Induction of tuber gene expression in solanaceous species by methyl jasmonate and wounding

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Induction of tuber gene expression in solanaceous species by methyl jasmonate and wounding

by

Tsu-Hwie Annie Liu

A Dissertation Submitted to the
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Evaluation of gene expression in solanaceous species by methyl jasmonate and wounding

Tsu-Hwie Annie Liu

Major Professors: David J. Hannapel and Loren C. Stephens

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Two major research approaches have been used to study the tuber-related gene expressions in different Solanaceous species. The first part of the research is using *Agrobacterium*-mediated gene transformation to study potato proteinase inhibitor II (pinII) gene which highly expressed in developing tuber in a wild nontuberizing species *Solanum brevidens*. To establish the transformation system, leaf pieces of in-vitro cultured *S. brevidens* were cocultivated with *Agrobacterium tumefaciens* that contained nptII and GUS genes on the disarmed plasmid pBI121. Independent transgenic shoots were regenerated from both solidified and liquid medium with kanamycin. *Agrobacterium* strains GV2260 resulted in a higher transformation frequency than LBA4404. All kanamycin-resistant, putatively transformed plantlets were confirmed positive by GUS assays. Southern analysis of randomly selected transgenic plants showed that each transgenic plant contained at least one copy of the GUS gene. *S. brevidens* were then cocultivated with *Agrobacterium tumefaciens* strain GV2260 with pinII-GUS fusion gene on the disarmed plasmid pRT210. Analysis of polymerase chain reaction (PCR) products followed by Southern blot hybridization demonstrated the presence of GUS genes in three of the transformants. Transgenic plants were tested for methyl jasmonate, sucrose, and
wounding induction of the pinII-GUS gene at the RNA and protein level using leaf-petiole cuttings and whole plants. Northern blot analysis showed the pinII-GUS transgene in the transgenic plant can be induced by methyl jasmonate and sucrose. Wounding of transgenic *S. brevidens* leaves on whole plants resulted in high expression of GUS activity both locally and systemically.

The second approach is to study the expression of cathepsin D inhibitor genes which is also expressed in developing tuber in both tuberizing and nontuberizing species using blot hybridization. Three Solanaceous species, tomato (*Lycopersicon esculentum*), potato (*S. tuberosum* cv. Superior) and *S. brevidens*, were tested for MJ induction at the RNA level using a petiole/leaf system. The results of this study showed that the cathepsin D inhibitor is induced by MJ. A dose response test was performed for MJ induction of CDI in the leaves of petiole/leaf cuttings. For Superior and tomato, both had highest level of expression at 50 μM. The maximum expression level for *S. brevidens* was at 500 μM MJ. The induction of MJ was also dependent on the duration of exposure. Twelve hours of incubation were enough to give relatively abundant accumulation for all three species. There is no light/dark effect on MJ induction from 0 to 48 hours for either Superior or *S. brevidens*. But it has a slight decrease of expression using tomato leaf samples in the same treatment. Leaf samples of Superior and *S. brevidens* were also treated with 50 μM of GA either before or after MJ treatment to observe the interaction of these two hormones. GA had no effect on the MJ induction of CDI expression in either Superior or *S. brevidens*. Immunoblot analysis showed CDI proteins were induced in 'Superior' leaves after MJ treatment.
The fear of the Lord is the beginning of wisdom,
all who follow his precepts have good understanding,
To him belongs eternal praise.
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CHAPTER 1. GENERAL INTRODUCTION

The family Solanaceae includes about 90 genera and over 3000 species (Hawkes, 1979). It contains a number of economically valuable crops and vegetables such as potato (Solanum tuberosum), tomato (Lycopersicon esculentum), peppers (Capsicum spp) and tobacco (Nicotiana tabacum). Others such as Petunia and ornamental tobacco are cultivated as flower crops. However, among those species, only approximately 160 wild and 7 cultivated species within the genus Solanum have the ability to form tubers (Hawkes, 1979). Many wild Solanum species, both tuberizing and nontuberizing, like Solanum brevidens, S. spegazzinii, S. demissum, S. stoloniferum, S. chacoense and S. integrifolium contain valuable disease resistance or stress tolerance traits (Foldo, 1987). Although they are not agronomically important, they can still be used as potential breeding materials in traditional breeding programs for cultivated solanaceous species or specific genes in their germplasm could be manipulated through genetic engineering. Some wild Solanum species such as S. integrifolium, S. verrucosum, S. hjertingii, S. stoloniferum and S. demissum have been transformed with reporter genes (Rotino, et al. 1992, Kumar, et al. 1995). These transformation systems provide not only an opportunity to put new genetic markers into the wild species for breeding purposes, and but also the expression of the same gene construct can be compared between more than one species of Solanum. Therefore, the availability of a transformation system for these wild species will provide a valuable tool for future research in the analysis of useful genes from cultivated species and in understanding their modes of expression.
One approach to studying gene expression in plants is to monitor the expression of the same gene in several species, and to compare gene expression between those species. Many genes that are involved in environmental and developmental changes have been isolated and studied from potato and tomato using molecular biology techniques. The proteinase inhibitors, for example, serve as major tuber storage proteins of potato and as part of the plant defense mechanism against invading pests of both tomato and potato (Johnson, et al. 1990). Some wild solanaceous species are also known to contain DNA sequences homologous to those of the potato these proteinase inhibitors. For example, a wild tomato (L. peruvianum) contains proteinase inhibitor I of tomato (Wingate, et al. 1989) and several wild nontuberizing Solanum species, S. brevidens and S. etuberosum, contain cathepsin D inhibitor of potato (Hansen and Hannapel, 1992). However, the transcript accumulation patterns of the cathepsin D inhibitor in potato and wild nontuberizing Solanum species and tomato are different. Sucrose can induce cathepsin D inhibitor in tuberizing species but not nontuberizing species (Hansen and Hannapel, 1992). The reason for this difference is not clear, but, by comparing the expression of proteinase inhibitor genes from those solanaceous species, we may learn more about the factors that control expression.

The objectives of this study are (i) to establish a dependable transformation system for S. brevidens, a nontuberizing species, using Agrobacterium tumefaciens. This system can be used as a model to study any tuber-related gene which is not present or activated in a nontuberizing Solanum species. (ii) To test the inducible gene expression in this transformation system by using a potato proteinase inhibitor II promoter and GUS gene
construct. (iii) To use blot hybridization techniques to further characterize the expression pattern of a cathepsin D inhibitor gene which is present in both tuberizing and nontuberizing solanaceous species and is induced by methyl jasmonate. These studies will also provide more information on the factors that regulate the expression of the potato proteinase inhibitor genes.

**Dissertation Organization**

This dissertation is arranged in an alternate format consisting of three papers suitable for publication. The first paper, "Transformation of *Solanum brevidens* using *Agrobacterium tumefaciens*" is accepted for publication in Plant Cell Reports. The second paper, "Expression of a chimeric proteinase inhibitor II-GUS gene in transgenic *Solanum brevidens* plants" is prepared to be submitted to the Journal of Plant Physiology, and the third paper, "Induction of cathepsin D inhibitor gene expression in response to methyl jasmonate" is submitted to Physiologia Plantarum. Tsu-Hwie Annie Liu was the principal investigator under the supervision of Drs. David J. Hannapel and Loren C. Stephens on all research reported herein, and is the first author on the three papers. The three papers are preceded by the General Introduction and followed by the General Conclusion. Literature Cited in the General Introduction and General Conclusion are listed in alphabetical order according to author's name following General Conclusion. The arrangement of the papers follows the guidelines set forth by each journal in their Instruction to Authors.
CHAPTER 2. TRANSFORMATION OF *Solanum brevidens* USING *Agrobacterium tumefaciens*

A paper accepted by *Plant Cell Reports*

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**Abstract**

Leaf pieces of *in vitro*-cultured plantlets of the wild potato species *Solanum brevidens* Phil. were cocultivated with *Agrobacterium tumefaciens* that contained *nptII* and *uidA* genes on the disarmed plasmid pBI121. Independent transgenic shoots were regenerated from solidified and liquid medium that contained 50 mg·L⁻¹ kanamycin. Two *Agrobacterium* strains were investigated for transformation efficiency. GV2260, which contained p35SGUSINT, resulted in a 11% transformation frequency, compared with 1% using LBA4404. Transformation rates were 7% in liquid culture and 3% on solidified medium. All kanamycin-resistant, putatively transformed plantlets were confirmed positive by histochemical GUS assays. GUS activity in 22 independently transformed plants was
quantified by fluorometric assay. Southern analysis of randomly selected transgenic plants showed that each transgenic plant contained at least one copy of the uidA gene.

**Key words:** Solanum brevidens, Agrobacterium tumefaciens, plant regeneration

**Abbreviations:** GUS, β-glucuronidase, MS, Murashige-Skoog medium, BA, 6-benzylaminopurine, 2ip, 6-(γ,γ-dimethylallylamino)purine, IAA, indole-3-acetic acid, GA3, gibberellic acid, npt II, neomycin phosphotransferase II, NOS, nopaline synthase, MUG, 4-methyl umbelliferyl glucuronide, MU, 7-hydroxy-4-methylcoumarin, X-gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid

**Introduction**

*Solanum brevidens* Phil. is a wild species of potato that does not bear tubers but has resistance to potato leaf roll virus (PLRV), potato virus X and potato virus Y (Jones et al. 1990). It is, however, sexually incompatible with the domestic potato, *S. tuberosum*. Somatic hybrids of *S. brevidens* + *S. tuberosum* are PLRV and late blight (*Phytophthora infestans*) resistant (Helgeson et al. 1986) and have shown a novel resistance to tuber soft rot caused by *Erwinia* spp. (Austin et al. 1988). Because these interspecies somatic hybrids are fertile, they have great potential for use in potato breeding programs (Helgeson et al. 1993). One method that has facilitated the selection of *S. tuberosum* + *S. brevidens* somatic hybrids is transformation of *S. brevidens* with the nptII selectable marker before protoplast fusion (Puete and Schaar 1993). Another potentially important use for transgenic *S. brevidens* is the
preferential inclusion of chromosomes in asymmetric fusion products between irradiated transgenic *S. brevidens* and *S. tuberosum* (Puite and Schart 1993). In addition to using transgenic *S. brevidens* in somatic hybridization, Jones et al. (1989) showed that genes from *S. tuberosum* could be studied in the nontuberizing *S. brevidens*.

Transformation of *S. brevidens* was first reported using electroporation (Jones et al. 1989). However, regeneration to whole plants has not been reported and protoplast-derived plants are sensitive to somaclonal variation (Karp et al. 1989). Transgenic *S. brevidens* plants and hairy root clones were obtained by Puite and Schart (1993) using *Agrobacterium tumefaciens* and *A. rhizogenes*, respectively, but the frequency of obtaining transgenic plants was relatively low. *Agrobacterium* strains have shown differences in their capacity to infect *S. tuberosum* (Higgins et al. 1992). Also, Puite and Schart (1993) rooted transgenic shoots in liquid medium, and Dodds (1989) showed that liquid medium was beneficial for potato stem culture. Therefore, objectives in this study were: 1) to test two different *Agrobacterium* strains for *S. brevidens* transformation; 2) to test liquid culture as a more efficient means of selection; and 3) to establish a predictable rate of production of independent transformants.

**Materials and Methods**

*Agrobacterium strains.* All *Agrobacterium tumefaciens* strains were maintained on MGL (An 1986) plates with 50 mg l\(^{-1}\) kanamycin at 4°C in dark. LBA4404 (Ooms et al. 1982) harboring two different disarmed binary vectors, pBI121 and p35SGUSINT, both derived from pBIN19, were used separately. Plasmid pBI121 contains the neomycin phosphotransferase II (*nptII*) gene and the β-glucuronidase (*uidA*) gene. The *nptII* gene is under the control of the nopaline
synthase (NOS) promoter, and the uidA gene is under the control of the cauliflower mosaic virus (CaMV35S) promoter. The plasmid p35SGUSINT has an nptII gene and a chimeric uidA gene with potato ST-LS1 intron under the control of the CaMV35S promoter (Vancanneyt et al. 1990). *Agrobacterium GV2260* originated from the C58 strain (Deblaere et al. 1985), and it contains the binary plasmid p35SGUSINT.

*Plant material and regeneration.* Several shoot cultures derived from seedlings of the same accession of *Solanum brevidens* were grown *in vitro* in plastic Magenta GA-7 jars (Sigma Chemical Co., USA) on MS (Murashige and Skoog 1962) basal medium supplemented with 3% (w/v) sucrose and 0.15% (w/v) Gelrite without hormones. Shoots were subcultured every 4 to 6 weeks by transferring stem segments with a single axillary bud. All plants were propagated under fluorescent light (7.2 μmol·s⁻¹·m⁻²) at growth chamber temperature of 25°C under a 16 h daylength.

*Transformation.* Stem and leaf explants were taken 4 to 6 weeks after subculturing axenic stock plants. The explants were cut in 5- x 3-mm pieces, soaked in 20 ml sterile distilled water with 500 μl overnight culture of *Agrobacterium* suspension (concentration of bacteria approx. 4 x 10⁹/ml) for about 20 minutes, and blotted dry on a sterile paper towel. Treated explants were placed on solidified callus induction medium (MS basal salts with 3% sucrose, 0.5 mg·l⁻¹ IAA, 2.2 mg·l⁻¹ 2ip and 0.7% agar) without antibiotics in sealed petri dishes. After 2 days of cocultivation, the explants were transferred to fresh callus induction medium with 250 mg·l⁻¹ claforan and 50 mg·l⁻¹ kanamycin. All explants were subcultured every other week.
After four weeks, explants were transferred onto either solidified or liquid medium that contained the same components except agar. Both cultures were subcultured in 2- or 3-week intervals until light-green shoots appeared on the callus or leaf surface. Then, they were transferred to either 10 ml liquid shoot elongation medium (MS basal salts with 3% sucrose, BA 5 mg·l⁻¹, GA₃ 5 mg·l⁻¹) in 125 ml flasks or 20 ml solidified medium in 15 cm Petri dishes that contained claforan (250 mg·l⁻¹) and kanamycin (50 mg·l⁻¹). All solidified plates were incubated at 25°C, 16 h photoperiod. Liquid cultures were maintained on a shaker at 80 rpm and 25°C under constant fluorescent light (0.3 μmol·s⁻¹·m⁻²). After six weeks, regenerated shoots were removed from explants and micropropagated on MS medium supplemented with 3% sucrose and 50 mg·l⁻¹ kanamycin and 250 mg·l⁻¹ claforan without hormones to allow root formation. All putative transformants that did not root in the rooting medium were considered escapes. All regenerated shoots were subcultured at 3 to 4 week intervals and underwent at least two cycles of micropropagation on solidified MS medium with no antibiotics. Shoot cultures were monitored for morphological normality, rooting ability, bacterial contamination, and histochemical GUS activity before transplanting to the greenhouse. An interval of four months was required to obtain transgenic plants from leaf-disk explants.

**GUS assays.** The histochemical GUS assay was used to assay qualitatively positive independent transformants from large numbers of regenerated shoots, and the fluorometric GUS assay was used to quantify GUS activity of each selected transgenic plant (Jefferson, 1987). For the histochemical assay, a young leaf from each putative transgenic plantlet was
taken as a sample. Leaf samples were incubated in buffer solution that contained 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) overnight at 37°C and bleached in 95% ethanol. For the fluorometric assay, leaves from independent transgenic plants in the greenhouse were homogenized in extraction buffer that contained 10 mM β-mercaptoethanol, 50 mM sodium phosphate, 10 mM EDTA, 0.1% Triton X-100 and 0.1% lauroyl sarcosine. Protein concentrations were determined by the Bradford (1976) assay. Leaf extract equivalent to 100 ng/μl total protein from each sample was incubated with 4-methylumbelliferyl glucuronide (MUG) solution. The GUS activity was determined by the turnover rate of 4-MUG to 7-hydroxy-4-methylcoumarin (MU) after 30 min incubation, by using a Hitachi fluorescence spectrophotometer model F-2000. GUS activity of each independent plant was the mean from 3 separate leaves.

**DNA extraction and Southern hybridization.** Genomic DNA was extracted from fresh leaves of untransformed and transformed plants using the CTAB (cetyltrimethylammonium bromide) method described by Rogers and Bendich (1985). For Southern blots, 10 μg genomic DNA was digested with *EcoRI*, subjected to electrophoresis in 0.7% agarose gel in TAE (tris acetic acid-EDTA) buffer and blotted onto a nylon membrane (Micron Separations Inc.). The membrane filter was hybridized overnight at 42°C with a 1.0 kb uidA probe, that was 32P-labeled by nick translation. The hybridization buffer and conditions were the same as described by Hansen and Hannapel (1992).
Chi-square analysis. Chi square tests were performed using the CHISQ option of the FREQ procedure (SAS Institute Inc. 1985).

Results and Discussion

Comparison of two A. tumefaciens strains. Two strains of A. tumefaciens, LBA4404 and GV2260, were compared in a regeneration test on solidified medium. Both strains harbor p35SGUSINT, a plasmid that contains an intron that prevents expression in Agrobacterium and that allows testing for GUS activity as soon as shoot regeneration occurs. GV2260 had a greater transformation frequency than LBA4404. Plants taken from 11 of the 96 explants transformed with GV2260 tested positive for GUS expression for a transformation frequency of 11% (Table 1). Only 1 of the 95 explants transformed with LBA4404 tested positive for a frequency of 1% (Table 1).

Table 1. Transformation frequency of S. brevidens leaf disks on solidified medium using two different A. tumefaciens strains. Both strains contain the binary vector p35SGUSINT. The histochemical assay was performed on one regenerated shoot per explant.

<table>
<thead>
<tr>
<th>Agrobacterium strain</th>
<th>No. of explants</th>
<th>No. of regenerating GUS(+) shoots</th>
<th>Transformation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA4404</td>
<td>95</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>GV2260</td>
<td>96</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>
Both helper plasmids are disarmed vectors of the octopine type, but LBA4404 has a much larger deletion region (Ooms et al. 1982). Additionally, each is derived from a different source, with LBA4404 being an Ach5 derivative, whereas GV2260 is derived from C58 (Deblaere et al. 1985). Either of these factors may explain why GV2260 is a more efficient vector for transformation of S. brevidens.

Solidified vs. liquid culture medium. Because the transformation frequency was so low with LBA4404 on solidified medium, we tried to improve the frequency using different culture methods. The regeneration frequency of transformed shoots in liquid medium was greater than twice that of the frequency on solidified medium, and was significant by chi square test (Table 2). One possible explanation is that the liquid medium contacts all surfaces of the explants and thereby promotes more transformed cell growth, whereas explants on solidified medium typically contact the medium in only a few places. Puite and Schaart (1993) obtained 13 transgenic plants from 350 plated explants, but they did not report the regeneration rate per explant. Of those shoots tested for rooting ability in kanamycin, the frequency of rooting was nearly twice as great among shoots transferred from liquid culture compared with shoots transferred from solidified culture, and was highly significant by chi square test (Table 2). No untransformed shoots rooted in kanamycin-containing medium, and we concluded that shoots from treatments not rooting in kanamycin were escapes. By this criteria, the selection against escapes was twice as efficient in liquid as in solidified medium, assuming that all shoots would root without kanamycin. One difference between liquid and solidified culture was that during shoot elongation, there were fewer but more vigorous shoots produced in liquid medium,
suggesting that kanamycin efficiently inhibited growth of untransformed shoots in liquid medium. Because leaf explants on solidified medium curled and did not maintain contact with the medium over the entire surface (data not presented), more escapes may have occurred. One disadvantage of liquid medium was that shoot regeneration was delayed three weeks on average. Also, discoloration of the medium by senescing leaf cells under kanamycin selection necessitated frequent transfer to fresh medium. However, when transformed shoots emerged from the callus, they grew more rapidly in liquid culture, consistent with the results of Ottaviani et al. (1990).

Table 2. Medium type comparison for *S. brevidens* transformation efficiency, using *A. tumefaciens* LBA4404/pBI121. Both shoot regeneration and rooting was in the presence of 50 mg·l⁻¹ kanamycin. Data were collected from three separate experiments.

<table>
<thead>
<tr>
<th>Medium type</th>
<th>No. of explants</th>
<th>No. of shoot-regenerating calli</th>
<th>Regeneration frequency (%)</th>
<th>No. of shoots tested for rooting ability</th>
<th>No. of rooted shoots</th>
<th>Rooting frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>248</td>
<td>16</td>
<td>7</td>
<td>60</td>
<td>44</td>
<td>73</td>
</tr>
<tr>
<td>Solidified</td>
<td>280</td>
<td>8</td>
<td>3</td>
<td>32</td>
<td>14</td>
<td>44</td>
</tr>
</tbody>
</table>

Chi square*  
P=0.036        P=0.005

*At P < 0.05, the response frequencies of the 2 treatments were significantly different.
Based on results of Wenzler et al. (1989), working with *Solanum tuberosum*, we used 50 mg·l\(^{-1}\) kanamycin for selection to minimize escapes. Our results showed, however, that even a kanamycin concentration as low as 10 mg·l\(^{-1}\) was effective in selecting *S. brevidens* transformants, although there was also a higher escape rate (data not shown). Wenzler et al. (1989) showed that 25 mg·l\(^{-1}\) kanamycin led to a high escape rate, whereas 75 mg·l\(^{-1}\) was no more effective than 50 mg·l\(^{-1}\). However, the *S. brevidens* transformed shoots regenerated about three weeks later than untransformed controls, whether we used 10 or 50 mg·l\(^{-1}\) kanamycin. We also tested preculture and postculture treatments similar to those of Wenzler et al. (1989) and Visser et al. (1989), respectively. Neither pretreating explants in medium before cocultivation nor postponement of selection showed any difference from those explants given no preculture or postculture treatments (data not shown).

**Analysis of transformed plantlets.** Twenty-four kanamycin-resistant calli from liquid or solidified cultures, which produced shoots and rooted in kanamycin, were subjected to the histochemical GUS assay, and all tested positive (data not shown). Twenty-two of a total of 24 shoots, each from a separate transgenic callus line, also tested positive in the GUS fluorometric assay (Fig. 1). The remaining two GUS-positive callus lines did not produce shoots large enough to test with the GUS fluorometric assay. The range of GUS activity was from 0.067 to 12.88 n mole MU·min\(^{-1}·mg^{-1}\) protein. This range (approximately 200-fold) compares with a 500-fold range reported by Ottaviani et al. (1990) for *S. tuberosum*. The untransformed control plants ranged from 0.008-0.01 n mole·min\(^{-1}·mg^{-1}\) protein, well below the values reported for the lowest expressing transgenic plants.
Five transformed plants (4-1, 8-6, 13-4, 18-1, and 21-6) were selected randomly for DNA analysis, with one additional (4-2) being chosen intentionally from the same callus (4-1). This last sample (4-2) was chosen to test whether shoots from the same explant were from independent transformation events. All samples from transformed plants showed that the *uidA* gene was stably integrated in at least one site of the nuclear genome (Fig. 2). Each sample possessed a different pattern, because there was one *EcoRI* site present in the T-DNA and another in the plant DNA at a site which depended on where the T-DNA integrated into the plant chromosome. Integration of GUS DNA was in unique sites in each transgenic plant, except for samples 4-1 and 4-2, (Fig. 2, lanes 2 and 3) which were apparently from the same transformation event. Fig. 2 also shows that transgenic plants 4-1 (or 4-2), 8-6 and 21-6, each with two bands, received more than one copy of the GUS gene. The darker bands of 4-1 and 4-2 might result from tandem repeats of T-DNA fragments that lack the *EcoRI* restriction site, but retain multiple copies of the *uidA* gene. T-DNA borders can be lost before integration (Deroles and Gardner 1988), and the *EcoRI* site would seem a prime candidate for loss because of its location near the LB region of the T-DNA strand.

One rooted transgenic plant per single selected callus was transplanted into soil and grown in the greenhouse. These plants were compared with untransformed plants, and all were phenotypically normal. Five plants were grown to maturity, three flowered and were able to produce viable seeds, but only one produced enough seeds for an inheritance test during the course of this study. Seedlings from selfing this plant (18-1) were germinated aseptically in a petri dish and tested with the histochemical GUS assay. The ratio of GUS positive to negative seedlings was 22 to 10. A chi square test (P > 0.1) showed that the gene
segregated in a 3:1 Mendelian ratio. This, combined with the Southern data (Fig. 2, lane 6), shows that the uidA gene is present as one copy at a single locus in this plant. Our results show that using A. tumefaciens strain GV2260 and liquid culture greatly improves transformation efficiency of the wild potato species S. brevidens.

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Fig. 1. Fluorometric GUS assays of 22 transgenic *S. brevidens*. CK is a control, untransformed plant. Data were collected from three repeated experiments. GUS assays were performed as described in Materials and Methods.
Fig. 2. Southern blot analysis of genomic DNA from *S. brevidens* plants. DNA (10 μg) was digested with *EcoRI*, subjected to electrophoresis in 0.7% agarose, transferred to a nylon membrane, and probed with radiolabelled *uidA* fragment. Lane 1 (C) was a digest of DNA from an untransformed plant and lanes 2-7 were digests of DNA from transgenic plants.
CHAPTER 3. EXPRESSION OF A CHIMERIC PROTEINASE INHIBITOR II - GUS GENE IN TRANSGENIC Solanum brevidens PLANTS

A paper to be submitted to the Journal of Plant Physiology

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Abstract

A wild nontuberizing potato species Solanum brevidens Phil. was transformed with Agrobacterium tumefaciens strain GV2260 containing chimeric neomycin phosphotransferase and potato proteinase inhibitor II (pinII) promoter-GUS (uidA) genes on the disarmed plasmid pRT210. Several independent transgenic plantlets were regenerated on kanamycin-containing medium. Analysis of polymerase chain reaction products followed by Southern blot hybridization confirmed the presence of uidA genes in three of the transformants. Transgenic plants were tested for methyl jasmonate, sucrose, and wound induction of the pinII-GUS genes at the RNA and protein levels using leaf-petiole cuttings and whole plants. Northern blot analysis showed that the pinII-GUS transgene can be induced by methyl jasmonate and sucrose. Methyl jasmonate-induced GUS activity in transgenic plants was up to fifteen times higher than basal levels after 12 h incubation and up to twenty-three times
higher than basal levels after 24 h. Wounding of transgenic *S. brevidens* leaves on whole plants resulted in a substantial induction of GUS activity both locally and systemically.

**Key words:** *Solanum brevidens*, gene expression, proteinase inhibitor II, transformation

**Abbreviations:** CDI, cathepsin D inhibitor; GUS, β-glucuronidase; MS, Murashige-Skoog medium; MUG, 4-methyl umbelliferyl glucuronidase; MU, 7-hydroxy-4-methylcoumarin; 
nptII, neomycin phosphotransferase; PCR, polymerase chain reaction; pin II, proteinase inhibitor II; X-gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid

**Introduction**

*Solanum brevidens* Phil. is a wild potato species which does not bear tubers and is sexually incompatible with the domestic potato *S. tuberosum*. Genetic analysis of *S. brevidens* has shown that it contains valuable germplasm including resistance to potato leaf roll virus, potato virus X and virus Y (Jones et al. 1990). Somatic hybrids of *S. brevidens* + *S. tuberosum* were fertile and showed resistance to potato leaf roll virus, late blight and soft rot (Helgeson et al. 1986, Austin et al. 1988). Transformation of *S. brevidens* was also established using electroporation (Jones et al. 1989) and *Agrobacterium*-mediated transformation (Puite and Schaart 1993, Liu et al. 1995). Because of its valuable germ plasm and facility for transformation and regeneration, *S. brevidens* has great potential in potato cultivar enhancement programs. In addition, transgenic *S. brevidens* can serve as a valuable model system for studying the regulation of genes expressed in *S. tuberosum*. Sequences
homologous to a potato patatin and cathepsin D inhibitor (CDI) gene were found in *S. brevidens* genomic DNA. The putative patatin gene sequences in *S. brevidens* could not be induced under any conditions. (Hannapel 1990), whereas the *S. brevidens* CDI exhibited a different pattern of expression than the *S. tuberosum* CDI gene. The *S. tuberosum* CDI gene is wound and sucrose inducible, whereas the *S. brevidens* CDI was wound-inducible only (Hansen and Hannapel 1992). Jones et al. (1989) showed that the patatin promoter from *S. tuberosum* was functional in leaf protoplasts of *S. brevidens* using transient gene expression assays, but not in stably transformed plants. Through blot hybridization, pinII sequences have been detected in *S. brevidens* genomic DNA, but there is no evidence that these sequences represent a functional pinII gene (Hannapel, unpublished results).

Proteinase inhibitors are produced in plants as part of a defense mechanism in response to wounding caused by insect attack (Green and Ryan 1972). The most abundant of the proteinase inhibitors is the serine proteinase inhibitor, pinII (Green and Ryan 1972). Expression of this protein has been induced in both tomato and potato wounded leaf tissue (local response) as well as in nonwounded leaves distant from the local wound site (systemic response) (Graham et al. 1986, Peña-Cortes et al. 1988). PinII can also be induced in leaves by plant hormones like abscisic acid (ABA) and methyl jasmonate (MJ) as part of the wound signal transduction pathway and by plant cell wall fractions, chitosan, and sucrose (Peña-Cortes et al. 1992, Sanchez-Serrano et al. 1986, Kim et al. 1991). In addition to pinII's induction in leaf tissue, its expression is also developmentally regulated in tubers, fruits, and flower organs (Lorberth et al. 1992). PinII can make up a large proportion of the soluble
protein present in a mature potato tuber, whereas no detectable expression occurs in other organs of a nonwounded potato plant.

Transgenic expression studies using a chimeric gene consisting of the 5'- and 3'-flanking regions of a potato pinII gene with a reporter gene confirmed that the fusion gene was constitutively expressed in the stolons and tubers of transgenic potato plants, while the expression in leaves was wound-inducible (Keil et al. 1989). Sanchez-Serrano et al. (1987) reported a pinII-GUS gene in tobacco was induced by wounding and enhanced by sucrose and glucose. These results demonstrated that although no pinII homologous sequence was detected in tobacco, the transgenic plant was still capable of expressing the potato gene after induction. In transformed rice plants, wounding, MJ, and ABA induced both a local and systemic response of a chimeric potato pinII promoter-GUS fusion gene, demonstrating that the wound response of pinII is conserved in both dicots and monocots (Xu et al. 1993). To continue the study of potato gene expression in nontuberizing Solanum species, this report documents the induction of a chimeric potato pinII promoter-GUS transgene in S. brevidens by MJ, wounding, and sucrose.

Materials and Methods

Agrobacterium strains. All Agrobacterium tumefaciens strains were maintained on MGL (An 1986) plates with 50 mg·l⁻¹ kanamycin at 4°C in dark. Agrobacterium GV2260 which originated from the C58 strain (Deblaere et al. 1985), was used as the host strain and harbored two different disarmed binary vectors separately, pBI121 and pRT210. Plasmid pBI121 contains the nptII gene and the uidA gene. The nptII gene is under the control of the nopaline
synthase (NOS) promoter, and the uidA gene is under the control of the cauliflower mosaic virus (CaMV35S) promoter. The plasmid pRT210 was obtained from Dr. Robert Thornburg and has an nptII gene and a chimeric uidA gene with potato proteinase inhibitor II promoter.

Plant material and transformation. The culture of in vitro stock plants and transformation of S. brevidens were according to the methods described previously (Liu et al. 1995).

Methyl jasmonate, sucrose, and wounding treatments. Leaf-petiole cuttings from transgenic S. brevidens plants were used for MJ and sucrose treatments. Leaves of transgenic S. brevidens were detached from the plants by cutting the petiole and subsequently, the cut ends were placed in a solution of either 50 μM MJ (Bedoukian Industries, Darbury, CT) or 60 mM sucrose. MJ was dissolved in 95% ethanol and diluted in sterile deionized water to make a final concentration of 50 μM MJ. All treatments contained 3 leaf-petiole cuttings each. The MJ and sucrose treatments were kept inside a negative-pressure fume hood at 25°C with light, up to 48 hours. The MJ treatments were kept separate from other treatments to prevent exposure to volatile MJ derivatives. After the incubation periods, leaf samples from the treatments were harvested, and either analyzed immediately or frozen in liquid nitrogen, and stored at -80°C.

Transgenic S. brevidens plants growing in the greenhouse were wounded as described by Hansen and Hannapel (1992). The third and fourth leaves down from the apex were wounded by a hemostat. Wounded leaves (local response) and nonwounded leaves above the
wounded leaves (systemic response) were collected 24 h after treatment and analyzed immediately after harvest by histochemical and fluorometric GUS assays.

**GUS assays.** Both histochemical and fluorometric GUS assays (Jefferson, 1987) were used to determine the GUS activity of each selected transgenic plant. For the histochemical assay, a young leaf from each treated transgenic plant was taken as a sample. Leaf samples were incubated in buffer solution that contained 1.0 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) overnight at 37°C and bleached in 95% ethanol. For the fluorometric assay, several leaves from each treated transgenic plant were homogenized in extraction buffer that contained 10 mM β-mercaptoethanol, 50 mM sodium phosphate, 10 mM EDTA, 0.1% Triton X-100 and 0.1% lauroyl sarcosine. Protein concentrations were determined by the Bradford (1976) assay. Leaf extract equivalent to 50 μg/μl total protein from each sample was incubated with 4-methyl umbelliferyl glucuronide (MUG) solution. The GUS activity was determined by the turnover rate of 4-MUG to 7-hydroxy-4-methylcoumarin (MU) after 30 min incubation, using a Hitachi fluorescence spectrophotometer model F-2000. GUS activity of each sample was the average mean from 2 independent experiments.

**DNA extraction, PCR and Southern hybridization.** Genomic DNA was extracted from fresh leaves of untransformed and transformed plants using the CTAB (cetyltrimethylammonium bromide) method (Rogers and Bendich 1985) and DNA miniprep method (Gentra System Inc.). PCR was carried out using 21-mer oligonucleotides position 400-420 and 1599-1579 from the GUS gene (Hamill et al. 1991). The amplification cycle consisted of denaturation at
94°C for 1 min, primer annealing at 61°C for 1 min, and primer extension at 72°C for 2 min. After 31 cycles, amplification products were analyzed on 1% agarose gel in TAE (tris acetic acid-EDTA) buffer. Gels were stained with ethidium bromide, visualized, and photographed under UV light. For Southern hybridization, after denaturation and neutralization, gels were blotted onto a nylon membrane (Micron Separations Inc.). The membrane filter was hybridized overnight at 42°C with a 2.2 kb uidA probe, that was \(^{32}\)P-labeled by nick translation. The hybridization buffer and conditions were the same as described by Hansen and Hannapel (1992).

**RNA extraction and northern hybridization.** Total leaf RNA from transgenic *S. brevidens* was isolated by the phenol/chloroform method described by Suh et al. (1991). For northern blots, RNA was subjected to electrophoresis in 1.4% agarose gels containing 5 mM methylmercuric hydroxide (Alfa Products, Danvers, MA, USA) in tris-borate buffer (pH 7.5) and blotted onto nylon membranes. RNA was detected with ethidium bromide under UV light to ascertain consistent loading of gels and efficient transfer to nylon membranes. Blots were hybridized with a 2.2 kb uidA probe. RNA blots were hybridized in 50% (v/v) formamide, 5x SSC, 0.07% polyvinylpyrrolidone, 0.07% Ficoll, 0.07% bovine serum albumin, 25 mM sodium phosphate (pH 6.8), 0.5% SDS, and 250 μg/ml sheared salmon sperm DNA. After hybridization overnight at 42°C, blots were washed once with 1x SSC/0.1% SDS at room temperature for 15 min. Filters were then washed twice in 0.1x SSC/0.1% SDS at room temperature and 65°C for 30 min each wash. Hybridized probe was detected by exposure of the filters to X-OMAT AR film for 7 days.
Results and Discussion

Transformation of S. brevidens. Eight independent kanamycin-resistant transformants containing the pinII-GUS construct were obtained from Agrobacterium-mediated transformation. All putative transformants were subcultured at 3 to 4 week intervals in kanamycin-containing medium and then underwent at least two cycles of micropropagation on solidified MS medium with no antibiotics. This step is necessary to confirm that the transgenic plantlets were free of Agrobacterium contamination. Total genomic DNA was prepared from the leaves of three putative transgenic plantlets and a control plant that was not treated with Agrobacterium. The DNA was subject to PCR using oligonucleotide primers specific for uidA DNA (Hamill et al. 1991). An approximately 1.2 kb PCR product was amplified from the DNA of the three transgenic plantlets as well as the positive control GUS sequence isolated from pBI221 (Fig. 1A). Following hybridization of PCR products with a uidA DNA probe, the presence of uidA DNA fragments was observed in all 3 transgenic plantlet (Fig. 1B). No DNA band was visualized nor signal was detected in the negative control from untransformed S. brevidens genomic DNA (Fig. 1, lane N).

Northern blot analysis of pinII-GUS gene expression. To determine whether this chimeric pinII-GUS gene is functioning in the transgenic S. brevidens plants, leaf-petiole cuttings from transgenic plants were incubated 24 h in either distilled water, 50 μM MJ, or 60 mM sucrose. Northern blot hybridization showed that GUS transcripts accumulated in response to both sucrose and MJ treatment after 24 h incubation (Fig. 2, lanes 4 and 5, respectively). Under the same conditions, a transgenic S. brevidens with constitutive CaMV promoter driving a
GUS gene (Liu et al. 1995) did not show any induction after 24 h MJ treatment (Fig. 2, lanes 1, 2). These results suggest that the increase in the GUS mRNA levels was probably due to the activation of the pinII promoter during the incubation period and are also consistent with a previous study showing that pinII gene was induced in detached potato leaves by exogenous treatments of MJ and sucrose at the RNA level (Peña-Cortes et al. 1992). Methyl jasmonate is a lipid derivative from the oxidative pathway of linolenic acid and is present in many plant species (van den Berg and Ewing, 1991). Methyl jasmonate and its deesterified derivative, jasmonate, act like phytohormones, and are known to be involved in mediating the wound response in potato leaves and in activating several wound-inducible defense-related genes (Ishikawa et al. 1994, Peña-Cortes et al. 1992). Kim et al. (1992) showed that a wound response element is located between -625 and -570 of the 5'-flanking region of the pinII gene and that a sequence which is essential for methyl jasmonate induction, designated the G-box, was identified on a pinII promoter from potato. Sucrose was also shown to mediate its stimulatory effect via a sucrose responsive region located between -570 and -500 on the pinII promoter (Kim et al. 1991). The G-box sequence was not required for sucrose induction, suggesting that the MJ induction mechanism was different from that of sucrose (Kim, et al. 1992).

**Fluorometric GUS assays of MJ induced pinII-GUS expression.** To determine the effect of MJ treatment on the induction of the pinII-GUS gene, leaf-petiole cuttings from three transgenic and one untransformed *S. brevidens* plant were treated with 50 μM MJ and analyzed at 12 h intervals up to 48 h. Treatment of the cuttings from all 3 transgenic plants
with MJ resulted in the induction of GUS activity in leaves after the 12 h incubation (Table 1), with a steady increase of activity through 24 or 48 h. Although each transformant had different basal levels of GUS expression before MJ incubation, overall GUS activities increased by 10- to 20- fold from original 0 h basal readings in all 3 transgenic plants (Table 1). Untransformed controls showed almost no increase in GUS activity. Xu et al. (1993) also reported that individual transgenic rice plants with a pinII-GUS construct had different basal levels of GUS activity and that GUS activity increased 10- to 15- fold in leaves 24 h after wounding. One of the reasons for the variation of GUS activity in each independent transformant may be a reflection of the position effect of transgene insertion (Peach and Velten 1991). It has been demonstrated that the local chromosomal environment at the insertion site can affect the expression of a transgene (Henikoff, 1990).

Table 1 Time course of the increase of GUS activity in leaves of transgenic *S. brevidens* leaf-petiole cuttings. Each cutting was treated with 50 μM MJ for 12, 24, or 48 h. Experiments were carried out with three independent transformants. Each experiment was repeated twice. GUS assays were performed as described in Materials and Methods. The total increase of MJ induced GUS activity was calculated by magnitude of increase from 0 h to 48 h.

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<th>Transgenic lines</th>
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Wound-inducible expression of the pinII-GUS gene in transgenic S. brevidens. Leaves from whole transgenic S. brevidens plants were mechanically wounded and subjected to both fluorometric and histochemical GUS assays for the induction of GUS enzyme activity both locally and systemically. Fig. 3 shows that GUS activity was induced 5- to 10- fold in the wounded leaves (L24h) of all three transgenic plants as well as nonwounded leaves on wounded plants 24 h after wounding (S24h). The level of visible GUS expression was also estimated according to histochemical results (Fig. 3). Even though leaves from transgenic plant no. 1 showed only faint levels of blue coloration, these leaves still exhibited a five- to six-fold increase of GUS activity in both locally wounded and systemically nonwounded leaves as measured by fluorometric assay. The blue coloration of GUS staining was not distributed evenly in wounded and nonwounded systemic leaves of transgenic S. brevidens with the pinII-GUS transgene. The pattern of staining appeared in different size sectors on the leaves of wounded plants with various intensities of blue color. The distribution of GUS staining in wounded leaves of transgenic potato plants containing a chimeric pinII-GUS gene was more homogenous with an accumulation of blue coloration along the veins in nonwounded systemic leaves (Keil, et al. 1989). Both our results and those of Keil et al. (1989) showed that the expression of the pinII-GUS transgene was associated with vascular tissue such as the stem and leaf veins. Keil et al. (1989) suggested that the signal which mediates the wound induction response is transported through vascular tissue. A proposed model for the signal transduction pathway that regulates both systemic and localized wound-induced expression of proteinase inhibitor genes was proposed by Ryan (1992). In this model, MJ would serve as a secondary messenger for induction through a mechanism involving an intracellular interaction
with a signal molecule that can be transported through the vascular tissue. Systemin, an 18-amino acid polypeptide, can readily move through the phloem (Pearce et al. 1991) to systemically activate proteinase inhibitor gene expression. Because systemin accumulation is induced by MJ (Farmer and Ryan, 1992), systemin could serve as the signaling molecule that is activated at the site of wounding or insect damage and transported to other plant organs to induce the systemic response.

In this study, we report that even though *S. brevidens* does not have a functional pinII gene present in its genome, the *S. brevidens* genome transformed with a chimeric pinII-GUS gene is still capable of responding to specific exogenous inducers and environmental changes in transformed *S. brevidens* plants. Transgenic expression in a nontuberizing *Solanum* species can be used for comparative induction studies of *S. tuberosum* genes and, clearly, is a practical model system for studying the regulation of potato gene expression.

**Acknowledgments**

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References


Fig. 1 Detection of the *uidA* gene in genomic DNA from leaves of transgenic plants by PCR analysis. (A) Ethidium bromide-stained PCR products separated on a 1% agarose gel. The primers used were specific to the *uidA* gene sequence. (B) Southern blot hybridization of the PCR products. The filter was hybridized with $^{32}$P-labeled GUS insert from pBI221. Template for the PCR came from genomic DNA extracted from transgenic plants no. 1, 2, and 3 (lanes 1, 2, 3, respectively) containing the pinII-GUS transgene. Positive control (lane P) template was the 2.2 kb *BamHI/EcoRI* GUS fragments from pBI221 and genomic DNA from an untransformed plant was used as a negative control (lane N).
Fig. 2 Northern blot hybridization of GUS mRNA accumulation in leaves of leaf-petiole cuttings of transgenic *S. brevidens* at 24 h after treatment with 50 μM MJ and 60 mM sucrose. Total RNA was extracted from transgenic plant no. 1 transformed with the pinII-GUS transgene. Leaf-petiole cuttings were harvested at the time treatments were initiated (0 h) or incubated in either 60 mM sucrose (SU), 50 μM MJ (MJ), or distilled water as a negative control (H₂O) for 24 h. Total RNA from a transgenic plant with CaMV-GUS transgene was extracted from leaf-petiole cuttings of 0 h or 24 h after 50 μM MJ treatment. The filter was hybridized with ³²P-labeled GUS insert from pBI221.
Fig. 3  Systemic and local induction of specific GUS activity in leaves of transformed *S. brevidens* plants 24 h after wounding. Fluorometric GUS assays were performed with three independent transformants no. 1, 2, and 3 (bars 1, 2, 3) and an untransformed *S. brevidens* plant as a negative control (bar N), for both systemic induction from nonwounded leaves (S24h) and local induction from directly wounded leaves (L24h). Each experiment was repeated twice. GUS assays were performed as described in Materials and Methods. Different levels of GUS were detected colorimetrically by histochemical analysis and scored according to blue coloration in the leaf tissue: (-) no blue color stain; (-/+) less than 10% blue color stain in total area of tested leaf samples; (+) 10-25% blue color stain; (++) 25-50% blue color stain.
CHAPTER 4. INDUCTION OF CATHEPSIN D INHIBITOR GENE EXPRESSION IN RESPONSE TO METHYL JASMONATE

A paper submitted to Physiologia Plantarum

T.-H. Annie Liu, David J. Hannapel and Loren C. Stephens


Abstract

Three plant species, tomato (Lycopersicon esculentum cv. Rutgers), potato (Solanum tuberosum cv. Superior) and a nontuberizing potato species (Solanum brevidens), were tested for methyl jasmonate induction of the potato cathepsin D inhibitor at the RNA level using a leaf-petiole cutting system. All three species had previously shown accumulation of cathepsin D inhibitor transcripts in leaves in response to wounding. Methyl jasmonate, a hormone-like, lipid-derived molecule, is considered to be a secondary messenger in wound signal transduction pathway and is a potent inducer of proteinase inhibitor II. A dose response test was performed for methyl jasmonate induction of cathepsin D inhibitor in the leaves of leaf-petiole cuttings. Transcript induction levels were directly related to the concentration of methyl jasmonate. Tomato had the highest level of RNA expression at 50 μM methyl
jasmonate, whereas the maximum expression level for both 'Superior' and *S. brevidens* was at 500 μM methyl jasmonate. Induction by methyl jasmonate was also dependent on the duration of exposure. Twelve hours of incubation were enough to produce abundant accumulation for all three species. Methyl jasmonate induction was light independent from 0 to 48 hours for both 'Superior' and *S. brevidens*. Cathepsin D inhibitor expression in the tomato leaf samples, however, was enhanced by light treatment. Leaf samples of 'Superior' and *S. brevidens* were also treated with 50 μM gibberellic acid either before or after methyl jasmonate treatment to observe the interaction of these two hormones. Gibberellic acid had no effect on the methyl jasmonate induction of cathepsin D inhibitor expression in either 'Superior' or *S. brevidens*. Immunoblot analysis showed cathepsin D inhibitor proteins were induced in 'Superior' leaves after methyl jasmonate treatment.

Key Words - cathepsin D inhibitor, gene expression, methyl jasmonate

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**Introduction**

Jasmonate (JA) and its methyl ester form, methyl jasmonate (MJ) have been identified in many plant species and have demonstrated hormone-like properties (Vick and Zimmerman 1984). Endogenous JA and MJ have a structure similar to that of prostaglandin in animals, and in plants are synthesized from linolenic acid by an oxidative pathway (Vick and Zimmerman
JA and its derivatives are known to have an effect on many physiological changes in plants such as leaf senescence and abscission, chlorophyll degradation, inhibition of photosynthesis, seed germination, induction of stomata closure, and tuber formation (Sembdner and Parther 1993). Exogenous treatments of MJ were reported to regulate the expression of several specific proteins such as serine proteinase inhibitor I and II from potato and tomato (Farmer et al. 1992, Hildmann et al. 1992, Peña-Cortés et al. 1992). The accumulation of these proteinase inhibitors is developmentally regulated in growing tubers and environmentally regulated in response to wounding in leaves (Sanchez-Serrano et al. 1986). Proteinase inhibitor genes are not only activated at the wound site in leaves, but are also systemically activated in nonwounded leaves of wounded plants (Peña-Cortés et al. 1988). Since MJ can mimic both wound responses in the induction of proteinase inhibitor genes in potato and tomato leaves (Farmer et al. 1992, Hildmann et al. 1992), it has been proposed that both JA and MJ act as secondary messengers in the wound signal transduction pathway (Ryan 1992).

The potato cathepsin D inhibitor (CDI) is a member of the complex family of 22 kD proteinase inhibitors (Mareš et al. 1989) which inhibits both cathepsin D and trypsin activity. Like proteinase inhibitor I and II, CDI is wound-inducible in potato leaves both locally and systemically (Suh et al. 1991). CDI also functions as one of the major tuber storage proteins and accumulates in developing potato tubers and can be detected in stem and root tissue depending on genotype and stage of development (Hannapel 1991). Hansen and Hannapel (1992) showed that the DNA sequences homologous to a potato CDI cDNA are present in nontuberizing potato species such as Solanum brevidens and S. etuberosum and in tomato
(Lycopersicon esculentum). These heterologous CDI genes were wound-inducible for all solanaceous species assayed (Hildmann et al. 1992, Hansen and Hannapel 1992), but only the S. tuberosum CDI types were sucrose-inducible (Hansen and Hannapel 1992). This study reports the effect of a proposed secondary messenger, MJ, on the induction of CDI genes that are differentially expressed in tuberizing and nontuberizing solanaceous species.

Abbreviations- Cathepsin D inhibitor, CDI; Gibberellic acid, GA₃; jasmonate, JA; methyl jasmonate, MJ; proteinase inhibitor II, pinII

Materials and Methods

Plant Material

Potato plants (Solanum tuberosum cv. Superior) were grown from certified seed tubers obtained from Tatro Potato Seed Farms, Antigo, WI. All plants were grown under standard greenhouse conditions with a long-day photoperiod. The nontuberizing potato species (Solanum brevidens) was grown from seeds obtained from the Potato Introduction Station, Sturgeon Bay, WI and germinated under aseptic conditions and maintained on MS medium (Murashige and Skoog 1962) containing 3% sucrose and 1.5% gelrite. Plants were then transplanted to the greenhouse and maintained as stock plants. Tomato seeds (Lycopersicon esculentum cv. Rutgers, Excel Seed Co. Dowers Grove, IL, USA) were germinated and grown in the greenhouse.
MJ and GA₃ treatments

Leaf-petiole cuttings from 6-8 week old potato, tomato and S. brevidens plants were used for methyl jasmonate (Bedoukian Industries, Danbury, CT, USA) and gibberellic acid (GA₃) treatments. Leaves of potato cv. Superior, tomato cv. Rutgers and S. brevidens were detached from the plants by cutting the petiole close to the stem and placing the cut ends in 300 ml water containing either MJ or GA₃. Both MJ and GA₃ were dissolved in 95% ethanol as stock solutions, and diluted in sterile deionized water to make final concentrations of 0.5, 5, 50, or 500 μM for MJ and 50 μM for GA₃. Controls received an equivalent concentration of ethanol. Each treatment contained 5 leaf-petiole cuttings. The time-course treatments were kept inside a negative pressure fume hood at 25°C with or without light, up to 48 hours. All other treatments were incubated under the same conditions under constant light for 24 hours. The MJ treatments were kept separate from other treatments to prevent exposure to volatile MJ derivatives. After the incubation periods, leaf samples from the treatments were harvested, frozen in liquid nitrogen, and stored at -80°C until analyzed.

Nucleic Acid Analysis

Total leaf RNA was isolated by the phenol/chloroform method described by Suh et al. (1991). For northern blots, RNA was subjected to electrophoresis in 1.4% agarose gels containing 5 mM methylmercuric hydroxide (Alfa Products, Danvers, MA, USA) in tris-borate buffer (pH 7.5) and blotted onto nylon membranes. Each sample was loaded on duplicate gels to check hybridization results. Total RNA was detected with ethidium bromide under UV light to ascertain consistent loading of gels and efficient transfer to nylon. Blot filters were hybridized
with a gel-purified, $^{32}$P-labelled insert (800 nucleotides in length) from the cathepsin D inhibitor cDNA, p749 (Hannapel 1993), which has 94% homology to an aspartic proteinase inhibitor cDNA (Štrukelj et al. 1990), representing a tuber storage protein with a molecular mass in its mature form of approximately 22 kD. The deduced amino acid sequence of p749 has 92% homology to a purified potato CDI (Mares et al. 1989). RNA blots were hybridized in 50% (v/v) formamide, 5X SSC, 0.07% polyvinylpyrrolidone, 0.07% Ficoll, 0.07% bovine serum albumin, 25 mM sodium phosphate (pH 6.8), 0.5% SDS, and 250 μl/ml sheared salmon sperm DNA. After hybridization for 16 hours at 42°C, blots were washed once with 1X SSC/0.1% SDS at room temperature for 15 min. Filters were then washed twice in 0.1X SSC/0.1% SDS at room temperature and 65°C for 30 min each. Hybridized transcripts were detected by exposure of the filters to X-OMAT AR film for 12 to 17 hours.

**Immunoblotting**

Total protein from 'Superior' and *S. brevidens* leaf extracts were determined by Bradford assay (Bradford 1976) standardized with bovine serum albumin. Gel electrophoresis was carried out using a 0.75 mm, 12% SDS-polyacrylamide gel and run for 60 min at 150 V. The gel was rinsed in transfer buffer (25 mM tris, 192 mM glycine in 20% methanol, pH 8.3) and electroblotted onto a nitrocellulose membrane (BioRad, Hercules, CA. USA) using a semi-dry electroblotting apparatus (BioRad, Hercules, CA. USA), at 10 V for 30 min. The first antibody produced in rabbit against potato cathepsin D inhibitor was diluted 1:200 in TTBS buffer (20 mM tris, 500 mM NaCl, and 0.05% Tween-20). The secondary antibody consisted of a goat anti-rabbit IgG-horseradish peroxidase conjugate and was used in a concentration of
1:3000. The blots were stained in 0.05% 4-chloro-1-naphthol in 4 ml of ice cold methanol, plus 0.015% hydrogen peroxide in 20 ml TBS buffer (20 mM tris and 500 mM NaCl). Prestained molecular weight markers were used to estimate the size of visible bands.

Results

Effect of MJ concentration on mRNA accumulation in leaves.

Leaf-petiole cuttings from potato 'Superior', S. brevidens and tomato 'Rutgers' were treated with various concentrations of MJ (0, 0.5, 5, 50 and 500 μM) to determine the optimum concentration for the induction of CDI. Cathepsin D inhibitor mRNA accumulated in leaves from all 3 plant species in response to MJ (Fig. 1). Induction was directly related to the concentration of MJ for both 'Superior' and S. brevidens with the highest levels of transcripts occurring in response to 500 μM and the lowest levels to 0.5 μM MJ. 'Superior' was the most sensitive of the three with detectable transcript levels beginning at 0.5 μM MJ and substantial inductions at 5 μM MJ. For S. brevidens and tomato 'Rutgers', the accumulation of CDI transcripts can be first detected only at 5 μM MJ. Accumulation of tomato CDI transcripts occurred at the lowest levels of any of the three species and does not appear to be dependent on MJ dosage. Using a potato CDI probe to detect tomato CDI transcripts may also result in a lower signal (Hannapel 1993). Based on previous studies (Hildmann et al. 1992, Ishikawa et al. 1994b), and because of the relatively high level of transcript accumulation in both potato 'Superior' and tomato 'Rutgers' leaves in response to the 50 μM MJ treatment, this MJ concentration was used for all subsequent experiments. For S. brevidens, 500 μM MJ
treatments were used for all subsequent experiments. The ethanol-treated controls showed no induction of CDI transcript accumulation.

**Effect of time course of MJ treatment in leaves.**

To determine the effect of the length of time of MJ treatment on the induction of CDI transcripts, leaf-petiole cuttings of potato 'Superior', *S. brevidens* and tomato 'Rutgers' were incubated in sterile deionized water with either 50 μM ('Superior' and 'Rutgers') or 500 μM (*S. brevidens*) MJ for up to 48 h. The control treatments to which no MJ was added showed that CDI transcripts were not detected for *S. brevidens* and tomato 'Rutgers' even after 48 h incubation. For potato 'Superior', a slight induction occurred after the 48 h incubation (Fig. 2A). A detectable level of CDI mRNA accumulation after 6 h of MJ treatment was present for all three species (Fig. 2B). Twelve hours of MJ treatment were enough to give relatively abundant accumulation for all three species. Transcript levels reached their maximum at 24 h and then decreased for 'Superior'. For *S. brevidens*, the rapid accumulation of transcripts was slower than for 'Superior', reaching the highest level after 48 h. The slowest rate of CDI transcript accumulation occurred in the tomato 'Rutgers' leaves (Fig. 2B).

**Light/dark effect on MJ induction.**

To investigate whether light plays a role in the induction of CDI expression by MJ, leaf-petiole cuttings were incubated for up to 48 h in darkness. By comparing Fig. 2B and Fig. 3, it is evident that MJ induction is independent of light/dark effects in potato 'Superior' and *S. brevidens*. In the dark, transcript levels induced by MJ treatment are equivalent to the levels
induced under light for both *Solanum* species. For tomato, however, no CDI transcript induction is detectable under the dark conditions.

**GA interaction with MJ induction.**

To study the dynamics of any potential effect of GA$_3$ on MJ induction, both a pre- and posttreatment of GA$_3$ were used. Regardless of the timing of the GA$_3$ treatment, there was no effect of GA$_3$ on induction by MJ of CDI transcript accumulation for either species (Fig. 4, compare lanes G/M and M/G to lane M). Both control treatments showed no CDI transcript induction (lanes C and G). As expected, both *Solanum* species showed a strong induction after 24 h MJ treatment (Fig. 4, lane M). These results indicate that GA$_3$ does not interact with MJ induction of CDI gene expression in this leaf-petiole cutting system.

**Immunoblotting analysis of MJ treatment in leaves.**

To examine whether the level of protein accumulation was consistent with transcript accumulation, immunoblot analyses were performed on MJ treated 'Superior' leaf extracts. Because CDI bands were barely detectable on the immunoblot after 48 h treatments, MJ treatment of the leaf-petiole cuttings was extended to 72 and 96 h (Fig. 5). Antibody specific to the tuber CDI protein reacted with three bands in total tuber protein extract (Fig. 5, lane Tu) and two bands in 72 and 96 h MJ treated 'Superior' leaf samples (Fig. 5). The tuber forms are approximately 20, 21, and 22 kilodaltons whereas the leaf forms are approximately 20 and 22 kilodaltons. The estimated size of CDI protein ranges from 20.5 to 22 kilodaltons (Mareš et al. 1989, Ritonja et al. 1990, Rupova et al. 1977). High levels of CDI transcripts
accumulate as early as 12 h after MJ treatment (Fig. 2) but Fig. 5 shows that the CDI protein is not detectable until 48 h. For the *S. brevidens* immunoblot, the pattern of CDI protein accumulation was similar to that of 'Superior' (data not shown). These results show that exogenous treatments of MJ can induce the accumulation of CDI proteins in leaves of a leaf-petiole cutting system.

**Discussion**

Like other potato proteinase inhibitors, CDI serves a dual role during potato development as a storage protein accumulating to very high levels in the potato tuber (Hannapel 1991), and as a part of the plant defense system against invading pests that feed on the leaves (Johnson et al. 1990). CDI gene expression in potato tubers is developmentally regulated as accumulation of CDI mRNA is correlated with the growth of the tuber (Hannapel 1991). In leaves, CDI expression can be induced by various factors such as sucrose (Hansen and Hannapel 1992), chitosan (Hansen and Hannapel 1992), wounding (Hildmann et al. 1992, Ishikawa et al. 1994a, Suh et al. 1991) and MJ (Hildmann et al. 1992, Ishikawa et al. 1994b). There are reports that homologs of the potato proteinase inhibitors, including CDI proteins, are widespread among plant species, particularly those in the Solanaceae family (Hannapel 1993, Hansen and Hannapel 1992, Werner et al. 1993). There is evidence, however, showing that patterns of expression for these proteinase inhibitors may differ among species. A CDI gene expressed in tomato 'Rutgers' and a nontuberizing potato species *S. brevidens* were induced by chitosan treatment and wounding but not by 300 mM sucrose treatments (Hansen and Hannapel 1992).
Here we test the effect of MJ treatments on this same set of solanaceous species. MJ and its deesterified derivative, JA, are known to be involved in mediating the wound response in potato leaves and in activating the expression of several wound-induced defense-related genes. Our results showed that the CDI genes are induced by MJ at the RNA level in the leaf-petiole cuttings of potato 'Superior', S. brevidens and tomato 'Rutgers', although we found that each plant species responded differently to various MJ concentrations, incubation time, and photoperiod treatments. Among these three species, S. tuberosum 'Superior' appears the most sensitive, reaching higher levels of induction more rapidly and responding to a lower MJ concentration than either S. brevidens or 'Rutgers'. Since it has been previously established that the CDI genes from S. brevidens and 'Rutgers' are expressed differently than those from 'Superior', it is not surprising that there is a difference in MJ sensitivity. All of the northern hybridizations of tomato total RNA showed a lower degree of hybridization to p749, probably because of probe mismatch as the probe used was isolated from a potato 'Superior' tuber library (Hannapel 1993). Werner et al. (1993) found that the deduced amino acid sequence of a tomato CDI gene is only 80% homologous to the sequence of potato p749 protein. The results reported here for MJ concentration and time course were consistent with those of Ishikawa et al. (1994b) using a detached potato 'Danshaku' leaf-petiole system. In their study, the CDI gene had 92% DNA sequence homology to p749 but was not sucrose inducible (Ishikawa et al. 1994a). However, Ishikawa et al. (1994b) reported no CDI transcript accumulation at 0.5 μM MJ. One explanation for the different results of the two studies could be genotype: the potato cultivar Superior may be more sensitive to MJ treatment than the cultivar Danshaku.
It has been previously shown that light enhances the sucrose induction of pinII genes in detached potato leaves (Peña-Cortés et al. 1992), but sucrose induction has been shown to be independent of light for CDI expression (Hansen and Hannapel 1992). We found that MJ induced CDI expression was independent of light effects in both Solanum species. This result was similar to MJ induction of pinII expression in detached potato leaves (Peña-Cortés et al. 1992). For tomato, in contrast to Solanum species, the level of CDI transcripts was reduced in response to dark (Fig. 2B and Fig. 3). Bolter (1993) reported that a papain inhibitor of tomato that was induced by MJ had significantly lower levels of inhibitory activity in the dark than in the light. These results imply that illumination may play an role in the MJ induction of some proteinase inhibitor genes of tomato.

Because both JA and GA$_3$ are involved in regulating tuber formation (Ewing 1990, Koda et al. 1991), the possibility exists that these two hormones could interact. JA has been shown to induce tuber formation and inhibit growth stimulated by GA$_3$ in dwarf rice and lettuce seedlings (Yamane et al. 1981). GA$_3$ has a well-documented history as a potent inhibitor of potato tuberization (Ewing 1990). GA$_3$ can block the chitosan induction of the proteinase inhibitor II (pinII) gene in detached potato leaves (Peña-Cortés et al. 1989). Previous research has shown that GA$_3$ inhibited mRNA accumulation induced by 150 mM sucrose for patatin, pinII and CDI genes in the detached petiole leaf system of potato 'Superior' (Hannapel, unpublished data). Our results show that GA$_3$ has no effect on MJ induction of CDI expression. GA$_3$'s lack of effect on MJ induction of CDI expression supports the proposed idea that MJ acts as a secondary messenger in signal transduction pathway independent of other hormones. Hildmann et al. (1992) suggested that MJ or JA
may serve as mediators that bypass the preliminary recognition steps requiring a hormone, and then trigger the induction of genes in the absence of the hormone. They found that JA strongly induced pinII, CDI and other wound-responsive genes in both wild-type and ABA-deficient mutant tomato. Exogenous treatments of MJ may supersede the effects of hormones like GA3 and ABA and activate gene expression downstream on the signal transduction pathway.

In the last part of the study, we confirmed that the CDI protein accumulates in response to MJ treatment. The CDI antibody reacts with three protein forms in the potato tuber extract and two forms in leaf samples. There are two possible explanations; (i) the different size bands could represent isoforms which belong to a multigenic family. Such isoforms were observed during the characterization of the 22-kD potato Kunitz-type inhibitor (Suh et al. 1990, Stiekema et al. 1988). Mares et al. (1991) reported two closely related cathepsin D isoinhibitors isolated from potato tuber. (ii) The different size bands could represent products of the same gene which are modified posttranslationally, either by glycosylation or cleavage of the signal peptides. Using transgenic tobacco, Nakamura et al. (1993) studied the processing of a sweet potato storage protein, sporamin, which has 35% sequence homology with potato CDI. Transport of prosoporamin, a higher molecular weight precursor of sporamin, from the ER to the vacuole occurs via the Golgi apparatus. Posttranslational cleavage of prosoporamin's signal peptide occurs during this transport. Nakamura et al. (1993) identified the same vacuole targeting motif in the signal peptide of both sporamin and CDI. The higher molecular weight CDI bands detected in the immunoblot analysis may represent precursor forms of the protein with the signal peptide intact. A similar
mechanism of posttranslational modification has been identified for the Kunitz-type potato proteinase inhibitor (Suh et al. 1995).

Previous studies indicated that the S. *tuberosum* CDI gene's pattern of expression is distinct from those of *S. brevidens* and tomato (Hansen and Hannapel 1992). Here we report that the CDI gene in each plant species is responsive to MJ induction but that there are differences in sensitivity among these species. The response of the *S. brevidens* CDI (a wound-inducible only type of CDI) to MJ treatment clearly shows that MJ can mimic leaf wound induction, strengthening the proposition that MJ acts as a secondary messenger in the signal transduction pathway of the wound response.

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**References**


Fig. 1. Northern blot analysis of CDI mRNA in leaves of potato 'Superior', *S. brevidens* and tomato 'Rutgers' after treatment with various concentrations of MJ. Leaf-petiole cuttings of these 3 species were treated for 24 h under constant light at 25°C. Lanes 0, 0.5, 5, 50, 500 indicate the concentration (μM) of MJ. Two micrograms of total RNA from *Solanum tuberosum* cv. Superior tubers (lane Tu) were used as a positive control. Ten micrograms of total RNA were loaded in all other lanes. The filter was hybridized with $^{32}$P-labeled insert from the potato CDI cDNA, p749 (Hannapel 1993). Sup: *S. tuberosum* cv. Superior, Bv: *S. brevidens*, Tm: *Lycopersicon esculentum* cv. Rutgers.
Fig. 2. Northern blot analysis of CDI mRNA in leaves of potato 'Superior', *S. brevidens* and tomato 'Rutgers' at various time courses after treatment with MJ. Leaf-petiole cuttings of these 3 species were treated (A) without MJ or (B) with 50 μM MJ (potato 'Superior' and tomato 'Rutgers') or 500 μM MJ (*S. brevidens*) from 0 to 48 h under light at 25°C. Lanes 0, 6, 12, 24, 48 indicate the incubation time of MJ treatment. Two micrograms of total RNA from *Solanum tuberosum* cv. Superior tubers (lane Tu) were used as a positive control. Ten micrograms of total RNA were loaded in all other lanes. The filter was hybridized with $^{32}$P-labeled insert from the potato CDI cDNA, p749 (Hannapel 1993). Su: *S. tuberosum* cv. Superior, Bv: *S. brevidens*, Tm: *Lycopersicon esculentum* cv. Rutgers.
Fig. 3. Northern blot analysis of CDI mRNA in leaves of potato 'Superior', *S. brevidens* and tomato 'Rutgers' at various time courses after treatment with MJ in the dark. Leaf-petiole cuttings of these 3 species were treated with 50 μM MJ (potato 'Superior' and tomato 'Rutgers') or 500 μM MJ (*S. brevidens*) from 0 to 48 h under light at 25°C. Lanes 0, 6, 12, 24, 48 indicate the incubation time of MJ treatment. Two micrograms of total RNA from *Solanum tuberosum* cv. Superior tubers (lane Tu) were used as a positive control. Ten micrograms of total RNA were loaded in all other lanes. The filter was hybridized with 32P-labeled insert from the potato CDI cDNA, p749 (Hannapel 1993). Sup: *S. tuberosum* cv. Superior, Bv: *S. brevidens*, Tm: *Lycopersicon esculentum* cv. Rutgers.
Fig. 4. Northern blot analysis of CDI mRNA accumulation in leaves of potato 'Superior' and *S. brevidens* at 24 h after treatment with 50 \( \mu \)M MJ under light. Control leaves (lane C) were incubated in sterile deionized water without any hormone for 24 h. Leaf samples were treated with either a pre- or posttreatment of 50 \( \mu \)M GA\(_3\). The pretreatment consisted of 50 \( \mu \)M GA\(_3\) for 6 h, then 18 h with 50 \( \mu \)M MJ (lane G/M). The posttreatment consisted of 50 \( \mu \)M MJ for 6 h, then 18 h with 50 \( \mu \)M GA\(_3\) (lane M/G). Leaf samples were treated with sterile deionized water for 18 h after an initial 6 h of incubation in either 50 \( \mu \)M GA\(_3\) (lane G) or 50 \( \mu \)M MJ (lane M). Two micrograms of total RNA from *Solanum tuberosum* cv. Superior tubers (lane Tu) were used as a positive control. Ten micrograms of total mRNA were loaded in all other lanes. The filter was hybridized with \(^{32}\)P-labeled insert from the potato CDI cDNA, p749 (Hannapel 1993). Su: *S. tuberosum* cv. Superior, Bv: *S. brevidens*. 
Fig. 5. Immunoblot analysis of the CDI proteins induced in leaves of potato 'Superior' at various time courses after MJ treatment. Leaf-petiole cuttings of 'Superior' were treated with 50 μM MJ from 0 to 96 h under light at 25°C. Lanes 0, 6, 12, 24, 48, 72, and 96 indicate the incubation time of MJ treatment. Lane Tu indicates proteins from 100-day field grown tuber as a positive control. Forty micrograms of total leaf protein were loaded in each lane. Polyclonal antibody specific to purified potato tuber CDI protein was used for immunodetection. Molecular weight markers are indicated in the far left lane (KD).
CHAPTER 5. GENERAL CONCLUSION

Many genes involved in potato tuber development have been isolated and the function of these genes has been identified. Most of these studies were concentrated on genes from potato *Solanum tuberosum*. The major objectives of this research were to compare the expression of potato genes in different solanaceous species with the expression patterns of *S. tuberosum*.

The first part of this study was to establish a dependable transformation system in a nontuberizing potato species. *Solanum brevidens* was chosen for this study because it is a nontuberizing species closely related to *S. tuberosum*, and contains valuable germplasm with utility in a potato breeding program (Jones et al. 1990). An *Agrobacterium*-mediated transformation system for a nontuberizing wild potato species *Solanum brevidens* was established. Two types of culture methods and two strains of *Agrobacterium tumefaciens* were compared to find the optimum transformation conditions for *S. brevidens*. Transformation rates were 7% in liquid culture and 3% on solidified medium. However, liquid medium also slowed down the shoot regeneration process about three weeks. *Agrobacterium* strain GV2260 resulted in an 11% transformation frequency in comparison with 1% using LBA4404. Twenty-two out of 24 kanamycin-resistant putative transformed plants were confirmed transgenic by fluorometric GUS assays. Southern analysis of randomly selected transgenic plants showed that each contained at least one copy of the GUS gene. Progeny analysis on a transgenic plant with one copy of the GUS gene showed that the gene was stably incorporated into the plant genome and segregated in a 3:1 Mendelian ratio.
To determine whether a potato gene can be activated in this *S. brevidens* transformation system, a potato proteinase inhibitor II gene (pinII) was selected as a model gene. There are three reasons to use the pinII gene: (i) although *S. brevidens* has DNA sequences which are homologous to pinII, there is no evidence showing these sequences are functional (Hannapel, unpublished data). (ii) pinII is involved in both tuber-developing and plant defense mechanism (Lorberth et al. 1992). (iii) The pinII gene has been studied extensively and considerable information is available (Peña-Cortes et al. 1992). A plasmid construct with a potato proteinase inhibitor II (pinII) promoter and a GUS gene was introduced into *Agrobacterium* strain GV2260 and then subsequently co-cultivated with leaf pieces of *S. brevidens*. Three transformed plants out of eight putative kanamycin-resistant plantlets were confirmed to be transgenic by the polymerase chain reaction (PCR) using GUS specific primers. The transgenic plants were subjected to methyl jasmonate, and sucrose treatment and wounding and sucrose which was known to activate the pinII promoter. Northern blot analysis showed that the pinII-GUS chimeric gene can be induced by methyl jasmonate and sucrose. The fluorometric GUS activity was 10- to 20-fold higher than the control with methyl jasmonate treatment for 48 h. Wounding of a leaf from transgenic *S. brevidens* also led to a high expression of GUS activity in leaves from a whole plant, both locally and systemically. These results were consistent with previous studies on potato pinII expression. This model system could be used to study other genes associated with tuber development.

Another approach for studying gene expression is using homologous genes present in different plant species. DNA sequences homologous to a potato cathepsin D inhibitor were
present in both nontuberizing species *Solanum brevidens* and *Lycopersicon esculentum* (Hansen and Hannapel, 1992). All three species had previously shown accumulation of cathepsin D inhibitor transcripts in leaves in response to wounding (Hansen and Hannapel, 1992). Since methyl jasmonate is considered to be a secondary messenger in the wounding signal transduction pathway and is a potent inducer of proteinase inhibitor II, exogenous methyl jasmonate was used for induction experiments in this part of the research. All methyl jasmonate induction treatments were performed using potato *S. tuberosum*, *S. brevidens* and tomato in a leaf-petiole cutting system. Transcript accumulation was directly related to the concentration and duration of exposure of methyl jasmonate for *S. tuberosum* and *S. brevidens*. Methyl jasmonate induction was light independent for both *S. tuberosum* and *S. brevidens*. For the tomato leaf samples, cathepsin D inhibitor transcript accumulation was enhanced by light. Gibberellic acid, a potent inhibitor of potato tuber formation, had no effect on methyl jasmonate induction in either *S. tuberosum* or *S. brevidens*. Immunoblot analysis showed cathepsin D inhibitor protein was induced by methyl jasmonate in *S. tuberosum* and *S. brevidens* after a 48 h incubation.

In summary, a transformation system established in *S. brevidens* can be a model to study potato tuber-related genes which are not functional or present in a nontuberizing *Solanum* species. The leaf-petiole cutting system with blot hybridization can be used for functional homologous gene expression analysis among related species. Combining these two experimental approaches, provides the means for a broad study of potato gene expression.


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