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FUNCTIONAL ANALYSES OF SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE FAMILY IN MULTIPLE SIGNALING PATHWAYS IN ARABIDOPSIS

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FUNCTIONAL ANALYSES OF SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE FAMILY IN MULTIPLE SIGNALING PATHWAYS IN ARABIDOPSIS

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This dissertation is dedicated to my wife,

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LIST OF ABBREVIATIONS

2D-DFE:	two-dimensional difference gel electrophoresis
ABA:	abscisic acid
ACC:	1-Aminocyclopropane-1-carboxylic acid
ACS:	ACC synthase
AOX:	alternative oxidase
APN1:	ARABIDOPSIS NPK1-LIKE PROTEIN KINASE1
APX:	ascorbate peroxidase
BAK1:	BRI1 ASSOCIATED RECEPTOR KINASE 1
BES1:	BRI1-EMS-SUPPRESSOR1
BFA:	brefeldin A
BKI1:	BRI1 KINASE INHIBITOR1
BKK1;	BAK1-LIKE 1
BIM:	BES1-INTERACTING MYC-LIKE PROTEIN
BIN2*:*	BRASSINOSTEROID INSENSITIVE2
BL:	brassinolide
BPCS:	biotin-tagged photoaffinity CS
BR:	brassinosteroid
BR6ox1:	BR-6-oxidase1
BRI1:	BRASSINOSTEROID INSENSITIVE 1
BRL1:	BRI1-LIKE 1
BRS1*:*	*BRI1* SUPPRESSOR 1
BRZ:	brassinazole
BSK:	BR-SIGNALING KINASE
BSU1:	*BRI1* SUPPRESSOR1
BZR1:	BRASSINAZOLE-RESISTANT1
CaMV 35S:	Cauliflower mosaic virus 35S
CAT:	catalase
CDC:	CELL DIVISION CYCLE

CFP:	cyan fluorescent protein
cGMP:	cyclic guanosine monophosphate
CPD:	CONSTITUTIVE PHOTOMORPHOGENESIS ANDDWARFISM
CS:	castasterone
CT:	cathasterone
DAB:	3, 3’-diaminobenzidine
DAG:	day after germination
DET2:	DE-ETIOLATED 2
DHA:	dehydroascorbate
DHAR:	DHA reductase
DWF4:	DWARF4
EDS1:	ENHANCED DISEASE SUSCEPTIBILITY 1
EFR:	EF-Tu RECEPTOR
EF-Tu:	elongation factor thermo unstable
ER:	endoplasmic reticulum
FLS2:	FLAGELLIN SENSITIVE2
GA:	gibberellins
GC:	guanylyl cyclase
GC-MS:	gas chromatography-mass spectrometry
GFP:	green fluorescent protein
GPX:	glutathione peroxidase
GR:	glutathione reductase
GSH:	glutathione
GSK3:	glycogen synthase kinase-3-like protein
GST:	glutathione S-transferase,
GTP:	guanosine-5'-triphosphate
GUS:	β-glucuronidase
HR:	hypersensitive response
IP:	immunoprecipitation
KAPP:	kinase-associated protein phosphatase
KD:	kinase domain

LRR:	leucine-rich repeat
LSD1:	LESION SIMULATING DISEASE 1
MAPK:	mitogen-activated protein kinase
MAPKK:	mitogen-activated protein kinase kinase
MAPKKK:	mitogen-activated protein kinase kinase kinase
MBP:	maltose binding protein
MDA:	monodehydroascorbate
MDAR:	MDA reductase
mRNA:	messenger RNA
NADP:	nicotinamide adenine dinucleotide phosphate
NahG:	nd6 salicylate hydroxylase gene
PAD:	PHYTOALEXIN DEFICIENT
PAMP:	pathogen-associated molecular pattern
PCD:	programmed cell death
PCR:	polymerase chain reaction
PR:	pathogen-related
PrxR:	peroxiredoxin
PS:	photosystem
PTI:	PAMP-triggered immunity
RLK:	receptor-like kinase
ROS:	reactive oxygen species
ROT3:	ROTUNDFOLIA3
RT:	reverse transcription
SA:	salicylic acid
SAM:	shoot apical meristem
SEM:	scanning electron microscopy
SERK:	SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE
SOC:	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
SOD:	superoxide dismutase
T-DNA:	transferred DNA
TE:	teasterone

TRIP1:	TGF-β receptor interacting protein 1
tRNA:	transfer RNA
TTL:	TRANSTHYRETIN-LIKE
UDP:	uridine diphosphate
UGT:	UDP-glycosyltransferase enzyme
UGGT:	UDP-glucose:glycoprotein glucosyltransferase

Chapter I

Introduction

1 Brassinosteroid signal transduction pathway

1.1 BRs are a new class of plant hormones

In 1970, Mitchell et al. reported unknown compounds extracted from rape (*brassica napus* L.) pollen could stimulate plant growth; and the compounds were named as brassins (Mitchell et al., 1970). It was not clear what the nature of brassins was and how brassins promoted plant growth. After the first brassin, brassinolide (BL) (Figure 1.1) was purified and the structure of BL was determined by X-ray analysis subsequently (Grove et al., 1979), researchers were able to analyze the roles of brassins in regulating plant growth and development. To date, more than 60 branssins have been identified and they are collectedly named as brassinosteroids (BRs) (Fujioka et al., 2003). The essential roles of BRs have been demonstrated by severe phenotypes of numerous mutants in either BR biosynthesis pathway or BR signaling pathway (Figure 1.1) (Li et al., 1996; Clouse et al., 1996; Szekeres et al., 1996). Lately, the identification of the BR receptor BRI1 (BRASSINOTEROID INSENSITIVE 1) (Li et al., 1997) in Arabidopsis not only dramatically accelerated the research of BR signal transduction, but also indisputably supported BRs as a new class of plant hormones, together with other well-known plant phytohormone classes including auxins, gibberellins (GAs), abscisic acid (ABA), cytokines and ethylene. This section will focus on the progress in studies on BR signaling pathway, including the processes from ligand binding, receptor activation, downstream signaling and induction of BR response genes.

1.2 BRI1 is the ligand-binding receptor of BRs

1.2.1 BRI1 protein structure

Arabidopsis BRI1 belongs to a protein kinase family called leucine-rich repeat receptor-like kinase (LRR-RLK) family with at least 223 members (Shiu et al., 2001). BRI1 consists of an extracellular domain, a single-pass transmembrane domain and a cytoplasmic kinase domain (Figure 1.2). As a typical LRR-RLK, BRI1 has twenty-five leucine-rich repeats in the extracellular domain. LRRs form paralleled β-sheets connected by α-helix, proving conformational structure for protein-protein interaction (Bella et al., 2008). Although the LRRs in BRI1 suggest BRI1 might interact with other proteins in extracellular space, there is still no direct evidence showing BRI1 has any extracellular protein interactors. LRR21 and LRR22 is separated by a 70-amino acid island. In the cytosol, there is a Thr/Ser kinase domain, containing 11 conserved subdomains. Activation of BRI1 kinase domain triggered by perception of BR signals plays a central role in initiating BR signaling cascade.

1.2.2 BRs bind to BRI1

Null *BRI1* mutants show complete insensitivity to the BL treatment (Clouse et al., 1996), implicating the essential function of BRI1 in BR perception. By using biotin-tagged photoaffinity BL precursor castasterone, BPCS, it was demonstrated that a 94 amino acid region including the 70 amino acid island domain and its flanking LRR22 within the BRI1 extracellular domain is responsible for the direct BR binding (Kinoshita et al., 2005). Thus, BRI1 has been confirmed as a ligand-binding receptor in BR signaling.

1.2.3 Activation of BRI1

The kinase activity of BRI1 is negatively regulated by its cytoplasmic C-terminal tail. Deletion of a 41 amino acid fragment at the C-terminal enhanced BRI1 kinase activity, as was revealed by its capability to suppress BR biosynthesis mutant *det2* (Wang

et al., 2005a). Phosphorylation at S/T residues at C-terminal is believed to play a positive role in BRI1 activation; mutations of certain S/T residues to D in distal C-terminal domain, mimicking a phosphorylation effect, dramatically increased BRI1 kinase activity, suggesting the mechanism of inhibition of BRI1 activity by the C-terminal domain and mechanism of BRI1 activation by phosphorylation in the C-terminal domain (Wang et al., 2005a).

1.2.4 Guanylyl cyclase activity of BRI1

Near the C-terminal region, BRI1 contains a guanylyl cyclase (GC) domain. This GC activity of BRI1 was confirmed by an experiment using the recombinant BRI1 GC domain containing 114 amino acids can catalyze GTP to cGMP (Kwezi et al., 2007). Since cGMP acts as a secondary messenger in multiple signaling pathways such as stresses and hormones, it suggests cGMP may play a role in BR signaling.

1.2.5 BRI1 endocytosis

BRI1 was observed to be endocytosed from plasma membrane into endosomes (Russinova et al., 2004). The endocytosis of BRI1, however, is independent of BR treatment or BR deficiency. Brefeldin A (BFA), a protein transport inhibitor, treatment resulted in an accumulation of BRI1 in endosomes. The BR signaling, however, was not blocked, indicating endosomal BRI1 is active (Geldner et al., 2007). A mutated BRI1 protein, bri1-9, is retained in endoplasmic reticulum (ER), leading to a typical BR mutant phenotype (Jin et al., 2007). With the help of a UGGT (UDP-glucose:glycoprotein glucosyltransferase), bri1-9 protein was relocated to plasma membrane, which suppressed the *bri1* mutant phenotype. This result implicated BRI1 can initiate BR signaling in

endosomes but not in ER (Jin et al., 2007). However, the reason of BRI1 endocytosis is still unknown.

1.3 BRI1-interactors

1.3.1 BAK1

1.3.1.1 Identification and structure of BAK1

BAK1 (BRI1 ASSOCIATED RECEPTOR KINASE 1) was identified as a BRI1 kinase domain interactor by a yeast-two hybrid screen (Nam et al., 2002) and a *bri1-5*, a *BRI1* weak mutant, genetic suppressor by an activation tagging screen (Li et al., 2002). Like BRI1, BAK1 is also a LRR-RLK, containing an extracellular domain with only five LRRs, a transmembrane domain and a cytoplasmic Thr/Ser kinase domain (Figure 1.3). Adjacent to the signal peptide, there is a leucine-rich domain called leucine zipper with the pattern Lx6Lx6Lx6L. Leucine zipper motif contains leucine residues at every seven amino acid residues, providing the conformational structure to form protein-protein interaction through α-helix (Landschulz et al., 1988). SERK1, a BAK1 paralog, interacts with another SERK1 molecule to form a homodimer. The homodimerization of SERK1 is reduced when the extracellular leucine zipper is deleted, indicating leucine zipper is essential for homodimerization. Following the leucine zipper motif, there are five LRRs. There is a unique proline-rich region between five LRRs and the transmembrane domain. Proline-rich motif creates flexibility to extracellular domain during the signal perception. BAK1 protein has a typical Thr/Ser protein kinase domain, containing 11 characteristic subdomains.

1.3.1.2 BAK1 interacts with BRI1 to regulate BR signaling

Overexpression of *BAK1* suppressesd the dwarfed phenotype of a weak *bri1-5* allele but not a null *bri1-4* allele (Li et al., 2002), suggesting the role of *BAK1* in mediating BR signaling is dependent on a functional *BRI1*. The *in vivo* interaction between BRI1 and BAK1 was demonstrated by the dominant negative phenotype resulted from the overexpression of a kinase dead form of *BAK1* (*mBAK1*) in *bri1-5* (Li et al., 2002); and the co-immunoprecipitation result showing BRI1 interacted with BAK1 *in vivo* (Li et al., 2002; Nam et al., 2002). BAK1 and BRI1 can also phosphorylate each other *in vitro* and *in vivo*. Further analyses indicated the interaction between BRI1 and BAK1 was dramatically enhanced by BL treatment and the phosphorylation levels of BRI1 and BAK1 were also stimulated by BL (Wang et al., 2005b). Since a *BAK1* single mutant did not show the dwarfed phenotype as severe as *bri1* null mutant does (Li et al., 2004; He et al., 2007), it was assumed that there are *BAK1* homologous genes playing redundant roles with *BAK1* in BR signaling.

1.3.1.3 *BKK1*, the closest paralog of *BAK1*, plays a redundant role with *BAK1* in BR signaling

BKK1 (BAK1-LIKE 1), also known as *SERK4*, was proven to function in the BR signaling pathway in a way similar to *BAK1* (He et al., 2007). BKK1 is the closest paralog of BAK1, sharing 82% amino acid identity. BKK1 also interacts with BRI1 *in vivo* and the interaction is stimulated by BL. In addition, the kinase activity of BKK1 also can be regulated by BL. Although *bak1* and *bkk1* single mutants do not obviously show a typical *bri1* mutant phenotype, *bak1 bkk1* double mutant exhibits a de-etiolation phenotype with opened cotyledons when grown in dark, a typical BR mutant response, further suggesting the roles of *BAK1* and *BKK1* in BR signaling.

1.3.1.4 The interplays of BR signaling and other pathways regulated by *BAK1* and its paralogs

1.3.1.4.1 *bak1 bkk1* double null mutant is lethal

Besides showing some *bri1* mutant phenotype, *bak1 bkk1* double mutant also exhibits a spontaneous cell death phenotype that is not observed in any other BR mutants (He et al., 2007), including BR deficient and BR signaling mutants. The cell death phenotype of *bak1 bkk1* double mutant is not observed until 5 days after germination (DAG). Lesions on the cotyledons of the *bak1 bkk1* start emerging around 7 DAG. Accompanied with the cell death are the accumulation of ROS, deposit of callose, and up-regulation of defense-related genes. Introduction of NahG gene partially suppresses *bak1 bkk1* cell death phenotype, suggesting the salicylic acid (SA) signaling pathway is partially related to the cell death in the double mutant (He et al., 2007).

1.3.1.4.2 The cell death in *bak1 bkk1* double mutant is independent of BR signaling pathway

The spontaneous cell death phenotype seen in *bak1 bkk1* double mutant is opposite to BR mutant phenotype that usually shows dark-green leaves, delayed senescence, and prolonged life span. The up-regulation of defense-related genes, such as *PR1*, *PR2*, *PR5*, *ACS2*, and *ACS6*, observed in *bak1-4 bkk1-1* double mutant was not seen or showed opposite expression patterns in *bri1-4* mutant (He et al., 2007). These observations implicate the *BAK1/BKK1*-controlling cell death is BR signaling-independent. *bak1* single mutant developed a runaway cell death (RCD) phenotype upon bacterial or fungal pathogen infection, whereas the BL treatment cannot rescue the disease phenotype, also

supporting that the *BAK1*-controlling cell death pathway is BL-independent (Kemmerling et al., 2007).

1.3.1.4.3 *BAK1* is involved in an *FLS2*-mediated innate immunity response pathway

The recognition of pathogen-associated molecular patterns (PAMPs) by corresponding cell surface receptors initiates PAMP-triggered immunity (PTI). FLS2 functions as the receptor of bacterial flagellin. A 22-amino acid peptide conserved in flagellin, flg22, is sufficient to trigger PTI through FLS2-mediated pathway (Gomez-Gomez et al., 2000). Recently, two groups reported simultaneously that BAK1 was involved in FLS2-mediated plant defense pathway. BAK1 interacted with FLS2 *in vivo* upon flg22 treatment and *bak1-4* showed reduced sensitivity to the flg22 treatment (Chinchilla et al., 2007; Heese et al., 2007).

1.3.2 BKI1

Through a yeast-two hybrid screen, BKI1 was identified as an interactor of BRI1 kinase domain (KD) via BKI1's C-terminal region (Wang et al., 2006). Overexpression of BKI1 resulted in a dwarfed phenotype similar to a *bri1* mutant and showed reduced accumulation of phosphorylated BES1, a BR downstream signaling transcription factor. In absence of BRs, BKI1 interacts with BRI1 *in vivo*; whereas, BKI1 is rapidly disassociated from BRI1 complex upon BL treatment. The BL-dependent release of BKI1 from BRI1 is thought to be an essential process to activate BRI1, which allows BAK1 to associate with BRI1. Transphosphorylation between BRI1 and BAK1 kinase domains activates their downstream substrates, triggering intracellular BR signaling.

1.3.3 BSKs

By using two-dimensional difference gel electrophoresis (2D DIGE), BSK1 and BSK2 were identified as early BR-regulated proteins (Tang et al., 2008). BSK1 and BSK2 belong to a receptor-like cytoplasmic kinase subfamily RLCK-XII. Co-immunoprecipitation assay showed the interaction of BRI1 and BSK1. Furthermore, BSK1 Ser-230 was phosphorylated by BRI1 *in vitro*, suggesting BSK1 is a substrate of BRI1. Lost-of-function of *BSK3*, a paralog of *BSK1* and *BSK2*, showed reduced BR sensitivity; overexpression of *BSK3* partially suppressed *bri1* null mutant but not *bin2* mutant, indicating BSKs function as downstream components of BRI1 but upstream components of BIN2. However, no evidence has proved BIN2 is the direct substrate of BSKs; and whether and how BR signaling is transduced from BSKs to BIN2 is still not understood.

1.3.4 TTL

Transthyretin-Like (TTL) protein was identified as a BRI1-interacting protein through a yeast-two hybrid screen by using BRI1 kinase domain as bait (Nam et al., 2004). N-terminus of TTL is involved in the interaction with BRI1. Overexpression of *TTL* resulted in a growth inhibition, whereas the *ttl* mutant showed opposite growth-promoting phenotype. Although *TTL* overexpression line and *ttl* mutant show some different phenotype compared to wildtype, more evidence is needed to prove TTL is involved in *BRI1*-mediated BR signaling.

1.3.5 TRIP-1

In mammals, TRIP-1 is involved in TGP-β signaling, acting as the substrate of TGF-β type II receptor kinase. Arabidopsis TRIP-1 can be phosphorylated by BRI1 on three sites, Thr-14, Thr-89 and Thr-197/Ser-198 (Ehsan et al., 2005). Co-

immunoprecipitation array revealed TRIP-1 had interaction with BRI1 *in vivo*. However, like TTL, there is no evidence showing TRIP-1 connects BR receptor BRI1 with BR downstream components BIN2 or BSU1; and the function of TRIP-1 in BR signaling pathway is not confirmed in plants.

1.4 BR downstream signaling

BZR1 and BES1 are two BR downstream transcription factors, positively regulated by BR signaling. Through DNA binding domain, BZR1 directly interacts with the CGT(T/G)G sequence of the promoters of BR feedback-regulated biosynthesis genes, such as *DWF4*, *CPD*, *ROT3* and *BR6OX*, acting as a repressor (Wang et al., 2002; He et al., 2005). As a paralog of BZR1, BES1 binds to BIM1, a basic helix-loop-helix transcription factor, and its homologs, BIM2 and BIM3. The BES1/BIM complex binds to the E box (CANNTG) of the promoters of BR-induced genes (Yin et al., 2005). BZR1 and BES1 are regulated by a glycogen synthase kinase-3 (GSK)-like kinase named BIN2 (BRASSINOSTEROID-INSENSITIVE 2) in a post-translational level (Yin et al., 2002; He et al., 2002). BIN2 is negatively regulated by BR signaling; and *bin2-1*, a *BIN2* dominant mutant, shows a *bri1*-like dwarfed phenotype and blocked feedback regulation of BR biosynthesis genes (Mathur et al., 1998). BIN2 phosphorylates BZR1 and BES1 and the phosphorylated BZR1 and BES1 are unstable and are likely recognized by E3 ligase then degraded through an E3-mediated ubiquitin-dependent protein degradation pathway. Phosphorylated BZR1 also binds to phosphopeptide-binding proteins, 14-3-3, leading to accumulation in the cytoplasm and inhibition of BZR1 function (Gampala et al., 2007). Playing an opposite role with BIN2, a Thr/Ser phosphatase, BSU1,

dephosphorylates BZR1 and BES1 and stabilizes both transcription factors (Mora-Garcia et al., 2004).

1.5 Current model for BR signaling

BRs are perceived by two plasma membrane-localized LRR-RLKs, BRI1, and BAK1. BRI1 functions as a ligand-binding receptor; whereas BAK1 acts as a co-receptor. BR binding to BRI1 releases BKI1, a BRI1 kinase domain-binding protein, from BRI1 complex and the conformational change subsequently leads to BRI1 autophosphorylation. The phosphorylation activates BRI1 kinase domain that interacts with BAK1 kinase domain to form BRI1-BAK1 heterodimmer complex. Transphosphorylation between BRI1 and BAK1 initiates BR signaling cascade. BIN2 phosphorylates two BR-regulating transcription factors, BZR1 and BES1. Phosphorylated BZR1 and BES1 are degraded through E3-mediated ubiquitin-dependent protein degradation pathway. A phosphatase, BSU1, dephosphorylates BZR1 and BES1, stabilizes both transcription factors and maintains their normal functions. BZR1 acts as a repressor in regulating BR biosynthesis genes; and BES1 promotes the expression of BR-induced genes. Although a variety of proteins have been identified to interact with BRI1, such as TTL, TRIP-1 and BSKs, there is still no evidence to show any BRI1-interacitng proteins connecting BRI1/BAK1 with BIN2 or BUS1. (Figure 1.4)

1.6 Perspectives

Identification of a secreted serine carboxypeptidase, BRS1, in the BR signaling pathway suggested a proteolytic protein modification proceeding BR binding to BRI1 may be necessary (Li et al., 2001). One possible function of BRS1 is to degrade a steroid-binding protein MSBP1 and releases free BRs that can be used to trigger BR signaling. It

is equally possible that BRS1 is to degrade a BRI1-binding protein occupying the BR-binding site in order to make BR perception possible (Figure 1.4). Nevertheless, these hypotheses need to be further tested in the future.

One of the major questions in BR signal transduction is what the relationship between BRI1 and BAK1 is. As a co-receptor of BRs, BAK1 does not seem to interact with BRs directly. This raises a question that whether BAK1 functions as an essential regulator in BR signaling or alternatively BAK1 only serves as an enhancer of BRI1 kinase activity. Wang et al. reported even in the *bak1 bkk1* double mutant, BRI1 was still active and BR signaling pathway was intact. The authors proposed that although BAK1 can enhance BR singling pathway by tansphosphorylating BRI1, it is not essential for BR signaling pathway. Nevertheless, given the fact that *BAK1* has three additional paralogs besides *BKK1*, and they might play redundant roles with *BAK1* in BR signaling as well, it prompts us to investigate the functions of other *BAK1* paralogs in BR signaling.

Although a number of proteins were identified as BRI1 substrates or BRI1-interactors, such as TTL, TRIP-1 and BSKs, the immediate upstream regulator of BIN2, however, is not identified yet. The development of new approaches, such as 2D-DFE which was recently used to successfully identify BSKs (Tang et al., 2008), in BR signaling research provide alternative opportunities to discover new regulatory components, filling gaps of the BR signaling pathway.

2 ROS in plants

ROS (reactive oxygen species), which include superoxide radical, hydrogen peroxide and singlet oxygen, are ubiquitous molecules produced as a consequence of normal cellular metabolisms. In green plants, ROS are continuously produced as

byproduct of several physiological processes, such as photorespiration and photosynthesis (Foyer et al., 1994). In addition, ROS also act as signaling molecules produced during biotic and abiotic tresses, regulating plant responses to various environmental challenges (Elstner et al., 1991; Malan et al., 1990; Prasad et al., 1994; Tsugane et al., 1999).

2.1 Generation of ROS

2.1.1 ROS are byproducts of normal physiological processes

ROS are produced continuously during photosynthesis, a plant unique and essential physiological process. In chloroplasts, H_2O loses an electron and is oxidized to O_2 in photosystem II (PSII). Through electron transport, the PSII complex passes electrons to photosystem I (PSI), where the electrons are used to oxidize O_2 to $O^{2\cdot-}$. $O^{2\cdot-}$ is unstable and is rapidly catalyzed by superoxide dismutase to form H_2O_2. PSI also transfers electrons to $NADP^+$ to generate NADPH and eventually produce glycolate. Glycolate is relocated to peroxisome where it is oxidized by glycolate oxidase to produce H_2O_2. (Figure 1.5)

In mammals, respiration is the major resource of ROS that are produced in the mitochondria. However, the contribution of mitochondria to ROS generation is relatively low in plants due to two reasons: one is the production of ROS in chloroplasts is relative high in green plants (Purvis et al., 1997); the second reason is the presence of alternative oxidase (AOX) in plant mitochondria, catalyzing the reduction of O_2 generated during electron transport (Wagner et al., 1995; Maxwell et al., 1999).

2.1.2 ROS are triggered by stresses

As signaling molecules, ROS are rapidly generated in response to various stresses: biotic stresses (attacks by other organisms, such as bacteria, fungi and virus) and abiotic tresses (environmental challenges, such as high/low temperature, drought, wounding and high light etc.).

2.1.2.1 Biotic stresses

In plants, recognitions of PAMPs by corresponding receptors triggered innate immunity responses. In Arabidopsis, cell surface RLKs were identified as pathogen receptors, such as FLS2, a receptor of bacterial flagellin (Gomez-Gomez et al., 2000); and EFR, a receptor of bacterial PAMP EF-Tu (Zipfel et al., 2006). The activation of disease resistance response pathways lead to enhanced activity of plasma membrane localized enzyme NADPH-oxidase that utilizes $NADP^+$ to produce O^{2-} (peroxide radicals) in apoplast (Sagi et al., 2001). Since O^{2-} is a strong oxidizer and highly toxic to pathogens, plants are able to protect themselves from biological invasion by using ROS as weapons. O^{2-} is unstable and is rapidly dismutated into H_2O_2 that can diffuse into the cell. This rapid accumulation of ROS is called oxidative burst (Apostol et al., 1989). The highly accumulated H_2O_2 in the cell ultimately cause programmed cell death (PCD) (Bolwell et al., 1999; Dangl et al., 2001), known as hypersensitive response (HR) (Wohlgemuth et al., 2002). By this strategy, on one hand, plants use ROS to kill invading pathogens; on the other hand, ROS work as signaling molecules to trigger HR, sacrificing infected areas to protect surrounding tissues and limit pathogen movement and spreading (Figure 1.6a). In some cases, if a plant fails to control HR in a limited area, the cell death will be spread out to the whole tissue, known as runaway cell death (RCD).

2.1.2.2 Abiotic stresses

In abiotic stresses, intracellular ROS level is also enhanced. ROS are mainly produced in chloroplasts and mitochondria in the electron transport. In contrary to biotic stresses, the increased ROS levels are negatively regulated by ROS scavengers that are involved in the removal of ROS in cytosol and specific organelles. ROS-scavenging pathway is up-regulated by ROS signaling, suggesting a feedback regulation of ROS signaling in abiotic stresses. Cell death can also be triggered if the ROS-scavenging system fails to detoxify excessively accumulated ROS. (Figure 1.6b)

2.2 ROS-scavenging pathways

As strong oxidizers, ROS are toxic to plant cell. Therefore, there must be a system continuously removing ROS to maintain a steady state redox (reduction/oxidation) homeostasis in a plant cell. Different mechanisms contribute to ROS detoxification, including antioxidants, such as glutathione and ascorbate, and ROS-scavenging enzymes. In Arabidopsis, five major groups of ROS-scavenging enzymes are involved in ROS metabolism, including SOD, CAT, APX, GPX and PrxR. (Figure 1.7)

2.2.1 SOD (superoxide dismutase)

SOD catalyzes superoxide, which is highly toxic and unstable, into hydrogen peroxide (H_2O_2). H_2O_2 is more stable and steadily diffuses membranes and can be further reduced by other mechanisms. No antioxidants are needed in this process.

2.2.2 CAT (catalase)

CAT reduces H_2O_2 into H_2O and O_2. There are no antioxidants involved in CAT-mediated H_2O_2 removal.

2.2.3 APX (ascorbate peroxidase)

By using an antioxidant ascorbate, APX catalyzes H_2O_2 to H_2O, monodehydroascorbate (MDA) and dehydroascorbate(DHA). MDA and DHA are catalyzed by MDA reductase (MDAR) and DHA reductase (DHAR) respectively to regenerate ascorbate.

2.2.4 GPX (glutathione peroxidase)

With the help of antioxidant glutathione (GSH), GPX catalyzes H_2O_2 to H_2O and oxidized glutathione (GSSG). GSH is regenerated from GSSG, catalyzed by glutathione reductase (GR)

2.2.5 PrxR (peroxiredoxin)

Using H_2O_2 as oxidant regent, PrxR oxidizes the thiol groups of cysteine residues to form disulfides. The state of thiol-disulfide of proteins regulated by ROS is believed to be essential for redox sensing mechanism.

2.3 ROS signaling pathway

2.3.1 Two-component histidine ROS sensor in prokaryotes

In prokaryotes, ROS signals are perceived by a two-component signaling system, including a plasma membrane localized histidine kinase (as a ROS sensor) and a response regulator (Quinn et al., 2002; Whistler et al., 1998). Upon oxidative stimuli, histidine kinase is autophosphorylated at a histidine (H) residue and subsequently activates a response regulator by transferring the phosphoryl group to the aspartate (D) residue of the response regulator. The activated (phosphorylated) response regulator acts as transcription factors, promoting the expression of ROS-response genes. In plants, although there are two-component histidine kinases identified (Hwang et al., 2002), it is, however, still unknown whether these histidien kinases are involved in ROS sensing.

2.3.2 MAP kinases are involved in ROS signaling in plants

Mitogen-activated protein kinases (MAPKs) are involved in multiple signal transduction, including stress signaling, hormone signaling and disease resistant pathways in yeast (Gustin et al., 1998). In Arabidopsis, two MAPKs, MPK3 and MPK6, showed responses to H_2O_2 treatment (Kovtun et al., 2000). H_2O_2 treatment rapidly increased the kinase activity of MPK3 and MPK6. The activation is mediated by a mitogen-activated protein kinase kinase kinase (MAPKKK), APN1, an upstream regulator of MPK3 and MPK6. Constitutively active ANP1 mimics H_2O_2 effect, promoting the expression of H_2O_2-inducible stress-response genes, indicating a central role of MAPK cascade in ROS signaling.

ANP1-MPK3/MPK6 cascade initiated by H_2O_2 eventually regulates the expression of defense genes and blocks auxin signaling, suggesting the interplay between oxidative stress and hormone signaling pathway. Arabidopsis NPR1 protein, essential for SA signaling, is regulated by redox homeostasis (Mou et al., 2003). NPR1 proteins form oligomers, localized in cytoplasm and connected by intermolecular disulfide bonds at cysteine residues. Antioxidant glutathione, ROS scavenger and byproduct of ROS metabolism, reduce disulfide bonds to thiol groups, disuniting NPR1 oligmers to NPR1 monomers that are moved into nuclear and lead to induction of pathogen-related (PR) genes and initiation of oxidative burst.

2.4 Conclusions and perspectives

Different from animals, plants are not capable of escaping from undesirable environments. During the evolution, plants have developed unique ways to survive in hostile conditions. As side product of normal plant physiological processes, as well as

signals triggered during a variety of environmental stresses, ROS play essential roles in orchestrating essential physiological metabolisms and plant-to-environment communications. ROS, toxic to plant cells when they exceed certain levels, are continuously produced during photosynthesis, photorespiration and respiration. On the other hand, the produced ROS by different metabolisms are rapidly detoxified by different mechanisms with the help of a variety of antioxidants as well as ROS scavenging-enzymes, maintaining a steady state redox homeostasis in a cell. The balanced redox homeostasis can be impaired by rapid accumulation of ROS, a response to a variety of biotic and abiotic stresses, known as oxidative burst. Thus, ROS play as signal messengers involved in multiple signaling pathways, modulating enzyme activity, regulating gene expression and ultimately showing stress responses such as callose deposit, induction of PR genes and hypersensitive response. The crosstalks between ROS signaling and plant hormone signaling pathways, such as auxin and brassinosteroid (BR) pathways, suggest the hormones are engaged in regulating intracellular redox state in plant growth and development.

Although MAPK cascade is known to be involved in ROS signaling, the sensing mechanisms of ROS are largely unknown in plants. The mechanisms how ROS are sensed can be further investigated. One possible mechanism might be involved in oxidation of thiol groups in cysteine residues to form disulfide bonds that may be intra-molecular or inter-molecular. Subsequent structure conformational changes or formation of hetero/homo-polymers activate downstream signaling mediated by APN1-MPK3/6 and ultimately regulate the expression of ROS-inducing genes. A number of RLKs, including

BAK1 and BKK1, contain cysteine pairs in the extracellular domain, providing a novel avenue to examine the mechanism of ROS-sensing in plants.

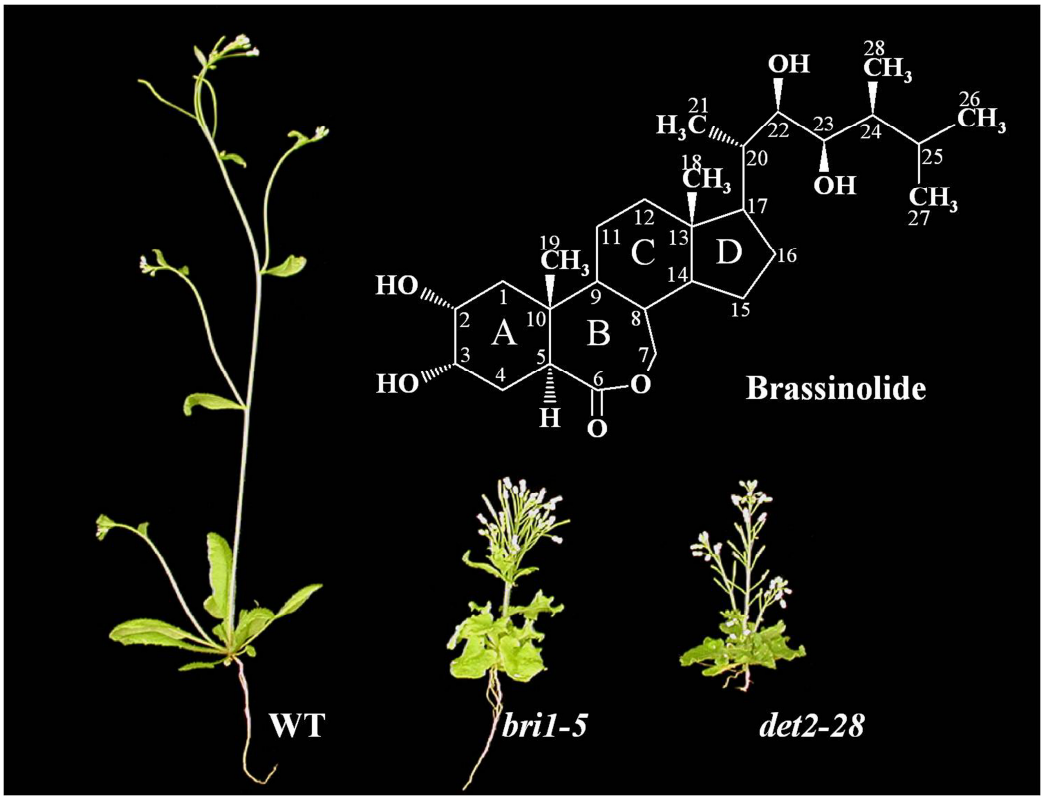

Figure 1.1 BL structure and representative BR mutants (Adapted from Li and Gou, 2007). More than 60 BRs are identified in plant. Brassinolide, the most active BR, is a C28 steroidal lactone. BR mutants, either signal transduction mutants (*bri1-5*) or biosynthetic mutants (*det2-28*) show similar defective phenotypes.

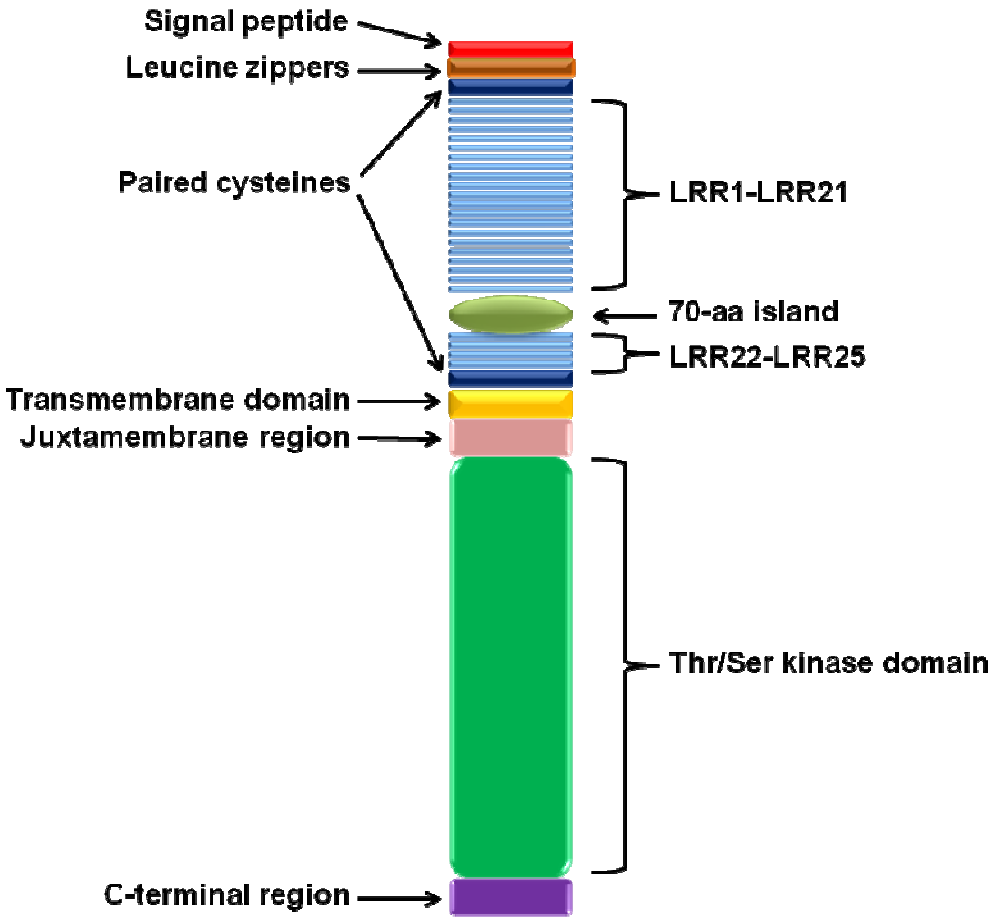

Figure 1.2 Structure of BRI1 protein. BRI1 is a LRR-RLK (1196 aa). It contains 25 LRRs in extracellular domain, separated by a 70-amino acid island. The 70-amino acid island with C-terminal flanking LRR22 is responsible for ligand-binding. BRI1 contains a typical Thr/Ser kinase domain. C-terminal region negatively regulates BRI1 kinase activity.

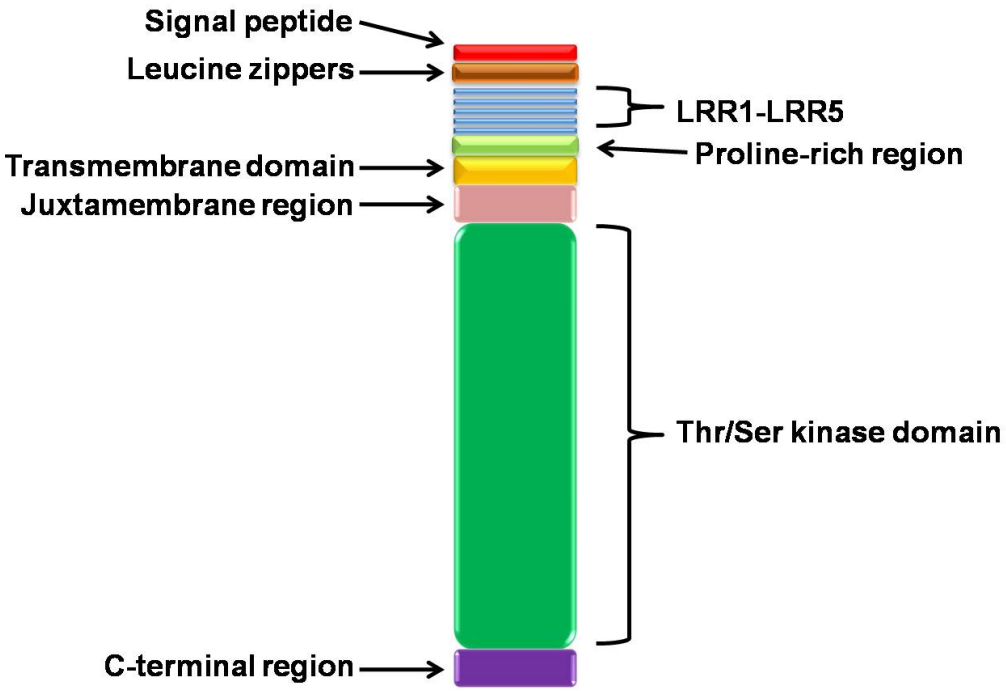

Figure 1.3 Structure of BAK1 protein. BAK1 is a LRR-RLK (615 aa). It consists of an extracellular domain with only five LRRs, a transmembrane domain and a cytoplasmic kinase domain. Leucine zipper motif provides the structural basis for protein-protein interaction. Unique proline-rich region creates flexibility to extracellular domain during the signal perception. BAK1 has a typical Thr/Ser kinase domain.

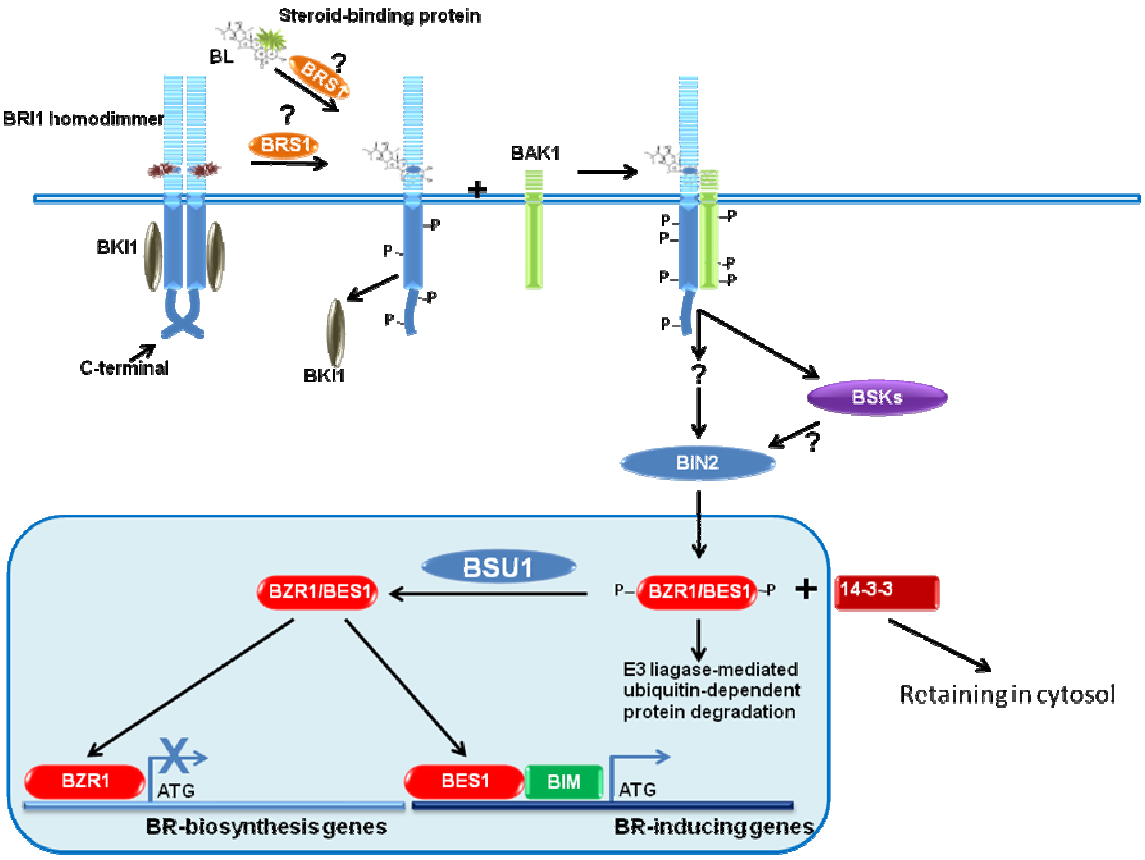

Figure 1.4 Current model for BR signaling pathway. BRs are perceived by BRI1 and BAK1. BR binding to BRI1 releases BKI1 from BRI1 complex and leads to BRI1 autophosphorylation. The phorphorylation activates BRI1 kinase domain that interacts BAK1 kinase domain to form heterodimmer complex. Interaction and transphosphorylation between BRI1 and BAK1 initiate BR signaling cascade. BIN2 phosphorylates BZR1 and BES1, BR-signaling-regulating transcription factors, and the phosphorylated BZR1 and BES1 are degraded through E3-mediated ubiquitin-dependent protein degradation pathway. BSU1 dephosphorylates and stabilizes BZR1 and BES1. The gap between BRI1/BAK1 to BIN2/BSU1 is still mysterious. A secreted serine carboxypeptidase, BRS1, is hypothesized to function in the modification preceding BR binding to BRI1, either by degrading a steroid-binding protein or by degrading a BRI1-binding protein occupying BR-binding site.

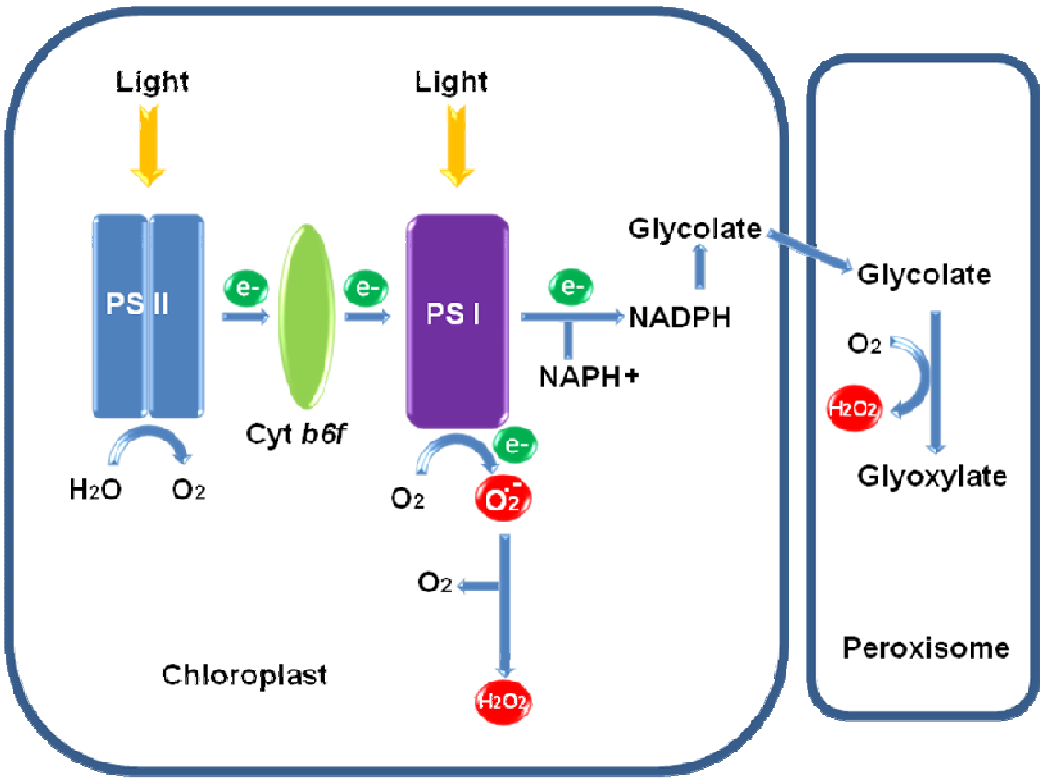

Figure 1.5 ROS are generated in chloroplast and peroxisome during photosynthesis. Under light condition, H_2O loses electrons in PSII. Electrons are transferred to PSI, where O_2 is oxidized into superoxide radicals ($O^{\cdot-}_2$) and the superoxide radicals are dismutated into hydrogen peroxide (H_2O_2). Glycolate, one photosynthesis product, is relocated into peroxisome where glycolate can produce H_2O_2.

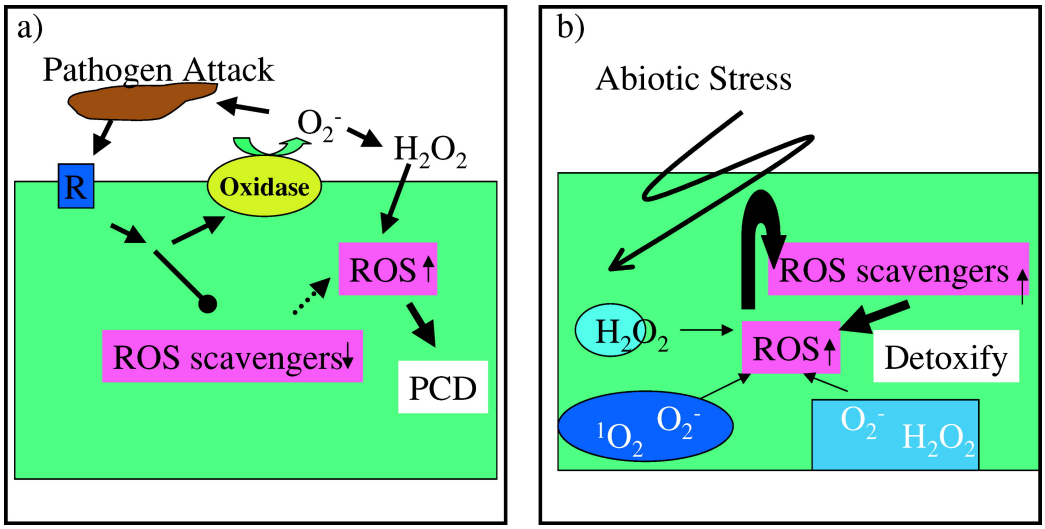

Figure 1.6 ROS are triggered under biotic and abiotic tresses (Adapted from *Apel and Hirt, 2004*). (A) Recognitions of invading pathogens by cell surface receptors activate NADPH-oxidase that produces O_2^- in apoplast, trying to kill the pathogens. O_2^- is dismutated into H_2O_2 that can diffuse into the cell. The highly accumulated H_2O_2 in the cell ultimately cause programmed cell death (PCD), known as hypersensitive response (HR). The down-regulation of ROS-scavengers facilitates this process. (B) In abiotic stresses, intracellular ROS level is also enhanced. The increased ROS levels are negatively regulated by ROS scavengers, which are up-regulated in this process. Cell death can also be triggered if the ROS-scavenging system fails to detoxify excessively accumulated ROS.

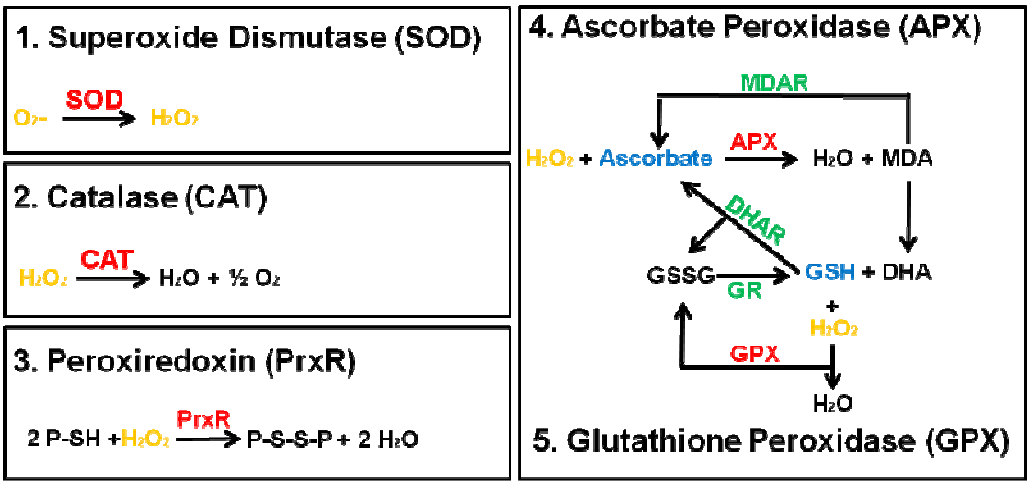

Figure 1.7 ROS scavenging system (Modified from *Apel and Hirt, 2004*). ROS are removed by five groups of ROS-scavenging enzymes: SOD, CAT, PrxR, APX and GPX. ROS are presented in orange and enzymatic ROS-scavengers are presented in red. ROS scavengers reduce ROS with the help of antioxidants (presented in blue). The regeneration of antioxidants is catalyzed by the enzymes presented in green.

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Chapter II

BAK1 and BKK1, Two Arabidopsis LRR Receptor-Like Protein Kinases, Regulate BR-mediated Growth and BR-Independent Cell Death Pathways

Key words: BRI1, BAK1, BKK1, brassinosteroids, cell death

1 Summary

Brassinosteroids (BRs) are phytosteroid hormones controlling various physiological processes critical for normal plant growth and development. BRs are perceived by a protein complex containing two transmembrane receptor kinases, BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Li et al., 1997; Li et al., 2002; Nam et al., 2002). *BRI1* null mutants exhibit a dwarfed-stature with epinastic leaves, delayed senescence, reduced male-fertility, and altered light responses. *BAK1* null mutants, however, only show a subtle phenotype, suggesting functionally redundant proteins may be present in the *Arabidopsis* genome. Here we report BAK1-LIKE 1 (BKK1) functions redundantly with BAK1 in regulating BR signaling. Surprisingly, rather than the expected *bri1*-like phenotype, *bak1 bkk1* double mutants exhibit a seedling lethality phenotype due to constitutive defense gene expression, callose deposition, reactive oxygen species (ROS) accumulation, and spontaneous cell death under sterile growing conditions. Our detailed analyses demonstrate that BAK1 and BKK1 have dual physiological roles: positively regulating a BR-mediated plant growth pathway, and negatively regulating a BR-independent cell death pathway. Both BR signaling and developmentally-controlled cell death are critical to optimal plant growth and development, but the mechanisms regulating early events in these pathways are poorly understood. This study provides novel insights into the initiation and crosstalk between both signaling cascades.

2 Results

The model plant *Arabidopsis* contains a large family of proteins called leucine-rich repeat receptor-like protein kinases (LRR-RLKs). A typical LRR-RLK contains an extracellular LRR domain and a cytoplasmic serine/threonine kinase domain separated by a single-pass transmembrane domain. The extracellular domain is mainly involved in ligand binding and signal perception, and the cytoplasmic domain is responsible for downstream signal transduction. There are at least 223 LRR-RLKs in the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000), only a handful of which have been functionally characterized. Those characterized have been shown to play critical roles in regulating various processes directly modulating growth and development, as well as immunity responses (Becraft et al., 2002; Dievart et al., 2004; Morillo et al., 2006). The discoveries of two LRR-RLKs, BRI1 and BAK1, in controlling the early event of the BR signaling pathway, suggested that the heterodimerization of distinct LRR-RLKs following ligand binding could be a central paradigm in the activation of LRR-RLK-mediated signaling cascades (Li et al., 1997; Li et al., 2002; Nam et al., 2002).

The biological significance of BRI1 has been well documented by a series of elegant genetic and biochemical studies. Genetic analyses indicated that *bri1* null alleles are extremely dwarfed and insensitive to exogenous brassinolide (BL), the most active BR, but remain sensitive to other known phytohormones (Clouse et al., 1996). Binding analyses demonstrated that the 70-aa "island" segment and the 22nd LRR of the BRI1 extracellular domain together confer BR binding activity (Wang et al., 2001; Kinoshita et al., 2005), whereas the kinase domain of BRI1 activates downstream components by protein phosphorylation. BAK1 is a member of a distinct LRR-RLK subfamily that contains only 5 LRRs and lacks the "island" region within its LRRs. BAK1 physically

interacts with BRI1 *in vivo* (Li et al., 2002; Nam et al., 2002; Russinova et al., 2004). In addition, BRI1 and BAK1 are able to phosphorylate each other. Both the interaction and the phosphorylation of BRI1 and BAK1 are BR-dependent (Wang et al., 2005). Unlike BRI1, however, null alleles of BAK1 display subtle *bri1*-like phenotypes, suggesting at least one additional protein in *Arabidopsis* is functionally redundant with BAK1 (Li et al., 2002; Nam et al., 2002).

To identify the presumed BAK1 functionally redundant protein(s), the BAK1 sequence was used to search the *Arabidopsis* database for related paralogs. BAK1 belongs to the LRR type II subfamily containing 14 members (Shiu et al. 2001), five of which were previously named SERK1 to SERK5 (Figure 2.1A; (Hecht et al., 2001)) due to their protein structure similarity to the carrot DcSERK (Schmidt et al., 1997). Phylogenetic analysis indicated that SERK4 and SERK5 are the two closest paralogs of BAK1/SERK3 (Figure 2.1A), and they may have biological functions similar to BAK1. Since overexpression of *BAK1* is able to suppress a weak *bri1* allele, *bri1-5* (Li et al., 2002), it was predicted that a *BAK1* redundant gene would also suppress *bri1-5* phenotypes when overexpressed. *SERK4* and *SERK5* were subsequently transformed into *bri1-5* to test whether they can suppress *bri1-5* upon overexpression. Like *BAK1, SERK4,* but not *SERK5,* was able to partially rescue *bri1-5* when overexpressed (Figure 2.1B and 2.1C). *SERK4* was subsequently renamed *BKK1*. Further analyses indicated that Col-0 SERK5 bears an amino acid substitution of Leu for Arg at position 401 within the critical "RD" kinase motif which may block its function in the BR signaling pathway (Figure 2.6; (Dardick et al., 2006)). Interestingly, the same mutation was not found in SERK5 from other *Arabidopsis* ecotypes such as WS2 (data not shown).

To examine the genetic significance of BAK1 and BKK1 in a real physiological setting, single T-DNA knock-out lines were isolated from SALK T-DNA insertion pools. Several knock-out lines were obtained for both genes. Only the lines directly relevant to this work are shown (Figure 2.2A). Whereas a *BAK1* null allele, *bak1-4*, showed a subtle *bri1*-like phenotype, a *BKK1* null allele, *bkk1-1*, did not exhibit any defective phenotypes (Figure 2.2B to 2.2E). The double null mutants, *bak1-4 bkk1-1*, however, illustrated extremely dwarfed phenotypes, distinct from typical *bri1* null mutants such as *bri1-4*. During the first 4 days after germination, the double null mutant showed no observable defective phenotypes compared to wild-type plants. A week after the germination, however, shoot apical meristem (SAM) growth of the double mutant almost completely ceased (Figure 2.7). Ten days after germination, the seedlings showed early senescence symptom starting at the cotyledons. Careful analysis of embryos from the progeny of *bak1-4* (+/-) *bkk1-1* (-/-) or *bak1-4* (-/-) *bkk1-1* (+/-) plants failed to identify any abnormal embryos during embryogenesis. It was expected that 25% of the embryos would be aberrant if *bak1-4 bkk1-1* double mutations did affect zygotic embryogenesis. The onset of seedling lethality is, therefore, a post-embryonic phenotype likely controlled by unknown developmental cues. The phenotypes observed in the double mutant were reproduced using different *bak1* and *bkk1* allele combinations from Col-0 background (Figure 2.8), and were restored to wild-type-like seedlings by expressing either *BAK1* or *BKK1* (Figure 2.2F to 2.2G). Various genetic segregation analyses further demonstrated that the observed phenotype was solely caused by the double *BAK1* and *BKK1* knock-outs (Table 2.1).

The overexpression of *BKK1* suppresses *bri1-5* phenotypes suggesting that BKK1 has a role in BR signal transduction. To further substantiate the function of BKK1 in the BR pathway, a series of tests were conducted. To begin, a *BKK1* kinase-dead mutant *mBKK1* (K322E) was generated and introduced into *bri1-5*. Over 50% of the transgenic plants displayed dominant-negative phenotypes, with phenotypic severity positively correlated with the expression levels of the transgene (Figure 2.3A to 2.3B). The most severe plants resembled the phenotypes of null *bri1*, characterized by extreme dwarfism and complete male sterility (data not shown). The observed dominant-negative phenotype suggested that BKK1 may interact with BRI1 *in vivo*, which was subsequently confirmed by a co-immunoprecipitation analysis using transgenic plants harboring *35S-BKK1-GFP* and *35S-BRI1-FLAG* (Figure 2.3C). The interaction was greatly enhanced by exogenously applied BL. Moreover, biochemical analysis indicated that the *in vivo* phosphorylation levels of BKK1 were also regulated by exogenously applied BL (Figure 2.3D), similar to that of BAK1 (Wang et al., 2005) and BRI1 (Figure 2.3E). Depletion of endogenous BRs by treating *Arabidopsis* seedlings (*BKK1-GFP* in *bak1-4 bkk1-1*, or *BRI1-GFP* in Col-0) with the specific BR biosynthesis inhibitor brassinazole (BRZ, (Asami et al., 2000)) for a week showed basal levels of threonine phosphorylation in both BKK1 and BRI1. A 90-minute treatment of these BRZ-pretreated-seedlings with BL greatly increased phosphorylation levels on their threonine residues. These results demonstrated that BKK1 is truly a functionally redundant protein of BAK1. To examine whether the BR signaling is completely blocked in the *bak1-4 bkk1-1* mutant, the double mutant seedlings grown in darkness were carefully analyzed and measured. The double mutant seedlings from darkness showed a typical de-etiolationphenotype including

opened cotyledons and shortened hypocotyls (Figure 2.9). The hypocotyls, however, were considerably taller than that of *bri1-4*, indicating that the BR signaling was not entirely blocked in the double mutant. These results suggested that there should be additional proteins, besides BAK1 and BKK1, involved in BR signal transduction. Consistently, it was recently reported that SERK1 was part of the BRI1 and BAK1 complex and may also participate in BR signal transduction (Karlova et al., 2006). Since *bak1-4 bkk1-1* double mutant showed a seedling lethality phenotype, a triple mutant was not generated in this study.

To investigate the molecular mechanisms leading to the seedling lethality phenotype of *bak1-4 bkk1-1*, Affymetrix arrays were employed to compare the global gene expression patterns of the 8-day-old double mutant with Col-0 wild-type. Among the most significantly up-regulated genes were those involved in defense responses. Representative up-regulated defense and senescence related genes, *PR1*, *PR2*, *PR5*, *ACS2*, *ACS6*, and the down-regulated gene, *PDF1.2*, were further analyzed by RT-PCR to examine whether the expression patterns were similar to those shown in *bri1-4*. If BAK1 and BKK1 are involved exclusively in BR signaling, one would expect these genes to show similarly dramatic expression changes in both *bri1-4* and *bak1-4 bkk1-1*. Interestingly, these defense- and senescence-related genes showed either no or opposite expression changes in *bri1-4* (Figure 2.4A). Consistent with these observations, aniline blue (Dietrich et al., 1994) staining analysis of the 8-day-old seedlings indicated that only the double mutant showed irregularly-distributed blue fluorescence spots due to the accumulation of callose, a known spontaneous defense response. None of the single mutants or wild-type showed similar staining patterns (Figure 2.4B to 2.4G). Since

extreme dwarfism, constitutive defense gene expression, early senescence, and seedling lethality are common phenotypes of cell death mutants (Lam et al., 2004; Ichimura et al., 2006), trypan blue staining (Shirasu et al., 1999) was used to test whether cell death was the culprit in seedling lethality. Only the 8-day-old double mutants showed a spontaneous cell death phenotype in cotyledons without pathogen treatments. None of the wild-type and single mutant plants showed any cell death phenotypes at this developmental stage (Figure 2.4H to 2.4M). In mammalian systems, the accumulation of reactive oxygen species (ROSs) was one of the most common mechanisms triggering apoptosis (cell death). To test whether the observed cell death in *bak1-4 bkk1-1* is caused by the accumulation of H_2O_2, 8-day-old seedlings were stained with 3, 3'-diaminobenzidine (DAB, (Thordal-Christensen et al., 1997)). The unique brownish patterns were only observed in *bak1-4 bkk1-1* but not in other plants tested (Figure 2.4N to 2.4S). The resulting H_2O_2 staining patterns consistently and closely mimicked the cell death patterns revealed by the trypan blue assay. The H_2O_2-accumulated cells were often clustered as groups that typically emanated from areas adjacent to vascular tissues. Many cell death mutants such as *lsd1* showed a cell death phenotype in a salicylic acid (SA)-dependent manner (Aviv et al., 2002). To test whether the cell death phenotype observed in *bak1-4 bkk1-1* was also SA dependent, Bacterial *NahG* gene was introduced into the double mutant by genetic crossing with a transgenic Col-0 *Arabidopsis* plant expressing *NahG* (Delaney et al., 1994). *NahG* encodes a salicylate hydroxylase that convert SA to catechol. The fact that *bak1-4 bkk1-1 NahG* seedlings were much healthier and bigger than the double mutant suggested that the cell death phenotype in the double mutant was likely SA-dependent (Figure 2.4T-2.4V).

3 Discussion

Our extensive genetic and biochemical analyses demonstrated that BAK1 and BKK1 are not only involved in BRI1-mediated pathway, but also involved in a BR-independent signaling pathway. The two pathways mediated by BAK1 and BKK1 showed some antagonistic features. For example, null *bri1* mutants usually displayed prolonged life spans and a dark green phenotype, whereas *bak1 bkk1* double mutant plants exhibited shortened life span, early cotyledon senescence, and a microscopic cell death phenotype. Expression of senescence-related genes such as *ACS2* and *ACS6* are reduced in null *bri1* mutant but enhanced in *bak1 bkk1* double null seedlings, relative to their wild-type backgrounds. The seedling lethality phenotype of the double mutant is likely a consequence of the blocking of the second unknown, BR independent pathway.

We hypothesize that BAK1 and BKK1 regulate BR-dependent and BR-independent pathways via an alternating interaction with either BRI1 and/or another defense-related LRR-RLK (Figure 2.5). In wild-type plants, BAK1 and BKK1 positively regulate the BR-mediated cell growth pathway, and negatively regulate a defense-related cell death pathway. In *bak1-4 bkk1-1*, the BR signaling pathway is interrupted, and the cell death pathway is constitutively activated, which leads to the accumulation of ROS and spontaneous cell death. This hypothesis is partially supported by an independent study from Nürnberger and colleagues (Kemmerling et al., 2007), who recently found that *BAK1* expression was up-regulated in response to pathogen treatment, and that BAK1 knock-out alleles, *bak1-3* and *bak1-4*, were more susceptible than wild-type to several different pathogens. They similarly concluded that BAK1 was likely involved in a BR-independent immunity pathway (Kemmerling et al., 2007). This model can also be

used to explain several early observations. For example, it was reported that overexpression of *CPD*, a key gene regulating multiple steps in BR biosynthesis, could induce the expression of a number of defense-related genes (Szekeres et al., 1996). Early experiments also indicated that application of BL induced senescence. It is likely that when the BR signaling pathway was enhanced, its antagonistic pathway was automatically reduced which led to the up-regulation of defense-related genes (Figure 2.5). Post-embryonic seedling lethality observed in *bak1-4 bkk1-1* may have been caused by the activation of this constitutive defense response and cell death pathway. Developmentally controlled programmed cell death is critical for normal plant growth and development, as well as defense against numerous biotic and abiotic stresses. It would be interesting to further define the second signaling pathway in which BAK1 and BKK1 are involved. If the hypothesis is correct, the proposed candidate LRR-RLKs should have roles in plant defense and/or cell death control. Up to date, at least two LRR-RLKs are known to be involved in plant defense against pathogens in *Arabidopsis*. For instance, ERECTA, known to regulate plant growth and development (Torii et al., 1996), was found to be responsible for plant immunity against pathogens (Godiard et al., 2003). FLS2 is another LRR-RLKs controlling plant immunity response (Gomez-Gomez et al., 2000). In the future, it will be particularly intriguing to investigate whether BAK1 and BKK1 can dimerize with ERECTA or FLS2 to mediate their corresponding signaling pathways.

4 Experimental procedures

4.1 Materials and Plant Growth Conditions

bri1-5 is in ecotype Wassilewskiji 2 (WS2). Knockout lines, *bak1-4* (SALK_116202) and *bkk1-1* (SALK_057955), and *NahG* transgenic plants used for crossing, were also in ecotype Col-0 background. Double nulls used for these analyses were isolated from the progenies of self-pollinated *bak1-4(+/-) bkk1-1 (-/-) or bak1-4(-/-) bkk1-1 (+/-)* plants. All plants or seedlings were grown at 22°C under 16 h light/8 h dark, unless otherwise specified.

4.2 Gene Cloning and *Arabidopsis* Transformation

BAK1 expression vector used was the same as reported by Li et al. (Li et al., 2002). Full length cDNAs of *BKK1* and *AtSERK5* were amplified by RT-PCR from Col-0. The primers used for RT-PCR were BKK1-fw 5'TCTAGATCTATGGAACAAAGATCACTCCTTTGCT, BKK1-rv 5' TCTAGATCTTTATCTTGGACCCGAGGGGTAATCGT, *AtSERK5*-fw 5'TCTAGATCTATGGAACATGGATCATCCCGTGGCT, *AtSERK5*-rv 5'-TCTAGATCTTTATCTTGGCCCCGAGGGGTAATCGT. The PCR products were cloned into the *Kpn*I site of the binary vector *pBIB-BASTA-35S*. Constructs were transformed into the *Agrobacterium tumefaciens* line *GV3101* by electroporation and subsequently transformed into *bri1-5* by the floral dipping method (Clough et al., 1998).

Full length *BAK1* and *BKK1* cDNAs were also cloned into the *Kpn*I and *BamH*I sites of the binary vector *pBIB-BASTA-35S-GFP* using the primers *BAK1*-fw 5'TCTAGATCTATGGAACGAAGATTAATGATCCCT, *BAK1*-rv 5'TCTGGATCCTCTTGGACCCGAGGGGTATTCGTT, *BKK1*-fw and BKK1-rv2 5'TCTGGATCCTCTTGGACCCGAGGGGTAATCGT. The constructs were sequenced

to ensure correct sequences and in-frame fusions. The resulting constructs were transformed into both *bri1-5* and Col-0 plants.

4.3 RT-PCR Analysis

Two μg total RNA was reverse transcribed in a 20-μl volume with Superscript III reverse transcriptase (Invitrogen). Two-μl first strand cDNA was used for RT-PCR with Ex Taq polymerase (Takara). A preliminary experiment was performed to determine the exponential range of each individual gene. *EF1α* was used as a quantitative control. For semi-quantitative RT-PCR analysis, PCR cycles used were, *BAK1* 22, *BKK1* 22, *AtSERK5* 28, *CPD* 22, *PR1* 30, *PR2* 30, *PR5* 22, *ACS2* 26, *ACS6* 22, *PDF1.2* 30, and *EF1α* 19, respectively. Primers used for amplifying *BAK1* and its paralogs were the same primers used for cloning. The primers used to amplify *CPD*, defense-related genes, and senescence-related genes were the same as reported by others (Tanaka et al., 2005; Oh et al., 2005; Yamagami et al., 2003).

4.4 Protein Extraction and Immunoprecipitation

Five grams of liquid cultured seedlings of Col-0, 35S-*BKK1-GFP* in *bak1 (-/-)/bkk1 (-/-)* and 35S-*BRI1-GFP* in Col-0 plants were ground in liquid N_2 as described by Wang et al. (Wang et al., 2005). Various seedling treatments with 1 μM BRZ or 1 μM BL and membrane protein isolation was the same as reported by Li et al. and Wang et al. (Li et al., 2002; Wang et al., 2005). BKK1-GFP was immunoprecipitated from solubilized total membrane protein using anti-GFP mouse antibody (Invitrogen) followed by a pull-down process using protein G beads (Roche).

4.5 Western Blot Analysis

GFP-immunoprecipitated membrane proteins were separated using 10% SDS polyacryamide gel electrophoresis. Various antibodies of α-GFP, α-FLAG, α-phosphothreonine and Western analysis procedures were all the same as previously described (Li et al., 2002; Wang et al., 2005).

4.6 Site-Directed Mutagenesis

BKK1 was cloned into the Gateway donor vector pDONR/zeo (Invitrogen) to generate pENTR-*BKK1*. PCR was conducted by using pENTR-*BKK1* as a template and primers AATCTAGTGGCTGTCGAAAGGCTAAAAGAAGAA and TTCTTCTTTTAGCCTTTCGACAGCCACTAGATT. PCR product was digested with *Dpn*I overnight followed by a heat shock transformation into *E. coli* DH5α. The plasmids were isolated and the mutation confirmed by sequencing analysis. The obtained pENTR-*mBKK1* was further cloned into pBIB-BASTA-35S-*GFP* using a Gateway strategy for plant transformation.

4.7 Various tissue stainings

Tissue stainings with aniline blue (0.01%, Sigma), trypan blue (1.25mg/ml, Sigma), and 3, 3'-diaminobezidine (DAB, 1mg/ml, Sigma) were the same as reported (Dietrich et al., 1994; Shirasu et al., 1999; Thordal-Christensen et al., 1997).

4.8 *bkk1-3* isolation and *bak1-3 bkk1-1* generation

bkk1-3, SALK_034523, was isolated from SALK T-DNA insertion lines. The double mutant *bak1-3 bkk1-1* was generated via crossing homozygous *bak1-3* with homozygous *bkk1-1* single mutants. The homozygous double mutant was isolated in F_3 generation. The genotype was confirmed by regular PCR and RT-PCR.

4.9 Scanning Electronic Microscopy analysis

Eight-day-old seedlings from ½ MS salts supplemented with 1% sucrose and 0.6% agar were harvested and immediately fixed in 2.5% glutaraaldehyde prepared in 0.1 M cacodylate buffer (pH 7.4) for 4 h at room temperature. The samples were dehydrated via a gradient ethanol series, dried in a critical-point drier. The pretreated samples were coated with carbon in a vacuum evaporator. The samples were analyzed using a JEOL JSM-800 high resolution scanning electron microscope.

5 Acknowledgements

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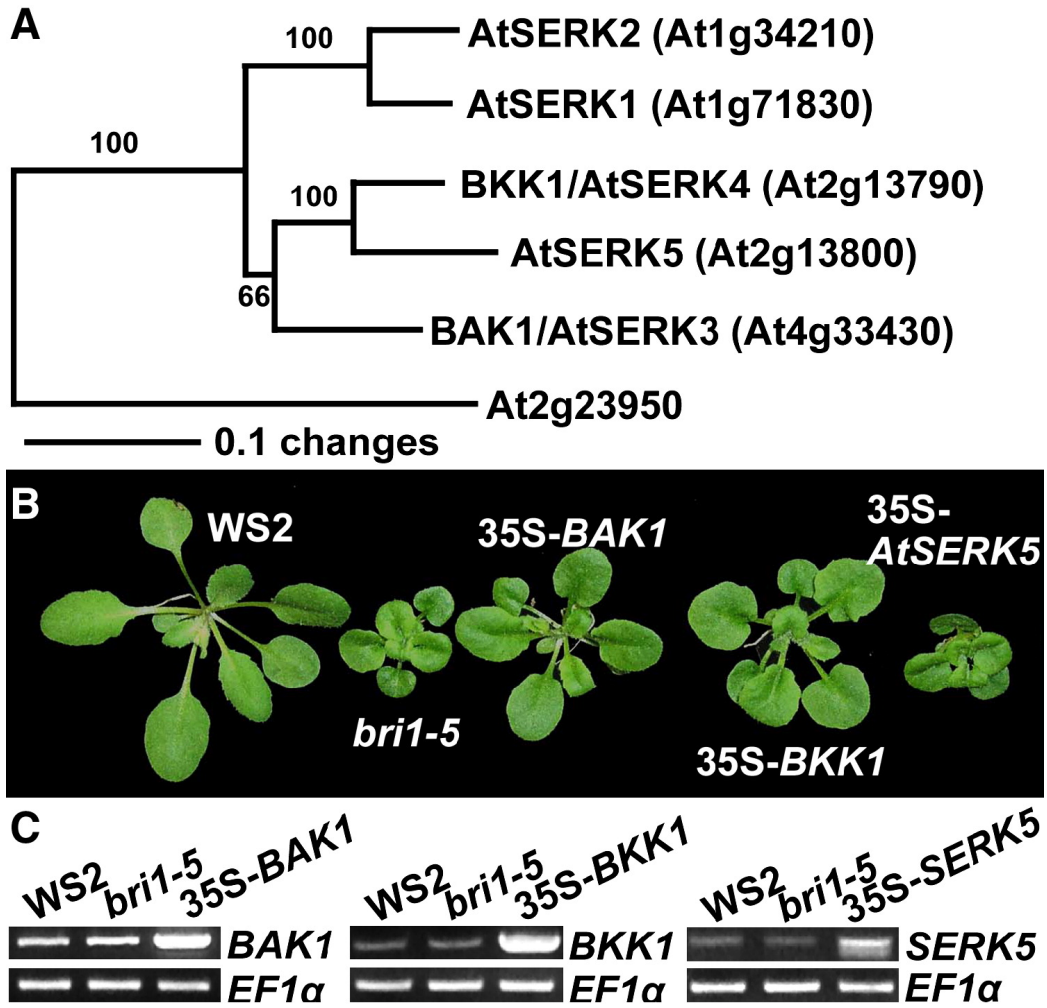

Figure 2.1 *BKK1* plays a redundant role with *BAK1* in suppressing *bri1-5* when overexpressed. (A) Phylogenetic analysis suggesting BKK1/SERK4 is the closest paralog of BAK1 in *Arabidopsis*.(B) Overexpression of *BAK1*, or *BKK1*, but not *SERK5*, suppresses the phenotypes of *bri1-5*. (C) RT-PCR analyses to confirm the elevated expression of the transgenes in the transgenic plants.

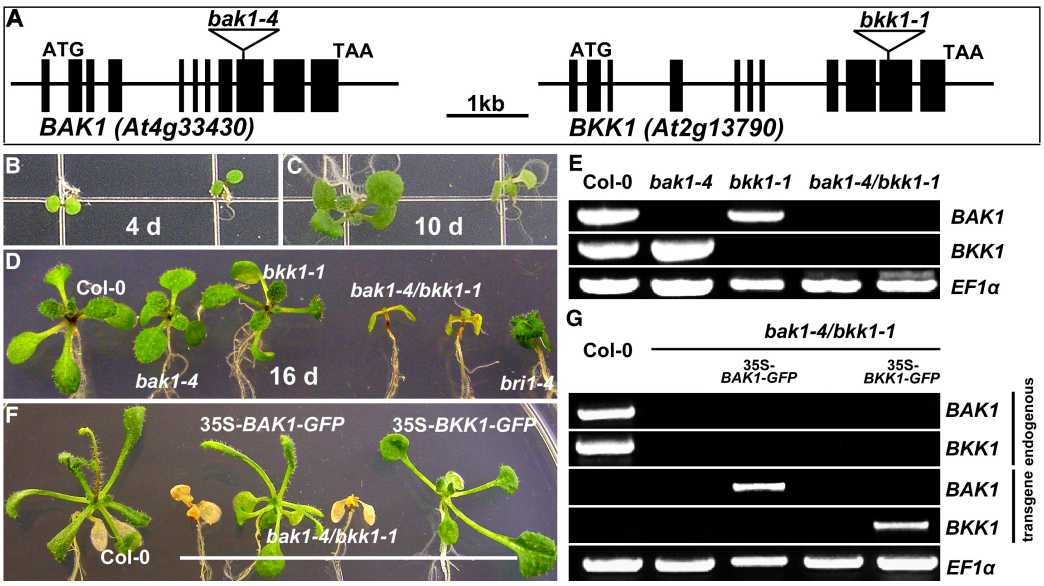

Figure 2.2 A *bak1-4 bkk1-1* double null mutant shows a seedling lethal phenotype at an early developmental stage. (A) T-DNA insertion sites of single knock-out lines, *bak1-4* (SALK_116202) and *bkk1-1* (SALK_057955). Both are in Col-0 background. (B-C) Phenotypes of wild-type (in Col-0, left side) and double null mutant (right side) at different developmental stages after germination. (D) Phenotypes of wild-type, *bak1-4* and *bkk1-1* single mutants, double mutant, and *bri1-4* mutant seedlings. (E) RT-PCR analyses to confirm the genotypes shown in (E). Genotypic analysis of *bri1-4* is not included. (F) Overexpression of either *BAK1* or *BKK1* driven by 35S promoter completely rescues the lethal *bak1-4 bkk1-1* double null phenotypes. (G) RT-PCR analysis verifies the genotypes of the plants shown in (K).

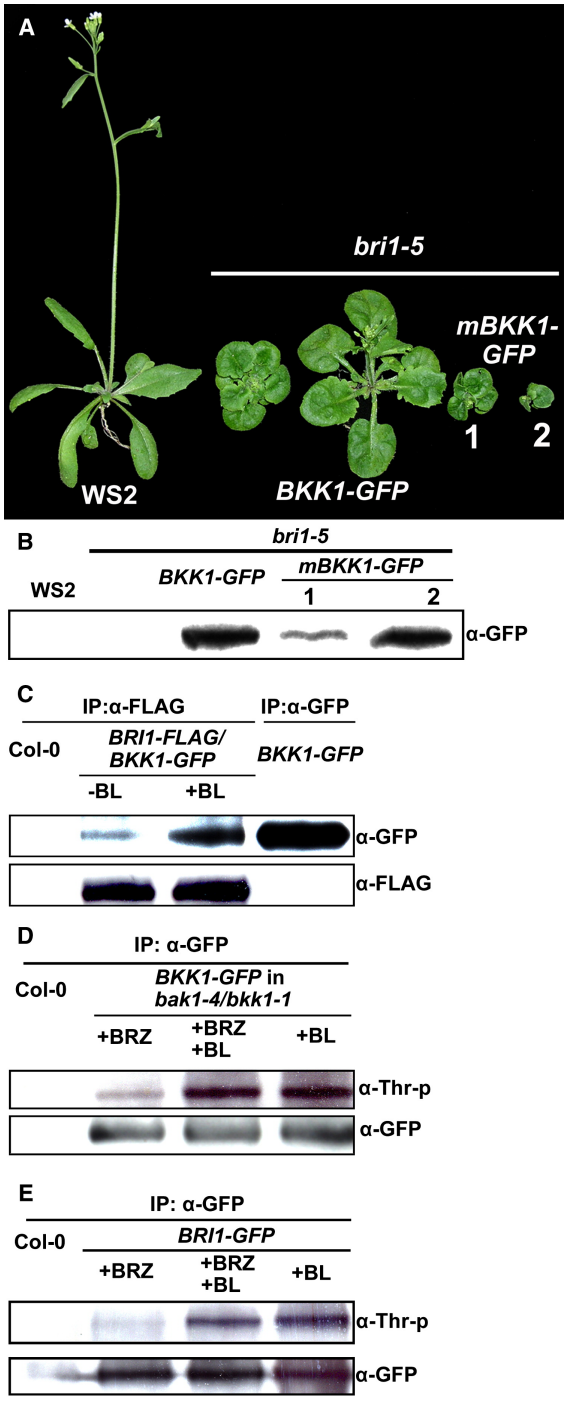

Figure 2.3 BKK1 interacts with BRI1 and mediates BR signal transduction. (A) Overexpression of *BKK1-GFP* suppresses *bri1-5* phenotypes; whereas overexpression of *mBKK1-GFP* results in a dominant negative effect, which greatly enhances *bri1-5* defective phenotypes. (B) Severity of transgenic plant phenotype is apparently correlated to the mBKK1-GFP protein levels. Equal amount of total protein was used for each sample for a Western analysis to show differently expressed mBKK1-GFP levels.(C) BKK1 interacts with BRI1 *in vivo* and the interaction is BL-enhanced. Wild-type and transgenic seeds harboring 35S-*BRI1-FLAG* and 35S-*BKK1-GFP* in Col-0 were grown in

liquid culture (Wang et al., 2005). A week after germination, one flask of seedlings was treated with mock (DMSO), and a duplicated flask of seedlings were treated with 1 μM BL. The membrane fractions from these two treatments were extracted and immunoprecipitated with α-FLAG. The co-immunoprecipitated BKK1-GFP was detected by a Western blot using α-GFP. A duplicated blot was hybridized with α-FLAG to confirm equal loading of the immunoprecipitated BRI1-FLAG (bottom panel). BKK1-GFP, immunoprecipitated with α-GFP from single transgenic plants harboring 35S-BKK1-GFP, was used as a size reference.(D) Exogenous application of BL increases the phosphorylation level of BKK1. Liquid- cultured seedlings harboring 35S-*BKK1-GFP* in *bak1-4 bkk1-1* double null background were either treated or untreated with 1 μM BL, after treatment with 1 μM BRZ to deplete endogenous BRs in liquid culture. Levels of threonine phosphorylation were detected with anti-phosphoThr antibody (upper panel). The same amount of transgenic seedlings, as well as non-transgenic Col-0 as a negative control, were harvested and immunoprecipitated with anti-GFP antibody. Equal amounts of BKK1-GFP proteins were used, as demonstrated in a duplicated immunoblot with an anti-GFP antibody as shown in the lower panel. (E) Exogenous BL application elevates the phosphorylation level of BRI1. Seedlings harboring *35S-BRI1-GFP* in Col-0 were used for the experiments. The treatments and detection were similar to what is described in (D).

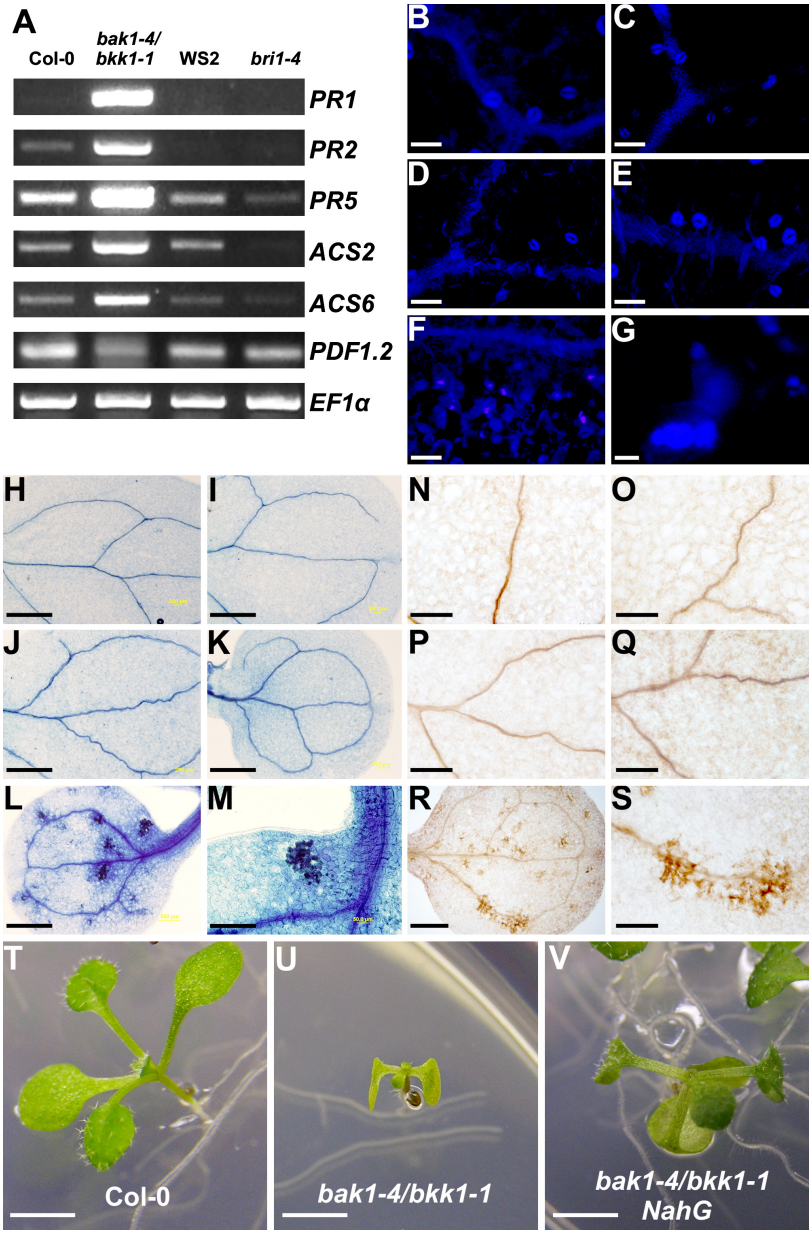

Figure 2.4 BAK1 and BKK1 are also involved in BR-independent cell death signaling pathway. (A) A number of defense-related genes are either up- or down-regulated in *bak1-4 bkk1-1*, relative to its background Col-0 plants; whereas these genes are not affected or oppositely regulated in *bri1-4*, compared to its background, WS2. (B-G) Aniline blue stained cotyledons of 8-day-old seedlings grown on ½ MS plates under sterile conditions. Only vascular tissues and guard cells were stained in wild-type (Col-0, B), *bak1-4* (C), *bkk1-1* (D), and *bri1-4* (E). Additional clustered signals on *bak1-4 bkk1-1* (F, G) double mutant were stained, indicating callose accumulation, a typical spontaneous defense response. (H-M) Trypan blue stained cotyledons of 8-day-old seedlings grown on ½ MS medium under sterile conditions. No cell death was found in cotyledons of wild-type (Col-0, H), *bak1-4* (I), *bkk1-1* (J), and *bri1-4* (K). Significant microscopic mesophyll cell death (shown as blue-stained cells) was detected in the

cotyledons of the *bak1-1 bkk1-1* double mutants (L, M). (N-S) DAB stained cotyledons of 8-day-old seedlings grown on ½ MS medium under sterile conditions. H_2O_2 was only detected in the vascular tissues of wild-type (Col-0, N), *bak1-4* (O), *bkk1-1* (P), and *bri1-4* (Q) and relatively little in their mesophyll cells. H_2O_2 accumulation was observed in the clustered mesophyll cells of the *bak1-1 bkk1-1* double mutants near the vascular tissues (R, S). (T-V) Expression of bacterial *NahG* in the double mutant partial rescues its seedling lethality phenotype. Phenotypes of 9-day-old Col-0 (T), *bak1-4 bkk1-1* (U), and *bak1-4 bkk1-1 NahG* (V) seedlings. Size bars, B-F = 50 μm, G = 10 μm, H-K = 40 μm, L = 150 μm, M = 40 μm, N-R = 1mm, and S = 200 μm. T-U = 2mm.

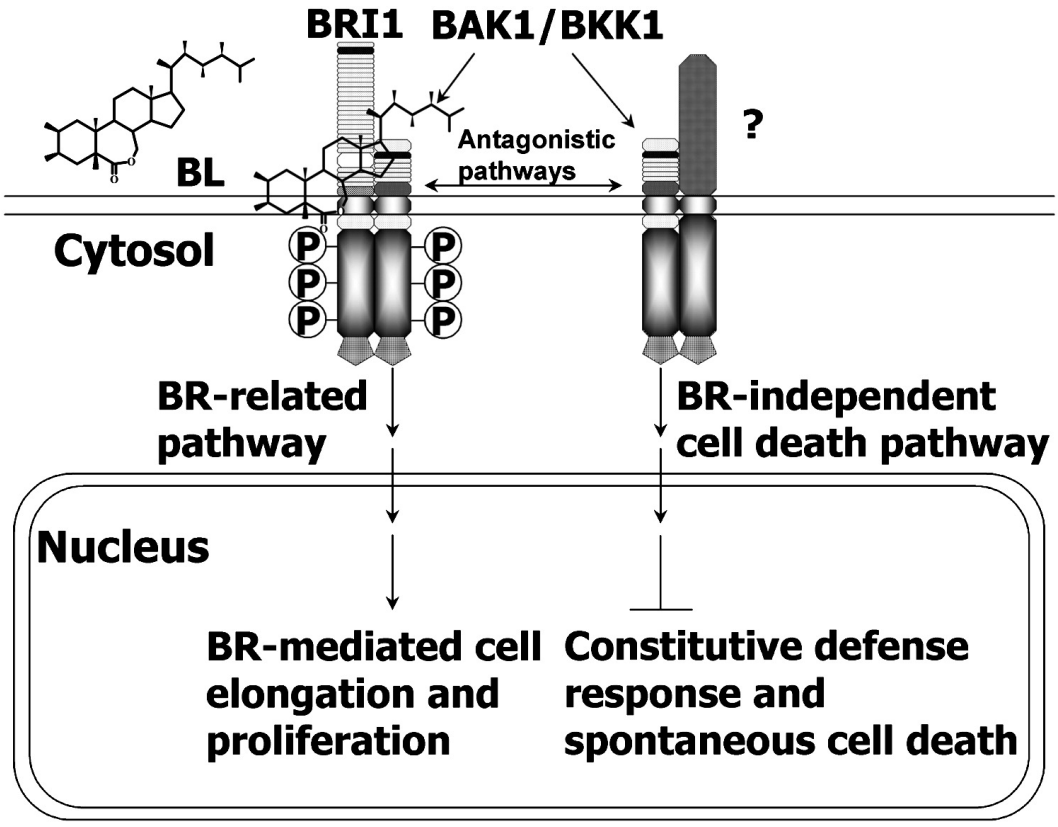

Figure 2.5 A proposed model indicating that BAK1 and BKK1 positively regulate BR signaling pathway, and negatively regulate a spontaneous cell death pathway. Cell death was observed on *bak1-4 (-/-) bkk1-1 (-/-)* seedlings grown under sterilized conditions, suggesting that the death signal is produced by the plant itself. The production of this cell death signal is likely controlled by unknown developmental cues.

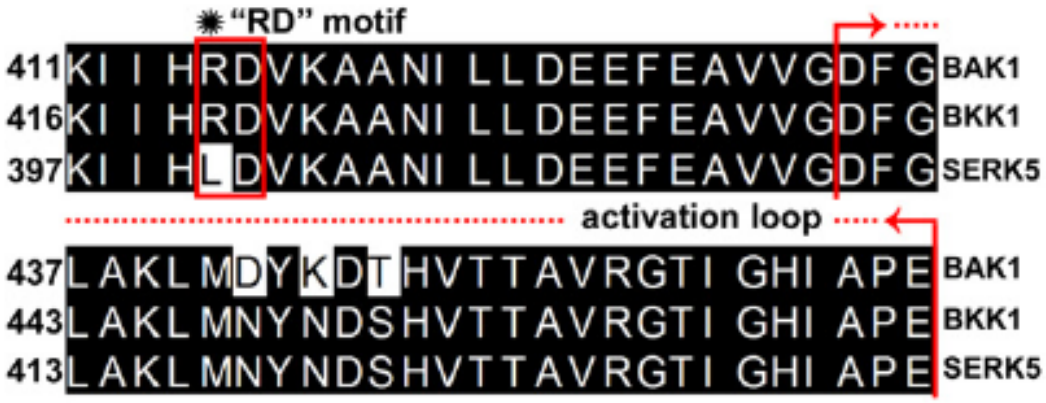

Figure 2.6 Partial sequence alignment of BAK1, BKK1/SERK4, and SERK5. In ecotype Col-0, SERK5 is likely not a functional kinase due to a single Arg401Leu single amino acid substitution compared to the same gene from WS2.

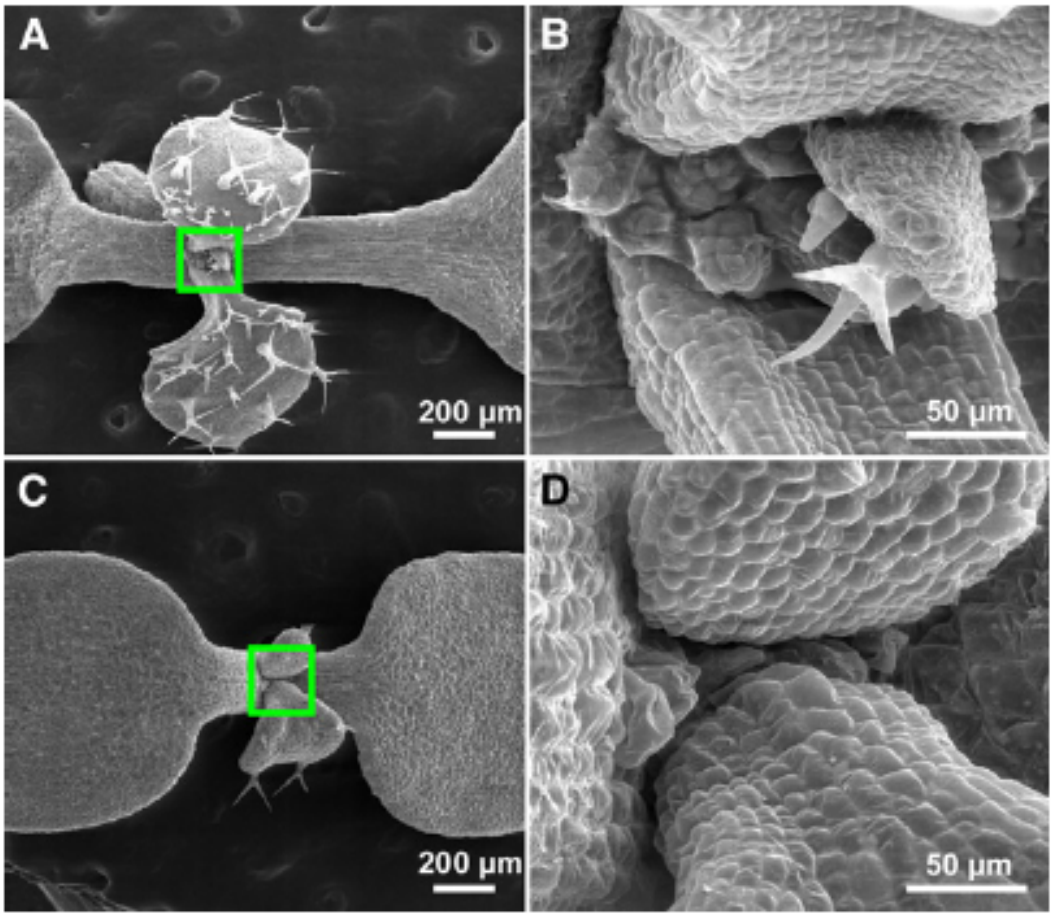

Figure 2.7 Shoot apical meristems (SAMs) of wild-type (Col-0) and *bak1-4 bkk1-4* seedlings revealed by Scanning Electron Microscopy (SEM). (A-B) Eight-day-old Col-0 wild-type seedlings at low magnification (A) and a higher magnification (B) view of shoot apical meristem region (as an inserted boxed area of A). (C-D) SEM analysis showing meristem structure of 8-day-old *bak1-4 bkk1-1* seedlings at low magnification (C) and higher magnification (D) view of shoot apical meristem region (as an insert of boxed area of C).

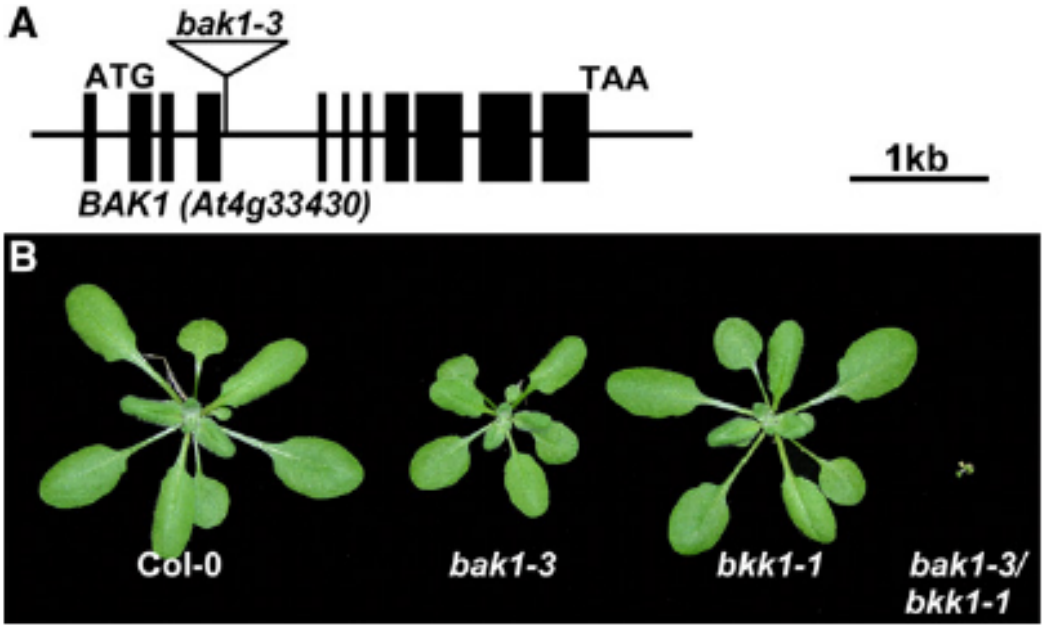

Figure 2.8 *bak1-3 bkk1-1* show early senescence and seedling lethality phenotype. (A) In *bak1-3*, a single T-DNA fragment was inserted in the fourth intron of the *BAK1* genomic sequence,(B) Phenotypes of 3-week-old soil-grown Col-0, *bak1-3*, *bkk1-1*, and *bak1-3 bkk1-1*.

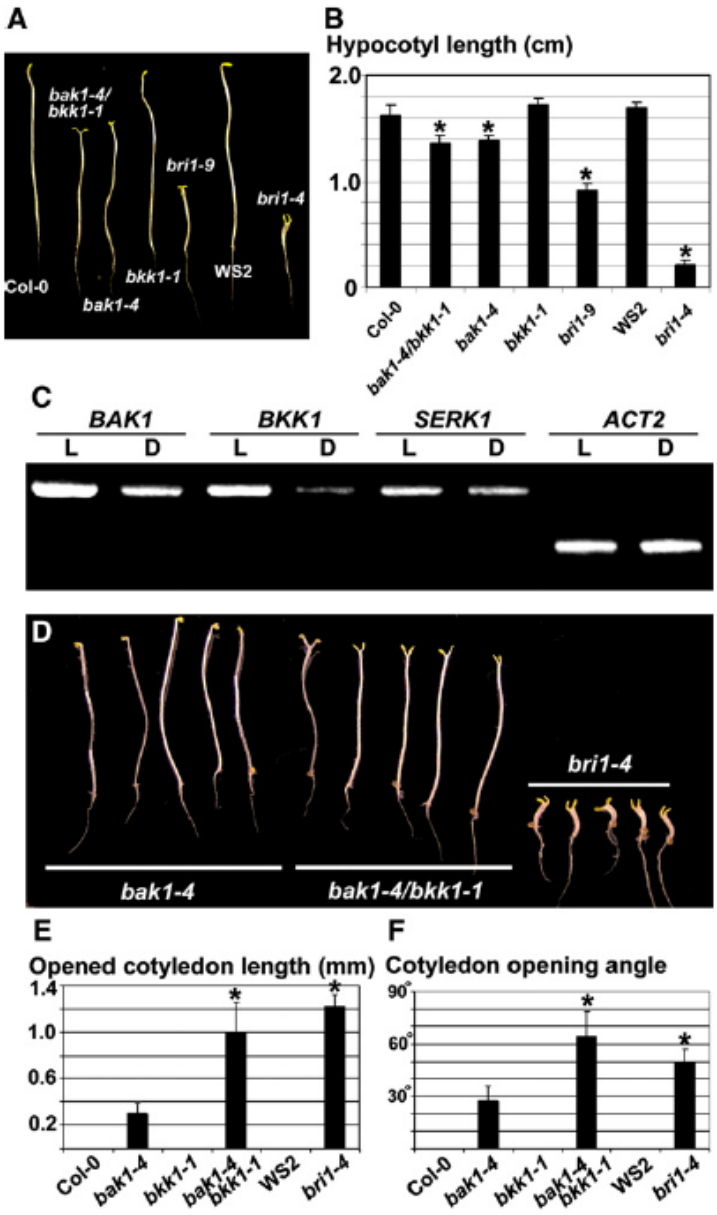

Figure 2.9 In *bak1-4 bkk1-1* double mutant, BR signaling pathway is partially blocked. (A) Five-day-old dark-grown seedling phenotypes of Col-0, *bak1-4 bkk1-1*, *bak1-4*, *bkk1-1*, *bri1-9*, WS2, and *bri1-4*.(B) The measurements of the dark-grown seedlings shown in (B).

Genotype	Survival Plants	Lethal Plants	Predicted Ratio[a]	Observed Ratio[a]	χ_c^2, p
Col.	248	0	N/A	N/A	N/A
bak1-4$^{+/-}$ *bkk1-1*$^{-/-}$	212	72	3:1	2.94:1	0.005, 0.94
bak1-4$^{-/-}$ *bkk1-1*$^{+/-}$	254	88	3:1	2.89:1	0.062, 0.80
bak1-4$^{+/-}$ *bkk1-1*$^{+/-}$	205	14	15:1	14.64:1	0.003, 0.96
35S-*BAK1* in *bak1-4*$^{-/-}$ *bkk1-1*$^{-/-}$[b]	146	46	3:1	3.17:1	0.063, 0.80
35S-*BKK1* in *bak1-4*$^{-/-}$ *bkk1-1*$^{-/-}$[b]	102	31	3:1	3.29:1	0.123, 0.73

[a] Ratio of survival plants:lethal plants.
[b] The transgenic plants used for analysis contains only one insertion. The seedlings for counting are from the T_2 generation.

Table 2.1 Genetic segregation analysis to confirm that the lethality is caused by the loss-of-function recessive mutations of both BAK1 and BKK1.

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Note

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Chapter III

Arabidopsis Leucine-Rich Repeat Receptor-Like Kinases, BAK1 and BKK1, Regulate the Levels of Reactive Oxygen Species in Chloroplasts

Key Words: BAK1, BKK1, ROS, ROS-scavenging, cell death

1 Abstract

BAK1 and *BKK1* regulate a *BRI1*-mediated BR signaling and a *BRI1*-independent cell death control pathways. *bak1-4 bkk1-1* double null mutant shows a spontaneous cell death phenotype, which is not seen in any other BR mutants. A weak double mutant, *bak1-3 bkk1-1*, is identified to show much milder cell death symptom compared to null double mutant. The cell death in *bak1-3 bkk1-1* is highly environment-dependent. Light is proven to be responsible for triggering the cell death. Microarray results reveal that *BAK1* and *BKK1* positively regulate a number of chloroplast-localized ROS-scavengers in transcription levels. Under a light condition, *bak1 bkk1* double mutant likely fails to detoxify ROS produced in chloroplasts, leading to cell death; whereas overexpression of *BAK1* or *BKK1* dramatically delays leaf senescence.

2 Introduction

To regulate growth and development under a normal condition and to respond various environmental challenges, plants have developed unique strategies to balance physiological processes and biotic/abiotic stress responses. In chapter 2, it is demonstrated that two Arabidopsis leucine-rich repeat receptor-like kinases (LRR-RLKs), *BAK1* and *BKK1*, are simultaneously regulating two distinct pathways: a *BRI1*-mediated BR signaling pathway and a cell death control pathway (chapter 2; He et al., 2007). BRI1 physically interacts with BAK1 and BKK1, *in vivo* (Li et al., 2002; Nam et al., 2002; Wang et al., 2005, He et al., 2007). Activation of BRI1 and BAK1 initiates the downstream signaling cascade. Unexpectedly, double null mutant of *BAK1* and *BKK1*, the closest paralog of *BAK1*, showed a spontaneous cell death and seeding lethality

phenotype observed about one week after germination (He et al., 2007). However, the defense-related genes up-regulated in *bak1-4 bkk1-1* did not show same expression patterns and even showed opposite patterns in *bri1-4* mutant; and the cell death phenotype is not seen in any BR lost-of-function and gain-of-function mutants, suggesting *BAK1* and *BKK1* negatively regulate a cell death pathway independent of BR signaling pathway (He et al., 2007). In addition, it was recently reported that BAK1 also functions as an FLS2-interactor in an innate immunity response pathway upon bacterial pathogen attacks (Chinchilla et al., 2007; Heese at al., 2007). Thus, by modulating the interplays among diverse signaling pathways, BAK1 and BKK1 act as central regulators integrating internal and external signals and eventually trigger the responses to a variety of challenges.

To elucidate the detailed mechanism of *BAK1*/*BKK1*-regulating cell death pathway, we investigated whether environmental factors contributed to triggering cell death. This study revealed the role of light as an elicitor of triggering cell death in *bak1 bkk1*. The cell death was suppressed by growing *bak1-4 bkk1-1* in a continuous dark condition. Further analyses indicated *BAK1* and *BKK1* positively regulate the expression of chloroplast-localized ROS-scavengers.

ROS include superoxide radical, hydrogen peroxide and singlet oxygen. In green plants, ROS are continuously produced as byproduct of several physiological processes, especially in the photosynthesis (Foyer et al., 1994). ROS also act as signaling molecules produced during biotic and abiotic tresses, regulating plant responses to various environmental challenges (Tsugane et al., 1999). ROS are toxic to plant cells when they exceed certain levels. Therefore, there must be a system to detoxify ROS. A variety of

ROS scavenging-enzymes, as well as antioxidants, are involved in removal of ROS to maintain a steady state redox homeostasis in a cell. *BAK1* and *BKK1* likely positively regulate ROS-scavenging genes fundamentally. Loss-of-function of *BAK1* and *BKK1* leads to down-regulation of ROS scavengers and accumulation of ROS in chloroplasts, and ultimately causes cell death.

3 Results

3.1 The cell death in *bak1-4 bkk1-1* is postembryonic

T-DNA disrupted alleles of *BAK1* and *BKK1* were screened from SALK pools. Two *BAK1* T-DNA insertion alleles, *bak1-3* and *bak1-4*, and two *BKK1* T-DNA insertion alleles, *bkk1-1* and *bkk1-2* were generated (Figure 3.1A). *bak1* or *bkk1* single mutant plants do not show obvious defective phenotype; whereas *bak1-4 bkk1-1* double mutant exhibits severe spontaneous cell death symptom (Chapter 2). When grown on the sterilized ½ MS medium, *bak1-4 bkk1-1* double mutant showed no defective phenotype compared to Col-0 wildtype 3 days after germination (DAG). Around 5 DAG, *bak1-4 bkk1-1* ceased growing and started showing lesions on the cotyledons at 8 DAG. The cell death phenotype was clearly seen at 13 DAG (Figure 3.1B). The lethality of *bak1-4 bkk1-1* was recapitulated by another double T-DNA insertion combination mutant, *bak1-4 bkk1-2*, which showed identical cell death phenotype *to bak1-4 bkk1-1* (Figure 3.1C). It takes at least one week to detect cell death in *bak1-4 bkk1-1*, implicating the embryonic development of *bak1-4 bkk1-1* is normal and the cell death is a postembryonic process.

3.2 *bak1-3 bkk1-1* is a weak mutant

bak1-4 bkk1-1 is completely lethal, making it less useful for further genetic studies. Besides *bak1-4*, we were able to identify another *BAK1* T-DNA insertion allele, *bak1-3*,

containing the T-DNA insertion at the fourth intron, the largest intron, of *BAK1* gene (Figure 3.1A). *bak1-3* mutant is able to express minute amount of *BAK1* transcripts. By using RT-PCR, the *BAK1* transcripts cannot be detected in *bak1-3* and *bak1-4* at 22 PCR cycles. The *BAK1* expression, however, was observed in *bak1-3* but not in *bak1-4* when the PCR cycles increased to 32 (Figure 3.2A). The *BAK1* expressed in *bak1-3* was sequenced to be wildtype *BAK1*. It is possible that the T-DNA located at the largest intron of *BAK1* was spliced out in a less efficient manner. Since *bak1-3* is a weak allele, it makes possible to generate *bak1 bkk1* weak double mutant showing a less severe phenotype, more valuable for elucidating the detailed mechanism of cell death in *bak1 bkk1* mutant.

3.3 Cell death in the weak double mutant *bak1-3 bkk1-1* is environment-dependent

Unlike null *bak1-4 bkk1-1* double mutant, weak double mutant *bak1-3 bkk1-1* shows much milder defective phenotype. Grown on sterilized ½ MS medium for 17 days, *bak1-3 bkk1-1* was perfectly healthy and phenotypically identical to a wildtype plant (Figure 3.2B). When grown in the potting soil in greenhouse condition, the weak double mutant seedlings showed an accelerated early senescence phenotype (Figure 3.2C), similar to lesion mimic mutants, such as *lsd1*(Aviv et al., 2002). The leaf cell death symptom of *bak1-3 bkk1-1* is highly environment-dependent, suggesting cell death is unlikely caused by developmental defects, such as vascular tissue defect. These observations indicated that certain death signal(s) is/are triggered by unknown environmental factors including biotic or abiotic stresses and the double mutant is more susceptible to the stresses.

3.4 ROS are accumulated in *bak1-3 bkk1-1*

Cotyledons and the first three pairs of true leaves from 28-day old Col-0 wildtype plants and *bak1-3 bkk1-1* mutant plants were compared. In contrary to Col-0, which still maintained healthy leaves, *bak1-3 bkk1-1* showed an accelerated senescence phenotype: the cotyledons and first pair of true leaves were completely dead; the second pair of true leaves showed senescence but less severe; while the third pairs of true leaves were almost healthy. This result suggested the cell death in *bak1-3 bkk1-1* was accumulative: older leaves showed more severe cell death symptoms (Figure 3.3C). Associated with cell death, ROS are usually triggered. Previous study indicated, as a typical defense response, H_2O_2, one of ROS, was accumulated in *bak1-4 bkk1-1*, accompanying the cell death symptom (He et al., 2007). DAB staining showed similar ROS accumulation in *bak1-3 bkk1-1* and the accumulated levels of H_2O_2 depend on the ages of leaves (Figure 3.3A). Higher H_2O_2 accumulation was detected in older leaves, consistent with phenotypes observed. For example, by comparing first five pairs of leaves, besides the cotyledons of *bak1-3 bkk1-1* that were already completely dead, the first and second pairs of true leaves of *bak1-3 bkk1-1* showed highest H_2O_2 accumulation (Figure 3.3B). Considering the cell death is a progressive effect in the weak double mutant and ROS can be produced and accumulated after germination (Elstner et al., 1991; Malan et al., 1990; Prasad et al., 1994; Tsugane et al., 1999), it is logical to hypothesize the accumulated ROS may contribute to the cell death.

3.5 The cell death in *bak1-4 bkk1-1* is triggered by light

The signal triggering the cell death in *bak1 bkk1* mutant is accumulated after germination and eventually causes cell death when it reaches to a certain limit. Given the fact that ROS are produced continuously under a light condition (Foyer et al., 1994), it is

possible that light might be involved in triggering cell death via ROS. Cotyledons from eight-day-old Col-0 and *bak1-4 bkk1-1* seedling grown in the dark and long-day lighting condition were stained by trypan blue and DAB. *bak1-4 bkk1-1* seedlings grown in a long-day lighting condition showed severe cell death symptoms and high H_2O_2 accumulation (Figure 3.4A.B). Conversely, *bak1-4 bkk1-1* plants grown in dark did not show any cell death symptom or H_2O_2 accumulation (Figure 3.4A.B). Even at 12 DAG, dark-grown *bak1-4 bkk1-1* plants did not show cell death. However, when transferred from dark to light condition at 9 DAG, *bak1-4 bkk1-1* started showing cell death in 3 days (Figure 3.4C). These results suggested that the cell death signal is likely triggered by light. *bak1-4 bkk1-1* might fail to protect plant from killing caused by the signal, or may have lost a mechanism to get rid of the cell death signal.

3.6 ROS are accumulated in chloroplasts

In plants, ROS can be largely produced as byproducts during photosynthesis and photorespiration (Foyer et al., 1994). ROS accumulation is also a typical response to biotic and abiotic tresses. Knowing ROS were accumulated in *bak1 bkk1* double mutant, we then examined the subcellular localization of accumulated ROS in *bak1-4 bkk1-1*. DAB staining clearly indicated H_2O_2 was mainly localized in the chloroplasts, where photosynthesis is taken place (Figure 3.5A).

3.7 Chloroplast-localized ROS-scavengers are down-regulated in *bak1-4 bkk1-1*.

ROS, as signaling molecules, can be rapidly produced in response to stresses, known as oxidative burst. On the other hand, ROS can also be produced during PCD. Therefore, there is a critical question that needs to be answered: is the accumulation of ROS in *bak1-4 bkk1-1* the cause of cell death or the consequence of cell death? By

analyzing microarray data comparing global gene expression profile between 9-day old Col-0 and *bak1-4 bkk1-1*, ROS-scavenging genes were examined. In Arabidopsis, there are five major ROS-scavenging gene groups: SOD, APX, GPX, CAT and PrxR, involving in detoxifying ROS by reducing ROS with the help of antioxidants (chapter 1). The microarray data revealed, out of total 39 major ROS-scavenging genes in Arabidopsis, 9 genes were down-regulated at least two-fold in *bak1-4 bkk1-1* (Table 3.1). Interestingly, except CSD3 localized in perioxisome, all other 8 down-regulated ROS-scavengers are chloroplast-localized (Table 3.1). RT-PCR was used to confirm the down-regulation ROS-scavenging genes in *bak1-4 bkk1-1* (Figure 3.5B). The data strongly suggested *BAK1* and *BKK1* positively regulate ROS-scavengers in chloroplasts. *bak1-4 bkk1-1* likely fails to sense the redox state in chloroplasts and to detoxify excessive amount of ROS. Uncontrolled ROS eventually trigger cell death.

2.8 Overexpression of *BAK1* and *BKK1* delays leaf senescence

bak1 bkk1 double mutant shows a severe cell death phenotype, while the plants overexpressing *BAK1* and *BKK1* exhibit delayed senescence. Driven by a constitutive active *35S* promoter, *BAK1* and *BKK1* were overexpressed in *bak1-4 bkk1-1*. The transgenic plants were still undergoing vegetative growth, producing rosette leaves not inflorescences when they were three-week old (Figure 3.6A). Col-0 wildtype plants at the same age started producing inflorescences and flowers. Four-week old Col-0 plants started showing lesions on the fifth pairs of true leaves, while the same leaves from four-week old *35S:BAK1/BKK1* plants were still completely healthy (Figure 3.6B). *bak1-3 bkk1-1* mutant plants, on the other hand, showed early senescence compared to wildtype plants. The opposite phenotypes shown by lost-of-function and gain-of-function

BAK1/BKK1 mutants indicated the role of *BAK1* and *BKK1* in negatively regulating cell death.

3.9 *bak1-3 bkk1-1* resembles *lsd1* phenotype

The environment-dependent cell death phenotype seen in *bak1-3 bkk1-1* resembles that of a lesion mimic mutant, *lsd1* (*LESION SIMULATING DISEASE1*) (Aviv et al., 2002). *LSD1* encodes a transcription factor of the C2C2 zinc finger family. LSD1 is an important regulator of plant PCD through several interactions with PAD4 and EDS1 (Rusterucci et al., 2001). *EDS1* and *PAD4* are required for accumulation of salicylic acid (SA), a phenolic defence-potentiating molecule in plants. Null mutations in *PAD4* and *EDS1* block *lsd1*-conditioned runaway cell death triggered by light or supply of the signaling molecule SA (Jabs et al., 1996; Rusterucci et al., 2001; Mateo et al., 2004). *lsd1* mutant shows spontaneous lesion on the leaves, similar to that of *bak1-3 bkk1-1*. Grown in the soil under greenhouse condition for three weeks, both *lsd1* and *bak1-3 bkk1-1* showed accelerated leaf senescence phenotype, starting from older leaves (Figure 3.6A). The comparison in the fifth pairs of true leaves from four-week old Col-0, *lsd1*, and *bak1-3 bkk1-1* showed similar leaf lesion phenotype in *lsd1* and *bak1-3 bkk1-1* (Figure 3.6B). The cell death of *lsd1* can be rescued by growing the plants under a short-day condition. Similarly, the cell death symptom in *bak1 bkk1* is also light-triggered and can be suppressed in darkness. In addition, *LSD1* play crucial role in SA signaling pathway in disease resistant pathway. SA pathway was previously reported to partially participate in *BAK1/BKK1*-mediating cell death control. Therefore, current genetic studies provide an interesting potential connection between *BAK1/BKK1*-regulating pathway and *LSD1*-mediated pathway.

3.10 Current model for *BAK1*/*BKK1*-mediated pathways

BAK1 and *BKK1* are involved in at least three signaling pathways: BR signaling, innate immunity response and cell death control pathways. Acting as co-receptors, BAK1 and/or BKK1 interact with ligand-binding receptors, BRI1 and FLS2, in regulating BR and pathogen defense pathways respectively. Based on this pattern, BAK1 and BKK1 may have other interacting RLK(s) for their functions in cell death control pathway. Specific ligand(s) may be involved in triggering this protein-protein interaction. One possible ligand could be described as "survival signal(s)" that will be synthesized as metabolic products or polypeptide and serve to maintain the activation of *BAK1/BKK1*-medaiting ROS scavenging pathway, protecting plants from ROS toxicity. The other possible ligand could be ROS themselves. The levels of ROS and redox state are continuously checked by BAK1 and BKK1; and the levels of ROS are regulated by a feedback manner. Excessive ROS enhance *BAK1/BKK1*-mediated ROS-scavenging. In prokaryotes, two-component histidine kinases work as the ROS cell surface sensors and the downstream signaling is mediated by a MAP kinase cascade. In Arabidopsis, although the ROS sensors or receptors have not been identified, a MAPKKK, ANP1, together with its two MAPK downstream regulators, MPK3 and MPK6, were found to be rapidly activated by H_2O_2 treatment (Kovtun et al., 2000). We hypothesize if BAK1 and BKK1 can sense ROS signals, ANP1 and MAPK3/6 might function as downstream components of BAK1/BKK1.

4 Discussion

4.1 *BAK1/BKK1*-regulating cell death is independent of BR signaling and *FLS2*-mediated signaling

The spontaneous cell death phenotype showed in *bak1-4 bkk1-1* double mutant is not seen in any BR biosynthesis mutants or BR signaling mutants, suggesting the cell death control pathway is independent of BRI1-mediated BR signaling pathway. Up-regulation of pathogen-related genes and senescence-related genes in *bak1-4 bkk1-1* was not noticed in *bri1-4* null mutant. Therefore, evidence indicated the cell death control mediated by *BAK1* and *BKK1* is BR signaling-independent.

Upon bacterial pathogen attacks, FLS2 recruits BAK1 and triggers innate immunity responses, such as ROS accumulation and PR gene expression. Since BAK1 is only recruited to FLS2 complex when exogenous pathogen ligands are present, FLS2 has no physical interaction with BAK1 in normal growth conduction (or in sterilized condition) (Chinchilla et al., 2007; Heese et al., 2007). *bak1-4 bkk1-1* shows spontaneous cell death phenotype even when grown in sterilized medium, implicating *BAK1* and *BKK1* protect plants from cell death without pathogen elicitors. FLS2, therefore, has no contribution to regulating the cell death due to lack of pathogen ligands in normal condition.

4.2 *BAK1* and *BKK1* regulate BR signaling and cell death control pathways under normal condition

As essential plant hormones, brassinosteroids control many aspects of plant growth and development, such as cell expansion and division, vascular development, senescence, fertility, skotomorphogenesis and photomorphogenesis, and biotic/abiotic stress responses. As BR co-receptor, BAK1 regulates BR signaling pathway under a normal growth condition, by promoting plant growth and development. Considering that BRs cannot be transported for long distance, the synthesis and utility of BRs is limited to local areas. Similar to *BRI1*, promoter:GUS staining result revealed *BAK1* has high expression

levels in almost all stages and all tissues, suggesting the fundamental function of *BAK1* in BR signaling. Yeast-two hybrid analysis revealed the physical interaction between the kinase domains of BRI1 and BAK1 without BR ligand; and BRI1 KD (kinase domain)-MBP fusion protein interacts with BAK1 KD-GST and phorsphorylate each other *in vitro*. These results indicate, although BR treatment dramatically enhances BRI1-BAK1 interaction and phosphorylation, the basal BRI1-BAK1 interaction is likely ligand-independent.

The cell death is seen in *bak1-4 bkk1-1* even grown in the sterilized medium, suggesting the *BAK1* and *BKK1* protect plant from cell death under normal condition; and similar to *BRI1*-mediated BR signaling, the involvement of *BAK1* and *BKK1* in cell death control pathway does not need exogenous ligands, such as pathogen ligands needed in *FLS2*-meidated innate immunity response pathway. Like in BR signaling, *BAK1* and *BKK1* also regulate cell death control pathway under normal condition.

Although the cell death control pathway regulated by *BAK1* and *BKK1* is BR signaling-independent, previous study showed BL treatment promoted senescence. As essential plant hormone, BRs promote growth and development, which conflicts with the observation of BR triggering senescence. It is explained by our model that BAK1 and BKK1 are shared by BR signaling and cell death control pathways and the balance between two pathways has been built up under normal growth condition. Excessive application of exogenous BL dramatically activates BRI1 and the activated BRI1 recruits more BAK1 and BKK1 from cell death control pathway, resulting in the attenuation in cell death control pathway and eventually leading to senescence and cell death.

4.3 BAK1 is mobilized to FLS2 upon pathogen attacks

BAK1 is not recruited to FLS2 complex until the pathogen ligand is perceived by FLS2 receptor. The ligand-independent BRI1-BAK1 interaction and ligand-dependent FLS2-BAK1 interaction indicate that the primary function of BAK1 in BR signaling pathway under normal condition and participation of BAK1 in disease resistance by joining in FLS2 complex upon pathogen attacks by a "distribution–according-to-need" manner. Plants utilize this strategy to maintain hormone signal transduction to promote normal growth and development; and temporally sacrifice normal physiological processes and mobilize components to fight against pathogen invasion. *BAK1*, therefore, controls multiple pathways by an economic way.

4.4 Accumulated ROS cause cell death in *bak1-4 bkk1-1*

Our results indicate the accumulation of ROS in *bak1-4 bkk1-1* chloroplasts is resulted from the large-scale down-regulation of chloroplast-localized ROS-scavengers. This result strongly suggested the accumulated ROS caused cell death in *bak1-4 bkk1-1*. It is apparent that *BAK1* and *BKK1* positively regulate a signaling that continuously check the redox state and detoxify ROS through ROS-scavengers. Although the ligand of BAK1/BKK1 in cell death control pathway is still mysterious, it is most likely that BAK1 and BKK1 regulate a fundamental ROS detoxification pathway.

5 Perspectives

BAK1 and *BKK1* may positively regulate major chloroplast-localized ROS-scavengers in transcription level. In plants, the sensor of ROS is still unknown. It will be a very interesting study to test whether BAK1 and BKK1 are serving as ROS sensors (receptors), regulating intracellular ROS levels and controlling cell death. The regulation of ROS could be a feedback way. The accumulated ROS, such as H_2O_2, act as

BAK1/BKK1 ligand, activate BAK1/BKK1, positively regulating ROS-scavengers to remove excessive ROS. Previous report showed the kinase activities of MPK3 and MPK6 were rapidly enhanced by H_2O_2 treatment (Kovtun et al., 2000). To test whether ROS are the ligands of BAK1 and BKK1, it is necessary to investigate the responses of MPK3 and MPK6 to H_2O_2 treatment in *bak1-4 bkk1-1*. It is also possible that ROS are not the ligands of BAK1 and BKK1. Some other signaling molecules trigger cell death control pathway through BAK1 and BKK1.

The patterns of BAK1 functioning in BR signaling and immunity response pathways indicate BAK1 may function as a co-receptors. It is possible that in cell death control pathway, BAK1 also need other interacting RLK(s), ligand-binding receptor(s), to function normally. However, the hypothesized ligand-triggering BAK1-RLK interaction makes it impossible to screen the BAK1-interactor through regular yeast-two hybrid screen or genetic screen such as activation tagging. The new discoveries perhaps rely on newly developed methods and strategies. Nevertheless, it cannot be excluded that BAK1 functions solely in cell death control pathway by directly perceiving the ligand.

6 Methods

6.1 RT-PCR

Total RNA was isolated from 9-day old Col-0 plants and *bak1-4 bkk1-1* mutant plants grown under long-day lighting condition. 2 µg total RNA from both samples was reverse-transcribed into 1st strand cDNA. cDNA equivalent to 100 ng RNA was used for each PCR reaction. The primers used to check the expression of ROS scavenging genes were: *GPX1* ATGGTTTCCATGACTACTTCAT and

AGCGGCAAGCAACTTCTGGAT,

APX4 ATGGGAGGAGTGTCCTTCCTCT and TAGCTTGAGTTTGCTCAGATT,

tAPX ATGTCTGTTTCTCTCTCCGCC and GAAACCAGAGAAATCGGAGTT,

CAT2 ATGGATCCTTACAAGTATCGTCCAGCT and

GATGCTTGGTCTCACGTTCAGA,

CSD3 ATGGAAGCTCCTAGAGGAAAT and TAGTTTAGCATCCGCAGATGAT,

2-cys PrxR A ATGGCGTCTGTTGCTTCTT and AATAGCTGAGAAGTACTCT,

2-cys PrxR B ATGTCAATGGCGTCTATAGCT and GATAGCTGAAAAGTATTCT,

PrxR Q ATGGCTGCTTCATCTTCCT and

AGCAGCTTTGAGAAACTTCA,

Type 2 PrxR E ATGGCGACTTCTCTCTCCGT and GAGAGCTTTAAGCATATCCT,

Ef1α CAGGCTGATTGTGCTGTCCT and

CAACGTTGTCACCTGGAAGT.

6.2 H_2O_2 detection by DAB staining

Prepare 1mg/ml 3, 3'-diaminobenzidine (DAB) (Sigma D8001), adjust the pH to 3.8 to solubilize DAB. Place the leaves in DAB solution and incubate in the growth chamber for 8 hours. Clear the tissue by placing into boiling 95% ethanol for 15 minutes. Transfer and store the cleared tissue in 95% ethanol. Make slides and check under light microscope.

6.3 Cell death detection by trypan blue staining

1 part Solution I (Phenol 50g, Lactic Acid 50ml,Glycerin 50ml,H_2O 50ml,Trypan Blue 100mg) is diluted with 2 parts 95% ethanol (1 Solution I :2 ethanol). Place fresh leaves in an EP tube and add enough diluted solution I immerging the plant tissue. Place the samples in the freshly boiled water bath for 30 minutes. Drain Solution I, add

Solution II (1 ml H_2O per 2.5g chloral hydrate) to double the tissue depth in the tube. Gently shake overnight and change the solution. Make slides and check under the light microscope.

6.4 *BAK1* and *BKK1* overexpression plants

bak1-4 bkk1-1 double mutant plants harboring *35S:BAK1-GFP* and *35S:BKK1-GFP* transgenes shown in figure 3 were previously describe in (*He et. al.,* 2007)

6.5 Identification of *bak1 bkk1* T-DNA insertion alleles

Homozygous T-DNA insertion disrupted mutants, *bak1-3*, *bak1-4*, *bkk1-1* and *bkk1-2*, were screened from SALK pool. LBb1 primer base on the T-DNA insertion left border sequence and two gene specific primers, RP and LP, flanking the T-DNA insertion, were used to identify mutants. The primers used were:

bak1-3 LP GCCTAACCACCAATACAAAAAGAG and

bak1-3 RP GCCTAACCACCAATACAAAAAGAG;

bak1-4 LP CTGTCTCTTTGGCCAATCAAG and

bak1-4 RP CAAATTCCTCTGTGTTCATGTTTC;

bkk1-1 LP GGTAGAGGTGGTTTTGGTAAAGTG and

bkk1-1 RP TTTTCGATAAGGGAATTGGAATAG;

bkk1-2 LP GATTGCAAGTAACATGAAACCAAG and

bkk1-2 RP AAAAATAAATTTGGCGGGAAAC;

LBb1 GCGTGGACCGCTTGCTGCAACT.

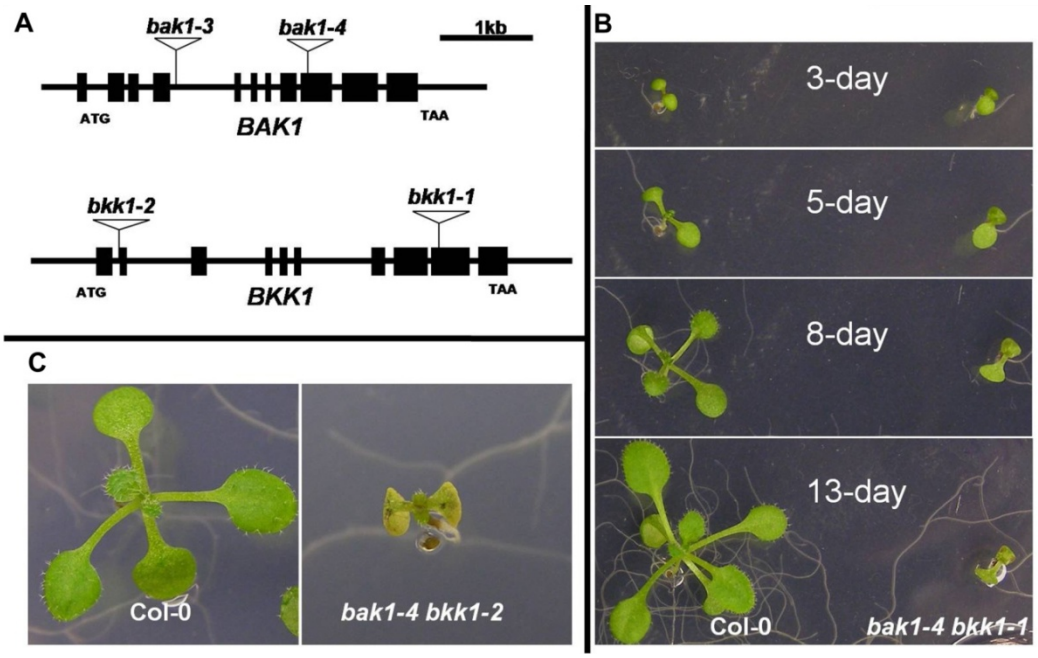

Figure 3.1 *bak1 bkk1* double mutants are lethal. (A) Multiple T-DNA insertion mutants for *BAK1* and *BKK1*. (B) *bak1-4 bkk1-1* double mutant seedling is lethal around one week after germination. (C) *bak1-4 bkk1-2* shows identical cell death phenotype to *bak1-4 bkk1-1*.

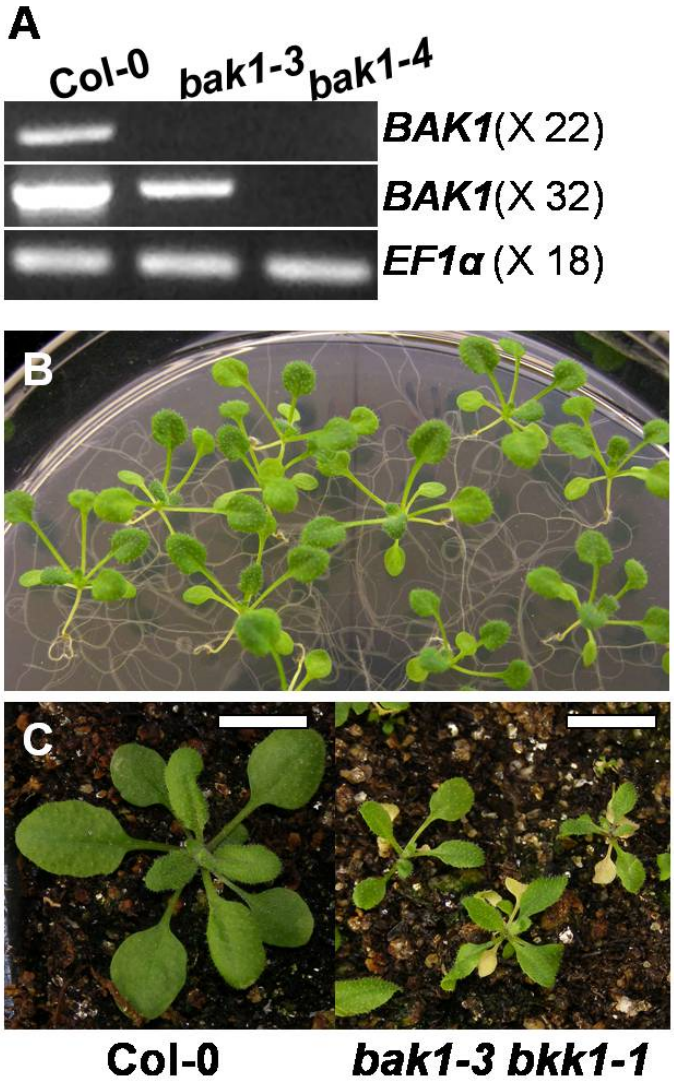

Figure 3.2 Cell death in *bak1-3 bkk1-1* weak mutant is environment-dependent. (A) *bak1-3* is a weak allele, expressing minute amount of *BAK1* transcripts, whereas *bak1-4* is null allele. (B) 17-day old *bak1-3 bkk1-1* grown on the sterilized medium shows perfectly healthy phenotype identical to wildtype. (C) When grown in the potting soil under greenhouse condition, 17-day old *bak1-3 bkk1-1* shows leaf cell death symptom, while wilderype plant is healthy.

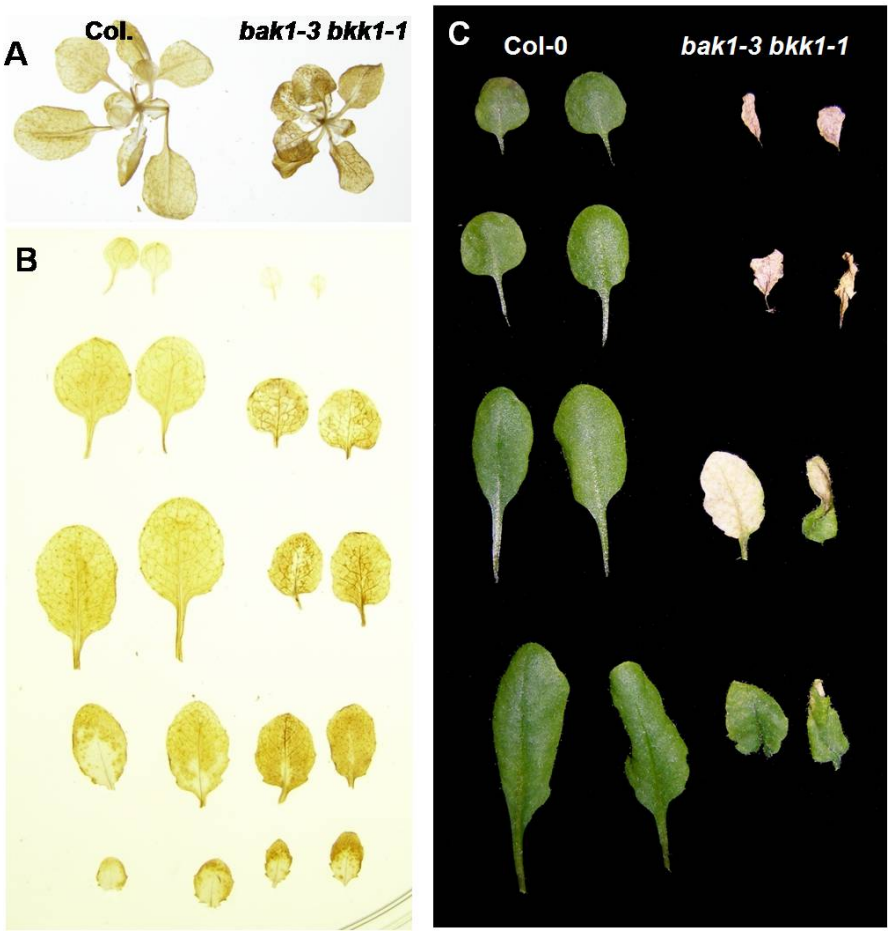

Figure 3.3 ROS product and cell death effect is accumulated in *bak1-3 bkk1-1*. (A) 17-day old *bak1-3 bkk1-1* plant accumulated more H_2O_2 compared to Col-0 wildtype plant. (B) Higher H_2O_2 accumulation is detected in older leaves of *bak1-3 bkk1-1*. (C) In 28-day old Col-0 wildtype plant, first four pairs of leaves still maintain healthy but in *bak1-3 bkk1-4*, older leaves show earlier senescence.

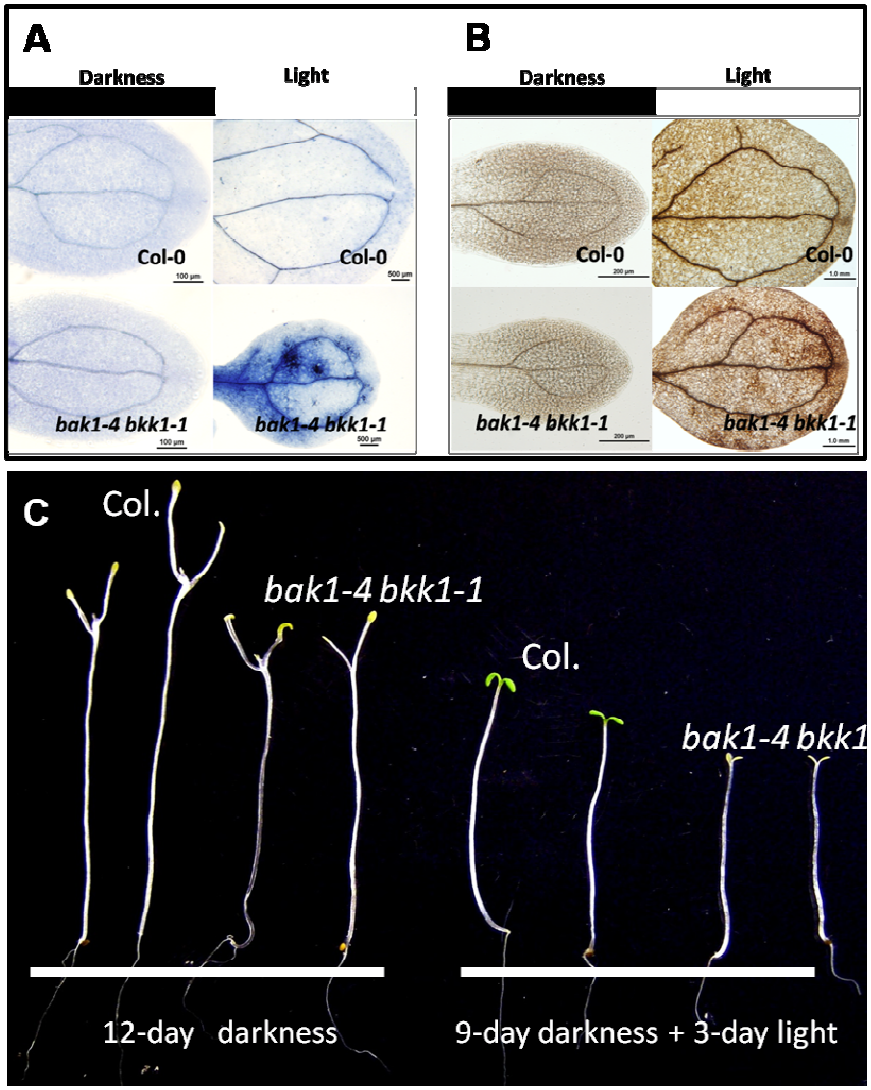

Figure 3.4 Light triggers cell death in *bak1-4 bkk1-1*. (A) Cell death in *bak1-4 bkk1-1* is suppressed by growing in dark. (B) ROS accumulation is not detected in *bak1-4 bkk1-1* grown in dark. (C) 12-day old *bak1-4 bkk1-1* grown in dark is still healthy. Transferred from dark condition to light condition, *bak1-4 bkk1-1* shows cell death in three days.

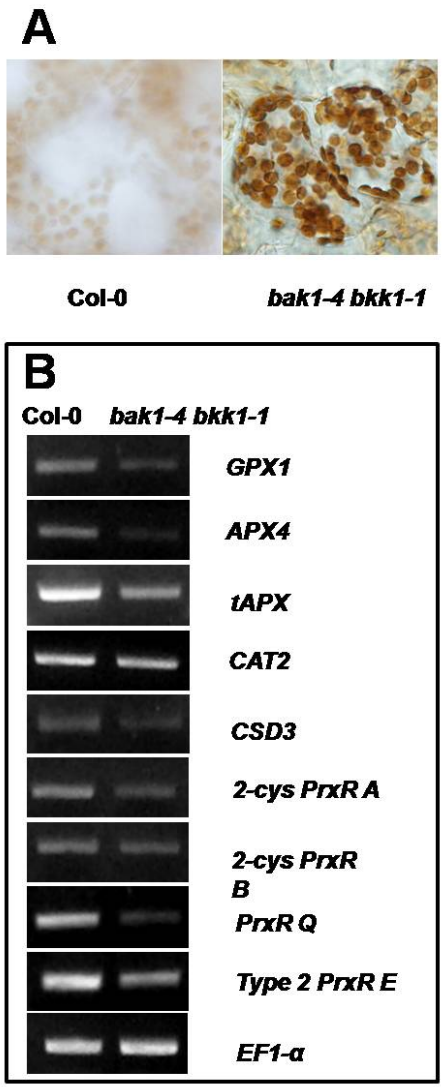

Figure 3.5 ROS accumulated in chloroplast due to down-regulation of ROS-savaging genes in *bak1-4 bkk1-1*. (A) H_2O_2 is mainly accumulated in the chloroplasts of *bak1-4 bkk1-1*. (B) RT-PCR conforms 9 ROS-scavenging genes are down-regulated in *bak1-4 bkk1-1*.

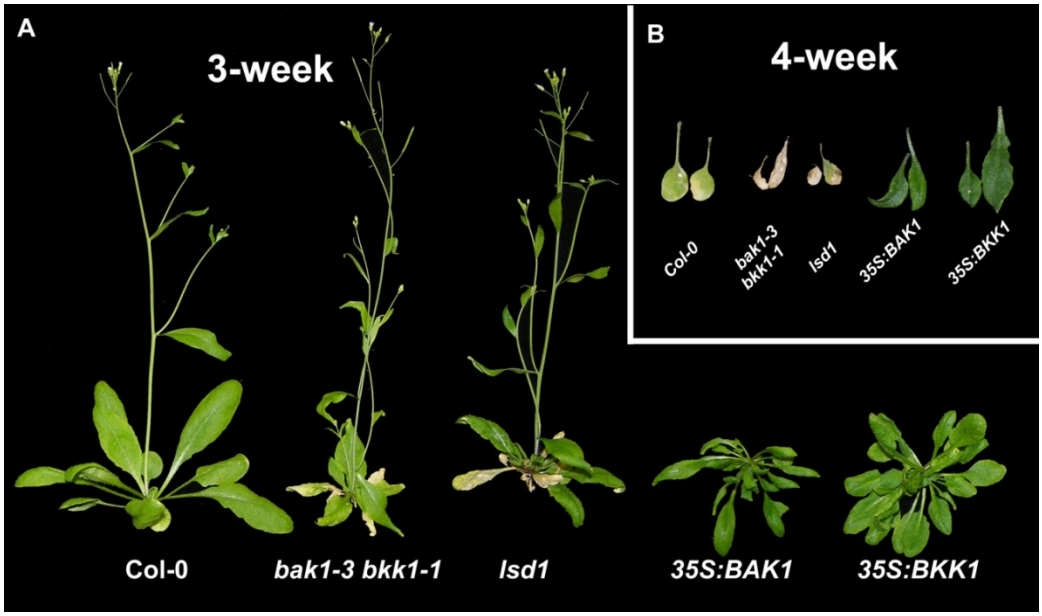

Figure 3.6 *bak1-3 bkk1-1* shows accelerated leaf senescence. (A) 3-week old *bak1-3 bkk1-1* shows leaf cell death symptom similar to *lsd1*. Overexpression of *BAK1* or *BKK1* leads to dark-green leaves and delayed flowering phenotype. (B) The fifth pairs of true leaves from 4-week old *bak1-3 bkk1-1* are completely dead, identical those from *lsd1*. The leaves from Col-0 wildtype plants start showing lesions, but the leaves from *BAK1* or *BKK1* overexpression plants are still dark-green and maintain healthy.

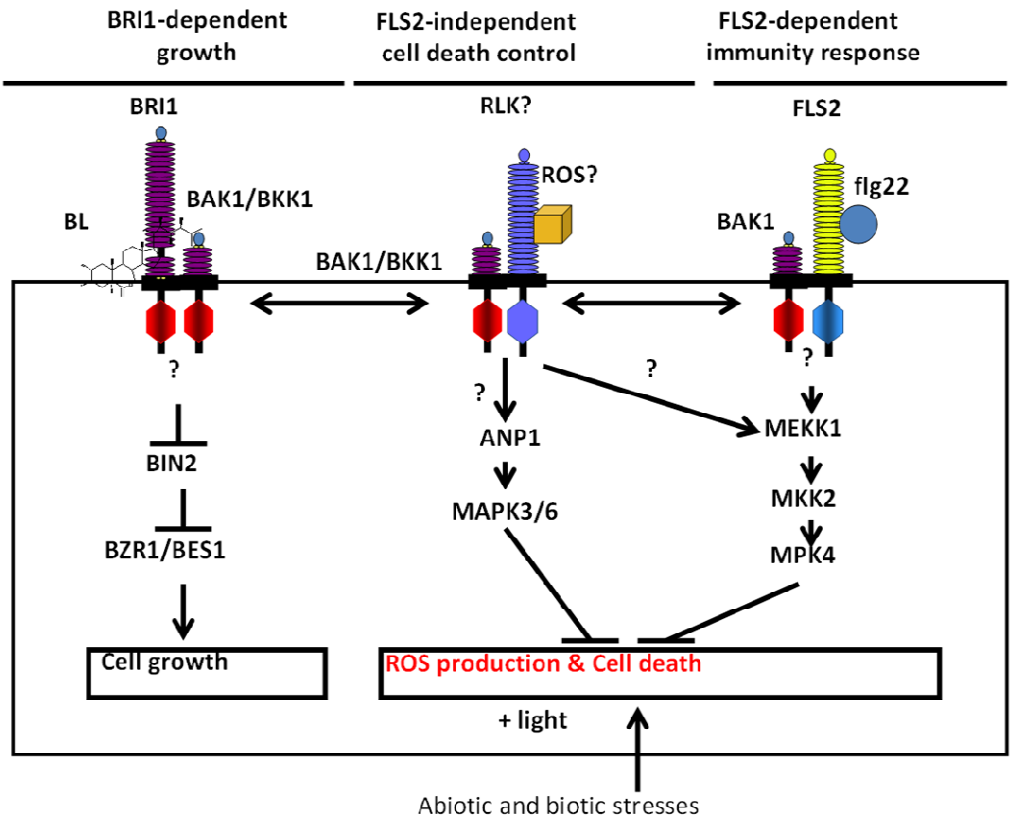

Figure 3.7 Current model for *BAK1/BKK1*-controlling pathways. *BAK1* and *BKK1* are involved in at least three pathways: BR signaling, innate immunity response and cell death control pathways. BAK1 and BKK1 may have other interacting RLK(s) in to function in cell death control pathway and specific ligand(s) may be involved. One possible ligand could be "survival signal(s)" that will be synthesized as metabolic products or polypeptide and serve to maintain the activation of *BAK1/BKK1*-medaiting ROS scavenging pathway. The other possible ligand could be ROS themselves. The levels of ROS and redox state are continuously checked by BAK1 and BKK1. In Arabidopsis, a MAPKKK, ANP1, together with its two MAPK downstream, MPK3 and MPK6, show response to ROS treatment. It is hypothesized if BAK1 and BKK1 can sense ROS signals, ANP1 and MAPK3/6 might function as downstream components of BAK1/BKK1.

Enzyme	Gene name	AGI code	localization
Superoxide dismutase (SOD)	FeSOD (FSD1)	At4g25100	Chl
	FeSOD (FSD2)	At5g51100	Chl
	FeSOD (FSD3)	At5g23310	Chl
	Cu/ZnSOD (CSD1)	At1g08830	Cyt
	Cu/ZnSOD (CSD2)	At2g28190	Chl
	Cu/ZnSOD (CSD3) ↓2.3	At5g01810	Per
	MnSOD (MSD1)	At3g10920	Mit
	MnSOD-like	At1g08830	Sec
Ascorbate peroxidase (APX)	APX1	At1g07890	Cyt
	APX2	At3g09640	Cyt
	APX3	At4g35000	Per,Chl
	APX4 ↓4.1	At4g09010	Chl
	APX5	At4g35970	Per
	APX6 ↓2.2	At4g32320	Cyt,chl,mit
	APX7	At1g33660	Mit
	Stromal-APX (s-APX)	At4g08390	Chl,mit
	Thylakoid-APX (t-APX) ↓5.5	At1g77490	Chl
Catalase (Cat)	Cat1	At1g20630	Per
	Cat2	At4g35090	Per
	Cat3	At1g20620	Per
Peroxinredoxin (PrxR)	1-cys PrxR	At1g48130	Nuc
	2-cys PrxR A ↓2.7	At3g11630	Chl
	2-cys PrxR B ↓2.3	At5g06290	Chl
	2-cys PrxR E	At3g06050	Mit
	PrxR Q ↓3.8	At3g26060	Chl
	Type 2 PrxR-related	At3g11630	Cyt
	Type 2 PrxR A	At1g65990	Mem,chl
	Type 2 PrxR B	At1g65980	Cyt
	Type 2 PrxR C	At1g65970	Cyt
	Type 2 PrxR D	At1g60740	Cyt
	Type 2 PrxR E ↓2.9	At3g52960	Chl,mit
Glutathione peroxidase (GPX)	GPX1 ↓2.5	At2g25080	Chl
	GPX2	At2g31570	Cyt,chl
	GPX3	At2g43350	Mit
	GPX4	At2g48150	Cyt
	GPX5	At3g63080	ER
	GPX7	At4g31870	Chl

Table 3.1 Nine ROS-scavenging genes are down-regulated in *bak1-4 bkk1-1*. Nine out total thirty-nine ROS scavenging genes are down-regulated in *bak1-4 bkk1-1*. Except CSD3, which is localized in peroxisome, eight down-regulated ROS scavengers are localized in chloroplasts.

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Chapter IV

Somatic Embryogenesis Receptor-Like Kinase (SERK) Family Controls Multiple Signaling Pathways in Arabidopsis

Key words: SERK, BR, cell death, FLS2, fertilization

1 Abstract

Arabidopsis *SERK* genes encode cell surface-localized LRR-RLKs, regulating multiple critical signaling pathways. *SERK* family contains five members named *SERK1* to *SERK5*. Previous studies demonstrated *SERK1* and *SERK2* control pollen development and maturation; *SERK1, SERK3 (BAK1)* and *SERK4 (BKK1)* act as BR co-receptor in plant hormone signal transduction; *SERK3 (BAK1)* and *SERK4 (BKK1)* regulate a light-dependent cell death control pathway; SERK3 (BAK1) is recruited by FLS2 upon pathogen attacks to initiate innate immunity response. This study revealed additional functions of *SERK* genes in corresponding pathways: *SERK2* in BR signaling pathway; *SERK1* and *SERK2* in cell death control pathway; and *SERK1, SERK2 and SERK3 (BAK1)* in regulating embryogenesis. More importantly, the phenotypes of multiple *SERK* gene mutants exhibit identical BR mutant phenotype to *bri1* null mutant, strongly supporting the notion that the *SERK* genes are essential for BR signaling.

2 Introduction

In carrot (*Daucus carota*), *SERK* (*Somatic Embryogenesis Receptor-Like Kinase*) gene encodes a leucine-rich repeat receptor-like kinase (LRR-RLK). It was found as a molecular marker for the development of somatic embryo from competent cells in culture (Schmidt et al., 1997). One ortholog of *DcSERK* in Arabidopsis was identified and named as *AtSERK1* (Hecht et al., 2001). *AtSERK1* belongs to a small subfamily in LRR-RLK II subfamily, containing five members: *AtSERK1, AtSERK2, AtSERK3 (BAK1), AtSERK4 (BKK1)* and *AtSERK5* (Baudino et al., 2001). *AtSERK1* is seen to be expressed in

developing ovules during magasporogenesis and in developing embryo cells until heart stage (Hecht et al., 2001). A kinase-associated protein phosphatase (KAPP) was identified as a SERK1-interactors (Rienties et al., 2005). The interaction, however, was only detected in the intracellular vesicles but not at the plasma membrane. KAPP dephosphorylated SERK1 at the activation loop, leading to internalization (Shah et al., 2002). These results suggested the SERK1 kinase activity regulated endocytosis. The detailed function of KAPP in modulating SERK1 is yet unknown. A cell division cycle (CDC) 48 protein (AAA ATPase CDC48A) was identified to interact with SERK1 protein, and the interaction only occurred in very restricted plasma membrane domain, but not in endosomes (Aker et al., 2006; Aker et al., 2007). The function of CDC48A in SERK1-mediated pathway is also not understood.

SERK2 is the closest paralog of SERK1, sharing 90% amino acid identity (Hecht et al., 2001). Both *SERK1* and *SERK2* are found to be expressed in the anther during pollen maturation. *SERK1* and *SERK2* are expressed in anther primordial and then in the tapetum. Single knockout mutant of *SERK1* or *SERK2* does not exhibit any defective phenotype. The *serk1 serk2* double knockout, however, fails to produce seeds and shows a complete male sterility phenotype (Albrecht et al., 2005; Colcombet et al., 2005). Further analyses of *serk1 serk2* double mutant show the microspore defect is caused by failing to develop meiosis and an absence of tapetal cell layer, which is essential for pollen maturation in the anther. The critical roles of *SERK1* and *SERK2* in taptum development and microspore maturation indicate the *AtSERK* genes control pathway(s) other than the firstly proposed function in embryogenesis like *DcSERK* does.

BAK1 (SERK3) and *BKK1 (SERK4)* were previously identified to function in both hormone brassinosteroid (BR) singling pathway and a cell death control pathway (Li et al., 2002; Nam et al., 2002; Wang et ak., 2005; He et al., 2007; Kemmerling et al., 2007). The interaction of BAK1 and BKK1 with BRI1 is enhanced by the treatment of BL, the most active BR. However, *bak1 bkk1* double mutant shows a spontaneous cell death, revealing the role of *BAK1* and *BKK1* in regulating an additional cell death pathway.

SERK1 was also identified to function as a redundant gene with *BAK1* in BR signaling (Karlova et al., 2006). BRI1 and BAK1 were found to be components in SERK1 complex. BAK1 could interact with SERK1 directly or indirectly through BRI1 complex. Introduction of T-DNA knockout of *SERK1* into *bri1* mutant enhances *bri1*-like dwarfed phenotype, providing genetic evidence that *SERK1* functions in BR signaling.

In Col-0 ecotype, SERK5 protein contains a natural mutation at the highly conserved "RD" motif in kinase subdomian VIa. RD motif is preceding the activation loop in subdomain VII. The R in RD motif is positively charged and forms ionic bridge with negatively charged D in activation loop, maintaining activation loop an appropriate structural conformation. Because R is substituted by an L, SERK5 is a non-RD protein in Col-0 (He et al., 2007).

Thus, previous studies revealed *SERKs*, except *SERK5*, are involved in at least four pathways: *SERK1*, *SERK3 (BAK1)* and *SERK4 (BKK1)* in BR signaling; *SERK3 (BAK1)* in *FLS2*-mediated response; *SERK3 (BAK1)* and *SERK4 (BKK1)* in cell death control; and *SERK1* and *SERK2* in microsporgensis regulation. This study provided more evidence that all *SERK* genes except *SERK5* are involved in regulating BR pathway, and

cell death control. In addition, although AtSERKs are identified as orthorlogs of somatic embryogenesis receptor-like kinase in carrot, there was no direct evidence showing *AtSERKs* are involved in embryogenesis. This study provided preliminary evidence that *AtSERK* genes might regulate and control the embryonic development and maturation.

The pattern that BRI1 acts as a ligand-biding receptor and SERKs act as co-receptors in BR signaling pathway provides an open question that whether SERK proteins work as an enhancer of BRI1 or are essential for BR signal transduction. In this study, the essentiality of *SERKs* in BR signaling has been demonstrated by genetic mutant analyses. It is the first genetic evidence to prove the SERKs not only activate BRI1 but also play essential role in mediating BR signaling.

3 Results

3.1 AtSERK subfamily consists of five leucine-rich repeat receptor-like kinases

In Arabidopsis, leucine-rich repeat receptor-like kinase (LRR-RLK) family is the largest subfamily in receptor-like kinase (RLK), containing at least 223 members (Shiu et al., 2001). LRR-RLK II subfamily consists of 14 members, including SERK family with five members. As typical LRR-RLKs, SERK proteins contain an extracellular domain with five LRRs, a transmembrane domain and a cytoplasmic Thr/Ser kinase domain, containing characterized 11 subdomains (Hanks et al., 1988; Stone and Walker, 1995) (Figure 4.1).

SERK1 and SERK2 are the closet paralogs to each other, sharing 90% amino acid identity. SERK4 and SERK5 are encoded by tandem repeat genes sharing 83% amino acid identity, and SERK4 is the closet paralog of BAK1, showing 82% amino acid

identity. Although the extracellular domains show some diversity, all SERK kinase domains are highly conserved with at least 95% amino acid identity.

3.2 All *SERKs* except *SERK5* are involved in cell death control pathway

To investigate whether other *SERKs* are involved in cell death control pathway regulated by *SERK3* and *SERK4*, all five *SERK* genes are cloned by a gateway strategy. All *SERKs* are transformed through Agrobacterium-mediated plant transformation method into *bak1-4 bkk1-1* null double mutant and *bak1-3 bkk1-1* weak double mutant. Driven by constitutively active *35S* promoter, all the overexpressed *SERK* genes except *SERK5* suppressed cell death symptom in *bak1 bkk1* null and weak mutants (Figure 4.2), suggesting the function of *SERK1, SERK2, SERK3* and *SERK4* in cell death control.

3.3 Expression patterns of *SERK* genes

Since *SERK1* and *SERK2* play a redundant role with *SERK3* and *SERK4* in cell death control, it should be investigated why *serk3 serk4* double mutant shows extremely severe cell death phenotype. To this end, the promoter regions of all *SERK* genes except *SERK5* were cloned and fused with the *GUS* reporter gene and transformed into Col-0 wildtype plants. GUS staining results indicated the expression patterns of *SERKs* are completely distinct. In 2-day old plants, *SERK3* and *SERK4* show high expression levels (Figure 4.3C-D) whereas *SERK1* and *SERK2* are expressed in low levels, in vascular tissue and root (*SERK1*) and shoot apical meristem (SAM) (*SERK2*) (Figure 4.3A-B). In 5-day old plants, *SERK3* and *SERK4* are still expressed predominantly (Figure 4.3G-H) while the low expression of *SERK1* can only be detected in the root and SAM (Figure 4.3E). *SERK2* has slightly stronger expression compared to *SERK1* (Figure 4.3F). Interestingly, all *SERKs* are clearly expressed in SAM (Figure 4.3I-L). Considering that

serk3 serk4 double mutant shows a SAM defect when grown in the light, it will be necessary to test whether *SERK* genes are involved in SAM development and maintenance. Thus, the promoter-GUS analyses of four *SERK* genes showed high expression level of *SERK3* and *SERK4* in Arabidopsis seedling. *SERK1* and *SERK2* have very low expression and are limited to certain tissues, such as root and SAM. It explained why the intact *SERK1* and *SERK2* in *serk3 serk4* mutant are not sufficient to complement the function of *SERK3* and *SERK4*. The overexpression of *SERK1* and *SERK2*, however, successfully rescued *serk3 serk4* doubly mutant, suggesting all the four *SERK* genes play redundant role in cell death.

3.4 All *SERK* genes except *SERK5* are involved in BR signaling pathway

Previous studies revealed three SERKs, SERK1, SERK3 and SERK4, are regulating BR signaling pathway by binding to BRI1 and acting as BR co-receptors. To test whether other *SERK* genes are also involved in BR signaling pathway, all five *SERK* genes driven by *35S* promoter were transformed into *bri1-5*. All overexpressed *SERKs* except *SERK5* were able to suppress *bri1-5* phenotype by elongating the petioles of rosette leaves (Figure 4.4A) and increasing the height of inflorescences (Figure 4.4C). The mutated *SERK* genes encoding kinase-death proteins, containing a mutation from K to E at the highly conserved ATP-binding site, were transformed into *bri1-5*. The obtained transgenic plants showed a *bri1*-like dominant-negative phenotype (Figure 4.4B). The result implicated mSERK proteins may interact with BRI1 and may have poisoned BRI1 complex, blocking normal BR signaling. Except *SERK5*, overexpression of *SERK* genes in Col-0 resembled the phenotype of *BRI1*-overexpressing plant: elongated and curled rosette leaves (Figure 4.4D), early flowering and early senescence

(data not show). Thus, *SERK1*, *SERK2*, *SERK3* and *SERK4* play redundant role in *BRI1*-mediated BR signaling pathway.

3.5 Identification of *SERK* T-DNA mutants

To investigate the detailed mechanisms of *SERK* genes regulating multiple pathways, T-DNA insertion mutants for each *SERK* gene were identified from SALK pools (Figure 4.5). RT-PCR results showed the lines used in this study were null alleles (data not shown). Single-, double- and triple- *SERK* gene knockout lines were generated with different combinations.

3.6 *sek1 serk3* shows reduced fertility

Previous reports revealed the essential role of *SERK1* and *SERK2* in microsporgenesis. *serk1 serk2* double mutant fails to produce seeds. Further analysis indicated the tapetum layer was missing during pollen development, leading to complete male sterility in *serk1 serk2*. Interestingly, our result showed besides *serk1 serk2*, *serk1 serk3* double mutant also showed some sterile phenotype. However, the sterility in *serk1 serk3* is not complete and seeds can be produced in some siliques. Observed under microscope, wildtype flower produces pollen and the style is elongated after pollination and eventual develops to siliques (Figure 4.6A-B). Although *serk1 serk2* and *serk1 serk3* are both sterile, the mechanisms are different. *serk1 serk2* mutant failed to produce any pollen (Figure 4.6C-D), whereas *serk1 serk3* mutant did produce normal pollen and the reduced fertility was caused by the pre-elongated style (Figure 4.6E-F). The chance that mature pollen contact with stigma is drastically decreased, leading to sterility. Manual pollinating *serk1 serk3* stigma by its own pollen rescued fertility phenotype (Figure 4.6G),

indicating the microaporgenesis in *serk1 serk3* is intact and the mechanism of fertility in *serk1 serk3* is distinct from *serk1 serk2*.

3.7 *SERK* genes are essential for BR signaling

In BR signaling pathway, the interaction between BRI1 and BAK1 after BR-binding enhances BRI1-BAK1 activity by transphosphorylation. The activated BR receptors initiate downstream signaling, regulating gene expression. Whether *SERKs* are essential for activating BR signaling is still not understood. Wang et al. reported a sequential transphosphorylation model that BRI1 is activated by BR-binding and then phosphorylates BAK1; inversely, the activated BAK1 phosphorylates BRI1 to turn on BR signaling subsequently (Wang et al., 2008). The authors propose that the function of BAK1 is dependent on BRI1 but BRI1 regulates BR pathway independent of BAK1's function, which suggests that SERKs only function as "enhancers" of BRI1. Our results, however, suggested the SERKs play essential roles in mediating BR signal transduction.

BR mutants show a typical de-etiolation phenotype when grown in dark. The severity of the de-etiolation phenotype depends on how much the BR signaling is blocked. *bri1-4* null mutant shows extremely de-etiolation phenotype, featured as shortened and swelling hypocotyls and opened cotyledons (He et al., 2007). The de-etiolation phenotype seen in *bri1-4*, therefore, can be used as an indicator to estimate whether BR signaling is completely blocked. To investigate the function of *SERK* genes in BR signaling, *SERK* single and multiple T-DNA insertion mutants were grown in the dark. Compared to *bri1-4*, all *SERK* single mutants did not exhibit obvious de-etiolation phenotype (Figure 4.7A). In double mutants, only *serk1 serk3* showed enhanced de-etiolation phenotype compared to *serk1* or *serk3* single mutant, exhibiting typical opened cotyledon and shortened

hypocotyls (Figure 4.7C). This result indicated that although all *SERKs* except *SERK5* are involved in BR signaling, *SERK1* and *SERK3* may play major role. The de-etiolation phenotype of *serk1 serk3*, however, is not as severe as that of *bri1-4*, suggesting additional redundant genes are still functioning in *serk1 serk3*. Triple mutants *serk1 serk2 serk4* and *serk2 serk3 serk4* did not show obvious de-etiolation phenotype probably due to the intact *SERK1* or *SERK3*, major BR signaling regulators. Compared to *serk1 serk3*, the triple mutant *serk1 serk3 serk4* showed more severe de-etiolation phenotype that is almost identical to the de-etiolation phenotype of *bri1-4* (Figure 4.7E). Also, compared to *serk1 serk3*, the triple mutant *serk1 serk2 serk3* not only showed de-etiolation phenotype, but also showed additional defective phenotype. *serk1 serk2 serk3* plants lack fully developed cotyledons and roots (Figure 4.7E). Therefore, these results not only confirmed the function of *SERK2* and *SERK4* in BR signaling pathway, but also indicated the *SERKs* are essential for BR signaling. Furthermore, the additional defects observed in *serk1 serk2 serk3* triple mutant is likely resulted from abnormal embryonic development, suggesting the functions of *SERK1*, *SERK2* and *SERK3* in embryogenesis, which was proposed for a long time but not supported by any experimental evidence.

Grown under long-day light condition, *SERK* single mutants did not show any obvious defective phenotype (Figure 4.7B). Double mutant *serk1 serk3* showed a weak *bri1*-like phenotype and *serk3 serk4* showed a cell death phenotype as previously reported (Figure 4.7D). All triple mutants containing both *serk3* and *serk4* showed cell death symptom. Triple mutant *serk1 serk2 serk3* showed severe defects: extremely dwarfed and compacted, and died a few days after germination (Figure 4.7F).

The multiple *SERK* mutants show typical BR mutant phenotype and some of them resemble the complete de-etiolation phenotype of *bri1-4*, strongly supporting *SERKs* function in BR singling pathway as essential regulators.

3.8 *SERK1* and *SERK3* play major role in BR signaling

Although all *SERK* genes except *SERK5* are involved in BR signaling pathway, the more severe phenotype of *serk1 serk3* in dark and in light suggests *SERK1* and *SERK3* play major role in BR signaling pathway. *serk1 serk3* then was used to test its response to BL treatment. Grown on ½ MS medium, *serk1 serk3* was compared with Col-0 by BL-based root inhibition analysis. The results indicated that, like *bri1-4* null mutant, *serk1 serk3* double mutant showed insensitivity to 100nM BL treatment (Figure 4.8). Although *SERK2* and *SERK4* are also involved in BR signaling, it is possible that prior functions of *SERK2* and *SERK4* are other than regulating BR signaling.

3.9 A current model for *SERK*-regulating signaling pathways

In SERK family, SERK5 might not be functional due to a natural point mutation at a highly conserved "RD" motif. Experimental data also support this notion because no specific function of *SERK5* has been identified by genetic evidence. All other *SERK* genes, *SERK1* to *SERK4*, are involved in at least four distinct signaling pathways. Previous studies indicated the function of *SERK1* and *SERK2* in microsporgenesis; *SERK1*, *SERK2*, *SERK3* and *SERK4* in BR signaling pathway; *SERK1*, *SERK2*, *SERK3* and *SERK4* in cell death control pathway; and *SERK3* in disease resistance pathway. More importantly, the multiple *SERK* knockout mutant phenotypes support the essential role of *SERKs* in BR signaling. The GUS reporter gene driven by *SERK* promoters indicates the high expression levels of *SERK3* and *SERK4* in the seedling stage,

consistent with the observation that only *serk3 serk4* double mutant shows cell death symptom, and conformed the major role of *SERK3* and *SERK4* in cell death control. Therefore, in this model, we hypothesize although all *SERKs* play redundant role to each other, some *SERKs* play major roles in certain pathways. *SERK1* and *SERK2* play major role in regulating microsporgenesis; *SERK1* and *SERK3* in BR signaling pathway; *SERK3* and *SERK4* in cell death control pathway. Whether other *SERKs* besides *SERK3* are involved in *FLS2*-mediated defense response pathway is still under investigation (Figure 4.9). In addition, *SERK1*, *SERK2* and *SERK3* are probably involved in the regulation of embryogenesis.

4 Discussion

BRI1 and BAK1 are two cell surface BR receptors. The understanding of the roles of BAK1 is still limited and under debating. Argument claims BAK1 is not essential and only acts as a BRI1-enhancer due to the observation that *bak1* null mutant shows much milder phenotype, in contrary to the extremely dwarfed phenotype shown by *bri1* null mutant. However, this argument obviously oversees *BAK1* has multiple redundant genes that are still functioning in *bak1-4* single mutant. For example, two *BAK1* paralogs, *SERK1* and *SERK4*, are identified to function redundantly with *BAK1* in BR signaling pathway. Recently, Wang et al. demonstrated that the activity of BRI1 is still regulated by exogenous BL treatment in *serk3 serk4* double mutant, and they supported that BAK1 or BKK1 can only activate BRI1 by transphosphorylation but not essential for the initiation of BR signal cascade (Wang et al., 2008). However, according to this study, *serk3 serk4* mutant still maintains *SERK* function due to intact *SERK1* and *SERK2* in the double mutant. In addition, this study provided genetic evidence that BR signaling is

completely blocked in certain multiple *SERK* mutants, indicating the essential role of *SERKs* in mediating BR signaling.

Recently, Albrecht et al. reported that *SERK* genes serve in BR-dependent and BR-independent signaling pathways (Albrecht et al., 2008). The authors demonstrated that *SERK* genes function in multiple pathways in pairs only: *SERK1* and *SERK3 (BAK1)* in BR signaling pathway; *SERK1* and *SERK2* in male microsporgenesis; *SERK3* and *SERK4* in cell death control. They excluded the role of *SERK4 (BKK1)* in the BR signaling pathway which is not consistent with our previous report (He et al., 2007). The reason they drew this conclusion was because they did not find enhanced phenotype when they introduced *serk4* into other *serk* mutants to make double or multiple mutants. However, the *BAK1* T-DNA insertion allele they used, *bak1*-3, was confirmed to be a weak allele. *bak1-3* still expresses wildtype *BAK1* transcripts although the transcription level is lower than that in wildtype plants. Considering that *BAK1* plays major role in BR signaling pathway, even minute amount of *BAK1* expression will drastically interference with the results when using any multiple mutant containing *bak1-3* in any BR response analyses. In addition, the measurement of root inhibition analysis they used is not sensitive enough to distinguish subtle BR response change. For example, *serk1 serk3* is almost completely insensitive to 100nM BL treatment, exactly like *bri1-4*, whereas when grown in the dark, *serk1 serk3* shows much milder de-etiolation phenotype compared to *bri1-4*, suggesting the BR signaling is not completely blocked. Starting with a *BAK1* null allele, *bak1-4*, we use a more accurate and sensitive method that is focused on comparison of the de-etiolation phenotype among different mutants and is able to successfully identify *bkk1-1* enhanced *bak1-4* de-etiolation phenotype by showing opened cotyledons. The function of

SERK4 in BR signaling was confirmed by the lost-of-function phenotype clearly demonstrating *serk1 serk3 serk4* triple mutant resembles *bri1-4* phenotype. In addition, the overexpression of *SERK2* suppressed *bri1-5* phenotype; while overexpression of a kinase-death form of *SERK2* enhances *bri1-5* phenotype, showing a dominant negative phenotype, suggesting *SERK2* is also involved in BR signaling. Overexpression of *SERK1* and *SERK2* suppresses cell death phenotype in *bak1-4 bkk1-1* null mutant and *bak1-3 bkk1-1* weak mutant, suggesting besides *BAK1* and *BKK1*, *SERK1* and *SERK2* are also functioning in cell death control pathway. Therefore, other than the model that *SERK* genes function in multiple pathways in pairs, all *SERK* genes, except *SERK5*, are involved in BR signaling and cell death control pathway.

Although all *SERK* genes except *SERK5* are involved in regulating BR signaling pathway and cell death control pathway, they do function primarily in pairs. Among all *SERK* single mutants, *serk3* is the only one showing BR-mutant-like phenotype, suggesting the predominant role of *BAK1* in BR signaling. Among all double *SERK* mutants, only *serk1 serk3* shows obvious *bri1*-like de-etiolation phenotype and is insensitive to BL treatment, implicating the secondary major role of *SERK1* in BR signaling. Lost-of-function of *SERK1* and *SERK3* leads to blocking most BR signaling. The cell death phenotype seen in *serk3 serk4* indicates the predominant role of *SERK3* and *SERK4* in cell death control pathway. Thus, although SERK genes are not functioning “only” in pairs, but they do function primarily in pairs.

SERK1 and *SERK2* are identified to be essential for microsporgensis and pollen maturation. *serk1 serk2* double mutant fails to produce any seeds. The mechanism of *SERK1* and *SERK2* in regulating male fertility should be further investigated. Firstly,

although it is clearly that *SERK1* and *SERK2* function redundantly in this regulation, it is necessary to test whether other *SERKs*, such *SERK3* and *SERK4*, are also involved in this process. Secondly, the detailed mechanism should be clarified: whether the developmental defect of pollen in *serk1 serk2* is caused by certain unknown signaling pathway or by the interruption of known pathways regulated by *SERKs*, such as BR signaling pathway or cell death pathway.

Arabidopsis *SERK* genes are first identified as orthologs of carrot *SERK* gene which is involved in regulating embryogenesis, providing the possibility *AtSERK* genes also control embryonic development and maturation. The potential embryonic defects seen in *serk1 serk2 serk3* triple mutant supported this notion. Nevertheless, the further investigation should be conducted to reveal the detailed mechanism of *AtSERKs* in this process.

5 Methods

5.1 Plant growth condition

For light condition, all plants were grown under long-day (18h light/6hr dark) light condition either in the soil in greenhouse condition or on sterilized ½ MS medium in a growth chamber condition. For dark condition, the plants were subjected to light treatment for 8 hours before wrapped with foil. The growth temperature was 22^{o}C for both light and dark treatment.

5.2 Identification of *SERK* T-DNA knockout lines

Homozygous *SERK* single T-DNA insertion mutants were screened by LP primers, RP primers and LBb-1 primer. The primers used were:

serk2 LP, GTTTTGAAAGACAAAGCCAATTTC and *serk2* RP, GCACTGTCTCTGTTCTTCAAAAAG; *serk1* LP, TGCTCTCTCTCAATTACTTTGACG and *serk1* RP, GAAAGGGTTTTTGTAGCAAAACAG; *serk3 (bak1-4)* LP, CTGTCTCTTTGGCCAATCAAG and *serk3 (bak1-4)* RP, CAAATTCCTCTGTGTTCATGTTTC; *serk4 (bkk1-1)* LP, GGTAGAGGTGGTTTTGGTAAAGTG and *serk4 (bkk1-1)* RP, TTTTCGATAAGGGAATTGGAATAG; *serk5* LP, TGGAAGTGTTGCTTCTTGTTTAAG and *serk5* RP, TTTTTAAATTAGGGGTTATAAAGCAAC; *bak1-3* LP, GCCTAACCACCAATACAAAAAGAG and *bak1-3* RP, AAAAATAAATTTGGCGGGAAAC; *bkk1-1* LP, TCTTTCATAATGCCATGGTTCTAG and *bkk1-2* RP, ATAGATTCGACTTTTTGTTCTGGG; and LBb-1, GCGTGGACCGCTTGCTGCAACT.

T-DNA insertions were identified by using priers RP and LBb-1 and primers RP and LP were used to determine the copy of T-DNA to identify homozygous *SERK* T-DNA knockout lines.

5.3 Root inhibition analysis

Col-0, *bri1-4* and *serk1 serk3* plants were grown on the sterilized ½ MS medium with exogenous applied 100nM BL or mock (DMSO). The roots from at least 20 plants from each genotype were measured.

5.4 *SERK* promoter-GUS: constructs

pSERK3:GUS T2 plant is from Dr. Jia Li's previous studies (unpublished). The promoter regions of *SERK1*, *SERK2* and *SERK4* were amplified by using primers:

SERK1, AAAAAGCAGGCTCACTCATTGGCAGCTGATTTAG and GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCAAACAACAATGCTAAATTTC;

SERK2, AAAAAGCAGGCTTGGTATGTGTTGTGTTCACGTGAG and GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACCAAAAAAAAGCAAATTTCT;

SERK4, GGGGACAAGTTTGTACAAAAAAGCAGGCTATCCCTTGTAAACCATTCCAATGTCA and GGGGACCACTTTGTACAAGAAAGCTGGGTCAGAGTAAAAGCAAAGCCATTATTA.

PCR products were cloned into donor vector pDNOR-Zeo to make enrtry clones. Enrty clones were further subcloned into destination vector pBASTA-GUS to make pBASTA-*pSERK*:-GUS constructs. pBASTA-*pSERK*:-GUS were transformed into Col-0 plants through Agrobacterium-mediated transformation. The transgenic plants were selected by spaying herbicide.

5.5 Promoter:GUS activity analyses

T2 transgenic pants harboring p*SERKs*-GUS were grown on ½ MS medium under long-day condition. Plant seedlings from different ages were incubated with X-Gluc solution overnight. 70% ethanol was used to destain the plants. The GUS-staining plants were observed and taken images under light microscope.

5.6 35S: *SERKs* constructs

Full length CDs of all five *SERK* genes were amplified by RT-PCR. The primers used for *SERK* cloning were:

SERK1,

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGTCGAGTTATGTGGTG

TTTA and

GGGGACCACTTTGTACAAGAAAGCTGGGTCCCTTGGACCAGATAACTCAACG;

SERK2, AAAAAGCAGGCTTCATGGGGAGAAAAAAGTTTGAAGCT and

AGAAAGCTGGGTCTCTTGGACCAGACAACTCCATAGCA;

SERK3,

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAACGAAGATTAATGATC

CCT and

GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTTGGACCCGAGGGGTATTCG

T;

SERK4,

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACAAGTTCAAAAATGGA

ACA and

GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTTGGACCCGAGGGGTAATCG

T;

SERK5, AAAAAGCAGGCTTCATGGAACATGGATCATCCCGTG and

AGAAAGCTGGGTCTCTTGGCCCCGAGGGGTAATCGT.

PCR products were cloned by a Gateway Cloning strategy described in 5.4. *SERKs* were cloned into destination vector pBASTA-*35S-FLAG* to make pBASTA-35S:*SERKs-FLAG*.

5.7 Site-Directed Mutagenesis

PCR was conducted by using pENTR-*SERKs* as a template and primers:

SERK1, ACTCTTGTTGCTGTCGAGAGACTGAAGGAAG and

CTTCCTTCAGTCTCTCGACAGCAACAAGAGT;

SERK2, ACACTTGTTGCAGTCGAACGGCTTAAAGAAG and

CTTCTTTAAGCCGTTCGACTGCAACAAGTGT;

SERK3, ACTTTAGTGGCCGTTGAAAGGCTAAAAGAGG and

CCTCTTTTAGCCTTTCAACGGCCACTAAAGT;

SERK4, AATCTAGTGGCTGTCGAAAGGCTAAAAGAAG and

CTTCTTTTAGCCTTTCGACAGCCACTAGATT;

SERK5, ACTCTAGTGGCTGTGGAACGGCTAAATGAAG and

CTTCATTTAGCCGTTCCACAGCCACTAGAGT.

PCR product was digested with *Dpn*I overnight followed by a heat shock transformation into *E. coli* strain DH5α. The plasmids were isolated and the mutations were confirmed by NDA sequencing. The obtained pENTR-*mSERKs* was further cloned into pBIB-BASTA-*35S-FLAG* using a Gateway strategy for plant transformation.

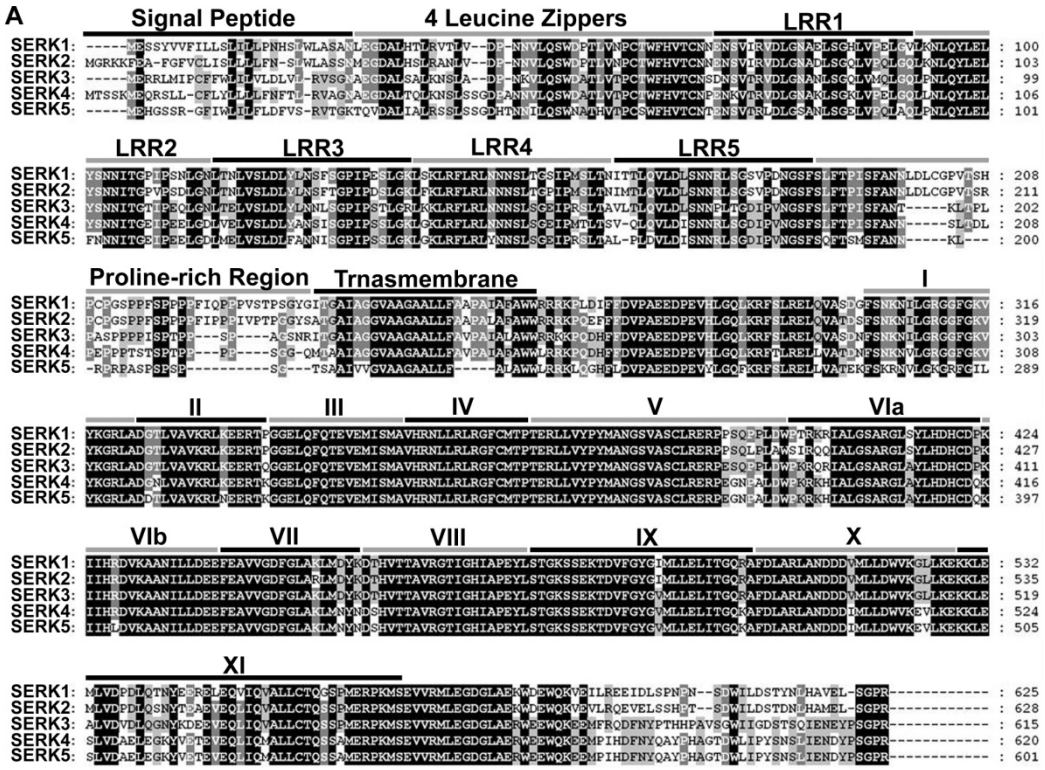

Figure 4.1 SERK proetin sequcne allignment. SERK proetins are typical LRR-RLKs. A SERK contains an extracellular domain, a single-pass tansmembrane domian and a Thr/Ser kinase domain. In extracellualr domian, signal peptide is cleavged in mature portiens. Leucine zippers are involed in protien-protein interaction. SERKs have only five LRRs and a unique proline-rich regoin. The typical Thr/Ser kinase domian contains 11 subdomians.

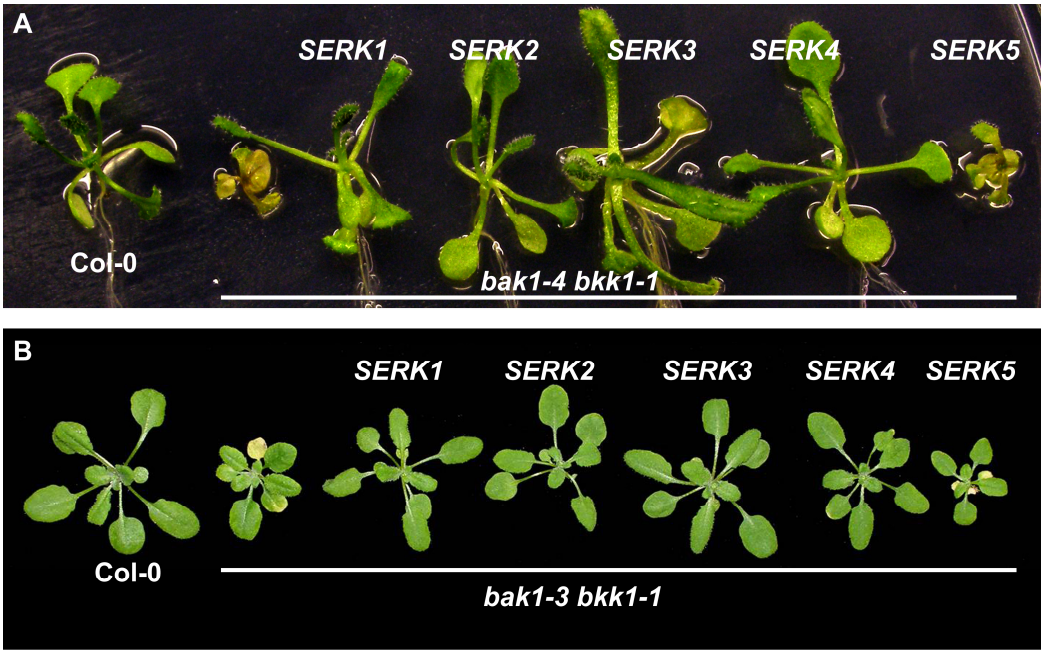

Figure 4.2 Overexpression of *SERK* genes rescues cell death phenotype in *bak1 bkk1*. (A) Overexpression of *SERK* genes, except *SERK5*, in null double mutant *bak1-4 bkk1-1* rescues lethality phenotype. Two-week old plants are examined. (B) Overexpression of *SERK* genes, except *SERK5*, in weak double mutant *bak1-3 bkk1-1* suppresses cell death symptom completely. Two-week old plants are examined.

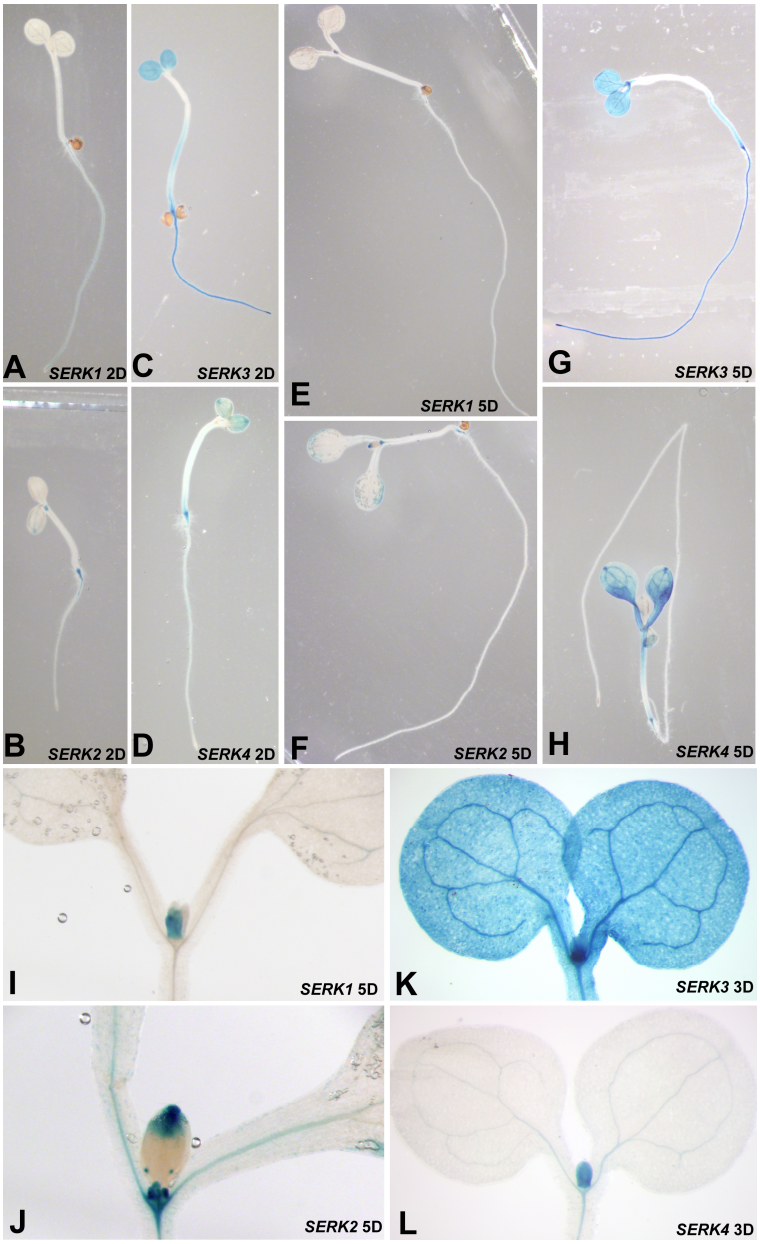

Figure 4.3 Expression patterns of *SERK* genes. (A-D) Expression patterns of *SERK* genes in 2-day old plants. (E-H) Expression patterns of *SERK* genes in 5-day old plant. (I-J) *SERK1* and *SERK2* are expressed in SAM in 5-day old plants. (K-L) *SERK3* and *SERK4* are expressed in 3-day old plants.

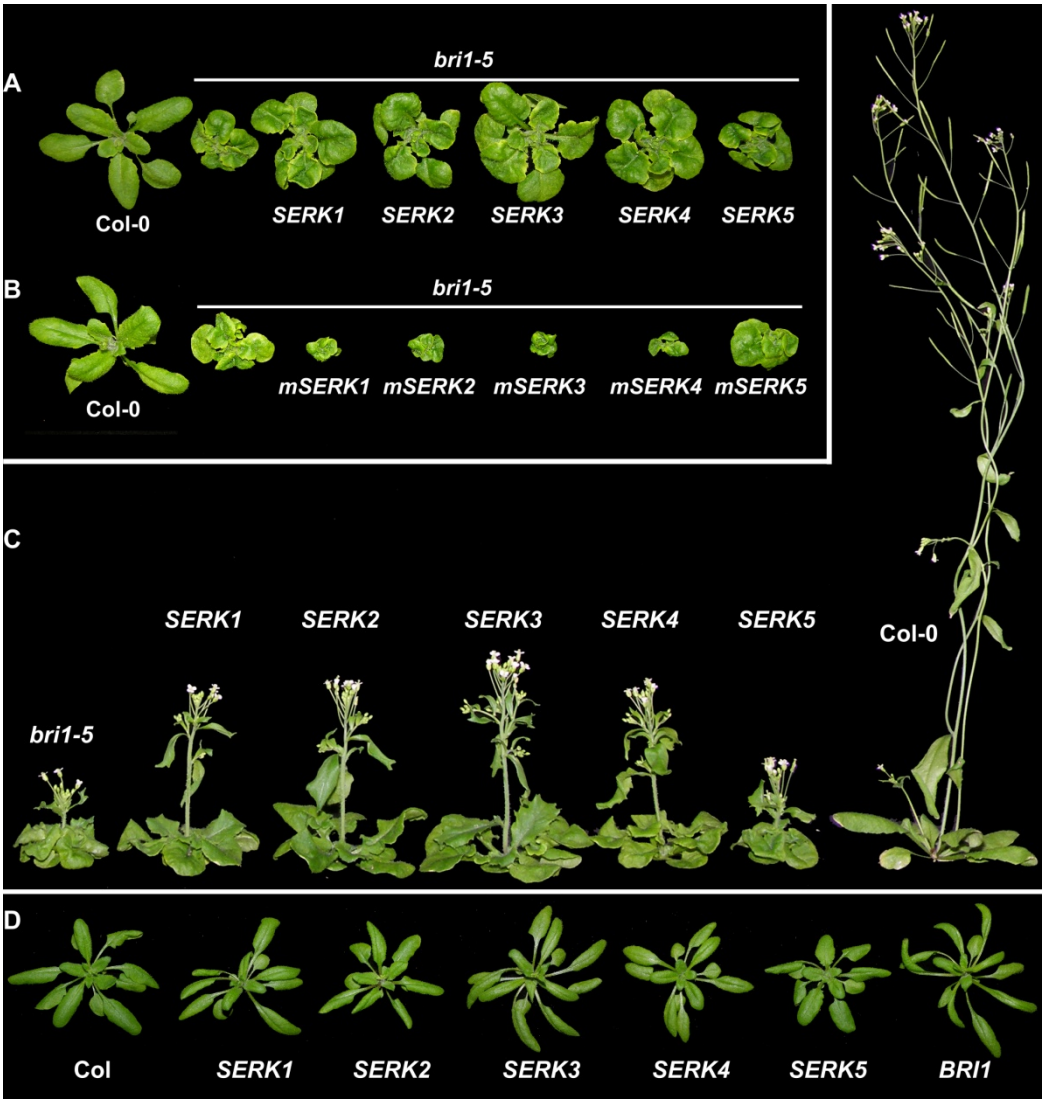

Figure 4.4 *SERK* genes play redundant roles in regulating BR signaling pathway. (A) Overexpression of *SERK* genes except *SERK5* partially suppresses *bri1-5* mutant by elongating rosette leaf petiole. (B) Overexpression of kinase death *mSERK* genes except *mSERK5* shows dominant-negative phenotype in *bri1-5*. (C) Overexpression of *SERK* genes except *SERK5* partially suppresses *bri1-5* mutant by increasing plant height. (D) Overexpression of *SERK* genes except *SERK5* in Col-0 wildtype resembles *BRI1* overexpression phenotype.

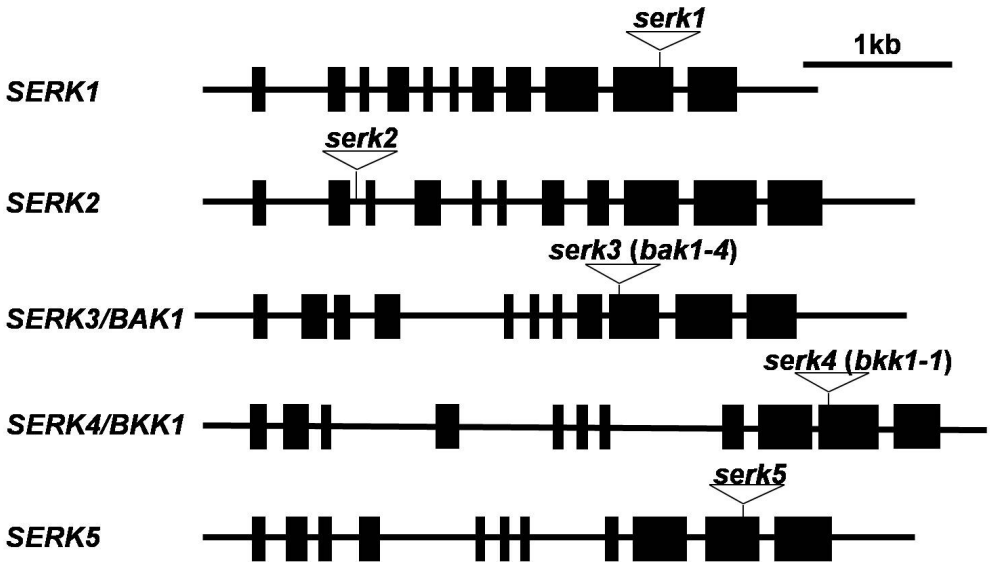

Figure 4.5 *SERK* gene T-DNA insertion mutants. Homozygous T-DNA disrupted mutants for all five *SERK* genes are identified from SALK pools. The *SERK* mutants used are *serk1* (SALK_071511), *serk2* (SALK_058020), *serk3* (*bak1-4*, SALK_116202), *serk4* (*bkk1-1*, SALK_057955) and *serk5* (SALK_089460).

Figure 4.6 Double mutants *serk1 serk2* and *serk1 serk3* are sterile. (A) and (B) Wildtype flower shows mature anthers with pollen. (C) and (D) *serk1 serk2* double mutant flower shows anther defect, no mature pollen produced. (E) and (F) *serk1 serk3* double mutant flower has elongated styles and normal anthers with mature pollen. (G) *serk1 serk2* is completely sterile. Some siliques in *serk1 serk3* are sterile (a). Pollination of *serk1 serk3* stigma by its own pollen rescues sterility phenotype (b).

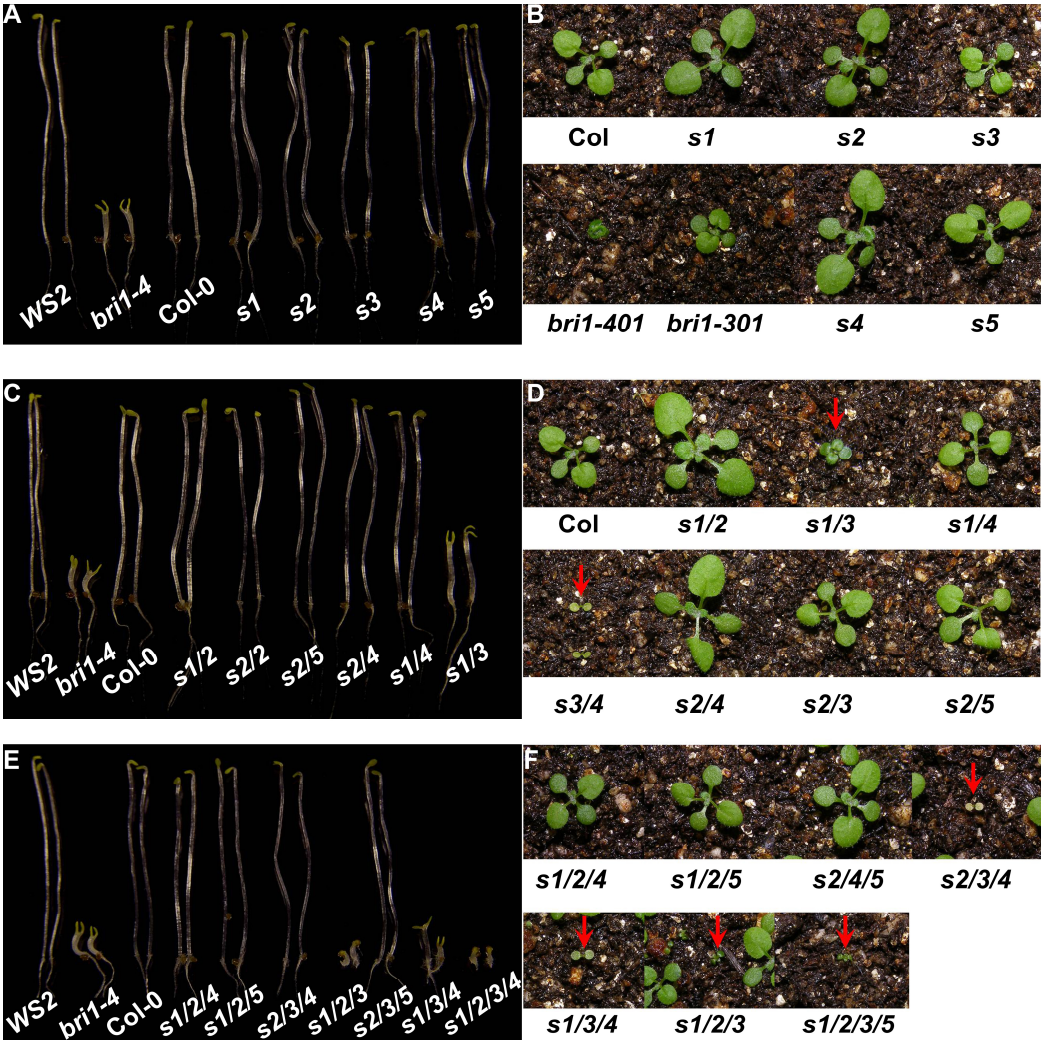

Figure 4.7 Phenotypes of *SERK* mutants. (A) Single *SERK* mutants have no obvious phenotype when grown in dark. *bri1-4* shows de-etiolation phenotype. (B) Single *SERK* mutants have no obvious phenotype when grown in light condition. (C) Only *serk1 serk3* shows de-etiolation phenotype similar to *bri1-4*. (D) *serk1 serk3* shows typical BR mutant phenotype. *ser3 serk4* are lethal when grown in the light. (E) *serk1 sek3 serk4* triple mutant shows de-etiolation phenotype almost identical to *bri1-4*. *serk1 serk2 serk3* mutant shows embryonic defect. (F) *serk1 serk2 serk3* and *serk1 serk3 serk4* show extremely defective phenotype even more severe than *bri1-4*.

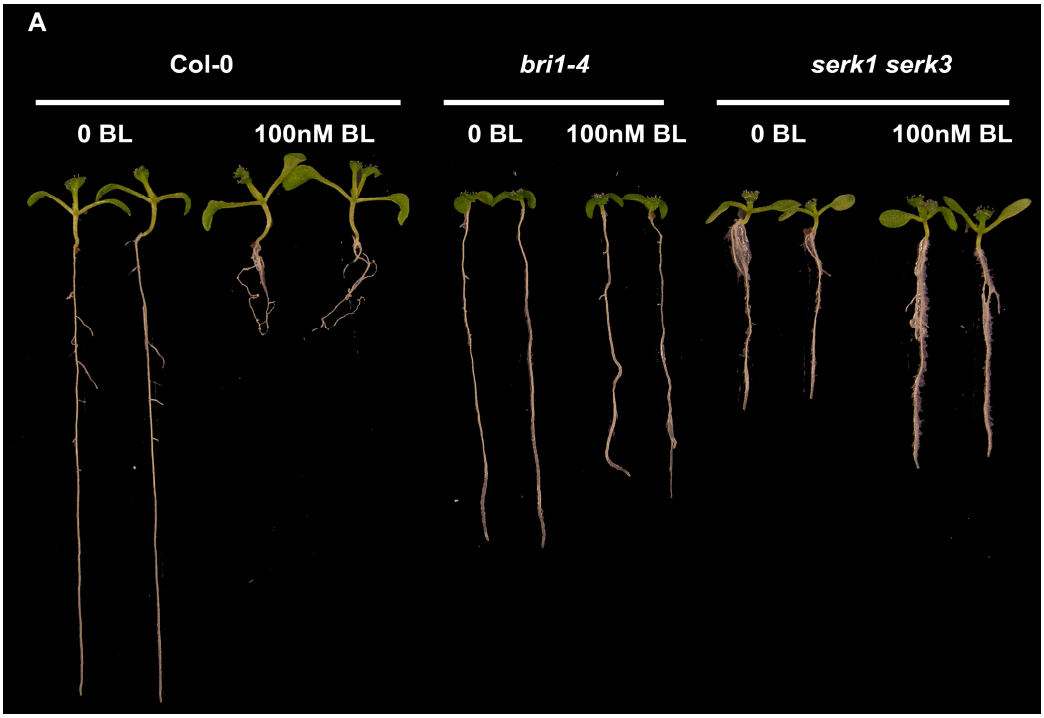

Figure 4.8 *serk1 serk3* is insensitive to BL treatment. Root inhibition analysis shows Col-0 is sensitive to 100nM BL treatment, while *bri1-4* and *serk1 serk3* are insensitive to BL treatment.

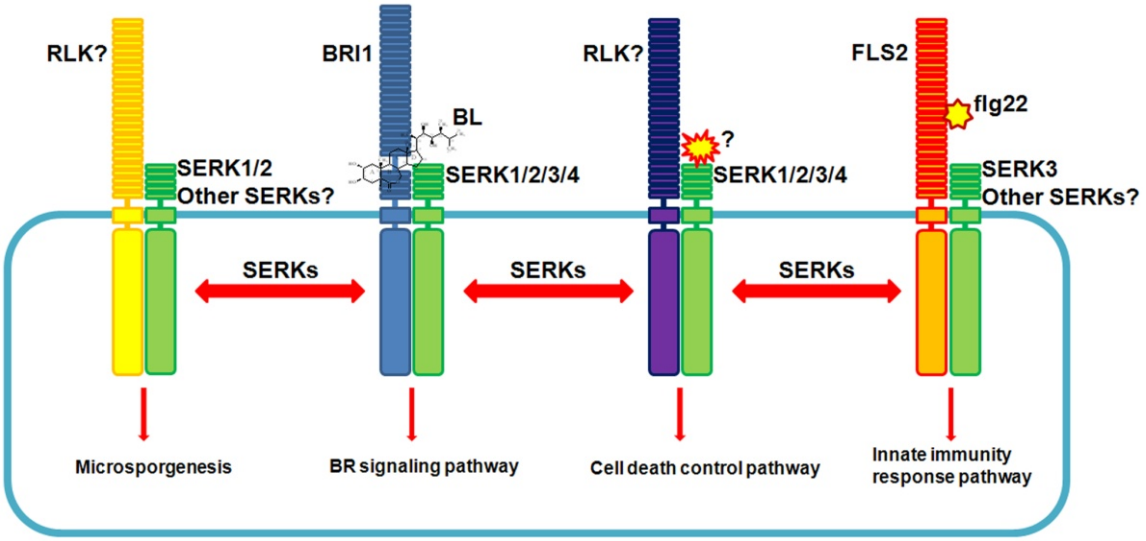

Figure 4.9 *SERKs* regulate multiple singling pathways. Arabidopsis *SERK* subfamily contains five members, named as *SERK1* to *SERK5*. *SERK5* might not be functional due to a natural point mutation at RD motif. All other *SERK* genes are involved in at least four distinct signaling pathways: *SERK1* and *SERK2* in microsporgenesis; *SERK1*, *SERK2*, *SERK3* and *SERK4* in BR signaling pathway; *SERK1*, *SERK2*, *SERK3* and *SERK4* in cell death control pathway; and *SERK3* in disease resistance pathway. We hypothesize although all *SERKs* play redundant role to each other in some pathways, some *SERKs* play major roles in certain pathways. *SERK1* and *SERK2* play major role in microsporgenesis; *SERK1* and *SERK3* in BR signaling pathway; *SERK3* and *SERK4* in cell death control pathway. Whether other *SERKs* besides *SERK3* are involved in *FLS2*-mediated defense response pathway is still under investigation.

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Note

Kai He and Xiaoping Gou contribute equally to the work in this chapter. I have obtained the permission from Xiaoping Gou to use the data in this dissertation.