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J. Plant Physiol. 161. 621–629 (2004)
http://www.elsevier.de/jplhp

JOURNAL OF PLANT PHYSIOLOGY

Tobacco PR-2d promoter is induced in transgenic cucumber in response to biotic and abiotic stimuli

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Received December 20, 2002 · Accepted June 4, 2003

Summary

The PR-2d promoter/*uidA* (GUS) gene construct was introduced into the cucumber (*Cucumis sativus* L.) genome and several transgenic lines were produced. Activation of the PR-2d promoter was investigated in these plants in response to inoculation with fungal pathogens and after salicylic acid (SA) or cold treatments. Treatment with exogenous SA increased GUS activity 2 to 11 fold over that of the control. Endogenous SA and its conjugate salicylic acid glucoside (SAG) rose in parallel after inoculation with the fungal pathogen *Pseudoperonospora cubensis*, with SAG becoming the predominant form. The free SA levels increased 15 fold above the basal level at 5 dpi and preceded the induction of the PR-2d promoter by five days, which occurred at 10 dpi with a 12 fold increase over the control. Inoculation with another fungal pathogen, *Erysiphe polyphage*, increased GUS activity 4 to 44 fold over that of the control. During normal development of flowers in the cucumber, the PR-2d/*uidA* gene expressed in the floral organs was similar to that of the primary host. In addition, we present the first evidence that the PR-2d promoter was induced (624 fold) under cold stress. We demonstrate that in the heterologous state the gene construct was expressed according to the signalling pattern of the native species and was stably transmitted to progeny over four generations.

Key words: Cold – *Cucumis sativus* L. (transgenic) – Pathogen – PR-2d promoter – Salicylic acid – Salicylic acid glucoside

Abbreviations: dpi = days post inoculation. – GUS = β-glucuronidase. – MS = Murashige and Skoog. – PR = pathogenesis related. – SA = salicylic acid. – SAG = salicylic acid glucoside. – *uidA* = β-glucuronidase gene

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0176-1617/04/161/05-621 $ 30.00/0

Introduction

Earlier findings have demonstrated that salicylic acid (SA) functions as an endogenous transduction signal (Malamy et al. 1990, Métraux et al. 1990). Its synthesis and accumulation are important requirements for the activation of several plant defence responses, including expression of a diverse group of defence-related genes (Klessig and Malamy 1994, Durner et al. 1997). Low concentrations of endogenous SA is sufficient for the induction of pathogenesis-related (PR) proteins (Malamy et al. 1990, Yalpani et al. 1991, Enyedi et al. 1992, Klessig and Malamy 1994). Moreover, *in vivo* labelling studies indicated that SA served as the systemic signal that translocated over long distances from the inoculated leaf to upper uninoculated leaves (Meuwly et al. 1995, Shulaev et al. 1995, Mölders et al. 1996).

Application of exogenous SA induces a range of defence genes, many of which encode PR proteins including acidic isoforms of PR proteins in healthy tobacco leaves (Ward et al. 1991, Klessig and Malamy 1994). Some of the PR proteins are hydrolytic enzymes, such as β-1,3-glucanase (Kauffmann et al. 1987, Meins and Ahl 1989, Stintzi et al. 1993). Most of these proteins have the ability to enhance disease resistance when over-expressed in transgenic plants (Ryals et al. 1996, Wobbe and Klessig 1996).

Previously, the promoter of PR-2d, a β-1,3-glucanase gene of *Nicotiana tabacum*, was well characterized by Hennig and co-workers (1993). These authors demonstrated that the *cis*-acting elements necessary for induction by SA, tobacco mosaic virus (TMV), and developmental signals were localized in the region between −321 bp and −607 bp upstream of the transcription start site. Later, Shah and Klessig (1996) reported that the sequences from 364 to 288 bp of the same promoter conferred high level activation by SA in transgenic tobacco plants. Since cucumber has been a useful model system to study the action of SA and SAG, we explored the possibility of creating a plant equipped with a reporter gene system that might be helpful for future investigations. We produced transgenic cucumber plants bearing a reporter system consisting of the PR-2d (-1706) promoter fused to the *uidA* gene (GUS).

Materials and Methods

Cucumber transformation

The PR-2d (-1706)/*uidA* gene construct (Hennig et al. 1993) was introduced into a highly inbred line of *C. sativus* L. cv. Borszczagowski via *Agrobacterium tumefaciens* strain LBA4404/pGA482 (An 1986) using a previously described method (Szwacka et al. 1996). The plantlets (designated T_0 generation) regenerated from the kanamycin resistant calli were transferred into the soil and cultivated under the greenhouse conditions until seed set (Burza and Malepszy 1995).

Plant material

The primary transgenic cucumber plants bearing the PR-2d (-1706)/*uidA* gene construct and their subsequent self-pollinated generations were used. The non-transgenic cucumber plants of Borszczagowski were used as a control. Cucumber seeds were germinated on an MS medium (Murashige and Skoog 1962) supplemented with 200 mg/L of kanamycin. The kanamycin-resistant plants were used in the further experiments. The seed population that showed no segregation for kanamycin resistance was regarded as the homozygous line, while the population that segregated was regarded as the heterozygous line. All of the plant materials were cultivated under the greenhouse conditions as described by Malepszy (1988). The transgenic line refers to the sexual progeny of one transgenic plant. None of the lines used in this study showed growth or morphological abnormalities.

DNA isolation and Southern hybridization analysis

Total genomic DNA was extracted from leaf tissues of T_0, T_1, and T_2 plants according to Dellaporta et al. (1983). DNA (10 µg) was digested with *Hind*III or *Hind*III/*Eco*RI and Southern blot analysis was carried out as described previously (Koetsier et al. 1993). A ^{32}P-labeled *uidA* fragment (1795 bp) amplified by PCR using specific primers was used as a probe.

Exogenous SA application

T_1, T_2, T_3, and T_4 generations of four primary transformants were used for GUS assay following treatment with SA. Leaf discs were punched out from young leaves of 4-week-old plants and floated in a Petri dish on water or 1 mmol/L SA, pH 6.7. Leaf samples were collected at time 0 and 24 hours after treatment and were stored at −80 °C for GUS fluorometric measurements.

Fungal inoculations

Induction of PR-2d and SA were determined after inoculation with the fungal pathogens *P. cubensis* and *E. polyphage*. Two transgenic lines in the T_3 and T_4 generation were inoculated *in vivo* with a suspension of *P. cubensis* spores in water and kept in highly humid conditions. For mock infection, plants were sprayed with water. Leaf samples were taken at 0, 5, 7, and 10 dpi and stored at −80 °C for GUS fluorometric measurements and determination of SA level.

Three T_2 transgenic lines were inoculated *in vivo* with a suspension of *E. polyphage* spores in water and kept six hours in highly humid conditions. Non-inoculated plants were used as a control. Leaf samples were taken at 5, 7, and 10 dpi and stored at −80 °C for GUS fluorometric determination.

Cold treatment

Four-week-old plants of two transgenic T_3 lines were kept at 14 °C in a cold chamber for 48 hours and then maintained at 25 °C (16 h/8 h light/dark regime). Leaf discs were collected at 0, 5, and 7 days after treatment and stored at −80 °C for GUS fluorometric measurements.

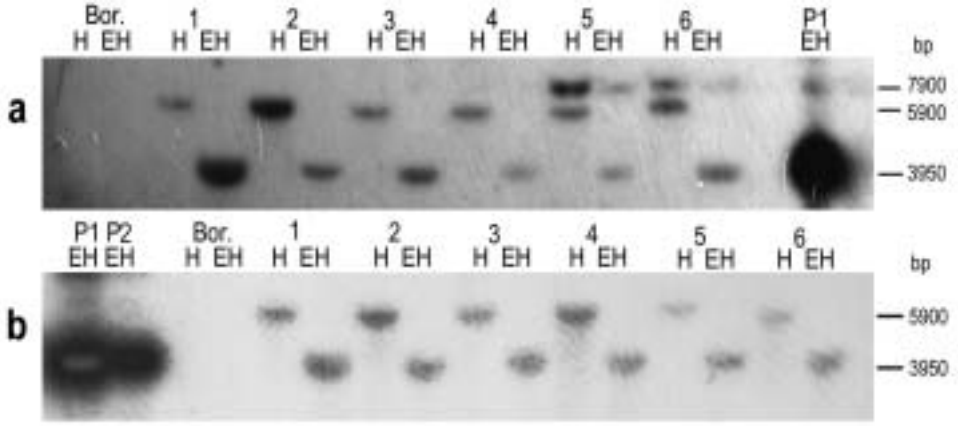

Figure 1. Southern blot hybridization of *uidA* gene in T_0, T_1 and T_2 plants bearing a R-2d/*uidA* gene fusion. 10 µg of genomic DNA was digested with *Hind*III (H) or with *Hind*III/*Eco*RI (EH). Electrophoresis was carried out in 0.8 % agarose gel. The gel was blotted on a nylon membrane under alkaline conditions and cross-linked by UV exposure. Hybridization was carried out using a ^{32}P-labeled *uidA* fragment (1795 bp) amplified by PCR using specific primers. P1 and P2: 200 ng and 20 ng of plasmid DNA pGA482 containing PR-2d/*uidA* construct, respectively. Bor.: Genomic DNA of non-transgenic Borszczagowski plants. a 1–6: Genomic DNA of T_0 plants TC2, TC3, TC4, TC5, TC6, TC7, respectively. b 1–3: Genomic DNA of T_2 plants TC3/26/3, TC4/11/3, TC5/12/8, respectively. b 4–6: Genomic DNA of T_1 plants TC3/26, TC4/11, TC5/12, respectively.

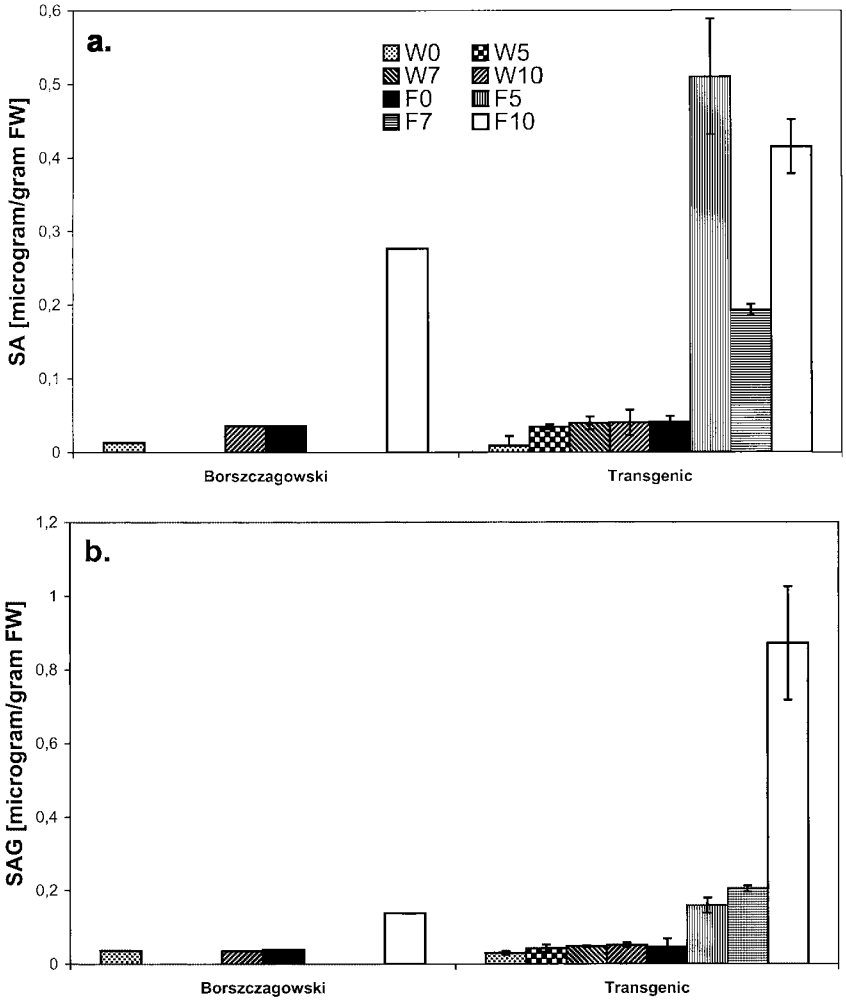

Figure 2. Accumulation of SA and SAG in *P. cubensis* inoculated leaves of transgenic and non-transgenic cucumber plants. Salicylic acid (SA) and salicylic acid glucoside (SAG) levels were determined in the fungus- (F) or mock-inoculated (W) leaves of transgenic (TC3/26/3-T_4) and non-transgenic control (Borszczagowski) plants. Leaf samples were taken at 0 (immediately following inoculation), 5, 7 and 10 days after fungal (*P. cubensis*) inoculation (F0, F5, F7, and F10, respectively) or water treatment (W0, W5, W7 and W10, respectively). Results are the mean calculated from two plants for transgenic and one plant for Borszczagowski. Data for control at 5 and 7 dpi are not shown. Error bars represent the standard deviations.

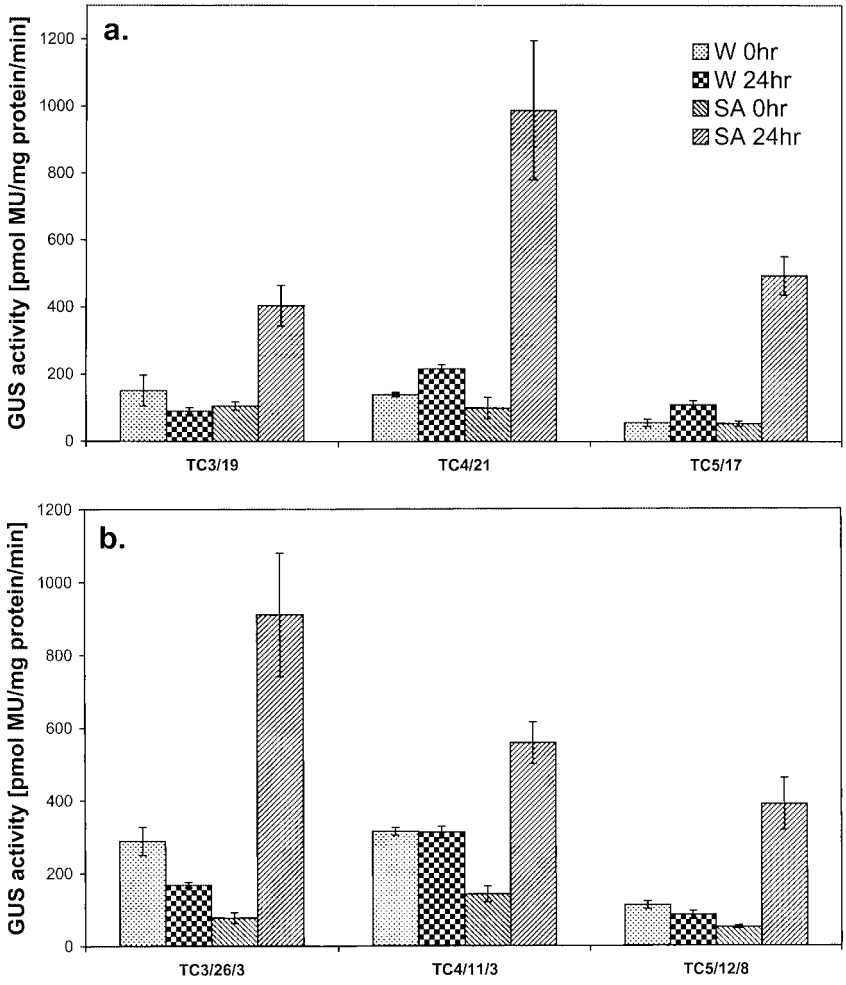

Figure 3. a + b.

GUS assays

GUS activity was quantified by a fluorometric method according to Jefferson et al. (1987). Protein content was measured by the Bradford (1976) method using bovine serum albumin as a standard. GUS activity was calculated as pmol of 4-methyl umbelliferone (4-MU) produced per minute per milligram of soluble protein.

Female flowers 14 mm in length and male flowers 4 mm in length from both T_0 and Borszczagowski plants were used for X-Gluc (5-bromo-4-chloro-3-indolyl β-glucuronide) histological staining. Hand-cut tissue sections were vacuum infiltrated with a staining solution and then fixed as described by Hennig et al. (1993).

Quantification of SA and SAG

SA and its conjugate SAG were extracted according to Malamy et al. (1992) with modifications. One gram of frozen tissue was used for organic extraction of free SA. SAG was re-extracted from water phases and released free using acidic lysis at 60 °C. Separation and quantification of SA were performed using a HPLC system and detected by fluorescence detector.

Statistical analysis

Multifactorial analysis of variance for GUS activity was performed using the computer software «Statgraphics Plus».

Results

Genomic hybridization in the primary transformants and their progeny

To confirm T-DNA integration pattern into genomic DNA, a Southern blot analysis was performed on total genomic DNA isolated from leaves of the primary transformants (T_0) and the kanamycin-resistant plants of their subsequent selfed progenies (T_1 and T_2). Genomic DNA was digested with *Hind*III (yielding border fragments which include a portion of the inserted T-DNA and genomic DNA) or with *Hind*III/*Eco*RI (releasing the expression cassette containing the PR-2d/*uidA* gene fusion from the inserted T-DNA) and probed with a fragment of the *uidA* gene. Digestion with *Hind*III revealed the transgene copy number from the number of hybridizing

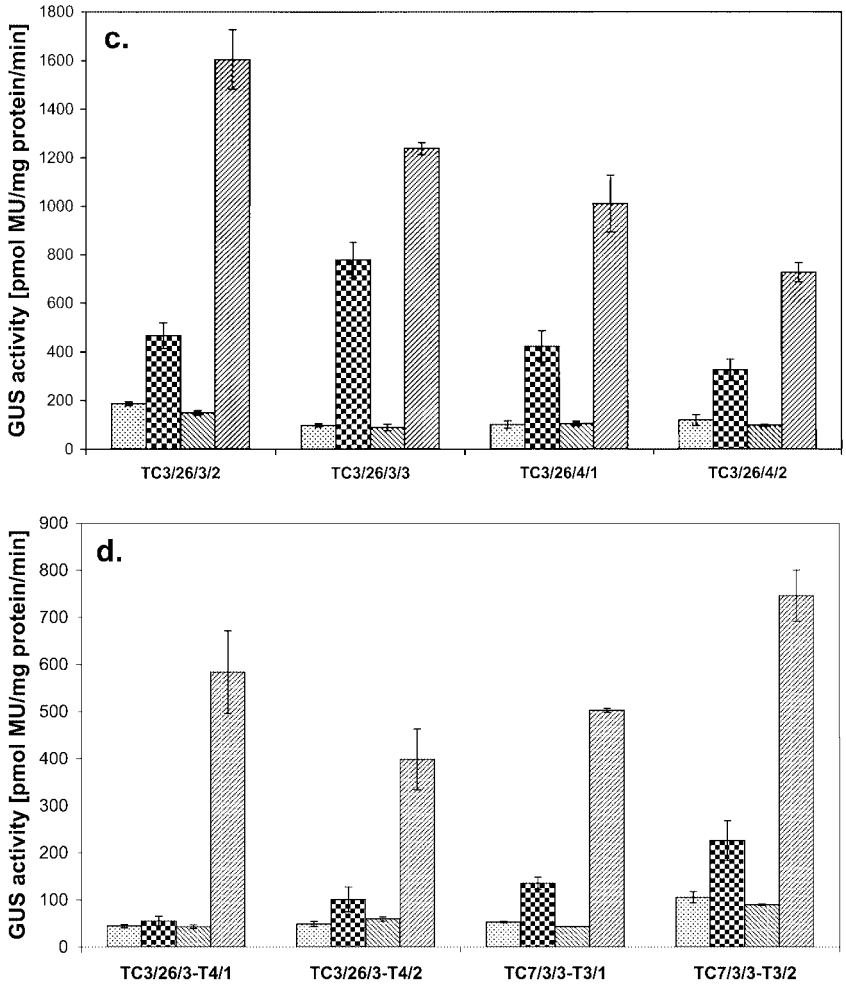

Figure 3. Induction of GUS activity in transgenic plants by SA. GUS activity was determined in SA (SA) and water (W) treated leaves. Leaf discs 1 cm in diameter were punched out from young leaves of 4-week-old plants and floated in a Petri dish on water or 1 mmol/L SA. Leaf samples were collected at time 0 (immediately following treatment) and 24 hours after treatment. Experiments were performed over four generations. The result for each plant is the mean calculated from three independent measurements. Error bars represent the standard deviations. a, T_1; b, T_2; c, T_3; d, T_3 and T_4.

bands and independent transformation events from the hybridization patterns. Two different sets of transgenic plants could be identified by Southern blot analysis data (Fig. 1 a–b). The hybridization pattern of the *uidA* gene in the T_0 plants: TC2, TC3, TC4, TC5; the T_1 plants: TC3/26, TC4/11, TC5/12; and the T_2 plants: TC3/26/3, TC4/11/3, TC5/12/8 was the same, consisting of a single band that was 5.9 kb in size (Fig. 1 a–b). This suggests that the transgene integrated with one copy of the *uidA* gene. The hybridization pattern of the *uidA* gene in T_0 plants TC6 and TC7 was the same, consisting of two bands that were 7.9 kb and 5.9 kb in size, which suggests that the transgene integrated with two copies of the *uidA* gene. For digestion with *Eco*RI/*Hind*III, the expected 3.95 kb fragment was observed in all tested plants (Fig. 1 a–b), which suggests that the transgene integrated into the cucumber genome without any truncation or rearrangement of the *uidA* gene.

Accumulation of SA and SAG after *P. cubensis* inoculation

The endogenous levels of SA and its conjugate SAG increased dramatically following *P. cubensis* inoculation. The concentration of SA rose 15 and 10 fold over that of water-treated controls at 5 dpi and 10 dpi, respectively (Fig. 2 a). The concentration of SAG increased in time up to 17 times that of basal levels by 10 dpi and was approximately two times higher than the concentration of SA (Fig. 2 b). Only very low levels of SA conjugates were detected in water-treated con-

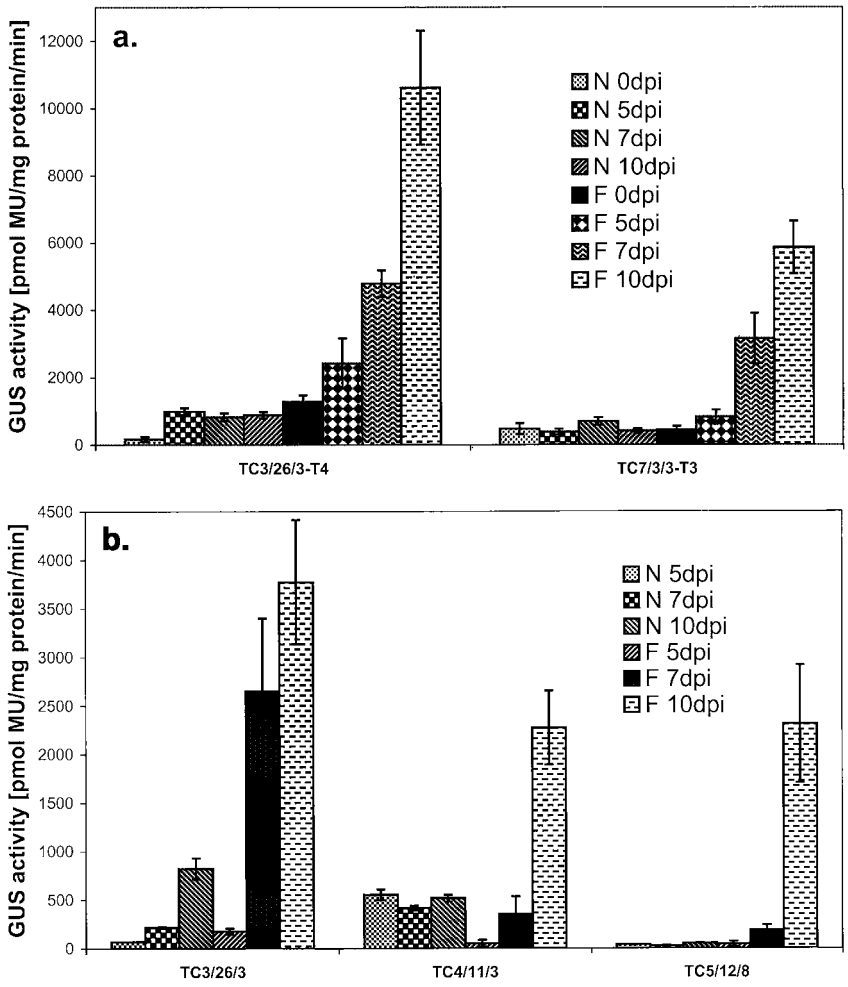

Figure 4. Induction of GUS activity in infected PR-2d transgenics. GUS activity was determined in fungi- (F) or mock-infected (N) plants. Plants were inoculated *in vivo* with a suspension of fungi spores in water and kept 6 hours under highly humid conditions. For *P. cubensis* (a) inoculation, T_3 or T_4 transgenic lines were used. Leaf samples were taken at 0 (immediately following inoculation), 5, 7 and 10 days after inoculation (dpi). Results were the mean calculated from two plants. Water-treated plants were used as a control. For *E. polyphage* inoculation (b), three T_2 transgenic lines were used. Leaf samples were taken at 5, 7 and 10 dpi. Results were the mean calculated from 3 vegetatively propagated plants. Non-inoculated transgenic plants were used as a control. For each plant, GUS activity was the average of three independent measurements. Error bars represent the standard deviations.

trol plants up to 10 dpi, indicating that both free and bound SA were produced *de novo* following the inoculation.

Induction of PR-2d promoter in transgenic cucumbers in response to different stimuli

T_1 to T_4 progeny plants derived from four primary transformants (TC3, TC4, TC5, and TC7) were used for the induction of PR-2d by SA. Figures 3 a, b, c and d show average GUS activities of leaf discs at time 0 and 24 hours after 1 mmol/L SA or water treatment. The induction factor was calculated as the ratio of GUS activity in the SA-treated (24 hr) discs to the activity in water-treated discs. We found considerable variations in basal GUS activity, as well as its induction by SA, between plants derived from the different primary transformants. The transgenic cucumber plants exhibited a 2 to 11 fold increase in GUS activity upon SA treatment, the highest inducibility by SA for T_4 homozygous plant TC3/26/3-T4/1. The SA inducibility was stably transmitted to the T_4 generation.

PR-2d/*uidA* homozygous (TC3/26/3) and heterozygous lines exhibiting high levels of SA inducibility were chosen for analysis of PR-2d induction by fungal pathogens. *P. cubensis* inoculation increased GUS expression levels 12 fold and 14 fold at 10 dpi over that of the controls (Fig. 4 a). The highest inducibility by *P. cubensis* was detected for the T_3 line TC7/3/3-T3, which carried two copies of the integrated *uidA* gene. As shown in Figure 4 b, the gene construct also responded to *E. polyphage* inoculation. However, the variations in the GUS basal level between the lines, as well as its induction, were

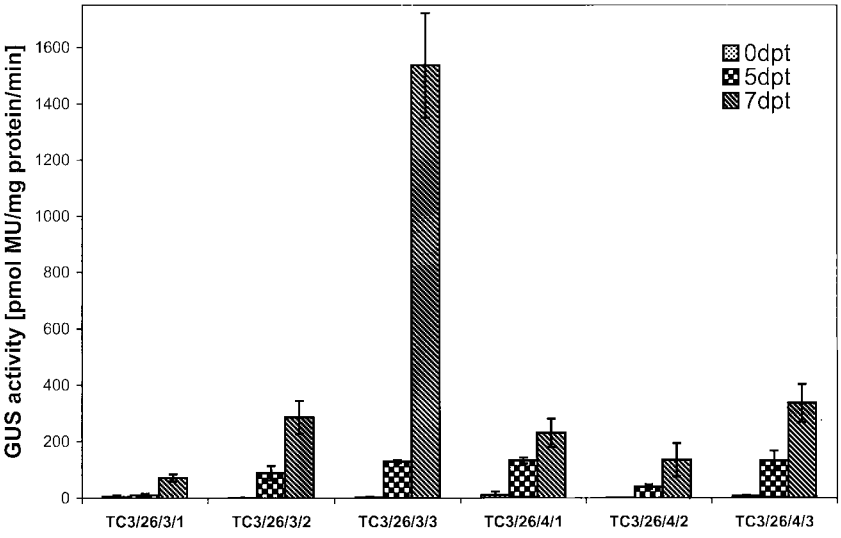

Figure 5. Effect of cold shock (14 °C) on GUS expression. GUS activity was determined in homozygous transgenic lines TC3/26/3 and TC3/26/4 after cold treatment. Four-week-old seedlings were kept at 14 °C for 48 hours and then transferred to 25 °C. Leaf samples were taken at 0 (immediately following treatment), 5 and 7 days after treatment (0 dpt, 5 dpt and 7 dpt, respectively). For each plant, GUS activity was the average of three independent measurements. Error bars represent the standard deviations.

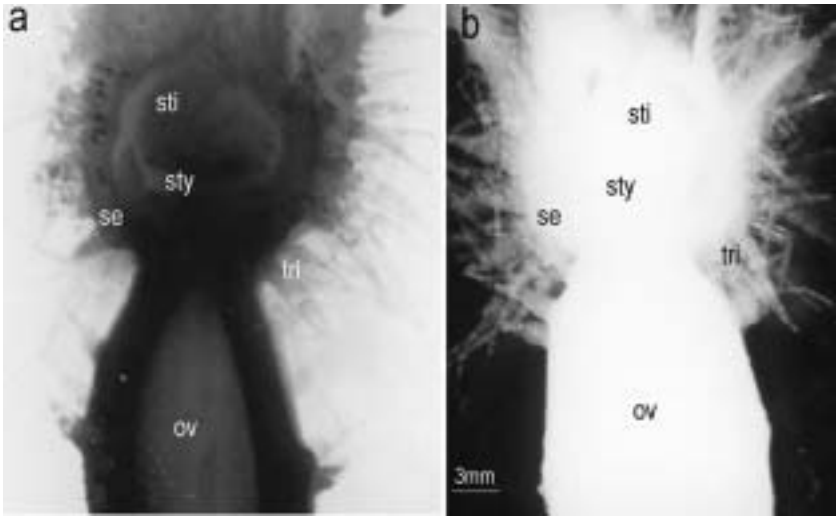

Figure 6. Histochemical GUS staining of female flower tissues of the primary cucumber transformants. Patterns of the PR-2d/*uidA* gene expression were studied in (a) female flower tissues (14 mm in length) of the primary transformant TC2, and (b) female flower tissues (13 mm in length) of the non-transgenic Borszczagowski plant. ov, ovary; sty, style; sti, stigma; se, sepals; tri, the trichomes of the sepals.

observed. By 10 dpi there was a 4 to 44 fold increase of GUS activity depending on the line. The highest inducibility by *E. polyphage* was observed in the heterozygous T_2 line TC5/12/8.

Furthermore, two homozygous T_3 lines were tested for induction by cold stress. As shown in Figure 5, a 16 to 624 fold increase in GUS activity was detected at seven days after treatment as compared with samples measured at time 0. The levels of cold inducibility varied between the sibling plants of the homozygous line TC3/26/3. The highest inducibility by cold was detected for plant TC3/26/3/3.

Expression of a PR-2d/*uidA* gene fusion in floral organs

Histochemical staining showed highly elevated levels of GUS activity in both female (14 mm in length) and male (4 mm in length) flowers. In the female flower, GUS activity was evident in the pedicel (not shown), ovary, style, stigma, sepals and the trichomes of the sepals (Fig. 6 a). In the male flower, the *uidA* gene was expressed in the pedicel, anthers, and sepals (not shown). In contrast, no activity of the reporter gene was detected in floral organs of the non-transgenic Borszczagowski plant (Fig. 6 b).

Discussion

In this study, we describe the expression of a PR-2d/*uidA* gene fusion in transgenic cucumber plants in response to biological, chemical, and physical inducers. Moreover, tissue-specific expression of this gene during normal development of plants was characterized.

SA, pathogen invasion and the PR-2d induction

White (1979) provided the first hint that SA might be involved in plant defence since injection of aspirin or SA into tobacco leaves enhanced resistance to subsequent infection by tobacco mosaic virus. Exogenous SA treatment also induced the accumulation of PR protein or mRNA of the PR genes (Antoniw and White 1980, Ward et al. 1991). In addition to tobacco, SA was found to induce the biosynthesis of certain PR proteins in a wide range of both dicotyledonous and monocotyledonous plants (Klessig and Malamy 1994).

Van Loon (1983) postulated that SA acts by mimicking an endogenous phenolic signal that triggers PR gene expression and disease resistance. The signal was further confirmed to be SA itself (Malamy et al. 1990). A dramatic increase (10- to 20-fold) in levels of endogenous SA was detected following TMV inoculation and preceded the induction of PR-1a mRNA accumulation by 2 hr and necrotic lesion formation by 3 to 5 hr (Malamy et al. 1990, 1992).

The SA signalling is not unique to tobacco. Increases in endogenous SA levels in pathogen infected tissues of cucumber, *Arabidopsis thaliana*, and potato have also been documented (Métraux et al. 1990, Rasmussen et al. 1991, Smith et al. 1991, Summermatter et al. 1994, 1995, Coquoz et al. 1995). A dramatic rise in SA levels (10- to 100-fold) was detected in the cucumber plants inoculated with tobacco necrosis virus (TNV), *Colletotrichum lagenarium* or *Pseudomonas syringae* pv *syringae*. These increases of SA in the phloem preceded both the appearance of systemic acquired resistance (SAR) and the induction of peroxidase activity.

Exogenously applied SA induces PR gene expression in a dose-dependent manner. Similarly, the levels of endogenous SA increase in parallel or slightly before the expression of PR genes in pathogen infected plants (Yalpani et al. 1991).

The data obtained using a SA-inducible promoter of tobacco in the heterologous cucumber plant shows a similar correlation. The PR-2d promoter can be induced by SA treatment or biotic and abiotic stress, which then leads to SA accumulation. These results provide additional evidence for the involvement of endogenous SA in the signalling of the plant defence responses to pathogen attack. However, in transgenic cucumber a clear induction of GUS in water-treated tissue was observed in all T_3 lines tested. After water treatment the high level of GUS activity (778 pmol MU/mg protein/min) was detected in one line only (i.e. TC3/26/3/3). In other lines of this generation, this level was 2–3 times lower. In its primary host, the PR-2d promoter was also induced in water-treated transgenic tobacco and the GUS level reached 280–300 pmol MU/mg protein/min (Hennig et al. 1993 and Hennig unpublished data).

Cold induced PR-2d expression

Cold treatment is another inducer of the PR-2d promoter. Seven days after treatment, there was a 624-fold induction of GUS activity in the transgenic cucumber. This is the first report on PR-2d induction by cold stress.

There is no definite information in the literature about cold stress induced salicylic acid dependent PR gene expression in tobacco. In other species, like oilseed rape (*Brassica napus* L.), cold stress induces phenylpropanoid pathways – PAL (phenylalanine ammonia-lyase) and CHS (chalcone synthase) gene expression and increases the ROS (reactive oxygen species). The content of phenolic compounds, including salicylic acid precursors, increases (Solecka et al. 1999). In the case of heat-acclimation in mustard (*Sinapis alba* L.) the salicylic acid and antioxidant levels were increased (Dat et al. 1998). On the other hand the salicylic acid treatment enhanced the resistance to biotic and cold stress in bean (*Phaseolus vulgaris* L.) and tomato (*Lycopersicon esculentum* L.) (Senaratna et al. 2000). In maize (*Zea mays* L.) cold stress caused an increase in ACC (1-aminocyclopropane–1-carboxylic acid) content. However, this increase was less pronounced in cold acclimated or SA pre-treated plants (Szalai et al. 2000). The pre-treatment of maize plants with SA induces antioxidant enzymes that lead to increased cold tolerance (Janda et al. 1999).

The use of the chimeric gene PR-2d/GUS in transgenic tobacco increases the inducibility by a factor of 2.5 after cold stress. GUS activity reached 580 ± 35 pmol MU/mg protein/min, while the level in the control was 170 ± 10 (Hennig, unpublished data). In transgenic cucumber, high level induction (624-fold) by cold stress was detected in a single T_3 plant (TC3/26/3/3) and GUS activity reached 1537 pmol MU/mg protein/min. For the same plant the induction factor by SA treatment was 1.6-fold and GUS activity reached 1239 pmol MU/mg protein/min. In the other six plants of this generation, the induction factors by cold ranged from 16 to 406-fold and GUS activities from 61 to 336 pmol MU/mg protein/min. On this basis, we conclude that the PR-2d/*uidA* construct can be induced by cold stress in transgenic cucumber.

Developmentally regulated PR-2d expression

The expression of glucanase genes in tobacco exhibits tissue-specific and developmental regulation (Côté et al. 1991, Hennig et al. 1993). In this study, we confirmed the previously localized PR-2d induction in the different floral organs during normal development of flowers (Hennig et al. 1993). This provided additional support for the physiological function(s) of PR-2d gene expression in flowering.

We demonstrated that the expression of the tobacco PR-2d promoter in a heterologous plant followed the same pattern as in the primary host. This opens up the possibility of using the PR-2d/*uidA* in various heterologous species.

References

An G (1986) Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. Plant Physiol 81: 86–91

Antoniw JF, White RF (1980) The effects of aspirin and polyacrylic acid on soluble leaf proteins and resistance to virus infection in five cultivars of tobacco. Phytopath Z 98: 331–341

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Burza W, Malepszy S (1995) Direct plant regeneration from leaf explants in cucumber (*Cucumis sativus* L.) is free of stable genetic variation. Plant Breeding 114: 341–345

Coquoz JL, Buchala AJ, Meuwly P, Métraux JP (1995) Arachidonic acid in potato plants induces local but not systemic synthesis of salicylic acid and confers systemic resistance to *Phytophthora infestans* and *Alternaria solani*. Phytopathology 85(10): 1219–1224

Côté F, Cutt JR, Asselin A, Klessig DF (1991) Pathogenesis-related acidic β-1,3-glucanase genes of tobacco are regulated by both stress and developmental signals. Mol Plant-Microbe Interact 4: 173–181

Dat JF, Foyer CH, Scott IM (1998) Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. Plant Physiol 118: 1455–1461

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA mini-preparation: Version II. Plant Mol Biol Rep 1: 19–21

Durner J, Shah J, Klessig DF (1997) Salicylic acid and disease resistance in plants. Trends Plant Sci 2(7): 266–274

Enyedi AJ, Yalpani N, Silverman P, Raskin I (1992) Localization, conjugation and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc Natl Acad Sci USA 89: 2480–2484

Hennig J, Dewey RE, Cutt JR, Klessig DF (1993) Pathogen, salicylic acid and developmental dependent expression of a β-1,3-glucanase/GUS gene fusion in transgenic tobacco plants. Plant J 4(3): 481–493

Janda T, Szalai G, Tari I, Páldi (1999) Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. Planta 208: 175–180

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907

Kauffmann S, Legrand M, Geoffroy P, Fritig B (1987) Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have β-1,3-glucanase activity. EMBO J 6: 3209–3212

Klessig DF, Malamy J (1994) The salicylic acid signal in plants. Plant Molec Biol 26: 1439–1458

Koetsier PA, Schorr J, Doerfler W (1993) A rapid optimized protocol for downward alkaline Southern blotting of DNA. BioTechniques 15(2): 260–262

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci 10: 123–150

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science 250: 1002–1004

Malamy J, Hennig J, Klessig DF (1992) Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. Plant Cell 4: 359–366

Malepszy S (1988) Cucumber (*Cucumis sativus* L.). In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry. Vol 6 Crops II. Springer, Berlin, Heidelberg pp 277–293

Meins JF, Ahl P (1989) Induction of chitinase and β-1,3-glucanase in tobacco plants infected with *Pseudomonas tabaci* and *Phytophthora parasitica* var. nicotianae. Plant Sci 61: 155–161

Meuwly P, Mölders W, Buchala A, Métraux JP (1995) Local and systemic biosynthesis of salicylic acid in infected cucumber plants. Plant Physiol 109: 1107–1114

Métraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science 250: 1004–1006

Mölders W, Buchala A, Métraux JP (1996) Transport of salicylic acid in tobacco necrosis virus-infected cucumber plants. Plant Physiol 112: 787–792

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473–497

Rasmussen JB, Hammerschmidt R, Zook MN (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. Plant Physiol 97: 1342–1347

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell 8: 1809–1819

Senaratna T, Touchell D, Bunn E, Dixon K. (2000) Acetyl salicylic acid (Aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. Plant Growth Regulat 30(2): 157–161

Shah J, Klessig DF (1996) Identification of a salicylic acid-responsive element in the promoter of the tobacco pathogenesis-related β-1,3-glucanase gene, *PR-2d*. Plant J 10(6): 1089–1101

Shulaev V, León J, Raskin I (1995) Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell 7: 1691–1701

Smith JA, Hammerschmidt R, Fulbright DW (1991) Rapid induction of systemic induction of systemic resistance in cucumber by *Pseudomonas syringae* pv *syringae*. Physiol Mol Plant Pathol 38: 223–235

Solecka D, Boudet AM, Kacperska A (1999) Phenylpropanoid and anthocyanin changes in low-temperature treated winter oilseed rape leaves. Plant Physiol Biochem 37(6): 491–496

Stintzi A, Heita T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie 75: 687–706

Summermatter C, Meuwly P, Mölders W, Métraux JP (1994) Salicylic acid levels in *Arabidopsis thaliana* after treatment with *Pseudomonas syringae* or synthetic inducers. Acta Hortic 381: 367–370

Summermatter C, Sticher L, Métraux JP (1995) Systemic responses in *Arabidopsis thaliana* infected and challenged with *Pseudomonas syringae* pv *syringae*. Plant Physiol 108: 1379–1385

Szalai G, Tari I, Janda T, Pestenacz A, Páldi E (2000) Effects of cold acclimation and salicylic acid on changes in ACC and MACC contents in maize during chilling. Biol Plant 43(4): 637–640

Szwacka M, Morawski M, Burza W (1996) *Agrobacterium tumefaciens*-mediated cucumber transformation with thaumatin II cDNA. J Appl Genet 37A: 126–129

Van Loon LC (1983) The induction of pathogenesis-related proteins by pathogens and specific chemicals. Neth J Plant Path 89: 265–273

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Métraux JP, Ryals JA (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3: 1085–1094

White RF (1979) Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology 99: 410–412

Wobbe KK, Klessig DF (1996) Salicylic acid – an important signal in plants. In: Dennis ES et al (eds) Plant Gene Research, Springer, pp 167–196

Yalpani N, Silverman P, Wilson TM, Kleier DA, Raskin I (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell 3(8): 809–818