Functional Analysis of a Plant Metacaspase in Negative Regulation of Innate Immunity

by

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Abstract

Programmed cell death (PCD) plays a central role in innate immune responses in plants. Plant metacaspases are distant relatives of animal caspases which have previously been shown to play important roles in regulating PCD. The *Arabidopsis thaliana* genome encodes three type I and six type II metacaspases (MCs). In this study, we showed that *Arabidopsis* type I metacaspase MC2 functions as a negative regulator of plant immunity. A loss-of-function mutant of *MC2* exhibits dwarf morphology, constitutively activated defense responses including enhanced resistance to virulent pathogens and constitutive defence marker *PATHOGENESIS RELATED* (*PR*) gene expression. On the other hand, overexpression of *MC2* leads to enhanced disease susceptibility. The autoimmune phenotype of *mc2* is dependent on the adaptor receptor-like kinases (RLKs) BAK1/BKK1 and SOBIR1, suggesting that MC2 functions upstream of these RLKs to negatively regulate plant immunity.

Lay summary

Plant protection in general and the protection of crops against plant disease in particular have an obvious role to play in meeting the growing demand for the global food security. Thus, it is of great importance to study plant microbe interaction. In this thesis, our main goal was to study the inner regulation mechanism of plant itself to fight against pathogen upon attack. In more specific, the whole research was based on the study of one novel gene *MC2* which was first reported to be involved in programmed cell death. Through both forward and reverse genetic studies, we showed that MC2 functions as a negative regulator of plant immunity and it functions upstream of several plasma-membrane localized receptors to negatively regulate plant immunity.

Preface

The work described in this thesis is an accumulation of research from Dr. Yuelin Zhang's Lab. This project benefited from the contributions made by Ms. Fang Gao and with the help and suggestions from other Zhang lab members.

Fang Gao made the cross and got the F1 seeds for *bak1-4 mc2* and *agb1-2 mc2* and conducted the mutagenesis of *mc2* in 2015. I followed up with the characterization of *mc2* single mutant and overexpression analysis of *MC2*. I generated and characterized all the double and triple mutants in the epistasis analysis. Fang Gao helped with the mutagenesis of *mc2* and I carried out the suppressor screen, which led to the isolation of ten suppressor lines.

In this thesis, I summarized the results from the work listed above and developed it into a full research story. Dr. Yuelin Zhang contributed to the discussion preparation and thesis revision.

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List of abbreviations

AGB1	Arabidopsis G Protein β-Subunit 1
AGG1/2/3	Arabidopsis G Protein γ-Subunit 1/2/3
Ave1	Avirulence protein from fungal pathogen *Verticillium dahliae*
Avr	Avirulent or Avirulence
AvrB	Avirulence protein from *Pseudomonas syringae*
AvrPto	Avirulence protein from *Pseudomonas syringae* pv. *tomato*
AvrRpm1	Avirulence protein from *Pseudomonas syringae* pv. *maculicola*
AvrRpt2	Avirulence protein from *Pseudomonas syringae* pv. *tomato*
BAK1	BRI1-Associated Receptor Kinase 1
BIK1	Botrytis-Induced Kinase 1
BIR1	BAK1-Interacting Receptor-like kinase 1
BKK1	BAK1-Like 1
BR	Brassinosteroid
BRI1	Brassinosteroid Insensitive 1
BTH	Benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester
CERK1	Chitin Elicitor Receptor Kinase 1
Cf-4	The tomato R gene that confers resistance to the fungus *Cladosporium fulvum* expressing the corresponding *Avr4* gene
Cf-9	The tomato R gene that confers resistance to the fungus *Cladosporium fulvum* expressing the corresponding *Avr9* gene
cfu	Colony forming unit
Col-0 or Col	Columbia, an Arabidopsis ecotype
C-terminal	Carboxyl terminal
DAMP	Damage Associated Molecular Pattern
EFR	EF-Tu Receptor
EF-Tu	Bacterial Elongation Factor Tu
EIX	Ethylene-Inducing Xylanase
elf18	An N-acetylated peptide comprising the first 18 amino acids of EF-Tu
EMS	Ethyl Methanesulfonate
ETI	Effector-Triggered Immunity

flg22	Flagellin conserved peptide 22
FLS2	Flagellin-Sensitive 2
GPA1	Arabidopsis G Protein α-Subunit 1
GPCRs	G-Protein-Coupled Receptors
H.a.	*Hyaloperonospora arabidopsidis*
HR	Hypersensitive Response
LRR	Leucine Rich Repeat
LSD1	Lesion Simulating Disease 1
MAPK	Mitogen-activated protein kinase
MC	Metacaspase
MS medium	Murashige and Skoog medium
NADPH oxidase	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NLP	Necrosis and Ethylene-Inducing Peptide 1-like Proteins
NLR	Nucleotide-Binding Leucine-Rich-Repeat Protein
N-terminal	Amino-terminal
PAMP	Pathogen-Associated Molecular Pattern
PCD	Programmed Cell Death
Pep1	an endogenous 23-amino-acid peptide
PEPCK1	Phosphoenolpyruvate Carboxykinase 1
PEPR1/2	Pep Receptor 1/2
PM	Plasma Membrane
PR	*Pathogenesis-Related*
PRR	Pattern Recognition Receptor
P.s.m	*Pseudomonas syringae* pv. *maculicola*
P.s.t	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP-Triggered Immunity
Pto	The tomato R gene that encodes the immune receptor that confers resistance to *Pseudomonas syringae* containing *AvrPto*
pv.	Pathovar
RBOHD	Respiratory Burst Oxidase Homolog D
RIN4	RPM1-Interacting Protein 4
RLCK	Receptor Like Cytoplasmic Kinase

RLK	Receptor Like Kinase
RLP	Receptor Like Protein
ROS	Reactive Oxygen Species
RPM1	Resistance to *Pseudomonas syringae* pv. *maculicona* 1
R protein	Resistance protein
RPS2	Resistant to *Pseudomonas syringae* 2
SA	Salicylic Acid
SARD1	SAR Deficient 1
SERK	Somatic-Embryogenesis- Receptor-Like Kinase
SOBIR1	Suppressor of *bir1-1*, 1
T-DNA	Transfer DNA
TTSS	Type III Secretion System
Ve1	The tomato R gene that encodes the immune receptor that confers resistance strains of the soil-borne vascular wilt fungi *Verticillium dahliae* and *Verticillium albo-atrum*
WT	wild type
XLG2	Extra-Large GTP-Binding Protein 2

Acknowledgement

This project would not have been possible without the assistance of many outstanding people. First, I would like to thank my supervisor, Dr. Yuelin Zhang for his guidance and support throughout my research here. Also, I would also like to thank my committee members, Dr. George Haughn and Dr. Xin Li for their precious suggestions and comments during committee meetings and on my thesis writing.

I would like to thank my lab member Fang Gao for training me in research techniques and assisting me on my project. I would also like to thank everyone in Zhang lab for providing me with many good suggestions on my project.

Thanks to the Botany Department for their financial support through teaching assistantships. I would like to give special thanks to Dr. Celeste Leander and Dr. Santokh Singh for being inspirational instructors. Moreover, I would like to thank UBC Biol 140 teaching team for providing the lab course instructor position for me to expand my learning not just on research but also on teaching.

Finally, I would like to thank my family members and friends for their support and encouragement.

Chapter 1 Introduction

The main objective of my master research was to study the role of type I metacaspase AtMC2 in regulating plant innate immunity and to determine how it relates to the action of several important receptor-like kinases involve in pathogen-associated molecular patterns triggered immunity. The first chapter is a brief overview of plant innate immunity and several important regulators in plant defense.

1.1 Introduction of plant innate immunity

In their natural environment, plants are exposed to a range of pathogenic organisms such as bacteria, fungi and viruses. These parasites can, at times, seriously compromise food security (Strange and Scott, 2005). To ensure healthy and sustainable agriculture, it is of great importance to study how plants stop pathogen growth, how pathogens overcome plant disease resistance and the molecular mechanism behind these plant-pathogen interactions (Grierson et al., 2011). It is now clear that there are conceptually two layers of the plant immune system (Fig. 1-1). In the first layer of defense, plants use transmembrane pattern recognition receptors (PRRs) to detect slowly evolving pathogen-associated molecular patterns (PAMPs), which results in PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). A number of PRRs have been identified. For example, Flagellin-Sensitive 2 (FLS2) and Elongation Factor-Tu Receptor (EFR) recognize bacterial flagellin and elongation factor Tu respectively (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006). Another well characterized PRR, Chitin Elicitor Receptor Kinase 1 (CERK1), is required for the perception of fungal cell wall component chitin and bacteria cell

wall peptidoglycan (Miya et al., 2007; Willmann et al., 2011). In addition to sensing invading microbes by means of PAMPs recognition, plants can also sense endogeneous damage-associated molecular patterns (DAMPs) to initiate defense response（Matzinger, 2007). A good example is AtPep1, an endogenous 23-amino-acid peptide of Arabidopsis perceived by the plasma membrane leucine rich repeats (LRRs) receptor kinase PEP Receptor 1 (PEPR1) and its close homologue PEPR2 (Huffaker, Pearce and Ryan, 2006; Yamaguchi, Pearce and Ryan, 2006; Yamaguchi et al., 2010).

Following activation of PRRs, defense responses in PTI often involve the activation of mitogen-activated protein (MAP) kinase cascades, production of reactive oxygen species (ROS), induced expression of defense marker *Pathogenesis-Related* (*PR*) genes, calcium ion influx, and accumulation of salicylic acid (SA) (Jones and Dangl, 2006).

To counteract the plant defense responses, pathogenic microbes have evolved effectors to suppress PTI. The best characterized effectors come from phytopathogenic bacteria. For example, gram-negative bacterial pathogens have acquired a type III secretion systems (TTSS) which enables the bacteria to deliver effector proteins into plant cells to suppress PTI (Chisholm et al., 2006). To defend pathogens that have overcome PTI, plants have evolved a second layer of immunity that is triggered by pathogen effectors, known as effector-triggered immunity (ETI). When pathogens deliver effectors into the plant cell, plant resistance（R）proteins can recognize specific effectors and activate a range of strong defense responses, which often leads to localized cell deaths known as hypersensitive responses (HR) (Jones and Dangl, 2006;

Chisholm et al., 2006).

R proteins perceive effectors through either direct or indirect interactions (Van Der Biezen and Jones, 1998). A direct interaction example has been demonstrated between the tomato bacterial speck resistance gene product, Pto, and the corresponding avrPto avirulence gene product of *Pseudomonas syringae* pv. *tomato* (Scofield et al., 1996; Tang et al., 1996). More Often, R proteins detect effectors in an indirect manner. For example, RPM1-interacting protein 4 (RIN4) is a plasma membrane localized conserved protein of 211 amino acids. RPM1 can recognize AvrRpm1 and AvrB induced RIN4 phosphorylation and trigger RPM1-dependent immunity. Another R protein RPS2 recognizes AvrRpt2-induced RIN4 cleavage and activate RPS2-dependent immunity (Mackey et al., 2002; Mackey et al., 2003).

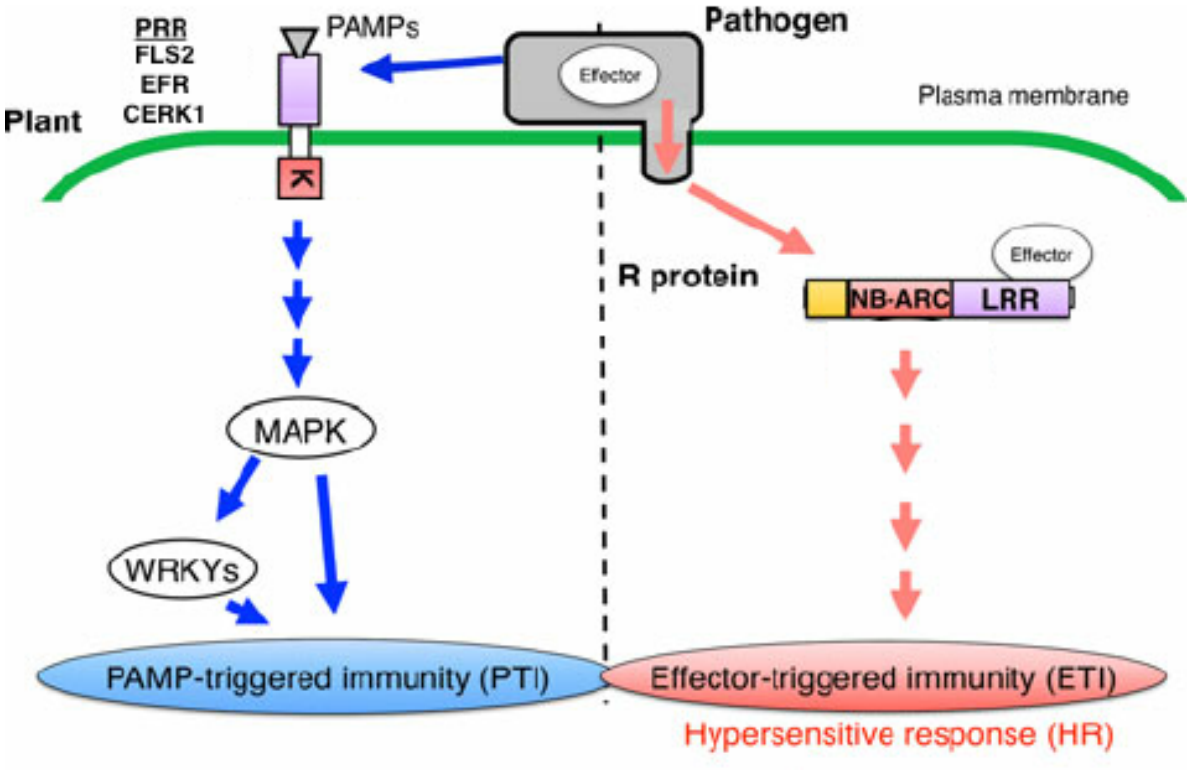

Figure 1-1 Simplified Model of plant innate immunity. Plasma membrane localized PRRs are the first layer of defense against pathogen. PAMPs are perceived by PRRs and then trigger PTI. R proteins act as intracellular receptors for the direct or indirect recognition of specific pathogen secreted effectors (also called avirulence (Avr) proteins) and induce ETI. (Modified from Islas-Flores et al., 2015).

1.2 Receptor-like kinases (RLKs) and receptor-like proteins (RLPs)

In plants, membrane bound immune receptors function as PRRs that perceive corresponding PAMPs. Currently known plant PRRs are either receptor-like kinases (RLKs), which possess a ligand-binding ectodomain, such as LRRs, lectin or lysine motif (LysM), a single transmembrane spanning region, and an intracellular kinase domain to transduce signal, or receptor-like proteins (RLPs), which share the same overall structure with RLKs but lack an intracellular kinase domain. Because RLPs do not have any obvious signaling domains in their short intracellular region, they most likely function in combination with one or several RLK-type receptors to transduce ligand binding into intracellular signaling (Zipfel, 2014). The best

studied RLPs involved in plant defense are the Cf proteins in tomato like Cf-4 and Cf-9, which confer resistance to the fungal tomato pathogen *Cladosporium fulvum*. (Thomas, 1997; Jones et al., 1994).

1.3 The RLK BAK1 serves as a PTI central regulator

PRRs usually recruit other components to form a complex and transduce the signal (Monaghan and Zipfel, 2012). One good example of PRR complex is the well-studied FLS2 complex. Upon perception of flagellin or the conserved 22 amino acid peptide flg22, FLS2 rapidly associates with another RLK, Brassinosteroid Insensitive 1 (BRI1)-Associated Receptor Kinase1 (BAK1) (Chinchilla et al., 2007).

BAK1, also known as SERK3, is a small LRR-RLK which was originally identified as an interactor and positive regulator of the brassinosteroid (BR) receptor BRI to play essential roles in plant development and growth (Li et al., 2002; Clouse, 2011). BAK1 belongs to a small protein family termed Somatic-Embryogenesis-Receptor-Like Kinases (SERKs) that were shown to be important in plant defense against diverse pathogens (Roux et al., 2011). Upon perception of the conserved 22 amino acid peptide flg22 or another conserved peptide elf18 from bacteria elongation factor Tu, BAK1 rapidly forms ligand-induced complexes with FLS2 and EFR (Boller and Felix, 2009). There is currently no evidence showing that BAK1 is required for the defense responses mediated by CERK1.

In addition to BAK1, another SERK member, BAK1-Like1 (BKK1), can also interact with

FLS2 and EFR in a ligand-dependent manner (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011). Moreover, He et al (2007) revealed that BKK1 functions redundantly with BAK1 in regulating BR signaling and the *bak1 bkk1* double mutants exhibit a seedling-lethality phenotype due to constitutive activated defense response and spontaneous cell death. All this evidence demonstrates that BAK1 and BKK1 have dual physiological roles in regulating plant growth, defense and cell death pathway.

Another BAK1 mutant allele *bak1-5*, which contains a point mutation (C408Y) in the kinase domain, strongly impairs the PTI response but not the BR signaling, and the *bak1-5 bkk1-1* shows normal growth morphology. In a *bak1-5 bkk-1* double mutant, EFR- and FLS2-dependent defense responses were significantly compromised, confirming that both BAK1 and BKK1 play an important role in PTI signaling (Roux et al., 2011; Schwessinger et al., 2011).

One reported component that connects the PRRs on the plasma membrane and downstream defense responses in the cytosol is a receptor-like cytoplasmic kinase (RLCK) named Botrytis-Induced Kinase 1 (BIK1). BIK1 was originally identified as a positive regulator of plant defense against necrotrophic fungal pathogens (Veronese et al., 2006). It was later shown that BIK1 associates with both BAK1 and FLS2 (Lu, Wu et al., 2010). BIK1 is rapidly phosphorylated upon flg22 treatment in a BAK1- and FLS2-dependent manner. Based on in vitro kinase assay, BIK1 was suggested to transphosphorylate FLS2/BAK1 to propagate flagellin signal (Lu et al., 2009). Recent studies showed that activated BIK1 could phosphorylate the NADPH oxidase RBOHD (Li et al., 2014), which leads to the production of ROS.

1.4 SOBIR1 plays a central role in signaling by receptor-like proteins

Another RLK, Suppressor of *bir1-1*, 1 (SOBIR1) was originally identified in a genetic screen searching for suppressors of the *bir1* (BAK1-Interacting RLK 1) (Gao et al., 2009). BIR1 is a receptor-like kinase that interacts with BAK1 (Gao et al., 2009; Wang et al., 2011). A T-DNA insertion mutant of *BIR1*, *bir1-1*, shows a constitutive defense phenotype and extreme dwarfism. It was proposed that BIR1 functions as a negative regulator of defense-associated LRR-RLKs. A suppressor screen of *bir1-1* yielded mutations in the LRR-RLK SOBIR1 that restore the growth and defense phenotype (Gao et al., 2009). It suggests that SOBIR1 acts as a positive regulator of defense, which is normally inhibited by BIR1 in wild type Arabidopsis. In agreement with this hypothesis, overexpression of *SOBIR1* in Arabidopsis resulted in a constitutive defense phenotype and elevated *PR* gene expression (Gao et al., 2009). However, no direct interaction between BIR1 and SOBIR1 was detected (Gao et al., 2009).

Multiple *bak1* alleles were also identified from the *bir1-1* screen. BAK1 was shown to be required for the cell death phenotype in *bir1-1* (Liu et al., 2016). Moreover, overexpression of *BAK1* or *BKK1* could cause detrimental effects on plant development along with stimulated immune responses, which are SOBIR1-dependent (Domínguez-Ferreras et al., 2015).

SOBIR1 expression is induced upon perception of the bacterial flagellin-derived peptide flg22 ligand by FLS2 in Arabidopsis cell cultures and seedlings（Navarro, 2004). Likewise, *SOBIR1* is induced upon activation of the EFR receptor (Tintor et al., 2013). However, Arabidopsis *sobir1* mutants are not compromised either in flg22-induced or elf18-induced PTI

responses (Zhang et al., 2013), suggesting that SOBIR1 is not directly involved in FLS2- and EFR- mediated immunity.

Recently, several studies revealed that SOBIR1 play important roles in immunity mediated by LRR-RLPs. In Arabidopsis, RLP30 is required for resistance against necrotrophic fungal pathogens and loss-of-function of *SOBIR1* greatly compromises RLP30-mediated immunity (Zhang et al., 2013). Another LRR-RLP receptor protein, RLP23, binds to a conserved 20-amino-acid fragment found in most necrosis and ethylene-inducing peptide 1-like proteins (NLPs) to activate a downstream PTI response. RLP23 forms a constitutive, ligand-independent complex with SOBIR1, and recruits BAK1 into a tripartite complex upon ligand binding (Albert et al., 2015). In tomato, there are two redundant SOBIR1 homologues, SlSOBIR1 and SlSOBIR1-like. The Cf proteins Cf-4 and Cf-9 form a constitutive complex with SOBIR1 and recruit BAK1 upon their activation by the cognate effectors (Liebrand et al., 2013). In addition, tomato SOBIR1 homologues also interact with tomato RLPs Ve1 and Eix2, where Eix2 acts as a receptor of Ethylene-Inducing Xylanase (EIX) and Ve1 recognizes secreted effector Ave1 from the vascular pathogen *Verticillium dahliae* and *Verticillium albo-atrum* (Liebrand et al., 2013; de Jonge et al., 2012; Zhang et al., 2013; Ron, 2004). A recent study shows that SOBIR1 contains a typical "glycine zipper" (GxxxGxxxG) motif which is essential for the interaction with RLPs (Bi et al., 2015). All of this evidence suggests that SOBIR1 plays an important role in RLP-mediated immune signaling by acting as a common adaptor for the LRR-RLPs immune-receptors.

1.5 Heterotrimeric G proteins serve as a point of convergence in plant defense signaling

In fungi and metazoans, extracellular signals are often perceived by cell-surface localized G-protein-coupled receptors (GPCRs) and transduced through heterotrimeric G-protein complexes to downstream targets. In *Arabidopsis*, heterotrimeric G proteins, which consist of one G α-subunit (G Protein α-Subunit 1 [GPA1]), one G β-subunit (Arabidopsis G Protein β-Subunit 1 [AGB1]), and three G γ-subunits (Arabidopsis G Protein γ-Subunit 1 [AGG1/2/3]), have been shown to be involved in a wide range of biological processes (Temple and Jones, 2007). AGB1 was identified from the SOBIR screen. Mutations in *AGB1* suppress the cell death and defense responses in *bir1-1* and transgenic plants overexpressing *SOBIR1* (Gao et al., 2009; Liu et al., 2013). In addition, *agb1* mutant plants are severely compromised in immunity mediated by other RLKs including FLS2, EFR and CERK1, suggests that heterotrimeric G proteins serve as a point of convergence in plant defense signaling activated by multiple RLKs (Liu et al., 2013).

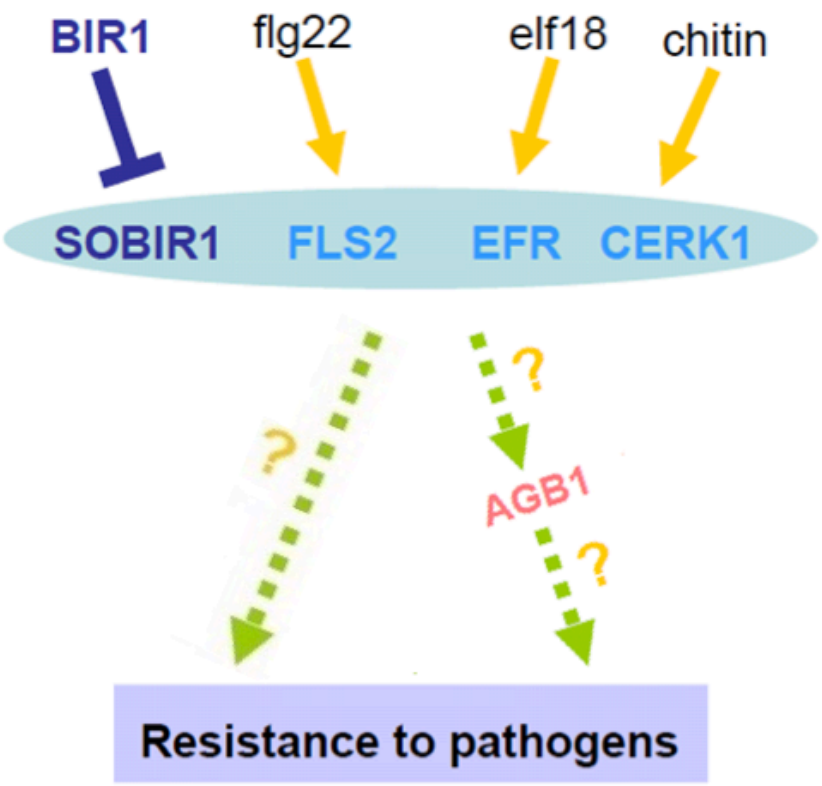

Figure 1-2 AGB1 serves as a point of convergence in plant defense signaling activated by multiple RLKs

AGB1 is localized at the plasma membrane (PM), which indicates that it may function together with RLKs. However, initial experiments failed to demonstrate an interaction between AGB1 and the kinase domains of SOBIR1, BAK1, FLS2, CERK1, or EFR (Liu et al., 2013). A more recent study reported that heterotrimeric G proteins modulates flagellin-triggered immunity by directly coupling to the FLS2-BIK1 receptor complex. Heterotrimeric G proteins function to stabilize the BIK1 protein to ensure that it is ready to respond in the absence of an infection. Upon activation of the FLS2-BIK1 complex by corresponding PAMP flagellin, BIK1 directly phosphorylates Extra-Large GTP-Binding Protein 2 (XLG2) at the its N terminus to regulate flagellin-induced ROS production (Liang et al., 2016).

1.6 Metacaspases

Protein degradation, or proteolysis, is the catabolism process by hydrolysis of peptide bond via enzymes known as proteases (Cooper and Hausman, 2000). There are different types of proteases and the largest protease families include serine, aspartate, and cysteine proteases (Beers, Jones and Dickerman, 2004). It has been demonstrated that proteases can have different substrate specificities, along with different protease cleavage preferences after specific amino acids (García-Lorenzo, 2007). In the case of cysteine proteases, a nucleophilic cysteine is first activated by a basic side chain usually a histidine residue via deprotonation and the cysteine nucleophile cleaves the peptide bond of the substrate (van der Hoorn, 2008). An asparagine residue is present in order to orient the histidine side chain in the appropriate direction (García-Lorenzo, 2007).

Caspases are cysteine dependent aspartate-specific proteases playing an important role in apoptosis or programmed cell death (PCD) in animals (Lee et al., 2010). Caspases are first synthesized as an inactive precursor, procaspase, and then activated following autoprocessing (autocleaving) process, which generates a large (p20) and a small (p10) subunit and brings conformational changes to allow access of the substrate to the active site for cleavage (Lee et al., 2010). Plants can possess different types of caspase-like activities (Bonneau et al., 2008). Caspase-like activities have also been detected in several different locations of the cell, such as the nucleus, cytosol and vacuole. Currently, it remains unclear whether the caspase-like activities observed in plants are directly involved in PCD and what roles they may play (Bonneau et al., 2008).

Homologous proteases were identified in other organisms following the discovery of caspases in animals. Bioinformatic analysis also identified two groups of proteins that are structurally similar to caspases, the paracaspases (PCs) and the metacaspases (MCs) (Uren, 2000). PCs are present in metazoans, while MCs can be found in plants, bacteria, fungi, chromista, and protozoa (Uren, 2000). There are two types of MCs, type I MC and type II MC, which are classified based on their overall protein structures and the level of sequence similarity (Uren et al., 2008). Both type I and type II metacaspases have a putative conserved caspase-like catalytic domain composed of 20 kDa (p20) and 10 kDa (p10) subunits (Ojha et al., 2010), which contain the catalytic amino acid dyad histidine/cysteine. The type I metacaspases, but not type II, exhibit an N-terminus extension that usually contains a zinc-finger motif as well as a prodomain rich in proline and may or may not contain a gliutamine-rich region, the metacaspase domain is located at the C-terminal region of the protein (Lam and Zhang, 2012). Type II metacaspases do not have the prodomain and the zinc finger motif, but carry an insert (linker) between the p20 and p10 subunits (Fagundes et al., 2015) (Fig.1-3).

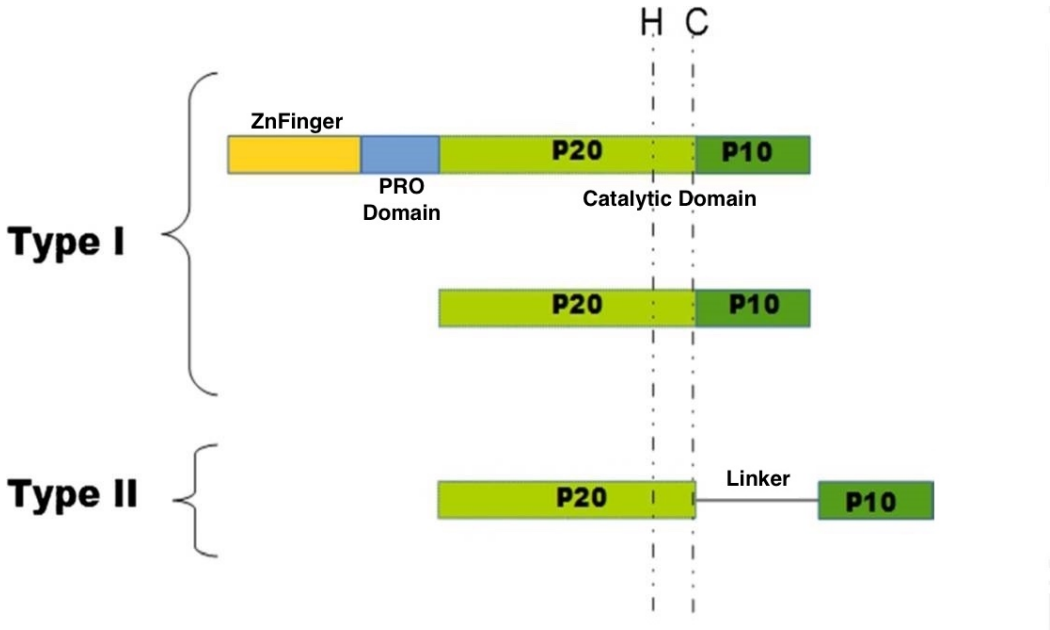

Figure 1-3 Protein structure of two types of metacaspases. The type I metacaspases may or may not have a prodomain rich in proline and a zinc finger motif in the N-terminus region, and have a caspases-like domain at the C-terminus region. Type II metacaspases do not contain the proline rich domain and the zinc finger motif, but feature an insert (linker) between the p20 and p10 subunits. The dotted lines indicate the catalytic amino acid regions containing histidine (H) and cysteine (C) residues. (Modified from Fagundes et al., 2015)

One common character among caspases and both types of MCs is the presence of a histidine/cysteine (His/Cys) dyad (Cambra, Garcia and Martinez, 2010). Similar to caspases, MCs also form a heterodimer made up of the p20 and p10 subunits (Woltering, 2002; Vercammen, 2004). Studies have shown that, just like caspases, MCs also need to be activated via autoprocessing in a cysteine dependent manner (Vercammen, 2004; Belenghi et al., 2006; Watanabe and Lam, 2011).

Even though the fact that caspases and MCs show a similar overall structure, both contain a caspase-specific catalytic diad of histidine and cysteine, and share a basic mechanism of catabolism, still, there are defining differences between the two. One of the largest differences is the cleavage preference (Bonneau et al., 2008). Caspases cleave their substrate after an

aspartate residue. In contrast, MCs cleave after either a basic lysine (Lys) or arginine (Arg) residue at P1 position (refer to the N-terminus direction from the cleaved bond), which is why they cannot be defined as caspases (Vercammen, 2004; Watanabe and Lam, 2005; Watanabe and Lam, 2011). A second major difference is the low sequence homology between the two protease families (Cambra, Garcia and Martinez, 2010). While MCs possess two cysteine residues in their sequence, caspases only have one (Belenghi et al., 2006). Due to these differences, it is considered that MCs are unlikely the homologs of caspases (Vercammen, 2004; Bonneau et al., 2008), despite the fact that MCs have similar structure as caspases.

In *Arabidopsis*, there are a total of nine MC (AtMC) genes making up a gene family which consists of three MC type I (AtMC1-3) and six MC type II genes (AtMC4-9) (Fig.1-4). Recently, the the substrate specificity of MC9 was identified through assessing the degradome of the arabidopsis MC9 (Tsiatsiani et al., 2013). Phosphoenolpyruvate carboxykinase 1 (PEPCK1), a key enzyme in gluconeogenesis, was identified as the physiological substrates of MC9 in *Arabidopsis thaliana* on the proteome-wide level. In addition, cleavage of PEPCK1 by AtMC9 in vivo and in vitro at Lys-19 and Arg-101 were characterized by means of positional proteomics (Tsiatsiani et al., 2013).

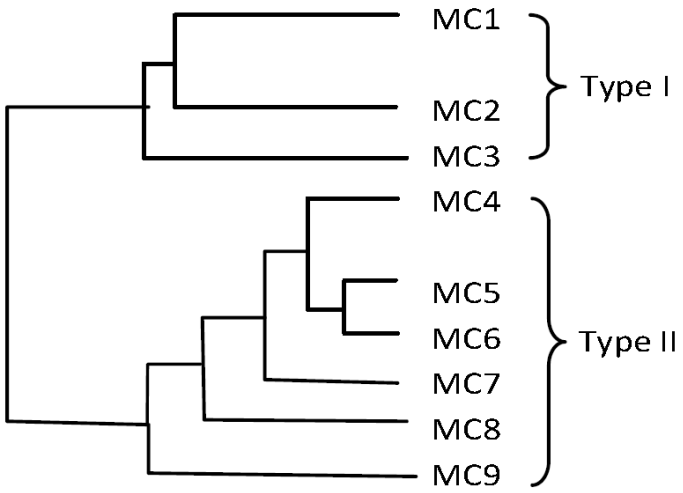

Figure 1-4 Phylogenetic tree of AtMCs (Vercammen, 2004).

Another study focusing on the role of type I metacaspases in Arabidopsis, demonstrated that MC1 and MC2 function antagonistically in controlling programmed cell death. MC1 acts as a positive regulator of cell death triggered by activation of different plant intracellular NLR innate immune receptors and requires conserved caspase-like putative catalytic residues for its function (Coll et al., 2010). MC1 and MC2 are both type I MCs that contain a N-terminal zinc-finger motif with sequence similarities to that found in the Lesion Simulating Disease 1 (LSD1). LSD1 encodes a small zinc finger protein and acts as a negative regulator of PCD (Dietrich et al., 1997). The *lsd1* mutant shows abnormal cell death triggered by ROS and SA, and presents a runaway cell death (RCD) phenotype (Dietrichet al., 1994; Jabs et al., 1996; Kliebenstein et al., 1999). MC1 was shown to be able to bind strongly to LSD1 protein as well as weakly with itself to form homo-dimers via its N-terminal region, whereas MC2 did not appear to interact with either LSD1 or MC1 proteins. Loss of function of *MC1* was found to suppress cell death activation in *lsd1*, by contrast, loss of *MC2* dramatically increased the level of cell death upon

salicylic acid analog benzo-(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) application. Conversely, overexpression of *MC1* can cause spontaneous cell death in the wild-type Arabidopsis background without BTH application while overexpression of *MC2* can suppress BTH-induced cell death in the *lsd1* background. These genetic studies showed that MC1 is a pro-death regulator, whereas MC2 acts to suppress cell death induction downstream of the LSD1/MC1 step (Coll et al., 2010). Other than this study, not much have been focused on type I MCs in regulating plant defense against pathogens.

1.7 Thesis objective

MC2 is a type I metacaspase with a pro domain and zinc finger motif. A T-DNA insertion allele of *MC2* which was isolated from a reverse genetic screen shows constitutively activated defense phenotypes including enhanced disease resistance to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*) ES4326 and a dwarf stature. My thesis research focused on the role of MC2 and its relationship to other known components in the pathway of pathogen-associated molecular patterns triggered immunity. Another main objective of my master's research was to clarify the details of MC2-mediated defense signaling through conducting a genetic suppressor screen.

Chapter 2 Arabidopsis metacaspase AtMC2 serves as a negative regulator in plant immunity in a BAK1/BKK1- and SOBIR1-dependent manner

2.1 Introduction

To identify new key regulators involved in plant immunity, a reverse genetic screen was carried out two years ago by another lab member, MS. Fang Gao. In this screen, lines with mutations in each of two closely related genes which were identified as targets of a master transcription factor of plant immunity constructed. One line used in this screen, the *mc1 mc2* double mutant, showed enhanced disease resistance to the bacterial strain *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*) ES4326 and a dwarf stature. The main objective of my thesis was to clarify the involvement of these two type I metacaspases in plant defense signaling. In the results part, I show here that the enhanced disease resistance phenotype is caused by the *MC2* mutation and MC2 functions upstream of BAK1/BKK1 and SOBIR1 to negatively regulate PAMP-triggered immunity.

2.2 Results

2.2.1 Loss of function of *MC2* leads to constitutive defense responses

To test whether the enhanced pathogen resistance observed in the *mc1 mc2* double mutant is caused by *mc1* or *mc2*, the *mc1* and *mc2* single mutants was isolated by backcrossing the double mutant with wild-type Col-0. The *mc2* single mutant was found to exhibit dwarf morphology and curly dark green leaves, which is often associated with autoimmune phenotype (Fig. 2-1a). In contrast, the *mc1* single mutant showed normal growth phenotype

like wild-type Col-0.

To test whether *mc2* mutant has enhanced pathogen resistance, *mc2* plants were challenged with two virulent bacterial strains, *Pseudomonas syringae* pv. *tomato* (*P.s.t.*) DC3000 and *P.s.m*. ES4326. As shown in Figure 1b and 1c, *mc2* plants displayed strong enhanced resistance against *P.s.t.* DC3000 and *P.s.m.* ES4326 (Fig. 2-1b and 1c). Quantitative RT-PCR (qRT-PCR) analysis revealed that the expression of defense marker genes *PR-1* (Fig. 2-1d) and *PR-2* (Fig. 2-1e) was constitutively expressed in the *mc2* mutant plants. These data suggest that defense responses were constitutively activated in the *mc2* mutant plants.

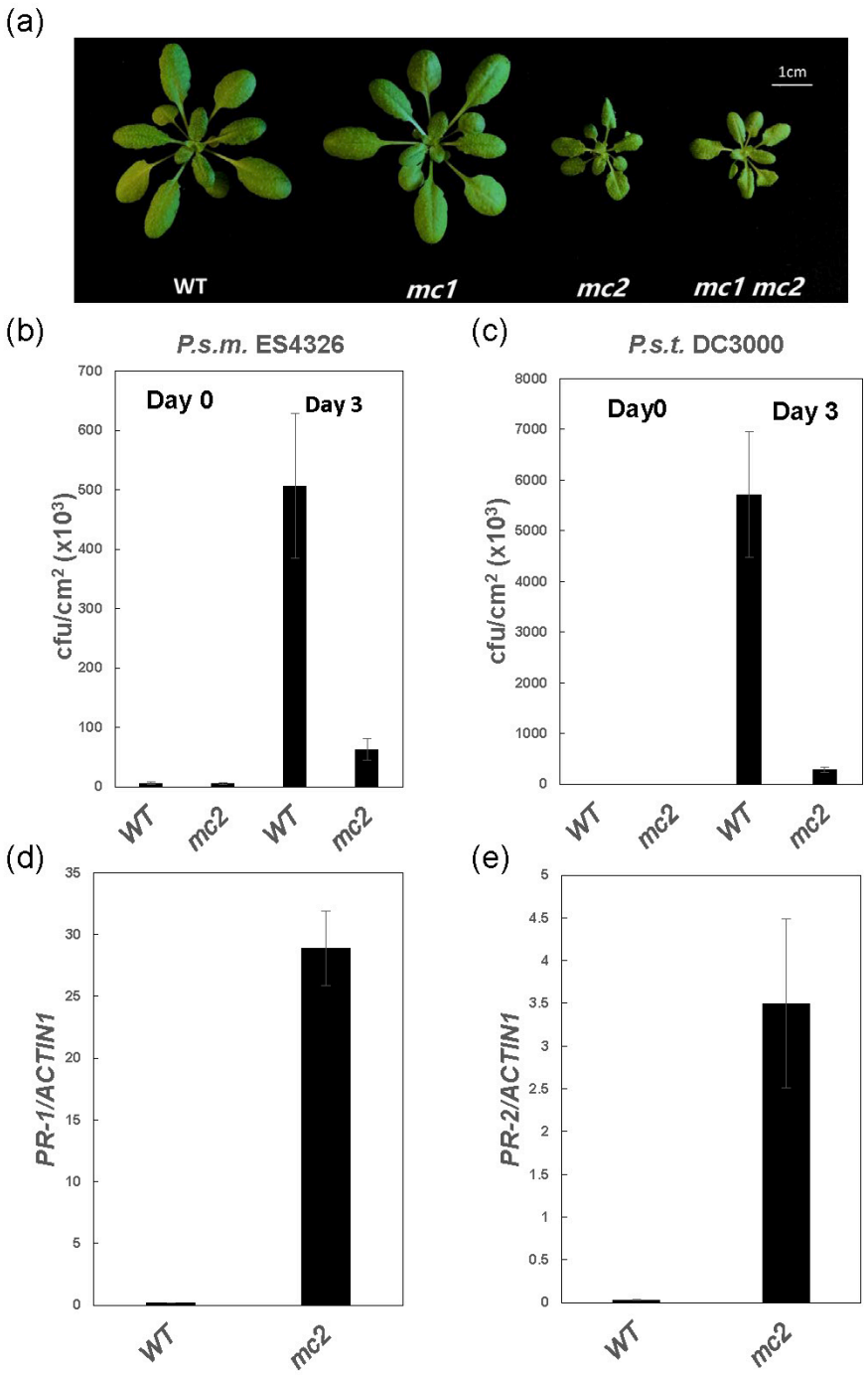

Figure 2-1 Characterization of the *mc2* mutant.
(a) Morphology of Col-0 (wild-type, WT) and *mc2*. Plants were grown on soil at 23°C and photographed three weeks after planting.
(b) Growth of *P.s.m.* ES4326 on wild-type and *mc2*. Leaves of four-week-old plants were infiltrated with a bacterial suspension at OD_{600} = 0.0001. The values presented are averages of five replicates ± SD.
(c) Growth of *P.s.t.* DC3000 on wild-type and *mc2*. Leaves of four-week-old plants were infiltrated with a bacterial suspension at OD_{600} = 0.0002. The values presented are averages of five replicates ± SD.
(d, e) Expression levels of *PR1* (d) and *PR2* (e) in wild-type and *mc2* seedlings compared to *ACTIN1*. Error bars represent SD from averages of three measurements. Total RNA was extracted from two-week-old seedlings grown on half-strength MS plates.

To test whether the *mc2* mutant phenotypes are caused by the T-DNA insertion in *MC2*, *mc2* plants were transformed with a construct expressing *MC2* with a C-terminal 3xHA tag driven by cauliflower mosaic virus 35S promoter and three representative transgenic lines were further characterized. As shown in Figure 6a, *MC2* fully complements the dwarf morphology of *mc2*. In addition, the expression of *PR2* in the transgenic plants was also reverted to wild-type levels (Fig. 2-2b). Thus, *mc2* mutant phenotypes were complemented by *MC2*.

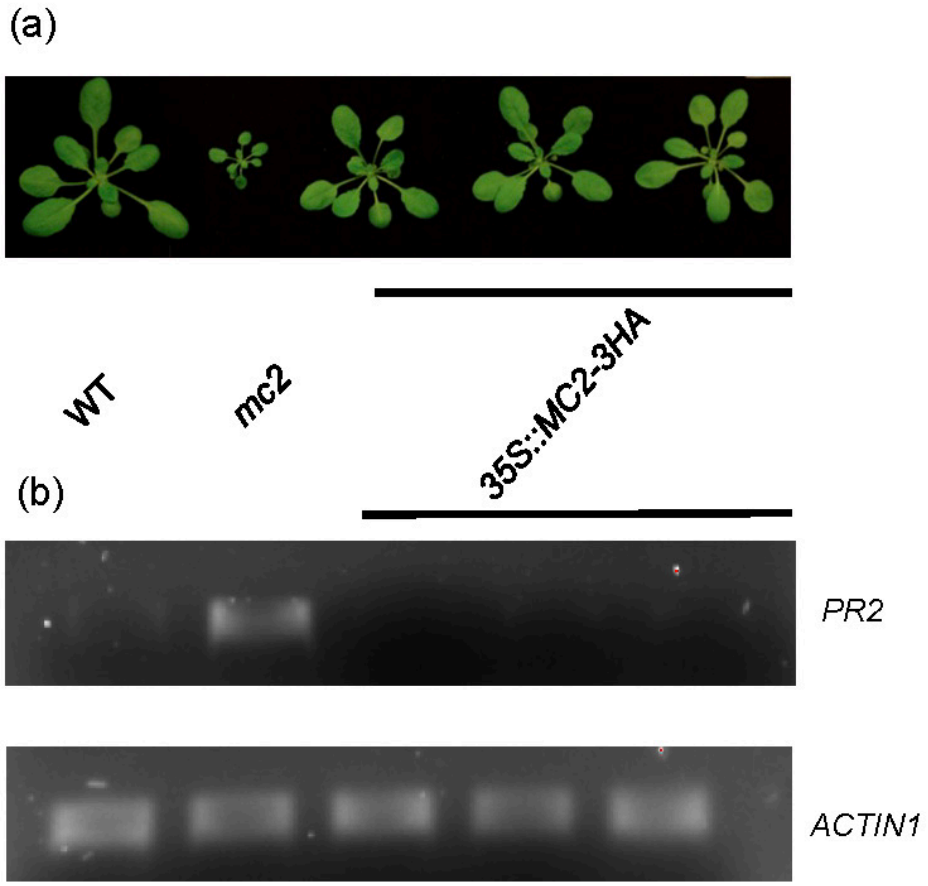

Figure 2-2 Analysis of 35S:*MC2* transgenic lines in the *mc2* mutant background.
(a) Phenotypes of wild-type, *mc2*, and three lines of *mc2* transformed with a 35S:*MC2-3xHA* construct. Plants were grown on soil at 23°C and photographed three weeks after planting.
(b) *PR2* gene expression in wild-type, *mc2* and three transgenic lines as determined by RT-PCR. Both *PR2* and *ACTIN1* were amplified with 32 cycles.

2.2.2 Overexpression of *MC2* leads to enhanced disease susceptibility

To determine whether overexpression of *MC2* suppresses defense responses, transgenic plants expressing *MC2* under the 35S promoter in wild-type Col-0 background were generated. Four representative lines with different level of MC2 protein accumulation were analyzed in detail (Fig. 2-3a). All four *MC2* overexpression lines were found to display enhanced growth of *P.s.t.* DC3000 (Fig. 2-3b), suggesting that MC2 functions as a negative regulator of plant immunity.

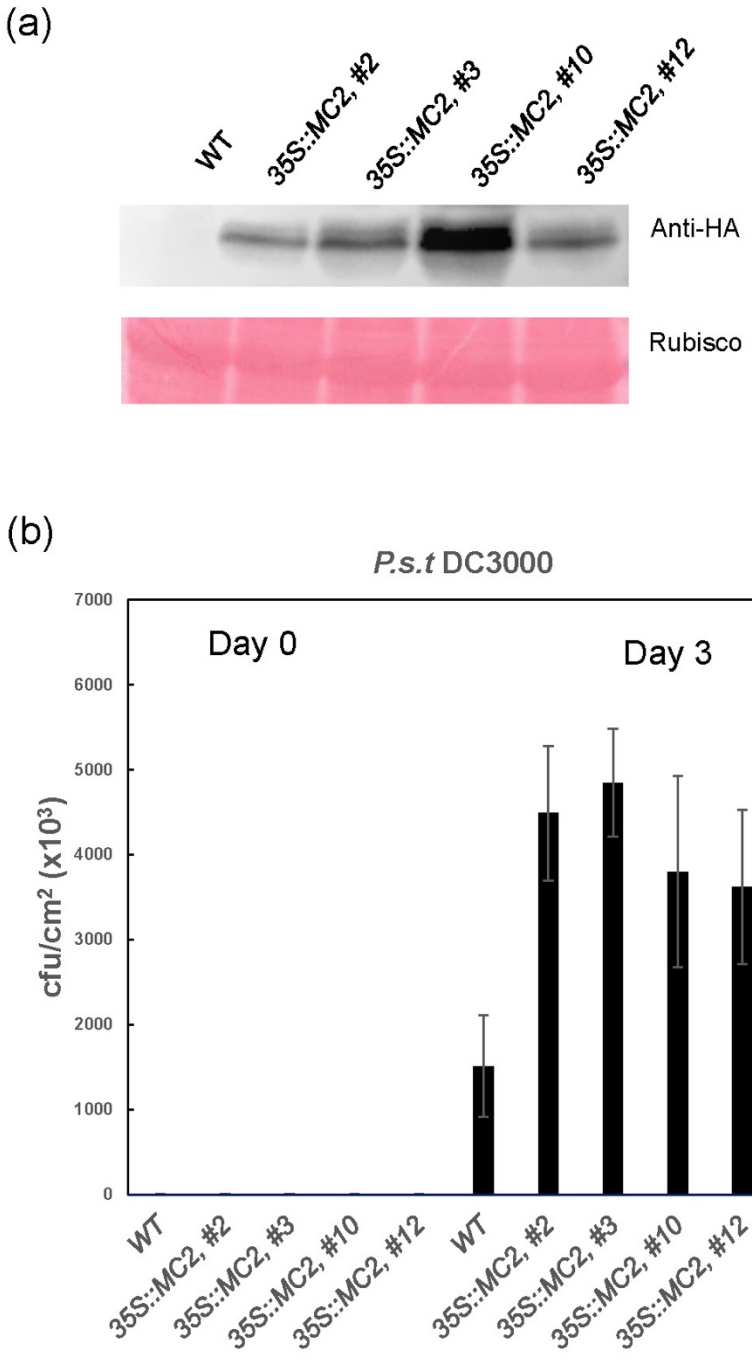

Figure 2-3 Analysis of transgenic plants overexpressing *MC2*.
(a) Expression of *MC2-3xHA* was detected by Western blot using an anti-HA antibody.
(b) Growth of *P.s.t.* DC3000 on wild-type and *MC2* overexpression lines. Leaves of four-week-old plants were infiltrated with a bacterial suspension at OD_{600} = 0.0002. The values presented are averages of five replicates ± SD.

2.2.3 Loss of function of *MC1* does not suppress or enhance the constitutive defense response in *mc2*

To determine whether the constitutive defense responses are affected by the mutation in *mc1*, we compared *PR1* gene expression and resistance against the oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 in *mc1, mc2* and *mc1 mc2*. As shown in Figure 2-4, both *PR1* expression and growth of *H.a.* Noco2 are comparable in *mc2* and *mc1 mc2*, but not *mc1* suggesting that loss function of *MC1* does not suppress or enhance the autoimmune phenotype of *mc2*.

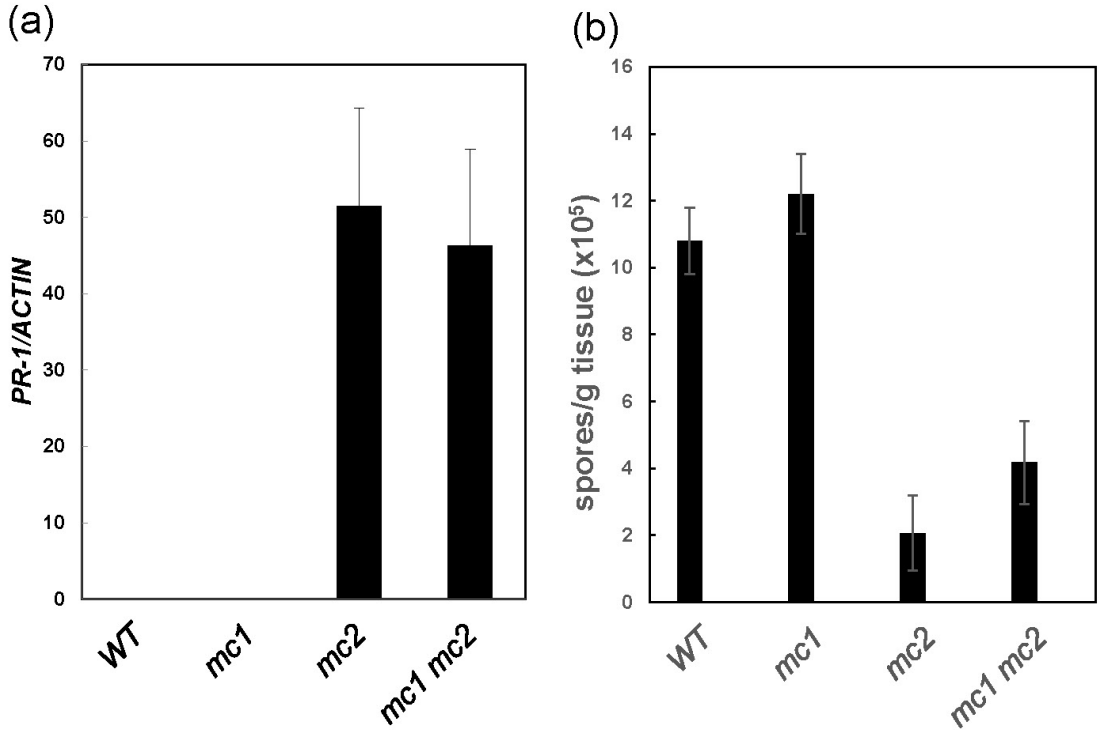

Figure 2-4 Characterization of *mc1 mc2*.
(a) Expression levels of *PR1* in wild-type, *mc1*, *mc2* and *mc1 mc2* seedlings compared to *ACTIN1*. Total RNA was extracted from two-week-old seedlings grown on half-strength MS plates.
(b) Growth of *H. a.* Noco2 on wild-type, *mc1*, *mc2* and *mc1 mc2* seedlings.
Error bars in (b)-(c) represent the standard deviation of three measurements.

2.2.4 Constitutive defense responses in *mc2* are dependent on *BAK1* and *BKK1*

BAK1 and BKK1 have been shown to play important roles in PTI. To determine whether loss of function of *BAK1* or *BKK1* can suppress the defense responses in the *mc2* mutant, we crossed *bak1-4*, a T-DNA knockout mutant of *BAK1*, into *mc2* to obtain the *bak1-4 mc2* double mutant. We also crossed *bak1-5 bkk1-1*, which carries a point mutation in the kinase domain of *BAK1* and a T-DNA insertion in *BKK1* to obtain the *bkk1-1 mc2* double and the *bak1-5 bkk1-1 mc2* triple mutant. As shown in Figure 9a, both the *bak1-4 mc2* and *bkk1-1 mc2* double mutant are bigger than the *mc2* single mutant, but smaller than the wild type plants in size, whereas the *bak1-5 bkk1-1 mc2* triple mutant is wild-type like. Further analysis of defense responses in the double and triple mutants showed that expression of *PR1* (Fig. 2-5b) and *PR2* (Fig. 2-5c) and resistance to *H.a.* Noco2 (Fig. 2-5d) in *mc2* are also suppressed. These data suggest that both BAK1 and BKK1 are required for the constitutive defense responses in *mc2.*

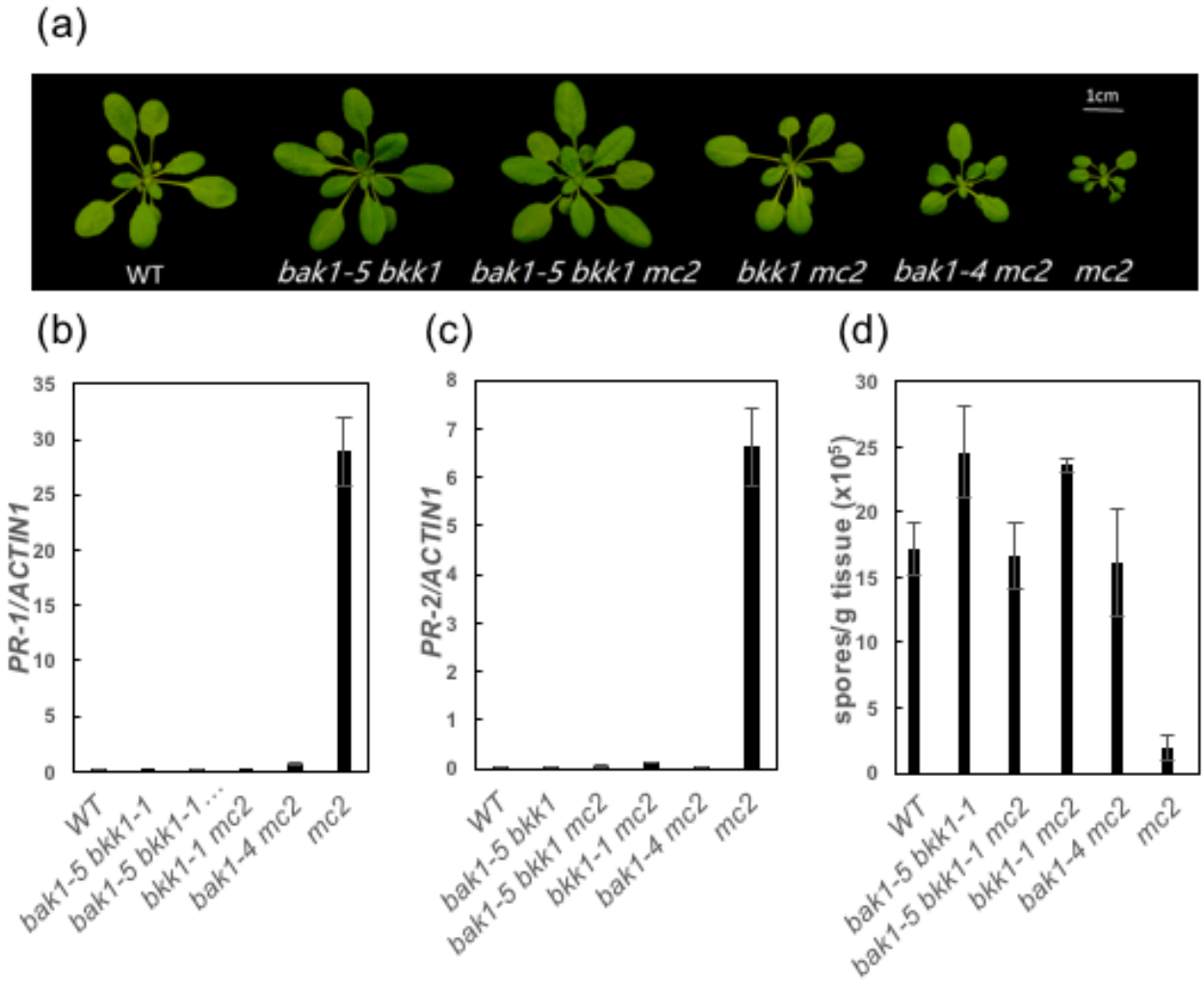

Figure 2-5 Characterization of *bak1-5 bkk1-1 mc2*, *bkk1-1 mc2 and bak1-4 mc2* mutants.

(a) Morphology of Col-0 (wild-type, WT), *bak1-5 bkk1-1*, *bak1-5 bkk1-1 mc2*, *bkk1-1 mc2*, *bak1-4 mc2* and *mc2* plants. Plants were grown on soil at 23°C and photographed three weeks after planting.

(b, c) Expression levels of *PR1* (b) and *PR2* (c) in wild-type, *bak1-5 bkk1-1*, *bak1-5 bkk1-1 mc2*, *bkk1-1 mc2*, *bak1-4 mc2* and *mc2* seedlings compared to *ACTIN1*. Total RNA was extracted from two-week-old seedlings grown on half-strength MS plates.

(d) Growth of *H. a.* Noco2 on wild-type, *bak1-5 bkk1-1*, *bak1-5 bkk1-1 mc2*, *bkk1-1 mc2*, *bak1-4 mc2* and *mc2* seedlings.

Error bars in (b)-(d) represent the standard deviations of three measurements.

2.2.5 Constitutive defense responses in *mc2* is dependent on *SOBIR1*

SOBIR1 has been shown to function as an adapter kinase required for immunity mediated by BAK1 and multiple RLPs. To determine whether loss of function of *SOBIR1* can suppress the defense responses in the *mc2* mutant, we isolated the *sobi1-12 mc2* double mutant from the F2 progeny of a cross between a T-DNA insertion mutant *sobi1-12* and *mc2*. As shown in Figure 2-6a, *sobir1-12 mc2* is much bigger than *mc2*, but slightly smaller than wild type. qRT-PCR analysis showed that the expression of *PR1* (Fig. 2-6b) and *PR2* (Fig. 2-6c) in *sobir1-12 mc2* is dramatically reduced compared to that in *mc2*. In addition, growth of *H.a.* Noco2 in *sobir1-12 mc2* is also significantly higher than in *mc2* and is comparable to that in the wild-type and *sobir1-12* single mutant (Fig. 2-6d). These data suggest that the constitutive defense responses in *mc2* are SOBIR1-dependent.

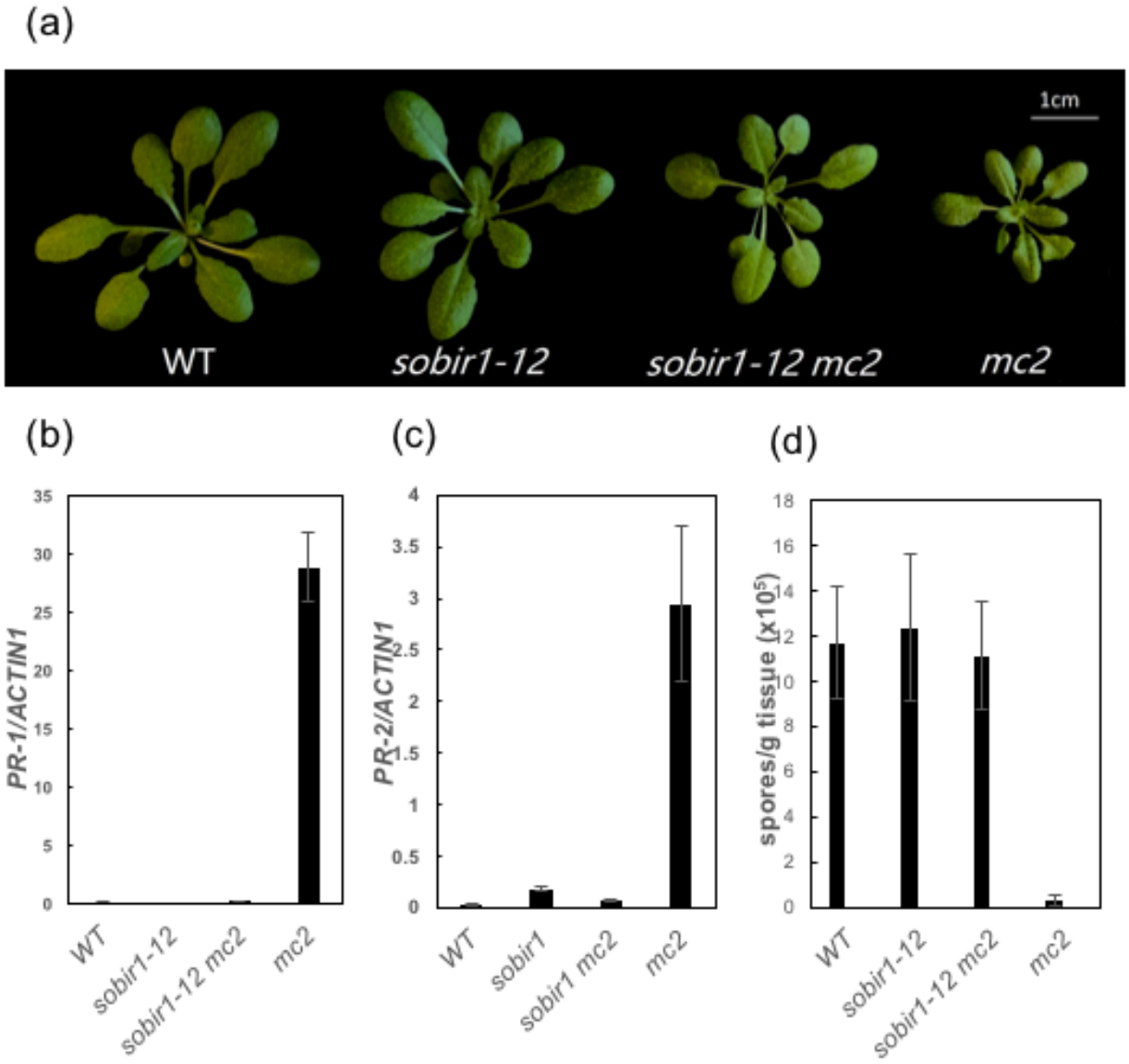

Figure 2-6 Characterization of the *sobir1-12 mc2* double mutant.

(a) Plant morphology of Col-0 (wild-type, WT), *sobir1-12*, *sobir1-12 mc2* and *mc2*. Plants were grown on soil at 23°C and photographed three weeks after planting.

(b, c) Expression levels of *PR1* (b) and *PR2* (c) in wild-type, *sobir1-12*, *sobir1-12 mc2* and *mc2* seedlings compared to *ACTIN1*. Total RNA was extracted from two-week-old seedlings grown on half-strength MS plates.

(d) Growth of *H. a.* Noco2 on wild-type, *sobir1-12*, *sobir1-12 mc2* and *mc2* seedlings.

Error bars in (b)-(d) represent the standard deviations of three measurements.

2.2.6 *agb1-2* partially suppresses the defense responses in *mc2*

AGB1 functions as a point of convergence in plant defense signaling activated by RLKs and functions downstream of BAK1/BKK1 and SOBIR1-mediated defense pathways. To determine whether loss-of-function of *AGB1* can suppress the *mc2* mutant phenotype, we crossed *agb1-2*, a T-DNA insertion mutant allele of *AGB1*, into *mc2*. The *agb1-2 mc2* double mutant is considerably larger than *mc2* in size (Fig. 2-7a). In *agb1-2 mc2*, the expression of *PR1* and *PR2* is partially reduced compared to the *mc2* mutant (Fig. 2-7 b&c). In addition, the enhanced resistance to *H.a.* Noco2 in *mc2* was also partially blocked in *agb1-2 mc2* (Fig. 2-7d). These data suggest that the constitutive defense responses in mc2 are partially dependent on AGB1.

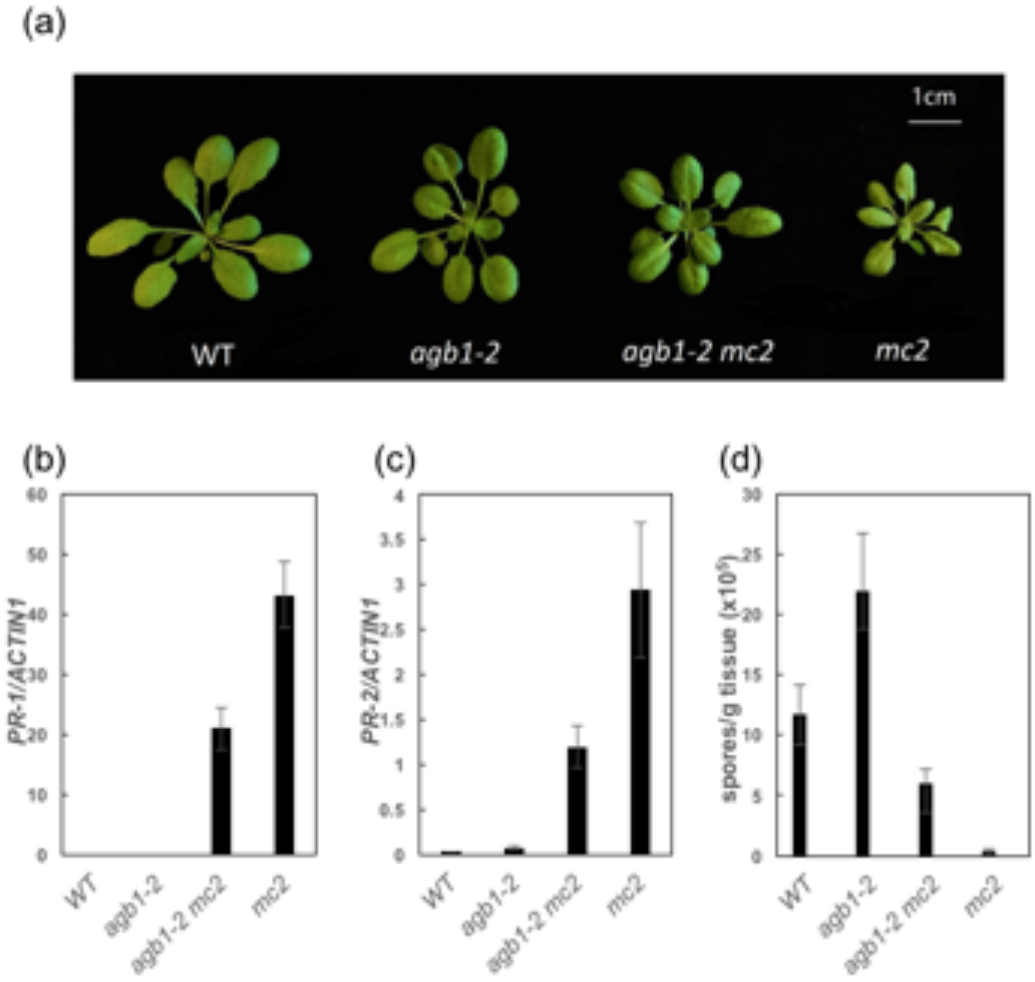

Figure 2-7 Characterization of the *agb1-2 mc2* double mutant.

(a) Plant morphology of Col-0 (wild-type, WT), *agb1-2*, *agb1-2 mc2* and *mc2*. Plants were grown on soil at 23°C and photographed three weeks after planting.

(b, c) Expression levels of *PR1* (b) and *PR2* (c) in wild-type, *agb1-2*, *agb1-2 mc2* and *mc2* seedlings compared to *ACTIN1*. Total RNA was extracted from two-week-old seedlings grown on half-strength MS plates.

(d) Growth of H. a. Noco2 on wild-type, *agb1-2*, *agb1-2 mc2* and *mc2* seedlings.

Error bars in (b)-(d) represent the standard deviations of these measurements.

2.2.7 Identification and characterization of suppressor mutants of *mc2*

Suppressor mutants have been successfully used to study signal transduction pathways activated in autoimmune mutants (van Wersch, Li and Zhang., 2016). To search for regulatory and signaling components required for MC2-mediated defense response, a forward genetic screen was carried out to search for mutants that suppress the autoimmunity of *mc2* using suppression of the stunted growth of *mc2* as a proxy during the primary screen. Approximately 10,000 *mc2* seeds were treated with 0.25% aqueous EMS for 16 hours and subsequently plated on MS plates. About 7,500 M1 plants were later transplanted to soil for self-fertilization. M2 seeds were harvested and planted to look for mutants that suppress the dwarf morphology of *mc2*. Candidate mutants were then subjected to a secondary screen, in which *PR1* expression and resistance to the virulent oomycete strain *H.a.* Noco2 were examined. Mutants that displayed reduced *PR1* expression and enhanced susceptibility to *H.a.* Noco2 compared to *mc2* were selected for further characterization.

Out of 60,000 M2 plants screened, twelve suppressor lines showed dramatic suppression of the dwarf morphology of *mc2* (Fig. 2-8a). Consistent with the morphological suppression, 10 out of 12 line showed significantly enhanced susceptibility to *H.a.* Noco2 (Fig. 2-8c). In addition, expression of *PR1* was significantly reduced in these ten mutants compared to *mc2* (Fig. 2-8b), which is consistent with the increased growth of *H.a.* Noco2. Taken together, these data suggest that these ten mutants contain mutations that suppress the autoimmune phenotypes of *mc2*.

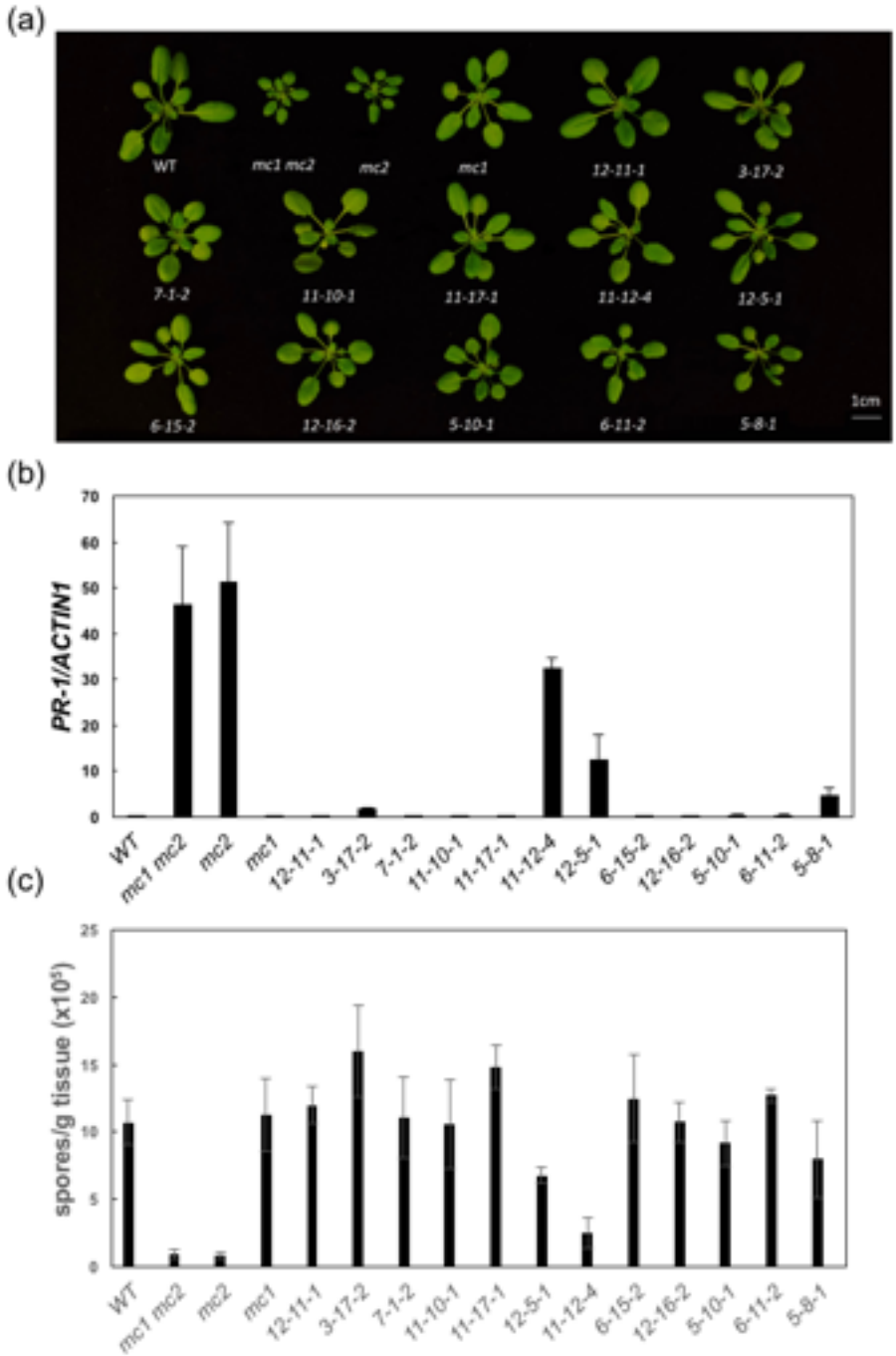

Figure 2-8 Characterization of *mc2* suppressor mutants.

(a) Plant morphology of Col-0 (wild-type, WT), *mc2*, *mc1 mc2* and 12 *mc2* suppressor mutant lines. Plants were grown on soil at 23°C and photographed three weeks after planting.

(b) Expression levels of *PR1* in wild-type, *mc2*, *mc1 mc2* and 12 suppressor mutant seedlings compared to *ACTIN1*. Total RNA was extracted from two-week-old seedlings grown on half-strength MS plates.

(c) Growth of *H. a.* Noco2 on wild-type, *mc2*, *mc1 mc2* and 12 suppressor mutants seedlings.

Error bars in (b)-(c) represent the standard deviations of three measurements.

Chapter 3 Discussion, conclusion and future directions

Metacaspases have previously been shown to play important roles in regulating programmed cell death in plants. In this study we showed that *Arabidopsis* MC2 functions as a negative regulator of PTI. A loss-of-function mutant of *MC2* exhibits dwarf morphology, constitutive *PR* gene expression and enhanced resistance to pathogens. Conversely, overexpression of *MC2* leads to enhanced disease susceptibility. The autoimmune phenotype of *mc2* are dependent on the adaptor RLKs BAK1/BKK1 and SOBIR1, which play critical roles in PTI, suggesting that MC2 functions upstream of these RLKs to negatively regulate plant immunity (Fig. 4-1). The exact molecular mechanism of how MC2 regulates plant immunity is currently unclear.

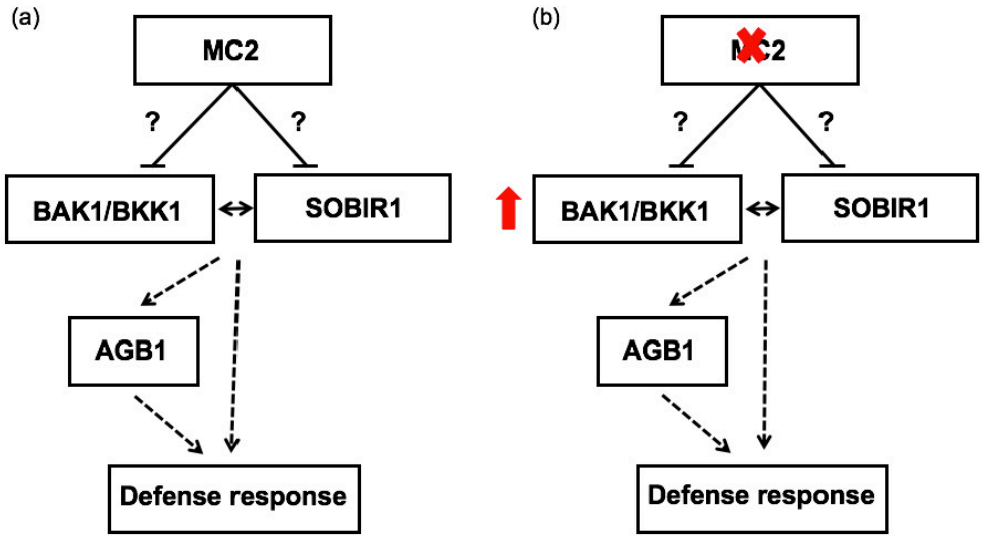

Figure 13. Proposed working model for the MC2-mediated defense signaling. MC2 acts as a negative regulator in plant defense through regulating of adaptor BAK1/BKK1 or SOBIR1 in either a direct or indirect way (a). When MC2 is gone, as in *mc2* mutant (b), it will cause the over-accumulation of BAK1/BKK1 protein to activate a SOBIR1 and AGB1 dependent autoimmunity.

Activation of PTI leads to various downstream defense responses such as oxidative burst, activation of MAP kinase cascades, Ca^{2+} influx and increased SA synthesis. It will be interesting to determine whether these responses are affected in the *mc2* mutant and transgenic plants overexpressing *MC2*. It is also important to determine whether MC2 has a specific role in regulating defense responses activated by specific PAMPs or it functions as a general regulator of PAMP-triggered immunity.

Another important question remained to be addressed is whether the protease activity of MC2 is required for its function in regulating plant immunity. Mutant constructs expressing *MC2* carrying mutations in critical residues in the predicted catalytic sites can be transformed into the

mc2 mutant to test whether they can complement the autoimmune phenotype of *mc2*. If the protease activity of MC2 is required for its function, the next question that needs to be addressed is what the target protein(s) of MC2 is. Since BAK1/BKK1 and SOBIR1 are required for activation of defense responses in *mc2* and overexpression of either BAK1 or SOBIR1 results in autoimmunity, BAK1/BKK1 and SOBIR1 could be potential targets of MC2.

From a suppressor screen of *mc2*, we identified 10 mutants that suppress the autoimmune phenotype of *mc2*. Some of these mutants may contain mutations in potential target proteins of MC2 and others may carry mutations in signaling components downstream of the RLKs BAK1/BKK1 and SOBIR1. Further characterization of these mutants and identification of the mutant genes by mapping and sequencing will help us better understand how PTI is regulated in plants.

Chapter 4 Material and methods

4.1 Plant material

The *mc1 mc2* (CS2102711) mutant was obtained from the European Arabidopsis Stock Centre (NASC) collection and they were reported before (Bolle et al., 2013). All plants were grown at 23°C under 16hr light/8hr dark. To isolate the *mc1* and *mc2* single mutant, *mc1 mc2* was crossed with Col-0 plants. In the F2 population, *mc1* and *mc2* single mutant were isolated by PCR genotyping.

bak1-4 (SALK_116202), *bak1-5 bkk1-1*, *sobi1-12* (SALK_050715) and *agb1-2* (CS6536) have been described previously (Kemmerling et al., 2007; Schwessinger et al., 2011; Joo, 2005; Wang, Narendra and Fedoroff, 2007). All plants were grown at 23°C under 16h light/8h dark regime. To generate the *sobir1-12 mc2* and *agb1-2 mc2* double mutants, *mc2* was crossed with *sobir1-12* and *agb1-2* respectively. In the F2 population, *sobir1-12 mc2* and *agb1-2 mc2* double mutant were isolated by PCR-based genotyping. To generate *bak1-4 mc2*, *bkk1-1 mc2* double mutant and *bak1-5 bkk1-1 mc2* triple mutant, *mc2* was crossed with *bak1-4* and *bak1-5 bkk1* respectively. *bak1-4 mc2*, *bkk1-1 mc2* and *bak1-5 bkk1-1 mc2* were identified in the F2 population by PCR genotyping. The primers used for genotyping are listed in Table 1.

For complementation analysis of *mc2*, a 2 kb fragment of the coding region of *MC2* was amplified from wild type genomic DNA by PCR using primer MC2-sfiI F and MC2-sfiI R (Table 1) and cloned into a modified pGreenHan229 vector to obtain pGreenHan229-*MC2*-3xHA which expresses *MC2* with a C-terminal 3xHA tag under the 35S promoter. The plasmid

was transformed into *Agrobacterium tumefaciens* and subsequently into *mc2* mutant by floral dipping (Clough and Bent, 1998). Transgenic plants were selected by spraying the herbicide BASTA.

For overexpression analysis, the pGreenHan229-*MC2*-3xHA construct was introduced into wild-type (Col-0) background through floral dipping and successful transgenic lines were selected with resistance to BASTA.

4.2 Mutant characterization

H.a. Noco2 infection assay was performed on two-week-old seedlings. The seedlings were sprayed with spore suspensions at a concentration of 50,000 spores/mL water. The plants were covered with a clear dome and kept at 18℃ under 12h light/12h dark cycles in a growth chamber. The humidity in the growth chamber was about 95%. Infection results were scored seven days later by counting the spores using a hemocytometer under a microscope as described previously (Bi et al., 2010).

Bacterial infection was performed by infiltrating two leaves of four-week-old plants grown under short-day conditions (12 h of light/12 h of dark) with *P.s.m.* ES4326 or *P.s.t.* DC3000 at OD_{600} = 0.0001 or 0.0002. Leaf discs within the infiltrated area were taken immediately (day 0) and 3 days after inoculation (day 3) to measure the bacterial growth in those leaves.

For gene expression analysis, total RNA was extracted from 12-day-old seedlings grown on half-strength MS plates using EZ-10 Spin Column Plant RNA Mini-Preps Kit (Bio Basic Inc). The extracted RNA was reverse transcribed into total cDNA using Easy Script

Reverse Transcriptase (Applied Biological Materials Inc). Real-time PCR was performed using SYBR Premix Ex Taq Ⅱ (Takara). Total cDNA was used as a template to determine the expression level of target genes with *ACTIN1* as control. Primers used for real-time PCR analysis of *ACTN1*, *PR1* and *PR2* are listed in Table 1.

4.3 Protein analysis of MC2

Western blot analysis of the MC2-3xHA protein was performed on total proteins extracted from two-week-old seedlings grown on 1/2 strength MS plates using the anti-HA M2 antibody (Sigma-Aldrich). The target protein was detected by chemiluminescence using the SuperSignal Sensitivity Substrate (Thermo Scientific).

4.4 *mc2* suppressor screen

Seeds of *mc2* were mutagenized with 0.25% aqueous EMS for 16 hr. About 60,000 M2 plants were screened to identify mutants with wild type morphology. Putative mutants were subsequently tested for *PR1* expression and *H.a.* NOCO2 growth in the M3 generation.

Table 1 Primers information

Primers used for plasmid constructions	
MC2-SfiI F	5′-GGCCGTCAAGGCCATGTTGTTGCTGGTGGACTG-3′
MC2-SfiI R	5′-GGCCCATGAGGCCTAAAGAGAAGGGCTTCTCATATAC-3′
Primers used for real-time PCR	
PR1 F	5′-GTAGGTGCTCTTGTTCTTCCC-3′
PR1 R	5′-CACATAATTCCCACGAGGATC-3′
PR2 F	5′-CAGATTCCGGTACATCAACG-3′
PR2 R	5′-AGTGGTGGTGTCAGTGGCTA-3′
ACTIN1 F	5′-CGATGAAGCTCAATCCAAACGA-3′
ACTIN1 R	5′-CAGAGTCGAGCACAATACCG-3′
Primers used for genotyping	
bak1-4 F	5′-GGCCACTAAAGTACCATCAG-3′
bak1-4 R	5′-CCTCTCACCGGAGATATTCCT-3′
sobir1-12 F	5′-CTCCCATTGTTAGCACACG-3′
sobir1-12 R	5′-CCAGAAGCATCTGTTCATC-3′
bkk1-1 NF	5′-CCAGCCATTGCGTTTGCTTG-3′
bkk1-1 NR	5′-GCGTACAGCAGTTGTCACA-3′
bak1-5 F	5′-AAGAGGGCTTGCGTATTTACATGATCAGT-3′
bak1-5 R	5′-GAGGCGAGCAAGATCAAAAG-3′
agb1-2 F	5′-GTCGGCGGTTCTCACAGCCGGC-3′
agb1-2 R	5′-GGTCCTCGGTCTTGAGTGATACCA-3′
mc1 F	5′-TGATCGTACTTTCTGTTGGTTCC-3′
mc1 R	5′-CCAATAGTAAGGGAACTTCAGCC-3′
mc2 F	5′-CGAACACGAACAGCGGAAATCGTCAA-3′
mc2 R	5′-CTAGAGGAAACTTACCAATCTCTCCA-3′
SALK Lba1	5′-TGGTTCACGTAGTGGGCCATCG-3′
GABI-KAT LB	5′-GGGCTACACTGAATTGGTAGCTC-3′

Reference

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