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Expression of a Chinese Cabbage Cysteine Proteinase Inhibitor, BrCYS1, Retards Seed Germination and Plant Growth in Transgenic Tobacco Plant

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Phytocystatins are plant cysteine proteinase inhibitors that regulate endogenous and heterologous cysteine proteinases of the papain family. A cDNA encoding the phytocystatin BrCYS1 (*Brassica rapa* cysteine proteinase inhibitor 1) has been isolated from Chinese cabbage (*B. rapa* subsp. *pekinensis*) flower buds. In order to explore the role of this inhibitory enzyme, tobacco plants (*Nicotiana tabacum* L. cv. Samson) containing altered amounts of phytocystatin were generated by over-expressing *BrCYS1* cDNA in either the sense or the antisense configuration. The resulting plants had *in vitro* enzyme inhibitory activities that were over 10% of those detected in wild type plants. The transgenic plants exhibited retarded seed germination and seedling growth and a reduced seed yield, whereas these properties were enhanced in antisense plants. These data suggest that BrCYS1 participates in the control of seed germination, post-germination and plant growth by regulating cysteine peptidase activity.

Keywords: cysteine proteinase, gene expression, inhibitory activity, phytocystatin, sense and antisense, transformation

Cysteine proteinase inhibitors (CPIs) are ubiquitous among microorganisms, animals, and plants (Turk and Bode, 1991). CPIs have been categorized into four major families based on their sequences and chemical properties. These families include the stefins, cystatins, kininogens, and the phytocystatins (PhyCys) (Barrett, 1987; Kondo et al., 1991; Misaka et al., 1996).

The PhyCys, CPIs of plant origin, are widely distributed and have been found in several different species of higher plants (Abe et al., 1987; Abe et al., 1992; Fernandes et al., 1993; Song et al., 1995; Lim et al., 1996; Misaka et al., 1996; Pernas et al., 1998; Gaddour et al., 2001; Kuroda et al., 2001). Margis et al. (1998) suggested that the PhyCys proteins could be divided into three groups (groups-I, -II and -III) based on their divergent amino-terminal consensus sequences and molecular masses. In addition, PhyCys group-I could be subdivided into three subgroups: subgroup-Ia, which contains members with a single copy of the inhibitory domain; subgroup-Ib, which contains multicystatin; and subgroup-Ic, which contains members with the inhibitory domain linked to a second domain of similar size but of unknown function located in the carboxyl (C)-terminal region. Among these subgroups, group-Ia has been extensively studied. Members of this subgroup have been linked to multiple physiological processes, including the regulation of seed ripening and/or germination (Abe et al., 1987; Abe et al., 1994; Hong et al., 2007), inhibition of exogenous proteinases such as digestive enzymes in the gut of insect pests and nematodes (Irie et al., 1996; Botella et al., 1996; Vain et al., 1998; Lecardonnel et al., 1999), regulation of the proteolytic enzyme activity of cysteine proteinases (CPs) in programed cell death (Solomom et al., 1999), and protection of cytosolic metabolism from the effects of the intercellular release of CPs through incidental rupturing of protein bodies (Kondo et al., 1990; Turk and Bode, 1991). Despite this knowledge, the PhyCys subgroup-Ic has been studied in far less detail, and neither the physiological functions of members of this group nor the role of the C-terminal region have been fully investigated. In addition, most of the transgenic studies have focused on biodefensive roles against insects and all of the transgenic plants were transfomed by subgroup-Ia genes (Masoud et al., 1993; Benchekroun et al., 1995; Irie et al., 1996; Bonade-Bottino et al., 1999).

In order to determine the effects of PhyCys subgroup-Ic expression in transgenic plants, a cDNA of *BrCYS1* under the control of the cauliflower mosaic virus (CaMV) 35S promoter, was transferred to tobacco plants. The effect of this inhibitor on normal plant development was examined.

MATERIALS AND METHODS

Generation of Transgenic Tobacco Plants

In order to construct the transgenic tobacco plants (*Nicotiana tabacum* cv. Samson), *BrCYS1* cDNA (GenBank accession number L41355) was ligated into a binary vector, *pBI121* (Clontech, USA), under the control of the CaMV 35S promoter in a sense (*BrCYS1-S*) and antisense orienta-

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tion (*BrCYS1-AS*) (Hong et al., 2007). Each of the recombinant plasmid and control vectors were introduced into *Agrobacterium tumefacience* LBA4404, and transformed tobacco plants were obtained by the leaf disc transformation method (Jin et al., 2005). The presence of transgenes in kanamycin-resistant plants was verified by PCR using genomic DNA (Jin et al., 2002) with the primers *BrCYS1-F* (5′-ACGACATGGCCATGCTCG-3′) and *BrCYS1-R* (5′-ACTAGTCATGGTGTTGCTG-3′). T_3 homozygous lines were derived as described by Koo et al. (2002). Plants expressing a high level of BrCYS1 were used for the experiments and were maintained at 25°C during the day and 20°C at night with a 16 hr photoperiod.

Western Blot Analysis

In order to determine the expression level of BrCYS1 proteins, unpurified antisera was used for immunoblot experiments as described previously (Hong et al., 2003). Proteins were extracted from seeds and seedlings, and supernatant proteins were separated by 12.5% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Protein bands were detected using a western blotting detection kit (ECL kit, Amersham Bioscience, UK) after incubation with horseradish peroxidase-conjugated anti-goat IgG (ICN Biomedicals, USA).

Seed Germination Assay

Seed lots to be compared were harvested on the same day from individual transgenic plants grown in identical environmental conditions. All of the germination experiments were performed in 9-cm Petri dishes on water soaked filter paper. Each line was sown in triplicate (100 seeds from one individual plant per 9-cm Petri dish). The average percentage of germinated seeds was determined after 2-days of incubation in a climate-controlled room (25°C, 16 hr light/day). For seedling growth comparisons, seedlings were incubated for 7 days prior to the measurement of hypocotyl length (Hong et al., 2007).

Inhibitory Activity Assay

To determine the inhibitory activity of the BrCYS1 protein in transgenic tobacco plants, papain (EC 3.4.22.2) was used for inhibitory experiments as described by He and Kermode (2003) with minor modifications. Fresh or frozen tobacco leaves and seeds were ground in liquid nitrogen and extracts were generated using a buffer containing 100 mM Tris-HCl [pH 7.5], 150 mM NaCl. The recombinant BrCYS1 protein has a strong inhibitory activity at high temperatures (Hong et al., 2008); therefore, the extracts were heated to 80°C for 10 min, and soluble proteins were recovered by centrifugation. Supernatants were transferred into tubes, and protein concentrations were determined using a Bradford protein assay kit (BioRad, USA) with bovine serum albumin as the standard. Two hundred microliters of protein were incubated with 0.1 mL of a papain solution (25 mM sodium phosphate buffer, 25 $\mu g\ mL^{-1}$ papain, pH 6.0) at 37°C for 10 min. The reaction was started by adding 0.2 mL 1 mM α-N-benzoyl-DL-arginine-2-naphthylaminde (BANA, Sigma) as the chromogenic substrate (Zhao et al., 1996), and incubation continued for another 10 min. The reaction was terminated by the addition of 1 mL 2% (v/v) HCl/ethanol. The colored product was developed by the addition of 0.06% (v/v) ρ-dimethyl-aminocinnamaldehyde (Sigma)/ethanol and the absorbance of the reaction mixture was measured at 540 nm. The reaction rate was expressed as Δ_{A540} $mL^{-1}\ h^{-1}$.

RESULTS AND DISCUSSION

Establishment of Transgenic Tobacco Plants

Following co-cultivation of *N. tabacum* with *A. tumefacience* containing *BrCYS1-S* and *BrCYS1-AS* (Hong et al., 2007), we were able to isolate 51 independent sense and antisense lines (T_1) on selective media containing kanamycin. Genomic PCR analysis of the plants from these 51 independent lines revealed the presence of both *npt II* and the *BrCYS1* sequence in each plant (data not shown). To determine the number of *BrCYS1* copies in the transgenic plants, T_1 plants were self-pollinated and the progeny (T_2) were segregated on selection media. Among 51 independent transgenic lines, the progeny of 44 lines exhibited a 3:1 ratio (resistant to susceptible) on selection media, indicating that the transgene integrated at a single locus (Katiyar-Agarwal et al., 2003). Following self-pollination of the single locus T_2 lines, four independent homozygous T_3 lines (i.e., Nts-②, Nts-③, Ntas-⑤, and Ntas-⑧) were selected for further study. These transformants produced no wild-type (WT) *CYS* band by PCR with the *BrCYS1* specific primers verifying that they are homoplastomic (Fig. 1a).

Transgenic lines that contained the *BrCYS1* sequence were analyzed for protein expression by Western blot analysis (Fig. 1b). Two protein bands of apparent molecular mass 23 and 24 kDa were observed in samples of heated seedling extracts from two independent sense transformants; however, the 23 kDa BrCYS1 band was absent in comparable extracts from untransformed and antisense plants. Results of the immunoblot analysis revealed that BrCYS1 accumulated as a mature protein in heterologous tobacco plants and suggested that the BrCYS1 polyclonal antibody cross-reacted with another 24 kDa tobacco CYS (GenBank accession no. DV158822). The nucleic acid sequence is highly homologous to tobacco cystatin sequence (Fig. 1c). BrCYS1 exhibits sequence identity of 53.3% with tobacco.

Over-expressed *BrCYS1* Retards Seed Germination

Previously, Martníez et al. (2003) reported that in barley, expression of the cathepsin B-like cysteine proteinase gene (*CatB*) increased in the aleurone upon germination, whereas that of a PhyCys encoding gene (*Icy*) decreased. If the Chinese cabbage PhyCys, BrCYS1, is also involved in seed germination, it should have same expression pattern as the barley PhyCys, *Icy*. We therefore examined germination rates using transgenic *BrCYS1* plants. The visible consequence of germination is the protrusion of the radical tip through the seed envelope (Debeaujon et al., 2000). Out of these T_3 homozygous lines (Nts-②, Nts-③, Ntas-⑤, and

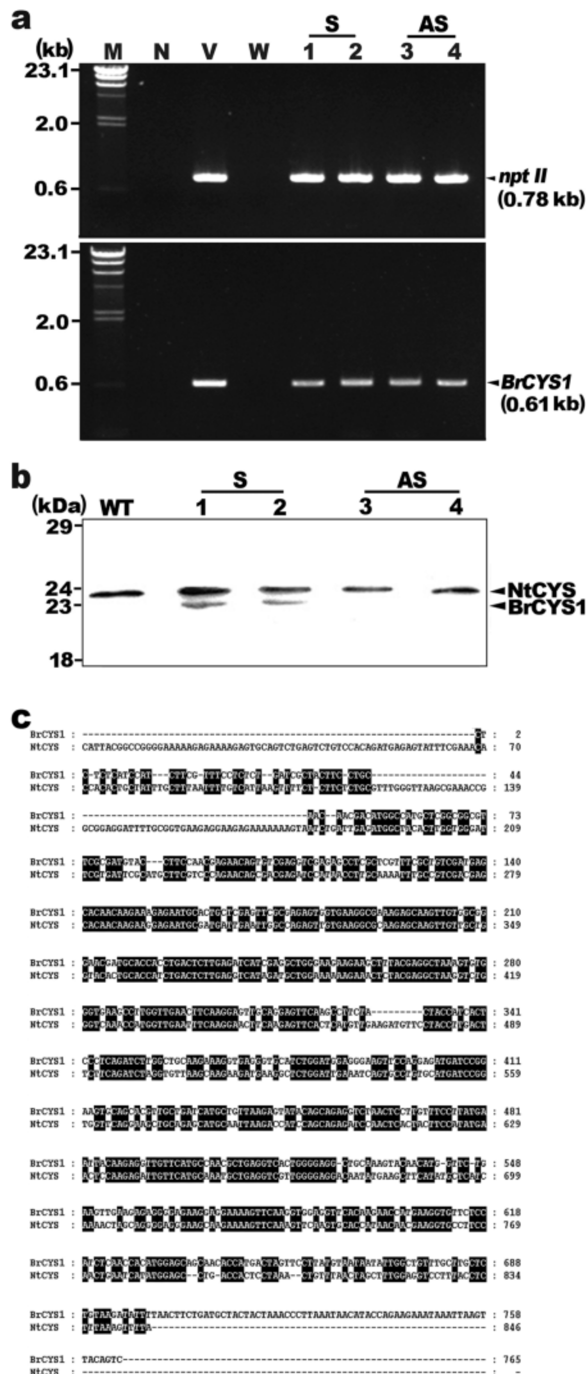

Figure 1. The expression of BrCYS1 in transgenic tobacco plants. (**a**) PCR analysis of WT and transgenic plants. The presence of *npt II* (upper panel) and *BrCYS1* (lower panel) was verified by PCR amplification. Lanes: M, molecular size marker; N, double-distilled water was used as template; V, *35S:BrCYS1-S* vector; W, untransformed WT; lanes 1 and 2, sense lines Nts-② and Nts-③; lanes 3 and 4, antisense lines Ntas-⑤ and Ntas-⑧. Arrowheads indicate positions of *npt II* and *BrCYS1* amplicons. (**b**) Western blot analysis of BrCYS1 in the seedlings of transgenic tobacco plants. WT, untransformed wild-type; lanes 1 and 2, sense lines Nts-② and Nts-③; lanes 3 and 4, antisense lines Ntas-⑤ and Ntas-⑧; NtCYS, tobacco CYS (GenBank accession no. DV158822). (**c**) Comparison of the BrCYS1 nucleic acid sequence with tobacco cystatin (NtCYS). Gaps have been introduced to maximize sequence similarity. Identical nucleic acids are indicated by white letters on a black background. The alignment was determined using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

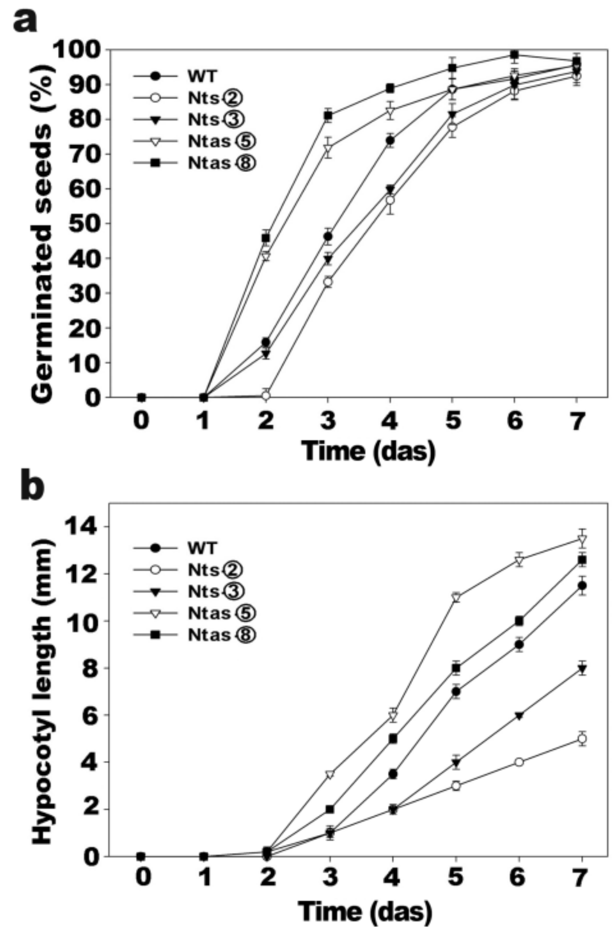

Figure 2. Comparison of germination rate and seedling growth in transgenic plants. (**a**) Time course of germination in days after stratification (das) for same seed batch. Germination was scored following the emergence of radicles. Average germination percentage was calculated with standard errors (S.E.s) of triplicates. Untransformed wild-type (●), sense lines Nts-② (○) and Nts-③ (▼), antisense lines Ntas-⑤ (▽) and Ntas-⑧ (■). (**b**) Hypocotyl lengths from 12 plants in each line. Plants were grown on control medium and scored. Data are means ± S.E. from at least three independent experiments.

Ntas-⑧), the sense lines (Nts-② and Nts-③) germinated at a slower rate than the WT, whereas antisense lines (Ntas-⑤ and Ntas-⑧) germinated slightly faster (Fig. 2a). In addition, the growth rate of seedlings from the sense lines was slower than that of the WT, whereas antisense seedlings exhibited slightly faster growth than the WT (Fig. 2b). These data indicate that introduction of *BrCYS1* retards seed germination and early seedling growth.

BrCYS1 Inhibits CP Activity During Seed Germination

CPs are responsible for the mobilization of most seed storage proteins during germination and their activity is regulated by inhibitors of papain-like cysteine endopeptidases (Abe et al., 1991; Müntz et al., 2001; Schlereth et al., 2001). In this regard, we considered the possibility that BrCYS1 may inhibit stored CP activity, as our transgenic plants over-expressing *BrCYS1* displayed retarded seed germination and seedling growth. Hence, we measured endog-

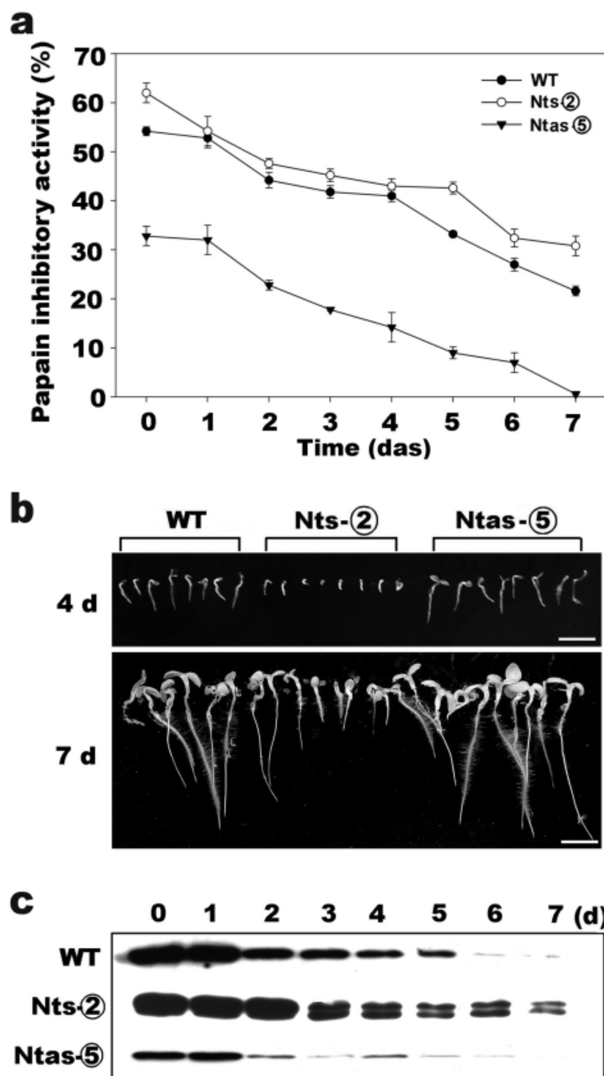

Figure 3. Endogenous CP activity and phenotypes of seedlings. (**a**) Analysis of endogenous CP activity during seed germination and seedling growth. 0, dry seeds; 1 - 7, dry seeds that were incubated on germination medium for 1-7 days after stratification (das). (**b**) Germination at 4 and 7 das for freshly harvested seeds of untransformed wild-type (WT), sense (Nts-②) and antisense (Ntas-⑤) lines. Seeds used in each experiment originated from the same seed batch. (**c**) Western blots analyses during germination of total protein for presence of BrCYS1 using BrCYS1 polyclonal antibody. Lane 0, dry seeds; lane 1 - 7, dry seeds were imbibed for 1 to 7 days.

enous CP activity in seeds and seedlings. Out of four transgenic lines (Nts-②, Nts-③, Ntas-⑤, and Ntas-⑧), we germinated seeds from T_3 homozygous sense (Nts-②) and antisense (Ntas-⑤) lines, since each of the sense and antisense lines showed the similar phenotypes (Fig. 2). We confirmed the occurrence of papain inhibitory activity during germination and/or post-germination by assaying papain inhibition in total protein samples extracted from transgenic and untransformed WT seeds and seedlings (Fig. 3a). In seeds from the sense line, endogenous CP activity was lower than that of the WT or antisense line. Following germination, inhibitory activity decreased in the sense line; however, the inhibitory activity was higher than that of the WT and antisense line. In addition, sense line (Nts-②) seedlings exhibited growth retardation compared with WT seedlings at the same stage. In contrast, seedlings from the antisense line (Ntas-⑤) exhibited slightly faster growth than the WT (Fig. 3b).

We investigated the BrCYS1 expression pattern during germination and post-germination using the anti-BrCYS1 polyclonal antibody and found that BrCYS1 levels decreased gradually over 5 days and had disappeared mostly by day 6. In the over-expressing line, BrCYS1 only decreased slightly throughout the sampling period and continued to be detected at day 7, whereas in the antisense line, the level of BrCYS1 decreased rapidly and was only weakly detected by day 3 (Fig. 3c). The slightly lower CP enzymatic activity and expression patterns might be caused by retarded germination in the sense line due to an accumulation of BrCYS1. This suggests that over-expression of *BrCYS1* caused the decrease in stored CP activity during germination, retarded germination and early seedling growth (Hong et al., 2007).

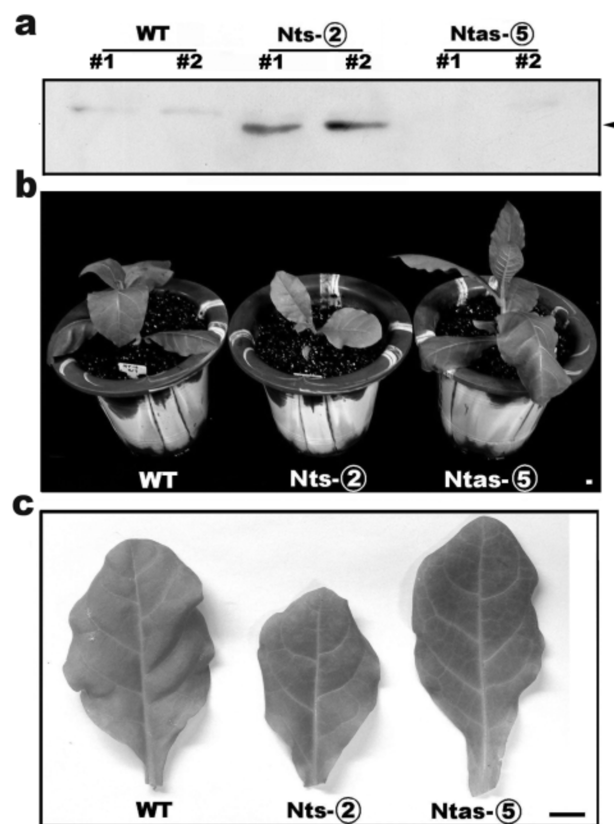

Figure 4. Phenotypes of the young plants. (**a**) Total proteins from the leaves of wild-type (WT) or transgenic plants were isolated. The expression of BrCYS1 was analyzed by western blotting. The elevated level of a single 23 kDa band in transgenic line Nts-②. (**b**) Whole-plant morphology of 4-week-old wild-type (WT) and transgenic plants of same age is shown. Phenotypic differences in stunting can be seen in the sense line plant (Nts-②). (**c**) Leaf morphology of the 4-week-old wild type (WT), sense (Nts-②) and antisense (Ntas-⑤) plants. Bars = 1 cm in (**b**) and (**c**).

Morphology of Transgenic Plants

Since transgenic tobacco plants over-expressing *BrCYS1*

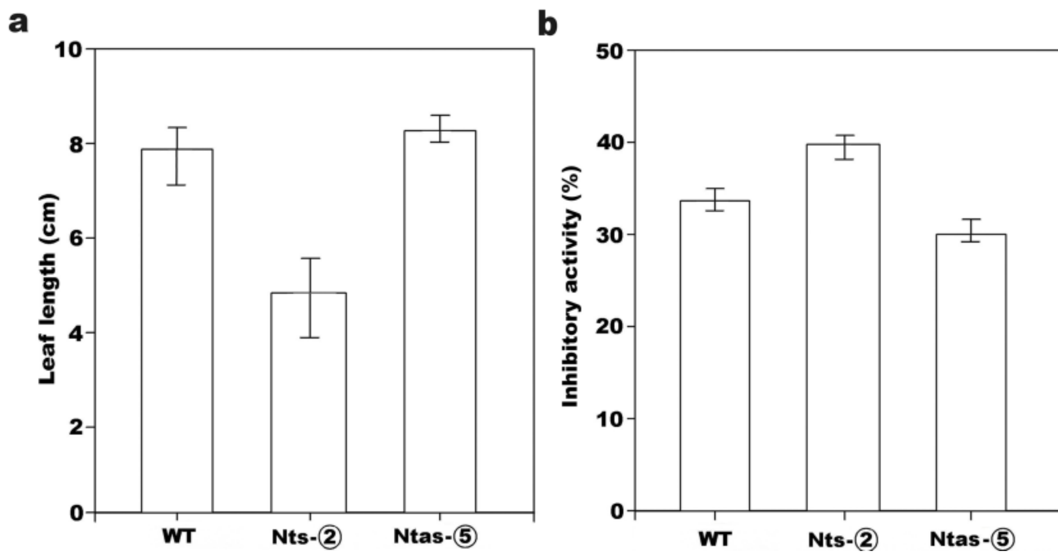

Figure 5. Comparison of leaf length and the endogenous CP activity. (**a**) Leaf length of 5-week-old fifth leaves from the initiation of primordial to full expansion. All measurements were performed on > 10 samples in each line. Data are means ± S.E. from at least three independent experiments. (**b**) Analysis of endogenous CP activity in 5-week-old fifth leaves. One millimolar BANA was used as substrate. WT, untransformed wild-type; Nts-② , sense line; Ntas-⑤ antisense line.

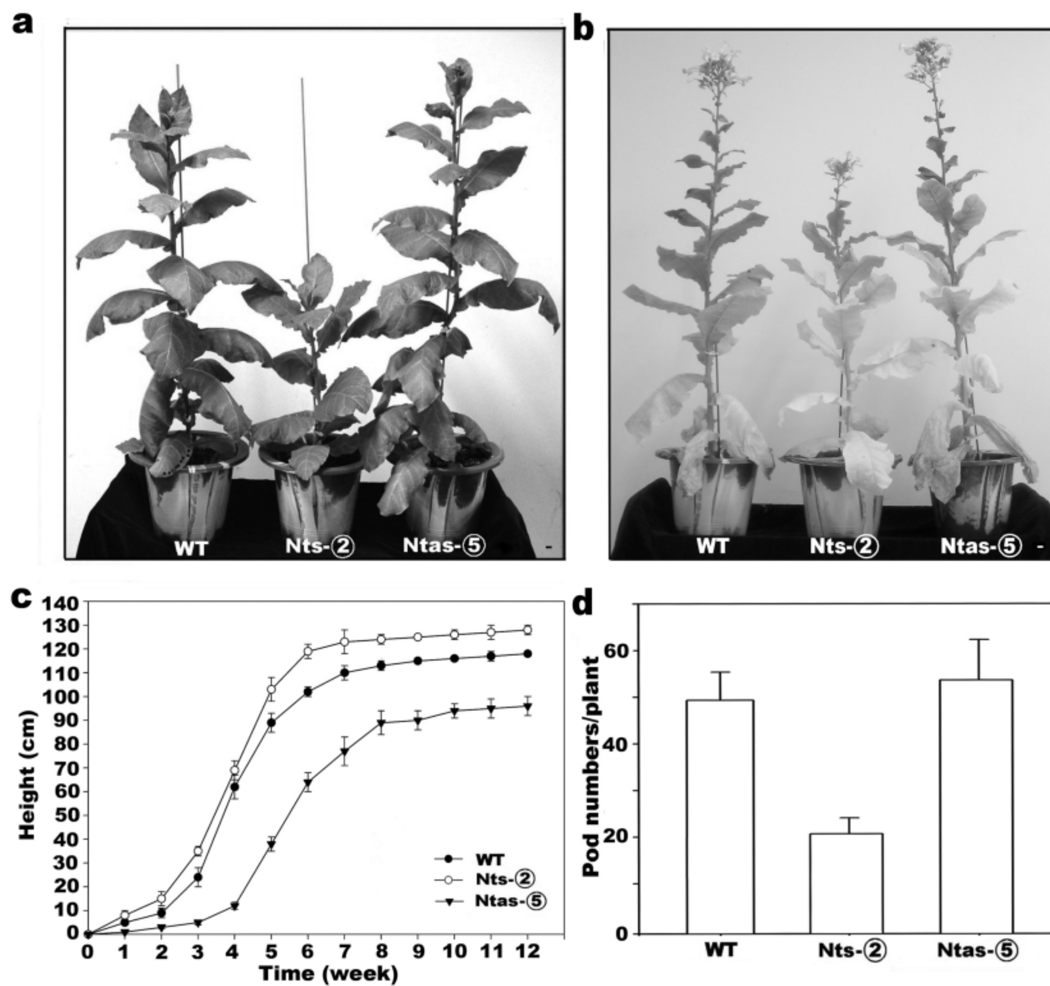

Figure 6. Phenotypes of the mature plants. (**a**) Whole-plant morphology of 16-week-old wild type (WT) and transgenic plants is shown. Phenotypic differences in stunting can be seen in the sense line plants. (**b**) Whole-plant morphology of 20-week-old WT and transgenic plants. (**c**) Compared the height of transgenic plants. (**d**) Pod numbers were reduced in sense line (Nts-②) at same age. Bars = 1 cm in (**a**) and (**b**).

displayed retarded germination and seedling growth, we assessed whether constitutive over-expression of *BrCYS1* in mature plants also leads to growth retardation. Transgenic plant seedlings were transplanted into soil and their protein expression levels were examined by Western blot analysis (Fig. 4a). BrCYS1 exhibited different expression levels in the same lines; however, it was readily detectable. As shown in Fig. 4b, the growth of the sense line plant (Nts-②) was stunted in comparison to the WT, whereas the antisense line (Ntas- ⑤) exhibited slightly enhanced growth at same stage. In order to characterize the fundamental features of growth, we compared the leaf morphology of transgenic plants under the same culture conditions. In the sense line Nts- ②, the leaves were small and had shorter petioles than the WT and antisense lines (Fig. 4c). Detailed examination indicated that the length of the leaf was also reduced in the sense line Nts-② (Fig. 5a), and the quantified endogenous CP activity in sense line leaves was lower than that of the WT or antisense line (Ntas-⑤) (Fig. 5b). This lower CP enzymatic activity might be caused by retarded growth in the sense line due to an accumulation of BrCYS1. In addition, the sense plants exhibited a significantly smaller pod size and number compared to the control plants and antisense lines (Fig. 6).

Our data suggests that the inhibitory effect of *BrCYS1* over-expression on seed germination and seedling growth caused stunting in mature plants. Detailed information about the regulatory roles of BrCYS1 on endogenous CP during later developmental stages awaits further experiments.

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