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BIOTIC AND ABIOTIC STRESS

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Abiotic and biotic stress differentially stimulate *as-1* element activity in *Arabidopsis*

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Abstract *As-1*-type *cis*-elements enhance the expression of putative plant protective genes in response to xenobiotic chemical stress. Here we investigate the effect of developmental changes on *as-1*-dependent transcription activities under basal and stimulus-induction conditions. We show that different xenobiotic stress cues give rise to distinct, differential patterns of *as-1* activity in planta, which are further effected by developmental control. Moreover, transcription through *as-1* is potentiated by infection with a virulent strain of *Pseudomonas syringae*, in line with recent genetic evidence indicating a role for this element and its cognate TGA transcription factors in host-defense gene responses. Our results here are consistent with the notion that inputs from both developmental and stimulus induction pathways define the complex spatial and temporal expression pattern of *as-1* activity in plants.

Keywords *as-1* element · *Arabidopsis* · Xenobiotic stress · Infection

Introduction

Originally described as promoter regulatory elements of plant viral and agrobacterial pathogens (Benfey et al. 1989), activation sequence-1 (*as-1*) and its homologues (e.g., *ocs*, *nos*, and *mas*) have increasingly been identified as key regulators of an array of plant nuclear genes (Bouchez et al. 1989; Ellis et al. 1993; Droog et al. 1995; Guevara-Garcia et al. 1998; Strompen et al. 1998; Chen and Singh 1999). When placed upstream of the cauliflower mosaic virus (CaMV 35S) minimal promoter (–46 to +4 bp) sequence, *as-1*-type elements have been shown to confer expression on a downstream β-glucuronidase (GUS) reporter gene in transgenic tobacco and *Arabidopsis* seedlings and mature plants (Benfey et al. 1989; Zhang and Singh 1994). In seedlings, *as-1*-type elements largely confer weak constitutive GUS reporter gene expression to root tips. Treatment of intact seedlings or leaves with micromolar amounts of any one of a number of xenobiotic stress compounds, such as biologically active or inactive analogues of auxin, salicylic acid, and methyl jasmonate plant hormones, can enhance *as-1*-dependent transcription in either leaves or roots, depending on the type of chemical inducer used (Liu and Lam 1994; Qin et al. 1994; Xiang et al. 1996; Klinedinst et al. 2000). These data suggest that tissue-specific and stimulus-responsive pathways are likely to collectively modulate *as-1* activity in planta. Here we show that plant development makes strong contributions to tissue-specific and stimulus-responsive patterns of activity of *as-1* and that this activity in *Arabidopsis* leaves is also potentiated by at least one type of biotic cue (i.e., infection with a virulent, but not avirulent, strain of a plant pathogenic bacterium). Together these findings demonstrate that *as-1*-dependent transcription is dynamically regulated by diverse abiotic and biotic stimuli within a plastic program of development.

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Materials and methods

Plants

Ti plasmid pBI101 (Clontech, Palo Alto, Calif.) was used as previously described to construct all GUS reporter gene constructs (Klinedinst et al. 2000). The –90- to +8-bp region of the CaMV 35S promoter, which contains a single *as-1* element located upstream of the minimal promoter (Benfey et al. 1989), was used to assess *as-1*-dependent transcription. A partially truncated form of this promoter that lacks the upstream binding half-site of

as-1 was used as a negative control for *as-1* activity. Promoter fragments were subcloned into pBI101 upstream of the *uidA* reporter gene, which encodes for the GUS reporter enzyme (Jefferson et al. 1987). These Ti plasmid constructs, termed *–90-GUS* (wild-type *as-1*) and *–76-GUS* (mutant *as-1*), respectively, were then transferred to *Agrobacterium tumefaciens* strain GV3101 by tri-parental mating. As a control for some experiments, a Ti vector containing the full-length CaMV 35S promoter and *uidA* (GUS) reporter gene (pBI121; Clontech) was also transformed into *Arabidopsis*. In all cases, germinal tissues of flowering *Arabidopsis thaliana* (Col.) plants were infiltrated and transformed with *Agrobacterium* strains containing these Ti plasmids by vacuum-infiltration as described. Mature seeds from these plants were collected, surface-sterilized, and germinated at 24°C on half-strength MS (Murashige and Skoog 1962) agar medium that contained minimal vitamins and 50 µg/ml kanamycin, under a photoperiod of 16/8-h (day/night) with light supplied by fluorescent lighting (approx. 700 foot candles). Kanamycin-resistant T1 seedlings were transferred to soil and allowed to set seed. Individual lines of T2 progeny that were homozygous for the transgene were identified by segregation analysis and used in experiments.

In all studies, seeds from transgenic *Arabidopsis* were surface-sterilized, washed with water, and germinated in the dark on sterile Whatman paper that was placed on a thin layer of agar containing half-strength MS salts and minimal vitamins. Two days post-germination, uniformly sized seedlings were transferred to 8-well tissue-culture plates (Costar) containing 2 ml of sterile half-strength MS salts and incubated at 24–26°C under a 16/8-h (day/night) photoperiod. As indicated, seedlings and plants were treated for 20 h in half-strength MS salts supplemented with either only 0.1% ethanol as carrier solvent (mock) or 100 µ*M* of each compound to be tested in carrier solvent. The compounds to be tested were: 2,4-dichlorophenoxyacetic acid (2,4-D); 2,3-dichlorophenoxyacetic acid (2,3-D); naphthaleneacetic acid (NAA); indole-3-acetic acid (IAA); salicylic acid (SA), and Dual 8E herbicide (kind gift of Dr. Gary Schnappinger, Novartis Agrochemicals). Seedlings and plants were vacuum-infiltrated, fixed in 1% (w/v) paraformaldehyde for 30 min at room temperature, and processed for GUS histochemical staining as described (Jefferson et al. 1987). Stained material was photographed using a Zeiss stereomicroscope with illumination by tungsten light.

Bacterial cultures and inoculations

Pseudomonas syringae strains PsmES4326 (virulent) and PstDC3000 (avirulent) were obtained from the American Type Tissue Collection (ATCC). Bacteria were cultured on LB agar without antibiotics and then grown in KB broth, washed in 10 m*M* $MgSO_4$, and adjusted with same to a final concentration of 10^6 cfu/ml. Plants were germinated directly in a commercial soil mix (Metro Mix) and grown to the four-leaf stage under the above conditions. One lower leaf from each plant was then hand-inoculated with a bacterial suspension or 10 m*M* $MgSO_4$ (mock control) using a syringe until a water-soaked spot of 5–7 mm in diameter was apparent. At 48-h post-inoculation, when plant symptoms had just begun to occur, the inoculated leaf was harvested, frozen in liquid nitrogen, and stored at –80°C until assayed as described below.

Methyl umbelliferone glucuronidate assay

Frozen leaves (more than ten) were extracted to obtain soluble protein, which was quantified as previously described (Jefferson 1987). GUS activity was assessed using a standard methyl umbelliferone glucuronidate (MUG) assay, and the results were analyzed for statistical significance.

Results and discussion

Developmental changes in *as-1*-dependent transcription

GUS activity was histochemically detected in the roots and shoots of homozygous *–90-GUS* transgenic seedlings and young plants (Fig. 1). In contrast, we were unable to detect any GUS activity in transgenic seedlings or plants bearing a single copy of *–76-GUS* (data not shown), a construct that contains the GUS reporter gene under the control of a mutant *as-1* element, which lacks the upstream half-site, and the 35S minimal promoter. GUS histochemical activity in mock-treated control and 2,4-D-treated seedlings that bear *–90-GUS*, a GUS reporter gene construct under the control of an intact *as-1* element and the same minimal promoter, was differentially expressed between 3 days and 14 days post-germination with respect to its tissue-specific pattern and degree of 2,4-D induction (Fig. 1). Thus, at 3 days post-germination, basal GUS activity of mock-treated plants was primarily restricted to the vascular system of the cotyledons (Fig. 1a), while 2,4-D-treated seedlings showed comparatively strong GUS activities in cotyledons, stems, and roots (Fig. 1b). In contrast, at 7 days post-germination, basal GUS activity was largely restricted to a small region in the cotyledon and, to a more variable extent, to the primary root (Fig. 1c). In response to 2,4-D, GUS activity was stimulated in the root and stem vascular system and in the primary root tip but was generally absent from much of the cotyledon (Fig. 1d). Relative to findings with seedlings at 7 days post-germination, little or no GUS activity was observed in mock- or 2,4-D-treated plants at 10 days post-germination (Fig. 1e, f), apart from faint GUS activity in the vascular system of the first true leaves. A similar overall pattern of GUS staining was also seen in mock-treated plants at 14 days post-germination (Fig. 1g), although in this case 2,4-D treatment stimulated GUS activity in both the root and shoot vascular systems (Fig. 1h). This staining was largely absent from the meristem and root cap of primary roots (Fig. 1i, arrow), whereas strong staining of the meristems of emerging lateral roots was commonly observed (Fig. 1j). In contrast, seedlings containing a single copy of the full-length 35S promoter upstream of the GUS reporter gene in pBI121 (Fig. 1k) showed strong constitutive GUS activity under either basal (left seedling) or 2,4-D-induced (right seedling) conditions.

Tissue-specific differences in *as-1* activity occur in response to diverse xenobiotic agents and defense cues

Differential patterns of *as-1*-dependent GUS reporter activity in seedlings at 7 days post-germination occurred in response to micromolar concentrations of plant auxin hormones (IAA, NAA, and 2,4-D), a biologically "inactive" auxin analogue (2,3-D), a plant defense hormone salicylic acid (SA), and the herbicide Dual (Fig. 2). Among the auxin analogues, 2,4-D and 2,3-D showed

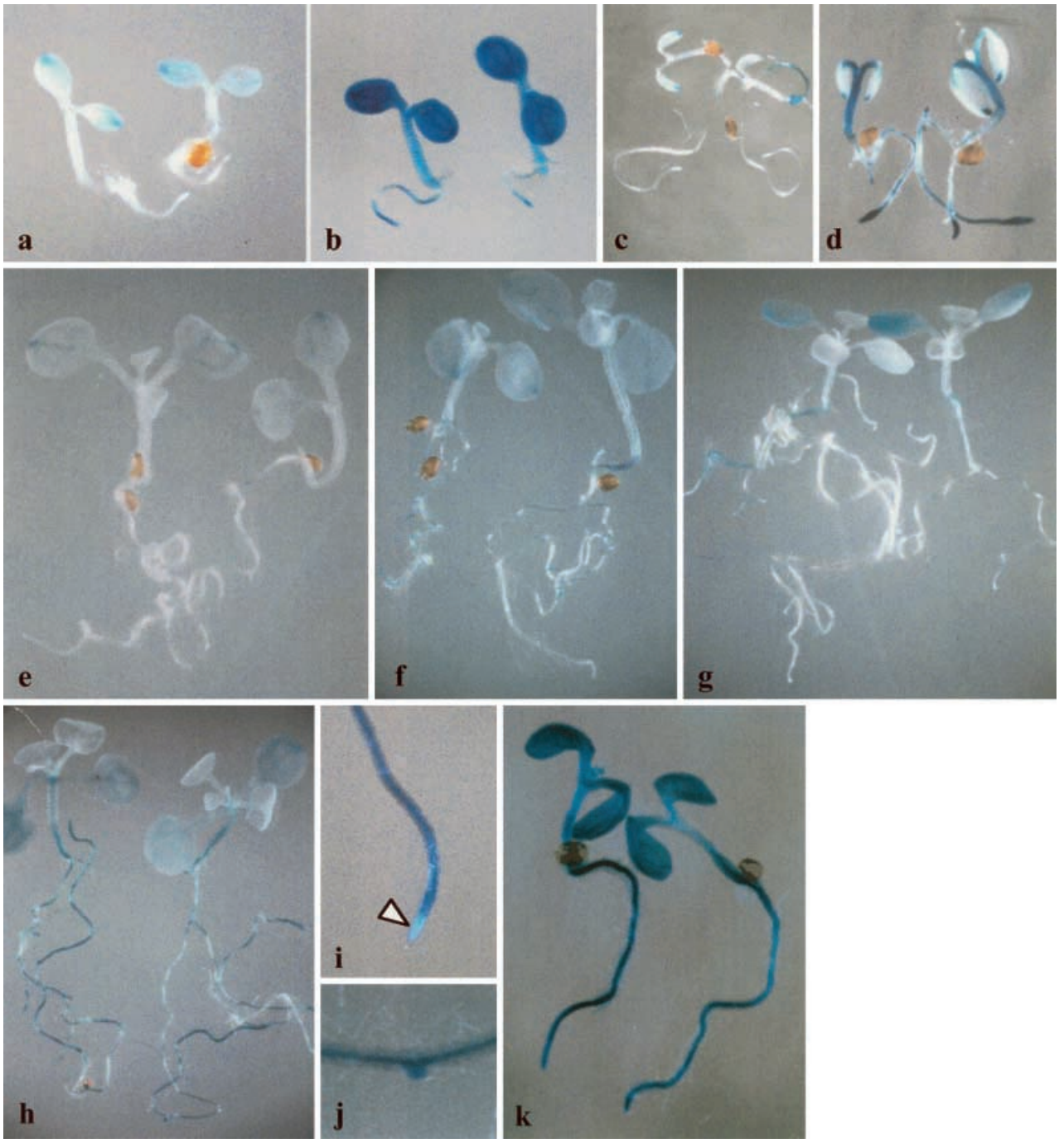

Fig. 1a–k Plant development and *as-1*-dependent transcription. Transgenic *–90-GUS* seedlings or young plants at 3 days (**a**, **b**), 7 days (**c**, **d**), 10 days (**e**, **f**), and 14 days (**g–j**) post-germination were either mock- (**a**, **c**, **e**, **g**) or 2,4-D-treated (**b**, **d**, **f**, **h–j**) for 6 h, fixed, and stained for GUS activity. For comparative purposes, the remaining panel (**k**) shows the staining of 14-day-old transgenic seedlings containing the full-length CaMV 35S promoter and GUS reporter gene. The *left* seedling was mock-treated, whereas the *right* seedling was treated with 2,4-D as above

similar patterns of GUS expression. Because 2,3-D lacks auxin hormone activity, its effect on *as-1*-dependent gene expression has been attributed to a mechanism involving xenobiotic chemical stress (Ulmasov et al. 1995). We surmise that treatments with physiologically high (i.e., micromolar) concentrations of NAA may similarly activate *as-1* through a mechanism involving xenobiotic stress, as physiological (i.e., nanomolar) concentrations of this hormone failed to potentiate transcription through this element (data not shown). However, changes in *as-1* activity induced by micromolar concentrations of a different active auxin, IAA, were limited to the cotyledons, whereas treatments with the plant defense hor-

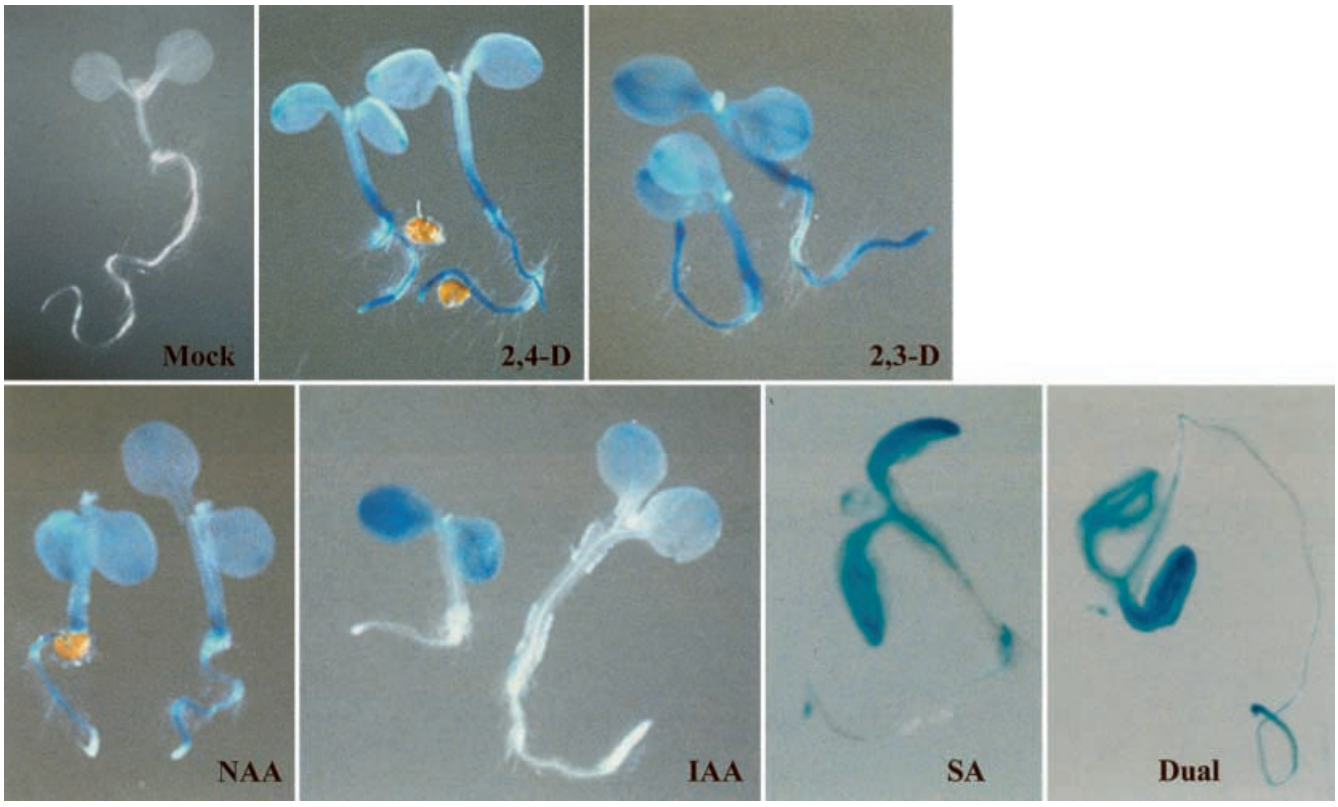

Fig. 2 Tissue-specific effects of different chemical stress cues on *as-1* activity. Transgenic *–90-GUS* seedlings 7 days post-germination were treated for 6 h with carrier solvent alone (*mock*) or with 2,4-D, 2,3-D, NAA, IAA, SA, and Dual (herbicide) as indicated, fixed, and then stained to detect GUS activity

mone SA and the herbicide Dual led to strong GUS expression throughout the cotyledons, stem, and the root vascular system and tip. Thus, the evidence here suggests that plant growth or defense hormones and xenobiotic stress cues may operate through different uptake or response pathways in plants to potentiate *as-1*-dependent transcription.

As-1-dependent transcription is activated by a virulent, but not avirulent, bacterial pathogen

The expression of *–90-GUS* transgenic plants (14 days post-germination) inoculated with either 10 m*M* $MgS0_4$ (mock) or avirulent and virulent strains of *P. syringae* was determined by use of a fluorometric MUG assay. Forty-eight hours post-treatment, mock-treated leaves appeared to be normal in appearance, whereas leaves inoculated with avirulent or virulent strains of *P. syringae* showed, respectively, either a hypersensitive response, as reflected by initial browning and cell death at the infection site, or a susceptible interaction as evidenced by the presence of an incipient yellow chlorotic halo. Results of MUG assays showed that mock-treated leaves and leaves that had been infected with the avirulent strain (PstDC3000) of this bacterium had similar levels of expression of the GUS reporter gene (Table 1). In contrast, leaves from *–90-GUS* plants infected with the virulent strain (PsmES4326) had fourfold higher GUS activity than that seen with mock controls, while GUS activity was undetected in mock or inoculated *–76-GUS* plants (data not shown). These findings indicate that the GUS activity of *–90-GUS* plants requires the *as-1* element. The differences in reporter gene activity noted above were not due to an effect of infection on either GUS enzyme activity or stability, as no differences were seen in GUS activity of mock or inoculated pBI121 transgenic plants. This activity is under the control of the full-length 35S promoter which, unlike *–90-GUS*, is highly expressed throughout the plant (Fig. 1). Thus, these results indicate that leaf-specific *cis*-regulatory sequences, such as the GT-1 element in the full-length 35S, are likely to have masked the comparatively weaker activity of *as-1* in this promoter.

The occurrence of functional *as-1*-type elements in the promoters of plant pathogen genes and in nuclear genes involved in plant protection has led to the suggestion that these elements may have opposing roles in regulating the expression of genes that modulate the host-pathogen outcomes (Bouchez et al. 1989). Plant genes that are regulated by *as-1* elements include members of the glutathione S-transferase (GST) and pathogenesis-related protein (PR) gene families, which have been implicated in host detoxification and defense, respectively (Mauch and Dudler 1993; Ulmasov et al. 1995; Xiang et al. 1996; Strompen et al. 1998; Chen and Singh 1999). A common trait of these *as-1*-regulated genes is that their

Table 1 Differential effects of avirulent and virulent *Pseudomonas syringae* on *as-1* activity. GUS reporter activity in leaves of intact plants following treatment with either 10 m*M* $MgSO_4$ (mock) or with avirulent (PstDC3000) or virulent (PsmES4326) strains of *P. syringae*, and incubation for 2 days. Inoculated leaves were harvested, extracted for protein, and analyzed for GUS enzyme activity using a fluorometric MUG assay. Mean and standard error of results of two to three independent experiments are shown. GUS activity was expressed in arbitrary units (fluorescent units per microgram protein per minute) that were normalized in each data set to the mock-treated sample values

Transgenic line	Treatment	GUS activity
–90-Gus	Mock	1.0 (0.3)
	PstDC3000	0.9 (0.4)
	PsmES4326	4.0 (0.3)
pBI121	Mock	1.0 (0.3)
	PstDC3000	0.9 (0.2)
	PsmES4326	0.4 (0.2)

expression is up-regulated in response to infection (PR and GST genes) or chemical stress (GST genes). Accumulating evidence indicates that the activation of these protective genes through *as-1* in response to abiotic or biotic stress involves one or more cognate plant TGA transcription factors (Pascuzzi et al. 1998; Zhang et al. 1999; Despres et al. 2000; Niggeweg et al. 2000; Zhou et al. 2000; Johnson et al. 2001b). However, despite our growing knowledge of *as-1*-dependent transcription, prior studies have not to date systematically examined the role that development plays in the response of this element to stimulus-induction. As evidenced here with transgenic *Arabidopsis*, cross-talk between tissue-specific or developmental controls with chemical induction pathways is likely to play an important part in governing spatial and temporal expression of *as-1* activity. Although the exact molecular basis for differential patterns of *as-1* activity in planta is at present unknown, such differences may involve developmentally associated changes in the rate of transport, uptake or metabolism of chemical inductive cues, or result from tissue-specific differences involving signal intermediates upstream of this transcription response pathway. With respect to the latter possibility, a nuclear intermediate termed p120 has been recently implicated in the regulation of TGA1a, a tobacco TGA factor that governs *as-1* activity in response to xenobiotic chemical stress (Johnson et al. 2001a). To help discriminate among these possibilities, we are currently using genetic screens of *Arabidopsis* to identify genes that selectively discriminate between developmental and stimulus-induction activities of *as-1*.

Findings here indicate that *as-1* imparts a complex and dynamic pattern of activity on a minimal promoter in response to abiotic and biotic cues within a plastic program of plant development. In *Arabidopsis* and tobacco, these changes closely match the patterns of expression of several known *as-1*-regulated GST genes, such as *GST6* of *Arabidopsis*, *GNT1*, *GNT35* and *ParA* of tobacco, and *GH2/4* of soybean (Droog et al. 1995; Ulmasov et al. 1995; Xiang et al. 1996; Chen and Singh 1999). When placed upstream of a GUS reporter gene, these plant promoters show patterns of cellular expression similar to those observed both here and elsewhere (Fromm et al. 1989, l991) with a single *as-1*-type element and minimal promoter. In all cases, the presence of a functional *as-1* element was essential, for conferring a transcriptional response to defense cues or xenobiotic stress compounds and for the enhanced target gene activity seen in leaves following infection with a virulent strain of *P. syringae*. This latter response is particularly interesting, given that *as-1*-regulated PR and GST genes may have early protective roles in host-defense against pathogens (Dixon et al. 1998; Edwards et al. 2000; Mauch and Dudler 1993; Strompen et al. 1998; Zhang et al. 1999). As inoculation with an avirulent strain of *P. syringae* induced a classical hypersensitive response by the host but failed to enhance *as-1* activity, we suggest that this element plays little or no role in mediating the expression of genes involved in this type of host-defense response. Alternatively, accumulating evidence suggests a potential role for this element in systemic-acquired resistance-mediated activation of PR gene expression in tobacco and *Arabidopsis* (Lebel et al. 1998; Zhang et al. 1999).

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