A Reporter Gene Expression Under the Control of a Pea Phenylalanine Ammonia Lyase-gene Promoter

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Summary

High yields of viable pea protoplasts were produced from suspension cultured cells derived from calli formed from embryogenic tissues or leaves and the conditions for the optimum expression of chloramphenicol acetyltransferase (CAT) fused to the phenylalanine ammonia-lyase gene of *Pisum sativum* (pPAL1-15) were investigated by transient assay after electroporation. A fungal elicitor isolated from a pea pathogen, *Mycosphaerella pinodes*, and the reduced from of glutathione induced the expression of PAL promoter but orthovanadate, a plasma membrane ATPase inhibitor, considerably suppressed the gene expression. Rice protoplasts were also prepared from the suspension cultured cells derived from embryonic tissues, and the effects of elicitors on the expression of CAT in pPAL1-15-electroporated rice protoplasts were examined. No distinctive induction of CAT activity was observed by the treatment of rice protoplasts with a chitosan oligomer elicitor.

Introduction

Pycnospore germination fluid of *Mycosphaerella pinodes* (Berk. *et* Blox.) Stone, a fungus pathogenic on pea, contains both elicitors (high molecular mass glucans or glycoproteins [>70,000 Da]) and suppressors (low molecular mass glycopeptides [< 5,000 Da]) for the accumulation of the pea phytoalexin, pisatin[18,19,23]. Purification of the suppressor from the low molecular mass fraction in the germination fluid by gel filtration and thin-layer chromatography gave two active components, F2 and F5[23]. The concomitant presence of F5 with the elicitor negates the activity of the elicitor ; that is, the induction of pisatin biosynthesis as well as phenylalanine ammonia-lyase (PAL) activity are suppressed in the mesophyll tissue of pea leaves [8]. We have also shown that the concomitant presence of the suppressor with an elicitor results in delays of host defense reactions including a 3-hr delay in the accumulation of PAL- and chalcone synthase (CHS)-mRNAs, a 6-hr delay in the increase of PAL enzyme activity, and a 6- to 9-hr suppression of pisatin accumulation[25]. Very recently, we have shown that the suppressor specifically inhibits pea plassma membrane (PM)-ATPase but does not inhibit other types of ATPase[27]. Inhibitors for pea PM-ATPase such as orthovanadate or verapamil also function in the same manner as the *M. pinodes* suppressor with respect to blocking phosphorylation of pea PM-ATPase. Furthermore the concomitant presence of orthovanadate or verapamil with a fungal elicitor results in delaying the induction of

pisatin accumulation[27] and delays the accumulation of PAL- or CHS-mRNA in a similar manner to a fungal suppressor[28].

Currently, attention in this area is being focused on the analysis of the *cis*- and *trans*-acting factors involved in the activation and deactivation of these defense response genes[7,13].

To establish the system of the functional analysis of the *cis* element involved in the plant active defense gene expression such as PAL or CHS, conditions for the cultured cell lines of pea (*Pisum sativum* L. Midoriusui) was investigated. At present, transient assay after electroporation of the genes of interest into pea protoplasts can be the only means that provide the informations of *cis*-acting elements residing in the proximity of the promoter. The information is provided by means of introducing the external recombinant DNA, in which the control region of the gene is fused to the reporter gene, such as chloramphenicol acetyltransferase (CAT), β-D-glucuronidase (GUS), since techniques for stable gene transformation and plant regeneration systems in pea have not been satisfactorily established.

In this communication, we describe the simple and rapid methods of preparing protoplasts from suspension cultured cells starting with calli derived from scutellar tissues of pea seeds and the conditions introducing chimereic DNA constructs with a bacterial chloramphenicol acetyltransferase (CAT) gene by electroporation into the protoplasts. We also analyzed the expression of CAT in the chimeric gene, in which the putative promoter fragment of pea *PSPAL1* trnslationally fused to the CAT cassette in the presence of the fungal elicitor to characterize the *cis* element necessary for the induction of the expression of pea PAL gene as a model for a plant active defense gene. We also analyzed the chimeric gene expression in rice protoplasts.

Materials and Methods

Callus Induction and Suspension Cultured Cell Lines in pea Seeds of *Pisum sativum* L. cv. Midoriusui or *Oryza sativa* L. cv. Nihonbare, sterilized by 70 % ethanol for 5 min, 2.5 % NaClO for 10 min followed by exhaustive washing with sterilized water, were placed on a MS agar medium[16] containing 2,4-D (2.0 mg/l) and 0.2 % Gellungum. They were incubated at 22℃ in the dark for 2 months and calli formed from embryo were excised and replaced on B5 medium[5] containing 1.5 mg/l 2,4-D and 0.2 % casein hydrolysate followed by incubation at 22℃ under light emission of 10 μEinstein/m^2/sec. with continuous agitation (100 rpm). Every other week, suspension cultured cells were subcultured into freshly prepared medium after the simple process called "Uragoshi"[20]. "Uragoshi" is the process that the aggregated suspension cultured cells are ground with the back of a sterilized spatula and filtered through a stainless filter with 20 mesh. This subculturing and Uragoshi were repeated every other week.

Preparation of elicitor from M. pinodes Crude elicitor fractions were prepared from spore germination fluid of *M. pinodes*, OMP-1 (ATCC #42741) by the method as previously described[25]. A final concentration of elicitor fraction was adjusted to 6 mg/ml glucose equivalent.

Protoplasts Preparations Protoplasts were prepared from leaves or suspension cultured

cells and the efficiencies of protoplasts formation and CAT gene expressions in transient assay were compared. To prepare protoplasts from pea leaves, *Pisum sativum* L. cv. Midoriusui was grown at 22℃ under continuous white light (25 μEinstein/㎡/sec) for 2 -3 weeks. Leaves were sterilized by 70 % ethanol and 1 % NaClO for 30 sec and washed with sterilized water. Epidermis of sterilized leaves were peeled off and cut into 1 ㎠ sections. The leaf pieces were washed with electroporation solution (ES; 0.5 M mannitol, 0.1 mM $MgSO_4$) and treated with the enzyme solution (1% Cellulase Onozuka R-10 (Yakult), 0.5 % Macerozyme R-10 (Yakult), 0.05% potassium dextran sulfate, 0.01 % $CaCl_2$, 9 % mannitol, pH 5, 6), and infiltrated *in vacuo* for a few minutes. Leaves were incubated at 30℃ for 60 min with gentle agitation (30 rpm) followed by incubation for 120 min without shaking. This solution was filtrated through a sheet of gauze and centrifuged at 110×g for 60 sec. The upper layer of the solution was removed, washed twice with ES and used for electroporation.

To prepare protoplasts from pea or rice suspension cultured cells, suspension cultured cells grown for 2 months after successive subculturing with the Uragoshi process were washed once by ES and treated with enzyme solution (0.05 % Pectolyase Y-23 (Seishin), 2.0 % Cellulase Onozuka RS (Yakult), 0.01 % $CaCl_2$, 0.05 % Potassium dextran sulfate, 9 % mannitol)[20], and incubated at 30℃ for 60 min with gentle agitation (30 rpm) followed by incubation for 120 min without shaking. This solution was filtrated through 30 μm nylon mesh and centrifuged at 110×g for 60 sec. The upper solution was removed, washed twice with ES and used for electroporation. Protoplasts were kept on ice until electroporation.

Construction of the chimeric gene pPAL1-15 contains a 480 bp fragment of the *PSPAL1*, corresponding to the sequences −340 to +140 relative to the transcriptional initiation site[9] fused to the coding region of the bacterial CAT gene in p35SCAT-N (a gift from Dr. Hirochika) (Fig. 1). That is, an anchored polymerase chain reaction (PCR) was carried out with two partly matching synthetic 30-mer oligonucleotides, 5'-GCGCATGCGT CATGGTCATG CATGGTTGCT-3' and 5'-GCGGATCCGT TATGG-CTGCT GCTACTGTTT-3' which include the *Sph* I and *BamH* I restriction sites at the 5'- end and 3'- end in addition to the dinucelotide, GC, respectively, in the presence of the recombinant plasmid carrying *Eco*R I-*Bam*H I (5 kb) fragment containing the 5' -distal to the PAL 1 promoter to the coding sequence[26]. PCR product was cloned in *Sph* I and *Bam*H I sites of p35SCAT-N after digestion with respective endonucleases to replace the CaMV 35 S promoter (Fig. 1). pCaMVCN, carrying CaMV 35S prtomoter-*cat-nos* terminator, was purchased from Pharmacia Inc,. As a control, pHSG-CN which lacks CaMV 35 S promoter was constructed by inserting *Bam*H I-*Bgl* II fragment from pCaMVCN containing bacterial *cat* and nopaline synhase gene (nos) terminator into *Bam*H I site of pHSG299 (Fig. 1).

Electroporation pCaMVCN (Pharmacia) was used to determine the optimum conditions for electroporation. Protoplasts suspension ($1-2\times10^6/4-500$ μl) were mixed with varying quantities of circular plasmid DNA in the presence or absence of 50 ㎍ of sonicated herring DNA as a carrier, and the total volume was adjusted to 800 μl with ES. The mixture was placed in an electroporation cuvette (0.4 ㎝ path length) and subjected

to electroporation. The DNA-protoplasts mixture was inverted 3 times and electroporated with a Shimazu GTE-10 electroporation apparatus, which generates an exponential decay pulse.

Treatment of electroporated-protoplasts with elicitor Protoplast suspension was mixed with 50 μg of pPAL1-15 and electroporated as described above. Electroporated protoplasts were collected by centrifugation (110×g, room temperature, 3 min). After decanting the supernatant, the protoplast pellet was suspended in 1 ml of LP medium[1] and incubated at 22℃ in the dark for 1h followed by the addition of various concentrations of elicitor isolated from *M. pinodes*. As a control, electroporated-protoplasts were treated with equal volume of LP medium. For reference assay, 50 μg of pCaMVCN or pHSG-CN were also electroporated and treated with fungal elicitor.

PAL assay PAL activity in pea protoplasts treated with or without fungal elicitor was determined as described elsewhere[25].

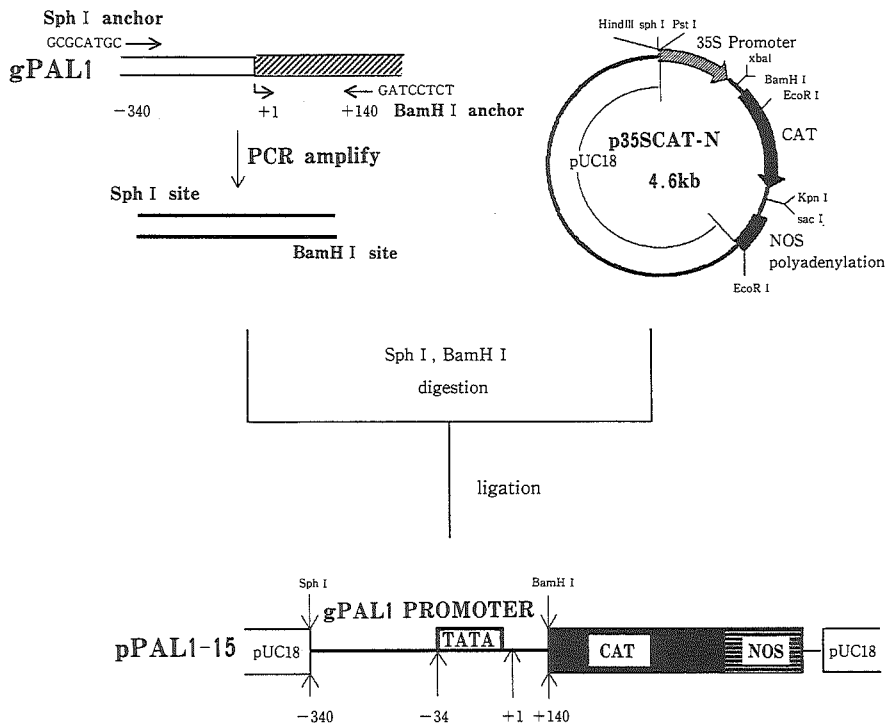

Fig. 1 Construction of pHSG-CN and pPAL1-15. A map of pPAL1-15. A pea PAL promoter fragment generated by a polymerase chain reaction (PCR) (position at −340 to +140) which creates the *Sph* I and *Bam*H I restriction sites was cloned in p35CaMV[9]. As a result, an additional octapeptide, METVAAA I, encoded from gPAL1 was presumably fused in frame to the CAT cassette. Amp refers to the gene encoding β-lactamase that conters ampicilin-resistance in pUC18 ; Km refers to the gene encoding acetylphosphotransferase that confers kanamycin and neomycin-resistance in pHSG299 (Takara Inc,). MCS refers to a multicloning site. Relative positions of the putative TATA box and the start of transcription (+1) on gPAL1 (Yamada et al. in press) are indicated.

CAT Assay CAT activity was determined by the methods of Gorman et al[6]. with a slight modification[25].

Results and Discussion

Effects of protoplasting from the difference of plant maturation was examined. The secondary and tertiary leaves from a pea plant with four developing leaves were used to prepare protoplasts. The yields of protoplasts from the secondary leaves was 3.3×10^6/leaf, and that from the tertiary leaves was 2.5×10^6/leaf, respectively. The survival rate (%) of the protoplasts was also examined with respect to the time of incubation after protoplasting by staining protoplasts with fluorescin diacetate (FDA). The number of vigorous protoplasts rapidly decreased with respect to the time (in hours) of incubation and reached approximately zero% 48-60 h after the incubation (Fig. 2).

The effects of light on protoplast preparation was also examined by incubating pea leaves grown in the dark for two days before the treatment with lytic enzymes for protoplasts preparation and the ones grown under continuous white-light (25 μEinstein/m^2/sec). A distinctive effect was not detected with or without the light irradiation (2.9×10^6 protoplasts/leaf and 3.3×10^6/leaf, respectively). The survival rate (%) of these protoplasts showed a pattern similar to Fig. 2 (data not shown).

To examine the effects of the fungal elicitor to pea protoplasts and to estimate the

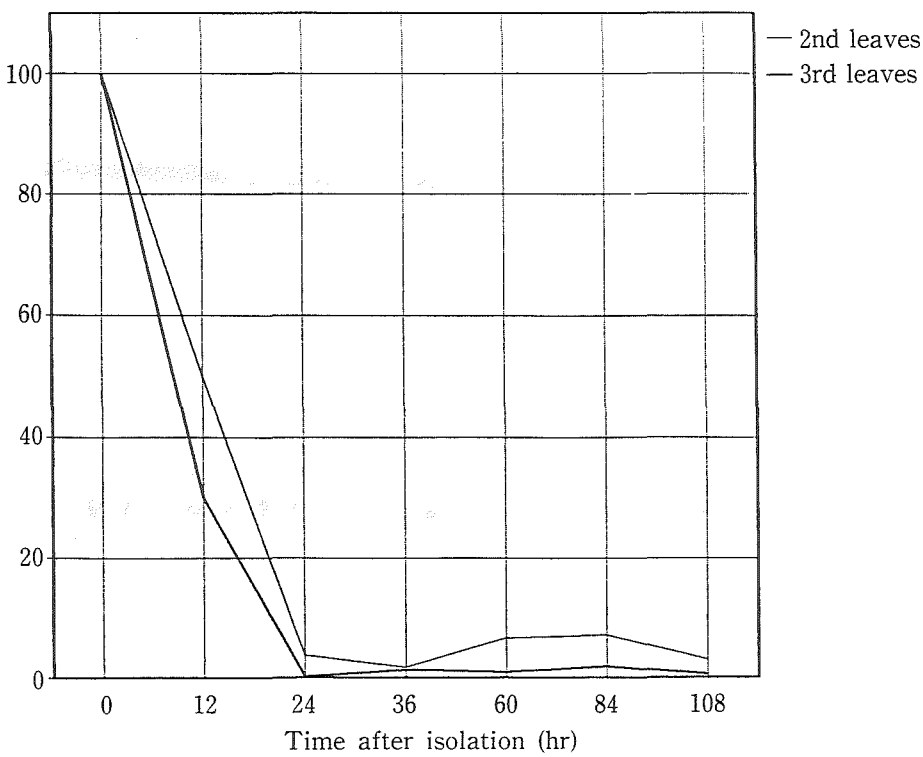

Fig. 2 Relative percentage of the protoplasts' survival after isolation. The viability of protoplasts was estimated by staining protoplasts with FDA and the number of viable protoplasts/total number of protoplasts was determined by examination under microscope.

time of the appearance of gene expression products in protoplasts, PAL activity was determined with respect to the time of incubation of protoplasts after the elicitor treatment (100 μg glucose equivalent/ml). PAL activity was estimated by the rate of ^{14}-C incorporation from [^{14}C]-L-phenylalanine into cinnamic acid as described[25]. A rapid increase of the accumulation of [^{14}C]-cinnamic acid was observed 6 h after the elicitor treatment, while the lower amount of the accumulation was observed 10 h after in mock-treated (Fig. 3). The accumulation of [^{14}C] -coumaric acid and naringenin chalcone was also induced by the treatment with the fungal elicitor.

Optimal conditions in electroporation using protoplasts from leaves and suspension cultured cells were examined by comparing CAT activities in transient assay using pCaMVCN. First, the effects of various electric field and time constant on electroporation were examined by comparing the level of CAT activity in transient assay. A maximum CAT activity was obtained under the electroporation condittion of 1,000 V/cm electric field, 0.5ms time constant, 5 times pulse with 50μg of pCaMVCN and 50 μg of sonicated herring carrier DNA in a solution containing 0.5 M mannitol and 0.1 mM $MgSO_4$ when protoplasts from leaves were used[9]. On the other hand, a maximum CAT activity was obtained under almost identical conditions except a time constant of 2.0 ms instead of 0.5 ms when protoplasts from suspension cultured cells were used[9].

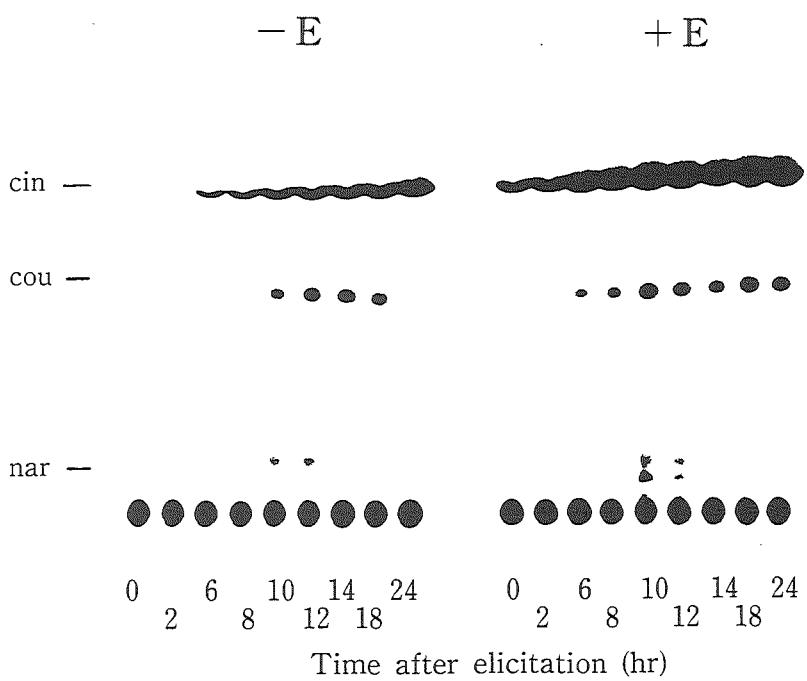

Fig. 3 Induction of PAL activity in pea protoplasts. A conversion of ^{14}C-Phenylalanine into ^{14}C-cinnamic acid in pea protoplasts treated with elicitor (+E) or mock-treated (−E) with respect to the time of incubation was analyzed on TLC. The positions of *t*-cinnamic acid (cin), *p*-coumaric acid (cou), and naringenine chalcone (nar) are indicated.

Dosage effects of pCaMVCN in electroporation was also examined. One to 50 μg of pCaMVCN was electroporated into $1\text{-}5\times10^6$ protoplasts prepared from suspension cultured cells in the presence or absence of 50 μg of sonicated herring DNA as a carrier. The more the DNA electroporated, the higher the CAT activity on transient assay obtained[9]. Moreover, higher CAT activity was obtained in the presence of carrier DNA than in the absence.

The level of CAT activities on transient assay with respect to the time of incubation after electroporation of pCaMVCN was examined by using protoplasts from leaves and those from suspension cultured cells. Higher level of CAT activity was observed after 13 h with protoplasts from leaves.[9] CAT activity was not detected when a promoter-less plasmid, pHSG-CN (Fig. 1) which lacks CaMV 35S promoter was electroporated under the same condition.[9] In case of suspension cultured cells, CAT activity was detected after 9 h of incubation until 48 h and maximal CAT activity was observed after 27-30 h of incubation[9].

The effects of the elicitor concentration on the induction of CAT activity in the suspension cultured-protoplasts electroporated with pPAL1-15 that contains a chimeric gene in which the putative promoter fragment from *PSPAL1* was tranlationally fused to bacterial *cat* gene (Fig. 1) was examined with respect to the time of incubation upon the treatment with fungal. Distinctive induction was observed by the treatment of elicitor (100μg and 1,000 μg/ml glucose equivalent) 12 h after the treatment (Fig. 4). Furthermore, the induction of CAT activity in the suspension cultured-protoplasts electroporated with pPAL1-5 was examined with respect to the time of incubation upon the treatment with fungal elicitor (100μg/ml glucose equivalent), a reduced form of glutathione

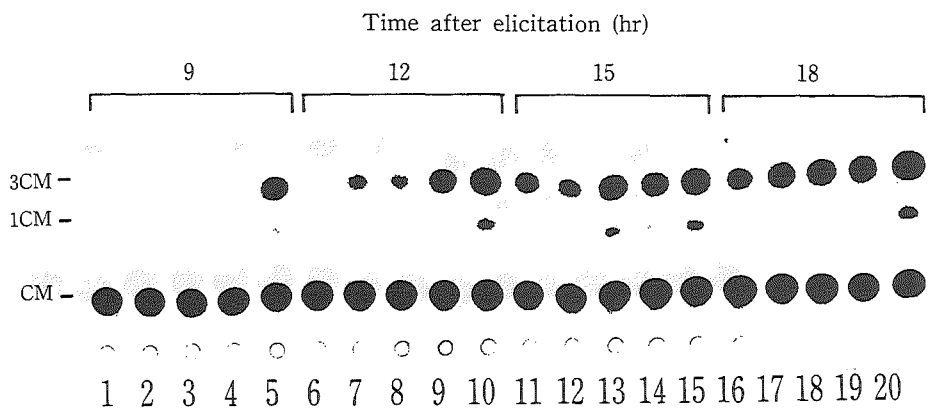

Fig. 4 Effects of elicitor concentration on transient CAT activity in pea protoplasts. A transient CAT activity in pPAL1-15-electroporated pea protoplasts treated with the various concentration of elicitor with respect to the incubation time (in h) were determined by the conversion of ^{14}-CM into acetylated chloramphenicol, Lane 1, 6, 11, 16 ; no elicitor treatment : Lane 2, 7, 12, 17 ; 10 μg/ml gulucose equivalent elicitor : Lane 3, 8, 13, 18, 50 μg/ml elicitor : Lane 4, 9, 14, 19 ; 100 μg/ml elicitor ; Lane 5, 10, 15 20 ; 1,000 μg/ml elicitor. *The numbers on top indicate the incubation time (h) after the* elicitor treatment. Positions of chloramphenicol (CM), 1-acetylchloramphenicol (1CM), 3-acetylchloramphenicol (3CM) and 1, 3-acetylchloramphenicol (1, 3-CM) are indicated.

(GSH) (50 μM), and elicitor in the concomitant presence of orthovanadate (Fig. 5). CAT activity was induced by the treatment with fungal elicitor and GSH 6-9 h after the treatment, but it was considerably suppressed in the concomitant presence of orthovanadate (Fig. 5).

These results indicate that the putative promoter fragment spanning -340 to+140 relative to the transcriptional start site of *PSPAL1*[11,12] is at least partly responsible for the activation of the PAL gene induced by fungal elicitor or GSH. In this region, conserved motifs of box I-IV sequences found in the promoter fragments of the genes involved in phenylpropanoid pathway are present[26].

We have examined the effects of the induction on gPAL1-15-electroporated rice protoplasts by the treatment with a hexamer of N-acetylglucosamine (1 μg/ml), known as an elicitor for rice. CAT activity was not induced by the treatment with rice elicitor under the same conditions as described in the case of pea (data not shown). It is presumed that the mechanisms of the induction of the defense gene in pea and rice is fundamentally different.

Although a large number of studies have been performed regarding the functional analysis of *cis* DNA elements in plant genes, including genes encoding phenylpropanoid pathway, genes subjected to light-regulation, etc, by creating trangenic plants with a reporter gene construct or by DNA footprinting analyses, very few successful cases have been reported so far[2,4,24,25,27,22]. Our results should be the first case to present the elicitor-respossiveness of PAL gene by transient transformations with reporter gene constructs.

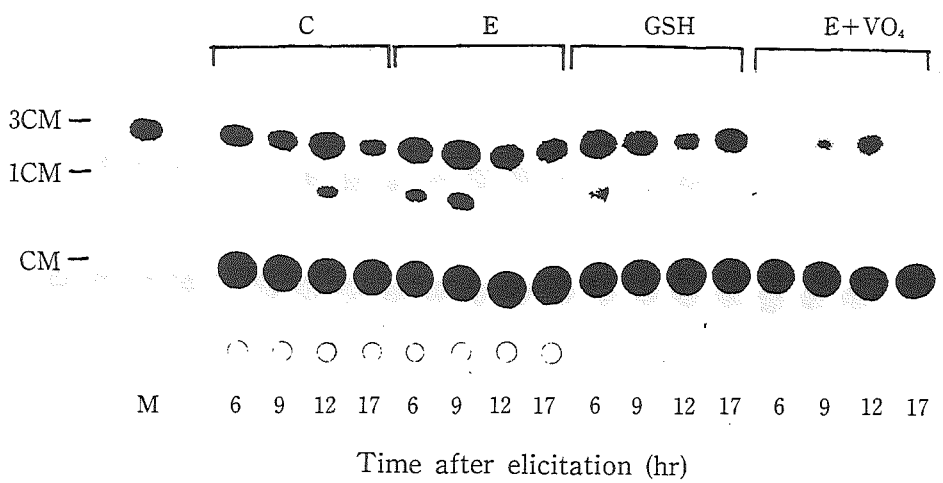

Fig. 5 Effects of the fungal elicitor, a reduced form of GSH and orthovanadate on CAT activity in pPAL1-15-electroporated pea protoplasts. CAT activity in pPAL1-15-electroporated pea protoplasts were examined 6, 9, 12 and 17 h after the treatment with 1,000μg/ml of elicitor (E), 50μM GSH (GSH) and a fungal elicitor in the concomitant presence of 1 mM orthovanadate (E+VO_4). Mock-treated protoplasts are indicated as a control (C). The numbers below indicate the incubation time (h) after the treatment. Positions of CM, 1CM, 3CM and 1, 3-CM are indicated.

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エンドウプロトプラスト細胞における植物プロモータの発現

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エンドウのフェニールアラニンアンモニアリアーゼ (CAT) をコードする遺伝子のプロモータを含むDNA断片を，クロランフェニコールアセチルトランスフェラーゼ (CAT) をレポーター遺伝子としてもつプラスミドに連結した組換え体キメラプラスミド (pPAL1-15) を構築し，種子胚由来カルス，あるいは葉内細胞から調製したエンドウプロトプラストにエレクトロポーレーション法を用いて導入し，CATのトランジエントな発現を調べた．エンドウ褐紋病菌由来エリシターや，還元型グルタチオンはCATの発現を誘導したが，原形質膜ATpase阻害剤であるオルトバナジン酸では，エリシターによる誘導が抑えられた．種子胚由来カルスから調製したイネプロトプラスト細胞に，pPAL1-15をエンドウと同様に導入し，イネに働くエリシターであるキトサンのオリゴマーを処理したところ，CAT活性の誘導が見られなかった．以上の結果はエンドウとイネのプロロプラスト細胞の遺伝子発現を含めた外界シグナルに対する応答機構が異なる可能性を示唆している．

謝　　辞

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