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WRKY transcription factors: from DNA binding towards biological function

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WRKY proteins comprise a large family of transcription factors. Despite their dramatic diversification in plants, *WRKY* genes seem to have originated in early eukaryotes. The cognate DNA-binding site of WRKY factors is well defined, but determining the roles of individual family members in regulating specific transcriptional programs during development or in response to environmental signals remains daunting. This review summarises the recent advances made in starting to unravel the various functions controlled by WRKY proteins.

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Abbreviations

EST	expressed sequence tag
FRK1/SIRK	*FLG22-INDUCED RECEPTOR-LIKE KINASE1/ SENESCENCE-INDUCED RECEPTOR KINASE*
MAPK	mitogen-activated protein kinase
PcPR1-1	Petroselinum crispum *PATHOGENESIS-RELATED 1-1*
RRS1	*RESISTANCE TO RALSTONIA SOLANACEARUM1*
SA	salicylic acid
TMV	Tobacco Mosaic Virus
TTG2	*TRANSPARENT TESTA GLABRA2*

Introduction

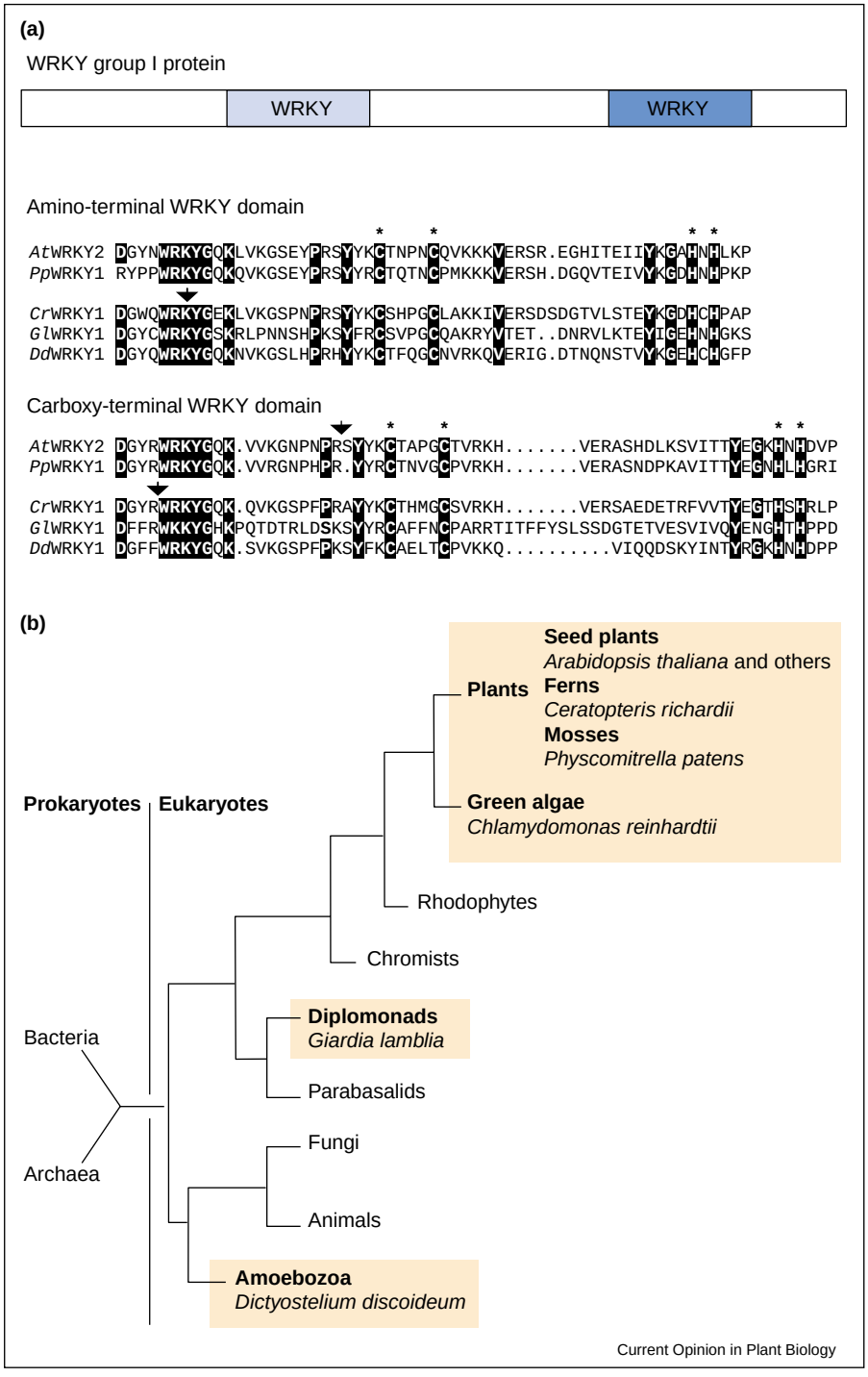
Ten years ago, Ishiguro and Nakamura [1] identified a novel DNA-binding protein from sweet potato, designated SWEET POTATO FACTOR1 (SPF1). Similar proteins were subsequently found in several plant species (reviewed in [2]). Common to these proteins is a DNA-binding region of approximately 60 amino acids in length (the WRKY domain), which comprises the absolutely conserved sequence motif WRKY adjacent to a novel zinc-finger motif. This conservation led us to rename this transcription factor family 'WRKY' [2,3]. The WRKY family has 74 members in *Arabidopsis* (http://www.mpiz-koeln.mpg.de/~somssich/wrky_webpage/wrky_family/wrky_family_index.html) and more than 90 members in rice. WRKY factors show high binding affinity to a DNA sequence designated the W box, (C/T)TGAC(T/C) [2], although altered binding preferences have also been observed ([4]; I Ciolkowski, IE Somssich, unpublished). W-box-dependent binding activity requires both the invariable WRKY amino-acid signature and the cysteine and histidine residues of the WRKY domain, which tetrahedrally coordinate a zinc atom [5].

Very little is known about the physiological processes and developmental programs that require the functions of WRKY proteins. Being transcriptional regulators, WRKY factors should act by directing the temporal and spatial expression of specific genes, thereby ensuring proper cellular responses to internal and external stimuli. Since the WRKY transcription factors were last reviewed [2], a substantial number of WRKY-related publications have appeared. Furthermore, the availability of microarray data (http://ssbdjc2.nottingham.ac.uk/narrays/experimentbrowse.pl; http://www.*Arabidopsis*.org/tools/bulk/microarray/analysis/index.jsp) describing gene expression on a global basis is providing valuable information concerning the altered expression patterns of *WRKY* genes under defined experimental conditions [6,7].

Origin of the *WRKY* genes

Until recently, WRKY proteins appeared to exist exclusively in plants. All of the higher plants analysed to date contain numerous members of the three major WRKY groups, groups that differ in the number of WRKY domains and in the pattern of the zinc-finger motif [2]. In lower plants, WRKY expressed sequence tags (ESTs) have been identified from ferns (*Ceratopteris richardii*) and mosses (*Physcomitrella patens*). We have isolated additional WRKY representatives from *P. patens*, revealing that this moss contains at least 12 distinct *WRKY* genes (D Wanke, P Giavalisco, IE Somssich, unpublished). Interestingly, although group-III members comprise about 20% of the family in higher plants, none have been found in *P. patens*. In *Arabidopsis*, nearly all group-III members respond to diverse biotic stresses [8•,9•], suggesting that this group has evolved late in land plants, perhaps as a consequence of increasing environmental pressures. The green alga *Chlamydomonas reinhardtii* contains only one *WRKY* gene (a group-I gene that encodes two WRKY domains [Figure 1]), which, based on the existence of ESTs, appears to be expressed. GenBank database searches now reveal the existence of WRKY group-I-like

Figure 1



Group I *WRKY* genes in eukaryotes. **(a)** Schematic representation of a WRKY group I protein and the deduced amino-acid sequences of the amino- and carboxy-terminal WRKY domains from *Arabidopsis thaliana* (*At*), *Physcomitrella patens* (*Pp*), *Chlamydomonas reinhardtii* (*Cr*), *Giardia lamblia* (*Gl*) and *Dictyostelium discoideum* (*Dd*). Absolutely conserved amino-acid residues are highlighted by black boxes; asterisks mark the invariant cysteines and histidines that are required to form the zinc-finger motif. Arrows indicate the positions of introns within the WRKY domain of the respective genes. **(b)** The distribution of genes is plotted on a highly schematic phylogeny of relevant eukaryotic groups as described by Simpson and Roger [51]. The distribution of *WRKY* genes in eukaryotic groups is indicated in bold and boxed text.

sequences in two non-photosynthetic eukaryotes, one in the slime mold *Dictyostelium discoideum* (accession **AAO52331**) and one in the unicellular protist *Giardia lamblia* (accession **EAA40901**; Figure 1). These findings imply that group-I *WRKY* genes may represent the ancestral form and, importantly, that *WRKY* genes originated some 1.5–2 billion years ago in eukaryotes, that is, before the divergence of the plant phyla. Why they have expanded so enormously in plants but appear to have been lost in yeast and animal lineages is unclear.

Biological functions of WRKY factors

Expression modes of *WRKY* genes

The transcription of *WRKY* genes is strongly and rapidly upregulated in response to wounding, pathogen infection or abiotic stresses in numerous plant species [2]. Infection of tobacco with Tobacco Mosaic Virus (TMV) or bacteria, or treatment with fungal elicitors, salicylic acid (SA) or H_2O_2, strongly induce several *WRKY* genes [10–13]. Pathogen-mimicking treatments also lead to the selective upregulation of similar genes in rice [14,15], potato [16,17], sugarcane [18], and camomile [19]. In *Arabidopsis*, 49 out of 72 tested *WRKY* genes respond to bacterial infection or SA treatment [8•], and it is very likely that an even higher percentage is activated throughout the overall plant defence response [9•]. This percentage of gene-family members responding to biotic stress is high compared to those of other multigene families that encode plant transcription factors, suggesting that biotic stresses may have played a key role in the expansion of the *WRKY* family. Abiotic stresses such as wounding, drought, cold adaptation and heat-induced chilling tolerance also induce the expression of *WRKY* genes in plants [20–23]. The expression of several *Arabidopsis WRKY* genes is strongly upregulated during plant senescence [6,22,24,25]. In fact, *WRKY* transcripts constitute the second largest group of *Arabidopsis* transcription factors in the transcriptome of senescing leaves [26•].

Role of *WRKY* genes in defence signaling

Although the transcriptional regulation of defence gene expression is pivotal for induced disease resistance [27], direct evidence for the involvement of WRKY proteins in this process remains limited. Circumstantial evidence from microarray studies revealed an over-representation of W-box elements (i.e. WRKY-binding sites) within the promoters of a cluster of genes that are co-expressed during systemic acquired resistance [28,29]. Recently, however, the role of specific WRKY factors that are associated with defence-induced mitogen-activated protein kinase (MAPK) signaling cascades has become apparent [30,31]. Two *Arabidopsis* WRKY factors (*At*WRKY22 and *At*WRKY29) have been identified as important downstream components of a MAPK pathway that confers resistance to both bacterial and fungal pathogens [31]. Expression of *AtWRKY29* in transiently transformed *Arabidopsis* leaves led to reduced disease symptoms, supporting the importance of *AtWRKY29* in this signaling event. In tobacco, TMV resistance is mediated by the resistance gene *N* [32]. When a candidate gene approach was used, downregulation of Nicotiana tabacum *MAPK KINASE1* (*NtMEK1*) and *NtNTF6* (encoding a MAPK kinase and a MAPK, respectively) and three tobacco *WRKY* genes all compromised *N*-mediated resistance, suggesting a vital role of WRKY factors in coordinating defence gene responses in this pathway [33•]. Induction of *WRKY* and defence genes, together with increased W-box binding activity, was also observed during the activation of a tobacco MAPK cascade that involves the *Nt*MEK2 kinase kinase and SA-induced protein kinase (SIPK) [34•]. Further support for the involvement of specific WRKY factors in pathogen-activated MAPK signaling was obtained in parsley [35•] and barley (K-H Kogel, pers. comm.). In tomato, evidence for direct phosphorylation of *Lp*WRKY1 by two fungal-elicitor-induced protein kinases was also observed (T Roitsch, pers. comm.).

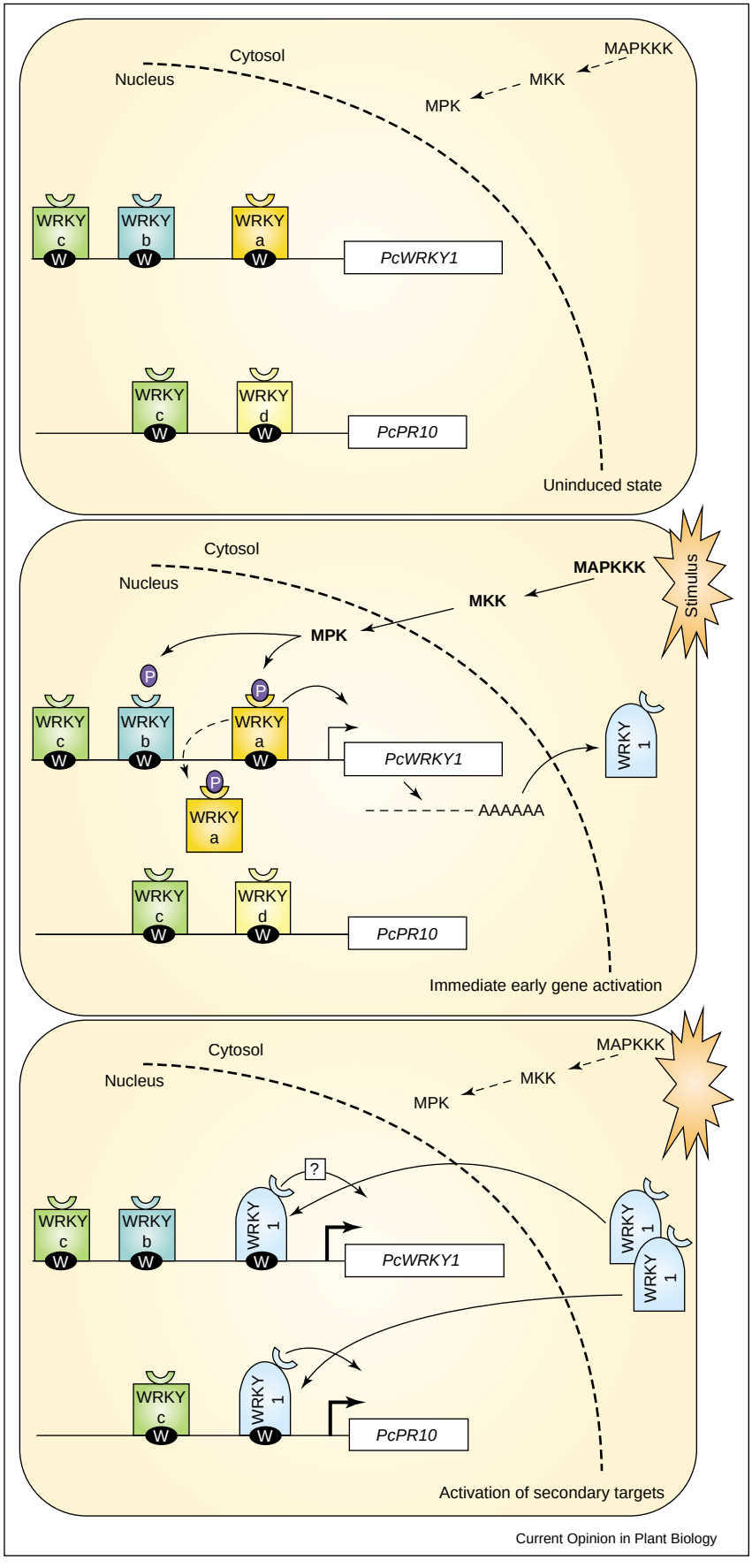
Recently, *Arabidopsis* WRKY70 was identified as a common regulatory component of SA- and jasmonic acid (JA)-dependent defence signaling, mediating cross-talk between these antagonistic pathways [36•]. In addition, *AtWRKY70* overexpression increased resistance to virulent pathogens and led to the constitutive expression of SA-induced genes. Similarly, expression of a key regulator of SA-dependent defence responses, *NONEXPRESSOR OF PR1* (*NPR1*), is controlled by WRKY factors [37]. *Arabidopsis* plants that expressed *AtWRKY18* showed elevated levels of resistance towards a bacterial pathogen, albeit in a development-dependent manner [38].

Genetic evidence linking a *WRKY* gene with resistance came from studies of the interaction of *Arabidopsis* with *Ralstonia solanacearum*, the causal agent of bacterial wilt. Cloning of the resistance gene *RESISTANCE TO RALSTONIA SOLANACEARUM1* (*RRS1*) revealed it to encode a protein that combines structural motifs similar to those of other resistance proteins (TIR-NBS-LRR [27]) with a WRKY domain [39]. RRS1/*At*WRKY52 physically interacts with the corresponding bacterial avirulence gene product, PopP2, and both colocalise to the plant cell nucleus [40••]. Whether this atypical WRKY factor acts as a transcriptional regulator remains to be determined.

Role of *WRKY* genes in development

TRANSPARENT TESTA GLABRA2 (*TTG2*)/*AtWRKY44*, the first *WRKY* gene whose function was unequivocally determined, plays a key role in trichome development [41••]. *ttg2* mutants have unbranched trichomes that are reduced in number, in addition to reductions in mucilage production and tannin synthesis in the seed coat. WRKY factors most probably co-regulate other developmental programs such as senescence as well, but hard core evidence to support this is scarce. In roots, numerous

Figure 2


Cytosol
Nucleus
MAPKKK
MKK
MPK
WRKY c
WRKY b
WRKY a
W
PcWRKY1
WRKY c
WRKY d
PcPR10
Uninduced state
Cytosol
Nucleus
MAPKKK
MKK
MPK
Stimulus
P
WRKY c
WRKY b
WRKY a
PcWRKY1
WRKY 1
AAAAAA
WRKY a
WRKY c
WRKY d
PcPR10
Immediate early gene activation
Cytosol
Nucleus
MAPKKK
MKK
MPK
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WRKY c
WRKY b
WRKY 1
PcWRKY1
WRKY c
WRKY 1
PcPR10
Activation of secondary targets
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Arabidopsis WRKY genes are expressed in a specifically localised domain, hinting that they have a specialised role in cell maturation [42•]. A role in hormone signaling was observed for the rice WRKY factor *Os*WRKY71, which acts as a transcriptional repressor of gibberellin-responsive genes [43]. In pepper, *CaWRKY1* expression is strongly upregulated in red fruit and may play an important role in fruit maturation (CH Harn, pers. comm.). The high abundance of *WRKY* ESTs present in plant cDNA libraries generated from floral and embryonic material could indicate that WRKY transcription factors have vital functions in these tissues. Finally, a 64-kDa antifreeze protein (STHP-64) from bittersweet nightshade was identified as a WRKY transcription factor on the basis of its cross-reactivity with a polyclonal antibody [20].

Defining WRKY functions *in vivo*

To date, traditional genetic approaches have not uncovered functions for individual *WRKY* genes apart from *RRS1/AtWRKY52*. When DNA-insertion lines are available, they provide an alternative to traditional approaches that greatly facilitates the identification of numerous *Arabidopsis* loss-of-function mutants (http://www.*Arabidopsis*.org/links/insertion.jsp). For transcription factor families, however, such mutants seldom exhibit altered phenotypes, most probably because of partly overlapping functional redundancy among individual members [44]. We have identified more than 40 *WRKY* knockout lines but rarely observe phenotypic alterations under standard growth conditions (B Ülker, N Kamphaus, A Zhou, IE Somssich, unpublished). Nevertheless, these mutants are a valuable source for the generation of multiple *WRKY* knockout lines and for extensive phenotypic profiling under defined stress and altered environmental conditions.

Ectopic overexpression can also provide information to help define gene function [44]. How useful this approach will be for WRKY factors remains unclear. For instance, overexpression of *AtWRKY6*, *AtWRKY18*, *AtWRKY53* or *AtWRKY70* always resulted in small stunted transgenic plants. Nearly all such lines showed altered leaf morphologies and changes in flowering time ([25,36•,38]; U Zentgraf, pers. comm.). On the other hand, clear differences could be observed that were related, in particular, to the expression of defense-associated marker genes and to the response of some of these plants to certain pathogens. Furthermore, varying sets of downstream candidate target genes were identified in the lines overexpressing the different WRKY transgenes, suggesting a certain degree of specificity for the individual factors. Still, the pleiotropic alterations seen in these plants will limit the possibilities for interpreting *in-vivo* function(s) from phenotypes.

Defining *in-vivo* target genes of WRKY factors

Identifying downstream target genes of WRKY factors will be crucial in understanding their biological functions. Currently, our knowledge rests mainly on the strong ectopic expression of *WRKY* genes in transgenic plants, protoplasts or leaves. Transient overexpression of *PcWRKY1* in parsley protoplasts led to the activation of a reporter gene driven by the promoters of three potential target genes, namely Petroselinum crispum *PATHOGENESIS-RELATED 1-1* (*PcPR1-1*), *PcWRKY1* and *PcWRKY3* [45]. Similarly, transient expression of *AtWRKY29* and *AtWRKY22* in *Arabidopsis* mesophyll-derived protoplasts resulted in the activation of their own promoters as well as that of the *Arabidopsis* receptor-like kinase gene *FRK1/SIRK*, and in downregulation of *GLUTATHIONE S-TRANSFERASE6* (*GST6*) and *RD29A* [31]. cDNA-AFLP analysis revealed putative target genes for *At*WRKY6 that are involved in leaf senescence, including *FRK1/SIRK*, in transgenic plants that overexpressed *AtWRKY6* [25]. Moreover, using microarrays, potential *At*WRKY70 targets were detected on the genome scale [36•]. A set of 42 genes showed marked differences in expression in transgenic lines ectopically expressing *AtWRKY70* in the sense or antisense orientation compared to control plants.

Clearly, the major caveat of such experiments is that the concentration of the respective transcription factor may significantly exceed that found under physiological conditions, thereby enabling interactions with otherwise low-affinity binding sites. The chromatin immunoprecipitation (ChIP) technique offers an attractive alternative for monitoring DNA–protein and protein–protein interactions *in vivo* under natural conditions and in a dynamic manner [46]. Applying this method to parsley cells, we confirmed that *PcWRKY1* and *PcPR1-1* are indeed *in-vivo* targets of *Pc*WRKY1 (F Turck, A Zhou, IE Somssich, unpublished). Surprisingly, however, these studies revealed that whereas recruitment of *Pc*WRKY1 to W-box-promoter elements is transient and fungal-elicitor dependent, the same elements are constantly occupied by other WRKY factors even in the non-induced state. This observation hints towards a mechanism in which the initial immediate-early activation of such target genes occurs through a stimulus-triggered modification of the pre-bound WRKY factors, rather than by the recruitment of WRKY factors to the transcription complex at these

(Figure 2 Legend) Hypothetical model for WRKY/W-box-mediated transcriptional gene regulation. In non-induced parsley cells, W-box promoter elements of direct target genes are already bound by a set of WRKY factors that are inactive or participate in actively repressing basal gene expression. Upon receptor-mediated recognition of a pathogen, a MAPK cascade (MAPK kinase kinase [MAPKKK]—MAPK kinase [MKK]—MAPK [MPK]) is rapidly activated and ultimately results in the translocation of the protein kinase (MPK) to the nucleus [47]. The activity of this kinase directly modifies certain WRKY factors at the promoter of immediate-early-type genes such as *PcWRKY1*, thereby derepressing/activating the expression of these genes. Consequently, WRKY1 protein levels in the cell increase, resulting in the autoregulation of *PcWRKY1* and in the activation of secondary target genes such as *PcPR10*.

promoters (Figure 2). The subsequent recruitment of new WRKY factors might fortify the expression of these genes or, alternatively, might downregulate their expression. As mentioned above, WRKY functions have been linked to pathogen-induced MAPK cascades, and parsley MAPKs are translocated to the nucleus upon elicitation [47]. Thus, it is conceivable that protein kinases modify WRKY factors that are already bound at promoter sites in analogy to recent findings for the Hog1 MAPK in yeast [48]. How general such a mechanism might be *in planta* remains to be substantiated.

Conclusions

Unravelling WRKY functions remains an ambitious long-term endeavour. We believe that the diversification of this gene family was mainly in response to environmental factors, particularly to pressures imposed by diverse phytopathogens. Of imminent importance is to uncover WRKY-interacting proteins that assist in regulating the transcription of genes and, furthermore, to identify the key components of upstream signal transduction pathways with which they physically communicate. Specific MAPKs and possibly also calcium-dependent protein kinases (CDPKs) [49] can be expected to be partners that modify distinct WRKY factors in such pathways. Global expression arrays, together with the use of *wrky* mutant lines, will certainly aid in uncovering potential target genes. Taking this approach one step further, microarrays that contain all intergenic DNA regions, whose generation is now feasible for *Arabidopsis* and rice, could be combined with ChIP assays to probe for specific WRKY DNA-binding sites at a genome-wide level. Integrating such data with similarly obtained information on other transcription factors will allow us to identify combinatorial gene expression programs, and to establish transcriptional regulatory networks in plants like those developed for yeast [50]. Finally, although the primary sequences of hundreds of WRKY proteins from numerous plant species are known, full appreciation of how these factors assemble at DNA-binding sites to modulate transcription will require structural information at atomic resolution. Considering the size of this gene family, there is little doubt that WRKY factors will keep us both fascinated and busy in the coming years.

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