ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2006, **38**(1): 46–52 **CN** 31-1940/Q

Roles of Salicylic Acid-responsive *Cis*-acting Elements and W-boxes in Salicylic Acid Induction of *VCH3* Promoter in Transgenic Tobaccos

Hai-Yan LI[1,2], Wei WEI[2&], and Yu LI[1]*

[1] *Institute of Mycology, Jilin Agricultural University, Changchun 130118, China;*
[2] *College of Biotechnology, Jilin Agricultural University, Changchun 130118, China*

Abstract A salicylic acid (SA)-inducible *VCH3* promoter was recently identified from grapevine (*Vitis amurensis*) that contains two inverse SA-responsive *cis*-acting elements and four W-boxes. To further demonstrate the roles of these elements, four fragments with lengths from −1187, −892, −589, −276 to +7 bp were fused with the β-glucuronidase (GUS) reporter gene and transferred to *Nicotiana tobacum*, together with another four *VCH3* promoter fragments with mutation in the two inverse SA-responsive elements. The functions of each promoter fragment were examined by analysis of GUS activity in the transgenic tobacco root treated with SA. Enhanced GUS activity was shown in the roots of transgenic tobaccos with the *VCH3* (−1187)-*GUS* construct containing two SA-responsive *cis*-acting elements and four W-boxes. However, GUS activity directed by the *VCH3* (−892)-*GUS* construct, containing one SA *cis*-acting element and four W-boxes, was reduced by up to 35% compared with that in tobaccos transformed with the *VCH3* (−1187)-*GUS* construct, indicating that the SA *cis*-acting element plays an important role in SA induction of the *VCH3* promoter. Neither the *m2VCH3* (−1187)-*GUS* nor the *mVCH3* (−892)-*GUS* construct, with mutation on the SA-responsive elements, abolished the expression of GUS activity, demonstrating that the W-boxes in the *VCH3* promoter are also involved in SA induction. Histochemical analysis of GUS activity directed by each of the eight *VCH3* promoter fragments showed that GUS was expressed specifically in vascular tissue. It was concluded that both the SA-responsive *cis*-acting elements and the W-boxes are important for the SA induction of the *VCH3* promoter. This promoter might have a potential use in plant genetic engineering.

Key words *VCH3* promoter; salicylic acid (SA)-responsive *cis*-acting element; W-box; site-directed mutagenesis; SA induction; transgenic tobacco

Salicylic acid (SA) is one of the important signal molecules involved in disease resistance in plants [1,2]. An increased level of SA is required to activate the transcription of defense genes and to develop an efficient pathogen resistance response [3]. In addition, the accumulation of SA and the activation of defense genes, such as pathogenesis-related (PR) genes, have been reported to occur after exposure of plants to ozone, ultraviolet radiation, cold or high salinity [4–6]. Therefore, it is considered that SA plays a crucial role under either biotic or abiotic stress, and that SA-inducible promoters might have an important use in engineering stress-tolerant plants to drive gene expressions when necessary.

To date, many plant gene promoters induced by SA have been reported, such as soybean *IFS* promoter, tobacco *PR-1a* and *PR-2d* promoters, *Gastrodia elata GAFP-2* promoter and *Arabidopsis GST6* promoter [7–

Received: July 25, 2005 Accepted: September 19, 2005
This work was supported by the grants from the National Natural Science Foundation of China (30400300), the Fund for Distinguished Young Scholars of Jilin Province and the Science and Technology Development Projects of Jilin Province (20050117, 20030553-1 and 20040209-2)
&Present address: Department of Biology, Graduate School of the Chinese Academy of Science, Beijing 100039, China
*Corresponding author: Tel, 86-431-4510966; Fax, 86-431-4510971; E-mail, yuli966@126.com

DOI: 10.1111/j.1745-7270.2006.00126.x

11]. Of these promoters, the SA-responsive *cis*-acting element TGACG, which belongs to the family of activation sequence-1 elements, is reported to function as a transcriptional enhancer conferring SA inducibility to reporter genes in transgenic plants [8–10]. The W-box (T)TGAC(C/T), another *cis*-acting DNA element found frequently in the promoter of defense-related genes [12–14], is recognized specifically by pathogen- or SA-induced WRKY DNA binding protein [15–17]. Recently, Rocher *et al*. reported the W-box was required for full expression of the SA-responsive gene *SFR2* [18]. We previously isolated a 1216 bp *VCH3* promoter from grapevine (*Vitis amurensis*), which contains two inverse SA-responsive *cis*-acting elements and four W-boxes, and is strongly induced by SA.

In the present work, we performed deletion analysis and site-specific mutagenesis to further demonstrate the roles of these promoter elements in response to SA induction in the *VCH3* promoter. The tissue-specific expression patterns of the β-glucuronidase (GUS) reporter gene directed by the wild-type and mutant *VCH3* promoter fragments are also discussed.

Materials and Methods

Materials

Escherichia coli strain DH5α, *Agrobacterium tumefaciens* strain LBA4404, binary vector pBI121 and tobacco (*Nicotiana tobacum*) cv. NC89 were used.

Site-directed mutagenesis of SA-responsive *cis*-acting element

Polymerase chain reaction (PCR)-based site-directed mutagenesis of the two SA-responsive *cis*-acting elements (TGACG) in the *VCH3* promoter was performed using the TaKaRa MutanBest kit (TaKaRa, Dalian, China). For the downstream SA *cis*-acting element present at –293 bp relative to the transcriptional start site, PCR was performed using the wild-type *VCH3* promoter-inserted plasmid as the template, a mutant sense primer A, 5′-ATGCGGTTAACTCTTCCTAAG-3′, and an antisense primer B, 5′-GCATTTTCTGACTCATTTCTC-3′. The single mutagenized nucleotide in the sense primer (underlined) resulted in the site mutation of the SA *cis*-acting element in the *VCH3* promoter. After being blunted and 5′ end phosphorylated, the resulting PCR products were then self-ligated and transformed into the *E. coli* cells, which resulted in the mutant *VCH3* promoter containing one mutant SA *cis*-acting element. Similarly, the single point mutation was introduced in the upstream SA *cis*-acting element, which was located at –1181 bp relative to the transcriptional start site. PCR was performed using the mutant *VCH3* promoter-insert plasmid as the template, a mutant sense primer C, 5′-TTGCGGTGTACTTTGGTTTTTG-3′, and an antisense primer D, 5′-TGTGCTTGATTAATGTGTGTGAG-3′. Both mutations of the SA *cis*-acting elements in the *VCH3* promoter were verified by sequencing.

Generation of the *VCH3* promoter deletion fragments and plasmid construction

To generate *VCH3* promoter-*GUS* chimera, four wild-type and mutant *VCH3* promoter deletion fragments were amplified by PCR using the wild-type or mutant *VCH3* promoter-inserted plasmid as the template. A total of eight *VCH3* promoter fragments from –1187, –892, –589, –276 to +7 bp relative to the transcriptional start site were generated using a common 3′ oligonucleotide and different 5′ oligonucleotides. The various deletion fragments of the *VCH3* promoter were then cloned upstream of the GUS coding sequence in the binary vector pBI121. Briefly, PCR products of the *VCH3* promoter fragments were blunted using Klenow fragment and were subsequently digested with *Bam*HI. Plasmid pBI121 was cleaved with *Pst*I, blunted using Klenow fragment, and then digested with *Bam*HI. The blunt/*Bam*HI *VCH3* promoter fragments were inserted in the blunt/*Bam*HI pBI121.

Transformation of tobacco

The GUS expression cassettes in the binary vector pBI121 were mobilized into *A. tumefaciens* strain LBA4404, which was kindly provided by the College of Life Sciences, Shandong Agricultural University. Leaf discs from *N. tobacum* cv. NC89 were transformed and plants were regenerated by standard methods [19]. For each construct, the presence of the *GUS* gene in transformed plants was verified by Southern blot analysis. Twelve independent transformant lines containing one copy of the chimeric gene were allowed to self-fertilize and seeds were collected and germinated on MS agar medium with 300 μg/ml kanamycin sulfate. Kanamycin-resistant T2 seedlings were used in the following induction.

SA-induction treatments

T2 seedlings at the 4–5 leaf stage were used in SA induction treatments. The tobacco plant roots were submerged in the MS medium without sucrose but supplemented with 1 mM SA at room temperature for 24 h be-

fore GUS activity was analyzed. In control plants, SA was replaced by distilled water.

Fluorometric assay of GUS activity

Root samples were homogenized in 0.6 ml chilled lysis buffer containing 100 mM sodium phosphate (pH 7.0) and 1 M EDTA to obtain the crude homogenates, and 10 µl aliquots of the homogenates were used for measuring GUS activity by fluorometric assay as described by Jefferson *et al.* [20]. The activity was expressed as the specific activity in the crude homogenates, that is, pmol 4-methyl umbelliferone·(mg soluble protein)$^{-1}$·min^{-1}. Protein concentration was measured by the Bradford method [21] using bovine serum albumin as standard.

Histochemical analysis of GUS activity

Histochemical analysis of GUS activity was performed as described by Stomp [22]. Tobacco roots 1–2 cm long were fixed by immersing them for 30 min in a fixing solution containing 100 mM sodium phosphate (pH 7.0), 0.1% formaldehyde, 0.1% Triton X-100 and 0.1% 2-mercaptoethanol. Fixed samples were stained by immersing the roots in a GUS staining solution containing 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM K ferrocyanide, 1 mM X-glucuronide and 0.1% Triton X-100. Tissues were vacuum infiltrated in the staining solution in order to assure homogeneous infection of the substrate. After dehydration in 100% ethanol, the tissues were incubated in toluene for 2 h at room temperature then embedded in paraffin for sectioning (8–10 µm in thickness). Pictures were taken from thin sections of tobacco roots under a microscope (BX 50; Olympus, Tokyo, Japan) equipped with the PM-30 automatic photomicrographic system (Olympus, Tokyo, Japan).

Results and Discussion

Promoter elements in the *VCH3* promoter and generation of site-directed mutagenesis of the SA *cis*-acting elements

The 1216 bp promoter sequence of chitinase gene *VCH3* (GenBank accession number AF441123) was isolated and the transcriptional start site was identified by primer extension analysis [23]. Sequence analysis revealed that the *VCH3* promoter contains two inverse SA *cis*-acting elements (TGACG) located at –293 and –1181 bp relative to the transcriptional start site (**Fig. 1**). In addition, four W-boxes [(T)TGAC(C/T)] [17–19] were found at –76, –591, –595 and –767 bp upstream of the transcriptional start site (**Fig. 1**).

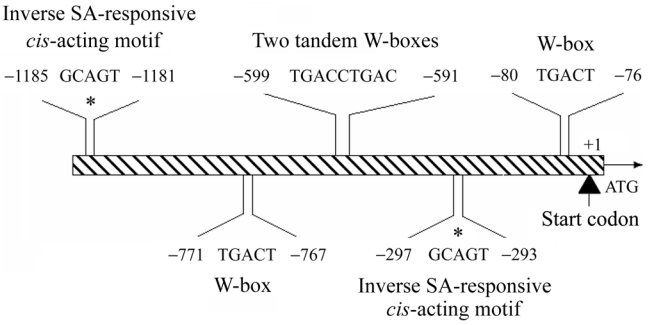

Fig. 1 Promoter elements in the *VCH3* promoter

The two inverse salicylic acid (SA) *cis*-elements (TGACG) and four W-boxes [(T)TGAC(C/T)], as well as their locations, are shown in the scheme of the *VCH3* promoter. * The introduced single point mutation in the two SA *cis*-elements.

To generate the mutant *VCH3* promoter deletion fragments, PCR-based site-directed mutagenesis of the two SA *cis*-acting elements in the *VCH3* promoter was performed (**Fig. 2**). The single mutagenized nucleotide present in the two sense primers resulted in the site mutation of the two inverse SA *cis*-acting elements (see "Materials and Methods"). Both mutations of the inverse SA *cis*-acting elements, from GC**A**GT to GC**g**GT in the *VCH3* promoter, were verified by sequencing.

Construction of the *VCH3* promoter fragment-*GUS* chimera and tobacco transformation

Four wild-type *VCH3* promoter fragments and the corresponding four mutants with one or two mutated SA *cis*-acting element(s) were constructed upstream of the GUS coding region. This resulted in a total of eight constructs (**Fig. 3**), of which *VCH3* (–1187)-*GUS* represented the maximal wild-type fragment containing two SA *cis*-acting elements and four W-boxes. *m1VCH3* (–1187)-*GUS* and *m2VCH3* (–1187)-*GUS* were the corresponding mutants which contained one and two mutated SA *cis*-acting element(s), respectively. Both the *VCH3* (–892)-*GUS* and *VCH3* (–589)-*GUS* fragments contained one SA *cis*-acting element, and four and one W-box(es), respectively, and *mVCH3* (–892)-*GUS* and *mVCH3* (–589)-*GUS* were the corresponding mutants, within which the SA *cis*-acting element was replaced by the mutated one. *VCH3* (–276)-*GUS* represented the minimal fragment that contained only one W-box. All the constructs were transferred to *N. tobacum* cv. NC89 by *A. tumefaciens*-mediated leaf discs transformation, and 12 independent transgenic lines were obtained for each construct.

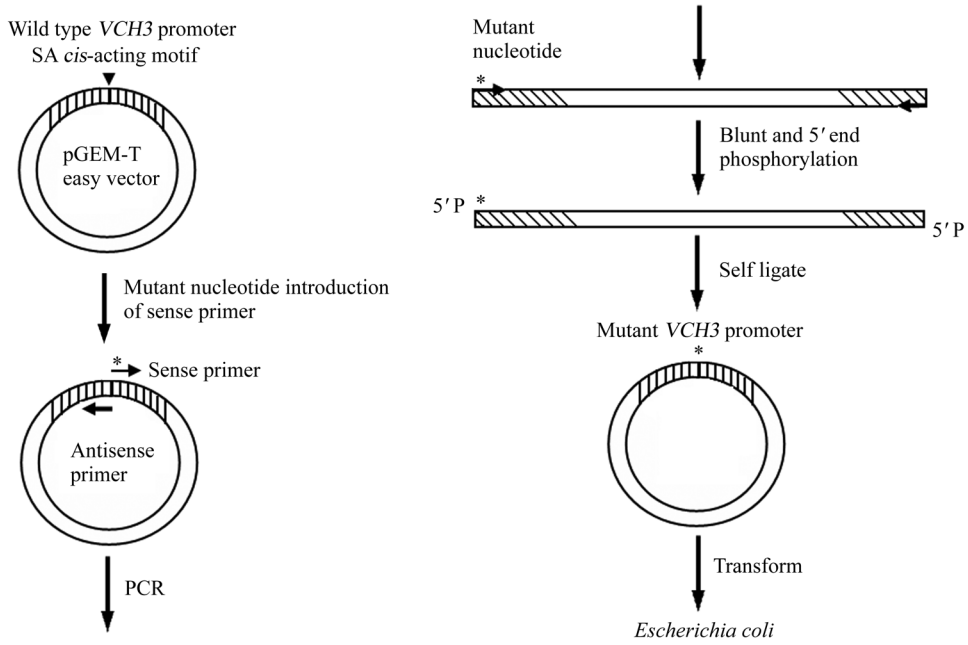

Fig. 2 Scheme of the polymerase chain reaction (PCR)-based site-directed mutagenesis for the inverse salicylic acid (SA) *cis*-acting element TGACG

The *VCH3* promoter inserted into the pGEM-T easy vector is indicated by vertical stripes. * The introduced single point mutation in the sense primer.

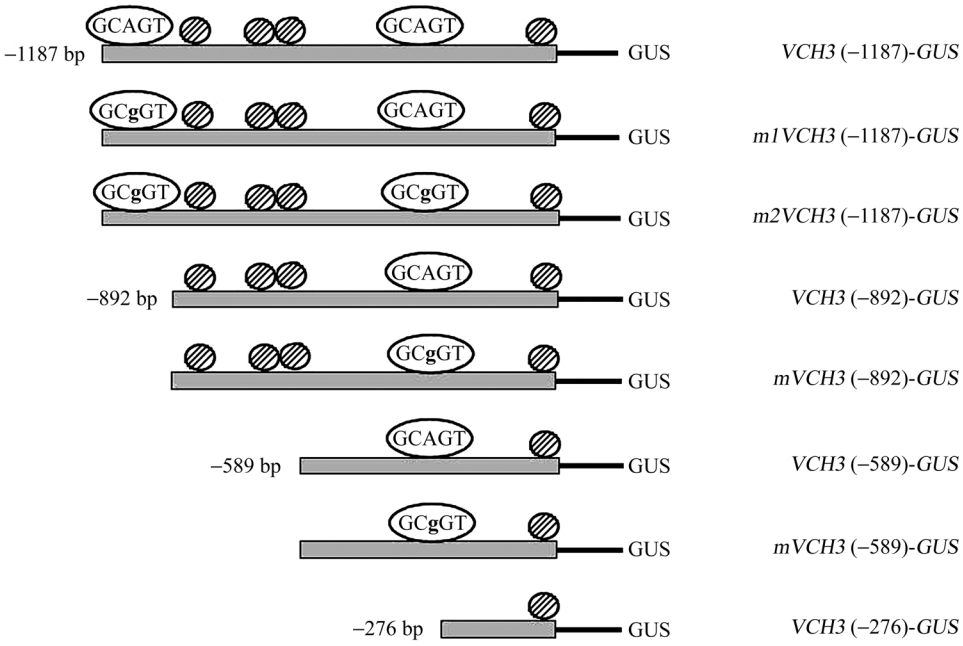

Fig. 3 Structures of wild-type and mutant *VCH3* promoter fragment-β-glucuronidase (*GUS*) chimera

The 5′ end point of each *VCH3* promoter fragment is indicated in the figure. *VCH3* (−1187)-*GUS* contains two salycilic acid (SA) *cis*-acting elements and four W-boxes, whereas *m1VCH3* (−1187)-*GUS* and *m2VCH3* (−1187)-*GUS* contain one and two mutant SA *cis*-acting element(s) and four W-boxes, respectively. Both *VCH3* (−892)-*GUS* and *VCH3* (−589)-*GUS* contain one SA *cis*-acting element and four and one W-box(es), and the *mVCH3* (−892)-*GUS* and *mVCH3* (−589)-*GUS* contain one mutant SA *cis*-acting element and four and one W-box(es), respectively. *VCH3* (−276)-*GUS* contains only one W-box. The 1 bp mutation within the inverse SA *cis*-acting elements (TGACG) is indicated by lowercase letters, and the W-boxes are indicated by ovals with diagonal lines.

Fluorometric analysis of GUS activities in transgenic tobacco roots induced by SA

GUS activity directed by the different *VCH3* promoter fragments was investigated in the SA-treated roots of transgenic tobaccos transformed with the *VCH3* promoter fragment-*GUS* constructs. Enhanced GUS activity was detected in the roots of the transgenic tobaccos transformed with the *VCH3* (−1187)-*GUS* construct containing two SA-responsive *cis*-acting elements and four W-boxes (**Fig. 4**). However, GUS activity, directed by the −892 bp promoter fragment containing one SA *cis*-acting element and four W-boxes, decreased by up to 35% compared with that in tobaccos transformed with the *VCH3* (−1187)-*GUS* construct. In addition, the *m1VCH3* (−1187)-*GUS* construct, with mutation on one of the SA *cis*-acting elements within the *VCH3* promoter fragment, reduced GUS activity to a level close to those found in transgenic plants transformed with the *VCH3* (−892)-*GUS* construct, indicating the SA *cis*-acting element plays an important role in SA induction of the *VCH3* promoter. It was noted that GUS activity in tobaccos transformed with the *VCH3* (−589)-*GUS* construct containing one SA *cis*-acting element and one W-box showed a strong reduction compared with that in tobaccos transformed with the *VCH3* (−892)-*GUS* construct, suggesting that the W-boxes also conferred the SA inducibility of the *VCH3* promoter. However, the *m2VCH3* (−1187)-*GUS* and *mVCH3* (−892)-*GUS* constructs, with mutations on all of the SA *cis*-acting element(s) in their promoter fragment, did not abolish but showed a similar expression of GUS activity, further demonstrating that the W-boxes in the *VCH3* promoter are involved in the activity of SA inducibility. Taken together, these results demonstrated that both the SA-responsive *cis*-acting elements and the W-boxes confer the SA induction of the *VCH3* promoter.

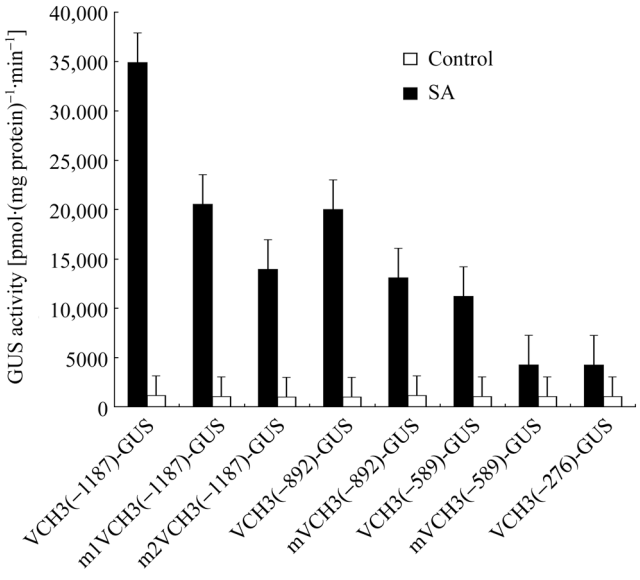

Fig. 4 Induction of β-glucuronidase (GUS) activity in the roots of transgenic tobacco treated by salicylic acid (SA)

Transgenic tobacco roots were used for the GUS assays 24 h after treatment with 1 mM SA. At least 12 independent T_2 transformed plant lines were used for each test, and bars represent standard errors.

The TGA family of transcription factors is known to bind to SA-responsive elements in the *PR-1* promoter [9]. In addition, numerous *in vitro* and *in vivo* experiments have demonstrated that WRKY proteins specifically bind to the W-box in the promoters of those early defense-response genes [12–14]. Our present study demonstrated that the two SA-responsive elements and four W-boxes located in the *VCH3* promoter (**Fig. 1**) were related to the activity of SA induction. Therefore, we suggest that some TGA family members of transcription factors and WRKY proteins might bind to the SA-responsive elements or W-boxes in the *VCH3* promoter. Further analysis will be needed to decide how these *cis* elements interact with the TGA family of transcription factors or WRKY proteins to mediate the SA inducibility of the *VCH3* promoter.

Histochemical analysis of GUS activity in transgenic tobacco roots induced by SA

The transgenic tobacco roots expressing the *VCH3* promoter fragment-*GUS* construct were treated with SA, and cross-sections were made from the resultant SA-treated tobacco transgenic roots (**Fig. 5**). GUS activities directed by all of the *VCH3* promoter fragments were observed to be more active in vascular tissue than that in outer and inner cortexes, including the mutant *VCH3* promoter deletion fragments containing one or two mutant SA *cis*-acting element(s). To date, many plant gene promoters induced by SA have been characterized [7–11]. However, few promoters have been reported to confer both SA-inducible and tissue-specific expression patterns [8]. In this study, we showed that the *VCH3* promoter was strongly induced by SA and specifically expressed in tobacco vascular tissue, which indicated that efficient genetic engineering could be performed to design an appropriate gene expression system by using this SA induction of the *VCH3* promoter to drive gene expression in vascular tissues when required. Also, and especially, this could drive the expression of plant disease-resistant genes, because many pathogens can spread rapidly throughout the plant once they penetrate the vascular system. Hence, this

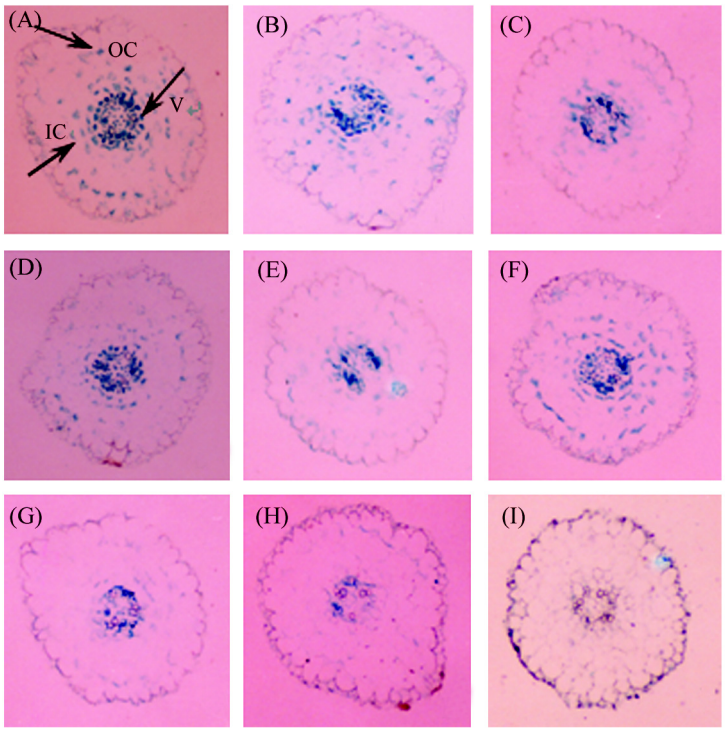

Fig. 5 Histochemical localization of β-glucuronidase (GUS) activity in cross-sections of transgenic tobacco roots induced by salycilic acid (SA)

Pictures are taken from thin sections of transgenic tobacco roots carrying: (A) *VCH3* (−1187)-*GUS* construct; (B) *m1VCH3* (−1187)-*GUS* construct; (C) *m2VCH3* (−1187)-*GUS*; (D) *VCH3* (−892)-*GUS* construct; (E) *mVCH3* (−892)-*GUS* construct; (F) *VCH3* (−589)-*GUS* construct; (G) *mVCH3* (−589)-*GUS* construct; (H) *VCH3* (−276)-GUS construct; (I) SA-untreated control. OC, outer cortex; IC, inner cortex; V, vascular system. Magnification, 30×.

expression pattern of the *VCH3* promoter might enhance the protection of such vulnerable tissues in response to pathogen infection.

Acknowledgements

We acknowledge Mr. Cheng-Chao ZHENG (Shandong Agricultural University, China) and Mr. Mark Andrew Hanson (Northeast Normal University, China) for their critical comments on the manuscript, and we would also like to express our appreciation to Mrs. Xiao-Lan NIE (Jilin Agricultural University, China) for her assistance with the histochemical analysis of GUS activity.

References

1 Alvarez ME. Salicylic acid in the machinery of hypersensitive cell death and disease resistance. Plant Mol Biol 2000, 44: 429–442

2 Desveaux D, Subramaniam R, Despres C, Mess JN, Levesque C, Fobert PR, Dangl JL *et al*. A "Whirly" transcription factor is required for salicylic acid-dependent disease resistance in *Arabidopsis*. Dev Cell 2004, 6: 229–240

3 Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T *et al*. A central role of salicylic acid in plant disease resistance. Science 1994, 266: 1247–1250

4 Yalpani N, Enyedi AJ, León J, Raskin I. Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta 1994, 193: 372–376

5 Stressmann M, Kitao S, Griffith M, Moresoli C, Bravo LA, Marangoni AG. Calcium interacts with antifreeze proteins and chitinase from cold-acclimated winter rye. Plant Physiol 2004, 135: 364–376

6 Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y *et al*. Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis*-related halophyte salt cress using Arabidopsis microarray. Plant Physiol 2004, 135: 1697–1709

7 Chen W, Singh KB. The auxin, hydrogen peroxide and salicylic acid induced expression of the *Arabidopsis GST6* promoter is mediated in part by an ocs element. Plant J 1999, 19: 667–677

8 Sa Q, Wang Y, Li W, Zhang L, Sun Y. The promoter of an antifungal protein gene from *Gastrodia elata* confers tissue-specific and fungus-inducible expression patterns and responds to both salicylic acid and jasmonic acid. Plant Cell Rep 2003, 22: 79–84

9 Gruner R, Strompen G, Pfitzner AJ, Pfitzner UM. Salicylic acid and the hypersensitive response initiate distinct signal transduction pathways in tobacco that converge on the *as-1*-like element of the *PR-1a* promoter. Eur J Biochem 2003, 270: 4876–4886

10 Subramanian S, Hu X, Lu G, Odelland JT, Yu O. The promoters of two

isoflavone synthase genes respond differentially to nodulation and defense signals in transgenic soybean roots. Plant Mol Biol 2004, 54: 623–639
11 Yin Z, Hennig J, Szwacka M, Malepszy S. Tobacco *PR-2d* promoter is induced in transgenic cucumber in response to biotic and abiotic stimuli. J Plant Physiol 2004, 161: 621–629
12 Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE. Early nuclear events in plant defence signalling: Rapid gene activation by WRKY transcription factors. EMBO J 1999, 18: 4689–4699
13 Du L, Chen Z. Identification of genes encoding receptor-like protein kinases as possible targets of pathogen- and salicylic acid-induced WRKY DNA-binding proteins in *Arabidopsis*. Plant J 2000, 24: 837–847
14 Yu D, Chen C, Chen Z. Evidence for an important role of WRKY DNA binding proteins in the regulation of *NPR1* gene expression. Plant Cell 2001, 13: 1527–1540
15 Chen C, Chen Z. Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced *Arabidopsis* transcription factor. Plant Physiol 2002, 129: 706–716
16 Dong J, Chen C, Chen Z. Expression profiles of the *Arabidopsis* WRKY gene superfamily during plant defense response. Plant Mol Biol 2003, 51: 21–37
17 Li J, Brader G, Palva ET. The WRKY70 transcription factor: A node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell 2004, 16: 319–331
18 Rocher A, Dumas C, Cock JM. A W-box is required for full expression of the SA-responsive gene SFR2. Gene 2005, 344: 181–92
19 Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT. A simple and general method for transferring genes into plants. Science 1985, 227: 1229–1231
20 Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 1987, 6: 3901–3907
21 Bradford MM. Rapid and sensitive methods for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Annal Biochem 1976, 72: 248–254
22 Stomp AM. Histochemical location of β-glucuronidase. In: Gallagher SR ed. GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression. San Diego: Academic Press 1992
23 Li HY, Qi J, Shu HR, Zheng CC, Li Y. Isolation and characterization of a chitinase gene *VCH3* promoter from grapevine (*Vitis amurensis*). J Plant Physiol Mol Biol 2005, 31: 485–491

Edited by
Zu-Hua HE