エンドウの推定ZnフィンガーDNA結合性タンパク質のcDNAクローニング

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Molecular Cloning of a cDNA Encoding a Putative DNA-Binding Zinc-Finger Protein in Pea

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We constructed a cDNA library from pea epicotyls treated with fungal elicitor for 5hrs, and performed differential screening using the individual ^{32}P-cDNA probe derived from poly (A)$^+$ RNA prepared from elicitor- and water-treated epicotyls. As a result of the screening, we have isolated about 90 cDNA clones as candidates for elicitor-inducible genes, and their nucleotide sequences have been partially determined. One of these clones, E31 was a pea homolog of the putative zinc-finger proteins, Ljpzf in *Lotus japonicus* and Gmpzf in soybean. E31 possesses 1,716bp insert, and encodes an open reading frame corresponding to position 82 amino acids from N-terminus to the C-terminal end in Ljpzf. The protein product of E31 was designated as Pspzf. Pspzf also possesses nuclear localization signal (NLS), HKRK, and Cys3His2Cys3 (RingH2) motif at the same position to Ljpzf and putative C-terminal end of the deduced amino acid sequences, respectively. Since zinc-finger motif is one of the well-known DNA-binding domains, Ljpzf and Pspzf might be able to bind to a particular DNA sequence and regulate transcriptional activity in plants.

Key words : DNA-binding protein, Elicitor, Ring H2 finger protein, Transcription factors, Zinc-finger protein

Introduction

Plants respond to the attack of microbial phytopathogens with a variety of defense reactions. Most of these are accompanied with *de novo* gene expressions, so-called active defense genes. The genes are involved in the reinforcement of the plant cell walls and production of PR-proteins and phytoalexins[7]. It has been well known that plants recognize the molecular signals produced by microbial phytopathogens, such as defense response inducing substances, elicitors, and their inhibiting substances, suppressors. After the recognition of elicitor molecules, plants initiate the activation of the defense genes, and express resistance. To elucidate the molecular mechanisms of the induced plant-defense responses, and to identify the genes related to defense reaction, we have attempted to isolate cDNA clones whose expressions are induced by elicitor-treatment. For the isolation of cDNA clones which are differentially expressed, we performed differential hybridization with a cDNA library constructed from elicitor-treated

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Abbreviations
CHS, chalcone synthase; Gmpzf, a putative zinc-finger protein in *Glycine max*; Ljpzf, a putative zinc-finger protein in *Lotus japonicus*; Pspzf, a putative zinc-finger protein in *Pisum sativum*; PAL, phenylalanine ammonia-lyase; RingH2 motif, Cys3His2Cys3 motif; RingH2 protein, Cys3His2Cys3 Ring finger motif possessing protein

The nucleotide sequence data reported will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number AB018422.

pea epicotyls for 5hrs. Among 90 cDNA clones, we have observed the homologous cDNA clones encoding PR-proteins, proteins for reinforcement of plant cell walls and phytoalexin biosynthesis, and putative transcription factors by partial sequencing analysis (unpublished).

Since E31, one of the cDNA clones, had a sequence homology to the putative zinc-finger proteins, we have chosen E31 as one of the candidates for further analysis as a novel elicitor-inducible cDNA clone. Zinc-finger proteins possess "zinc-finger motifs" that have in common the property of binding zinc ions in order to stabilize the protein structure and function of the activity of proteins, and most zinc-finger proteins are well known as DNA binding transcription factors, such as GAL4 in yeast, eukaryotic transcription factor (TFIIIA) and steroid hormone receptors in mammals[6,9]. Although a variety of defense genes have been isolated in a wide range of plant species, little is known about the transcription factors that activate defense gene expression. There are only a limited number of reports of the isolation of cDNA clones encoding transcription factors. For example, cDNA clone encoding BPF-1 (box-P binding factor) was isolated from parsley by Southwestern method[8]. Box-P was found in the promoter of parsley *PAL1* gene, and its sequence homolog, Box-II also exists in pea *PAL1*, *PAL2*, *CHS1* and *CHS2* genes[1,15]. In both plants, Box-P (Box-II) is known as one of the elicitor-responsive cis-elements required for maximal activity of elicitor-induced transcription[8,12]. However, to explain transcriptional activation of various kinds of defense genes, information is still limited about the defense-response related transcription factors.

In this paper, we report the molecular cloning of E31 cDNA which encodes putative zinc-finger protein. The product of E31 (Pspzf) might be a transcription factor involved in elicitor-mediated transcriptional activation of some defense genes.

Materials and Methods

Plant Material and elicitor preparation — Pea (*Pisum sativum* L. cv. Midoriusui) was grown in darkness as described[15], and etiolated epicotyls were treated with water or elicitor prior to the extraction of total RNA. Elicitor was prepared from the pycnospore germination fluid of *M. pinodes* as described[15], and used at 100 μg/ml glucose equivalent as a final concentration.

RNA extraction and construction of cDNA library — Pea epicotyls were frozen in liquid nitrogen, and total RNA was extracted by single-step method[3]. Poly $(A)^+$ RNA was further purified using polyATtract mRNA Isolation System (Promega, Madison WI, USA) according to the manufacturer's specifications. A cDNA library was constructed from poly $(A)^+$ RNA extracted from pea epicotyls 5h after the elicitor treatment with ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA), and recombinant phage DNAs were packaged with Gigapack III Gold Packaging Extract (Stratagene).

Differential screening — Twenty thousand independent cDNA clones were subjected to differential hybridization. Two sets of nylon membranes blotted with the phage DNAs were made, and each membrane was hybridized with ^{32}P-labeled cDNA probe which was prepared by reverse transcriptase, ^{32}P-dCTP and poly $(A)^+$ RNAs of elicitor or water-treated epicotyls for 5 hrs. Phage clones whose expressions seemed to be induced by elicitor treatment were isolated and corresponding plasmid DNAs were prepared by standard procedure[10].

DNA sequencing and homology search — The nucleotide sequence of E31 plasmid DNA was determined with Thermo Sequenase pre-mixed Cycle Sequencing Kit and Hitachi DNA Sequencer SQ5500 (Hitachi, Tokyo, Japan). Homologies

at the DNA and deduced amino acid sequence levels were analyzed with Blast search protocol on the Internet.

Results and Discussion

As a result of one round of differential hybridization from 20,000 independent cDNA clones, we have isolated 90 clones as candidates for elicitor-responsive genes. As shown in Fig5. 1 and 2, one candidate, E31 possesses an insert DNA of 1,716 bp in length and deduced amino acid sequence showed a striking homology to the putative zinc-finger proteins, Ljpzf and Gmpzf, isolated from *Lotus japonicus* and soybean, respectively[11]. However, a non-homologous 68 nucleotide sequence to Ljpzf and Gmpzf was found at the 5' end of the insert. It might be explained that the 68 bp region was ligated by chance to the 5' end of the cDNA encoding Ljpzf homolog in pea by an unknown artificial mechanism. The homologous region of E31 to Ljpzf started from the nucleotide at position 69 which corresponds to the amino acid at position 81 in Ljpzf. Thus there is one open reading frame consisting of 472 amino acids showing 57.5% amino acid sequence homology to that of Ljpzf. The protein product of this open reading frame was designated as Pspzf. As a characteristic feature of Pspzf, as well as Ljpzf,

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CATCCTCAGGCCCCAGCATTGAAGTGCCTCCTCCACATCCTCCTCCTCCATCACCTCCACCACCTCTTCCTCAGCAATTGGACACACCAGGCACTTCTAA  100
                                                                  P  Q  Q  L  D  T  P  G  T  S  N   [11]

CGATCATTTTATGCATACATCTAATGCTGGACCTTTCTTTGCAGTATCTGAAAATTATGTACACCCGCCTCCGTCTTCTTCCAATTATGACAGACATGCA  200
 D  H  F  M  H  T  S  N  A  G  P  F  F  A  V  S  E  N  Y  V  H  P  P  P  S  S  S  N  Y  D  R  H  A   [44]

TTTCATGTTGATAGTGGTTTTATTGATCTTACAATGGGAAGTGGACGGGGGCATCACAAGAGGAAGAGCCCTGGAATTCCTTCAGTTTACGAGAGAGGCA  300
F  H  V  D  S  G  F  I  D  L  T  M  G  S  G  R  G  H  H  K  R  K  S  P  G  I  P  S  V  Y  E  R  G  S   [78]

GTTCAAGTGGATATTTCAATGCTGGGAGCTCAACCGATCTTCCTACACCTCCAGAATCATGGCCAGAGAAACCAAATATGAATTCTCAATATTTGCCCTG  400
  S  S  G  Y  F  N  A  G  S  S  T  D  L  P  T  P  P  E  S  W  P  E  K  P  N  M  N  S  Q  Y  L  P  W   [111]

GGATCATGTTGCTATGACACCCACATTCAGAGGTGCTGGCCTCTCAATGAAGGGTGAGAGCTCTGTACGGAATGTAAGGAGCCGTTCTGCACTTGATCTG  500
 D  H  V  A  M  T  P  T  F  R  G  A  G  L  S  M  K  G  E  S  S  V  R  N  V  R  S  R  S  A  L  D  L   [144]

GAATCCAACCTGTCTAGGACCCATTTACCAATTAACCATTCACTCAACTCGTACCTTACTGCCTCACCATTCGGCCATTGTAGCTTGGCGGATCTTTCAG  600
E  S  N  L  S  R  T  H  L  P  I  N  H  S  L  N  S  Y  L  T  A  S  P  F  G  H  C  S  L  A  D  L  S  A   [178]

CTCAGGTTACTACCCCTTTGACAAGGGATTGGAGCCAAATGAATGTAACTCCTGCTAATGGAAGAGTGTTATTACCAGATGCTAGTACTTCTGGTCTTGA  700
  Q  V  T  T  P  L  T  R  D  W  S  Q  M  N  V  T  P  A  N  G  R  V  L  L  P  D  A  S  T  S  G  L  E   [211]

GACAAGTCACTTCCCTGTTGGAAATGCTGCTACGGCTTCTAGTAATGCTCCTGCAGATGTTGGAAGCTTCCATCATGAATTTGGTACAAGCAGAAATCCT  800
 T  S  H  F  P  V  G  N  A  A  T  A  S  S  N  A  P  A  D  V  G  S  F  H  H  E  F  G  T  S  R  N  P   [244]

ACTACTGCTCAAAGTTTTCAAAATTTGACTCAACCTGCTAGGGGAACTCGAAGTAATTACTCTCAGAGATCCACTCCAGCTTTCAGGGCTTCTTCAAACT  900
T  T  A  Q  S  F  Q  N  L  T  Q  P  A  R  G  T  R  S  N  Y  S  Q  R  S  T  P  A  F  R  A  S  S  N  L   [278]

TGCGCATGTCCCATGCGGTACCTTCAGATAATGGACTGCCTATGGTAGCTGAAGGTTACTCTTCTAGACATCCAAGGCCGTTGACCACTGTTGGCTGGCG  1000
  R  M  S  H  A  V  P  S  D  N  G  L  P  M  V  A  E  G  Y  S  S  R  H  P  R  P  L  T  T  V  G  W  R   [311]

GAACGGTGACAGAAATGGAAGGTCTAGAATTTCGAGTGAAAGATATAGATCGTTGGCTGATGAGGCTGGGCTCCATGCTAGATTTTCCTCTGAGGTACCG  1100
 N  G  D  R  N  G  R  S  R  I  S  S  E  R  Y  R  S  L  A  D  E  A  G  L  H  A  R  F  S  S  E  V  P   [344]

GGCTTCATGATTGTTGAGCGTGCTTCACTGTATGGCTCTAGGAATATACTTGATCAGCACAGAGACATGAGAATGGACGTAGATAACATGAGCTATGAGG  1200
G  F  M  I  V  E  R  A  S  L  Y  G  S  R  N  I  L  D  Q  H  R  D  M  R  M  D  V  D  N  M  S  Y  E  E   [378]

AACTTCTTGCACTTGGGGAGAGGATTGGCCAGGTGAACACAGGATTGTCTGAGGATGTGCTTTCCAAGTGTTTGACAGAAACAATATATTGTTCATCTGA  1300
  L  L  A  L  G  E  R  I  G  Q  V  N  T  G  L  S  E  D  V  L  S  K  C  L  T  E  T  I  Y  C  S  S  D   [411]

CCAATGCCAGGATGAAGGAAGCTGTGTGATCTGTCTGGAAGAGTACAAGAATATGGACGATGTTGGGACACTTAAAACTTGTGGACATGACTATCATGTG  1400
 Q  C  Q  D  E  G  S  C  V  I  C  L  E  E  Y  K  N  M  D  D  V  G  T  L  K  T  C  G  H  D  Y  H  V   [444]

AGCTGCATTAAAAAGTGGTTATCTATGAAGAAACTATGTCCTATCTGCAAATCTTCTGTTTTGCCTGAGGATAAGAAGGATAAATAACTTAAACTTTANT  1500
S  C  I  K  K  W  L  S  M  K  K  L  C  P  I  C  K  S  S  V  L  P  E  D  K  K  D  K  *                 [472]

ATTTATTAATCAATCTCCTTTGTATATATATTTAGAAATCCTCCAAAAGCAGAAGGAAACAGACTATAGCTTTGGAAATGTCTTGACAAGAATGCTACTT  1600

CATGTCAATTTGTTTGGCTTCGATGTCAATTTAGTGAAGCCTGGAAATTATTATGTGGATTTGAAGCAGTTCTGGATTGCTTCTATTTTTAAATAAAAAA  1700

AAAAAAAAAAAAAAA                                                                                    1716
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Fig. 1 Nucleotide sequence and deduced amino acid sequence of pea E31 cDNA.
The nucleotide sequence of E31 between *Pst*I and *Xho*I sites is shown and numbered from *Pst*I side. A non-homologous sequence to that of Ljpzf at the 5' end of E31 cDNA is shown with waved underline. Deduced amino acid sequence homologous to that of Ljpzf is numbered in brackets. Nuclear localization signal (NLS), HKRK at the position 63 to 66 of amino acid is boldly underlined. Core amino acids for Ring H2 motif are doubly underlined. The stop codon, TAA is marked by an asterisk.

Pspzf also possesses a putative nuclear localization signal, HKRK at position 63 to 66 and Cys3His2Cys3 (Ring H2) finger motif at the C-terminal end. Schauser *et al.* reported that 2.1 kb of mRNA for soybean Gmpzf was detected in all organs tested, although they didn't show the results[11]. We have also detected the transcripts corresponding to Pspzf with about 2 μg of poly $(A)^+$ RNA prepared from wounded and elicitor-treated pea epicotyls. However, the size of the transcript in pea was about 2.7 kb and different from the observation by Schauser et al.

The amino acid sequence motif, Cys3His2Cys3 (Ring H2) is similar to the Cys3HisCys4 Ring finger motif. Ring finger motif is relatively better characterized than Ring H2 motifs, and some of them, such as *Arabidopsis* COP1, are identified as Zn-finger DNA-binding transcription factors[4,5,6,9]. In the case of Ring H2 proteins, there is a significant accumulation of sequence reports in the protein data bank so far, such as G1 regulatory protein in fruit fly[2] and C-RZF in chicken[14]. Although there is no absolute evidence that G1, C-RZF and Ljpzf have DNA-binding activity, these Ring H2 proteins have typical amino acid sequence motifs for DNA-binding transcription factors, such as nuclear localization signal, a luecine zipper motif or a stretch of acidic amino acids, suggesting a novel type of DNA-binding proteins.

Ljpzf was isolated from a nodule cDNA library, and it was reported that the expression of Ljpzf-mRNA was slightly enhanced in nodules, although the authors didn't show the experimental result[11]. Elicitor-inducibility of Pspzf is still unknown yet. However, the isolation of cDNA clones encoding Ring H2 proteins, such as a rice EL5 as early elicitor-inducible gene[13] and chickpea INR132 from *Ascochyta rabiei*-inoculated leaves in resistant cultivar (our unpublished result) led us to speculate that some kinds of Ring H2 proteins might be elicitor-inducible. Thus, Pspzf might be responsible for the elicitor-treatment and be a novel type of DNA-binding

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E31                                                                    PQQLDTPGTSNDHFMHTSN  19
LjPZF  MGHRHLYNTTPLFEGEPDQNWNHMHTDQHYVNLGRTSTAENGSYFYPVENVSIDSTSFPSHWNPATRSNGYASSSLNIEVPPHQSDTSGASNDHFIHSSS 100

                                                  ####
E31    AGPFFAVSENYVHPPPSSSNYDRHAPHVDSGFIDLTMGSGRGHHKRKSPGIPSVYERGSSSGYFNAGSSTDLPTPPESWPEKPNMNSQYLPWDHVAMTPT 119
LjPZF  AGAFFAVSNNCV-PQPPAANYDRPPFQVDGGFIDLTMGSGRGPHKRKSPGIPSVYERGGSSRYFNAGSSTDVPISSESRPEKPNIDSQYMPWDHVPMTST 199
Gmpzf                                                  IPPVCERGSTSRYSNATNAADRPTSSELQPEKPNMDSLYMPWDHVNMTPT  50
                                                       ** * ***  * * **    * *   *  *****  * * ***** ** *

E31    FRGAGLSMKGESSVRNVRSRSALDLESNLSRTHLPINHSLNSYLTASPFGHCSLADLSAQVTTPLTRDWSQMNVTPANGRVLLPDASTSGLETSHFPVGN 219
LjPZF  VRGAGHSIKGESSLRNVRSRSALDLESNLSRTHLPSNHSHNSYSTIPPVDHSSMMDLSGQISTSLTGDWSQMNISPANGRVLLPDAGAYGLESSHFLAGS 299
Gmpzf  FRGRGLSIRGEGPLRNVRSRSALDLESNLARTHLSNTHPHNSYSTGLPIDHSNMVDLSTQTSGTLTRDWSPMSICPPHGRVSSSDANAFNHEASHFLIG- 149
        ** * *  **   ********************* ****   *  *** *  *  *    *** *    ** *** *   *  ***   **     * ***  *

E31    AATASSNAPADVGSFHHEPGTSRNPTTAQSF-QNLTQPARGTRSNYSQRSTPAFRASSNLRMSHAVPSDNGLPMVAEGYSSRHPRPLTTVGWRNGDRNGR 318
LjPZF  GATA-SNASVDVGSFHHEFGTSRNPTGPQSF-HNLTQTARGIRSNYSQRSAPTFRASSSTHLGHVTPLDDGLPMVAESYSSRHQRPLSSIGWRNSDRNGR 397
Gmpzf  --SSSSNAPVDVGGFHNEPGTSRNPTAPQSFHNNLTQTARGVHSNYSQRSTPTFRASS-LRLGRVAPSDDGLHMVSESYSSRQPRPLTTVGWQNNNRNGR 246
          ***  *** ** **********  ***  **** ***  ******* * *****        * * ** ** * ****  ***   ** *  ****

E31    SRISSERYRSLADEAGLHARFSSEVPGFMIVERASLYGSRNILDQHRDMRMDVDNMSYEELLALGERIGQVNTGLSEDVLSKCLTETIYCSSDQCQDEGS 418
LjPZF  ARISSERYRSLANEAGLHGRISPEV--FMIVDRASMYGSRNMLDQHRDMRMDIDNMSYEELLALGERIGHVNTGLSEDSFSQCMTETIYCSSEQGQDEGS 495
Gmpzf  SRIPSDRYRSL--D---D-----E--GFMIVDRTSLYGPRNMLDRHRDMRMDIDNMSYEELLALGERIGHVSTGLSEDLISKYLTETIYCSSEQSQEEEA 334
        ** * *****           *   **** * * ** ** ** ******* ********************* * ******  *   ******** * * *

       + +              + + + +         + +
E31    CVICLEEYKNMDDVGTLKTCGHDYHVSCIKKWLSMKKLCPICKSSVLPEDKKDK                                              472
LjPZF  CVICLEEYKNMDDVGTLKTCGHDYHVNCIKKWLSMKKLCPICKASVMPEDKMNK                                              549
Gmpzf  CAICLEEYKNMDYVGTLKACGHDYHVGCIRKWLSMKKVCPICKASALPEDKKGK                                              388
       * ********** ***** ******* ** ******* ***** *  ****  *
       ___________________________________
       C3H2C3 Ring H2 Finger Domain
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Fig. 2 Alignment of the deduced amino acid sequences of pea E31 cDNA, Ljpzf in *Lotus japonicus* and Gmpzf in soybean.
The deduced amino acid sequences are aligned and numbered from the putative N-terminal methionine in Ljpzf and the amino acids corresponding to each 5' end of the homologous sequence to Ljpzf in E31 and Gmpzf. The identical amino acids in three proteins are marked by asterisks under the sequences. Nuclear localization signals (NLS), HKRK in E31 and Ljpzf are marked by sharps (#). The region of Ring H2 finger domain is boldly underlined, and core amino acids of Cys and His are marked by pluses (+).

protein.

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エンドウの推定 Zn フィンガー DNA 結合性タンパク質の cDNA クローニング

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エリシターを処理したエンドウの上胚軸から cDNA ライブラリーを作成し，水処理上胚軸をコントロールに differential screening を行い，エリシター応答性 cDNA の候補として90個のクローンを単離した．それらのクローンの１つE31は1,716bp のインサートを有し，ミヤコグサ，ダイズで cDNA がクローニングされている Ljpzf，Gmpzf のエンドウにおけるホモローグをコードしていると考えられ，その推定翻訳産物を Pspzf と命名した．E31は，Ljpzf の推定アミノ酸配列の82アミノ酸目からカルボキシル末端側をコードしていると考えられた．Ljpzf や Pspzf の推定アミノ酸配列中には核移行シグナル HKRK が存在していたこと，カルボキシル末端側には Cys3His2Cys3 をモチーフとする Ring H2 finger ドメインが存在していたことより，Ljpzf や Pspzf は，核内に局在する DNA 結合性の Zn fingerタンパク質の１種として遺伝子発現の制御に機能している可能性が示唆された．