REGULATION OF SOBIR1 ACCUMULATION AND DEFENCE ACTIVATION IN AN AUTOIMMUNE MUTANT BY SPECIFIC COMPONENTS OF ER QUALITY CONTROL

by

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Abstract

Plants are sessile organisms that are surrounded by pathogens. To stay healthy, they need a complex and sensitive immune system. Specific pattern-recognition receptors (PRRs) localized on the plasma membrane can recognize conserved motifs from pathogens and transduce the signal into the cell to initiate defence responses. The receptor-like kinase BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (BIR1), functions as a negative regulator of plant immunity. \textit{bir1-1} exhibits spontaneous cell death and constitutive defence responses that are dependent on SUPPRESSOR OF BIR1,1 (SOBIR1) and PHYTOALEXIN DEFICIENT 4 (PAD4). Here I present the evidence that ER-quality control, a collective mechanism ensuring that only native proteins are produced by the secretary pathway, plays important roles in regulating defence responses in \textit{bir1-1}. Five components in ER-quality control pathways, including CRT3, UGGT, STT3a, ERdj3b and SDF2, are all required for the immune responses in \textit{bir1-1}. Western blot analysis showed that mutations in CRT3, ERdj3b and UGGT lead to reduced accumulation of SOBIR1 protein. The data suggest that ER-quality control plays an important role in the accumulation of SOBIR1 and is required for the defence responses in \textit{bir1-1}. 
Preface

The work described in this thesis is an accumulation of research from Dr. Yuelin Zhang’s Lab. Below is a list of manuscripts (published or in preparation) that comprise this thesis, and the contribution made by the candidate.

Chapter 2 – Regulation of SOBIR1 accumulation and activation of defence responses in bir1-1 by ER-quality control components CRT3, ERdj3b and SDF2 was modified from the manuscript:


The suppressor screen of bir1-1 pad4-1 was carried out by Dr. Minghui Gao. Dr. Yaxi Zhang performed the cloning of CRT3 with help from technicians (Yujun Han and Yue Zhang). ERdj3b and SDF2 were identified by Tongjun Sun with help from Xia Liu and Yaling Wu. The candidate and Tongjun Sun characterized sobir3-1 bir1-1pad4-1, sobir4-1 bir1-1 pad4-1 and sobir5-1 bir1-1 pad4-1, and generated and characterized the double mutants bir1-1 crt3-1, bir1-1 erdj3b-1 and bir1-1 sdf2-2. The candidate analyzed the protein accumulation of SOBIR1-FLAG in crt3-1 and erdj3b-1. Dr. Yuelin Zhang, Tongjun Sun and the candidate prepared the manuscript.
Chapter 3 - Regulation of SOBIR1 accumulation and activation of defence responses in bir1-1 by ER-quality control components UGGT and STT3a was modified from a prepared manuscript:

Zhang, Q., Sun, T. and Zhang, Y. ER quality control components UGGT and STT3a are required for activation of defense responses in bir1-1.

The suppressor screen of bir1-1 pad4-1 was carried out by Dr. Minghui Gao. The candidate performed the following experiments: isolation and characterization of bir1-1 sobir6-1 double mutant; generation and characterization of bir1-1 stt3a-2 and bir1-1 pad4-1 stt3a-2; analysis of the protein accumulation of SOBIR1 in sobir6-1 and stt3a-2 backgrounds. Dr. Yaxi Zhang performed the map-based cloning of SOBIR6 with help from technicians (Yujun Han and Yue Zhang). Dr. Yuelin Zhang and the candidate prepared the manuscript.
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List of abbreviations

BAK1 - Brassinosteroid Insensitive 1 (BRI1)-Associated Receptor Kinase 1

BIK1 - Botrytis-Induced Kinase 1

BiP - Binding immunoglobulin Protein

BIR1 - BAK1-Interacting Receptor-like kinase 1

BR - Brassinosteroid

CERK1 - Chitin Elicitor Receptor Kinase 1

*Cf-4* - The tomato R gene that confers resistance to the fungus *Cladosporium fulvum*

expressing the corresponding *Avr4* gene

CNX - calnexin

CRT - calreticulin

EFR - Elongation Factor-Tu

EIX2 - ET- Inducing Xylanase 2

ER - endoplasmic reticulum

ER-QC - ER-quality control

ERdj3b - ER-localized DnaJ-like protein 3b

ETI - Effector-triggered immunity

FLS2 - Flagellin-sensitive 2

GI and GII - Glucosidase I and II

HR - Hypersensitive Response
IRK - Induced Receptor-like Kinase
LPS - lipopolysaccharide
MAPK - Mitogen-activated protein kinase
OST – oligosaccharyltransferase
PAD4 - Phytoalexin Deficient 4
PAMP - Pathogen-associated molecular pattern
PDI - Protein Disulfide Isomerase
PRR - Pattern-recognition receptor
PR1/2 - Pathogenesis Related 1/2
PTI - PAMP-triggered immunity
R protein - Resistance protein
RIN4- RPM1 Interacting Protein 4
RLCK - Receptor-Like Cytoplasmic Kinase
RLK - Receptor-like kinase
RLP - Receptor-like protein
ROS - Reactive Oxygen Species
SA - salicylic acid
SDF2 - Stromal-Derived Factor-2
SERK - Somatic Embryogenesis Receptor-like Kinase
SOBIR1 - Suppressor of bir1-I, 1
STT3a - Staurosporin and Temperature Sensitive 3-like a

UGGT - UDP-Glucose: Glycoprotein Glucosyltransferase

*Ve1* - the tomato R gene that encodes the immune receptor that confers resistance to race 1 strains of the soil-borne vascular wilt fungi *Verticillium dahliae* and *Verticillium albo-atrum*
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Chapter 1: Introduction

1.1 Plant immunity

Plants are sessile organisms that are surrounded by pathogens. To stay healthy, they need a complex and sensitive immune system to protect themselves. Defects in plant immune system make them fail to mount effective defence responses and they become susceptible to pathogen infection. Establishing effective plant defence responses is especially important in agriculture, because susceptible crops will lead to problems with food availability and great economic loss. Uncovering the mechanisms of the plant immune system may contribute to sustainable agriculture.

Plant immune system can be divided into two layers. The first layer is activated upon recognition of conserved pathogen-associated molecular patterns (PAMPs). Specific pattern-recognition receptors (PRRs) localized on plasma membrane are responsible for the ligand binding and recognition (Jones and Dangl 2006). Two well-studied PRRs are FLAGELLIN-SENSITIVE 2 (FLS2) and ELONGATION FACTOR-TU RECEPTOR (EFR) that can recognize bacterial flagellin and Elongation Factor-Tu, respectively (Gomez-Gomez and Boller 2000, Zipfel, Kunze et al. 2006). Another PRR, CHITIN ELICITOR RECEPTOR KINASE1 (CERK1), is required for the perception of fungal cell wall component chitin and bacteria cell wall component peptidoglycan (Miya, Albert et al. 2007, Willmann, Lajunen et al. 2011). Downstream of these activated PRRs, a series of defence responses can be activated, such as production of reactive oxygen
species (ROS), calcium influx, defence-related gene expression, mitogen-associated protein kinase (MAPK) activation and callose deposition (Boller and Felix 2009). This layer of plant immunity is called PAMP-triggered immunity (PTI). However, successful pathogens are able to secrete effectors into plant cells to interfere with PTI to promote pathogen growth (Jones and Dangl 2006). Often plants evolve resistance (R) proteins to recognize these pathogen effectors either directly or indirectly and elicit defence responses termed effector-triggered immunity (ETI). For example, the flax R protein L directly recognizes its corresponding rust fungal protein AvrL (Dodds, Lawrence et al. 2006). Other effectors like AvrRpt2, AvrB and AvrRpm1 are recognized by plant R proteins indirectly. RPM1 recognizes AvrRpm1 and AvrB induced RIN4 phosphorylation and trigger RPM1-dependent immunity. PRS2 recognizes AvrRpt2 induced RIN4 cleavage and trigger PRS2-dependent immunity (Mackey, Holt et al. 2002, Mackey, Belkhadir et al. 2003). ETI can often lead to hypersensitive response (HR) which may contribute to limiting the growth of pathogen (Jones and Dangl 2006).

Whereas both PTI and ETI contribute to plant resistance to pathogens, there are considerable differences between them. PTI is activated by perception of PAMPs by receptors on the plasma membrane, and the ligands of the PAMP receptors are usually conserved pathogen molecules, such as bacterial lipopolysaccharide (LPS), flagella, fungal chitin (Boller and Felix 2009). ETI is initiated mostly in the cytoplasm upon recognition of effectors by R protein directly or indirectly. The effectors or the
modification by the effectors are changing over time, so ETI is under evolutionary pressure from pathogens (Jones and Dangl 2006). In terms of the strength of defence responses, PTI is generally weaker and transient, while ETI is stronger and lasts longer.

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

PRRs localized on the plasma membrane are key elements in plant immune system. They function like guards that can sense the presence of invading pathogen at first encounter. PRRs can be classified into two groups according to their structures. One group comprises receptor-like kinases (RLKs) that consist of an extracellular domain for recognition of PAMPs, a transmembrane domain and a cytoplasmic kinase domain for signal transduction. The other group is receptor-like proteins (RLPs), which also have an extracellular domain for ligand binding and a transmembrane domain. However, they do not have a cytoplasmic kinase domain, and they often need a partner with a cytoplasmic kinase domain for signal transduction. The best known RLPs involved in plant defence are the Cf proteins in tomato (Kruijt, MJ et al. 2005).

PRRs usually recruit other components to form a complex and transduce the signal (Monaghan and Zipfel 2012). One good example of PRR complexe is the well-studied FLS2 complex. Upon perception of flagellin or the conserved 22 amino acid peptide flg22, FLS2 rapidly associates with another RLK, BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE1 (BAK1) (Chinchilla, Zipfel et al. 2007). BAK1 was originally identified to be involved in brassinosteroid (BR) signaling that
plays essential roles in plant development and growth (Li, Wen et al. 2002). It belongs to a small protein family termed SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASEs (SERKs) that were shown to be important in immunity against diverse pathogens (Roux, Schwessinger et al. 2011). BAK1 is also required for the defence responses mediated by EFR, but not CERK1 (Monaghan and Zipfel 2012). Analysis of crystal structure of FLS2-BAK1 complex showed that BAK1 functions as a co-receptor of FLS2 to mediate downstream defence responses (Sun, Li et al. 2013).

One reported component that connects the PRRs on the plasma membrane and downstream defence responses in the cytosol is a receptor-like cytoplasmic kinase (RLCK) named BOTRYTIS-INDUCED KINASE1 (BIK1). BIK1 was originally identified as a positive regulator of plant defence against necrotrophic fungal pathogens (Veronese, Nakagami et al. 2006). It was later shown that BIK1 associates with both BAK1 and FLS2 (Lu, Wu et al. 2010). BIK1 is rapidly phosphorylated upon flg22 treatment in a BAK1- and FLS2-dependent manner. Based on in vitro kinase assay, BIK1 was suggested to transphosphorylate FLS2/BAK1 to propagate flagellin signal (Lu, Wu et al. 2010). Recent studies showed that activated BIK1 could phosphorylate the NADPH oxidase RBOHD (Li, Li et al. 2014), which leads to the production of ROS and helps to prevent the growth of pathogen.

1.3 Genetic analysis of defence responses regulated by BIR1 and SOBIR1

Two genetic approaches have been used to find new components in plant immune
system. One is forward genetics, starting by generating random mutations in an organism and screening for mutants with a specific phenotype. Cloning genes that are responsible for the phenotype may reveal new components in the pathway of interest. On the other hand, reverse genetics starts with genes with unknown functions. By generating mutants of the candidate genes and analyzing the phenotype of the mutants, the function of the genes can be inferred. Although forward genetics is straightforward and a known phenotype can provide clear clues about the function of the protein encoded by the mutated gene, it takes time and effort to clone the gene. What’s more, genetic redundancy and lethality make it very difficult to study certain genes using forward genetics, while the problems can be addressed using reverse genetics.

Several years ago, a reverse genetic screen was carried out in our lab to find new components that play important roles in plant immunity. Based on microarray data, genes that are up-regulated upon pathogen infection were selected and analyzed for function in plant defence. Initial analysis of homozygous T-DNA knockout lines of these genes identified a mutant with seedling lethal phenotype. Further analysis showed that this T-DNA knockout mutant has curly leaves, accumulates high levels of salicylic acid (SA), exhibits severe cell death and elevated PATHOGENESIS RELATED (PR) 1/2 expression (Gao, Wang et al. 2009), suggesting that immune responses are constitutively activated in this mutant and that the protein encoded by the gene is a negative regulator of plant immunity. A mutation in PHYTOALEXIN DEFICIENT 4 (PAD4), a positive regulator in
plant immunity (Jirage, Tootle et al. 1999), can partially block the constitutive defence responses in this T-DNA mutant (Gao, Wang et al. 2009). The protein encoded by this gene was named BAK1-INTERACTING RECEPTOR-LIKE KINASE1 (BIR1) because it was later found to associate with BAK1 on the plasma membrane (Gao, Wang et al. 2009).

To dissect the immune pathways regulated by BIR1, a forward genetic screen was carried out to find suppressors of bir1-1. Because bir1-1 pad4-1 can set seeds, which makes the screen doable, bir1-1 pad4-1 seeds were treated with EMS and used in the mutant screen instead of bir1-1. Usually, immune responses are under strict control, so most energy can be used for plant developmental processes. But when a mutant has constitutive defence responses like in bir1-1, more energy is distributed to immunity, which impairs the development of plants. Based on this rationale, it is anticipated that mutations that block the immune responses in bir1-1 pad4-1 will make the mutant regain wild-type growth phenotype. Thus, we searched for mutants with bigger size than bir1-1 pad4-1 in the suppressor screen. It is likely that these plants carry mutations that can completely or partially block the immune responses in bir1-1.

The first SOBIR (Suppressor of bir1-1) gene identified is SOBIR1, which encodes another RLK with four leucine rich repeats (LRRs) (Gao, Wang et al. 2009). Mutations in SOBIR1 and PAD4 together can revert bir1-1 to wild type-like (Gao, Wang et al. 2009). Overexpression of SOBIR1 leads to cell death and high PR gene expression (Gao, Wang
et al. 2009). However, no direct interaction between BIR1 and SOBIR1 was detected (Gao, Wang et al. 2009).

Recently, SOBIR1 was shown to interact with several RLPs and is required for the RLP-mediated immune responses (Liebrand, van den Berg et al. 2013, Zhang, Fraiture et al. 2013). In tomato, there are two redundant SOBIR1 homologues, Sl/SOBIR1 and Sl/SOBIR1-like. Both SOBIR1 homologues interact with Cf proteins in *N. benthamiana* (Liebrand, van den Berg et al. 2013). In addition, tomato SOBIR1 homologues also interact with tomato RLPs Ve1 and Eix2 (Liebrand, van den Berg et al. 2013). Silencing of the tomato SOBIR1 homologues compromised Cf-4- and Ve1-mediated immune responses in tomato. Similarly, silencing of two NbSOBIR1 homologues also compromised Cf-4-mediated HR (Liebrand, van den Berg et al. 2013). In Arabidopsis, RLP30 is required for resistance against a necrotrophic fungal pathogens and loss of function of SOBIR1 greatly compromises RLP30-mediated immunity (Zhang, Fraiture et al. 2013). These data suggest an important role of SOBIR1 in RLP-mediated immune responses. It was proposed that SOBIR1 may function as a signaling partner for multiple RLPs (Liebrand, van den Burg et al. 2014).

**1.4 ER-QC in plant immunity**

Both RLK and RLP types of PRRs are localized on the plasma membrane and play important roles in immune responses. Their folding and modification are important processes that can greatly affect immune responses. It is known that the folding and
modification of transmembrane proteins occurs with help of ER-resident chaperons through ER-quality control (ER-QC), which refers to a collective mechanism in the ER ensuring that only completely folded and modified proteins are produced by the pathway (Trombetta and Parodi 2003). Events monitored by ER-QC include ligand binding, post-translational modification, and even exposure of polar amino acids in transmembrane domains (Trombetta and Parodi 2003). Proteins that fail to pass ER-QC will be retained in the ER to go through additional rounds of folding or degraded through ER-associated degradation (Trombetta and Parodi 2003).

In yeast and mammals, three ER-QC pathways were reported. The first pathway relies on direct binding between chaperones and the hydrophobic backbone of the client proteins to assist the folding processes (Trombetta and Parodi 2003). Known chaperones in this pathway include Hsp70 family member BINDING IMMUNOGLOBULIN PROTEIN (BiP) and Hsp40 family member ER-J proteins (Trombetta and Parodi 2003). The second pathway starts with Asn (N)-glycosylation of the client protein that is catalyzed by oligosaccharyltransferase (OST) complex (Trombetta and Parodi 2003). Then the two outermost Glc residues are trimmed by glucosidases I and II (GI and GII) to produce the mono-glucosylated glycan-conjugated protein that can be recognized by ER-resident lectin-like chaperones calreticulin (CRT) and calnexin (CNX) (Trombetta and Parodi 2003). The CRT/CNX assisted folding forms a reaction cycle driven by GII-mediated removal and UDP-GLUCOSE: GLYCOPROTEIN
GLUCOSYLTRANSFERASE (UGGT)-mediated addition of the terminal Glc (Trombetta and Parodi 2003, Dejgaard, Nicolay et al. 2004). The third pathway ensures proper disulfide bond formation between free thiol groups on client proteins, which is mediated by protein disulfide isomerases (PDIs) (Trombetta and Parodi 2003). These pathways can either work together to assist the folding of one protein, or work independently.

Although our knowledge on ER-QC is mostly from study on yeast and mammals, multiple genes involved in ER-QC have been characterized in plants. In *Arabidopsis*, the retention of defective BR receptor bri1-9 protein was found to require CRT3 and UGGT (Jin, Hong et al. 2009). Also, CRT3 was found to interact with ER-localized bri1-9 protein in a glycan-dependent manner (Jin, Hong et al. 2009).

There is also increasing evidence suggesting the importance of ER-QC in plant immunity. Most of it came from studies on the RLK EFR in *Arabidopsis*. Two independent groups have found different ER-QC components involved in EFR signaling. Mutations in both CRT3 and UGGT reduce EFR accumulation and signaling (Li, Zhao-Hui et al. 2009). One of the subunits of the OST complex, STT3a, is also required for normal EFR signaling (Nekrasov, Li et al. 2009). Similarly, mutations in Gα and β subunit, which function in the same ER-QC pathway, cause reduced accumulation of EFR and attenuate EFR-mediated immune responses (Lu, Tintor et al. 2009). Components in another ER-QC pathway, including Hsp40 family member
ER-LOCALIZED DnaJ-LIKE PROTEIN 3b (ERdj3b) and STROMAL-DERIVED FACTOR-2 (SDF2), were also shown to be required for EFR biogenesis and signaling (Nekrasov, Li et al. 2009). A modified model for the ER-QC of the receptor EFR is shown in Figure 1-1 (Saijo 2010).

![Figure 1-1 A modified model for the ER-QC of the Arabidopsis receptor EFR.](image)

When EFR polypeptide is translocated into ER, a series of folding reactions happen and each step is numbered with a roman number. I, Asn (N)-glycosylation catalyzed by STT3a-containing OST complex. II, GII-mediated trimming of the two outmost glucose residues. III, CRT3-assisted folding process. IV, UGGT mediated mono-glycosylation. V, BiP/SDF2/ERdj3b mediated folding.

The importance of ER-QC in plant immunity was also demonstrated in tomato and tobacco. In tomato, silencing of CRT3a leads to impaired Cf-4 and Ve1-mediated immunity (Liebrand, Kombrink et al. 2013). In tobacco, silencing of ER-resident chaperones affects the accumulation of the tobacco protein INDUCED RECEPTOR-LIKE KINASE (IRK) and N gene mediated resistance against TMV.
My thesis study focused on several ER-QC components and their roles in defence responses activated in *bir1-1*. Mutations in *CRT3, SDF2, ERdj3b, UGGT* and *STT3a* can completely or partially block the immune responses in *bir1-1 pad4-1*. In addition, in *crt3-1, erdj3b* and *uggt* mutant plants, the accumulation of SOBIR1 is reduced, suggesting that ER-QC plays an important role in the accumulation of SOBIR1 and the immune responses in *bir1-1*. 
Chapter 2: Regulation of SOBIR1 accumulation and activation of defence responses in bir1-1 by ER-quality control components CRT3, ERdj3b and SDF2

2.1 Introduction

Transmembrane receptor-like kinases (RLKs) play indispensable roles in the recognition of pathogens and initiation of plant immune responses. For example, three RLKs, FLAGELLIN-SENSITIVE 2 (FLS2), ELONGATION FACTOR-TU RECEPTOR (EFR) and CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1), are well-established pathogen-associated molecular pattern (PAMP) receptors. FLS2 and EFR recognize bacterial flagellin and EF-Tu, respectively (Gomez-Gomez and Boller 2000, Zipfel, Kunze et al. 2006). CERK1 is involved in the perception of the fungal cell wall component chitin, as well as bacterial cell wall component peptidoglycan (Miya, Albert et al. 2007, Wan, Zhang et al. 2008, Gimenez-Ibanez, Hann et al. 2009, Willmann, Lajunen et al. 2011). Another RLK, Brassinosteroid Insensitive 1 (BRI1)-associated receptor kinase 1 (BAK1), serves as a co-receptor for FLS2 and EFR (Chinchilla, Zipfel et al. 2007, Heese, Hann et al. 2007).

Secreted and membrane proteins are synthesized on ER-bound ribosomes and folded in the ER lumen with the assistance of chaperones. One well-studied pathway for retention and folding of proteins in ER relies on BiP (Binding immunoglobulin protein) and ERdj3 (ER-localized DnaJ-like protein 3) (Hebert and Molinari 2007, Anelli and Sitia 2008). BiP is an ER luminal chaperone of the Hsp70 family. It contains an
N-terminal ATPase domain and a C-terminal substrate-binding domain. Binding of Hsp70 proteins to their substrates can prevent protein aggregation and assist with protein folding and assembly. ERdj3 functions as a co-chaperone of BiP and binds directly to unfolded substrates. After binding to the substrate, ERdj3 recruits BiP to the complex and activates the ATPase activity of BiP, which induces a conformational change leading to interaction between the substrate and the substrate-binding domain of BiP.

Another well-characterized protein folding pathway known as the calnexin/calreticulin (CNX/CRT) cycle is specific to glycoproteins, which involves the ER luminal enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT) and the lectin-like chaperones CNX and CRT (Dejgaard, Nicolay et al. 2004). In ER, CNX and CRT recognize client proteins bearing mono-glucosylated glycans (Glc\textsubscript{1}Man\textsubscript{9}GlcNAc\textsubscript{2}) and facilitate their folding. Trimming of the outermost glucoses by a glucosidase allows client proteins to be released from CNX and CRT. Whereas proteins that achieved their native conformation subsequently exit ER, the incompletely folded proteins are recognized by UGGT, which adds glucose residues back to the Man\textsubscript{9}GlcNAc\textsubscript{2} side chains and allows the mono-glucosylated proteins to interact with CNX/CRT and repeat the folding process.

In *Arabidopsis*, both UGGT and CRT3 are involved in the retention of the defective bri1-9 protein in the ER (Jin, Yan et al. 2007, Jin, Hong et al. 2009). Increasing evidence suggests that ER-quality control (ER-QC) also plays important roles in plant immunity.
Multiple components of ER-QC are required for the accumulation of Arabidopsis EFR (Li, Zhao-Hui et al. 2009, Lu, Tintor et al. 2009, Nekrasov, Li et al. 2009, Saijo, Tintor et al. 2009). In tobacco, ER-resident chaperones are shown to be required for accumulation of the tobacco INDUCED RECEPTOR-LIKE KINASE (IRK) and N gene mediated resistance against tobacco mosaic virus (Caplan, Zhu et al. 2009). In tomato, silencing of CRT3a affects defence responses mediated by the resistance proteins Cf-4 and Ve1 (Liebrand, Smit et al. 2012, Liebrand, Kombrink et al. 2013). Silencing genes encoding ER luminal protein receptors ERD2a and ERD2b in Nicotiana benthamiana also alters pathogen-induced programmed cell death (Xu, Li et al. 2012). More recently, the ER-QC component SDF2 was shown to be required for immunity mediated by the RLK Xa21 in rice (Park, Sharma et al. 2013).

*Arabidopsis* BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (BIR1) functions as a negative regulator of cell death and defence responses (Gao, Wang et al. 2009). To dissect signaling pathways activated in bir1-1, we carried out a suppressor screen and obtained a large number of suppressor mutants of bir1-1. Positional cloning of *SOBIR1* (*SUPPRESSOR OF BIR1, 1*) and *SOBIR2* showed that *SOBIR1* encodes an RLK that positively regulates cell death and defence responses (Gao, Wang et al. 2009), whereas *SOBIR2* encodes the β subunit of heterotrimeric G protein, which functions downstream of *SOBIR1* (Liu, Ding et al. 2013). In this study, we report that several sobir mutants contain mutations in CRT3, ERdj3b and SDF2, suggesting that ER-QC plays an
important role in the activation of cell death and defence responses in bir1-1.

2.2 Results

2.2.1 Identification and characterization of sobir3-1 bir1-1 pad4-1

The sobir3-1 mutant was identified from a suppressor screen of bir1-1 pad4-1 described earlier (Gao, Wang et al. 2009). As shown in Figure 2-1A, the dwarf morphology of bir1-1 pad4-1 is almost completely suppressed by sobir3-1. RT-PCR analysis showed that the expression levels of defence marker genes PATHOGENESIS-RELATED 1 (PR1) (Figure 2-1B) and PR2 (Figure 2-1C) are significantly lower in sobir3-1 bir1-1 pad4-1 than in bir1-1 pad4-1. Whereas bir1-1 pad4-1 plants display strong resistance to Hyaloperonospora arabidopsidis (H.a.) Noco2, the enhanced resistance against H.a. Noco2 is abolished in sobir3-1 bir1-1 pad4-1 (Figure 2-1D). These data indicate that SOBIR3 is required for the constitutive defence responses observed in bir1-1 pad4-1.
Figure 2-1 Characterization of sobir3-1 bir1-1 pad4-1.

(a) Morphology of Col-0 (wild-type, WT), bir1-1, bir1-1 pad4-1 and sobir3-1 bir1-1 pad4-1. Plants were grown on soil at 23°C and photographed 3 weeks after planting.

(b, c) Expression levels of PR1 (b) and PR2 (c) in wild-type, bir1-1, bir1-1 pad4-1 and sobir3-1 bir1-1 pad4-1 seedlings compared to ACTIN1. Total RNA was extracted from 2-week-old seedlings grown on half-strength MS plates.

(d) Growth of H. a. Noco2 on wild-type, bir1-1, bir1-1 pad4-1, and sobir3-1 bir1-1 pad4-1 seedlings.

Error bars in (b)-(d) represent the standard deviation of three measurements.
2.2.2 SOBIR3 encodes CALRETICULIN 3

To map the sobir3-1 mutation, a segregating mapping population was generated by crossing sobir3-1 bir1-1 pad4-1 (in the Columbia ecotype background) with Ler-0. Crude mapping using the F2 progeny showed that the sobir3-1 mutation is located between marker T7A14 and F13K23 on Chromosome 1. Further fine mapping narrowed down the mutation to a 130 kb region between markers T27G7 and F22O13 (Figure 2-2A). This region in sobir3-1 was amplified by PCR and sequenced. A single C to T mutation was identified in At1g08450, which encodes the ER-quality control component CRT3. The mutation changes the Ser-28 of CRT3 to Phe-28. To determine whether any of the other sobir mutants also contain mutations in the gene, AT1g08450 was amplified by PCR from 18 other sobir mutants and sequenced. Four additional mutants were found to contain mutations in At1g08450 (Figure 2-2B and 2-2C). These mutations results in either amino acid changes or alteration of the splicing pattern. All these data suggest that SOBIR3 is AT1g08450.
Figure 2-2 Map-based cloning of SOBIR3.

(a) Map position and location of the sobir3-1 mutation. WT, wild-type.

(b) Morphology of sobir3-1 bir1-1 pad4-1 alleles. Plants were grown on soil at 23°C and photographed 3 weeks after planting.

(c) Mutations identified in the sobir3 alleles. The positions of mutated nucleotide in the coding sequence are listed.
2.2.3 CRT3 is required for cell death and defence responses in \textit{bir1-1}

To determine whether loss of function of \textit{CRT3} can suppress cell death and defence responses in \textit{bir1-1} without the presence of \textit{pad4-1}, we crossed \textit{crt3-1}, a T-DNA knockout mutant of CRT3, into \textit{bir1-1} to obtain the \textit{crt3-1 bir1-1} double mutant. As shown in Figure 2-3A, the \textit{crt3-1 bir1-1} double mutant is much bigger than the \textit{bir1-1} single mutant, only slightly smaller than the wild type plants. To determine whether cell death was blocked in the \textit{crt3-1 bir1-1} double mutant, trypan blue staining was carried out on the mutant seedlings. As shown in Figure 2-3B, no obvious cell death was observed in \textit{crt3-1 bir1-1}. Expression of \textit{PR-1} (Figure 2-3C) and \textit{PR-2} (Figure 2-3D) and resistance to \textit{H.a. Noco2} are all dramatically reduced in \textit{crt3-1 bir1-1} (Figure 2-3E). These data suggest that CRT3 is required for spontaneous cell death and constitutive defence responses in \textit{bir1-1}.
Figure 2-3 Characterization of the *crt3-1 bir1-1* double mutant.

(a) Morphology of Col-0 (wild-type, WT), *bir1-1, crt3-1 bir1-1*. Plants were grown on soil at 23°C and photographed 3 weeks after planting.

(b) Trypan blue staining of wild-type, *bir1-1* and *crt3-1 bir1-1* mutant seedlings. Plants were grown at 23°C on half-strength MS plates for 2 weeks.

(c, d) Expression levels of *PR1* (b) and *PR2* (c) in wild-type, *bir1-1, crt3-1 bir1-1* seedlings compared to *ACTIN1*. Total RNA was extracted from 2-week-old seedlings grown on half-strength MS plates.

(e) Growth of *H. a. Noco2* on wild-type, *bir1-1, crt3-1 bir1-1* seedlings.

Error bars in (c)-(e) represent the standard deviation of three measurements.
2.2.4 Identification of an allele of *erdj3b* that suppresses *bir1-1 pad4-1* mutant phenotypes

Another mutant, *sobir4-1*, was mapped to the bottom of chromosome 3, closely linked to marker MAA21. As shown in Figure 2-4A, *sobir4-1* almost completely suppresses the dwarf morphology of *bir1-1 pad4-1*. Expression of both *PR1* (Figure 2-4B) and *PR2* (Figure 2-4C) in *sobir4-1 bir1-1 pad4-1* is much lower than that in *bir1-1 pad4-1*. In addition, the enhanced resistance to *H.a. Noco2* observed in *bir1-1 pad4-1* is also lost in *sobir4-1 bir1-1 pad4-1* (Figure 2-4D).

Since *ERdj3b*, which encodes another component of ER-QC (Nekrasov, Li et al. 2009), is located in the region where *sobir4-1* was mapped to, we sequenced *ERdj3b* in *sobir4-1 bir1-1 pad4-1*. Comparison with wild type sequence revealed a single G to A mutation at the junction of intron 6 and exon 7 in *sobir4-1*. Transforming a genomic clone of *ERdj3b* into *sobir4-1 bir1-1 pad4-1* resulted in transgenic plants with dwarf morphology like *bir1-1 pad4-1* (Figure 2-4E), suggesting that the mutation in *ERdj3b* is responsible for the suppression of *bir1-1 pad4-1* mutant phenotype in *sobir4-1 bir1-1 pad4-1*. When *erdj3b-1*, a T-DNA allele of *ERdj3b*, was crossed into *bir1-1 pad4-1*, the dwarf morphology and resistance to *H.a. Noco2* in *bir1-1 pad4-1* were also suppressed (Figure 2-4F and 2-4G), further confirming that *SOBIR4* is *ERdj3b*. 

Figure 2-4 Characterization and cloning of sobir4-1.

(a) Morphology of Col-0 (wild-type, WT), bir1-1, bir1-1 pad4-1 and sobir4-1 bir1-1 pad4-1.

(b, c) Expression levels of PR1 (b) and PR2 (c) in wild-type, bir1-1, bir1-1 pad4-1 and sobir4-1 bir1-1 pad4-1 seedlings compared to ACTIN1. Total RNA was extracted from 2-week-old seedlings grown on half-strength MS plates.

(d) Growth of H. a. Noco2 on wild-type, bir1-1, bir1-1 pad4-1, and sobir4-1 bir1-1 pad4-1 seedlings.

(e) Morphology of sobir4-1 bir1-1 pad4-1 plants expressing wild-type ERdj3b. Four independent transgenic lines are shown.

(f) Morphology of wild-type, bir1-1, bir1-1 pad4-1 and erdj3b-1 bir1-1 pad4-1.

(g) Growth of H.a. Noco2 on wild-type, bir1-1, bir1-1pad4-1 and erdj3b-1 bir1-1pad4-1.

Plants in (a), (e) and (f) were grown on soil at 23°C and photographed 3 weeks after planting. Error bars in (b)-(d) and (g) represent the standard deviation of three measurements.

2.2.5 ERdj3b is required for cell death and defence responses in bir1-1

To determine whether erdj3b-1 can suppress cell death and defence responses in bir1-1 without the presence of pad4-1, we constructed the erdj3b-1 bir1-1 double mutant. The double mutant is much bigger than bir1-1, but slightly smaller than the wild type plants (Figure 2-5A). Trypan blue staining showed that cell death in bir1-1 was largely blocked by erdj3b-1 (Figure 2-5B). As shown in Figure 2-5C and 2-5D, expression of PR-1 and PR-2 in erdj3b-1 bir1-1 is considerably lower comparing to bir1-1, but much higher than in the wild type (Figure 2-5C and 2-5D). In erdj3b-1 bir1-1, H.a. Noco2 grows much more than in bir1-1, but considerably less than in the wild type (Figure 2-5E). Taken together, the cell death and constitutive defence responses in bir1-1 are partially dependent on ERdj3b.
Figure 2-5 Characterization of the erdj3b-1 bir1-1 double mutant.

(a) Morphology of Col-0 (wild-type, WT), bir1-1, erdj3b-1 bir1-1. Plants were grown on soil at 23°C and photographed 3 weeks after planting.

(b) Trypan blue staining of wild-type, bir1-1 and erdj3b-1 bir1-1 mutant seedlings. Plants were grown at 23°C on half-strength MS plates for 2 weeks.

(c, d) Expression levels of PR1 (b) and PR2 (c) in wild-type, bir1-1, erdj3b-1 bir1-1 seedlings compared to ACTIN1. Total RNA was extracted from 2-week-old seedlings grown on half-strength MS plates.

(e) Growth of *H. a. Noco2* on wild-type, bir1-1, erdj3b-1 bir1-1 seedlings.

Error bars in (c)-(e) represent the standard deviation of three measurements.
2.2.6 Identification of an allele of sdf2 that supresses bir1-1 pad4-1 mutant phenotypes

The sobir5-1 mutant also suppresses the dwarf morphology of bir1-1 pad4-1 (Figure 2-6A). In comparison with bir1-1 pad4-1, expression of PR1 (Figure 2-6B) and PR2 (Figure 2-6C) is clearly reduced in sobir5-1 bir1-1 pad4-1. Enhanced resistance to H.a. Noco2 in bir1-1 pad4-1 is also blocked by sobir5-1 (Figure 2-6D). The sobir5-1 mutation was mapped to a region between marker F27D4 and T9J22 on chromosome 2, a region of about 1 Mb that contains SDF2, which encodes another component of ER-QC (Nekrasov, Li et al. 2009). Sequencing of SDF2 from sobir5-1 bir1-1 pad4-1 showed that it carries a single G to A mutation at the junction of exon 2 and intron 2.

To determine whether the mutation in SDF2 is responsible for the suppression of the bir1-1 pad4-1 phenotypes in sobir5-1 bir1-1 pad4-1, we crossed sdf2-2, a T-DNA insertion mutant allele of SDF2, into bir1-1 pad4-1. The sdf2-2 bir1-1 pad4-1 triple mutant is much larger than bir1-1 pad4-1 (Figure 2-6E) and completely lost the enhanced resistance to H.a. Noco2 as observed in bir1-1 pad4-1 (Figure 2-6F), suggesting that SOBIR5 encodes SDF2.
Figure 2-6 Characterization and cloning of *sobir5-1*.

(a) Morphology of Col-0 (wild-type, WT), *bir1-1*, *bir1-1 pad4-1* and *sobir5-1 bir1-1 pad4-1*.

(b, c) Expression levels of *PR1* (b) and *PR2* (c) in wild-type, *bir1-1*, *bir1-1 pad4-1* and *sobir4-1 bir1-1 pad4-1* seedlings compared to *ACTIN1*. Total RNA was extracted from 2-week-old seedlings grown on half-strength MS plates.

(d) Growth of *H. a. Noco2* on wild-type, *bir1-1*, *bir1-1 pad4-1*, and *sobir4-1 bir1-1 pad4-1* seedlings.
(e) Morphology of wild-type, bir1-1, bir1-1 pad4-1 and sdf2-2 bir1-1 pad4-1.

(f) Growth of H. a. Noco2 on wild-type, bir1-1, bir1-1 pad4-1, and sdf2-2 bir1-1 pad4-1 seedlings.

Plants in (a) and (e) were grown on soil at 23°C and photographed 3 weeks after planting. Error bars in (b)-(d) and (f) represent the standard deviation of three measurements.

2.2.7 SDF2 is required for cell death and defence responses in bir1-1

To determine whether sdf2-2 can suppress cell death and defence responses in bir1-1 without the presence of pad4-1, we isolated the sdf2-2 bir1-1 double mutant from the F2 progeny of a cross between sdf2-2 and bir1-1 pad4-1. As shown in Figure 2-7A, sdf2-2 bir1-1 is much bigger than bir1-1, but considerably smaller than wild type (Figure 2-7A). Trypan blue staining showed that cell death is reduced, but still present in sdf2-2 bir1-1 (Figure 2-7B). RT-PCR analysis showed that the expression of PR-1 (Figure 2-7C) and PR-2 (Figure 2-7D) in sdf2-2 bir1-1 is reduced compared to bir1-1, but still much higher than in wild type. Growth of H.a. Noco2 in sdf2-2 bir1-1 is significantly greater than in bir1-1, but much lower than in wild type (Figure 2-7E). These data suggest that cell death and defence response activation in bir1-1 are partially dependent on SDF2.
Figure 2-7 Characterization of the *sdf2-2 bir1-1* double mutant.

(a) Morphology of Col-0 (wild-type, WT), *bir1-1, sdf2-2 bir1-1*. Plants were grown on soil at 23°C and photographed 3 weeks after planting.

(b) Trypan blue staining of wild-type, *bir1-1* and *sdf2-2 bir1-1* mutant seedlings. Plants were grown at 23°C on half-strength MS plates for 2 weeks.

(c, d) Expression levels of *PR1* (b) and *PR2* (c) in wild-type, *bir1-1, sdf2-2 bir1-1* seedlings compared to *ACTIN1*. Total RNA was extracted from 2-week-old seedlings grown on half-strength MS plates.

(e) Growth of *H. a. Noco2* on wild-type, *bir1-1, sdf2-2 bir1-1* seedlings.

Error bars in (c)-(e) represent the standard deviation of three measurements.
2.2.8 CRT3 and ERdj3b are required for the accumulation of SOBIR1

Since cell death and activation of defence responses in bir1-1 are dependent on the RLK SOBIR1, we hypothesized that suppression of bir1-1 mutant phenotypes by mutations in the ER-quality control components could be caused by reduced accumulation of SOBIR1. To test whether ER-QC is critical for SOBIR1 accumulation, we generated transgenic lines expressing the SOBIR1-FLAG fusion construct in wild type plants. One of the transgenic lines was crossed with crt3-1 and erdj3b-1 to introduce the SOBIR1-FLAG transgene into these mutant backgrounds. As shown in Figure 2-8A, the SOBIR1-FLAG protein level is much lower in crt3-1 than in wild type background. In erdj3b-1, accumulation of SOBIR1-FLAG is also reduced (Figure 2-8B) in comparison with that in the wild type. These data indicate that CRT3 and ERdj3b are required for the accumulation of SOBIR1.

Figure 2-8 Accumulation of SOBIR1 protein is reduced in crt3-1 and erdj3b-1 mutants.
(a) Western blot analysis of SOBIR1-FLAG protein level (top) and RT-PCR analysis of SOBIR1-FLAG transcript accumulation (bottom) in wild-type and crt3-1 mutant background.
(b) Western blot analysis of SOBIR1-FLAG protein level (top) and RT-PCR analysis of SOBIR1-FLAG transcript accumulation (bottom) in wild-type and erdj3b-1 mutant background.
Protein and RNA samples were extracted from 2-week-old seedlings grown on half-strength MS plates at 23°C. Rubisco was used as protein loading control, and ACTIN1 was used as RNA control.
2.3 Discussion

Despite the importance of ER-QC in folding and maturation of secreted and membrane proteins, very few client proteins have been identified to be affected in mutants defective in ER-QC. Here we report that the ER-QC components CRT3 and ERdj3b are required for the accumulation of the RLK SOBIR1, suggesting that ER-QC plays an important role in the accumulation of SOBIR1.

In Arabidopsis, loss of function of BIR1 leads to activation of two defence pathways, one dependent on PAD4 and the other dependent on SOBIR1 (Gao, Wang et al. 2009). Our current studies on the suppressor mutants of bir1-1 pad4-1 reveal that CRT3, ERdj3b and SDF2 are required for activation of cell death and defence responses in bir1-1. Suppression of the auto-immune phenotypes of bir1-1 by mutations in CRT3 and ERdj3b is at least partially caused by the reduced accumulation of SOBIR1 in the crt3 and erdj3b mutants. As accumulation of SOBIR1 is reduced but not abolished in crt3-1 and erdj3b-1, whereas suppression of the mutant phenotypes of bir1-1 pad4-1 by crt3 and erdj3b mutants is fairly complete, it is possible that these mutants also affect the biogenesis of additional yet to be identified components involved in activation of cell death and defence responses in bir1-1.

In ER-QC, CRT3 functions as a lectin-like chaperone in the CNX/CRT cycle to facilitate the folding of glycoproteins (Dejgaard, Nicolay et al. 2004), whereas ERdj3b and SDF2 form complexes with the ER luminal chaperone BiP to assist with protein
folding and assembly (Dejgaard, Nicolay et al. 2004). Analysis of double mutants between bir1-1 and the ER-QC mutants showed that crt3-1 appears to have a stronger effect on suppressing cell death and defence responses in bir1-1 than erdj3b-1 and sdf2-2. Reduction in SOBIR1 protein accumulation also appears to be more severe in crt3-1 than in erdj3b-1. These data suggest that protein retention by the BiP-ERdj3b-SDF2 complex may be less critical than CRT3-based ER-QC in the biogenesis of SOBIR1.

In addition to its function in regulating cell death and defence responses in bir1-1, SOBIR1 plays a critical role in innate immunity against necrotrophic fungi (Zhang, Fraiture et al. 2013). In tomato, SOBIR1 orthologs interact with RLP-type resistance protein Cf-4 and Ve1 and are required for hypersensitive response and immunity mediated by Cf-4 and Ve1 (Liebrand, Smit et al. 2012, Liebrand, Kombrink et al. 2013). Interestingly, silencing tomato CRT3a also affects Cf-4 and Ve1-mediated defence responses. It will be interesting to determine whether silencing CRT3a affects the accumulation of SOBIR1 in tomato and the reduced accumulation of SOBIR1 causes the compromised resistance responses mediated by Cf-4 and Ve1.

Previous studies showed that ER-QC is required for the biogenesis of PAMP receptor EFR, but not the closely related FLS2 (Li, Zhao-Hui et al. 2009, Lu, Tintor et al. 2009, Nekrasov, Li et al. 2009, Saijo, Tintor et al. 2009). However, it is unclear what determines the differential requirements of ER-QC for the biogenesis of different proteins. One difference between FLS2 and EFR is that FLS2 is a conserved protein present in
higher plants, whereas EFR is newly evolved and only found in *Brassicaceae*, which leads to a hypothesis that newly evolved proteins may have lower stability and thus require extra assistance of ER-QC for their folding (Li, Zhao-Hui et al. 2009, Nekrasov, Li et al. 2009). Our studies showed that the evolutionarily conserved SOBIR1 also requires CRT3 and ERdj3b for its folding and accumulation. It seems that whether a protein requires ER-QC for its biogenesis depends on its conformation and ability to fold properly rather than its evolutionary history.

2.4 Experimental procedures

2.4.1 Plant material

*bir1-1, bir1-1 pad4-1* and the identification of suppressor mutants of *bir1-1 pad4-1* were described previously (Gao, Wang et al. 2009). *crt3-1* (SALK_051336), *erdj3b-1* (SALK_113364) and *sdf2-2* (SALK_141321) were obtained from Arabidopsis Stock Center and they were reported before (Li, Zhao-Hui et al. 2009, Nekrasov, Li et al. 2009). All plants were grown at 23°C under 16hr light/8hr dark. To generate various double and triple mutants, *crt3-1, erdj3b-1* and *sdf2-2* mutant plants were crossed with *bir1-1 pad4-1.*

*crt3-1 bir1-1, crt3-1 bir1-1 pad4-1, erdj3b-1 bir1-1, erdj3b-1 bir1-1 pad4-1, sdf2-2 bir1-1* and *sdf2-1 bir1-1 pad4-1* were identified from the F2 progeny by PCR genotyping.

For complementation analysis of *sobir4-1*, a 4 kb genomic DNA fragment of *ERdj3B* was amplified from wild type genomic DNA by PCR using primers

5’-cgcgagctcaatgtagtcccagcttttaggaccatcatgttct-3’ and 5’-cccaagcttttaggaccatcatgttct-3’ and cloned
into pCAMBIA1305 vector. The plasmid was transformed into *Agrobacterium tumefaciens* and subsequently into *sobir4-1* by floral dipping (Clough and Bent 1998).

Transgenic plants were selected on 1/2 strength Murashige and Skoog (MS) plates containing hygromycin.

### 2.4.2 Mutant characterization

Trypan blue staining was performed on two-week-old seedlings grown on 1/2 strength MS plates. 1 ml lactophenol trypan blue solution (10 mg trypan blue, 10 g phenol, 10 mL lactic acid, 10 mL glycerol and 10 mL water) diluted 1:1 in ethanol was added to a 1.5 mL microcentrifuge tube containing the seedlings. After boiling for 1 min, the staining solution was removed and the samples were de-stained with 1.5 mL de-staining buffer (2.5 g per mL water) on an orbital shaker for 2 h. The samples were further destained overnight with new destaining buffer before they were examined by microscopy.

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

For gene expression analysis, RNA was extracted from two-week-old seedlings grown on 1/2 strength MS plates using Isol-RNA Lysis Reagent (5 PRIME). The
extracted RNA was reverse transcribed into total complementary DNA using M-MuLV Reverse Transcriptase (New England Biolabs). Real-time PCR was carried out using the total complementary DNA as template to determine the expression levels of target genes. Primers used for real-time PCR analysis of Actin1, PR1 and PR2 were described previously (Zhang, Tessaro et al. 2003).

2.4.3 Analysis of SOBIR1 protein levels in crt3-1 and erdj3b-1 mutant plants

To express the SOBIR1 protein with a 3xFLAG tag, a 3.2 kb genomic DNA fragment containing SOBIR1 without the stop codon was amplified by PCR using primers 5’-cgggtaccatatagcttgccgtagaacctc-3’ and 5’-cgcggatccgtgcttgatctgggacaac-3’. The fragment was cloned into a modified pCAMBIA1305 vector with a 3xFLAG tag to obtain pCAMBIA1305-SOBIR1-FLAG. The plasmid was transformed into Agrobacterium tumefaciens and subsequently into wild type plants by floral dipping. Expression of the FLAG-tagged SOBIR1 protein in the transgenic lines was confirmed by western blot. A selected transgenic line was crossed with crt3-1, erdj3b-1 and sdf-2. Homozygous crt3-1, erdj3b-1 and sdf-2 mutants carrying the SOBIR1-FLAG transgene were identified in the F2 progeny by PCR.

Western blot analysis of the SOBIR1-FLAG protein was performed on total proteins extracted from two-week-old seedlings grown on 1/2 strength MS plates using the anti-flag M2 antibody (Sigma-Aldrich). The target protein was detected by chemiluminescence using the SuperSignal Sensitivity Substrate (Thermo Scientific).
Chapter 3: Regulation of SOBIR1 accumulation and activation of defence responses in bir1-1 by ER-quality control components UGGT and STT3a

3.1 Introduction

Eukaryotic cells have evolved several quality control mechanisms to monitor the folding of secretory proteins in the endoplasmic reticulum (ER) (Hebert and Molinari 2007, Anelli and Sitia 2008). Correctly folded proteins are allowed to traffic to their final destinations, whereas misfolded proteins are retained in the ER for additional folding process, or degraded by the ER-associated degradation pathway.

One of the well-studied protein folding pathways specific for secreted glycoproteins is the calnexin (CNX)/calreticulin(CRT) cycle, which involves ER-localized lectin-like chaperones CNX/CRT and the UDP-glucose:glycoprotein glucosyltransferase (UGGT) (Dejgaard, Nicolay et al. 2004). Following protein translation, preassembled glycan chains (Glc$_3$Man$_9$GlcNAc$_2$) are transferred to the Asn (N)-residues within the N-X-Ser/Thr consensus sequences in acceptor proteins by the oligosaccharyltransferase (OST) complex. Trimming of two glucose residues from the glycan chain by glucosidases generates proteins with monoglucosylated glycans (GlcMan$_9$GlcNAc$_2$). CNX and CRT interact with these proteins and assist with their folding in the ER. Subsequent removal of the remaining glucose from GlcMan$_9$GlcNAc$_2$ leads to
dissociation of the client protein from CNX and CRT. Proteins that attained their native conformation can then enter the secretory process, whereas improperly folded proteins are recognized by UGGT and a glucose residue is added back to the Man9GlcNAc2 by the enzyme. The monoglucosylated proteins subsequently associate with CNX and CRT to go through another round of folding.

UGGT and CRT3 have been shown to play important roles in the biogenesis of transmembrane receptors in plants. Retention of the defective brassinosteroid receptor bri1-9 protein in the ER requires both UGGT and CRT3 (Jin, Yan et al. 2007, Jin, Hong et al. 2009). In Arabidopsis uggt and crt3 mutant plants, accumulation of the receptor-like kinase (RLK) EFR, which recognizes bacterial EF-Tu, is reduced (Li, Zhao-Hui et al. 2009, Saijo, Tintor et al. 2009). Expression of the tobacco protein INDUCED RECEPTOR-LIKE KINASE was also shown to be dependent on NbCRT3 (Caplan, Zhu et al. 2009). In tomato, silencing of CRT3a affects the biogenesis of Cf-4 and leads to loss of pathogen resistance mediated by Cf-4 (Liebrand, Smit et al. 2012). In addition, loss of function mutations in STT3a, which encodes a subunit of the OST complex, also cause reduced EFR protein levels and impair its function in plant immunity (Nekrasov, Li et al. 2009, Saijo, Tintor et al. 2009).

In Arabidopsis, BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (BIR1) negatively regulates cell death and defence responses mediated by the RLK SOBIR1 (SUPPRESSOR OF BIR1, 1) (Gao, Wang et al. 2009). Previous studies showed that
activation of defence responses in bir1-1 is also dependent on the β and γ subunits of heterotrimeric G protein, as well as several ER-quality control (ER-QC) components including CRT3, ERdj3b and SDF2 (Liu, Ding et al. 2013, Sun, Zhang et al. 2014). Here we report that additional ER-QC regulators, UGGT and STT3a, also play important roles in the regulation of defence responses in bir1-1.

3.2 Results

3.2.1 Identification and characterization of sobir6-1 bir1-1 pad4-1

To identify defence pathways activated in bir1-1, a suppressor screen was carried out in the bir1-1 pad4-1 mutant background (Gao, Wang et al. 2009). sobir6-1 is one of the mutants identified from the screen. In the sobir6-1 bir1-1 pad4-1 triple mutant, the dwarf morphology of bir1-1 pad4-1 is almost completely suppressed (Figure 3-1A). Analysis of the expression levels of defence marker genes PATHOGENESIS-RELATED 1 (PRI) (Figure 3-1B) and PR2 (Figure 3-1C) in sobir6-1 bir1-1 pad4-1 showed that PR2 expression is significantly lower than in bir1-1 pad4-1. In addition, sobir6-1 bir1-1 pad4-1 supports much higher growth of the oomycete pathogen Hyaloperonospora arabidopsidis (H.a.) Noco2 than bir1-1 pad4-1 (Figure 3-1D). These data indicate that the constitutive defence responses observed in bir1-1 pad4-1 are largely suppressed by sobir6-1.
3.2.2 **SOBIR6 encodes UGGT**

The *sobir6-1* mutation was mapped to a region between marker T2E12 and T8K14 on Chromosome 1 using a mapping population generated by crossing *sobir6-1 bir1-1 pad4-1* (in the Columbia ecotype background) with *Ler*-0. Further fine mapping analysis narrowed down the mutation to a 70 kb region between markers F23N20 and F26A9.
(Figure 3-2A). To identify the sobir6-1 mutation, PCR fragments covering this region were amplified from genomic DNA of sobir6-1 bir1-1 pad4-1 and sequenced. A single G to A mutation was identified in AT1G71220, which encodes the ER-QC component UGGT. The mutation is located at the junction between the 29th intron and 30th exon of UGGT.

In the same mutant screen, we also identified two additional alleles of sobir6. Both of them failed to complement sobir6-1 (Figure 3-2B). Sequence analysis of UGGT in sobir6-2 and sobir6-3 showed that they also contain mutations in this gene. In sobir6-2, a C to T mutation changes Ala1426 to Val. In sobir6-3, a G to A mutation introduces a stop codon in the coding region (Figure 3-2C). These data suggest that SOBIR6 is UGGT.
Figure 3-2 Map-based cloning of SOBIR6.

(A) Mapping of the sobir6-1 mutation. Positions of the mapping markers, the gene structure of SOBIR6 and the mutation site in sobir6-1 are shown. The exons are indicated with boxes and introns with lines. The mutation site is located at the junction between the 29th intron and 30th exon. The lower case letters represent nucleotides in the intron and the uppercase letters represent nucleotides in the exon.

(B) Morphology of sobir6 bir1-1 pad4-1 alleles and representative F1 plants of indicated crosses for the complementation test. Plants were grown on soil at 23°C and photographed three weeks after planting.

(C) Mutations identified in the sobir6 alleles. aa, amino acid. ¹The positions of mutated nucleotides in the coding sequences are listed.
3.2.3 UGGT is required for constitutive defence responses in *bir1-1*

To test whether *sobir6-1* can suppress the constitutive defence responses in *bir1-1* in the absence of *pad4-1*, we isolated the *sobir6-1 bir1-1* double mutant from the F2 population of a cross between *sobir6-1 bir1-1 pad4-1* and wild type. *sobir6-1 bir1-1* plants are much bigger than *bir1-1*, but smaller than wild type (Figure 3-3A). In *sobir6-1 bir1-1*, expression of both *PR1* and *PR2* is greatly reduced compared to that in *bir1-1* (Figure 3-3B and 3-3C). As shown in Figure 3-3D, resistance to *H.a. Noco2* is also considerably reduced in *sobir6-1 bir1-1* (Figure 3-3E). These data suggest that UGGT is required for the constitutive defence responses in *bir1-1*. This is consistent with the requirement of another component of the CNX/CRT cycle, CRT3, for the autoimmune phenotype in *bir1-1* (Sun, Zhang et al. 2014).
Figure 3-3 Characterization of the sobir6-1 bir1-1 double mutant.

(A) Morphology of wild type (WT), bir1-1 and sobir6-1bir1-1 plants. Plants were grown on soil at 23°C and photographed three weeks after planting.

(B-C) Expression levels of PR1 (B) and PR2 (C) in WT, bir1-1 and sobir6-1 bir1-1 seedlings as normalized with ACTIN1. Total RNA was extracted from 12-day-old seedlings grown on half-strength MS plates.

(D) Growth of H. a. Noco2 on WT, bir1-1 and sobir6-1 bir1-1 seedlings. Error bars in (B-D) represent standard deviations of three measurements.

3.2.4 The autoimmune phenotype of bir1-1 is partially suppressed by stt3a-2

Since STT3a is involved in co-translational N-glycosylation of nascent proteins before they enter the CNX/CRT cycle (Dejgaard, Nicolay et al. 2004), we tested whether STT3a is required for the constitutive defence responses in bir1-1 by crossing stt3a-2 with bir1-1 pad4-1 and isolating the stt3a-2 bir1-1 pad4-1 triple mutant and the stt3a-2
*bir1-1* double mutant in the F2 generation. As shown in Figure 3-4A, *stt3a-2 bir1-1 pad4-1* is larger than *bir1-1 pad4-1*, but considerably smaller than wild type. In *stt3a-2 bir1-1 pad4-1*, the expression of both *PR1* and *PR2* is lower than in *bir1-1 pad4-1* (Figure 3-4B and 3-4C). *H.a.* Noco2 growth on *stt3a-2 bir1-1 pad4-1* is much higher than on *bir1-1 pad4-1*, but significantly lower than on wild type (Figure 3-4D). The *stt3a-2 bir1-1* double mutant retained the dwarf morphology of *bir1-1*, but is larger in size (Figure 3-5A). In *stt3a-2 bir1-1*, the expression of both *PR1* and *PR2* is greatly reduced compared to that in *bir1-1* (Figure 3-5B and 3-5C). There is a small amount of *H.a.* Noco2 growth on the *stt3a bir1-1* double mutant compared to almost no growth of the pathogen on *bir1-1* plants (Figure 3-5D). Taken together, the autoimmune phenotype of *bir1-1* is partially dependent on STT3a. Our data suggest that STT3a-dependent N-glycosylation is also critical for activation of defence responses in *bir1-1*.  


Figure 3-4 Characterization of the *stt3a-2 bir1-1 pad4-1* triple mutant.

(A) Morphology of wild type (WT), *bir1-1, bir1-1 pad4-1* and *stt3a-2 bir1-1 pad4-1* plants. Plants were grown on soil at 23°C and photographed 3 weeks after planting.

(B-C) Expression levels of *PR1* (B) and *PR2* (C) in WT, *bir1-1, bir1-1 pad4-1* and *stt3a-2 bir1-1 pad4-1* seedlings as normalized with *ACTIN1*. Total RNA was extracted from 12-day-old seedlings grown on half-strength MS plates.

(D) Growth of *H. a. Noco2* on WT, *bir1-1, bir1-1 pad4-1* and *stt3a-2 bir1-1 pad4-1* seedlings.

Error bars in (B-D) represent standard deviations of three measurements.
3.2.5 Accumulation of SOBIR1 is reduced in sobir6-1 but unaffected in stt3a-2

Because the constitutive defence responses in bir1-1 are dependent on the RLK SOBIR1 and the accumulation of SOBIR1 is dependent on the ER-QC component CRT3 (Sun, Zhang et al. 2014), we further tested whether UGGT and STT3a are also required for SOBIR1 accumulation. A transgenic line expressing the SOBIR1-FLAG fusion protein in wild type background was crossed into sobir6-1 or stt3a-2. As shown in Figure 3-6A, the SOBIR1-FLAG protein level is considerably lower in sobir6-1 than in wild
type background, suggesting that UGGT is also required for the accumulation of SOBIR1.

In contrast, the SOBIR1-FLAG protein levels are similar in stt3a-2 and wild type background (Figure 3-6B).

![Figure 3-6 Accumulation of SOBIR1 protein in sobir6-1 and stt3a-2.](image)

(A) Western blot analysis of SOBIR1-FLAG protein level (top) and RT-PCR analysis of SOBIR1-FLAG expression (bottom) in wild type (WT) and sobir6-1 mutant background.

(B) Western blot analysis of SOBIR1-FLAG protein level (top) and RT-PCR analysis of SOBIR1-FLAG expression (bottom) in WT and stt3a-2 mutant background.

Protein and RNA samples were extracted from 12-day-old seedlings grown on half-strength MS plates at 23°C. Rubisco was used as protein loading control and ACTIN1 was used as RNA control.

### 3.3 Discussion

In addition to its role in cell death and defence activation in bir1-1, increasing evidence suggests that SOBIR1 functions as a critical component of receptor-like protein (RLP)-mediated immunity (Liebrand, van den Berg et al. 2013, Liebrand, van den Burg et al. 2014). SOBIR1 proteins in tomato interact with two RLPs, Cf-4 and Ve1, and are required for Cf-4 and Ve1 mediated immunity. In addition, SOBIR1 functions together with Arabidopsis RLP30 in defence against necrotrophic fungi (Zhang, Fraiture et al. 2013).
Our study provided additional evidence that ER-QC plays important roles in the biogenesis of SOBIR1 and reduced accumulation of SOBIR1 contributes to the suppression of \( bir1-1 \) mutant phenotypes by mutations in \( UGGT \). Because biogenesis of SOBIR1 in Arabidopsis is dependent on multiple components of ER-QC, it is likely that accumulation of SOBIR1 proteins in tomato also relies on ER-QC. The compromised Cf-4 and Ve1-mediated immune responses observed in tomato plants when CRT3a was silenced (Liebrand, Smit et al. 2012, Liebrand, Kombrink et al. 2014) might be partially due to reduced accumulation of tomato SOBIR1.

Compared to almost complete suppression of the autoimmune phenotype in \( bir1-1 \) \( pad4-1 \) by \( uggt \) and \( crt3 \) mutants, \( stt3a-2 \) has a much smaller effect on the morphology as well as defence responses in \( bir1-1 \) \( pad4-1 \). This can probably be explained by genetic redundancy. In Arabidopsis, there is a close homolog of STT3a named STT3b (Koiwa, Li et al. 2003). It is likely that STT3b can partially compensate the loss of the function of STT3a in N-glycosylation.

As SOBIR1 accumulation is not affected in \( stt3a-2 \), the mechanism of how \( stt3a-2 \) suppresses the phenotypes of \( bir1-1 \) remains to be determined. It is possible that the contribution of STT3a to the biogenesis of SOBIR1 is masked by genetic redundancy between STT3a and STT3b. Since SOBIR1 usually functions together with RLPs, it is likely that one or more RLPs might be involved in the activation of cell death and defence responses in \( bir1-1 \), and the suppression of \( bir1-1 \) mutant phenotypes by \( stt3a-2 \).
might be caused by reduced accumulation of the RLPs.

3.4 Experimental procedures

3.4.1 Plant material

*bir1-1*, *bir1-1 pad4-1* and the identification of suppressor mutants of *bir1-1 pad4-1* have been described previously (Gao, Wang et al. 2009). *sst3a-2* (SALK_058814) was obtained from the Arabidopsis Biological Resource Center and has been described previously (Koiwa, Li et al. 2003). All plants were grown at 23°C under 16h light/8h dark. To isolate the *sobir6-1* single mutant and *sobir6-1 bir1-1* double mutant, *sobir6-1 bir1-1 pad4-1* was crossed with a Col-0 plant. In F2, *sobir6-1* single mutant and *sobir6-1 bir1-1* double mutant were isolated by PCR genotyping. To generate *sst3a-2 bir1-1* double mutant and *sst3a-2 bir1-1 pad4-1* triple mutant, *sst3a-2* was crossed with *bir1-1 pad4-1*. *sst3a-2 bir1-1* and *sst3a-2 bir1-1 pad4-1* were identified in F2 by PCR genotyping.

3.4.2 Mutant characterization

*H. a.* Noco2 infection was performed on 12-day-old seedlings. The seedlings were sprayed with spore suspension at a concentration of 50,000 spores per ml water. Sprayed plants were covered with a clear dome and kept at 16°C under 12h light/12h dark cycles in a growth chamber. The humidity in the growth chamber was approximately 95%. Infection results were scored seven days later as previously described (Bi, Cheng et al. 2010).
For gene expression analysis, RNA was extracted from 12-day-old seedlings grown on half-strength MS plates using EZ-10 Spin Column Plant RNA Mini-Preps Kit (Bio Basic Inc). The extracted RNA was reverse transcribed into total cDNA using Easy Script Reverse Transcriptase (Applied Biological Materials Inc). Real-time PCR was performed using SYBR Premix Ex Taq II (Takara). Total cDNA was used as a template to determine the expression level of target genes with ACTIN1 as control. Primers used for real-time PCR analysis of ACTN1, PR1 and PR2 have been described previously (Zhang, Tessaro et al. 2003).

### 3.4.3 Analysis of SOBIR1 protein levels in sobir6-1 and stt3a-2 mutant plants

A transgenic line expressing the SOBIR1 protein with a 3xFLAG tag (Sun, Zhang et al. 2014) was crossed with sobir6-1 and stt3a-2. In F2, plants that were homozygous for sobir6-1 and stt3a-2 and carried the SOBIR1-FLAG transgene were identified by PCR. Western blot analysis of the SOBIR1-FLAG protein was carried out using the anti-flag M2 antibody (Sigma-Aldrich).
Chapter 4: Future directions and conclusions

Upon pathogen infection, diverse immune responses are turned on to fight against invaders. Immune responses are under strict control to avoid unnecessary energy consumption when there is no pathogen around. One of the negative regulators of plant immune system is BIR1. Knocking out \textit{BIR1} leads to constitutive defence responses and greatly impairs plant development. From the \textit{bir1-1} suppressor screen, we found that SOBIR1 is an important positive regulator of immune responses in \textit{bir1-1}. Interestingly, cloning of several other \textit{SOBIR} genes revealed that mutations in specific ER-QC components result in completely or partially blocked immune responses in \textit{bir1-1}. In some of these mutants, SOBIR1 protein accumulation is reduced, suggesting that the ER-QC regulates the accumulation of SOBIR1 and reduced SOBIR1 protein level contributes to suppression of the \textit{bir1-1} mutant phenotype. However, we can still detect significant SOBIR1 protein accumulation in \textit{crt3-1} despite that \textit{crt3-1} can almost completely block the immune responses in \textit{bir1-1}. The decrease in SOBIR1 protein may not account for all of the suppression effect on defence responses by defects in ER-QC.

It is still not very clear how mutations in \textit{SOBIR1} suppress the immune responses in \textit{bir1-1}. To answer this question, it is critical to identify the component that BIR1 is negatively regulating in wild type plants. In a previous study, it was shown that BIR1 kinase activity is important for its function (Gao, Wang et al. 2009). Failure of phosphorylation of the substrate of BIR1 in \textit{bir1-1} may activate the immune responses in
It is interesting to identify to the substrate of BIR1 and test the role of the substrate in activating immune response in bir1-1.

Recent studies showed that SOBIR1 is a signal partner of multiple RLPs (Liebrand, van den Berg et al. 2013, Zhang, Fraiture et al. 2013). It is likely that there are one or more RLPs that are involved in the activation of defence responses in bir1-1, which function together with SOBIR1 in the activation of defence responses in bir1-1. However, we didn’t find any RLP in our forward genetic screen. It is probably because of redundancy and weak suppression phenotypes caused by a mutation in a single RLP. To identify RLPs involved in activation of cell death in bir1-1, online protein-protein interaction database such as MIND (Membrane-based Interactome Database), or immune-precipitation and mass spectrometry analysis may help us to find possible RLP candidates. Further genetic analysis of the RLP candidates will reveal which RLPs are involved in the defence responses in bir1-1. Identifying the RLPs that work together with SOBIR1 to transduce the immune signal in bir1-1 is critical in understanding how BIR1 negatively regulates defence responses.
References:


