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Target genes for OBP3, a Dof transcription factor, include novel basic helix-loop-helix domain proteins inducible by salicylic acid

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Summary

Overexpression of a salicylic-acid (SA)-inducible *Arabidopsis* DNA binding with one finger (Dof) transcription factor, called OBF-binding protein 3 (OBP3; AtDof3.6), has previously been shown to result in growth defects. In this study, suppressive subtraction hybridization (SSH) was used to isolate genes induced in an OBP3-overexpression line and several putative clones, called OBP3-responsive genes (*ORGs*), were isolated. The link with the induced expression levels of these genes and OBP3 overexpression was confirmed by analysing additional OBP3-overexpression lines. *ORG1* through *ORG4* are novel genes, while *ORG5* is an extensin gene, *AtExt1*. While ORG4 has no similarity with other proteins in the database, ORG1 has weak similarity in different regions of the predicted protein with CDC2 and fibrillin. ORG2 and ORG3 share 80% overall identity in their deduced amino acid sequences and contain a basic helix-loop-helix DNA-binding domain, suggesting that ORG2 and ORG3 may be transcription factors. The expression of the *ORG1, ORG2* and *ORG3* genes was co-regulated under all conditions examined including upregulation by SA and down-regulation by jasmonic acid (JA). Fifteen *OBP3*-silenced lines were generated to further explore the function of OBP3. Although there were no visible phenotypic changes in any of these lines, the expression of *ORG1*, *ORG2* and *ORG3* was reduced. Among the ORG genes, *ORG1*, *ORG2* and *ORG3* contained the highest number of potential Dof-binding sites in the promoter region, and their expression was significantly increased within 3 h after induction of OBP3 expression using an inducible promoter system, and closely reflected the expression levels of the exogenous OBP3 protein. The results from the gain-of-function and loss-of-function experiments suggest that the *ORG1*, *ORG2* and *ORG3* genes are direct target genes of OBP3.

Keywords: Dof, basic helix-loop-helix, salicylic acid, jasmonic acid, AtDof3.6.

Introduction

Although there is considerable similarity in the regulation of transcription between animals and plants, there is growing evidence for transcription factor families that are unique to a particular kingdom. One class of transcription factors unique to plants is a novel type of zinc finger protein called DNA binding with one finger (Dof) proteins. Dof proteins contain a highly conserved 52-amino acid region called the Dof domain, which contains a single $CX_2CX_{21}CX_2C$-type zinc finger motif (reviewed in Yanagisawa, 2002). The zinc finger in the Dof domain is essential for DNA binding as specific mutations in cysteine residues within the zinc finger abolish DNA binding as do zinc chelaters (see Yanagisawa, 2002 and references within). Binding sites for Dof proteins typically contain (A/T)AAAG or its complementary sequence (Yanagisawa and Schmidt, 1999). Some Dof proteins have also been shown to interact specifically with other types of transcription factors including bZIP proteins (Kang and Singh, 2000; Vincente-Carbajosa *et al.*, 1997; Zhang *et al.*, 1995), HMG1 (Yanagisawa, 1997), Myb proteins (Diaz *et al.*, 2002) and other Dof proteins (Yanagisawa, 1997).

Dof proteins have been isolated in a number of plant species by screens for DNA-binding proteins to specific promoter elements, by protein–protein interaction screening or by sequence homology (see Yanagisawa, 2002 and references within). As Dof proteins have only been found in plants, it has been speculated that they may play plant-specific roles. To date, Dof proteins have been implicated in seed-specific gene expression and seed germination (Diaz *et al.*, 2002; Gualberti *et al.*, 2002; Isabel-LaMoneda *et al.*, 2003; Mena *et al.*, 1998, 2002; Papi *et al.*, 2002; Vicente-Carbajosa *et al.*, 1997; Washio, 2001), the expression of genes involved in carbon metabolism (Yanagisawa, 2000; Yanagisawa and Sheen, 1998), response to plant hormones (Baumann *et al.*, 1999; De Paolis *et al.*, 1996), guard cell-specific gene expression (Plesch *et al.*, 2001) and plant defence/stress gene expression (Chen *et al.*, 1996; Kang and Singh, 2000). In some cases, closely related Dof proteins have been shown to have quite opposite functions. For example, the *Arabidopsis* DAG1 and DAG2 proteins play opposite regulatory roles in committing the seed to germinate (Gualberti *et al.*, 2002).

We have focused on a subgroup of the 37 *Arabidopsis* Dof proteins called OBF-binding proteins (OBPs; Kang and Singh, 2000; Zhang *et al.*, 1995). The first OBP protein (OBP1) was isolated as a protein that interacts with an ocs-element-binding factor, OBF4 (Zhang *et al.*, 1995). ocs elements are found in both pathogen and plant promoters, where they function as stress-responsive elements (see Singh *et al.*, 2002 and references within). While the OBP proteins showed similar *in vitro* DNA binding, protein–protein interaction and transcription-activation properties, they had distinct RNA-expression patterns with some family members showing a strong correlation with the activity of the ocs element (Kang and Singh, 2000). A range of phenotypes was observed in plants overexpressing OBP3, also called AtDof3.6, based on its chromosomal position (Yanagisawa, 2002). These phenotypes included retarded growth in both roots and aerial parts of the plant that, in the most severe cases, led to death (Kang and Singh, 2000).

To gain insight into the function of OBP3, we isolated genes induced in an OBP3-overexpression background using suppressive subtraction hybridization (SSH; Diatchenko *et al.*, 1996). Given that OBP3 is a DNA-binding protein with transcriptional activation activity, we reasoned that the phenotypes observed from OBP3-overexpression lines might result from constitutively expressing OBP3 target genes. Using SSH, five OBP3-responsive genes (*ORGs*) were isolated. Expression of three of these genes – *ORG1,* which encodes a protein of unknown function, and *ORG2* and *ORG3*, which encode closely related basic helix-loop-helix domain (bHLH) proteins – was co-regulated under all conditions examined, including upregulation by salicylic acid (SA) and downregulation by jasmonic acid (JA). To initiate loss-of-function experiments with OBP3, we generated fifteen *OBP3*-silenced lines using intron-spliced hairpin (ihp) RNA. The *OBP3*-silenced lines had no visible phenotypic changes in contrast to what was seen with the *OBP3*-overexpression lines. However, the expression of the *ORG1, ORG2* and *ORG3* genes was reduced in the *OBP3* silenced lines. *ORG1, ORG2* and *ORG3* expression was also significantly increased within 3 h after induction of OBP3 expression using an inducible promoter system, and closely reflected the expression levels of the exogenous OBP3 protein. Our results strongly suggest that the *ORG1, ORG2* and *ORG3* genes are direct target genes for OBP3.

Results

Screening for OBP3-responsive genes using suppressive subtraction hybridization

In an effort to identify induced genes in the OBP3-overexpressing plants, we used SSH. SSH was chosen for the following reasons: (i) SSH does not require very much plant tissue. This is important because the OBP3-overexpression lines are small in size and are difficult to propagate because of fertility problems. (ii) Low-abundance transcripts can be isolated with SSH. Total RNA from an OBP3-overexpression line and a wild-type line was isolated, and a cDNA library enriched for genes upregulated in an OBP3-overexpressing line was prepared. Differential screening of this library, which consisted of 198 clones with subtracted probes representing the OBP3-overexpressing line or the wild-type line, revealed a number of genes whose expression was enhanced in the OBP3 line. We initially focused on eight clones whose expression appeared to be upregulated the strongest in the OBP3 line. As shown in Table 1,

Table 1 Isolation of OBP3-responsive genes (ORGs)

Genes	Frequency	Function	Gene locus	OBP-binding motifs (%)
ORG1	3	Unknown	At5g53450	9 (231)
ORG2	2	bHLH protein	At3g56970	10 (256)
ORG3	1	bHLH protein	At3g56980	10 (256)
ORG4	1	Unknown	At2g06010	7 (179)
ORG5	1	Extensin	At1g76930	5 (128)

This Table includes the frequency with which independent clones were isolated through suppressive subtraction hybridization (SSH), putative function of genes, gene locus, and number of OBP-binding motifs (CTTT (A/T) and (A/T)AAAG; Yanagisawa and Schmidt, 1999) located within 1-kb upstream of cDNA start site. Percentages in parentheses compare the number of OBP-binding motifs in the *ORG* promoter regions with the number of sites that is statistically expected from a 1-kb random sequence (less than four OBP-binding sites).

these clones were derived from five independent genes (*ORG1–ORG5*).

OBP3-responsive gene expression correlates with the level of OBP3 expression in OBP3-overexpression lines under constitutive or inducible promoters

The expression characteristics of the five genes were initially analysed using a virtual Northern blot analysis, and all the clones showed enhanced expression in the OBP3-overexpression background. The *ORG* gene induction by OBP3 was tested in three additional OBP3-overexpression lines – a strongly overexpressing OBP3 line (#1) similar to the original line used for the SSH screen (#5), an intermediate OBP3 line (#7) and a weakly overexpressing OBP3 line (#3) – using RT-PCR, as shown in Figure 1(a). All the *ORG* genes were induced to different degrees in the OBP3-overexpression lines that had high or intermediate levels of *OBP3* transgene expression, with *ORG2* and *ORG4* showing the largest and the smallest induction, respectively. In contrast, in line #3 in which *OBP3* RNA levels were similar to that in the wild type, and where there were no significant effects on plant growth and development, there was also no change in *ORG* gene expression.

The OBP3-overexpression lines used in Figure 1(a) employed the constitutive cauliflower mosaic virus (CaMV) 35S promoter to express OBP3. We also checked the expression pattern of the ORG genes following an increase in OBP3 expression from a glucocorticoid-inducible promoter system (Aoyama and Chua, 1997; Kang *et al.*, 1999). The system consists of two components: a glucocorticoid-regulated chimeric transcription factor with the DNA-binding domain of the yeast transcription factor GAL4 called GVG, and the gene of interest transcribed from a promoter containing GAL4 DNA-binding sites. Transgenic plants containing the system only express the gene of interest following glucocorticoid treatment, as GVG is only activated upon binding glucocorticoid (for further details, see Aoyama and Chua, 1997). In Figure 1(b), expression of the *ORG* and *OBP* genes were analysed 6 h after the expression of OBP3 had been induced following treatment with dexamethasone (Dex), a chemical inducer for this system. We also examined *ORG* expression in a control line that

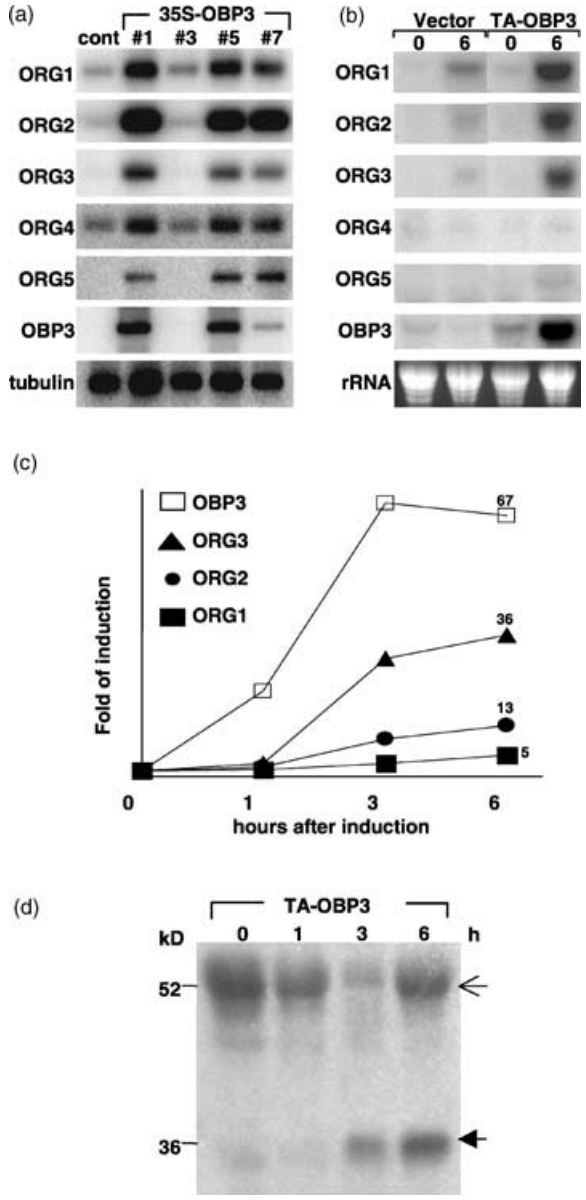

Figure 1. Analysis of OBP3-responsive gene (ORG) and OBF-binding protein 3 (OBP3) expression in OBP3-overexpression lines.
(a) RNA was isolated from four OBP3 transgenic lines under the control of the CaMV 35S promoter and control plants (cont) at the 12 to 16-day-old stage. Seedlings were grown on MS solid medium with kanamycin for 12 through 16 days. RT-PCR was performed using primers specific for each *ORG* gene. In the case of OBP3 (exo), only the *OBP3* transgene was amplified. β-tubulin was used as a control for equal loading. The lane labelled cont is an unrelated kanamycin-resistant line that was used as a wild-type control for this experiment.
(b) RNA was isolated from a dexamethasone (Dex)-inducible line for OBP3 with/without induction. The line was grown for 3 weeks and then spayed with 30 μM of Dex. RNA was isolated from the lines before the induction (0) and 6 h after the induction (6). 25 μg of RNA was used for Northern blot analysis, and rRNA stained with ethidium bromide was shown as a control for equal loading. In the case of *OBP3*, the *OBP3* transgene was shorter than the endogenous *OBP3* gene in size, resulting in two slightly different-sized bands.
(c) RT-PCR analysis with RNA isolated from a 3-week-old Dex-inducible line for OBP3 (TA-OBP3-#48) at 1, 3 and 6 h after treatment with 30 μM of Dex. Expression levels were normalized to β-tubulin and the fold induction was graphed with 0 h serving as a reference point for each time point. The fold of induction at 6 h is shown next to each symbol.
(d) Immunoblot analysis with the same samples as used in (c). Monoclonal anti-hemagglutinin (HA) antibody was used to detect the exogenous OBP3 protein, which was tagged with an HA epitope (thick arrow). Cross-reacting Rubisco protein was used as a loading control (thin arrow). Approximately 20 μg of protein was loaded into 12% SDS–PAGE, except for the 3-h time point which was underloaded. Size markers are shown on the left of the panel in kilodaltons.

contained only the empty vector. *ORG1, ORG2* and *ORG3* showed a large increase in expression following induction of *OBP3*, whereas *ORG4* and *ORG5* had little to no change in expression. In the empty vector line, we also observed a small induction in *ORG1*, *ORG2* and *ORG3* expression, although the level of induction was considerably less than that observed in the OBP3-inducible line.

To determine if OBP3 expression was enhanced before the increase in *ORG* expression, we examined earlier time points following Dex treatment. As shown in Figure 1(c), *OBP3* RNA levels had already increased significantly by 1 h, continued to increase at 3 h and appeared to have peaked by 6 h. In contrast, there was no change in *ORG* expression at 1 h, with an increase in expression of the three *ORG* genes first being observed at 3 h. ORG expression increased further at 6 h, although the induction appeared to be levelling off. The OBP3 construct had been tagged with a hemagglutinin (HA) epitope and so changes in the levels of the exogenous OBP3 protein following Dex treatment could be analysed. As shown in Figure 1(d), the increase in OBP3 protein levels lagged a little behind the increase in RNA levels as expected, with little change at 1 h, but a significant increase by 3 h, when *ORG* RNA levels were first observed to have increased. Thus, the increase in expression of the *ORG1*, *ORG2* and *ORG3* genes follows soon after and parallels the increase in expression of OBP3, strongly suggesting that they are direct targets of OBP3.

The promoter regions of the *ORG* genes extending 1 kb from the cDNA start site were examined to see if they contain potential Dof-binding sites. As shown in Table 1, all five *ORG* genes contain multiple Dof-binding sites in this region, ranging from 10 for *ORG2* and *ORG3* to 5 for *ORG5*. The frequency of these Dof-binding sites was higher than the number that is statistically expected from a 1-kb random sequence, which would be less than 4. Interestingly, the three *ORG* genes (*1*, *2* and *3*) that were significantly induced within 6 h when OBP3 was expressed off an inducible promoter, also contained the highest number of potential Dof-binding sites in the promoter region.

Classification of the OBP3-responsive genes

One of the *ORG* genes had been previously characterized. *ORG5* is *AtExt1*, an extensin gene that codes for a class of hydroxyproline-rich glycoproteins that are abundant in plant cell walls (Merkouropoulos *et al.*, 1999). *ORG1–ORG4* are novel genes. While *ORG1, ORG2* and *ORG3* share some similarity with known genes, *ORG4* does not have any similarity with genes in the database. The *ORG1* gene comprises 13 exons and 12 introns and codes for a predicted protein of 670 amino acids. The ORG1 protein appears to be novel, although database searches revealed weak similarity in different regions of the predicted ORG1 protein with fibrillin proteins and some cdc2 proteins.

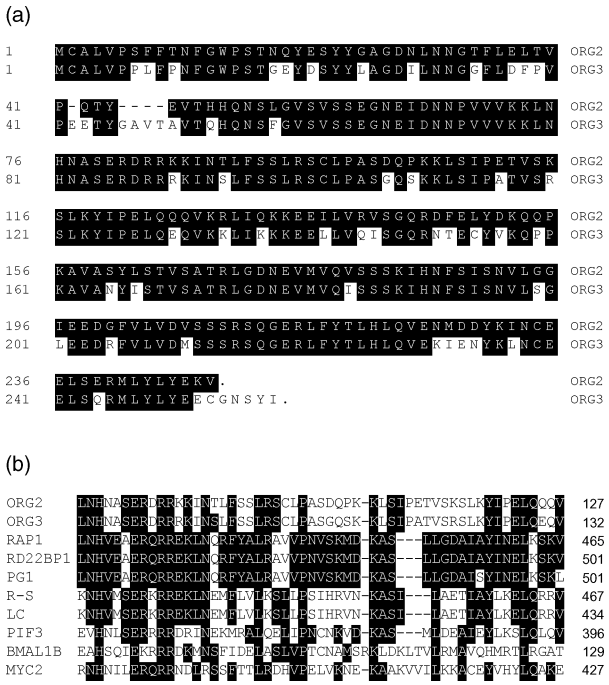

Figure 2. OBP3-responsive gene 2 (ORG2) and ORG3 are basic helix-loop-helix (bHLH) proteins.
(a) Alignment of predicted ORG2 and ORG3 full-length amino acid sequences.
(b) Alignment of the bHLH domain in ORG2 and ORG3 with bHLH proteins from different organisms ranging from plants to mammals. Accession numbers and origin species in parentheses for each gene are: RAP1 (*Arabidopsis*), X99548; RD22BP1 (*Arabidopsis*), AB000875; PG1 (bean), U18348; R-S (maize), P13027; Lc (Maize), X57276; PIF3 (*Arabidopsis*), AAC95156; bMAL1B (human), AB000812; MYC2 (woodchuck), S11511. For both (a) and (b), identical amino acids are shown as white text on black box.

ORG2 and ORG3 were closely related to each other as shown in Figure 2(a), and have 79.8% overall identity in their deduced amino acid sequences. Both proteins contain a bHLH domain, which is known to be a DNA-binding domain present in plant and animal transcription factors. The alignment of the bHLH domains with other bHLH proteins from different organisms is shown in Figure 2(b). The deduced amino acid sequence of the bHLH domain in the *ORG2* and *ORG3* genes are diverged from other plant Myc proteins. In particular, three amino acids were inserted in the second helix region, which resembles animal Myc proteins more (compare BMAL1B and MYC2 with ORG2 and ORG3 in Figure 2b).

Salicylic and jasmonic acid responsiveness of the OBP3-responsive genes

As *OBP3* is an SA-responsive gene (Kang and Singh, 2000), we tested whether the novel *ORG* genes can also be induced by SA treatment. The SA-responsiveness of the *ORG* genes was analysed in aerial parts and roots

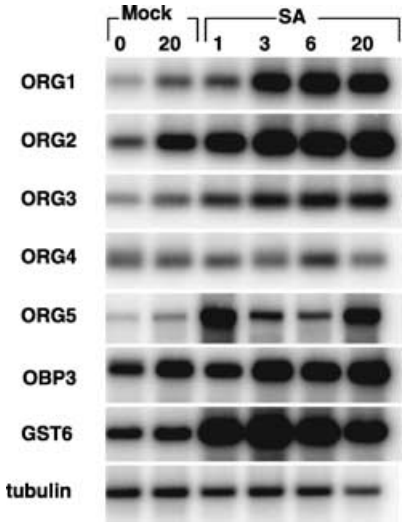

Figure 3. Analysis of OBP3-responsive gene (*ORG*) RNA levels following treatment with salicylic acid (SA).
Eight-day-old seedlings were transferred to fresh medium (mock) or medium that contained SA (100 μM) for the indicated time in hours. RNA was then isolated from the roots and analysed using RT-PCR and primers specific for each gene. *GST6* also known as *GSTF8*, which is induced in response to SA, and β-tubulin were used as controls.

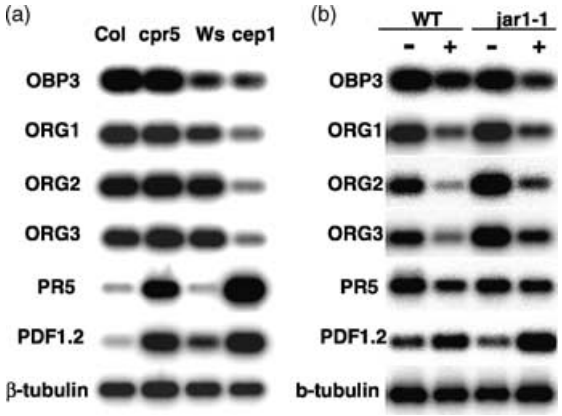

Figure 4. Analysis of *OBP3, ORG1, ORG2* and *ORG3* RNA levels in mutants with altered responses to defence-signaling molecules and/or pathogens.
(a) RT-PCR analysis of RNA samples from 2-week-old *Arabidopsis* seedlings of the Columbia (Col) or Wassilewskija (Ws) ecotype and *cpr5-2* (Bowling *et al.*, 1997) and *cep* (Silva *et al.*, 1999) mutants. *PR5* and *PDF1.2*, which are upregulated by salicylic acid (SA; Ward *et al.*, 1991) and methyl jasmonate (MeJA; Penninckx *et al.*, 1998), respectively, were used as controls.
(b) RT-PCR analysis of RNA samples from 2-week-old *Arabidopsis* seedlings of the Columbia (WT) ecotype and the *jar1-1* mutant (Staswick *et al.*, 1998) treated for 6 h with 0.1% (v/v) ethanol (−) or 100 μM MeJA solution in 0.1% (v/v) ethanol (+).

separately. As shown in Figure 3, *ORG1*, *ORG2* and *ORG3*, like *OBP3*, were SA-inducible in roots, while *ORG4* did not respond to SA within the time period tested. None of the *ORG* genes showed significant SA responses in aerial tissue (data not shown), similar to what was seen with *OBP3* (Kang and Singh, 2000).

The RNA expression patterns of *OBP3* and the SA-inducible *ORG* genes were analysed in two different *Arabidopsis* mutants, *cpr5-2* (Bowling *et al.*, 1997) and *cep1* (Silva *et al.*, 1999), which constitutively express plant-defence responses. The *CPR5* gene encodes a novel, putative transmembrane protein (Kirik *et al.*, 2002), and *cpr5-2* plants have elevated levels of SA, enhanced levels of *PR-1*, increased *PDF1.2* gene expression and enhanced resistance to virulent strains of *Pseudomonas syringae* and *Peronospora parasitica* (Bowling *et al.*, 1997). The *cep1* mutant has higher levels of SA as well as higher levels of *PR* and *PDF1.2* gene expression (Silva *et al.*, 1999; Oñate-Sánchez and Singh, 2002). As shown in Figure 4(a), there was no difference in the expression levels of *OBP3* or *ORG1, ORG2* and *ORG3* between the wild type and the *cpr5-2* mutant, while there was a clear induction of *PR5* and *PDF1.2* expression, which are SA- and JA-responsive genes, respectively. While there was again no difference in the expression of *OBP3* between the wild-type plants and the *cep1* mutant, there was a significant reduction in *ORG1, ORG2* and *ORG3* expression in this mutant background.

As the high levels of *PR5* and *PDF1.2* expression in the *cpr5-2* and *cep1* mutants suggest activated SA- and JA-signalling pathways, we analysed the expression of *OBP3,* and *ORG1, ORG2* and *ORG3* after treatment with MeJA in a JA-resistant mutant, *jar1-1* (Staswick *et al.*, 1998). JAR1 appears to belong to the acyl adenylate-forming firefly luciferase superfamily (Staswick *et al.*, 2002) and *jar1-1* plants have decreased sensitivity to JA inhibition of root elongation and are more susceptible to the soil fungus *Pythium irregulare* (Staswick *et al.*, 1992, 1998). As shown in Figure 4(b), the expression of both *OBP3* and the *ORG1, ORG2* and *ORG3* genes were repressed in response to MeJA in the wild type and the *jar1-1* mutant. *PDF1.2* gene expression was used as a control for the MeJA treatment, and its expression was induced both in the wild type and in the *jar1-1* mutant as seen previously by Oñate-Sánchez and Singh (2002).

ORG1 to ORG3 RNA levels are decreased in OBP3-silenced lines

We initiated loss-of-function experiments using gene constructs encoding ihpRNA (Wesley *et al.*, 2001) against OBP3 to further analyse the function of OBP3 and the role it plays in regulating the expression of the *ORG1, ORG2* and *ORG3* genes. The ihpOBP3 construct contained 525 bp of *OBP3*-specific sequence as determined by BLAST search against the *Arabidopsis* genome. Fifteen independent lines were generated, and none of these lines had any visible phenotype. Seven of these lines contained single T-DNA-insertion sites as determined by segregation analysis, and we focused on these lines. T_3 lines were further characterized

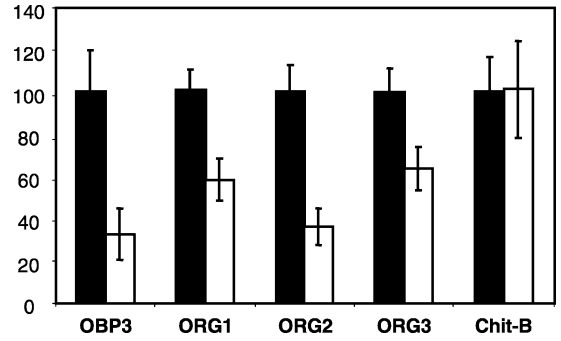

Figure 5. OBP3-responsive gene 1 (*ORG1*), *ORG2* and *ORG3* RNA levels are reduced in OBP3-silenced lines.
RNA was isolated from seedlings from seven independent lines and five wild-type (Columbia) replicates grown on MS solid medium for 8 days. RT-PCR was performed using primers specific for *OBP3, ORG1, ORG2, ORG3, OBP3* and *chitinase*, and normalized to β-tubulin. The relative average with wild type set at 100% and standard error of the five wild-type replicates (black columns) and seven independent hpOBP3 lines (white columns) are graphed.

by RT-PCR. As shown in Figure 5, the ihpOBP3 lines contained on average approximately 30% OBP3 RNA compared to the wild type. These ihpOBP3 lines also had reduced levels of *ORG1, ORG2* and *ORG3*. *Chitinase B* was used as a control to show that the reduction in *ORG1, ORG2* and *ORG3* expression was a specific effect and not a general stress response. These results are consistent with the results obtained with the OBP3-overexpressing lines under constitutive or inducible promoters, and show that the expression of O*RG1*, *ORG2* and *ORG3* is strongly correlated with OBP3 expression.

Discussion

To gain insight into the function of OBP3 *in planta*, we screened for genes that are induced in an OBP3-overexpression line using SSH, a cDNA subtraction method. One of the *ORG* genes, *ORG4*, encodes for an unknown protein, while another, *ORG5/AtExt1*, is an *Arabidopsis* extensin gene (Merkouropoulus *et al.*, 1999). Extensins are hydroxyproline-rich glycoproteins that are very abundant in plant cell walls where they may function to increase the mechanical strength of the cell wall (see Bucher *et al.*, 2002; Kieliszewski and Lamport, 1994). Extensins may also be involved in plant-defence responses as pathogen infection and treatment with elicitors has been shown to induce their expression (Benhamou *et al.*, 1990; Corbin *et al.*, 1987; Mazau and Esquerre-Tugaye, 1986; Showalter *et al.*, 1985). *ORG5/AtExt1* was shown previously to be induced by SA, wounding and other defence-related compounds such as MeJA (Merkouropoulus *et al.*, 1999).

The other three *ORG* genes encode proteins with similarity to other proteins in the database. *ORG1* has weak similarity in different regions of the protein to fibrillin proteins, which are major components of the extracellular microfibrils, and some cdc2 proteins, which are involved in cell-cycle control. However, no clones with similarity to both cdc2 and fibrillin genes were present in the database, indicating that *ORG1* may represent a novel class of genes. Although the region of highest similarity between ORG1 (230–302 amino acids) and the rice CDC2 protein (102–174 amino acids) scores 49% similarity with 30% amino acids being identical, ORG1 lacks the signature motifs of the cdc2 protein family: the PSTAIR motif (EGVP-STAIREISLLKE; 42–57 amino acids in the yeast CDC2), which binds to a cyclin, and the GEGTYG motif (GEGTYG: 11–16 amino acids in the yeast CDC2), which is in the amino-terminal ATP-binding domain. Therefore, it is not clear whether ORG1 has a cell cycle regulatory function merely based on the amino acid similarity, and further work will be required to address this question.

ORG2 and *ORG3* both encode proteins that contain a bHLH domain, share approximately 80% overall identity, are located next to each other in the genome and are responsive to SA with similar kinetics to *OBP3*. Myc proteins with a bHLH domain have been shown to be potent inducers of both cell proliferation and apoptosis in animals (see Eisenman, 2001 and Pelengaris *et al.*, 2000 for reviews). Plant Myc homologs appear to function in a number of different areas. For example, in maize, the Myc proteins encoded by the *R*-gene family including the R, Lc, B and Sn proteins regulate the anthocyanin biosynthesis pathway. Another plant Myc-like protein is the *Arabidopsis* RD22BP1 protein, which is induced by ABA, a stress signal in plants, and by dehydration stress (Abe *et al.*, 1997). It is noteworthy that the deduced amino acid sequence of the bHLH domain in ORG2 and ORG3 are diverged from other plant Myc-like proteins, suggesting that the ORG2 and ORG3 DNA-binding profiles and function may be different from these proteins.

The expression patterns of the *ORG1*, *ORG2* and *ORG3* genes were very similar in all the situations examined. All three genes were coordinately induced in an OBP3-inducible line within 3 h following treatment with the chemical inducer Dex, although the absolute levels of induction differed. The increase in *ORG1*, *ORG2* and *ORG3* expression occurred shortly after *OBP3* RNA levels started to increase, and closely reflected the expression levels of the exogenous OBP3 protein. The *ORG1*, *ORG2* and *ORG3* genes also showed a small induction in an empty vector line following treatment with Dex, although the levels of induction were small compared to the OBP3-inducible line. The expression of some stress-responsive genes (*PDF1.2* and *PR-5*) has also been shown to be induced in empty vector lines following Dex treatment, although in these cases, induction was observed later, 24 or 48 h after Dex treatment (Kang *et al.*, 1999).

The *ORG1*, *ORG2* and *ORG3* genes were also inducible by SA and downregulated by JA. The interaction between the

SA and JA signalling pathways is known to be complex with both antagonistic and synergistic interactions reported (reviewed in Glazebrook, 2001), for example, a SA-responsive *PR1* gene was repressed by JA (Rao *et al.*, 2000). In another study, 8 out of 2300 genes were found to be induced by SA and repressed by JA using microarray hybridizations (Schenk *et al.*, 2000). However, antagonistic response to these hormones is clearly not universal, as Schenk *et al.* (2000) also found that a significantly higher number of genes were co-induced or co-repressed by SA and JA. Interestingly, the expression of the *ORG1*, *ORG2* and *ORG3* genes was not affected in the *cpr5-2* mutant and even reduced in the *cep1* mutant, although both mutants are known to have elevated SA levels (Bowling *et al.*, 1997; Silva *et al.*, 1999).

OBP3 was also often expressed in a similar manner to the *ORG1, ORG2* and *ORG3* genes. One exception was in *cep1*, where the expression of *OBP3* was not affected, while the expression of the *ORG1, ORG2* and *ORG3* genes was significantly reduced. Moreover, while the induction of *OBP3* by SA is not reduced by cycloheximide (Kang and Singh, 2000), this is not the case with *ORG1* (our unpublished observation). These results demonstrate that *OBP3* is not co-regulated with the *ORG1, ORG2* and *ORG3* genes and are consistent with *OBP3* being upstream of *ORG* regulation, and that other unknown factors also influence *ORG*-expression levels.

The transgenic lines overexpressing OBP3 under the control of the CaMV 35S promoter had a range of phenotypes, the severity of which correlated with the levels of OBP3 expression (Kang and Singh, 2000). These phenotypes included growth defects involving retarded growth in both roots and aerial parts of the plant. In the most severe cases, these growth defects led to death. At this stage, it remains unclear whether one or more of the ORGs isolated in this study contributed to any of these phenotypes. *ORG1* and *ORG4* encode proteins of unknown function, and *ORG2* and *ORG3* encode potential transcription factors but where the identity of target genes is unknown. In the case of *ORG5*, overexpression or antisense repression of other extensin genes did not give rise to any visible phenotypes (Bucher *et al.*, 2002; Memelink *et al.*, 1993).

In contrast to the *OBP3*-overexpressing lines, the *OBP3*-silenced lines had no obvious visible phenotypes, although they did lead to specific changes in gene expression. One possibility is that other proteins such as OBP1 and/or OBP2, which are also responsive to SA and have similar DNA-binding and protein–protein interaction properties as OBP3 (Kang and Singh, 2000), may be able to compensate for the reduction in OBP3 expression. It will be interesting to examine if the *ORG* genes are affected in other *OBP*-mutant backgrounds. Another possibility is that complete silencing of OBP3 was not achieved with the ihpRNA approach (Wesley *et al.*, 2001). Although this silencing approach was shown to result in well over 80% suppression of target genes tested (Wesley *et al.*, 2001), we have failed to obtain similarly efficient silencing lines not only for OBP3, but also for other transcription factors including TGA2 (Kang *et al.*, unpublished results). The difficulty to get efficient silencing lines for OBP3 may be as a result of lethality caused from the loss of OBP3.

The promoter regions of the *ORG* genes were examined, and were found to contain a higher frequency of potential Dof-binding sites than statistically expected for a random sequence of the same size. For example, the *ORG2* and *ORG3* promoter regions each had ten Dof-binding sites and the *ORG1* promoter region had nine sites, whereas a random sequence would be predicted to have less than four. Other gene promoters have also been shown to contain multiple Dof-binding sites, including the maize *cyppdk1* promoter (Yanagisawa, 2000), the *Arabidopsis GST6* promoter (Chen *et al.*, 1996) and the potato *KST1* promoter (Plesch *et al.*, 2001). With whole-genome sequences available for some model systems, it was proposed that genes regulated by a transcription factor could be identified by a cluster of the corresponding binding sites (Wagner, 1999). Computational methods looking for a cluster of transcription-factor-binding sites more frequent than expected by chance alone revealed downstream genes for several transcription factors in *Drosophila* (Berman *et al.*, 2002; Rebeiz *et al.*, 2002). Moreover, microarray analysis of systemic acquired resistance (SAR)-induced genes in *Arabidopsis* showed that W-boxes, binding sites for WRKY transcription factors, are highly enriched in the promoter regions (Maleck *et al.*, 2000). These results suggest that the clustering of binding sites for some transcription factors could be a common scheme for gene regulation. Interestingly, all three *ORG* genes whose expression was significantly increased in the OBP3-inducible lines, also had the highest frequency of Dof-binding sites in the promoter region. However, which of these Dof-binding sites in the *ORG* promoters are functional remains to be determined.

The combined results of the gain-of-function and loss-of-function experiments strongly suggest that the *ORG1*, *ORG2* and *ORG3* genes are direct target genes of OBP3. Thus, some of the direct target genes of OBP3 may encode regulatory factors. Further studies will be required to determine the function of the *ORG1*, *ORG2* and *ORG3* genes and the role they play in the growth defects observed in OBP3-overexpressing lines.

Experimental procedures

Plant materials, growth conditions and treatments

Arabidopsis thaliana (ecotype Columbia) seeds were surface sterilized with 70% ethanol for 15 min, dried on autoclaved paper, stratified at 4°C for 2 days and grown on MS (Sigma, St Louis, MO,

USA) medium or on autoclaved soil. Plants were grown at 22°C with a day length of 16 h. For treatments with MeJA and SA, *Arabidopsis* seedlings were grown on MS solid medium for 8 days. The plants were treated in MS liquid medium containing 100 μM SA (Sigma) or 100 μM MeJA (Aldrich, Milw, WI, USA). Total seedlings or roots of the plants were collected and frozen in liquid nitrogen prior to RNA isolation. For Dex treatment, *Arabidopsis* seeds were geminated on MS solid medium with 20 μg ml^{-1} hygromycin B to select transgenic lines. After 1 week on the MS medium, seedlings were transferred to autoclaved soil and grown for two more weeks. 30-μM Dex solution in 0.01% Tween-20 was sprayed onto seedlings, which were then subjected to a vacuum treatment for 2 min. The plants were collected and frozen in liquid nitrogen prior to RNA isolation.

Preparation of double-strand cDNAs for RACE (rapid amplification of cDNA ends), virtual Northern blot, and suppression subtractive hybridization

One microgram of cDNA was denatured with 10 pmol of the following primers in 5 μl of total volume at 72°C for 2 min and cooled at 4°C for 2 min: cDNA synthesis primers (5′-AAGCAGTGGTAACAACGCAGAGTACT$_{30}$N$_{-1}$N-3′) and SMARTII oligonucleotide (5′-AAGACGTGGTAACAACGCAGATGTACGCGGG-3′). A mixture containing 2 μl of the supplied buffer (Gibco BRL, Rockville, MD, USA), 20 nmol of DTT, 10 nmol of dNTP and 200 units of MMLV reverse transcriptase (Gibco) in 10 μl final volume was added and incubated at 42°C for 1 h, followed by addition of 40 μl of TE (10 mM Tris (pH 7.6), 1 mM EDTA). The synthesized double-strand cDNA was then heat-denatured at 72°C for 7 min and stored at −80°C.

One microlitre of double-stranded cDNA, synthesized as described in the previous section, was amplified in a final volume of 20 μl, which contained 10 nmol of dNTP, 1× Advantage2 polymerase mix (Clontech, Palo Alto, CA, USA), the supplied buffer (Clontech) and the following primers synthesized specific to the gene of interest. This primer was designed to bind to the junction of two consecutive exons (this suppresses an amplification from genomic DNA).

In the case of 3′ RACE, 10 pmol of oligo dT_{18} was used and for 5′ RACE, a mixture of two primers (0.2 pmol of 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT-3′ and 1 pmol of 5′-CTAATACGACTCACTATAGGGC-3′) was used. The following PCR programme was used: 5 cycles of 94°C for 5 sec and 72°C for 3 min, followed by 5 cycles of 94°C for 5 sec, 70°C for 10 sec and 72°C for 3 min, and again followed by 27 cycles of 94°C for 5 sec, 68°C for 10 sec and 72°C for 3 min. In the case of 3′ RACE, the PCR products were then subcloned into an appropriate vector and sequenced. In the case of 5′ RACE, 0.1 μl of the PCR products was subsequently amplified with a secondary PCR in a final volume of 20 μl that contained 10 nmol of dNTP, 1× Advantage2 polymerase mix (Clontech), the provided buffer (Clontech), 10 pmol of the nested primer for 5′ cDNA end (5′-AAGCAGTGGTAACAACGCAGAGT-3′) and 10 pmol of the nested primer for the gene of interest. The following PCR programme was used: 25 cycles of 94°C for 5 sec, 68°C for 10 sec and 72°C for 3 min. The PCR product was then cloned and sequenced.

One microliter of double-strand cDNA was amplified with a mixture containing 1× Advantage 2 polymerase mix (Clontech), the supplied buffer (Clontech), 20 nmol of dNTP, 20 pmol of PCR primer (5′-AAGCAGTGGTAACAACGCAGAGT-3′) in a final volume of 100 μl using the following PCR programme: 95°C for 1 min, followed by 20–25 cycles of 95°C for 5 sec, 65°C for 5 sec and 68°C for 6 min (for SSH, two identical reactions were performed to obtain enough material for the subsequent step). The amplification was stopped before the PCR reached the saturation phase: this was checked by aliquotting PCR samples at different cycles. 2 μl of 0.5M EDTA was added to terminate the PCR reactions. 10 μl of the amplified products were then loaded on a 1.5% agarose gel for a virtual Northern analysis, followed by the standard procedure for Southern blot analysis (Sambrook *et al.*, 1989).

Two hundred microliters of amplified products from a virtual Northern blot analysis was purified using Concert Rapid PCR Purification System (Gibco) according to the manufacturer's instruction, resulting in a 50-μl TE solution. The solution was then digested with 30 units of *Rsa*I in 300 μl final volume at 37°C for 4 h, followed by purification using Concert Rapid PCR Purification System (Gibco) according to the manufacturer's instruction, resulting in a 50-μl TE solution. The solution was then precipitated with 2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and re-suspended to 0.3 μg μl^{-1} with water. The digested double-stranded cDNA was then used for SSH, using the PCR-Select cDNA Subtraction Kit (Clontech) according to the manufacturer's instruction. The resulting products from the PCR-Select cDNA Subtraction Kit (Clontech) were subcloned into the pT-Adv vector (Advantage PCR cloning kit, Clontech). Plasmid DNAs of pT-Adv containing each product from the hybridization were prepared and used in a dot-blot analysis to screen for differentially expressed clones using a standard protocol (Sambrook *et al.*, 1989).

Generation of hpOBP3 lines

To produce hairpin constructs, a 525-bp OBP3 fragment was amplified using the following primers: 5′-GGGGATCGATGGTACCTGATAATACTACTAGTA-3′ and 5′-GGGGTCTAGACTCGAGGCCTGTTGAGTTGTTGG-3′. The PCR product was digested with the appropriate restriction enzymes, purified and ligated into the *Xba*I/*Cla*I site and the *Xho*I/*Kpn*I site of pHANNIBAL (Wesley *et al.*, 2001). The *Not*I cassette was then transferred to the binary vector pART27. *Arabidopsis* was transformed with this construct using standard infiltration techniques and selection for kanamycin-resistance.

RNA Isolation, Northern and RT-PCR analysis

Total RNA was isolated from *Arabidopsis*, using the Purescript reagent (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instruction, except for the protein precipitation step in which RNA samples were centrifuged twice instead of once. Purified RNA for RT-PCR analysis was subject to further purifications: Purified RNA was treated with 2 units of RNase-free DNase (Promega, Madison, WI, USA) at 37°C for 60 min and was re-purified again with the Purescript reagent (Gentra Systems).

Northern blot analysis and synthesis of random primed probes were performed as described earlier (Shah *et al.*, 1999). Random primed probes were synthesized from PCR fragments from *Arabidopsis* cDNA with oligonucleotides used for RT-PCR. Northern gel blot hybridization was performed as described previously by Kachroo *et al.* (1995).

For cDNA synthesis, 1 μg of RNA in 5 μl of the total volume was denatured at 65°C for 10 min and mixed with 0.5 μg of oligo dT_{18}, 12 nmol of dNTP, 2 units of AMV reverse transcriptase (Promega) and 10 units of RNase inhibitor (Promega) in 25 μl of the supplied buffer. The mixture was then incubated at 37°C for 1 h, denatured at 65°C for 10 min and stored at −80°C until the PCR reactions were performed.

All PCR reactions were performed with an annealing temperature of 60°C, 0.5 unit of Taq polymerase (Invitrogen, Carlsbad, CA, USA), 4 nmol of dNTP and a pair of primers (10 pmol each) in a

final volume of 20 μl of the buffer recommended by the supplier. For each primer condition, the cycle was chosen as two cycles prior to visualization on an ethidium bromide-stained agarose gel. The following are the specific primer pairs used for the RT-PCR: Numbers in parentheses indicate the number of PCR cycles completed for each target gene.

- ORG1 (22): 5′-CCTTGTACATGGGTGCCATGGAAGT-3′
  5′-ACTGTAAGCGTTGCTAGTACTTGG-3′
- ORG2 (20): 5′-ACCACAGACTTATGAAGTGA-3′
  5′-CCGATACTCGTACCAAAATT-3′
- ORG3 (22): 5′-TGGAGCTGTTACAGCGGTGA-3′
  5′-CTGAAATTTGCACCAAGAGC-3′
- ORG4 (22): 5′-CAATAACCTTGTCCTTGATTAAGC-3′
  5′-AGACATGAACGGAGAGATGAGAAC-3′
- ORG5 (23): 5′-ATGGCCTCTTTCCTTGTCTTAGCA-3′
  5′-CCATTAGAGACGATTAAGTAATCTA-3′
- GST6 (15): 5′-CCCCGTCGATATGAGAGC-3′
  5′-GAGAGAGGGTCACTACTGCTTCTGG-3′
- OBP3 (26): 5′-AGTAAGGGAACCAACATCAG-3′
  5′-GCTTGAGTCACATCTAGGGC-3′
- Chitinase (22): 5′-CGGTGGTACTCCTCCTGGACCCACCGGC-3′
  5′-CGGCGGCACGGTCGGCGTCTGAAGGCTG-3′
- β-tubulin (20): 5′-ATCACAGCAATACAGAGCCTTAACC-3′
  5′-GCTGTTGTTATTGCTCCTCCTGCA-3′
- PR5 (20): 5′-ATGGCAAATATCTCCAGTATTCACA-3′
  5′-ATGTCGGGGCAAGCCGCGTTGAGG-3′
- PDF1.2 (27): 5′-AATGGATCCATGGCTAAGTTTGCTTCCATC-3′
  5′-AATGAATTCAATACACACGATTTAGCACC-3′

The PCR products were run on a 1.5–2% agarose gel, transferred to a nylon membrane (Bio-Rad, Hercules, CA, USA) and probed with the corresponding random-primed ^{32}P-labelled probe. Amplification products were imaged using a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA) or Cyclone phosphoimager (Packard, Meriden, CT, USA). IMAGEQUANT software (Molecular Dynamics) with the default parameters was used for quantizing RT-PCR products.

Immunoblot analysis

Immunoblot was analysed as described previously using an enhanced chemiluminescence method (Amersham, Pharmacia Biotech, Piscataway, NJ, USA; Lin *et al.*, 1995) with the following modification. The blot, a PVDF membrane (Millipore, Bedford, MA, USA), was probed with anti-HA antibody (Sigma, diluted 1/1000) and then with anti-mouse antibody conjugated to horseradish peroxidase (Sigma, diluted 1/5000).

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