Role of WRKY Transcription Factors in *Arabidopsis* Development and Stress Responses

Jing Li

Division of Genetics  
Department of Biosciences  
Faculty of Biological and Environmental Sciences  
University of Helsinki, Finland

And

Helsinki Graduate Program in Biotechnology and Molecular Biology  
University of Helsinki, Finland

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences, University of Helsinki, in the auditorium 1041 of the Biocenter 2, Viikinkaari 5, Helsinki, on the 7th of March, 2014, at 12 o’clock noon
Cover image: Upper image is \textit{wrky54wrky70} double mutant of Arabidopsis infected by \textit{Pectobacterium carotovorum}. Lower images are stomatal aperture (left) and senescence leaf (right) of \textit{wrky54wrky70} double mutant plant.
To the memory of my grandmother
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This thesis is based on the following publications, which are referred to in the text by their roman numerals. The publications have been reprinted with the kind permission of their copyright holders.


My contribution to the above publications:

I: JL participated in the design of the experiments, identified the homozygous knock-out lines, analysed the interaction network of transcription factors and characterized the gene expression profile by qRT-RCR, and took part in the revision of the article, performing all additional experiments requested by the reviewer.

II: JL participated in the design of the experiments. JL performed the crossing for the triple mutants and identification of homozygous mutant lines, abiotic stress treatments, microarray and gene expression profiling, proline measurements, and stomatal work. JL and NS measured hormone levels. JL and PT analyzed the microarray data. JL wrote the manuscript and carried out the revision after review.

III: JL participated in the experiments design, carried out all the experiments and analysis of results, and wrote the manuscript.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ABFs</td>
<td>ABA responsive element binding factors</td>
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<tr>
<td>ABI</td>
<td>abscisic acid insensitive</td>
</tr>
<tr>
<td>ABRE</td>
<td>ABA-responsive element</td>
</tr>
<tr>
<td>AIP</td>
<td>Arabidopsis potassium ion transporter 1 interacting protein phosphatase</td>
</tr>
<tr>
<td>AOS</td>
<td>allene oxide synthase</td>
</tr>
<tr>
<td>AP2/ERF</td>
<td>APETALA 2/ethylene-responsive element binding factor</td>
</tr>
<tr>
<td>AREBs</td>
<td>ABA responsive element binding proteins</td>
</tr>
<tr>
<td>BiFC</td>
<td>bimolecular fluorescence complementation assays</td>
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<tr>
<td>BWMK</td>
<td>blast-and wounding-activated MAP kinase</td>
</tr>
<tr>
<td>CaBD</td>
<td>Ca$^{2+}$-dependent calmodulin-binding domain</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>COI</td>
<td>coronatine insensitive</td>
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<tr>
<td>EDS</td>
<td>enhanced disease susceptibility</td>
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<tr>
<td>EIL</td>
<td>ethylene insensitive like</td>
</tr>
<tr>
<td>EIN</td>
<td>ethylene insensitive</td>
</tr>
<tr>
<td>ESR</td>
<td>epithiospecifying senescence regulator</td>
</tr>
<tr>
<td>ET</td>
<td>ethylene</td>
</tr>
<tr>
<td>ETI</td>
<td>effector-triggered immunity</td>
</tr>
<tr>
<td>GA</td>
<td>gibberellins</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>H3K4</td>
<td>histone H3 tail lysine 4</td>
</tr>
<tr>
<td>HAB</td>
<td>homology to ABI</td>
</tr>
<tr>
<td>HDA</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HR</td>
<td>hypersensitive response</td>
</tr>
<tr>
<td>ISR</td>
<td>induced systemic resistance</td>
</tr>
<tr>
<td>JA</td>
<td>jasmonic acid</td>
</tr>
<tr>
<td>JAR</td>
<td>jasmonic acid resistant</td>
</tr>
<tr>
<td>JAZ</td>
<td>jasmonate-zim domain</td>
</tr>
<tr>
<td>LOX</td>
<td>lipoxygenase</td>
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MAMP/PAMP microbe/pathogen-associated molecular pattern
MAP mitogen-activated protein
MeJA methyl jasmonic acid
MPK MAP kinase
NAC no apical meristem, ATAF1/2, CUP-SHAPED COTYLEDON 2
NahG a bacterial gene encoding SA hydroxylase
NCED 9-cis-epoxycarotenoid dioxygenase
NPR nonexpresser of PR genes
OST open stomata
PAD phytoalexin deficient
PDF plant defensin
PEG polyethylene glycol
PP2C type 2C protein phosphatase
PR pathogenesis related
PTI PAMP-triggered immunity
PYR/PYL/RCAR pyrabactin resistance/PY-like/regulatory component of ABA receptor
R resistance
RD responsive to dessication
ROS reactive oxygen species
SA salicylic acid
SAR systemic acquired resistance
SIB sigma factor-interacting protein
SID SA induction deficient
SIPK salicylic acid-induced protein kinase
SIRK senescence-induced receptor-like serine/threonine protein kinase
SLAC slow anion channel-associated
SnRK SNF1-related protein kinase
SP serine residue followed by proline residue
TF transcription factor
TGA TF which can recognize the TGACG element in promoters
VSP vegetative storage protein
WIPK wound-induced protein kinase
WRKY transcription factor containing a highly conserved WRKY domain
ABSTRACT

It has been well established that environmentally induced alterations in gene expression are mediated by transcription factors (TFs). One of the important plant-specific TF groups is the WRKY (TFs containing a highly conserved WRKY domain) family, which is involved in regulation of various physiological programs including biotic and abiotic defenses, senescence and trichome development. Two members of WRKY group III in Arabidopsis thaliana, WRKY54 and WRKY70, are demonstrated in this study to be key components in cooperative regulation of developmental senescence, osmotic stress response as well as specific pathogen defenses.

As revealed by molecular studies, we found that WRKY54, the closest homologue of WRKY70, exhibited a similar expression pattern as WRKY70 and also functioned in leaf senescence. Disruption of both WRKY54 and WRKY70 resulted in clearly enhanced premature senescence, suggesting that WRKY54 and WRKY70 co-operate as negative regulators of senescence. In addition, yeast two-hybrid analysis showed that WRKY54, WRKY70 and WRKY53 could independently interact with WRKY30. Moreover, the phytohormone salicylic acid (SA) positively affected the expression of WRKY54, WRKY70, WRKY53 and WRKY30. Additionally, WRKY53 and WRKY30 but not WRKY54 and WRKY70 were responsive to reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$), a central factor in senescence. All of these data suggest that WRKY54, WRKY70 and WRKY53 act as critical regulators in modulating the process of senescence through independent interaction with WRKY30.

The involvement of WRKY54 and WRKY70 in abiotic stress responses was also explored in this study. The transient induction of WRKY54 and WRKY70 by osmotic stress implicated that they might play roles in the abiotic stress response. The wrky54wrky70 double mutant showed enhanced tolerance to osmotic stress compared to the corresponding single mutants and wild-type plants, indicating that these two TFs cooperate as negative regulators of the osmotic stress response. Although the tolerance to osmotic stress was improved in the wrky54wrky70 double mutant, neither the expression of osmotic stress-related genes nor the accumulation of the osmolyte proline was enhanced. The suppressed gene expression in the wrky54wrky70 double mutant is SA-dependent, but the osmotic stress tolerance results more directly from the involvement of both negative regulators WRKY54 and WRKY70. In addition, abscisic acid (ABA) signaling was also involved in this suppression. The final analysis showed that the enhanced tolerance in the wrky54wrky70 double mutant was correlated with improved water retention and enhanced stomatal closure. Consequently, the crosstalk between SA-mediated biotic and ABA-mediated abiotic stress responses is modulated by WRKY54 and WRKY70.

The contribution of both WRKY54 and WRKY70 to plant disease resistance remains unclear although the role of WRKY70 in biotic stress has been previously characterized. Non-stressed wrky54wrky70 double mutant exhibited constitutively expressed defense-related genes and accumulation of H$_2$O$_2$, resulting in pre-formed defense to necrotrophic pathogens such as Pectobacterium carotovorum and Botrytis
*Fusarium cinerea*. However, this pre-formed resistance was compromised in non-stressed *wrky54wrky70sid2-1* triple mutant due to the reduced level of SA. These results suggest that increased SA leads to accumulation of H$_2$O$_2$ which is required to activate antimicrobial defenses to pathogens. Furthermore, genes encoding cell wall-related peroxidases and cell wall modification proteins were up-regulated in the *wrky54wrky70* double mutant but not in the *wrky54wrky70sid2-1* triple mutant, indicating that the cell wall-associated defense to necrotrophs could result from the elevated SA level in the *wrky54wrky70* double mutant. However, this cell wall-associated resistance in *wrky54wrky70* did not contribute to the defense against biotrophs. This might require additional defense measures controlled by WRKY54 and WRKY70 which are not activated in the double mutant, although the SA responsive genes are up-regulated by the accumulation of H$_2$O$_2$. 


1. INTRODUCTION

1.1 WRKY transcription factors: key regulators of plant processes

1.1.1 WRKY transcription factors

Transcription is the first step in gene expression and this process is regulated by transcription factors (TFs) which cause either activation or repression. More than 1500 TFs have been reported in Arabidopsis thaliana since 2000. The TFs contain a DNA-binding domain (DBD) which specifically recognizes the target DNA sequence forming a transcriptional complex and thus regulate gene expression (Riechmann et al., 2000; Guo et al., 2005; Mitsuda and Ohme-Takagi, 2009). In Arabidopsis, TFs are categorized into many groups according to the conserved DBD domain, such as AP2/ERF (APETALA 2/ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR), B3 (the third basic domain in the maize gene VIVIPAROUS1), NAC (NO APICAL MERISTEM, ATAF1/2, CUP-SHAPED COTYLEDON 2), SBP (SQUAMOSA-PROMOTER BINDING PROTEIN) and WRKY (TF containing a highly conserved WRKY domain) superfamilies. One of the largest TF families is the WRKY superfamily, comprising plant specific TFs involved in many biotic and abiotic stress responses as well as plant growth and development (Rushton et al., 2010). They show similarity with animal TFs, which contain the DNA-binding domain known as the GCM domain. TFs containing the GCM domain are classified together with WRKY TFs into the WRKY-GCM1 superfamily (Yamasaki et al., 2013). To date, WRKYs are not restricted to higher plants such as Arabidopsis and other flowering plants, but are also found in, for example, ferns, slime mold and unicellular green algae, indicating the origin of WRKYs in primitive eukaryotes (Ulker and Somssich, 2004; Agarwal et al., 2011; Yamasaki et al., 2013).

1.1.2 WRKY domains and W-box

More than 70 representatives of WRKY TFs are found in Arabidopsis. WRKY family members appear to be involved in the regulation of various physiological programs that are unique to plants, including pathogen defense, abiotic stress responses, senescence and trichome development (Rushton et al., 2010). The WRKY domain is defined by the conserved amino acid sequence WRKYGQK at its N terminus, together with a novel zinc-finger-like motif. The construction of the zinc-finger motif is either Cx4–5Cx22–23HxH (C2H2 type) or Cx7Cx23HxC (C2HC type) (Fig. 1). The WRKY domain is considered as the DNA-binding domain (Eulgem et al., 2000; Yamasaki et al., 2012; Yamasaki et al., 2013). Previous evidence has shown that WRKY members bind specifically to various W box elements, which contain the invariant TTGAC(C/T) sequence for WRKY binding. However, the binding selectivity of WRKY TFs to DNA is dependent on the flanking sequence outside the W box motif. A recent study has shown that a G base upstream of the core motif of the W box is most preferred by Arabidopsis
proteins WRKY6 and WRKY11 rather than other WRKYs. In addition, the R residue in the conserved WRKYGQK motif is able to form hydrogen bonds with the G base (Ciolkowski et al., 2008; Agarwal et al., 2011; Yamasaki et al., 2013).

AtWRKY1......**WRKYGQK**LVKGNFVRSYYRCTHPNCKAKKQLERSAGGQVDTVYFGH**...**DH......
Consensus ..**WRKYGQK**..........................C N4-7.C...........................22-23........................HNH/C.
........................................................................ about 60 a.a. ........................................

**Figure 1.** The structure of the WRKY domain. AtWRKY1 is used as an example. Red letters represent the conserved amino acid sequence WRKYGQK; the green ones indicate the conserved Cys/His residues located in the C terminus. At, *Arabidopsis thaliana*.

In Arabidopsis, WRKY TFs have been arranged into three distinct groups (Table 1), depending on the number and type of their WRKY domains and features of the zinc-finger-like motif. WRKY proteins with two WRKY domains belong to group I, while those with one WRKY domain belong to groups II and III. However, the zinc-finger motif in groups I and II belongs to the C2H2 type, whereas the C2HC type zinc-finger motif is found in group III. These three WRKY groups can be further clustered into subgroups. For instance, group II has been clustered into five subgroups (a-e) based on the additional amino acid motifs outside the WRKY domain and their phylogenetic distance. Group III consists of 13 members and can be divided into two subgroups group IIIa and IIIb. Only members of group IIIa share a highly conserved region of about 50 amino acids located at the N terminus, but this is not found in group IIIb members (Eulgem et al., 2000; Zhang and Wang, 2005; Agarwal et al., 2011; Yamasaki et al., 2013).

**Table 1.** Distinct groups of WRKY TFs in Arabidopsis (At standing for *Arabidopsis thaliana*).
1.1.3 WRKY transcription factors in biotic stresses

WRKYs play a very important role in plant innate immunity. According to recent studies, the plant immune system is mainly responsive to two functional patterns: microbial/pathogen-associated molecular patterns (MAMPs/PAMPs) and effector associated patterns. Recognition of these two patterns by plants leads to MAMP or PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively (Jones and Dangl, 2006). PTI and ETI initiate both local and systemic acquired resistance (SAR), but ETI is associated with the hypersensitive response (HR) while PTI inhibits HR-induced programmed cell death (Jones and Dangl, 2006; Pandey and Somssich, 2009; Crabill et al., 2010; Ishihama and Yoshioka, 2012). In Arabidopsis, more than 40 WRKY TFs have been reported to be involved in pathogen responses and phytohormone e.g. salicylic acid (SA)-mediated signaling pathways (Dong et al., 2003). Genetic analysis has shown that WRKYs either positively modulate plant defense responses or negatively affect them (Eulgem and Somssich, 2007). Induction of SAR by pathogens is accompanied by accumulation of a hormone signal, SA. Many WRKYs are positively regulated by SA through the receptors NPR1 and its paralogues NPR3 and NPR4 (Wang et al., 2006; Fu et al., 2012; Wu et al., 2012). Eight WRKYs in Arabidopsis have been reported to be the targets of NPR1 during SAR, including WRKY18, WRKY38, WRKY53, WRKY54, WRKY58, WRKY59, WRKY66, WRKY70 (Wang et al., 2006; Ishihama and Yoshioka, 2012). WRKY70 has already been identified as the node of convergence for SA-mediated and jasmonic acid (JA)-mediated defense signaling pathways, and it positively regulates SAR towards specific bacteria and fungi (Li et al., 2004; Li et al., 2006; Wang et al., 2006; Hu et al., 2012). Moreover, WRKY70 and WRKY54 play dual roles in repressing SA synthesis and transducing the SA signal. WRKY70 and WRKY53 are considered as positive regulators to plant defense against Pseudomonas syringae pv. maculicola (Psm) ES4326 (Wang et al., 2006). In addition, WRKY33 in Arabidopsis is another example, which is considered as a positive regulator of resistance to the necrotrophic fungi Alternaria brassicicola and Botrytis cinerea although unidentified components might also be involved (Zheng et al., 2006; Birkenbihl et al., 2012). Two structurally similar WRKYs, WRKY3 and WRKY4 have been found to be positive regulators in plant resistance to necrotrophic pathogens (Lai et al., 2008).

In addition to acting as positive regulators in Arabidopsis defense, WRKY TFs can also function as negative regulators. For instance, WRKY11 and WRKY17 negatively regulate basal defense in Arabidopsis (Journot-Catalino et al., 2006). Disruption of WRKY38 and WRKY62 enhanced plant defense while overexpression of these two genes compromised resistance to P. syringae, suggesting that they act as negative regulators in disease resistance to bacteria (Mao et al., 2007; Kim et al., 2008). Loss of function and gain of function studies showed that WRKY25 and WRKY48 negatively influenced SAR through altering the expression of PATHOGENESIS-RELATED GENE 1 (PR1) (Zheng et al., 2007; Xing et al., 2008). Group II member WRKY23 was reported to be the negative player in response to the cyst nematode Heterodera schachtii (Grunewald et al., 2008). Apart from the WRKYs mentioned above, additional ones in Arabidopsis such as WRKY18, WRKY40, WRKY60, WRKY27, WRKY41, WRKY53 and WRKY58 also...
negatively affect plant defense responses (Wang et al., 2006; Xu et al., 2006; Higashi et al., 2008; Hu et al., 2008; Mukhtar et al., 2008; Pandey and Somssich, 2009; Schön et al., 2013).

Many WRKYs in Arabidopsis display dual roles in plant defense, either positive or negative depending on the type of pathogen. WRKY53 was reported to show different functions to distinct pathogens. It positively regulated the plant response to \textit{P. syringae} while negatively affected plant defense to \textit{Ralstonia solanacearum} (Murray et al., 2007; Hu et al., 2008). Three WRKY TFs WRKY18, WRKY40 and WRKY60 had redundant roles in basal plant defense. Loss of function studies showed that \texttt{wrky18wrky40} and \texttt{wrky18wrky60} double mutants and the \texttt{wrky18wrky40wrky60} triple mutant were substantially more resistant to \textit{P. syringae} but more susceptible to \textit{B. cinerea}. Recent studies also imply that WRKY18 and WRKY40 negatively affect pre-invasion of the powdery mildew fungus, \textit{Golovinomyces orontii} (Xu et al., 2006; Pandey et al., 2010). Li et al., (2006) showed that up-regulation of WRKY70 caused enhanced resistance to the biotroph \textit{Erysiphe cichoracearum} but increased susceptibility to the necrotroph \textit{A. brassicicola}, this supplement the previous findings that WRKY70 is a positive regulator in SAR (Li et al., 2004). This dual function was also discovered for WRKY41. Opposite phenotypes were found when overexpressing WRKY41 to \textit{P. syringae} and \textit{Pectobacterium carotovora}. Moreover, mutating the WRKY8 gene in Arabidopsis resulted in increased resistance to \textit{P. syringae} but less resistance to \textit{B. cinerea}. In contrast, the ectopic expression of WRKY8 resulted in susceptibility to \textit{P. syringae} but resistance to \textit{B. cinerea}. In addition, WRKY8 was also found to negatively affect crucifer-infecting tobacco mosaic virus (TMV-cg) accumulation in infected leaves (Chen et al., 2010; Chen et al., 2013).

The research on WRKYs is not limited to Arabidopsis. Numerous WRKY TFs have been identified as participating in defense in other plant species, indicating the importance of WRKY TFs in plant innate immunity. The genome of rice (\textit{Oryza sativa}) contains 109 WRKY genes (Ross et al., 2007), many of which are involved in biotic stress and response to phytohormones (Ryu et al., 2006). Constitutive expression of \texttt{OsWRKY13}, \texttt{OsWRKY31}, \texttt{OsWRKY45}, \texttt{OsWRKY53} and \texttt{OsWRKY47} led to enhanced resistance to \textit{Magnaporthe oryzae} (Chujo et al., 2007; Qiu et al., 2007; Shimono et al., 2007; Zhang et al., 2008; Tao et al., 2009; Wei et al., 2013). Overexpression of \texttt{OsWRKY89} resulted in increased wax deposition on the leaf surface and more tolerance to rice blast fungus, while the resistance was compromised in an \texttt{OsWRKY89} silenced line (Wang et al., 2007). Ectopic expression of a PAMP-responsive gene \texttt{OsWRKY28} caused decreased resistance to \textit{M. oryzae}, indicating the negative role of \texttt{OsWRKY28} in PTI (Chujo et al., 2013). In addition, transgenic Arabidopsis overexpressing \texttt{OsWRKY77} was able to inhibit the growth of the bacterial pathogen \textit{P. syringae}, revealing that \texttt{OsWRKY77} might play a positive role in plant basal defense (Lan et al., 2013). Similarly, HvWRKY1 and HvWRKY2 in barley (\textit{Hordeum vulgare}) were repressors of PTI and this negative effect was suppressed by interfering with intracellular mildew A (MLA) protein, de-repressing PAMP-triggered basal defense (Shen et al., 2007; Chang et al., 2013). A loss of function study implied that HvWRKY10, HvWRKY19, and HvWRKY28 positively regulated ETI in response to \textit{Blumeria graminis} (Meng and Wise, 2012). CaWRKY1 from pepper
Capsicum annuum) is considered as a negative regulator as a result of the evidence shown in virus-induced silencing lines and overexpressor lines (Oh et al., 2008). CaWRKY30 was induced by various pathogens and SA, but repressed by virulent Meloidogyne incognita and the hormone methyl jasmonic acid (MeJA), suggesting that CaWRKY30 might be involved in SA and JA mediated plant defense (Zheng et al., 2011). Two other WRKYs in pepper, CaWRKY40 and CaWRKY58, were identified as positive and negative regulators respectively, in the resistance of pepper to R. solanacearum (Dang et al., 2013; Y., Wang et al., 2013). Additionally, an increasing number of WRKY TFs involved in mediating plant immunity have been found in different plant species. For example, VvWRKY1 and VvWRKY2 from grapevine (Vitis vinifera); GhWRKY11 and GhWRKY15 in cotton (Gossypium hirsutum L.); CIWRKY70 from watermelon (Citrullus lanatus) as well as SIWRKY70 in tomato (Solanum lycopersicum). They are characterized as important players in plant defense responses (Marchive et al., 2007; Mzid et al., 2007; Atamian et al., 2012; Cho et al., 2012; Sun et al., 2012; Yu et al., 2012; Marchive et al., 2013).

1.1.4 WRKY transcription factors in abiotic stresses

Abiotic stresses such as osmotic stress, drought, salt, heat, nutrient deficiency as well as reactive oxygen species (ROS), are defined as non-living, intangible and naturally occurring factors. They are harmful and threaten plant growth and product yields. To adapt to such adverse conditions, plants have evolved many strategies, which mainly occur at the cellular and molecular levels, and involve a number of transcription factors. The WRKY family is one of the regulatory protein families involved also in abiotic stress responses. Although involvement of WRKY TFs in abiotic studies are lagging behind biotic research, more attention is being paid to this field nowadays (Rushton et al., 2010; Agarwal et al., 2011; Chen et al., 2012).

In Arabidopsis, at least 17 out of 74 members of the WRKY family are currently reported to be involved in abiotic stress responses. For example, two closely related WRKY TFs, WRKY25 and WRKY33, are considered as positive regulators in response to salt stress. Their overexpression increased tolerance to NaCl but sensitivity to ABA (abscisic acid). Only slightly increased NaCl-sensitivity was discovered in both the wrky33 null mutant and the wrky25wrky33 double mutant, indicating functional redundancy with other TFs (Jiang and Deyholos, 2009). WRKY18, WRKY40 and WRKY60 in Arabidopsis have been investigated in biotic stress responses (Xu et al., 2006), but additional studies showed that they also acted in abiotic stress signaling, including the ABA signaling pathway. WRKY40 could directly inhibit the expression of ABA responsive genes such as ABA RESPONSIVE ELEMENT BINDING FACTOR 4 (ABF4), ABA INSENSITIVE 4 (ABI4), ABA INSENSITIVE 5 (ABI5), DEHYDRATION RESPONSE ELEMENT BINDING 1A (DREB1A), MYB2 (MYB DOMAIN PROTEIN 2) and RESPONSIVE TO ABA 18 (RAB18). WRKY40 interacts with two other antagonists WRKY18 and WRKY60 in the effect of plant sensitivity to ABA and abiotic stress (Chen et al., 2010; Shang et al., 2010). Ren et al. (2010) showed that the ABA overly sensitive
mutant, abo3/wrky63, was hypersensitive to ABA and less drought tolerant than wild type due to less efficient stomatal closure. Furthermore, other WRKYs such as WRKY15, WRKY57, WRKY8 and WRKY28 in Arabidopsis were identified as important regulators in abiotic stress responses, including osmotic stress, drought, salinity and oxidative stress (Jiang et al., 2012; Vanderauwera et al., 2012; Babitha et al., 2013; Hu et al., 2013).

There is also evidence that WRKYs function in abiotic stress responses in other plant species. Previous studies indicated that 10 of 13 WRKY genes and 8 of 15 WRKY genes in rice and wheat (Triticum aestivum), respectively, responded to NaCl, PEG (polyethylene glycol), cold or heat (Qiu et al., 2004; Wu et al., 2008). Many WRKY overexpressor lines have been employed in rice studies. The OsWRKY11 gene, for instance, which was induced by both heat and drought stresses, played a role in heat and desiccation tolerance when overexpressed under the HSP101 (HEAT SHOCK PROTEIN 101) promoter in rice (Wu et al., 2009). OsWRKY45 and OsWRKY72 were also identified to be involved in salt and drought stress responses in 35S:OsWRKY45 and 35S:OsWRKY72 Arabidopsis, resulting in alteration in expression of stress-related genes (Qiu et al., 2009; Yu et al., 2010). Additionally, two alleles of OsWRKY45 (OsWRKY45-1 and OsWRKY45-2) had opposite functions in ABA signaling, whereas only OsWRKY45-2 negatively regulated the rice response to salt stress (Tao et al., 2011). Overexpression of OsWRKY30 in rice dramatically increased drought tolerance, which was attributed to the activation of OsWRKY30 by MAP (MITOGEN-ACTIVATED PROTEIN) kinases (Shen et al., 2012). Overexpression of the TaWRKY10 from wheat in tobacco (Nicotiana tabacum L.) led to enhanced drought and salt stress tolerance, which demonstrated that TaWRKY10 acts as a positive factor in modulating osmotic balance and transcription of stress related genes in wheat (Wang et al., 2013). Nuclear proteins TaWRKY2 and TaWRKY19 were able to directly bind to the promoters of downstream genes related to various abiotic stresses. Transgenic Arabidopsis overexpressing TaWRKY2 or TaWRKY19 exhibited tolerance to salt, drought, and freezing stresses (Niu et al., 2012). Soybean (Glycine max) WRKY-type transcription factors GmWRKY13, GmWRKY21 and GmWRKY54, acted differently to various abiotic stresses. Ectopic expression of GmWRKY21 in Arabidopsis plant conferred tolerance to cold stress while overexpression of GmWRKY54 showed salt and drought tolerance through the control of DREB2A (DEHYDRATION RESPONSE ELEMENT BINDING 2A) and STZ/Zat10 (SALT TOLERANCE ZINC FINGER) expression. However, overexpression of GmWRKY13 decreased the tolerance to salt and osmotic stresses (Zhou et al., 2008).

In addition to the involvement of WRKYs in the abiotic stresses such as drought, osmotic stress, cold and salt, WRKYs also participate in the nutrient deficiency response, ROS signaling and wounding. Both AtWRKY6 and AtWRKY75 regulate the Pi (phosphate)-deficiency response but in different regulatory pathways (Devaiah et al., 2007; Chen et al., 2009). AtWRKY6 was the first reported representative to function as a positive regulator in the low-boron response (Kasajima et al., 2010). Transcriptome profiling showed that AtWRKY45 and AtWRKY65 regulated gene expression during carbon starvation, indicating the role of these two WRKYs in the nutrient deficiency response (Conte et al., 2004). In barley, HvWRKY46/SUSIBA2 was identified as being involved in the sugar signaling pathway (Sun et al., 2003; Sun et al., 2005).
Furthermore, ROS, a natural byproduct of many abiotic stresses, has important roles in cell signaling. WRKYs are also involved in ROS signaling. For instance, AtWRKY6, AtWRKY18, AtWRKY25, AtWRKY33, AtWRKY40, AtWRKY46, AtWRKY54, AtWRKY60 and AtWRKY70, were induced in a T-DNA knockout mutant of the key ROS-scavenging gene APX1 (ASCORBATE PEROXIDASE 1) during light stress, which implied their possible roles in ROS signaling (Rizhsky et al., 2004; Davletova et al., 2005; Ciftci-Yilmaz et al., 2007). Additionally, previous studies showed that WRKYs also take part in the early stage of wounding response (Hara et al., 2000; L., Chen et al., 2010).

1.1.5 WRKY transcription factors in plant growth and development

In addition to the roles of WRKY TFs in biotic and abiotic stress responses, they are also involved in the regulation of many plant growth and development processes, such as trichome development (Johnson et al., 2002), seed development (Luo et al., 2005), embryogenesis (Lagacé and Matton, 2004), senescence (Robatzek and Somssich, 2001; Robatzek and Somssich, 2002; Miao et al., 2004; Zentgraf et al., 2010; Zhou et al., 2011), dormancy (Pnueli et al., 2002) and metabolic pathways (Willmott et al., 1998).

Plant trichomes are defined as the protective screen from attack by predators. The Arabidopsis gene TTG2 (TRANSPARENT TESTA GLABRA 2), corresponding to WRKY44, was the first WRKY gene reported to control morphogenesis. Mutation of WRKY44 led to the disruption of trichome development and tannin and mucilage production in the seed coat (Johnson et al., 2002). In seed development, AtWRKY10 encoded by MINI3 (MINISEED 3) was associated with seed size. The wrky10 mutant produced significantly smaller seeds than wild-type due to reduced growth and early cellularization of the endosperm (Luo et al., 2005). Additionally, a recent study showed that AtWRKY10 recruited the protein SHB1(SHORT HYPOCOTYL UNDER BLUE1) to trigger endosperm proliferation and seed cavity enlargement (Kang et al., 2013). Seed germination and post germination growth are also important in seed development and these processes are also mediated by WRKY TFs. For example, AtWRKY2-mediated seed germination and post-germination developmental are arrested by ABA, and OsWRKY78 might act as a regulator in stem elongation and seed development (Jiang and Yu, 2009; Zhang et al., 2011). In the wild potato species (Solanum chacoense), ScWRKY1 has been found to be expressed in ovules bearing late torpedo-staged embryos, indicating the role of WRKY in embryogenesis (Lagacé and Matton, 2004).

Much progress has been made in senescence processes mediated by WRKY TFs compared to the other processes of plant development. In Arabidopsis, WRKY6 was found to activate one target gene termed SIRK (SENESCENCE-INDUCED RECEPTOR-LIKE SERINE/THREONINE PROTEIN KINASE), which was induced significantly during leaf senescence. The wrky6 mutant showed less green leaves while overexpression of WRKY6 resulted in the opposite phenotype (Robatzek and Somssich, 2002). A number of WRKY53 candidate target genes were identified including other WRKys and SENESCENCE-ASSOCIATED GENES (SAGs). Mutation and overexpression of WRKY53 displayed delayed and accelerated senescence phenotypes, respectively, and altered the
expression of target genes, suggesting WRKY53 plays a pivotal role in controlling plant senescence and regulating target gene expression (Miao et al., 2004). In addition, WRKY53 has been reported to mediate crosstalk between pathogen resistance and senescence with the epithiospecifying senescence regulator (ESR), governed by the JA and SA equilibrium in Arabidopsis (Miao and Zentgraf, 2007). WRKY53 was considered as a positive regulator during senescence (Miao et al., 2004; Zentgraf et al., 2010). By contrast, WRKY70 acted as a negative regulator in the senescence process (Ulker et al., 2007). Loss of the WRKY70 gene promoted both developmentally and dark-induced senescence (Ulker et al., 2007). Overexpression or knockout of WRKY22 displayed accelerated or delayed senescence phenotypes when dark treated and the expression of senescence-associated genes was altered. Additionally, mutual regulation existed between both WRKY22 and WRKY6, and, WRKY53 and WRKY70 in Arabidopsis (Zhou et al., 2011).

1.2 Interactions of WRKYs and their partners

1.2.1 Regulation of WRKY-dependent signaling pathways

1.2.1.1 Auto-regulation and cross-regulation

WRKY proteins are involved in diverse biological processes in plants, controlling the expression of downstream genes related to biotic or abiotic stress responses either positively or negatively. The regulation of WRKY-dependent signaling pathways is very extensive and complex. Many aspects of this regulation have been investigated recently, including transcriptional regulation, DNA-binding affinity as well as post-translational regulation (Ishihama and Yoshioka, 2012; Chi et al., 2013). WRKY genes are responsive to internal and external stimuli. Consequently, they integrate the signals and trigger the expression of target genes through binding to their W box-containing promoters. In fact, many WRKY genes themselves are enriched for W boxes in their promoters, suggesting the possibility of auto-regulation by themselves or cross-regulation by other WRKY TFs (Eulgem and Somssich, 2007; Rushton et al., 2010). For example, WRKY1 from parsley (Petroselinum crispum) has been reported to bind to not only the W box of its native promoter but also the promoters of PcWRKY3 and the marker gene PcPR1, as revealed by chromatin immunoprecipitation (ChIP) (Eulgem et al., 1999; Turck et al., 2004). PAMP-triggered early responses recruited PcWRKY1 to the three synergistically acting W boxes (W_ABC), which were occupied constitutively by pre-bound WRKY repressors. Meanwhile, PcWRKY1 was also employed to bind to the W box in the promoter of the target gene, PcPR1 (Fig.2), leading to the repression of PcWRKY1 itself and activation of PcPR1 (Turck et al., 2004). Thus, PcWRKY1 was involved in PAMP-induced regulation of a WRKY gene and its target gene through a negative feedback loop and direct activation (Fig.2). In addition, ChIP studies also showed that in Arabidopsis, pathogen-inducible WRKY33, whose expression was regulated by the MITOGEN-ACTIVATED PROTEIN
KINASES 3/6 (MPK3/6), could bind to its own promoter in vivo for auto-regulation through a potential positive feedback loop (Mao et al., 2011). WRKY33 was also reported to be involved in thermo-tolerance in cooperation with WRKY25 and WRKY26. Any of these three genes was positively regulated by the other two during heat stress, indicating the cross-regulation between WRKY25, WRKY26 and WRKY33 (Li et al., 2011). Furthermore, ChIP and gel shift assays indicated that the ABA signaling regulators in Arabidopsis, WRKY18, WRKY40 and WRKY60, directly bound to the W box regions in the promoters of their respective genes WRKY18, WRKY40 and WRKY60, allowing the repression of the expression of all three WRKY genes (Chen et al., 2010; Yan et al., 2013).

Taken together, these findings for the auto-regulation and cross-regulation of various WRKY TFs imply a potential mechanism to keep the homeostasis of the transcription repression and activation in different WRKY-dependent signaling pathways in response to biotic and abiotic stresses.

**Figure 2.** A schematic model which describes the involvement of *PcWRKY1* in PAMP (pathogen-associated molecular pattern)-induced regulation of the *WRKY* gene and its target gene through a negative feedback loop or directly binding to the W box of *PcPR1* gene. W_A, W_B and W_C represent a specific arrangement of the W box in the promoter of *PcWRKY1*, interacting with constitutively expressed WRKY proteins (modified from Turck et al., 2004).

### 1.2.1.2 MPKs in regulation

MPK cascades are also involved in regulation of WRKY TFs (Fiil et al., 2009; Rushton et al., 2010; Ishihama and Yoshioka, 2012; Chi et al., 2013). The MPKs in Arabidopsis as
well as WIPK (WOUND-INDUCED PROTEIN KINASE) and SIPK (SALICYLIC ACID-INDUCED PROTEIN KINASE) in other plant species, have been identified and studied (Zhang and Klessig, 2000; Pitzschke et al., 2009). In Arabidopsis, it has been shown that WRKY33, required for pathogen-induced production of phytoalexin, functioned in the nucleus as a result of release from the MPK4-MKS1 (MPK4 SUBSTRATE)-WRKY33 complex. Upon bacterial infection, the activated MPK4 phosphorylated MKS1, and WRKY33 was released, leading to the binding of WRKY33 to the promoter of the target gene, PHYTOALEXIN DEFICIENT 3 (PAD3), which was required for the synthesis of antimicrobial camalexin (Qiu et al., 2008). Moreover, WRKY33 could be phosphorylated by two other MPKs, MPK3 and MPK6, which led to binding to its own and the PAD3 promoters in response to B. cinerea. In the wrky33 mutant, the induction of camalexin production was abolished, and the mutation of MPK3/MPK6 phosphorylation sites in WRKY33 had the same effect. These findings suggested that WRKY33 acted downstream of the MPK3/MPK6 cascade and the phosphorylation of WRKY was crucial in the regulation of pathogen-induced camalexin production (Mao et al., 2011). In tobacco, WRKY8 was identified as substrate of SIPK and its partner NTF4 (a tobacco MPK) and WIPK. Phosphorylation of WRKY8 by MPKs was dependent on the D domain, a MPK interacting motif. Phosphorylation increased the DNA binding activity of WRKY8 to the W box of target genes, resulting in defense response to pathogens in tobacco (Ishihama et al., 2011). Likewise, the MPK in rice, in terms of BWMK1 (BLAST AND WOUNDING-ACTIVATED MAP KINASE 1), has been identified to phosphorylate OsWRKY33, thereby enhancing the DNA-binding ability of OsWRKY33 to PR gene promoters. SA-dependent expression of the GUS-reporter gene driven by the W box and the PRI promoter was elevated when OsBWMK1 and OsWRKY33 were co-expressed in Arabidopsis protoplasts. This indicated that OsBWMK1 mediated SA-dependent defense responses through phosphorylation of WRKY TFs in rice (Koo et al., 2009). However, the phosphorylation of WRKYs involved in abiotic stress tolerance by MPK cascades is less well-studied when compared to those in biotic stress responses. OsWRKY30 is one of the recent examples related to the function of phosphorylation in abiotic stress response. Overexpression of OsWRKY30 led to tolerance to drought stress, whereas the mutation of the SP (serine residue followed by proline residue) site phosphorylated by MPK cascades resulted in the abolishment of tolerance to drought. This illustrates the importance of phosphorylation of OsWRKY30 in tolerance to drought stress (Shen et al., 2012). WRKY TFs are downstream of MPK cascades and phosphorylated by these kinases, increasing the capacity of WRKYs to bind to the promoters of target genes which are involved in the plant defense and environmental stress responses (summarized in Fig.3).
Figure 3. The involvement of MPKs cascades in regulation of WRKY transcription factors. Three examples of pathways are shown in this model, including responses to different stresses in different plant species. In Arabidopsis, MPK4 is activated by infection and phosphorylates MKS1; WRKY33 is released and binds to the W box of the target gene, PAD3, which is required for the synthesis of camalexin. In tobacco (Nicotiana tabacum L.), WRKY8 is phosphorylated by SIPK, NTF4 and WIPK on the D domain, which is a MPK interacting motif, leading to increased DNA binding activity to the defense related genes. In rice (Oryza sativa), the corresponding MPKs are BWMK1 and MPK3 involved in wounding and drought stresses, respectively. WRKY33 and WRKY30 are the two important transcription factors resulting in SA-dependent defense response and drought tolerance, separately. Abbreviations: MPKs, MITOGEN-ACTIVATED PROTEIN KINASES; MPK4, MITOGEN-ACTIVATED PROTEIN KINASE 4; MKS1, MPK4 SUBSTRATE; PAD3, PHYTOALEXIN DEFICIENT 3; SIPK, SALICYLIC ACID-INDUCED PROTEIN KINASE; NTF4, redundant partner of SIPK; WIPK, WOUND-INDUCED PROTEIN KINASE; BWMK1, BLAST AND WOUNDING-ACTIVATED MAP KINASE 1; MPK3, MITOGEN-ACTIVATED PROTEIN KINASE 3 (adapted from Qiu et al., 2008; Koo et al., 2009; Ishihama et al., 2011; Mao et al., 2011; Shen et al., 2012).

1.2.2 WRKY and their partners

1.2.2.1 Histone-modifying complex and WRKY

The regulation of gene expression requires changes in the chromatin structure (Jaskiewicz et al., 2011; Chi et al., 2013). It is known that the basic unit of chromatin is nucleosome, which constitutes DNA (~147bp) and a histone octamer. The histone octamer is composed of two copies of each of four core histones: H2A, H2B, H3 and H4 wrapped by
the DNA and locked by a linker histone H1. When a gene is transcribed, the promoter of the gene is bound by the core transcriptional machinery proteins (RNA polymerase, transcription factors, and activators or repressors), allowing transcription to occur. DNA in the nucleus is tightly packaged, with the help of packaging proteins, forming a condensed chromatin structure. This does not easily interact with transcription machinery proteins to allow transcription. Thus, alteration of chromatin structure by chromatin remodeling proteins is the prerequisite for gene regulation (Glatt et al., 2011). Histone-modifying complex, one of the chromatin remodeling proteins which modify the histone by acetylation, methylation, phosphorylation and ubiquitination, either loosens or tightens the DNA, allowing activation or repression of gene expression, respectively (Glatt et al., 2011).

In addition to auto-regulation, cross-regulation and MPKs, histone-modifying complex is also involved in the regulation of WRKYs. In Arabidopsis, a few WRKY TFs have been reported to be involved in histone modifications, for example, WRKY70 was activated by the Arabidopsis homolog of trithorax (ATX1), through which the nucleosomal histone H3K4 trimethylation (me3) was established. Moreover, the SA-responsive gene, PR1, and the JA-responsive gene, THI2.1 (THIONIN2.1) also contain the H3K4me3. These findings suggest that the downstream genes such as PR1 and THI2.1 are controlled by the epigenetic regulation of WRKY70 (Alvarez-Venegas et al., 2007). Additionally, WRKY53 in Arabidopsis, a key regulator of senescence, was also related to epigenetic regulation by histone methyltransferase. When WRKY53 was activated during senescence, an increasing level of H3K4me2 and H3K4me3 was found at both the 5’end and the coding regions of the gene. These methylation marks indicated the actively transcribed WRKY53 during senescence (Ay et al., 2009). One more example of histone modification focused on two structurally similar type III WRKY TFs in Arabidopsis, WRKY38 and WRKY62. They both interacted with HDA19 (HISTONE DEACETYLASE 19), as identified by yeast two-hybrid screens. HDA19 has an opposite role compared to those of WRKY38 and WRKY62 in basal resistance to bacterial pathogens. Interestingly, the activation abilities of WRKY38 and WRKY62 were reduced by overexpression of HDA19, as HDA19 removed acetyl groups from histone tails, leading to tightly packaged chromatin structure of WRKY38 and WRKY62 genes (Kim et al., 2008). The latest report related to the interaction between chromatin remodeling protein and WRKYs showed that the linker histone H1 gene termed HIS1 in banana (Musa acuminate), was induced by ethylene during fruit postharvest ripening. Yeast two-hybrid and bimolecular fluorescence complementation assays (BiFC) showed that the protein MaHIS1 could interact with MaWRKY1 in banana (Wang et al., 2012).

1.2.2.2 WRKY and WRKY proteins interaction

Increasing evidence indicates that WRKY proteins interact with each other to form homo- or hetero-complexes in many stress responses (Chi et al., 2013). The most extensively studied WRKYS involved in self- and mutual-interactions were three group II WRKY proteins in Arabidopsis, WRKY18, WRKY40 and WRKY60 (Xu et al., 2006; Chen et al.,
2010; Shang et al., 2010). In plant defense, single mutants of wrky18, wrky40 and wrky60 showed no or very small alterations in response to the pathogens P. syringae and B. cinerea, whereas wrky18wrky40 and wrky18wrky60 double mutants and wrky18wrky40wrky60 triple mutants exhibited enhanced tolerance to P. syringae but reduced tolerance to B. cinerea. These mutant studies suggest that these three genes were cooperative and partially redundant in plant basal defense (Xu et al., 2006). Moreover, overexpression of WRKY18 led to improved tolerance to P. syringae, whereas co-expression of WRKY18 and WRKY40 or WRKY18 and WRKY60 resulted in reduced resistance to both P. syringae and B. cinerea, illustrating the antagonistic role of these three proteins in response to pathogens (Xu et al., 2006). In addition, co-immunoprecipitation assay provided the evidence for the interactions of WRKY18 with itself and with WRKY40 and WRKY60 in vivo. The hetero-complexes of WRKY18 and WRKY40 enhanced the DNA binding activity when compared to the binding activity of the homo-complexes of WRKY18 or WRKY40, while the interaction of WRKY18 and WRKY60 showed selectively enhanced DNA binding activity to the promoters containing the W-box arranged in specific manner. In contrast, the DNA binding activity of WRKY40 was reduced when mixed with WRKY60 (Xu et al., 2006). Not only do the three WRKYs play a role in plant defense, but also they are important in ABA signaling-related seed germination and post-germination growth (Chen et al., 2010; Shang et al., 2010). In protoplasts, WRKY60 could be recognized and activated by WRKY18 and WRKY40. Thus, WRKY60 might be a direct target gene of WRKY18 and WRKY40 in ABA signaling. On the other hand, the ABA hypersensitive phenotypes in different combinations of wrky mutants showed a strong phenotype of ABA hypersensitivity in wrky40 single, wrky40wrky18 double and wrky40wrky18wrky60 triple mutants rather than the other combinations, suggesting that WRKY40 had a more important role than the other two WRKYs in ABA signaling. Nevertheless, WRKY60 acted as a regulator to balance the negative roles of WRKY40 and WRKY18 in ABA signaling, as revealed by the repressed ABA hypersensitive phenotypes in wrky40wrky60 and wrky18wrky60 mutants (Chen et al., 2010; Shang et al., 2010).

WRKY-WRKY interactions have also been found in other plant species. In rice, the interactions of OsWRKY71 proteins themselves and OsWRKY71 and OsWRKY51 were verified by BiFC assay in the nuclei of aleurone cells. The synergistic interaction of these two WRKYs played an essential role in the regulation of gene expression concerning cross-talk between ABA and GA (gibberellins) signaling (Xie et al., 2006).

1.2.2.3 WRKY and other factors

Calmodulin (CaM) is another factor which can bind to the conserved structural motif termed Ca$^{2+}$-dependent CaM-binding domain (CaBD) in WRKY group members (Park et al., 2005). With the help of several techniques, such as site-directed mutagenesis, gel mobility shift assay, split-ubiquitin assay and competition assay, WRKY7 in Arabidopsis was shown to bind to CaM specifically through the CaBD in WRKY7 itself. Moreover, it has been shown that the CaBD of WRKY7 was also conserved in group IIId of the WRKY
members, which suggests that WRKY group IId members might be regulated by CaM to some extent (Park et al., 2005). Increased Ca\(^{2+}\) concentration in the cells by a variety of signals triggered the interaction of CaM and WRKY group II members. The CaM-WRKY interaction was more preferential than the WRKY-WRKY interaction if the interaction domains in WRKYS were too close or overlapping, which provided a possible way to regulate WRKY-WRKY interaction in the presence of Ca\(^{2+}\) (Knight, 2000; Chi et al., 2013). One of the most widespread proteins in plants, termed 14-3-3 protein, which specifically binds to phosphoserine and phosphothreonin, regulates a wide range of plant development and stress responses (Roberts, 2003; Denison et al., 2011). 14-3-3 proteins bind directly to transcription factors and other signaling protein components and control many processes. Based on identification by the tandem affinity purification tag assay, 7 WRKY family members in Arabidopsis, including WRKY6, WRKY16, WRKY18, WRKY19, WRKY27, WRKY32 and WRKY40, were shown as putative interaction partners for 14-3-3 proteins. These results suggest that 14-3-3 proteins might have potential roles in regulating biotic and abiotic stress responses through WRKYS involved in these responses (Chang et al., 2009; Rushton et al., 2010; Chi et al., 2013). Recently, it was shown that a group of cofactors designated VQ proteins, containing a short VQ (FxxxVQ\(xLTG\)) motif, could also cooperate with WRKY proteins. In Arabidopsis, WRKY33 interacted with two VQ proteins, SIGMA FACTOR-INTERACTING PROTEIN1 (SIB1) and SIB2 through the C terminal of WRKY domains. These interactions with VQ proteins activated the DNA-binding activity of WRKY33 (Lai et al., 2011). Furthermore, additional studies showed that 34 VQ proteins found in Arabidopsis, exclusively cooperated with the C-terminal of the WRKY domains of group I and group IIC WRKY proteins. These results demonstrate that VQ proteins are crucial cofactors in regulating WRKY-mediated gene expression (Cheng et al., 2012; Chi et al., 2013). In addition, resistance (R) proteins inactivated the repressing function of WRKY proteins in ETI, leading to activation of defense genes in the nucleus triggered by the effectors. In barley, HvWRKY1/2 interacted with the CC domain of intracellular mildew A (MLA) R protein, therefore the repressing function of HvWRKY1/2 was interfered with and the basal defense was activated (Shen et al., 2007).

The regulation of WRKY TFs is evidently complex. WRKYs are able to auto-regulate themselves or cross-regulate each other. In addition, a wide range of protein partners including MPKs, chromatin remodeling proteins, other WRKY proteins, CaM, 14-3-3 proteins, VQ proteins as well as R proteins, participate in this complex network of WRKY-mediated transcriptional reprogramming. The interactions between WRKYs and their partners provide knowledge of a dynamic regulatory and functional network.

1.3 Crosstalk in phytohormone mediated signaling pathways

Phytohormones are involved in many aspects of signaling pathways of plants in response to a variety of biotic and abiotic stresses. In nature, these phytohormones such as SA, JA, ethylene (ET) and ABA are not working alone, they interact either synergistically or antagonistically in the development of plant responses to pathogens or environmental
stresses. (Gazzarrini and McCourt, 2003; Robert-Seilaniantz et al., 2011; Cui and Luan, 2012; Proietti et al., 2013). Furthermore, the convergence between biotic and abiotic stress signaling is governed by crosstalk among hormone signaling pathways. Several molecules such as transcription factors and kinases are involved in this crosstalk mediated by SA, JA, ET and ABA (Fujita et al., 2006; Atkinson and Urwin, 2012).

1.3.1 Crosstalk involved in SA and JA/ET signaling pathways

Plants encounter various microbial pathogens and herbivorous insects during their lifetime. Thus, plants have evolved effective defense systems to recognize and activate a series of responses to deal with the attack from numerous invaders. To elucidate the complexity in regulation of defenses to pathogens and herbivores, many important defense-related hormones SA, JA and ET have been identified (Kunkel and Brooks, 2002; Koornneef and Pieterse, 2008; Thaler et al., 2012; Proietti et al., 2013). Evidence has shown that SA, JA and ET are the key players in controlling plant resistance responses. Although SA-, JA- and ET-mediated signaling pathways are apparently independent, they influence each other via a complex network of defense responses (Kunkel and Brooks, 2002; Koornneef and Pieterse, 2008). Given that plant pathogens can be divided into biotrophs and necrotrophs based on their lifestyles, the function of the hormone-mediated defense is specific in most cases. The SA-mediated signaling pathway is triggered when plants are attacked by biotrophic pathogens, whereas JA/ET mediated responses are involved in protection from invasion of necrotrophic pathogens and herbivorous insects (Glazebrook, 2005; Koornneef and Pieterse, 2008; Thaler et al., 2012). However, plants always confront multiple enemies rather than a single offender in nature. It is obvious that the corresponding defense signaling pathways mediated by phytohormones need to cooperate to effectively cope with the adverse environment (Koornneef and Pieterse, 2008). This crosstalk between different signaling pathways can be shown to be either antagonistic or synergistic, leading to negative or positive interactions. Moreover, crosstalk is considered as a potential benefit for plants, allowing them to fine-tune the plant defense to invaders by choosing an optimal defense pathway with low energy costs (Kunkel and Brooks, 2002; Koornneef and Pieterse, 2008).

1.3.1.1 SA and JA/ET mediated signaling pathways

Over the past few years, the SA-mediated pathway has been shown repeatedly to play a key role in plant defense against pathogens. Upon infection by pathogens, SA accumulates in plants. Furthermore, application of exogenous SA to the plant tissues causes enhanced resistance to certain types of plant pathogens (Kunkel and Brooks, 2002; Koornneef and Pieterse, 2008; Thaler et al., 2012). Increased SA levels lead to rapid activation of defense responses during which defense related genes such as PR genes are activated. This activation is critical for local defense and is able to induce whole-plant adaptive responses to pathogens termed systemic acquired resistance (SAR) (Kunkel and Brooks, 2002; Koornneef and Pieterse, 2008).
Brooks, 2002; Koornneef and Pieterse, 2008). SAR was described by the restriction of pathogen growth and the enhancement of disease resistance in the whole plant systematically after local infection (Lawton et al., 1995; Cao et al., 1997; Kunkel and Brooks, 2002). Several mutants in Arabidopsis which lack the ability to accumulate SA, including eds1, eds4, eds5 (enhanced disease susceptibility 1,4,5), pad4 (phytoalexin deficient 4) and sid2 (SA induction deficient 2) show reduced resistance to several biotrophic pathogens such as *Peronospora parasitica* and *P. syringae* (Glazebrook et al., 1996; Nawrath and Métraux, 1999; Feys and Parker, 2000; Kunkel and Brooks, 2002). Moreover, transgenic lines expressing *NahG* (a bacterial gene encoding SA hydroxylase) was also susceptible to the pathogens mentioned above due to reduced levels of SA (Lawton et al., 1995). The common characteristic of these mutants and transgenic lines was the abolishment of SAR, which indicated the importance of SA in defense signaling. In contrast to mutants in the SA-mediated pathway, the mutants in Arabidopsis such as coi1 (coronatine insensitive 1) and jar1 (jasmonic acid resistant 1) that are impaired in JA perception, display reduced resistance to a series of necrotrophic pathogens, for example, *A. brassicicola*, *B. cinerea* and *P. carotovora* (Thomma et al., 1998; Norman-Setterblad et al., 2000; Kunkel and Brooks, 2002). Likewise, the mutant ein2 (ethylene insensitive 2) which is involved in the ET signaling pathway, showed the same susceptibility as JA signaling mutants to *B. cinerea* and *P. carotovora* (Thomma et al., 1999; Norman-Setterblad et al., 2000) although enhanced resistance of the *ein2* mutant to some specific pathogens has been also reported (Bent et al., 1992). JA and ET were both required for the establishment of induced systemic resistance (ISR) mediated by rhizobacteria and the wounding response (Pieterse and van Loon LC, 1999).

1.3.1.2 Crosstalk between SA and JA signaling pathways

The crosstalk between SA and JA mediated defense pathways is well established. Although several studies indicate that SA and JA interaction is both synergistic and antagonistic, the SA-JA antagonism appears prominent (Kunkel and Brooks, 2002; Koornneef and Pieterse, 2008). The presence of SA-JA antagonism has been reported in 17 plant species, and appears to have originated before the split of gymnosperms and angiosperms (Thaler et al., 2012). The antagonistic effect between SA- and JA-mediated signaling pathways has been revealed by many genetic studies. NPR1, the famous regulatory protein has most recently been identified as the receptor for SA (Wu et al., 2012). *NPR1* was induced by SA treatment, and at the protein level, NPR1 could be localized to the nucleus in the presence of SA in Arabidopsis. Here, it interacted with TGA TFs (TF which can recognize the TGACG element in promoters), and led to the activation of SAR through expression of SA responsive *PR* genes (Dong, 2004). In the *npr1* mutant, the transduction of the SA signal is blocked and SAR is not established, leading to the activated JA synthesis and signaling (Spoel et al., 2003; Dong, 2004). Intriguingly, nuclear localization of NPR1 is necessary for *PR* gene activation, but not for the prevention of the JA signaling pathway. NPR1 inhibited the JA mediated defense either by binding to the positive regulator or negative regulator in JA signaling (Spoel et
Similarly, the SA-JA crosstalk mediated by NPR1 has also been found in tobacco. NaNPR1 negatively regulates SA production, allowing the unaltered JA signaling pathway to cope with attack by herbivores (Rayapuram and Baldwin, 2007; Thaler et al., 2012).

WRKY TFs are crucial mediators in the SA-JA interaction (Koornneef and Pieterse, 2008; Thaler et al., 2012). One of the representatives of the WRKY family, WRKY70 in Arabidopsis, modulates the selection of SA-dependent and JA-dependent signaling pathways in plant defense. WRKY70 has been shown to be induced by SA but repressed by JA. Constitutive overexpression of WRKY70 enhances the expression of SA-induced pathogenesis-related genes. By contrast, the JA-responsive plant defensin gene PDF1.2 is activated when WRKY70 is silenced. Thus, WRKY70 is considered as the node of convergence between the SA and JA signaling pathways (Li et al., 2004; Li et al., 2006). Moreover, WRKY11 and WRKY17 in Arabidopsis are also involved in SA-JA crosstalk. In the regulation of basal defense to P. syringae, WRKY11 and WRKY17 positively regulate the pathogen-induced JA related genes, LOX2 and AOS, which are the key genes encoding enzymes for JA biosynthesis. In addition, they negatively regulate the expression of WRKY70 that is considered as a central player between the SA and JA signaling pathways (Li et al., 2004; Journot-Catalino et al., 2006; Li et al., 2006). In addition to Arabidopsis, the ortholog of WRKY70 in rice, OsWRKY13, positively regulates SA-induced responses but negatively controls JA-induced responses. Overexpression of OsWRKY13 activates the SA synthesis genes and SA-responsive genes but reduces the expression of JA related genes (Qiu et al., 2007).

Glutaredoxin 480 (GRX480), one of the members in the glutaredoxin family, belongs to a superfamily of redox proteins which mediate redox regulation of proteins by catalyzing disulfide transitions (Bouarab et al., 2009). Overexpression of GRX480 results in near wild-type expression of marker genes for the SA response but reduced expression level of the JA-dependent gene PDF1.2, indicating the role of GRX480 in SA-JA crosstalk (Ndamukong et al., 2007). MPKs are also involved in the SA-JA crosstalk of plant defense (Ligterink et al., 1997; Innes, 2001). Previous studies showed that one of the MAP kinases in Arabidopsis, MPK4, acted as a negative regulator of SA signaling but a positive regulator of JA signaling (Petersen et al., 2000; Brodersen et al., 2006). In the knockout mutant mpk4, SA dependent defense was constitutively activated, while the induction of the JA defense response was repressed. Two of the defense regulators EDS1 and PAD4 also participated in the signaling pathway of mpk4 since mutations in these genes resulted in abolishment of activation of SA signaling and suppression of the JA signaling pathway in mpk4 (Brodersen et al., 2006). Hence, EDS1, PAD4 and MPK4 work together to control the SA-JA crosstalk, although EDS1 and PAD4 act oppositely to MPK4 (Brodersen et al., 2006). Recently, new data suggested a potential role of JAZ (JASMONATE-ZIM DOMAIN) proteins in SA-JA crosstalk (Kazan and Manners, 2012). JAZ proteins are co-receptors of JA but repressors of the JA signaling pathway in Arabidopsis. When the JA level is low or JA signaling not activated, JAZ suppresses the expression of the JA-responsive ethylene-signaling gene EIN3/EIL1, which in turn encodes suppressors of SA biosynthesis through blocking SID2. Upon infection by necrotrophic pathogens, JAZ proteins are degraded, eliminating suppression of
EIN3/EIL1, resulting in the activation of EIN3/EIL1 and blocking SA signaling (Kazan and Manners, 2012). Taken together, several factors contribute to the antagonistic interactions between the SA and JA signaling pathways, which commonly occur in wide variety of plants.

Despite the prominence of antagonistic interactions between SA- and JA-mediated defenses in plant immunity, synergistic crosstalk exists as well, as revealed by microarray analysis (Schenk et al., 2000). Upon treatment with a combination of SA and JA, a large number of genes were co-induced or co-repressed, suggesting the synergistic cooperation of the SA and JA signaling pathways (Schenk et al., 2000). Moreover, it has been shown that the antagonism and synergism of SA-JA are dependent on the treatment times and the concentration of the hormones used (Mur et al., 2006).

1.3.1.3 Crosstalk between SA and ET signaling pathways

ET is the gaseous hormone that is involved in plant growth and fruit ripening. In addition, ET also functions in plant defense signaling upon pathogen attack and wounding (van Verk et al., 2009). Similar to the SA-JA interaction, SA and ET can cooperate either positively or negatively. The positive crosstalk between SA and ET has been identified in both Arabidopsis and tomato. In Arabidopsis, ET had a crucial role together with SA in cell death and resistance, as revealed by genetic analysis of the lesion mimic mutant vad1-1 (vascular associated death1-1) (Bouchez et al., 2007). Likewise, SA and ET acted in an equivalent manner in response to Xanthomonas campestris pv. vesicatoria infection in tomato. The alteration of ET synthesis or perception influenced the SA accumulation greatly in infected tissues of tomato in response to Xcv (O’Donnell et al., 2001). These examples suggested a positive cooperation between SA and ET in plant defense. In contrast, antagonism in SA-ET crosstalk has also been verified. In mutant the cev1 (cellulose synthase gene CeSA3), JA and ET responses were constitutively activated, and the JA responsive genes PDF1.2 and VSP2 (VEGETATIVE STORAGE GENE 2) were not suppressed by SA. Further pharmacological assays and mutant studies illustrated that ET signaling could render the JA signaling insensitive to the suppression of SA upon multi-attacker invasion (Leon-Reyes et al., 2010). This might suggest that the JA and ET signaling pathways take the precedence over SA mediated defense pathways in plant defense (Leon-Reyes et al., 2010).

Taken together, the studies of crosstalk between SA, JA and ET suggest a complex network of different signaling pathways, allowing plants to fine-tune the responses to different stresses, especially to biotic stresses (Fig.4).
1.3.2 Crosstalk involved in ABA, SA and JA/ET signaling pathways

1.3.2.1 ABA-mediated signaling pathway

The first evidence for ABA was found in young fruit. ABA increased greatly when fruit approached abscission (Addicott, 1982). Nowadays, an increasing number of studies have shown that ABA serves as an endogenous messenger involved in biotic and abiotic stress responses as well as seed germination and further development (Christmann et al., 2006; Adie et al., 2007; Hirayama and Shinozaki, 2007; Raghavendra et al., 2010). The well-established predominant role of ABA is in abiotic stress signaling, including responses to drought/osmotic and salt stresses (Xiong et al., 2002; Jakab et al., 2005; Zhang et al., 2006; Ren et al., 2010). ABA accumulates when plants confront drought/osmotic, and salt stresses, but this can be rapidly catabolized following the relief of stresses (Taylor et al., 2000; Xiong et al., 2002). Increasing endogenous ABA levels trigger stomatal closure through the regulation of guard cell movement due to desiccation resulting from osmotic or salt stresses (Zhu, 2002). Simultaneously, many stress-related genes are activated by ABA, improving the cellular dehydration tolerance in plants (Xiong et al., 2002; Shinozaki and Yamaguchi-Shinozaki, 2007; Tuteja, 2007). Additionally, both exogenous application of ABA and accumulated endogenous ABA can induce cold tolerance in plants, indicating that ABA also plays a critical role in the low temperature stress
In ABA signaling, three types of proteins constitute the central signaling pathway in the early event of ABA-mediated plant response to stresses. The first important protein class is the ABA receptor, PYR/PYL/RCAR (PYRABACTIN RESISTANCE/PY-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR) family, which belongs to START (STAR-RELATED LIPID-TRANSFER) proteins (Ma et al., 2009; Park et al., 2009). Although Ma et al. (2009) and Park et al. (2009) used different methods, they both discovered that ABA directly bound PYR/PYL/RCAR proteins, indicating that they acted as ABA receptors. Moreover, it was reported that PYR/PYL/RCAR was able to directly interact with type 2C protein phosphatases (PP2Cs) such as ABI1 (ABSCISIC ACID INSENSITIVE 1), HAB1 (HOMOLOGY TO ABI1) and AIP1 (AKT1 INTERACTING PROTEIN PHOSPHATASE 1) that were considered as the second central components in ABA signaling (Saez et al., 2004; Ma et al., 2009; Park et al., 2009; Lim et al., 2012). In the presence of ABA, PYR/PYL/RCAR firstly binds to ABA and then interacts with PP2Cs to inhibit the function of PP2Cs on downstream signal transduction (Miyazono et al., 2009; Yin et al., 2009). The third protein class consists of protein kinases functioning in protein phosphorylation and dephosphorylation, such as SnRK2s. These are suppressed by PP2C in the absence of ABA, but de-repressed by inhibiting the phosphatase activity of PP2C via interaction with PYR/PYL/RCAR in the presence of ABA, allowing SnRK2s to activate the target proteins (Ma et al., 2009; Park et al., 2009). The regulation of stomatal closure by ABA provides a well-studied model of ABA signaling involving PYR/PYL/RCAR-PP2C- SnRK2s complex interaction. When the level of ABA is low, a SnRK2-type kinase, for example, OST1, is inhibited by PP2C phosphatases. The components of S-type anion channels, SLAC1 and SLAH3, are not phosphorylated and quite stable under low ABA content. However, in the presence of high levels of ABA as a result of drought or other stresses, the interaction between PYR/PYL/RCAR and PP2C is promoted by binding ABA, releasing the SnRK2 (OST1) to phosphorylate its target SLAC1, and activate anion efflux through anion channels. Through this process, the turgor pressure of guard cells is reduced, resulting in stomatal closure (Geiger et al., 2009; Lee et al., 2009; Geiger et al., 2010; Geiger et al., 2011; Lee and Luan, 2012).

1.3.2.2 TFs in ABA signaling

Several groups of TFs have been characterized by having roles in ABA signaling (Yoshida et al., 2010; Antoni et al., 2011; Lindemose et al., 2013; Nakashima and Yamaguchi-Shinozaki, 2013). The bZIP-type ABFs/AREBs (ABA RESPONSIVE ELEMENT BINDING PROTEINS/FACTORS) are positive regulators in ABA signaling. Three members of this family, AREB1/ABF2, AREB2/ABF4 and ABF3, have been characterized by genetic analysis of the mutants or the transgenic lines with the corresponding genes, indicating their pivotal role in the ABA mediated response to drought or osmotic stress (Kang et al., 2002; Fujita et al., 2005; Yoshida et al., 2010). The triple mutant of areb1areb2abf3 in Arabidopsis was insensitive to ABA and showed
reduced drought tolerance. Furthermore, several stress-related genes were not expressed in the areb1areb2abf3 triple mutant, suggesting that these three TFs acted coordinately in controlling gene expression and stress tolerance mediated by ABA (Yoshida et al., 2010). Moreover, overexpression of AREB1/ABF2 or AREB2/ABF4 or ABF3 in Arabidopsis displayed enhanced ABA sensitivity and more resistance to drought (Kang et al., 2002; Fujita et al., 2005). In contrast to the TFs mentioned above, other members in the bZIP group such as AB15, EEL (ENHANCED EM LEVELS), bZIP67, and AREB3 have been shown to function mainly in seed development rather than in vegetative tissues (Finkelstein and Lynch, 2000; Bensmihen et al., 2002; Bensmihen et al., 2005; Fujita et al., 2011; Lindemose et al., 2013). They also regulate the ABA-mediated ABRE-dependent gene expression during seed germination and maturation (Nakashima and Yamaguchi-Shinozaki, 2013).

Interestingly, WRKY group members have also been implicated in ABA-mediated signaling, although they are widely considered as regulators in plant defense or SA signaling (Dong et al., 2003; Ulker and Somssich, 2004). Recent evidence has shown that WRKY TFs are also key components in ABA signaling (Antoni et al., 2011; Chen et al., 2012; Rushton et al., 2012; Lindemose et al., 2013). WRKY40 in Arabidopsis, for instance, was identified as a negative regulator of ABA signaling in seed germination and an inhibitor of expression of important ABA-responsive genes, interacting with the antagonists WRKY18 and WRKY60 (Chen et al., 2010; Shang et al., 2010). Similarly, disruption of WRKY63/ABO3 in Arabidopsis enhanced ABA sensitivity but reduced tolerance to drought stress due to impaired ABA-induced stomatal closure in the mutant. In addition, as revealed by gene expression analysis, the ABF2/AREB1 level was lower in the abo3 mutant than in the wild type, which was consistent with the binding ability of WRKY63 to the promoter of ABF2/AREB1 in vitro. In summary, WRKY63 could play an important role in the complex network of ABA-dependent gene expression and drought stress response (Ren et al., 2010). Recent studies showed that WRKY57 conferred drought tolerance in Arabidopsis by increasing the level of ABA (Jiang et al., 2012). Further ChIP assays confirmed that WRKY57 directly bound to the W-box of RD29A (RESPONSIVE TO DESSICATION 29A) and NCED3 (9-CIS-EPOXYCAROTENOID DIOXYGENASE 3) promoters and that overexpression of WRKY57 led to drought tolerance through up-regulation of ABA responsive genes (Jiang et al., 2012). In addition to Arabidopsis, WRKYs involved in ABA signaling have also been reported in other crop plants. For example, two alleles OsWRKY45-1 and OsWRKY45-2 in rice played negative and positive roles in the ABA signaling pathway, respectively (Tao et al., 2011). OsWRKY45-2 rather than OsWRKY45-1 negatively regulated the rice response to salt stress. However, these two alleles acted similarly in drought and cold stress responses (Tao et al., 2011). Additionally, TF families such as AP2/ERF, NAC, C2H2 ZF (Cys2His2-TYPE ZINC FINGERS), MYB as well as bHLH (BASIC HELIX-LOOP-HELIX) have also been demonstrated to be involved in ABA signaling and the abiotic stress response (Lindemose et al., 2013).
1.3.2.3 Crosstalk among ABA, SA and JA/ET signaling pathways

Although ABA is defined as a key hormone controlling abiotic stress responses, ABA appears to also have an important role in biotic stress responses. Although the hormones SA, JA/ET are the most important players associated with SAR, ISR and resistance to a variety of pathogens, fine-tuning the plant responses to multiple stresses requires a network of cross-talk which connects ABA, SA and JA/ET together, resulting in either synergistic or antagonistic interactions (Fujita et al., 2006; Atkinson and Urwin, 2012; Lee and Luan, 2012).

Recent observations have shown that ABA is capable of affecting biotic stress signaling in both negative and positive ways (Fujita et al., 2006; Asselbergh et al., 2008; Yasuda et al., 2008; Lee and Luan, 2012). The application of exogenous ABA or drought stress to Arabidopsis reduced the tolerance to an avirulent P. syringae. However, aba1-1, an ABA-deficient mutant, exhibited more tolerance to P. parasitica (Mohr and Cahill, 2003). Moreover, SAR induction is suppressed by ABA through inhibition of the pathways both upstream and downstream of SA, as revealed by several mutants together with SAR-inducing chemicals in Arabidopsis (Yasuda et al., 2008). This suppression by ABA is also observed in other plant species, such as tomato and rice (Audenaert et al., 2002; Koga et al., 2004; Asselbergh et al., 2008). In PAMP signaling, ABA is employed by pathogens to suppress the SA mediated pathway (Boatwright and Pajerowska-Mukhtar, 2013). For instance, coronatine, a bacterial toxin, triggered ABA accumulation, resulting in the suppression of SA synthesis (de Torres Zabala et al., 2009). Additionally, high levels of ABA accompanied by enhanced coronatine levels promoted bacterial growth and increased susceptibility of the plants (Seo and Park, 2010; Boatwright and Pajerowska-Mukhtar, 2013). Thus, hormone signaling pathways could be modulated by pathogens, facilitating pathogen growth and virulence to the plants (Boatwright and Pajerowska-Mukhtar, 2013). By contrast, ABA also interacts with SA synergistically depending on the type of pathogen and its method of entry into host cells (Ton et al., 2009). PAMP-induced stomatal closure in Arabidopsis is a good example which links plant defense and ABA signaling pathways (Melotto et al., 2006). Genetic studies showed that the ABA signaling kinase mutant ost1 and the ABA-deficient mutant aba3-1 could not close stomata under PAMP treatment, indicating that an intact ABA signaling pathway was essential for stomatal closure in plant defense. Likewise, mutant plants and transgenic lines with low levels of SA, for instance, eds5-1, sid2, npr1 as well as NahG were not able to close their stomata in response to PAMPs, MAMPs and even osmotic stress (Melotto et al., 2006; Zeng and He, 2010). Intriguingly, the stomata in the ABA-deficient mutant aba2-1 could not be closed when treated with SA, but no alterations in stomatal closure were found in the sid2 mutant or the NahG line in response to ABA. These findings suggest that ABA and SA are indispensable in stomatal related defense, but SA seems to function upstream of ABA (Zeng and He, 2010; Montillet and Hirt, 2013).

The antagonistic effect of SA on ABA signaling also exists. Yasuda et al. (2008) showed a suppressive effect of SAR on the ABA signaling pathway by using several SA signaling-related mutants of Arabidopsis. The expression of ABA biosynthesis and ABA
responsive genes was repressed by the induction of SAR and involved the contribution from NPR1 or signaling downstream of NPR1. Likewise, the suppressive effect of SA on abiotic stress responses has also been shown in other plant species. For example, drought tolerance decreased when maize (*Zea mays*) was pre-treated by SA (Németh *et al.*, 2002). These previous findings illustrate the negative effect of SA on ABA-mediated abiotic stress responses. However, the role of SA on abiotic stress responses is not limited to the negative side. SA also plays a positive role in certain stress responses depending on the dosage of SA and how severe the stresses are (Yuan and Lin, 2008; D. *et al.*, 2011).

For the interaction between ABA and JA/ET signaling pathways, Anderson *et al.* (2004) has shown that the JA/ET-related defense genes were repressed by ABA in Arabidopsis. Consistent with this result, disruption of the ABA biosynthesis related genes *ABA1* and *ABA2* led to enhanced expression of JA/ET responsive genes. Moreover, disease resistance to the fungal pathogen *Fusarium oxysporum* was enhanced in mutants associated with ABA signaling genes. Likewise, the *jar1* and *jin4* (*jasmonic acid insensitive 4*) mutants in JA signaling showed hypersensitivity to ABA inhibition of germination (Anderson *et al.*, 2004). Furthermore, transcript levels of ABA responsive genes *KIN1*, *VSP2*, *RD22* (*RESPONSIVE TO DESSICATION*) and *MYC2/JAI1* (*JASMONATE INSENSITIVE 1*) were significantly up-regulated in the ET signaling mutants *etr1-1* (*ethylene receptor 1*), *ein2-1* and *ein3-1* (Anderson *et al.*, 2004). These results illustrate the mutual antagonism between ABA and JA/ET mediated signaling pathways. Intriguingly, Adie *et al.* (2007) have claimed controversially that ABA played a positive role in activation of defense gene expression in Arabidopsis. This was based on the result that approximately one-third of the plant genes induced by *Pythium irregulare* were up-regulated by ABA in a genome wide analysis. This was contradictory to the results from Anderson *et al.* (2004), who showed that some JA/ET related genes, for instance, *PDF1.2*, *HEL* (*HEVEIN-LIKE*) and *b-CHI* (*BASIC CHITINASE*) were repressed by ABA. The explanation for this discrepancy was that ABA might prevent a small group of JA/ET regulated genes, but not all. Moreover, the role of ABA, either negative or positive, in plant defense also depends on the timing of infection and the characteristics of the pathogen (Adie *et al.*, 2007; Ton *et al.*, 2009).

In summary, ABA is considered as a central player which integrates signals from abiotic and biotic stresses involving SA, JA and ET. In addition, many components such as TFs, ROS, and small RNAs, participate in this interplay, precisely controlling the different stress responses in plants (Ton *et al.*, 2009; Atkinson and Urwin, 2012). The model for the crosstalk between biotic and abiotic signaling pathways is described in Fig. 5.
Figure 5. The plant hormones (SA, ABA and JA/ET) integrating the crosstalk between biotic and abiotic stress responses. ABA is the major component which controls the switch in priority among different stress responses, allowing plants to fine-tune the activation of defense or tolerance to adverse conditions. Arrows indicate induction or positive regulation, dashed lines imply repression or negative regulation. Abbrevation: SA, salicylic acid; ABA, abscisic acid; JA, jasmonic acid; ET, ethylene; PR, pathogenesis-related; SAR, systemic acquired resistance (modified from Atkinson and Urwin, 2012).

1.4 Roles of two closely related WRKY TFs, WRKY70 and WRKY54 in Arabidopsis

The role of the plant-specific TF WRKY70 in plant immunity has been demonstrated by Li et al., (2004). They found that the expression of WRKY70 was induced by defense related signals such as bacterial elicitors and the phytohormone SA. Overexpression of WRKY70 enhanced plant resistance to virulent bacterial pathogens, accompanied with constitutive activation of a subset of defense-related genes involved in SA mediated SAR. By contrast, the expression of WRKY70 was suppressed by JA and a subset of JA-inducible genes were up-regulated in the miRNA silencing line of WRKY70. These results indicate that WRKY70 acts as an activator of SA-induced genes and a repressor of JA-related genes. Furthermore, Li et al., (2006) showed that a WRKY70 knockout mutant improved the JA-mediated resistance to a fungal pathogen, whereas overexpression of WRKY70 resulted in enhanced resistance to a fungal pathogen involving SA-mediated signaling. These findings suggest that WRKY70 acts as a node of convergence for balancing the crosstalk between SA-dependent and JA-dependent signaling pathways. As
revealed by the analysis for the transcriptional cascade leading to SAR, several WRKYs including WRKY70 and its closest homolog WRKY54 in Arabidopsis were found to be direct targets of NPR1 (Wang et al., 2006). Moreover, the double mutant wrky54wrky70 displayed a significantly higher level of free SA, which was consistent with the SA biosynthesis gene \textit{ICS1} (\textit{ISOCHORISMATE SYNTHASE1}) that was up-regulated in the double mutant (Wang et al., 2006). Therefore, WRKY70 and WRKY54 are considered as repressors of SA biosynthesis. Although elevated SA levels were found in the wrky54wrky70 double mutant, it did not show heightened resistance to a biotroph \textit{Psm ES4326}, which might indicate a dual role of WRKY70 and WRKY54 in transducing the SA signal (Wang et al., 2006). Nevertheless, the data shown in these articles mentioned above were quite limited and several necrotrophic pathogens have not yet been tested to challenge the wrky54wrky70 double mutant. Likewise, the elevated SA level in the double mutant may also trigger many downstream events, possibly involved in plant defense responses. Thus, these unanswered questions still need to be elucidated. Although WRKYs are considered as defense associated regulators, there are less the studies involving WRKY TFs in abiotic stress responses. Recent progress has begun to reveal the roles of WRKY TFs in plant response to abiotic stresses (Chen et al., 2012). Thus, the involvement of defense-related WRKY70 and WRKY54 in abiotic stress response would be an interesting topic for further study.

Additionally, the role of WRKY70 in plant development has also been investigated previously (Ulker et al., 2007). Loss of \textit{WRKY70} function promoted both developmentally and dark-induced leaf senescence, indicating that WRKY70 played a role as a negative regulator in leaf senescence. However, it still remains to be investigated what other components, such as other WRKYs, are involved in cooperatively regulating the senescence process as well as the network between SA-mediated signaling and senescence.
2. AIMS OF THE STUDY

The aims of the project were to elucidate the role of the transcription factors (TFs) WRKY54 and WRKY70 in controlling development and adaptation to different stress conditions in Arabidopsis. TFs are key components that govern plant growth and tolerance to biotic and abiotic stress conditions. Genetic and molecular approaches were used for analyzing the functions of these two TFs in senescence, osmotic stress as well as some specific pathogens responses.

The specific objectives of this study focused on three work packages:

1) To elucidate the function of \textit{WRKY54} and \textit{WRKY70} in the leaf senescence process during plant growth and development.
2) To explore the mechanism by which \textit{WRKY54} and \textit{WRKY70} regulate abiotic stress adaptation (osmotic stress).
3) To illustrate the cooperation of \textit{WRKY54} and \textit{WRKY70} in response to pathogen attack.
3. MATERIALS AND METHODS

Materials
The biological materials used in this study are described in the original publications (I, II, III). Briefly, we used Arabidopsis thaliana wild type, mutants and transgenic lines as plant material. In addition, pathogens, including Pectobacterium carotovorum subsp. carotovorum SCC1, Botrytis cinerea B05.10 and Pseudomonas syringae pv. tomato DC3000, were employed as pathogenic materials.

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4. RESULTS AND DISCUSSION

4.1 WRKY54 and WRKY70 function redundantly as negative regulators of senescence in Arabidopsis (I)

4.1.1 WRKY70 collaborates with WRKY54 to negatively regulate leaf senescence in Arabidopsis

Leaf senescence is a natural developmental stage in plants, controlled by multiple developmental and environmental factors (Lim et al., 2003). Senescence regulation is controlled by a complex network, integrating internal and external factors into the senescence process. The WRKY superfamily, one of the plant-specific TF families, has been considered to be involved in the process of senescence in Arabidopsis. For example, WRKY53 and WRKY70 have been demonstrated as positive and negative regulators, respectively, during leaf senescence (Miao et al., 2004; Ulker et al., 2007). In this study, we investigated the overlapping role of WRKY70 and its closest homolog WRKY54 in regulation of leaf senescence in Arabidopsis. As revealed by single and double mutant studies, the \textit{wrky54} single mutant showed no clear early senescence symptoms, whereas the \textit{wrky70} single mutant exhibited an early senescence phenotype when compared to wild type and the \textit{wrky54} single mutant. Interestingly, the \textit{wrky54wrky70} double mutant presented a drastically enhanced senescence phenotype compared to that in the \textit{wrky70} single mutant (I, Fig. 5A and B). These results suggest that WRKY70 and WRKY54 function redundantly as negative regulators of senescence in Arabidopsis. To further investigate how the senescence-related genes altered in the double mutant \textit{wrky54wrky70} during development of leaves, we monitored the expression of \textit{CAB} (CHLOROPHYLL A/B), \textit{SAG12} (SENESCENCE-ASSOCIATED GENE 12) and \textit{SEN1} (SENESCENCE-ASSOCIATED PROTEIN DIN1) genes. Compared to the wild-type plant, the expression of \textit{CAB} in the double mutant decreased earlier, and the induction of \textit{SAG12} and \textit{SEN1} was premature (I, Fig. 3B and 5C). These alterations of senescence-associated genes are in accordance with the observed early senescence phenotype in \textit{wrky54wrky70} double mutant.

4.1.2 Interaction of WRKY group III TFs in leaf senescence

There are 13 members of the WRKY group III TF family including WRKY54 and WRKY70, which are involved in many aspects of plant defense responses and physiological processes including senescence (Rushton et al., 2010). Based on their expression patterns induced by specific stress conditions, we assumed that these TFs might interact in a regulatory network (Berri et al., 2009). To verify this hypothesis, we investigated the protein-protein interactions among WRKY group III TFs through yeast two-hybrid analysis. Interestingly, we found that WRKY54 and WRKY70 could interact with WRKY30 independently. Additionally, WRKY53 was observed to interact with
WRKY30 (I, Fig. 2). WRKY70 and WRKY53 have previously been reported as regulators in leaf senescence (Miao et al., 2004; Ulker et al., 2007), thus WRKY54 and WRKY30 might be two new regulators in leaf senescence.

When comparing the expression profiles of WRKY30 and WRKY54 to those of WRKY53 and WRKY70, we found WRKY30 showed a similar induction pattern to WRKY53, with a retained high level of expression during the senescence process. However, the expression pattern of WRKY54 closely resembled WRKY70, showing a transient induction at the onset of senescence (I, Fig. 3A). These similar expression patterns between WRKY30/WRKY53 and WRKY54/WRKY70, suggest possible roles of WRKY30 and WRKY54 in leaf senescence. Moreover, the differences in these two expression patterns also implicate different phases for the action of WRKY regulators in the process of senescence. During the early development of leaves, the positive regulators of senescence are not induced whereas the negative regulators are slowly up-regulated. This is due to the fact that premature senescence has to be prevented in the beginning of leaf growth to guarantee effective nutrient recycling in the plant. At the onset of leaf senescence, positive and negative regulators of the senescence are co-induced, and during the last stage of senescence, the expression of positive regulators dominates over the negative factors to finalize the life cycle of the leaves. The co-operation of WRKY54 and WRKY70 negatively controls the senescence process, and is fine-tuned by the positive regulator WRKY53. Although the miRNA-silenced line for WRKY30 did not show alterations in the senescence phenotype compared to wild type, WRKY30 could play a role as a positive binding factor, interacting with WRKY54, WRKY70 and WRKY53 independently. Also it could integrate both positive and negative signals at the transcriptional level to control leaf senescence. Moreover, the preferential binding activity of WRKY30 to either the positive regulator WRKY53 or the negative regulators WRKY54 and WRKY70 would depend on the expression ratio between WRKY54/WRKY70 and WRKY53 to some extent. Either displacement or heterodimer formation would allow adjustment of the outcome of the leaf senescence process.

4.1.3 The expression of WRKY53, WRKY70, WRKY54 and WRKY30 is partially SA-dependent

As WRKY group III genes are responsive to SA (I. Fig. 1A and B), we further investigated whether the induction of WRKY30, WRKY53, WRKY54 and WRKY70 were also SA dependent during the senescence process. Transcript accumulation of each WRKY gene was drastically reduced in the sid2-1 mutant compared to those in wild type plants (I. Table 1). The SA-deficient sid2-1 mutant (Nawrath and Métraux, 1999) showed induction values of only 25-55% compared to the corresponding wild-type values. These results indicate that the full induction of WRKY30, WRKY53, WRKY54 and WRKY70 during leaf senescence is partially SA dependent. Simultaneously, it also suggests that SA plays a pivotal role in regulating leaf senescence. As revealed by previous results, SA level in the wrky54wrky70 double mutant is constitutively high (Wang et al., 2006). Consequently,
this could be the main factor triggering early senescence in the double mutant, with premature induction of WRKY53 and senescence-related genes (I, Fig. 5).

In addition to SA, ROS are also very important in senescence and cell death and WRKY53 has been reported to be induced by hydrogen peroxide (H$_2$O$_2$) (Miao et al., 2007). Similarly, WRKY30 was induced rapidly and transiently by H$_2$O$_2$. Moreover, both WRKY53 and WRKY30 could be highly induced by ozone. In contrast, neither H$_2$O$_2$ nor ozone could induce WRKY54 and WRKY70 (I, Fig. 6). These results indicate that ROS are an important inducer for the positive regulators WRKY53 and WRKY30, but not for the negative regulators WRKY54 and WRKY70. Moreover, the interaction between SA and ROS here was further supported in study III. The elevated SA level in the wrky54wrky70 double mutant potentiated the accumulation of ROS, resulting in the activation of WRKY53 and WRKY30 and early senescence in the double mutant.

In conclusion, leaf senescence is a complex process which involves regulation of a variety of genes and many metabolic and signaling pathways (Gan, 2008). The WRKY group III TFs, WRKY53, WRKY54 and WRKY70 participate in this regulatory network possibly through interaction with WRKY30, integrating both positive and negative signals to fine-tune the senescence process. In addition, expression profiling showed that at the transcriptional level, WRKY70 influenced the expression of WRKY53 and vice versa (Dong et al., 2003; Li et al., 2004; Miao et al., 2004). Thus cross-regulation among WRKYs is very common, and this cross-modulation indicates that WRKY TFs cooperate with each other to take part in a regulatory network rather than a linear signaling pathway during the process of leaf senescence (Miao et al., 2007). Furthermore, other groups of TFs such as NAC, C2H2-type zinc finger, AP2/EREBP, MYB and RAV (RELATED TO ABI3/VP1) proteins were demonstrated to be involved in the regulation of leaf senescence in Arabidopsis (Guo et al., 2004; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005; Balazadeh et al., 2008). Therefore, WRKY TFs, along with other TFs, constitute a complex web in regulating the process of leaf senescence.

4.2 WRKY54 and WRKY70 negatively modulate osmotic stress tolerance by regulating stomatal aperture in Arabidopsis (II)

4.2.1 Defense related WRKY54 and WRKY70 are induced by osmotic stress

It is known that the TFs WRKY54 and WRKY70 play an important role in plant defense to biotic stresses (Li et al., 2004, 2006; Wang et al., 2006). Their additional roles in plant development, for example senescence, have been demonstrated (Ulker et al., 2007). To investigate the possible involvement of WRKY54 and WRKY70 in abiotic stress responses, we first characterized the expression of the corresponding genes in wild-type Arabidopsis in response to osmotic stress (15% PEG6000). Interestingly, WRKY54 and WRKY70 displayed similar but transient induction by osmotic stress (II, Fig. 1). The specificity of the response of these two TFs to osmotic stress was further characterized by comparison with seven additional WRKYs which are responsive to osmotic stress.
The expression of these genes in wild type showed that WRKY54 and WRKY70 were indeed unique in their response to osmotic stress among the tested WRKYS, with rapid and transient induction patterns (II, Fig. S1). As a comparison, two of the tested WRKY genes, WRKY63 and WRKY40 were also induced prominently by osmotic stress but showing delayed and persistent patterns (II, Fig. S1). The induction of WRKY63 and WRKY40 by osmotic stress was also consistent with the recent findings that they are involved in the plant response to ABA and abiotic stresses (Ren et al., 2010; Shang et al., 2010). Likewise, the inducibility of WRKY54 and WRKY70 by osmotic stress also implied that they could have important roles in the abiotic stress response.

4.2.2 WRKY54 and WRKY70 negatively regulate plant tolerance to abiotic stresses

To investigate the involvement of WRKY54 and WRKY70 in osmotic stress tolerance, wild type plants, wrky54 and wrky70 single, wrky54wrky70 double mutants as well as a WRKY70 overexpressor line (S55) were exposed to osmotic stress. Both the phenotypes of the plants exposed to stress and the stress damage quantified by ion leakage showed that the wrky54wrky70 double mutant exhibited enhanced tolerance to osmotic stress and very low ion leakage. In comparison, wild-type plants and the wrky54 single mutant displayed equivalent symptoms of wilting and increased electrolyte leakage. The wrky70 single mutant presented an intermediate phenotype, whereas the WRKY70 overexpressor line showed less tolerance to osmotic stress, with clearly wilted leaves and considerably higher electrolyte leakage than the other lines (II, Fig. 2). These results indicate that loss of function of both WRKY54 and WRKY70 leads to enhanced tolerance to osmotic stress, suggesting these two TFs may act redundantly as negative regulators in osmotic stress tolerance. Additionally, the observed intermediate phenotype of the wrky70 single mutant compared to wrky54wrky70 and wild type plants, together with weak or no phenotypic differences found between wrky54 and wild-type demonstrate that WRKY70 rather than WRKY54 plays a more prominent role in altered response to osmotic stress.

Osmotic stress can be caused by many natural abiotic stresses, including drought, high salinity and low temperature (Verslues et al., 2006). To further elucidate if this tolerance to osmotic stress caused by inactivation of WRKY54 and WRKY70 also applies to other environmental factors, the expression of WRKY54 and WRKY70 in wild type in response to high salt, drought, cold and exogenous ABA was explored. Similar to the induction by PEG, these two TFs showed transient induction patterns, especially in response to salt and ABA (II, Fig. S3). In addition, the enhanced tolerance of the wrky54wrky70 double mutant to drought stress and high salinity was elucidated (II, Fig. S4 and S5). These interesting findings suggest that inactivation of WRKY54 and WRKY70 results in tolerance to not only osmotic stress but also to other abiotic stresses. Moreover, the altered expression of WRKY54 and WRKY70 to ABA treatment implicates the possible roles of these two TFs in ABA signaling.
4.2.3 Involvement of SA and ABA in WRKY54 and WRKY70-dependent osmotic stress tolerance

Osmotic stress tolerance is accompanied by up-regulation of abiotic stress response genes and accumulation of osmoprotectants (Delauney and Verma, 1993). However, the enhanced tolerance in the wrky54wrky70 double mutant to osmotic stress could not be explained by the enhanced induction of osmotic stress-related genes or enhanced accumulation of protective osmolytes, such as proline. The majority of the osmotic stress-induced genes were suppressed in the wrky54wrky70 double mutant compared to those in wild-type plants, as revealed by microarray and qRT-PCR (quantitative reverse transcription-polymerase chain reaction) (II, Table 1, Fig. 3 and 4). It appears that inactivation of WRKY54 and WRKY70 blocks the induction of abiotic stress responsive genes.

WRKY54 and WRKY70 are well known to be involved in negative regulation of SA biosynthesis, and this negative feedback loop leads to elevated level of SA in the wrky54wrky70 double mutant (Wang et al., 2006). SA and SA-mediated signaling have been demonstrated to be involved in an antagonistic interaction with ABA-mediated abiotic stress responses (Yasuda et al., 2008). In maize, pre-treatment with SA increased the sensitivity of plants to drought stress (Németh et al., 2002). In Arabidopsis, the growth of wild-type plants was inhibited by SA accumulation at chilling temperature but this inhibition was not found in NahG plants, which were unable to accumulate SA (Scott et al., 2004). Moreover, NahG plants showed increased resistance to NaCl due to lack of SA induced production of oxidative damage in Arabidopsis seedlings (Borsani et al., 2001). These findings indicated that SA negatively affected the environmental stress responses. Thus, in our case, the elevated level of SA in the wrky54wrky70 double mutant might be the reason for the suppression of osmotic stress-induced gene expression in the double mutant. This hypothesis was further verified by suppression of osmotically induced gene expression by exogenous SA in wild-type Arabidopsis and the partial abolishment of the suppression via introduction of the sid2-1 allele into the wrky54wrky70 background (II, Fig. S2 and Table S3).

ABA is the central hormone, which always acts as a positive messenger in response to various environmental stresses (Tuteja, 2007; Yasuda et al., 2008). Under drought and salt stresses, ABA-deficient mutants readily wilt and grow poorly. Additionally, ABA is also required for freezing tolerance for the induction of dehydration tolerance genes (Xiong et al., 2001). Numerous abiotic stress-induced genes are responsive to ABA (Tuteja, 2007), such as RAB18, LTI78 (LOW TEMPERATURE INDUCED 78) and KIN1, which are not only responsive to ABA, but also to drought and low temperature (Kurkela and Franck, 1990; Lång and Palva, 1992; Nordin et al., 1993). Additionally, NCED3 encodes a key enzyme in ABA biosynthesis (Iuchi et al., 2001). In the wrky54wrky70 double mutant, expression of the NCED3 gene was reduced as a result of the high SA level, suggesting impaired ABA accumulation that consequently could lead to reduced expression of ABA target genes. However, this hypothesis was not supported by the determination of ABA levels in the double mutant during the early response to drought
stress (II, Fig. S7). Since the antagonistic crosstalk between the SA-mediated defense signaling and the ABA-mediated signaling of environmental stress responses occurs in a complex manner in multiple steps, several mechanisms must exist in the regulation of crosstalk (Yasuda et al., 2008). Therefore, the negative effect of SA does not appear to occur in the early accumulation of ABA in response to abiotic stress in the wrky54wrky70 double mutant.

Although osmotic stress-induced gene expression was suppressed in the wrky54wrky70 double mutant due to the elevated SA level, the enhanced osmotic stress tolerance of the double mutant was not caused by the high SA level. When the sid2-1 allele was introduced into the wrky54wrky70 background, the SA level was reduced accordingly but the osmotic stress tolerance in the wrky54wrky70sid2-1 triple mutant was not abolished although a slight reduction in enhanced tolerance was observed (II, Fig. 5 and 6). This suggests that the tolerance in the wrky54wrky70 double mutant might be explained by the direct effect of inactivation of WRKY54 and WRKY70, but not by the increased SA level.

4.2.4 WRKY54 and WRKY70 cooperatively regulate stomatal aperture in early response to osmotic stress

The osmotic stress tolerance in wrky54wrky70 was caused by neither enhanced expression of abiotic stress-related genes nor the accumulation of proline. Most abiotic stresses ultimately result in desiccation of the cell and water imbalance (Shinozaki and Yamaguchi-Shinozaki, 2000). The osmotic tolerance of the wrky54wrky70 double mutant may be linked to the water content in the leaves, controlled by the stomatal aperture. To verify this hypothesis, the water loss and stomatal conductance in wrky54wrky70 were characterized, and the results indeed showed drastically reduced water loss and stomatal conductance in the double mutant (II, Fig. 7A and B). This suggests that the reduced water loss regulated by stomata was the main reason for tolerance to osmotic stress in the wrky54wrky70 double mutant. Furthermore, inactivation of WRKY54 and WRKY70 resulted in reduction of stomatal conductance. In contrast, overexpression of WRKY70 showed enhanced stomatal conductance in both unstressed and osmotically stressed plants (II, Fig. 7C), indicating that WRKY54 and WRKY70 cooperate as negative regulators of stomatal closure in Arabidopsis (II, Fig. 10).

Stomatal aperture movement is controlled by both extra- and intra-cellular signals. A variety of environmental factors including drought, light, humidity as well as biotic stresses are able to affect stomatal closure (Acharya and Assmann, 2009). In addition, phytohormones play pivotal roles in regulating stomatal aperture movement (Acharya and Assmann, 2009). Among the phytohormones, ABA is the key player in restricting water loss by promoting stomatal closure in the osmotic stress response. This stomatal regulation through control of the ABA level was supported by the WRKY70 overexpressor line, where the reduced ABA level resulted in more open stomates and reduced stomatal closure upon stress (II, Fig. 7C and S7). Moreover, generation of the triple mutant wrky54wrky70abi1-1 resulted in abolishment of the osmotic stress tolerance of
wrky54wrky70 due to introduction of the dominant negative abi1-1 (abscisic acid-insensitive 1-1) allele (II, Fig. 9A and B). However, although osmotic stress tolerance was lost in the abi1-1 background, the stomatal conductance in the wrky54wrky70abi1-1 triple mutant (II, Fig. 9C) showed that both WRKY54 and WRKY70 still negatively regulated stomatal closure over that of the abi1-1 mutant itself, and consequently attenuated the ABA-mediated processes in stomatal regulation.

In addition to ABA, it has been reported that SA acts as a positive factor in regulation of stomatal closure. Transgenic NahG and sid2-2 mutant plants were both deficient in SA, and their stomatal closure was suppressed (Melotto et al., 2008). Khokon (2011) also demonstrated that application of SA induced accumulation of ROS and nitric oxide (NO), leading to stomatal closure in Arabidopsis. The same was observed in the wrky54wrky70 double mutant with high SA level, under both non-stressed and osmotically-stressed conditions, showing decreased stomatal conductance. Conversely, stomatal conductance was clearly increased in the sid2-1 mutant as well as in the wrky54wrky70sid2-1 triple mutant, in which the SA level was reduced (II, Fig. 7). These results confirm the positive function of SA in regulating stomatal closure, in agreement with previous findings (Melotto et al., 2006; Acharya and Assmann, 2009; Khokon et al., 2011). Nevertheless, the negative effect of WRKY54 and WRKY70 on stomatal closure was also evident, because when the plants were treated osmotically, stomatal closure in the triple mutant wrky54wrky70sid2-1 was still enhanced (II, Fig. 7C). Therefore, the enhanced stomatal closure observed in the wrky54wrky70 double mutant was not caused directly by the accumulated SA, but by the absence of the negative regulators WRKY54 and WRKY70. These two TFs may regulate stomatal closure redundantly through two signaling pathways. On one hand, WRKY54 and WRKY70 negatively regulate SA biosynthesis, keeping SA levels down and preventing SA-induced stomatal closure. On the other hand, they have a more direct, but SA-independent negative effect on stomatal closure by reducing ABA levels (II, Fig. 10).

Interestingly, other members of the WRKY TF family have also been demonstrated to participate in abiotic stress responses and ABA signaling. For instance, WRKY40 in Arabidopsis directly targeted a number of ABA-responsive genes by binding to the W box-containing promoters (Shang et al., 2010). WRKY63 positively regulated ABA-induced stomatal closure, hence a wrky63 mutant (abo3) showed enhanced sensitivity to drought stress. In addition, WRKY63 could bind to the promoter of ABF2, positively regulating ABF2 expression in Arabidopsis (Ren et al., 2010). A recent report also showed that WRKY57 in Arabidopsis improved drought tolerance by increasing the ABA level and expression of stress-related genes (Jiang et al., 2012). Similarly, defense-related WRKY54 and WRKY70 are also involved in osmotic stress responses. They might work, however, as negative regulators of an early step of stomatal closure in the plant response to osmotic stress rather than the later stages of ABA-mediated gene expression and accumulation of osmoprotectants. This conclusion was supported by the comparison of rapid regulation of stomatal aperture among wild-type plants and mutants, induced by both ABA and PEG treatment (II, Fig. 8).

In this study, the demonstrated involvement of defense-related TFs WRKY54 and WRKY70 in abiotic stress responses shed light on the interaction between biotic and
abiotic stress response pathways. This cross-talk always appears to be orchestrated by different hormone signaling pathways which either cooperate or antagonize each other (Atkinson and Urwin, 2012). The parallel SA-mediated biotic and ABA-mediated abiotic signaling pathways appear antagonistically related through involvement of WRKY54 and WRKY70. Both WRKY54 and WRKY70 were similarly induced by osmotic stress, and they negatively regulated the early response of ABA-controlled stomatal closure. Simultaneously, these two WRKYs also negatively governed the SA level which had a positive effect on stomatal closure, and consequently provided the indirect negative effect on stomatal closure. However, despite the evidence of modulation of these two WRKYs in biotic and abiotic stress response interactions, details of the molecular mechanism in controlling stress cross-talk mediated by WRKYs still need to be addressed in the future.

4.3 WRKY70 cooperates with WRKY54 to regulate the resistance to necrotrophic pathogens in Arabidopsis (III)

4.3.1 WRKY54 and WRKY70 cooperate as negative regulators in basal defense to necrotrophs

Previous studies have demonstrated that WRKY70 is a key component in the crosstalk between SA- and JA-mediated signaling pathways (Li et al., 2004; 2006). Additionally, WRKY70 and WRKY54 are considered as negative regulators of SA biosynthesis (Wang et al., 2006). However, the role of the cooperation of WRKY70 and WRKY54 in plant defense is not fully elucidated. Intriguingly, a set of defense related genes found from the microarray data in study II, were up-regulated in the unstressed wrky54wrky70 double mutant compared to unstressed wild-type plants (III, Table S1). Further qRT-PCR and western blot analysis confirmed the expression of SA-inducible PR1,2,5 and PAD4 genes, JA/ET responsive PR3,4, PDF1.2 and PAD3 genes as well as the H2O2 responsive gene GST1. They were up-regulated in the wrky54wrky70 double mutant compared to the other lines (III, Table 1 and Fig. 1), although up-regulation of the five genes (PR2, PR5, PAD4, PR3, PR4) was not limited to the double mutant. However, the expression level of these genes was reduced to basal levels in both the sid2-1 single and the wrky54wrky70sid2-1 triple mutants (III, Fig. 1). These results suggest that the enhanced SA level in wrky54wrky70 is the main reason for the constitutive expression of defense-related genes. Moreover, since GST1 is controlled by H2O2 (Alvarez et al., 1998), the up-regulation of GST1 indicated the possibility of H2O2 associated events occurring in the wrky54wrky70 double mutant. Interestingly, H2O2 already accumulated in the wrky54wrky70 double mutant under control conditions, whereas no accumulation of H2O2 could be visualized in the wrky54wrky70sid2-1 triple mutant, as revealed by DAB (3,3′-diaminobenzidine) staining (III, Fig. 2D). This suggests that the increased SA could trigger production of ROS (H2O2) in wrky54wrky70. This is supported by previous findings indicating that SA potentiated the production of ROS (H2O2) (Chen et al., 1993; Shirasu et al., 1997).

It is well known that ROS are ubiquitous molecules of redox pathways which induce plant resistance through either facilitating cell death or triggering antimicrobial
activity (Kotchoni and Gachomo, 2006; Mengiste, 2012). Indeed, the wrky54wrky70 double mutant with accumulation of H$_2$O$_2$ induced by elevated SA level showed enhanced resistance to the necrotrophic pathogens $P$. carotovorum and $B$. cinerea. In contrast, this resistance was lost in the wrky54wrky70sid2-1 triple mutant, most likely due to the reduced level of SA (III, Fig. 2 and 3). These results suggest the importance of SA-induced H$_2$O$_2$ accumulation in plant defense. Nevertheless, the slightly enhanced resistance in the wrky70 single mutant and susceptibility in the WRKY70 overexpressor line also indicates the direct and negative roles of WRKYs in plant defense to necrotrophs.

4.3.2 The accumulation of ROS (H$_2$O$_2$) induced by SA in the wrky54wrky70 double mutant triggers cell wall-associated antimicrobial defense to necrotrophs

The involvement of ROS in plant-microbe interactions have been demonstrated previously. The downstream events mediated by ROS in plant cells exposed to pathogens depend on the intensity of the ROS signals (Kotchoni and Gachomo, 2006). High dosages of ROS lead to HR and oxidative damage in cells, whereas moderate and balanced levels of ROS induce the expression of a set of defense genes, production of antimicrobial compounds, and cell wall fortification through oxidative cross-linking (Brisson et al., 1994; Lamb and Dixon, 1997; Brown et al., 1998; Kotchoni and Gachomo, 2006). In unstressed wrky54wrky70 double mutants, a number of genes encoding cell wall-bound peroxidases and cell wall modification proteins were up-regulated (III, Fig. S1 and Table S1, S2), implicating that the source of H$_2$O$_2$ production and the cell wall fortification of wrky54wrky70 might be mediated by H$_2$O$_2$. This preformed fortified layer in the wrky54wrky70 double mutant might be the first protective barrier against the penetration of plant cell walls by several necrotrophic pathogens, for example, $P$. carotovorum and $B$. cinerea (III, Fig. 6), whose virulence strategies mainly rely on cell wall-degrading enzymes (CWDEs) for disrupting plant cell wall integrity (Toth and Birch, 2005; van Kan, 2006). This is supported by the previous findings that elimination of H$_2$O$_2$-mediated cell wall modification led to increased penetration success of fungi in plant cells, suggesting H$_2$O$_2$ was one of the most critical determinants of pathogen penetration failure in invading epidermal cells (Mellersh et al., 2002).

Although H$_2$O$_2$-mediated disease resistance of the wrky54wrky70 double mutant effectively prevents the growth of necrotrophic pathogens, it did not stop the infection by a biotrophic pathogen $P$. syringae (III, Fig. 4). In addition to the role of ROS in cell wall modification of the wrky54wrky70 double mutant, ROS are widely considered as signaling molecules which trigger programmed cell death in challenged cells (Levine et al., 1994). Moreover, programmed cell death is beneficial for plant resistance to biotrophic pathogens but promotes the virulence of necrotrophs (Govrin and Levine, 2000). According to these data, the wrky54wrky70 double mutant should be resistant to $P$. syringae but susceptible to $P$. carotovorum and $B$. cinerea. Nevertheless, the results were the opposite. Why? The wrky54wrky70 double mutant did not show any cell death symptoms as visualized by trypan blue staining under control conditions, although the accumulation of H$_2$O$_2$ was revealed by DAB staining in the double mutant (III, Fig. 2D
and 3C). In contrast, although the uninfected overexpressor of WRKY70 did not show cell death, enhanced cell death symptoms compared to others were found after infection, which was consistent with the fast activation of the oxidative burst and HR after pathogen infection in the WRKY70 overexpressor (III, Fig. 2D and 3C). These results suggest that accumulation of ROS in non-stressed wrky54wrky70 only results in cell wall-mediated resistance to necrotrophs rather than cell death induced defense to biotrophs. Evidently, the ROS produced in both wrky54wrky70 and S55 acted differently and this difference resulted from the different time point and intensity of the signals. In the wrky54wrky70 double mutant, the accumulation of ROS was associated with pre-accumulated SA level due to inactivation of WRKY54 and WRKY70, and this level of ROS was probably not high enough to induce the HR. Overexpression of WRKY70 resulted in fast reaction to external stimuli and produced high dosages of ROS which were toxic to plant cells, leading to rapid cell death which was detrimental for the growth of biotrophs. Thus, plant responses to ROS and outcomes of defense to pathogens are highly dependent on the dosage of ROS (Kotchoni and Gachomo, 2006). A similar phenomenon has been found in tomato (Asselbergh et al., 2007). The ABA-deficient mutant showed early accumulation of H$_2$O$_2$ in epidermal cell walls, causing modification by protein cross-linking, and enhanced resistance to the necrotrophic pathogen B. cinerea (Asselbergh et al., 2007). Healthy ocp3 (overexpressor of cationic peroxidase 3) mutants of Arabidopsis displayed increased accumulation of H$_2$O$_2$ and expression of GST1 and PDF1.2 marker genes constitutively, leading to increased resistance to the necrotrophs B. cinerea and Plectosphaerella cucumerina (Coego et al., 2005).

4.3.3 JA/ET-mediated signaling dominates over SA-mediated signaling in preformed defense in the wrky54wrky70 double mutant

Mild dosages of ROS-induced pathogen defense accompanied with constitutive expression of defense-related marker genes in the wrky54wrky70 double mutant suggested that the major signaling pathways mediated either by SA or JA/ET were activated in this pre-alerted state of defense. This activation could be explained by the interaction of the balanced levels of ROS and SA, which were implicated to form a feedback loop in function against pathogens (Chen et al., 1993). Additionally, both WRKY54 and WRKY70 appeared to negatively regulate the expression of JA/ET responsive genes. The JA/ET related marker genes were up-regulated especially in the wrky70 single and wrky54wrky70 double mutants in non-stressed condition and their induction after necrotroph infection were not blocked in any of the wrky mutants, while overexpression of WRKY70 led to a reduction in expression of these marker genes under both control and infected conditions (III, Fig. 1A and 5). These results indicate that WRKY54 and WRKY70 cooperate as negative regulators in controlling the expression of JA/ET responsive genes.

To date, the antagonistic cross-talk between SA and JA signaling has been widely studied. The SA-mediated signaling pathway usually suppresses the JA-responsive genes and vice versa (Pieterse and Van Loon, 2004; Bostock, 2005; Nomura et al., 2005;
Koornneef et al., 2008; Koornneef and Pieterse, 2008). Nevertheless, both SA- and JA-responsive genes were activated by the combination of SA and ROS in the \textit{wrky54wrky70} double mutant. This seems to be a contradiction compared to previous findings. However, Koornneef (2008) described that the antagonistic effect of SA to JA-responsive genes was not obvious when SA signaling was activated before the onset of JA signaling. Therefore, it is possible that both SA- and JA-responsive genes are activated at the same time due to the continually high level of SA induced ROS production in the unstressed \textit{wrky54wrky70} double mutant.

Although SA and JA/ET dependent signaling pathways were both activated, unstressed \textit{wrky54wrky70} double mutants only showed resistance to necrotrophic pathogens but not to the biotroph \textit{P. syringae}. Thus, it seems that some unknown WRKY54 and WRKY70 controlled processes are not activated in the double mutant although most SA-dependent genes were up-regulated. Consequently, the missing processes requiring WRKY54 and WRKY70 might be necessary for development of resistance to biotrophs. Additionally, \textit{P. syringae} can deliver effectors, whose job is to mess the signaling pathways in plants (Lindeberg et al., 2012). This complicates the interpretation of the phenotypes in \textit{wrky54wrky70} double mutants. Moreover, when plants encounter a pathogen attack, different signaling pathways are activated to allow plants to fine-tune their defense response, thus the appropriate defense mediated by a specific hormone is employed while the inappropriate one is shut down accordingly (Kunkel and Brooks, 2002). For this reason, the antagonistic interaction between SA and JA take place after specific pathogen attack rather than in a preformed defense system. On the other hand, post-translational modification might occur in SA-dependent defense-related proteins, but this still needs to be investigated in the future.
5. CONCLUSIONS AND FUTURE PERSPECTIVES

The studies included in this thesis have revealed novel data about the cooperation of two structurally related TFs WRKY54 and WRKY70 in the regulation of Arabidopsis development, abiotic stress and biotic stress responses. Since WRKYs are considered as key components in various physiological programs (Ulker and Somssich, 2004), the achieved considerable progress in elucidating the roles of WRKY54 and WRKY70 in developmental senescence, osmotic stress as well as plant defense responses could provide invaluable tools for improving plant adaptability and even resistance to different stresses. The conclusive model for the function of these two TFs is described in Fig. 6.

The data provided by study I highlighted that WRKY54 and WRKY70 cooperated as negative regulators of leaf senescence in Arabidopsis. In addition, WRKY30 interacted independently with these two negative regulators, as well as with the positive regulator WRKY53 (Miao et al., 2004), to fine-tune the outcome of leaf senescence. Heterodimer formation would allow adjustment of the activity of regulators to either positively or negatively affect the onset and progression of leaf senescence. Moreover, the expression of WRKY54, WRKY70, WRKY53 and WRKY30 during senescence was partially SA dependent as revealed by reduced induction of these genes in a sid2 mutant compared to wild type. Following the identification of WRKY54 and WRKY70 as negative regulators during leaf senescence, future work would focus on the target genes downstream of WRKY54 and WRKY70. For instance, the senescence-associated genes CAB, SAG12 and SEN1 were early induced in the wrky54wrky70 double mutant compared to that of wild-type plants, suggesting that they were negatively regulated by WRKY54 and WRKY70. How does this happen? Do WRKY54 and WRKY70 directly regulate these senescence-associated genes or indirectly through some other factors? These questions remain unanswered. Additionally, MAP kinases are the pivotal factors during leaf senescence, as shown by the interaction between WRKY53 and MEKK1 (MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE) (Miao et al., 2007; Zentgraf et al., 2010). Thus, the interactions between MAP kinases and WRKY54 /70 need to be investigated, possibly through yeast one-hybrid analysis. Another open question refers to the uncharacterized WRKY30. Despite its similar expression pattern to WRKY53 and interactions with other WRKYs during senescence, silencing of WRKY30 did not present a senescence phenotype. Therefore, further investigation of the influence of WRKY30 on other WRKYs during senescence is needed, and could be facilitated by using T-DNA insertion mutants instead of miRNA lines.

Studies II and III of this thesis characterized the roles of WRKY54 and WRKY70 in abiotic stress and biotic stress responses. The novel data provided genetic evidence for illustrating how the genetic manipulation of these TFs could improve plant tolerance or resistance to abiotic stress or biotic stress, respectively. Firstly, inactivation of WRKY54 and WRKY70 enhanced tolerance to osmotic stress and this was shown to be SA-independent. Nevertheless, the improved tolerance in the wrky54wrky70 double mutant did not arise from induction of osmotic stress-related genes or accumulation of the osmoprotectant proline, but was due to the improved water retention and enhanced
stomatal closure. These findings suggest that WRKY54 and WRKY70 cooperate as negative regulators in the early events of abiotic stress response, but not the later stages of ABA signaling and stress-induced gene expression. Recent progress showed WRKY TFs were key nodes in ABA-mediated abiotic stress signaling networks (Rushton et al., 2012) in spite of their roles in biotic stress responses. WRKYS can bind to the W-box sequence in the promoters of downstream genes, for example, RAB18, KIN1, LTI78 and NCED3 which contain several W-box sequences in their promoters. Although the suppression of abiotic stress related genes in the wrky54wrky70 double mutant mainly resulted from the elevated SA level; WRKY54 and WRKY70 also had some effects on this suppression. Therefore, it is worth investigating whether these two TFs directly bind to the downstream gene promoters or not. If not, what other factors may also participate in the regulation of gene expression? In addition, the early regulation of stomatal aperture by WRKY54 and WRKY70 was independent of SA, which raises the open question as to how WRKYs regulate stomatal closure and what molecular mechanism is involved. On the other hand, several kinases such as OST1 are activated by osmotic stress as well as ABA and play a critical role in the control of stomatal closure (Yoshida et al., 2006). Thus, whether WRKY54 and WRKY70 can be phosphorylated by OST1 during the regulation of stomatal closure needs to be addressed in future work.

Secondly, although the central role of WRKY70 in integrating biotic stress responses has been demonstrated, the contribution of both WRKY70 and its homologue WRKY54 in plant defense to pathogens is not fully understood. Previous studies have shown that disruption of WRKY54 and WRKY70 results in accumulation of SA (Wang et al., 2006). This elevated SA level triggered the accumulation of ROS in non-stressed wrky54wrky70 double mutants. ROS accumulation activated the early antimicrobial defense to pathogens such as the necrotrophs P. carotovorum and B. cinerea and was accompanied by constitutive expression of defense related genes in the wrky54wrky70 double mutant, including both SA and JA/ET responsive marker genes. In addition, the genes encoding cell wall-related peroxidases and cell wall-modification proteins were up-regulated in the double mutant. These data indicate that the ROS triggered defense in the wrky54wrky70 double mutant is cell wall-associated. However, resistance to the biotrophic pathogen P. syringae pv tomato DC3000 in the wrky54wrky70 double mutant was not enhanced. Although ROS-induced cell death was harmful to the plants in response to necrotrophic pathogens (Mengiste, 2012), the early accumulation of ROS in specific locations such as epidermal cells was believed to be beneficial for plant defense to necrotrophs (Asselbergh et al., 2007). In accordance with this, the data shown in study III indicated that the timing, quantity and localization of ROS determine the outcome of the interaction between plant and pathogens. Nevertheless, in the wrky54wrky70 double mutant, both the SA-dependent and JA/ET dependent signaling pathways appeared to be constitutively activated due to the up-regulation of defense-related marker genes, but showed resistance to necrotrophs rather than biotrophs. This might be related to the unknown mechanism controlled by WRKY54 and WRKY70 which is not activated in the double mutant towards biotrophs. Thus, these missing processes still need to be investigated in the future.
Figure 6. The involvement of two transcription factors WRKY54 and WRKY70 in developmental senescence, osmotic stress as well as plant defense responses. The arrows indicate induction or positive modulation; the blunt-end arrows indicate block or suppression. SA, salicylic acid; JA, jasmonic acid; ABA, abscisic acid; ROS, reactive oxygen species.
ACKNOWLEDGEMENTS

The work for my thesis has been carried out at the Department of Biosciences, Faculty of Biological and Environmental Sciences, University of Helsinki. Research funding was provided by the Academy of Finland Center of Excellence programme, the project of Academy of Finland (257644), the European Research Area in Plant Genomics (ERAPGFP/06.023a) and the Helsinki Graduate Program in Biotechnology and Molecular Biology (GPBM).

I express my deepest gratitude to my supervisor Professor Tapio Palva, for his excellent guidance, patience, caring and continuous support of my PhD research. Without his brilliant mind and encouragement over the years, finishing this work would have been much more difficult. I am also grateful to Docent Pekka Heino, his enthusiasm on science and sense of humor have given me a deep impression. His involvement in discussion about my manuscript is greatly acknowledged.

I want to thank Docent Minna Pirhonen and Docent Suvi Taira for kindly reviewing my thesis. Their suggestions and comments are rather important for finalizing my thesis. Special thank goes to Dr. Karen Sims-Huopaniemi for her conscientious language checking on my thesis. Dr. Tarja Kariola is sincerely acknowledged for her invaluable comments on my third manuscript.

I wish to thank my follow-up group members Günter Brader, Mikael Brosché and Kurt Fagerstedt for following my PhD study, and great discussions during the past few years. I also would like to thank the coordinator and the communications officer of GPBM, Erkki Raulo and Anita Tienhaara, for organizing many interesting activities and creating a nice academic atmosphere for PhD students in the graduate school.

Great thanks are given to all the co-authors, Liisa Holm, Hannes Kollist, Sébastien Besseau, Petri Törönen, Nina Sipari, for your great help and contributions to the publications. I am grateful to my present colleagues, Tarja Kariola, Outi Niemi, Mantas Survila, Ville Pennanen, Maria Piisilä, Mehmet Ali Keceli, Pär Davidsson, Anne Leino and Martin Broberg. I really enjoyed good moments with you all during these years. I also want to acknowledge all the former members in Tapio Palva’s group, especially Markku Aalto, Zhubing Hu, Anzu Minami, Elina Helenius, Nina Von Numers, Kukka Aho, Anne Kujanpää, Solveig Sjöblom, Heidi Harjunpää, Diana Tulea, Umamaheswari Ramjee. Thank you for all the help over the years. Dr. Kirk Overmyer is acknowledged for his help in growth facility. I wish to thank Arja Välimäki and Arja Ikävalko for your generous help with all sorts of things. I am grateful to Leila Miettinen, Hanne Mikkonen and Olga Jurgens for their skilled technical assistance.

I am feeling lucky that I am not along during this long scientific journey. A lot of friends are around me and have helped me through these years. My great appreciation and friendship go to these non-Chinese speaking friends, especially Heidi Harjunpää, Outi Niemi, Maral Jamshidi, Netta Mäkinen, who constantly encourage and stand up for me when I am in struggles and frustrations. Your friendship makes me feel warm during my life in Finland.
I am also thankful to my Chinese friends around, Guo Deyin, Li Chunyang, Chen Qiu zhen, Tan Xuezhou, Yan Ping, Cui Fuqiang, Xu Enjun, Wang Changfang, Sun Hui and Wang Wei. We shared quite a lot of nice moments together, and these precious memories will be never forgotten. I am particularly grateful to my beloved Xianbao. Your life attitude always gives me strength and encouragement of being confident and optimistic.

Finally, I am deeply and forever indebted to my loving parents. We have weekly video chatting during all these years, without interruption! Mom and dad always show a concern for my daily life and research progress. They keep faith in me and give me freedom to choose what I desired. I could not even imagine my life without their love, support and encouragement. They are the only people in this world whom I love and cherish from the bottom of my heart!!!

Jing Li  Helsinki  Feb. 2014
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