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 wall[1]. In leguminous plants, phytoalexins are iso-flavonoid 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, Mucor fasciatus pisodes[2]. 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[3]. CHS mRNA is also known to be inducible within 1 hr by the treatment of etiolated pea epicotyls with elicitor of M. fasciatus as well as phenylalanine amonialyase (PAL) genes[4]. Recently we found that the accumulation of PAL- and CHS mRNAs depend on transcriptional activation[5]. CHS genes constitute a multigene family in pea[6]. 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; RT, nucleotides; PAL, phenylalanine amonialyase; PSSPAL-2, gene coding for phenylalanine amonialyase in Pisum sativum; PSCHS, gene coding for chalcone synthase in Pisum sativum
expression of each member in pea\(^1\). 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\(^3\). Two of these elicitor-inducible CHS genes, PSCHS1 and PSCHS2, are tandemly clustered in about 9 kb\(^4\). 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\(^5\). 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\(^6\) (Fig. 1). Since G-Box-like sequence is an indispensable element for the activation of basal expression level as well as elicitor-induction in PSCHS2, 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.

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**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 upwcheckees 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 Hinf I 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 electropropated pea protoplasts.
Sequential 5'-nested deletions of the PSCHS2 promoter fragment, translationally fused to a CAT reporter gene, were created and electropropated into pea protoplasts. Numbers in the left panel denote the 5'-deletion end points and the position of the translatable initiation codon (+83), respectively. Relative CAT activity in elicitor-treated (black bars) and mock-treated (dotted bars) pea protoplasts electropropated with individual chimeric constructs is depicted to that observed in mock-treated protoplasts electropropated 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. Midori,unai) was grown in darkness as described12, and eliolated epicotyls were treated with water or elicitor prior to the preparation of nuclear extracts6. Pea suspension culture was described previously46.

Preparation of fungal elicitor

Elicitor was prepared from the pycnomore germination fluid of M. pinodes as described12, 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 gene51. 5'-nested deletions were made from pCNC2 with unidirectional deletion procedure using Enosexmucease 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 32P-dNTPs by filling-in reaction with a Klenow enzyme or 32P-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 electroporation6. The activity of CAT was measured as described39.

Gel mobility shift assay

Preparation of nuclear extract and the gel mobility shift assay were carried out as described previously46.

Results and Discussion

As we have observed previously45, 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 -275 gave about 3-fold basal CAT expression and about 2-fold induction upon elicitor-treatment (Fig. 2). However deletion up to -275,
−180 and −130 significantly and sequentially reduced basal CAT activity as well as elicitor-inducibility. Interestingly, the elimination of the promoter distal region from −188 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 −486 to +83 was divided into three portions and used as DNA probes. All three DNA fragments (F1: −486 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*, it is the 61 bp DNA fragment of *PSCHS1*, 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 factor(s) for the elicitor-mediated transcriptional activation.
the induction and accumulation of pisatin upon elictor-treatment\(^{11}\). That is, it is very plausible that the elictor-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\(^{[4,5]}\). Recently we have identified elictor-responsive members among pea CHS gene family, and compared nucleotide sequences of promoters, exons and introns\(^{[6]}\). The nucleotide sequences in the promoter of all elictor-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 elictor-responsibility\(^{[6]}\). These results indicate that in \(PSCHS2\), G-Box-like sequence is not stringently necessary for elictor-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 elictor-responsive CHS genes in pea\(^{[6]}\). 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-
section, 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 PSCH2, LMC formation was increased upon elicitor-treatment and DNA-protein complex was further analyzed with the competition assay(s). LMC formation was blocked by the addition of an excessive amount of DNA fragment not only in 119 and 152 from PSPL2, but also in 135 from PSCH1, and 127 and 189 from PSCH2. Among these competitive DNA fragments, 135 from PSCH1, 127 (parts of F1) and F2 in Fig. 3 and 189 (F2 in Fig. 3) from PSCH2 were observed to form LMCs (Fig. 3). Thus, common nuclear factors (at least in part) may be involved in elicitor-mediated activation of elicitor-inducible genes in the phenylpropyranoid pathway. DNA fragment F3 exhibited similar LMC formation as observed in F2, 152 in PSPL2 and 135 in PSCH1 (Fig. 4). However, two separated DNA fragments, 107 and 135, of F3 from PSCH2 haven’t shown any competitive activity in the LMC formation of 152 from PSPL2. Although it is not clear why DNA fragments 107 and 135 are not effective competitors, 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 PSCH2 is at least partly different.

At the moment, the fine mechanism for elicitor-activation and deactivation of elicitor-responsve CHS gene family in pea by the suppressor still remains to be elucidated. However, since the elicitor-inducible CHS genes, PSCH1 and PSCH2 are tandemly clustered, the 5‘-upstream regulatory sequence for PSCH2 between 3’-end of PSCH1 and the transcription start site of PSCH2 is restricted within about 7 kb. Therefore a transcriptional study of PSCH2 might be a good model for the elucidation of the plant gene expression.

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References

エンドウカプス合成酵素遺伝子 PSCHS2 のエリシターによる転写活性化に関与する
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エンドウカプス合成酵素遺伝子, 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抑制シスエレメントとトランスに働く核内DNA 細胞タンパク質について解析した．PSCHS2 の転写開始点より-471 までの配列を持つプロモーターは, 高井ベーサウン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幅した．また, エリシター処理したエンドウトマト種から調製された枝抽出物は PSCHS2 の+83から+484 までの異なるDNA 断片をグルシトアミドで移動度の変化を示す新たなエリシターを形成した．さらに, LMC の形成は枝抽出物をアルカリフリッカーで処理することにより抑制されることより, 何らかの核タンパク質のリン酸化が LMC 形成を促進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抑制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, その結果によって転写が活性化することを示唆している．