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Review article

WRKY transcription factors: key components in abscisic acid signalling

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Summary

WRKY transcription factors (TFs) are key regulators of many plant processes, including the responses to biotic and abiotic stresses, senescence, seed dormancy and seed germination. For over 15 years, limited evidence has been available suggesting that WRKY TFs may play roles in regulating plant responses to the phytohormone abscisic acid (ABA), notably some WRKY TFs are ABA-inducible repressors of seed germination. However, the roles of WRKY TFs in other aspects of ABA signalling, and the mechanisms involved, have remained unclear. Recent significant progress in ABA research has now placed specific WRKY TFs firmly in ABA-responsive signalling pathways, where they act at multiple levels. In Arabidopsis, WRKY TFs appear to act downstream of at least two ABA receptors: the cytoplasmic PYR/PYL/RCAR-protein phosphatase 2C-ABA complex and the chloroplast envelope–located ABAR–ABA complex. *In vivo* and *in vitro* promoter-binding studies show that the target genes for WRKY TFs that are involved in ABA signalling include well-known ABA-responsive genes such as *ABF2*, *ABF4*, *ABI4*, *ABI5*, *MYB2*, *DREB1a*, *DREB2a* and *RAB18*. Additional well-characterized stress-inducible genes such as *RD29A* and *COR47* are also found in signalling pathways downstream of WRKY TFs. These new insights also reveal that some WRKY TFs are positive regulators of ABA-mediated stomatal closure and hence drought responses. Conversely, many WRKY TFs are negative regulators of seed germination, and controlling seed germination appears a common function of a subset of WRKY TFs in flowering plants. Taken together, these new data demonstrate that WRKY TFs are key nodes in ABA-responsive signalling networks.

Introduction

WRKY transcription factors (TFs) have mostly been studied with regard to their roles in regulating plant responses to pathogens. They are key regulators, both positive and negative, of the two partly interconnected branches of plant innate immunity: microbe/pathogen-associated molecular pattern-triggered immunity (MTI/PTI) and effector-triggered immunity (ETI) (Rushton *et al.*, 2010). More recently, it has become clear that WRKY TFs also play key roles in responses to abiotic stresses such as cold and high temperature, water stress, high CO_2 levels, high ozone concentrations and salt stress (Rushton *et al.*, 2010). Many of these stress responses are regulated by the plant hormone abscisic acid (ABA). Two of the first identified WRKY TFs (AfWRKY1/ABF1 and AfWRKY2/ABF2) were implicated in the regulation of gene expression during seed germination (Rushton *et al.*, 1995), a process that is regulated jointly by the plant hormones gibberellin (GA) and ABA. Although notable progress has been made determining the involvement of WRKY TFs in seed germination (Zhang *et al.*, 2004, 2009; Zou *et al.*, 2004, 2008; Xie *et al.*, 2005, 2006), the role of WRKY TFs in ABA signalling in general has remained obscure. This situation has now changed with the recent publication of several reports that place specific WRKY TFs into signalling networks that respond to ABA (Jiang and Yu, 2009; Ren *et al.*, 2010; Shang *et al.*, 2010). Some of these WRKY TFs appear to regulate the expression of other TFs (for example, bZIPs, MYBs and ERFs) that were already known to be regulators of ABA responses. These new insights into the mechanisms of ABA signalling have important consequences for the manipulation of processes such as drought responses and seed germination in crop plants.

Abscisic acid signalling

Abscisic acid was identified in the 1960s and is a plant hormone that coordinates responses to stresses such as drought, extreme temperature and high salinity, as well as regulating nonstress responses including seed maturation, seed germination and bud dormancy (Shang *et al.*, 2010; Umezawa *et al.*, 2010). ABA functions through a complex web of signalling networks, and many parts of these pathways have been identified in recent years (Umezawa *et al.*, 2010). These signalling components include TFs of various classes, E3 ligases, phospholipases C/D, G proteins, receptor-like kinases and other classes

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14. ABSTRACT

WRKY transcription factors (TFs) are key regulators of many plant processes, including the responses to biotic and abiotic stresses, senescence, seed dormancy and seed germination. For over 15 years, limited evidence has been available suggesting that WRKY TFs may play roles in regulating plant responses to the phytohormone abscisic acid (ABA), notably some WRKY TFs are ABA-inducible repressors of seed germination. However, the roles of WRKY TFs in other aspects of ABA signalling, and the mechanisms involved, have remained unclear. Recent significant progress in ABA research has now placed specific WRKY TFs firmly in ABA-responsive signalling pathways, where they act at multiple levels. In Arabidopsis, WRKY TFs appear to act downstream of at least two ABA receptors: the cytoplasmic PYR ⁄ PYL ⁄ RCARprotein phosphatase 2C-ABA complex and the chloroplast envelope?located ABAR?ABA complex. In vivo and in vitro promoter-binding studies show that the target genes for WRKY TFs that are involved in ABA signalling include well-known ABA-responsive genes such as ABF2 ABF4, ABI4, ABI5, MYB2, DREB1a, DREB2a and RAB18. Additional well-characterized stressinducible genes such as RD29A and COR47 are also found in signalling pathways downstream of WRKY TFs. These new insights also reveal that some WRKY TFs are positive regulators of ABA-mediated stomatal closure and hence drought responses. Conversely, many WRKY TFs are negative regulators of seed germination, and controlling seed germination appears a common function of a subset of WRKY TFs in flowering plants. Taken together, these new data demonstrate that WRKY TFs are key nodes in ABA-responsive signalling networks.

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of protein kinases and phosphatases (Shang *et al.*, 2010; Umezawa *et al.*, 2010). However, plant scientists laboured for decades to understand how plant cells sense ABA (Pennisi, 2009), and the elusive ABA receptors remained to be found. Starting in 2006, several potential ABA receptors were proposed, although a role as a true ABA receptor remains, in many cases, controversial. The first reported ABA receptor was an RNA-binding protein called FCA (Razem *et al.*, 2006), although the data associated with the report proved to be unreliable (Risk *et al.*, 2008). In the same year, a second potential ABA receptor was proposed (Shen *et al.*, 2006). The protein was the magnesium-protoporphyrin IX chelatase large subunit (Mg-chelatase H subunit [CHLH]/putative ABA receptor [ABAR]). ABAR is a chloroplast/plastid protein and has multiple functions in plant cells. Doubts have also been expressed about the role of ABAR as an ABA receptor as knockout mutations in the barley homologue, XanF, appear to have no effect on ABA signalling (Muller and Hansson, 2009). This lack of a detectable phenotype may, however, be a consequence of the presence of duplicated or multiple ABA receptors in plants. Nevertheless, recent additional work on ABAR provides not only more data in support of the protein as an ABA receptor but also a mechanism of action with a near-complete signalling pathway (Shang *et al.*, 2010). ABAR spans the chloroplast envelope, and the cytosolic C-terminus interacts with a group of WRKY TFs (AtWRKY40, AtWRKY18 and AtWRKY60) that function as negative regulators of ABA signalling. Other reports of possible ABA receptors have centred on plasma membrane–located G protein-coupled receptors. Two have been implicated in ABA responses. First, an unconventional G protein-coupled receptor GCR2 (Liu *et al.*, 2007) and secondly, GTG1 and GTG2, members of a novel class of G protein-coupled receptor (Pandey *et al.*, 2009). The role of both proteins as ABA receptors, again, remains controversial (Johnston *et al.*, 2007; Pennisi, 2009).

Recently, PYR/PYL/RCAR proteins, members of the START domain superfamily, were reported to function as cytosolic ABA receptors by directly inhibiting type 2C protein phosphatases (Ma *et al.*, 2009; Park *et al.*, 2009). Shortly after these first reports, six independent groups simultaneously defined the structural and functional mechanisms by which ABA is sensed (Fujii *et al.*, 2009; Melcher *et al.*, 2009; Miyazono *et al.*, 2009; Nishimura *et al.*, 2009; Santiago *et al.*, 2009; Yin *et al.*, 2009). In the absence of ABA, a phosphatase PP2C acts as a constitutive negative regulator of a family of kinases (SnRK2) whose autophosphorylation is required for kinase activity towards downstream targets. The binding of ABA by the PYR/PYL/RCAR receptor facilitates binding of the receptor to PP2C and this represses PP2C activity. This sequestration of PP2C in the ABAR-receptor complex allows autoactivation of the SnRK2 kinase which then phosphorylates downstream TFs leading to the transcriptional activation of ABA-responsive genes (Sheard and Zheng, 2009). This elegant mechanism of ABA perception and signal transduction likely represents a major component of ABA signalling, although other receptors such as ABAR are probably additional features of a complex signalling web with both multiple inputs and multiple outputs.

WRKY transcription factors

WRKY proteins comprise one of the largest families of TFs found in plants (Rushton *et al.*, 2010). As with most TFs, the defining feature, or signature, of WRKY proteins is their DNA-binding domain. This is called the WRKY domain after the almost invariant WRKY amino acid sequence (Rushton *et al.*, 1996, 2010; Eulgem *et al.*, 2000). The WRKY domain is about 60 residues in length and has two components. At the N-terminal end is the WRKY amino acid signature, and this is followed by a zinc finger structure at the C-terminus. The amino acid sequence of the zinc finger in the WRKY domain is $CX_{4\text{-}7}CX_{22\text{-}23}HXH/C$, and the exact amino acid sequence of the finger reflects the subfamily of WRKY genes to which the protein belongs. The bipartite nature of the WRKY domain is underlined by the observation that in many subfamilies of WRKY genes (groups I, IIc, IId, IIe and III), the two component parts of the WRKY domain are separated by an intron (Eulgem *et al.*, 2000). Some of the important outstanding questions about the WRKY domain were answered by an NMR solution structure of a WRKY domain (Yamasaki *et al.*, 2005) followed 2 years later by a crystal structure determination (Duan *et al.*, 2007). Both the solution structure and the crystal structure revealed that the WRKY domain consists of a four-stranded β-sheet, with a zinc-binding pocket formed by the conserved Cys/His residues. The WRKYGQK residues form the most N-terminal β-strand and appear to enter the major DNA groove and form contacts with an approximately 6-bp region of the DNA. This 6-bp region of interaction is consistent with the length of the W box, (Yamasaki *et al.*, 2005), which is the core binding site for most WRKY proteins (Rushton *et al.*, 1996, 2010; Eulgem *et al.*, 2000).

Outside of the WRKY domain, WRKY proteins contain characteristic features of TFs such as nuclear localization signals, activation/repression domains and domains associated with protein–protein interactions such as leucine zippers (Eulgem *et al.*, 2000; Rushton *et al.*, 2010). Although some of these domains are conserved between members within a subfamily, it is only the WRKY domain itself that is shared by all WRKY TFs. The availability of the complete genome sequence of several flowering plant species has facilitated the classification of WRKY TFs into seven major subfamilies called groups I, IIa, IIb, IIc, IId, IIe and III (Eulgem *et al.*, 2000). Phylogenetic analyses have then more accurately divided the WRKY family into groups I, IIa + b, IIc, IId + e and III with the group II genes not being monophyletic. Group IIa and IIb genes form two closely related clades, as do group IId and IIe genes (Zhang and Wang, 2005; Rushton *et al.*, 2008, 2010).

Fifteen years of research have confirmed that the conservation of the WRKY domain in WRKY proteins is mirrored by a remarkable conservation of its cognate binding site, the W box (TTGACC/T) (Rushton *et al.*, 1996, 2010; Eulgem *et al.*, 2000). Both bioinformatic-based and functional studies of plant promoters have found clusters of W boxes in stress-inducible promoters and, in some cases, multiple W boxes appear to have a synergistic effect on transcription (Eulgem *et al.*, 1999). Almost all WRKY TFs bind preferentially to W boxes, and this raises the question as to how they show specificity for the promoters of their target genes (Rushton *et al.*, 2010). Data concerning the binding-site specificity of WRKY proteins are surprisingly rare, but Ciolkowski *et al.* (2008) showed that although the W box core is required, adjacent sequences also play a role in determining binding-site preference (Ciolkowski *et al.*, 2008). The binding of WRKY proteins to W boxes is a feature of both biotic and abiotic stress responses. The presence of functional W boxes in the promoters of abiotic stress–inducible genes has recently been clearly demonstrated in

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Arabidopsis using both chromatin immunoprecipitation (ChIP) and gel retardation assays (Shang *et al.*, 2010).

WRKY proteins can activate or repress transcription, and some WRKY TFs appear to possess both functions (Rushton *et al.*, 2010). The mechanisms of activation and repression require further elucidation; however, an increasing number of proteins have been shown to interact with WRKY TFs (Rushton *et al.*, 2010). These include both proteins that direct epigenetic changes, such as histone deacetylases, and signalling components, such as MAP kinases, MAP kinase kinases, 14-3-3 proteins and calmodulin (Rushton *et al.*, 2010). The discovery of interacting partners facilitates the reconstruction of signalling pathways that contain WRKY proteins and identifies both inputs and outputs for these TFs.

The importance of WRKY TFs in plant stress signalling is illustrated by two recent reports concerning interacting partners of the group IIa proteins HvWRKY1, HvWRKY2, AtWRKY18, AtWRKY40 and AtWRKY60 (Shen *et al.*, 2007; Shang *et al.*, 2010). In barley, ETI to barley powdery mildew requires the recognition of the fungal avirulence AVR10 effector by the resistance protein MLA. This occurs in the cytoplasm and leads to a subsequent association of the MLA resistance protein with HvWRKY1 and HvWRKY2 inside the nucleus (Shen *et al.*, 2007). This association of WRKY TFs with resistance proteins is exciting, as it reveals a direct signalling mechanism involving two well-characterized components of plant stress responses. Another interaction occurs between the Arabidopsis group IIa WRKY proteins, AtWRKY18, AtWRKY40 and AtWRKY60, and the chloroplast/plastid-localized ABA receptor, ABAR (Shang *et al.*, 2010). These data reveal direct interactions between WRKY TFs and important components of plant-signalling webs such as resistance proteins and receptors and underlines the importance of WRKY TFs in these signalling networks.

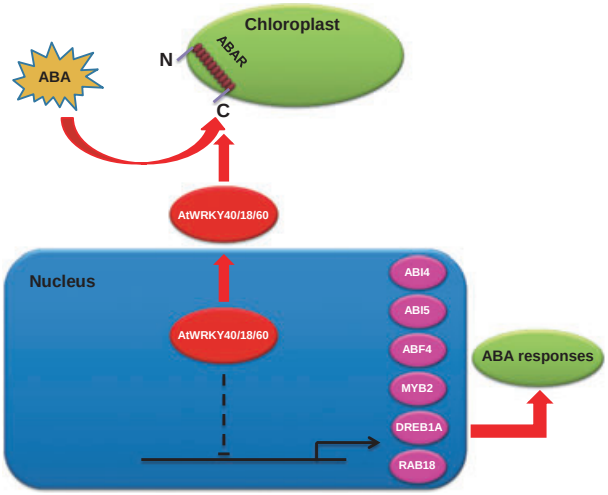

Figure 1 A schematic representation of the mechanism by which AtWRKY40, AtWRKY18 and AtWRKY60 regulate ABA responses. During seed germination and postgermination growth, the Mg-chelatase H subunit/putative ABA receptor (ABAR) and AtWRKY40, AtWRKY18 and AtWRKY60 regulate ABA responses by a de-repression mechanism that removes the WRKY repressor proteins from the nucleus. ABAR spans the chloroplast envelope and the cytosolic C-terminus binds ABA. Binding of ABA by ABAR recruits AtWRKY40, AtWRKY18 and AtWRKY60 from the nucleus to the cytoplasm where they also bind to the C-terminus of ABAR. These WRKY transcription factors (TFs) function as negative regulators of ABA signalling, and this results in a de-repression of ABA-signalling pathways. As a result, expression of ABA-responsive genes such as *ABF4*, *ABI4*, *ABI5*, *DREB1A*, *MYB2* and *RAB18* is induced and ABA responses occur. The dotted line denotes de-repression of gene expression as the WRKY TFs are removed from the nucleus. Abbreviations: ABA, abscisic acid; ABAR, Mg-chelatase H subunit/putative ABA receptor.

WRKY transcription factors in ABA-signalling networks

Several recent publications have not only placed WRKY TFs in ABA-induced signalling networks but have also provided novel insights into both the ABA receptors that lie upstream of the WRKY TFs and the target genes that are downstream. New data concerning the ABA receptor, ABAR, provide more information in support of the protein as an ABA receptor and also a mechanism of action that includes at least three group IIa WRKY TFs (Shang *et al.*, 2010). In an extensive set of studies using ABAR-GFP fusion proteins and immunodetection, it was shown that ABAR spans the chloroplast envelope and that both the N- and C-terminal portions of the protein are exposed to the cytoplasm. Previous work had shown that it is the C-terminal part of ABAR that binds ABA (Wu *et al.*, 2009a), and this C-terminus (amino acids 692–1381) was used as a bait in a yeast two-hybrid screen. These experiments led to the identification of AtWRKY40 as a protein that interacts with the C-terminus of ABAR. The other two Arabidopsis group IIa proteins, AtWRKY18 and AtWRKY60, also interact with ABAR, albeit with a lower affinity. The interaction of ABAR and AtWRKY40 was confirmed by both coimmunoprecipitation and luciferase complementation imaging (Shang *et al.*, 2010). This interaction is stimulated by ABA, and ABA also recruits AtWRKY40 from the nucleus to the cytoplasm. This suggests a mechanism of ABA signalling that operates by the removal of AtWRKY40 from the nucleus (Figure 1). Further evidence provided a clearer insight into this mechanism. Knockout mutants of *AtWRKY18*, *AtWRKY40* and *AtWRKY60* all show ABA-hypersensitive phenotypes in ABA-induced postgermination growth arrest, and inhibition of seed germination and the mutant analyses suggest that the three WRKY TFs cooperate to negatively regulate ABA signalling. The ABA-induced movement of AtWRKY40 from the nucleus to the cytoplasm therefore represents a de-repression of ABA signalling and reveals the first stages in a novel mechanism where ABA induces gene expression (Figure 1).

Regardless of the exact role of ABAR in ABA perception, it is nevertheless clear that the three Arabidopsis group IIa WRKY TFs play important roles in ABA signalling (Chen *et al.*, 2010; Shang *et al.*, 2010). The expression of a large number of known ABA-responsive genes is altered in *AtWRKY40* or *AtWRKY40/AtWRKY18* knockout lines. These genes include *ABF4*, *ABI1*, *ABI2*, *ABI4*, *ABI5*, *DREB1A*, *DREB2A*, *MYB2*, *PYL2/RCAR13*, *PYL2/RCAR11*, *RAB18*, *PYL2/RCAR9*, *PYL2/RCAR7*, *SnRK2.2* and *SnRK2.3* (Shang *et al.*, 2010). ChIP experiments show that AtWRKY40 directly targets a number of these genes as the protein binds *in vivo* to W box-containing fragments of the promoters of the *ABI4*, *ABI5*, *ABF4*, *MYB2*, *DREB1A* and *RAB18* genes (Table 1) (Shang *et al.*, 2010). These data place AtWRKY40, AtWRKY18 and AtWRKY60 upstream of other known ABA-responsive TFs such as the AP2/ERF genes *DREB1A* and *ABI4*, the MYB gene *MYB2* and the bZIP genes *ABI5* and *ABF4*. ABI5 controls seed germination and

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Table 1 Abscisic acid-signalling components that are direct targets of AtWRKY40

Target gene	Type of gene	Evidence
ABI4	AP2/ERF transcription factor	Binds to the promoter *in vivo* (ChIP). Yeast one-hybrid analyses Gel shifts
ABI5	bZIP transcription factor	Binds to the promoter *in vivo* (ChIP) Yeast one-hybrid analyses Gel shifts
ABF4	bZIP transcription factor	Binds to the promoter *in vivo* (ChIP) Yeast one-hybrid analyses Gel shifts
MYB2	MYB transcription factor	Binds to the promoter *in vivo* (ChIP) Yeast one-hybrid analyses Gel shifts
DREB1A	AP2/ERF transcription factor	Binds to the promoter *in vivo* (ChIP)
DREB2A	AP2/ERF transcription factor	Binds to the promoter *in vivo* (ChIP)
RAB18	Rab-related protein	Binds to the promoter *in vivo* (ChIP)
AtWRKY60	WRKY transcription factor	Gel shifts Cotransfection (together with AtWRKY18)

ChIP, chromatin immunoprecipitation.

postgermination growth and is one of the most important and genetically well-characterized ABA-signalling regulators (Finkelstein and Lynch, 2000; Finkelstein *et al.*, 2002). Both cotransfection experiments using the *ABI5* promoter and AtWRKY40 and the analysis of *ABI5* expression in *AtWRKY40* knockout lines suggest that AtWRKY40 directly represses *ABI5* expression (Shang *et al.*, 2010). This observation that these WRKY TFs regulate known ABA-responsive TFs is strong evidence that they are early nodes in the ABA-signalling web.

The observation that WRKY TFs regulate the expression of ABF genes necessitates a cautionary note here on the naming of genes. The name ABF has been used several times to describe TFs. Human ABF-1 is a bHLH transcription factor (Mitchell *et al.*, 2000). Yeast ABF-1 is a trans-acting factor involved in the regulation of transcription and in DNA replication (Rhode *et al.*, 1989). More importantly, the names ABF1 and ABF2 had already been used to describe plant TFs before a subfamily of bZIP genes were called ABFs (Choi *et al.*, 2000). *ABF1* and *ABF2* from wild oat were among the first WRKY genes isolated, and *ABF2* was the first group IIa gene described (Rushton *et al.*, 1995). Both ABF1 and ABF2 have been implicated in GA and ABA signalling (Rushton *et al.*, 1995). Wild oat *ABF2* appears to be an orthologue of Arabidopsis *AtWRKY40* and may therefore be a negative regulator of wild oat ABF genes (bZIPs) in a similar way to its Arabidopsis counterpart. The potential for confusion is clear. We propose the names *AfWRKY1/ABF1* and *AfWRKY2/ABF2* to avoid any confusion caused by the multiple use of the name ABF.

An additional recent report also demonstrated that AtWRKY40, AtWRKY18 and AtWRKY60 are involved in plant responses to ABA and abiotic stress (Chen *et al.*, 2010) and also provides some additional information about the complexity of the regulation of ABA responses by these three WRKY proteins. Through analysis of single, double and triple mutants and overexpression lines, it appears that AtWRKY40 does indeed negatively regulate ABA responses during seed germination and postgermination growth. By contrast, it was suggested that *AtWRKY18* and *AtWRKY60* have a positive effect on ABA responses and also enhance plant sensitivity to salt and osmotic stress. Both *AtWRKY40* and *AtWRKY18* are rapidly induced by ABA, whereas induction of *AtWRKY60* is slower (Chen *et al.*, 2010). It appears that *AtWRKY60* might be a direct target of AtWRKY40 and AtWRKY18 because induction of *AtWRKY60* is almost completely abolished in *wrky18* and *wrky40* mutants, and both AtWRKY40 and AtWRKY18 proteins recognize a cluster of W box sequences in the *AtWRKY60* promoter. The authors suggest that a AtWRKY18/AtWRKY40 heterocomplex may regulate the expression of the *AtWRKY60* gene and that homo- and heterodimer complexes of these three group IIa WRKY proteins then regulate ABA responses (Chen *et al.*, 2010). This cross-regulation at the transcriptional level and the involvement of homo- and heterodimer WRKY complexes add an extra level of complexity to the ABA-signalling network.

Recent mutant analysis has presented direct evidence that WRKY TFs are components of other parts of ABA-induced signalling networks (Jiang and Yu, 2009; Ren *et al.*, 2010). It is relatively rare to identify mutants in WRKY genes that have detectable phenotypes, largely because of functional redundancy (Eulgem and Somssich, 2007; Rushton *et al.*, 2010). Nevertheless, two recent reports provide details of WRKY knockout mutants that are hypersensitive to ABA responses during seed germination and postgermination growth. The ABA-hypersensitive mutant, *abo3*, was found to be caused by a T-DNA insertion in *AtWRKY63* (*At1g66600*). The *abo3* mutant was hypersensitive to ABA in both seedling establishment and seedling growth. Conversely, stomatal closure was less sensitive to ABA, and the mutant was therefore also less drought tolerant than the wild type (Ren *et al.*, 2010). AtWRKY63 is a member of the group III subfamily of WRKY TFs and is not only a different type of WRKY protein from AtWRKY40, AtWRKY18 and AtWRKY60 but it is also to be found in a different part of the ABA-signalling web (Figure 2). The *abo3* mutation impaired the expression of *ABF2* and downstream genes such as *RD29A* and *COR47*, but the levels of *ABF3*, *DREB2A*, *RD22* and *KIN1* did not differ (Ren *et al.*, 2010). Additionally, the transcriptional induction of *AtWRKY63/ABO3* by ABA was impaired in *abi1*, *abi2* and *abi5* mutant lines. This places the *AtWRKY63* gene downstream of *ABI1*, *ABI2* and *ABI5* but upstream of *ABF2*, *RD29A* and *COR47* (Figure 2). Interestingly, AtWRKY40 appears to act upstream of the bZIP transcription factor *ABI5* (Shang *et al.*, 2010), whereas AtWRKY63 acts downstream of it in seed germination and postgermination growth (Figure 2) (Ren *et al.*, 2010; Shang *et al.*, 2010). Taken together, this suggests that ABA induces a cascade of transcription factor activation with AtWRKY40 repressing *ABI5* gene expression in the absence of ABA. Upon ABA perception by ABAR, PYR/PYL/RCAR or other receptors, de-repression of *ABI5* leads to activation of *AtWRKY63* at the transcriptional level. AtWRKY63 then activates downstream genes such as *RD29A* and *COR47*. As each of these TFs appears to have multiple target genes, this

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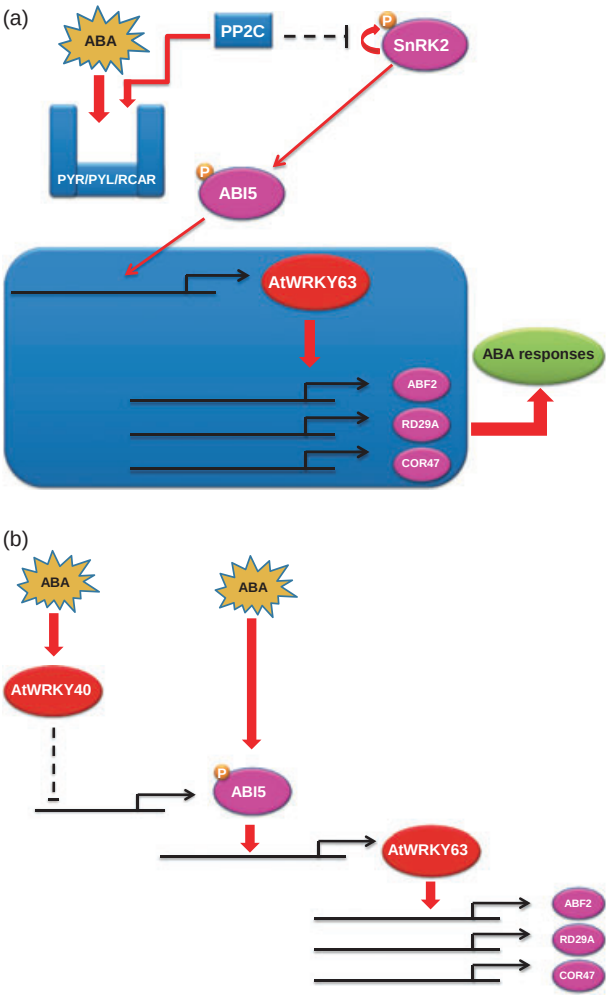

Figure 2 (a) The role of AtWRKY63 in the ABA-signalling network. Upon ABA perception by PYR/PYL/RCAR, activation of ABI5 occurs following phosphorylation by the SnRK2 kinase. This leads to transcriptional activation of the *AtWRKY63* gene by ABI5. AtWRKY63 then activates known ABA response genes such as *RD29A*, *ABF2* and *COR47*. The dotted line denotes de-repression of SnRK2 autophosphorylation. Abbreviations: ABA, abscisic acid; PP2C, type 2C protein phosphatase; SnRK2, SNF1-related protein kinase 2. (b) An AtWRKY40-ABI5-AtWRKY63 module is part of the ABA-signalling network. WRKY transcription factors operate at multiple levels in the ABA-signalling network. During seed germination and postgermination growth, ABA is perceived by both PYR/PYL/RCAR and ABA receptor (ABAR). ABA perception by ABAR results in movement of the repressor protein AtWRKY40 out of the nucleus. This leads to de-repression of *ABI5* at the transcriptional level. The produced ABI5 is activated following phosphorylation as a result of ABA perception by PYR/PYL/RCAR. Activation of ABI5 leads to transcription of the *AtWRKY63* gene, and AtWRKY63 then activates further downstream target genes such *RD29A*, *ABF2* and *COR47*. The dotted line denotes de-repression of *ABI5* gene expression as AtWRKY40 is removed from the nucleus.

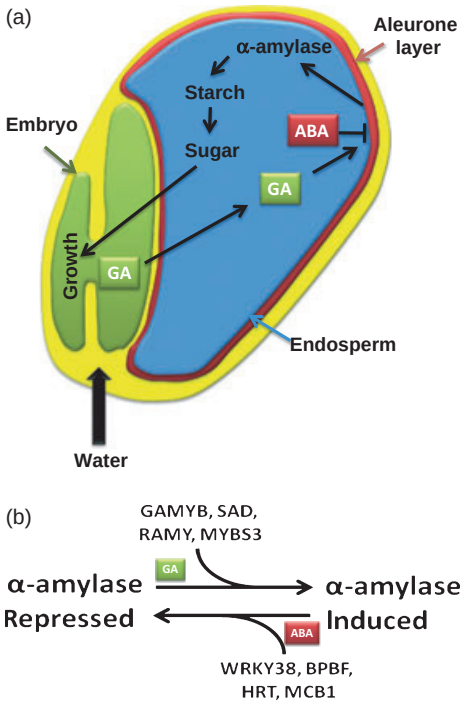

Figure 3 The enhanceosome and repressosome model for the regulation of α-amylase gene expression during barley seed germination. (a) A schematic representation of seed germination. Before germination, expression of genes required for the mobilization of storage compounds is repressed by abscisic acid (ABA). Upon imbibition of water, gibberellin (GA) is synthesized by the embryo and translocated to the aleurone layer. This promotes germination and expression of genes encoding hydrolytic enzymes such as α-amylase. Stored starch is broken down by α-amylase, and soluble sugars are used by the germinating embryo. (b) ABA promotes the formation of a repressosome on the α-amlyase promoters consisting of HvWRKY38, HvBPBF, HvHRT and HvMCB1. Upon perception of GA, this repressosome is replaced by an enhanceosome consisting of HvGAMYB, HvSAD, HvRAMY and HvMYBS3. This results in transcription of the α-amylase genes.

pathway is therefore only a small module within a complex web of gene activation and repression that ABA perception sets in motion.

Another knockout mutation that affects seed germination and postgermination growth was reported in *AtWRKY2* (Jiang and Yu, 2009). The knockout mutant has a similar phenotype to the *abo3/AtWRKY63* mutation except that there appears to be no effect on stomatal closure. *wrky2* knockout mutants displayed delayed or decreased expression of *ABI5* and *ABI3* and increased or prolonged expression of *Em1* and *Em6*. Analysis of *AtWRKY2* expression levels in ABA-insensitive and ABA-deficient mutants indicated that ABA-induced *AtWRKY2* accumulation during germination and postgermination early growth requires *ABI5*, *ABI3*, *ABA2* and *ABA3* (Jiang and Yu, 2009). This suggests a feedback loop involving *ABI3* and *ABI5* in *AtWRKY2* gene expression and also places *AtWRKY2* upstream of both genes in the initial ABA response. Taken together, these analyses suggest a role for *AtWRKY2*, *AtWRKY40*, *AtWRKY18* and *AtWRKY60* upstream of *ABI5*, whereas *AtWRKY63* acts downstream of it.

WRKY transcription factors, abscisic acid, drought responses and stomatal opening

Drought stress is one of the major causes of crop loss (Cominelli and Tonelli, 2010; Nakashima and Yamaguchi-Shinozaki, 2010), and improving plant responses to drought stress is a major aim of plant biotechnology. Drought stress induces the accumulation

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of ABA, which leads to stomatal closure. Closing of the stomata helps to maintain the water status of cells within the plant under water deficit conditions by reducing water loss as a result of transpiration (Schroeder *et al.*, 2001). The *abo3* mutation impairs ABA-induced stomatal closure, and the plants are therefore more sensitive to drought stress than wild-type plants, suggesting that ABO3/AtWRKY63 functions in ABA-mediated drought stress response pathways (Ren *et al.*, 2010). This positive role of ABO3/AtWRKY63 in ABA-mediated stomatal closure contrasts with its negative role in seed germination and root growth. Interestingly, it has also been proposed that WRKY TFs are part of a mechanism of stomatal closure that is induced by pathogens (Schulze-Lefert and Robatzek, 2006). An elegant set of experiments showed that stomata close upon detection of potential microbial pathogens to prevent infection of the plant (Melotto *et al.*, 2006). In the continual battle between plants and their pathogens, pathogenic bacteria have evolved strategies to suppress this stomatal closure mechanism and it was suggested that WRKY TFs play a role in this suppression by bacteria (Schulze-Lefert and Robatzek, 2006). The possibility that WRKY TFs are involved in stomatal closing as a response to both biotic and abiotic stress is an area that requires more research but more evidence is slowly appearing. Ectopic overexpression of the ABA-inducible *OsWRKY45* gene in Arabidopsis conferred a number of properties to the transgenic plants, including enhanced disease resistance, enhanced tolerance to salt and drought stress, decreased sensitivity to ABA during seed germination and postgermination growth and enhanced induction of stress-related genes (Qiu and Yu, 2009). Importantly, under water stress conditions, the *OsWRKY45*-overexpressing plants had a lower rate of water loss than control plants and this appeared to be because these plants had a greater number of closed stomata. The reduced transpiration rate because of greater stomatal closure allowed the *OsWRKY45*-overexpressing plants to maintain a more favourable water balance and resulted in greater drought tolerance. There are other recent reports of WRKY genes regulating water stress responses. Rice lines overexpressing *OsWRKY11* showed significant desiccation tolerance and induction of raffinose synthase and galactinol synthase genes (Wu *et al.*, 2009b). Induction of these genes and accumulation of raffinose as an osmoprotectant are both well-characterized responses to water stress and this suggests a regulatory role for *OsWRKY11* in these processes. More evidence that WRKY genes regulate galactinol synthase induction has come from the resurrection plant *Boea hygrometrica* (Wang *et al.*, 2009). The *BhGolS1* gene is inducible by both dehydration and ABA. The *BhGolS1* promoter contains four W boxes, and ChIP showed that it is bound *in vivo* by the early dehydration and ABA-inducible BhWRKY1 (Wang *et al.*, 2009). These data provide direct evidence linking a dehydration-inducible WRKY TF with a downstream target gene that plays an important role in drought responses.

Other data suggest that manipulation of WRKY TF expression may lead to improved drought responses through changes, not in stomata but in root architecture. Heterologous overexpression of the rice WRKY gene *OsWRKY08* in Arabidopsis improved osmotic tolerance. Although not overexpressed in rice, these data are of interest as they suggest a physiological basis for the increased drought tolerance. 35S:OsWRKY08 transgenic Arabidopsis plants have increased lateral root number and primary root length during root development (Song *et al.*, 2009).

Gibberellin, abscisic acid and WRKY transcription factors in seed germination

By far, the best studied role of WRKY TFs in regulating ABA responses is their role as ABA-inducible repressors of seed germination. This has a history that goes back to the discovery of AfWRKY1/ABF1 and AfWRKY2/ABF2 over 15 years ago (Rushton *et al.*, 1995). ABA is integral to establishing and maintaining seed dormancy. In this capacity, ABA and GA participate in antagonistic crosstalk; while GA initiates germination, ABA prevents this initiation (Finkelstein *et al.*, 2008). In cereals, upon imbibing, the embryos of nondormant seeds produce GA. This GA is transported to the aleurone, a thin layer of cells surrounding the endosperm. Upon arrival in aleurone cells, GA interacts with the receptor, GID1 (Murase *et al.*, 2008). Through a series of signal transduction events (Ueguchi-Tanaka *et al.*, 2007; Murase *et al.*, 2008), a number of TFs, including GAMYB (Gubler *et al.*, 2002), initiate the processes involved in germination. These processes include the induction of α-amylase production, allowing this enzyme to be secreted into the endosperm, where it mobilizes starch reserves to fuel embryo growth (Gubler *et al.*, 1997) (Figure 3a). ABA inhibits this pathway through a number of mechanisms, including the repression of GAMYB expression (Gomez-Cadenas *et al.*, 2001), and possibly through the inhibition of GA biosynthesis (Gubler *et al.*, 2005).

Several WRKY TFs have been shown to be involved in this suppression of germination by ABA. In rice, OsWRKY45 and OsWRKY24 inhibit transcription from the promoter of the ABA-responsive gene, *HVA22,* under ABA treatment (Xie *et al.*, 2005). In contrast, another two, OsWRKY72 and OsWRKY77, enhance transcription from the *HVA22* promoter by ABA. Similar experiments in barley aleurone cells, using a reporter construct containing the GA-inducible *Amy32b* α-amylase promoter (Lanahan *et al.*, 1992) showed that OsWRKY71, a homologue of wild oat AfWRKY2/ABF2 (Rushton *et al.*, 1995), antagonizes the activation of the reporter construct by GA and GAMYB (Zhang *et al.*, 2004). Further studies indicate that OsWRKY71 works with another WRKY family member, OsWRKY51, to mediate crosstalk between GA and ABA in the control of α-amylase production (Zhang *et al.*, 2004; Xie *et al.*, 2006). Promoter-binding studies suggest that OsWRKY71, but not OsWRKY51, binds to the O2S element (containing two W boxes) of the *Amy32b* α-amylase promoter. Interestingly, bimolecular fluorescence assays demonstrate that the two proteins interact in the nuclei of barley aleurone cells, (Xie *et al.*, 2006) and interaction of the two TFs increases the binding of OsWRKY71 to the W box. The current model of ABA and GA action sees the OsWRKY51 and OsWRKY71 TFs form a heterotetramer that binds the *Amy32b* promoter and prevents the activation of the promoter by OsGAMYB. The OsWRKY71/51 heterotetramer, along with other proteins in a repression complex, appears to play a role in preventing early release of the dormancy induced by ABA. Another interesting aspect of this mechanism is that GA promotes the degradation of OsWRKY71 (but not OsWRKY51), resulting in disintegration of the repression complex, expression of genes such as those encoding α-amylases and hence seed germination (Zhang *et al.*, 2004; Xie *et al.*, 2006).

It appears that this repression of seed germination by a subset of WRKY TFs is a conserved feature of flowering plants, as barley *HvWRKY38* appears to play a similar role to its rice orthologue *OsWRKY71* (Zou *et al.*, 2007). In barley,

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additional components of the transcriptional network have been uncovered including the DOF transcription factor HvBPBF that acts as a negative regulator of GA response in aleurone cells (Mena *et al.*, 2002). HvWRKY38 is capable of forming a homodimer as well as interacting with HvBPBF to form a heterodimer. Another DOF protein, SAD, interacts with HvGAMYB to positively regulate GA responses in aleurone cells (Diaz *et al.*, 2005). Cobombardment studies with the transcriptional activators HvGAMYB and SAD and the transcriptional repressors HvWRKY38 and HvBPBF suggest a possible mechanism of competition between these four TFs (Figure 3). Acting as a homodimer, HvWRKY38 blocks induction of *Amy32b* by HvGAMYB or SAD, although coexpression of these transcriptional activators overcomes the repression by HvWRKY38. Conversely, combination of HvWRKY38 and HvBPBF strengthens the ability of HvWRKY38 to block the induction of *Amy32b* expression even in the presence of both HvGAMYB and SAD. The interaction between repressors and activators of *Amy32b* suggest regulatory control by a repressosome, which includes proteins such as HvWRKY38 and HvBPBF, and an enhanceosome, which includes proteins such as HvGAMYB and SAD (Rushton *et al.*, 2010). The *Amy32b* promoter also contains an AMY box, which can be bound by the repressor HvMCB1 or the activator HvMYBS3 (Rubio-Somoza *et al.*, 2006), and these proteins may also play a part in the repressosome and enhanceosome, respectively (Figure 3). In addition, the HRT zinc finger protein can bind the GA response element and has been found to act as a repressor (Raventós *et al.*, 1998), and the RAMY zinc finger protein can bind the W box element (Peng *et al.*, 2004). Addition of these proteins to the repressosome and enhanceosome model produces a possible mechanism whereby *Amy32b* expression is controlled by the balance between four enhanceosome proteins, RAMY, SAD, HvGAMYB and HvMYBS3, and four repressosome proteins, HvWRKY38, HvBPBF, HRT and HvMCB1 (Figure 3). Use of such a mechanism might allow the fine control of genes involved in seed germination and postgermination growth.

WRKY TFs from dicots that play roles in regulating ABA-mediated seed dormancy and germination are increasingly being reported. As already mentioned, ABA induces the expression of *AtWRKY2* in wild-type plants, and the ABA-signalling mediators ABI3 and ABI5 are required for its induction by ABA (Jiang and Yu, 2009). The seeds of *AtWRKY2* knockout plants show an increased delay in germination in the presence of ABA, compared with wild-type plants (Jiang and Yu, 2009). This indicates that AtWRKY2 acts as a negative regulator of the ABA-mediated maintenance of dormancy. Together with the data presented previously concerning AtWRKY40, AtWRKY18, AtWRKY60 and AtWRKY63, it is clear that WRKY TFs constitute multiple nodes in signalling webs that regulate seed germination in Arabidopsis (Table 2).

This widespread connection between WRKY TFs and seed germination is underlined by the observation that it is not just a single subgroup of WRKY proteins that appear to regulate this process, but rather that WRKY TFs from across the superfamily are involved. For example, AtWRKY2 is a group I WRKY protein, whereas AtWRKY63 is a member of group III, and AtWRKY40, AtWRKY18 and AtWRKY60 are found in group IIa. The widespread occurrence of WRKY TFs that regulate seed germination suggests that this is a common feature of seed germination in flowering plants.

Table 2 Arabidopsis WRKY genes and their positions in abscisic acid-signalling networks

WRKY gene	Process	Apparent signalling network position
AtWRKY2	Seed germination Seedling establishment	Downstream of *ABI5, ABI3, ABA2* and *ABA3*
AtWRKY18	Seed germination Postgermination growth	Upstream of *ABF4, ABI1, ABI2, ABI4, ABI5, DREB1A, DREB2A, MYB2, PYL2/RCAR13, PYL2/RCAR11, RAB18, PYL2/RCAR9, PYL2/RCAR7, SnRK2.2* and *SnRK2.3* May function as a heterodimer with AtWRKY40 to activate *AtWRKY60*
AtWRKY40	Seed germination Postgermination growth	Directly targets *ABI4, ABI5, ABF4, MYB2, DREB1A* and *RAB18* Also upstream of *ABI1, ABI2, ABI4, DREB1A, DREB2A, PYL2/RCAR13, PYL2/RCAR11, RAB18, PYL2/RCAR9, PYL2/RCAR7, SnRK2.2* and *SnRK2.3*
AtWRKY60	Seed germination Postgermination growth	May target some of the same genes as AtWRKY18 and AtWRKY40 Possibly activated by a AtWRKY18/AtWRKY40 heterodimer
AtWRKY63	Seedling establishment Seedling growth Stomatal closure	Downstream of *ABI1, ABI2, ABI3* and *ABI5* Upstream of *ABF2*, *COR47* and *RD29A*

WRKY transcription factors that mediate ABA responses also form parts of other signalling networks

One of the most important recent insights into the role of WRKY TFs is the realization that a single WRKY protein may be involved in regulating several seemingly disparate processes (Rushton *et al.*, 2010). We have already encountered the Arabidopsis group IIa WRKY proteins, AtWRKY18, AtWRKY40 and AtWRKY60 with respect to their roles in mediating ABA responses, but these TFs also play other important roles in Arabidopsis. AtWRKY18 and AtWRKY40 play a major and partly redundant role in PAMP-triggered basal defence (Pandey *et al.*, 2010), where they negatively affect pre-invasion host defence. Using ChIP, direct *in vivo* interactions of WRKY40 with promoter regions of the regulatory gene *EDS1*, the AP2-type transcription factor gene *RRTF1* and *JAZ8*, a member of the JA-signalling repressor gene family, were recently demonstrated (Pandey *et al.*, 2010). The data suggest that WRKY18/40 negatively modulate the expression of positive regulators of defence such as *CYP71A13*, *EDS1* and *PAD4*, but positively modulate the expression of some key JA-signalling genes. Taken together, these data suggest that AtWRKY18, AtWRKY40 and

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AtWRKY60 represent nodes in Arabidopsis signalling networks that take inputs from numerous stimuli and that they are involved in mediating responses to numerous phytohormones including salicylic acid, jasmonic acid, ABA and GA. These roles in multiple signalling pathways may in turn partly explain the pleiotropic effects commonly seen when TF genes are overexpressed. In the past, these pleiotropic effects have often been attributed to the binding of TFs to promoters of genes that are not normally targets because of the increased concentration of these DNA-binding proteins. This explanation may not always hold true.

The involvement of WRKY TFs that regulate ABA responses in regulating other processes can also be seen in monocots, where the barley gene *HvWRKY1/38* is involved in regulating cold and drought responses (Marè *et al.*, 2004) while also being a repressor of basal defence that directly interacts with the MLA resistance protein (Shen *et al.*, 2007). These data clearly show not only that these WRKY genes can be regulators of several different processes such as biotic stress responses, abiotic stress responses and seed germination but may also partly explain the mechanisms of crosstalk between ABA signalling and other signalling pathways. A WRKY gene that mediates ABA responses can be a node or hub in signalling that takes inputs from other phytohormones and stimuli.

New insights reveal that WRKY TFs are key nodes in ABA-responsive signalling networks

Until very recently, the role of WRKY TFs in ABA responses was obscure, and their possible roles were normally overlooked in reviews into ABA signalling (Hirayama and Shinozaki, 2007; Seki *et al.*, 2007; Agarwal and Jha, 2010; Urano *et al.*, 2010). The recent significant advances in the study of ABA signalling discussed in this review show that WRKY TFs are key nodes in ABA-responsive signalling networks. These ABA-regulated plant processes include seed germination and dormancy, postgermination growth and also the opening and closing of stomata. The real significance of the recent work is that it places specific WRKY TFs into signalling networks that respond to ABA. At the molecular level, WRKY TFs act downstream of at least two ABA receptors: the cytoplasmic PYR/PYL/RCAR-protein phosphatase 2C-ABA complex and the chloroplast envelope–located ABAR–ABA complex. WRKY genes also operate at multiple levels in the ABA-signalling networks. For example, in Arabidopsis, ABA induces a cascade of transcription factor activation. In the initial absence of ABA, AtWRKY40 represses expression of the bZIP transcription factor *ABI5*. Upon ABA perception by ABAR, PYR/-PYL/RCAR or other receptors, de-repression of *ABI5* leads to activation of *AtWRKY63*. AtWRKY63 then activates downstream genes such as *RD29A* and *COR47*. This part of the network of ABA-inducible gene activation is therefore characterized by a WRKY-bZIP-WRKY module, with WRKY TFs active at multiple levels (Figure 2b). These new WRKY-containing signalling modules can now be investigated as parts of the larger signalling networks.

Conclusions

A recent burst of activity in WRKY TF research has clearly demonstrated that WRKY TFs are components of ABA signalling at several different levels. Some, such as AtWRKY40, are early components of signalling pathways and repress ABA responses through a novel mechanism that involves the ABA receptor ABAR. Others, such as AtWRKY63, are further downstream and target known response genes such as *RD29A* and *COR47*. These new insights also show that some WRKY TFs represent major hubs in plant signalling as they take input signals from multiple stimuli. This has major implications for the use of WRKY genes in crop improvement. On the one hand, it may make the manipulation of a single plant process difficult, but on the other, it may be possible to improve several different stress responses (for example, both biotic and abiotic stresses) using a single gene. The future of WRKY research will certainly hold some interesting surprises.

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