CHAPTER 6

OVEREXPRESSION OF ATWRKY50 IS CORRELATED WITH ENHANCED PRODUCTION OF SINAPIC DERIVATIVES IN ARABIDOPSIS

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ABSTRACT

Several WRKY proteins, members of a plant-specific class of transcription factors, were overexpressed in Arabidopsis. To investigate their influences on the metabolites of Arabidopsis, an NMR spectroscopy-based metabolomic approach was applied. Multivariate data analysis, such as principal component analysis, hierarchical cluster analysis and partial least square-discriminant analysis of ^{1}H NMR data have been conducted. The results showed that the metabolome of transgenic Arabidopsis overexpressing AtWRKY50 was quite different from wild type Arabidopsis and transgenic Arabidopsis overexpressing other WRKY genes. Amongst other metabolites, especially sinapic acid and sinapoyl glucose were the most prominent differentiating metabolites and increased to levels 2 to 3 fold higher in the AtWRKY50 overexpressors. Our results indicate a possible involvement of AtWRKY50 on secondary metabolite production in Arabidopsis, in particular hydroxycinnamates such as sinapic acid and sinapoyl glucose. However, whether regulation takes place at the level of the genes encoding enzymes of the biosynthesis pathway or at higher levels of signal transduction is not clear and requires further study.

INTRODUCTION

Plants are under continuous threat of attack by fungal, viral and bacterial pathogens. Upon pathogen attack, the plant may respond by activating defense measures through signaling hormones including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Malamy et al., 1990; Vlot et al., 2009). SA is typically involved in mediating defense against biotrophic pathogens, while JA

and ET mediate resistance responses against necrotrophic pathogens (Glasebrook et al., 2005). The accumulation of SA in systemic leaves leads to the onset of systemic acquired resistance (SAR). SAR is a broad-spectrum plant defense mechanism that is engaged upon a diversity of plant/pathogen interactions (Ryals et al., 1996). SAR is tightly correlated with the expression of several classes of genes, including genes encoding proteins collectively called pathogenesis-related (PR) proteins (Uknes et al., 1992; Uknes et al., 1993b; Ward et al., 1991). *PR* genes are conserved across the entire plant kingdom, including tobacco and Arabidopsis (Uknes et al., 1992). The enzymatic activity of various PR proteins is well-characterized and functionally correlates with resistance against pathogens, although the function of other PR proteins is still not understood. Among these is PR-1, which is generally used as marker for SAR.
WRKY proteins belong to a plant-specific class of transcription factors. There are 74 *WRKY* genes identified in Arabidopsis (Eulgem et al., 2000). In Arabidopsis and other plants many WRKY proteins are involved in responses to stress, especially to biotic stress. They may act either as transcriptional activators or as repressors of genes that play roles in the stress response (Asai et al., 2002; Dong et al., 2003; Journot-Catalino et al., 2006; Kim et al., 2006; Li et al., 2006; Wang et al., 2006; Eulgem and Somssich, 2007). Transcriptional regulation of gene expression is largely mediated by the specific recognition of *cis*-acting promoter elements by *trans*-acting sequence-specific DNA-binding transcription factors. The WRKY proteins are characterized by the presence of the WRKY domain, a DNA-binding domain consisting of a conserved WRKYGQK sequence followed by a zinc-finger (Eulgem et al., 2000).

We are interested in the transcriptional activation of defense during SAR and have used the tobacco and Arabidopsis *PR-1* genes as model genes in our studies. Previous work by others has indicated the importance of a region in the promoter of the *PR-1* gene for SA-induced expression (Lebel et al., 1998,

Pape et al., 2010). The region contains several potential binding sites for transcription factors that could be involved in the induced expression. Members of the TGA proteins, a group of proteins with conserved amino acid sequences that belong to the class of bZIP transcription factors, were found to be important for transcriptional regulation of the *PR-1* genes. However, evidence indicates that also other transcription factors are important for regulation of gene expression (Lebel et al., 1998; Pape et al., 2010). A number of studies have suggested the involvement of Arabidopsis WRKY transcription factors in induced *PR* gene expression, although no direct evidence has been presented for specific WRKY-*PR* promoter interactions (Chen and Chen, 2002; Robatzek and Somssich, 2002; Kim et al., 2006). Recently, our studies on the transcriptional activation of the tobacco *PR-1a* gene have indicated that tobacco WRKY transcription factor NtWRKY12 is important for gene expression (van Verk et al., 2008). Based on these results we started a study of the putative WRKY proteins that might be involved in expression of the Arabidopsis *PR-1* gene. Arabidopsis WRKY50 is the closest homologs of NtWRKY12. The *AtWRKY50* gene is induced by pathogen infection and SA, and the protein localizes to the nucleus, supporting a role as transcription factor. We have shown that AtWRKY50 binds to the *PR-1* promoter at two positions in close proximity to the TGA binding sites. Protoplast transactivation assays have indicated that AtWRKY50 is able to activate *PR-1* gene expression and that co-expression with TGA2 or TGA5 further enhances expression. Together with the finding that AtWRKY50 physically interacts with the TGAs, this supports the idea that the transcription factors co-operate in the regulation of *PR-1* expression.

As mentioned above, the function of PR-1 in defense is not known. Overexpression of the protein in transgenic plants did not elevate defense against virus infection and also defense against other types of pathogens has

not been substantiated (Linthorst et al., 1989; Alexander et al., 1993; Niderman et al., 1995; Hussain et al., this thesis). Although PR-1 proteins are produced at high levels in infected plants and partly accumulate in the apoplast, properties that are not in support of an enzymatic function, we cannot exclude that their expression has direct or indirect effects on the metabolome. Furthermore, in addition to its function in activation of *PR-1* gene expression, AtWRKY50 may act in the transcriptional regulation of other genes involved in defense, like genes encoding enzymes of biosynthesis pathways for metabolites with defense properties, cell-wall strengthening, etc. Here we have investigated the effects of overexpression of several WRKY proteins, including AtWRKY50, on the metabolome using NMR spectroscopy.

RESULTS AND DISCUSSION

Transgenic WRKY overexpressor plants

This study was aimed at investigating the effects of WRKY transcription factors on metabolite production in Arabidopsis. Therefore we made use of the *AtWRKY50* and *AtWRKY28* overexpression lines that were generated in the previous chapter and in addition generated transgenic Arabidopsis lines overexpressing *AtWRKY51* and *AtWRKY46*. To this end, the *AtWRKY51* and *AtWRKY46* coding regions were amplified by RT-PCR on total RNA isolated from SA-treated Arabidopsis and cloned behind the *35S* promoter. After flower-dip transformation of Arabidopsis Col-0, 20 primary, hygromycin-resistant seedlings were selected for further analysis. The seedlings were transferred to soil and grown through flowering and seed set. T2 generation plants were grown for 19 of the AtWRKY51 lines and 20 AtWRKY46 lines. None of the lines produced plants that were phenotypically different from wild

type Arabidopsis. The Northern blots of Fig. 1 show the expression levels of the transgenes in the plants of the T2 generation. The absence of bands in wild type Arabidopsis indicates that the expression levels of the *AtWRKY51* (Fig. 1, Panel A) and *AtWRKY46* (Fig. 1, Panel B) genes are below the level of detection, whereas a band of varying intensity corresponding to *AtWRKY51* mRNA is visible in most AtWRKY51 overexpression lines and similarly is *AtWRKY46* mRNA present in most of the AtWRKY46 overexpression lines. This demonstrates that the transgenes are expressed in most lines, although the expression levels vary considerably. For further analyses *AtWRKY51* overexpression lines W51 #1, #8 and #11 and AtWRKY46 overexpressor lines W46 #5, #8 and #9 were selected. In addition to these WRKY overexpressor lines also overexpressor lines of *AtWRKY50* and *AtWRKY28* described in the previous chapter were used for the metabolomic analyses. These were lines W50#2, #3 and #8, and W28#2, #4 and #12.

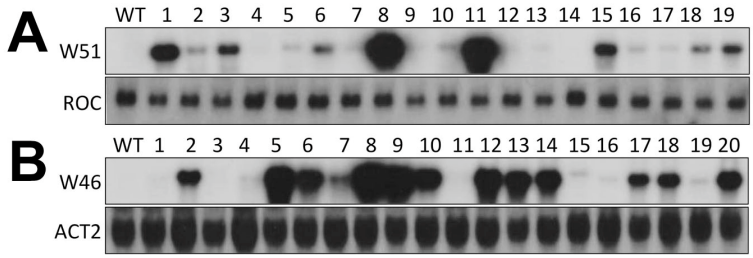

Figure 1. Transgene expression levels in transgenic plants. Northern blots containing total RNA extracted from hygromycin resistant seedlings generated from flower-dip transformed Arabidopsis were hybridized to cDNA probes corresponding to the respective transgenes *AtWRKY51* (A) and *AtWRKY46* (B). To check equal loading, identical blots were hybridized with probes corresponding to constitutive house-keeping genes *At4G38740* encoding rotamase cyclophilin (ROC) and *At3G18780* encoding actin 2 (Actin), respectively. Numbers above the lanes indicate the transgenic line. Samples from non-transformed Arabidopsis were electrophoresed in lanes WT.

Principal component analysis

Selected transgenic Arabidopsis lines overexpressing *WRKY* genes (*AtWRKY28*, *AtWRKY46*, *AtWRKY50*, *and AtWRKY51*) were examined for their metabolites using ^{1}H NMR spectroscopy. ^{1}H NMR spectroscopy can detect all metabolites containing hydrogen, and therefore it is suitable to obtain broad range metabolome snapshots of the given samples. In general ^{1}H NMR spectra produce large numbers of variables (usually more than 200 signals), which makes it necessary to perform multivariate data analysis. The most common unsupervised multivariate data analysis is principal component analysis (PCA). As the first step of multivariate data analysis, PCA of ^{1}H NMR spectra was performed to discriminate the transgenic Arabidopsis WRKY overexpression plants. The PCA score plot showed that PC1 and PC2 explained 34% and 31%, respectively (Fig. 2). Most prominent differences among the tested Arabidopsis plants were found in the two lines that overexpressed AtWRKY50, notably lines W50#3 and W50#8. Line W50#3 was separated by PC1 compared to wild type Arabidopsis, while line W50#8 separated both PC1 and PC2 (Fig. 2a).

The loading plot of PC1 (Fig. 2b) indicated that sinapic acid, sinapoylglucose, sucrose, glucose, alanine and threonine had high levels in both W50#3 and W50#8 in comparison to wild type. In contrast, levels of other phenolic compounds were decreased in the W50#3 and W50#8 plants. The loading plot of PC2 (Fig. 2b) indicated that glucose, glutamate and the signals of δ 2.70, δ 2.95, δ 3.10 were increased in W50#3, compared to W50#8, while hydroxycinnamates and amino acids, in particular, threonine, were higher in W50#8, compared to wild type and W50#3 plants.

Hierarchical cluster analysis

Although the PCA analysis provides some clues for the differences between the Arabidopsis overexpression lines, the available PCs are limited, because only

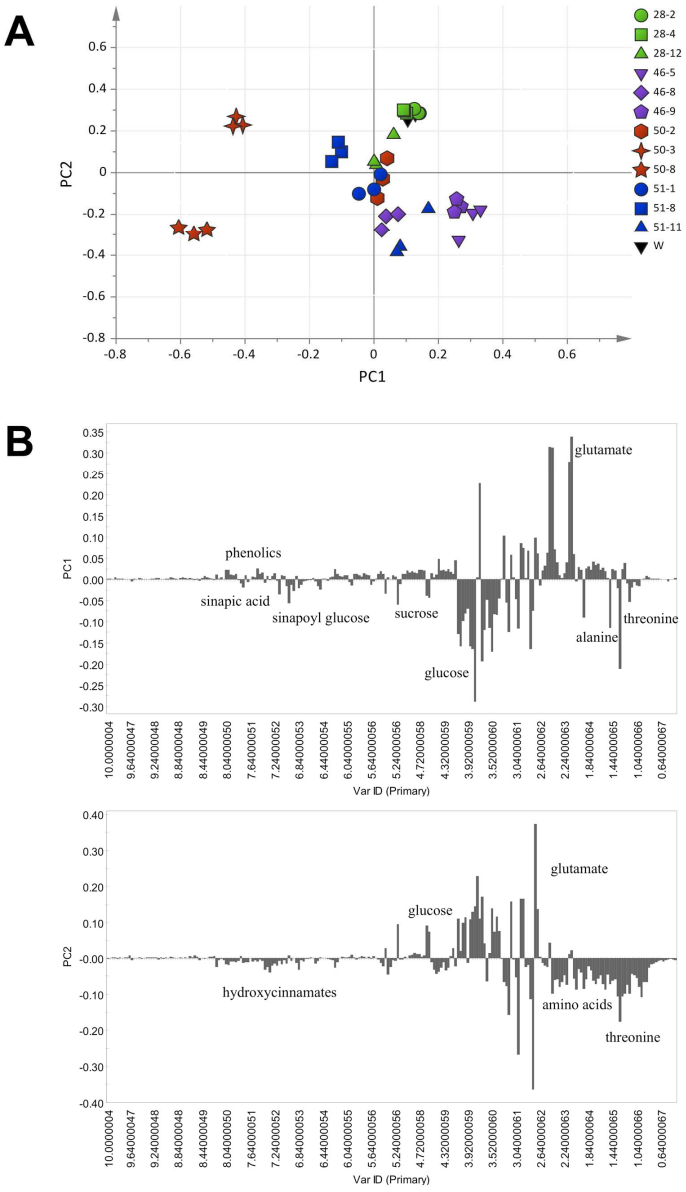

Figure 2. Principal component analysis of ^{1}H NMR spectra from Arabidopsis overexpressing WRKY28, WRKY46, WRKY50 and WRKY51 and wild type Arabidopsis. Score plot (a) and loading plots (b) of PC1 and PC2.

two or three PCs can be visualized. Besides, the score plot does not provide detailed information on the closeness between differently overexpressed plants and wild type plants. Applying hierarchical cluster analysis (HCA) allows obtaining further information on these aspects. For the HCA, 7 PCs reduced from the original ^{1}H NMR signals were used, which explained almost 95% of variables. The HCA showed that Arabidopsis overexpressing AtWRKY28 is very similar to wild type (group A), while plants overexpressing AtWRKY46 and AtWRKY51 were clustered in a different group (group B) (Fig. 3). It was obvious that Arabidopsis overexpressing AtWRKY50 was quite different (group C) from wild type Arabidopsis and other WRKY overexpressors, as was also shown in PCA.

Partial least square–discriminant analysis

To further analyze which metabolites contribute for the differentiation of each group, partial least square–discriminant analysis (PLS-DA) was applied. As a type of PLS, PLS-DA uses discrete class matrix, in contrast to PCA, which only uses the information of one matrix (Berrueta et al., 2007). In the PLS-DA, only two groups (group A and B) were used as Arabidopsis overexpressing AtWRKY50 most obviously differed from wild type by the PCA analysis. In the PLS-DA score plot, Arabidopsis overexpressing AtWRKY46 and AtWRKY51 were clearly differentiated by PLS component 1 (Fig. 4a). The model diagnostics for the first component showed an explained variation (R^2Y) of 0.85 and a goodness of fit (Q^2Y) of 0.76. Model validation using 200 permutations showed a negative slope of the regression line suggesting that there is no model overfit. To find out precisely which metabolites contributed to the discrimination between two groups, a PLS-DA loading plot was generated (Fig. 4b). In the loading plot, positive values of wc*[1] were associated with group A, while negative values were associated with group B. Investigation of the loading plots

suggested that glucose (δ 5.20, δ 4.60), sucrose (δ 5.40), glutamate (δ 2.12, δ 2.16, δ 2.48), and *cis*-sinapic acid (δ 5.96) were higher in group A, whereas flavonoids and other hydroxycinamates (*trans*-sinapic acid, sinapoyl glucose) and amino acids (alanine, phenylalanine) were higher in group B.

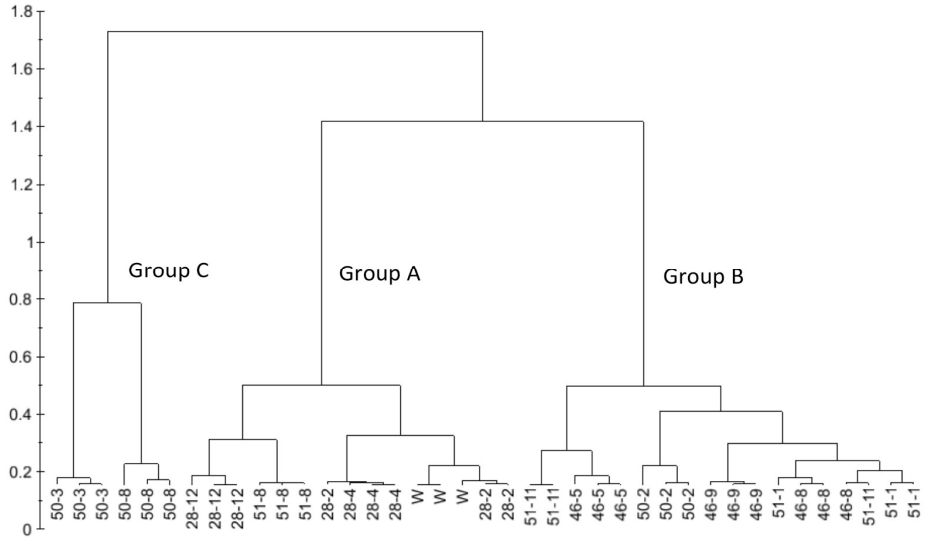

Figure 3. Dendrogram resulting from hierarchical cluster analysis showing three distinct groups A, B and C.

To summarize the results, Arabidopsis overexpressing AtWRKY50 (W50#3, W50#8) had increased amounts of sinapic acid, sinapoyl glucose, glucose, sucrose and amino acids (especially threonine in W50#8), compared to wild type. Group B plants overexpressing AtWRKY46 and AtWRKY51 (W51#1, W51#11) showed increased levels of sinapoyl glucose, sinapic acid, flavonoids and amino acids compared to wild type. The metabolome's of Arabidopsis overexpressing AtWRKY28 and AtWRKY51 (W51#8) were similar to wild type Arabidopsis. This study shows that the levels of sinapic acid and sinapoyl glucose were increased in Arabidopsis plants overexpressing AtWRKY50.

Quantitative analysis of sinapic acid and sinapoyl glucose of all samples clearly indicated the level of both compounds in Arabidopsis plants overexpressing AtWRKY50 were increased by 2-3 folds compared to wild type (Fig. 5).

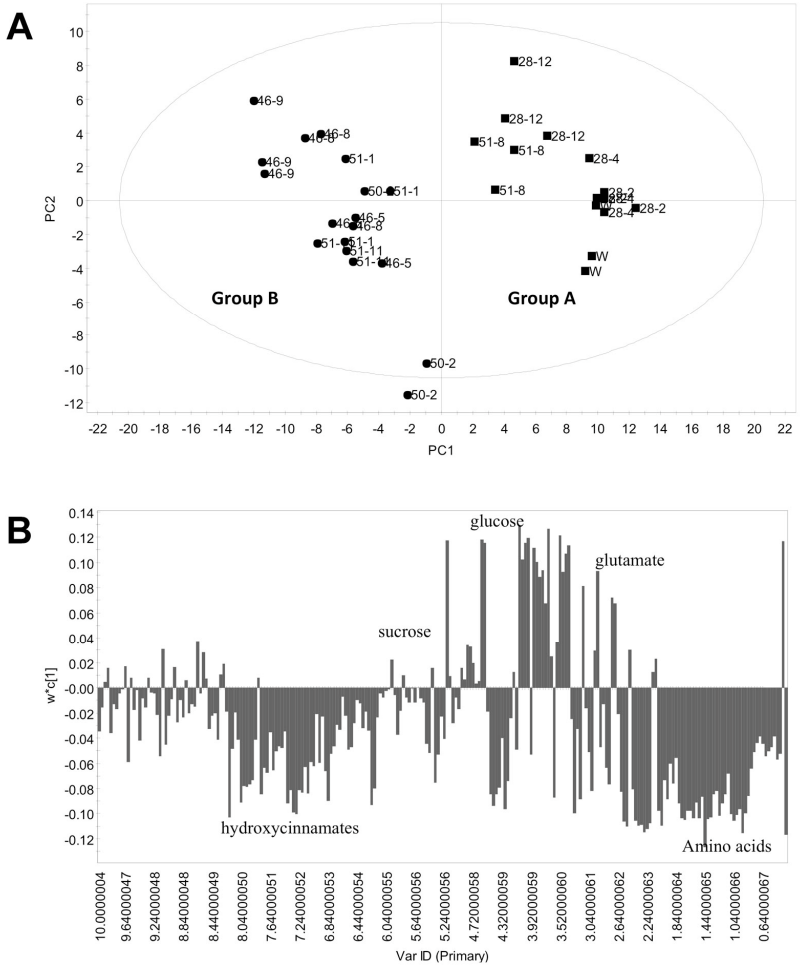

Figure 4. Partial least square-discriminant analysis of overexpressors of WRKY28, WRKY46, WRKY50 and WRKY51. The score plot (a) and loading plot (b) of PC1. Only two groups, group A (green) and group B (blue) have been analyzed for PLS-DA. Validation has been tested using permutation methods.

Sinapic acid is a common hydroxycinammate found in many plants, including Arabidopsis and Brassica, and is mostly present in a conjugated form. In the leaves, sinapoyl malate is the dominant form and sinapoyl glucose and sinapoyl choline mostly occur in the seeds of Arabidopsis (Chappel et al., 1992). Sinapoyl glucose is synthesized from sinapate by UDP-glucose:sinapic acid glycosyltransferase (Wolfram et al., 2010) and it is known to function in protection against UV radiation (Landry et al., 1995; Sheahan, 1996).

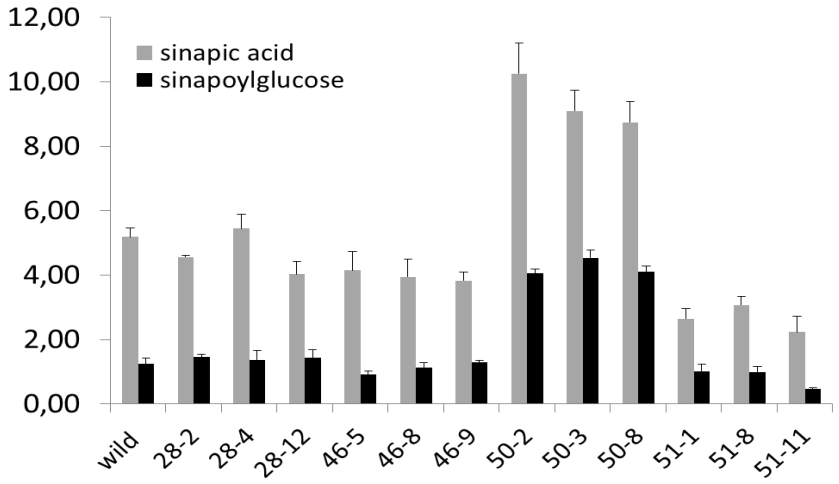

Figure 5. Quantitative analysis of sinapic acid and sinapoyl glucose in Arabidopsis plants. Peak intensities of the signals of sinapic acid (δ 7.00) and sinapoyl glucose (δ 7.02) were expressed relative to the peak area of the internal standard TSP. Mean value of each peak intensity was shown with standard deviation.

Moreover, derivatives of the related compounds sinapic acid, coumaric acid and ferulic acid are constituents of the cell wall strengthening phenolic polymer lignin, emphasizing the importance of these hydroxycinnamates for plant defense. Indeed, plants are able to adapt lignin structure to remedy particular types of stress by regulating expression of genes encoding enzymes involved in hydroxycinnamate biosynthesis. An example is the gene for ferulate-5-hydroxylase (F5H), the enzyme involved in the conversion of ferulate to

sinapate, which is regulated by biotic and abiotic types of stress (Kim et al., 2006; Hruz et al., 2008). Although WRKY transcription factors have been suggested to be especially involved in defense, a function of WRKYs in the biosynthesis of hydroxycinnamates has not been reported thus far. In this context it is interesting to note that the promoter of the Arabidopsis *F5H* gene contains the sequence GACTTTTC that we identified as an AtWRKY50 binding site in the *PR-1* promoter.

In conclusion, our results show that WRKY transcription factors effect secondary metabolite production, such as sinapic acid and sinapoyl glucose in Arabidopsis. However, whether regulation takes place at the level of the genes encoding enzymes of the biosynthesis pathway or at higher levels of signal transduction is not clear and requires further study.

MATERIALS AND METHODS

Plant vectors and transformation

For the construction of transgenic lines constitutively overexpressing WRKYs, the Cauliflower mosaic virus (*CaMV*) *35S* cassette containing the *WRKY's* ORF in sense orientation was obtained from pRT101 and cloned in pCAMBIA1300 (acc. no. Af234296). Binary vectors pCAMBIA1300-*WRKY* was introduced into *Agrobacterium tumefaciens*. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on solid MA medium containing 100 mg/L timentin and 20 mg/L hygromycin. Transgenic plants from T2 generations were selected on MA medium containing only 20 mg/L hygromycin.

Growth of plant materials

Arabidopsis thaliana ecotype Columbia (Col-0) seeds were surface sterilized by incubation for 1 min in 70% ethanol, 15 min in 50% bleach, and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chorine gas for 3-4 hours. Surface-sterilized seeds were sown on plates containing MA (Masson and Paszkowski, 1992) medium supplemented with 0.6% plant agar. Following stratification for 3 days at 4^0C, seeds were incubated at 21°C in a growth chamber (16 h light/8 h dark, 2500 lux) for 10-12 days. Immediately after harvesting, the material was frozen in liquid nitrogen and kept at -80^0C until use.

RNA extraction and Northern blot analyses

Total RNA was extracted from the 10-12 days old seedlings that were frozen in liquid nitrogen by phenol/chloroform extraction followed by overnight precipitation with 8M lithium chloride, washed with 70% ethanol, and re-suspended in water. For RNA blot analysis 10µg RNA samples were subjected to electrophoresis in 1.5% agrose/1% formaldehyde gels and blotted to Gene Screen nylon membranes (Perkin-Elmer Life Sciences). All probes were ^{32}P-labelled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed to X-ray film (Fuji, Tokyo). DNA fragments used as probes were PCR-amplified from sets of primers shown in Table 1.

Sample extraction for NMR analysis

Twenty mg of freeze-dried material were transferred to a microtube (2 ml) to which 1.5 ml of 50% methanol-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.05% TSP (trimethyl silyl propionic acid sodium salt, w/v) was added. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min,

and centrifuged at 13,000 rpm at room temperature for 5 min. Eight hundred μL of the supernatant was transferred to a 5 mm NMR tube.

Table 1.

AtWRKY28 (At4g18170)	F	GTCACTCGAGATGTCTAATGAAACCAGAGATCTCTAC
	R	CAGTGGATCCTCAAGGCTCTTGCTTAAAGAAAATTG
AtWRKY46 (A2g46400)	F	GTCACTCGAGATGATGATGGAAGAGAAACTTGTG
	R	CAGTTCTAGACTACGACCACAACCAATCCTGTC
AtWRKY50 (At5g26170)	F	ATAGCTCGAGGTATGAATGATGCAGACACAAACTTG
	R	GCCTCTAGACGAGTCTTAGTTCATGCTTGAGTGATTGTG
AtWRKY51 (At5g64810)	F	AAACGAATTCAAATGAATATCTCTCAAAACCCTAGCC
	R	GATGAGGTACCTGGATTAAGATCGAAGAAGGTGTTG
Actin2 (At3g18780)	F	CTGTGCCAATCTACGAGGGTT
	R	GGAAACCTCAAAGACCAGCTC
ROC1 (At4g38740)	F	CGGGAAGGATCGTGATGGA
	R	CCAACCTTCTCGATGGCCT

NMR measurements

^{1}H NMR spectra were recorded at 25^{o}C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH-d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30^o (11.3 μsec), and relaxation delay (RD) = 1.5 sec. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker). 2D J-resolved NMR spectroscopy, ^{1}H-^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). 2D J-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of

5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin-spin coupling constant axis). A 1.5 sec relaxation delay was employed, giving a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. J-resolved spectra tilted by 45^{o}, was symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). The COSY spectra were acquired with 1.0 sec relaxation delay, 6361 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 sec relaxation delay, 6361 Hz spectral width in F2 and 27 164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectrum except for 30183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

NMR Data Analysis

The ^{1}HNMR spectra were automatically reduced to ASCII file. Spectral intensities were scaled to the total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.4 – δ 10.0. The region of δ 4.75 – δ 4.90 and δ 3.28 – δ 3.34 was excluded from the analysis because of the residual signal of HDO and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Principal component analysis (PCA) was performed with the SIMCA-P software (v. 12.0, Umetrics, Umea, Sweden) with scaling based on Pareto method. PLS-DA was performed same way except using UV scaling method.

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