Functional analysis of *Arabidopsis thaliana* matrix metalloproteinases and MORC in plant immunity

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List of Abbreviation

ald1	*agd2-like defense response protein 1*
APMA	4-aminophenyl mercuric acetate
AzA	Azelaic acid
AZI1	Azelaic acid induced 1
BAP	6-benzylaminopurine
BTH	Benzothiadiazole S-methyl ester
cDNA	Complementary DNA
CFU	Colony forming units
Col	Collagenase-like protein
CRT1	Compromised recognition of TCV
Cs	Cytosolic
Cys	Cysteine array
D	Aspartic acid
DIR1	Defective in induced resistance 1
dko	*atmorc1/atmorc2* double knock-out
DMSO	Dimethyl sulfoxide
dpi	day post inoculation
E	Glutamic acid
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EFR	Elongation factor Tu receptor
EF-Tu	Elongation factor Tu
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
Fg	*Fusarium graminearum*
flg22	A 22-amino-acid-long peptide derived from flagellin
FLS2	FLAGELLIN-SENSITIVE 2
Fn	Fibronectin repeat
Fr	Furin-cleavage site
G3P	Glycerol-3-phosphate
GHKL	Gyrase, Hsp90, histidine kinase, MutL
GPI	Glycosylphosphatidylinositol
h	Hour
HPX	Hemopexin
HR	Hypersensitive response
IL	Interleukin
INA	2, 6-dichloroisonicotinic acid
JA	Jasmonic acid
K	Lysine
LB	Left border primer
LP	Left primer
LRR	Leucine-rich-repeat
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen activated protein kinase
MBP	Myelin basic protein
MeSA	Methyl salicylic acid

min	Minutes
MMPs	Matrix metalloproteinases
MORC	Microrchidia
NASC	European *Arabidopsis* stock center
NB-LRR	Nucleotide-binding-leucine-rich repeat proteins
NMMP1	*Nicotiana benthamiana* matrix metalloprotease 1
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death
PR genes	Pathogenesis-related genes
PRRs	Pattern recognition receptors
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP/pattern-triggered immunity
RdDM	RNA-directed DNA methylation
R gene	Resistance genes
RMG1	*resistance methylated gene 1*
ROS	Reactive oxygen species
RP	Right primer
R protein	Resistance protein
RT-PCR	Real-time PCR
SA	Salicylic acid
SAR	Systemic acquired resistance
SDC	*suppressor of drm2 cmt3*
SDS	Sodium dodecyl sulfate
SH	Thiol group
SMEP1	Soybean metalloendoproteinase 1
SP	Signal peptide
TBF1	TL1-binding factor 1
TCV	Turnip crinkle virus
TEs	Transposable elements
TNF	Tumour-necrosis factor
Xcv	*Xanthomonas campestris pv. vesicatoria*
Zn	Zinc

1. Introduction

1.1 Plant immune system

Plants have to fight with various pathogens in whole life for their survival. Biotrophic, hemi-biotrophic and necrotrophic pathogens employ different strategies to infect plants, biotrophic pathogens infect plants and proliferate in living tissues; necrotrophic pathogens can be axenically cultured and secret toxins to infect host plants. In order to prevent from pathogens infection, plants have evolved two layers of innate immunity, which is termed as pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006).

1.1.1 Pattern-triggered immunity (PTI)

The first layer of plant innate immunity is pattern-triggered immunity (PTI). When plants are stimulated by biotic stresses, PTI is triggered through the recognition of pathogen-associated or microbe-associated molecular patterns (PAMPs/MAMPs) by membrane-localized pattern recognition receptors (PRRs) (Macho and Zipfel, 2015; Rajamuthiah and Mylonakis, 2014; Boller and Felix, 2009; Jones and Dangl, 2006). PTI in plants is very similar to innate immunity in animals (Boller and Felix, 2009; Chisholm et al., 2006; Jones and Dangl, 2006; Smith et al., 2003). For example, in plants, the flagellin of bacteria is perceived as a MAMP through the leucine-rich-repeat (LRR) domains of the membrane receptor FLAGELLIN-SENSITIVE 2 (FLS2) (Bohm et al., 2014; Chinchilla et al., 2006; Gómez-Gómez and Boller, 2000). In mammals, the Toll-like receptor TLR5 could also perceive bacterial flagellin through its LRR domain (Hayashi et al., 2001; Smith et al., 2003). Another well known MAMP is elongation factor Tu (EF-Tu) which is recognized by the

kinase EFR receptor (elongation factor Tu receptor) (Zipfel, 2014; Zipfel et al., 2006). The typical PTI responses include the accumulation of reactive oxygen species (ROS), activation of MAP kinase cascades, induction of defense genes expression and occurrence of callose deposition (Bigeard et al., 2015; O'Brien et al., 2012; Ahuja et al., 2012; Bednarek, 2012; Torres and Dangl, 2005; Zipfel, 2008; Gómez-Gómez and Boller 2000)

1.1.2 Effector-triggered immunity (ETI)

Effector-triggered immunity (ETI), which was formerly called *R*-gene-based or vertical resistance, is the second layer of plant innate immunity (Martin et al., 2003; Nimchuk et al., 2003; Boller and He, 2009). When confronting with intruders, efficient PTI could trigger host resistance to circumvent the pathogen attacks. During the process, successful pathogens evolved strategies, such as secretion of virulence effectors, to overcome PTI and achieve compatibility (Abramovitch et al., 2006; Block et al., 2008; Block and Alfano, 2011; Chisholm et al., 2006; Jones and Dangl, 2006). Nevertheless, plants would not stop making effort to protect them from infection. They further evolved effector-triggered immunity, in which the effectors are directly or indirectly recognized by the resistance (R) proteins encoded by the resistance genes (*R* genes) (Dangl et al., 2013; Win et al., 2012; Spoel and Dong, 2012). Most of the *R* genes encode nucleotide-binding leucine-rich repeat proteins (NB-LRR) (Ellis et al., 1999; Wei et al., 1999). The *R* gene-mediated plant resistance is only active against specific isolates of a pathogen. NB-LRR activation involves intra- and intermolecular conformational changes and inappropriate NB activation seems to be tightly controlled by the autoinhibition of LRR domains (Takken et al., 2006). Several NB-LRR proteins indirectly recognize type III effectors, by detecting products of their action on host targets, consistent with the 'guard hypothesis' (Dangl and Jones, 2001;

Rajamuthiah and Mylonakis, 2014; Hurley et al., 2014), and this is more frequently compared with direct recognition of effectors. The outcome of ETI is the increase of plant resistance to invading pathogens. The typical event is the strong defense reaction called the hypersensitive response (HR), which is characterized by rapid apoptotic cell death and local necrosis at the infection site to limit pathogen proliferation and disease symptoms (Strauss et al., 2012; Boys et al., 2012; Bozkurt et al., 2012; Thirugnanasambandam et al., 2011). ETI is regarded as a faster and stronger immune response (Cui et al., 2015; Tao et al., 2003; Truman et al., 2006). More recently, proteomic approaches have been used to study plant ETI signaling (Hurley et al., 2014; Parker et al., 2013; Elmore et al., 2012; Dunham et al., 2012; Rodríguez-Herva et al., 2012; Ntoukakis et al., 2013).

Based on the co-evolution during the interaction between a plant and a microbe, a four phased 'zigzag' model was proposed and accepted as a current concept of the plant immune system (Fig. 1-1).

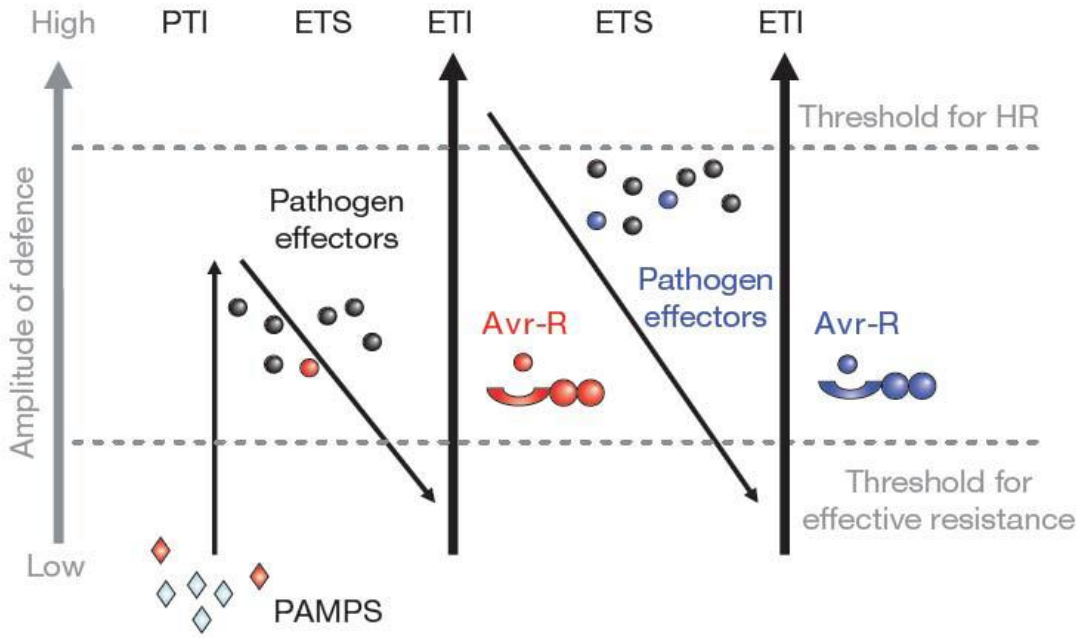


Figure. 1-1. Zigzag model of the plant immune system (Jones and Dangl, 2006).

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, which activates PTI to 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.

1.1.3 Systemic acquired resistance (SAR)

The invasion of pathogens not only triggers the local defense response, but also induces the generation of specific signals in plants, such as salicylic acid (SA), methyl salicylic acid (MeSA), azelaic acid (AzA), and glycerol-3-phosphate (G3P) (Gao et al., 2015; Shah and Zeier, 2013; Kachroo and Robin, 2013; Gao et al., 2014; Chaturvedi et al., 2012; Chanda et al., 2011; Jung et al., 2009; Park et al., 2007). The accumulation of these signals leads to the expression of *PR* genes (pathogenesis-related genes) in the uninfected tissue to protect the rest of the plant from subsequent infections (Yan and Dong, 2014; Durrant and Dong, 2004). This process is termed systemic acquired resistance (SAR). SAR can also be induced by 2, 6-dichloroisonicotinic acid (INA) which is synthetic analogs of SA and benzothiadiazole S-methyl ester (BTH) (Görlach et al., 1996; Durrant and Dong, 2004). Wang et al (2014) showed that nitric oxide (NO) and reactive oxygen species (ROS) play an important role in inducing SAR. SAR leads to long-lasting, broad- spectrum resistance against pathogen infection

(Wendehenne et al., 2014; Fu and Dong, 2013). Pajerowska-Mukhtar et al (2012) suggested that SAR signaling is associated with changes in amino acid homeostasis induced by ETI. TBF1 (TL1-binding factor 1), which is required for the growth-to-defense transition upon pathogen challenge, is derepressed within 30 min of the pathogen infection, suggesting that it might be one of the earliest responses triggered by SAR (Pajerowska-Mukhtar et al., 2012). Characterization of the *Arabidopsis ald1* (*agd2-like defense response protein 1*) mutant also showed that an amino acid–derived defense signal is generated upstream of SA synthesis (Song et al., 2004). DIR1(defective in induced resistance 1) was discovered in a genetic screen designed specifically to identify SAR signals, which encodes a putative lipid-transfer protein, is probably involved in the synthesis or transport of a lipid molecule, which is a signal for SAR (Maldonado et al., 2002). AZI1 (azelaic acid induced 1), encoding a predicted secreted protease-inhibitor/seed-storage/lipid-transfer family protein, which regulates the production or translocation of a mobile SAR signal together with DIR1 (Jung et al., 2009). Another important signal for SAR is jasmonic acid (JA). The level of JA increased significantly at 6 h after *P. syringae* pv. *tomato* (*Pst*) DC3000/AvrRpm1 inoculation and returned to normal level 11 h after *Pst* infection (Truman et al., 2007). The SAR is induced by exogenous application of JA. However, SAR is compromised in JA-insensitive mutant *sgt1b/jai4*, JA-biosynthesis mutant *opr3*, and JA-response mutant *jin1* plants (Attaran et al., 2009). Another required signal for SAR is glycerol-3-Phosphate (G3P), which showed accumulation within 6 h after pathogen infection (Chanda et al., 2011). It can be produced through the activity of the G3P dehydrogenase GLY1 (Mandal et al., 2011; Chanda et al., 2008).

1.1.4 Nonhost resistance

Nonhost resistance is defined as a resistance of an entire plant species to all isolates of a pathogen (Stam et al., 2014; Heath, 2000; Mysore and Ryu, 2004; Nürnberger and Lipka, 2005). It is a broad-spectrum plant defense. Most of nonhost resistance is associated with a broad range of mechanisms that are regulated by multiple genes (Uma et al., 2011; Fan and Doerner, 2012; Ham et al., 2007; Mysore and Ryu, 2004). A pathogen capable of infecting other plant species but incapable of invading a nonhost plant is referred as a nonhost pathogen. Nonhost pathogens which land on the plant surface are exposed to a wide range of preformed plant defenses (Heath, 2000; Hückelhoven, 2007). Some nonhost pathogens are able to penetrate into apoplastic space through stomata or wounds of the plant surface. The apoplast is therefore the major battleground during the plant-microbe interactions (Alfano and Collmer, 1996). Nonhost defense responses can be induced by PTI or ETI (Senthil-Kumar and Mysore, 2013). Physical restriction and chemical inhibition of pathogens are the important components of nonhost resistance. The cuticle layer, the epidermis, and the cell wall act as physical barriers for the entry of pathogens. The callose and lignin deposition is induced and reinforce the cell wall after nonhost pathogen invasion (Bestwick et al., 1995, 1997). Plants also produce some antimicrobial compounds which inhibit host- and nonhost pathogen growth (Che et al., 2011; Lee et al., 2008). Several secondary metabolites have antimicrobial properties and involve in restricting the growth of invading pathogens (Fan et al., 2011; Aires et al., 2009; Filippone et al., 1999). In *Arabidopsis thaliana*, sulfur- and nitrogen-containing secondary metabolite compounds glucosinolates play an important role in plant defense against variety of pathogens (Baskar et al., 2012; Bednarek, 2012). Induced defense response is another main part of nonhost resistance of plants against bacterial pathogens (Tao et al., 2003). During the nonhost resistance, ROS, salicylic acid (SA), and other hormones

play an important role as signaling molecules. These defense responses against nonhost-pathogen growth through producing structural barriers, inducing biosynthesis of antimicrobial chemicals, and activating several defense pathways at the molecular level, the growth of nonhost-pathogen could be inhibited.

1.1.5 Reactive oxygen species (ROS) in plant-microbe interaction

The production of reactive oxygen species (ROS) is one of the earliest plant defense responses during microbial infection. Previous studies showed that ROS may be involved in plant defense directly through the antimicrobial activity or mediating cell wall cross-linking, the induction of defense genes expression and the induction of cell death (Torres, 2010; Torres and Dangl, 2005; Boller and Felix, 2009; Bolwell, 1999; Lamb and Dixon, 1997; Levine et al., 1994; Torres et al., 2006; Zurbriggen et al., 2009; Hückelhoven and Kogel, 2003). The production of ROS requires the prior accumulation of Ca^{2+} (Kadota et al., 2014; Li et al., 2014b; Grant and Loake, 2000; Grant et al., 2000), and is dependent on the activity of membrane-localized NADPH oxidases (respiratory burst oxidase homologs, Rboh) (Ranf et al., 2011; Kobayashi et al., 2006; Torres et al., 2006). Different lifestyles of pathogens have distinct responses to ROS generated in the host plants (Heller and Tudzynski, 2011). For example, biotrophic and hemibiotrophic fungi depend on the prevention of a strong oxidative burst response and the hypersensitive response to achieve infection (Molina and Kahmann, 2007; Shetty et al., 2007). Thus, the oxidative burst accumulation of host plants is an effective strategy to combat biotrophic pathogens.

1.1.6 Callose deposition

Callose is an amorphous, high–molecular weight β-(1, 3)-glucan polymer. It is contained in cell-wall appositions that are effective barriers induced at the penetration sites during early stages of pathogenic infection (Piršelová and Matušíková, 2013; Li et al., 2012). Callose deposition is triggered by MAMPs (Luna et al., 2011; Brown et al., 1998; Gómez-Gómez et al., 1999a), and is a hallmark of FLS2-mediated PTI. For example, the MAMP flagellin (Gómez-Gómez and Boller, 2000), elongation factor EF-Tu (Elf18) (Kunze et al., 2004), and chitin, a β-(1,4)-linked polymer as well as other molecules from fungal cell walls can induce callose deposition (Poliakovskiy and Dmitriev, 2011; Iritri and Faoro, 2009).

1.2 The section of matrix metalloproteinases

1.2.1 Matrix metalloproteinases (MMPs) in mammals

Matrix metalloproteinases (MMPs) are a family of highly conserved endopeptidases containing zinc ion in the active site, which was first found in mammals in 1962 (Gross and Lapiere, 1962). The MMP family is widely distributed throughout all kingdoms of life. There are 23 MMPs found in humans (Nagase et al., 2006). MMPs are secreted or attached to the cell surface. In mammals, MMPs play a key role in many important physiological and pathological processes, such as remodeling of the extracellular matrix (ECM), regulation of cell migration, proliferation, adhesion and signaling, by limited proteolytic processing of substrate proteins (Butler and Overall, 2009; Birkedal-Hansen et al., 1993; Stamenkovic, 2003; Vu and Werb, 2000; Parks et al., 2004).

1.2.1.1 Structure of MMPs in mammals

The structure of mammalian MMP contains a signal peptide, a prodomain, a catalytic domain, a linker peptide and a hemopexin (Hpx) domain (Nagase et al., 2006). In the homologues of *MMP*, the catalytic domain possesses a zinc binding motif HEXXHXXGXXH, in which the zinc atom is located in the active site. The propeptide domain maintains the “cysteine switch” sequence PRCGXPD. All of the MMPs share this common domain structure (Nagase and Woessner, 1999).

The conserved cysteine residue coordinates with the active zinc ion to inhibit catalytic activity. When the propeptide domain is removed, the MMPs have the activity to cleave substrate (Andrea et al., 2007). Most MMP members also contain a hemopexin domain in the C-terminal by a flexible hinge. The hemopexin domain encodes a four-bladed β-propeller structure which mediates protein–protein interactions. This domain also has a function in modulating substrate specificity, activation of the enzyme, protease localization, internalization and degradation (Overall, 2002; Parks et al., 2004). MMPs are subdivided into different groups according to differences in domain composition (Fig.1–2). One clear division is between MMPs that are secreted and anchored to the cell surface by an intrinsic motif, including a transmembrane domain, a glycosylphosphatidylinositol (GPI) anchor or an amino N-terminal signal anchor (Parks et al., 2004).

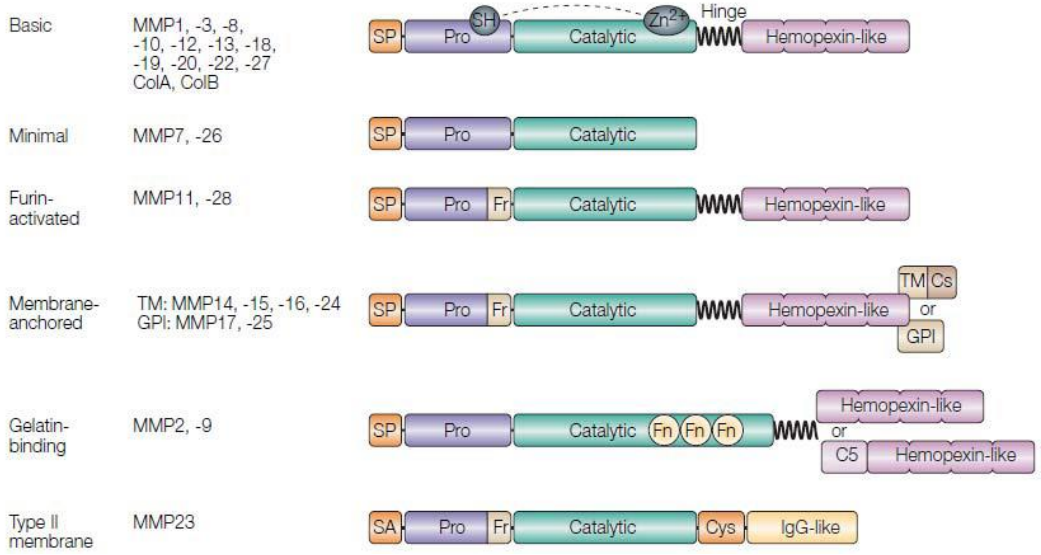


Figure. 1–2. Domain structure of the mammalian MMP family.

MMPs are subdivided into groups on the basis of differences in domain composition. 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 (according to Parks et al., 2004).

1.2.1.2 Function of MMPs in mammals

MMPs were thought to be responsible for degradation of extracellular matrix (ECM) molecules in the tissue. All isolated MMPs have been shown to be capable of degrading various protein components in the ECM (Sternlicht and Werb, 2001). Consequently, MMPs family plays a role as enzymes responsible for the turnover, degradation, catabolism and destruction of the ECM. In addition, some non-ECM molecules are also possible substrates of MMPs (Nagase et al., 2006). Since MMPs are secreted or anchored to the cell surface, their potential substrates include all membrane proteins and proteins in the secretory pathway and extracellular space.

In most cases, MMP-deficient mice mutants showed no or a minor phenotype

under unchallenged condition. Nevertheless, the MMP14-deficient mice mutant showed severe bone deformations (Holmbeck et al., 1999; Zhou et al., 2000). These indicate that some MMPs might not have a direct role in the turnover of ECM proteins. After challenge, such as injury, cancer, inflammation or infection, MMP-deficient mice mutant displayed various phenotypes, indicating that these enzymes have specific roles in tissue repair, angiogenesis, host defence, tumour progression and inflammation (Table.1-1). Taken together, MMPs may have evolved to respond to environmental pressures (Fig.1-3) (Parks et al., 2004).

Additionally, MMPs are of importance in modulating inflammatory processes. Many *MMPs* are increased or mis-regulated in any disease that is characterized by or associated with inflammation. The role of MMPs as matrix degrading proteinases justifies their inclusion as important components of the host response to traumatic, infectious, toxic or autoimmune insults.

Several MMPs were induced at injury sites and crucial for wound closure (Parks, 1999). For example, the catalytic activity of MMP1 is required for the repair of skin wounds. Moreover, MMP7 and MMP9 are implicated in wound repairing (Dunsmore et al., 1998; McGuire et al., 2003; Pilcher et al., 1997). MMP3-deficient mice mutant showed impaired immunity to intestinal bacterial infection suggesting that MMP3 is important to resist bacteria (Li et al., 2004). It is different from other MMPs which are expressed in response to injury or inflammation, *MMP7* is expressed in healthy epithelium indicating its function in common homeostatic processes, such as resistance to microorganisms and apoptosis. In mice, MMP7 activates intestinal pro-α-DEFENSINS which is evidenced by the impaired ability to battle pathogens *Escherichia coli* and *Salmonella typhimurium* in the MMP7-deficient mice mutant (Wilson, et al., 1999). Furthermore, the induction of MMP7 in mucosal epithelium is highly sensitive to the presence of virulent bacteria, further suggesting a role of this MMP in innate immunity (López-Boado, et al., 2000, 2001). Besides, MMP2 and MMP9 also showed early immune response to against *Streptococcus*

pneumoniae infection (Hong et al., 2011).

Mouse	Phenotypes that indicate a function in innate or acquired immunity, including repair-related processes
Mmp2	Decreased allergic inflammation
	More-severe immune-mediated arthritis
	Reduced tumour, corneal, retinal and choroidal neovascularization
Mmp3	More-severe immune-mediated arthritis
	Reduced contact hypersensitivity
	Reduced neutrophil influx in immune-mediated lung injury
	Reduced number of macrophages in atherosclerotic plaques
	Reduced cartilage-derived macrophage-chemotactic activity
Mmp7	Lack of activation of cell-surface TNF displayed on macrophages
	Lack of active intestinal α-defensins and impaired ability to kill enteric pathogens
	Spatially constrained chemokines and reduced neutrophil influx in injured lung
	Markedly impaired re-epithelialization and reduced shedding of E-cadherin in injured lung
	Reduced shedding of active FAS ligand (CD95L) and epithelial-cell apoptosis
	Increased neovascularization in corneal wounds
Mmp8	Marked increased in chemically induced skin tumours*
Mmp9	Prolonged contact hypersensitivity
	Reduced experimental autoimmune encephalomyelitis
	Protection against endotoxin-mediated shock
	Reduced dendritic (Langerhans)-cell migration
	Reduced antigen-mediated blister formation
	Reduced angiogenesis in developing bone, tumours* and ischaemic tissues
	Impaired bronchiolization after acute lung injury
	Altered chemokine gradients in models of allergen-induced airway inflammation
	Altered leukocyte influx in models of allergen-induced airway inflammation
	Modulation of IL-13-induced lung inflammation
	Less-severe experimental arthritis
	Impaired macrophage infiltration in atherosclerosis
	Protection against macrophage-induced aneurysm formation*
	Reduced ischaemia-induced cerebral injury
Mmp2,Mmp9	Reduced choroidal neovascularization
Mmp12	Reduced macrophage migration and influx in smoke-induced emphysema
	Reduced spinal cord injury
	Reduced release of active TNF from smoke-exposed macrophages
	Reduced neutrophil influx and epithelial permeability in immune-mediated lung injury
	Modulation of IL-13-induced lung inflammation
Mmp28	Markedly reduced inflammation in models of lung injury

Table. 1-1. Inflammatory and immune phenotypes of *Mmp*-null mice

*These phenotypes were reversed following transplantation of wild-type bone marrow, indicating that the effect observed in knockout mice was caused by the lack of the matrix metalloproteinase (MMP) in an inflammatory cell or group of inflammatory cells. E-cadherin, epithelial cadherin; IL, interleukin; TNF, tumour-necrosis factor (Parks et al., 2004).

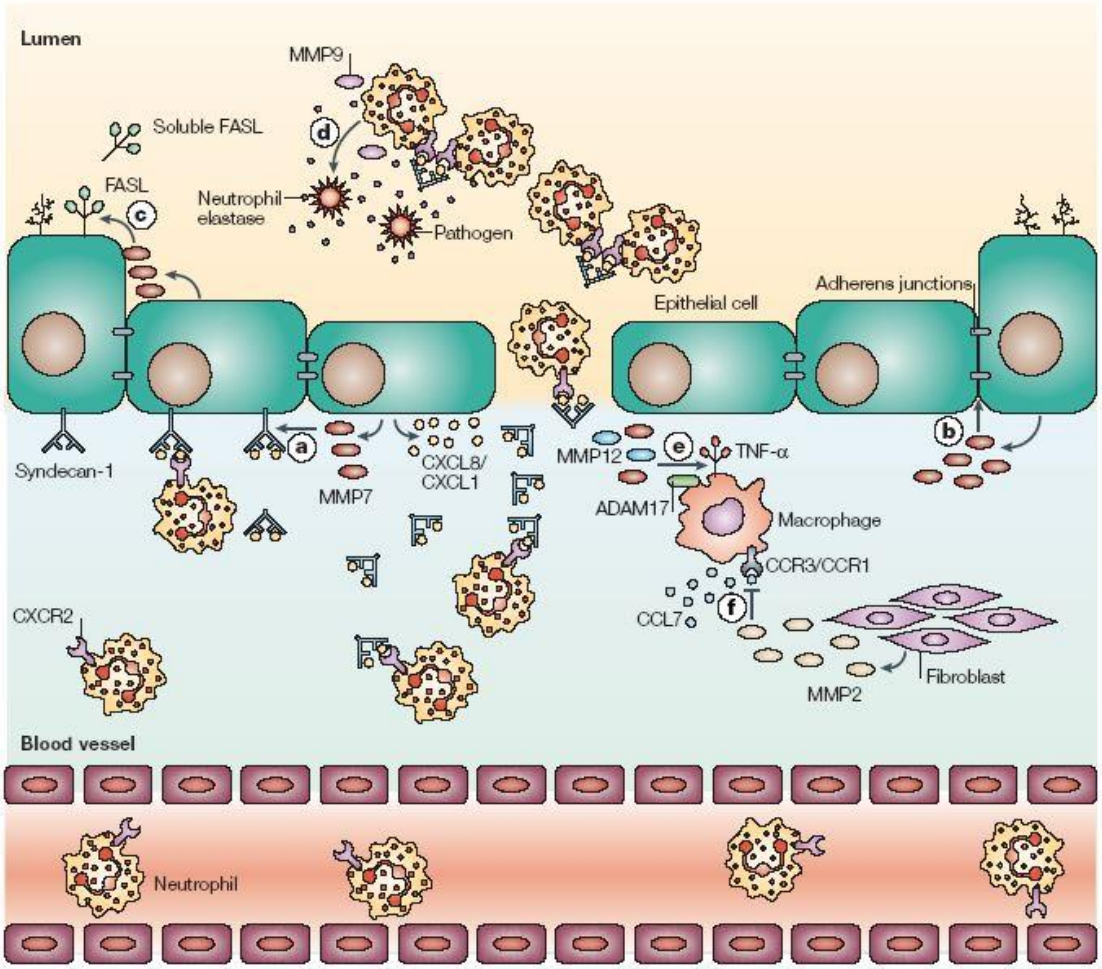


Figure. 1–3. MMPs in inflammation in response to tissue injury.

Injury initiates a programmed, coordinated series of responses to both repair the damaged tissue and to defend against infection. Almost all resident cells, particularly epithelial cells, endothelial cells and fibroblasts, participate in these processes and contribute to the regulation of inflammation. This occurs partly through the specific activity of a variety of matrix metalloproteinases (MMPs) that are produced by these cells. **a**: Soon after injury, epithelial cells at the wound edge produce a chemokine (in humans, CXC-chemokine ligand 8, also known as interleukin-8, and in mice CXCL1, also known as KC) that accumulates on the heparan sulphate chains of syndecan-1, a transmembrane proteoglycan. At the same time, these cells release MMP7, which sheds the ectodomains of syndecan-1, thereby establishing a local chemokine gradient that controls the influx and activation of neutrophils. **b**:

Later on in the repair process, epithelial-derived MMP7 cleaves the ectodomains of epithelial (E)-cadherin, thereby disrupting adherens junctions and, in turn, facilitating cell migration. Re-epithelialization is also facilitated by the action of other MMPs, such as MMP1 in skin and MMP9 in lung cells. **c**: MMP7 also sheds and activates FAS ligand (FASL, also known as CD95L) that is produced by epithelial cells, thereby mediating apoptosis, which is a potential innate defence mechanism (discussed in text). **d**: After activation, neutrophils release several proteases. Among them, neutrophil elastase, a serine protease that is exclusively produced by neutrophils, which has direct antimicrobial activity. Mice deficient in this enzyme have an impaired ability to defend against Gram-negative bacteria98. Activated neutrophils also release MMP9, which degrades and neutralizes the serine protease inhibitor α1-antiproteinase99, a potent inhibitor of neutrophil elastase. In this setting, MMP9 provides cover for the antimicrobial activity of neutrophil elastase, thereby assigning it an indirect role in innate immunity. **e**: The activation of the latent form of tumour-necrosis factor (TNF) on the surface of cells such as macrophages is due to metalloproteinase-mediated proteolysis. In addition to ADAM17 (a disintegrin and metalloproteinase; also known as TNF-converting enzyme, TACE), MMP7 and MMP12 can activate latent TNF (TABLE 1). **f**: The influx of inflammatory cells is mainly directed by specific chemokines that are released by resident cells. In addition to indirect effects on chemokine activity, as discussed in a, MMPs also directly act on chemokines, either enhancing or abrogating their activity. For example, MMP2, which is typically produced by mesenchymal cells, can cleave and inactivate CC-chemokine ligand 7 (also known as macrophage-chemotactic protein 3, MCP3). CCR, CC-chemokine receptor; CXCR, CXC-chemokine receptor (according to Parks et al., 2004).

1.2.2 MMPs in plants

In comparison to the importance of MMPs in mammals, the function of MMPs is still less well-studied in plants. So far, there are only a few MMPs being reported, and MMPs have been isolated from plant species, including *Arabidopsis* (Giada et al., 2014; Lenger et al., 2012; Maidment et al., 1999), tobacco (Kang et al., 2010), soybean (Cho et al., 2009; Ragster and Chrispeels, 1979), cucumber (Delorme et al., 2000) and Loblolly pine (Ratnaparkhe et al., 2009). In plant, the MMPs function to degrade the extracellular matrix (ECM). Moreover, they involved in several physiological processes during plant growth and development, such as the germination of seeds (Ratnaparkhe et al., 2009), programmed cell death (PCD) (Delorme et al., 2000), senescence (Golldack et al., 2002) and expansion of leaf (Graham et al., 1991). Besides, MMPs were reported to play an important role when confronting with biotic or abiotic stresses (Schiermeyer et al., 2009; Liu et al., 2001; Combier et al., 2007; Flinn, 2008).

1.2.2.1 Structure of plant MMPs

In *Arabidopsis thaliana*, there are five MMPs (Maidment et al., 1999). Similar to the structure of mammalian MMPs, the plant MMPs possess a signal peptide, a propeptide domain and a catalytic domain (Fig.1-4). In the propeptide domain, there exists a conserved cysteine switch sequence PRCGXXD; while the catalytic domain contains the zinc-binding motif (HEIGHXLGLXH) followed by the conserved methionine residue of the Met turn (Rawlings et al., 2010). The most important site determining the specificity of MMP cleavage site is S1, which is located directly to the right of the catalytic zinc ion and well suited to accommodate hydrophobic residues, such as leucine or isoleucine (Giada et al., 2014).

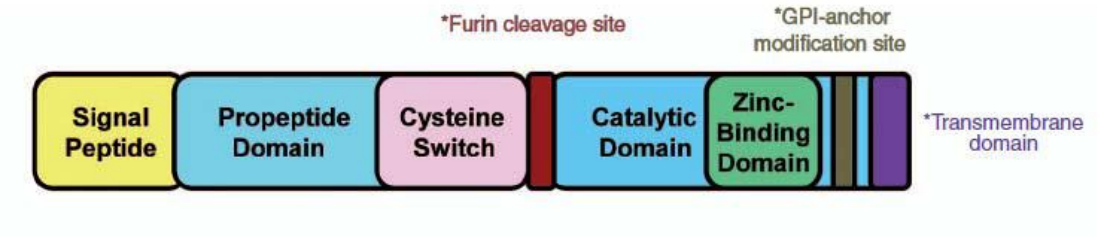


Figure. 1– 4. General structure of plant MMPs.

Relevant domains identified are color-coded. Signal peptide, propeptide domain, cysteine switch, catalytic domain, zinc-binding domain, positions of putative furin cleavage sites, GPI-anchor modification sites and C-terminal transmembrane domains (according to Flinn, 2008).

1.2.2.2 Activation of the plant MMPs activities

As mentioned, the activity of mammalian MMPs requires a mechanism to disrupt the Cys-Zn^{2+} interaction between the conserved cysteine residue and the active zinc site (Parks et al., 2004; Sternlicht et al., 2001). Similarly, the plant MMPs exhibit no activity in original situation; but they require physical delocalization to achieve the proteolytic activity by cleaving off the cysteine switch (Flinn, 2008). In *Arabidopsis*, all five recombinant MMPs were able to cleave the substrates (Giada et al., 2014), among which the At1-MMP can be activated by the activator 4-aminophenyl mercuric acetate (APMA) to cleave the propeptide domain (Maidment et al., 1999).

1.2.2.3 Function of plant MMPs

1.2.2.3.1 Tissue remodeling

One of the most important roles of plant MMPs is to remodel the plant tissues. The first plant metalloproteinase activity was described in soybean leaves (Ragster and Chrispeels, 1979). They reported that the protein possesses an

Azocollase-A activity in soybean leaf extracts. This proteinase was thereafter purified and named SMEP1 (**S**oybean **me**talloendo**p**roteinase 1) (Graham et al., 1991, McGeehan et al., 1992). The accumulation of SMEP1 began from the leaf emergence, while less amount of SMEP1 was observed in the younger leaves in comparison to the older leaves (Pak et al., 1997). In *Arabidopsis*, *At2-MMP* was expressed in young and developing rosettes, young flowers and mature siliques; while the *At5-MMP* showed constitutive expression during the development in all tissues (Flinn, 2008).

1.2.2.3.2 Seed germination and development

The data from analysis of gene expression using Genevestigator (Hruz et al., 2008) demonstrated that *At4-MMP* was highly expressed during seed germination. *At1-MMP* and *At4-MMP* exhibited a similar expression profile during seed/silique development (Flinn, 2008). In Loblolly pine (*Pinus taeda*), the expression of *Pta1-MMP* increased in embryo and megagametophyte from proembryo to early cotyledonary stage, but it is reduced during late embryogenesis and maturation drying (Ratnaparkhe et al., 2009). Taken together, these data indicated that the plant MMPs had a potential role in seed germination and development.

1.2.2.3.3 Senescence and programmed cell death (PCD)

Plant MMPs also affect the senescence and programmed cell death. In the *Arabidopsis at2-mmp* mutant, earlier senescence and cell death were observed (Golldack et al., 2002). In cucumber (*Cucumis sativus* L. cv Marketmore), the expression of *Cs1-MMP* was detected in late senescence and early programmed cell death (PCD). In soybean (*Glycin max*), the matrix metalloproteinase *Slti114* exhibited an age-dependent manner in cotyledon

(Cho et al., 2009). Taken together, these studies suggest that the plant MMPs involved in senescence and PCD.

1.2.2.3.4 Biotic and abiotic stresses

Based on the gene expression analysis, the role of plant MMPs in response to biotic and abiotic stresses have been reported (Schiermeyer et al., 2009; Liu et al., 2001; Combier et al., 2007; Flinn, 2008). In soybean, *GmMMP2* was induced by wounding and dehydration (Liu et al., 2001). Likewise, the expression of *At2-MMP* in *Arabidopsis* root was induced by NaCl and stimulated by cadmium treatment in leaves (Golldack et al., 2002). In *Arabidopsis*, four enzymes, At1-MMP, At2-MMP, At3-MMP and At5-MMP, displayed maximal activity at pH between 7.0 and 8.0. In addition, the proteolytic activity of At-MMPs was affected by temperature. For example, the activity of At3-MMP and At5-MMP reach a maximum at 35°C; for At1-MMP, At2-MMP and At4-MMP, 45–55°C is the best temperature for their activity (Marino et al., 2014). Taken together, these data suggested that the plant MMPs play a role in the adaptation to abiotic stresses.

On the side of biotic interactions, *GmMMP2* transcript levels were increased in compatible and incompatible interactions of soybean tissues with the oomycete pathogen *Phytophthora sojae*, as well as the bacterial pathogen *Pseudomonas syringae* pv. *glycinea*. In accordance with the *GmMMP2* activation, a metalloproteinase activity was increased in suspension cells following the bacterial infection (Liu et al., 2001). In *Arabidopsis*, *At3-MMP* was induced after flg22 treatment in seedlings (Zipfel et al., 2004). In addition, meta-profile heat map analysis of *At-MMPs* gene expression in response to different pathogen stresses indicated that *At2-MMP* and *At3-MMP* were up-regulated (Flinn, 2008). These data suggested that plant MMPs participate in the response to biotic and abiotic stresses.

1.3 The section of MORC

1.3.1 Morc family

The turnip crinkle virus (TCV) is a positive sense RNA virus which belongs to the carmovirus group. It is able to infect most *Arabidopsis* ecotypes. The R protein HRT confers resistance to TCV (Cooley et al., 2000). MORC was formerly termed CRT1 (compromised recognition of TCV). It was identified through a genetic screening of mutants carrying HRT that were compromised for the recognition of TCV (Kang et al., 2008). The MORC family is a subfamily of microrchidia (MORC) GHKL ATPases (Gyrase, Hsp90, histidine kinase, MutL) superfamily (Dutta and Inouye, 2000). The first MORC protein was isolated from mouse, which is required for meiotic nuclear division (Watson et al., 1998). Thereafter, *MORC* genes have been identified in mammals (Pastor et al., 2014) and *Caenorhabditis elegans* (Moissiard et al., 2012). Besides, MORC have also been isolated from different plant species, including *Arabidopsis* (Kang et al., 2008), barley, tobacco and potato. There are seven members of MORC identified in *Arabidopsis thaliana* and five in barley (Langen et al., 2014). Previous studies demonstrated that MORC is involved in plant immunity (Kang et al., 2008, 2010, 2012; Langen et al., 2014).

1.3.2 The role of MORC in plant-pathogen interaction

Sequence analysis showed that the MORC family contains a combination of a gyrase, histidine kinase, MutL (GHKL) and S5 domains. It is a subfamily of microrchidia (MORC) GHKL ATPases superfamily. The previous data indicated that MORC is required for multiple layers of plant immunity. RNAi-mediated silencing of *MORC2* and *MORC3*, which are the two closest homologues of *MORC1*, results in higher susceptibility to TCV infection

compared with the wild-type (Kang et al., 2008). The *ssi4* mutant contains a mutation of a TIR-NBS-LRR type R protein which leads to the activation of defense responses, such as HR, SA accumulation and defense-related gene expression (Shirano et al., 2002). While *morc1* mutant reduced the spontaneous cell death induced by a constitutively active R protein (*ssi4*). Additionally, MORC1 also altered HRT-induced defense responses as well as *ssi4*, and MORC1 protein interacted with other NBS-LRR proteins such as HRT and SSI4 (Kang et al., 2008). The *Arabidopsis* knockout *morc1/ morc2* double mutant, which was produced from the Col-0 background, displayed higher susceptibility to avirulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) and the oomycete *Hyaloperonospora arabidopsidis* (Kang et al., 2010; Langen et al., 2014). *Arabidopsis* MORC1 and MORC2 have been previously demonstrated to be required for various types of disease resistance including basal resistance, nonhost resistance SAR and ETI (Kang et al., 2008; 2010; 2012). In barley, there are five MORCs isolated, all of which are involved in resistance. Knock-down (KD) of barley *MORC2* plant displayed reduced numbers of fungal colonies compared with control after powdery mildew fungus *Bgh* race A6 (*Bgh*A6) infection. Moreover, the overexpression line of barley *MORC1* exhibited more *Bgh* colonies. Additionally, silencing of barley *MORC2* increased the resistance to the root rot-causing necrotrophic fungus *Fusarium graminearum* (*Fg*). Furthermore, barley MORCs were also involved in ETI (Langen et al., 2014). Taken together, MORCs are involved in plant resistance. However, *Arabidopsis* and barley MORC resulted in opposite effects on plant immunity.

1.3.3 The role of MORC in gene silencing

DNA methylation, DNA repeats and histone methylation are the common ways to achieve epigenetic gene silencing. *Arabidopsis* MORC1 and MORC6

were involved in gene silencing, which cause derepression of DNA-methylated genes and transposable elements (TEs) but no losses of DNA or histone methylation (Moissiard et al., 2012). A modest reduction of DNA methylation and repressive histone marks at specific RNA-directed DNA methylation (RdDM) target sites in *atmorc6* mutant suggested that *AtMORC6* play a role in RdDM pathway (Brabbs et al., 2013; Lorkovic et al., 2012). Moissiard et al (2014) reported that the AtMORC6 physically interacts with AtMORC1 and AtMORC2 to enforce gene silencing, moreover, real-time PCR (RT-PCR) from RNA extracted from *atmorc1* and *atmorc2* mutants indicated that *SDC* was derepressed in *atmorc1* mutant but not in *atmorc2* mutant, the expression of two transposons, *ATCOPIA28* and *ROMANIAT5*, showed an increased derepression in the *atmorc1/atmorc2* double mutant compared with *atmorc1* and *atmorc2* single mutants, indicating that AtMORC1 and AtMORC2 act redundantly in transposon silencing, suggesting that AtMORC2 acts redundantly with AtMORC1 to achieve gene silencing. In *Caenorhabditis elegans*, knockdown of the single *MORC* gene is also required for silencing (Moissiard et al., 2012). Additionally, *MORC1*-deficient mice showed that MORC1 is required for transposon repression in the male germline (Pastor et al., 2014). Taken together, MORC family plays an important role in gene silencing in both plants and mammals.

1.4 Objectives

Plants are confronted with various biological challenges in their life. In order to ensure proper development and reproduction, plants have evolved defense strategies to pathogenic infection. To understand the mechanisms of plant resistance to pathogens is of importance to sustainable agriculture and food security.

The mammalian MMPs have been reported to function in resistance to pathogens. However, the function of plant MMPs in disease resistance remained less well understood. In my study, the model plant *Arabidopsis thaliana* was intensively employed to investigate the function of plant MMPs in plant immunity. For this, a set of Arabidopsis *MMP* single, double and triple mutants were produced and employed to analyze how MMPs affect the plant basal defense responses, such as the production of reactive oxygen species (ROS) and callose deposition with the treatment of MAMPs. Next, I investigated the function of MMPs in resistance in way of analyzing the disease phenotypes in various Arabidopsis MMPs mutants and transgenic lines during the infection of necrotrophic fungal pathogen *Botrytis cinerea* and biotrophic fungal pathogen powdery mildew.

The MORC1 and MORC2 were previously demonstrated to function in plant immunity. Moreover, the Arabidopsis MORC1 and MORC6 were reported to involve in gene silencing. However, whether the other homologs of Arabidopsis *MORC* have similar functions in resistance and gene silencing remained elusive. Since the MORC proteins exhibit ATPase activity and putative endonuclease activity, whether these enzymes activity is necessary for the function of MORC is unclear. To address these questions, I identified Arabidopsis *MORC* single mutants of *atmorc1*, *atmorc2*, *atmorc5*, *atmorc6*, *atmorc7* and produced set of transgenic Arabidopsis plants containing mutations in ATPase domain and putative endonuclease domain of *AtMORC1*. Thereafter, the function of MORC in plant immunity was investigated through analyzing the disease phenotype in all *AtMORCs* mutants and transgenic plants during the infection of *Pseudomonas syringae* pv. *tomato* (*Pst*). Furthermore, the role of MORC in gene silencing was investigated in way of examining the expression profile of a transposon gene (*ATCOPIA28*) and silencing-related gene *SDC* (*suppressor of drm2 cmt3*). Subsequently, the expression of the *R* gene *RMG1* (*resistance methylated gene 1*), which is regulated by RdDM (RNA-directed DNA methylation), was analyzed in

AtMORCs mutants and transgenic plants in order to investigate the function of MORC in relating the plant resistance and gene silencing.

2. Material and methods

2.1 Plant growth condition

To surface sterilize the seeds, the seeds of *Arabidopsis thaliana* were washed with ddH_2O for 2 min to remove the inflorescence and clean the seeds surface then centrifuge for 1 min at 4,000 rpm and discard the supernatant. Then add 1 ml 70% ethanol into the tube and shake for 1 min and discard the supernatant. Afterwards, seeds were surface sterilized with 1 ml 3% NaOCl (Stock solution is 12%, 1 ml stock+3 ml H_2O) for 10 min under shaking. Then centrifuge for 1 min at 4,000 rpm and discard the supernatant. The seeds were then rinsed 4-5 times with ddH_2O, pipette the seeds with water on sterile filter paper to dry. Then, put the sterilized seeds on 1/2 MS medium (0.22% salts, 0.5% agar, 1% sucrose, PH 5.4 with KOH) for germination. In order to enhance germination, the seeds in the plates were first placed in the dark at 4°C for 2 days. Afterwards, the plates were transferred into the growing chamber under short-day condition (8 h light/16 h darkness). After 1 week, the seedlings were transferred to soil (soil: sand = 3:1 (v/v)). To keep the high humidity, the plants were covered with a plastic pane for 1 week.

2.1.1 Matrix metalloproteinases (MMPs) protein assays

For the *Arabidopsis* MMPs protein assay, the plants were grown under short-day condition (8 h light/16 h darkness) and 22°C at day/18°C at night and 60% humidity. Four-week-old plants were used for different pathogen inoculation, oxidative burst assay and callose deposition response to MAMP treatment.

2.1.2 Microrchidia (MORC) assays

For the study of the *MORC* genes in plant immunity, the *Arabidopsis morc* plants were grown under long-day condition (14 h light/10 h darkness). Four-week-old plants were used for *Pseudomonas syringae* pv. *tomato* (*Pst Avr Rpt2*) pathogen inoculation and transposons expression assay.

2.2 Extraction of DNA/RNA

2.2.1 DNA extraction

Genomic DNA was extracted from *Arabidopsis* leaves using a quick method for identify T-DNA insertion mutants and transgenic plants. Put one glass beads in 2 ml eppendorf tube and harvested leaves from each plant. Samples were frozen in liquid nitrogen and leaf disks were crushed using TissueLyser II (manufactured by Retsch). Then add 500 µl DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) to the collection tubes and vortex vigorously, after that incubate the samples at room temperature for 5-10 min, add 500 µl chloroform to the same tubes and vortex vigorously. Then centrifuge the samples at 13,000 rpm for 10 min. Take 500 µl of the supernatant into 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 and discard the supernatant. The pellet was kept and washed with 500 µl 70% ethanol by vigorous vortex. Then centrifuge the samples again at 13,000 rpm for 5 min. Discard the supernatant and dry the pellet, then dissolved the pellet in 100 µl double distilled water and incubate at room temperature for 10-20 min and vortex vigorously. Spin down the DNA shortly and the supernatant can be directly used for genotyping.

2.2.2 RNA extraction

For RNA extraction, 4-week-old Arabidopsis leaves were harvested and frozen in liquid nitrogen. Leaf samples were crushed to powder with pre-cooled mortars and pestles in liquid nitrogen. The leaf powder was transferred into 2 ml eppendorf tubes and 1 ml trizol was added to each tube, then vortexed for 15 seconds and kept at room temperature for 5 min. After incubation for 5 min, 200 µl chloroform was added to each tube and vortexed the samples for 15 seconds, then they were incubate at room temperature for 3 min. After that, the samples were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was transferred in a new eppendorf tube (1.5 ml), then 500 µl chloroform was added and vortexed briefly. After that, the samples were kept at room temperature for 10 min. Then samples were then centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was discarded and 1 ml 75% ethanol (treated with DEPC) was added to the pellet and vortex for 15 seconds. Then a centrifugation was performed at 4°C and 13,500 rpm for 5 min. The supernatant was discarded and the pellet was dried under the clean bench. Then 30 µl H_2O (treated with DEPC) was added to dissolve the pellet. After this, the samples were incubated at 65°C for 10-15 min. The concentration of RNA 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.

2.2.2.1 cDNA synthesis

RT-PCR was performed with Fermentas reagents. RNA extraction was performed as described above. Two microgram RNA from each sample was treated with DNase I and RNase Inhibitior. Each sample was added with a

mixture containing 2 µl DNase I (1U/µl, Fermantas, Germany), 2 µl 10×DNase I buffer and 0.5 µl RNase I inhibitor (40 U/µl). DEPC-treated MilliQ H_2O was added to each sample to reach 20 µl final volumes. After 30 min incubation at 37°C, 1 µl EDTA 50mM was added to each sample and incubated for 10 min at 65°C. Ten µl of RNA was used for cDNA synthesis with 1 µl oligo (dT) 18 primer (100 µM) and 1µl Random hexmaer primer (100 µM). After 5 min incubation at 70°C, samples were placed on ice. A second mixture containing 4 µl 5×reaction buffer and 0.5 µl RNase Inhibitor (40 U/l) and 2 µl dNTP's was added to each sample. Then transfer the second mixture to the first mixture. The reactions were incubated in a Professional thermocycler (Aviso GmbH, Germany) following the program of 25°C for 10 min, 42°C for 60 min and 70°C for 10 min. Then the PCR product was kept on ice and 80 µl H_2O (DEPC treated) was added to the sample. Store the cDNA samples at -20°C prior to use.

2.2.2.2 Check the quality of cDNA

After cDNA synthesis, semi-quantitative PCR was performed to check the quality of cDNA synthesis using primers amplify the *Arabidopsis* housekeeping gene Ubiquitin. Then the cDNA were used for quantitative PCR.

25 µl PCR reaction included the following components:

2.5 µl 10X BD Buffer (DNA Cloning Service, Hamburg, Germany)

2.5 µl 2 mM dNTPs

1.5 µl 25 mM MgCl2 (DNA Cloning Service, Hamburg, Germany)

1 µl Ubi5-4 forward primer

1 µl Ubi5-4 reverse primer

0.2 µl DCS Taq Polymerase (5U/µl; DNA cloning services, Hamburg, Germany)

4 µl template cDNA were added, make up the final volume to 25 µl with nuclease free water and set up the PCR reaction. Separate the PCR products in 1% agarose gel at 120 V for 1 h.

PCR reaction for checking cDNA

Temperature(℃)	Time	
Initial denaturation 95	3 min	
Denaturation 95	30 sec	35 cycles
Annealing 58	30 sec	
Elongation 72	30 sec	
Final Elongation 72	5 min	

2.3 Use of *Arabidopsis* knock-out mutants

2.3.1 Identification of T-DNA mutants (MMPs and MORCs)

2.3.1.1 Identification of *at1-mmp* mutant

The *at1-mmp* mutant (GABI-Kat mutant GK-575B01, NASC code N753809-N753820) was generated from GABI-Kat population. For identification of homozygous lines, the seeds were germinated on selective medium (1/2 MS+sulfadiazine), then the method for genomic DNA extraction as described in Chapter 2.2.1. T-DNA insertion was confirmed by PCR using a T-DNA border primer and a gene-specific primer (right primer). Another PCR was performed to identify homozygous plants by the gene-specific primer. The procedure followed to identify the homozygous population is described in figure 2-1 and the primers used in Table S1. The total volume of PCR reaction are 25 µl which contains 2.5 µl 10 x buffer, 2.5 µl 2 mM dNTPs, 1.5 µl 25 mM $MgCl_2$, 0.6 µl forward and reverse primer, 0.2 µl DNA polymerase, 1 µl template DNA (≈50 ng) and 16.1 µl of H_2O. PCR reactions are initiated with

95°C for 3 min and then 35-40 cycles for amplification including 95°C for 30 seconds, 30 seconds for annealing time (temperature is depending on the gene), and 72°C for 30-90 seconds (depending on the size of the band), a final extension of 5 min at 72°C.

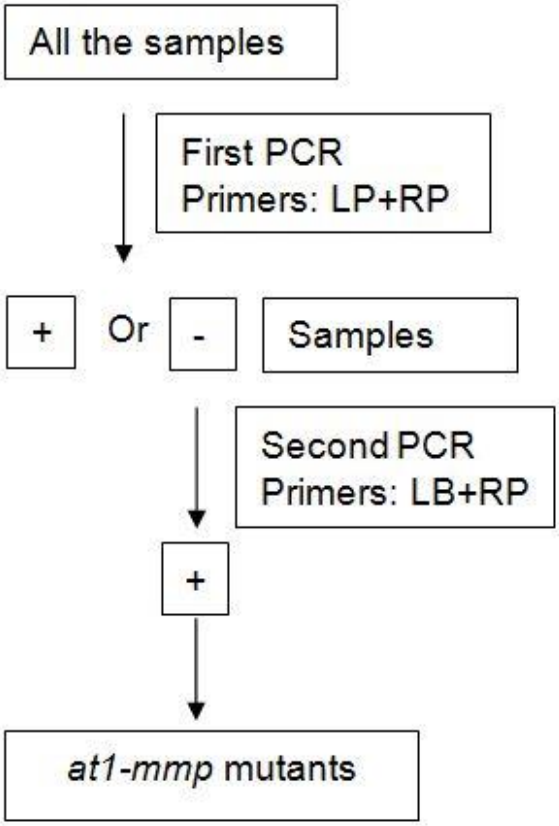


Figure. 2-1. Identification of the *at-mmp1* homozygous mutant.

Two PCR reactions were performed with LP, RP and LB primers. PCR1 was performed with LP and RP primer of *At-MMP1*. PCR1 negative plants were tested by PCR 2 with LB and RP primer of *At-MMP1*. LP: Left primer. RP: Right primer. LB: Left border primer.

2.3.1.2 Identification of *atmorc* mutants

Molecular phylogenetic analysis showed that there are seven homologs in *Arabidopsis* genome. We have used five *AtMORCs* mutants *atmorc1*, *atmorc2*, *atmorc5*, *atmorc6* and *atmorc7* to study the potential function of AtMORCs in

plant immunity system. T-DNA insertion lines for *AtMORCs* were ordered from NASC (European *Arabidopsis* stock center). These mutant lines were segregating and thus required identification of homozygous lines. For the identification, first the seeds were germinated on selection medium (1/2 MS medium+ sulfadiazine), identified most promising candidate lines according to the growth condition. The genomic DNA was extracted follow the method described in Chapter 2.2.1. Finally, the homozygous mutants were identified by PCR with the gene specific primer and T-DNA border primer, the method is the same as described in Chapter 2.3.1.1. The primer sequences and product size for genotyping are described in Table 1-2.

Table. 1-2. Primers for genotyping of *atmorc* mutants

Genotype	Insertion line	LP	RP	Expected size LP/RP(bp)	Expected size LBb1.3/RP(bp)
AtMORC1	SAIL_1239_C08	CGTATCTCAGCCGCTAACTTG	AAGCAGCTGCAGTGGATTATG	1152	545-845
AtMORC2	SALK_072774C	CTACTCAGAGCGTTGGCATTC	GTTGTAGCTGTATGGGGCTTG	1017	432-732
AtMORC5	SALK_021871C	CTGTGTCGGACTGAAACAGTG	GTTAGTGATCGGAGACATGGC	1092	575-875
AtMORC6	GK_599B06	ACATCTTCCAATGGCTGAATC	GCTGGTGTCACTTCTTCATCC	1067	534-834
AtMORC7	SALK_151643C	AAAATAATCCGGTCTTCCACG	ATGATGTTTTGCGTCCTTCAC	1165	572-872
LBb1.3		ATTTTGCCGATTTCGGAAC			

2.3.2 Production and identification of *at-mmp* triple mutant

2.3.2.1 Crossing of *Arabidopsis* T-DNA mutants

Triple mutant was generated from crossing of one single mutant *at5-mmp* and double mutant *at2-mmp/at3-mmp* (Table. 2-1). Seeds of single mutant and double mutants were grown on 1/2 MS medium for germination and the

seedlings were transplanted into soil and grown under short-day condition. After 4-5 weeks, the plants were transferred to long-day condition to promote flowering. For crossing, the mature siliques as well as open flowers and buds from mother plants were removed with sterilized forceps. The meristems with too small buds were also removed, keeping only flower buds of suitable size 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. Between different crosses, forceps were cleaned with 70% ethanol then washed with H_2O and dried with tissue paper. After 4-5 weeks, siliques with the hybrid seeds were mature and harvested.

Table. 2-1. Combination of crossing to produce triple mutant (*atmmp2/atmmp3/atmmp5*)

Single mutant (*at5-mmp*)	Double mutant (*at2-mmp*, *at3-mmp*)	Crossing combination
HM237	F3-Ⅱ-7	HM237×F3-Ⅱ-7
		HM237×F3-Ⅱ-11
HM246	F3-Ⅱ-11	HM246×F3-Ⅱ-7
		HM246×F3-Ⅱ-11

2.3.2.2 Identification of triple mutant (*at2-mmp/at3-mmp/at5-mmp*)

The seeds produce in Chapter 2.3.2.1 were collected and grown under short day condition. DNA extraction was performed as described in Chapter 2.2.1. Triple mutant was identified by PCR with specific primer in Table S1 from parent lines. Six serial PCR were carried out for identification of homozygous

triple mutant described in figure 2-2. LP and RP primers from *At2-MMP* were used for the first PCR (PCR1). The samples not showing amplicon after PCR 1 were selected for further test in PCR 2, using LP and RP primer from *At3-MMP*. Likewise, the samples with no amplicon after PCR 2 were used for PCR 3, using LP and RP primer from *At5-MMP*. The samples which did not show product were used for PCR4. These samples were tested in PCR4 using the LB and RP primer (from *At2-MMP*), the samples showing amplicon in PCR4 were used for further test in PCR5. LB and RP primer (from *At3-MMP*) were used in PCR5, the samples showing amplicon were used for PCR6. The primers LB and RP (from *At5-MMP*) were used in PCR6 being positive the samples that show amplicon. The triple mutant should not produce any amplicon with LP and RP primers in PCR 1, PCR 2 and PCR 3, but they should show amplicon in PCR 4, PCR 5 and PCR 6.

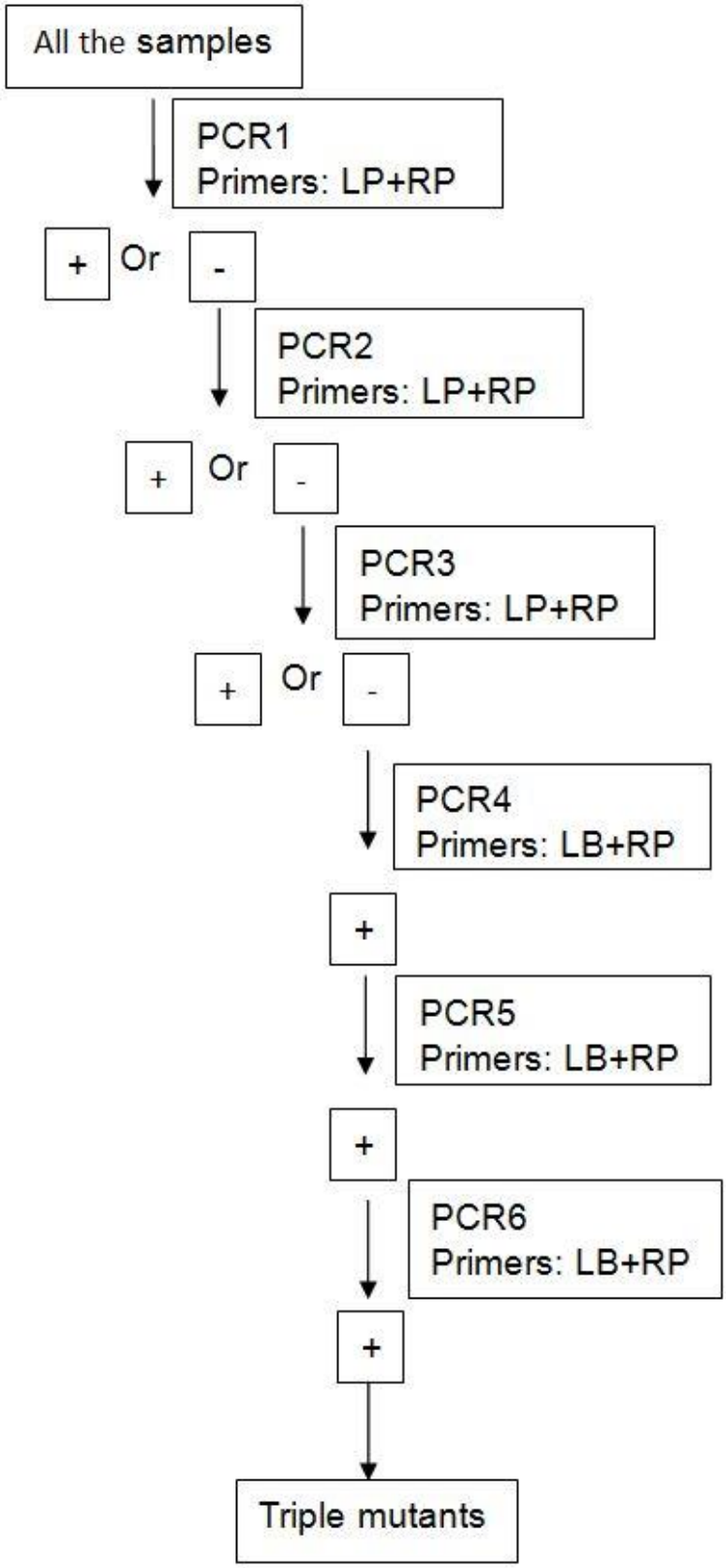


Figure. 2-2. Identification of homozygous *at2*-mmp/*at3-mmp*/*at5-mmp* triple mutant.

Six PCR reactions were performed with LP, RP and LB primers. PCR1 was

performed with LP and RP primer of *At2-MMP*. PCR1 negative plants were tested by PCR 2 with LP and RP primer of *At3-MMP*. Both PCR1 and PCR2 negative plants were tested by PCR 3 with LP and RP primer of *At5-MMP*. The plants which are negative for PCR 1, PCR 2 and PCR 3 were used for PCR 4 with LB and RP primer of *At2-MMP*, the positive plants were then verified by PCR 5 with LB and RP primer of *At3-MMP*. Both PCR4 and PCR5 positive plants were tested by PCR 6 with LB and RP primer of *At5-MMP*. LP: Left primer. RP: Right primer. LB: Left border primer.

2.4 Generation of Arabidopsis over-expression stable transformants

2.4.1 Cloning and construction of transformation vectors

To generate the *AtMORC1 mutations* constructs, the genomic DNA of the *Arabidopsis* was used to amplify the full length sequence by PCR. Primer pairs (Table. S1) were used for amplification of *AtMORC1* D111K, *AtMORC1* EDE108/115/116KKK, *AtMORC1* D366K, *AtMORC1* E441K, *AtMORC1* E450K, respectively. The numbers denote amino acid position in AtMORC1 structure, D denotes aspartic acid, E denotes glutamic acid and K denotes lysine. I replaced the aspartic acid/glutamic acid with lysine. The fragment was first cloned into pET28a vector and sequenced by LGC Genomics (Berlin, Germany). For cloning, 10 µl of ligation reaction was added to 50 µl competent cells of *E. coli*. Then the sample was incubated on ice for 30 min, heat shock the sample at 42°C for 50 to 60 seconds. The sample was kept on ice quickly after heat shock, 400 µl LB medium was added and shaked for 1.5 h in 37 °C. The cells were spread on LB-Agar plates with Spectinomycin (50 µg/ml). The plates were incubated at 37°C for one day, and then the colonies were picked and confirmed by PCR. The positive colony was selected for Miniprep and *Agrobacterium* transformation. In order to get enough plasmid, Minprep extraction of the plasmid was performed using Pure Yield Plasmid

Miniprep System (Promega). The transformation was performed following the instructions from the manufacturer. The construct pET28a:AtMORC1 was digested with HindIII, and the 1920 bp fragment containing AtMORC1 was purified in agarose gel. The plasmid p35S-BM was also digested with HindIII and the 5' phosphates were removed with CIAP (Calf intestinal alkaline phosphatase) to avoid rejoining. The fragment containing AtCRT1 was ligated into p35S-BM and subcloned into the SfiI sites of the *Agrobacterium* transformation vector pLH6000 in sense orientation under control of *35S* promoter. The construct was then transformed into the *Agrobacterium* strain GV3101 by electroporation using Gene Pulser Xcell Electroporation system (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacture indications. Around 1 mg of plasmid was added to the competent cells GV3103 (50 µl) and mixed, then they were incubated on ice for 10 min. After incubation the cells were introduced into a electroporation cuvette and the electro shock was applied. 600 µl of LB medium was added to the cells in the cuvette after transformation, and the cells were transferred to an eppendorf tube and incubated at 28°C for 1.5 h. The cells were then plated on LB medium containing 25 µg/ml Rifampicin, 50 µg/ml Gentamicin and 50 µg/ml Spectinomycin. After 2 days of incubation at 28°C, the colonies were picked and confirmed by PCR. The positive colonies were selected to prepare for the agro-transformation described in Chapter 2.4.2.d

2.4.2 Floral dip transformation

Agrobacterium-mediated transformation of *A. thaliana* was carried out by the floral dip method (Clough and Bent, 1998). The background *Arabidopsis* plants *atmorc1/atmorc2* mutant were grown in soil under short-day conditions. There are 8-10 plants per pot. After 3 weeks, the plants were put into long-day conditions. The first inflorescence shoots were removed to induce the growth

of more inflorescence. Plants were used for transformation after about 1 week, when the secondary inflorescence emerged. Three days prior to plant transformation, a 5 ml liquid pre-culture of *Agrobacterium* carrying a suitable binary vector was prepared and incubated at 28°C with vigorous agitation. The liquid culture consisted of LB medium containing antibiotics (Rifampicin 25 µg/ml, Gentamicin 50 µg/ml). Two days before the infiltration, 200 ml of YEB medium (1%Bacto-Peptone, 1%Yeast extract, 0.5% NaCl) was inoculated with 1 ml of the pre-culture and incubate again with vigorous agitation for additional 48 h at 28°C. Use YEP medium for higher Agrobacterium density. Stop watering the plants and allow the soil to dry out a little, so that it will be less prone to falling out of the pots during dipping. After two days, the agrobacterium was centrifuged at 6000 rpm for 10 min at room temperature and the cell pellet resuspended in 400 ml of infiltration medium(1/2 MS salts including vitamins, 5% sucrose, pH 5.7) supplemented with 0.04% 6-benzylaminopurine (BAP, 10 ul L^{-1} of a 1 mg mL^{-1} stock in dimethyl sulfoxide (DMSO)) and 0.02% Silwet L-77. A glass bell jar connected via a condensation trap to a Leybold Trivac oil pump (type S8B/AF 4-8) was used for vacuum infiltration. A glass tray filled with 400 ml of the *Agrobacterium* suspension was placed in the jar. The inflorescence shoots were dipped into the suspension, a pressure of 16 mbar was used for 2 min to allow the submersion of the inflorescence shoots in the suspension. After 2-3 min treatment the vacuum was immediately released and the infiltration step was repeated. The 400 ml bacterial suspension was re-used for three pots. After the infiltration treatment, the plants were covered with a transparent cover for 2 days. After 2 days, the cover was removed and the plants transferred to a long-day growth chamber. Mature seeds were collected in bags after about 3-4 weeks.

2.4.3 Presence of construct

Seeds from T0 plants after floral dip were sowing in 1/2 MS containing 30 mg/l hygromycin and incubated at 4°C refrigerator for 2 days before being transferred to a short day condition growth chamber. Transformants were selected by their hygromycin resistance. After 2 weeks, green seedlings with long roots were transformed to soil. Two weeks later, these plants were tested by PCR to confirm the presence of construct.

2.4.4 Expression of the construct

2.4.4.1 Transcript levels (RNA)

After cDNA synthesis, the cDNA samples were used for checking the transcript level by semi-quantitative PCR with AtMORC1 full length primers (described in Table S1). In order to get clear results, we used the Phusion High-Fidelity DNA Polymerase, and the PCR reaction as flow:

10 µl 5X Phusion HF Buffer

5 µl 2 mM dNTPs

0.5 µl AtMORC1 forward primer

0.5 µl AtMORC1 reverse primer

0.5 µl Phusion DNA Polymerase

5 µl template cDNA were added, make up the final volume to 50 µl with nuclease free water and set up the PCR reaction. Separate the PCR products in 1% agarose gel at 120 V for 1.2 h.

PCR reaction for transcript level assay

Temperature(°C)	Time	
Initial denaturation 98	30 sec	
Denaturation 98	10 sec	35 cycles
Annealing 58	30 sec	
Elongation 72	2 min	
Final Elongation 72	5 min	

2.4.4.2 Protein expression of generated constructs

2.4.4.2.1 Protein extraction

For protein extraction, 4-week-old *Arabidopsis* leaves from *AtMORC1* overexpression line with myc-tag were harvested and frozen in liquid nitrogen. Leaf samples were crushed to powder with pre-cooled mortars and pestles in liquid nitrogen. The leaf powder was transferred into 2 ml eppendorf tubes and 200-300 µl Laucus buffer (1 tablet of protease-inhibitor + 10 ml Laucus buffer) was added to the sample powder and vortexed for 1 min, then centrifuged at 13,000 rpm at 4°C for 20 min. The supernatant was transferred into 1.5 ml eppendorf tubes for Bradford analysis. Take 10 µl of supernatant add 990 µl Bradford-Reagent, using 10 µl of Laucus buffer add 990 µl Bradford-Reagent for blanking. Then the protein can be used for western blot.

2.4.4.2.2 Western blot

Before western blot, the proteins as described in Chapter 2.4.4.2.1 were boiled in a water bath for 5 min at 95°C, and then the samples were used for loading. For western blot, proteins were separated by SDS-polyacrylamide gel

electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane (Roti®-PVDF, pore size 0.4 um, ROTH, Germany) with semi-dry electrophoretic transfer cell (Bio-Rad) at 0.3 A for 1 h. The PVDF membrane was incubated 1 min in methanol afterwards in 1× Towbin buffer (25 mM Tris, 192 mM glycine and 20% [v/v] methanol) for 20 min. After protein transfer, the PVDF membrane was washed three times with TBS buffer (1.21% Tris, 8.76% NaCl, adjust pH to 7.9), 5 min per time. Non-specific binding was blocked using 5% (w/v) milk powder (ROTH, Germany) in TBS buffer at room temperature for 2 h. After three times washing with TBS buffer, the membrane was incubated in 5% milk powder contained c-myc antibody (1: 3000) overnight at 4°C on a shaker. The membrane was washed three times for 5 min/time in TBS buffer and incubated with western blotting detection reagent luminol enhancer solution and peroxide solution (GE Healthcare) for 1 min at room temperature. After that, the blot was developed using Amersham Hyperfilm ECL (GE Healthcare).

2.5 Pathogen infection

2.5.1 *Botrytis cinerea* inoculation

B. cinerea strain B05.10 was grown on HA agar medium (1% malt extract, 0.4% yeast extract, 0.4% glucose, 1.5% agar, pH 5.5. Plant leaves were detached from 4-week-old *Arabidopsis* plants and placed in petri dish containing 0.5% agar medium. To infect plants, conidia were collected from 14-day-old culture plate, and the spore density was adjusted in 12 g L^{-1} potato dextrose broth (PDB, Duchefa Biochemie, Haarlem, The Netherlands) to $5x10^4$ conidiospores ml^{-1} for pathogen resistance assay. Inoculation was performed by placing 5 µl of spore suspension in the leaf center. Cover the petri dish and incubate the leaves at room temperature. Depending on the

symptom development rate, three to six days after infection, the leaf samples were photographed and measured lesion size with ImageJ software.

2.5.2 Powdery mildew (*Golovinomyces orontii*) inoculation

For *G. orontii* inoculation, the conidia spores were collected from heavily infected plants with Tween H_2O (1:20,000). The density of spore suspension was adjusted to 20,000-40,000 conidia ml^{-1} and sprayed on 4-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 short day condition under 22°C. For quantification of the fungal growth, the infected plants were harvested when clear symptom is appearing. The fresh weight was measured and the plants were rinsed with Tween H_2O to collect the conidia spores. The number of conidia per mg of fresh weight was determined to quantify the fungal growth.

2.5.3 *Pseudomonas syringae* pv. *tomato* DC3000 inoculation

Pst Avr Rpt2 are streaked out from a –80°C glycerol stock onto a plate of King's B medium (1% protease peptone, 0.15% anhydrous K_2HPO_4, 1.5% glycerol, 1.5% Agar, pH 7.0) containing 50 mg/l Rifampicin and Kanamycin 25 mg/l and grown for 2 or 3 days at 28°C. After three days, the bacterial were scrapped off with sterile 10 mM $MgCl_2$ using a glass spatula and the optical density were adjusted to 0.2. The Optical Density (OD) of the bacterial cell suspension is quantified using a spectrophotometer set at 600 nm. For *Pst* DC3000 an OD_{600}=0.2 is approximately 1 x 10^8 colony-forming units/ml. Injection of dense bacterial suspensions (~10^8cfu/ml) of avirulent bacteria is used to elicit a confluent hypersensitive response. A lower level of inoculum (OD600=0.0002 of *Pst* DC3000 is 1 x 10^5cfu/ml) is used for infiltration. The

inoculation was done by pressure infiltration. A leaf is selected and marked so that it can be identified later. A 1ml needleless syringe containing a bacterial suspension is used to pressure infiltrate the leaf intracellular spaces. Before infiltration, spray the estradiol (30 µM), because the Arabidopsis MORC1 and barley MORC1 overexpression in *atmorc1/atmorc2* double mutant are under control of the estradiol inducible promoter (Zuo et al., 2000). Leaf disks (0.5 cm^2) were harvested at 72 h after infiltration and ground in 10 mM $MgCl_2$. After grinding of the tissue, the samples were thoroughly vortex-mixed and diluted in a 10 fold serial dilution (10^1 to 10^5). Samples were finally plated on King's B medium supplemented with 50 mg/l Rifampicin and Kanamycin 25 mg/l. Plates were incubate for 2 days at room temperature and the colony-forming units were counted.

2.6 Oxidative burst assay

For ROS detection, 5 mm leaf disks were cut from 4-week-old healthy plants with sharp puncher and were floated overnight in 200 µl H_2O in 96-well plates in dark to minimize wounding effect. For ROS measurement, the water was replaced with 200 µl master mix aqueous containing 30 µg/ml luminol (Sigma, 15 mg/ml stock in DMSO) and 20 µg/ml horseradish peroxidase (10 mg/ml in water, Sigma, P6782) under low-light condition. Elicitor flg22 (100 nM) was injected by TECAN Infinite®F200 microplate reader (TECAN, Switzerland) to trigger ROS production. Luminescence was measured for 60 cycles, 60 seconds per cycle.

2.7 Callose deposition assay

For detection of callose deposition, 5-week-old *Arabidopsis* plant leaves were infiltrated with 1 µM flg22 for 24 h. The leaves were collected and fixed in

ethanol: glacial acetic acid (3:1) for several hours, under shaking. Then the solution was discarded and put fresh solution to incubate overnight at room temperature until the leaves were transparent. The next day, the leaves were re-hydrated in 70% ethanol for 15 min, then in 50% ethanol for 15 min, and washed several times with water. After several water washes, leaves were incubated in 150 mM K_2HPO_4 (PH9.5) solution containing 0.01% aniline blue for 1 to 2 h in darkness. Callose was observed under the microscope.

2.8 Transposon expression assay (qPCR)

For the measure of transposons expression, the leaves of 3-week-old plants were harvested 6 h after estradiol spraying (30 μM). Then RNA extraction and cDNA synthesis were performed as previously described in Chapter 2.2.2. Quantitative PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma) to amplify the target genes. Primer sequences used for transposon expression are described in Table 2-2 refer to the publication of Moissiard et al (2014).

Table. 2-2. Sequences of primers used for transposon expression

Primers	Sequence
ACTIN 7_LP	TCGTGGTGGTGAGTTTGTTAC
ACTIN 7_RP	CAGCATCATCACAAGCATCC
SDC_LP	AATGTAAGTTGTAAACCATTTGAACGTGACC
SDC_RP	CAGGCATCCGTAGAACTCATGAGC
ATCOPIA28_LP	AGTCCTTTTGGTTGCTGAACA
ATCOPIA28_RP	CCGGATGTAGCAACATTCACT
RMG1_LP	TCGGTATCTGTCACCTCATCTG
RMG1_RP	GCTTTCCTCTTGGTCTACATGG

3. Results

3.1 Identification of *at-mmp* mutants

3.1.1 Identification of *at1-mmp* mutant

Molecular phylogenetic analysis indicated that there are five *MMP* homologs in the Arabidopsis genome. The four *at-mmp* mutants *at2-mmp*, *at3-mmp*, *at4-mmp* and *at5-mmp* are available in this study except for the *at1-mmp* mutant. To investigate the potential function of all At-MMPs in plant immunity, the T-DNA insertion lines for *at1-mmp* were ordered from NASC. These mutants were segregated and thus required the identification of homozygous lines. The seeds were firstly germinated on selection medium (1/2MS medium+sulfadiazine). According to the growth condition of the seeds and the ration of survival / died seedlings (Table.3-1), the most promising candidate lines of N753809, N753811, N753815, N753818, N753819 and N753820 were selected (Fig. 3-1). These lines were further screened for the homozygous plants by the PCR as described in Chapter 2.3.3.1, method which is described in Figure 2-1. Finally, the homozygous mutants were identified by PCR (Fig. 3-2). In PCR1 (Fig. 3-2 A), the LP and RP primers were used to amplify the wild-type of *at1-mmp*, all of the eighteen plants did not show fragment. In PCR2 (Fig. 3-2 B), the primer LBb1.3 and RP primer were used to detect the insertion, twelve plants (2, 3, 4, 5, 8, 9, 10, 12, 13, 14, 15, 16) showed amplification. Amplification of a fragment in PCR2, but not in PCR1 implies that the corresponding line is homozygous, so these twelve plants are homozygous of *at1-mmp*, 4 plants (plant 2, 3, 4, 5) from line N753811, 8 plants (plant 8, 9, 10, 12, 13, 14, 15, 16) from line N753820 (Table. 3-2).

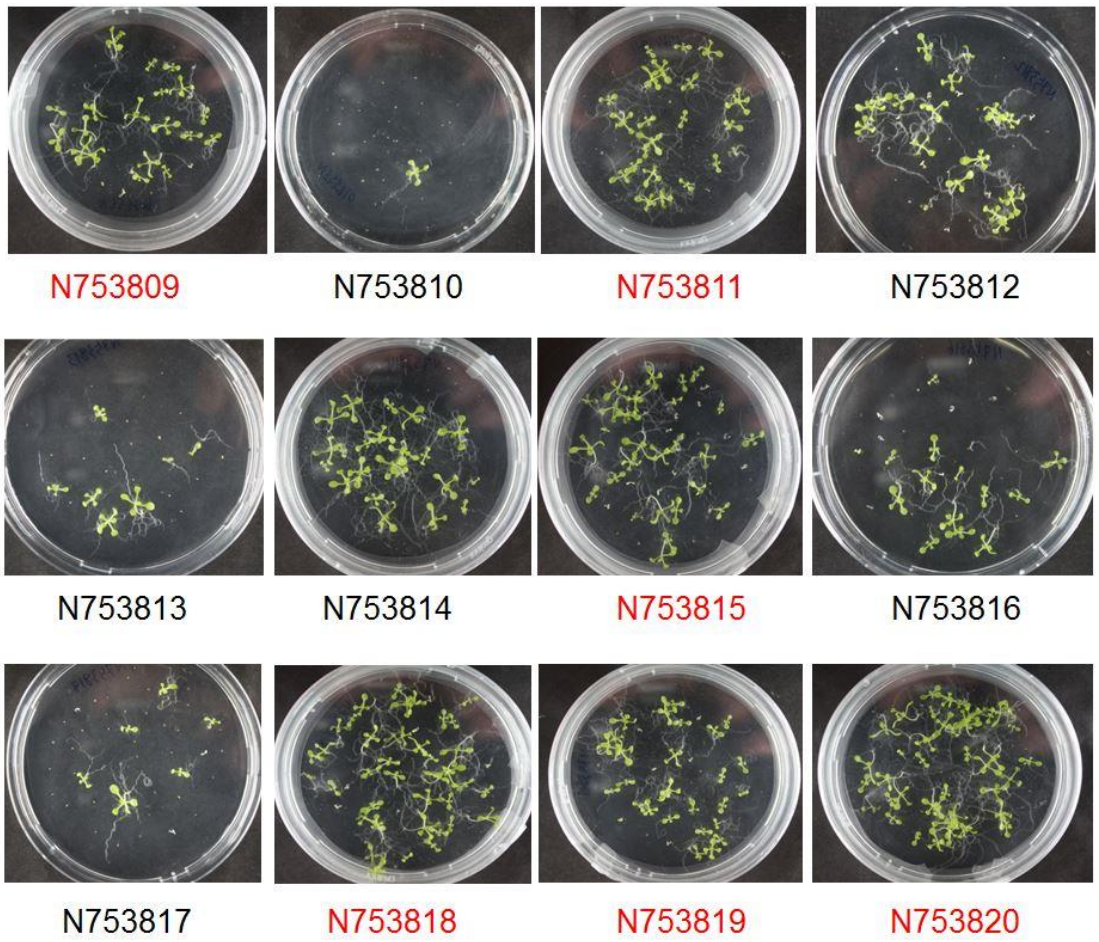


Figure. 3-1. The growth of *at1-mmp* seedlings on selective medium.

The sterilized seeds were germinated on selective medium (1/2 MS medium+sulfadiazine). After 10 days, the number of growing seedlings was counted.

Table. 3-1. The growth condition of seeds in selection medium

At-MMP	NASC Code	Name	Survive/Die
At1-MMP	N753809	GK-575B01.01	22:5
At1-MMP	N753810	GK-575B01.01	1:25
At1-MMP	N753811	GK-575B01.01	22:8
At1-MMP	N753812	GK-575B01.01	24:18
At1-MMP	N753813	GK-575B01.01	8:18
At1-MMP	N753814	GK-575B01.01	21:5
At1-MMP	N753815	GK-575B01.01	38:8
At1-MMP	N753816	GK-575B01.01	19:9
At1-MMP	N753817	GK-575B01.01	9:39
At1-MMP	N753818	GK-575B01.01	39:6
At1-MMP	N753819	GK-575B01.01	32:5
At1-MMP	N753820	GK-575B01.01	32:12

A

B

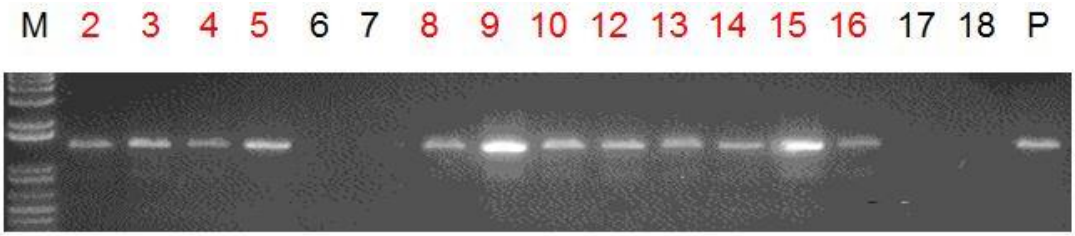


Figure. 3-2. Identification of homozygous T-DNA insertion *at1-mmp* mutants.

The seedlings were transferred from selection medium to soil: sand (3:1) pot, genomic DNA was extracted from each line used for PCR. **A**: The gene specific primer LP and RP were used to detect the presence of the wild-type version, all of the eighteen plants have no amplified PCR products. **B**: The border primer LBb1.3 and RP primer were used to detect the T-DNA insertion, twelve plants with red marker (2, 3, 4, 5, 8, 9, 10, 12, 13, 14, 15, 16) showed amplification.

Table. 3-2. Homozygous mutant of *at1-mmp*

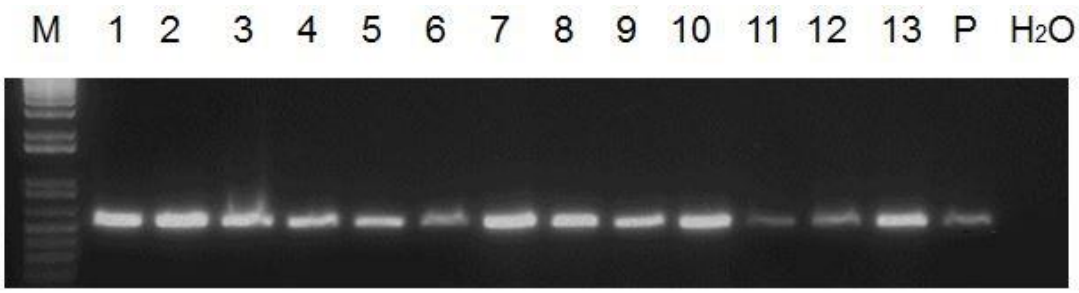
At-MMP	NASC Code	Number of plants tested	Number of homozygous plants
At1-MMP	N753811	10	8
At1-MMP	N753820	8	4

3.1.2 Generation of *at2-mmp at3-mmp at5-mmp* triple mutant

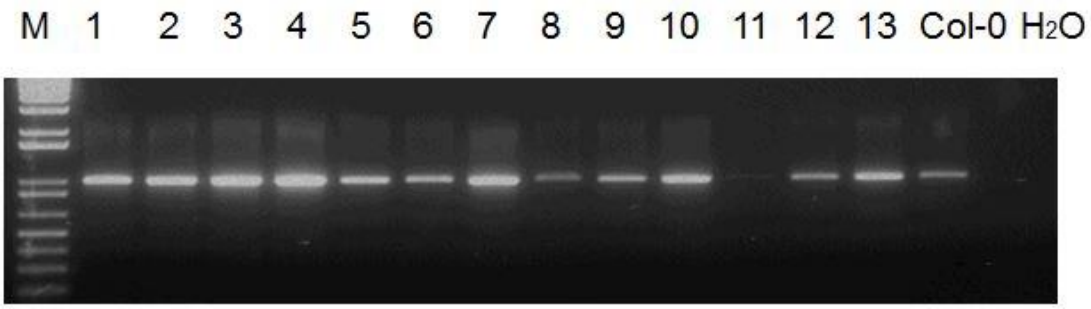
The triple mutant was produced through crossing of *at5-mmp* single mutant and *at2, 3-mmp* double mutant. All the lines of F1 generation are heterozygous. Thereafter, the F1 hybrids were self-crossed in order to select the homozygous mutants. The workflow for identification of homozygous triple mutant is as described in Chapter 2.3.2.2. Homozygous plants have no amplified PCR products with LP/RP primers but show amplification with LB/RP primers (Fig. 3-3). In principle, six PCR reactions were used to identify the homozygous lines in the crossing combination. The *at2, 3-mmp* double mutant and *at5-mmp* single mutant were both used as father parent and mother parent to produce *at2, 3, 5-mmp* triple mutant. After the PCR selection in three generations, the homozygous *at2, 3, 5-mmp* triple mutant were

identified out of 698 plants (Table 3-3). In the first generation, the gene specific primers LP and RP were used to confirm the lines which are heterozygous (Fig. 3-3 A and B). In F2 generation, the T-DNA border primer LBb1.3 and gene specific primer RP were used to screen out that the line #161 is homozygous for *at3-mmp* and *at5-mmp*, but heterozygous for *at2-mmp* (Fig. 3-3 C). In the F3 generation of the line #161, the line #698 is detected to be homozygous for all the mutants of *at2-mmp*, *at3-mmp* and *at5-mmp* (Fig. 3-3 D).

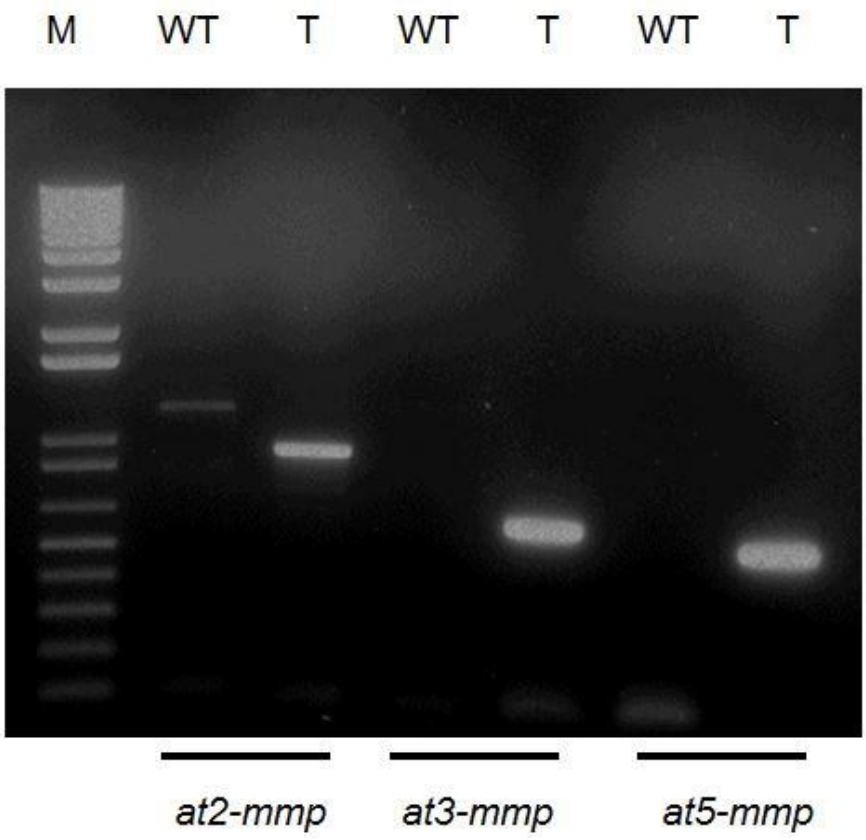
A



B



C


M
WT
T
WT
T
WT
T
at2-mmp
at3-mmp
at5-mmp

D

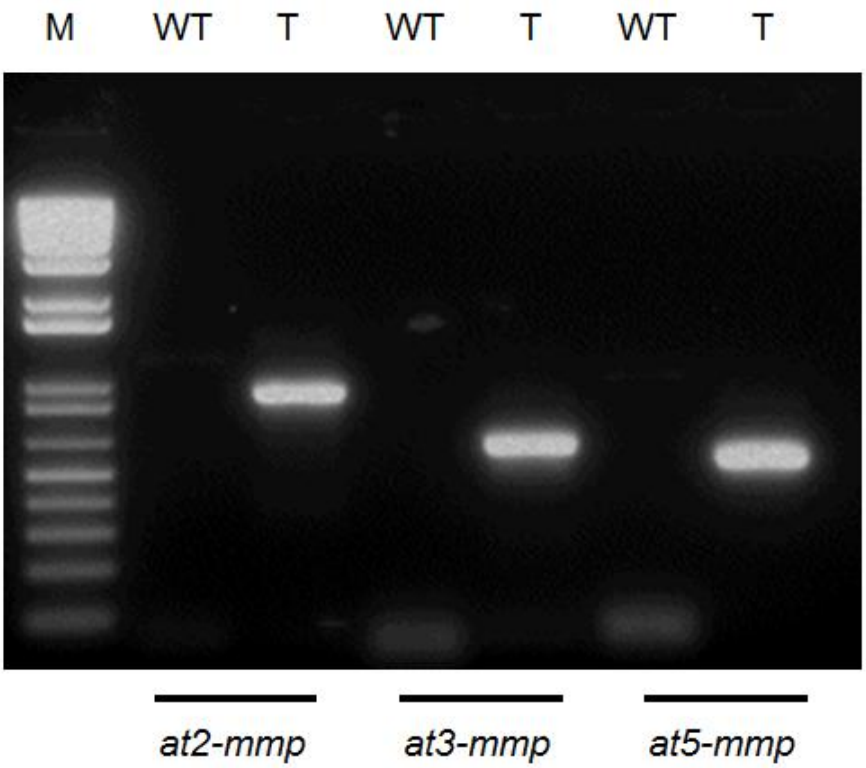

M
WT
T
WT
T
WT
T
at2-mmp
at3-mmp
at5-mmp

Figure. 3-3. Identification of homozygous triple mutant *at2, 3, 5-mmp*.

Genomic DNA was extracted from 3-week-old Arabidopsis plant leaves, used for PCR to screen out the homozygous mutant. **A**: The gene specific primer LP and RP were used to detect the presence of the wild-type version of *at2-mmp*, all of the thirteen plants have amplified PCR products. **B**: The gene specific primer LP and RP were used to detect the presence of the wild-type version of *at3-mmp*, all of the thirteen plants have amplified PCR products as *at1-mmp*. **C**: The LP and RP primers were used to detect the presence of the wild-type version of *at2-mmp*, *at3-mmp* and *at5-mmp*, and the border primer LBb1.3 and RP primer were used to detect the T-DNA insertion of *at2-mmp*, *at3-mmp* and *at5-mmp*, the plant #161 is homozygous for *at3-mmp* and *at5-mmp*, but heterozygous for *at2-mmp*. **D**: The same combination of primers was used to detect the next generation of plant #161. The plant #698 has no amplification for LP and RP primers of *at2-mmp*, *at3-mmp* and *at5-mmp*; meanwhile, it has amplification for LBb1.3 and RP primers of *at2-mmp*, *at3-mmp* and *at5-mmp*. It indicated that the plant #698 is homozygous for all the mutants of *at2-mmp*, *at3-mmp* and *at5-mmp*. WT: wild-type, T: T-DNA.

Table. 3-3. Homozygous mutant of *at2, 3, 5-mmp*

Crossing combinations	Number of tested F1 plants	Number of heterozygous lines for *at2-mmp* and *at3-mmp*	Number of tested F2 plants	Number of homozygous lines for *at3-mmp* and *at5-mmp*	Number of tested F3 plants	Number of homozygous lines for *at2-mmp*, *at3-mmp* and *at5-mmp*
♀HM237(*at5-mmp*) ♂F-II-7(*at2,3mmp*)	13	13	148	1 (Line #161)	537	1 (Line #698)

3.2 At-MMPs are required for pattern-triggered immunity

3.2.1 Callose response to MAMP in *at-mmp* mutants and *At-MMP* OE transgenic plants

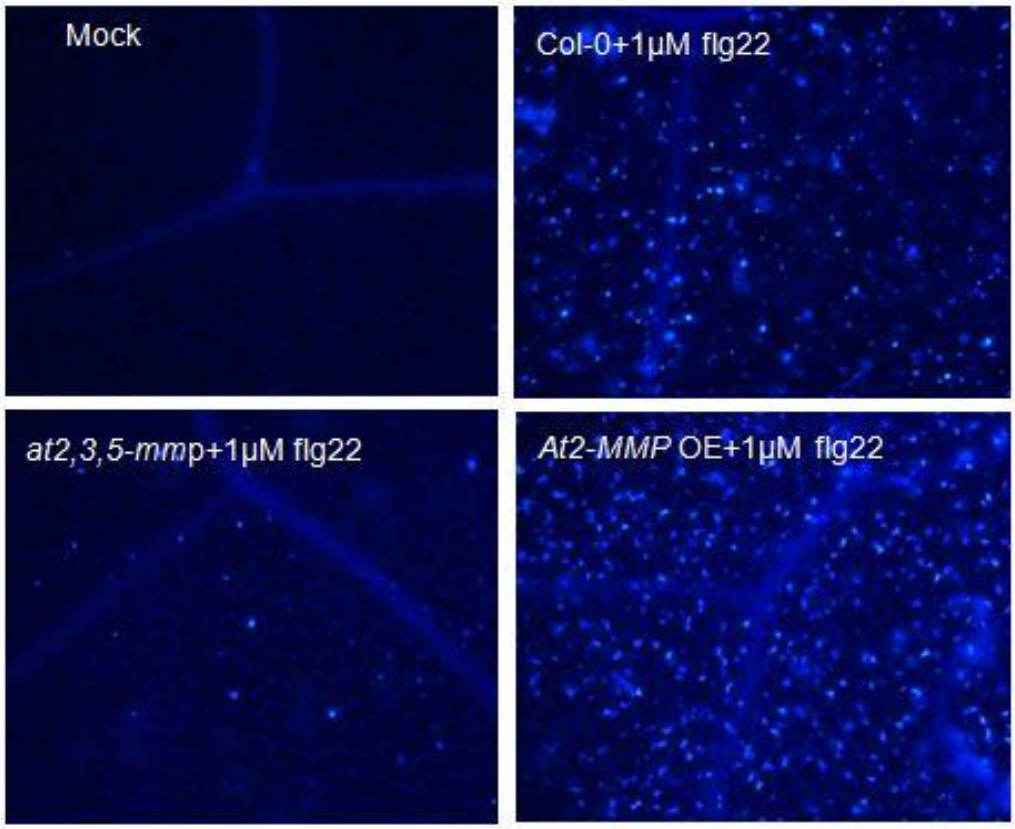
To assess whether At-MMPs participate in plant basal defense, I monitored series of hallmark events of MAMP-triggered immunity in WT, *at2, 3, 5-mmp* triple mutant and *At2-MMP* overexpression line. Callose deposition is known to be a hallmark of FLS2-mediated PTI (Brown et al. 1998; Gómez-Gómez et al. 1999a). Therefore, the callose deposition of the plants with the treatment of flg22 was firstly observed. The results showed a regular callose deposition in the leaf of wild-type plant; however, in comparison to wild-type, the callose deposition was significantly reduced in *at2, 3, 5-mmp* triple mutant. As expected, the reduction was able to be complemented in the *At2-MMP* overexpression line, which exhibited a comparable level of callose deposition to the wild-type (Fig. 3-4 A).

3.2.2 ROS response to MAMP in *at-mmp* mutants and *At-MMP* OE transgenic plants

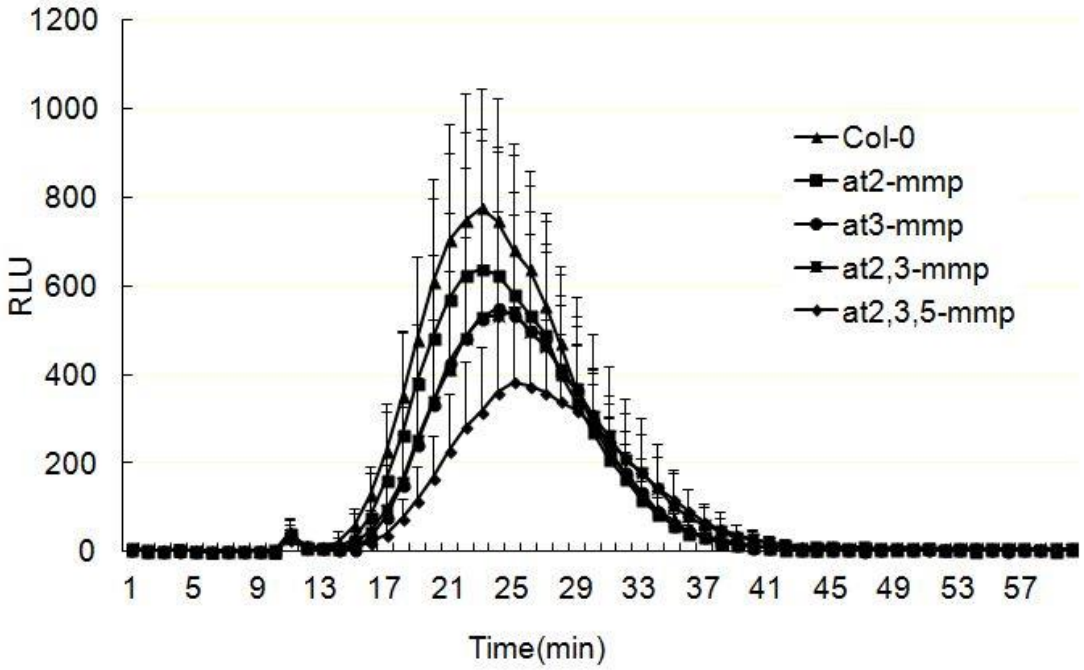
Subsequently, I compared the flg22-induced ROS production in wild-type, single *at-mmp* mutants, *at2, 3-mmp* double mutant, *at2, 3, 5-mmp* triple mutant and *At2-MMP* overexpression line. The mutants of *at2-mmp*, *at3-mmp* showed slight less ROS production compared with wild-type. Nevertheless, *at2, 3-mmp* double mutant displayed around 30% less ROS accumulation than wild-type (Fig. 3-4 B). Notably, *at2, 3, 5-mmp* triple mutant exhibited significant reduction of ROS production, which was nearly 50% lower than that in wild-type at 30 min after flg22 treatment (Fig. 3-4 B). On the contrary, the reduction of ROS generation was complemented in the *At2-MMP* overexpression line, which showed even higher level of ROS generation

compared with wild-type (Fig. 3-4 C). Moreover, I further investigated the flg22-induced ROS production in other single mutants of *at1-mmp, at4-mmp* and *at5-mmp*, which showed no, if any, slight lower ROS level compared with wild-type (data not shown). Taken together, these data indicated that At-MMPs are required for the MAMP-triggered immunity in plant.

A



B



C

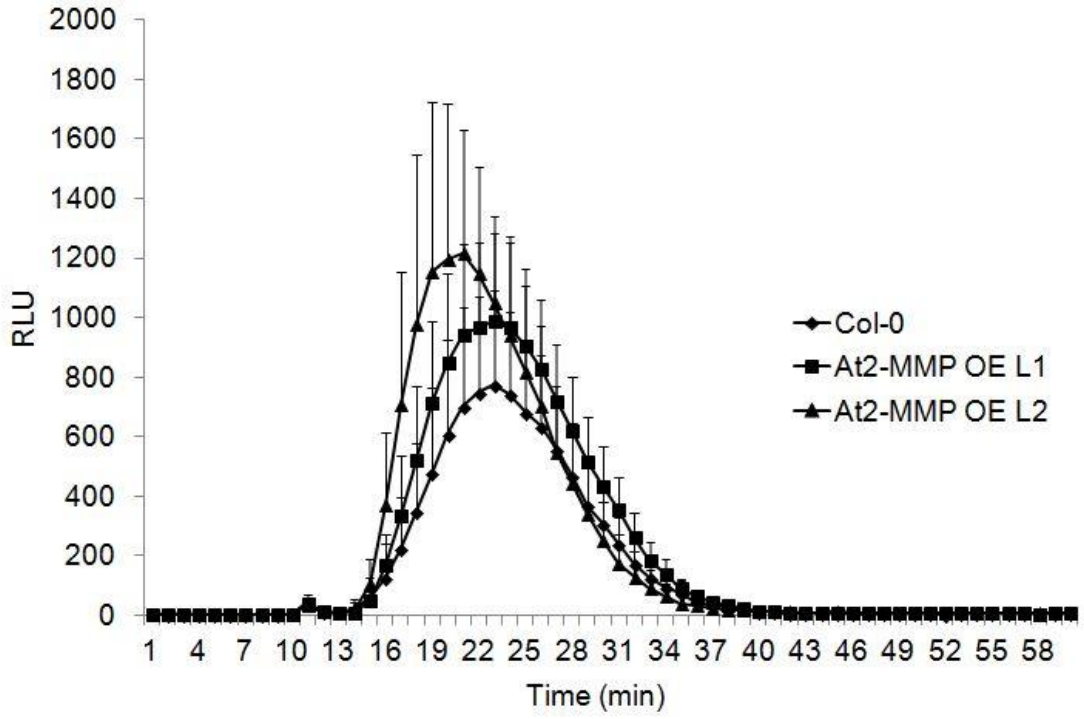


Figure. 3-4. Responses of *at-mmp* mutants and *At-MMP* OE transgenic plants to MAMP.

A: Four-week-old plants were treated with 1μM flg22 or mock (water). At 24 h

after treatment (hat), the leaves were fixed with ethanol-to-glacial acetic acid for several h and stained by aniline blue for 1.5 h. The callose deposition was observed by epifluorescence microscopy. Scale bar = 20 μm. **B**, **C**: Oxidative burst induced by 100nM flg22 was measured in relative light units (RLU) in the leaves. The leaf disks 5 mm in diameter were cut from 4-week-old plants wild-type, *at2-mmp*, *at3-mmp*, *at2, 3-mmp*, *at2, 3, 5-mmp*, EV(empty vector), overexpression line (line1and line2) of mmp2. Results are mean ± SE (n = 8). Three independent experiments were performed showing the similar results.

3.3 At-MMP is required for basal resistance to necrotrophic and biotrophic fungal pathogens

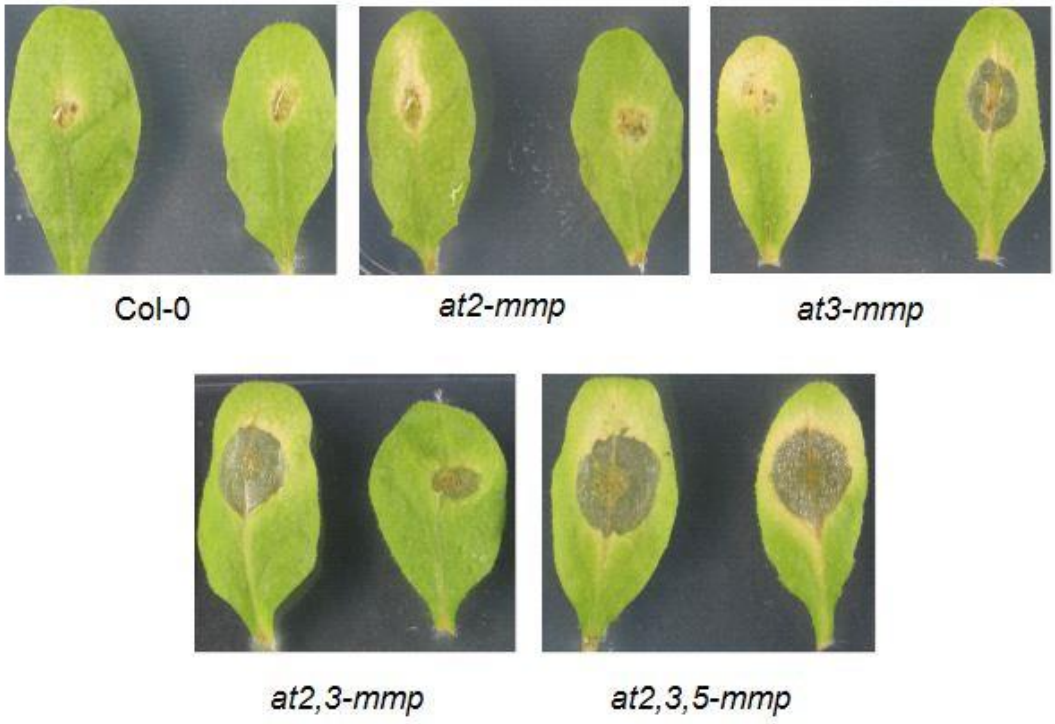
3.3.1 At-MMP is required for basal resistance to necrotrophic fungal pathogen *Botrytis cinerea*

Since expression of *at2-mmp* and *at3-mmp* is induced by *B. cinerea* infection in wild-type plants (Zhao, 2011), this prompted us to study the At-MMPs function in *at-mmp* mutants during the *B. cinerea* infection. For this, the detached leaves were cut from 4-week-old plants, and symptomatic chlorosis were taken at 3-4 days post inoculation (d.p.i). The lesion size was measured with Image J. The results demonstrated that single knockout mutant *at2-mmp* and *at3-mmp* showed bigger lesion size and significantly more susceptible to *B. cinerea* than wild-type plants (Fig. 3-5 A, B). To further assess whether At2-MMP, At3-MMP and At5-MMP have the similar function during *B. cinerea* infection, the phenotypic analyses were performed on *at2, 3-mmp* double mutant and *at2, 3, 5-mmp* triple mutant after *B. cinerea* infection. In comparison to wild-type, *at2, 3-mmp* double mutant showed significantly higher susceptibility. This susceptible symptoms were even slightly more than those in *at2-mmp* and *at3-mmp* mutants (Fig. 3-5 B). Notably, the highest

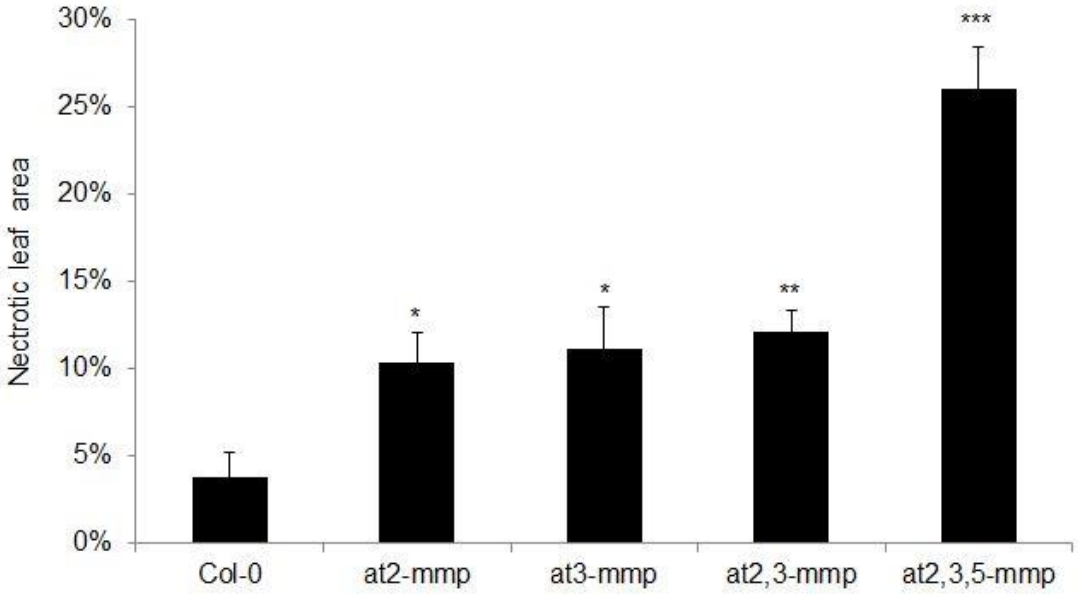
susceptibility was observed in *at2, 3, 5-mmp* triple mutant, which exhibited approximatly double level of susceptibitiy compared with wild-type (Fig. 3-5 A, B). These data implied an additive effect of the mutations in *at2-mmp*,*at3-mmp* and *at5-mmp* on the plant resistance to *B. cinerea.*

To confirm the role of MMPs during necrotrophic pathogen *B. cinerea* infection, the responses to *B. cinerea* infection in two indepent overexperssion lines of *At2-MMP* (line1 and line2) were subsequently analyzed. Expectedly, the susceptible symptoms in *at-mmp* mutants were complemented in both overexperssion lines of *At2-MMP*, exhibiting more than 50% lesion size compared with wild-type and EV (Fig. 3-5 C), which demonstrated more resistance to *B. cinerea* than wild-type plants (Fig. 3-5 D). Taken together, the data suggested that At-MMPs play an important role in resistance to necrotrophic pathogen *B. cinerea*.

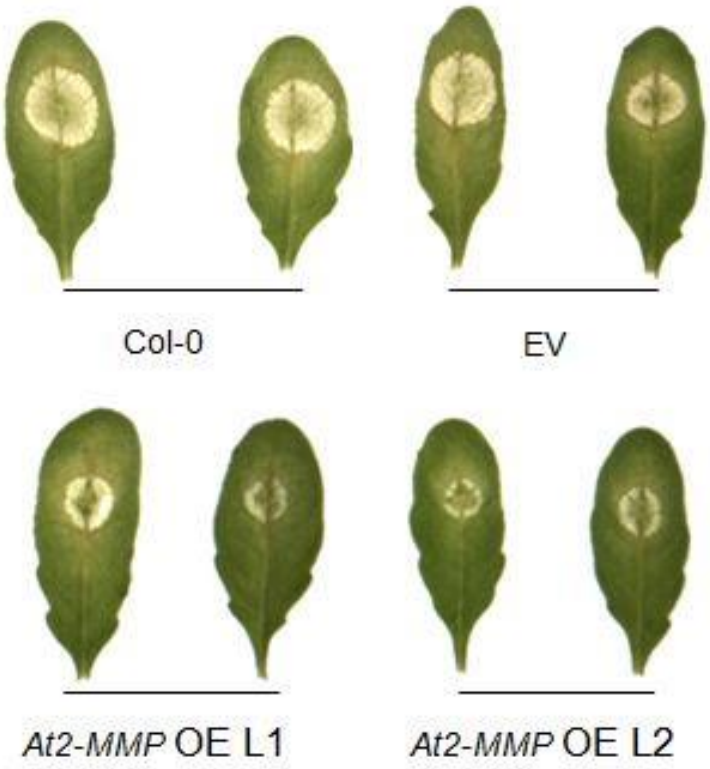
A



B


30%
25%
20%
15%
10%
5%
0%
Nectrotic leaf area
*
*
**
***
Col-0
at2-mmp
at3-mmp
at2,3-mmp
at2,3,5-mmp

C


Col-0
EV
At2-MMP OE L1
At2-MMP OE L2

D

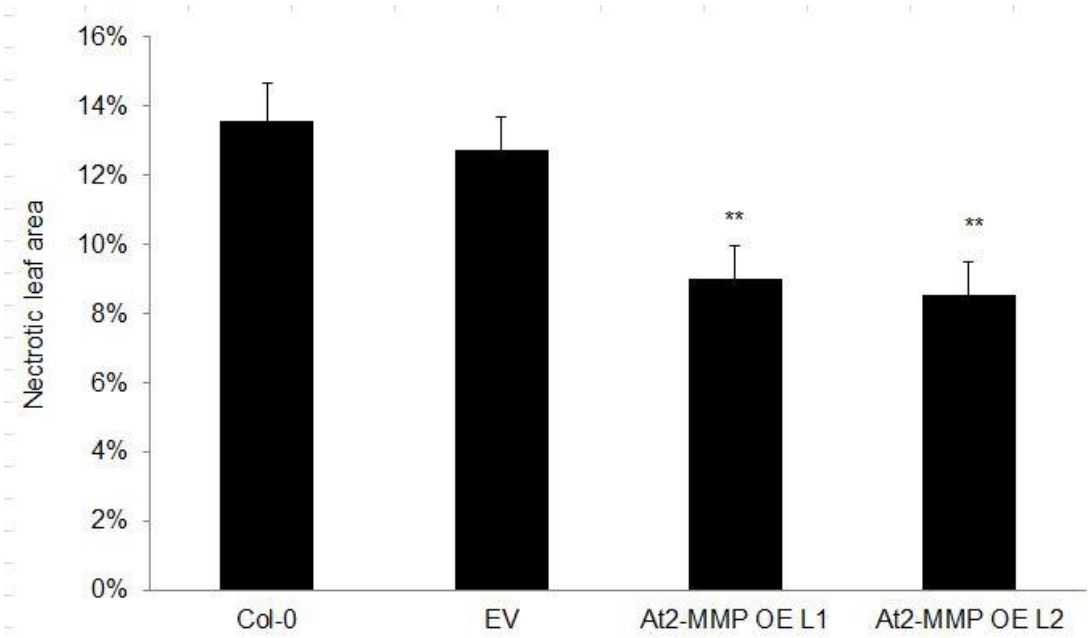


Figure. 3-5. At-MMPs is required for basal resistance to *Botrytis cinerea*. The detached leaves from 4-week-old plants wild-type, *at2-mmp*, *at3-mmp*, *at2, 3-mmp*, *at2, 3, 5-mmp*, EV(empty vector), *At2-MMP* OE L1 and L2 were inoculated with *Botrytis cinerea*. Five µl spore suspension adjusted to 50,000 conidia per ml were placed on the middle vein. **A**: Disease symptoms of wild-type, single mutants of *at2-mmp* and *at3-mmp*, double mutant *at2, 3-mmp* and triple mutant *at2, 3, 5-mmp* were photographed at 3 d.p.i. **B**: Disease symptoms on *at-mmp* mutants were evaluated by Image J at 3 d.p.i. **C**: Disease symptoms of wild-type, EV and *At2-MMP* overexpression line were photographed at 4 d.p.i. **D**: Disease symptoms were evaluated on *At2-MMP* OE line by Image J at 4 d.p.i. Experiments were repeated three times with similar results. The mean ± SE of one experiment. Significant differences are marked as: *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-Test).

3.3.2 At-MMP is required for resistance to biotrophic fungal pathogen powdery mildew

Given that At-MMPs are required for necrotrophic fungal resistance, I further investigated whether At-MMPs also exhibited a wider resistance to other pathogens. For this, the powdery mildew fungal spores were inoculated on the 4-week-old leaves of *at2-mmp*, *at3-mmp*, *at2, 3-mmp* and *at2, 3, 5-mmp* mutants. The infection phenotype was quantified by the amount of conidia per mg of leaf fresh weight at 11 days post inoculation. Although the single mutants *at2-mmp* and *at3-mmp* showed slightly more fungal colonies compared to wild-type, *at2, 3-mmp* double mutant and *at2, 3, 5-mmp* triple mutant demontrated significantly higher susceptibility, which increased by approximately 30% and 110% respectively in comparison to wild-type (Fig. 3-6). Moreover, the numbers of fungal colonies reduced in the *At2-MMP* OE line. Taken together, these data implied that At-MMPs play an important role in plant resistance to various invading microbes.

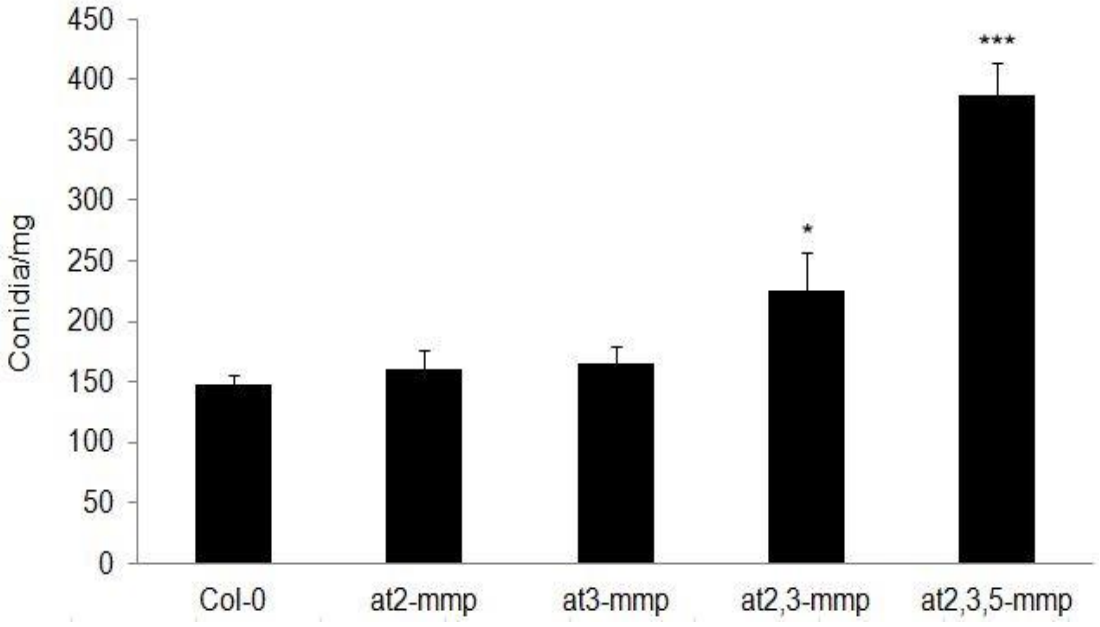


Figure. 3-6. Analysis of disease symptoms of *at-mmp* mutants and Col-0

plants with the infection of fungal pathogen powdery mildew.

Four-week-old plants were spray-inoculated with 25,000 conidia per 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 from at least 10 individually treated plants. The error bars indicate the standard error. Experiments were repeated three times with similar results. Significant differences are marked as: *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-Test).

3.4 Identification of *atmorc* mutants

Molecular phylogenetic analysis showed that there are seven *MORC* homologs in Arabidopsis genome. In my work, five of the identified *atmorc* mutants, *atmorc1*, *atmorc2*, *atmorc5*, *atmorc6* and *atmorc7* were used to study the potential function of MORC in plant immunity. T-DNA insertion knock-out mutants of *atmorc1*, *atmorc2*, *atmorc5*, *atmorc6* and *atmorc7* were obtained from the NASC. Since these mutants segregated, the identification of homozygous lines was the first step in the following work. For this, seeds were germinated on selection medium (1/2 MS medium+sulfadiazine). The plants resistant to sulfadiazine were screened for homozygosity by PCR method as described in Chapter 2.3.1.2. After genotyping, the homozygous lines for each mutant were propagated for further experiments. The numbers of homozygous plants are described in Table 3-4, the primer sequences for genotyping are described in Table S1.

Table. 3-4. Homozygous mutant of At-MORC

At-MORC	Insertion line	Number of plants tested	Number of homozygous plants
At-MORC1	SAIL_1239_C08	7	6
At-MORC2	SALK_072774C	10	10
At-MORC5	SALK_021871C	10	10
At-MORC6	GK_599B06	10	8
At-MORC7	SALK_151643C	10	8

3.5 Arabidopsis MORCs affect plant resistance against pathogens

Previous studies have shown that Arabidopsis MORC1 and its homolog MORC2 were required for both basal resistance and ETI against various pathogens (Kang et al., 2008; 2010; 2012). To support this, our previous studies confirmed that *atmorc1* and *atmorc1/atmorc2* (dko) mutants showed significantly more susceptible to hemi-biotrophic bacterium *Pst* than the wild-type plant. Moreover, the complementation of the *atmorc1/atmorc2* dko mutant with AtMORC1 could restore the plant resistance to *Pst* (Langen et al., 2014). This clearly demonstrated that AtMORC1 has positive functions in plant resistance. However, whether the other MORC members have similar resistance function remained less well understood. To assess whether the other *MORC* homologs are required for resistance, 4-week-old Arabidopsis knock-out mutants *atmorc2*, *atmorc5*, *atmorc6* and *atmorc7* were inoculated with *Pst AvrRpt2*. Three days after inoculation, leaf disks were collected and *Pst* colony forming units (CFU) were counted as described in Chapter 2.5.3. Consistent with previous reports (Kang et al., 2010; Langen et al., 2014), *atmorc1* and *atmorc1/atmorc2* mutants were significantly more susceptible to

Pst AvrRpt2 compared with the wild-type plant, which exhibited respectively around 50% and 60% higher susceptibility levels in *atmorc1* mutant and *atmorc1/atmorc2* dko mutant in comparison to that in wild-type plant (Fig. 3-7). Interestingly, similar to the disease phenotype displayed in *atmorc1* and *atmorc1/atmorc2* mutants, *atmorc6* exhibited a strong increase in susceptibility to *Pst AvrRpt2*, which showed around 60% more CFUs than that in wild-type (Fig. 3-7). In contrast, *atmorc2*, *atmorc5* and *atmorc7* mutants showed a similar *Pst AvrRpt2* susceptibility level as wild-type (Fig. 3-7).

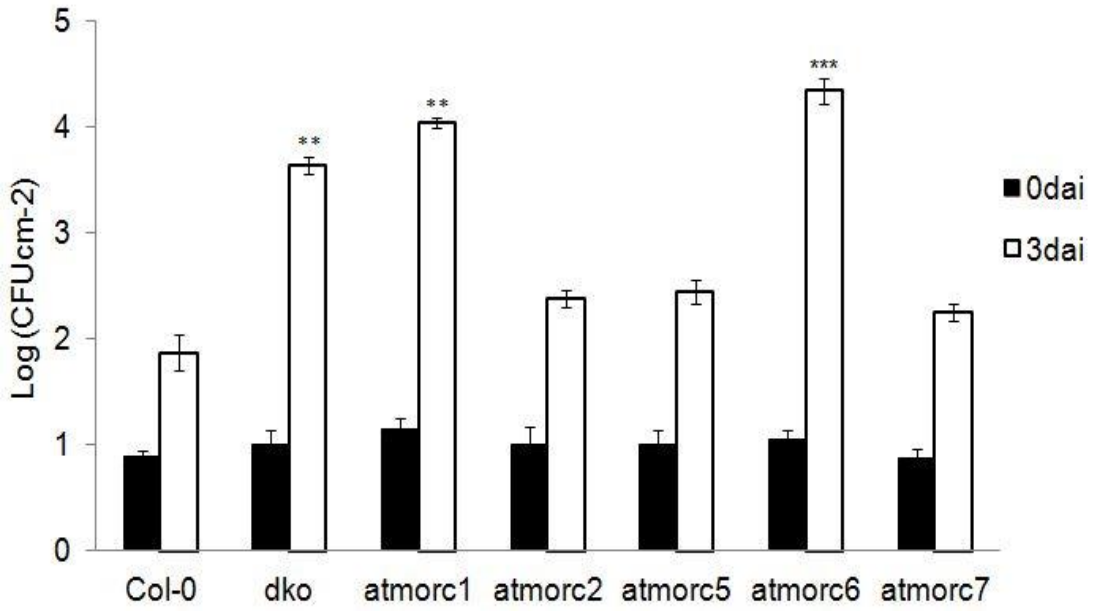


Figure. 3-7. Effect of AtMORCs in resistance to *Pseudomonas syringae* pv. *tomato* carrying the avirulence gene *AvrRpt2*.

The leaves from 4-week-old Arabidopsis plants were infiltrated with *Pst AvrRpt2* at 10^5 c.f.u. ml^{-1}, and the leaves were collected at 0 (black bars) and 3 dai (white bars). The colony forming units (CFU) were counted. Col-0: Columbia wild-type Arabidopsis; dko: *atmorc1/atmorc2* double knock-out mutant; atmorc1: *morc1* single knock-out mutant; atmorc2: *morc2* single knock-out mutant; atmorc5: *morc5* single knock-out mutant; atmorc6: *morc6* single knock-out mutant; atmorc7: *morc7* single knock-out mutant. The error bars indicate the standard error. Experiments were repeated three times with

similar results. Significant differences are marked: *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-Test).

3.6 Production of transgenic plants containing site mutations in MORC1

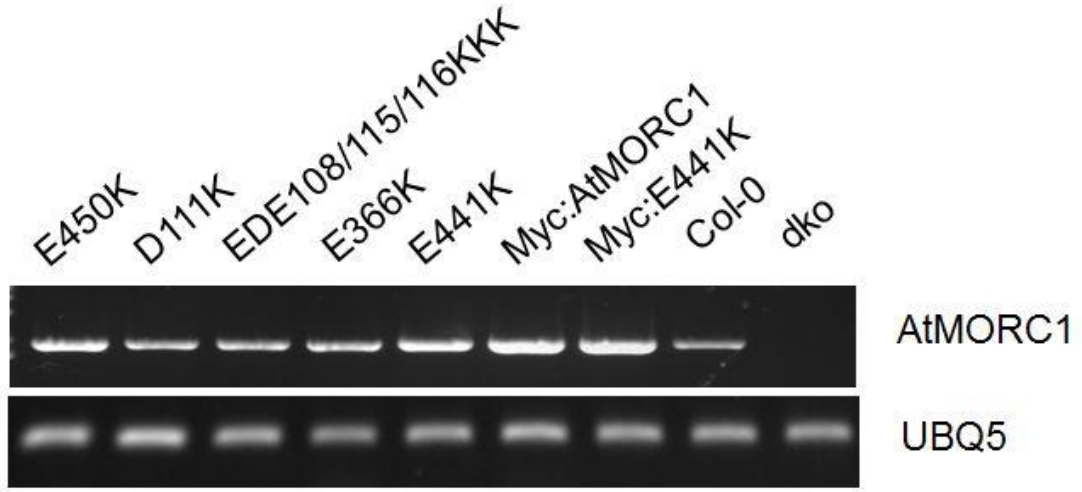
Arabidopsis MORC1 is involved in multiple layers of disease resistance against various pathogens (Kang et al., 2012; Langen et al., 2014), and exhibited activities of ATPase and endonuclease (Kang et al., 2008; 2010; 2012; Lorkovic et al., 2012). In order to further investigate the function of ATPase activity and putative endonuclease activity in AtMORC1 during plant defense, *AtMORC1* site mutations were generated. These mutations were targeted in the ATPase site (AtMORC1 D111K and AtMORC1 EDE108/115/116KKK) and a putative endonuclease site (AtMORC1 D366K, AtMORC1 E441K and AtMORC1 E450K) of AtMORC1. These mutated versions of AtMORC1 were used to complement into *atmorc1/atmorc2* dko mutant. For this, the transgenic plants were generated from the *atmorc1/atmorc2* dko background through floral dip transformation as described in Chapter 2.4.2. The positive transgenic lines containing mutations are described in Table 3-5. The expression level of *AtMORC1* was tested by semi-quantitative RT-PCR. The result indicated that all the transgenic lines containing the different mutated MORC1 versions were transcribed to mRNA (Fig. 3-8 A). Next, to confirm whether the mRNA is translated into protein, the total protein was extracted from transgenic plants which additionally contain the myc-tag (only constructs: myc: AtMORC1, myc: E441k) and was forwarded to the immune-blot assay with anti-myc antibody. The data showed a 70 kDa product, indicating the expression of AtMORC1 protein in the transgenic lines (Fig. 3-8 B). Furthermore, in order to check whether the myc tag might affect the function of MORC in resistance, I compared the disease phenotypes with the infection of *Pst AvrRpt2* in Arabidopsis *MORC1*

overexpression line dko-AtMORC1 and the myc-tagged overexpression lines of *AtMORC1* (myc: AtMORC1) and mutated *AtMORC1* (myc: E441K). The data showed that both myc: *AtMORC1* and myc: E441K lines remained certain levels of restoration of resistance to *Pst AvrRpt2* in comparison to *atmorc1/atmorc2* dko mutant (Fig. 3-8 C), which indicated that the myc tag does not affect the MORC function in plant resistance to *Pst AvrRpt2*. Thereafter, the restoration of resistance to *Pst AvrRpt2* was examined in the produced transgenic lines containing specific site mutations of *MORC1*.

Table. 3-5. Positive transgenic lines containing *AtMORC1* mutations

Mutations	Number of positive plants in F1	Number of positive plants in F2	Number of positive plants in F3
AtMORC1 D111K	9	10	14
AtMORC1 EDE108/115/116KKK	9	10	12
AtMORC1 D366K	10	8	13
AtMORC1 E441K	10	9	9
AtMORC1 E450K	9	10	10
myc: AtMORC1	9	10	9
myc: E441K	8	9	11

A



B

C

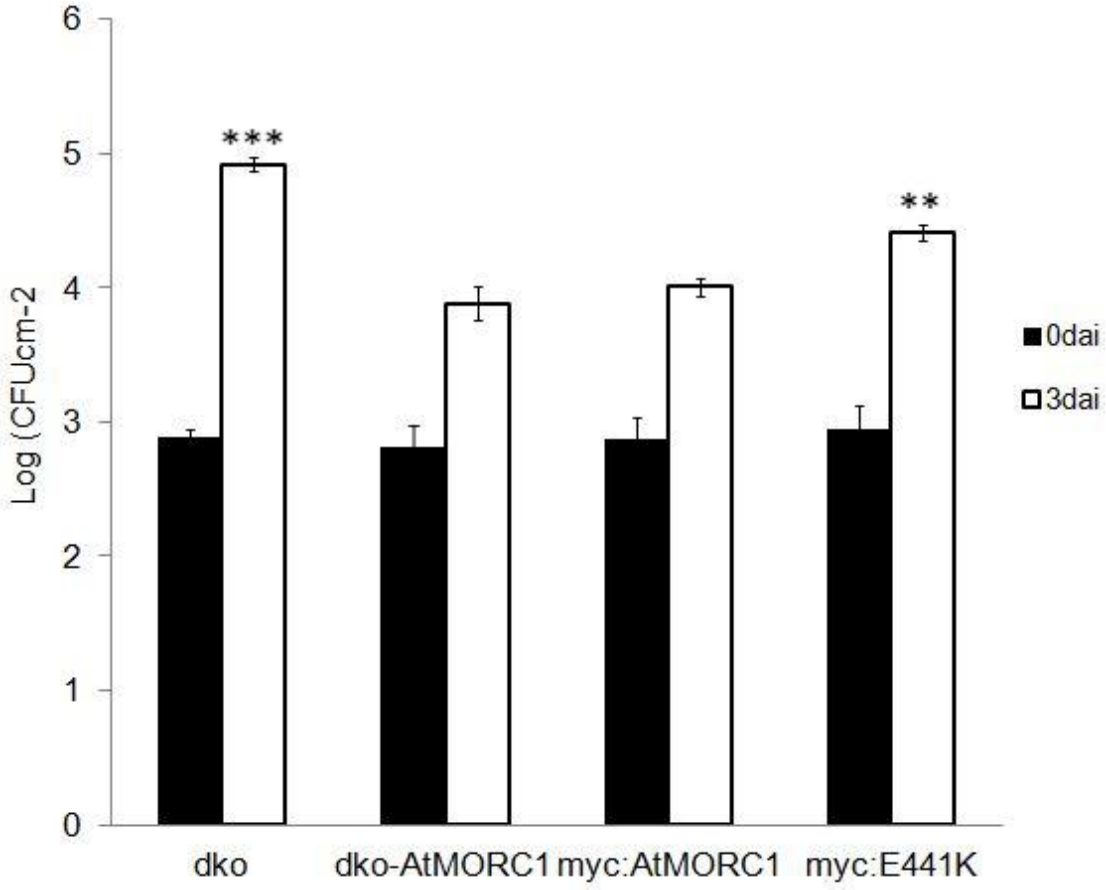


Figure. 3-8. Expression of *AtMORC1* in transgenic plants.

A: Semi-quantitative PCR showing the transcription level of *AtMORC1* in the

different transgenic plants and control pants by PCR. The cDNA samples were extracted from the leaves of 4-week-old Arabidopsis plants. **B**: Expression of AtMORC1 protein in transgenic plants expressing the construct myc: E441K or myc: AtMORC1. Control is myc: HvMORC1. For western blot, the leaves were collected from 4-week-old plants for protein extraction, 10 µg total protein per sample was separated by 10% SDS-PAGE, and myc-MORC1 protein expression was detected by anti-myc antibody. The staining with Ponceau S indicates equal loading of all samples (data not show). **C**: The phenotype of transgenic plants (myc: E441K and myc: *AtMORC1*) in resistance to *Pseudomonas syringae* pv. *tomato* carrying the avirulence gene *AvrRpt2*. The leaves of 4-week-old plants were infiltrated with *Pst AvrRpt2* at 10^5 c.f.u. ml^{-1}, and collected at 0 (black bars) and 3 dai (white bars). The colony forming units (CFU) were counted. Col-0: Columbia wild-type Arabidopsis; dko: *atmorc1/atmorc2* double knock-out mutant; dko-AtMORC1: *AtMORC1* overexpression line in dko; D111K and EDE108/115/116KKK are transgenic lines containing mutations in ATPase domain of AtMORC1; D366K, E441K and E450K are transgenic lines containing mutations in putative endonuclease domain of AtMORC1. The numbers denote amino acid position in AtMORC1 structure: D denotes aspartic acid; E denotes glutamic acid and K denotes lysine. The aspartic acid/glutamic acid was replaced by lysine. The error bars indicate the standard error. Experiments were repeated three times with similar results. Significant differences are marked: *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-Test).

3.7 Mutations in *AtMORC1* reduces plant resistance against the hemi-biotrophic bacterium *P. syringae* pv. *tomato*

To assess whether the ATPase domain and/or endonuclease domain of AtMORC1 might play an important role in plant resistance, the transgenic

lines containing mutations of *AtMORC1* were employed to examine their response to *Pst*. In comparison to the *AtMORC1* overexpression line in the *atmorc1/atmorc2* background (dko-AtMORC1), the transgenic lines containing mutations (D111K and EDE108/115/116KKK) in ATPase domain of AtMORC1 showed significantly more susceptible to *Pst AvrRpt2* (Fig. 3-9). Similarly, the transgenic lines containing mutations (E450K, E366K and E441K) in putative endonuclease domain of AtMORC1 also exhibited significantly higher susceptibility to *Pst AvrRpt2* in comparison to the dko-AtMORC1 (Fig. 3-9). Since the transcriptional level of *AtMORC1* (Fig. 3-8 A) and the expression of AtMORC1 protein (Fig. 3-8 B) were confirmed in the above transgenic lines containing mutations, it is speculated that the alteration of disease phenotypes are due to the mutations at ATPase domain and/or endonuclease domain of AtMORC1.

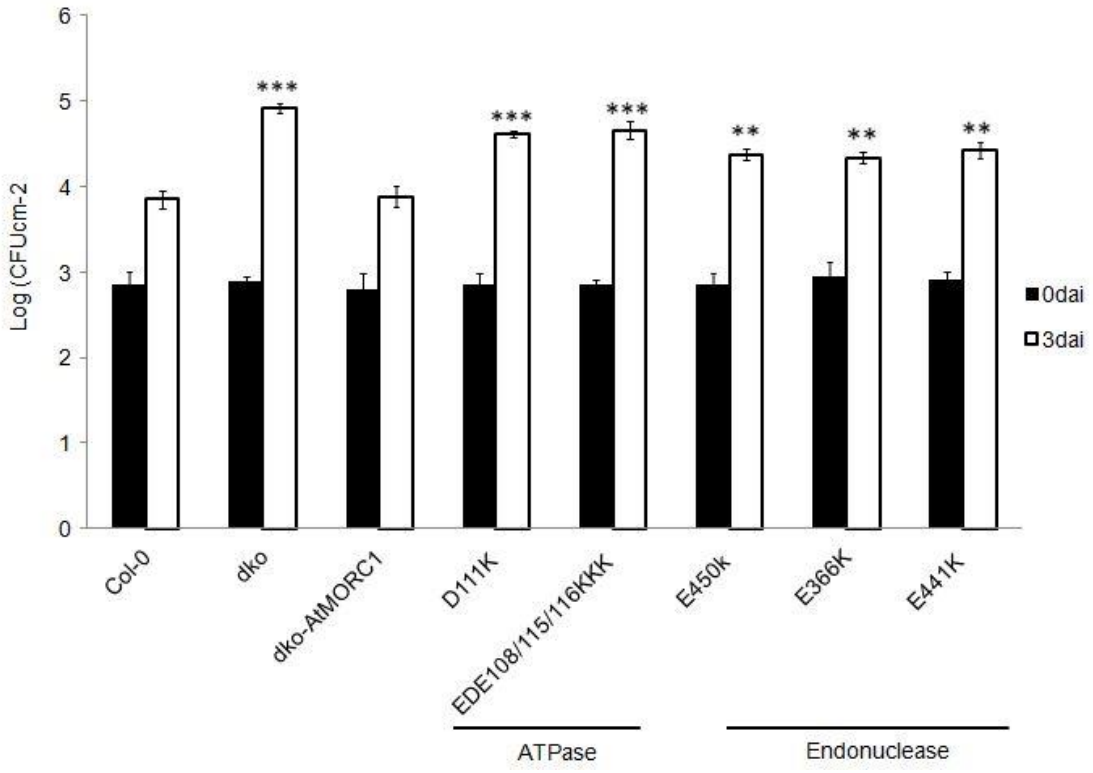


Figure. 3-9. Mutations in ATPase domain and putative endonuclease domain in AtMORC1 affects resistance against bacterium *Pst AvrRpt2*.

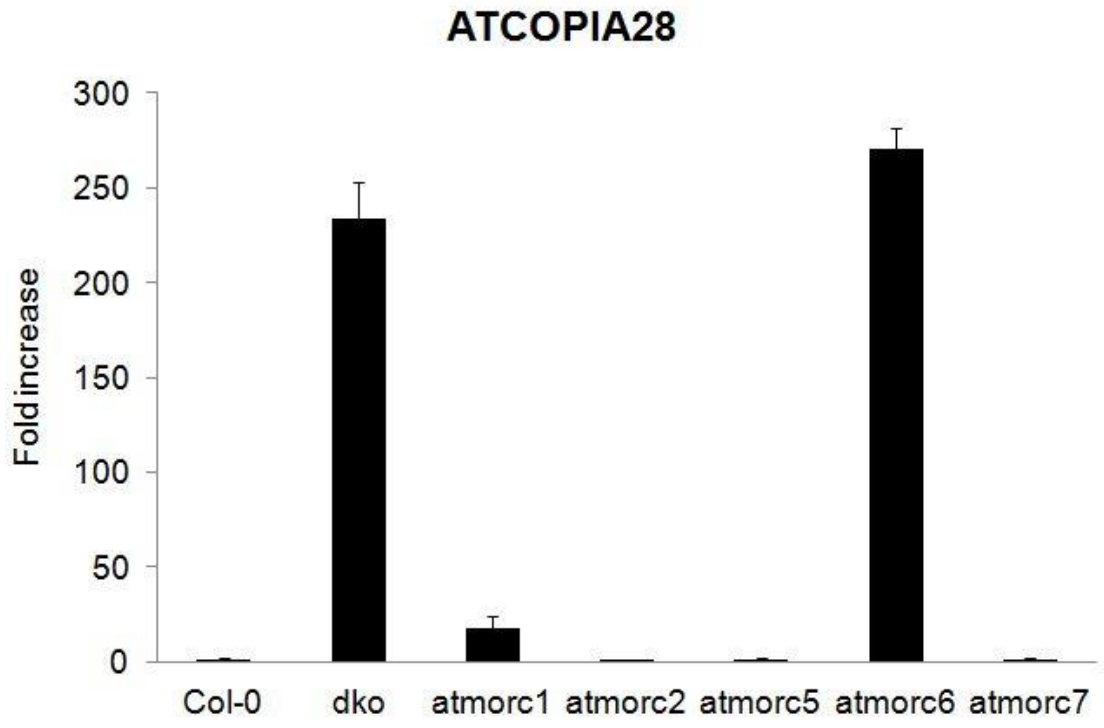
The leaves from 4-week-old plants were infiltrated with *Pst AvrRpt2* at 10^5 c.f.u. ml^{-1}, and the leaves were collected at 0 (black bars) and 3 dai (white bars). The colony forming units (CFU) were counted. Col-0: Columbia wild-type Arabidopsis; dko: *atmorc1/atmorc2* double knock-out mutant; dko-AtMORC1: *AtMORC1* overexpression line in dko; D111K and EDE108/115/116KKK are transgenic lines containing mutations in ATPase domain of AtMORC1; D366K, E441K and E450K are transgenic lines containing mutations in putative endonuclease domain of AtMORC1. The numbers denote amino acid position in AtMORC1 structure: D denotes aspartic acid; E denotes glutamic acid and K denotes lysine. The aspartic acid/glutamic acid was replaced by lysine. The error bars indicate the standard error. Experiments were repeated three times with similar results. Significant differences are marked: *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-Test).

3.8 *AtMORCs* knock-out mutants show enhanced expression of a transposon and genes related to silencing mechanisms.

Not only functioning in plant resistance to pathogens, recent research demonstrated that Arabidopsis *MORC1* and *MORC6* could also suppress transposons expression (Moissiard et al., 2012; 2014). To investigate whether other Arabidopsis *MORC* homologs are involved in gene silencing, the expression of a transposon gene (*ATCOPIA28*) and genes involved in silencing *SDC* (*suppressor of drm2 cmt3*) were measured in *atmorc1*, *atmorc2*, *atmorc5*, *atmorc6* and *atmorc7* knock-out mutants as well as *atmorc1/atmorc2* dko mtuant. The total RNA was extracted from 3-week-old Arabidopsis plant leaves, which were used for cDNA synthesis. Thereafter, the genes expression level was analyzed by real-time PCR (RT-PCR) using gene specific primers described in Table 2-2. The results showed that

expression of *ATCOPIA28* was dramatically induced in *atmorc6* and *atmorc1/atmorc2* dko mutants, which displayed more than 200-fold induction in comparison to wild-type. The *atmorc1* mutant also exhibited around 10 fold induction of *ATCOPIA28* (Fig. 3-10 A). In contrast, *atmorc2*, *atmorc5* and *atmorc7* showed little or no induction of *ATCOPIA28*, which was similar to that displayed in wild-type (Fig. 3-8 A). *SDC* encodes an F-box protein and possesses seven promoter tandem repeats, which show a unique silencing based on non-CG DNA methylation directed redundantly by histone methylation and siRNAs. The enhancement of *SDC* gene expression means the alteration of the mechanisms regulating its promoter. In the mutants of *atmorc1*, *atmorc6* and *atmorc1/atmorc2* dko, the expression level of *SDC* was significantly up-regulated, which displayed respectively around 80-fold, 130-fold and 110-fold induction in comparison to the level in wild-type (Fig. 3-10 B).

A



B

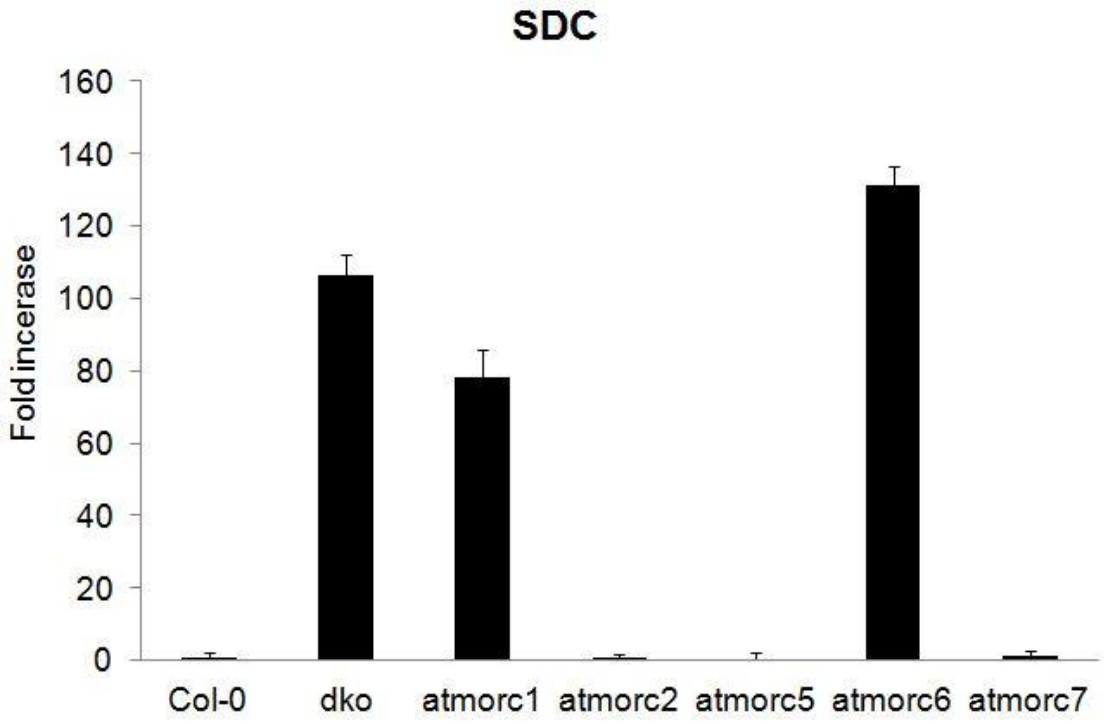


Figure. 3-10. AtMORCs affect the expression of transposon and silencing-related genes.

The cDNA samples from the leaves of 3-week-old Arabidopsis plants were used for real-time PCR (RT-PCR). Data represent the Ct thresholds of the indicated candidate genes relative to the Ct thresholds of the housekeeping gene actin using the ΔΔCt method. Relative fold increase of one transposon (**A**) *ATCOPIA28* and one gene involved in silencing (**B**) *SDC* (*suppressor of drm2 cmt3*) was calculated**.** Col-0: Columbia wild-type Arabidopsis; dko: *atmorc1/atmorc2* double knock-out mutant; atmorc1: *morc1* single knock-out mutant; atmorc2: *morc2* single knock-out mutant; atmorc5: *morc5* single knock-out mutant; atmorc6: *morc6* single knock-out mutant; atmorc7: *morc7* single knock-out mutant. The error bars indicate the standard error. Experiments were repeated three times with similar results.

3.9 Transgenic plants containing mutations of AtMORC1 mutations show no or strong derepression of transposons.

To further support the importance of AtMORC1 in gene silencing, the transposon genes expression was examined in *AtMORC1* overexpression line dko-AtMORC1. Expectedly, the dramatic induction of one transposon marker gene *AtCOPIA28* and gene involved in silencing *SDC* (*suppressor of drm2 cmt3*) disappeared in dko-AtMORC1, which showed comparable expression levels to wild-type plant (Fig. 3-11). Nevertheless, significant inductions of both *AtCOPIA28* and *SDC* were observed in dko-HvMORC1 line, in which barley *MORC1* was overexpressed in *atmorc1/atmorc2* background. The level of transposons gene expression in dko-HvMORC1 was even similar to that displayed in *atmorc1/atmorc2* dko mutant (Fig. 3-11).

Next, to assess whether the activity of ATPase and endonuclease is necessary for gene silencing in MORC1, the expression of transposon genes in the transgenic lines containing mutations of AtMORC1 was further investigated. Similar to that observed in *atmorc1/atmorc2* mutant, the transgenic lines containing the mutations (D111K; EDE108/115/116KKK) in ATPase domain of AtMORC1 exhibited significant induction of *AtCOPIA28* and *SDC* expression (Fig. 3-11). Nevertheless, in the transgenic lines containing the mutations (E366K; E450K; E441K) in the putative endonuclease domain of AtMORC1, the expressions of *AtCOPIA28* and *SDC* exhibited comparable level to that in wild-type plant and dko-AtMORC1 line, although the transgenic lines with the mutations of E366K and E450K showed slightly induction of both genes (Fig. 3-11).

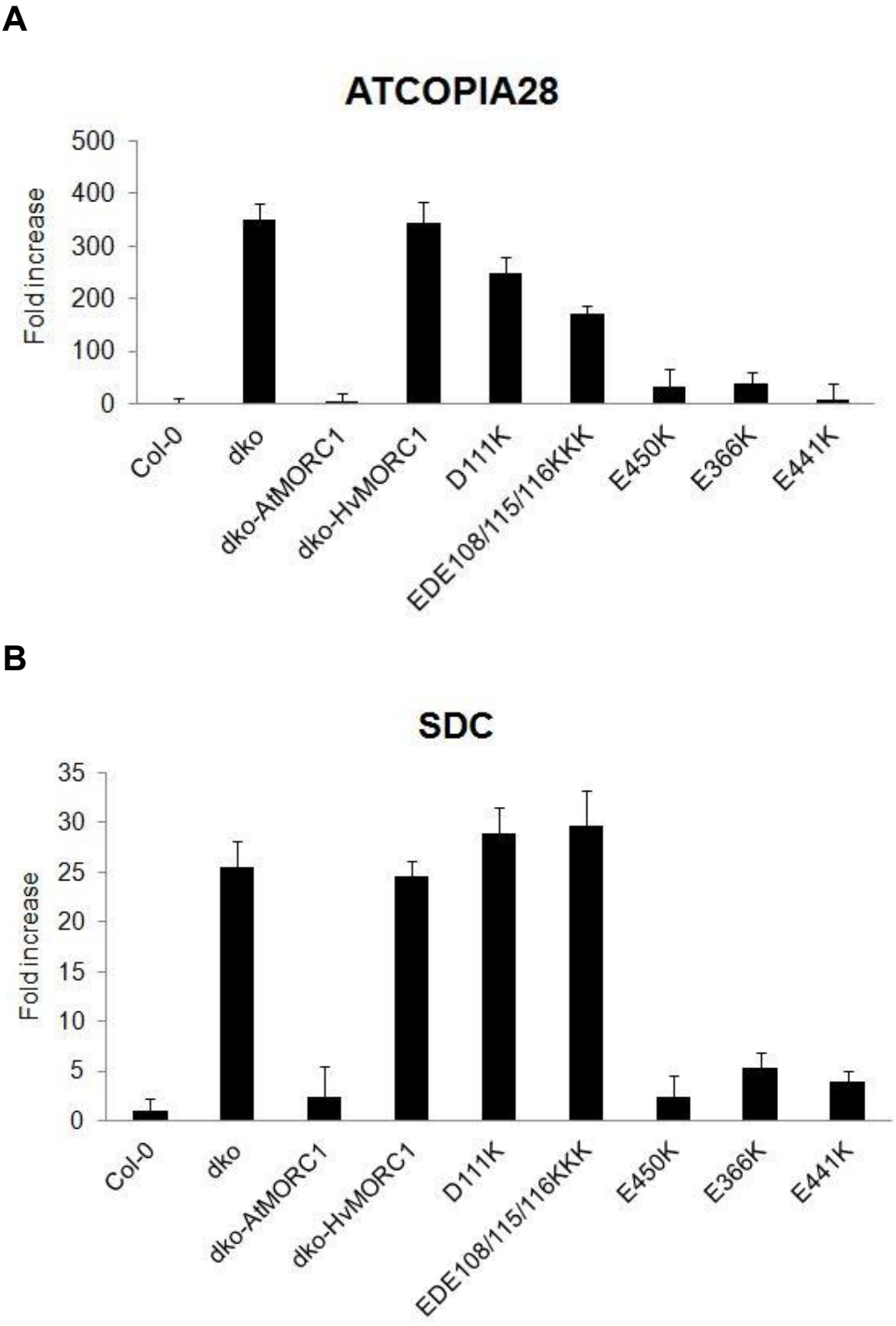


Figure. 3-11. Effects of AtMORC1 mutations on silencing.

The expression of a transposon and a gene controlled by silencing was measured in the different lines expressing the mutated AtMORC1 constructs

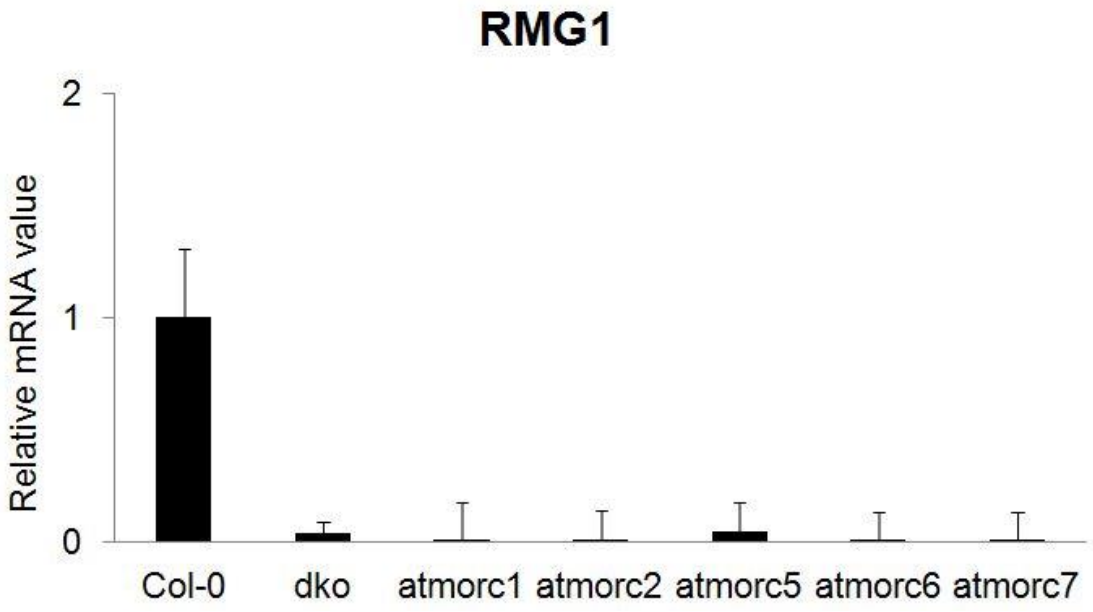
and dko-HvMORC1 (*HvMORC1* overexpression in dko). RT-PCR was performed from RNA extracted from 3-week-old leaf tissue. Data represent the Ct thresholds of the indicated candidate genes relative to the Ct thresholds of the housekeeping gene actin using the ΔΔCt method. **A**: Relative expression level of a transposon *ATCOPIA28*, whose repression depends on silencing mechanism. **B**: Relative expression level of a gene controlled by silencing *SDC* (*suppressor of drm2 cmt3*). Col-0: Columbia wild-type Arabidopsis; dko: *atmorc1/atmorc2* double knock-out mutant; dko-AtMORC1: *AtMORC1* overexpression line in dko; dko-HvMORC1: *HvMORC1* overexpression line in dko; D111K and EDE108/115/116KKK are transgenic lines containing mutations in ATPase domain of AtMORC1; D366K, E441K and E450K are transgenic lines containing mutations in putative endonuclease domain of AtMORC1. The numbers denote amino acid position in AtMORC1 structure: D denotes aspartic acid; E denotes glutamic acid and K denotes lysine. The aspartic acid/glutamic acid was replaced by lysine. The error bars indicate the standard error. Experiments were repeated three times with similar results.

3.10 AtMORCs show effect on the expression of *R* gene *RMG1* (*resistance methylated gene 1*)

MORCs are involved in both resistance and gene silencing. To assess whether MORC might function in plant resistance through gene silencing, I tested how MORC affects the expression of *R* gene *RMG1* (*resistance methylated gene 1*). The *RMG1* has transposon element (TE) remnants in its promoter that attracts the RNA-directed DNA methylation (RdDM) machinery. In comparison to wild-type, most of the tested *morc* mutants exhibited no induction of *RMG1*, except for the slight expression shown in mutants of *atmorc2*, *atmorc5* and *atmorc1/atmorc2* dko (Fig. 3-12 A). Similar to that

observed in *morc* mutants, the transgenic lines containing the AtMORC1 mutations (D111K; EDE108/115/116KKK) in ATPase domain and the mutations (E366K; E450K; E441K) in the putative endonuclease domain exhibited comparable expression level of *RMG1* to that in *atmorc1/atmorc2* dko mutant, which exhibited slight induction of *RMG1* expression in comparison to wild-type plant. However, the dko-AtMORC1 and dko-HvMORC1 lines exhibited significant induction of *RMG1* expression in comparison to that displayed in *atmorc1/atmorc2* dko mutant (Fig. 3-12 B).

A



B

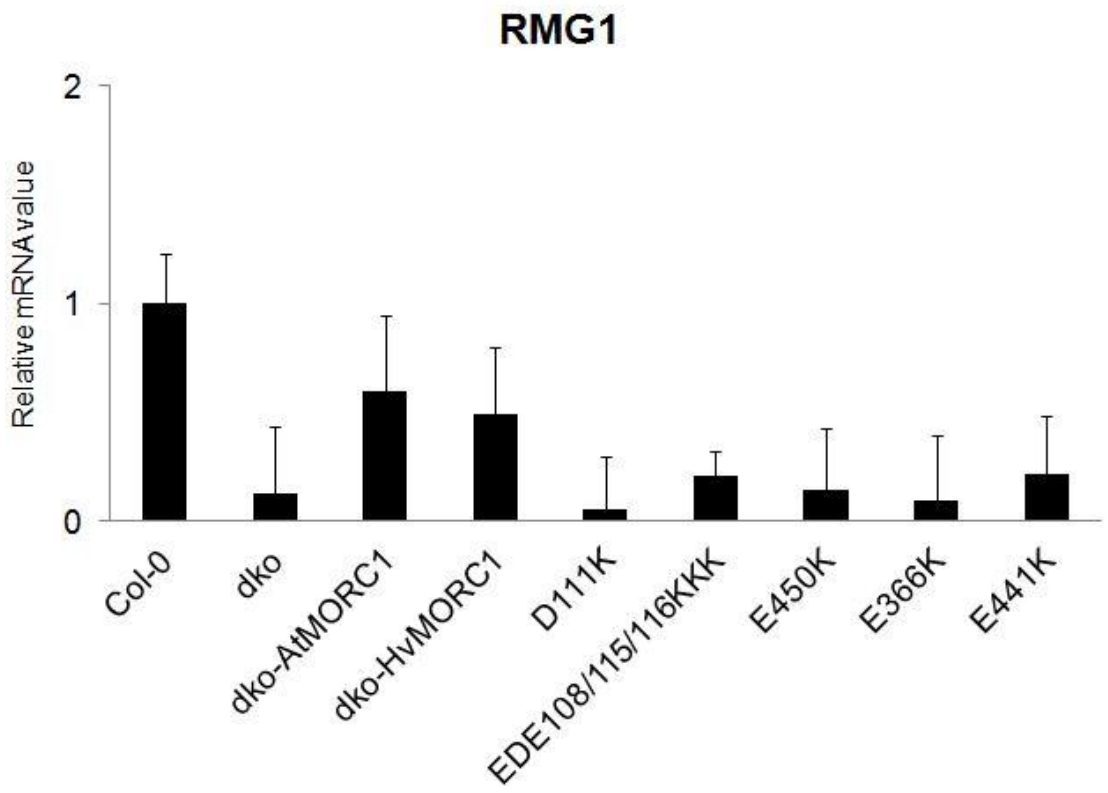


Figure. 3-12. Effects of AtMORC on resistance gene (*RMG1*) expression.

The expression level of resistance gene *RMG1* (*resistance methylated gene 1*) was analyzed in different *AtMORC* knock-out mutants and mutation lines. RT-PCR was performed from cDNA samples extracted from 3-week-old Arabidopsis leaves. Data represent the Ct thresholds of the indicated candidate genes relative to the Ct thresholds of the housekeeping gene actin using the ΔΔCt method. **A**: Relative expression level of *RMG1* was analyzed in *AtMORC* knock-out mutants and wild-type plant. Col-0: Columbia wild-type Arabidopsis; dko: *atmorc1/atmorc2* double knock-out mutant; atmorc1: *morc1* single knock-out mutant; atmorc2: *morc2* single knock-out mutant; atmorc5: *morc5* single knock-out mutant; atmorc6: *morc6* single knock-out mutant; atmorc7: *morc7* single knock-out mutant. **B**: Relative expression level of *RMG1* was analyzed in AtMORC1 mutations line and dko-HvMORC1 (*HvMORC1* overexpression in dko). Col-0: Columbia wild-type Arabidopsis; dko: *atmorc1/atmorc2* double knock-out mutant; dko-AtMORC1: *AtMORC1*

overexpression line in dko; dko-HvMORC1: *HvMORC1* overexpression line in dko; D111K and EDE108/115/116KKK are transgenic lines containing mutations in ATPase domain of AtMORC1; D366K, E441K and E450K are transgenic lines containing mutations in putative endonuclease domain of AtMORC1. The numbers denote amino acid position in AtMORC1 structure: D denotes aspartic acid; E denotes glutamic acid and K denotes lysine. The aspartic acid/glutamic acid was replaced by lysine. The error bars indicate the standard error. Experiments were repeated three times with similar results.

4. Discussion

4.1 The section of MMPs

4.1.1 The MMPs family

In mammals, MMPs have been reported to play an important role in the regulation of inflammation and wounding (Parks et al., 2004; Page-McCaw et al., 2007), in bone modeling and remodeling (Page-McCaw et al., 2007), and especially in the formation of diseases. In healthy adult tissue, *MMP7* is expressed only in mucosal epithelia; but it is up-regulated by bacterial in epithelia (Lopez-Boado et al., 2000). Consistently, mouse *mmp7* mutant is much easier to get infected by intestinal bacteria. The precursor of cryptdin is found in intestinal epithelia from *mmp7* mutant mice (Wilson et al., 1999). Moreover, the MMP7 can mediate wound-induced epithelial migration by cleaving E-cadherin (McGuire et al., 2003). In wounded *mmp7* mutant mice, epithelial cells do not migrate and E-cadherin cleavage does not occur (Dunsmore et al., 1998). In addition, MMP3 functions in epidermal wound healing. The skin wounds of *mmp3* mutant mice heal more slowly compared with control mice. These data indicated that the MMPs involved in innate immunity and wound healing (Bullard et al., 1999).

Compared with MMPs in mammals, the function of MMPs in plants is less well known. The first plant MMP was described from soybean SMEP1 (Ragster and Chrispeels, 1979; Graham et al., 1991). Thereafter, the plant MMPs were identified in more plant species, including cucumber (Delorme et al., 2000), Arabidopsis (Maidment et al., 1999), tobacco (Kang et al., 2010) and Medicago truncatula (Combier et al., 2007). The majority of MMPs are secreted into the ECM. Through regulating ECM, MMPs might function in plant development and growth. Several reports have shown that the expression of *MMPs* is associated with aging. For instance, the expression of

soybean *SMEP1* is increased in young leaves, but there is no change in the next one month (Pak et al., 1997). The other soybean *GmMMP2*, however, displayed a higher transcript level in mature leaves compared with the senescence stage (Liu et al., 2001). The expression of *MMPs* in not only associated with aging but also reacting to the outer abiotic and biotic stresses. It has been reported that the soybean *GmMMP2* could accumulate in leaves with either wounding stress or in response to pathogen invasion (Liu et al., 2001). In Arabidopsis, the five *MMPs* exhibited different expression patterns in different part of plant (Maidment et al., 1999).

Not only participating in plant development and growth, the MMPs also play a key role in plant-microbe interactions. The cucumber *Cs1-MMP* presents at the boundary of senescence and cell death indicating its function in programmed cell death (PCD) (Delorme et al., 2000). The soybean SMEP1 possibly contributes to the resistance to pathogen challenges (Pak et al., 1997). Nevertheless, these studies are mainly based on the MMP protease activity. In my study, I employed genetic, molecular and biochemical analyses to provide further clear evidences to the function of MMPs in plant disease resistance.

4.1.2 Reactive oxygen species and the role in plant immunity

The oxidative burst, a rapid production of huge amount of reactive oxygen species (ROS), is one of the earliest defense events when plants respond to various environmental threats, including biotic and abiotic stresses. The ROS mainly occurred in the plant cell surface. In normal situation, the plants employ certain protective mechanisms to keep a lower level of ROS. However, when confronting stresses, the balance level of ROS in plants will be significantly altered by the high amount of ROS production. This reaction has been known to support the plant resistance to pathogen invasion through

strengthening the cell wall, inducing pathogenesis-related (PR) genes and cell death. Zurbriggen et al (2009) treated the tobacco wild plant with a nonhost pathogen *Xanthomonas campestris pv. vesicatoria* (*Xcv*), and they found that the accumulation of ROS is higher in chloroplast. In addition, the localized cell death was also significantly increased in comparison to the plants expressing the flavodoxin, which is an electron shuttle. When expressed in chloroplasts, the flavodoxin could prevent ROS formation under abiotic stress. These results indicated that generation of ROS is important for the cell death process during nonhost pathogen invasion.

4.1.3 At-MMPs mediated oxidative burst

In order to understand the functional mechanism of At-MMPs in resistance and whether At-MMPs affect the oxidative burst, I analyzed the ROS production in *at-mmp* mutants after the treatment with flg22. As expected, the single mutants *at2-mmp*, *at3-mmp*, double mutant *at2, 3-mmp* and triple mutant *at2, 3, 5-mmp* exhibited a lower level of ROS production compared with wild-type (Fig. 3-4 B). To further check whether the reduction of ROS production could be complemented, I further detected the oxidative burst in *At2-MMP* overexpression line after the treatment with flg22. The data demonstrated indeed a higher level of ROS production in *At2-MMP* overexpression line (Fig. 3-4 C). The alteration of oxidative burst in single mutants *at2-mmp*, *at3-mmp*, double mutant *at2, 3-mmp* and triple mutant *at2, 3, 5-mmp* and *At2-MMP* OE line is actually consistent with the phenotype shown in the pathogen test (Fig. 3-5). Thus, it indicated that At-MMPs function in affecting plant immunity in a way of regulating the MAMP triggered ROS production.

4.1.4 Callose deposition and the role in plant immunity

Callose is a β-(1, 3) glucan polymer, whose structure and chemical composition are slightly different in various cell wall types. It is an important part of the cell plate during cell division, vascular bundles and pollen wall exine. Callose is produced in specific site and developmental stages of plant cell. Callose synthesis is catalyzed by different subunits of enzyme complex, which is associated with the plasma membrane (Piršelová and Matušíková, 2013). The callose accumulation is regulated by distinct signaling pathways, depending on the outside conditions (Luna et al., 2011). For example, the heat and cold can induce the callose deposition in the sieve plates of the phloem (Furch et al., 2007; Bilska and Sowinski, 2010). Callose could also accumulated around cell wall, supporting the cell wall to against different environment stresses. Under hypoxic conditions, callose deposition can maintain the mechanical stability of cell wall (Albrecht and Mustroph, 2003). In roots, callose might help to protect the sink function of organ and store carbohydrates (Biemelt et al., 1999).

Callose deposition occurs at the site of pathogen attack at the cell wall. These cell walls are thought to function as a physical barrier to restrict pathogen invasion (Stone and Clarke, 1992). In tobacco, the *β-1, 3-glucanases* mutant induced more callose deposition and showed more resistance to different viruses (Iglesias and Meins, 2000). The callose deposition is also observed at the fungi and bacteria penetration sites (Poliakovskiy and Dmitriev, 2011; Yun et al., 2006). As one of the popular markers, plant early immune response to pathogen invasion can be quantified by callose deposition. Since the callose deposition can be triggered by MAMPs, such as Flg22 (Gómez-Gómez and Boller, 2000), bacterial elongation factor EF-Tu (Elf18) (Kunze et al., 2004) and chitin, a β-(1, 4)-linked polymer of *N*-acetylglucosamine (Iritri and Faoro, 2009). MAMP induced callose deposition in plant roots or leaves has become an important marker response, together with other signaling events, such as

the accumulation of reactive oxygen species (ROS) and anion fluxes, to detect the PTI signaling pathways.

4.1.5 At-MMPs mediate callose deposition

In order to understand whether At-MMPs would affect the callose deposition, I analyzed the occurrence of callose deposition in *at-mmp* mutants after the treatment with flg22. Similar to the ROS production assay, all the mutants exhibited less callose deposition in comparison to wild-type. To further check whether the reduction of callose deposition could be complemented in overexpression line, the callose deposition in *At2-MMP* overexpression line after the treatment of flg22 was further observed. Expectedly, the highest occurrence of callose deposition was detected in *At2-MMP* OE line.

Combining the ROS production assay, the data implied that *at-mmp* mutants showed reduction of both ROS production and callose deposition with the MAMP treatment; while this reduction could be complemented in overexpression lines, exhibiting a restored higher level of ROS production and callose deposition (Fig. 3-4 A, C). These phenotypes are also consistent with the function of ROS in flg22-induced callose deposition (Zhang et al., 2007; Galletti et al., 2008; Estrella et al., 2011), indicating the function of At-MMPs in resistance to pathogens. Indeed, in the pathogen test, *at-mmp* mutants exhibited higher susceptibility to necrotrophic fungal pathogen *Botrytis cinerea* and biotrophic fungal pathogen powdery mildew. Nevertheless, the *At2-MMP* OE lines demonstrated more resistance to *Botrytis cinerea* compared to wild-type (Fig. 3-5 D). Taken together, it is speculated that At-MMPs play a positive role in PTI. More specifically, At2-MMP, At3-MMP and At5-MMP might function in immune response in way of enhancing the ROS production and callose deposition.

4.1.6 The role of plant MMPs in immune system

The previous studies have indicated that plant MMPs could function in response to abiotic stresses, such as NaCl treatment, cadmium treatment, wounding and temperature stress (Liu et al., 2001; Golldack et al., 2002; Golldack et al., 2002; Cho et al., 2009). Moreover, recent researches further demonstrated that the plant MMPs are also of importance to plant immunity. In tobacco, the expression of *Nicotiana benthamiana* matrix metalloprotease 1 (*NMMP1*) was up-regulated by *Pseudomonas syringae* pv. *tomato* T1(*Pst1*) or *P. syringae* pv. tabaci (*Psta*) infection. The transient *NMMP1* over-expression in *N. benthamiana* showed less CFUs compared with the control plant after *Psta* inoculation; while, the *NMMP1* silenced leaves exhibited significantly higher number of CFUs than the control leaves (Kang et al., 2010). In *Arabidopsis*, the *At2-MMP* overexpression line appeared to be more resistant to *Botrytis cinerea* infection in comparison to wild-type (Zhao, 2011). According to the phylogenetic analysis, there are five MMPs in *A. thaliana* (At1-MMP to At5-MMP), among which At2-MMP, At3-MMP and At5-MMP are close to each other and appear short genetic distance based on sequence alignment (Maidment et al., 1999; Zhao, 2011). Marino et al (2014) expressed and purified the catalytic domains of all five At-MMPs with His-tagged proteins in *Escherichia coli* cells to delineate the biochemical function which demonstrated that all five At-MMPs are proteolytically active enzymes and each enzyme showed specific protease activity according to the recombinant At-MMPs by three independent methods: the recombinant At-MMPs were incubated individually with: (i) MBP and β-casein as typical model protease substrates, with activity assayed by SDS/PAGE; (ii) quenched fluorescent peptides using fluorescence generation as readout; and (iii) an *A. thaliana* leaf proteome-derived peptide library, followed by MS-based identification of cleaved peptides. To assess whether these three MMPs (At2-MMP, At3-MMP and At5-MMP) have the same function in defense response, the double

mutant *at2, 3-mmp* and triple mutant *at2, 3, 5-mmp* were produced, and their responses to the infection by different patterns of pathogens were further analyzed. With the infection by necrotrophic fungus *Botrytis cinerea*, the double mutant *at2, 3-mmp* showed bigger lesion size compared with the parental single mutant *at2-mmp* and *at3-mmp* or the wild-type (Fig. 3-5 A, B), which is consistent with the former studies (Zhao, 2011). Although the triple mutant *at2, 3, 5-mmp* exhibited a similar phenotype in comparison to the single mutants *at2-mmp*, *at3-mmp* and double mutant *at2, 3-mmp*, yet it appeared to be more susceptible to *B. cinerea* than the double mutant *at2, 3-mmp* (Fig. 3-5 B). This indicated an additive effect of the mutations in At2-MMP, At3-MMP and At5-MMP. Furthermore, the phenotype in response to *B. cinerea* infection in *At2-MMP* overexpression line was analyzed. Consistent with the previous studies, the *At2-MMP* overexpression line demonstrated increased resistance to *B. cinerea* when comparing to the wild-type (Fig. 3-5 C, D). Taken together, the results suggest that At-MMPs play an important role in plant immune response and function to defend the invasion by necrotrophic fungus *B. cinerea*.

Given that At-MMPs were required for necrotrophic fungus resistance. To assess whether At-MMP are also required for the resistance to biotrophic fungus, I analyzed the infection phenotype in mutants of *at2-mmp*, *at3-mmp*, *at2, 3-mmp* and *at2, 3, 5-mmp* by the biotrophic fungus powdery mildew. Similarly, the single mutants *at2-mmp* and *at3-mmp* showed slightly increased numbers of fungal colonies compared to wild-type (Fig. 3-6). While the *at2, 3-mmp* and *at2, 3, 5-mmp* mutants appeared to be significantly more susceptible to the fungal infection, which increased by approximately 30% and 110% respectively in comparison to wild-type and single mutants (Fig. 3-6). Taken together, the data clearly implied that At2-MMP, At3-MMP and At5-MMP play positive roles in defense responses to nectrophic and biotrophic fungal invasion. Furthermore, they might have similar functions in plant immunity.

4.2 The section of MORC

4.2.1 The MORC family

MORC (microrchidia) was first described in mouse where it encodes a 108 kDa protein. This protein accumulated specifically in male germ cells (Inoue et al., 1999). The MORC family widely exists in eukaryotic and prokaryotic organisms (Iyer et al., 2008). The protein sequences revealed that MORC contains nuclear localization signals coiled-coil domain and GHL (GyraseB, Hsp90, MutL) ATPase (Inoue et al., 1999). MutL and its homologs are essential for cell growth and viability, being involved in mismatch repair, gene conversion and chromosome segregation (Williamson et al., 1985; Hunter and Borts, 1997). Hsp90 functions in the conformational activation of eukaryotic protein kinases and regulating the expression of genetic variation (Dutta and Inouye, 2000; Rutherford and Lindquist, 1998). In order to test the localization of MORC protein, Inoue et al (1999) expressed epitope-tagged mouse MORC in COS7 cells and determined its subcellular localization by multicolor immunofluorescence. They found that the MORC expressed in the nuclear. Based on the localization of MORC and the phenotypes of other mouse *morc* mutants, it was indicated that MORC might function in the regulation of transcription, cell division, meiotic chromosome dynamics or DNA repair (Inoue et al., 1999; Okabe et al., 1998). Moreover, its coiled-coil domain demonstrates that MORC could interact with other proteins. The GHL (GyraseB, Hsp90, MutL) ATPase domain in the N-terminus of MORC implies that the ATP hydrolysis might be required for MORC function (Inoue et al., 1999).

Interestingly, several recent studies have demonstrated that MORC protein could also function in plant immunity. Kang et al (2008; 2010; 2012) identified the MORC family in Arabidopsis. The data implied that AtMORC1 increased the plant resistance to the necrotrophic fungal pathogen *P. syringae* pv.

tomato (*Pst*). Moreover, five *MORC* genes have been identified in the barley genome (Langen et al., 2014). The *morc* genes knock-down barley plants and *MORC* overexpression barley lines were infected by powdery mildew fungus *Bgh* race A6. The results indicated that the *morc2* knock-down plants exhibited less fungal colonies in comparison to control; while the *MORC1* OE plants displayed higher susceptibility. In addition, the *morc2* knock-down plants also showed increased resistance to necrotrophic fungus *Fusarium graminearum* (Langen et al., 2014). In my study, the pathogens test in Arabidopsis *morc* mutants supported that, similar to its function in barley, MORC also plays an important role in the immune responses to pathogens in Arabidopsis. Notably, different from the phenotypes in barley, the Arabidopsis *morc1* mutant seemed more susceptible to *Pst* in comparison to control (Fig. 3-7). Moreover, the resistance to *Pst* could be restored in Arabidopsis *MORC1* overexpression line dko-AtMORC1 (Fig. 3-10). These data implied that MORC might have distinguished or alternative mechanisms to function in plant immunity among different hosts.

In addition, recent reports demonstrated that MORC1 is responsible for transposon repression in the male germline in mouse (Pastor et al., 2014). In plant, Arabidopsis MORC1 and MORC6 were also reported to suppress the expression of transposons (Moissiard et al., 2012). These clearly indicated that the MORC family could play a role in gene silencing. In my study, I tested the gene expression profile of a transposon (*ATCOPIA28*) and genes involved in silencing *SDC* (*suppressor of drm2 cmt3*) and *RMG1* (*resistance methylated gene 1*) which is regulated by RNA-directed DNA methylation (RdDM) in Arabidopsis *morc* mutants and transgenic lines containing AtMORC1 mutations. The data showed that mutants of *atmorc1/atmorc2*, *atmorc1* and *atmorc6* exhibited most significantly different expression profile in comparison to Col-0, which was the up-regulation of *ATCOPIA28* and *SDC* (Fig. 3-10), and the down-regulation of *RMG1* (Fig. 3-12 A) in the above Arabidopsis *morc* mutants. The results are similar to the previous reports,

indicative of the participation of MORC in gene silencing in Arabidopsis.

4.2.2 Production and identification of AtMORC1 mutations

The structure of MORC showed that MORC possesses ATPase domain and endonuclease domain, which is supported by the ATPase and endonuclease activity exhibited in AtMORC1 and AtMORC6 (Kang et al., 2012; Lorkovic et al., 2012). Interestingly, AtMORC1 and AtMORC6 were reported to function as repressors of transposons and DNA methylated genes (Moissiard et al., 2012; 2014). Additionally, MORC1 also repressed transposons in the male germline in mouse (Pastor et al., 2014). These indicated that MORC might link to gene silencing. In order to address the question of whether the activities of ATPase and endonuclease are necessary for the resistance and gene silencing, I produced point mutations which are highly conserved in AtMORC1 according to the database. Furthermore, amino acids in both ATPase domain (D111K; EDE108/115/116KKK) and putative endonuclease domain (E366K; E441K; E450K) were mutated, in which the amino acids were replaced from negative charge to positive charge, resulting in the deactivation of ATPase and endonuclease. Thereafter, the transgenic plants in Arabidopsis dko background with/without myc-tag were produced. These stable transgenic plants were used for phenotypic analysis of disease resistance against *P. syringae* pv. *tomato* (*Pst*), and the expression analysis of a transposon gene *ATCOPIA28*, a silencing-related gene *SDC* (*suppressor of drm2 cmt3*) and *RMG1* (*resistance methylated gene 1*) which is regulated by RNA-directed DNA methylation.

4.2.3 The role of MORC in resistance

The previous studies have indicated that MORC involved in resistance to

pathogen invasion (Kang et al., 2008; 2010; 2012; Langen et al., 2014). For instance, *AtMORC1* overexpression line increased the resistance to necrotrophic fungus *Botrytis cinerea* (Langen et al., 2014). Similarly, my studies demonstrated a higher growth level of hemi-biotrophic bacterium *P. syringae* pv. *tomato* which carries the avirulence gene *AvrRpt2* (*Pst AvrRpt2*) in Arabidopsis single *morc1* and double *morc1/morc2* mutants in comparison to wild-type (Fig. 3-7). However, the resistance to *Pst AvrRpt2* could be restored in the overexpression line of *AtMORC1* dko-AtMORC1 (Fig. 3-9). This pathogen phenotypic analysis was further performed on Arabidopsis *morc2* mutant, and it showed similar disease symptom of *Pst AvrRpt2* to wild-type (Fig. 3-7). Taken together, it is speculated that Arabidopsis MORC1 has functions in disease resistance. Nevertheless, MORC2 might not directly affect the resistance against pathogens, indicative of a different function from MORC1 in plant immunity, although they are the closest homologs out of seven *MORC* homologs in Arabidopsis genome, sharing 80.9% amino acid sequence identity (Kang and Klessig, 2008; Kang et al., 2008, 2010; Langen et al., 2014; Mossiard et al., 2014). Interestingly, overexpression of barley *MORC1* in *atmorc1/atmorc2* background exhibited similar phenotype with the infection of *Pst AvrRpt2* to that in *atmorc1/atmorc2* mutant. However, the barley *MORC1* OE line demonstrated more susceptibility to powdery mildew fungus *Bgh* race A6 (*Bgh*A6) compared to the control (Langen et al., 2014). Combing the opposite phenotype to pathogens infection in Arabidopsis and barley *morc* transgenic plants, it is suggested that MORC could involve in disease resistance in both Arabidopsis and barley. However, MORC might employ different mechanisms to affect plant immunity in dependence of host.

Besides, I further tested how *atmorc5*, *atmorc6* and *atmorc7* single mutants respond to the infection of *Pst AvrRpt2.* Similar to the phenotype on *atmorc2* mutant, *atmorc5* and *atmorc7* mutants exhibited comparable level of susceptibility to that displayed in wild-type plant (Fig. 3-7), indicative of a less significant function in resistance. By contrast, *atmorc6* mutant showed similar

phenotypes to *atmorc1* and *atmorc1/atmorc2* mutants, which exhibited significantly higher susceptibility to *Pst AvrRpt2* in comparison to the wild-type plant (Fig. 3-7). Among them, *atmorc6* mutant even displayed the highest susceptible level (Fig. 3-7). These data suggested that the MORC6 might have similar or comparable significant functions to MORC1 in plant defense response. Up to date, the functional mechanisms of MORC1 and MORC6 in plant immunity remained elusive. Therefore, the next challenge is to decipher in-depth their mode of action.

To further investigate the MORC1 function, I specifically tested the disease phenotype with the infection of *Pst AvrRpt2* in Arabidopsis transgenic lines (D111K; EDE108/115/116KKK), in which the conserved region of ATPase domain in AtMORC1 was mutated. The transgenic plants showed more susceptible than control line (dko-AtMORC1) (Fig. 3-9), which demonstrated that the mutations in ATPase domain of AtMORC1 might disrupt the resistance complementation. Similarly, the transgenic lines containing three AtMORC1 mutations (E366K; E441K; E450K) in the putative endonuclease domain also exhibited higher susceptibility to *Pst AvrRpt2* than control (dko-AtMORC1) (Fig. 3-9), suggesting that the AtMORC1 mutations in endonuclease domain might not improve the resistance. Since the MORC1 protein was correctly expressed in the transgenic lines with mutations (Fig. 3-8 B), it is speculated that the specific mutations induce the plant susceptibility to *Pst AvrRpt2* infection. Considering that these mutated amino acids are highly conserved in MORC1, they might play an important role in biological function of MORC1. Alternatively, these mutations might reduce or even disrupt the biological activity of ATPase or endonuclease, which further lead to the loss-of-function of MORC1 in plant immunity.

4.2.4 The role of MORC in gene silencing

The *MORC1* gene was firstly found to be essential for male primordial germ cell development in mice (Inoue et al., 1999; Watson et al., 1998). Interestingly, MORC1 could also repress transposons in the male germline of mice (Pastor et al., 2014). In plant, several reports have shown that Arabidopsis MORC family involved in transposon repression and gene silencing (Brabbs et al., 2013; Lorkovic et al., 2012; Moissiard et al., 2012, 2014). For instance, AtMORC1 could interact with AtMORC6, playing a similar role in gene silencing (Moissiard et al., 2012). Consistently, my studies demonstrated that the expression levels of one transposon marker gene *ATCOPIA28* and silencing-related gene *SDC* were dramatically up-regulated in *atmorc1* and *atmorc6* mutants (Fig. 3-10). These data further supported the potential function of AtMORC1 and AtMORC6 in the regulation of transposon expression and gene silencing. By contrast, the expression of *SDC* and *AtCOPIA28* was not induced in *atmorc2, atmorc5* and *atmorc7* mutants together with wild-type (Fig. 3-10). Notably, a high induction of transposon marker genes expression was detected in *atmorc1/atmorc2* double mutant (Fig. 3-10), suggesting that AtMORC2 might be redundant for AtMORC1 to function in gene silencing. But, AtMORC5 and AtMORC7 might have less significant function in transposons repression and gene silencing.

To further support the importance of AtMORC1, the transposon genes expression was examined in *AtMORC1* overexpression line dko-AtMORC1. Expectedly, the dramatic induction of transposon gene *ATCOPIA28* and silencing-related gene *SDC* disappeared in dko-AtMORC1, which showed comparable expression levels to wild-type. This clearly supported that AtMORC1 functions in the regulation of transposon expression and gene silencing. Nevertheless, significant inductions of both *AtCOPIA28* and *SDC* were observed in dko-HvMORC1 line, in which barley *MORC1* was overexpressed in *atmorc1/atmorc2* background. The level of transposons

gene expression in dko-HvMORC1 was even similar to that displayed in *atmorc1/atmorc2* dko mutant (Fig. 3-11), indicating that barley MORC1 might not directly affect the regulation of gene silencing. Consistent to this result, Langen et al (2014) have demonstrated that barley *MORC1* silencing line has minor effect on the transposons expression. Taken together, it is suggested that, different from AtMORC1, barley MORC1 might not function in transposon silencing, indicating that the function of MORC1 might be in dependence of host.

Next, to assess whether the activity of ATPase and endonuclease is necessary for gene silencing in MORC1, the expression of transposon genes in transgenic plants containing AtMORC1 mutations in ATPase domain and endonuclease domain was further investigated. Similar to that observed in *atmorc1/atmorc2* mutant, the transgenic lines containing the AtMORC1 mutations (D111K; EDE108/115/116KKK) in ATPase domain exhibited significant induction of *AtCOPIA28* and *SDC* expression (Fig. 3-11). This indicated the mutation of ATPase domain or at least specific part of the ATPase domain is crucial for the function of AtMORC1 in transposons silencing. Alternatively, these mutations might disrupt ATPase activity, resulting in the effect on transposons expression. Nevertheless, in the transgenic lines containing the AtMORC1 mutations (E366K; E450K; E441K) in the putative endonuclease domain, the expressions of *AtCOPIA28* and *SDC* exhibited comparable level to that in wild-type and dko-AtMORC1 line, although the transgenic lines with the mutations of E366K and E450K showed slightly induction of both genes (Fig. 3-11). This suggested that the mutation of endonuclease domain or at least specific part of the endonuclease domain have minor effect on MORC1-regulated transposons silencing, although the endonuclease domain is highly conserved in MORC1. However, this argument needs to be further proved in future studies.

4.2.5 The role of MORC to link resistance and gene silencing

RNA-directed DNA methylation (RdDM) is an important pathway for transcriptional silencing of transposons in *Arabidopsis thaliana*. The SET domain proteins SUVH2 and SUVH9 are involved in the RdDM pathway in the way of interacting with the chromatin-remodeling complex DDR (DMS3, DRD1, and RDM1) and binding to methylated DNA at RdDM loci. Interestingly, AtMORC1 and AtMORC6 were shown to interact with SUVH2 and SUVH9 (Liu et al., 2014). This indicated that the AtMORC1 and AtMORC6 might indirectly function in RdDM-regulated gene silencing. Notably, RdDM might function in plant immunity through demethylation (*R* gene activation) and methylation (*R* gene repression). For instance, dampening RdDM could enhance Arabidopsis resistance to *Pst* (Pumplin and Voinet, 2013). *RESISTANCE METHYLATED GENE 1* (*RMG1*) was identified as one of the *R* genes in *Arabidopsis thaliana* which could be negatively regulated by RdDM (Pumplin and Voinet, 2013). *RMG1* has transposon elements in the vicinity of its promoter that attract the RdDM machinery and the activation of *RMG1* is regulated by active demethylation process. DNA glycosylase REPRESSOR OF SILENCING 1 (ROS1) is the demethylase in plant vegetative tissues which activates demethylation and further induces the expression of *R* genes. Since the Arabidopsis *ros1* mutant exhibited more susceptibility to *Pst,* and the induction of *RMG1* is compromised in *ros1* mutant (Pumplin and Voinet, 2013; Yu et al., 2013), it is speculated that the active demethylation of *RMG1* might at least partially induce immune response, although the role of *RMG1* in resistance remained elusive. Similar to that displayed in *ros1* mutant, the expression of *RMG1* was significantly down-regulated in all mutants of *atmorc1*, *atmorc2*, *atmorc5*, *atmorc6*, *atmorc7* and *atmorc1/atmorc2* (Fig. 3-12). This implied that MORC might function in the demethylation and the further activation of *RMG1*, which could consequently induce plant immune response. Moreover, the significant inductions of *SDC* and *ATCOPIA28* in

atmorc1 and *atmorc6* mutants further supported that AtMORC1 and AtMORC6 are involved in the regulation of transposon gene silencing (Fig. 3-10). Interestingly, since *atmorc1* and *atmorc6* mutants exhibited significantly higher susceptibility to *Pst AvrRpt2* infection (Fig. 3-7); while the resistance could be restored in dko-AtMORC1 line (Fig. 3-9), it is suggested that AtMORC1 and AtMORC6 function in plant immune response to pathogens infection. Combining together, AtMORC1 and AtMORC6 function as key regulators to mediate both gene silencing and disease resistance. One hypothesis is that AtMORC1 and AtMORC6 might repress transcriptional gene silencing through affecting RdDM and further induce specific pathogenesis-related genes, which could affect plant immune responses to microbial infection. Nevertheless, the MORCs exhibited different functions in dependence of host. For instance, the barley MORC1 was demonstrated to have an opposite function to Arabidopsis MORC1 in resistance to pathogen attack as well as in transposon silencing (Langen et al., 2014). These indicated that MORCs might play a role in both repression and activation of *R* genes and transposon elements. Furthermore, it is highly possible that MORCs function in a loci or pathway specific manner.

5. Summary

Plant matrix metalloproteinases (MMPs) have some function in development, but their function in plant immunity remained little understood. The aim of my study in this part is to analyze the role of *Arabidopsis thaliana* At-MMPs in plant immunity. The studies showed that triple mutant *at2, 3, 5-mmp* exhibited lower level of ROS production and callose deposition than wild type plants. Furthermore, the double mutant *at2, 3-mmp* and triple mutant *at2, 3, 5-mmp* exhibited more susceptibility to necrotrophic and biotrophic fungal pathogens than single mutants *at2-mmp* and *at3-mmp*, respectively. *At2-MMP* overexpression could restore the resistance to necrotrophic fungal pathogen and exhibited higher levels of ROS production and callose deposition than WT plants. These results indicated that At2-MMP plays an important role in plant disease resistance and might have an additive positive effect with At3-MMP and At5-MMP on the plant immunity, suggesting that At2-MMP, At3-MMP and At5-MMP have at least partially overlapping function in plant immunity, which might be due to the overlapping of enzyme activity.

MORC1 showed some function in plant immunity and gene silencing. However, whether the other homologs of Arabidopsis MORC have similar functions in resistance and gene silencing remained unclear. In this part, *atmorc1* and *atmorc6* mutants exhibited more susceptibility to *Pst AvrRpt2* than WT plants. In contrast, *atmorc2*, *atmorc5* and *atmorc7* mutants showed a similar phenotype to *Pst AvrRpt2* as WT. Similar to that displayed in DNA glycosylase *REPRESSOR OF SILENCING 1* (*ROS1*) mutant, the expression of *R* gene *RESISTANCE METHYLATED GENE 1* (*RMG1*), which is negatively regulated by RdDM, was down-regulated in *atmorc* mutants. It is speculated that AtMORC activate demethylation resulting in the induction of *R* genes. Meanwhile, the expression of a transposon gene (*ATCOPIA28*) and silencing-related gene *SDC* (*suppressor of drm2 cmt3*) were significantly up-regulated

in *atmorc1* and *atmorc6* mutants in comparison to WT. However, *atmorc2*, *atmorc5* and *atmorc7* showed little or no induction of transposon gene *ATCOPIA28* and silencing-related gene *SDC*, which was similar to that displayed in WT. Taken together, AtMORC1 and AtMORC6 might repress transcriptional gene silencing through affecting RdDM and further induce specific pathogenesis-related genes, which could affect plant immune responses to microbial infection.

Zusammenfassung

Pflanzliche Matrix-Metalloproteinasen (MMPs) besitzen verschiedene Funktionen in der Entwicklung, aber ihre Funktion in der Pflanzenabwehr ist bisher kaum verstanden. Das Ziel dieser Arbeit in diesem Abschnitt ist es, die Rolle von *Arabidopsis thaliana* At-MMPs in der pflanzlichen Abwehr zu untersuchen. Es konnte gezeigt werden, dass die Dreifach-Mutante *at2,3,5-mmp* niedrigere Werte für die ROS-Produktion und Kallose-Ablagerung als Wildtyp-Pflanzen aufweist. Des Weiteren besitzt die Doppel-Mutante *at2,3-mmp* und Dreifach-Mutante *at2,3,5-mmp* eine höhere Suszeptibilität gegenüber nekrotrophen und biotrophen pilzlichen Erregern als die Single-Mutanten *at2-mmp* beziehungsweise *at3-mmp.* Eine Überexpression von *At2-MMP* konnte die Resistenz gegenüber nekrotrophen pilzlichen Erregern wiederherstellen und besaß eine vermehrte ROS-Produktion und Kalloseablagerungen als Wildtyp-Pflanzen. Diese Ergebnisse deuten an, dass At2-MMP eine wichtige Rolle in der pflanzlichen Pathogenabwehr spielt und zusammen mit At3-MMP und At5-MMP einen additiven, positiven Effekt auf die Pflanzenimmunität hat. Daraus kann geschlossen werden, dass At2-MMP, At3-MMP und At5-MMP eine zum Teil überlappende Funktion in der Pflanzenimmunität besitzen, welche wahrscheinlich auf eine ähnliche Enzymaktivität zurückzuführen ist.

MORC1 besitzt verschiedene Funktionen in der Pflanzenimmunität und

„Gene-Silencing“. Ob die anderen Arabidopsis MORC Homologe ähnliche Funktionen besitzen, ist bisher kaum erforscht. In diesem Versuchsteil zeigten *atmorc1* und *atmorc6* eine höhere Anfälligkeit gegen *Pst AvrRpt2* als der Wildtyp. Im Gegensatz hierzu zeigten *atmorc2*, *atmorc5* und *atmorc7* einen ähnlichen Phänotyp nach Infektion mit *Pst AvrRpt2* wie der Wildtyp. Ähnlich wie in der DNA Glykosylase *REPRESSOR OF SILENCING 1(ROS1)* dargestellt, war die Expression des R-Gens *RESISTANCE METHYLATED GENE 1 (RMG1)*, welches negativ durch RdDM reguliert wird, geringer in den *atmorc* Mutanten. Es wird spekuliert, dass AtMORC die Demethylierung aktiviert und somit zu einer Induzierung von R-Genen führt. Außerdem wurde die Expression des Transposon-Gens *ATCOPIA28* und Silencing-verwandten Gens *SDC (suppressor of drm2 cmt3)* signifikant hochreguliert in den *atmorc1* und *atmorc6* Mutanten verglichen mit dem Wildtyp. Im Gegensatz dazu zeigten *atmorc2, atmorc5* und *atmorc7* keine oder eine geringe Induzierung dieser Gene. Zusammengefasst kann behauptet werden, dass AtMORC1 und AtMORC6 transkriptionelles Gene-Silencing durch eine Beeinträchtigung von RdDM unterdrückt und im Weiteren Pathogenitäts-zugehörige Gene, die die Antwort der Pflanzenimmunität nach mikrobieller Infektion beeinträchtigen können, induziert.

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7. Supplementary

Primer list

Primers	Sequence
N455117_F	TGTCAATCATATGGAGAGAGTTGG
N455117_R	TGCCGATTCCAGAGGACCAT
masprom	GTCTTGCGCACTGATTTGAA
term35S-R	GCTCAACACATGAGCGAAAC
GABI_416E03_F	TTTCCATTGGAATCATTCACC
GABI_416E03_R	TTACGTTTCCCTGTCGTGATC
GABI_LB	CCCATTTGGACGTGAATGTAGACAC
N115923_F	GAACCTTCTACCGAGGAATGG
N115923_R	TTTAACCGGTCCTTTACCACC
Spm32	TACGAATAAGAGCGTCCATTTTAGAGTGA
N593137_F	CGCCGCAGAATAATTTAACAG
N619909_R	CCTAAGAAACTACCATTAATGATGCTC
LBb1.3	ATTTTGCCGATTTCGGAAC
SAIL_LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
LBb1	GCGTGGACCGCTTGCTGCAACT
atmorc1-4_LP	ATCAAGGAGGCCCCTAAACTT
AtCRT1q_F	GGCTGGAGGCGAGGCTGAA
AtCRT1q_R	CAGAAGGCAAAGGCGAAGGATTGTA
morc1_RP1	TAGATTTTGACGACGATGATG
morc1_LP1	TTGCAGTTTGGAACCAAAATC
morc1_LP2	TCAGGACGAGAATATTGCGAC
morc1_RP2	ACACCATCAATGCCTTCAGTC
AtMORC1_F	GTAATGGCGAAAAATTACACAGTCGC
AtMORC1_R	CTTTTACTAAACTTGTTGCATCTCCTTC
Chimera 1	GAATTCCATGGCGAAAAATTACACAG

Chimera 2	TCCAGTAATCAGAGGTTATCCT
Chimera 5	GAATTCATGCCAGCGGCAATGGC
Chimera 6	TCCAGTACTCAATGATAATTTGTCTAAGTTTAG
Chimera8	CATATGCTAAACTTGTTGCATCTCCTTCTTC

Sequence information about *AtMORC* mutations

AtMORC1 D111K fw

GTGACGACGACAACGGAGGAGTAATAGGAATGGTTCCGAGCTTGGCATCTCTAATAGAGAATCAGAAAGTT
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AtMORC1 D111K rv

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AtMORC1 EDE108/115/116KKK fw

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AtMORC1 EDE108/115/116KKK rv

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AtMORC1 E366K fw

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AtMORC1 E366K rv

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8. 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 safe guarding of Good Scientific Practice”.

Giessen, Jan 2016

Signature:

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