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Stress responses in alfalfa (*Medicago sativa* L.) IV. Expression of defense gene constructs in electroporated suspension cell protoplasts

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Summary. We have investigated conditions for the uptake and expression of chimeric genes in protoplasts of alfalfa (Medicago sativa L.). Constructs containing the bacterial reporter gene chloramphenicol acetyltransferase (CAT) under the control of either the cauliflower mosaic virus 35S promoter or a bean chalcone synthase (CHS) promoter were introduced into protoplasts by electroporation in the presence of polyethyleneglycol. The extent of expression in the absence of added inducers depended on the conditions for isolation, electroporation and subsequent culture of the protoplasts. Expression of the CHS promoter construct was increased on exposure of the protoplasts to a fungal elicitor or reduced glutathione. The relative levels of induced expression in relation to either basal expression or the type of elicitor used depended on the age of the suspension cultures from which the protoplasts were isolated. Electroporation of protoplasts with a construct from which bean CHS antisense transcripts were synthesized under the control of the 35S promoter resulted in the inhibition of appearance of elicitor-induced endogenous alfalfa CHS activity. The suitability of the alfalfa protoplast system for analysis and potential identification of defense response genes is discussed.

Key Words: Medicago sativa L. - Protoplast - Chalcone synthase - Promoter - Transient assay - Antisense RNA

Abbreviations: CAT, chloramphenicol acetyltransferase; CHS, chalcone synthase; CLE, elicitor from Colletotrichum lindemuthianum; CM, acetylated chloramphenicol; GSH, reduced glutathione; MES, (2-[N-Morpholino] ethanesulfonic acid); NOS, nopaline synthase; PEG, polyethyleneglycol

INTRODUCTION

A major defense response of leguminous plants to fungal infection is the rapid accumulation of antimicrobial isoflavonoid phytoalexins in the area of attempted penetration (Dixon 1986). This has been shown in a number of systems to involve rapid transcriptional activation of the genes encoding phytoalexin biosynthetic enzymes (Dixon and Harrison 1990). Currently, attention in this area is being focused on the analysis of the cis- and trans-acting factors involved in the activation of these defense response genes (Dron et al. 1988; Lois et al. 1989) and on the identification of specific enzymes involved in the later stages of phytoalexin biosynthesis (Hinderer et al. 1987; Kochs and Grisebach 1986; Kessmann et al. 1990). Altering the expression of these enzymes in transgenic plants is one potential strategy for engineering improved disease resistance.

In alfalfa cell suspension cultures, synthesis of the isoflavonoid phytoalexin medicarpin is preceeded by rapid increases in the extractable activities of the eleven enzymes involved in its synthesis from L-phenylalanine (Dalkin et al. 1990; Kessmann et al. 1990). Among these enzymes are chalcone synthase (CHS), which is encoded by a multigene family in both bean and alfalfa (Ryder et al. 1987; K. Dalkin and R.A.D., unpublished results), and three cytochrome P450 activities (cinnamic acid 4-hydroxylase, isoflavone synthase and isoflavone 2'-hydroxylase). The cytochrome P450 enzymes are induced in both cell suspension cultures and protoplasts of the alfalfa cultivar Calwest 475 by an elicitor preparation from cell walls of the bean pathogen Colletotrichum lindemuthiamun (Kessmann et al. 1990).

The functional analysis of defense gene promoters in transgenic plants or by transient assay in electroporated protoplasts is most meaningfully performed in the homologous plant species, and extrapolation between transient assay and stable transformation data requires that the same species (and cell type) be analyzed in both types of experiment. To date bean (Phaseolus vulgaris L.) has been the subject for most of the molecular analysis of legume defense response genes (Dixon and Harrison 1990). However, there is at present no effective transformation system for bean. Among other legumes which share with bean common biosynthetic pathways to isoflavonoid phytoalexins, alfalfa is the most amenable to stable genetic transformation (Shahin et al. 1986, Chabaud, et al., 1988).

In order to develop alfalfa as a system for the functional analysis of defense response genes, we have established conditions for the efficient expression of a bean CHS promoter in electroporated protoplasts. We also describe the effects of expression of antisense CHS transcripts on CHS enzyme activity in these protoplasts, and thus define a potential method for the characterization of elicitor-induced cDNAs encoding enzymes for which probes are not readily obtainable via classical biochemical strategies.

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MATERIALS AND METHODS

Cell culture and isolation of protoplasts. The initiation and growth of cell suspension cultures of alfalfa (Medicago sativa L. cv. Calwest 475) and the optimized protocol for the isolation of protoplasts from cultures 5 days after subculture are described in an accompanying paper (Kessmann et al. 1990). Effects of modifications to the standard protoplast isolation/culture protocol are described in "Results".

Electroporation of protoplasts. Prior to electroporation, protoplasts were re-suspended at a density of 2.0 x 10^7/ml in either MaMg solution (0.4 M mannitol, 30 mM $MgCl_2$, 0.1% w/v MES, pH 5.8 (Negrutiu et al. 1987)) or MsMg solution (MS salts, 0.4 M mannitol, 30 mM $MgCl_2$, 0.1% w/v MES, pH 5.8). The protoplasts were heat-shocked for 5 min at 45°C, and then brought to room temperature by incubating on ice for 2 min. Aliquots (500 µl) of heat-shocked protoplasts were distributed in 1 ml plastic spectrophotometer cuvettes containing DNA samples (10-60 µg plasmid DNA plus 50 µg carrier calf thymus DNA) and incubated at room temperature for 10 min. Two hundred µl of 40% PEG (40% v/v PEG, 0.1% w/v MES, 0.4 M mannitol, 30 mM $MgCl_2$, pH 7.0) were then added, followed by incubation on ice for 10 min. Protoplasts were electroporated using a BTX Transfector 300 unit (BTX, San Diego, CA). In initial optimization experiments, the pulse was delivered from capacitors of 50 to 200 µF charged from 100-200 V. In all subsequent experiments, electroporation was at 150 V and 50 µF. Following electroporation, protoplasts were first incubated on ice for 10 min, and then at room temperature for an additional 10 min before serial dilution with 5 volumes of 0.2 M $CaCl_2$. Samples were centrifuged for 5 min at 50 x g, washed with protoplast culture medium (Kao and Michayluk 1980) and then cultured in 2 ml of protoplast culture medium at 25°C in the dark. Electroporated protoplasts were treated with elicitor from cell walls of Colletotrichum lindemuthianum (CLE) (50 µg glucose equivalents/ml final concentration) (Hamdan and Dixon 1986) or reduced glutathione (0.5 mM final concentration) at 2, 6 or 20 h after transfer to protoplast culture medium. Controls were treated with equal volumes of culture medium. Elicited protoplasts were harvested at 0, 2, 6, 8 and 10 h after elicitation for assay of CAT activity, and at 0, 6, 18 and 24 h for assay of CHS activity.

Enzyme assays. CAT activity was determined in protoplast sonicates essentially as described by Fromm et al. (1985). The assay mixture (final volume 150 µl) contained 4 µl acetyl coenzyme A (40 mM), 0.05 µCi [^{14}C]-chloramphenicol (54 mCi/mmol, Amersham) and 100 µl of extract (35 µg protein) in 40 mM Tris-HCl pH 7.5. After incubation for 2 h at 37°C, the reaction mixtures were extracted 2 x with equal volumes of ethyl acetate. The acetylated reaction products were separated by thin layer chromatography on silica plates [Merck GF254, 0.2 mm] developed in chloroform: methanol (24:1 v/v). After identification of reaction products by autoradiography, spots were scraped off the plates and radioactivity determined by liquid scintillation counting.

For assay of CHS, protoplasts were extracted by sonication on ice for 40 sec in 150 µl of 50 mM KH_2PO_4 pH 8.0 containing 20 mM ascorbic acid. The assay was perfomred using 50 µl of the supernatant as described by Edwards and Kessmann (1990). Protein was determined by the Bradford procedure (Bradford 1976).

Construction of plasmids. pCHC1 contained the 5' upstream region to -326 of the bean CHS15 promoter (Ryder et al. 1987; Dron et al. 1988) in a transcriptional fusion to the bacterial CAT gene, followed by the NOS 3' terminator, in pUC19 (Fig. 1). pDO400 was reconstructed by excising the CAT insert from pCHC1 with BamH1, gel-purifying the fragment and re-ligating into BamH1-cut pDO394, a plasmid containing the 35S promoter linked via the BamH1 site to the NOS 3' terminator, inserted between the HindIII and EcoR1 sites in the pUC19 polylinker. A full length bean CHS cDNA in pSP64 (pCHS17, Ryder et al. 1987) was cut with BspM1 to yield a 984 bp fragment containing the 5' half of the cDNA sequence. The fragment was blunt-ended by filling with dNTPs using Klenow polymerase, and re-ligated into pDO394 which had been cut with BamH1 and similarly blunt-ended. The orientations of the inserts in transformants were determined by restriction analysis, and plasmids designated as pCHSS984 (cDNA in "sense" orientation) and pCHSA984 (cDNA in the antisense orientation). A plasmid containing 387 bp of the 5' end of the CHS17 cDNA in the antisense orientation (pCHSA387) was constructed in a similar manner after excision of the 5' fragment from pCHS17 with EcoR1 and Pst1.

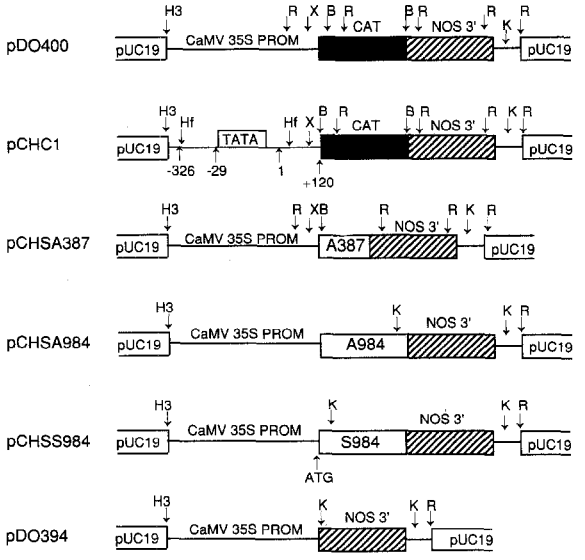

Figure 1. DNA constructs used in the present work (see Materials and Methods). H3 = HindIII, R = EcoR1, X = Xba1, B = BamH1, k = Kpn1, Hf = HinfIII.

RESULTS AND DISCUSSION

Optimization of electroporation conditions. Optimum conditions for protoplast electroporation vary greatly in higher plants. However, the most efficient uptake of DNA by protoplasts often occurs at the voltage which kills 30-50% of the protoplasts (Hofmann 1987). In the alfalfa protoplasts, a single 26 msec pulse at 150 V caused a decrease in viability from 90% to around 50% (data not shown). These conditions resulted in optimum levels of expression of introduced foreign chimeric DNA constructs and were used in all subsequent experiments.

To test the effect of different electroporation media on uptake and transient gene expression, we electroporated the constructs pDO400 and pCHC1 (Fig. 1). The expression of both constructs in the alfalfa protoplasts was dependent upon the electroporation medium (Fig. 2), being greatest when protoplasts were electroporated in MsMg medium compared to the MaMg medium in which $MgCl_2$ is the only salt present. The positive effect of inclusion of salts in electroporation media has been reported previously (Negrutiu et al. 1987).

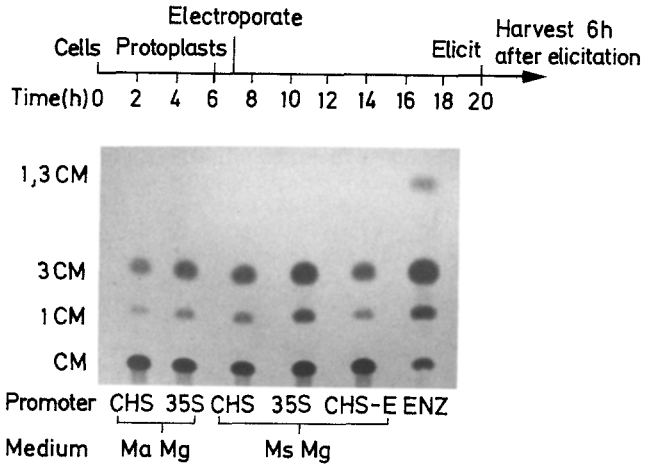

Figure 2. The effects of culture medium on the expression of CAT from CHS promoter-CAT-NOS 3' and 35S promoter-CAT-NOS 3' constructs in alfalfa protoplasts prepared and electroporated according to the schedule shown. See text for details of MaMg and MsMg media. CHS-E shows CAT activity in extracts from unelicited protoplasts containing the CHS promoter construct. 1-, 3- and 1,3-CM are the acetylated products of the CAT reaction resolved by thin layer chromatography and visualized by autoradiography.

The presence of polyethyleneglycol (PEG) in the electroporation medium is sometimes beneficial (Hashimoto et al. 1985; Boston et al., 1987) or even essential (Shillito et al. 1985; Negrutiu et al. 1987) for efficient DNA uptake. We also found inclusion of PEG to be beneficial in the alfalfa protoplasts. The presence of divalent cations (Mg^{2+} or Ca^{2+}) in the electroporation medium is generally considered necessary for good DNA uptake (Hofmann 1987; Negrutiu et al. 1987; Boston et al. 1987), although some reports suggest that Ca^{2+} may be ineffective (Nishiguchi et al. 1986) or even toxic (Watts et al. 1987). In our initial studies we tested separately the effects of both $MgCl_2$ and $CaCl_2$ in the electroporation medium and found $MgCl_2$ to be necessary for efficient electroporation leading to CAT expression; exchanging Mg^{2+} with Ca^{2+} resulted in approximately 10 times less expression.

Characteristics of the transient assay system. The promoter sequence of the bean CHS15 gene in the chimeric CHS-CAT-NOS construct pCHC1 comprises 326 bp upstream of the transcription start site and 102 bp of transcribed leader sequence (Fig. 1). This chimeric gene was expressed at high levels (comparable to expression from the 35S promoter) after electroporation into the alfalfa protoplasts. An increase in CAT activity above control levels was recorded from 6-8 h after addition of CLE with an increasing elicitor-mediated stimulation as a function of time up to 10 h (Fig. 3). The extent of expression increased as a function of the concentration of pCHC1 in the electroporation cuvette, both in the presence or absence of elicitor (Fig. 3, inset).

The protoplasts analyzed in the above experiments were from suspension cultures which had been through at least 10 serial subcultures from callus. As seen in Fig. 3, their behaviour was characterized by relatively high constitutive expression from the CHS promoter with a weak but reproducible stimulation of expression by fungal elicitor or reduced glutathione (GSH) (1.5-fold, 10 h after elicitation). Protoplasts from newly

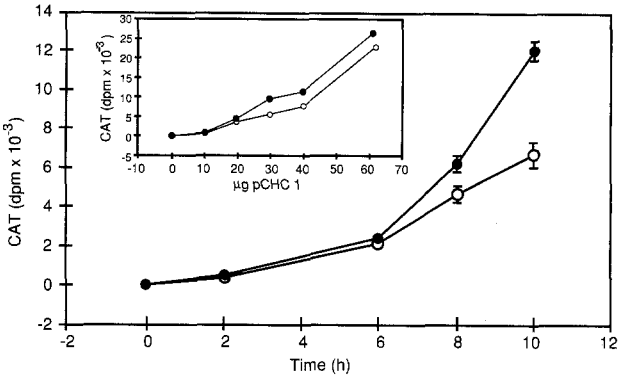

Figure 3. CAT expression as a function of time in elicitor-treated (●) and control (○) alfalfa protoplasts electroporated with pCHC1. 40 µg pCHC1 were present in the electroporation cuvette, and CLE (50 µg glucose equivalents/ml) was added at zero time (2 h after electroporation). Inset: CAT expression as a function of the amount of pCHC1 plasmid present in the electroporation cuvette. Controls (○) and protoplasts treated with CLE as above (●), were harvested after 10 h. The cultures from which the protoplasts were isolated had been through more than 10 passages since initiation from callus. Error bars represent spread of values in duplicate electroporations with protoplasts from the same culture batch (no error bar, s.d. <5%).

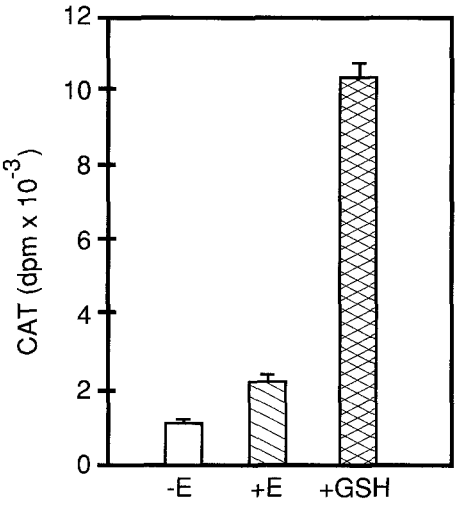

Figure 4. CAT expression in control alfalfa protoplasts (-E), or protoplasts treated with CLE (50 µg glucose equivalents/ml final concentration) (+E) or reduced glutathione (0.5 mM final concentration) (+GSH). Protoplasts were electroporated in the presence of 40 µg pCHC1, elicitors added 2 h later, and protoplasts harvested 10 h after elicitation. The cultures from which the protoplasts were isolated had been through 5 passages since initiation from callus. Error bars represent spread of values from duplicate determinations.

isolated suspension cultures (between 4-8 passages) behaved differently from the protoplasts from the older cultures. The basal expression of pCHC1 was relatively low, a weak stimulation was again observed in response to fungal elicitor, but an approximately 10-fold increase above basal levels was recorded in response to GSH (Fig. 4). Altering the length of time that the cells were exposed to protoplasting enzymes (5 h or overnight) or the timing of addition of elicitor (2,6 or 20 h after protoplast preparation), did not yield conditions in which the high basal relative to induced CAT expression could be reduced in protoplasts from older cultures. However, responsiveness to elicitor, although low, was always maintained in these cultures. The results indicate that the 326 bp 5' of the transcription start site of the bean CHS gene are sufficient to confer regulation by glutathione or fungal elicitor in alfalfa protoplasts, confirming recent results with soybean protoplasts (Dron et al. 1988).

The extent of expression of the CHS-CAT-NOS construct in electroporated alfalfa protoplasts was also found to vary with the elicitor used (data not shown). Among the different elicitors tested, GSH was found to be the most effective in inducing CAT expression, followed by CLE. The observation that GSH is inactive as an elicitor in alfalfa cells (Dalkin et al. 1989) but active in protoplasts may be explained by differences in uptake by cells and protoplasts (R. Edwards and R.A.D., unpublished results). Yeast extract elicited a very weak response, whereas oligogalacturonide fragments of various sizes were totally ineffective in stimulating expression. This suggests that the basal expression from the CHS promoter in the absence of added elicitor may not simply result from the effects of oligogalacturonide elicitors (Davis et al. 1987) released from the alfalfa cell walls during protoplasting.

The reproducibility of the transient assay system was assessed in terms of variability of pCHCI expression using (a) different batches of protoplasts and (b) identical treatments of the same protoplast stock. More than 10-fold differences were observed in the absolute levels of elicitor-induced CAT activity between different protoplast batches although the degree of induction was reproducible. In 7 independent experiments, each with a different protoplast batch, a fold-induction of 1.52 $\pm$ 0.23 was observed (for CAT activity measured 10 h after elicitation). However, variation between values for CAT expression from pCHC1 for replicate electroporations of the same protoplast batch was not greater than $\pm$ 4-5% (n = 4). The system is therefore highly reproducible providing all results are related to internal controls included with the same batch of protoplasts as used for the test electroporations. In view of the genotypic heterogeneity of alfalfa cultivars, some variability of response between different protoplast batches might be minimized by isolating the initial callus from a single plant. We have found, however, that most of the variability between protoplast batches probably reflects the metabolic state of the cells after protoplasting.

The above data indicate that electroporated alfalfa protoplasts can be used as a stimulus-responsive system for the transient assay of a plant defense gene promoter. Levels of expression are high and can be readily measured in small batches of protoplasts. Use of this system for the analysis of deletions in the bean CHS promoter has helped define cis-acting regulatory elements (M.J. Harrison, A.D.C., J.Kooter, C.J.L. and R.A.D., in preparation), and can now be complemented by functional analysis in transgenic alfalfa.

Expression of antisense constructs in alfalfa protoplasts. We have also tested the feasibility of using the alfalfa protoplast system for the characterization of unidentified cDNA clones by measuring the effects of expression of antisense RNA. Two plasmids were constructed for expression of CHS antisense transcripts. pCHSA387 contained 330 bp of the CHS 17 (Ryder et al. 1987) cDNA coding sequence including the translational start site and 57 bp 5' of this flanked by the 35S promoter and NOS 3' terminator (Fig. 1). In pCHSA984 the cDNA insert was extended to 984 bp. After electroporation into alfalfa protoplasts, expression of these antisense chimeric genes completely eliminated elicitor-induced expression of endogenous alfalfa CHS, as measured by enzyme activity (Fig. 5). This suggests that there must be considerable homology between bean and alfalfa CHS sequences. The effects of antisense RNA were exactly reproduced in subsequent experiments. Interestingly, the control sense construct pCHSS984 (Fig. 1) also reduced the expression of endogenous alfalfa CHS in some experiments (Fig. 5A), although the absolute extent of this effect was somewhat variable (see Fig 5B). It is conceivable that this inhibition could be the result of the formation of inactive CHS heterodimers between alfalfa CHS subunits and incomplete bean CHS

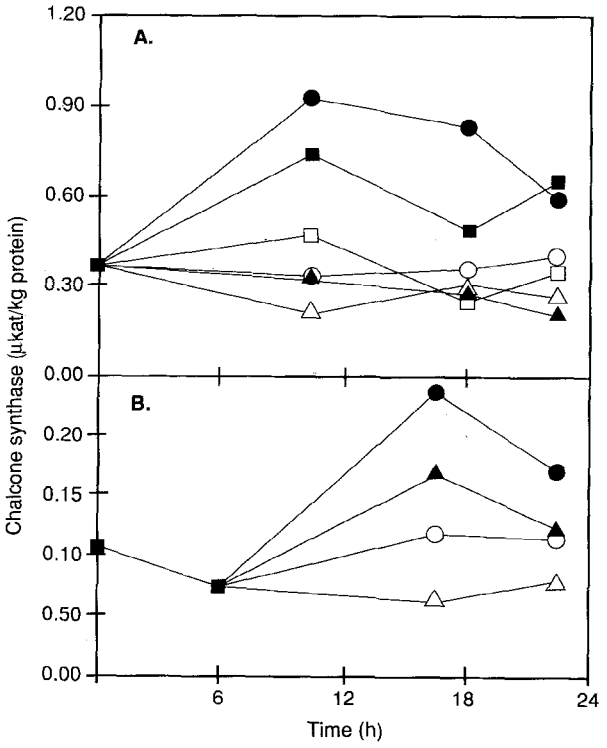

Figure 5. The effects of expression of bean CHS transcripts on elicitor-mediated induction of CHS activity in electroporated alfalfa protoplasts in 2 independent experiments (**A** and **B**). Control (unelectroporated) protoplasts were either untreated (○) or treated with GSH (2 h after electroporation) at a final concentration of 0.5 mM (●). Elicitor-treated protoplasts were first electroporated in the presence of 40 µg of the following plasmids: pDO394 (control with no CHS insert) (■); pCHSS984 (to produce a transcript with 984 bp of bean CHS, sense orientation) (▲); pCHSA984 (984 bp bean CHS, antisense orientation) (△); pCHSA387 (387 bp bean CHS, antisense orientation) (□).

subunits. The control construct pDO394 (35S-NOS 3' with no CHS insert) did not prevent induction of CHS activity when electroporated into the alfalfa protoplasts. Furthermore, electroporation of the CHS antisense construct did not inhibit the induction of the next enzyme in the flavonoid/isoflavonoid pathway, chalcone isomerase, in the alfalfa protoplasts.

We have recently shown that three cytochrome P450 enzymes involved in medicarpin biosynthesis (cinnamic acid 4-hydroxylase, isoflavone synthase and isoflavone 2'-hydroxylase) are induced in alfalfa protoplasts in response to fungal elicitor (Kessmann et al. 1990). These membrane-bound enzymes are labile and difficult to purify by classical biochemical methods. The inhibition of elicitor-mediated enzyme induction in the protoplasts by antisense RNA suggests that it may be feasible to characterize putative cytochrome P450 cDNAs (or cDNAs encoding any other enzyme involved in medicarpin biosynthesis) by electroporation of constructs expressing antisense transcripts and assaying for inhibition of elicitor-induced enzyme activity. Work is now in progress to obtain candidate clones for this approach by differential hybridization and PCR amplification strategies.

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