Plant Molecular Biology **22**: 573–588, 1993.

Systemic induction of a potato *pin2* promoter by wounding, methyl jasmonate, and abscisic acid in transgenic rice plants

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Received 22 December 1992; accepted in revised form 17 March 1993

Key words: defense response, foreign gene expression, intron effect, proteinase inhibitor, transgenic rice, wounding

Abstract

To address the question whether common signal(s) and transduction pathways are used to mediate a systemic wound response in monocot and dicot plants, a fusion of the potato proteinase inhibitor II gene (*pin2*) promoter and the bacterial β-glucuronidase gene (*Gus*)-coding region was introduced into rice. In transgenic rice plants, the expression of the *pin2-Gus* fusion gene displays a systemic wound response, although the expression level is relatively low. Incorporation of the first intron from the rice actin 1 gene (*Act1*) into the 5′-untranslated region of the *pin2-Gus* construct results in high-level, systemically wound-inducible expression of the modified construct in transgenic rice plants. Histochemical analysis shows that this high-level, wound-inducible expression is associated with the vascular tissue in both leaves and roots. Furthermore, the expression of the *pin2-Act1* intron-*Gus* fusion gene in transgenic rice plants can be systemically induced by both methyl jasmonate (MJ) and the phytohormone abscisic acid (ABA). These results suggest that the signal(s) mediating the observed systemic wound response and certain steps of the transduction pathways are conserved between dicot and monocot plants. Transient expression assays show that the *pin2-Act1* intron-*Gus* construct is also actively expressed in transformed cells and tissues of several other monocot plants. Thus, the wound-inducible *pin2* promoter in combination with the rice *Act1* intron 1 might be used as an efficient regulator for foreign gene expression in transgenic monocot plants.

Introduction

In response to insect attack (mechanical wounding) or pathogen infection, a battery of defense responses are coordinately activated in plants. Some defense responses are restricted to the local areas of wounding or infection, whereas other defense responses are induced throughout the plant that represent systemic gene activation [3]. Among many inducible defense-related genes which have been cloned from dicot plants, the potato proteinase inhibitor II gene (*pin2*) family is the best analyzed system of systemic gene activation [32, 33]. The search for systemic wound

signals and the elucidation of the signal transduction pathways have been the focus of intensive research efforts. Based on a massive body of information accumulated in the past 20 years, a working model for the wound signal transduction pathways has been suggested by Ryan and his colleagues [33].

In addition to being an excellent system for the study of wound signal transduction pathways, both the coding function of the proteinase inhibitor and the systemic wound inducibility of the *pin2* promoter make it a potentially useful tool for the genetic engineering of insect and/or pathogen resistance in transgenic plants [32]. More genes which confer insect or pathogen resistance are being isolated from plants or other organisms. The advantages of using a systemically inducible promoter to express these well-defined insect and/or pathogen resistance genes in transgenic plants are evident. Confining high-level expression of resistance genes to the period of actual insect or pathogen attack will not only conserve the metabolic energy and building blocks of plant cells, but may also minimize the selection pressure on insect pests and pathogens [9, 15].

Currently, research on plant defense in higher plants has focused on the more tractable dicot plants. So far, systemically wound-inducible genes have not been isolated in monocot plants. The expression of several defense-related genes isolated from monocots show only a limited and local wound response [8, 18]. However, two recent reports suggest that systemic responses can be induced in monocot plants by insect or pathogen attack. First, herbivorous injury of a single leaf in maize seedlings induced a systemic release of volatile terpenoids throughout the whole plant [42]. Second, inoculation of the first leaf of rice seedlings with *Pseudomonas syringae* induced systemic resistance to the rice fungal pathogen *Pyricularia oryzae* in the upper leaves [36]. These systemic responses in monocot plants have not been characterized at the molecular level.

In previous studies, many differences have been found between monocots and dicots in the molecular basis of transcriptional regulation, RNA splicing, and developmental patterns of gene expression and morphogenesis [13, 30, 31, 34]. It is also well known that the local wound response of most monocots is different from that of most dicots. Wounded dicot tissues respond by cell proliferation resulting in the production of wound callus. The cells of wounded tissues in most monocots, on the other hand, do not divide but differentiate into lignified or sclerified cells, thus producing a ring of hardened cells around the initial wound site [35]. It is not known whether this general difference in local wound response between monocot and dicot plants would lead to differences in their respective wound signal production, transmission, and systemic response in unwounded plant parts.

The current study was undertaken to determine whether common signal(s) and transduction pathways are used in mediating the systemic wound response in distantly related dicot and monocot plants. In the absence of a cloned, systemically wound-inducible gene from monocot plants, we have addressed this question by using the promoter from a well characterized, systemically wound-inducible potato *pin2* gene [41] as a molecular marker. We introduced the dicot *pin2* promoter fused to the bacterial β-glucuronidase gene (*Gus*) coding region into rice, and analyzed its functionality and its response to wounding and known chemical signals which have been shown to be involved in the wound response of dicot plants. Our experimental results suggest that the wound response signal(s) and certain steps of the transduction pathways are conserved between dicot and monocot plants. With the purpose of optimizing gene expression from the *pin2* promoter, we also analyzed the effects of the first intron of rice *Act1* gene on the gene expression level, pattern, and wound inducibility of the *pin2* promoter in transgenic rice plants.

Materials and methods

General recombinant DNA techniques

All recombinant DNA techniques were performed essentially as described by Maniatis *et al.*

[19], using modifications recommended by the enzyme manufacturers.

Construction of pin2-Gus *fusion constructs*

The *pin2* promoter was isolated from plasmid pRT41 [41] as a 1.0 kb *Pst* I-*Xba* I restriction fragment. Plasmid pBI221.1 [11] was digested with *Pst* I and *Xba* I to remove the 0.8 kb *Pst* I-*Xba* I fragment containing the CaMV 35S promoter, and the remaining fragment was ligated with the 1.0 kb *pin2* promoter to produce plasmid pDX106. Plasmid pBCG-A [22], which contains the CaMV 35S promoter, the first intron of rice *Act1* gene, the *Gus*-coding region, and *nos* 3′ end, was digested with *Pst* I and *Xba* I to remove the 0.8 kb *Pst* I-*Xba* I fragment containing the CaMV 35S promoter, and the remaining fragment was ligated with the 1.0 kb *pin2* promoter to create plasmid pDX107. The 3′ region of *pin2* was isolated from pRT41 as a 0.9 kb *Xba* I-*Pst* I restriction fragment, and its ends were blunted with Klenow DNA polymerase. To replace the *nos* 3′ end of plasmid pDX106 by the 3′ region of *pin2*, pDX106 was digested with *Eco* RI and *Sst* I to remove the 280 bp *nos* 3′ end, and the remaining fragment was blunted with Klenow DNA polymerase and ligated with the 0.9 kb blunted 3′ region of *pin2* to produce plasmid pDX108. The same method was used to replace the *nos* 3′ end on plasmid pDX107 by the 3′ region of *pin2* to create plasmid pDX109. All the newly created junctions and cloning orientations were confirmed by DNA sequence analysis.

Transient expression assay and production of transgenic rice plants

For transient expression assay, rice (*Oryza sativa* cv. Taipei 309) protoplasts were isolated from finely dispersed, embryogenic suspension cultures, transformed with supercoiled plasmid DNA, and cultured as previously described [46]. Extraction of protein from protoplasts or from rice plant tissues and fluorometric analysis of GUS activity were carried out essentially as described by Jefferson [11]. Transient expression assays in maize, wheat, barley, and sorghum were performed with suspension culture cells or immature embryos, using the particle gun method as described by Cao *et al.* [5]. GUS activity in the transformed cells was visualized by histochemical method as described by Jefferson [11].

For stable transformation, rice protoplasts were co-transformed with each construct and the selectable gene construct pDM307, which contains the CaMV 35S promoter fused to the bacterial *bar* gene and *nos* 3′ end [5]. Resistant colonies were selected with 4 mg/l of ammonium glufosinate (Crescent Chemical Co., Hauppauge, NY) in the medium as the selective agent. The resistant calli were transferred to regeneration medium [46] to regenerate into plants. Some resistant calli were subjected to visual GUS activity assay by incubating in X-gluc solution [11] to score the co-transformation efficiency. Plants regenerated from the same resistant callus were regarded as clones of the same transgenic lines. The presence of the transferred genes in transgenic rice plants was confirmed by DNA gel blot hybridization analysis as described by Zhang *et al.* [45].

Wounding, MJ, and ABA treatments

The position of the leaf to be wounded and the extent of wounding were dependent on the size of the transgenic plants. For most wounding experiments, greenhouse-grown transgenic rice plants at the 5- to 6-leaf stage were used, unless otherwise indicated. The first leaf from the bottom was cut off the plant before wounding (as unwounded sample), frozen in liquid nitrogen, and stored at −80 °C before analysis. The second and third leaves were wounded by gently crushing the leaf blade between two flat wooden files, or by making small cuts along both edges of the leaf blade without damaging the middle vein. After 24 h, the two wounded leaves, as well as the two unwounded leaves above the wounded leaves (as systemically induced sample), were collected, im-

mediately frozen in liquid nitrogen, and stored at −80 °C before analysis.

To establish the time course of wound-inducible gene expression, the wounding method of An *et al.* [1] was used. Leaves from several plants of the same transgenic line were excised, cut into 3–5 cm segments, and incubated on filter paper moistened with MS salts [24] under light at 27 °C. Duplicate samples were collected at various times after wounding, immediately frozen in liquid nitrogen, and stored at −80 °C before analysis.

For direct wounding of roots, roots on axenic transgenic rice plants were pinched with forceps and excised after 24 h for analysis. Alternatively, roots were excised from the transgenic plants, pinched with forceps, and incubated on filter paper moistened with MS salts in the dark at 27 °C for 24 h before analysis.

Plants were exposed to MJ (Bedoukian Research Inc., Danbury, CT) vapor, essentially as described by Farmer and Ryan [7], except that 5 μl of 1/10 dilution of MJ in ethanol was applied in a two-liter air-tight plastic chamber containing the plants. Control plants were exposed only to ethanol vapor under the same conditions. For spraying, a 25 μM MJ solution in sterile water/0.01% ethanol/0.125% Triton X-100 was applied to plants by aerial spraying twice during a period of 24 h. Leaves to be taken as systemically induced were covered by aluminium foil during spraying. Control plants were sprayed with the same solution without MJ.

A 100 μM racemic *cis-trans* ABA (Sigma Chemical Co., St Louis, MO) solution in sterile water/0.01% ethanol/0.125% Triton X-100 was applied to the leaf surface by spraying in the same way as in MJ treatment. Control plants were sprayed with the same solution without ABA. Leaf samples were collected for analysis at 24 h after the initial spraying.

Isolation and gel blot analysis of RNA

Total RNA was isolated from plant tissues essentially as described by Mitra and An [23]. RNA (40 μg per lane) was denatured by glyoxal and DMSO treatment, separated in a 1.2% agarose gel, blotted onto GeneScreen Plus membrane (NEN Research Products, Boston, MA), and hybridized with a radioactive probe. For slot blot analysis, 10 μg of total RNA per slot was applied onto a support membrane. The entire 1.8 kb *Gus*-coding region was used as the *Gus* probe. The rice actin 1 gene-specific probe was derived from the 3′ region as a 0.9 kb *Bam* HI-*Hin*d III fragment [21].

Histochemical analysis of GUS activity in transgenic rice tissues

Whole tissues or hand-cut thin sections were incubated in GUS substrate X-gluc solution for 16–24 h, as described by Jefferson [11]. GUS activity is visualized by the formation of blue precipitate in the cells. For incubation of whole young plants, 0.5% of Triton X-100 was included in the X-gluc solution. To exclude the possibility that *Gus* expression might be induced during this incubation, a 25 μM concentration of cycloheximide (Sigma Chemical Co., St Louis, MO) was also added to the X-gluc solution. Chlorophyll was removed from the tissues, sections, or whole plants by treating in 70% ethanol before microscopic observation.

Results

The potato pin2 *promoter is functionally active in transformed rice protoplasts and in transformed cells and tissues of other monocot plants*

Transient expression assays have been widely used to analyze the activity of promoters in both homologous and heterologous plant systems. To test the functionality of the *pin2* promoter in rice, we first constructed the *pin2-Gus* fusion construct, pDX108, which is shown in Fig. 1. We suspected that the dicot *pin2* promoter might not function efficiently in monocot plants such as rice. In a previous study, the first intron of the rice *Act1*

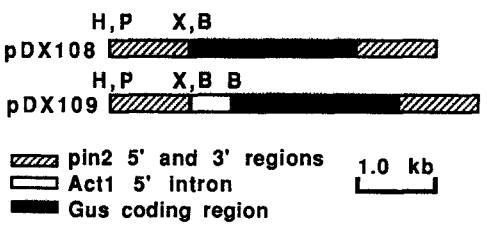


Fig. 1. Structure of the *pin2-Gus* and the *pin2-Act1* intron-*Gus* fusion constructs. The indicated restriction enzyme sites are abbreviated as follows: B, *Bam* HI, H, *Hind* III; P, *Pst* I; X, *Xba* I. Construction of these two fusion genes is described in Materials and methods.

gene has been shown to stimulate gene expression from a dicot promoter in transformed monocot cells [22]. Therefore, we incorporated the first intron of the rice *Act1* gene into the 5′-untranslated region of the *pin2-Gus* fusion gene to create the *pin2-Act1* intron-*Gus* fusion construct, pDX109 (Fig. 1).

The activity of the two fusion genes was first analyzed in transient expression assays of transformed rice protoplasts using the *Act1-Gus* fusion construct, *pAct1*-D [21], as a positive control. As shown in Table 1, the *pin2* promoter in pDX108 was active in rice protoplasts, although the GUS specific activity was only 4 to 6 times higher than that of non-transformed control protoplasts. Incorporation of the rice *Act1* intron 1 into the *pin2-Gus* fusion gene stimulated the expression level over 40-fold, and led to a level of GUS specific activity comparable with that of the *Act1-Gus* fusion gene. In our transient expression assays using the particle gun method [5], the *pin2-Act1* intron-*Gus* fusion construct, pDX109, was also introduced into suspension culture cells or immature embryos from several other monocot plants, including maize, wheat, barley, and sorghum. Histochemical assays of GUS activity showed that this fusion gene was also highly expressed in the transformed cells from these monocot plants (data not shown). These transient expression results encouraged us to carry out stable transformation experiments in rice using these gene constructs.

Table 1. GUS-specific activity in rice protoplasts transformed with *pin2-Gus* fusion genes.

Name of construct	GUS specific activity[a] (nmol 4-MU h^{-1} mg^{-1}) Mean ± SE	Visible *Gus* expression (blue cells)[b]
pDX108 (– intron)	3.4 ± 1.6	+/–
pDX109 (+ intron)	143.5 ± 32.6	+
p*Act1*-D	213.8 ± 41.7	+
Untransformed	0.6 ± 0.4	–

[a] The results of four independent transformation experiments were used to estimate the mean GUS specific activity in transformed rice protoplasts. 4-MU, 4-methylumbelliferone; SE, standard error.

[b] Transformed rice protoplasts were subjected to a visual *Gus* expression assay two days after transformation. + indicates that visible *Gus* expression (blue cells) was observed; – indicates that visible *Gus* expression was not observed; +/– indicates that visible *Gus* expression was barely observed.

The pin2 *promoter directs systemically wound-inducible gene expression in transgenic rice plants*

For stable transformation, the *pin2-Gus* or the *pin2-Act1* intron-*Gus* fusion construct was used together with the construct pDM307, which contains a selectable marker gene, CaMV 35S-*bar-nos* [5], to co-transform rice protoplasts. Resistant colonies were selected using 4 mg/l ammonium glufosinate. Some resistant calli were subjected to visual *Gus* expression assays by incubation in the GUS substrate, X-gluc [11]. As shown in Fig. 2A, no GUS-positive calli appeared when untransformed rice calli were subjected to this GUS staining assay. However, the *pin2* promoter was found to be active in rice callus, as shown in Fig. 2B.

Rice plants were regenerated from individual resistant calli. Multiple shoots regenerated from each of the resistant calli were separated and grown as clones from the same transgenic lines. DNA gel blot analyses were performed to identify those transgenic plants which contained intact transferred genes (data not shown).

Young plants regenerated from known GUS-positive resistant calli were subjected to GUS

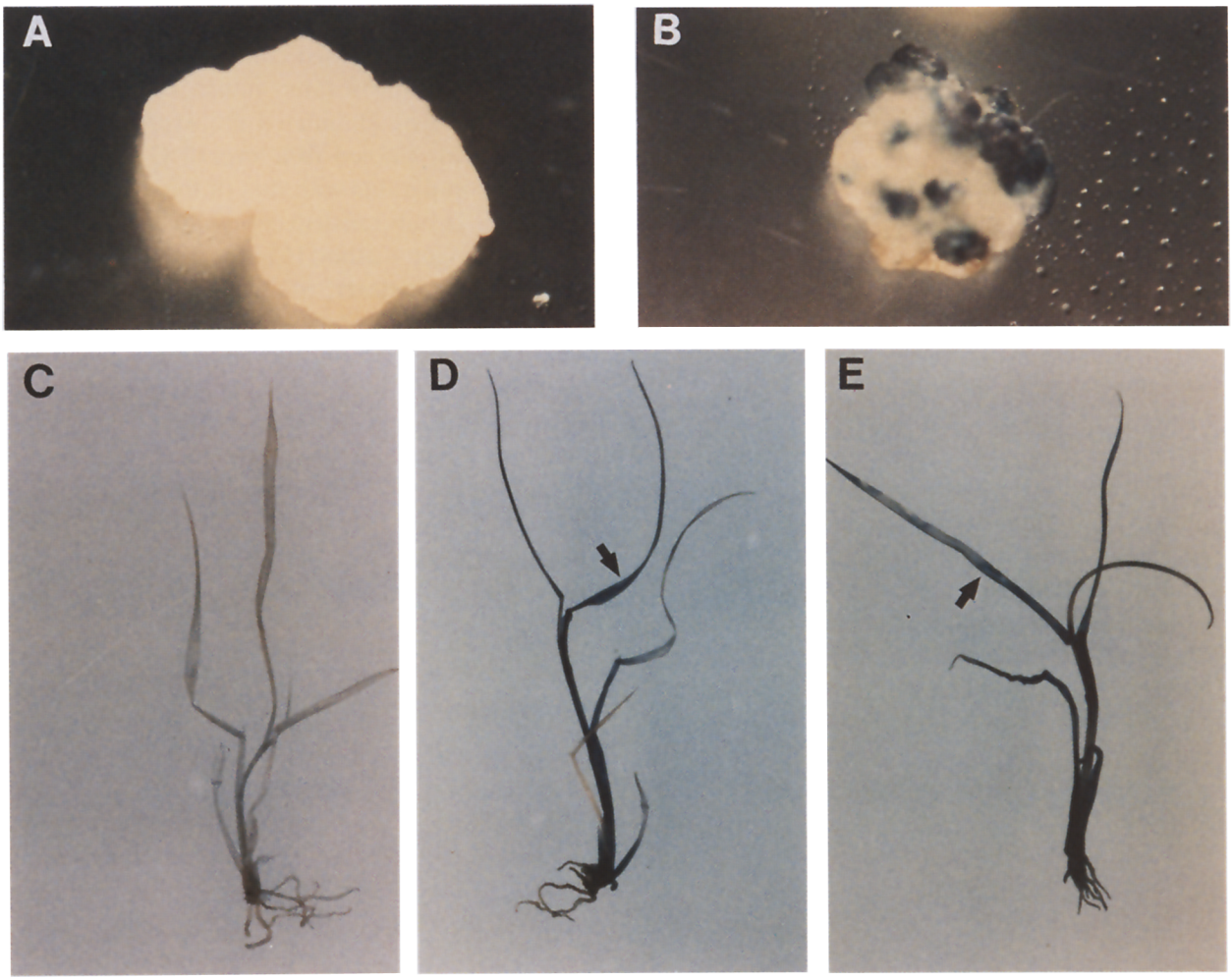


Fig. 2. Visualization of GUS activity in transformed rice calli, and systemic wound-induction in young transgenic rice plants, of the *pin2-Act1* intron-*Gus* construct, pDX109. Resistant calli were directly subjected to visual histochemical assay of GUS activity, and whole young transgenic rice plants were subjected to same kind of assay before and 24 h after wounding treatment. Plants were then cleared with 70% ethanol to remove chlorophyll before observation. A. Untransformed rice callus (×30). B. Transformed, GUS-positive rice callus (×30). C. Unwounded transgenic rice plant (×0.5). D and E. Wounded transgenic rice plant (×0.5 and ×0.5, respectively). Arrow indicates the wounded leaf.

staining by incubation in X-gluc solution before and 24 h after wounding treatment. Representative results from transgenic plants transformed with the *pin2-Act1* intron-*Gus* construct, pDX109, are also shown in Fig. 2. In non-wounded transgenic plants, no or very low GUS activity was observed (Fig. 2C). However, in wounded transgenic plants from the same transgenic line, high levels of GUS activity were induced not only in the directly wounded leaf but also in unwounded upper and lower leaves (Fig. 2D and 2E). Thus, wounding induced systemic *Gus* gene expression driven by the potato *pin2* promoter in transgenic rice plants. Transgenic plants transformed with the *pin2-Gus* fusion gene, pDX108, also displayed a systemic wound response, but *Gus* expression was relatively weak (data not shown).

Quantitative analysis of wound-induced expression of the pin2-Gus *and* pin2-Act1 *intron*-Gus *fusion genes in transgenic rice plants*

Transgenic rice plants, at the 5- to 6-leaf stage, were first analyzed for the induction of GUS enzymatic activity in wounded leaf tissues. Table 2 shows the GUS specific activity in leaf tissues before and 24 h after wounding. In the case of plants transformed with the *pin2-Gus* fusion gene, pDX108, the mean level of GUS specific activity in unwounded leaf tissue was only slightly higher than that of untransformed control plants. Wounding a leaf from these transgenic plants increased GUS specific activity by 5- to 10-fold. In unwounded plants transformed with the *pin2-Act1* intron-*Gus* fusion gene, pDX109, a higher basal level of GUS specific activity was detected, which was 5 to 15 times higher than that detected in untransformed control plants. This level of GUS specific activity was further increased 10- to 15-fold by wounding (Table 2).

Data in Table 2 also show that the levels of GUS specific activity in unwounded but systemically wound-induced leaf tissues (column SW) were similar to those in directly wounded leaf tissues (column W), suggesting that wounding triggered an efficient systemic wound response in transgenic rice plants.

Table 3 shows the data of the GUS enzymatic assay in root tissues. In roots of unwounded plants (column NW), a relatively high basal level of GUS specific activity was detected compared with that of untransformed control plants. In all wound-induction experiments, wounding of leaves did not further induce *Gus* expression in roots (column LW). However, GUS specific activity in roots was increased 10- to 20-fold by directly wounding the root tissues (column DW).

Transgenic rice plants transformed with an *Act1-Gus* fusion gene, p*Act1*-D, which were obtained from a previous study [45], were used as controls for constitutive expression in these wound-induction experiments. The results in Tables 2 and 3 clearly show that wounding had little effect on the expression of the *Act1-Gus* fusion gene, with very similar expression levels being detected in unwounded and wounded leaves.

Wound-inducible *Gus* gene expression was also analyzed at *Gus* mRNA level. Total RNA was isolated from leaf tissues of transgenic rice plants before and 24 h after wounding, and analyzed by RNA gel blot hybridization. Representative results from transgenic plants transformed with the *pin2-Act1* intron-*Gus* fusion construct are shown in Fig. 3A. The results of such RNA analysis clearly show that wounding led to a significant

Table 2. GUS specific activity in leaves of unwounded and wounded transgenic rice plants.

Name of construct	Transgenic lines	Mean GUS specific activity[a] (nmol 4-MU min^{-1} mg^{-1})		
		NW	W	SW
pDX108	P-1	0.3	2.7	2.1
(−intron)	P-2	0.5	3.8	2.6
	P-3	0.8	4.4	3.1
pDX109	PI-1	1.1	14.0	10.1
(+intron)	PI-2	2.0	22.9	17.3
	PI-3	2.6	32.1	24.5
p*Act1*-D	T-1	73.1	61.8	ND
Untransformed		0.2	0.2	ND

[a] The mean GUS specific activity of three independent wounding induction experiments is shown. Abbreviations are: NW, unwounded; W, wounded; SW, systemically wound-induced; ND, not determined.

Table 3. GUS specific activity in unwounded and directly wounded roots of transgenic rice plants.

Name of construct	Transgenic lines	Mean GUS specific activity[a] (nmol 4-MU min^{-1} mg^{-1})		
		NW	DW	LW
pDX109	PI-1	1.5	12.4	1.7
(+intron)	PI-2	1.1	19.0	1.8
	PI-3	1.4	29.4	1.0
p*Act1*-D	T-1	50.5	47.4	ND
Untransformed		0.4	0.4	ND

[a] The mean GUS specific activity of three independent wounding induction experiments is shown. Abbreviations are: NW, unwounded; DW, directly wounded; LW, roots from plants with wounded leaves; ND, not determined.

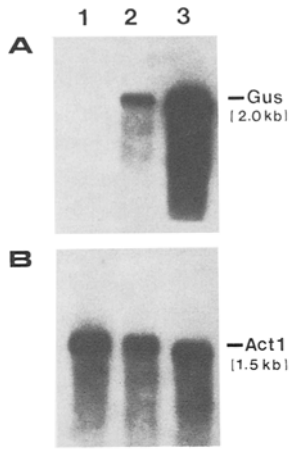


Fig. 3. Wound-inducible accumulation of *Gus* mRNA in transgenic rice plants transformed with the *pin2-Act1* intron-*Gus* construct, pDX109. Total RNA was isolated from unwounded leaves (lane 1), systemically induced leaves (lane 2), and directly wounded leaves (lane 3), and loaded (40 μg per lane) for RNA gel blot hybridization analysis. A. The blot was hybridized with a radiolabelled *Gus* gene probe. B. The same blot as in A was stripped and rehybridized with a radiolabelled *Act1* gene-specific probe.

increase in the abundance of *Gus* mRNA in both directly wounded leaves (lane 3) and systemically induced leaves (lane 2), suggesting that the increase of GUS specific activity upon wounding was probably a reflection of the *pin2* promoter activation. Little *Gus* mRNA was detected in the unwounded leaf tissues (lane 1). When the same RNA blot was hybridized with a rice *Act1* gene-specific probe, it was further demonstrated that wounding had little effect on the expression of this constitutive rice gene at the mRNA level, as shown in Fig. 3B. Wound-induced *Gus* mRNA level in transgenic plants transformed with the *pin2-Gus* fusion construct was much lower than that in transgenic plants transformed with the *pin2-Act1* intron-*Gus* fusion construct (data not shown).

Analysis of the kinetics of wound-inducible Gus *gene expression*

The time course of wound-induced gene expression was established at both the GUS enzymatic activity level and *Gus* mRNA level. Leaf tissues of transgenic rice plants were wounded and samples were collected for analysis at different times after wounding. Although the absolute magnitude of the wound response varied between different transgenic lines and among different wound-induction experiments, very similar kinetics were observed both for different transgenic lines and for different experiments. Data from a representative transgenic plant line are shown in Fig. 4. An increase in GUS specific activity was detectable 2–6 h after wounding, and the level of GUS specific activity increased steadily with the maximal level being detected around 24 h after wounding (Fig. 4A). At 48 h after wounding, the level of GUS specific activity was similar to that detected at 24 h after wounding (Fig. 4A), which probably reflected the extreme stability of the GUS enzyme.

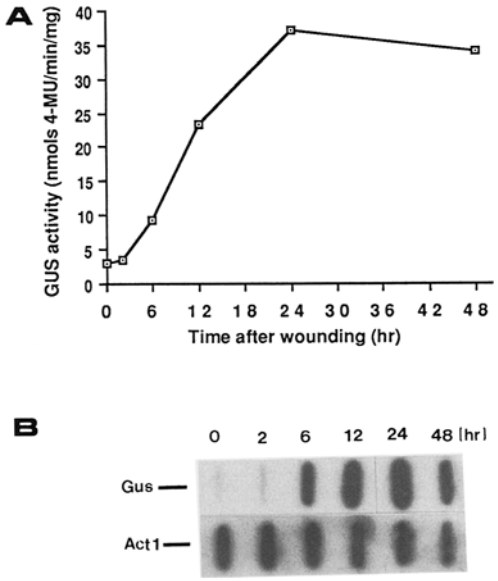


Fig. 4. Time course of wound-inducible expression of the *pin2-Act1* intron-*Gus* fusion gene in directly wounded leaf tissues of transgenic rice plants. Leaf tissues were wounded, samples were collected, and both GUS specific activity (A) and *Gus* mRNA level (B) were analyzed at indicated times after the wounding treatment, as described in Materials and methods. Only data from the representative transgenic line, PI-3, are shown. A. Time course of the wound-inducible increase of GUS specific activity. B. Time course of the wound-inducible accumulation of *Gus* mRNA.

Little *Gus* mRNA was detected in unwounded leaf tissue or even in wounded leaf tissues shortly (2 h) after wounding, as shown in Fig. 4B. Significant accumulation of *Gus* mRNA was detected at 6 h after wounding, and the maximal level was detected at 18–24 h after wounding. At 48 h after wounding, the *Gus* mRNA abundance significantly decreased, to a level similar to that detected at 6 h after wounding (Fig. 4B).

Both MJ and ABA systemically induce the expression of the pin2-Act1 *intron*-Gus *fusion gene in transgenic rice plants*

Having shown that the *pin2* promoter can direct systemic wound-inducible expression of the *Gus* reporter gene in transgenic rice plants, we were interested to know whether common chemical signal(s) can mediate the wound response in both monocot and dicot plants. Thus, we studied the effects of MJ and ABA on the expression of the *pin2-Act1* intron-*Gus* fusion gene in transgenic rice plants. Figure 5 shows the GUS specific activity (A) and *Gus* mRNA levels (B) in leaf tissue before and 24 h after application of either MJ or ABA. It was clearly shown that both MJ and ABA systemically induced the expression of the *pin2-Act1* intron-*Gus* gene at both the GUS enzymatic activity and *Gus* mRNA levels in transgenic rice plants. The time course of induction in response to either MJ or ABA was similar to that of wounding induction (data not shown). Several induction experiments were performed using the same transgenic line (line PI-3). At the concentrations tested, it was found that MJ was more effective than ABA for the induction of GUS activity in our spraying experiments (Fig. 5A). Exposure to MJ vapor also induced systemic *Gus* gene expression, but it was much less effective than direct spraying under our experimental conditions (data not shown). We also sprayed leaves with a solution containing both MJ (25 μM) and ABA (100 μM). No additive effect was observed in the induction of the *pin2*-driven *Gus* gene expression (data not shown). When a rice *Act1* gene-specific probe was used to hybridize with

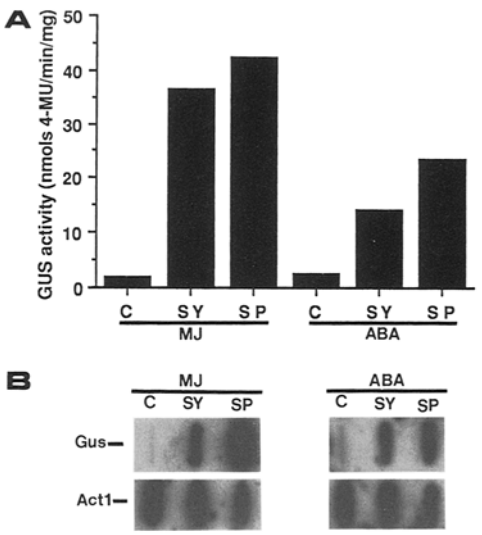
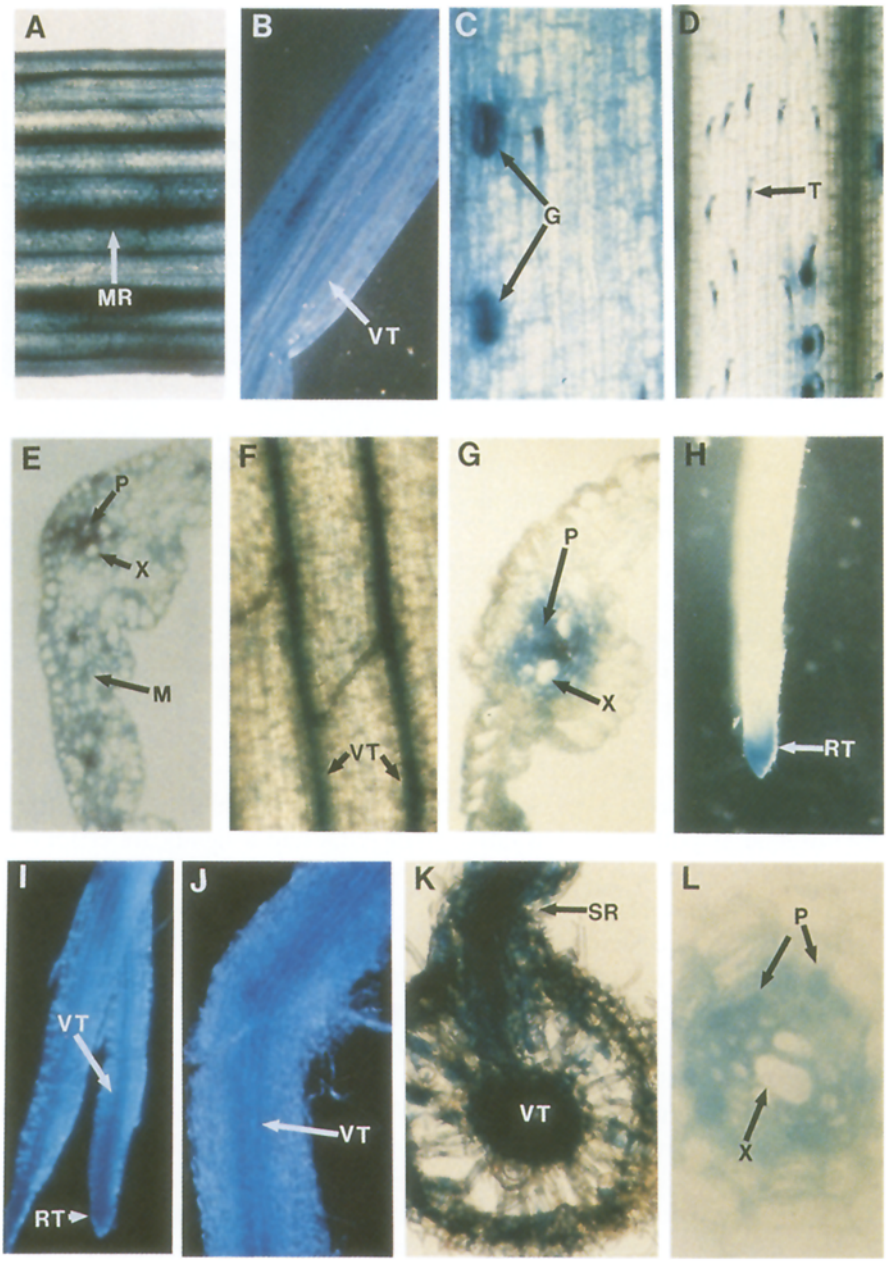


Fig. 5. Systemic induction of expression of the *pin2-Act1* intron-*Gus* fusion gene by MJ and ABA in leaves of transgenic rice plants. Plants were sprayed with a solution containing 25 μM MJ or 100 μM ABA twice during a period of 24 h. Leaf samples were collected 24 h after the initial spraying for analysis of *Gus* gene expression. Only data from the representative transgenic line, PI-3, are shown. A. Induction of GUS specific activity. B. Induction of *Gus* mRNA accumulation. Abbreviations are: C, unsprayed control; SY, systemically induced; SP, directly sprayed.

the same RNA blots, it was found that neither MJ or ABA had a significant effect on the expression of this constitutive rice gene (Fig. 5B).

Histochemical analysis of the spatial pattern of the pin2-Act1 *intron*-Gus *fusion gene expression in transgenic rice plants*

We also analyzed the tissue- and cell-specific pattern of the *pin2* promoter-directed *Gus* expression in transgenic rice plants before and after wounding as shown in Fig. 6. Several transgenic lines were used for histochemical analysis, and similar results were observed from all these plants. Transgenic rice plants transformed with the *pin2-Act1* intron-*Gus* construct show a very similar expression pattern as those transgenic plants transformed with the *pin2-Gus* construct. In the case of leaf tissue, no GUS activity could be vi-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sually detected in the leaf tissues of unwounded transgenic plants (see Fig. 2C). In directly wounded leaves, GUS activity was detected in the whole leaf tissues including leaf blades (Fig. 6A) and leaf sheaths (Fig. 6B). The highest intensity of GUS staining was detected in the leaf vascular tissue (Fig. 6A, 6B and 6E). High levels of GUS activity were also easily detected in specialized leaf epidermal cells, including epidermal guard cells (Fig. 6C) and leaf trichomes (Fig. 6D). GUS activity in other leaf epidermal cells and leaf mesophyll cells was relatively low but could also be easily detected (Fig. 6A, 6C, 6D and 6E). In leaf sheaths, which do not have a well-developed mesophyll tissue, the observed high level of GUS activity was mainly associated with the vascular tissues and with specialized epidermal cells (Fig. 6B). The expression pattern observed in systemically induced, unwounded leaves was generally the same as that in directly wounded leaves. High levels of GUS activity were always detected in the vascular tissues of systemically induced leaves, although the GUS activity detected in other cell types of leaf tissues varied among leaf samples from different wounding treatments (Fig. 6F and 6G). Microscopic observation of thin sections revealed that the high level of GUS activity in the vascular tissues was mainly associated with the phloem and cells in the vicinity of vascular bundles (Fig. 6E and 6G).

In unwounded roots, histochemical analysis revealed relatively high GUS activity in the root tip, while GUS activity was undetectable in other parts of the root (Fig. 6H). Roots from leaf-wounded plants displayed the same expression pattern as that observed in roots from unwounded plants (data not shown). Direct wounding of roots induced *Gus* gene expression in the whole root tissues (Fig. 6I). Again, the highest GUS activity was associated with the root vascular cylinder and root tip, although GUS staining was also detected in other cell types of the whole root tissues (Fig. 6J and 6K). Cross sections of roots revealed that the highest level of GUS activity in the vascular tissue was associated with the phloem (Fig. 6L).

Discussion

The wound response signal(s) and certain steps of the transduction pathways are conserved between dicot and monocot plants

Systemic induction of plant defense genes has long been documented in several dicot plants. A sustained question is what signal(s) trigger the systemic responses. The potato proteinase inhibitor II gene (*pin2*) family is probably the best studied system of systemic gene activation in plant defense responses. The search for signals which mediate the systemic wound response and the elucidation of the signal transduction pathway are active research areas [7, 14, 27, 29]. Based on the accumulated results from their own extensive studies and related information from other researchers, Ryan and his colleagues have postulated a working model of the signal transduction pathways which regulate the expression of wound- and pathogen-inducible potato proteinase inhibitor gene families [33].

Systemic induction of plant defense genes has rarely been documented in monocot plants. At present, no such systemically wound-inducible genes have been characterized in monocot plants. Some defense-related genes from monocot plants show only a limited and localized expression in response to wounding [8, 18]. Limited informa-

Fig. 6. Histochemical analysis of wound-induced expression of the *pin2-Act1* intron-*Gus* fusion gene in transgenic rice plants. A, C and D. Wounded leaf blade (× 33, × 132 and × 100, respectively). B. Wounded leaf sheath (× 15). E. Cross section of wounded leaf blade (× 100). F. Systemically wound-induced leaf blade (× 66). G. Cross section through the midrib of systemically wound-induced leaf blade (× 132). H. Unwounded root (× 15). I. Directly wounded root (× 15). J. An enlarged part of the elongating region of wounded root shown in I (× 33). K. Cross section of wounded primary root (× 100). L. Cross section of wounded primary root vascular cylinder (× 200). Abbreviations are: G, guard cell; M, mesophyll cell; MR, leaf midrib; P, phloem; RT, root tip; SR, secondary root; T, trichome; VT, vascular tissue; X, xylem.

tion suggests that in monocot plants systemic responses can indeed be induced by wounding or pathogen infection [36, 42]. We are interested to know whether common chemical signal(s) and signal transduction pathways are used to regulate these systemic responses in monocot and dicot plants. To this end, we have introduced a well-characterized, systemically wound-inducible potato *pin2* gene promoter fused to *Gus* reporter gene into rice, and analyzed its response to wounding and several wound-related signals in transgenic rice plants.

In our transient expression assays, the *pin2* promoter was found to be active in rice protoplasts, although the activity of the *pin2* promoter was much lower than that of the rice *Act1* promoter. This result is reminiscent of a previous report which found that the promoter of the wound-inducible *wun1* gene from potato was as active in rice protoplasts as in potato protoplasts in transient expression assays of a *wun1*-NPTII construct [16]. Presumably, the processes of preparation, transformation, and culture of rice protoplasts produce a physiological stress that is equivalent to wounding in potato tissue. In this study, more importantly, we found that the wound-inducible dicot *pin2* promoter could also direct systemically wound-inducible gene expression in transgenic rice plants. This finding suggests that there is also a systemic signalling mechanism(s) operating in rice to respond to mechanical wounding, and that the wound response signal(s) and certain steps of the transduction pathways are conserved between distantly related dicot and monocot plants.

In a previous study, a similar potato *pin2-Gus* fusion gene was analyzed in transgenic potato plants. It was found that wound-inducible *Gus* expression directed by the *pin2* promoter was associated with the vascular tissue [12]. This supports the hypothesis that the wound response signals are most likely transported both basipetally and acropetally via the vascular system [28]. Our histochemical analysis of transgenic rice plants also revealed that in both wounded and systemically induced leaves, as well as in directly wounded roots, the highest levels of *Gus* expression were associated with the vascular tissues. This result further suggests that the chemical nature of the signals mediating the systemic wound response, and their long-distance intercellular transmission pathways, might be similar in dicot and monocot plants.

In searching for the wound response signals in dicot plants, it was recently reported that jasmonic acid (JA) or its methyl ester, methyl jasmonate (MJ), induced systemic *pin2* gene expression in tomato plants when it was either applied to the leaf surface or present in the growth environment [7]. Jasmonate is derived from the lipoxygenase-dependent oxidation of linolenic acid. It has been suggested that JA arises from the release of cell membrane fatty acids through the action of lipase in response to wounding or pathogen infection, and thus JA might play a central role in the signalling process of plant stress response [33, 38, 43]. Convincing evidence has also been reported that the plant hormone ABA plays an important role in mediating wound-inducible expression of the *pin2* gene family in both potato and tomato plants [29]. Recently, ABA was suggested to play a general role in gene activation as a result of mechanical wounding because ABA also induces other wound-responsive genes [10].

A number of previous studies in dicot plants have shown that wounding induces the expression of several enzymes of the plant lipoxygenase pathway [2, 37]. A recent report has shown that the lipoxygenase pathway is also activated by wounding and fungal pathogen infection in rice [25]. In this study, we found that application of either MJ or ABA to the leaf tissue of transgenic rice plants induced systemic *Gus* gene expression driven by the *pin2* promoter. No additive effect was observed when both MJ and ABA were used together for induction, suggesting that these two signals might share a common transduction pathway for the final activation of the *pin2* promoter. Consistently, it has been reported that in barley leaf segments, JA parallels the effects of ABA in the promotion of senescence, leading to the synthesis of a common set of proteins [44]. Taken together, we conclude that the wound response

signal(s) and certain steps of the transduction pathways are conserved between dicot and monocot plants.

However, we also found several differences in the expression and wound induction of the *pin2* promoter between dicot plants and rice. First, in dicot plants potato, tomato, and tobacco, the *pin2* gene is not expressed in roots and wounding leaves does not induce *pin2* gene expression in roots [12, 28, 41]. In contrast, the *pin2* promoter shows high activity in the root tips of unwounded transgenic rice plants, and direct wounding induces high level expression of the *pin2-Act1* intron-*Gus* fusion gene throughout the whole roots. Second, compared with the timing of the wound induction of the *pin2* gene in dicots, the time course of the wound induction of the *pin2* promoter in transgenic rice plants is much slower. It is well known that the local wound responses differ between dicot and monocots [35]. These differences in wound response suggest that certain intervening steps in the signal transduction pathways might be divergent between dicot and monocot plants. These divergent steps might include the wound signal production at the local wound sites, transmission of wound signals to non-wounded plant parts, and final transcriptional activation of the *pin2* promoter. Many signals have been shown to induce the expression of proteinase inhibitor genes in dicot plants [33]. An important issue in plant biology is to understand the molecular mechanisms of signal transduction responsible for the activation of proteinase inhibitor gene transcription. Transgenic rice plants harboring the *pin2* promoter-*Gus* reporter gene fusions should be a useful system for studying wound-inducible gene expression in monocots.

The first intron of the rice Act1 *gene stimulates gene expression directed from the dicot* pin2 *promoter without overriding its wound inducibility in transgenic rice plants*

Several recent studies have found that dicot-specific promoters showed relatively low activity in transformed monocot cells or plants, and vice versa [6, 13, 21, 22, 30, 34]. Introns have been shown to be an important component for normal expression of some plant genes, and moreover, introns of several monocot genes have been shown to enhance gene expression from heterologous gene promoters when they are incorporated into the transcription units [4, 17, 20, 40]. In our previous study, we found that the first intron from the rice *Act1* gene greatly stimulated gene expression directed by a dicot promoter in transformed monocot cells [21, 22]. However, in all these previous studies the effects of introns on gene expression were examined in transient expression systems. Thus, it is not known whether the stimulating effects of introns on gene expression in transient assays reflect the real situation in intact transgenic plants. Furthermore, the effects of introns on the expression pattern and specific regulatory properties associated with the heterologous promoters have not been studied in transgenic cereal plants. This is mainly due to the difficulties in regenerating transgenic monocot plants.

In the present study, we analyzed the effects of the rice *Act1* intron 1 on the stable gene expression level, pattern, and wound inducibility of the potato *pin2* promoter in transgenic rice plants. We found that the rice *Act1* intron greatly increased gene expression directed by the potato *pin2* promoter without overriding either the wound inducibility or the general tissue specificity of the *pin2* promoter in transgenic rice plants. We also demonstrated that the increased level of GUS-specific activity in transgenic rice plants transformed with the *pin2-Act1* intron-*Gus* fusion gene could be correlated with an increased abundance of the *Gus* mRNA. This interesting result demonstrates the utility of the rice *Act1* intron 1 for increasing fusion gene expression without affecting the specific regulatory properties of the promoter under investigation. In addition to increasing gene expression for the purpose of genetic engineering, this feature of the *Act1* intron 1 should facilitate the study of gene expression and regulation from promoters which show weak activity in transgenic monocots.

Use of the pin2 *promoter for systemic wound-inducible foreign gene expression in transgenic monocot plants*

In any plant transformation system, strong and constitutive promoters are beneficial for generating a high level of expression of selectable genes which is necessary for efficient selection and generation of transgenic plants. Recently, a constitutive actin gene promoter [21, 45] and a light-regulated light harvesting chlorophyll *a/b*-binding protein gene promoter [39] were isolated from rice, and a constitutive ubiquitin gene promoter was isolated from maize [6]. These monocot gene promoters have been shown to be highly efficient regulators of foreign gene expression in transformed monocot cells or in transgenic monocot plants. However, constitutively active promoters are not always desirable for genetic engineering. The systemically wound-inducible *pin2* promoter is a potentially useful tool for genetic engineering of insect resistance. It has also been reported that the *pin2* gene can be systemically induced by infection of a bacterial pathogen in tomato [26]. In this study, by evaluating the activity and wound inducibility of the *pin2* promoter in transgenic rice plants, we have explored the potential utility of the *pin2* promoter for foreign gene expression in transgenic monocot plants. The levels of *Gus* gene expression in wounded rice plants directed by the *pin2* promoter in combination with the rice *Act1* intron 1 were comparable with those from the rice *Act1* promoter. In transient expression assays, we found that the *pin2-Act1* intron-*Gus* fusion gene was also actively expressed in transformed cells and tissues from other monocot plants, including maize, wheat, barley, and sorghum, suggesting that this wound-inducible promoter should be generally useful for foreign gene expression in transgenic monocot plants. Use of this efficient wound-inducible promoter to express natural insect and/or pathogen resistance genes should significantly decrease the investment of cellular metabolic energy and building blocks in transgenic plants, and may also minimize the selection pressure on insect pests, while still providing adequate protection against insect attack or pathogen infection.

Acknowledgements

We are grateful to Drs S.H. Howell, E.D. Earle and H. Moore for critical reading of this manuscript. We also wish to thank Dr N. Ayers for providing rice suspension cultures, Drs J. Cao and W. Zhang for advice in rice transformation, and Ms K.C. Kong for technical assistance. This research was supported by Grant RF91001 Allocation No. 136 from The Rockefeller Foundation. D.X. was supported by a predoctoral fellowship from The Rockefeller Foundation. D.M. was supported by a predoctoral fellowship from the Cornell University Biotechnology Program and the Cornell NSF Plant Science Center. The Cornell NSF Plant Science Center is a unit of the USDA-DOE-NSF Plant Science Centers Program and a unit of the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the US Army Research Office.

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