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The Nightshade Proteinase Inhibitor IIb Gene is Constitutively Expressed in Glandular Trichomes

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The best known property of plant proteinase inhibitor II (PIN2) genes is their wound-inducible expression in leaves and constitutive expression in flowers. Here we show by promoter analysis in transgenic plants and in situ reverse transcription–PCR (RT–PCR) analysis that *SaPIN2b*, a member of the PIN2 gene family of nightshade (*Solanum americanum*), is also constitutively expressed in glandular trichomes. *SaPIN2b* promoter and its deletions were cloned and fused upstream of β-glucuronidase (GUS) to transform the nightshade and tobacco (*Nicotiana tabacum*) plants. Histochemical staining assays indicated that *SaPIN2b:GUS* was expressed constitutively in glandular trichomes, predominantly in the gland cells, of both transgenic nightshade and tobacco plants. Constitutive expression of *SaPIN2b* in glandular trichomes was further confirmed by liquid phase in situ RT–PCR analysis of nightshade leaves. Deletion analysis from the 5′ end of the *SaPIN2b* promoter revealed that separate regulatory elements control *SaPIN2b* expression in gland cells and stalk cells of glandular trichomes. Fluorometric GUS assays showed that *SaPIN2b:GUS* expression was significantly increased in transgenic plant leaves after mechanical wounding or methyl jasmonate treatment. The *SaPIN2b* promoter sequence contains six MYB-binding motifs and an L1 box that are involved in trichome differentiation and development. Overexpression of *SaPIN2b* in tobacco resulted in a significant increase in glandular trichome density and promotion of trichome branching. These results suggest that, as well as being an induced defensive protein of the well-known PIN2 family, SaPIN2b could also play roles in trichome-based defense by functioning as a constitutive component of trichome chemical defense and/or by regulating the development of glandular trichomes.

Keywords: GUS — *Nicotiana tabacum* — Promoter — Protease inhibitor — *Solanum americanum* — Trichomes.

Abbreviations: EST, expressed sequence tag; GUS, β-glucuronidase; MeJA, methyl jasmonate; NGT, non-glandular trichome; PIN2, proteinase inhibitor II; RLM-5′ RACE, RNA ligase-mediated rapid amplification of 5′ cDNA ends; RT–PCR, reverse transcription–PCR; SGT, short glandular trichome; TGT, tall glandular trichome.

The nucleotide sequence reported in this paper has been submitted to GenBank under accession number AY749108.

Introduction

Proteinase inhibitor II (PIN2) is a serine proteinase inhibitor with trypsin and chymotrypsin inhibitory activities (Bryant et al. 1976), which was first found in Solanaceae plants tomato (Gustafson and Ryan 1976) and potato (Bryant et al. 1976). PIN2 occurs in various tissues, e.g. wounded leaves (Pearce et al. 1993), flowers (Atkinson et al. 1993), fruits (Richardson 1979, Pearce et al. 1988), tubers (Bryant et al. 1976) and stems (Xu et al. 2001). PIN2s are encoded by a small multigene family (Rosahl et al. 1986, Stiekema et al. 1988, Balandin et al. 1995, Xu et al. 2001). We have previously shown that the nightshade (*Solanum americanum*) PIN2 gene family contains two members, *SaPIN2a* and *SaPIN2b*, which are differentially expressed in plants (Xu et al. 2001). *SaPIN2a* was expressed most abundantly in the stem, and the protein was immunolocalized to the phloem (Xu et al. 2001). In contrast, like the previously characterized PIN2 genes from tomato (Graham et al. 1986; Pena-Cortes et al. 1991), potato (Pena-Cortes et al. 1988, Pena-Cortes et al. 1991) and tobacco (Pearce et al. 1993), *SaPIN2b* was constitutively expressed in flowers (Xu et al. 2001, Sin and Chye 2004) and was wound-induced in leaves (Xu et al. 2001, Schmidt et al. 2004). By using an RNA interference-based gene silencing strategy, Sin et al. (2006) demonstrated recently that SaPIN2a and SaPIN2b are essential for seed development.

The best known property of PIN2s is their wound-inducible expression in leaves (Graham et al. 1986, Lorberth et al. 1992, Pena-Cortes et al. 1995, Koiwa et al. 1997) and constitutive expression in flowers (Pena-Cortes et al. 1991.

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Lorberth et al. 1992, Atkinson et al. 1993, Pearce et al. 1993, Xu et al. 2001, Sin and Chye 2004). In this report, however, by using promoter–reporter gene (β-glucuronidase; *GUS*) fusion and in situ reverse transcription–PCR (RT–PCR) analysis, we have shown that one of the nightshade PIN2 genes, *SaPIN2b*, is also constitutively expressed in glandular trichomes. Furthermore, overexpression of *SaPIN2b* in tobacco resulted in a significant increase in glandular trichome density and promotion of trichome branching. The possible roles of SaPIN2b in trichome-based defense and glandular trichome development are discussed.

Results

Cloning and sequence analysis of the SaPIN2b promoter

Based on our previously reported partial cDNA sequence of *SaPIN2b* (Xu et al. 2001), a 1,943 bp genomic DNA fragment containing the *SaPIN2b* promoter region was obtained by genome walking (Siebert et al. 1995). The sequence of this fragment (GenBank accession No. AY749108) is shown in Fig. 1. To identify the transcription start site of the *SaPIN2b* gene, RNA ligase-mediated rapid amplification of 5′ cDNA ends (RLM-5′ RACE; Schaefer 1995) was carried out with total RNA from nightshade flower buds in which *SaPIN2b* mRNA is highly expressed (Xu et al. 2001). Sequence analysis of the amplified 5′ RACE product (347 bp) indicated that the 5′ terminus of the *SaPIN2b* transcript was located 30 nucleotides upstream of the translation initiation codon, and the corresponding position in the sequence was therefore defined as +1 (Fig. 1). Comparison of the 5′ genomic sequence of *SaPIN2b* with its cDNA sequence (Xu et al. 2001) revealed a single intron (102 bp) located in the 5′ end of the coding region. A putative TATA box sequence was found at −32 to −38, and two putative CAAT box sequences were located at −48 to −51 and −123 to −126, respectively (Fig. 1).

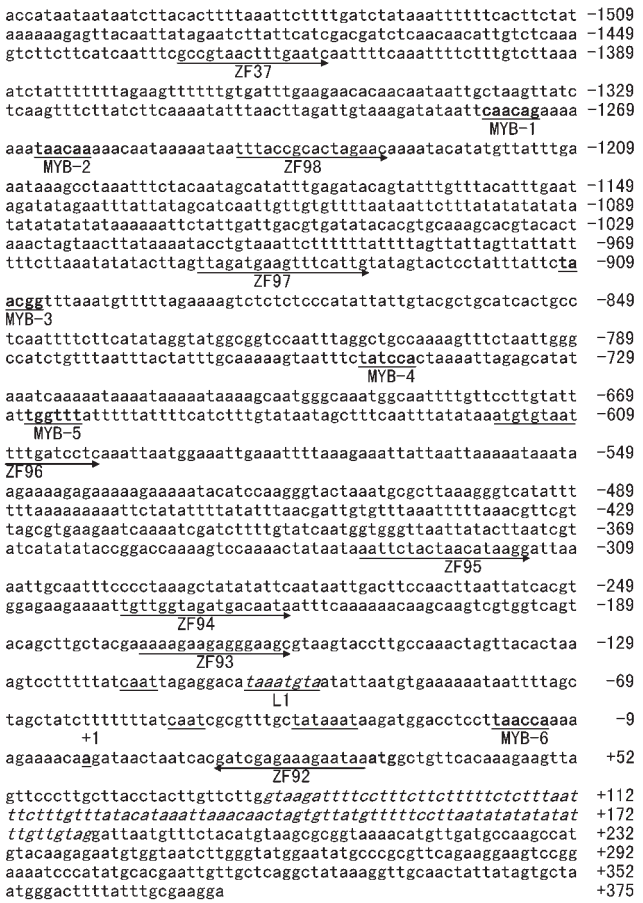

Fig. 1 Nucleotide sequence of the *SaPIN2b* 5′-flanking region. The transcription start site (+1) is in bold and underlined. A putative TATA box (−32 to −38) and two putative CAAT box (−48 to −51 and −123 to −126, respectively) sequences are underlined. The six putative MYB recognition sites (MYB-1 to MYB-6) are in bold and underlined. The putative L1 box (L1) is italicized and underlined. The ATG start codon is in bold. Intron sequence is shown in italics. The sequence and direction of the primers used in PCR amplification to generate the *SaPIN2b* promoters with different lengths are represented by long arrows.

Expression patterns of the SaPIN2b promoter–GUS constructs in transgenic plants

To visualize expression conferred by the *SaPIN2b* promoter, 1.5 kb of sequence upstream of the *SaPIN2b* coding region (Fig. 1, −1,430/+30) was fused upstream of the *GUS* gene in pBI101.1 (Jefferson et al. 1987) to create p2bP:GUS (Fig. 2). Nightshade and tobacco plants were transformed using the *Agrobacterium tumefaciens* LBA4404 derivative harboring p2bP:GUS.

Like other PIN2 promoters (Pena-Cortes et al. 1991, Lorberth et al. 1992), and also consistent with previous Northern blot analysis (Xu et al. 2001) and in situ hybridization (Sin and Chye 2004) of *SaPIN2b* mRNA, the *SaPIN2b* promoter constitutively drives GUS expression in flowers (data not shown). However, constitutive GUS expression was unexpectedly detected in short glandular trichomes (SGTs; Fig. 3a, b, arrowheads), predominantly in the gland cells of transgenic nightshade plants grown on MS medium. No GUS activity was detected in non-glandular trichomes (NGTs; Fig. 3a, arrow) and other leaf cells. This expression pattern was also found in soil-grown transgenic nightshade plants at different development stages (Fig. 3c–e). In transgenic tobacco plants, apart from SGTs (Fig. 3f, arrowhead), tall glandular trichomes (TGTs) that are not present in nightshade leaves also showed strong GUS expression (Fig. 3f, arrow).

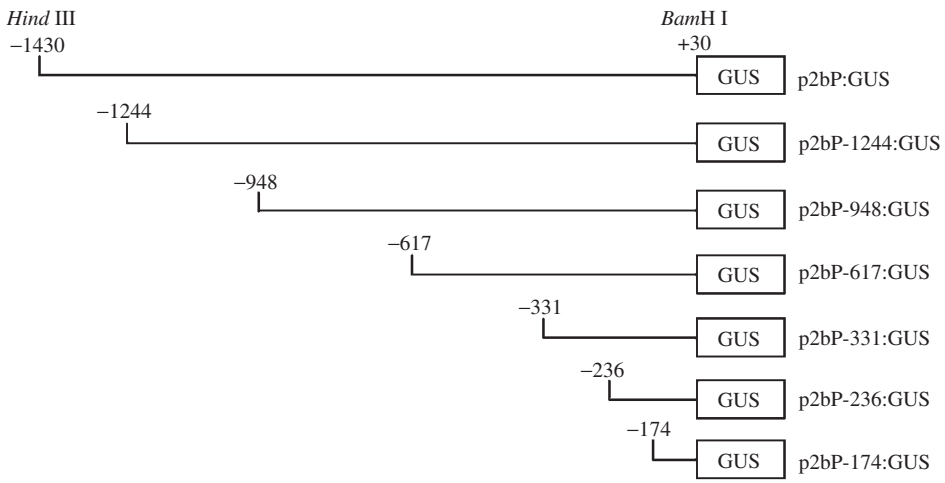

Fig. 2 Schematic representation of the *SaPIN2b* promoter–GUS constructs. The full-length (p2bP) and 5′-deleted promoter (p2bP-1244, p2bP-948, p2bP-617, p2bP-331, p2bP-236 and p2bP-174) fragments were generated by PCR amplification with the primers shown in Fig. 1, and fused with the GUS gene in pBI101.1.

SaPIN2b promoter deletion analysis

To determine the location of regulatory elements within the *SaPIN2b* promoter, a series of 5′ deletions of the *SaPIN2b* promoter were generated and fused upstream of the *GUS* reporter gene (Fig. 2). Transgenic tobacco plants carrying the 5′-deleted promoters were obtained and analyzed for GUS activity by fluorometric assay and histochemical staining. As shown in Fig. 4, deletion of sequences between −1,430 and −1,244 resulted in a significant increase in GUS activity, suggesting the presence of negative regulatory elements in this region. Further deletions to −331 resulted in a drastic decrease in GUS activity and are therefore indicative of the presence of positive regulatory elements between −1,244 and −331. GUS activity was lost with additional deletions to −236. Thus the basal *SaPIN2b* promoter is present between −331 and +30. Histochemical staining revealed that the expression pattern was maintained until deletions to −948 (Fig. 3g, h). Further deletions to −617 abolished GUS activities in most of the trichomes. A small portion of the trichomes, however, showed increased GUS activity in the stalk cells but not in gland cells when deletion proceeded to −617 (Fig. 3i, j). GUS staining disappeared with deletion to −331 (data not shown).

Induction of the SaPIN2b promoter by wounding and methyl jasmonate

Previous studies revealed that *SaPIN2b* expression in leaves was up-regulated in response to wounding (Xu et al. 2001) and attack from insects (Schmidt et al. 2004). To study the wound inducibility of the *SaPIN2b* promoter, GUS expression in leaves of transgenic plants in response to wounding and the wound signaling compound methyl jasmonate (MeJA) was investigated in this study. Fluorometric assay showed that GUS activities in leaves of both transgenic nightshade (Fig. 5a) and tobacco (Fig. 5b) significantly increased after wounding treatment. MeJA treatment also enhanced GUS expression in leaves of transgenic nightshade (Fig. 5c) and tobacco (Fig. 5d). Histochemical analysis of transgenic nightshade plants revealed widespread GUS activities in wounded leaves and prominent induced GUS activity in the area surrounding the incision wounding site (Fig. 3k), whereas in unwounded leaves GUS staining appeared only in trichomes (Fig. 3l). It is worth noting that *SaPIN2b* expression in nightshade trichomes was down-regulated after wounding (Fig. 3k), although the overall expression of *SaPIN2b* was greatly increased in wounded leaves (Fig. 5a).

In situ RT–PCR analysis of SaPIN2b mRNA in nightshade leaves

Histochemical analysis using promoter–GUS fusion gene constructs in transgenic plants indicates that *SaPIN2b* is expressed in SGTs of nightshade plants. *SaPIN2b* mRNA, however, was not detected in unwounded leaves by Northern blot analysis (Xu et al. 2001). We suspected that the restricted expression in leaf SGTs could explain the undetectable *SaPIN2b* mRNA levels found in Northern blot analysis of total RNA from complete leaves. Accordingly, a more sensitive RT–PCR assay in this study demonstrated that, although at low levels, *SaPIN2b* is indeed expressed in leaves (data not shown).

To confirm further *SaPIN2b* expression in SGTs, as revealed by promoter–GUS fusions and RT–PCR assay, whole-mount liquid phase in situ RT–PCR analysis

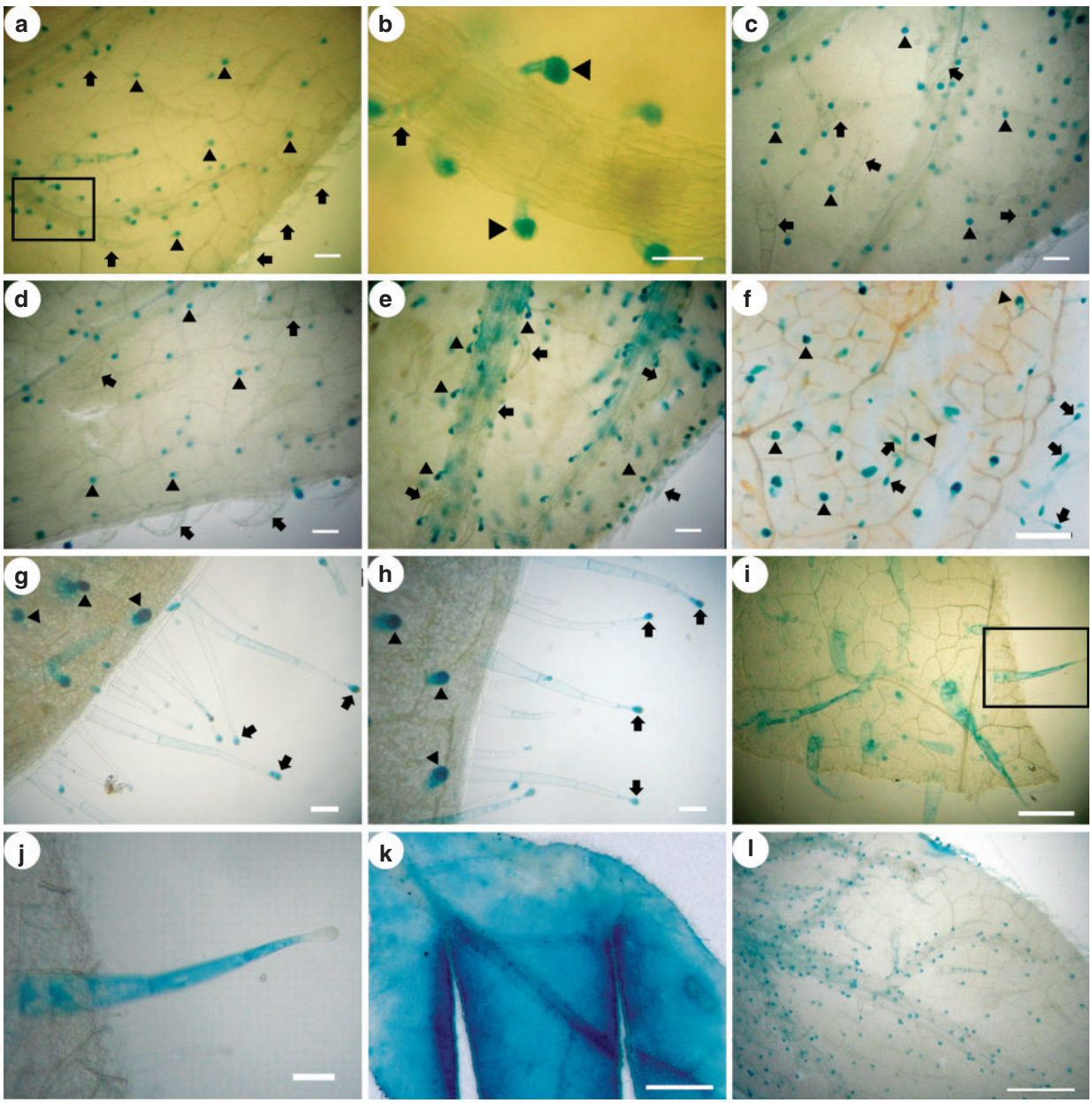

Fig. 3 Histochemical determination of the expression pattern of *SaPIN2b* promoter–*GUS* fusion genes in transgenic plants. (a–e) Transgenic nightshade plants with p2bP:GUS. (a) Transgenic nightshade plants grown on MS medium. The box indicates the zone magnified in b. (b) High magnification image of the boxed area in a. (c) Soil-grown transgenic nightshade plants (10 cm tall). (d) Soil-grown transgenic nightshade plants (30 cm tall). (e) Soil-grown transgenic nightshade plants (110 cm tall at the flowering stage). (f–j) Transgenic tobacco plants grown on MS medium with p2bP:GUS (f), p2bP-1244:GUS (g), p2bP-948:GUS (h) and p2bP-617:GUS (i and j). (j) High magnification image of the boxed area in i. (k) Mechanically wounded leaf of transgenic nightshade plants with p2bP:GUS. (l) Unwounded nightshade leaf. Arrowheads indicate short glandular trichomes (SGTs). Arrows indicate non-glandular trichomes (NGTs) in a–e or tall glandular trichomes (TGTs) in f–h. Bars, a, c–h and j, 100 µm; b, 50 µm; i, k, l, 500 µm.

(Koltai and Bird 2000) of *SaPIN2b* mRNA was carried out with nightshade leaves. *SaPIN2b* expression was localized in SGTs (Fig. 6a, b), as indicated by the strong green fluorescence, which did not occur in the control leaves (Fig. 6c, d).

Discussion

Although the expression patterns of several plant PIN2 genes have been studied extensively (Keil et al. 1990, Pena-Cortes et al. 1991, Kim et al. 1992, Lorberth et al. 1992,

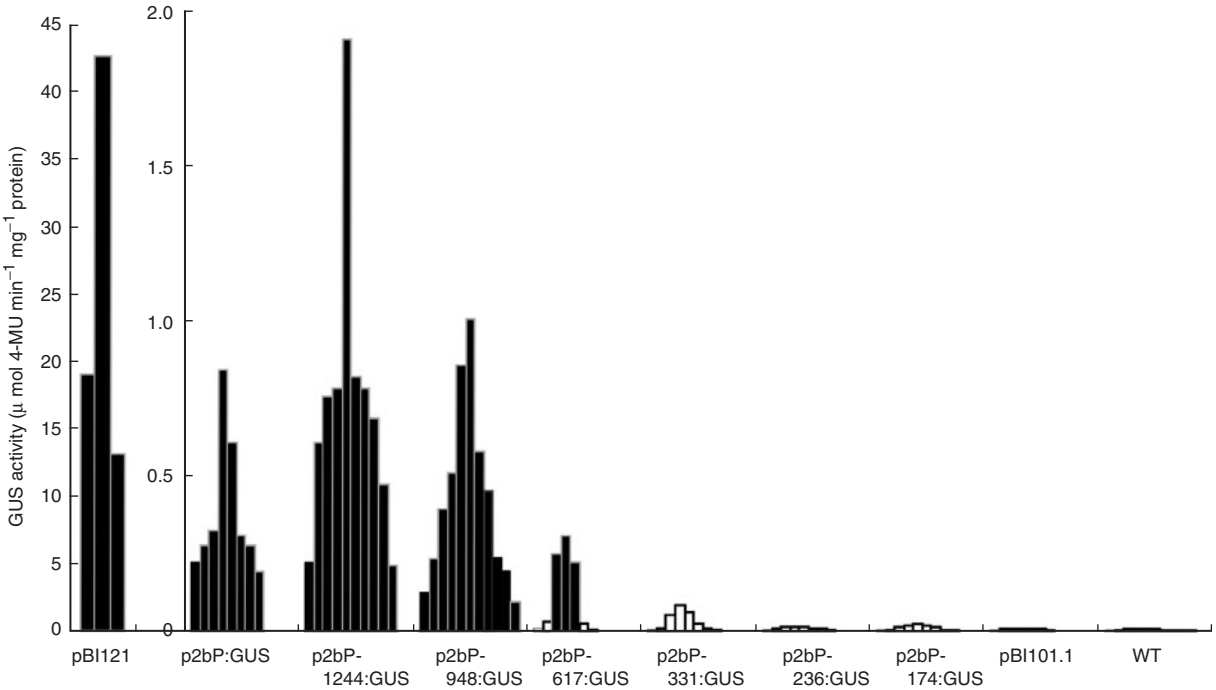

Fig. 4 GUS activity in leaves from individual transgenic tobacco plants with the full-length *SaPIN2b* promoter (p2bP:GUS) and various 5′-deleted promoters (shown in Fig. 2). Solid bars indicate the individual transgenic plants with visible GUS staining, while open bars denote the plants without clear GUS staining. The height of all bars denotes GUS activity in leaves of individual plants. pBI121 (containing the CaMV 35S promoter) and pBI101.1 (promoter-less) transgenic plants were included as positive and negative controls, respectively. WT, wild-type plants.

Xu et al. 1993, Zhang et al. 2004), to our knowledge, this is the first report of a PIN2 gene that is constitutively expressed in glandular trichomes. In agreement with this finding, an expressed sequence tag (EST; GenBank AW616253) with sequence similarity to tomato *PIN2* was found in wild tomato (*Solanum habrochaites*) trichomes.

Trichomes are specialized epidermal cells that function as a first line of defense against biotic and abiotic stresses by various physical and chemical mechanisms (Wagner 1991; Amme et al. 2005). Studies of the chemical-based trichome defense mechanism in plants have focused on secondary metabolites (Levin 1973, Wagner 1991, Laue et al. 2000, Wagner et al. 2004). Recent reports on the analysis of proteins and transcripts in trichomes (Amme et al. 2005, Aziz et al. 2005, Shepherd et al. 2005) suggest that defensive proteins are also involved in the chemical-based trichome defense. Here, we show that SaPIN2b, a member of the PIN2 family of well-known defensive proteins induced in response to insect and pathogen attacks (Ryan 1990), is constitutively present in glandular trichomes, suggesting that SaPIN2b could be an important component of trichome chemical defense.

There is growing circumstantial evidence that PIN2 may also be involved in the regulation of trichome development, in addition to as a component of trichome chemical defense. Insect feeding (Agrawal 1998, Traw and Dawson 2002), artificial damage (Traw and Bergelson 2003) and jasmonic acid (Traw and Bergelson 2003, Boughton et al. 2005), which all induce PIN2 expression (Koiwa et al. 1997, Ryan 2000), cause significant increases in trichome production of several plant species. Consistent with these observations, Li et al. (2004) found a significant reduction in the density of glandular trichomes on leaves of the tomato *jasmonic acid-insensitive1-1* (*jai1-1*) mutant that fails to produce PIN2 in leaves. In good agreement with these studies, we also observed that overexpression of *SaPIN2b* in transgenic tobacco led to a significantly increased density of glandular trichomes (TGTs and SGTs) on both adaxial and abaxial surfaces of leaves, whereas the density of NGTs was not affected in transgenic plants (Fig. 7). Furthermore, scanning electron microscopy revealed the presence of branched glandular trichomes on transgenic tobacco leaves (arrows in Fig. 7c, e), which occurs very occasionally on leaves of wild-type tobacco (Fig. 7b, d; Barrera and Wernsman 1966, Roberts et al. 1981, Johnson et al. 1985). These phenotypic changes are very similar to those resulting from ectopic expression of an *Antirrhinum* MYB-related transcription factor *MIXTA* in tobacco

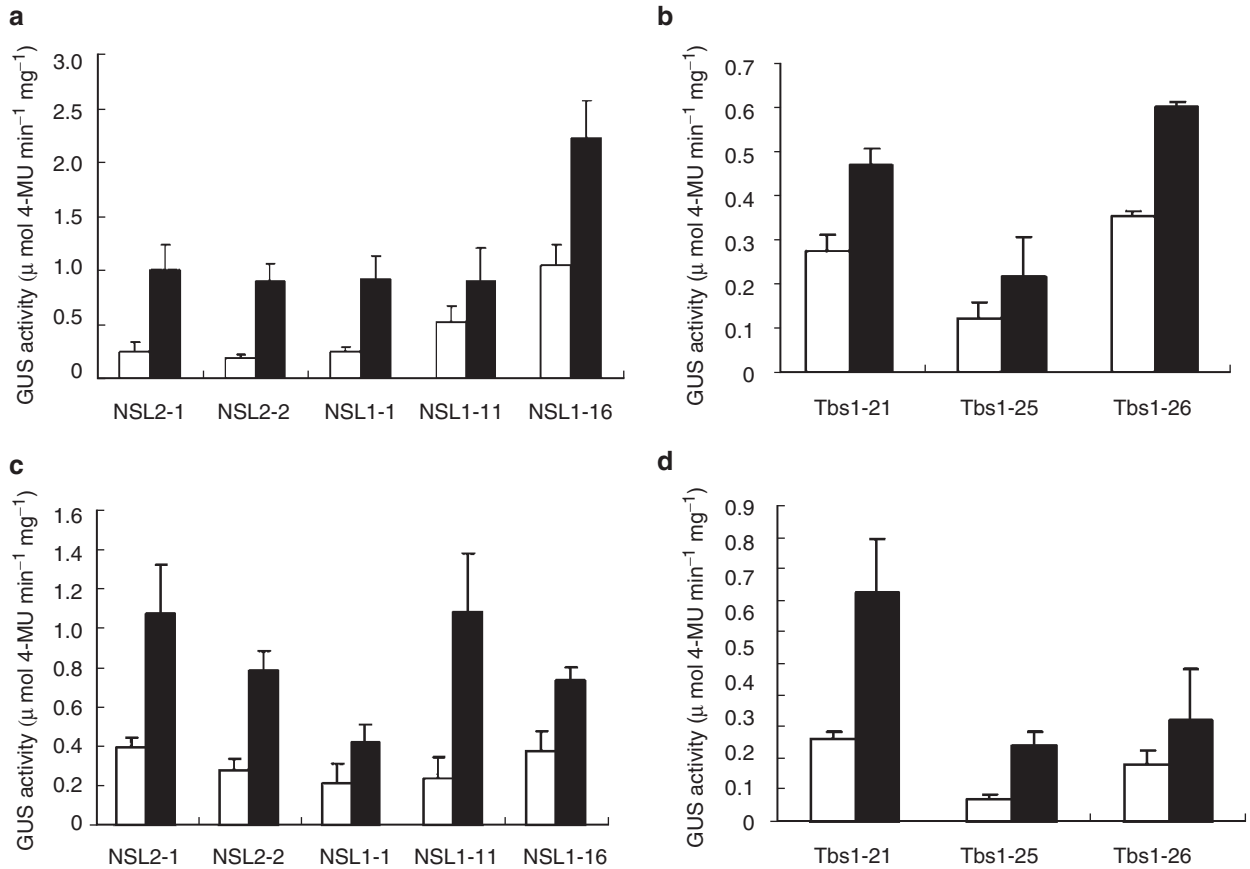

Fig. 5 Inducible expression of the GUS gene driven by the fulll-length *SaPIN2b* promoter in leaves of transgenic nightshade (a and c) and tobacco (b and d). (a and b) Wounding treatment; (c and d) MeJA treatment. GUS activity before and 24 h after treatment is indicated by open and solid bars, respectively.

(Glover et al. 1998), raising the possibility that *SaPIN2b* might be the target of MIXTA-like MYBs. Accordingly, a search by PLACE (http://www.dna.affrc.go.jp/PLACE; Higo et al. 1999) for regulatory elements in the *SaPIN2b* promoter sequence revealed that six MYB-binding motifs and an L1 box were present (Fig. 1). The MYB motif is recognized by MYB transcription factors (Stracke et al. 2001, Ito 2005), which are required for trichome differentiation and development (Oppenheimer et al. 1991, Kirik et al. 2001, Wang et al. 2004, Kasahara et al. 2005, Kirik et al. 2005, Matsui et al. 2005). The L1 box is the binding site of homeodomain proteins (Abe et al. 2001, Ohashi et al. 2003), which also play a role in trichome development (Rerie et al. 1994, Ohashi et al. 2002, Wang et al. 2004, Schellmann and Hulskamp 2005).

The function of plant serine proteases, the potential in vivo targets of PIN2, in plant differentiation and development has recently been explored (Schaller 2004, Antao and Malcata 2005). Two subtilisin-like serine proteases, SDD1 and ALE1, have been shown to be involved in regulation of plant epidermal differentiation and development (Berger and Altmann 2000, Tanaka et al. 2001, Von Groll et al. 2002). The SDD1 protease, which was identified by map-based cloning from the *Arabidopsis sdd1* mutant (*stomatal density and distribution 1-1*), is involved in the regulation of *Arabidopsis* stomatal density and distribution (Berger and Altmann 2000). ALE1 protease, which was isolated using a transposon-tagged allele *ale1-1* (*abnormal leaf shape1*), is required for cuticle formation on *Arabidopsis* embryos and juvenile plants (Tanaka et al. 2001). Both of these subtilisin-like serine proteases were suggested to play a role in processing a signal peptide or a receptor involved in cell–cell communication (Tanaka et al. 2001, Von Groll et al. 2002).

We propose that SaPIN2b might function in trichome development through inhibiting activities of proprotein convertase-like proteases, similar to SDD1 and ALE1 proteases. Consistent with this hypothesis, we found that *Escherichia coli*-expressed SaPIN2b is a potent proteinase inhibitor against subtilisin (data not shown). Our previous study also showed that the heterogeneously expressed SaPIN2a, a sister member of SaPIN2b in the nightshade

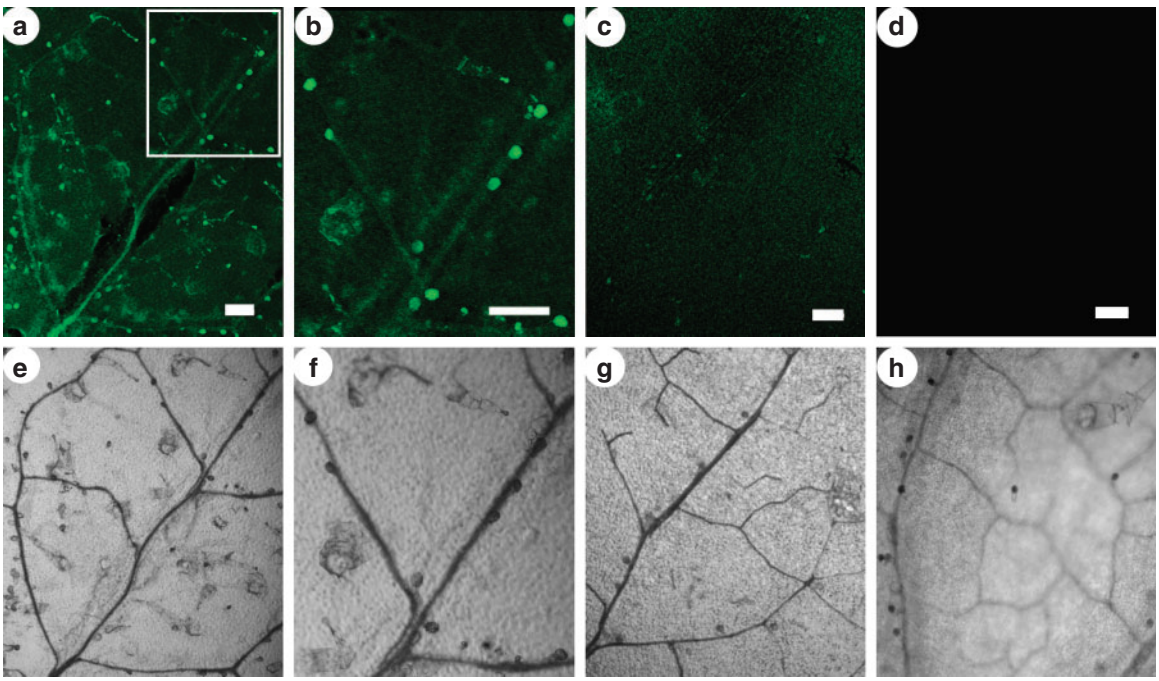

Fig. 6 Localization of *SaPIN2b* transcripts in nightshade leaves using whole-mount in situ RT–PCR. (a) Sample leaf. (b) High-magnification image of the boxed area in a. (c) The control without Taq DNA polymerase in the PCR. (d) The control without fluorescent dye in the PCR. (e–h) The bright field images of a–d, respectively. Bars, 100 μm.

PIN2 family, inhibited plant endogenous trypsin- and chymotrypsin-like activities in transgenic lettuce (Xu et al. 2004). Most recently, it has been demonstrated that SaPIN2b plays a crucial role in seed development, probably through regulating the activity of serine proteases (Chye et al. 2006, Sin et al. 2006). By transcriptome analysis, Aziz et al. (2005) have recently identified numerous ESTs with sequence similarity to various proteases and protease inhibitors in alfalfa glandular trichomes. The isolation of SaPIN2b and its target endogenous protease(s) from nightshade glandular trichomes will be necessary to determine the exact function of PIN2 in trichome development. In addition, the SaPIN2b promoter described in this study has potential applications in genetic engineering of plant trichomes for enhancing pest and disease resistance, heavy metal removal and molecular farming (Liu et al. 2000, Wang et al. 2002, Gutierrez-Alcala et al. 2005).

Materials and Methods

Plant material, wounding and MeJA treatments

Plant tissue cultures of nightshade (*S. americanum*) and tobacco (*Nicotiana tabacum* var. Xanthi) were maintained and propagated in a growth room at 25–28°C under a 16 h light/8 h dark regime. Plants in soil were grown under natural conditions in a greenhouse.

The 10 cm high T_1 progeny plants with 10 fully expanded leaves were employed for wounding and MeJA treatment. The third, fourth and fifth leaves (counted basipetally from the apex) of each plant were mechanically wounded with a forceps, across the midrib. At this time (time = 0), half of each leaf was excised with a razor blade along the midrib as the control (unwounded), and the other half left attached to the plants for wounding induction, which was harvested 24 h after wounding. For MeJA treatment, according to Xu et al. (1993), a 25 μM MeJA solution in sterile water/0.01% ethanol/0.125% Triton X-100 was applied to plants by aerial spraying twice with a 12 h interval. Control plants were sprayed with the same solution without MeJA. All samples harvested were directly placed into liquid nitrogen for protein extraction.

Cloning of the SaPIN2b promoter

The *SaPIN2b* promoter fragment was isolated using the Universal GenomeWalker Kit (Clontech, Palo Alto, CA, USA). Genomic DNA was isolated from leaves of *S. americanum* according to the procedure of Dellaporta et al. (1983). The genomic DNA was completely digested with four alternative restriction enzymes, *Dra*I, *Eco*RV, *Stu*I and *Pvu*II, and was ligated separately with the adaptor provided with the kit to obtain four restriction fragment libraries. PCRs were carried out with *SaPIN2b*-specific primers 2bGSP1 (5′-CTGTGTATCACATTCGAAGGTACAATC-3′) and 2bGSP2 (5′-GCAAGTATTTGGGTTTTTAGGGTCAGAACT-3′) and adaptor primers provided with the kit. The longest promoter fragment (1.9 kb) from the *Eco*RV restriction fragment library was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The resultant plasmid pTP2bE DNA was used as a template for sequencing.

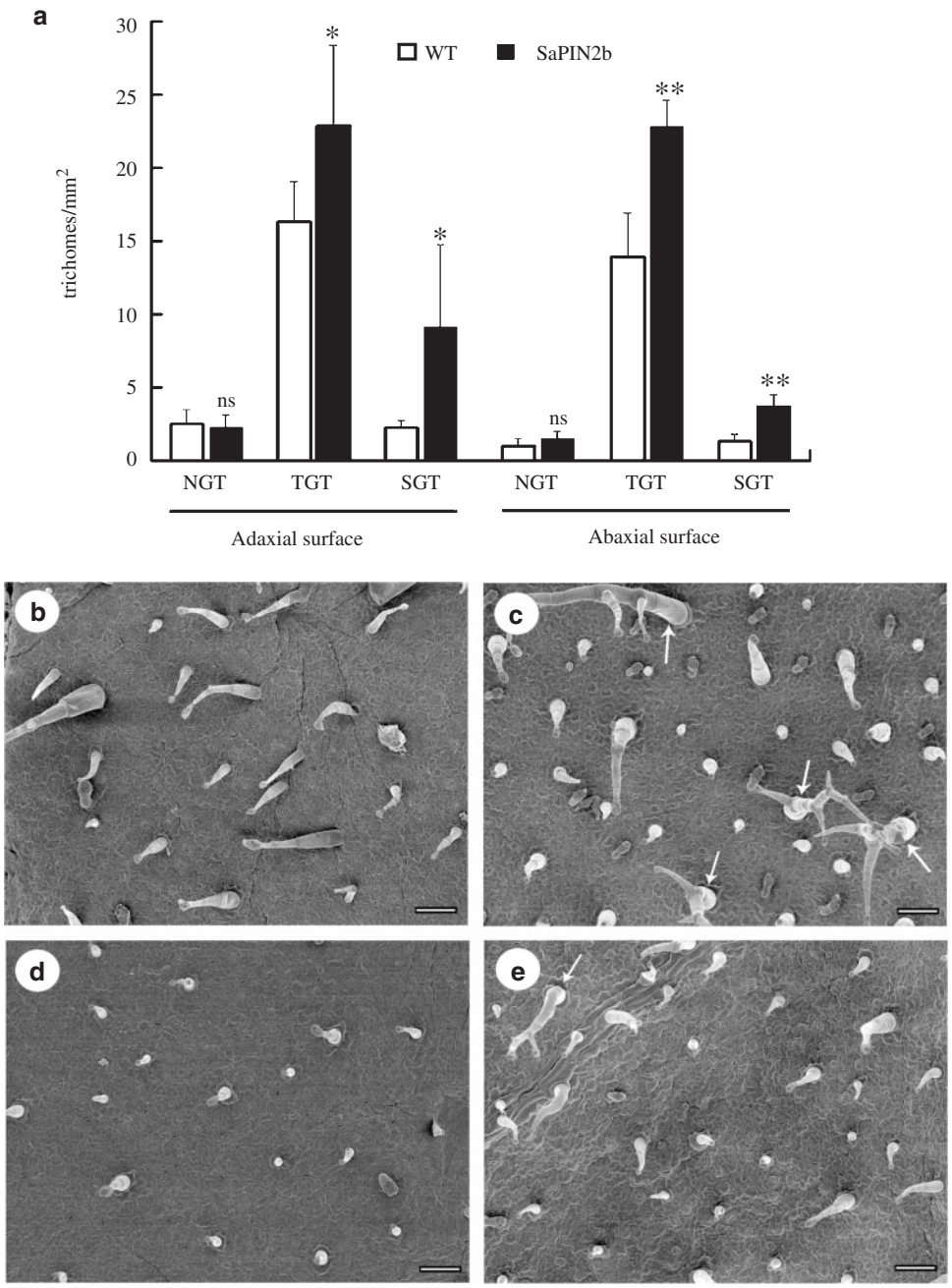

Fig. 7 Effects of overexpression of *SaPIN2b* on trichome development on tobacco leaves. (a) Densities of different types of trichomes from adaxial and abaxial leaf surfaces of wild-type (WT) and *SaPIN2b*-overexpressing (SaPIN2b) tobacco. Mean values plus standard errors are given. NGT, non-glandular trichome; TGT, tall glandular trichomes; SGT, short glandular trichomes; ns, not significant at the 5% level; * and **, significant at the 5 and 1% level, respectively. (b–e) Scanning electron micrographs of adaxial (b and c) and abaxial (d and e) leaf surfaces of wild-type (b and d) and *SaPIN2b*-overexpressing (c and e) tobacco. Arrows in c and e indicate branched glandular trichomes. Bars, 100 μm.

RNA ligase-mediated rapid amplification of 5′ cDNA end (RLM-5′ RACE)

Total RNA was extracted from *S. americanum* flower buds using TRIzol reagent (Invitrogen). The full-length 5′ cDNA of *SaPIN2b* was obtained by RLM-5′ RACE using a GeneRacer™ core Kit with Superscript™ II Module (Invitrogen, Carlsbad, CA, USA). The *SaPIN2b*-specific primer 2bGSP-1 was used for reverse transcription and 2bGSP-2 for PCR. The PCR product was cloned into pGEM-T easy vector (Promega). The resultant plasmid p2bTS-T DNA was used as a template for sequencing.

Construction of SaPIN2b promoter–GUS fusions

A 1.5 kb *SaPIN2b* promoter fragment (−1,430/+30) and its various deletions were PCR amplified using different primer pairs (Fig. 1) carrying a *Hin*dIII and a *Bam*HI restriction enzyme site. respectively. Each of the seven promoter fragments was digested with *Hin*dIII and *Bam*HI, and cloned into pBI101.1 (Jefferson et al. 1987), resulting in seven binary vectors (Fig. 2) that were transferred into *A. tumefaciens* LBA4404 and used for plant transformation.

Plant transfomation

The *Agrobacterium*-mediated leaf disc transformation method (Horsch et al. 1985) was employed to transform nightshade and tobacco plants with the binary vectors. Transformants were selected on MS medium (Murashige and Skoog 1962) containing kanamycin at 100 μg ml^{-1}.

Histochemical and fluorometric GUS assay

GUS analyses were carried out with the primary transformants (T_0 plants) and the progeny of the self-pollinated T_0 plants (T_1 plants). Plant tissues to be analyzed were submersed in GUS substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution overnight at 37°C as described by Matsuda et al. (2002), except using 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide instead. To exclude the possibility that GUS expression might be induced during overnight incubation, a 25 μM concentration of cycloheximide (Sigma, St Louis, MO, USA) was added to the X-gluc solution (Xu et al. 1993). The samples then were treated successively with 70–100% ethanol until chlorophyll was removed. The cleared samples were viewed with a microscope (Olympus BH2, Melville, NY, USA) and stored in 50% glycerol solution.

Fluorometric quantification of GUS gene expression in transgenic tissue was conducted according to Jefferson et al. (1987). The protein concentration was determined according to Bradford (1976). GUS specific activity [hydrolysis of 4-methylumbelliferone glucuronide (4-MU) mg^{-1} protein min^{-1}] was determined at 460 nm with a Hoefer® DyNA Quant® 200 (Amersham, Piscataway, NJ, USA) apparatus.

Liquid phase whole-mount in situ RT–PCR

Liquid phase whole-mount in situ RT–PCR was carried out following the procedures of Gray-Mitsumune et al. (2004) and Zhang et al. (2003). The whole leaves from soil-grown seedlings were fixed for 30 min in 1 : 1 heptane : fixation buffer [120 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 2.7 mM KCl, 0.1% Tween-20, 0.08 M EGTA, 5% formaldehyde and 10% dimethylsulfoxide (DMSO), pH 7.4], which was vigorously shaken for 15 min to mix the organic and water phases. Samples were subsequently dehydrated twice for 5 min in absolute methanol and three times for 5 min in absolute ethanol, and then stored for 1–3 d in ethanol at −20°C. The tissue was permeabilized by a rinse in absolute ethanol and an incubation for 30 min in 1 : 1 absolute ethanol : xylene. The tissue was then washed twice for 5 min in absolute ethanol, twice for 5 min in absolute methanol, and once for 5 min in 1 : 1 methanol : PBT [phosphate buffered saline (PBS) containing 0.1% Tween-20]. The tissue was post-fixed for 30 min in PBT containing 5% formaldehyde, followed by a rinse with PBS and two rinses with double-distilled water.

For the first strand cDNA synthesis, the samples were transferred into the PCR tubes containing reverse transcription (RT) buffer mixture [1 × RT buffer (Promega), 1 mM dNTP, 1 μM *SaPIN2b*-specific primer-1 (GSP-1, 5′-ggagccaagtcctcatggatgatc-3′), 1 U of RNase inhibitor (TAKARA SHUZO CO. LTD, Kyoto, Japan), 2.5 U of M-MLV reverse transcriptase (Promega)]. The tubes were then incubated at 42°C for 60 min. The PCR was first carried out without fluorescent nucleotides to amplify the cDNA and to saturate non-specific binding sites of fluorescent nucleotides. The RT mixture was replaced with PCR mixture [1 × Ex Tag buffer (TAKARA SHUZO CO. LTD), 0.2 mM dNTP, 1 μM each of forward primer (5′-gaaggagagagtgatgaacccaacgtt-3′) and reverse primer (GSP-1) and 0.1 U of Ex Tag polymerase (TAKARA SHUZO CO. LTD)]. The PCR was carried out for one cycle of 95°C/5 min; 30 cycles of 95°C/30 s, 62°C/30 s, 72°C/30 s; and one cycle of 72°C/10 min. The PCR mixture was then replaced with a new PCR mixture containing fluorescent nucleotides [1 × Ex Tag buffer, 0.2 mM each of dCTP, dGTP, dATP, 60 μM dTTP, 20 μM Alexa Fluor® 488-5-dUTP (Molecular Probes, Eugene, OR, USA), 1 μM each of primers and 0.1 U of Ex Tag polymerase]. The second PCR was performed with the same thermal regime as that of the first PCR except that the amplification cycles were reduced to 20 cycles. After the labeling reaction, samples were washed once in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), three times in PBS buffer, and stored in PBS buffer overnight at 4°C. The samples were observed under a confocal laser scanning microscope (Leica TCS SP2, Wetzlar, Germany) with an excitation wavelength of 488 nm and detection wavelengths between 510 and 520 nm.

Generation of transgenic tobacco plants overexpressing SaPIN2b

SaPIN2b cDNA was first cloned between the *Bam*HI and *Kpn*I sites of pHANNIBAL (Wesley et al. 2001) by replacing the sequence of the pdk intron, resulting in a construct pH2b. The expression cassette containing the cauliflower mosaic virus (CaMV) 35S promoter, *SaPIN2b* cDNA and the OCS terminator cut with *Not*I from pH2b was then inserted at the *Not*I site of the binary vector pART27 (Gleave 1992) to obtain the *SaPIN2b*-overexpressing construct pAH2b. *Agrobacterium tumefaciens* strain LBA4404 containing pAH2b was used for leaf disk transformation of *N. tabaccum* L. cv. Xanthi. Transgenic plants overexpressing *SaPIN2b* were identified by Northern blot analysis and were subjected to scanning electron microscopy.

Scanning electron microscopy

Leaf samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for 4 h at room temperature, washed in five changes of 0.1 M phosphate buffer (pH 7.2), and then post-fixed with osmium tetroxide (1% w/v) for 2 h at room temperature. Samples were then washed in five changes of 0.1 M phosphate buffer (pH 7.2), dehydrated in an ethanol series, and then displaced by 3-methylbutyl acetate for 10 min. Samples were critical-point dried with CO_2, followed by sputter coating with gold. Samples were examined with a Jeol field emission scanning electron microscope (Model JSM-6330F). Trichome counts were determined from photographs at low magnification (×100). Statistical analysis was carried out using the software SAS version 8.1.

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