Plant Molecular Biology **21**: 341–354, 1993.

Induction of a tomato anionic peroxidase gene (*tap1*) by wounding in transgenic tobacco and activation of *tap1*/GUS and *tap2*/GUS chimeric gene fusions in transgenic tobacco by wounding and pathogen attack

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Received 3 September 1992; accepted 30 September 1992

Key words: β-glucuronidase gene, pathogen induction, tomato anionic peroxidase gene promoters, transgenic tobacco, wound induction

Abstract

The anionic peroxidase genes of tomato, *tap1* and *tap2*, are induced by wounding in tomato fruits and by elicitor treatment in cell suspension cultures. These homologous genes code for anionic peroxidases that are postulated to cause polymerization of the phenolic residues into wall polymers in wound-healing and pathogen-infected tissues. An expression construct containing the entire TAP1 gene with its 5′ and 3′ flanking sequences was introduced into tobacco by *Agrobacterium tumefaciens*-mediated gene transfer. Also, constructs containing the 5′ upstream regions of *tap1* and *tap2* including sequences coding for their respective putative leader peptides fused translationally to the β-glucuronidase (GUS) reporter gene were made and introduced into tobacco. Northern blot analysis of transcripts from wound-healing leaf tissues of transformants containing *tap1* showed that the introduced gene was being transcribed in the heterologous host. The induction of *tap1* transcripts in the wound-healing transgenic tobacco tissues was observed by 48 h and increased over time period of 84 h. Wounding also led to expression of GUS in *tap1*/GUS and *tap2*/GUS transformants and GUS activity was localized to the wound site. Activation of the *tap1* and *tap2* promoters in wound-healing transgenic tobacco tissues showed a GUS expression profile that correlated with the postulated role for anionic peroxidases in phenolic polymerization in suberizing tissues. Inoculation of *tap1*/GUS and *tap2*/GUS transformant leaves with fungal conidia from *Fusarium solani* f. sp. *pisi* caused expression of GUS in locally inoculated regions, and GUS expression increased over a period of four days.

Introduction

Higher plants respond to stress such as wounding and pathogenic attack by eliciting a number of defense mechanisms. Lignification and suberization of the plant's cell walls have been suggested as being part of the plant's elaborate defense strategies. Like lignin, suberin comprises an aromatic polymer that is covalently attached to carbohydrates of the plant cell wall [18] and/or possibly attached to proteins like the hydroxyproline-rich glycoproteins (HRGPs). Suberin however also contains aliphatic components and associated waxes that are not present in lignin, which

together with the aromatic domain form a hydrophobic barrier to water-proof the wounded plant tissues [19]. The deposition of these phenolic polymers as barriers on the walls of the wound-healing or infected tissues has been postulated to prevent the entry or spread of phytopathogens [17]. The polymerization of the phenolic moieties of the aromatic domain are thought to be catalyzed by a cell wall-associated anionic peroxidase [2, 4, 9]. Suberin has been shown to be the wound-induced polymer in many plant organs from many plants and seems to be the universal barrier in wound-healing tissues of higher plants [5], that renders the cell walls highly resistant to mechanical and enzymatic disruption [19, 28]. More recently, we have shown that suberization is induced during a resistance response in tomatoes to fungal attack [33]. Using petioles from two near-isogenic tomato lines that were resistant and susceptible to the vascular wilt-causing fungus *Verticillium albo-atrum* it was demonstrated that, when challenged with fungal spores the resistant, but not the susceptible plants, were able to respond in a timely manner by inducing the deposition of suberin on the walls of the infected tissues.

Two tandemly located homologous genes in tomato encoding the highly anionic peroxidases, *tap1* and *tap2*, thought to be involved in suberization, were cloned and characterized [34]. Both the potato and tomato anionic peroxidase genes were found to be induced specifically only during suberization in wound-healing potato tubers and tomato fruits, respectively [35]. Although *tap1* is known to be induced by wounding, whether *tap2* is expressed is not known [34]. When tomato petioles from resistant and susceptible near-isogenic lines were challenged with spores from *V. albo-atrum*, only the resistant line responded in a timely manner by producing the anionic peroxidase transcripts [33].

In order to study the regulation of the tomato anionic peroxidases, we generated transgenic tobacco plants that were transformed with constructs containing the entire TAP1 gene together with its 5′ and 3′ flanking regions. Constructs containing the 5′ promoter region and coding sequences for the putative TAP-1 leader peptide fused translationally to the β-glucuronidase (GUS) reporter gene, and similarly the 5′ DNA sequences of *tap2*, that contained the promoter and putative TAP-2 leader peptide sequences also fused translationally to the GUS coding sequences, were introduced into tobacco. We report here, that *tap1* transformant tobacco plants expressed the tomato gene upon wounding. Also, *tap1*/GUS and *tap2*/GUS transformants when wounded induced localized production of GUS activity in the wound-healing tobacco plant tissues. GUS activity was also induced in leaves of transgenic *tap1*/GUS and *tap2*/GUS transformants upon inoculation with a fungus, *Fusarium solani* f. sp. *pisi*. Thus, we were able to ascertain that not only *tap1*, but also *tap2*, is expressed upon wounding and fungal attack.

Materials and methods

Vector construction and generation of transgenic tobacco plants

The DNA sequences of the TAP1 gene together with its 5′ and 3′flanking regions contained on two *Xba* I-*Pst* I restriction enzyme fragments [34] were ligated at the *Pst* I sites to reconstruct the gene and transferred into the *Xba* I site of pBIN19 [1] to produce TAP1[X-X]pBIN19. To clone the *tap1* and *tap2* gene promoters into a plant expression vector in frame with the GUS marker gene, an intermediate plasmid pGUS.2 was created so as to facilitate the cloning steps. All DNA manipulations were done according to standard procedures [26]. The plasmid pGUS.2 was constructed by cloning the *Hin*d III-*Eco* RI fragment that contained the DNA sequences of the multiple cloning site, GUS gene and polyadenylation signals of the NOS gene from the expression vector pBI101.2 [16] into the *Hin*d III-*Eco* RI sites of the plasmid pUC19. A 767 bp *Eco* RV-*Pvu* II DNA fragment derived from the 5′ region of *tap1* was cloned into the *Sma* I site within the multiple cloning region of pGUS.2 in frame with the GUS gene to produce TAP1[−461]-LP/GUS.

This *tap1* fragment contained the upstream promoter region and sequences encoding the N-terminal 83 amino acids of TAP-1 (74 amino acids putative leader sequence and 9 amino acids of the putative mature peptide). Similarly, a 1560 bp *Xba* I-*Pvu* II DNA fragment derived from the 5′ end of *tap2* [34] was cloned into the *Xba* I-*Sma* I sites of pGUS.2. The *tap2* fragment contained the upstream promoter region and sequences encoding the N-terminal 84 amino acids of TAP-2 (73 amino acid putative leader sequence and 11 amino acid of the putative mature peptide). The TAP-1 gene promoter-LP-GUS-NOS and TAP-2 gene promoter-LP-GUS-NOS chimeric constructs were excised with the restriction endonucleases *Hind* III and *Eco* RI and cloned back into the *Hind* III-*Eco* RI sites of the expression vector pBI101.2 (from which the GUS-NOS DNA fragment had been removed) to produce expression vectors TAP1[−461]-LP/pBI101.2 and TAP2[−1320]-LP/pBI101.2, respectively. The expression vectors were transferred into *Agrobacterium tumefaciens* LBA4404 and then into tobacco (*Nicotiana tabacum* cv. Havana) by the leaf-disc transformation method [14].

Southern blot analysis

Total DNA was isolated from leaf tissues of TAP1[−461]-LP/pBI101.2, TAP2[−1320]-LP/pBI101.2 and TAP1[X-X]pBIN19 transgenic tobacco plants by standard methods [37]. CsCl gradient-purified DNA was digested with the restriction enzymes as indicated in the figure legends, fractionated on a 0.9% agarose gel and blotted onto a Nytran membrane (Schleicher and Schuell). An 1800 bp ^{32}P-labeled GUS gene fragment was used as a probe for the *tap1*/GUS and *tap2*/GUS genomic blot, while a 491 bp *Cla* I-*Dra* I fragment isolated from the 5′ end of *tap1* was ^{32}P-labeled and used for the genomic blot containing the DNAs from TAP1[X-X]pBIN19 transformants. The blots were subjected to autoradiography at −80 °C after washes at stringent conditions (65 °C for 30 min) with the final wash solution containing 0.1 × SSPE, 0.1% SDS.

Wounding experiment

Stems of primary TAP1[X-X]pBIN19 transformants were wounded on the plant by gently rolling a circular file over the surface of the stem. Leaves were also wounded by using a circular file with a wooden block held under the leaf blade for support. This method has been used for wounding as it allows enough crushing to release wound signals but also leaves enough intact cells among the broken cells to express the wound-induced genes [29]. No senescence or wilting was observed when these plants were allowed to wound-heal in a humid atmosphere (80% at 25 °C) under standard growth chamber conditions. Stem tissues were harvested 3 days after wounding. Leaf tissues were harvested after various periods of time as mentioned in the text.

Excised leaves of R1 transgenic TAP1[−461]-LP/pBI101.2 and TAP2[−1320]-LP/pBI101.2 transformant plants were wounded with a 1 ml plastic tip (cut to provide a bore 3 mm in diameter). The leaf tissues were allowed to wound-heal for 0, 20, 33, 48, 72 and 92 h on moist filter paper in Petri plates and harvested by cutting four circular discs, 1 cm in diameter, using a cork borer each containing four 3 mm damaged areas of the leaf tissue.

Isolation of RNA and northern analysis

Total RNA from stem and leaf tissues was isolated by published procedure [3]. Poly(A)$^+$ RNA was selected from total RNA [26], and electrophoresed on a 1.5% agarose gel containing 0.67 M formaldehyde [8]. The RNA was blotted onto a Nytran membrane (Schleicher & Schuell), and hybridized to ^{32}P-labeled 491 bp *tap1* fragment. The blots were subjected to autoradiography at −80 °C.

Western-blot analysis

Protein (100 μg) samples from wound-healed leaf tissues of a *tap1*/GUS and a CaMV 35S/GUS

transformant were subjected to SDS-PAGE on a 10% polyacrylamide gel. The proteins were electroblotted onto a nytran membrane and immunoblot analysis was done using a polyclonal rabbit anti-GUS antibody (Clonetech, CA) according to the manufacturer's recommended methods in TSW buffer (10 mM Tris-Cl pH 7.4, 155 mM NaCl, 0.25% w/v gelatin, 0.1% w/v Triton X-100 and 0.02% w/v SDS). Detection of the protein-antibody hybrid was done using ^{125}I-labeled Protein A, followed by autoradiography.

Preparation of conidia from Fusarium solani *f. sp.* pisi *and inoculation of tobacco leaves*

Fusarium solani f. sp. *pisi* (*Nectaria hematococca*) was grown on PDA plates at 24 °C for 2 weeks and conidia harvested in water. The fungal spores were suspended at 10^6/ml or 8×10^7/ml. For histochemical staining, young leaves from R1 transformants were inoculated by placing 10 μl of 10^6 conidia/ml on the leaf surface. For the time-course analysis, leaves (with a leaf blade size of 10 cm long) from 8-week-old R1 transformants and from untransformed tobacco were inoculated with 50 μl of a suspension of 8×10^7 conidia/ml of *F. solani*. The inoculated leaves were incubated at 24 °C in Petri dishes on moist filter paper in a humid atmosphere for different time periods. Four leaf pieces, 1 cm in diameter centered over the inoculum, from each of two such inoculated leaves were collected after 0, 1, 2, 3, and 4 days. Control samples were similarly treated with water and harvested at the same time as the fungal inoculated ones.

Enzymatic and histochemical analysis for GUS activity

Tissues were homogenized for 1 min using a Mini BeadBeater apparatus (Biospec Products) with five 4.7 mm stainless steel balls (Small Parts, Inc.) in 2 ml screw-cap plastic tubes (Sarstead) containing GUS extraction buffer [16]. The homogenate was transferred to 1.5 ml Eppendorf tubes, centrifuged at $12000 \times g$ for 5 min and the supernatant was used for protein determination and GUS enzyme assays. GUS activity in tissue extracts was determined as per standard procedures [16]. Fluorescence was determined using a Perkin-Elmer LS-3B spectrofluorometer. Histochemical localization of the expression of GUS in wounded (48 h after wounding) and fungus -inoculated (72 h after inoculation) leaves was carried out by incubating the leaf tissues with the chromogenic substrate 5-bromo-4-chloro-3-indolyl glucuronide, first under gentle vacuum and then transferred to a 37 °C incubator for 8 to 10 h. The stained leaf samples were cleared in 70% ethanol before photography.

Results

Wound-induced expression of the tomato gene, tap1, *in transgenic tobacco plants*

To understand the regulation of anionic peroxidase gene activation in wound-healing plant tissues, a 7.7 kb genomic clone containing the coding and flanking regions of *tap1* (Fig. 1A) were transferred via *Agrobacterium* into tobacco. Primary putative transformants that rooted in kanamycin containing media (50 μg/ml) were selected and transferred to soil. Southern analysis of DNA isolated from several transformants confirmed the stable integration of the plasmid into the tobacco genome when DNA gel blots were probed with a *tap1* 5′-end-specific DNA probe. Restriction digests of genomic DNA from four such transformants using the enzymes *Xba* I, *Sal* I, and *Pst* I upon Southern blot analysis using a 491 bp *Cla* I-*Dra* I fragment derived from the 5′ end of *tap1* (see Fig. 1A) produced hybridizing bands of sizes 7.7 kb, 5 kb, and 3 kb, respectively (Fig. 2). The *tap1* construct was found not to have integrated into the tobacco genome in an intact manner in plant 33 since the 7.7 kb band (Fig. 2) that appeared in *Xba* I-digested genomic DNA blots of plants 5, 7 and 28 was absent, but the 5 kb band was present in all the *Sal* I-restricted DNA gel blots. Transgenic tobacco plants were then se-

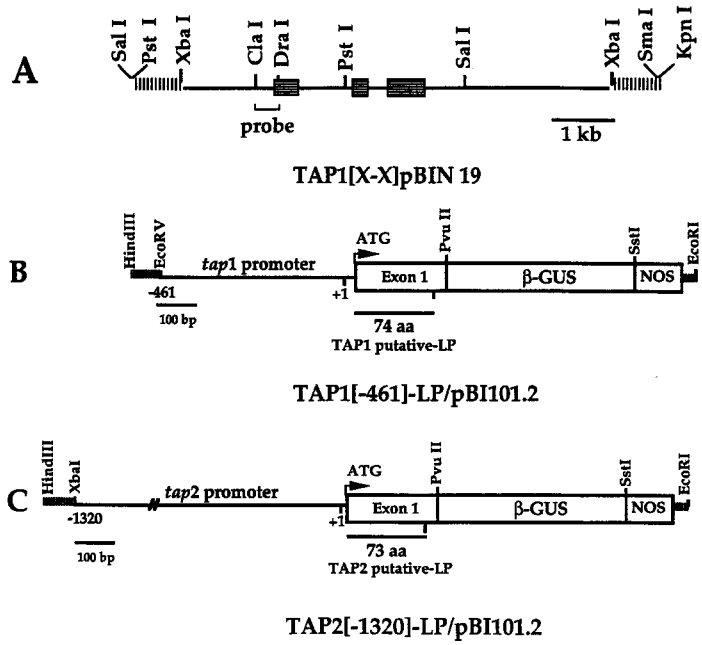

Fig. 1. Diagrammatic representation of the *tap1* genomic DNA and chimeric *tap1* and *tap2* promoter-GUS fusions introduced into tobacco. A. A partial restriction map of the 7.7 kb *Xba* I genomic fragment containing the entire TAP1 gene and its 5′ and 3′ flanking sequences is shown. This fragment was cloned into the binary vector pBIN19 to produce TAP1[X-X]pBIN19. B. A 767 bp 5′ restriction fragment of *tap1* was ligated to make a translational fusion with the coding sequences of GUS and represented as TAP1[−461]-LP/pBI101.2. C. A 1560 bp 5′-end fragment of *tap2* was ligated to make a translational fusion gene with GUS and is represented as TAP2[−1320]-LP/pBI101.2.

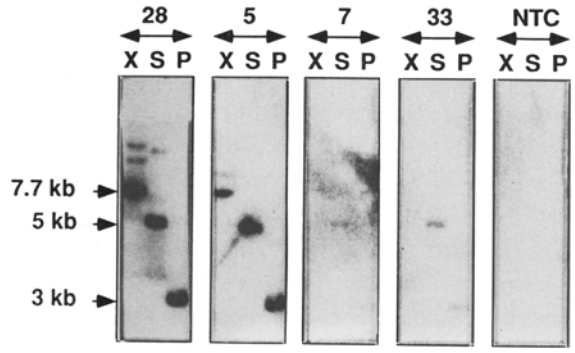

Fig. 2. Southern blot analysis of tobacco plants transformed with the TAP1 gene. Gel blot of 10 μg of restricted genomic DNA from TAP1[X-X]pBIN19 transformed tobacco was probed with a ^{32}P-labeled 491 bp *Cla* I-*Dra* I *tap1* fragment. NTC, non-transformed control tobacco. 5, 7, 28, and 33 represent individual transformants. X, *Xba* I; S, *Sal* I; P, *Pst* I.

lected for further analysis based on their ability to express *tap1* transcripts upon wounding. A 1.6 kb band hybridized on the northern blots when wound-induced transcripts from these four transformants were probed with the 491 bp *Cla* I-*Dra* I *tap1*-specific fragment (Fig. 3). No homologous transcripts could be detected in tissues from unwounded transgenic plants or untransformed tobacco controls. The data obtained from different tobacco plants clearly show that, in all cases, the expression of *tap1* occurred only in the wound-healing tissues. The DNA sequences downstream from the *Sal* I site at the 3′ end of the TAP-1 gene construct were found not to be necessary for wound induction, as plant 33 that lacked the extreme 3′ sequences produced *tap1* transcripts upon wounding (Fig. 3). Both the stem and leaf responded to wounding by yielding transcripts of the same size (Fig. 3A and B). The variations in the level of transcripts found among the individual transformants was reflected equally in both leaves and stems.

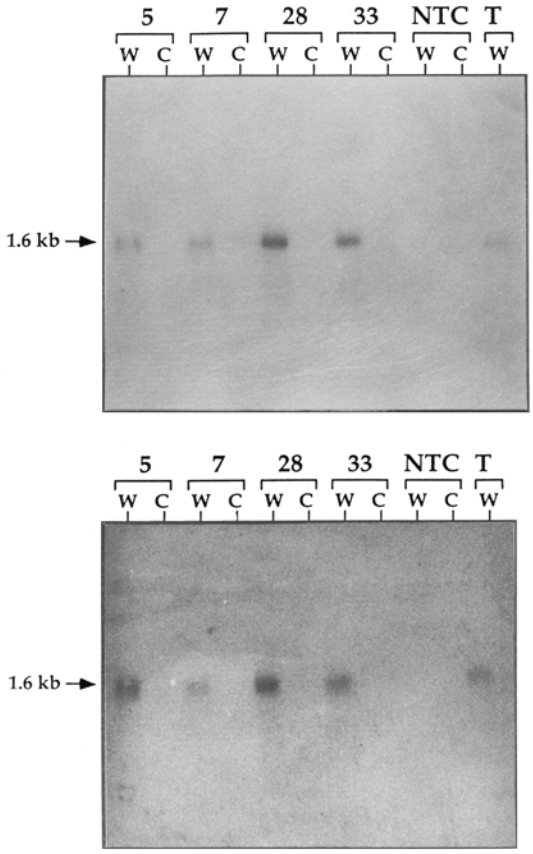

Fig. 3. Northern blot analysis of RNAs from wounded and unwounded transgenic tobacco stems and leaves. Leaves (A) and stems (B) from transgenic TAP1[X-X]pBIN19 plants indicated in Fig. 2 and from a non-transformed control (NTC) plant were wound-healed for 3 days. Poly(A)$^+$-enriched RNA (10 μg) from wound-healing (W) and adjacent unwounded control (C) tissues were subjected to northern blot analysis as described in the text. Total RNA (5 μg) from a 3-day wound-healed tomato fruit [27] was used as a control (T).

Time course of induction of tap1 *by wounding in transgenic tobacco*

A wound-healing time course analysis of *tap1* activation was done using transformant 5. Leaves were wounded and harvested at various periods extending to 96 h (Fig. 4). Northern blot analysis of RNAs from the wound-healing transgenic tobacco tissues was done using the same ^{32}P-labeled 5′ *tap1* probe as above. Transcripts that hybridized to the *tap1* probe could be detected 48 h after wounding. The levels of *tap1*-specific transcripts

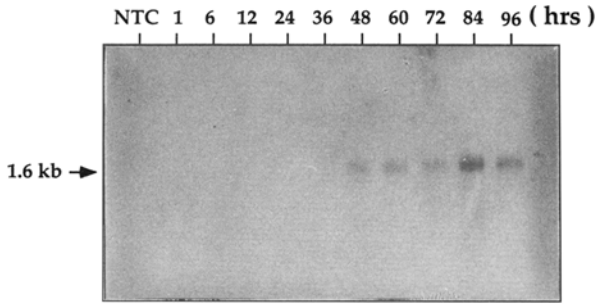

Fig. 4. Time-course of induction of *tap1* transcripts in wound-healing transgenic tobacco leaves. Tobacco leaves from a TAP1[X-X]pBIN19 transformant (plant 5) were wounded and allowed to heal for the indicated times and RNA isolated. The autoradiogram shows a northern blot of 10 μg poly(A)$^+$-enriched RNA per lane from wounded transgenic tobacco and from a 3-day wounded non-transformed control (NTC).

gradually increased to a maximum by 84 h, and then began to decrease by about 96 h.

Activation of tap1 *and* tap2 *promoters in transgenic tobacco plants by wounding*

To determine whether wound and pathogen response could be conferred by *tap1* and *tap2* 5′ upstream DNA sequences, gene fusion constructs were made by ligating a 767 bp *Eco* RV-*Pvu* II DNA fragment of *tap1* in frame with the GUS gene in the expression vector pBI101.2 to produce TAP1[−461]-LP/pBI101.2, and a 1560 bp *Xba* I-*Pvu* II *tap2* fragment was used to create TAP2[−1320]-LP/pBI101.2 in a similar manner (Fig. 1B and 1C).

Primary transformants of tobacco were screened by histochemical staining for GUS activity 48 h after wounding. Leaf tissues of twelve out of eighteen primary transformants stained positive for GUS activity when wounded with a pair of forceps. The stable integration of the introduced TAP1[−461]-LP/pBI101.2 and TAP2[−1320]-LP/pBI101.2 fusion constructs was confirmed when total DNA from leaves of R1 transformants were subjected to Southern blot analysis using a ^{32}P-labeled GUS gene probe (Fig. 5). In the particular plants subjected to this analysis, the TAP1[−461]-LP/pBI101.2 con-

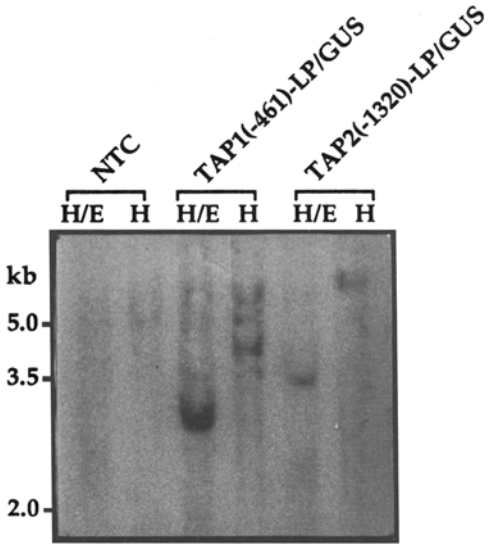

Fig. 5. Southern blot analysis of tobacco plants transformed with the *tap1*/GUS and *tap2*/GUS chimeric gene fusion constructs. Autoradiogram of a gel blot of 10 μg restricted DNA from *tap1*/GUS and *tap2*/GUS transformants that was probed with an 1800 bp ^{32}P-labeled GUS gene probe. NTC, non-transformed control; H, *Hin*d III; E, *Eco* RI.

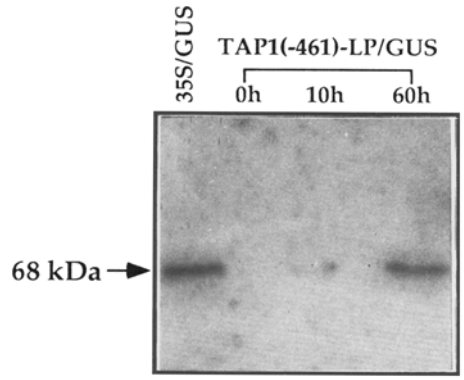

Fig. 6. Western blot analysis of wound-induced GUS expression in transgenic tobacco containing the *tap1*/GUS chimeric fusion gene. Protein samples (100 μg) from a leaf of a CaMV 35S/GUS transformant and from leaf tissues of a *tap1*/GUS transformant wound-healed for the indicated periods were subjected to 10% SDS-PAGE, blotted and probed with a GUS-specific antibody and ^{125}I-labeled Protein A.

struct in plant A-17 was found to have integrated at more than one locus, as indicated in the *Hin*d III restriction digest that produced multiple bands of higher molecular weight, whereas a single band was observed in the digests from the *tap2*/GUS transformants (plant 4). No correlation between number of copies of the introduced *tap1* or *tap2* constructs could be made with the level of wound-induced GUS activity.

When tissue extracts from wound-healing leaves of the *tap1*/GUS transformants were analyzed for GUS activity, it was found that they produced GUS at levels much lower than that reported in wound-healing tissues of transgenic plants containing promoter/GUS fusions of *win1* or *wun1*, the wound-inducible genes of potato [39, 40]. To test whether the GUS polypeptide was being synthesized in the wound-healing tissues, extracts from unwounded and wounded leaves of a *tap1*/GUS transformant were subjected to western blot analysis. As a control, leaf extracts from a tobacco transformant containing the CaMV 35S promoter/GUS construct was used. We were able to detect the GUS polypeptide in the leaf extracts of the 35S/GUS plants and in that of the 60 h wound-healed leaf samples from the *tap1*/GUS transformant. Bands of about equal intensity that hybridized with the GUS-specific antibody were detected (Fig. 6). Measurement of GUS activity in the tissue extracts from both transformants showed that the *tap1*/GUS wound-healing leaves produced about a 10-fold lower level of GUS compared to that produced by the 35S/GUS transformant (data not shown).

High GUS-producing *tap1*/GUS (plant A-17) and *tap2*/GUS transformants (plant 4) were selected for the time course analysis. Leaves from these plants were wounded and incubated on moist filter paper in a humidity chamber at 22 °C. The activation of the chimeric gene promoters was monitored by measuring GUS activity in extracts from the leaf discs after different periods of incubation. GUS activity in the wound-healing tissues from the *tap2*/GUS transformants increased steadily over time as indicated in Fig. 7. The unwounded control tissues had very little GUS activity over the wound-healing period, indicating that GUS expression was associated with the wound-response. *tap1*/GUS transformants showed a slightly different (biphasic) induction profile. GUS expression increased by 20 h, then remained constant through to 33 h,

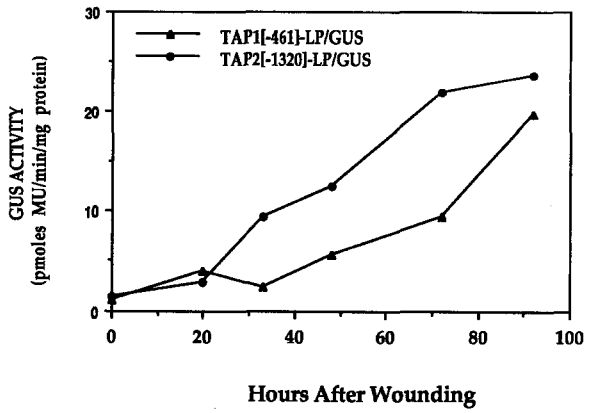

Fig. 7. Time-course of activation of the *tap1* and *tap2* promoters in wound-healing transgenic tobacco leaves. Leaf tissues of *tap1*/GUS and *tap2*/GUS transformants were wounded and allowed to heal as described in the text. The leaf tissues were harvested and assayed for GUS activity using 4-methyl umbelliferyl glucuronide (4-MUG) as substrate.

where upon a second burst of expression was observed that continued to increase to 92 h. The initial increase could be detected by 10 h in separate experiments and leveled off between 20 h and 33 h; this biphasic increase was consistently observed. The wound-induction profile of the *tap1*/GUS transformants paralleled that of the *tap2*/GUS transformants after the 33 h wound-healing point. GUS activity in wound-healing tissues was found to be greater in the *tap2*/GUS transformants than in the *tap1*/GUS transformants.

Localization of GUS activity in wound-healing tissues of tobacco transformed with the tap1*/GUS and* tap2*/GUS chimeric constructs*

To monitor the spatial expression of the *tap1*/GUS and *tap2*/GUS chimeric genes in wound-healing transgenic tobacco tissues, leaves from the respective transformants were wounded by pricking tiny holes using a needle or by cutting with a razor blade. Figure 8A shows that staining was observed only in tissues directly around the wounded sites of an injured leaf that was wounded by pricking holes into it using a needle. When cut with a razor blade, the induction of GUS activity was confined to a few cell layers along the wounded edge of a 48 h wound-healed leaf (Fig. 8B). Staining was not observed in the freshly cut leaf tissues (not shown). When leaves from different transgenic plants were tested, small variations in the intensity of staining were observed, but the spatial pattern of GUS expression was the same in all wounded leaf tissues of *tap1*/GUS and *tap2*/GUS transformants.

Time course of activation and localization of tap1 *and* tap2 *promoter activities in transgenic tobacco plants upon inoculation with* Fusarium *f. sp.* solani pisi

To determine whether *tap1* and *tap2* promoters would also respond to fungal attack, leaves from the respective transgenic tobacco plants were inoculated with spores of the fungus *Fusarium solani* f. sp. *pisi*. Although *F. solani* is not a pathogen of tobacco, a hypersensitive response could be observed upon leaf inoculation. After 1 day, the hypersensitive response was revealed as tiny brown speckles on the leaf surface (not shown). A substantial induction of GUS expression could be detected when tissue extracts from the inoculated leaves were analyzed for GUS activity one day after inoculation. This activation of GUS expression was observed in the leaf tissues of both *tap1*/GUS and *tap2*/GUS transformants. Two days after inoculation, GUS expression continued to increase in a steady manner that continued through day four (Fig. 9). The water controls did not show an increase in GUS expression over the time course (not shown). The *tap1*/GUS and *tap2*/GUS transformants showed a similar time course of GUS expression; however the levels of GUS activity were always slightly higher in the *tap2*/GUS transformants.

To determine the spatial pattern of GUS expression upon fungal inoculation, *tap1*/GUS and *tap2*/GUS transformants were challenged with conidia from *F. solani* f. sp. *pisi*. Young leaves from these R1 transgenic tobacco plants, 3 days after inoculation with 10^6 conidia/ml, when incubated in the chromogenic substrate, showed that

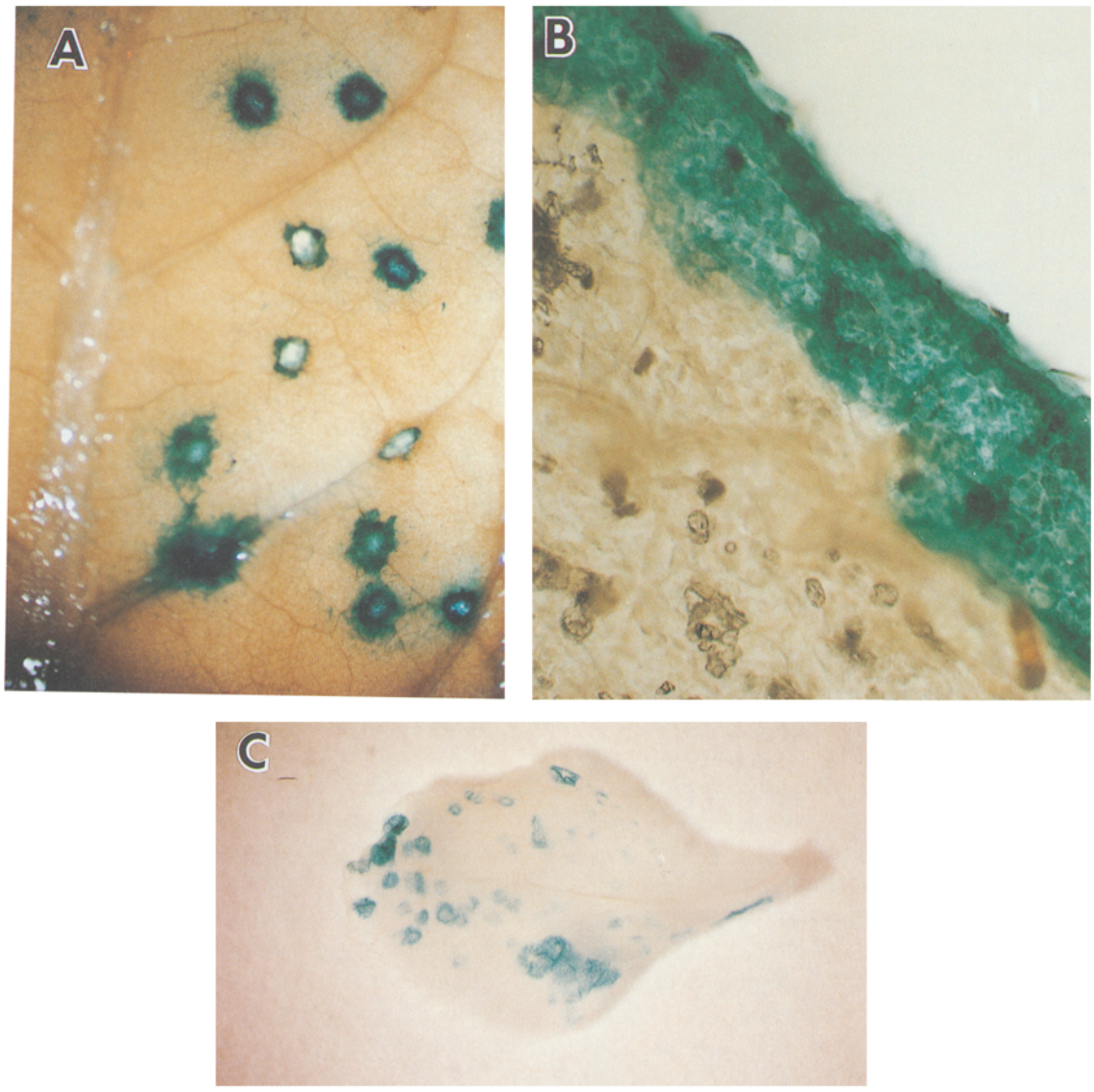

Fig. 8. Histochemical localization of GUS activity in wound-healing tissues and *F. solani* f. sp. *pisi* inoculated leaves of transgenic *tap1*/GUS and *tap2*/GUS tobacco plants. A. GUS activity staining in the areas around the pin-pricks made in the leaf of a *tap2*/GUS transformant (magnification × 9). B. A magnified view of the wound-healing tissue of a *tap1*/GUS transformant leaf cut with a razor blade seen under bright field (magnification × 23), stained 48 h after wounding. C. Localized expression of GUS activity in a *F. solani* inoculated (10^6 conidia/ml) *tap1*/GUS transgenic tobacco leaf that was stained 72 h after inoculation, cleared with ethanol and then photographed (magnification × 9).

GUS activity was confined to discrete regions around the sites of inoculation (Fig. 8C). Leaves from very young tobacco plants did not show a strong hypersensitive response as leaves from older plants. Nontransformed tobacco when inoculated with *F. solani* spores did not show GUS staining. There were no obvious differences in the staining pattern of *tap1*/GUS versus *tap2*/GUS transformants, and both showed only the localized host response to fungal inoculation.

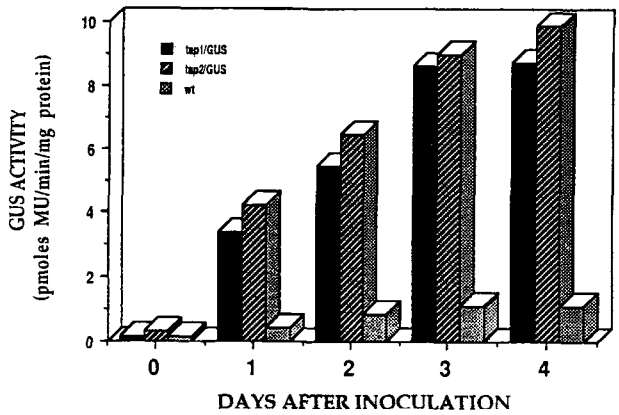

Fig. 9. Time-course of induction of the *tap1* and *tap2* promoters in transgenic tobacco leaves inoculated with *Fusarium solani* f. sp. *pisi*. The induction profile of GUS activity in transgenic *tap1*/GUS and *tap2*/GUS tobacco leaf tissues that were inoculated with 8×10^7 conidia/ml of *F. solani* f. sp. *pisi*. The inoculated leaf tissues were harvested after the indicated time periods and assayed for GUS activity using 4-MUG as substrate.

Discussion

Wound-healing plant tissues erect polymeric barriers on their walls to prevent moisture loss and attack by opportunistic phytopathogenic fungi [17]. Suberin is one such biopolymer that contains phenolic and aliphatic domains. Suberization of the walls of wound-healing plant tissues probably begins with the coupling of pre-existing phenolic esters to the wall components like carbohydrates and possibly wall proteins. The cross-linking of pre-existing wall-bound phenolics [25] and wall proteins [7, 38] has been found to occur soon after wounding. This early reaction is followed by the thoroughly studied large increase in phenolic biosynthesis that involves transcription of gene coding for enzymes involved in the phenylpropanoid biosynthetic pathway [12, 13, 23, 32, 42]. The elevated levels of such enzymes remain for several days providing monomers for the deposition of the phenolic matrix of suberin [4]. The polymerization of the phenolic domain of suberin has been postulated to involve a cell wall-associated anionic peroxidase [2, 4, 18]. The insolubilized phenolics could provide esterification sites for aliphatic components of suberin that could further cross-link the phenolic matrix. Such phenolic reinforcement could make the wall less susceptible to the pathogen-encoded extracellular degradative enzymes [17, 19]. Since wounding caused the induction of the anionic peroxidase in tomato fruits and potato tubers [27, 34, 35], we investigated whether *tap1* contained in a 7.7 kb fragment would respond to wound signals when transferred into a heterologous host, namely tobacco. No homologous anionic peroxidase genes could be detected in nontransformed tobacco (cv. Havana) when a *tap1*-specific DNA fragment was used to probe Southern blots. Northern-blot analysis of RNAs derived from wound-healing leaf and stem tissues also confirmed that *tap* homologous transcripts were not produced in non-transformed tobacco plants. Although it is puzzling that tomato and potato contain highly homologous anionic peroxidase genes while tobacco, another member of the Solanaceae family, does not contain a homologous gene, the absence of the endogenous gene makes tobacco a convenient host to test *tap1* expression. A 1.6 kb transcript was detected in the RNA from wound-healing stem and leaf tissues of the TAP1[X-X]pBIN19 transformed tobacco plants. The transcript was of the same size as that observed from wound-healing tomato fruits [34]. The *tap1* transcripts were found to be induced 48 h after wounding and were detected in only the wound-healing transgenic tobacco tissues. The time-course of appearance of *tap1* transcripts after wounding in transgenic tobacco was quite similar to that previously observed for wound-healing tomato fruits and potato tubers [35]. These observations indicate that the *tap1* sequences introduced into tobacco were sufficient to induce its expression upon wounding just as the gene is induced in its native host. In all transgenic tobacco plants tested, no expression of *tap1* was observed in unwounded leaf or stem tissues. In this study, we also were able to determine that DNA sequences at the extreme 3′ region of *tap1* were not necessary for the wound induction, as one transformant (plant 33) lacking the sequences downstream of the *Sal* I restriction site of *tap1* produced *tap1* transcripts in the wound-healing

tissues. Obviously, tobacco contains the trans-acting factors necessary to trigger expression of *tap1* by wounding and therefore is an appropriate host to study wound induced expression of *tap1*.

We next investigated whether the 5′-flanking region of *tap1* and that of *tap2* could confer reporter gene wound-induced expression in tobacco when introduced as translational fusions with the GUS gene. Indeed, tobacco plants transformed with these promoters showed GUS expression in leaf tissues upon wounding. Induced GUS activity in *tap2*/GUS transformants that was barely detected by 20 h after wounding, increased steadily over the wound-healing period till 72 h. Previously it was not known whether *tap2* was expressed in wound-healing tomato [34]. The present results demonstrate that *tap2* promoter is responsive to wound signals just as *tap1* is responsive to such signals.

Interestingly, *tap1* had a slightly different time-course of induction than that of *tap2*. *Tap1*-induced GUS expression showed a biphasic expression pattern after wounding. There was an early induction that started soon after wounding and peaked by 20 h or so and remained constant up to 33 h. The early phase of *tap1*-promoted GUS induction could represent anionic peroxidase activity involved in polymerization of pre-existing phenolics of the cell walls and for depositing on the wall phenolics that are well known to accumulate soon after wounding [12, 13, 32, 42]. Phenolic deposition on the walls of suberizing plant tissues takes place for a number of days after wounding [4] and could require such an activity during this time period. The increase in phenolics thought to be characteristic of suberin, the level of the wound-induced highly anionic peroxidase and mRNA level for this peroxidase reached maximal levels in 72 to 96 h after wounding of potato and tomato fruits [4, 35]. The second major phase of increase in GUS expression in the wounded transgenic tobacco plants followed a similar time-course and therefore probably represents the induction of *tap1* and *tap2* associated with the final phase of phenolic deposition for suberization. Since GUS expression driven by both *tap1* and *tap2* promoters showed a similar increase during this period, both genes are probably expressed during suberization in their native environment although direct evidence for this conclusion is lacking.

The level of GUS produced in wound-healing tissues of TAP1[−461]-LP/pBI101.2 transformants was found to be surprisingly low compared to the constitutive levels of GUS produced by transformants containing the CaMV 35S promoter/GUS chimeric gene. Comparison of the amount of GUS enzyme produced versus the levels of GUS activity obtained from wound-healing tissue extracts between *tap1*/GUS and CaMV 35S/GUS transformants, showed that both transformants produced comparable levels of the GUS polypeptide, but the activity was about 10-fold lower in extracts from wound-healing leaf tissues of the *tap1*/GUS transformants. Also, when *tap1* promoter/GUS fusion constructs lacking the leader peptide coding sequences were introduced into tobacco protoplasts, a 6- to 8-fold higher level of constitutive GUS activity was produced compared to similar *tap1* promoter/GUS chimeric gene constructs that contained the DNA sequences coding for the TAP1-LP (R. Mohan and P.E. Kolattukudy, unpublished). Inhibition of GUS activity in transgenic tobacco has been reported [15]. The authors determined that when the coding sequences for the patatin transit peptide was fused to GUS-coding sequences, GUS was presumably translocated via the glycosylation pathway. This led to glycosylation of the GUS polypeptide and hence inhibition of GUS enzyme activity. They also reported that the inhibition could be alleviated by treatment with tunicamycin, a known glycosylation inhibitor. Inhibition of GUS activity for a similar reason probably explains our results.

We found that the wound-activated *tap1* and *tap2* promoters responded to wound signals in a manner similar to that observed in the native host. Similarly, a time-course of induction of the potato wound-inducible gene *win2* promoter in transgenic potato, containing a *win2*/GUS chimeric fusion gene, showed substantial differences in the degree of induction and final levels of GUS and *win2* transcript level [40]. The authors also added

that the *win2* promoter/GUS fusion gene was not being regulated accurately when introduced into tobbaco, as compared to potato. In yet another study, the *wun1* promoter of another potato wound-inducible gene, when introduced into tobacco as a *wun1*/GUS chimeric gene fusion, was found to be regulated in a manner analogous to that found in its native host [24, 39]. Thus, most foreign gene promoters do seem to respond to wound signals in heterologous hosts such as tobacco by activating the fusion reporter gene, but the level of transcripts and gene products do not always truly represent the levels found in the native host.

Histochemical localization of wound-induced GUS expression showed that only the wound-healing cells directly bordering the wound expressed this activity [6]. Freshly wounded samples were not stained by the chromogenic substrate when placed along with the wound-healing samples. Wounding of tissues by gently crushing the leaf blade between the serrated end of a pair of forceps showed greater staining intensity than by cutting with a razor blade, indicating that a higher population of cells were activated to produce GUS when wounded by crushing. The *tap2*-promoted GUS expression was also confined to the wound-healing tissues, and this induced expression was detected 48 h after wounding, the staining being from moderate to intense depending on the individual transgenic plant. The activation of *tap*-promoted GUS expression in the few cell layers bordering the wound site is similar to the observed induced expression of GUS, driven by the promoter of *wun1* [39].

The activation of the GUS reporter gene by the 5′-flanking region of *tap1* and *tap2* in transgenic tobacco was also observed upon inoculation with *F. solani*. This observation suggests a possible role of the anionic peroxidases in host defense. The *tap1* and *tap2* promoters were induced when the fungus was used in the range of 10^6 to 10^7 conidia/ml. Staining for GUS activity 3 days after inoculation showed that the *tap1* and *tap2* promoters were activated only in the inoculated regions of transgenic leaves. Even though fully developed HR response was not always observed, GUS activation by fungal inoculation was observed in *tap1*/GUS and *tap2*/GUS transgenic plants. We also attempted to inoculate tobacco with a conidial suspension of *F. solani* by infiltrating the spores through the stomata. Such a method of infiltration of the fungal spores led to massive tissue damage by 2 days, with a concomitant loss of GUS activity, and therefore chose to inoculate the tobacco leaves by applying the conidial suspension on the surface of the leaf. The activation of *tap* expression in transgenic tobacco follows a spatial pattern of gene activation that is consistent with the localized defense responses that was observed in tomato plants infected by *V. albo-atrum* [33]. The activation of GUS expression in transgenic tobacco plants containing a chitinase/GUS chimeric fusion gene was observed when tobacco leaves were infected with *Rhizoctonia solani* [36].

Plant peroxidases are a class of enzymes, each individual enzyme probably with its own unique physiological and developmental role [9]. Recently, additional evidence for defense roles of anionic peroxidases has appeared. The anionic peroxidases have been implicated in the wall-associated defense processes like suberization [33], lignification [21] and extensin cross-linking [38]. The timely activation of such a wall fortification process to contain the pathogen to its initial site of entry, when the host is challenged, could be an essential part of the resistance response. The rapid accumulation of phenolic polymers and anionic peroxidases has been reported in soybean cotyledons when challenged with an elicitor preparation from *Phytophthora megasperma* cell walls [11]. The defense-related anionic peroxidases from tobacco associated with the cell wall were reported to be induced systemically upon inoculation with tobacco mosaic virus and with the fungus, *Peronospora tabacina* [41]. The *trans*-acting factors involved in such gene activation are probably involved in the activation of *tap* genes observed in transgenic tobacco in the present study. A fungus-induced anionic putative peroxidase (pI ca. 5.7) cDNA from wheat leaves infected with *Erysiphe graminis* was recently cloned and sequenced [31]. The presence of a

characteristic eukaryotic signal peptide sequences in the wheat peroxidase suggests that it is perhaps also secreted. It will be interesting to see whether the wheat peroxidase is also activated by wounding.

Lignin and suberin are cell wall-associated biopolymers, but their location within the cell wall is quite different. Lignin is found to be concentrated in the middle lamella, whereas suberin is found to be deposited on the walls towards the plasma membrane side in wound-healing or pathogen-infected plant cells. The distribution of these polymers within the wall may thus necessitate different anionic peroxidases with discrete functions in such cell wall-associated phenolic polymerization. The anionic peroxidase from tobacco with a putative function in lignin synthesis, and having only as much as 45% cDNA nucleotide sequence homology with *tap1* or *tap2*, has been cloned [22]. The anionic peroxidase (pI 4.1) from the seed coat of soybean, with a postulated role in providing a barrier to protect the enclosed embryo, was recently purified and found to be induced 20 days after anthesis [10]. An endosperm-specific peroxidase (pI ca. 8.5) cDNA has been cloned from barley and characterized [30]. Thus, the cloning and characterization of other peroxidase genes to study their spatial, temporal and developmental patterns of expression could give us clues about their function in plants.

Acknowledgements

This work was supported by National Science Foundation Grant DCB-8819008 and by a grant from the Midwest Plant Biotechnology Consortium. We also thank Monsanto Company for a Biotechnology fellowship granted to Royce Mohan. We thank Paula Pack for assistance in preparing the manuscript.

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