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Review

The role of WRKY transcription factors in plant abiotic stresses ☆

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ABSTRACT

The *WRKY* gene family has been suggested to play important roles in the regulation of transcriptional reprogramming associated with plant stress responses. Modification of the expression patterns of *WRKY* genes and/or changes in their activity contribute to the elaboration of various signaling pathways and regulatory networks. Furthermore, a single *WRKY* gene often responds to several stress factors, and then their proteins may participate in the regulation of several seemingly disparate processes as negative or positive regulators. WRKY proteins also function via protein–protein interaction and autoregulation or cross-regulation is extensively recorded among *WRKY* genes, which help us understand the complex mechanisms of signaling and transcriptional reprogramming controlled by WRKY proteins. Here, we review recent progress made in starting to reveal the role of WRKY transcription factors in plant abiotic stresses. This article is part of a Special Issue entitled: Plant gene regulation in response to abiotic stress.

1. Introduction

Being unable to move, plants constantly encounter various fluctuating abiotic environmental factors, such as water deficiency (drought), excessive salt (salinity), threshold temperatures (from freezing to scorching), decreased availability of essential nutrients (nutrient starvation) and variable light conditions. These factors can occur at multiple stages of plant development and often more than one stress simultaneously affects the plant, potentially restricting plant growth, plant development or even determining plant species distribution across different types of environments. Much attention had been paid to these abiotic factors because of their potential impact on agricultural production and quality.

Plants have evolved intricate mechanisms at multiple levels that increase tolerance in order to adapt to adverse conditions. For example, at the cellular level, closure of stomata and inhibition of vegetative growth help the plants survive in water-limited conditions [1]. At the molecular level, the induction of stress-responsive and stress-tolerance genes also contribute to the plants to adapt to unfavorable environmental conditions [2]. Recognition of stress cues and transduction of the signals to activate adaptive responses and regulation of stress-related genes are the key steps leading to plant stress tolerance [3]. Induction of stress-related genes occurs mainly at the transcriptional level, and modification of the temporal and spatial expression patterns of specific stress-related genes is an important part of the plant stress response [4]. Plants devote a large portion of their genome capacity to transcription, with the *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*) genome coding for more than 2100 and 2300 transcription factors respectively [5]. These transcription factor genes often belong to large gene families, which in some cases are plant-specific. Among them, the WRKY transcription factors compose one large family of regulatory proteins in plants.

Although discovered relatively recently, the WRKY transcription factors are becoming one of the best-characterized classes of plant transcription factors. Previous studies have demonstrated that WRKY transcription factors participated in various biotic stress responses [6, 7] and several developmental and physiological processes, including embryogenesis, seed coat and trichome development, leaf senescence, regulation of biosynthetic pathways, and hormone signaling [8–15]. Currently, some researchers have focused on the functional analysis of WRKY factors in plant responses to abiotic stress such as drought, cold and nutrient deficiency. Moreover, test materials were no longer restricted to model plants such as *Arabidopsis* (*A. thaliana*) but also applied to non-model plants, in particular crop species. In this review, we will emphasize on the roles of *WRKY* transcription factor genes in plant abiotic stresses.

2. Structure characterization and classification

WRKY proteins belong to the WRKY-GCM1 superfamily of zinc finger transcription factors that evolved from Mutator or Mutator-like (Mule) transposases [16, 17]. Although WRKY transcription factors are reported in some non plant species [18–20], they constitute a large family of transcription factors in plants [20]. A large number of *WRKY* genes have been identified from *Arabidopsis* (74) [20], rice (>100) [10], soybean (197) [21], papaya (66) [7], poplar (104) [7],

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sorghum (68) [7], *Physcomitrella patens* (38) [7], *Selaginella moellendorffii* (35) [22], pinus (80) [23], and Barley (>45) [24].

The WRKY protein family owes its name to the highly conserved 60 amino acid long WRKY domains, which contain a conserved amino acid sequence motif WRKYGQK at N-termini and a novel zinc-finger-like motif at C-termini [6]. The heptapeptide WRKYGQK motif showed slight variations in a few WRKY proteins [24–27]. Both of these two motifs are vital for the high binding affinity of WRKY transcription factors to the consensus cis-acting elements termed the W box (TTGACT/C), although alternative binding sites have been identified [28–31]. Almost all WRKY transcription factors show binding preference to the cognate cis-acting element, however, the binding site preferences are also partly determined by additional adjacent DNA sequences outside of the TTGACY-core motif [29]. Initially, based on both the number of WRKY domains and the features of their zinc-finger motif, the WRKY protein family can be categorized into three distinct groups [6]. The first group (I) has two WRKY domains; group II has one WRKY domain containing the same Cys2-His2 zinc-finger motif, and group III has one WRKY domain containing the different Cys2-His/Cys Cys2-His2 zinc-finger motif. The group II WRKY proteins are further divided into subgroups *a*–*e* based on additional conserved structural motifs outside the WRKY domain [6]. Later, based on more accurate phylogenetic analysis, Zhang and Wang [25] classified the WRKY factors into Groups I, IIa + IIb, IIc, IId + IIe, and III with the Group II genes not being monophyletic. In addition to both the highly conserved WRKY domain and the Cys2-His2 or Cys2-His/Cys Zinc-finger motif, the WRKY proteins still contain the following structures: putative basic nuclear localization signals, leucine zippers, serine–threonine-rich region, glutamine-rich region, proline-rich region, kinase domains and TIR-NBS-LRRs. Thereby, owing to such structure characterizations, the WRKY proteins can play their appropriate roles in regulation of gene expression.

3. Function in abiotic stresses

WRKY transcription factors function as important components in the complex signaling progresses during plant stress responses. However, compared with the research progress in biotic stresses, far less information is available to understand the function of WRKY proteins in abiotic stresses. Considering the relative large number of WRKY transcription factors from different plants and their unknown and diverse roles under complex environmental stimulations, it remains a big challenge to uncover their roles in abiotic stresses. Until recently, the possible involvement of WRKY proteins in the abiotic stress responses was deduced indirectly from transcription profiling; however, recent functional analyses have provided some direct evidence. The recent data presented here mainly summarized the function of most of WRKY transcription factors in regulating transcriptional reprogramming associated with plant abiotic responses (Table 1). The tight regulation and fine-tuning of WRKY proteins during plant stress responses contribute to the establishment of complex signaling webs and the important roles of WRKY proteins in plant abiotic stress responses make them potential candidates for imparting stress tolerance.

3.1. Expression pattern of WRKY genes under abiotic stresses

Numerous studies have demonstrated that many *WRKY* genes behave strongly and rapidly induced expression when respond to certain abiotic stresses, such as wounding, drought or salinity , indicating their regulatory function in these signaling pathways. Northern blotting analysis revealed that 10 of 13 *OsWRKY* genes differentially respond to NaCl, PEG, cold or heat treatment [32]. In wheat, 8 of 15 *WRKY* genes were also responsive to low temperature, high temperature, NaCl or PEG treatment [33]. Microarray profiling of NaCl-treated *Arabidopsis* roots revealed that there are 18 *AtWRKY*

Table 1
Function of WRKY transcription factors in abiotic stresses.

Gene	Locus	Induced by abiotic factors	Function in abiotic stress	Refs
AtWRKY2	At5g56270	NaCl, mannitol	Negative regulator in ABA signaling	[57,58]
AtWRKY6	At1g62300	H_2O_2, methyl viologen, Pi and B starvation	Negative regulator in low Pi stress and positive regulator in low B stress	[35,65,66]
AtWRKY18	At4g31800	ABA	ABA signaling, NaCl and mannitao tolerance	[60,61]
AtWRKY22	At4g01250	H_2O_2, dark	Enhanced dark-induced senescence	[13]
AtWRKY25	At2g30250	Ethylene, NO, NaCl, mannitol, cold, heat, ABA, cold	Tolerance to heat and NaCl, increased sensitivity to oxidative stress and ABA	[40,41,48]
AtWRKY26	At5g07100	Heat	Tolerance to heat	[41]
AtWRKY33	At2g38470	NaCl, mannitol, cold, H_2O_2, ozone oxidative stress, UV	Tolerance to heat and NaCl, increased sensitivity to oxidative stress and ABA	[41,48]
AtWRKY34	At4g26440	Cold	Negative regulator in pollen specific cold response	[44]
AtWRKY39	At3g04670	Heat	Tolerance to heat	[64]
AtWRKY40	At1g80840	ABA	ABA signaling	[60,61]
AtWRKY60	At2g25000	Wounding	ABA signaling, NaCl and mannitao tolerance	[60,61]
AtWRKY63	At1g66600	ABA	Negative regulator in ABA signaling while positive regulator in drought tolerance	[59]
AtWRKY75	At5g13080	Pi deprivation	Positive regulator in Pi starvation	[36]
OsWRKY08	05g50610	Drought, salinity, H_2O_2, ABA, NAA	Tolerance to osmotic stress	[54]
OsWRKY11	01g43650	Heat, drought	Tolerance to xerothermic stress	[51]
OsWRKY23	01g53260	Salinity, ABA, H_2O_2, Osmotic stress, dark	Enhanced dark-induced senescence	[82]
OsWRKY45	05g2577	Cold, ABA	Tolerance to salt and drought stress	[52]
OsWRKY72	11g29870	Salinity, heat, ABA, NAA, osmotic stress, sugar starvation	Negative regulator in ABA signaling and sugar starvation	[53]
OsWRKY89	11g02520	Salinity, ABA, UV-B, wounding	Tolerance to UV-B radiation	[81]
GmWRKY13	DQ322694	Salt, drought	Increased sensitivity to salt and mannitol while decreased sensitivity to ABA	[55]
GmWRKY21	DQ322691	Salt, drought, cold	Cold tolerance	[55]
GmWRKY54	DQ322698	Salt, drought	Salt and drought tolerance	[55]
TcWRKY53	EF053036	Salinity, cold, drought	Negative regulator in osmotic stress	[49]
HvWRKY34	DQ863118	Sugar	Sugar signaling	[70]
HvWRKY41	DQ863124	Sugar	Sugar signaling	[70]
HvWRKY46 (*SUSIBA2*)	AY323206	Sugar	Sugar signaling	[71–73]
NaWRKY3	AY456271	Wounding	JA signaling	[80]
NaWRKY6	AY456272	Wounding	JA signaling	[80]

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genes that were induced by 150 mM NaCl treatment [34]. Transcript levels of both *AtWRKY6* and *AtWRKY75* are enhanced during phosphate deprivation [35,36]. There are many additional studies to show that numerous *WRKY* genes respond to wounding, drought, heat, cold or heat pre-treated chilling [10,37–48]. Interestingly, the induced expression of *WRKY* genes is often extremely rapid and transient, and appears independent of de novo synthesis of regulatory factors [6,37]. The immediate-early expression behavior of *WRKY* genes assure the successful transduction of the signals to activate adaptive responses and regulation of stress-related genes, and finally result in plant stress tolerance. Furthermore, a single *WRKY* gene often simultaneously responds to several stress factors, indicating its diverse regulatory function during plant stress responses. *AtWRKY25* and *AtWRKY*33 respond to both heat and salt treatments [40,41,48]. *TcWRKY53* is also simultaneously induced by cold, salt and PEG treatments [49]. Thus, *WRKY* genes appear to be expressed under different abiotic stresses and could therefore participate in the control of signaling processes associated with transcriptional reprogramming when plants encounter various adversities. Based on their expression pattern under different abiotic stresses, a researcher may obtain some clues as to their regulatory functions toward particular stress conditions.

3.2. Drought, salinity, osmotic stress and ABA signaling

Drought is often associated with salinity, important abiotic stress factors, usually affecting plant growth, development, survival and crop productivity. Thus, understanding the complex mechanism of drought and salinity tolerance is important for agriculture production. Interestingly, several WRKY proteins were shown to be involved in plant drought and salinity stress responses [50]. For example, overexpression of *OsWRKY11* under the control of *HSP101* promoter led to enhanced drought tolerance, as showed by the slower leaf-wilting and increased survival rate of green plant parts [51]. Similarly, the altered salt and drought tolerance of *35S:OsWRK45* and *35S:OsWRK72 Arabidopsis* plants may be attributed to induction of ABA/stress-related genes [52,53]. *OsWRKY08*, whose transcripts was enhanced by PEG, NaCl or abscisic acid (ABA) treatment, improves the osmotic stress tolerance of transgenic *Arabidopsis* through positive regulation of the expression of two ABA-independent abiotic stress responsive genes, *AtCOR47* and *AtRD21* [54]. Plants which have over-expressed *GmWRKY54* showed enhanced salt and drought tolerance, possibly through the regulation of transcription factor *STZ/Zat10*, while overexpression of *GmWRKY13* led to increased sensitivity to salt and mannitol stresses [55]. Similarly, the expression of *TcWRKY53* was strongly induced by NaCl and drought stresses and transgenic tobacco plants overexpressing *TcWRKY53* showed depressed expression of two ERF family genes, *NtERF5* and *NtEREBP-1* [49]. In *Arabidopsis*, the transcripts of two closely related WRKY transcription factors (*At*WRKY25 and *At*WRKY33) were increased by ABA, drought or NaCl treatment and both the *Atwrky33* null mutants and *Atwrky25Atwrky33* double mutants showed moderately increased NaCl-sensitivity; however, overexpression of either *AtWRKY25* or *AtWRKY33* led to increased *Arabidopsis* NaCl tolerance [41]. These data provide evidence that different WRKY proteins play differential roles in specific abiotic stress responses.

ABA is a stress hormone and plays essential roles in plant responses to abiotic stresses. Previous research had demonstrated that WRKY proteins may act as activators or repressors in ABA signaling. *LtWRKY21* was shown to function as an activator to control ABA-regulated expression of genes [15]. Transient expression analysis showed that *Os*WRKY24 and *Os*WRKY45 act as repressors while *Os*WRKY72 and *Os*WRKY77 act as activators to the same ABA-inducible promoter [56]. *AtWRKY2* was induced by NaCl and mannitol treatments [57], and studies using *Atwrky2* T-DNA insertion mutants indicated that *AtWRKY2* possibly functioned as a negative feedback regulator of ABA-mediated arrest of seed germination and post-germination growth [58]. Analysis of T-DNA insertion mutant of *AtWRKY63* (*ABO3*) indicated that *AtWRKY63* play an important role in plant responses to ABA and drought stress. *AtWRKY63* was induced by ABA treatment and mutation of *AtWRKY63* rendered the mutants more sensitive to ABA in both seedling establishment and seedling growth and less drought tolerant. EMSA showed that the W-box sequence upstream of the *AtABF2* promoter could be bound by *At*ABO3, supporting its repressed expression in the *Atabo3* mutant plants. However, overexpression of *AtABO3* did not result in drought tolerance, thus *AtABO3* need either co-factors or some post-translational modifications to activate the downstream genes for stress tolerance [59]. Recently, two research groups reported their results about the function of a group of structurally related WRKY proteins, *At*WRKY18, *At*WRKY40 and *At*WRKY60, in ABA signaling. Shang et al. [60] showed that the three WRKY proteins function as negative regulators of ABA signaling in seed germination and postgermination growth. Further genetics analysis showed that *At*WRKY40 acts as a central negative regulator among the three WRKY proteins and could directly inhibit the expression of several important ABA-responsive genes, such as *AtABF4*, *AtABI4*, *AtABI5*, *AtDREB1A*, *AtMYB2* and *AtRAB18*, by directly binding to the W-Box sequences upstream of their promoters. High levels of ABA recruits *At*WRKY40 from the nucleus to the cytosol and promotes ABAR–WRKY40 interaction, thereby expression of ABA-responsive genes is relieved and finally ABA responses occur (Fig. 1). Another work on the three *WRKY* genes using both single, double, and triple mutants and overexpression lines showed that *AtWRKY18* and *AtWRKY60* have a positive effect on plant ABA sensitivity for inhibition of seed germination and root growth. They also increase plant sensitivity to salt and osmotic

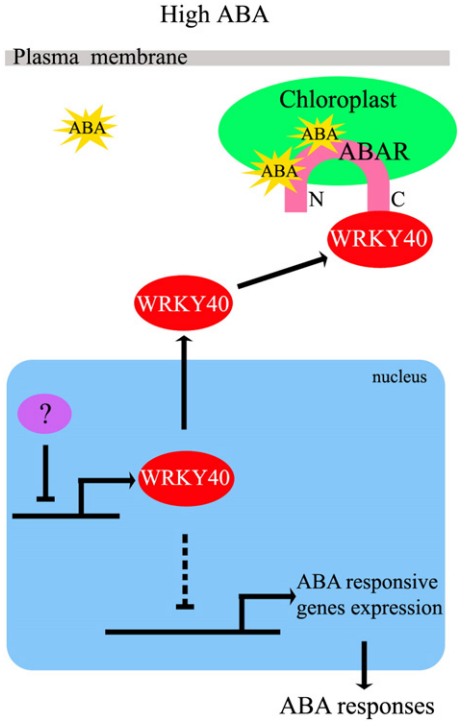

Fig. 1. *At*WRKY40 acts as a central negative regulator in ABAR-mediated ABA signaling pathway. ABAR spans the chloroplast envelope and the cytosolic C-terminus binds ABA and also interact with *At*WRKY40. In response to high levels of ABA signal that recruit *At*WRKY40 from the nucleus to the cytosol and promotes ABAR–WRKY40 interaction, expression of ABA-responsive genes, such as *At*ABF4, *AtABI4*, *AtABI5*, *AtDREB1A*, *AtMYB2* and *AtRAB18*, were relieved and finally ABA responses occur. The dotted line denotes de-repression of ABA-responsive gene expression as *At*WRKY40 is removed from the nucleus. The symbol "?" represents an unknown factor or signaling cascade that may inhibit the expression of *AtWRKY40* [60]. Abbreviations: ABA, abscisic acid; ABAR, Mg-chelatase H subunit/putative ABA receptor.

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stresses. While *AtWRKY40*, on the other hand, antagonizes *AtWRKY18* and *AtWRKY60* in the effect on plant sensitivity to ABA and abiotic stress in germination and growth assays [61]. These results indicate that WRKY proteins function as key components during ABA signaling.

3.3. Temperature stress

Temperature that exceeds an organism's optimal tolerance range is considered as an important abiotic stress factor. In agriculture, high or low temperature acts as a major negative factor limiting crop production. Thus, finding an effective strategy to enhance plant's adaptability to rapid and/or drastic changes in temperature is of particular importance for agricultural production. Tremendous work has been done in the past two decades to reveal the complex molecular mechanism in plants' responses to extreme temperature and there is increasing evidence that WRKY proteins are involved in responses to both heat and cold stresses. For example, a WRKY transcription factor in tobacco (*Nicotiana tabacum* L.) responds to a combination of drought and heat stress [38]. Another example is that transgenic *Arabidopsis* plants overexpressing *GmWRKY21* showed increased tolerance to cold stress when compared with wild-type plants [55]. Moreover, overexpression of *OsWRKY11* under the control of *HSP101* promoter led to enhanced heat tolerance [51]. The expression of *AtWRKY18*, *AtWRKY33*, *AtWRKY40*, and *AtWRKY46* is elevated in *AtMBF1c* over-expressing plants, which possess enhanced thermotolerance compared with wild-type plants [62]. Microarray analysis of *A. thaliana hsf1a/hsf1b* double knockout mutants has revealed that nine of 60 analyzed *AtWRKY* genes are regulated by heat stress and, among these nine genes, *AtWRKY7* is a HsfA1a/1b-dependent heat stress gene [63]. Our recent studies have shown that *At*WRKY25, *At*WRKY26, and *At*WRKY33, three types of group I WRKY proteins, were involved in the regulation of resistance to heat stress. They show distinct expression patterns upon high temperature treatment, with induced expression of *AtWRKY25* and *AtWRKY26* and repressed expression of *AtWRKY33*. Mutation of these three genes render the mutant plants more sensitive to heat stress, as can be seen from reduced germination, decreased survival, and elevated electrolyte leakage. In contrast, transgenic plants overexpressing *AtWRKY25*, *AtWRKY26*, or *AtWRKY33* showed enhanced resistance to heat stress. These three WRKY transcription factors participated in the heat response through modulating transcriptional reprogramming of heat-inducible genes (Fig. 2A). Interestingly, *At*WRKY25, *At*WRKY26, and *At*WRKY33 were also involved in regulation of the heat-induced ethylene-dependent response. Thus, these three proteins play overlapping and synergetic roles in plant thermotolerance through positively regulating the cooperation between the ethylene-activated and heat shock protein-related signaling pathways [41]. Our previous research also exhibited that heat stress-induced *AtWRKY39* positively regulates the cooperation between the SA- and JA-activated signaling pathways that mediate responses to heat stress [64]. Contrary to *AtWRKY25*, *AtWRKY26*, *AtWRKY33*, and *AtWRKY39*, *AtWRKY34* participates in the pollen-specific cold stress response. Promoter-GUS analysis revealed that *AtWRKY34* expression is pollen-specific and its expression is enhanced by cold treatment. Mutation of *AtWRKY34* make the pollen more insensitive to cold stress compared with that of wild type, while the pollen of *AtWRKY34* overexpressing plants is sterile even under normal growth conditions. The *At*WRKY34 transcription factor negatively mediates cold sensitivity of mature *Arabidopsis* pollen through regulating the expression of transcriptional activator CBFs (Fig. 2B) [44]. Taken together, WRKY proteins function in plants' adaption to temperature variations through transcriptional reprogramming of downstream stress-related genes.

3.4. Nutrient deficiency

Various nutrient elements are required for plant's normal growth and development, and deficiency in any necessary element will have a significant effect on plant architecture formation or even its adaptability to various adversities. Several studies have indicated that the WRKY transcription factors also participate in the nutrient deficiency response signaling pathways. *At*WRKY75 was the first WRKY member reported to be involved in regulating phosphate starvation. *AtWRKY75* was strongly induced in plant during Pi-deficiency and

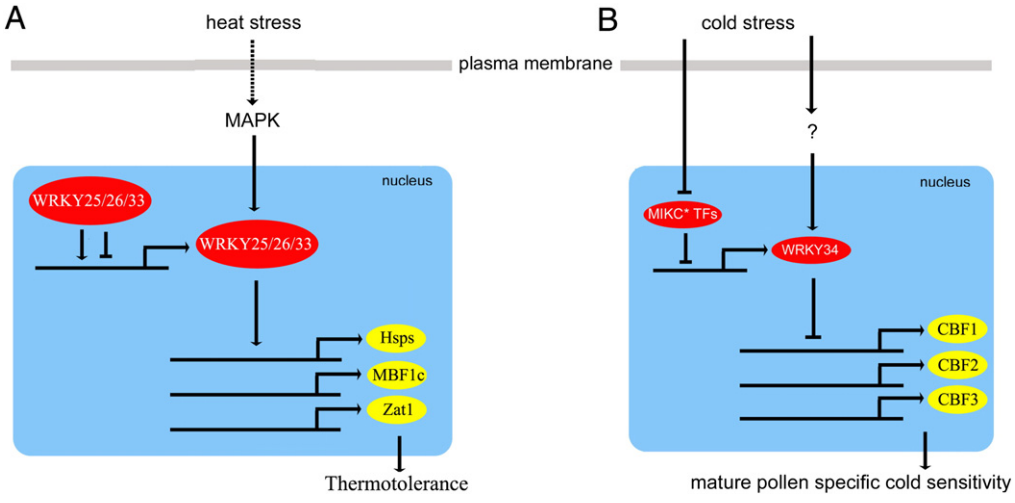

Fig. 2. WRKY transcription factors participated in the responses to heat and cold stresses. A. *AtWRKY25*, *AtWRKY26*, and *AtWRKY33* positively regulate heat defense-related genes. Any one of these three genes is positively regulated by the other two during heat stress, reflecting a synergistic interaction among *AtWRKY25*, *AtWRKY26*, and *AtWRKY33*; furthermore, *AtWRKY33* shows auto-regulation in a negative manner. *AtWRKY25*, *AtWRKY26*, and *AtWRKY33* coordinate induction of plant thermotolerance through modulating the expression of heat-inducible genes, such as *AtHsps*, *AtMBF1c*, *AtZats* [40,41]. Several studies had indicated that *At*WRKY25 and *At*WRKY33 were phosphorylated by several MAPKs [91,95], and thus they may also involve in heat stress responses. B. *At*WRKY34 negatively regulates transcriptional activator AtCBF genes. Pollen-specific MIKC* (MADS DNA-binding domain, intervening domain, keratin-like domain, and c-terminal domain) transcription factors are required for pollen maturation and tube growth, and could affect the expression of many genes specific to mature pollen grains in *Arabidopsis* [96–98]. The expression of five MIKC* transcription factors, including *AtAGL66*, *AtAGL65*, *AtAGL104*, *AtAGL30*, and *AtAGL94*, was strongly reduced under cold treatment and mutation of *AtAGL65*, *AtAGL66*, and *AtAGL104* enhanced the cold-induced expression of *AtWRKY34*. The expression of *AtCBFs* displayed tremendous induction in cold-treated mature pollen of *Atwrky34* mutants, while *At*CBF transcription showed no induction in that of WT [44].

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suppression of the *AtWRKY75* expression conferred the plant more susceptible to Pi stress and decreased Pi uptake during Pi starvation. Expression of several Pi-starvation associated genes, such as phosphatases, Mt4/TPS1-like genes and high affinity Pi transporters was decreased in *AtWRKY75* RNAi plants [36]. However, whether these genes were *At*WRKY75's direct target or not should be further determined. Thus identifying the gene(s) whose expression is specifically and directly regulated by *At*WRKY75 as well as its interacting partners will help us to clarify the complex mechanisms of plant responses to low Pi stress. Recently, another member, *AtWRKY6* was also determined to function in plant responses to low Pi stress through negatively regulating *Arabidopsis PHOSPHATE1* (*PHO1*) expression. Transgenic plants overexpressing *AtWRKY6* had a phenotype similar to the *Atpho1* mutant plants and were more sensitive to low Pi stress and accumulated less Pi in shoots when compared with wild-type or *Atwrky6-1* mutant plants. ChIP-qPCR analysis showed that *At*WRKY6 negatively regulated *AtPHO1* expression through directly binding to the two W-boxes upstream of the *AtPHO1* promoter in a Pi dependent manner. Furthermore, low Pi-induced release of *AtPHO1* repression may result from 26S proteosome-mediated proteolysis of *At*WRKY6 protein (Fig. 3). In addition, as an interacting partner of *At*WRKY6, *At*WRKY42 could also inhibit *AtPHO1* expression through directly binding to W-boxes of the *AtPHO1* promoter [35]. Taken together, both *At*WRKY75 and *At*WRKY6 participated in the Pi-deficiency response; however, they regulated the expression of different types of downstream target genes, thus they may function in different regulatory pathways during low Pi stress. *At*WRKY6 was also represented as the first transcription factor involved in the response to boron deficiency. However, opposite to low Pi stress, *At*WRKY6 functions as a positive regulator in low-boron response [65,66]. Thus, *AtWRKY6* seems to respond to several aspects of nutrient deficiency induced stresses, implicating its diverse functions in these signaling pathways.

In *Arabidopsis*, *WRKY45* and *WRKY65* are involved in the regulation of gene expression during carbon starvation [67]; and the *35S:OsWRKY72* transgenic *Arabidopsis* also revealed increased sensitivity to sugar-starvation stress [53]. Similarly, there are also three *OsWRKY* genes whose expression showed significant alternation in sucrose-starved rice suspension cells [68]. *AtNDPK3a*, which is induced by sucrose and glucose, was possibly regulated by *At*WRKY4 and *At*WRKY34 [69]. Interestingly, three *HvWRKY* genes involved in plant sugar signaling [70] and *HvWRKY46* (*SUSIBA2*) participated in sugar signaling by mediating the expression of *ISO1* and *SBEIIb* [71–73]. Thus, WRKY proteins may also be involved in the regulation of the many metabolic and stress-related proteins that were involved in sugar signaling.

3.5. ROS signaling

Various abiotic stresses always aggravate the production of reactive oxygen species (ROS) in mitochondria, via consumption of oxygen in a so-called oxidative burst in plants [74]. ROS like H_2O_2 act as important signal transduction molecules, mediating the acquisition of tolerance to various stresses [75]. In *Arabidopsis*, the expression of *AtWRKY30*, *AtWRKY75*, *AtWRKY48*, *AtWRKY39*, *AtWRKY6*, *AtWRKY53*, *AtWRKY22*, and *AtWRKY8* were significantly induced by H_2O_2 treatment [12,13,43,76]. Several important enzymes such as zinc finger proteins, ascorbate peroxidases (APX), and NADPH oxidases have revealed the key nodes in the ROS signal network. In zinc finger protein gene *Atzat12* mutant plants, the expression of *AtWRKY25* was unable to be enhanced after H_2O_2 treatment, suggesting that the induction of *AtWRKY25* during oxidative stress was dependent on *AtZat12* [77]. Also *AtWRKY70* was constitutively expressed in ROS-scavenging enzyme gene *Atapx1* mutant plants, suggesting its possible role in ROS signaling [78]. Under light stress, the expression of several other WRKY transcription factor genes including *AtWRKY6*, *AtWRKY18*, *AtWRKY25*, *AtWRKY33*, *AtWRKY40*, *AtWRKY46*, *AtWRKY54*, and *AtWRKY60*, was also elevated in *Atapx1* mutant plants when compared with wild type plants, implying the possible involvement of these WRKY proteins in ROS signaling [79]. Hence, WRKY transcription factors appear to play an important role in ROS signaling web.

3.6. Other abiotic stresses

Besides of the involvement of abiotic stresses mentioned above, WRKY proteins also participated in other abiotic stress responses, such as wounding and UV radiation. Numerous studies showed that a number of *WRKY* genes were induced by wounding treatment [37,43]. Both *NaWRKY3* and *NaWRKY6* respond to wounding, and interestingly, *NaWRKY3* is required for *NaWRKY6* elicitation by fatty acid–amino acid conjugates (FACs) in larval oral secretions that are introduced into wounds during feeding [80]. Silencing either or both genes make plants highly vulnerable to herbivores and reduce *M. sexta*-elicited JA and JA-Ile/-Leu levels (Fig. 4). The response to wounding and herbivore-specific signals (FACs) imply that the two WRKYs help plants to differentiate mechanical wounding from herbivore attack. In *Arabidopsis*, three *WRKY* genes were strongly induced by UV-B light treatment [45]. Similarly, *OsWRKY89* was strongly

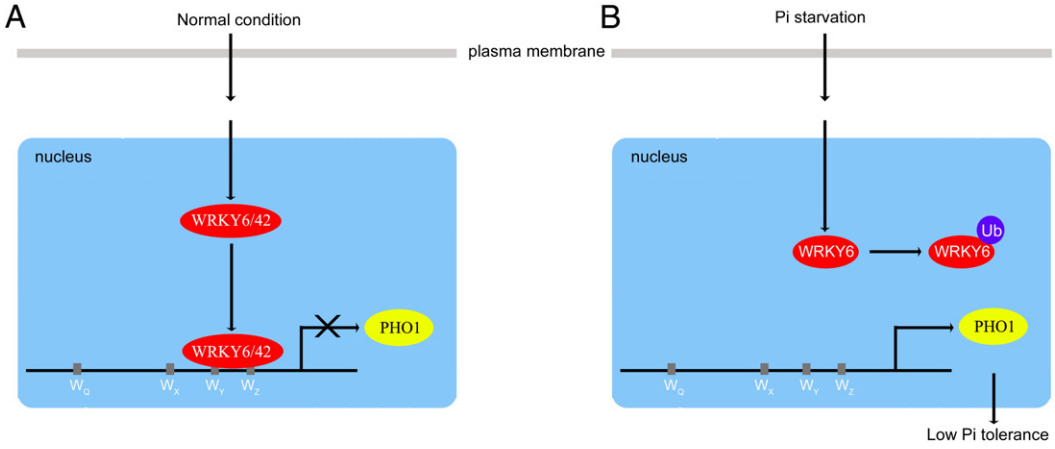

Fig. 3. The role of *At*WRKY6 transcription factor in signaling pathways during Pi starvation. The *AtPHO1* promoter contains six W-boxes and four of them were shown (Q, X, Y, and Z). Under normal conditions, the expression of *AtPHO1* was repressed by *At*WRKY6 through directly binding to the W-box motifs W_Y and W_Z within the *AtPHO1* promoter (left); while under low Pi conditions, *At*WRKY6 protein was degraded via a 26S proteosome-mediated proteolysis and then repression of *AtPHO1* transcription by *At*WRKY6 is relieved (right) [35].

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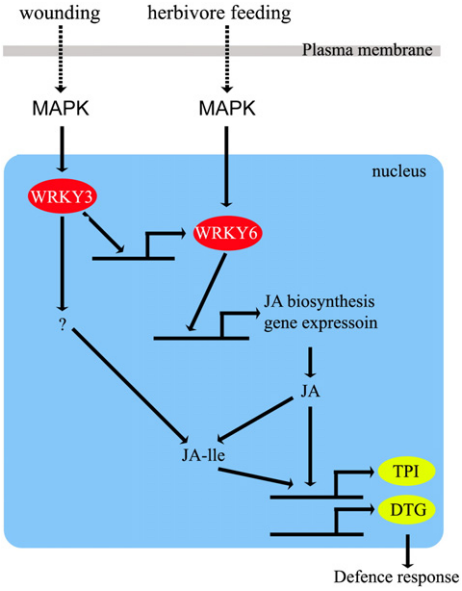

Fig. 4. Wounding induced *NaWRKY3* and *NaWRKY6* coordinate responses to herbivory. *NaWRKY3* is strongly induced by wounding treatment while *NaWRKY6* is only moderately induced. *NaWRKY3* is required for *NaWRKY6* elicitation by herbivore feeding which introduces fatty acid–amino conjugates (FACs) in *Manduca sexta* larval oral secretions into wounds. Both of them regulate expression of jasmonic acid (JA) biosynthesis genes (*LOX*, *AOS*, *AOC* and *OPR3*), and *NaWRKY3* may also regulate *JAR4* or unknown JA conjugating genes, thereby increasing the levels of JA and JA-Ile. This is turn enhances defense gene expression, such as *TPI* and *DTG*, and finally regulate plant defenses against herbivores [80]. The symbol "?" represents *JAR4* or unknown JA conjugating enzymes.

induced by UV-B radiation and over-expression of *OsWRKY89* enhanced tolerance to UV-B irradiation through increasing the wax deposition on leaf surfaces of transgenic plants [81]. In addition, we found that over-expressing *AtWRKY22* and *OsWRKY23* in *Arabidopsis* accelerated leaf senescence in darkness [13,82].

3.7. One WRKY toward multiple processes

Numerous studies have demonstrated that a single transcription factor may function in several seemingly disparate signaling pathways, as can be deduced from their induced expression profile by various stress factors. Recent research suggest that the three structurally related WRKY proteins, *At*WRKY18, *At*WRKY40 and *At*WRKY60, participate in at least three phytohormone-mediated signaling pathways (SA, JA and ABA) [60,61,83,84]. Pandey et al. demonstrate that WRKY18/40 negatively modulate EDS1 signaling but positively regulate JA-signaling when responding to *G. orontii* infection [84]. Two closely related WRKY transcription factors (*At*WRKY25 and *At*WRKY33) respond to both biotic and abiotic stresses, e.g., *P. syringae*, NaCl, cold and heat [41,48,85,86]. Both of them act as a negative regulator of the defense response to *P. syringae* while functioning as a positive regulator in NaCl and heat stress response. Studies on *At*WRKY6 showed that it at least functions in three different processes, including pathogen defense, senescence and phosphate (Pi) and boron (B) deficient responses [35,65,66,87]. These data demonstrated that a single WRKY gene can function as regulator of several different processes and may also mediate the crosstalk between different signaling pathways.

4. Defining direct target genes

In order to better understand WRKY transcription factors' biological functions and their possible signaling pathways, it is necessary to identify their downstream target genes. Through comparing the expression pattern of different genotypes using the microarray technology, certain potential targets of the *WRKY* gene could be obtained on the genome scale. For example, a set of 12 genes showed marked differences in mature pollen's expression between *Atwrky34-1* mutant and wild-type plants after treatment at 4 °C for 48 h [44]. Microarray analysis also confirmed that the expression of several ABA signaling pathway genes, such as *AtABI5*, *AtABI3*, was significantly enhanced in *Atwrky2* mutants compared with wild type [58]. cDNA-AFLP analysis provide us another method to identify putative target genes. For example, several downstream genes, including *FRK1*/*SIRK*, were identified to be *AtWRKY6*'s target gene, and these genes work together to play important roles in the plant leaf senescence [87]. This method could also be used to identify other *WRKY* gene's target gene involved in plant abiotic stresses. However, the methods shown above only provide us the candidate target genes, and whether these genes are WRKY proteins' direct target genes or not need to be further determined. Thus other advanced methods were needed to determine their direct target genes. One of them is the chromatin immunoprecipitation (ChIP) technique. The ChIP technique has been suggested to be an effective strategy in monitoring DNA–protein and protein–protein interactions in vivo under various conditions and in a dynamic manner [88]. Using this method, an increasing number of single *WRKY* gene's targets have been identified under certain abiotic stresses. For example, several important ABA responsive genes, such as *AtABF4*, *AtABI4*, *AtABI5*, *AtDREB1A*, *AtMYB2* and *AtRAB18*, have been confirmed to be bound by *At*AD1A (*At*WRKY40) in vivo through direct interaction with the W-box sequence upstream of their promoters [60]. Both *At*WRKY6 and *At*WRKY42 could also inhibit *AtPHO1* expression through directly binding to W-boxes of the *AtPHO1* promoter [35]. In *Boea hygrometrica*, chromatin immunoprecipitation showed that four W boxes upstream of the *BhGolS1* gene promoter was directly bound in vivo by the early dehydration and ABA-inducible *BhWRKY1* [89]. Identification of important components that are directly regulated by WRKY transcription factors will add to our knowledge on the understanding of stress-induced signaling pathways. Considering on the large number of WRKY proteins and their distinct roles in specific stress responses, much work is still needed to find out their target genes in corresponding stress pathways.

Interestingly, consistent with the substantial enrichment of W boxes in promoters of numerous *WRKY* genes, the WRKY transcription factors can directly bind to both their own and other WRKY transcription factors' promoters (autoregulation or crossregulation). Chromatin immunoprecipitation (ChIP) studies showed that *Pc*WRKY1 protein binds to the W boxes of its native promoter as well as to that of *Pc*WRKY3 [90]. Electrophoretic Mobility Shift Assays (EMSA) showed that *At*WRKY18 and *At*WRKY40 recognize the W-box sequences upstream of the *AtWRKY60* gene promoter and both of them activate *AtWRKY60* expression in protoplasts, indicating that *AtWRKY60* might be a direct target gene of *At*WRKY18 and *At*WRKY40 in ABA signaling [61]. Recently, ChIP-qPCR assay demonstrated that *At*WRKY33 could regulate its own expression through directly binding its own promoter [91]. These results imply that extensive autoregulation and cross-regulation among WRKY genes facilitate transcriptional reprogramming during plant stress responses.

5. Identifying Interacting partners

To understand how the WRKY proteins participated in various plant stress responses, it is necessary and urgent to identify their interacting proteins using yeast two-hybrid screens or other technologies. Many WRKY proteins were shown to be important components of specific signal pathways, however their interacting partners remain to be identified. Up to now, several reports have demonstrated that WRKY transcription factors carry out their diverse functions in various stress signaling pathways through physical interaction with different proteins, such as MAP kinases, MAP kinase kinases, histone

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deacetylases, calmodulin, etc. [22,92]. During stress response and signal transduction, WRKY proteins were phosphorylated by various MAPKs [93], ultimately regulating plant stress response gene activation. *At*WRKY38 and *At*WRKY62 interact with Histone Deacetylase 19 (HDA19) and finally fine-tune plant basal defense responses [94]. Thus, histone deacetylases may also play essential roles in plant stress responses through maintaining the appropriate acetylation state of histones. WRKY proteins can also form functional homo- or heterodimers among some WRKY proteins to perform their function. Interestingly, the heterodimer formation between different WRKY proteins may have positive or negative effects on their DNA binding activities [61,83]. In the abiotic area, some research also showed that WRKY proteins play their roles through protein–protein interaction. *At*WRKY6 interacts with at least a dozen proteins including its closest homolog *At*WRKY42 and co-overexpression of both resulted in stronger repression on *ProPHO1:GUS* expression. Furthermore, *At*WRKY6 protein was degraded by the 26S proteasome through polyubiquitination by interaction with unknown proteins under Pi-deficient conditions [35]. Besides their interaction, *At*WRKY18, *At*WRKY 40 and *At*WRKY 60 can also form complexes with the magnesium-protoporphyrin IX chelatase H subunit (CHLH/ABAR), a receptor for ABA in *A. thaliana* [60,83]. The identification of WRKY proteins' interacting partners contribute to the reconstruction of signaling webs that involve WRKY proteins.

6. Conclusions and future prospects

Thanks largely to the use of diverse technologies and approaches, including physiology, chemical genetics, molecular computational and informational biology, the field of plant signal transduction and gene regulation has shown rapid progress, which helps us to understand the complex mechanisms underlying various aspects of plant responses to abiotic stresses. Much progress in WRKY transcription factors' functional research has been obtained over the past 15 years. However, most of advances are related with the involvement in biotic stresses, and there are few examples of functional research into abiotic stresses. Furthermore, considering the size of this gene family, the identification of WRKY function in abiotic stresses will remain a big challenge in the coming years. In order to achieve a better understanding of their role during abiotic stresses, it is of vital importance to identify the interacting partner of WRKY proteins under a certain condition which cooperate in regulating the transcription of downstream target genes. This is also important to determine the key components of signal transduction pathways with which they physically cooperate. Second, combined with microarrays and chromatin immuno precipitation assays or even massive parallel sequencing, we could directly determine the specific WRKY DNA-binding sites on a global scale under certain abiotic stresses. In this way, we then may gradually come to understand the complex mechanisms of signaling and transcriptional reprogramming controlled by WRKY proteins and the plant processes in which they participate. One can expect that further molecular studies of WRKY transcription factors under abiotic stress will clarify the fine-tuning mechanisms that are controlled by WRKY proteins in plants, with significant benefits to agricultural production.

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