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Short Communication

Changes in in vivo DNA-protein Interactions in Pea Phenylalanine Ammonia-lyase and Chalcone Synthase Gene Promoter Induced by Fungal Signal Molecules

Hikaru Seki, Yumi Nagasugi, Yuki Ichinose, Tomonori Shiraishi and Tetsuji Yamada [1]

Laboratory of Plant Pathology & Genetic Engineering, College of Agriculture, Okayama University, Tsushima, Okayama, 700 Japan

Elicitor-induced protein bindings were detected on the several particular sequence motifs in two members of the pea *PAL* and *CHS* gene promoters by in vivo footprinting analyses. However, elicitor-induced changes rapidly reverted back to the uninduced pattern after the subsequent application of suppressor from *M. pinodes*.

Key words: Chalcone synthase (EC 2.3.1.74) — Elicitor — In vivo footprinting — Phenylalanine ammonia-lyase (EC 4.3.1.5) — *Pisum sativum* — Suppressor.

In the interaction between plants and phytopathogens, plants have evolved defense mechanisms such as the production of antifungal compound, phytoalexins, pathogenesis-related (PR) proteins and proteins to strengthen their cell walls (for review, see Dixon and Lamb 1990). Conversely, pathogens of compatible hosts are well equipped with sophisticated systems that allow them to circumvent the defenses of their hosts. Pycnospore germination fluid of *Mycosphaerella pinodes*, a pathogenic fungus on pea, contains both an elicitor and a suppressor for the plant defense responses such as the accumulation of phytoalexin (Shiraishi et al. 1991, 1994, Yamada et al. 1989).

Earlier, we have revealed by nuclear run-on assay that the transcriptional rates of the genes encoding phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), key enzymes involved in the biosynthesis of phytoalexin in pea (*Pisum sativum* L.), were rapidly activated in response to elicitor; in contrast, suppressor drastically and rapidly deactivated the elicitor-induced transcription of these genes (Kato et al. 1995, Wada et al. 1995). Furthermore, we have shown that the TATA-proximal regions of *PSPAL1, PSPAL2, PSCHS1* and *PSCHS2* contain the ability to respond to elicitor or suppressor in transient transfection assays (An et al. 1993, Murakami et al. 1997, Seki et al. 1996, 1997, Yamada et al. 1994). Sequence comparison revealed that these four genes shared a putative *cis*-acting element, an AC-rich element of Box I in their TATA-proximal region (Ito et al. 1997, Seki et al. 1997, Yamada et al. 1992, 1994). Box I (comparable to Box L in parsley *PAL* gene promoters, see Fig. 4) was initially identified as an elicitor- and UV-inducible in vivo DNA footprint on the parsley *PAL1* gene promoter by Lois et al. (1989). Sequences similar to Box I have been found in the promoters of several phenylpropanoid genes from different plant species including *Arabidopsis PAL* (Ohl et al. 1990), bean *PAL* (Cramer et al. 1989) and *CHS* (Loake et al. 1992), parsley 4-coumarate:CoA ligase genes (Douglas et al. 1991), and other parsley *PAL* genes (Logemann et al. 1995).

Recently, we have reported that the pea *CHS* genes form a small multigene family with at least eight members, which can be divided into two major groups, an elicitor-inducible (*PSCHS1*~*5* and *PSCHS8*) and a non-inducible group (*PSCHS6* and *PSCHS7*) (Ito et al. 1997). Interestingly, we found that only the elicitor-inducible group (*PSCHS1*~*5*) shared an adjacent G-Box and Box I at similar positions in TATA-proximal region (however, the G-Box is absent in *PSCHS2*) (Ito et al. 1997, Seki et al. 1997), although no information on the promoter sequence of *PSCHS8* was yet available. Box I is also highly homologous to the H-Box in the bean *chs15* gene promoter (Droge-Laser et al. 1997, Faktor et al. 1996, Loake et al. 1992) (see Fig. 4). The presence of the adjacent G-Box and Box I/H-Box like motifs near the TATA-box is conserved in numerous *CHS* gene promoters (for review, see Faktor et al. 1996), and their importance as regulatory motifs has been shown in some cases. In the parsley *CHS* gene, the unit of ACE^{CHS} (closely resembles G-Box) and MRE^{CHS} (highly homologous to the Box I in pea *PAL* and *CHS* genes, see Fig. 4) is sufficient to mediate light-induced expression (Feldbrugge et al. 1997, Weisshaar et al. 1991). The ACE^{CHS} and MRE^{CHS} were identified as UV-induced in vivo footprints (originally designated as Box II and Box I, respectively) in the parsley *CHS* gene promoter by Schulze-Lefert et al. (1989). A similar structure in the *CHS1* promoter of white mustard has also been demonstrated to be essential for light response (Kaiser et al. 1995).

Abbreviations: CHS, chalcone synthase; LMPCR, ligation-mediated polymerase chain reaction; PAL, phenylalanine ammonia-lyase.

[1] To whom correspondence should be addressed.

The combination of the G-Box and H-Box motifs is necessary for transcriptional activation of bean *chs15* gene by a phenylpropanoid-pathway intermediate, *p*-coumaric acid (Loake et al. 1992).

Herein we report the results of in vivo dimethyl sulfate (DMS) footprinting, coupled with the ligation-mediated PCR (LMPCR) method, in two pea *PAL* (*PSPAL1* and *PSPAL2*) and *CHS* (*PSCHS1* and *PSCHS2*) gene promoters, in particular, at the locations of the Box I or G-Box (Fig. 3). To our knowledge, the application of this in vivo footprinting method for the identification of elicitor-responsive elements has been reported only in very few cases, as in a member of parsley *PAL* (Lois et al. 1989) and *PR 1* genes (Meier et al. 1991).

Our present results may be the first to demonstrate elicitor- and suppressor-induced changes in in vivo DNA-protein interactions in two coordinately-regulated members of the *PAL* and *CHS* genes; our data clarified that the timing and duration of protein bindings to the conserved sequence motifs were strictly regulated in response to the elicitor, depending on the individual member of pea *PAL* and *CHS* genes.

Elicitor-induced changes in DNA-protein interactions in PSPAL gene promoters—To follow the appearance of the elicitor-induced footprints, a time-course experiment was performed (Fig. 1). Etiolated pea epicotyls were sliced longitudinally and treated with elicitor from *Mycosphaerella pinodes* (500 μg ml^{-1} glucose equivalent) for 0, 30, 60 or 180 min. In this study, we did not observe the distinctive protection at G residues but observed partial protection in elicitor-treated epicotyl tissues. This is presumed to be due to the heterogeneous cell population in the epicotyl tissues; all cells might not respond simultaneously to signal molecules in this system. In our preliminal results, water treatment instead of the elicitor did not cause any significant changes in DNA-protein interactions within 90 min after treatment (data not shown). Thus, the footprints we have observed are mainly due to the elicitor-treatment, not to the wounding itself (data not shown).

We mainly focused on examining the region where Box I is located, ranging from −131 to −67 of the *PSPAL1* (Fig. 3a), and −190 to −136 of the *PSPAL2* gene promoter (Fig. 3b). The footprints were induced on the Box I motif in both *PSPAL1* and *PSPAL2* gene promoters by elicitor-treatment. In *PSPAL1* (Fig. 1a), inducible footprints on the Box I were detected at 60 min after elicitor-treatment (compare lane 0 and E60); however, they were no longer evident at 180 min after elicitor-treatment. Densitometric scans of the lane 0 (filled line) and E60 (dotted line) given below provide more quantitative comparison of the relative band intensities.

In *PSPAL2* (Fig. 1b), footprints on Box I appeared most prominent at 180 min after elicitor-treatment (compare lane 0 and E180; densitometric scans of the lane 0 (filled line) and E180 (dotted line) are given below).

These observations, together with the conservation of the Box I motif in a number of phenylpropanoid genes, strongly suggest that Box I acts as one of the elicitor-responsive *cis*-elements in *PSPAL* gene promoters. We also found that G residues at positions −114 and −111 (upstream from Box I), and −84, −77 and −74 (downstream from Box I) in *PSPAL1*, appear to be protected at 60 min after elicitor-treatment (Fig. 1a). These sequences may also participate on the elicitor-mediated activation; complementary transient expression analysis, however, will be indispensable for evaluating their possible contribution.

Additionally, in *PSPAL1* (Fig. 1a), we also detected the protection without elicitor-induction (compare lane Naked and 0) at G residues at positions −123 and −120 (indicated by open arrowheads). Interestingly, the level of the protection as observed in the uninduced-state (lane 0) was significantly reduced by the elicitor-treatment (see lane E30 and E60). Moreover, elicitor-induced hyper-reactivity at A residue at position −122 (indicated by the open circle) was detected at 60 min after elicitor-treatment (see lane E60). DMS reacts preferentially with N7 position of guanines, but, in some cases, it also reacts, to a lower extent, with the N3 position of adenines (Maxam and Gilbert 1977, Strauss et al. 1992, Vernhettes et al. 1997). These observations lead us to speculate that the region around −122 might be involved in the negative regulation of the *PSPAL1* gene expression in the uninduced-state; however, the protein binding is reduced in response to the elicitor-mediated signal to facilitate the transcriptional activation.

Elicitor-induced changes in DNA-protein interactions in PSCHS gene promoters—We examined the DNA-protein interactions in the regions ranging from −136 to −41 of the *PSCHS1*, and from −120 to −50 of the *PSCHS2* gene promoter. As depicted in Fig. 3c and 3d, *PSCHS1* contains the G-Box and Box I, and *PSCHS2* also contains Box I at a similar position. In addition, we also found consecutive repeats of the AGCC sequence 5′-further upstream of Box I in both *PSCHS1* and *PSCHS2*.

In *PSCHS1* (Fig. 2a), elicitor-induced footprints were detected on Box I, the G-Box, and the AGCC motif. All these induced footprints were most prominent at 180 min after elicitor-treatment (compare lane 0 and E180; densitometric scans of the lane 0 (filled line) and E180 (dotted line) are given below).

Our previous work on the *PSCHS1* gene has demonstrated that mutations in either Box I (base substitution at nucleotide position −64 and −63) or the G-Box (base substitution at nucleotide position −84 and −83) completely abolished the elicitor-responsiveness and reduced the basal expression (Seki et al. 1997). This finding, together with the result obtained from in vivo footprint-

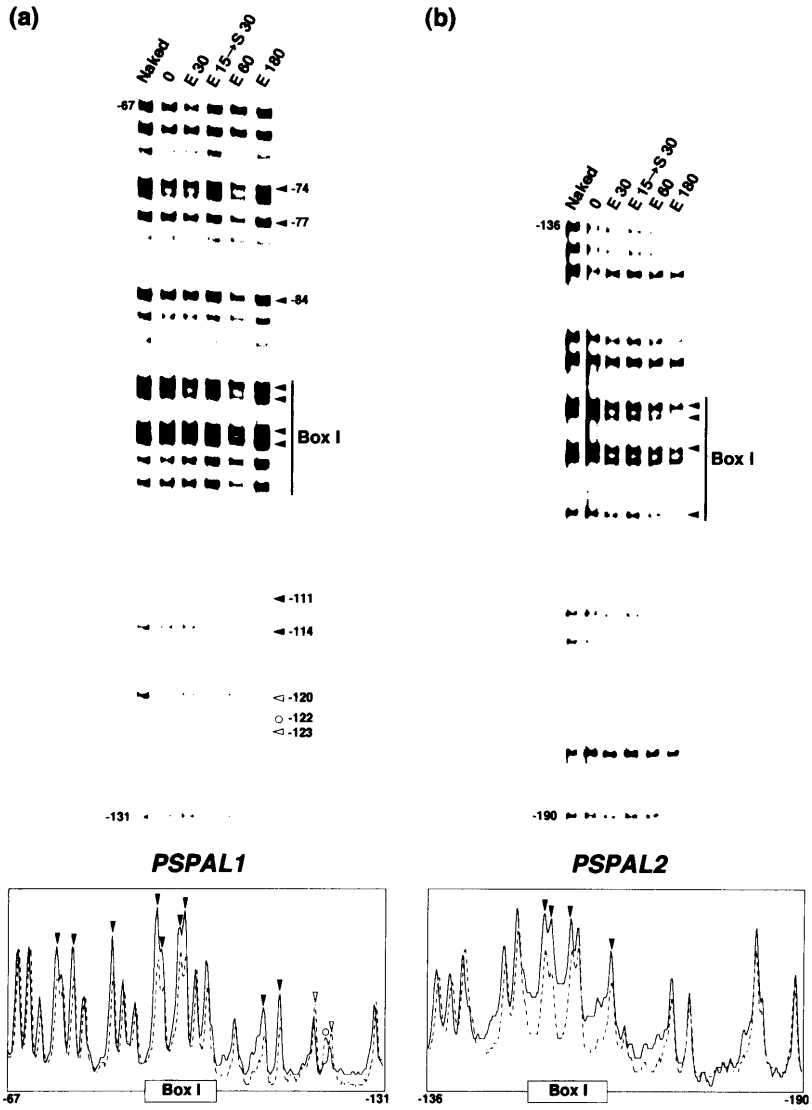

Fig. 1 Elicitor- and suppressor-induced changes in DNA-protein interactions in *PSPAL* gene promoters. Pea epicotyls were treated with DMS in vivo after 0 min (0, uninduced), 30 min (E30), 60 min (E60), or 180 min (E180) of elicitor-induction and after 15 min of elicitor-induction followed by 15 min of suppressor-treatment (E15 → S30). Lane, Naked represents the protein-free (naked) DNA treated with DMS in vitro. (a) The bottom strand from −131 to −67 of *PSPAL1* promoter was visualized. Scanning of the footprint is shown below (filled line represents lane 0 and the dotted line represents lane E60). Closed arrowheads represent the position of elicitor-induced protections from DMS methylation. Open arrowheads and the open circle represent the position of elicitor-induced enhancements of DMS methylation at G (−123 and −120) or A (−122) residue, respectively. (b) The bottom strand from −190 to −136 of *PSPAL2* promoter was visualized. Scanning of the footprint is shown below (filled line represents lane 0 and the dotted line represents lane E180. Closed arrowheads represent the position of elicitor-induced protections from DMS methylation. The extent of the putative *cis*-elements mentioned in the text are indicated with a bar at the right side of the sequence ladder. Numbers noted on the side of the sequence ladder refer to the position of the nucleotides relative to the transcription start site. The pea tissues were prepared and treated with elicitor and suppressor as described by Kato et al. (1995). Crude elicitor and suppressor fractions were prepared from the spore germination fluid of *M. pinodes* OMP-1 (IFO-30342, ATCC-42741) by the methods of Yamada et al. (1989). Segments of pea epicotyls were exposed to 1% DMS (Aldrich Chemical Company, USA) solution for 2 min at room temperature. Genomic DNA was isolated essentially as described by Dellaporta et al. (1983), followed by three cycles of phenol extraction and one phenol–chloroform–isoamylalcohol (24 : 24 : 1) extraction. An additional purification step, chloroform extraction in the presence of 1% CTAB (cetyltrimethylammonium bromide) and 0.7 M NaCl was performed as described by Murray and Thompson (1980). In vivo or in vitro methylated DNA was then cleaved at methylated guanines with 1 M piperidine as described by Mueller et al. (1992). LMPCR was carried out essentially as described by Mueller et al. (1992) with slight modifications. The third gene-specific primers were labeled using 5′ oligonucleotide Texas Red labeling kit (Amersham), and sequence ladders were analyzed on a Hitachi SQ-5500 (Hitachi, Tokyo, Japan). The densitometric scanning analyses were carried out with the FRAGLYS (Hitachi Electronics Engineering, Tokyo, Japan). Two independent footprinting analyses were performed; they showed similar results.

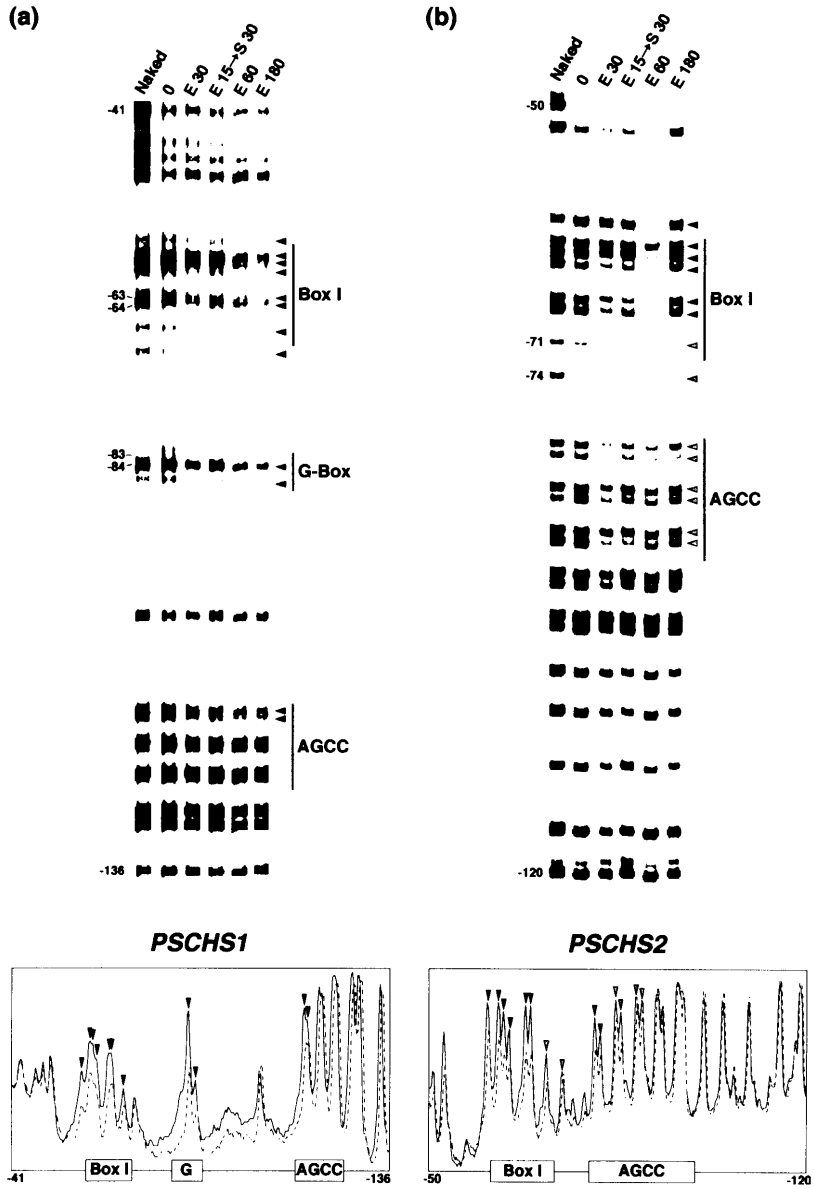

Fig. 2 Elicitor- and suppressor-induced changes in DNA-protein interactions in *PSCHS* gene promoters. (a) The bottom strand from −136 to −41 of *PSCHS1* promoter was visualized. Scanning of the footprint is shown below (filled line represents lane 0 and the dotted line represents lane E180). Closed arrowheads represent the position of elicitor-induced protections from DMS methylation. (b) The bottom strand from −120 to −50 of *PSCHS2* promoter was visualized. Scanning of the footprint is shown below (filled line represents lane 0 and the dotted line represents lane E60). Closed and gray arrowheads represent the position of elicitor-induced protections from DMS methylation; they were most prominent at 60 min (closed arrowheads) or 30 min (gray arrowheads) after elicitor-treatment, respectively. Lanes are the same as in Fig. 1. The extent of the putative *cis*-elements mentioned in the text are indicated with a bar at the right side of the sequence ladder. Two independent footprinting analyses were performed; they showed similar results.

ing analysis in *PSCHS1*, strongly suggests that the combination of Box I and the G-Box is significantly important in elicitor-mediated activation of *PSCHS1* gene. This proposal is supported by the results obtained with the bean *chs15* gene. Very recently, a soybean cDNA encoding a novel bZIP (basic-region leucine-zipper) protein, G/HBF-1, which binds to both the G-Box and the adjacent H-box (highly homologous to Box I, see Fig. 4) in the proximal region of the bean *chs15* promoter has been cloned (Droge-Laser et al. 1997). Although the level of the G/HBF-1 transcript and the subsequent protein product do not increase during the induction of *PAL* and *CHS* transcription, G/HBF-1 is rapidly phosphorylated in elicited cells and phosphorylation enhances binding to the *chs15*

promoter (Droge-Laser et al. 1997).

In *PSCHS2* (Fig. 2b), footprints were induced on the Box I and AGCC motifs; however, the timing of the appearance of these footprints differs between these two sequence motifs. As indicated by the closed arrowheads, induced footprints on Box I (except for the G residue at position −71) were most prominent at 60 min after elicitor-treatment (compare lane 0 and E60; densitometric scans of the lane 0 (filled line) and E60 (dotted line) are given below), but almost completely restored at 180 min after elicitor-treatment (see lane E180). On the other hand, as indicated by the gray arrowheads, footprints on AGCC motifs and on G residues at positions −74 and −71 were most prominent at 30 min after elicitor-treatment (compare lane 0 and E30), but nearly restored to the uninduced state within next 30 min (see lane E60). These results indicate that Box I and AGCC motifs are presumably protected by independent DNA-binding proteins and that binding is differentially regulated in response to the elicitor.

In these experiments, we found a novel sequence motif, AGCC repeated sequences (Fig. 3c, d); this is a candidate for one of the elicitor-responsive elements common to *PSCHS1* and *PSCHS2*. Interestingly, *PSCHS2* contains the AGCC repeated sequences instead of the G-Box in other elicitor-inducible *PSCHS* genes, at a neighboring region 5′-upstream of Box I (compare Fig. 3c, d). This finding leads us to speculate that the AGCC repeated sequences might complement the absence of G-Box in *PSCHS2* and confer a specific mode of expression for *PSCHS2*. We have recently grouped pea *CHS* genes by S1 nuclease protection assay depending on their modes of expression (Ito et al. 1997). Among the elicitor-inducible members of *PSCHS* gene family (*PSCHS1*～*5* and *PSCHS8*), only *PSCHS1, PSCHS2*, and *PSCHS8* were constitutively expressed in flower organs (Ito et al. 1997). Interestingly, AGCC repeats are found in the promoter region of *PSCHS1* and *PSCHS2* (information on the promoter sequence of *PSCHS8* is not available yet), but not in *PSCHS3*～*5*. Thus, the pea *CHS* gene family provides a valuable model for assessing the function of each putative *cis*-element by comparing the structural features of promoter region and the regulation in differential expression patterns of the individual member. However, detailed analysis may be required to evaluate the significance of AGCC repeats in the regulation of *PSCHS1* and *PSCHS2*.

Putative trans-acting factors involved in the elicitor-

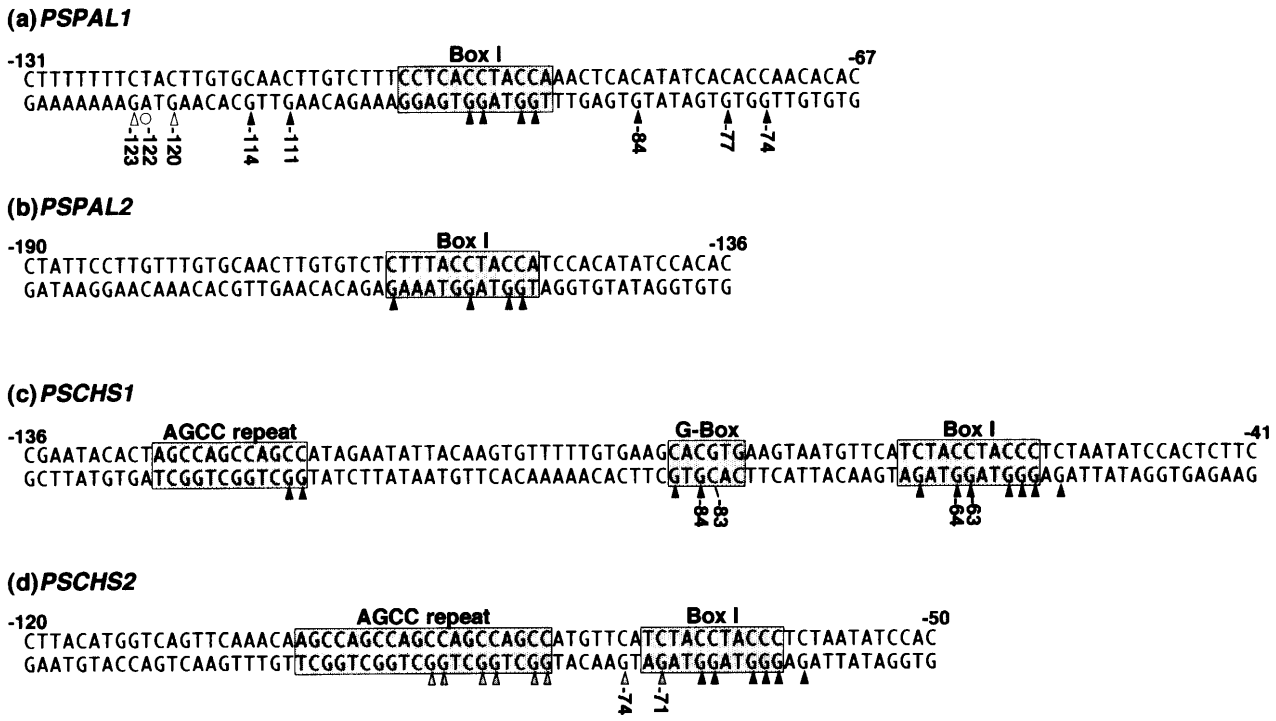

Fig. 3 Summary of the DNA-protein interactions in *PSPAL* and *PSCHS* gene promoters. Nucleotide sequences of the *PSPAL1* (a), *PSPAL2* (b), *PSCHS1* (c) and *PSCHS2* (d) promoter regions examined in in vivo footprinting analyses are listed. Sequences corresponding to Box I, the G-Box, and AGCC repeats are boxed in gray. Closed arrowheads represent the position of elicitor-induced protections from DMS methylation. Open arrowheads and the open circle in (a) represent the position of elicitor-induced enhancements of DMS methylation at G (open arrowheads) or A (open circle) residue, respectively. Closed and gray arrowheads in (d) represent the position of the induced protections from DMS; these were most prominent at 60 min (closed) or 30 min (gray) after the onset of elicitor-induction, respectively. Numbers refer to the position of the nucleotides relative to the transcription start site.

mediated activation—As mentioned above, we have identified multiple sequence motifs as candidates for elicitor-responsive *cis*-elements in pea *PAL* and *CHS* genes. We have also demonstrated that bindings of putative *trans*-acting factors to Box I or the AGCC motif are strictly regulated in vivo with respect to the timing and duration in response to the elicitor, depending on the individual member of the pea *PAL* and *CHS* gene groups. That is, protein bindings to Box I in *PSPAL2* (Fig. 1b) and in *PSCHS1* (Fig. 2a) are most prominent at 180 min after elicitor-treatment, but no longer obvious in *PSPAL1* (Fig. 1a) and in *PSCHS2* (Fig. 2b) at the same time point. Likewise, protein binding to the AGCC motif in *PSCHS1* (Fig. 2a) is most prominent at 180 min after elicitor-treatment, but no longer obvious in *PSCHS2* (Fig. 2b) at the same time point. It is tempting to speculate that different classes of binding proteins to Box I or the AGCC motif might be involved in the elicitor-mediated activation of pea *PAL* and *CHS* genes and confer highly specialized modes of expression for the individual pea *PAL* or *CHS* gene.

Recently, several Box I-related sequence motifs (see Fig. 4), MRE^{CHS} (light-responsive element) in parsley *CHS* (Feldbrugge et al. 1997), Box L in parsley *PAL1* (Logemann et al. 1995, Lois et al. 1989) and box P in bean *PAL2* (Sablowski et al. 1994), have been shown to be bound by plant Myb proteins. Feldbrugge et al. (1997) showed that MRE^{CHS} and Box L were bound by parsley Myb protein, *Pc*MYB1 and MYB 305 from *Antirrhinum majus*. Sablowski et al. (1994) demonstrated that MYB 305 bound to box P in the bean *PAL2* promoter. Interestingly, Sablowski et al. (1994) also demonstrated that activation from the bean *PAL2* promoter by MYB 305 requires a G-Box-like element in addition to box P. This is consistent with the observations in *PSCHS1*, in which the combination of Box I and G-Box is significantly important for the expression in pea protoplasts (Seki et al. 1997). The elicitor-induced footprints at both sequence motifs are observed in in vivo footprinting analysis (Fig. 2a, 3c). The interaction of a Myb protein with another transcription factor has been observed in some cases. For example, the maize Myb protein, C1, needs a Myc-type transcription factor (Goff et al. 1992), yeast Myb protein, BAS1, needs a homeodomain protein partner (BAS2) to activate the *HIS4* promoter (Tice-Baldwin et al. 1989). Likewise, it is possible that putative Box I binding protein(s) might choose their target genes depending on the availability of other factors, such as G-Box or AGCC motif binding factor(s), with which they cooperate.

PSPAL1 (Box I)	CCTCACCTACCA	-91
PSPAL2 (Box I)	CTTTACCTACCA	-151
PSCHS1 (Box I)	TCTACCTACCC	-57
PSCHS2 (Box I)	TCTACCTACCC	-62
PvCHS15 (H-Box)	CTCACCTACCC	-55
PcCHS (MRE^{CHS})	CCAACCTAACC	-131
PcPAL1 (Box L)	TCTCACCTACC	-107
PvPAL2 (box P)	GAACCTAACT	-246[a]

Fig. 4 Sequences related to Box I identified in promoters of phenylpropanoid biosynthetic genes. Nucleotide sequences with striking homology are shown in the black boxes. The numbers on the right indicate the position relative to the transcription start site. Abbreviations: *Ps, Pisum sativum*; *PAL1* and *PAL2* (Yamada et al., 1992, 1994), *CHS1* and *CHS2* (Ito et al. 1997, Seki et al. 1997), *Pv, Phaseolus vulgaris*; *CHS15* (Loake et al. 1992, Faktor et al. 1996), *PAL2* (Sablowski et al. 1994), *Pc, Petroselinum crispum*; *CHS* (Schulze-Lefert et al. 1989, Feldbrugge et al. 1997), *PAL1* (Lois et al. 1989, Logemann et al. 1995). [a] The sequence is found in the reverse orientation.

Suppressor-induced changes in DNA-protein interactions—To date, we have reported that suppressor from *M. pinodes* blocks the signal transduction pathway during the elicitor-mediated activation of the defense responses in pea by inhibiting cell wall-bound ATPases (Kiba et al. 1996), plasma membrane ATPase, and enzymes in polyphosphoinositide metabolism in plasma membrane (Shiraishi et al. 1994). Moreover, we have also revealed by nuclear run-on assay that the suppressor drastically deactivates the transcription of pea *PAL* and *CHS* genes within five to ten minutes, even when it is applied after elicitor-induced transcriptional rates reach their nearly maximal level (Kato et al. 1995, Wada et al. 1995).

To observe the changes in DNA-protein interactions during suppressor-mediated deactivation, suppressor was applied 15 min after elicitor-treatment of pea epicotyl tissues (see lane E15 → S30 of Fig. 1, 2). Consistent with the results from the nuclear run-on assay, subsequent application of suppressor reverted the elicitor-induced protections or enhancements to the uninduced pattern, although to some extent weakly (see lane E15 → S30 of Fig. 1, 2). The most illustrative example of this result could be seen in *PSCHS2* (compare lane 0, E30 and E15 → S30 in Fig. 2b). No obvious protein bindings specific for suppressor-mediated deactivation in these regions were detected. However, we cannot rule out the possibility that the suppressor-induced protein bindings occur at further 5′-upstream or 3′-downstream regions. It is also possible that suppressor-mediated signal deactivates the transcription machinery through the direct modification of the conformation of elicitor-induced binding complexes or RNA polymerase itself, although we do not have any evidence to support it.

At present, it is not conclusive whether suppressor initiates the independent signal transduction pathway, deactivating the defense genes as well as blocking the elicitor-mediated signal. However, initiation of an independent signal by suppressor is more plausible, because the changes in DNA-protein interactions as well as in transcriptional rates of *PAL* and *CHS* genes by suppressor-treatment were very rapid. Furthermore, suppressor is even capable of deactivating *PSPAL1* promoter induced by treatment with UV (Murakami et al. 1997). We believe, therefore, that identification of suppressor-responsive *cis*-elements and *trans*-acting factors will help in elucidating the mode of action of suppressors in compatible host-pathogen interactions.

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