Structural and Functional Analysis of VQ Motif-Containing Proteins in Arabidopsis as Interacting Proteins of WRKY Transcription Factors[1][W][OA]

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WRKY transcription factors are encoded by a large gene superfamily with a broad range of roles in plants. Recently, several groups have reported that proteins containing a short VQ (FxxxVQxLTG) motif interact with WRKY proteins. We have recently discovered that two VQ proteins from Arabidopsis (*Arabidopsis thaliana*), SIGMA FACTOR-INTERACTING PROTEIN1 and SIGMA FACTOR-INTERACTING PROTEIN2, act as coactivators of WRKY33 in plant defense by specifically recognizing the C-terminal WRKY domain and stimulating the DNA-binding activity of WRKY33. In this study, we have analyzed the entire family of 34 structurally divergent VQ proteins from Arabidopsis. Yeast (*Saccharomyces cerevisiae*) two-hybrid assays showed that Arabidopsis VQ proteins interacted specifically with the C-terminal WRKY domains of group I and the sole WRKY domains of group IIc WRKY proteins. Using site-directed mutagenesis, we identified structural features of these two closely related groups of WRKY domains that are critical for interaction with VQ proteins. Quantitative reverse transcription polymerase chain reaction revealed that expression of a majority of Arabidopsis *VQ* genes was responsive to pathogen infection and salicylic acid treatment. Functional analysis using both knockout mutants and overexpression lines revealed strong phenotypes in growth, development, and susceptibility to pathogen infection. Altered phenotypes were substantially enhanced through cooverexpression of genes encoding interacting VQ and WRKY proteins. These findings indicate that VQ proteins play an important role in plant growth, development, and response to environmental conditions, most likely by acting as cofactors of group I and IIc WRKY transcription factors.

WRKY proteins are a relatively recently identified class of sequence-specific DNA-binding transcription factors found almost exclusively in plants (Rushton et al., 2010). The characteristic structural feature of WRKY proteins is the highly conserved WRKY domain, which contains the almost invariant WRKYGQK sequence at the N terminus followed by a $Cx_{4-5}Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$ zinc-finger motif (Rushton et al., 2010). Genes encoding WRKY proteins have been identified in low photosynthetic and nonphotosynthetic eukaryotes, but they have greatly proliferated and form large superfamilies only in higher plants with more than 70 members in Arabidopsis (*Arabidopsis thaliana*; Zhang and Wang, 2005). Based on the number and structures of the conserved WRKY zinc-finger motifs, WRKY proteins were initially classified into three groups (Eulgem et al., 2000). The first group contains two $Cx_4Cx_{22-23}HxH$ zinc-finger motifs, the second group contains one $Cx_{4-5}Cx_{23}HxH$ zinc-finger motif, and the third group contains one $Cx_7Cx_{23}HxC$ zinc-finger motif. More recent analyses, however, have shown that group II WRKY proteins can be further divided into IIa, IIb, IIc, IId, and IIe subgroups (Zhang and Wang, 2005; Rushton et al., 2010). In the green alga *Chlamydomonas reinhardtii* as well as in the nonphotosynthetic slime mold *Dictyostelium discoideum* and unicellular protist *Giardia lamblia*, there is only a single group I WRKY gene (Zhang and Wang, 2005), suggesting that group I WRKY proteins with two WRKY domains are the ancestors to the other groups of WRKY proteins.

Since their initial identification (Ishiguro and Nakamura, 1994), plant WRKY transcription factors have been subjected to intensive analysis for their biological functions. Earlier studies from analysis of the expression patterns of isolated *WRKY* genes and the cis-acting elements in defense gene promoters suggested an important role of plant WRKY transcription

factors in plant defense responses. For example, we have previously analyzed the expression profile of the Arabidopsis *WRKY* gene superfamily and discovered that more than 70% of the gene family members are responsive to pathogen infection and salicylic acid (SA) treatment (Dong et al., 2003). The indirect evidence for the involvement of WRKY proteins in plant defense responses has been confirmed by studies using loss-of-function knockout/down mutants or overexpression lines of WRKY genes. These studies have shown that WRKY transcription factors can act as positive or negative regulators of plant defense responses (Li et al., 2004, 2006; Journot-Catalino et al., 2006; Kim et al., 2006, 2008; Xu et al., 2006; Zheng et al., 2006; Lai et al., 2008). Other studies have found that WRKY proteins play critical roles in plant hormone signaling (Chen et al., 2010; Shang et al., 2010), secondary metabolism (Wang et al., 2010b; Suttipanta et al., 2011), and plant responses to abiotic stresses (Jiang and Deyholos, 2009; Li et al., 2009, 2011). In addition, several WRKY proteins regulate plant growth and developmental processes including trichome (Johnson et al., 2002) and seed (Luo et al., 2005) development, germination (Jiang and Yu, 2009), and leaf senescence (Robatzek and Somssich, 2002; Miao et al., 2004). Thus, WRKY proteins have important roles in a broad range of biological processes in plants.

Despite the wide range of biological functions, almost all analyzed WRKY proteins recognize the TTGACC/T W-box sequences (de Pater et al., 1996; Rushton et al., 1996; Wang et al., 1998; Chen and Chen, 2000; Cormack et al., 2002). This raises the question of how the functional specificity of different members of the transcription factor superfamily is achieved. One potentially important specificity determinant could be the nucleotides adjacent to the TTGACC/T core sequence, which may influence both the binding specificity and binding affinity of WRKY proteins to the core W boxes (Ciolkowski et al., 2008). Other mechanisms may include differential expression of *WRKY* genes and difference in transcription-activating or -repressing activities of different WRKY proteins. As found with other transcription factors, differential DNA-binding and transcription-regulatory activities of many WRKY transcription factors could well be regulated by their interacting partners such as coactivators, chromatin remodelers, and enzymes that modify histone. For example, we have previously reported both physical and functional interactions between structurally related Arabidopsis WRKY18, WRKY40, and WRKY60 (Xu et al., 2006). The three pathogen-induced WRKY proteins formed both homocomplexes and heterocomplexes with significantly different DNA-binding activity (Xu et al., 2006). More recently, we have shown that Arabidopsis WRKY38 and WRKY62 interact with HISTONE DEACETYLASE19 (HDA19) in plant basal defense (Kim et al., 2008). Both WRKY38 and WRKY62 were transcriptional activators in plant cells but the activating activities were largely abolished by

overexpressed HDA19. Functional analysis using knockout mutants indicated that HDA19 had a positive role whereas WRKY38 and WRKY62 had negative roles in plant basal defense. The physical interaction of the functionally opposing WRKY proteins with HDA19 may, therefore, act to fine tune plant basal defense responses (Kim et al., 2008).

Over the past several years, several groups including ours have reported physical interactions of WRKY transcription factors with proteins containing a conserved FxxxVQxLTG or VQ motif. Arabidopsis WRKY25 and WRKY33 interact with MKS1, a VQ protein that also acts as a substrate of MAPK4 (MPK4) from Arabidopsis (Andreasson et al., 2005; Qiu et al., 2008). HAIKU1 (IKU1), another VQ protein important for endosperm growth and seed size, interacts with WRKY10, which also regulates Arabidopsis seed size (Wang et al., 2010a). Very recently, we have shown that WRKY33 interacts with two other VQ proteins, SIGMA FACTOR-INTERACTING PROTEIN1 (SIB1) and SIB2 (Lai et al., 2011). MKS1, SIB1, and SIB2 recognize the C-terminal WRKY domain and stimulate the DNA-binding activity of WRKY33 (Lai et al., 2011). Further analysis indicated that the conserved V and Q residues in the VQ motif of SIB1 are important for its interaction with WRKY33 (Lai et al., 2011). Based on these and additional analyses of the interacting WRKY and VQ proteins, we have proposed that the short VQ motif represents the core of a WRKY-interacting domain and the VQ proteins are important regulators of DNA binding and other molecular activities of WRKY transcription factors (Lai et al., 2011). Here we report our expanded structural and functional analysis of the 34 Arabidopsis *VQ* genes and their gene products. We have discovered that the structurally diverse VQ proteins interact with the C-terminal WRKY domains of group I WRKY proteins and the sole WRKY domains of group IIc WRKY proteins, which share a number of amino acid residues critical for interaction with VQ proteins. Additional analysis using gene expression, knockout, and overexpression lines strongly suggest that plant *VQ* genes play important roles in plant growth, development, and defense responses.

RESULTS

Identification of VQ Proteins from Arabidopsis

Five VQ motif-containing proteins from Arabidopsis have been previously reported and functionally analyzed. These known VQ proteins are MKS1 (Andreasson et al., 2005), IKU1 (Wang et al., 2010a), CALMODULIN-BINDING PROTEIN25 (CAMBP25; Perruc et al., 2004), SIB1, and SIB2 (Xie et al., 2010; Lai et al., 2011). Loss-of-function mutants and/or overexpression lines for the *VQ* genes are altered in seed size, tolerance to abiotic stress, or resistance to pathogen infection, indicating that members of this protein family play important roles in plant growth, development, and responses to environmental conditions. To understand the

extensiveness of VQ proteins in Arabidopsis, we analyzed the entire family of the 34 genes encoding proteins containing the FxxxVQxxLTG motif in Arabidopsis (Xie et al., 2010; Table I). A majority of the Arabidopsis *VQ* genes are intronless and encode relatively small proteins with fewer than 300 amino acid residues. VQ2 is the largest VQ protein with 430 amino acid residues and is predicted to be encoded by a gene with four introns. The N-terminal region of approximately 240 amino acids of VQ2 is highly homologous with VQ3, which is encoded by an intronless gene adjacent to VQ2 on chromosome I (Fig. 1). The remaining, C-terminal region of VQ2, which are encoded by the other three exons, contains a RNA-recognition motif that is absent in VQ3. It is likely that VQ2 and VQ3 were resulted from relatively recent gene duplication, after which VQ2 has acquired additional functions through fusion with other gene(s).

The previously characterized IKU1 (VQ14), CAMBP25 (VQ15), and MKS1 (VQ21) are localized exclusively in the nucleus. On the other hand, both SIB1 (VQ23) and SIB2 (VQ16) are dual targeted, interacting with both plastic SIG1 and nuclear WRKY proteins. Through search of the Subcellular Proteomic Database, which contains large proteomic and GFP localization sets and precompiled bioinformatics predictions for subcellular localization of proteins from Arabidopsis, we found that a majority of Arabidopsis VQ proteins are also localized in the nucleus (Table I). Interestingly, a substantial number of Arabidopsis VQ proteins also contain a putative chloroplast or mitochondrial targeting

signal at their N terminus, in addition to the nuclear localization signal (Table I). Thus, like SIB1 and SIB2, some members of the Arabidopsis VQ protein family appear to be dual targeted to both the nucleus and chloroplasts or mitochondria.

Apart from the short VQ motif, there is relatively low sequence homology among the Arabidopsis VQ proteins (Fig. 1). When the full-length protein sequences of the 34 VQ proteins were used to construct the phylogenetic tree, there were only four branches with bootstrap values larger than 95% (Fig. 2). A majority of the branches, particularly those deep ones had low confidence values (Fig. 2), likely due to the divergent sequences of the protein family.

Interacting Partners of VQ Proteins from Arabidopsis

Among the five Arabidopsis VQ proteins functionally analyzed, four have been shown to interact with WRKY proteins. In addition, we have previously reported that Arabidopsis VQ1 interacts with WRKY33 (Lai et al., 2011). Subsequent screens also identified VQ10 interacted with WRKY33 and closely related WRKY25 and WRKY26 (Table II). In this study, we first analyzed using yeast (*Saccharomyces cerevisiae*) two-hybrid assays whether CAMBP25/VQ15, one of the five functionally characterized VQ proteins, also interacted with WRKY proteins. For this purpose, we fused the *CAMBP25* gene with the AD domain of the Gal4 transcription factor in the yeast two-hybrid

Figure 1. Alignment of VQ motif sequences of Arabidopsis proteins. The highly conserved residues in the VQ motif are indicated in red.

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VQ1(AT1G17147):    GVRSEPMKVVFINTQYVQTDARSFKTIVQELTGKNAVVADGPYEFSGQGYGGKDSSQ
VQ2(AT1G21320):    RPPPGPVNIYTVTPRIIHTHPNNFMTLVQRLTGQTSTSTTSSSSSSSTSEPKDTSTM
VQ3(AT1G21326):    RPPPGPVIIYTVSPRIIHTHPNNFMTLVQRLTGKTSTSTTSSSYSSSTSAPKDASTM
VQ4(AT1G28280):    HVTTRSESGNPYPTTFVQADTSSFKQVVQMLTGSAERPKHGSSLKPNPTHHQPDPRS
VQ5(AT1G32585):    SNSVPQLAQTQPRVQVYIIDKNDFKSLVQQLTSPQPCDRLPQNIPKHQDIRPEPINW
VQ6(AT1G32610):    KIGKNIKKSPLPQPQGYSMSNNDFTSIVQQLTDSPSRESLPQPLPRNLLKPQQKIRP
VQ7(AT1G35830):    NPRKRTRASRRAPTTVLTTDTSNFRAMVQEFTGVPASPFSHPFSSTTRRFDIFRSPS
VQ8(AT1G68450):    HGRASPVIIYAHSPKVIHTRAEDFMALVQRLTGLDEIIRRNTSESSSSVVTEEVNVG
VQ9(AT1G78310):    PLQINQGNLHQHQPPVYNINKNDFRDVVQKLTGSPAHERISAPPQQPIHHPKPQQSS
VQ10(AT1G78410):   KVKSEPMKVVFINTQYVETDARSFKTVVQELTGKNAIVAAGPFDSPSAFDGRCYDGG
VQ11(AT1G80450):   SHQQPPSYATEPNTMFVQADPSNFRNIVQKLTGAPPDISSSSFSAVSAAHQKLPLTP
VQ12(AT2G22880):   WKKPVTGLPQRMHPKVYRVEPVNFKELVQRLTGAEDVEQEVHQVEAKPVKISDDTTT
VQ13(AT2G33780):   TTPTRYVKDDMYETTFIRTDPSSFKQVVQLLTGIPKNPTHQPDPRFPPFHSIPPIKA
VQ14(AT2G35230):   STSNGAAPRLQTQPQVYNISKNDFRSIVQQLTGSPSRESLPRPPQNNSLRPQNTRLQ
VQ15(AT2G41010):   AAKRRARASKKSQTTFITADPSNFRQMVQQVTGAKYIDDSSSFGIFDPIVKPEPLRF
VQ16(AT2G41180):   TTHKPIKVRYISNPMRVETCPSKFRELVQELTGQDAADLPPSPTTFTAVDLHRPCES
VQ17(AT2G42140):   KSKPKIRIIHIFAPEIIKTDVANFREIVQNLTGKQDHHHHDLPHQKGLKRNPRSRRS
VQ18(AT2G44340):   KIKPKIRIIHIFAPEVIKTDVKNFRSLVQSLTGKPAPGEAKTGKKRAKSRITTPQEP
VQ19(AT3G15300):   LHHHIITRSDHYPTTFVQADSSSFKQVVQMLTGSSSPRSPDSPRPPTTPSGKGNFVI
VQ20(AT3G18360):   AKPRHPVIIYTHTPRIIHTNPKDFMALVQKLTGMTHSDEDLGGGNAMTDPGVVKSIN
VQ21(AT3G18690):   YIPREPVVIYAVSPKVVHATASEFMNVVQRLTGISSGVFLESGGGGDVSPAARLAST
VQ22(AT3G22160):   PTRRRSRASRRTPTTLLNTDTSNFRAMVQQYTGGPSAMAFGSGNTTSAFSLTSSSDP
VQ23(AT3G56710):   STNKPIKVRYISNPMRVQTCASKFRELVQRLTGDAVDLQPEPIYSPSSDDHNLSPP
VQ24(AT3G56880):   TKRRSRVSNKKSQTTFITADAANFRQMVQQVTGAKFLGSSNSIFAPIVKPEPHRLAS
VQ25(AT3G58000):   KSKPKIRIIHIFAPEIIKTDVANFRELVQSLTGKPDDQRTSKAKPRRDMHRLHRQVQ
VQ26(AT3G60090):   KIKPKIRIIHIFAPEIINTDVKNFRTLVQSLTGKPEITKTGSKKKITNTNFPAPPGL
VQ27(AT4G15120):   PTRRRSRASRRTPTTLFNTDTANFRAMVQQFTGGPSAVAFGSSPSSGFSLTSSDPTA
VQ28(AT4G20000):   AGRRSRASRRAIPTTLLNANPSNFRAMVQKFTGRSAGGESNRRKGPVTLDFGSPTTI
VQ29(AT4G37710):   PAISSSSPLNPMHPHVYRVEPVNFKELVQRLTGAPEHETVQAKPLKTSDDAAKQSSS
VQ30(AT4G39720):   KTKKRSRASRRAPTTVLTTDTSNFRAMVQEFTGIPAPPLFNNNSIVNTTRLNTFLGL
VQ31(AT5G08480):   KGSQNVATTCKPVTTFVQTDNTFREIVQRLTGPTENNAAAATPEATVIKTAIQKRP
VQ32(AT5G46780):   DPPNQQNQQQNPQALVYNVNKTDFRSIVQQLTGLGSTSSVNPPQTNHPKPPNSRLVK
VQ33(AT5G53830):   HHDQHQHLSNPYPTTFVQADTSTFKQVVQMLTGSSTDTTTGKHHEAPSPVNNNNKGS
VQ34(AT5G65170):   NPKKRSRVSRRAPTTVLTTDTSNFRAMVQEFTGNPSTPFTGLSSSFPRSRFDLFGSS
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Table I. *Properties of the Arabidopsis VQ proteins*

AGI ID, Arabidopsis Genome Initiative Identification Number; CDS, coding DNA sequence; n, Nucleus; p, plastid; m, mitochondria.

Name	AGI ID	Other Name	No. of Amino Acids	Subcellular Localization[a]	Intron within CDS
AtVQ1	AT1G17147		99	p/n	0
AtVQ2	AT1G21320		430	n	4
AtVQ3	AT1G21326		239	n	0
AtVQ4	AT1G28280		247	n/m	0-1
AtVQ5	AT1G32585		220	n	0
AtVQ6	At1G32610		291	n	0-1
AtVQ7	AT1G35830		302	n/m	0
AtVQ8	AT1G68450		152	n/p	0
AtVQ9	AT1G78310		311	n/p	0
AtVQ10	AT1G78410		104	p/n	0
AtVQ11	AT1G80450		177	n/p	0
AtVQ12	AT2G22880		114	p/m	0
AtVQ13	AT2G33780		204	n/m	0
AtVQ14	AT2G35230	IKU1	402	n	0-1
AtVQ15	AT2G41010	CAMBP25	238	n	0
AtVQ16	AT2G41180	SIB2	141	n/p	0
AtVQ17	AT2G42140		172	p	0
AtVQ18	AT2G44340		188	n/p	0
AtVQ19	AT3G15300		219	n/p	0
AtVQ20	AT3G18360		285	n/p	0
AtVQ21	AT3G18690	MKS1	222	n	0
AtVQ22	AT3G22160		192	n/p	0
AtVQ23	AT3G56710	SIB1	151	p/n	0
AtVQ24	AT3G56880		245	n	0
AtVQ25	AT3G58000		175	n/m	0
AtVQ26	AT3G60090		157	n	0
AtVQ27	AT4G15120		193	n/p	0
AtVQ28	AT4G20000		208	n	0
AtVQ29	AT4G37710		117	p/n	0
AtVQ30	AT4G39720		290	n	0
AtVQ31	AT5G08480		174	p/n	0-1
AtVQ32	AT5G46780		237	n	0-1
AtVQ33	AT5G53830		243	p/n	0
AtVQ34	AT5G65170		363	n	0

[a]Based on prediction in the Subcellular Proteomic Database (http://suba.plantenergy.uwa.edu.au).

prey vector pAD. We also cloned cDNA fragments corresponding to the DNA-binding domains for WRKY25, WRKY33, WRKY51, WRKY70, and WRKY38 into the pBD bait vector. The fused pAD and pBD vectors were then cotransformed into yeast cells and tested for *LacZ* reporter gene expression through assays of β-galactosidase activity. Both assays showed that CAMBP25/VQ15 was capable of interacting with both WRKY25 and WRKY51 (Table II).

To determine whether other Arabidopsis VQ proteins are capable of interacting with WRKY proteins, we utilized a recently published proteome-wide binary protein-protein-interaction map of Arabidopsis (Arabidopsis Interactome Mapping Consortium, 2011). The map was generated by testing all pairwise combinations of a collection of approximately 8,000 Arabidopsis open reading frames with an improved high-throughput binary interactome mapping pipeline based on the yeast two-hybrid system. The interaction map contains approximately 6,200 highly reliable interactions between approximately 2,700 proteins.

Through searching the Arabidopsis interactome database, we found eight VQ proteins (VQ8–10, VQ12, VQ16, VQ20, VQ24, and VQ32) in the generated interactome database with interacting partners. Significantly, all these eight VQ proteins had WRKY proteins as interacting partners (Table II). These interacting WRKY proteins all belong to either group I or group IIc WRKY proteins (Table II). Several of these eight VQ proteins also interacted with other non-WRKY proteins. For example, VQ9 interacted with MPK3 (Table II), a key signaling regulator involved in a variety of biological processes including defense responses (Asai et al., 2002; Pitzschke et al., 2009; Mao et al., 2011). VQ32 also interacted with N-MYC DOWN-REGULATED-LIKE1 (NDL1; Table II), a positive regulator of auxin transport in a G-protein-mediated pathway (Mudgil et al., 2009).

To further examine the partnership between the WRKY and VQ proteins, we analyzed the remaining 20 VQ proteins from the protein family for interaction with the WRKY domains from various groups of the
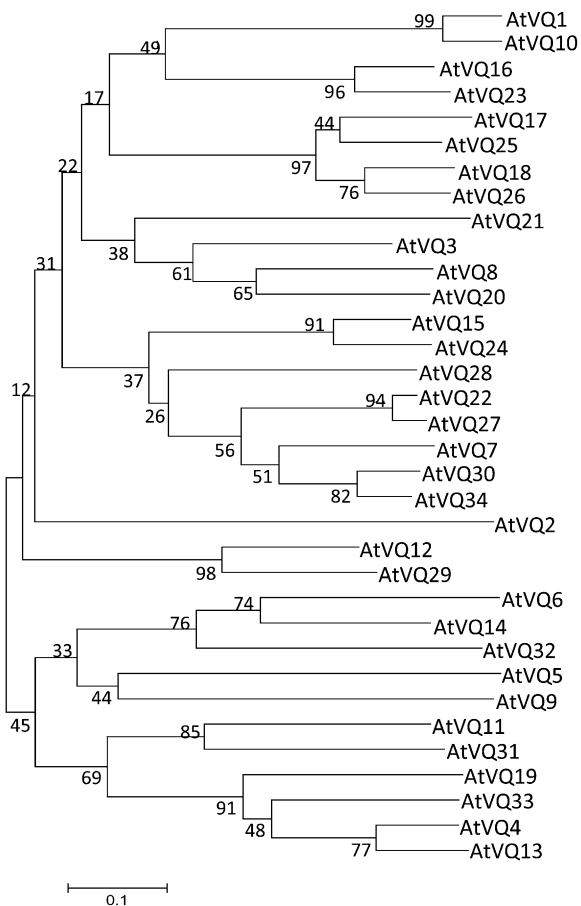
Figure 2. Phylogenetic trees of the VQ proteins from Arabidopsis. The tree was inferred using the neighbor-joining method. Phylogenetic analyses were conducted in MEGA5. Bootstrap values from 1,000 replicates were used to assess the robustness of the tree.

WRKY superfamily. These tested WRKY domains included the C-terminal WRKY domains of WRKY25 and WRKY33 (both group I) and the WRKY domains of WRKY11 (group IId), WRKY18 (group IIa), WRKY38 (group III), WRKY70 (group III), and WRKY51 (group IIc; Fig. 3). Again, we fused these *VQ* genes with the AD domain of the Gal4 transcription factor in the yeast two-hybrid vector pAD and cDNA fragments corresponding to the DNA-binding domains for the WRKY genes into the pBD vector. The fused pAD and pBD recombinant vectors were then cotransformed into yeast cells and tested for *LacZ* reporter gene expression through assays of β-galactosidase activity. As shown in Figure 3, a majority of the 20 VQ proteins interacted with the C-terminal WRKY domains of both WRKY25 and WRKY33, albeit with varying intensities based on the β-galactosidase activity. VQ6, VQ17, and VQ34 failed to interact with WRKY25 and only interacted weakly with WRKY33 based on the very low β-galactosidase activities detected (Fig. 3). About 50% of the 20 VQ proteins also interacted with group IIc WRKY51 based on the

positive β-galactosidase activity detected in the cotransformed yeast cells. On the other hand, none of the 20 VQ proteins interacted with the DNA-binding WRKY domains of group IId (WRKY11), group IIa (WRKY18), or group III (WRKY38 and WRKY70) WRKY proteins (Fig. 3). To determine whether VQ proteins recognized the other two groups of WRKY domains, we assayed the interactions of VQ5, VQ7, and VQ10 with the WRKY domains of WRKY6 (group IIb) and WRKY22 (group IIe) but failed to detect any significant β-galactosidase activity in yeast cells cotransformed with the fused bait and prey vectors (see Supplemental Fig. S1).

Structural Features of WRKY Domains Critical for Interaction with VQ Proteins

Assays of the 34 Arabidopsis VQ proteins in yeast cells revealed a clear pattern: They interacted with the C-terminal DNA-binding WRKY domains of group I and the sole WRKY domains of group IIc WRKY proteins, but not with the N-terminal WRKY domain of group I or the WRKY domain of group IIa, IIb, IId, IIe, or III WRKY proteins (Table II; Fig. 3; Supplemental Fig. S1). The consensus sequences of WRKY domains for each WRKY group in higher plants have been generated (Rushton et al., 2010). Comparison of the consensus sequences revealed that a basic amino acid (Arg or Lys) residue in the region immediately preceding the WRKYGQK signature sequence is unique to the C-terminal WRKY domain of group I WRKY proteins and the sole WRKY domain of group IIc WRKY proteins (Rushton et al., 2010; Fig. 4A). There are also two Asp residues in the region preceding the WRKYGQK signature sequence in the C-terminal WRKY domain of group I WRKY proteins and the sole WRKY domain of group IIc and III WRKY proteins (Rushton et al., 2010). For the C-terminal WRKY domain of WRKY33 (W33CT), these three amino acid residues correspond to Asp-359 (D359), Asp-362 (D362), and Arg-366 (R366; Fig. 4A). To test their role in interaction with VQ proteins, we changed Arg-366, which immediately precedes the WRKYGQK signature sequence of W33CT, to a Gln residue (W33CTR366Q), a consensus residue at the same position in group IIa and IIb WRKY proteins (Fig. 4A). We also changed Asp-359 and Asp-362 of W33CT to an Ala residue to generate the W33CTD359A and W33CTD362A mutant WRKY domains, respectively (Fig. 4A). The mutant C-terminal WRKY domains of WRKY33 were then tested for interacting with VQ10. As shown in Figure 4B, W33CTR366Q interacted with the VQ protein as strongly as wild-type C-terminal WRKY domain of WRKY33 (W33CT), indicating that the basic amino acid residue immediately preceding the WRKYGQK sequence is not critical for interaction with the VQ protein. By contrast, the W33CTD362A mutant WRKY domain failed to interact with the same VQ protein based on the β-galactosidase activity

Table II. *Proteins that interact with VQ proteins from Arabidopsis*

AGI ID, Arabidopsis Genome Initiative Identification Number; Last column: 1, This study; 2, Perruc et al. (2004); 3, Andreasson et al. (2005); 4, Morikawa et al. (2002); 5, Lai et al. (2011); 6, Wang et al. (2010a); 7, Arabidopsis Interactome Mapping Consortium (2011).

| VQ Protein | | VQ-Interacting Protein | | | | Source |
| | | WRKY Protein | | Non-WRKY Protein | | |
Name	AGI ID	Name	AGI ID	Name	AGI ID	
AtVQ1	AT1G17147	WRKY33(I)	AT2G38470			1
AtVQ8	AT1G68450	WRKY20(I)	AT4G26640	RACK1C	At3g18130	7
		WRKY24(IIc)	AT5G41570			
		WRKY34(I)	AT4G26440			
AtVQ9	AT1G78310	WRKY20(I)	AT4G26640	MPK3	At3g45640	
AtVQ10	AT1G78410	WRKY25(I)	AT2G30250			1
		WRKY26(I)	AT5G07100			
		WRKY33(I)	AT2G38470			
AtVQ12	AT2G22880	WRKY20(I)	AT4G26640	CSN5A	AT1G22920	7
		WRKY23(I)	AT2G47260			
		WRKY24(IIc)	AT5G41570			
AtVQ14	AT2G35230	WRKY10(IIc)	AT1G55610			6
AtVQ15	AT2G41010	WRKY25(I)	AT2G30250	CaM1	AT5G37780	1, 2
		WRKY51(IIc)	AT5G64810			
AtVQ16	AT2G41180	WRKY25(I)	AT2G30250			4, 5
		WRKY33(I)	AT2G38470			
AtVQ20	AT3G18360	WRKY20(I)	AT4G26640			7
		WRKY75(IIc)	AT5G13080			
AtVQ21	AT3G18690	WRKY25(I)	AT2G30250	MPK4	At4g01370	3
		WRKY33(I)	AT2G38470			
AtVQ22	AT3G22160	WRKY51(IIc)	AT5G64810			
AtVQ23	AT3G56710	WRKY3(I)	AT4G26640	SIGA	At1g64860	4, 5
		WRKY4(I)	AT1G13960			
		WRKY20(I)	AT4G26640			
		WRKY25(I)	AT2G30250			
		WRKY33(I)	AT5G64810			
AtVQ24	AT3G56880	WRKY20(I)	AT4G26640			7
		WRKY75(IIc)	AT5G13080			
AtVQ32	AT5G46780	WRKY20(I)	AT4G26640	NDL1	AT5G56750	7

assays (Fig. 4B). W33CTD359A interacted positively but weakly with VQ10, based on the relatively low β-galactosidase activities (Fig. 4B). Thus, Asp-362 is a critical residue for interaction with VQ proteins.
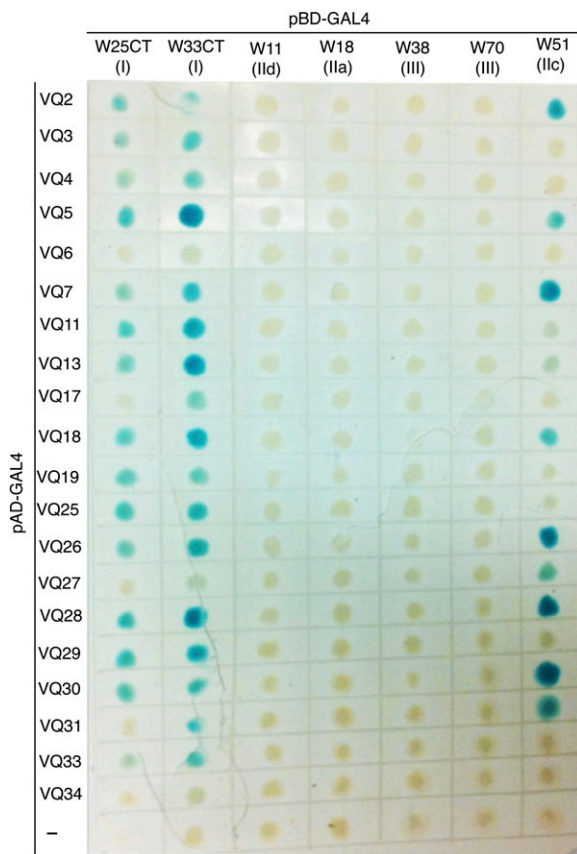
Comparison of the consensus sequences also revealed that there are only four amino acid residues between the two conserved Cys residues (Cx_4C) in the zinc-finger structure of the C- and N-terminal WRKY domains of group I WRKY proteins and the WRKY domains of group IIc WRKY domains (Rushton et al., 2010; Fig. 4A). On the other hand, there are five or seven residues between the conserved Cys residues in the zinc-finger structure of the WRKY domains of the other five groups of WRKY proteins (group IIa, IIb, IId, IIe, and III; Rushton et al., 2010; Fig. 4A). We inserted an Ala residue in the middle of the four residues between the conserved Cys residues in the zinc-finger structure of the C-terminal WRKY domain of WRKY33 (W33CTIns), as found in group IIa and IIb WRKY proteins (Fig. 4A). As shown in Figure 4B, the W33CTIns mutant WRKY domain failed to interact with VQ10 based on the β-galactosidase activity assays. This result indicated that the number of amino acid residues between the two conserved Cys residues

involved in zinc coordination is also critical for interaction with VQ proteins.

Quantitative Real-Time-PCR Analysis of *VQ* Genes Expression Profiles

We have previously analyzed the expression profile of the Arabidopsis *WRKY* gene family and showed that almost 70% of the *WRKY* genes in Arabidopsis were differentially regulated, mostly up-regulated, in response to pathogen infection or SA treatment (Dong et al., 2003). This observation strongly suggested that a major function of the *WRKY* gene superfamily is the regulation of genes associated with plant defense and stress responses. To examine their roles in plant defense, we also analyzed the expression levels of the 34 *VQ* genes from Arabidopsis. Arabidopsis plants were inoculated with a virulent strain of *Pseudomonas syringae* or treated with SA (1 mM). Leaf samples were collected at 0, 4, 12, and 24 h post pathogen inoculation (hpi) or SA treatment and the transcript levels of the *VQ* genes were quantified using quantitative real-time (qRT)-PCR. Cluster analysis of the expression profiles

Figure 3. Interaction of Arabidopsis VQ proteins with WRKY proteins in yeast cells. The Gal4 DNA BD-WRKY domain fusion bait vectors were contransformed with the activation domain (AD)-VQ fusion prey vectors into yeast cells and the transformant cells were assayed for *LacZ* reporter gene expression. The empty pAD prey vector was used as negative control (−).

than the statistically expected frequencies (Dong et al., 2003). The overrepresentation of the W boxes suggests extensive self and cross regulation among members of the *WRKY* gene family. To examine possible regulation of Arabidopsis *VQ* genes by WRKY transcription factors, we inspected 1.5-kb sequences upstream of the predicted translational start sites of the Arabidopsis *VQ* genes for the core W-box sequence (TTGAC). The average frequencies of the W boxes in the 1.5-kb promoters of the 34 *VQ* genes from Arabidopsis are 3.8 (Fig. 6), which is higher than the statistically expected frequency of 3.04. Among the 34 *VQ* genes, 16 (47%) contain four or more copies of the TTGAC pentamer sequence in their promoters (Fig. 6). VQ11 contains the highest number of WRKY-binding sites (11 copies of TTGAC), whereas VQ20 does not contain even a single WRKY-binding site in its promoter (Fig. 6). However, neither the induction/repression by pathogen or SA, nor the degree of either response, appeared to correlate well with the number of W boxes in the *VQ* gene promoters. A similar trend has been previously found with Arabidopsis *WRKY* genes (Dong et al., 2003).

Functional Analysis of *VQ* Genes from Arabidopsis

To analyze directly the biological functions of Arabidopsis *VQ* genes, we first sought a loss-of-function approach through characterization of T-DNA or transposon insertion mutants. Through screening of insertion lines obtained from various stock centers, we isolated T-DNA or transposon insertion mutants for eight new *VQ* genes (*VQ4, VQ5, VQ7, VQ8, VQ10, VQ20, VQ24,* and *VQ32*). Mutants for four additional *VQ* genes have been previously reported and characterized (Andreasson et al., 2005; Wang et al., 2010a; Lai et al., 2011). Thus, T-DNA or transposon insertion mutants exist only for less than 50% of *VQ* genes from Arabidopsis. This low frequency is likely due to the small gene sizes of *VQ* genes. For the mutants of the eight *VQ* genes, we examined their overall phenotypes in growth, development, and resistance to a virulent strain of the bacterial pathogen *P. syringae* and the necrotrophic fungal pathogen *Botrytis cinerea*. Except for the mutant for *VQ8*, we observed no major alteration in growth, development, or disease resistance in the mutants for the *VQ* genes.

VQ8 encodes a VQ protein with an N-terminal signal peptide predicted to be chloroplast targeting (Table I). A transposon-tagged line was identified and verified by PCR using primers flanking the insertion site in the gene (Fig. 7, A and B; Kuromori et al., 2006). The *vq8-1* mutant displayed pale-green and stunted-growth phenotypes throughout the entire life cycle (Fig. 7C). As expected for a loss-of-function mutant, the F1 progeny of the cross between *vq8-1* and No-0 wild-type plants were normal in growth and development. Furthermore, survey of F2 progeny showed that the mutant phenotypes were observed only in

divided the *VQ* genes into two groups (Fig. 5). In the first group, the levels of transcript of the *VQ* genes including VQ19 and VQ21 were overall reduced after SA treatment and at later hours after pathogen infection (12 and 24 hpi). In the other group, the transcript levels were up-regulated at 4 and 12 h post SA treatment (Fig. 4). Many of these SA-induced *VQ* genes were also up-regulated at earlier hpi (4 hpi). At later hours, particularly at 24 h post pathogen infection or SA treatment, the transcript levels of many of the induced *VQ* genes were not further increased and some of them even declined (Fig. 5). Thus, induction of many of the *VQ* genes by pathogen infection and SA was rapid but transient. Generally speaking, the transcript levels of Arabidopsis *VQ* genes changed quite dynamically upon pathogen infection or SA treatment.
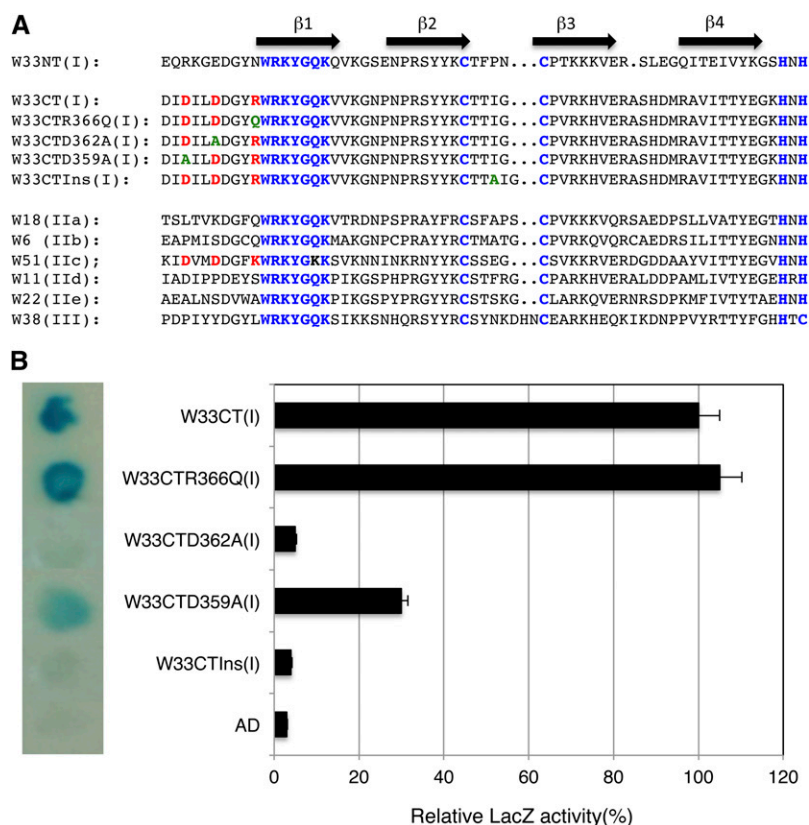
Analysis of VQ Gene Promoters from Arabidopsis

We have previously shown that the average frequencies of the W boxes in pathogen- and SA-regulated WRKY gene promoters from Arabidopsis are substantially higher

A

B

Relative LacZ activity(%)

Figure 4. Identification of critical amino acid residues of C-terminal WRKY domain of WRKY33 for interaction with VQ10. A, Sequence comparison of WRKY domains of WRKY33 (W33), WRKY18 (W18), WRKY6 (W6), WRKY51 (W51), WRKY11 (W11), WRKY22 (W22), WRKY38 (W38), which belong to group I, IIa, IIb, IIc, IId, IIe, and III WRKY proteins, respectively. Mutant C-terminal WRKY domains of WRKY33 (W33CT) with D395A, D362A, or R366Q substitution or with an Ala inserted between the two zinc-finger Cys residues (W33CTIns) are also shown. The WRKYGQK sequences and the Cys (C) and His (H) residues involved in zinc coordination are indicated in blue. The two Asp (D) residues and one basic residue in the region preceding the WRKYGQK sequence shared by the W33CT and W51 WRKY domains are in red. Resulted amino acid residues in the mutant W33CT proteins are in green. B, Yeast two-hybrid assays of interactions of VQ10 with wild-type and mutant forms of the C-terminal WRKY domains of WRKY33. pAD-VQ10 fusion vector was cotransformed with various pBD-W33CT constructs into yeast cells. Yeast transformants were analyzed for the *LacZ* reporter gene expression through assays of β-galactosidase activity on membrane using 5-bromo-4-chloro-3-indoly1-β-D-galactopyranoside (left section) or *o*-nitrophenyl-β-D-galactopyranose (right section) as substrate.

vq8-1/vq8-1 homozygous mutant plants, indicating that the *vq8-1* mutant is a recessive loss-of-function allele (Fig. 7B).

As a complementing approach for functional analysis of the *VQ* genes, we generated transgenic plants that constitutively overexpressed *VQ* genes. The coding sequences of the *VQ* genes were subcloned behind the cauliflower mosaic virus (CaMV) *35S* promoter in a binary plant transformation vector and transformed into Arabidopsis plants. RNA blotting was used to identify F0 transformant plants with elevated levels of transcripts for the transformed *VQ* gene (see Supplemental Fig. S2). Those transgenic F1 lines with increased *VQ* gene expression were analyzed for alterations in growth, development, and disease resistance. Due to the large number of transgenic lines for almost 30 *VQ* genes, our analysis of the transgenic lines was primarily focused on highly altered phenotypes that could be readily detected and verified. Generally speaking, overexpression of a majority of the *VQ* genes had no major effects on growth or development in transgenic plants. However, grossly altered growth was observed in transgenic plants overexpressing some of the *VQ* genes. Specifically, transgenic plants with elevated transcript levels for *VQ17*, *VQ18*, or *VQ22* were highly stunted in growth (Fig. 8A). Overexpression of *VQ29* didn't alter growth but substantially delayed the flowering time of the transgenic plants (Fig. 8B).

We also examined the responses of the VQ overexpression lines to *P. syringae* and *B. cinerea* and found relatively minor alteration in responses to the pathogens for a number of *VQ* genes. Overexpression of three *VQ* genes, however, had major effects on plant resistance to the two pathogens. Transgenic plants overexpressing *VQ5* displayed increased chlorosis after infection of *B. cinerea* (Fig. 9A), indicating enhanced susceptibility to the necrotrophic fungal pathogen. Transgenic plants overexpressing *VQ20* displayed enhanced disease symptoms after infection of either *B. cinerea* or *P. syringae* (Fig. 9), suggesting that VQ20 is a negative regulator in plant defense responses. In addition, transgenic plants overexpressing *VQ25* were also highly susceptible to *P. syringae* but were normal in resistance to *B. cinerea* (Fig. 9).

Functional Interactions of Interacting WRKY and VQ Proteins

VQ proteins interact physically with group I and IIc WRKY proteins and may regulate DNA binding and other molecular activities of the two groups of WRKY transcription factors. To determine possible functional interaction between VQ proteins and their interacting WRKY partners, we compared the phenotypes of transgenic plants overexpressing a single *VQ* or an interacting *WRKY* gene with those of transgenic plants

Figure 5. Cluster analysis of the expression profiles of Arabidopsis *VQ* genes. *VQ* gene expression was determined by qRT-PCR. *VQ* genes are represented as rows and treatment/time point as columns in the matrix. Red, black, and green elements indicate up-regulated, no change, and down-regulated *VQ* genes, respectively, in the matrix. Cluster analysis was performed on expression profiles of 34 *VQ* genes across six transients treatments (P, *P. syringae* infection; S, SA treatment) and time points (4, 12, and 24 h after pathogen inoculation or SA treatment). The horizontal and vertical dendrograms indicate the degree of similarity between expression profiles for *VQ* genes and conditions tested, respectively.
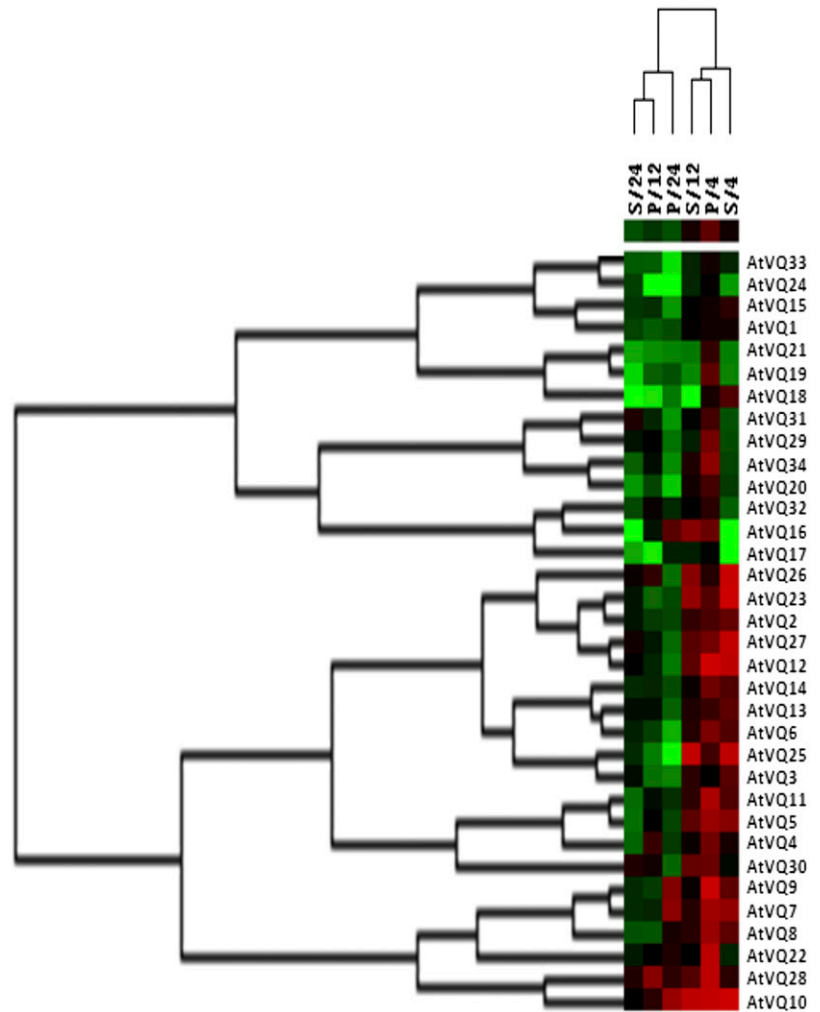

cooverexpressing both the interacting *VQ* and *WRKY* genes. For this purpose, we chose genes encoding *VQ10* and the *WRKY25*, *WRKY26*, and *WRKY33* interacting partners. These genes were selected for analysis of functional interactions because they were among the earliest identified interacting partners during our study of VQ proteins (Table II). As shown in Figure 10, transgenic plants overexpressing *VQ10*, *WRKY25*, *WRKY26*, or *WRKY33* were largely normal in growth as judged from the sizes of plants at mature stages. However, when a *VQ10*-overxpressing line was crossed with lines overexpressing the three WRKY genes, the F1 progeny were substantially altered in growth. Specifically, those F1 progeny cooverexpressing *VQ10* and either *WRKY25* or *WRKY33* were greatly stunted in growth as indicated by the drastically reduced plant sizes at mature stages (Fig. 10). The transgenic plants cooverexpressing *VQ10* and *WRKY26*, on the other hand, were not significantly reduced in sizes at mature stage when compared to those expressing either *VQ10* or *WRKY26* (Fig. 10). However, the leaves of the transgenic plants coexpressing the interacting *VQ10* and *WRKY26* genes

were substantially darker and rounder than those of wild-type and transgenic plants overexpressing a single *VQ10* or *WRKY26* gene (Fig. 10).

DISCUSSION

As a large protein superfamily, WRKY transcription factors are diverse in both structures and functions. As transcription factors, the diverse biological functions of WRKY proteins are due to their ability in regulating expression of a wide range of target genes through coordination with other DNA-binding or non-DNA-binding interacting proteins. Four VQ motif-containing proteins from Arabidopsis have been previously reported to interact with WRKY proteins and play critical roles in the biological processes in which their respective interacting WRKY proteins are involved (Andreasson et al., 2005; Qiu et al., 2008; Wang et al., 2010a; Xie et al., 2010; Lai et al., 2011). In this study, we expanded the study to the entire family of 34 VQ proteins in Arabidopsis and provided new important insights into the structures, interaction partnership
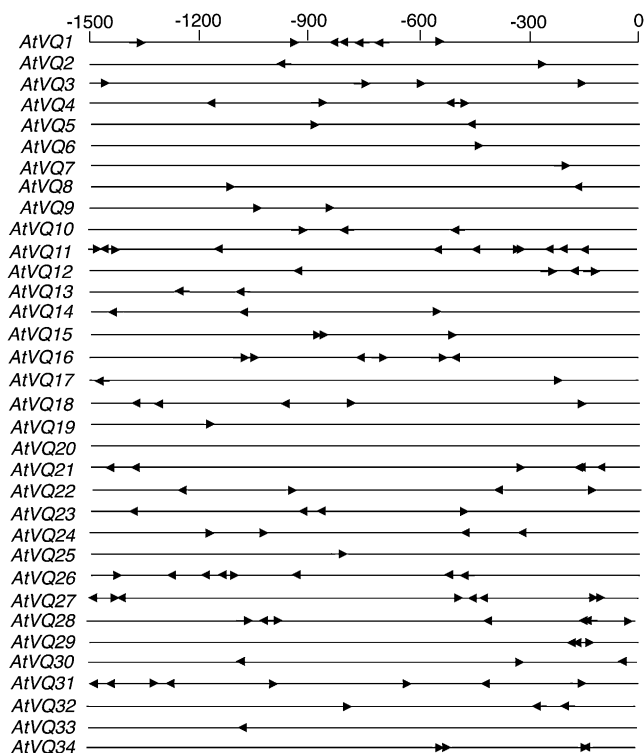
Figure 6. W-box elements in the promoter of Arabidopsis *VQ* genes. Numbering is from predicted translation start codons.

## VQ Proteins as Group I and IIc WRKY-Interacting Proteins

We have recently shown that Arabidopsis VQ proteins SIB1 (VQ23), SIB2 (VQ16), and MKS1 (VQ21) specifically recognize the C-terminal WRKY domain and stimulate the DNA-binding activity of WRKY33 (Lai et al., 2011). We have further revealed that the conserved V and Q residues in the short VQ motif of SIB1 are important for its physical interactions with WRKY33 and proposed that the short VQ motif is the core of the WRKY-interacting domain (Lai et al., 2011). To extend the analysis, we examined the entire family of 34 VQ proteins in Arabidopsis for interaction with WRKY proteins. Apart from the short VQ motif, there is relatively low sequence homology among the 34 Arabidopsis VQ proteins (Fig. 1). Despite the structural diversity, all 34 VQ proteins from Arabidopsis were capable of interacting with various WRKY proteins (Table II; Fig. 3). The WRKY proteins identified or assayed for interactions with the 34 VQ proteins included WRKY3, WRKY4, WRKY20, WRKY23, WRKY25, WRKY33, and WRKY34 (Table II; Fig. 3), which all belong to group I WRKY proteins with two WRKY domains (Eulgem et al., 2000). The interactions of these group I WRKY proteins with VQ proteins are likely through their C-terminal WRKY domains based on the inability of the N-terminal WRKY domain of

WRKY33 to interact with VQ proteins (Lai et al., 2011; Supplemental Fig. S1). Additional WRKY proteins identified to interact with VQ proteins include WRKY10, WRKY24, WRKY51, and WRKY75, which belong to group IIc WRKY proteins (Table II; Fig. 3). By contrast, the WRKY domains from group IIa (WRKY18), IIb (WRKY6), IId (WRKY11), IIe (WRKY22), and III (WRKY38 and WRKY70) failed to interact with VQ proteins (Fig. 3; Supplemental Fig. S1). Thus, VQ proteins appeared to interact only with group I and IIc WRKY transcription factors.

Using WRKY33 as a model, we have identified structural features unique to the two groups of WRKY proteins that are critical for their interaction with VQ proteins. These structural features include the presence of four amino acid residues between the two
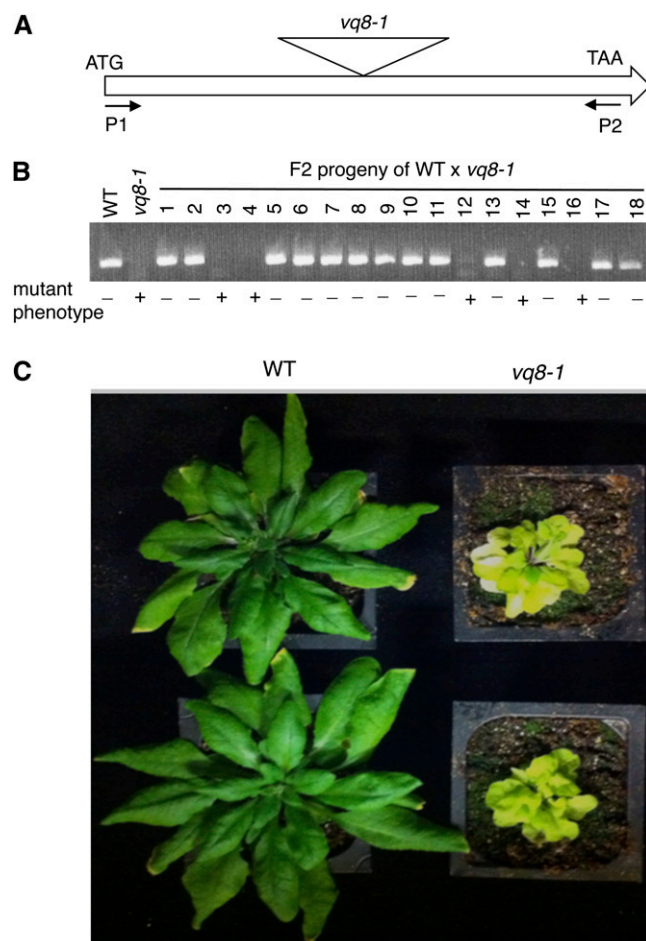
Figure 7. Identification and characterization of Arabidopsis *vq8-1* mutant. A, The structure of the intronless *VQ8* gene. The transposon insertion site of the *vq8-1* mutant is indicated. P1 and P2 are the two primers used for identification of homozygous *vq8-1* mutant plants. B, Genetic analysis of *vq8-1*. Col-0 wild type (WT) was crossed with the *vq8-1* and the F2 progeny were screened for homozygous *vq8* progeny and scored for the absence (−) or presence (+) of its mutant phenotypes. C, The phenotypes of the *vq8-1* mutant. The picture was taken at 7 weeks after germination.

with WRKY proteins, and biological functions of the new protein family.
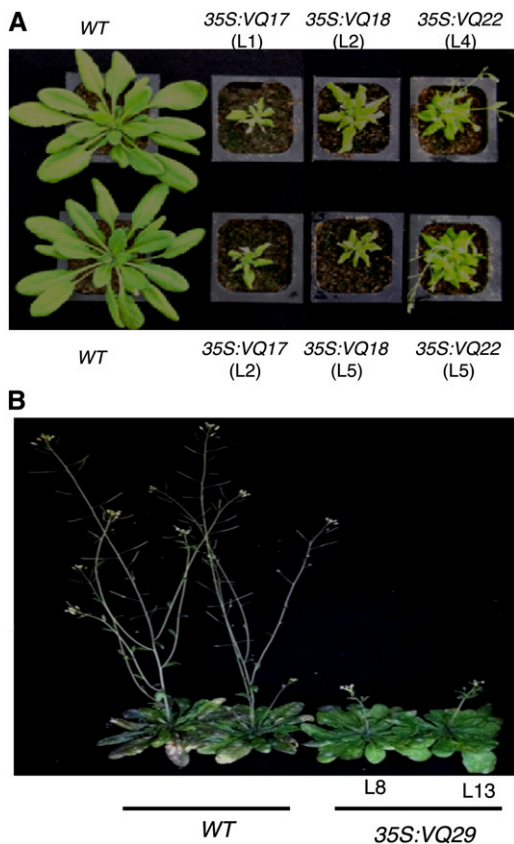
Figure 8. Growth and developmental phenotypes of transgenic VQ-overexpressing Arabidopsis plants. A, Stunted growth of transgenic plants overexpressing *VQ17*, *VQ18*, or *VQ22*. The picture of Col-0 wild type (WT) and two lines (L) of transgenic overexpression plants for each VQ gene was taken 7 weeks after germination. B, Delayed flowering of transgenic plants overexpressing *VQ29*. The picture of Col-0 wild type (WT) and two lines (L) of transgenic *VQ29* over-expression plants was taken 10 weeks after germination. The RNA-blotting analysis of the overexpressed *VQ* genes in the transgenic lines is shown in Supplemental Figure S2S.

conserved Cys residues involved in zinc coordination and two charged amino acid residues in the region that precede the WRKYGQK signature sequence (D359 and D362 for WRKY33). However, the N-terminal WRKY domains of group I also contain these similar structural features but failed to interact with VQ proteins (Supplemental Fig. S1). The N-terminal WRKY domains of group I WRKY proteins also have very low DNA-binding activity and, therefore, may lack certain unknown structural features necessary for strong DNA binding and interaction with VQ proteins.
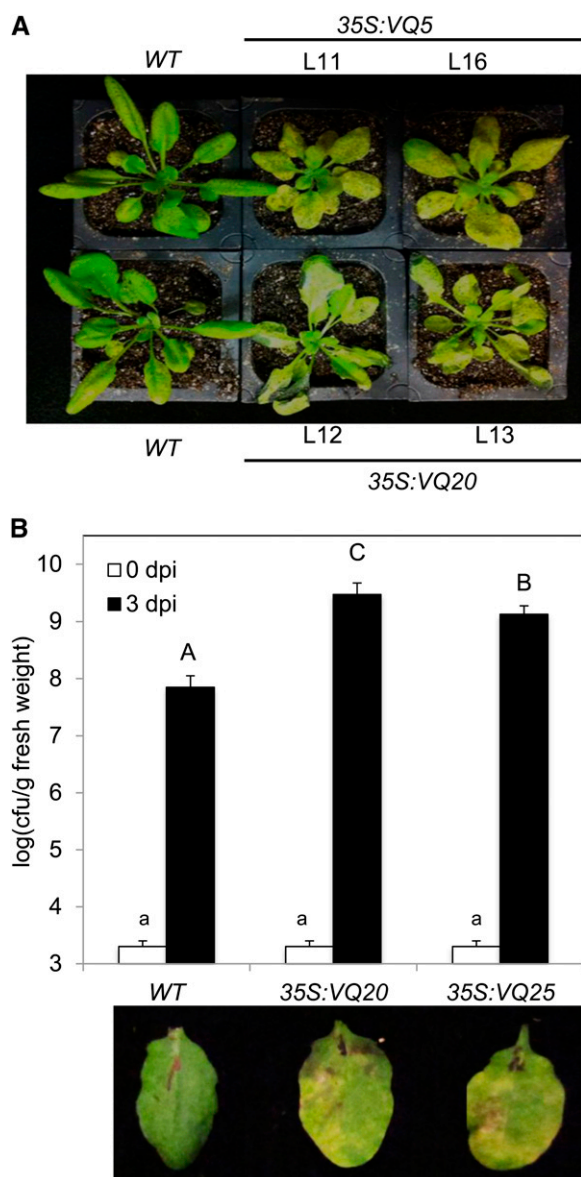
According to the structure solved by NMR, the C-terminal WRKY domain of WRKY4 is an antiparallel β-sheet forming from four β-strands (Yamasaki et al., 2012). For the longer C-terminal WRKY domain of WRKY4 or WRKY1 with more residues at the N terminus, there is actually another β-strand that is linked through a long bridging loop with the β-strand in which the WRKYGQK sequence is located (Duan et al.,

2007). Asp-262 of the C-terminal WRKY domain of WRKY33 would be expected to be located in the C-terminal end of the long bridging loop (Duan et al., 2007). Asp-262 of WRKY33 is also adjacent to the highly conserved Asp-263, which forms a salt bridge with the Trp residue in the invariant WRKYGQK sequence (Duan et al., 2007), which, in turn, is adjacently connected with the first Cys residue of the zinc-finger motif (Yamasaki et al., 2012). Therefore, Asp-262 and those residues located between the two Cys residues involved in zinc coordination, although well separated in the primary sequence, are in close proximity at one of the ends of the β-sheet, which may act as the interface for interaction with the small VQ motif. The recently solved solution structure of the C-terminal DNA-binding WRKY domain of WRKY4 in complex with the W-box DNA has further revealed that the β-sheet of the WRKY domain enters the major groove of DNA, nearly perpendicular to the DNA helical axis (Yamasaki et al., 2012). Residues located in the β-strand containing the conserved WRKYGQK motif are in direct contact with DNA bases (Yamasaki et al., 2012). Therefore, VQ proteins could potentially regulate the DNA binding of group I and IIc WRKY proteins by specifically recognizing the part of the β-sheets of their DNA-binding WRKY domains that directly contacts DNA molecules.

Biological Functions of *VQ* Genes

Due to the relatively large number of genes and the lack of knockout mutants for many of the gene family members, our functional analysis of the *VQ* gene family was by no means comprehensive but did provide a glimpse into the potential biological functions of the gene family in plant growth, development, and defense responses. We showed that overexpressing of some *VQ* genes alone or in combination with their interacting *WRKY* genes, led to altered phenotypes in growth and flowering (Figs. 8 and 10). Because overexpression could lead to physiologically irrelevant phenotypes, the inferred biological functions in plant growth and development should been considered with caution. On the other hand, *WRKY* genes with important roles in growth and development including trichome and seed development have been identified (Johnson et al., 2002; Wang et al., 2010a). In addition, many *WRKY* and *VQ* genes are highly responsive to pathogen infection and some interacting *VQ* and *WRKY* genes could be induced at sufficiently high levels locally, potentially to impact the expression of not only defense-related genes but also genes associated with growth and development. There is increasing body of evidence that plant growth is intrinsically linked with plant stress and defense responses (Kazan and Manners, 2009). Plant hormones including auxin and brassinosteroids, which are primarily associated with growth and development, have now been directly linked or implicated in plant defense responses

to a variety of microbial pathogens (Remans et al., 2006; Stotz et al., 2011; Belkhadir et al., 2012). Plant VQ and their interacting WRKY partners could play roles in interplay of plant growth, development, and stress responses.

Using qRT-PCR we showed that many of the *VQ* genes from Arabidopsis were responsive to pathogen infection and SA treatment (Fig. 5), suggesting possible roles in plant defense. In Arabidopsis, SA-mediated defense is important for resistance to biotrophic pathogens such as *P. syringae* and *Hyaloperonospora arabidopsidis*, whereas jasmonic acid (JA) often mediates plant defense against necrotrophic pathogens such as *B. cinerea* (Glazebrook, 2005). A number of studies have shown that the SA and JA signaling pathways are mutually antagonistic and, as a result, many genes involved in plant defense often play opposite roles in resistance to the two distinct types of plant pathogens (Kunkel and Brooks, 2002). Overexpression of *MKS1* led to increased expression of SA-regulated *PR1* gene expression but compromised plant resistance to necrotrophic *B. cinerea* (Andreasson et al., 2005; Petersen et al., 2010). In addition, the *mks1* mutants were compromised in resistance to biotrophic pathogens *P. syringae* and *H. arabidopsidis* (Petersen et al.,

Figure 9. Altered disease resistance of transgenic *VQ*-overexpressing Arabidopsis plants. A, Enhanced susceptibility to *B. cinerea*. Col-0 wild type (WT) and two independent lines (L) of transgenic plants overexpressing *VQ5* or *VQ20* were spray inoculated with *Botrytis* and the picture was taken at the third d post inoculation (dpi). B, Enhanced susceptibility to *P. syringae*. Col-0 wild type (WT) and transgenic plants overexpressing *VQ20* (lines 12 and 13) or *VQ25* (lines 7 and 8) were inoculated with a virulent strain of *P. syringae* pv tomato DC3000 ($OD_{600} = 0.0002$ in 10 mM $MgCl_2$). Samples were taken at 0 and 3 dpi to determine the growth of the bacterial pathogen (top section). According to Duncan's multiple range test ($P = 0.01$), means of colony-forming units (cfu) do not differ significantly at 0 dpi if they are indicated with the same lowercase letter and do not differ significantly at 2 dpi if they are indicated with the same uppercase letter. Images from representative inoculated leaves taken at 3 dpi are also shown (bottom section). The RNA-blotting analysis of the overexpressed *VQ* genes in the transgenic lines is shown in Supplemental Figure S2S.

Figure 10. Enhanced phenotypes in transgenic plants cooverexpressing interacting *VQ10* and *WRKY* genes. Transgenic plants overexpressing *VQ10* (line 1) were crossed with transgenic plants overexpressing *WRKY25* (line 1), *WRKY26* (line 1), or *WRKY33* (line 1). The F1 progeny of the crossing were compared with Col-0 and their parental lines for growth alteration. The images for the plants were taken 6 weeks after germination.

2010). These results indicated that MKS1 plays a positive role in SA-mediated defense against biotrophic pathogens but a negative role in JA-regulated defense against necrotrophic pathogens. On the other hand, the closely related SIB1 and SIB2 proteins act as coactivators of WRKY33 in JA-mediated plant defense against necrotrophic pathogens (Lai et al., 2011). Interestingly, overexpression of *SIB1* and *SIB2* also increased resistance to hemibiotrophic pathogen *P. syringae* (Xie et al., 2010). By contrast, overexpression of *VQ20* compromised resistance to both biotrophic and necrotrophic pathogens (Fig. 9). Thus, the impacts of mutations or overexpression of *VQ* genes on plant resistance to biotrophic pathogens are not always opposite to those on resistance to necrotrophic pathogens. Therefore, *VQ* genes have complex roles in plant defense responses, which could, in part, be due to their ability to interact with multiple WRKY proteins.

The Modes of Action of VQ Proteins

Out of the 72 members of the *WRKY* gene superfamily in Arabidopsis, there are a total of 32 group I and IIc WRKY genes (13 group I and 19 group IIc). This number is similar to the 34 *VQ* genes in the plant. Despite the similar numbers, the interaction partnership between the WRKY and VQ proteins is not highly specific. A single VQ protein interacted with multiple WRKY proteins and different VQ proteins had partially overlapping pools of interacting WRKY partners, which raised the question of how *VQ* genes achieve their functional specificity. In this regard, it is useful to consider several factors that could influence the formation and properties of the WRKY/VQ protein complexes in plant cells. First, members within the *VQ* and *WRKY* gene families differ in their spatial and temporal expression patterns. Because only coexpressed WRKY and VQ proteins can interact in vivo, the number of major WRKY/VQ complexes for a specific VQ protein in a given type of cell or tissue under a given condition may not be as large as indicated from the yeast two-hybrid assays. Identification of the major WRKY interaction partners for a VQ protein in the physiological context would, therefore, be key to understanding the biological functions of the *VQ* gene. Second, apart from the highly conserved short VQ motif, VQ proteins are highly divergent in flanking amino acid sequences, which could influence the specificity of interaction of a VQ protein with WRKY partners. Indeed, although a majority of the VQ proteins interacted with group I WRKY25 and WRKY33, only about half interacted with group IIc WRKY51 in yeast cells (Fig. 3). Thus, structurally divergent VQ proteins may be quite selective in interactions with group II WRKY proteins. Moreover, as VQ proteins appear to recognize the part of the WRKY domains that contacts DNA, they could differ substantially in their effects on DNA binding of their interacting WRKY partners. Unlike SIB1 and SIB2,

which stimulate the DNA-binding activity of WRKY33, other WRKY33-interacting VQ proteins might inhibit DNA binding of WRKY33, thereby antagonizing the positive roles of WRKY33, SIB1, and SIB2 in plant defense against necrotrophic pathogens. Another possibility is the potential varying effects of different VQ proteins on the DNA-binding specificity of their interacting WRKY partners, particularly to the nucleotides neighboring the conserved W boxes, which may play a critical role in determining target gene specificity of WRKY transcription factors (Ciolkowski et al., 2008). Third, there are other proteins besides WRKY proteins that interact with VQ proteins (Table I), some of which such as MPK3, MPK4, and CaM1 are regulatory proteins in signal transduction. MPK4 interacts with VQ21 (MKS1) and regulates the activity of the VQ21/WRKY33 complex (Qiu et al., 2008). Likewise, interaction of VQ9 and VQ15 with MPK3 and CaM1, respectively, might also alter the interactions of the VQ proteins with WRKY proteins and, as a consequence, WRKY-regulated gene expression.

Both SIB1 and SIB2 are targeted to chloroplasts and interact with a σ factor of chloroplast RNA polymerase (Morikawa et al., 2002). Interestingly, a large percentage of the VQ proteins from Arabidopsis contain chloroplast- or mitochondria-targeting signal peptides at their N terminus (Table I), raising the possibility that many of the VQ proteins might function in the two organelles. The *vq8* mutant plants were pale green (Fig. 8), likely due to defects in chloroplast development. Genetic and molecular analyses have identified genes that encode chloroplast (plastid)-localized proteins involved in the synthesis and signaling of plant defense signal molecules, such as SA and JA. Chloroplasts are also a major source of reactive oxygen species and redox-active soluble molecules that affect the expression of both chloroplast and nuclear genes associated with defense responses. In addition, it is known that plant disease resistance is strongly influenced by environmental factors such as light (Chandra-Shekara et al., 2006; Griebel and Zeier, 2008). The fact that many members of a WRKY-interacting protein family are targeted to chloroplasts or mitochondria raised the possibility that WRKY/VQ-regulated gene expression may be coordinated with certain processes in the two organelles during plant defense and stress responses.

In summary, we found that structurally divergent VQ proteins from Arabidopsis were all capable of forming complexes with group I and IIc WRKY proteins through physical interaction of conserved VQ and WRKY domains. Several highly conserved amino acid residues located at the end of the β-sheets of the DNA-binding domains of the two groups of WRKY proteins with direct contacts with DNA are critical for interaction with VQ proteins. *VQ* genes exhibited dynamic expression patterns in response to pathogen infection and SA treatment. Functional analysis of the *VQ* genes indicated that they play important roles in

plant growth, development, and defense responses. These results provided strong evidence that plant VQ proteins have evolved primarily as cofactors of WRKY proteins and play critical roles in the network of WRKY-mediated gene expression.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown in growth chambers or rooms at 22°C, 120 μE m^{-2} light with 12 h of light/12 h of darkness.

Yeast Two-Hybrid Assays

Interactions between WRKY domains and VQ proteins were assayed using Gal4-based yeast (*Saccharomyces cerevisiae*) two-hybrid system as described (Lai et al., 2011). pAD-Gal-VQ and pBD-Gal-WRKY fusion constructs were generated from PCR-amplified DNA fragments using the gene-specific primers listed in Supplemental Tables S1 and S2, respectively. The prey and bait plasmids were transformed to yeast strain YRG-2. After overnight culture, the transformants were plated on membrane and assayed for β-galactosidase activity using 5-bromo-4-chloro-3-indoly1-β-D-galactopyranoside as substrate. Quantitative assays of β-galactosidase activity of yeast transformants were performed using *o*-nitrophenyl-β-D-galactopyranose as substrate.

Site-Directed Mutagenesis

Changes of amino acid residues in the region preceding the WRKYGQK sequence of the C-terminal WRKY domain of WRKY33 (W33CT) were achieved through site-directed mutagenesis using either the QuikChange kit from Agilent or overlapping PCR with the following primers: W33CTD359A (gtagtgcagacaacgagtgatattgccattct), W33CTD362A (gtagtgcagacaacgagtgatattgccattct), and W33CTR366Q (cttgacgacggttaccaatggagaaaatacggccaga). Insertion of an Ala residue in the region between the two Cys residues in the zinc finger of the C-terminal WRKY domain of WRKY33 (W33CTIns) was performed using the QuikChange kit (primer: actacaagtgcacaaccgctatcggttgtccagtgagga).

qRT-PCR

Total RNA was isolated from 5- to 6-week-old pathogen-infected or SA-treated Arabidopsis Columbia-0 (Col-0) wild-type plants using the Trizol reagent according to the supplier's instruction. Extracted RNA was treated with DNase (remove contaminating DNA) and reverse transcribed using the SuperScript III first-strand synthesis system for reverse transcriptase-PCR (Invitrogen). qRT-PCR was performed with an iCycler iQ multicolor real-time PCR detection system (Bio-Rad). PCRs were performed using the SYBR Green PCR master mix (Applied Biosystems) and gene-specific primers listed in Supplemental Table S3. The PCR conditions consisted of denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Relative gene expression was calculated as previously described (Livak and Schmittgen, 2001). The Arabidopsis *ACT2* gene was used as internal control as previously described (Huang et al., 2010).

Expression patterns of the 34 *VQ* genes were used for cluster analysis. To determine the degree of regulation of each *VQ* gene after pathogen infection or SA treatment, we calculated the induction/repression fold by determining the relative expression levels compared to those in uninfected/untreated plants. The degree-of-regulation data across six transients (three time points × two treatments) produced a matrix from which a gene expression similarity metric (the Peterson correlation coefficient) was calculated using the implementation as previously reported (Eisen et al., 1998). The similarity metrics were analyzed to determine clusters of the *VQ* genes with similar expression profiles. The cluster analysis was performed using the software tools written by Eisen (Eisen et al., 1998; http://rana.lbl.gov/EisenSoftware.htm).

Identification of T-DNA/Transposon Insertion Mutants

T-DNA/transposon insertion mutants for the following eight new *VQ* genes were identified: *VQ4* (Sail_589_A07), *VQ5* (GABI_063E09), *VQ7* (Salk_099709),

VQ8 (Pst20793), *VQ10* (SK6114), *VQ20* (Salk_002300), *VQ24* (Salk_151487), and *VQ32* (Salk_035635). PCR for identification of homozygous mutants were performed using gene-specific primers listed in Supplemental Table S1.

Generation of Transgenic VQ and WRKY Lines

For generating transgenic VQ overexpression lines, the full-length coding sequences for *VQ* genes were first excised from the corresponding pAD-Gal-VQ recombinant vectors using appropriate restriction enzymes and inserted behind the *CaMV 35S* promoter in the plant transformation vector PFGC5941. The resulted plasmids were transformed into Col-0 wild-type plants using the *Agrobacterium*-mediated floral-dip procedure (Clough and Bent, 1998). Transformants were identified for resistance to Basta. Transgenic plants overexpressing the transformed *VQ* transgene were identified by northern blotting (Supplemental Fig. S2).

For generating transgenic WRKY overexpression lines, *WRKY25*, *WRKY26*, and *WRKY33* full-length cDNAs were inserted behind CaMV 35S promoter in the pOCA30 plant transformation vector and transformed into Arabidopsis Col-0 plants. Transformants were identified for resistance to kanamycin and transgenic lines overexpressing the transformed WRKY genes were identified by northern blotting (Supplemental Fig. S2).

RNA Gel-Blot Analysis

Total RNA was isolated from leaves and separated on 1.2% agarose-formaldehyde gels and blotted to nylon membranes according to standard procedures. Blots were hybridized with ^{32}P-dATP-labeled gene-specific probes. Hybridization was performed in PerfectHyb plus hybridization buffer (Sigma) overnight at 68°C. The membrane was then washed for 15 min twice with 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 1% SDS and for 10 min with 0.1× SSC and 1% SDS at 68°C.

Pathogen Inoculation and SA Treatment

The bacterial strain *Pseudomonas syringae* DC3000 were grown overnight in King's B medium containing rifampicin (50 μg/mL) and kanamycin (25 μg/mL). The bacterial cells were harvested and suspended in 10 mM MgCl$_2$. The cells were then diluted to OD$_{600}$ = 0.0001 and infiltrated into the abaxial surface of the leaves. Growth and spray inoculation of *Botrytis cinerea* were performed as previously described (Zheng et al., 2006).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Interaction of Arabidopsis VQ5, VQ7, and VQ10 with WRKY proteins in yeast cells.

Supplemental Figure S2. VQ and WRKY gene overexpression in transgenic plants.

Supplemental Table S1. Primers for pAD-Gal fusion and overexpression constructs.

Supplemental Table S2. Primers for pBD-Gal fusion constructs.

Supplemental Table S3. Primers for quantitative reverse transcriptase-PCR.

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