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Cis-Regulatory Elements and *Trans*-acting Factors Involved in the Activation of a Member of Elicitor-Responsive Pea Chalcone Synthase Gene Family, *PSCHS2*

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To elucidate the elicitor-mediated transcriptional activation in one of the chalcone synthase genes, *PSCHS2* in pea, we investigated the putative *cis*-regulatory elements in the promoter sequence and trans-acting nuclear DNA binding proteins. The promoter up to −471 from the transcription start site of *PSCHS2* gave considerable level of basal transcriptional activity. Nuclear extract from elicitor-treated pea epicotyls formed DNA-protein complexes with three independent DNA fragments spanning from +83 to −484 of *PSCHS2* with low mobility (LMC, low mobility complex) in the gel mobility shift assay. Since the LMC formation was blocked by the treatment of nuclear extract with alkaline phosphatase, the phosphorylation of some nuclear factor(s) assists LMC formation. These results indicate that the bindings of the putative positive nuclear factors to the multiple *cis*-regulatory elements in *PSCHS2* promoter region were enhanced by elicitor-treatment that might result in transcriptional activation.

Key words : chalcone synthase, *cis*-acting elements, DNA binding proteins, elicitor, promoter activity

Introduction

In the interaction between plants and phytopathogens, plants have evolved their defense mechanisms, such as the production of antimicrobial compounds, phytoalexins, PR-proteins and proteins to strengthen their cell walls[3]. In leguminous plants, phytoalexins are isoflavonoid compounds, and pisatin is a major phytoalexin in pea. We have analyzed the regulation of phytoalexin production by the signal molecules, elicitor and suppressor, produced by pea blight phytopathogen, *Mycosphaerella pinodes* [5,13]. Chalcone synthase (CHS, EC 2.3.1.74) is a key enzyme of pisatin production in pea, which catalyzes the formation of naringenin-chalcone from one molecule of 4-coumaroyl-CoA and three malonyl-CoA[2]. *CHS*-mRNA is also known to be inducible within 1 hr by the treatment of etiolated pea epicotyls with elicitor of *M. pinodes* as well as phenylalanine ammonia-lyase (*PAL*) genes[13]. Recently we found that the accumulation of *PAL*-and *CHS*-mRNAs depend on transcriptional activation[8,12].

CHS genes constitute a multigene family in pea[1,6,7]. Very recently, we have investigated the organization of *CHS* gene family and the specific

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Abbreviations
CHS, chalcone synthase; LMC, low mobility complex; nt, nucleotides; PAL, phenylalanine ammonia-lyase; *PSPAL*, gene coding for phenylalanine ammonia-lyase in *Pisum sativum*; *PSCHS*, gene coding for chalcone synthase in *Pisum sativum*

expression of each member in pea[7]. In the pea genome, there are at least eight members of *CHS* genes. Among them expression of six members of *CHS* genes (*PSCHS1*, *2*, *3*, *4*, *5* and *8*) is induced in etiolated pea epicotyls by treatment with elicitor[7]. Two of these elicitor-inducible *CHS* genes, *PSCHS1* and *PSCHS2*, are tandemly clustered in about 9 kb[1]. *Cis*-acting elements and putative *trans*-acting factors in one of these members, *PSCHS1*, have been relatively well analyzed so far, and we have identified that at least five distinct *cis*-acting elements located from −242 to +78 were required for maximal activation by the elicitor[9,10]. In comparison of the putative regulatory sequence in the promoters of elicitor-inducible *CHS* genes in pea, we have found that only *PSCHS2* does not carry the G-Box-like sequence in its promoter[7] (Fig. 1). Since G-Box-like sequence is an indispensable element for the activation of basal expression level as well as elicitor-induction in *PSCHS1*[9], loss of G-Box-like sequence in *PSCHS2* is a striking feature.

In this paper, we have investigated the *cis*-acting elements involved in activation by the elicitor-treatment and the putative *trans*-acting factors that bind to the promoter sequence in *PSCHS2*. Furthermore, we discuss *PSCHS2*-specific mechanisms in the coordinated transcriptional activation of *CHS* multigene family by the elicitor.

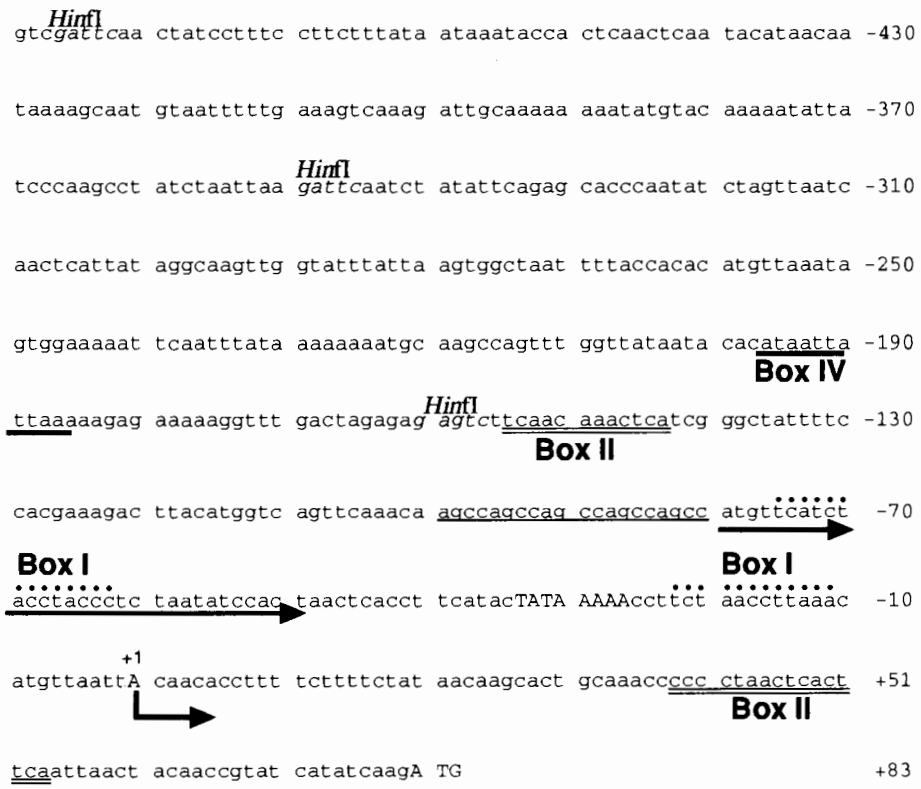

Fig. 1 The putative regulatory *cis*-acting elements in promoter sequence of *PSCHS2*.
TATA box, transcription start site (+1) and translation initiation codon, ATG, are shown with capital letters. The putative *cis*-regulatory elements, Box I, Box II and Box IV, are indicated as dots, double underlines and thick single underline, respectively. The identical 31 nucleotide sequence to the sequence of *PSCHS1* promoter is indicated with arrows below the sequence. Five repeats of AGCC sequence located just upstream from the identical 31 nucleotide sequence have a single underline. The restriction sites for *Hin*fI used in the preparation of labeled DNA fragments for gel mobility shift assay are shown with italic letters. Numbers on the right indicate the number of nucleotides from the transcriptional initiation site.

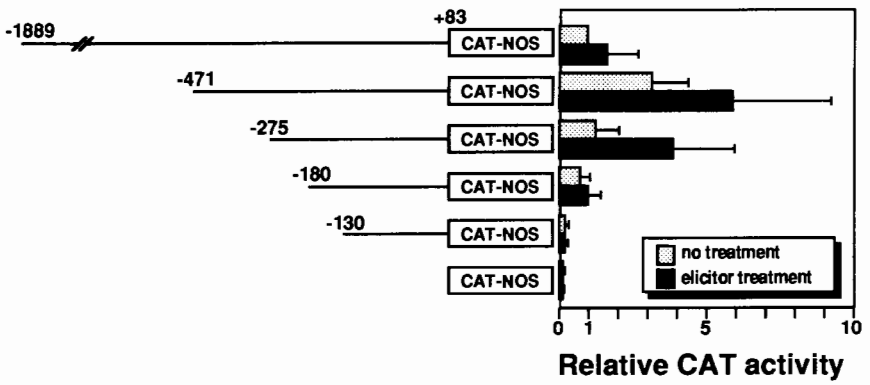

Fig. 2 Effect of 5'-deletions on the induction of CAT activity by the treatment with fungal elicitor in electroporated pea protoplasts.
Sequential 5'-nested deletions of the PSCHS2 promoter fragment, translationally fused to a CAT reporter gene, were created and electroporated into pea protoplasts. Numbers in the left panel denote the 5'-deletion end points and the position of the translational initiation codon (+83), respectively. Relative CAT activity in elicitor-treated (black bars) and mock-treated (dotted bars) pea protoplasts electroporated with individual chimeric constructs is depicted to that expressed in mock-treated protoplasts electroporated with the deletion up to -1889, with mock-treated being regarded as 1 in each individual experiment. Means and standard errors are from at least five independent experiments.

Materials and Methods

Plant Material

Pea (*Pisum sativum* L. cv. Midoriusui) was grown in darkness as described[10], and etiolated epicotyls were treated with water or elicitor prior to the preparation of nuclear extracts[8]. Pea suspension culture was described previously[4].

Preparation of fungal elicitor

Elicitor was prepared from the pycnospore germination fluid of *M. pinodes* as described[5,13], and used at 100 μg/ml as glucose equivalent as a final concentration.

Plasmid construction

pCNC2, a plasmid for transient transfection assay possessing a nearly full-length *PSCHS2* promoter and a bacterial chloramphenicol acetyltransferase (CAT) gene was constructed as a chimeric gene[1]. 5'-nested deletions were made from pCNC2 with unidirectional deletion procedure using Exonuclease III.

Preparation and labeling of DNA probes

For gel mobility shift assay, DNA fragments were prepared from *PSCHS2* promoter region after digestions with appropriate restriction enzymes and extraction from polyacrylamide gels subjected to electrophoresis, then labeled with ^{32}P-dNTPs by filling-in reaction with a Klenow enzyme or ^{32}P-ATP by T4 polynucleotide kinase after dephosphorylation.

CAT transient transfection assay

For transient transfection assay, protoplasts were prepared from pea suspension cultured cells, then plasmid DNAs possessing chimeric gene were introduced into the protoplasts by electroporation[4]. The activity of CAT was measured as described[10].

Gel mobility shift assay

Preparation of nuclear extract and the gel mobility shift assay were carried out as described previously[8,10].

Results and Discussion

As we have observed previously[1], we could confirm that a full-length promoter to −1889 gave a considerable level of CAT expression without elicitor treatment, and a deletion up to −471 gave about 3-fold basal CAT expression and about 2-fold induction upon elicitor-treatment (Fig. 2). However deletion up to −275,

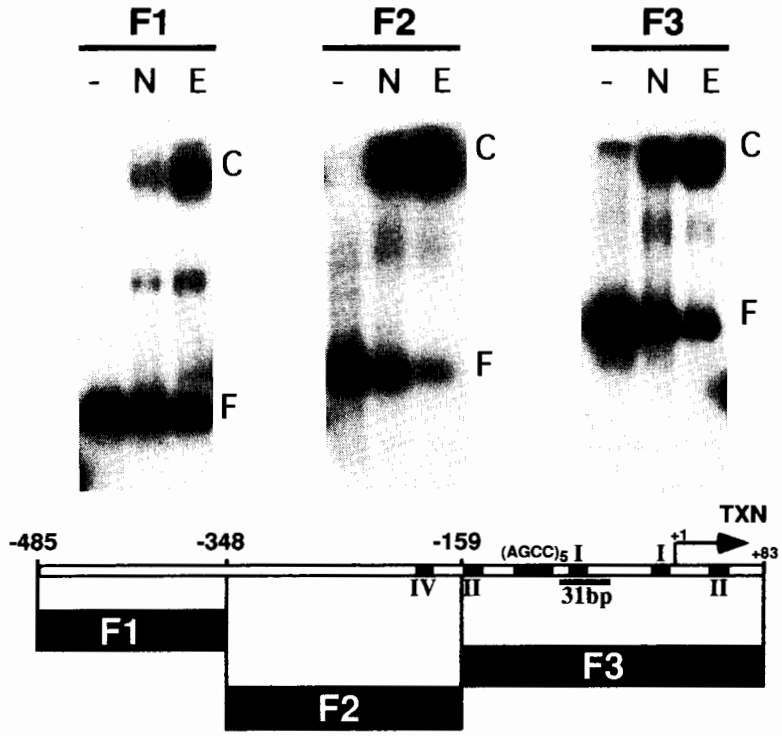

Fig. 3 DNA-binding activities in the 5'-upstream region of *PSCHS2*. Gel mobility shift assays were performed using crude nuclear extracts (2 μg of protein) prepared from non-treated (lane N) or elicitor-treated (lane E) pea epicotyl tissues incubated with individual fragments of the 5'-upstream region of *PSCHS2*. Lane "-" denotes no addition of nuclear extract. Nuclear extract prepared from pea epicotyls treated with or without elicitor for 1 hr was incubated with ^{32}P-labeled probes at pH 8.0 in the presence of 2 μg of poly(dI-dC) • poly(dI-dC). C and F denote the position of the retarded DNA-nuclear factor(s) complexes with low mobility (LMC) and free probes, respectively. F1, F2 and F3 are the DNA fragments used in gel mobility shift assay in the 5'-upstream region as shown in the boxes below. The horizontal bar indicates a promoter region of *PSCHS2*, and the numbers denote the positions of translation (+83) and transcription (+1) initiation sites and *Hin*fI sites used for the preparation of the labeled DNA fragments. TXN and arrow denote the direction of the transcription. Box I, Box II, Box IV and AGCC repeats are shown as black boxes in the bar. The identical 31 nucleotides sequence is indicated as thick bar.

−180 and −130 significantly and sequentially reduced basal CAT activity as well as elicitor-inducibility. Interestingly, the elimination of the promoter distal region from −1889 to −471 drastically increased CAT expression both with and without elicitor-treatment. A similar phenomenon was also observed in the case of the promoter analysis of *PSCHS1*. These results indicate that both distal regions of *PSCHS1* and *2* might possess a silencer-like element that negates the promoter activity located in its own downstream. Furthermore, the region between −471 to −130 might contain enhancer-like elements that are required for the activation of the basal level of promoter activity and elicitor-induction.

To detect nuclear factor(s) that specifically bind to the promoter region of *PSCHS2*, we carried out a gel mobility shift assay. The promoter region spanning from −485 to +83 was divided into three portions and used as DNA probes. All three DNA fragments (F1: −485 to −348; F2: −347 to −159; F3: −158 to +83) strongly formed DNA-nuclear factor(s) complexes with low mobility (LMC, low mobility complex) (Fig. 3). As previously observed in the probe 152 of *PSPAL1*[8] and in the 61 bp DNA fragment of *PSCHS1*[10], LMC formation was significantly enhanced upon elicitor treatment. Therefore the nuclear factor(s) that contribute to the formation of the LMC might be the positive regulatory factors for the elicitor-mediated transcriptional activation.

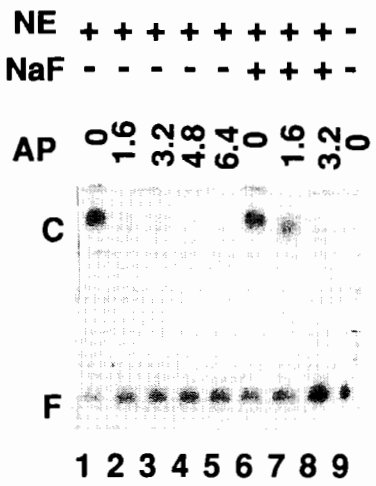

Fig. 4 The effect of dephosphorylation of pea nuclear extract on DNA-binding activity.
Nuclear extract (NE: 2 μg of protein) prepared from pea epicotyls treated with elicitor for 1 hr was incubated with ^{32}P-labeled probe F1 and with or without calf intestinal alkaline phosphatase (AP: final concentration 1.6-6.4 μg/ml) and sodium fluoride (NaF : final concentration 5 mM) at pH 8.0 in the presence of 2 μg of poly(dI-dC) • poly(dI-dC). C and F denote the position of the retarded DNA-nuclear factor(s) complex and a free probe, respectively.

When we treated the nuclear extracts with alkaline phosphatase prior to DNA gel mobility shift assay, the formation of DNA-protein complex with DNA fragment F1 was completely blocked in the concentration dependent manner (Fig. 4 : lane 2 - 5). However, when we added the inhibitor of alkaline phosphatase, sodium fluoride, the formation of DNA-protein complex recovered significantly (lane 7). This effect was lost by the addition of excess amount of alkaline phosphatase (lane 8). A similar result was also observed in the case of LMC formation of the probe 152 of *PSPAL1*[8]. These results suggest that the DNA binding factor(s) contribute to LMC formation might be increased and/or activated in their binding activity upon elicitor-treatment. Then the increase of LMC formation is strongly related to the phosphorylation of some DNA binding factor(s). Previously, we have observed that prior treatment of pea epicotyls with an inhibitor for protein kinase, K252a, suppresses the induction and accumulation of pisatin upon elicitor-treatment[11]. That is, it is very plausible that the elicitor-mediated transcriptional activation of the defense response-related genes, at least in some part, involves the activation of protein kinase in the signal transduction pathway.

CHS genes constitute a multigene family in pea as well as other plants, especially in leguminous plants[1,6,7]. Recently we have identified elicitor-responsive members among pea *CHS* gene family, and compared nucleotide sequences of promoters, exons and introns[7]. The nucleotide sequences in the promoter of all elicitor-responsive *CHS* genes analyzed have revealed the presence of a well conserved set of putative *cis*-acting elements, such as Box-I, Box-II and G-Box-like sequence, except for the absence of a G-Box-like sequence in *PSCHS2*. Although it has been observed that G-Box-like sequence as well as Box-I and Box-II elements, are indispensable *cis*-acting elements in *PSCHS1*, *PSCHS2* which does not carry G-Box-like sequence has shown strong elicitor-responsibility[7]. These results indicate that in *PSCHS2*, G-Box-like sequence is not stringently necessary for elicitor-mediated activation. Thus *PSCHS2* might possess other *cis*-acting element(s) in place of G-Box-like sequence. Interestingly, as shown in Fig. 1, we have found a unique nucleotide sequence just upstream of the distal Box-I element from the transcription start site. This is five repeats of the AGCC motif, and is located at the same position of the G-Box-like sequence in other elicitor-responsive *CHS* genes in pea[7]. This unique sequence might complement the loss of G-Box-like sequence in *PSCHS2*. However, a detailed analysis will be required for the evaluation of this sequence.

Since a longer promoter up to -471 resulted in higher relative CAT activity (Fig. 2), the promoter sequence might associate primarily with the positive transcription factor(s). In this con-

nection, the major and minor DNA-protein complexes observed in gel mobility shift assay (Fig. 3), seem to be formed with positive nuclear factor(s) for active transcription. It is not clear whether these DNA-protein complexes require particular nucleotide sequence with high stringency or not. In the gel mobility shift assay with probe 152 of *PSPAL1*, LMC formation was increased upon elicitor-treatment and DNA-protein complex was further analyzed with the competition assay[8]. LMC formation was blocked by the addition of an excessive amount of DNA fragment not only in 119 and 152 from *PSPAL1*, but also in 135 from *PSCHS1*, and 127 and 189 from *PSCHS2*[8]. Among these competitive DNA fragments, 135 from *PSCHS1*, 127 (parts of F1 and F2 in Fig. 3) and 189 (F2 in Fig. 3) from *PSCHS2* were observed to form LMC[10] (Fig. 3). Thus, common nuclear factors (at least in part) may be involved in elicitor-mediated activation of elicitor-inducible genes in the phenylpropanoid pathway. DNA fragment F3 exhibited similar LMC formation as observed in F2, 152 in *PSPAL1* and 135 in *PSCHS1*[8,10] (Fig. 4). However, two separated DNA fragments, 107 and 135, of F3 from *PSCHS2* haven't shown any competitive activity in the LMC formation of 152 from *PSPAL1*[8]. Although it is not clear why DNA fragments 107 and 135 are not effective competitors[8], plausible explanations are 1) separation of F3 into the two DNA fragments is fatal for LMC formation, or 2) the nuclear factor(s) involved in the similar LMC formation in F2 and F3 from *PSCHS2* is at least partly different.

At the moment, the fine mechanism for elicitor-activation and deactivation of elicitor-responsive *CHS* gene family in pea by the suppressor still remains to be elucidated. However, since the elicitor-inducible *CHS* genes, *PSCHS1* and *PSCHS2* are tandemly clustered, the 5'-upstream regulatory sequence for *PSCHS2* between 3'-end of *PSCHS1* and the transcription start site of *PSCHS2* is restricted within about 7 kb. Therefore a transcriptional study of *PSCHS2* might be a good model for the elucidation of the plant gene expression.

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エンドウカルコン合成酵素遺伝子 PSCHS2 のエリシターによる転写活性化に関与するシス制御配列とトランス因子

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エンドウのカルコン合成酵素遺伝子，*PSCHS2* のエリシターによる転写活性化機構を解明するために，*PSCHS2* プロモーターの転写制御シスエレメントとトランスに働く核内 DNA 結合タンパク質について解析した．*PSCHS2* の転写開始点より－471までの配列を有するプロモーターは，高いベーサルな転写活性を示すと共にエリシター処理により転写活性が増高した．また，エリシター処理したエンドウ上胚軸から調製された核抽出物は *PSCHS2* の＋83から－484までの異なる DNA 断片とゲルシフトアッセイで移動度の遅い複合体（LMC）を形成した．更に，LMC の形成は核抽出物をアルカリフォスファターゼで処理することにより阻害されたことより，何らかの核タンパク質のリン酸化が LMC 形成を促していると考えられた．以上の結果は，*PSCHS2* のプロモーター上にある複数の転写制御シスエレメントに対する正の転写活性化因子の結合はエリシター処理によって増加し，その結合によって転写が活性化することを示唆している．