A role for nuclear localised proteasomes in mediating auxin action

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Summary

A number of important cellular events in animals and yeast are regulated by protein degradation, and it is becoming apparent that such regulated proteolysis is involved in many facets of plant physiology and development. We have investigated the role of protein degradation by proteasomes in plants using NtPSA1, a tobacco gene that is predominantly expressed in young developing tobacco tissues and has extensive homology to yeast and human α-type proteasome subunit genes. The NtPSA1 cDNA was used to complement a lethal mutation of the yeast PRC1 α subunit gene indicating that NtPSA1 encodes a functional proteasome subunit, and transient expression of an NtPSA1::GUS protein fusion in onion cells confirmed that the nuclear localisation signal that is present in the NtPSA1 peptide sequence is active in plant cells. Plants transformed with an antisense NtPSA1 gene had reduced levels of NtPSA1 mRNA and exhibited reduced apical dominance. In addition, these low NtPSA1 plants displayed several morphological defects associated with auxin resistance such as reduced stamen length, and showed increased tolerance to high concentrations of auxin. These results support a role for nuclear localised proteasomes in floral development and auxin responses.

Keywords: proteasome, NLS, stamen, root, auxin, antisense.

Introduction

The 20S proteasome is a multisubunit protease found in both the nucleus and cytoplasm of all eukaryotic cells (Coux et al., 1996). It has a characteristic barrel shape created by the assembly of 28 subunits into four stacked rings; the two outer rings are each composed of seven α subunits and the two inner rings of seven β subunits. The β subunits form the proteolytic core of the 20S proteasome, entry to which is blocked by the N-terminal ends of the α subunits (Groll et al., 1997; Lowe et al., 1995). Substrate access to the catalytic sites of the proteasome is therefore achieved via interaction with other complexes (Baumesiter et al., 1996). For example, the 20S proteasome associates with the 19S complex to form the 26S proteasome, which is responsible for the degradation of ubiquitinated proteins.

The ubiquitin/26S proteasome system is considered to be the major non-lysosomal proteolytic pathway for the removal of intracellular proteins in eukaryotic cells. Protein substrates destined for degradation by this pathway are modified by the covalent attachment of a polyubiquitin chain, which serves as a recognition signal for the 26S proteasome. Ubiquitination is achieved by the action of three enzymes: an ATP dependent ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase (E3). The E3 protein ligases appear to be the major players in substrate recognition and thus determine the susceptibility of a protein to ubiquitination. The E3 enzymes can occur as single polypeptides however, there has been much evidence to suggest that many exist as multisubunit E3 complexes (Hershko and Ciechanover, 1998). One of the best characterised of these E3 complexes in plants is the SCF, which consists of four subunits a cullin, SKP1 and RBX1 and a F-box protein which interacts with the ubiquitinated substrate (Patton et al., 1998).

Recent studies in Arabidopsis have identified a number of F-box proteins acting in a variety of pathways these include: – TIR1 (TRANSPORT INHIBITOR RESPONSE 1),
which regulates auxin responses, UNUSUAL FLORAL ORGAN (UFO), which has an important role in determining floral organ identity, CORONATINE-INSENSITIVE 1 (COI1), which is essential for jasmonic acid regulated responses, ZEITLUPE (ZTL) and FLAVIN BINDING KELCH REPEAT (FKF1), which are, involved the circadian clock (del Pozo and Estelle, 2000). Such research has implicated the ubiquitin/proteasome pathway in a number of aspects of plant development. However, to date the most significant advances have been in the role this pathway plays in the response of plants to auxin. Significantly, two peptide aldehydes which are known to inhibit the chymotrypsin activity of eukaryotic proteasomes have recently been shown to inhibit AUX/IAA protein degradation (Ramos et al., 2001), and SCFTR1, has been demonstrated to interact with AUX/IAA proteins and promote their degradation (Gray et al., 2001).

Although there is accumulating evidence for the involvement of the ubiquitin/proteasome pathway in plant development, few studies have focused on the proteasome itself. The genes encoding the 20S proteasome of Arabidopsis have been identified (Fu et al., 1997) but the role of each of the subunits has yet to be determined. We have previously characterised a gene from tobacco, NtPSA1, which is homologous to the α proteasome subunit genes and is predominantly expressed in young dividing tissues (Bahrami and Gray, 1999). In this paper we present results to show that NtPSA1 can be translocated to the nucleus of plant cells and is able to rescue an α proteasome subunit mutation in yeast. We also describe the phenotype of transgenic plants with reduced NtPSA1 gene expression.

**Results**

The NtPSA1 cDNA complements a yeast α proteasome subunit mutant

The predicted NtPSA1 protein sequence (Bahrami and Gray, 1999) shows considerable homology to several α proteasome subunit sequences including that of the yeast *Saccharomyces cerevisiae*, encoded by the PRC1 gene (Lee et al., 1991). Disruption of PRC1 in yeast has been shown to be lethal (Balzi et al., 1989; Fujiwara et al., 1990). However, a mutant allele of this gene, named scl1-1, acts as an extragenic suppressor of the temperature sensitive (ts) lethal mutation, crl3 (Balzi et al., 1989; McCusker and Haber, 1988). To determine if NtPSA1 functions in a similar manner to Prc1p, we used the NtPSA1 cDNA to complement the yeast scl1-1 mutation. The full-length cDNA sequence of NtPSA1 containing the initiation codon and open reading frame was transformed into the double mutant scl1-1, crl3 (Y55-282) (Balzi et al., 1989; McCusker and Haber, 1988) and transformants selected on uracil minus minimal media. Northern analysis confirmed the expression of NtPSA1 in Y55-282 cells (Figure 1c). Control and NtPSA1 transformed cultures were grown on minimal media at both the permissive and restrictive temperatures of 28°C and 37°C. Control cultures grew at both temperatures, but the NtPSA1 transformants were only able to grow at 28°C and failed to grow at 37°C (Figures 1a,b). NtPSA1 is therefore able to complement the scl1-1 mutation in the Y55-282 double mutant, restoring the temperature sensitive phenotype of the crl3 mutation. Thus, the tobacco NtPSA1 gene encodes a protein that is able to function in an analogous manner to its counterpart in yeast.

NtPSA1 encodes an authentic nuclear localisation signal

Towards the N-terminus of the deduced NtPSA1 protein sequence there is a potential NLS motif of KKKMK (Bahrami and Gray, 1999), which matches the consensus motif of K(K/R)X(K/R), that is found in several other α subunits (Tanka et al., 1990). There are also several putative phosphorylation sites in the sequence surrounding the NLS. Such sites have also been reported in other proteasome subunits and in some cases these have been shown to be necessary, in conjunction with the NLS, to target the protein to the nucleus (Tanka et al., 1990). To investigate whether the putative NLS in the NtPSA1 peptide sequence functions in vivo we carried out transient gene expression studies using an NtPSA1::GUS protein fusion construct. This construct and controls of vector alone, and a GUS fusion with the NLS of the Opaque 2 protein (PNR2012) (Varagona et al., 1992), were bombarded into onion epidermis. Histochemical staining was then carried out to locate GUS activity in cells expressing the transgenes. Cells bombarded with the NtPSA1::GUS fusion construct showed much higher accumulation of GUS activity in their nucleus compared with that in the cytoplasm indicating that the fusion protein was present in the nucleus (Figure 2a). In control experiments with cells transformed with the vector alone, we found no difference between the cytoplasmic and nuclear GUS activity (Figure 2b). The positive control, cells transformed with the PNR2012 construct, showed nuclear localisation of GUS activity (Figure 2c). NtPSA1 therefore contains an NLS that is capable of targeting a peptide to the nucleus of onion cells.

**Antisense NtPSA1 plants**

To study the role of this proteasome subunit in plants NtPSA1 antisense plants were produced. The effect of the antisense constructs on NtPSA1 mRNA levels was examined in five transgenic lines (1, 2, 10, 11 and 13). RNA was extracted from seedlings and semiquantitative RT-PCR analysis was carried out to compare the mRNA levels of

NtPSA1 in the antisense and control seedlings. A reduction in the amount of amplified NtPSA1 fragment was apparent in all five antisense lines tested with the most severe reductions noted in lines 2 and 10 (Figure 3). Lines 2 and 10 were selected for further study and RNA gel blot analysis used to confirm the reduced levels of NtPSA1 mRNA in these plants (Figure 3c).

Antisense NtPSA1 transgenic plants have increased shoot branching and reduced fertility

The antisense NtPSA1 plants were shorter and more branched than the control plants suggesting that they had reduced apical dominance (data not shown). These plants also failed to set seed or produced very little seed. Closer examination of the T1 antisense plants (lines 2 and 10) revealed that their flowers possessed shorter stamens relative to the control plants (Figure 4). To quantify this apparent shortness the stamen from 15 different flowers from each line were measured (Figure 4). The average length of stamen from line 10 was 29.86 mm ± 0.86 (SEM), from line 2 flowers 30.53 mm ± 0.85 (SEM) and from control flowers 35.06 mm ± 0.63 (SEM). Using a T-test, stamens from line 2 and line 10 flowers were both...

Figure 1. Functional complementation of scl1-1 by NtPSA1 cDNA. Yeast mutant, Y55-282, transformed with pYES2 vector only (rows 1 and 3) grows at both 28°C (a) and 37°C (b). The strain complemented with NtPSA1 (rows 2 and 4) cannot grow at 37°C. (c) Northern blot confirms the expression of NtPSA1 mRNA in complemented yeast cells (lane 2) and not in the control line (lane 1). Ethidium bromide staining of 28S ribosomal RNA to confirm equal loading is shown in the panel below.

Figure 2. Histochemical localisation of GUS activity in bombarded onion epidermal cells. (a) Nuclear localisation of NtPSA1::GUS; (b) cytoplasmic location of GUS control bombarded with empty vector pBI221; and (c) nuclear location of Opaque2::GUS. Tissues were stained simultaneously with X-gluc (a, b and c) and nuclei-specific DAPI (d, e and f). nu: nucleus, bars = 50 μm.

Figure 3. Reduced levels of NtPSA1 mRNA in NtPSA1 antisense lines. (a) RT–PCR with NtPSA1 specific primers using antisense lines indicated below lanes, compared to control line transformed with empty vector pBIN19. (b) RT–PCR using ubiquitin specific primers to confirm equal level of cDNA template in each PCR. (c) RNA gel blot with antisense 2, 10 and control lines. (d) 25S RNA levels revealed by ethidium bromide staining to indicate loading.

found to be significantly shorter than controls ($P < 0.005$). This short stamen phenotype co-segregated with kanamycin resistance in 20 T1 generation plants suggesting that this phenotype is due to the presence of the NtPSA1 antisense transgene.

The resultant increased distance between the anthers and the stigma surface in these flowers may be the cause of their apparent infertility as these flowers were fertile when pollinated manually.

**NtPSA1 antisense transgenic plants show reduced sensitivity to auxin phytohormone**

The NtPSA1 antisense plants exhibited defects in apical dominance and elongation of stamens (Figure 4); both are processes that are associated with the action of the phytohormone auxin (Koning, 1983; Lincoln et al., 1990). We therefore carried out experiments to investigate whether other aspects of auxin action were affected in the NtPSA1 antisense plants.

First, we examined the gravitropic response of NtPSA1 antisense seedlings. Seedlings from line 10, line 2 and controls were grown on solid medium in a vertical position and after 7 days the dishes were rotated by $90^\circ$. Within 24 h after dish reorientation the root tips of controls had begun to bend but no change was observed in the orientation of the antisense root tips. 48 h after dish reorientation the root tips of the control plants had reorientated by $90^\circ$ to grow downwards, but the antisense NtPSA1 root tips had only just begun to reorientate (Figure 5). The antisense NtPSA1 roots had fully reorientated by 96 h. Both control and antisense roots elongated at the same rate throughout the experiment.

Next we examined the effect of various auxin concentrations on the growth of antisense NtPSA1 roots. At low concentrations auxin is known to promote growth whilst at high concentrations it is inhibitory. Seedlings from line 10, line 2 and the control line were grown on solid medium and transferred to media containing 0–10 $\mu$M 2, 4 Dichlorophenoxy acetic acid (2, 4 D). At concentrations of 10 $\mu$M root growth was completely inhibited in all plants. At lower auxin concentrations (0.01–5 $\mu$M 2, 4 D) root growth was inhibited in all lines tested in a dose dependent manner but the retardation of root elongation was much less severe in the roots of NtPSA1 antisense seedlings (Figure 6a). The lack of inhibition of root growth by auxin could also be clearly seen in older NtPSA1 antisense plants (Figures 6b,c).

These results indicate that the NtPSA1 antisense plants have increased tolerance to high levels of auxin (being tolerant to approximately 5-fold higher levels of 2, 4 D in the root growth experiment). Their phenotype has similarities with Arabidopsis mutant plants described as auxin resistant, e.g. axr1 (Lincoln et al., 1990).

**Discussion**

**NtPSA1 protein has the same function as its counterpart in yeast**

We have studied an $\alpha$ proteasome subunit from tobacco which has over 50% overall identity with its counterparts in yeast (Yc7u/PRC1) and humans (PRC1) (Bahrami and Gray, 1999). Our results indicate that this conservation of primary sequence is also maintained in the conservation of function of NtPSA1. The NtPSA1 protein is able to complement the scl1-1 mutation of its yeast counterpart (Figure 1). This would indicate that NtPSA1 functions in a similar manner to its ortholog Prc1p and assumes the same position within the 20S proteasome, thus reflecting recent studies, which have shown that the arrangements of subunits in yeasts and humans are conserved (Dahlmann et al., 1999). Other $\alpha$ subunits of the Arabidopsis 20S proteasome have previously been shown to complement mutations in their yeast counterparts (Fu et al., 1998). Thus our results, along with those of Fu et al. (1998), would suggest that the function of the $\alpha$ subunits has been conserved from yeast to plants and perhaps to humans.

**NtPSA1 protein has a functional NLS**

Several of the proteasome $\alpha$ subunits of yeast, human and Drosophila carry a putative NLS sequence (Knehl et al., 1996; Tanka et al., 1990; Tanka et al., 1992). The function of this NLS sequence has been examined using reporter protein experiments to demonstrate in vitro targeting of a peptide to the nucleus (Knehl et al., 1996; Nederlof et al., 1995). The deduced protein sequence of NtPSA1 also contains a putative NLS and several phosphorylation sites, which have been suggested to be involved in the nuclear translocation of the subunit (Tanka et al., 1990). We examined the subcellular location of this tobacco $\alpha$ subunit using a GUS-protein fusion and found that NtPSA1 is able to target the fusion protein to the nucleus of onion epidermal cells (Figure 2). The conservation of the NLS and phosphorylation sequences accompanied by functional activity in vitro would suggest that these subunits play an important role in controlling the entry of the proteasome to the nucleus.

Various studies have shown that the 20S and 26S proteasome are located in both nucleus and cytoplasm (Palmer et al., 1996; Schauer et al., 1993). The role that the proteasome plays in these different subcellular locations has yet to be determined. However, the proteasome is predominantly nuclear located in actively dividing cells (Klein et al., 1990; Pahl and Baeuerle, 1996) and is found at varying locations within the nucleus, dependent on the stage of the cell cycle (A mestramd et al., 1993; Kawahara et al., 1994).
and Yokosawa, 1992), suggesting that nuclear located proteasomes are involved in regulating the cell cycle.

In plants nuclear localisation of the proteasome may play an important role in photomorphogenesis. Levels of nuclear located transcription factor HY5 increase 15–20 fold in the light compared with the dark and this increase in HY5 levels can be directly correlated to the degree of photomorphogenesis (Osterlund et al., 2000). The alteration in HY5 abundance results from an increased proteasomal degradation of HY5 in the dark, a process that is thought to involve COP1 (Osterlund et al., 2000). COP1 shows similarities to Ring H2 E3 ligases proteins and can directly interact with HY5 (Ang et al., 1998). However, the COP1 protein is excluded from the nucleus during the light (Deng et al., 1992; von Arnim and Deng, 1994). Thus, in the darkness nuclear located COP1 targets HY5 for degradation, which is likely to occur via nuclear located proteasomes and may recruit the COP9 signalsome in this process. This would predict that NtPSA1 antisense plants would exhibit some degree of de-etiolation in the dark, a hypothesis that is currently being investigated.

**Antisense NtPSA1 plants have an auxin resistant phenotype**

In recent years several mutants in auxin action have been isolated which have provided new insights into the role of proteolysis in auxin action (Gray and Estelle, 2000). For example, the auxin resistant 1 (axr1) mutant of *Arabidopsis* exhibits a number of defects associated with reduced response to auxin (Lincoln et al., 1990). The AXR1
gene has been cloned and shown to encode a protein with high similarity to the N-terminal half of ubiquitin-activating E1 enzymes (Leyser et al., 1993) and has been shown to activate the ubiquitin like protein RUB1 (del Pozo et al., 1998). The auxin transport inhibitor resistant mutant (tir1) also exhibits a reduced response to auxin and is a member of the F-box protein family (Ruegger et al., 1998). It has been suggested that RUB activation by AXR1 leads to modification of the cullin in the SCF complex in plants favouring formation of SCF containing TIR1, which would promote degradation of repressors of the auxin response. In the absence of such degradation the auxin response pathway is not activated in the presence of auxin resulting in a reduced response to auxin (Gray et al., 2001). We show here that the antisense NtPSA1 plants also exhibit a reduced auxin response. However it is not known whether the tobacco auxin response pathway acts in a similar manner to that in Arabidopsis.

The Arabidopsis AXR1 protein is primarily located in the nuclei of dividing cells (del Pozo et al., 1998) leading to the suggestion that auxin responses could be mediated through RUB1 modification of certain nuclear proteins. NtPSA1 and the PS-IAA4-like protein family of early auxin induced genes all contain functional NLSs, indicating that a ubiquitin proteasome pathway could mediate auxin action via the degradation of short-lived auxin responsive proteins in the nucleus. Significantly, two peptide aldehydes, which are known to inhibit of the chymotrypsin activity of eukaryotic proteasomes, have recently been shown to inhibit Aux/IAA protein degradation (Ramos et al., 2001).

Together all these studies suggest a central role for the ubiquitin/proteasome pathway in the auxin response. This hypothesis is supported by our results. The NtPSA1 antisense plants possess shorter stemmers compared with control plants (Figure 4), a phenotype also displayed by the axr1 mutant and believed to be mediated by auxin (Lincoln et al., 1990). Further investigations revealed that the NtPSA1 antisense plants have a degree of insensitivity to auxin, the roots of the antisense plants were able to continue to grow at non-permissive auxin concentrations (Figure 6). In addition, NtPSA1 antisense seedlings exhibited retarded root gravitropism, taking 4 days rather than the 2 days taken by controls to reorientate (Figure 5). Targeted proteolysis has been previously implicated in auxin signalling, and our results provide further evidence for the involvement of proteolytic degradation by the proteasome in the auxin response.

In summary, the results presented in this paper and elsewhere lead us to suggest that a ubiquitin/26S proteasome-like pathway plays a role mediating a range of plant processes including responses auxin via the degradation of nuclear proteins.

Experimental procedures

Plant materials

Tobacco plants (Nicotiana tabacum cv. Wisconsin 38) were maintained as previously described (Li and Gray, 1997), and white onions were purchased and used fresh.

Complementation of yeast cells

Saccharomyces cerevisiae strain Y55-282 (HO, crl3-2, trp5-1, scl1-1, ade1-1, ura3-1) (McCusker and Haber, 1988) was used as scl1-1, crl3 host for complementation studies. NtPSA1 (Bahrami and Gray, 1999), nucleotide accession number Y16644 was isolated from a tobacco-style cDNA library (Li and Gray, 1997). The coding region of the NtPSA1 cDNA (nucleotides 57–586) including its initiation codon and stop codon was used as a DNA template for the constructs. This region was amplified by PCR using oligonucleotide primers (forward: AGCAATCTAGAAAATGAGTAGAGGCG and reverse: ATAGGATCTAGGCAACATACATTTC) and cloned into the K121T-cloning vector (MBI). The construct was excised by KpnI/BamHI restriction, ligated into pYES2 yeast expression vector downstream of the PGAL1 promoter, sequenced to ascertain the integrity of the coding region and transformed into competent cells prepared from the yeast mutant. This construct, and the pYES2 plasmid with no insert as a negative reference, were electroporated into the yeast mutant strain, Y55-282, using the Bio-Rad Gene Pulser (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), as previously described (Becker and Guarente, 1991).

Transformants were selected on synthetic minimal (SD) medium, minus uracil (Sherman, 1991). Physiological studies, to check the complementation of the mutant with the NtPSA1 construct, were carried out by treating the transformed cells at different temperatures. A single colony of the transformants, both control and NtPSA1 constructs, were grown for 2 days on minimal SD medium, without uracil and replacing glucose with galactose as carbon source, at 28°C. Four serial dilutions of the cultures (1 in 5), were prepared in the same medium. 10 μl of the diluted suspensions were spotted on to the same media, in separate rows and incubated at 28°C or 37°C. The growth of the NtPSA1 transformed mutants was compared with that of controls.

Nuclear localization by transient transformation

The NtPSA1 coding region (nucleotides 57–822) was amplified by PCR using internal primers (forward: AGCAATCTAGAAAATGAGTAGAGGCG and reverse: GACTGGATCTAGGCAACATACATTTC) cloning the K121T-cloning vector (MBI). The PCR product was digested at KpnI/BamHI restriction sites included at the 3' and 5'-ends and ligated into pBI221 plant expression vector (BD Biosciences Clontech, Oxford UK) downstream of the CaMV 35S RNA promoter and fused in frame upstream of the β-glucuronidase (GUS) reporter gene. The construct was checked by DNA sequencing.

A helium biolistic gene transformation system (Bio-Rad) was used to transiently transform onion cells with the NtPSA1::GUS fusion protein construct. Onion cells were also transformed with pBI221 without insert and pNVR2012, containing the efficient bipartite NLS B of the Opaque2 (O2) gene fused to the GUS gene (Varagona et al., 1992). Inner epidermal single cell layers of the onions were peeled and placed on MS104 solid medium. 5 μg of column (Qiagen Ltd., Crawley, West Sussex, UK) purified and water dialysed, plasmid DNA was precipitated onto 1.6 μm gold nanoparticles and electroporated into the onion cells using a Bio-Rad GenePulser. After electroporation, the constructs were incubated for 1 h at 4°C before incubation for 3 days at 30°C in MS medium, minus uracil (Sherman, 1991). Physiological studies, to check the complementation of the mutant with the NtPSA1 construct, were carried out by treating the transformed cells at different temperatures. A single colony of the transformants, both control and NtPSA1 constructs, were grown for 2 days on minimal SD medium, without uracil and replacing glucose with galactose as carbon source, at 28°C. Four serial dilutions of the cultures (1 in 5), were prepared in the same medium. 10 μl of the diluted suspensions were spotted on to the same media, in separate rows and incubated at 28°C or 37°C. The growth of the NtPSA1 transformed mutants was compared with that of controls.

particles (Bio-Rad) using, 2.5 mM CaCl$_2$ and 1 mM spermidine (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK). DNA coated particles were rinsed with absolute ethanol, resuspended in ethanol, and used for bombardment of the onion cells at 1300 psi. The bombarded tissue was incubated for approximately 18 h in the dark before histochemical analysis.

*NtPSA1::GUS* fusion protein was detected in transformed cells, by histochemically staining the bombarded onion epidermis with X-gluc (Jefferson *et al*., 1987). The position of the nucleus in each cell was determined by staining with 4', 6-diamidino-2-phenylindole (DAPI) (Varagona *et al*., 1992). X-gluc staining was detected and photographed in transformed cells under an Olympus (Southall, Middlesex, UK) Axiphot microscope with bright-field optics, and the DAPI stained nuclei of the same cells were visualised using fluorescence optics.

**Production of antisense *NtPSA1* plants**

Three regions of the *NtPSA1* coding region were used to produce antisense constructs. DNA fragments of 775 bp (nucleotides 172–447), and 197 bp (nucleotides 564–761), were used to specifically target suppression of *NtPSA1* over other α subunit genes. The full-length coding region (57–856) was also used. These regions were PCR amplified using internal primers (forward/reverse) ATACACCTCATTGGCGTCC/CGATGCTGTGTGGAACAC, respectively. The amplified cDNA fragments were ligated into pUC57-T (MBI Fermentas, Funabashi, Japan). The amplification product was subcloned into pBIN19 binary transformation vector.

Antisense constructs were electroporated into *Agrobacterium tumefaciens* LBA4404 using a Bio-Rad Gene Pulser (2.5 V; 400 μF; and 25 μF). Tobacco leaf discs were incubated with *Agrobacteria* (as described (Walkerpeach and Velten, 1992). Transgenic plants were regenerated on MS104K medium (4.4 gl±1 MS salts plus B5 as described (Walkerpeach and Velten, 1992). Transgenic plants released, with XbaI and BamHI and ligated into pDH51, between the CaMV 35S promoter and terminator, released with EcoRI and subcloned into pBIN19 binary transformation vector.

Antisense constructs were electroproporated into *Agrobacterium tumefaciens* LBA4404 using a Bio-Rad Gene Pulser (2.5 V; 400 μF; and 25 μF). Tobacco leaf discs were incubated with *Agrobacteria* as described (Walkerpeach and Velten, 1992). Transgenic plants were regenerated on MS104K medium (4.4 gl±1 MS salts plus B5 vitamins; 30 gl±1 sucrose; 0.1 gl± 1 naphthalene acetic acid; 1.0 gl±1 benzylaminopurine; 200 gl±1 kanamycin; 9 gl±1 agar), containing 500 mg l±1 carbenicillin and rooted on similar medium minus auxin. Leaf discs were transformed with *Agrobacterium* carrying the pBIN19 vector only, to provide control plants. Following selection on kanamycin the presence of the transgene was confirmed in each plant by genomic Southern blotting or PCR analysis (results not shown).

Independently transformed antisense plant lines were selected for study. Plants 1–8 contained the 278 bp construct, plants 9, 10 the 197 bp construct and plants 11–13 contained the complete open reading frame construct.

**Estimation of *NtPSA1* mRNA levels by Reverse Transcription–Mediated PCR and RNA gel blot analysis**

Seedlings (T2) were grown on solid 0.5x MS medium for 3 weeks. Total RNA was extracted using an RNA extraction kit (Qiagen) and RT–PCR performed using reverse transcriptase (Promega Ltd., Southampton, UK) and internal primers for *NtPSA1*, forward ATACACCTCATTGGCGTCC and reverse CCTCGGTAGTTAACCTGT, designed to flank introns on the tomato genomic DNA. At the same time a PCR was performed on genomic DNA and total RNA, without the reverse transcription reaction, as size marker and negative control, respectively (not shown). To confirm that equal amounts of RNA were present in reactions separate RT–PCR reactions were carried out on the same RNA samples using primers complementary to the tomato ubiquitin gene, which was determined not to reach saturation amplification levels (data not shown). Similar results were obtained on three separate sets of seedlings.

For Northern analysis total RNA was extracted from young tissues of tobacco plants using an RNA extraction kit (Qiagen). 10 μg of total RNA samples were electrophoresed on a 1% deaerinating gel agarose gel and transferred onto Hybond N membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) by capillary blotting in 10X SSC buffer. Filters were hybridised onto antisense digoxigenin labelled RNA probe as described (Bahrami and Gray, 1999).

**Physiological studies of transgenic tobacco plants**

The gravitropic response of *NtPSA1* antisense seedlings (lines 2 and 10, T2 generation) was tested. Antisense and control seedlings were grown on MS medium at 25°C under light in a vertical position with the roots downwards. After 7 days the root tip positions were marked, and the dish rotated by 90°. The root tip positions were compared between control and transgenic seedlings for 4 days. At least 23 seedlings were examined for each plant line.

The auxin sensitivity of *NtPSA1* antisense seedlings was tested (lines 2 and 10, T2 generation). Seedlings, grown as above were transferred to fresh MS plates containing 2, 4 D (Sigma), ranging in concentration from 0 to 10 μM using at least 16 seedlings per line at each auxin concentration. The rate of root growth was compared between transgenic and control seedlings.

To test whether the short stamen phenotype co-segregated with the presence of the transgene, seedlings (line 10, T1) were grown on compost and leaf discs from 20 plants were incubated on MS104K medium. Plants that were kanamycin resistant were identified by callus producing discs and scored as transgenic.

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