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ocs element promoter sequences are activated by auxin and salicylic acid in *Arabidopsis*

(plant hormones/gene regulation/glutathione *S*-transferases/stress response)

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ABSTRACT **ocs elements are a group of promoter elements that have been exploited by two distinct groups of plant pathogens, *Agrobacterium* and certain viruses, to express genes in plants. We examined the activity of single and multiple ocs elements linked to a minimal plant promoter and the *uidA* reporter gene in transgenic *Arabidopsis*. β-Glucuronidase activity was detected only in root tips and in callus tissue after auxin treatment. A more sensitive assay revealed that auxin treatment also increased ocs element activity in aerial parts of the plant, although the absolute levels of ocs element activity were greater in roots. The response of ocs elements to exogenous auxin began within 1 h. Salicylic acid, a disease-resistance signal in plants, also increased ocs element activity in both roots and aerial parts of the plant. The question of whether the induction in ocs element activity is mediated through auxin and/or salicylic acid signal transduction pathways or is part of a more general stress response is discussed.**

ocs elements are a family of related 20-bp DNA sequences that are important components of the promoters of a number of *Agrobacterium* genes expressed in plants (1). The expression of two of these genes, nopaline synthase (*nos*) and mannopine synthase (*mas*), has been shown to be inducible by the hormone auxin in transgenic plants (2, 3). Auxin plays major roles in many aspects of plant development including cell elongation, cell division, cell differentiation, apical dominance, senescence, and fruit ripening (reviewed in refs. 4 and 5). ocs elements are also important for the expression of certain plant viral promoters; for example, cauliflower mosaic virus (CaMV) contains an ocs element (1), also called *as-1* (6), in the CaMV 35S promoter. In transgenic tobacco plants, ocs element/*as-1* sequences direct tissue-specific expression patterns, which differ in developmental stages of the plant; in tobacco seedlings, the expression is primarily in the root tip (7–9). The interaction of ocs element/*as-1* sequences with plant DNA-binding proteins has been analyzed (1, 6, 10–12), and genes for ocs element/*as-1* binding factors (OBFs), belonging to the basic–leucine zipper class of transcription factors, have been isolated in tobacco, maize, wheat, and *Arabidopsis* (13–20).

The first plant gene containing a functional ocs element promoter sequence to be identified (12) was the soybean *Gmhsp26-A* gene. *Gmhsp26-A* belongs to a group of auxin-responsive genes that includes the tobacco *CNT/GNT, par*, and *pLS216* genes (21–25). These genes encode proteins that have significant homology to animal and plant glutathione *S*-transferases (GSTs), and the *parB* and *CNT103* proteins have GST activity *in vitro* (25, 26). The tobacco *GNT35/GNT1* and *par-1* genes (23, 27) also contain potential ocs element-like sequences in their promoters. In a number of cases the expression of the auxin-responsive GST genes is also inducible by other stimuli such as heavy metals, wounding, and salicylic acid (SA) (see ref. 28 and references within). SA is a disease-resistance signal in plants; exogenous application of SA stimulates resistance to a variety of lesion-inducing pathogens (29, 30). The expression of the promoters of the *Agrobacterium nos* and *mas* genes has also been shown to be inducible by wounding in transgenic plants (2, 3), and the *nos* promoter has been shown to be inducible by SA and methyl jasmonate (31). The presence of ocs elements in the promoters of auxin-responsive genes suggested a potential role as auxin-responsive elements. In this paper we demonstrate that ocs element sequences linked to minimal plant promoters are responsive to auxin as well as SA in transgenic *Arabidopsis* plants.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Treatment with Plant Hormones. Ti plasmid pGA472 constructs containing an ocs element tetramer linked to the −45 CaMV 35S promoter, the *uidA* reporter gene, and nopaline synthase 3′ flanking sequences or the −90 CaMV promoter fused to the *uidA* reporter gene and nopaline synthase 3′ flanking sequences were provided by Jeff Ellis (Plant Industry, CSIRO Australia). The ocs element tetramer contained the 20-bp ocs element sequence (AAACGTAAGCGCTTACGTAC) from the promoter of the octopine synthase (*ocs*) gene, flanked by 11 and 8 nucleotides at the 5′ and 3′ end, respectively. The pGA472 constructs in *Agrobacterium tumefaciens* strain AGL1 were introduced into *Arabidopsis* using the root transformation procedure of Valvekens *et al.* (32). Transgenic seedlings were grown on Murashige and Skoog (MS) medium with kanamycin (50 μg/ml) at 22°C with a day length of 16 h. Six- to 8-day-old seedlings were transferred to MS medium containing various amounts of 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma) or naphthylphthalmic acid (NPA; Pfaltz & Bauer) for the indicated hours and harvested for the β-glucuronidase (GUS) histochemical assay or RNA extraction for reverse transcriptase (RT)-PCR. Growth of the seedlings on auxin or NPA for 1 day did not result in any visible changes in the development of the seedlings. For SA treatment, seedlings were transferred to medium containing various amounts of SA (Sigma) and also initially sprayed with the SA solution. For abscisic acid (GIBCO), cytokinin [6-benzylaminopurine (Sigma)], and gibberellin [GA3 (GIBCO)] treatment, seedlings were transferred to medium containing the respective hormone at 1, 4.5, or 0.25 μg/ml.

Histochemical Assay, RNA Extraction, and RT-PCR. Histochemical GUS assays were performed as described by

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; GUS, β-glucuronidase; GST, glutathione *S*-transferase; NPA, naphthylphthalmic acid; SA, salicylic acid; CaMV, cauliflower mosaic virus; RT, reverse transcriptase; EpRE, electrophile-responsive element; OBF, ocs element binding factor.

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Jefferson *et al.* (33), on segregating populations of F_2 and F_3 plants. Seedlings were fixed in 0.3% formaldehyde in 50 mM sodium phosphate (pH 7.0) for 45 min, following washes with 50 mM sodium phosphate. Histochemical reactions with the GUS substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Clontech), were performed overnight with 1 mM substrate in 50 mM sodium phosphate (pH 7.0) at 37°C. Plant material was fixed in ethanol after GUS staining. All RNA analysis was carried out on segregating populations of F_3 plants. RNA from roots or aerial parts of the transgenic seedlings (predominantly leaves) was extracted using the guanidinium thiocyanate method (34). One microgram of total RNA was used for the first-strand cDNA synthesis. RNA was incubated in diethyl pyrocarbonate-treated water at 65°C for 5 min. RT reactions were performed in a volume of 25 μl that contained 100 μM poly(dT) primer, 50 mM Tris·HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 600 μM deoxynucleotide triphosphates, 2 units of avian myeloblastosis virus RT (Promega), and 35 units of RNAguard (Pharmacia) at 37°C for 1 h. The RT-PCR assay was based on the method of Kawasaki (35). The PCR reaction contained 2 μl of single-stranded cDNA, 0.25 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 200 ng of each oligonucleotide primer, 1 unit of Tfl polymerase (Epicentre Technologies, Madison, WI), 50 mM Tris·HCl (pH 9.0), and 20 mM ammonium sulfate. The PCR cycle consisted of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C. After 20, 27, and 35 cycles, aliquots were run on an agarose gel, transferred to a nylon membrane, and probed with the corresponding labeled probe. Amplification products were visualized by autoradiography. To enable quantitation, a cycle number was chosen for each primer pair that was in the exponential phase of the PCR reaction. Primer pairs were 5′-GTGCACGGGAATATTTCGCCACTGG-3′ and 5′-GCGGTTTTTCACCGAAGTTCATGCC-3′ for GUS, 5′-AATAACCTAATGTTCGATGAAGGGC-3′ and 5′-AGCACGTAACCGCAATGTATAATTG-3′ for OBF5, and 5′-CGCCTGGCTCTATCATAATCCCGAC-3′ and 5′-TCCCGGCTGCTACACTCTCTCTGCTAA-3′ for OBF4.

RESULTS

ocs Element Promoter Sequences Are Activated by Auxin. Transgenic seedlings containing an ocs element tetramer linked to a minimal plant promoter and the *uidA* reporter gene were transferred for 1 day to MS medium containing different concentrations of auxin (Fig. 1). With the GUS histochemical assay, no activity was detected in the transgenic seedlings in the absence of exogenous auxin as shown in Fig. 1*A* for the root. If the transgenic *Arabidopsis* seedlings are placed on medium with the synthetic auxin 2,4-D at 2.5 μM, GUS activity was observed in and immediately behind the root tip (Fig. 1*B*). No GUS activity was detectable in the root cap (Fig. 1 *B* and *C*) or other parts of the seedling (Fig. 1*C*). GUS activity was also detectable in lateral root tips after auxin treatment. With 13.5 μM 2,4-D, the GUS staining appeared more intense and GUS activity was detectable further back along the root. Treatment with abscisic acid, cytokinin, and gibberellin, respectively, did not result in any detectable GUS activity in the transgenic plants using the histochemical assay.

Results similar to those shown in Fig. 1 *A–C* were observed with four independent transformants. The natural plant auxin, indole-3-acetic acid, and another synthetic auxin, α-naphthalene acetic acid, also induced ocs element activity. The induction of GUS activity is seen at concentrations of 2,4-D as low as 0.2 μM. Control plants, which contained the −45 CaMV promoter fused to GUS, showed no GUS activity

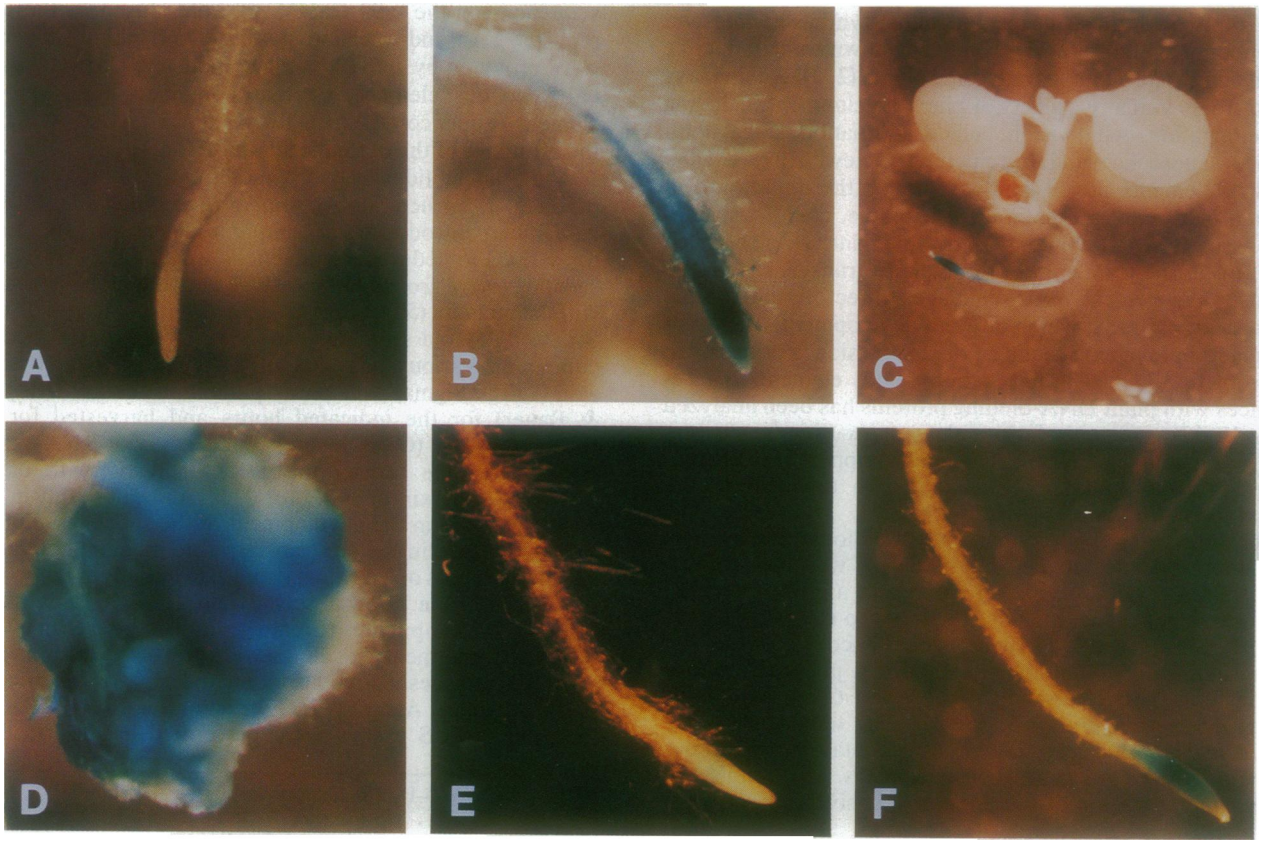

FIG. 1. Analysis of ocs element activity in transgenic *Arabidopsis* using the GUS histochemical assay. (*A*) 4ocs#1 root, no auxin. (*B*) 4ocs#1 root, 2.5 μM 2,4-D. (*C*) 4ocs#1 whole seedling, 2.5 μM 2,4-D. (*D*) 4ocs#1 callus tissue. (*E*) −90 CaMV#1 root, 2.5 μM 2,4-D. (*F*) −90 CaMV#1 root, 100 μM 2,4-D.

with or without the addition of exogenous auxin. The activity of the ocs element–GUS fusion was examined in callus tissue. Roots from transformant 4ocs#1 were placed on medium containing auxin, and callus tissue was generated. The ocs element–GUS fusion, which was expressed only in the root tip region *in planta*, was expressed throughout the callus (Fig. 1*D*). Callus generated from control plants containing the −45 CaMV promoter–GUS fusion showed no GUS activity by histochemical staining.

These experiments demonstrated that a tetramer of a 39-bp sequence containing an ocs element sequence was sufficient to confer responsiveness to exogenous auxin. To test if single ocs element sequences also acted as auxin-responsive elements, the expression of the truncated −90 CaMV 35S (Δ35S) promoter linked to GUS was examined in *Arabidopsis*. The −90 CaMV promoter contains an ocs element sequence from −82 to −63 (1, 6) that shares 14 out of 20 nucleotides with the ocs element from the promoter of the *ocs* gene. With plants containing the Δ35S promoter–GUS fusions and only one copy of the ocs element sequence, the amount of GUS activity was significantly reduced compared with plants with four copies of the ocs element sequence. With transformant −90 CaMV#1 and 2.5 μM 2,4-D, GUS activity was faintly visible in the root tip region (Fig. 1*E*). With 100 μM 2,4-D, strong GUS activity was visible in the root tip region (Fig. 1*F*). No GUS activity was detected histochemically in the absence of exogenous auxin.

To further understand the auxin-induced expression of the ocs element, we used RT-PCR to analyze the expression pattern. The RT-PCR technique is more sensitive and quantitative than the GUS histochemical assay. Two independent transformants were analyzed: 4ocs#1 and 4ocs#2; each contained the ocs element tetramer linked to the −45 CaMV promoter and the GUS reporter gene. As shown in Fig. 2*A* for 4ocs#2, a large induction in GUS RNA was seen in roots within 6 h after treatment with 50 μM 2,4-D. Due to the increased sensitivity of the PCR technique, it was possible to detect ocs element activity in roots that have not been treated with exogenous auxin. Auxin induction of ocs element activity in aerial parts of the plant was also detectable, although the overall levels of GUS RNA expression were lower than in roots (Fig. 2*A*, compare lanes 3 and 4 and lanes 5 and 6).

In *Arabidopsis*, the genes for two ocs element binding factors, OBF4 and OBF5 (20), as well as genes for the closely related aHBP1b and TGA1 proteins (17, 18) have been isolated. A simple model for ocs element activation would be an increase in the level of RNA encoding one or more of these transcription factors after auxin treatment. Using the RT-PCR assay and primers specific for each transcript, we checked the transcript levels of all four transcription factors and found no significant change after auxin treatment (shown in Fig. 2 *A* and *C* for OBF5 and Fig. 2*D* for OBF4).

For 4ocs#2, a 50- to 100-fold increase in GUS RNA levels was observed in roots (Fig. 2*B*) and aerial parts of the plant after treatment with 50 μM 2,4-D for 6 h. With the sensitive PCR assay, a low level of GUS RNA expression was detectable in the control plants containing the minimal −45 CaMV promoter fused to GUS. No change in GUS RNA levels was observed in response to auxin, ruling out the possibility that auxin may be activating the −45 CaMV minimal promoter or causing a posttranscriptional change in GUS RNA levels. To investigate the auxin induction pattern further, RNA was made from 4ocs#2 seedlings treated with 5 μM 2,4-D for 0, 1, 3, and 6 h. The auxin response began as early as 1 h after application of 2,4-D (Fig. 2*C*) and reached the maximum between 3 and 6 h. The levels of GUS RNA were also analyzed in 4ocs#1 after auxin treatment, and comparable results were obtained (Fig. 2*D*).

ocs Element Activity Can Be Altered After Treatment with the Auxin Transport Inhibitor NPA. Auxin is synthesized primarily in the shoot apex and leaf primordia and is then transported to the root tip. The auxin transport inhibitor NPA inhibits the transport of auxin to the roots and is thought to result in a build-up of auxin in the aerial parts of the plant (ref. 36 and references therein). NPA treatment may therefore result in an increase in ocs element activity in the aerial parts of the plant. We treated 4ocs#1 with NPA and used the PCR assay to analyze the level of GUS expression in the aerial parts of the plant. As shown in Fig. 3, treatment with 50 μM NPA for 24 h resulted in a large induction in GUS RNA levels in the aerial parts of the plant. The induction in GUS RNA levels was increased further with 100 μM NPA for 24 h. The transcript levels of OBF5 did not change after NPA treatment.

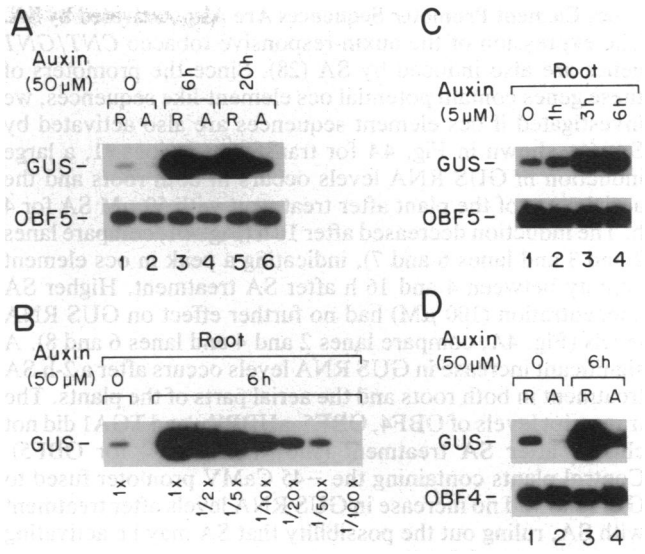

FIG. 2. RT-PCR analysis of GUS and control RNA levels after treatment of the transgenic seedlings 4ocs#1 and 4ocs#2 with auxin. (*A*) Eight-day-old 4ocs#2 seedlings treated with 50 μM auxin for 0, 6, and 20 h. (*B*) Quantitation of GUS RNA levels in the roots of 4ocs#2 seedlings after a 6-h treatment with 50 μM 2,4-D. A dilution series of the 6-h PCR product was generated and compared with the 0-h PCR product. (*C*) Eight-day-old 4ocs#2 seedlings treated with 5 μM auxin for 0, 1, 3, and 6 h. (*D*) Eight-day-old 4ocs#1 seedlings treated with 50 μM auxin for 0 and 6 h. For all panels, RNA was isolated from roots (R) or aerial parts (A) of the plant. The RNA was converted to single-stranded cDNA by reverse transcription with an oligo(dT) primer. Primers specific for the GUS and control transcripts (*A–C*, OBF5; *D*, OBF4) were used in the PCR reaction with cDNA from each pool. The constitutive expression of OBF5 and of OBF4 serve as controls for the RT-PCR technique. The results shown were obtained from the linear phase of the PCR reaction.

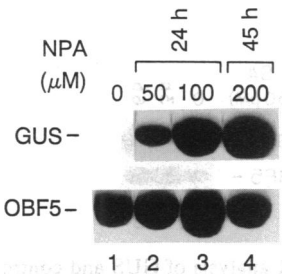

FIG. 3. Analysis of the effects of the auxin transport inhibitor NPA on ocs element activity. Eight-day-old 4ocs#1 transgenic seedlings were treated with different concentrations of NPA for the times indicated. RNA was isolated from the aerial parts of the seedlings and analyzed by RT-PCR with GUS and OBF5 primers as described in the legend to Fig. 2.

ocs Element Promoter Sequences Are Also Activated by SA. The expression of the auxin-responsive tobacco *CNT/GNT* genes are also induced by SA (28). Since the promoters of these genes contain potential ocs element-like sequences, we investigated if ocs element sequences are also activated by SA. As shown in Fig. 4*A* for transformant 4ocs#1, a large induction in GUS RNA levels occurs in both roots and the aerial parts of the plant after treatment with 50 μM SA for 4 h. The induction decreased after 16 h (Fig. 4*A*, compare lanes 2 and 3 and lanes 6 and 7), indicating a peak in ocs element activity between 4 and 16 h after SA treatment. Higher SA concentration (100 μM) had no further effect on GUS RNA levels (Fig. 4*A*, compare lanes 2 and 4 and lanes 6 and 8). A significant increase in GUS RNA levels occurs after a 2-h SA treatment in both roots and the aerial parts of the plants. The transcript levels of OBF4, OBF5, aHBP1b, and TGA1 did not change after SA treatment (shown in Fig. 4 for OBF5). Control plants containing the −45 CaMV promoter fused to GUS showed no increase in GUS RNA levels after treatment with SA, ruling out the possibility that SA may be activating the minimal −45 CaMV promoter or causing a posttranscriptional change in GUS RNA levels. The levels of GUS RNA following SA treatment were also analyzed in 4ocs#2 (Fig. 4*B*), and a 10- to 20-fold increase in GUS RNA was observed after treatment with 50 μM SA for 6 h. SA also resulted in an increase in the levels of GUS RNA in transformant −90 CaMV#1 (data not shown).

DISCUSSION

We have demonstrated that ocs element sequences are responsive to auxin and SA in transgenic *Arabidopsis* plants. The magnitude of the induction in ocs element activity after treatment with exogenous auxin is the same in the root and aerial parts of the plant. The higher overall levels of ocs element activity in roots may reflect increased levels of endogenous auxin in the root versus the aerial parts of the plant or an increased sensitivity of cells in the root to auxin. The expression patterns of the promoter of the auxin-inducible *GNT35* gene, when linked to GUS and analyzed in transgenic tobacco plants, were very similar to those observed with the ocs element–GUS fusions in *Arabidopsis*—

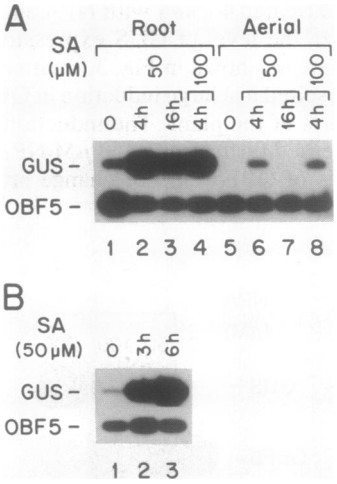

FIG. 4. RT-PCR analysis of GUS and control RNA levels after treatment of transgenic seedlings 4ocs#1 and 4ocs#2 with SA. (*A*) Eight-day-old 4ocs#1 transgenic seedlings were treated with different concentrations of SA for the times indicated. (*B*) Eight-day-old 4ocs#2 transgenic seedlings were treated with 50 μM SA for the times indicated. RNA was isolated from the roots (*A*) and aerial parts (*A* and *B*) of the seedlings and analyzed by RT-PCR with GUS and OBF5 primers as described in the legend to Fig. 2.

namely, auxin-induced expression in the root tips of young plants (27). The expression of the related tobacco *CNT103* and *CNT107* genes was also induced by both auxin and SA in tobacco tissue culture cells (28). The promoters of these genes contain potential ocs element sequences, which, either acting independently or in combination with other cis-acting elements, are likely to be responsible for the auxin and SA induction of these genes.

The trans-acting factor(s) that mediates the auxin and/or SA responsiveness of ocs elements remains to be determined. The *Arabidopsis* OBF4, OBF5, aHBP1b, and TGA1 proteins are reasonable candidates. Although auxin and SA treatment do not affect the transcript levels of these genes, they may be activated, for example, through changes in phosphorylation. *De novo* protein synthesis is not required for auxin-induced expression of a number of genes (22, 24, 37), and cycloheximide does not appear to inhibit the auxin induction of ocs element activity (unpublished results). It will be important to analyze other ocs element sequences to see how they respond to auxin and SA. A deletion analysis has demonstrated that a region of the *nos* promoter containing part of the ocs element is necessary for the auxin and SA induction of this promoter (31), although further studies are required to see if the *nos* ocs element is sufficient for mediating the auxin and SA response.

The response of ocs element sequences to both auxin and SA raises a number of interesting questions. Is the induction in ocs element activity mediated through auxin and/or SA signal transduction pathways? If so, do these pathways converge at the ocs element or at an earlier stage? Alternatively, the induction of ocs element activity by auxin and/or SA may be part of a stress response. Our data do not allow us to distinguish between these two possibilities, which are not mutually exclusive. One class of plant genes that uses ocs element sequences is GSTs. GSTs are multifunctional proteins that catalyze the conjugation of glutathione to a variety of hydrophobic electrophiles (38–40). GSTs are involved in the detoxification of cytotoxic products produced during xenobiotic metabolism, the biosynthesis of prostaglandins and leukotrienes, the protection of tissues against oxidative damage, and the intracellular transport of hydrophobic compounds. GST expression in animals is induced by a range of factors including growth factors, hormones, transforming oncogenes, and cellular stress-inducing agents. It has been proposed that these factors activate GST expression by inducing conditions of oxidative stress (40).

An electrophile-responsive element (EpRE) has been identified in the promoters of the rat and mouse GST *Ya* genes and the rat GST *Pi* gene (40). The EpRE and ocs element sequences share a number of features. The EpRE consists of two adjacent AP-1-like binding sites. The AP-1 site bears some resemblance to an ocs element half site and is the binding site for the Jun/Fos (AP-1) complex. The Jun/Fos proteins, like the OBF proteins, belong to the basic–leucine zipper class of transcription factors. Individually, the two AP-1-like sequences have low to no activity but act synergistically to form the EpRE (40). Similarly, both halves of the ocs element are required for *in vivo* transcriptional activity (1, 10, 12).

There is a direct correlation between the potency of electrophilic inducers of the EpRE and their ability to serve as GST substrates (40). Auxins, especially 2,4-D, and SA resemble GST substrates and may therefore be electrophilic inducers of the ocs element. In animals, GST gene expression is induced during cell proliferation resulting from growth factors or neoplastic transformation. Auxin, a plant growth factor, may also induce ocs elements by acting as a mitogen. In a tobacco tissue culture system, 2.2 μM 2,4-D was found to be optimal for stimulating cell division and inducing CNT103 RNA levels (22). Interestingly, a soluble auxin-

binding protein has been found to be a GST (41), raising the possibility that GSTs modulate auxin activity or are involved in the transport/storage of auxin. The ability of SA, a disease-resistance signal, to induce GSTs may also have biological relevance. An involvement of redox perturbation in early stages of plant defense has been proposed, and reduced glutathione specifically induces plant defense genes (42). A pathogen-induced wheat GST gene has been reported (43), which may play a role in limiting tissue damage during pathogen attack. NPA may also serve as an electrophilic inducer of the ocs element. The increase in ocs element activity in the aerial parts of the plant after NPA treatment may be the result of direct NPA induction and/or NPA-mediated changes in endogenous auxin levels.

The auxin responsiveness of ocs element sequences provides a possible explanation for why *Agrobacterium* utilizes ocs element sequences to express genes *in planta*. Both *A. tumefaciens* and *Agrobacterium rhizogenes* encode auxin biosynthetic genes that are expressed in transformed tumors or hairy roots and, at least for *A. tumefaciens*, are required for tumor formation (44). Another class of *Agrobacterium* genes, the opine biosynthetic genes, are expressed at high levels in tumor tissue and hairy roots, and a number of these genes have been shown to contain ocs element sequences in their promoters (1). The products of these genes, the opines, are secreted from the tumors and used by the invading bacteria as a metabolic source. The SA responsiveness of ocs element sequences may also be advantageous for *Agrobacterium*, which can initiate transformation only at a wound site. Endogenous SA levels have been shown to rise 50-fold and reach concentrations of up to 100 μM in tobacco leaves inoculated with tobacco mosaic virus (45, 46). SA may therefore be present at high levels at the wound site and in the tissues in and/or around the growing tumor. Therefore, *Agrobacterium* may have selected ocs elements in order to key into plant transcription systems that are inducible by auxin and SA and consequently active in transformed tissue.

We thank Dr. Jeff Ellis for the *Agrobacterium* constructs containing the 4ocs–GUS fusions and −90 CaMV–GUS fusions, Dr. Robert Goldberg for *Arabidopsis* seed containing the −45 CaMV–GUS fusion, Lu Huang for expert technical assistance, Dr. Rudy Dolferous for help with transforming *Arabidopsis*, and Drs. Rhonda Foley, Elaine Tobin, and George Laties for critical comments on the manuscript. B.Z. was supported in part by a University of California Biotechnology predoctoral training grant in plant molecular biology. This work was supported by U.S. Department of Agriculture Grant 91-37301-6369 to K.B.S.

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