

## Production of Transgenic Soybean to Eliminate the Major Allergen Gly m Bd 30K by RNA Interference-mediated Gene Silencing

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Gly m Bd 30K has been recognized as a major allergenic protein in soybean [*Glycine max* (L.) Merr.] seeds, and it exists in nearly all of the soybean cultivars. This study attempted to eliminate the immunodominant Gly m Bd 30K protein from soybeans using RNA interference (RNAi). **RESULTS:** A 395-bp fragment from the *Gly m Bd 30K* gene coding region was designed to target the *Gly m Bd 30K* gene. The 30K-specific RNAi transformation cassette was subcloned into a binary pCAMBIA3301 vector to construct the plasmid pCAMBIA-30K-RNAi. Transgenic soybeans were generated by infecting soybean cotyledonary node explants with *Agrobacterium tumefaciens* EHA105. Three independent transgenic soybean plants were confirmed by PCR and Southern blot analyses. Quantitative real-time PCR (qRT-PCR) analysis of gene expression in the transgenic seeds indicated that there was trace accumulation of *Gly m Bd 30K* mRNA. Western blotting demonstrated the absence of Gly m Bd 30K protein in the crude extracts of transgenic seeds. In addition, compared with wild-type plants, the RNAi plants showed no apparent phenotypic and no obvious developmental changes. **CONCLUSION:** Our study yielded new soybean germplasm with Gly m Bd 30K-null seeds and demonstrated the feasibility of alleviating soybean allergies using RNAi technology.

**Key words:** *Agrobacterium tumefaciens*; allergen Gly m Bd 30K; gene silencing; RNAi; soybean transformation.

Soybeans [*Glycine max* (L.) Merr.] are a major source of protein and vegetable oil, which are essential components of the human diet. With the increasing demand for food in the world, soybeans have become an important material in food production because of their high nutritional value and low price. However, soybeans rank among the “big eight” of the most allergenic foods that are responsible for 90% of all IgE-mediated food allergies (Hefle *et al.*, 1996). A food allergy is an abnormal immunological reaction resulting from

an exposure to antigens (generally proteins), which trigger the production of allergen-specific IgE antibodies, leading to an allergic reaction. Approximately 1-2% of adults and 5-7% of children are affected by food allergies (Mills and Breiteneder, 2005). Currently, there is no cure for food allergies. One effective method for managing a soybean allergy is to avoid the allergenic food after the food allergy has been confirmed (Sicherer and Sampson, 2006). In the case of soybeans, the avoidance of soybeans and soybean-containing products in a standard diet is very difficult because soy components have been increasingly used in a variety of food products (Tryphonas *et al.*, 2003).

Soybeans contain many types of proteins that are potentially allergenic. At least 16 allergenic proteins, with molecular weights ranging from

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approximately 70,000 to 14,000, were recognized by the IgE antibodies in soybeans (Ogawa *et al.*, 1991). Three of them, Gly m Bd 30K, Gly m Bd 28K and Gly m Bd 60K, are shown to be major allergenic proteins, and they are found in the 7S globulin fraction of soybean proteins (Ogawa *et al.*, 1991 and Ogawa *et al.*, 1993).

Gly m Bd 30K, known as P34, comprises less than 1% of the total seed protein and has been recognized as the predominant immunodominant allergen in the 7S globulin protein fraction of the soybean storage protein (Helm *et al.*, 2000). IgE binding assays using immunoglobulin from soybean-sensitive individuals indicated that P34 was identified in 65% of the soy-sensitive patients (Helm *et al.*, 2000 and Ogawa *et al.*, 1993) P34 showed considerable sequence similarity in its N-terminal amino-acid residues to the thiol proteinase of the papain family, but it displayed no enzymatic function (Kalinski *et al.*, 1990). A mature P34 protein is a monomeric, insoluble glycoprotein consisting of 257 amino acid residues. It exhibits post-translational modifications derived from a 46 to 47 kDa precursor protein produced by the partial removal of 122 N-terminal amino acid residues (Kalinski *et al.*, 1990 and Wilson *et al.*, 2005). Helm *et al.* (1998) elucidated a minimum of 12 linear IgE specific epitopes on the P34 protein. Using individual patient serum, five immunodominant epitopes were identified in this allergen.

A variety of strategies have been employed to reduce the allergenic proteins in soybean seeds. However, it is difficult to remove the allergenicity of soybean proteins completely with existing processing methods, which include enzymatic methods and physical treatments. An alternative strategy that minimizes allergic reactions involves the production of hypoallergenic soybean cultivars. This strategy is comprised of two useful methods. One method is to screen the germplasm lines in the absence or with a reduced content of specific allergenic proteins. Joseph *et al.* (2006) screened 16,266 soybean accessions from the USDA germplasm collection, and two *Glycine max* accessions (PI603570A and PI567476) were identified as P34 nulls. The other strategy is to employ a genetic transformation to silence the native genes that encode the allergenic proteins. Herman *et al.* (2003) have used gene silencing

without a hairpin structure to successfully eliminate the accumulation of P34 in transgenic soybeans. Therefore, the removal of Gly m Bd 30K by gene silencing is feasible.

RNAi is a mechanism that limits the transcript level by activating a sequence-specific RNA degradation process (Agrawal *et al.*, 2003). This process facilitates targeted post-transcriptional gene silencing (PTGS), and has been routinely used as a powerful tool to manipulate gene expression and study gene function by affecting the degradation of a targeted transcript (Fire *et al.*, 1998). The method for RNAi has been shown to be highly efficient, especially when a gene construct consists of an inverted repeat of a fragment of the targeted gene sequence separated by an intron (Smith *et al.*, 2000). Such a construct is called intron-spliced hairpin RNA (ihpRNA). The use of RNAi for suppressing gene expression has been applied to improve the quality of plants in many venues, such as changing the composition of seed storage proteins in soybeans (Kinney *et al.*, 2001 and Schmidt *et al.*, 2011), increasing the relative content of amylase in wheat grains, (Regina *et al.*, 2006) and reducing allergens (Dodo *et al.*, 2008; Gilissen *et al.*, 2005; Herman *et al.*, 2003; Tada *et al.*, 1996).

Efficient transformation and regeneration systems are necessary to successfully produce transgenic plants. As a successful and reproducible transformation protocol, cotyledonary node-based *Agrobacterium*-mediated transformation of soybeans has been applied (Olhoft *et al.*, 2003 and Zhang *et al.*, 1999).

The objectives of this study were to silence the *Gly m Bd 30K* gene by RNAi using a hairpin structure and to characterize the resulting transgenic soybeans.

## MATERIALS AND METHODS

### RNAi vector construction

#### Intermediate vector construction

A 190-bp PCR product, serving as the intron, was amplified from the plasmid pCAMBIA1301 (position 10-199, GenBank AF234297) using the following primer pair with built-in double restriction sites (underlined): 5' - ACCACGTGCTCGAGGTAATTTCTAGTTTTTCTCCT-3' (*Pml* I and *Xho* I) and 5' -

TATGGTGACCTTAATTAACCTGTAACATCATCATCAT-3' (*Bst*E II and *Pac* I). The intron was cloned into the pMD19-T vector (TaKaRa, Japan) to generate a new vector, pMD19-I (Fig. S1A). The pMD19-I vector was used to generate the RNAi constructs.

#### RNAi target design and amplification

A 395-bp fragment of the *Gly m Bd 30K* gene (fragment from coding sequence position 9-403, GenBank accession AB013289), which was used for the construction of an inverted repeat to silence the targeted gene, was amplified by PCR with cDNA prepared from soybean (cv. 'NY-1001') seeds (Fig. S1B). The sense fragment was amplified using the following primers: 5' -CCTTAATTAACCTGTGTTGCTTCTTTCTCC-3' and 5' -TATGGTGACCGATGGTCAACA GAATATTGTTCC-3' flanked by *Pac* I and *Bst*E II. The antisense fragment was amplified using the primers (forward: 5' -CCGCTCGAGCCTTG TGTGCTTC TTTTCTCC-3' and reverse: 5' -ACCACGTGGATGGTCAACAAGAATATTGTTCC-3') flanked by *Xho* I and *Pml*I. The correct sequence and orientation of the introduced fragments were confirmed by sequencing.

#### Plant expression vector construction

The two PCR fragments confirmed above were inserted as the *Pml*I-*Xho* I fragment and *Pac* I-*Bst*E II fragment, respectively, into the cloning sites of pMD19-I to create an inverted repeat construct separated by the intron pMD19-hpRNA (Fig. S1C). Double digestions were used to validate the pMD19-hpRNA vector (Fig. S1D), suggesting that the two gene-specific sequences had been ligated successfully to an intron in the antisense and sense orientations. The RNAi cassette was spliced out of pMD19-hpRNA using a *Pml*I/*Bst*E II restriction digestion and subcloned into the binary plasmid pCAMBIA3301, which shares identical promoter and terminator sequences with the *gus* gene (Fig. 1). The resulting RNAi vector, pCAMBIA3301-30K-RNAi, was mobilized in *A. tumefaciens* EHA105.

#### Plant transformation and regeneration

The soybean cultivar 'NY-1001' was used for the generation of transgenic plants using a *A. tumefaciens*-mediated transformation procedure as previously described by Olhoft *et al.* (2003) with a few modifications. Soybean seedlings were germinated aseptically on B5 medium (Gamborg *et*

*al.*, 1968). The cotyledonary node explants were obtained from 5-day-old seedlings and cultivated with the *A. tumefaciens* strain EHA105 on co-cultivation medium (CCM: 1/10 B5 medium, 1.67 mg•L<sup>-1</sup> BAP, 0.25 mg•L<sup>-1</sup> GA<sub>3</sub>, 200 μM acetosyringone, 20 mM MES, 3% sucrose, 1 mM dithiothreitol, 1 mM L-cysteine, 1 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.7% agar, pH 5.4) for 4 days at 25°C in the dark. After co-cultivation, the explants were first cultured on shoot induction medium (SIM: B5 salts and vitamins, 1.67 mg•L<sup>-1</sup> BAP, 500 mg•L<sup>-1</sup> carbenicillin, 3% sucrose, 3 mM MES, 0.7% agar, pH 5.6) without selection agents for 7 days. Next, the explants were cultured on the same SIM but were supplemented with 4 mg•L<sup>-1</sup> bialaphos for 3 weeks. After induction for 4 weeks, explants with differentiated shoots were transferred to shoot elongation medium (SEM: MS salts, B5 vitamins, 1 mg•L<sup>-1</sup> zeatin, 0.5 mg•L<sup>-1</sup> GA<sub>3</sub>, 0.1 mg•L<sup>-1</sup> IAA, 100 mg•L<sup>-1</sup> pyroglutamic acid, 50 mg•L<sup>-1</sup> asparagine, 300 mg•L<sup>-1</sup> carbenicillin, 2 mg•L<sup>-1</sup> bialaphos, 3% sucrose, 3 mM MES and 0.8% agar, pH 5.6). Elongated resistant shoots of approximately 3-4 cm height, which were detected by the half-leaf GUS assay method, were excised and transferred to rooting medium (RM: 1/2 B5 salts, 0.5 mg•L<sup>-1</sup> IAA, 3% sucrose and 0.8% agar, pH 5.6). Rooted plantlets were transferred into pots filled with artificial substrate (50% vermiculite/50% peat) and kept inside the greenhouse to mature.

#### PCR analysis of transgenic plants

Genomic DNA was extracted from the soybean leaf tissues of transgenic and wild-type control plants using the SDS protocol. (Ma and Sorrells, 1995) PCR reactions were performed to analyze the *bar* gene, *gus* gene and RNAi constructs not naturally present in soybeans. Primers for *bar* and *gus* genes were designed to amplify a 413-bp DNA fragment of the *bar* gene and a 944-bp DNA fragment of the *gus* gene (Choi *et al.*, 2007). Additionally, primers Fhp 5' -ACCACGTGGATGGTCAACAAGAAT-3' and Rhp 5' -GAAGCAACA CAAGGCTGTAACATCA-3' were designed to amplify a 621-bp DNA fragment of the ihpRNA cassette which was mentioned hereafter as ΔpRNA. The amplified products were separated by electrophoresis on a 1% agarose gel and were visualized with ethidium bromide.

#### Southern blot analysis

Genomic DNA from both transformed

soybeans and wild-type soybeans was digested with *EcoR* I. The digested genomic DNA (10 µg) was separated on a 0.8% agarose gel and transferred onto a Hybond-N<sup>+</sup> nylon membrane (GE Healthcare, UK). The 944-bp PCR products of the *gus* gene were purified and labeled with digoxigenin-dUTP and used as the probe for hybridization. Probe labeling and Southern hybridization were performed using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Germany) according to the manufacturer's instructions.

#### Segregation analysis and identification of RNAi homozygous lines

Segregation analysis was conducted on the progeny of three independent T<sub>0</sub> transgenic plants. Seeds (T<sub>1</sub>) from each T<sub>0</sub> plant were individually harvested, and the seedlings were raised. T<sub>1</sub> plants were evaluated with both a Basta resistance assay and a GUS histochemical assay using the leaves. To measure herbicide resistance, a 0.5% Basta solution was applied to the leaves with a cotton swab and scored after 7 days. GUS expression was conducted using the GUS histochemical assay. Segregation analyses of *gus* and *bar* genes were performed on the progenies of three T<sub>0</sub> plants by chi-squared ( $\chi^2$ ) analysis. The T<sub>2</sub> lines whose progeny plants all displayed herbicide resistance were considered to be the RNAi homozygous lines. The T<sub>3</sub> seeds from the homozygous T<sub>2</sub> plants were used for qRT-PCR and Western blot analyses.

#### Quantitative RT-PCR analysis

The transcript abundance of *Gly m Bd 30K* gene was analyzed by qRT-PCR. The isolated soybean seeds were immediately frozen in liquid nitrogen and then stored at -80°C. Total RNA was isolated from the developing seeds (4-5 mm in length) using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's protocol. First-strand cDNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa, Japan) and subsequently used as the template for quantitative RT-PCR amplification with the following specific primer pairs: FK (5'-GGTTCAGCGGATG GTGTAGATTA-3') and RK (5' -TGGTTGGGTATGAA GCGAAATAA -3') for the *Gly m Bd 30K* gene fragment, and Fcyp (5' -CGGGACCAGTG TGCTTCTTCA-3') and Rcyp (52 -CCCCTCC ACTACAAAGGCTCG-3') for the soybean *CYP2*

gene as an internal standard for normalization. The 2<sup>- $\Delta\Delta$ CT</sup> method was used to quantify the relative change in gene expression (Livak and Schmittgen, 2001).

The reaction was performed on the MX3005p QPCR System (Agilent, USA). The reaction mixture (20 µL) consisted of 2×SYBR® *Premix Ex Taq*<sup>TM</sup> (TaKaRa, Japan), 10 µM each of the forward and reverse primers, and 90 ng of cDNA. After 30 sec at 95°C for the activation of Hot-start Taq DNA polymerase, the reaction conditions included 40 cycles of 5 sec at 95°C, 20 sec at 60°C, followed by a dissociation curve analysis from 60°C to 95°C.

#### Western blot analysis

Seed protein extracts were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Total protein was extracted from soybean seeds at 4°C with 700 µL of protein extraction buffer [0.05 M Tris-HCl (pH 8.0), 0.2% SDS, 5 M urea] with 5 µL β-mercaptoethanol. Protein samples were resolved on a 12% SDS-PAGE gel, and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with a 5% milk solution in PBST for at least 1 h. The primary antibody reaction was performed using a monoclonal antibody specific to Gly m Bd 30K from AbMART Inc. (www.ab-mart.com.cn) in a 1:2000 ratio with a 5% milk solution in PBST buffer for at least 3 h at room temperature. The blots were each washed for 15 min in PBS buffer prior to incubation with the alkaline horseradish peroxidase (HRP)-coupled secondary antibody in a 1:4000 ratio in blocking buffer for at least 1 h. The presence of the Gly m Bd 30K was detected using the TMB color substrate system (Beyotime, China).

#### Morphological comparison of wild-type and RNAi lines

To discern if *Gly m Bd 30K* gene silencing caused phenotypic changes or adverse effects on plant growth, the transgenic plants containing the RNAi construct were compared morphologically with wild-type soybean lines. Both transgenic and wild-type plants were cultivated in pots (23 × 13 × 20 cm) with artificial substrate (50% vermiculite/50% peat). The shoot phenotypic characteristics of plant height, stem diameter, main stem node number, and first-flowering time were measured and recorded.

**Statistical analysis**

The data were presented as the mean of three replicates and were tested using an analysis of variance (ANOVA) with SPSS 20 software (SPSS Inc., Chicago, USA). The levels of significance were indicated either by Tukey’s test at  $P < 0.05$  for Table 2 or by Dunnett’s  $t$  test at  $P < 0.01$  for Fig. 6.

**RESULTS**

**Generation of transgenic soybean plants**

The *Agrobacterium tumefaciens*-mediated cotyledonary-node method was adopted for soybean transformation. The transformation process was illustrated in Fig. 2. Explants were obtained from 5-day-old aseptic seedlings (Fig. 2A), and then were infected and co-cultured in CCM for 4 days with *A. tumefaciens* EHA105 harboring the transformation vector pCAMBIA 3301-30K-RNAi (Fig. 2B). After co-cultivation, the explants were transferred to SIM and cultured without the selective agent for 1 week and then with 4 mg•L<sup>-1</sup> bialaphos for 3 weeks. The explants were initiated after shoot induction on SIM for 2 weeks (Fig. 2C). Significant death of the non-transformed shoots was observed after a 4-week shoot induction (Fig. 2D). After shoot induction

for 4 weeks, the explants with shoot differentiation were transferred onto SEM with 2 mg•L<sup>-1</sup> bialaphos. Shoots elongation was obvious (Fig. 2E); GUS expression was readily detectable in elongated shoots using the half-leaf GUS assay method (Fig. 2F). Elongated shoots with GUS activity reached 3-4 cm in height and were excised for rooting (Fig. 2G). Rooted plantlets were transferred into pots filled with artificial substrates and kept inside the greenhouse to mature (Fig. 2H).

**Identification of transgenic plants**

The regenerated T<sub>0</sub> plants (L1, L2 and L3) were first verified using an herbicide leaf-painting assay involving the application of a 0.5% Basta solution to the leaf’s upper surface and then evaluated by a progeny segregation analysis. Three transgenic plants demonstrated a tolerance to the herbicide Basta, remaining green for 10 days after application (Fig. 3A, left), whereas the leaf of the control plant exhibited herbicide damage (Fig. 3A, right). In addition, GUS expression in various tissues was assayed histochemically. All plants that showed Basta resistance were GUS positive. GUS expression was detected in the leaf (Fig. 3B), root (Fig. 3C), flower (Fig. 3D), anther (Fig. 3E), and pod (Fig. 3F).

**Table 1.** Progeny segregation analysis of T<sub>0</sub> RNAi soybeans

T <sub>0</sub> plant number	No. of T <sub>1</sub> plants				Segregation ratio	χ <sup>2</sup> value <sup>z</sup>	P-value <sup>y</sup>
	GUS <sup>+</sup>	GUS <sup>-</sup>	Bar <sup>+</sup>	Bar <sup>-</sup>			
L1	13	2	13	2	3:1	1.49	0.22
L2	9	3	9	3	3:1	0.11	0.74
L3	14	3	14	3	3:1	0.76	0.38

<sup>z</sup> Data were based on the GUS histochemical staining for the *gus* gene and the Basta-resistant analysis for the *bar* gene.

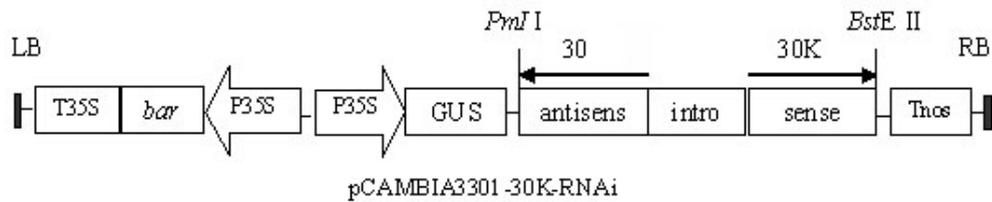
<sup>y</sup> A single degree of freedom was used to obtain the P-values.

**Table 2.** Comparison of the morphological traits of the RNAi and wild-type lines<sup>z</sup>

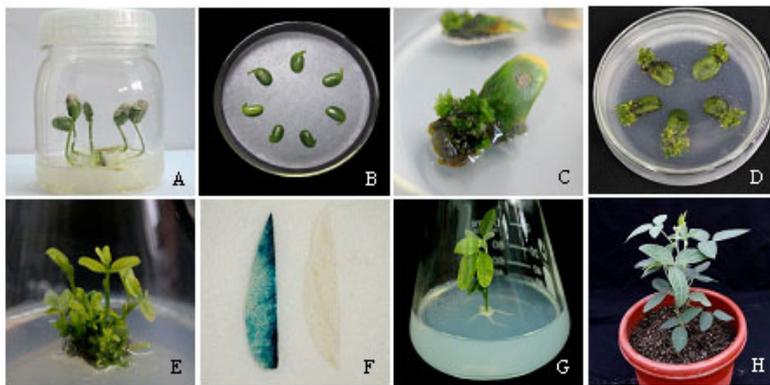
Plant line	Plant height (cm)	Stem diameter (mm)	Main stem node number	First-flowering time (d)
WT	37.00±4.54 a <sup>y</sup>	3.44±0.23 a	14.67±0.52 a	35.17±2.64 a
L1	38.47±8.82 a	3.54±0.25 a	14.17±1.33 a	36.33±1.86 a
L2	38.83±5.37 a	3.46±0.26 a	14.33±0.82 a	36.00±2.10 a
L3	37.28±6.35 a	3.33±0.24 a	14.16±0.98 a	35.67±2.66 a

<sup>z</sup> The means±SD were given for three individuals from the RNAi and wild-type lines.

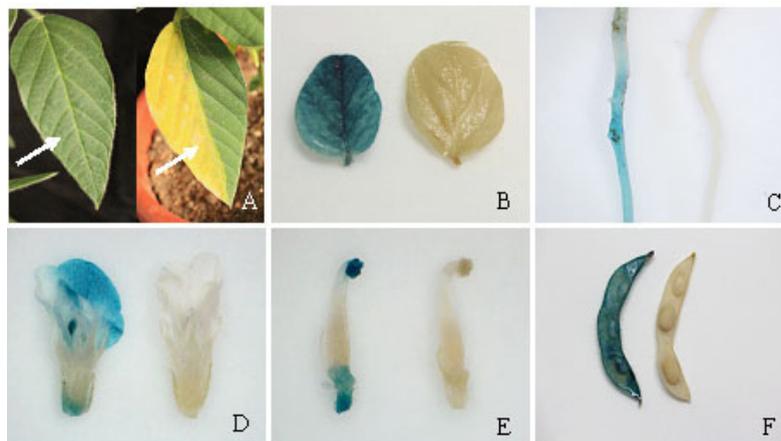
<sup>y</sup> The same small letter indicated no significant difference within the same column by Tukey’s test at  $P < 0.05$ .



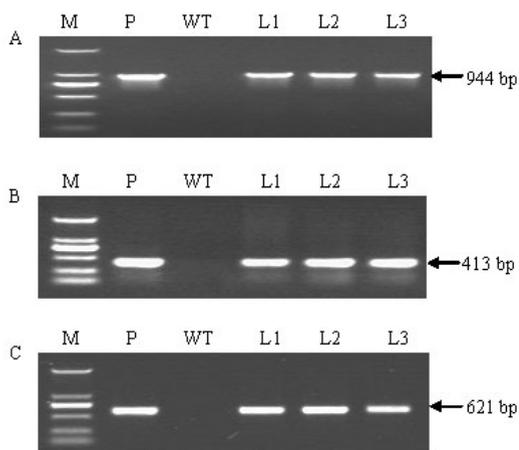
**Fig. 1.** The T-DNA region of the *Gly m Bd 30K* silencing vector, pCAMBIA3301-30K-RNAi. LB and RB, T-DNA left and right borders, respectively; P35S, CaMV 35S promoter; T35, CaMV 35S terminator; *bar*, bialaphos resistance gene; GUS,  $\beta$ -glucuronidase; Intron, castorbean catalase intron; Tnos, nopaline synthase terminator; 30K sense and antisense, the 395-bp inverted repeats of *Gly m Bd 30K* target sequence in reverse and forward orientations, respectively



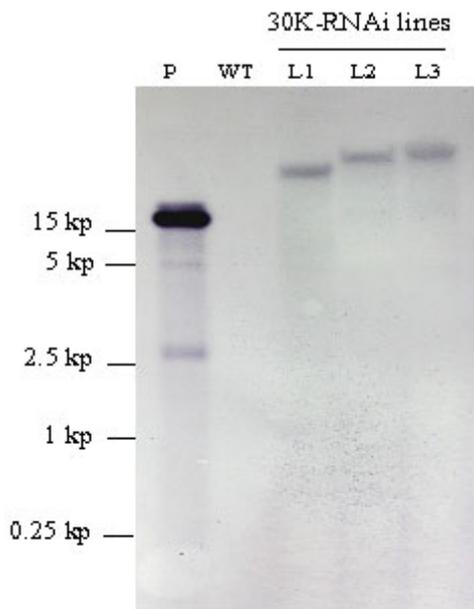
**Fig. 2.** Generation of *Gly m Bd 30K*-silenced transgenic soybeans by *Agrobacterium*-mediated cotyledonary nodes. (A) The 5-day-old aseptic seedlings of the soybean cultivar 'NY-1001'. (B) Explants co-cultivated with *Agrobacterium tumefaciens* EHA105. (C) Explants initiated after shoot induction on SIM for 2 weeks (the first 7 days without bialaphos). (D) Significant death of non-transformed shoots observed after a 4-week shoot induction. (E) Shoot elongation after 3 months of co-cultivation. (F) Elongated shoots detected with GUS expression by the half-leaf GUS assay method. (G) Rooting of GUS-positive shoots on rooting medium for 2 weeks. (H) Rooted plantlets transferred to an artificial substrate and kept in the greenhouse to mature



**Fig. 3.** Identification of putative transgenic soybeans by Basta painting and GUS staining in different organs of the transgenic soybeans. (A) Transgenic plants and wild-type control plants painted with a 0.5% Basta solution, demonstrating herbicide tolerance and herbicide damage, respectively. White arrow indicates the smearing site. (B) GUS expression in the leaves. (C) GUS expression in the roots. (D) GUS expression in the flowers. (E) GUS expression in the anthers. (F) GUS expression in the pods. For A-F, the left side shows the results for transgenic plants and the right side for wild-type plants



**Fig. 4.** PCR detection of transgenic soybean plants. (A) The 944-bp fragment of the *gus* gene. (B) The 413-bp fragment of the *bar* gene. (C) The 621-bp fragment of the hpRNA fragment. Lane M, DL2000 molecular Marker; lane P, pCAMBIA3301-30K-RNAi plasmid (positive control); lane WT, wild-type plant (negative control); lanes L1-L3, transgenic plants



**Fig. 5.** Southern blot analysis of genomic DNA from three individual  $T_0$  transgenic plants. Total genomic DNA (10  $\mu$ g) was digested with *EcoRI*. The 944-bp fragments of the *gus* gene were labeled with digoxigenin-dUTP and were used as the probe for hybridization. Lane P, pCAMBIA3301 digested with *EcoRI* (positive control); lane WT, genomic DNA from the wild-type plant (negative control); lanes L1-L3, genomic DNA from transgenic plants

Three putative, independently transformed plants were analyzed by PCR amplification using three specific primers for the *gus* (944 bp), *bar* (413 bp), and  $\Delta$ hpRNA (621 bp) gene fragments. All three plants yielded amplified bands of the expected sizes at the same positions as those amplified from the positive control vector pCAMBIA3301-30K-RNAi, whereas no corresponding PCR product was obtained from the genomic DNA of wild-type plants (Figs. 4A-C). Southern blotting was carried out to further confirm the integration of the hairpin-RNAi structure into the soybean genome. Genomic DNA from the three  $T_0$  transgenic plants L1, L2, and L3 were digested with *EcoRI*. As shown in Fig. 5, the three transgenic plants exhibited one strong hybridized band in the Southern blot analysis using a probe targeted to the *gus* gene, whereas no hybridization signal was observed in the non-transformed control plants. The number of hybridization signals showed that three  $T_0$  plants all had a single copy in their genomes, and were derived from independent events and contained one copy of the transgene.

All three transgenic plants were self-pollinated, and their seeds were harvested. Segregation analyses for GUS expression and Basta resistance were performed on the progenies of the three transgenic lines. The *gus* and *bar* genes were segregated in a 3:1 ratio in the progeny of three  $T_0$  transgenic plants (Table 1). These results are consistent with the Mendelian segregation of a single dominant gene.

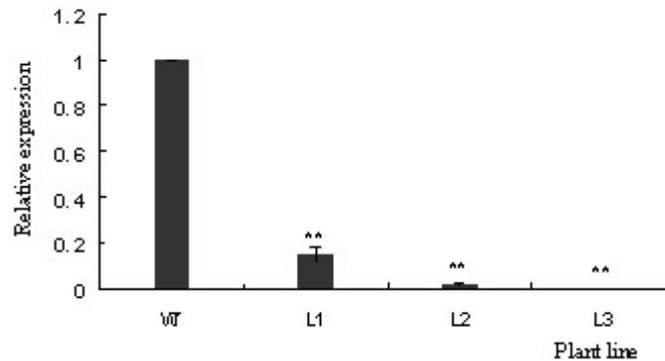
#### Suppression of Gly m Bd 30K synthesis in RNAi transgenic lines

The effectiveness and specificity of silencing induced by the ihpRNA, as expressed from the 35S promoter, was determined by the analysis of *Gly m Bd 30K* mRNA synthesis and protein by using qRT-PCR and Western blot analysis, respectively.

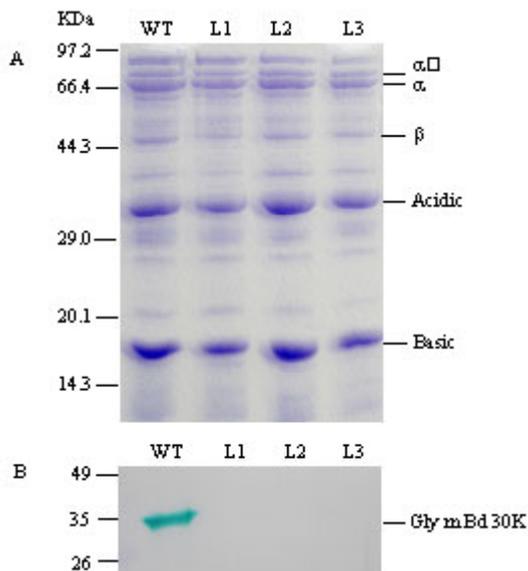
Three independent homozygous  $T_2$  transgenic lines were obtained through the GUS staining of leaves. Total RNA from the immature seeds ( $T_3$ ) of selected homozygous  $T_2$  lines was analyzed to measure the accumulation levels of *Gly m Bd 30K* mRNA. As shown in Fig. 6, only a trace accumulation of *Gly m Bd 30K* mRNA was detected in transgenic  $T_3$  seeds compared with the wild-type control, suggesting that the silencing was significantly efficient.

To examine the elimination of the Gly m Bd 30K protein in transgenic plants, a Western blot analysis was performed. Total proteins extracted from the seeds of soybean plants were probed with the Gly m Bd 30K antibody. As shown

in Fig. 7, a single 34-kDa band was observed in the protein extracts obtained from the wild-type control. However, this band could not be detected when the extracts of the RNAi lines were probed with the Gly m Bd 30K antibody.



**Fig. 6.** qRT-PCR analysis of the expression of *Gly m Bd 30K* gene in transgenic soybean seeds. A soybean cyclophilin 2 (*CYP2*) was used as an internal standard. *Gly m Bd 30K* expression in transgenic plants was normalized to the expression in wild-type (WT) plants. Normalized expression levels of less than one indicate gene silencing. Values represent the means $\pm$ SD from three replications. Double asterisks indicate a significant difference between each transgenic line and the wild-type control at  $P < 0.01$  (one-way ANOVA with Dunnett2 *s t* test)



**Fig. 7.** Western blot analysis of Gly m Bd 30K from the total storage proteins of seeds in the transgenic plants. (A) Total protein extracts from soybean seeds separated by SDS-PAGE. (B) Western blotting with the Gly m Bd 30K monoclonal antibody. Lane WT, wild-type control sample; lanes L1-L3, total protein extracts from RNAi-lines. The numbers on the left indicate the positions of the protein size markers in kDa

### Morphological comparison of wild-type and RNAi lines

To discern whether the manipulation of systemic RNAi had adverse effects on plant development, select homozygous transgenic RNAi lines were compared morphologically with the wild-type control. The shoot phenotypic characteristics of the plant height, stem diameter, main stem node number, and first-flowering time were measured and recorded (Table 2). No significant morphological differences were observed between the RNAi lines and the wild-type control.

### DISCUSSION

RNAi has become an essential tool in functional genomic studies and enables the genetic improvement of crops by recessive gene disruption and dominant gene silencing (Smith *et al.*, 2000). RNAi triggers gene-specific silencing based on the sequence homology-dependent degradation of cognate mRNA (Hannon, 2002 and Matzke *et al.*, 2001). The length and position of dsRNA are two critical factors that affect the efficiency of gene silencing in RNAi experiments. Wesley *et al.* (2001) reported that in ihpRNA construct-mediated

silencing, the silencing efficiency averaged approximately 90%, arms of 98-853 nt appeared to be stable and effective, and high levels of silencing were obtained with arm sequences derived from the coding region of the target gene. In our experiments, the fragment of Gly m Bd 30K that was selected for constructing the silencing vector consisted of 395 nucleotides and began exactly after the start codon (Fig. S1B), which was inserted as an inverted repeat to create a hairpin structure separated by the intron (Fig. S1C).

The *Agrobacterium*-mediated transformation protocol with bialaphos selection was successfully applied in the experiments to produce fertile transgenic plants (Fig. 2). We detected GUS expression in the resistant shoots at the stage of shoot elongation by using the half-leaf GUS assay method (Fig. 2F), which resulted in a shortening of the culture period for the early detection of transformed shoots.

The presence and integration of the Gly m Bd 30K transgene in the soybean genome was verified by PCR and Southern blotting of the T<sub>0</sub> bialaphos-resistant plants. *Agrobacterium*-mediated plant transformation is known for its stable inheritance and low copy transgenic integration (Hiei *et al.*, 1994). The integration of the *gus* gene into the soybean genome was confirmed by PCR (Fig. 4A) and Southern blotting analysis (Fig. 5). Because the RNAi cassette shared identical promoter and terminator sequences with the *gus* gene, and a part of the RNAi cassette was also amplified from the transgenic soybean genome (Fig. 4C), suggesting that the RNAi cassette was integrated into the soybean genome. The knockdown of the *Gly m Bd 30K* gene in transgenic soybean seeds was inheritable, and the transgenic plants expressed stable GUS activity and transmitted the phenotypes of *gus* and *bar* genes in a 3:1 ratio to their progenies (Table 1).

The efficiency of eliminating Gly m Bd 30K from transgenic soybean seeds was monitored by qRT-PCR and Western blot analyses. The *Gly m Bd 30K* mRNA levels were significantly suppressed in the three transformed lines (Fig. 6). Western blotting with the Gly m Bd 30K antibody further demonstrated that the synthesis of the P34 protein was completely suppressed (Fig. 7). RNAi is a process of post-transcriptional gene silencing, which leads to sequence specific mRNA

degradation in cytoplasm. As a result, the target mRNAs cannot accumulate in the cytosol, although they remain detectable (Fagard and Vaucheret, 2000).

*Gly m Bd 30 K*-silenced transgenic soybeans were obtained using RNAi. Our results showed that no significant morphological differences were observed between the RNAi lines and the wild-type control (Table 2), which was in agreement with previous reports (Herman *et al.*, 2003), indicating that Gly m Bd 30K protein was not required for plant growth and seed development.

In summary, fertile *Gly m Bd 30K*-silenced transgenic soybean plants were obtained from the cultivar 'NY-1001' by *Agrobacterium*-mediated transformation using RNAi. Bialaphos was used to select the transformants with successful genomic integration of the *bar* gene, which conferred herbicide resistance in the transgenic soybeans. The *Gly m Bd 30K*-silenced lines obtained in this study have established the foundation for further breeding of hypoallergenic soybean cultivars.

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