#227). LP, left primer; RP, right primer; LB, Left border.

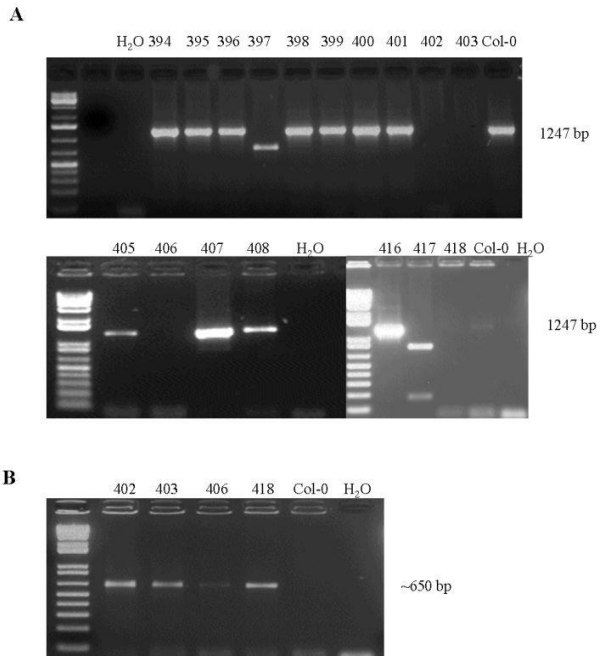


Fig.S 4. Identification of homozygous T-DNA insertion mutant line N115923 (*at3-mmp,* HM-402). Genomic DNA was extracted and amplified using REDExatract-N-Amp PCR kit. Size of the PCR products was shown at the right side. **A**, PCR amplification using the gene specific primer LP (#392) and RP (#393). **B**, PCR confirmation of T-DNA insertion using a combination of the border primer Spm32 (#443) and gene specific primer RP (#393). LP, left primer; RP, right primer; LB, Left border.

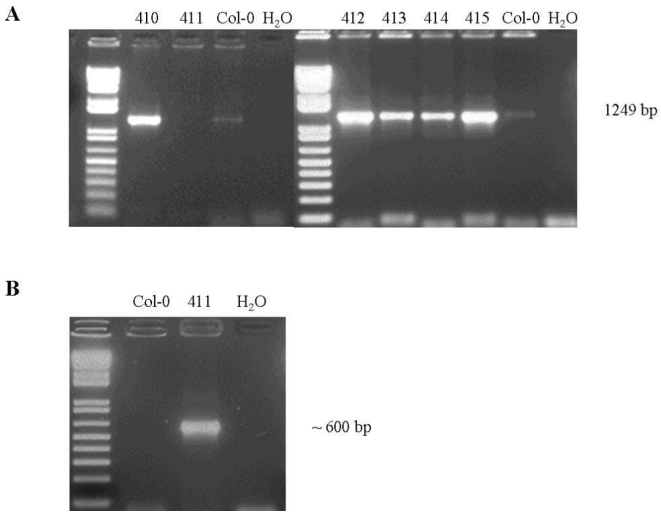


Fig.S 5. Identification of homozygous T-DNA insertion mutant line N103532 (*at3-mmp,* HM-411). Genomic DNA was extracted and amplified using REDExatract-N-Amp PCR kit. Size of the PCR products was shown at the right side. **A**, PCR amplification using the gene specific primer LP (#390) and RP (#391). **B**, PCR confirmation of T-DNA insertion using a

combination of the border primer Spm32 (#443) and gene specific primer RP (#391). LP, left primer; RP, right primer; LB, Left border.

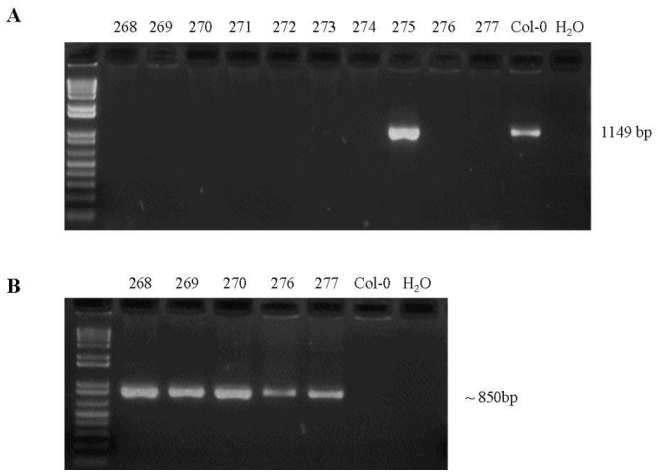


Fig.S 6. Identification of homozygous T-DNA insertion mutant line N327098 (*at4-mmp,* HM-268). Genomic DNA was extracted and amplified using REDExatract-N-Amp PCR kit. Size of the PCR products was shown at the right side. **A**, PCR amplification using the gene specific primer LP (#224) and RP (#225). **B**, PCR confirmation of T-DNA insertion using a combination of the border primer LB (#222) and gene specific primer RP (#225). LP, left primer; RP, right primer; LB, Left border.

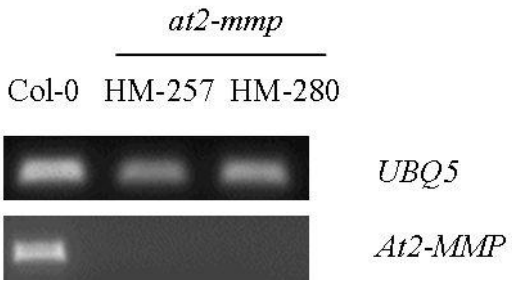


Fig.S 7. Absence of At2-MMP transcript in T-DNA insertion mutants. Primer pair #511 and #208 (Table S1) were used for the RT-PCR using the samples infected with *B. cinerea* for 48 h from wild-type Col-0 plant and *at2-mmp* mutants lines (HM-257 and HM-280). UBQ5 was used as an internal control.

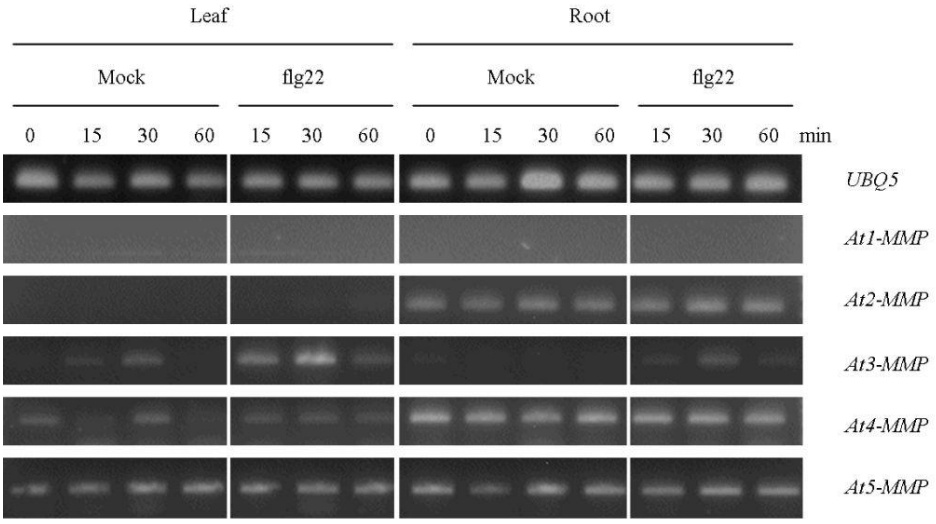
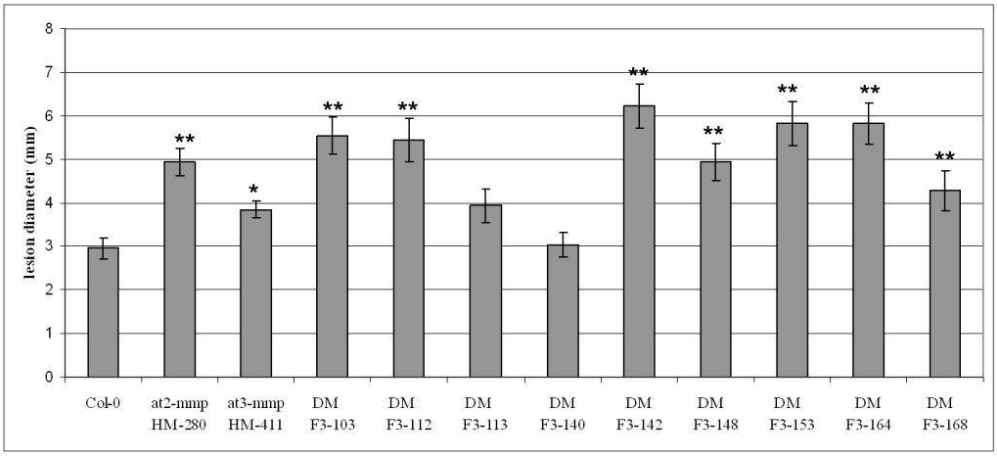


Fig.S 8. Expression profile of *At-MMPs* in *Arabidopsis* leaves and roots after the MAMP flg22 treatment. Three-week-old Col-0 seedlings grown on the ATS medium were sprayed with 1 μM flg22. The mock treatment was sprayed with ddH_2O. Leaf and root samples were harvested separately at the indicated time points after flg22 treatment and used for extraction of total RNA. RT-PCR was performed using UBQ5 as an internal control. min: minutes.

A



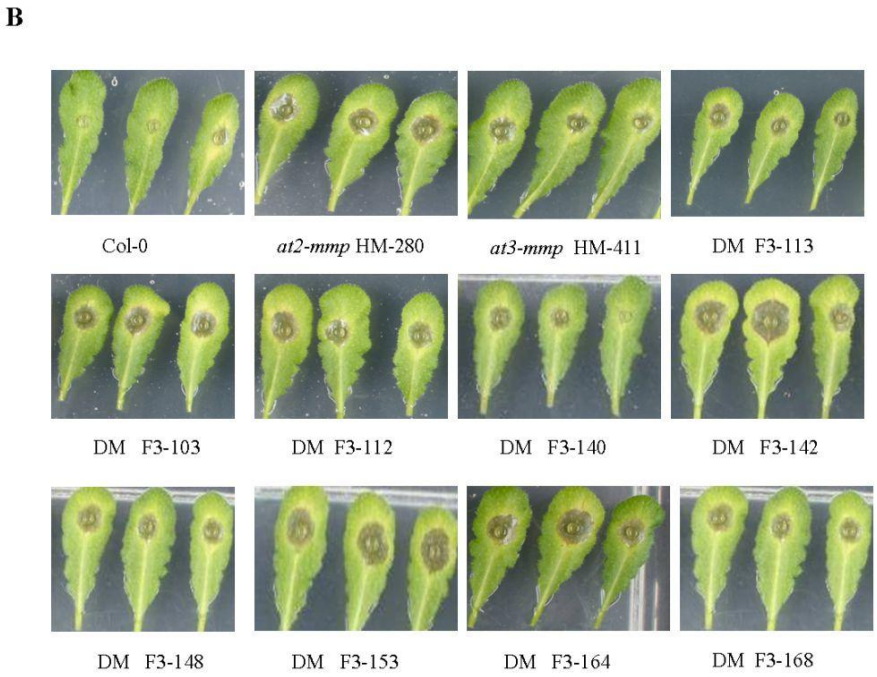


Fig.S 9. *at2-mmpat3-mmp* double mutants F_3-140, F_3-153, F_3-168, F_3-142, F_3-148, F_3-164, F_3-103, F_3-112, F_3-113 showed increased susceptibility to the necrotrophic pathogen *B. cinerea.* The rosette leaves were detached from 6-week-old plants and placed into plastic box containing 0.5% H_2O-Agar. The detached leaves were inoculated by placing 5 µL spore suspension (5×10^4 conidiospores/mL) on the middle vein. The photograph was taken at 2 days after inoculation. The lesion size was measured by Image J. **A**, Lesion diameters formed in leaves of double mutants F_3-XX, parents line *at2-mmp* (HM-280), *at3-mmp* (HM-411) and wild type Col-0 two days after inoculation with *B. cinerea.* Data represent average ± SE of at least of 20 lesions. Asterisks indicate significance difference between the mutants and wild-type plants according to Students's t-test (($P\leq 0.01$)
B, Disease symptoms on detached leaves of double mutants, the parental single mutant *at2-mmp* (HM-280), *at3-mmp* (HM-411) and Col-0 plants two days after infection.

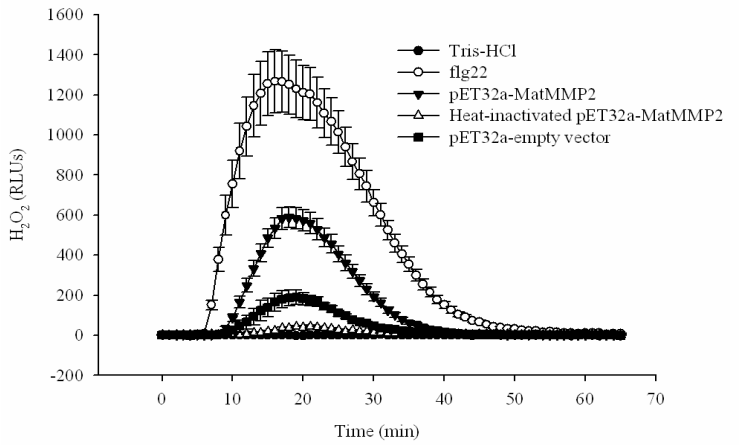


Fig.S 10 Recombinant Mat-MMP2 protein induced ROS production in *Arabidopsis*. Leaf disks were cut from seven-week-old soil-grown Arabidopsis plants under short day conditions. ROS production was measured in relative light units (RLU) in leaves disk after adding Tris-HCl, 500 nM flg22, 0.1 µM Mat-MMP2, 0.1 µM heat-inactivated Mat-MMP2 or 0.1 µM empty vector-derived recombinant protein (Thioredoxin-His-S tag). The Heat inactivation of Mat-MMP2 was done by boiling at 100 °C for 10 minutes. Results are mean ± SE (n = 12).

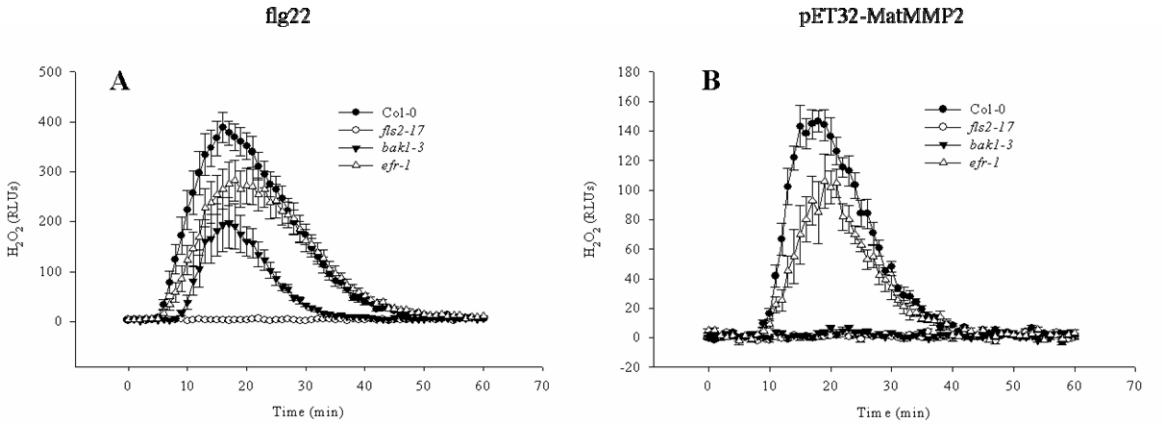


Fig.S 11 Mat-MMP2-induced ROS was abolished in *fls2-17* and *bak1-3* mutants. Leaf disks were cut from seven-week-old soil-grown *Arabidopsis* plants under short day conditions. Oxidative burst was measured in relative light units (RLU). Results are mean ± SE (n = 12).**A**, flg22 failed to triggered ROS production in the flg22 receptor mutant *fls2-17*. **B**, Mat-MMP2 protein (0.1 µM) triggers ROS production in Col-0 and *efr-1* mutants but not in fls2-17 and bak1-3 mutants.

Declaration

I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation.

I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me.

At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice".

Signature:
Date:

Acknowledgements

I am happy to write down this part which I could express my great appreciation from the bottom of my hart to everyone who helped me during this thesis work. Firstly I would like to thank China Scholarship Council (CSC) which provided me with living expenses during the first two years. I would like to thank the STIBET scholarship which was provided by Deutscher Akademischer Austausch Dienst (DAAD) and BASF.

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Giessen. My mother suffers a lot of burdens from the life but never complains and always shows her great optimism. She uses her own behavior encouraging me to do my utmost best and never give up. Thanks to my brothers for taking more responsibilities to take care of my parents during my stay in Germany.

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At last, I would like to say thanks again to every one who helped me during my stay in IPAZ.

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.