Plant Biotechnology 27, 409–420 (2010)

Original Paper

Application of somatic embryos to rapid and reliable analysis of soybean seed components by RNA interference–mediated gene silencing

Keito Nishizawa[1,a], Kyoko Takagi[1], Masayoshi Teraishi[2], Akiko Kita[1], Masao Ishimoto[1,3,*]

[1] National Agricultural Research Center for Hokkaido Region, Sapporo, Hokkaido 062-8555, Japan; [2] Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan; [3] National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan

* E-mail: ishimoto@affrc.go.jp Tel & Fax: +81-29-838-7452

Received June 15, 2010; accepted August 23, 2010 (Edited by T. Anai)

Abstract Soybean somatic embryos have attracted attention both as a model of zygotic embryos and as explants for the generation of transgenic plants. β-Conglycinin, which is composed of three subunits (α, α′, and β) that are encoded by a multigene family, is a major seed component of soybean, and accumulates in somatic embryos with their maturation. We subjected the somatic embryos to transformation with vectors encoding double-stranded RNA fragments of various sizes that correspond to the gene for the α′ subunit of β-conglycinin. These DNA fragments were put under the control of the promoter region of the gene for another major seed protein, the A2B1a subunit of glycinin. Transgenic somatic embryos were obtained within 2 months of transformation, and the resulting mature embryos manifested down-regulation of the α′ subunit of β-conglycinin in a manner dependent on the size of the RNAi vector insert. Accumulation of small interfering RNA and depletion of mRNA corresponding to the α′ subunit were indicative of RNAi. The amounts of α and β subunits of β-conglycinin, which share high sequence similarity with the α′ subunit, were also reduced in the mature somatic embryos. Moreover, the abundance of all β-conglycinin subunits was greatly reduced in the seeds of regenerated transgenic plants. Our results indicate that the application of RNAi to somatic embryos is a feasible and rapid option for functional studies of soybean seed components.

Key words: β-conglycinin, RNA interference, somatic embryo, soybean (*Glycine max*), transformation.

Soybean [*Glycine max* (L.) Merr.] is distinguished from other starchy grain crops by the large amounts of high quality protein (~40% by weight) and oil (~20% by weight) present in its seeds. Other components of soybean seeds are also economically important given that they are used in industrial, pharmaceutical, food and agricultural products. Additionally, consumption of foods containing soybean or soybean constituents has been suggested to provide protection against several chronic diseases (Birt et al. 2004). These various features render soybean as one of the most economically important grain crops. The recent availability of the genome sequence of soybean has allowed the application of functional genomics approaches to characterization of the genetic basis of important traits (Schmutz et al. 2010). The genome sequence represents ~85% of the predicted entire genome and reveals a highly duplicated genome structure, with nearly 75% of the genes present in multiple copies.

RNA interference (RNAi)–mediated gene silencing, which allows inhibition of the expression of one or more homologous genes depending on the sequence of the inducer (Lawrence and Pikaard 2003; Miki et al. 2005; Travella et al. 2006), will likely be an efficient tool for characterization of gene function in soybean. Seed components such as protein, oil, and secondary metabolites are synthesized de novo during seed development. Elucidation of the biosynthetic pathways and regulatory mechanisms underlying the accumulation of these components by the application of RNAi-mediated gene silencing to developing soybean seeds therefore has the potential to provide important information for improvement of seed quality by genetic engineering or marker-assisted selection (Flores et al. 2008; Nunes et al. 2006). However, it is not considered practical to verify the function of the large number of

Abbreviations: CaMV, cauliflower mosaic virus; CBB, Coomassie brilliant blue; hpRNA, hairpin RNA; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RNAi, RNA interference; RT, reverse transcription; siRNA, small interfering RNA; 3′-UTR, 3′ untranslated region.

[a] Present address: National Institute of Crop Science, Tsukuba, Ibaraki 305-8518, Japan

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genes implicated in the determination of seed components with the use of transgenic seeds because of the long regeneration time and life cycle of soybean (Finer and Mcmullen 1991; Ko et al. 2006; Olhoft et al. 2006).

Soybean somatic embryos have attracted attention both as a model of zygotic embryos and as explants for the generation of stable transgenic plants. Somatic embryos of soybean are induced from immature cotyledons cultured on medium containing a moderately high concentration of synthetic auxin, either 2,4-dichlorophenoxyacetic acid or α-naphthaleneacetic acid. These embryos are able to generate proliferative embryogenic cultures, and whole plants can be recovered from them through differentiation, maturation, and germination (Lazzeri et al. 1985; Ranch et al. 1985). Immature somatic embryos can retain regenerative properties for >1 year, with differentiation and development being readily induced when required (Finer and Nagasawa 1988; Parrott et al. 1988). Mature somatic embryos accumulate seed storage proteins with the same temporal and spatial regulation as developing seeds (Dahmer et al. 1992; Nishizawa and Ishimoto 2009), and the fatty acid composition of such embryos is similar to that of seeds (Dahmer et al. 1991; Shoemaker and Hammond 1988). In addition, soybean somatic embryos have been subjected to transformation (Finer and Mcmullen 1991; Ko et al. 2006; Sato S. et al. 1993), with transgenic embryos being obtained ~7 weeks after the introduction of exogenous genes by particle bombardment-mediated transformation (Khalafalla et al. 2005). Transgenic somatic embryos can also be maintained in culture, and homogeneous masses of such embryos can be readily and repeatedly subjected to induction of differentiation. Somatic embryos have therefore been used to screen for transgene expression and to examine transgene effects before recovery of whole plants (Cahoon et al. 1999; Cahoon et al. 2000; Cahoon et al. 2002; Chen et al. 2006; Herman et al. 2003). The combination of RNAi and somatic embryos might therefore be a feasible alternative option for the rapid validation of gene function in the synthesis of seed components.

Soybean seed storage proteins consist of two major globulins, β-conglycinin (7S globulin) and glycinin (11S globulin), which together account for about 70% of the total seed protein (Derbyshire et al. 1976; Utsumi et al. 1997). β-conglycinin is composed of at least three subunits, α, α′ and β subunits. α Subunit of β-conglycinin is a well-known allergen (Ogawa et al. 1995). In addition, β-conglycinin has a lower content of sulfur-containing amino acids and a lower gel forming ability than glycinin (Derbyshire et al. 1976; Utsumi et al. 1997). Therefore, a reduction of β-conglycinin is one of the important objects of soybean seed improvement, and many trials for removing β-conglycinin have been energetically carried out.

Here we have transformed soybean somatic embryos with constructs encoding self-complementary hairpin RNAs (hpRNAs) of different lengths that correspond to the gene for the α′ subunit of β-conglycinin. These DNA fragments were put under control of the promoter region of a glycinin subunit gene (*gy2*). The abundance of the α′ subunit as well as of homologous subunits of β-conglycinin was analyzed in transgenic embryos and seeds and compared among those resulting from transformation with the various hpRNA fragments.

Materials and methods

Gateway-based RNAi vector for particle bombardment-mediated transformation

An starting RNAi vector, pUHR:P11S-IR (Figure 1A), was constructed according to the following procedures. With the use of the polymerase chain reaction (PCR), a *Xho*I restriction site was inserted downstream of the 35S promoter of cauliflower mosaic virus (CaMV) in the T-DNA destination vector pH7GWIWG2(I) (Karimi et al. 2002). A DNA fragment containing the inverted Gateway sites separated by a 644-bp linker and connected to the 35S terminator of CaMV was then transferred from the modified pH7GWIWG2(I) vector to the pUHR plasmid (Nishizawa et al. 2006) with the use of *Xho*I and *Kpn*I sites [a *Kpn*I restriction site is present downstream of the 35S terminator of pH7GWIWG2(I)], yielding pUHR:7GWIWG2(I). The promoter sequence of the soybean A2B1a proglycinin gene (*gy2*) (Kitamura et al. 1990) was amplified by PCR with the pHV plasmid (El-Shemy et al. 2004) as the template and with primer 1 (5′-CGCGAGCTCGAATCCGTTGCATTCACTA-3′, underline indicates an introduced *Sac*I site) and primer 2 (5′-CGCCTCGAGGGTGATGAGTGTTCAAAGA-3′, underline indicates an introduced *Xho*I site). The resulting product was digested with *Sac*I and *Xho*I and then inserted into the corresponding sites of pUHR:7GWIWG2(I) [a *Sac*I site is present upstream of the *Xho*I site of pUHR:7GWIWG2(I)] to yield pUHR:P11S-IR.

RNAi target design and vector construction

The sequence used for construction of an inverted repeat to silence the gene for the α′ subunit of β-conglycinin was amplified by PCR with cDNA prepared from soybean (cv. Jack) developing seeds as the template. For construction of inverted repeats consisting of 93-, 103-, or 300-bp fragments of the α′ subunit gene (Figure 1C), we used primer 3 (5′-*AGAAAGCTGGGT*ATAAGTATGTAGTACTAAAATGTATGCTGTAATAG-3′) and primer 4 (5′-*AAAAAGCAGGCT*CATAGATGGAGCTCAAGTCATAGTTAAATAG-3′), primer 5 (5′-*AGAAAGCTGGGT*TTTTTACTGAATAAGTATGTAGTACTAAAATGTATGC-3′) and primer 4, and primer 6 (5′-*AGAAAGCTGGGT*TTCCTTGCAGGTTCGAAAGACAATGTGA-3′) and primer 4, respectively, with italics indicating adapter sequences. Adapter sequences were elongated by PCR performed with the product of the first PCR as the template and

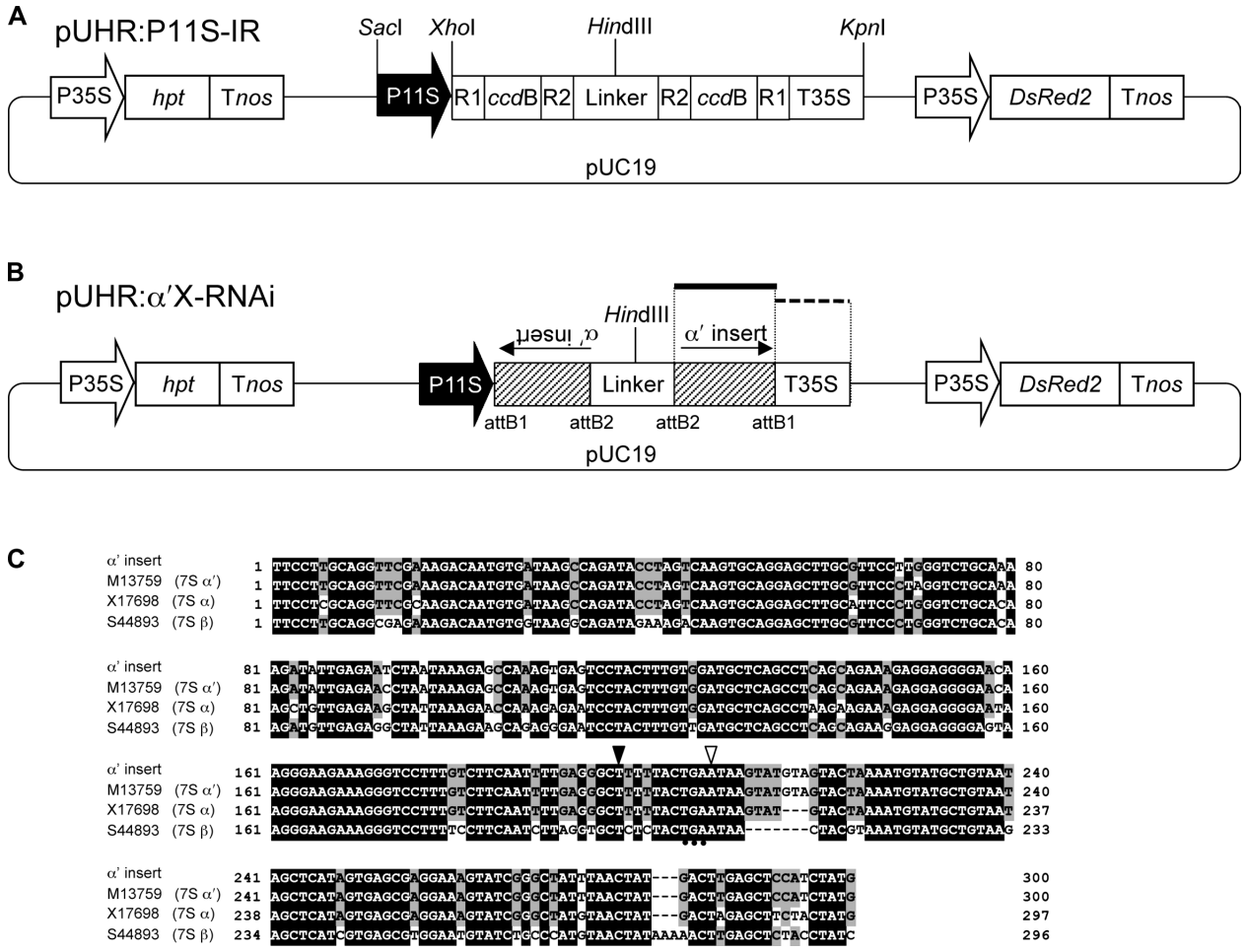

Figure 1. RNAi constructs for particle bombardment–mediated transformation. (A) The underlying RNAi destination vector, pUHR:P11S-IR, is a derivative of pUHR that contains the hygromycin phosphotransferase gene (*hpt*) and red fluorescent protein gene (*DsRed2*). The Gateway cassettes for introduction of the fragment of interest are positioned in opposite orientations at each end of a 644-bp linker sequence. The *ccd*B (control of cell death) gene, which is lethal to *Escherichia coli*, is used to ensure recombination at both Gateway cassettes. Expression of the hpRNA is controlled by the promoter of the soybean A2B1a proglycinin gene (P11S). Unique restriction sites originating from pUHR, and *Hin*dIII site used for Southern blot analysis are also shown. P35S, 35S promoter of CaMV; T*nos*, terminator of the nopaline synthase gene; T35S, 35S terminator of CaMV; R1 and R2, attR1 and attR2 sites that permit recombinational cloning of the gene of interest from a Gateway entry clone. (B) For construction of pUHR:α'X-RNAi, 93-, 103-, or 300-bp DNA fragments of the coding and 3′ untranslated regions of the gene for the α' subunit of soybean β-conglycinin were inserted into the destination vector in opposite orientations at each end of the linker sequence. The regions corresponding to a DNA probe used for detection of siRNA and to a probe used for Southern blot analysis are indicated by the solid and broken lines, respectively. attB1 and attB2 are attachment sites resulting from the LR recombination reaction. *Hin*dIII site used for Southern blot analysis is also shown. (C) Alignment of the RNAi insert in pUHR:α'300-RNAi with soybean cDNA sequences encoding β-conglycinin subunits. The RNAi insert was isolated and identified from developing seeds of cv. Jack. GenBank accession numbers for the subunit cDNAs are shown on the left. DNA fragments downstream of the closed and open arrowheads correspond to the RNAi inserts in pUHR:α'103-RNAi and pUHR:α'93-RNAi, respectively. Dots under the alignment indicate the stop codon.

with primer 7 (5′-GGGGACAAGTTTGTACAAAAAAGC-AGGCT-3′) and primer 8 (5′-GGGGACCACTTTGTAC-AAGAAAGCTGGGT-3′). The amplified DNA fragments were subcloned into the pDONR221 cloning vector (Invitrogen, Carlsbad, CA) by the BP clonase reaction to generate entry vectors. The DNA fragments were then transferred from the entry vectors to pUHR:P11S-IR with the use of the LR clonase reaction to generate the final RNAi plasmids pUHR:α'93-RNAi, pUHR:α'103-RNAi, and pUHR:α'300-RNAi (Figure 1B), all of which encode a self-complementary hpRNA corresponding to the 93-, 103-, or 300-bp fragments of the gene for the α' subunit of β-conglycinin (Figure 1C).

Soybean culture conditions and transformation

Somatic embryos were induced from immature cotyledons of soybean (cv. Jack) cultured on MSD40 medium (Finer and Nagasawa 1988), as previously described (El-Shemy et al. 2004), and were maintained in FNL medium (Samoylov et al. 1998). Somatic embryos (~20 mg) were matured on a rotary shaker at 100 rpm in histodifferentiation and maturation medium (FNL0S3S3 liquid medium) (Walker and Parrott 2001) at 25°C under white fluorescent light (12.1 μmol m^{-2} s^{-1}; 23-h-light, 1-h-dark cycle). Transformation of soybean by particle bombardment and subsequent plant regeneration were performed as previously described (Khalafalla et al. 2005).

Southern blot analysis

Total DNA (10 μg) isolated from young leaves of T_0 plants by the CTAB (cetyl trimethyl ammonium bromide) method was digested overnight with *Hin*dIII. The resulting fragments were fractionated by electrophoresis through a 1% agarose gel, transferred to a Hybond N+ membrane (GE Healthcare, Buckinghamshire, UK), and then subjected to hybridization with a probe for the 35S terminator of CaMV. The probe was prepared by PCR with pUHR:P11S-IR as the template and with primer 9 (5′-CGGCCATGCTAGAGTCCGCA-3′) and primer 10 (5′-AGGTCACTGGATTTTGGTTTTAGG-3′). Labeling and detection of the probe were performed with the use of an ECL kit (GE Healthcare).

Protein extraction

Somatic embryos matured in FNL0S3S3 medium were collected, flash-frozen in liquid nitrogen, and stored at –80°C until analysis. Proteins were extracted from the frozen somatic embryos at 4°C with 1 ml of protein extraction buffer [55 mM Tris-HCl (pH 8.0), 0.22% (w/v) SDS, 5.5 M urea, 2.2% (v/v) 2-mercaptoethanol, 2.75% (v/v) protease inhibitor cocktail for plants (Sigma-Aldrich, St. Louis, MO)] per 100 mg of fresh weight. The mixture was then boiled for 5 min and centrifuged at 17,800×*g* for 10 min at 4°C, and the resulting supernatant was used for analysis. Protein concentration was estimated with the use of a Protein Assay kit (Bio-Rad, Richmond, CA), with bovine serum albumin as a standard.

For analysis of the protein composition of dry seeds, total protein was extracted from seed flour with SDS sample buffer [50 mM Tris-HCl (pH 8.0), 0.2% (w/v) SDS, 5 M urea, 2% (v/v) 2-mercaptoethanol] at a ratio of 5 ml per 100 mg. The mixture was centrifuged at 17,800×*g* for 10 min at room temperature, and the resulting supernatant was used for the analysis after estimation of its protein concentration.

SDS-PAGE and immunoblot analysis

Protein samples were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) on a 10.5% gel, which was then either stained with Coomassie brilliant blue (CBB) or subjected to immunoblot analysis with rabbit antisera to the α subunit, α′ subunit (Nishizawa et al. 2003), or β subunit (Mori et al. 2004) of β-conglycinin or to glycinin (Teraishi et al. 2001).

Quantitative RT-PCR analysis

Total RNA (400 ng) isolated from somatic embryos with the use of a NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany) was subjected to reverse transcription (RT) with random hexamers (Applied Biosystems, Foster City, CA) and ReverTra Ace (Toyobo, Osaka, Japan) in a total volume of 20 μl. After the addition of 100 μl of H_2O to the reaction mixture, 1 μl of the diluted mixture was subjected to PCR in a reaction volume of 25 μl. PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) and an ABI PRISM 7000 sequence detection system (Applied Biosystems). An MGB (minor groove binder) probe labeled at the 5′ end with 6-carboxyfluorescein (FAM), 5′-FAM-AGCCAGATACCTAGTCAAG-MGB-3′, as well as primer 11 (5′-GCAGGTTCGAAAGACAATGTGA-3′) and primer 12 (5′-GCTGAGCATCCACAAAGTAGGAC-3′) were used to measure the amount of the mRNA for the α′ subunit of β-conglycinin. The PCR protocol included an initial incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The amount of the α′ subunit mRNA in transgenic somatic embryos was normalized by that of 18S rRNA, which was measured with Pre-Developed TaqMan Assay Reagent-Eukaryotic 18S rRNA (Applied Biosystems); it was then expressed relative to the normalized value for somatic embryos transformed with the empty vector (pUHR).

Detection of siRNA

Small interfering RNA (siRNA) was detected by Northern blot analysis of a small-RNA fraction with a digoxigenin-labeled probe. Small RNAs were extracted essentially as described (Senda et al. 2004). In brief, total RNA was isolated from mature somatic embryos (day 25) by the phenol-chloroform method. High molecular weight RNA was precipitated with LiCl, and small RNAs and genomic DNA remaining in the supernatant were precipitated with ethanol. The resulting pellet was dissolved in water, and genomic DNA was removed by precipitation with an equal volume of 20% (w/v) polyethylene glycol 8000 (MP Biomedicals, Irvine, CA) in 2 M NaCl (Goto et al. 2003). Small RNAs were then precipitated with ethanol, and the isolated fraction (20 μg) was dissolved in 15 μl of formamide. Electrophoresis, transfer, hybridization, and chemiluminescent detection were performed as described (Goto et al. 2003). A 300-bp DNA probe was prepared by PCR with cDNA for the α′ subunit of β-conglycinin as the template and with primer 13 (5′-TTCCTTGCAGGTTCGAAAGACAATGT-3′) and primer 14 (5′-CATAGATGGAGCTCAAGTCATAGTTA-3′). Digoxigenin-labeled dUTP was incorporated into the DNA probe in this step.

RT-PCR

Total RNA was isolated from somatic embryos with the use of a NucleoSpin RNA Plant kit (Macherey-Nagel). For synthesis of first-strand cDNA, total RNA was subjected to reverse transcription (RT) with random hexamers (Applied Biosystems) and QuantiTect Rev. Transcription kit (QIAGEN, Valencia, CA). The resulting cDNA was subjected to PCR analysis with the following primers; primer 15 (5′-CCAAACACAACAAGTGTCTCCAG-3′) and primer 16 (5′-GGGAATGGGAATTGACGTTCATCTTG-3′) for detection of α subunit cDNA, primer 17 (5′-CACAAGCAGGAAAAGCACCAAGGA-3′) and primer 18 (5′-TCCCCTTGTTGCTGCCCC-3′) for α′ subunit cDNA, primer 19 (5′-GAGAATAACCCTTTCTACTTTAGAAGC-3′) and primer 20 (5′-AAGTTTGATTATTTTGAGATTCTGGTGGTC-3′) for β subunit cDNA, and primer 21 (5′-ATTGAACCCCTTGTTTGCGA-3′) and primer 22 (5′-ATCAGGAAGCTCATGGCTTT-3′) for actin 3 cDNA.

Accession numbers

Nucleotide sequences relevant to the present study are available in GenBank under the accession numbers M13759 for the α′ subunit of soybean β-conglycinin and X15122 for soybean *gy2*.

Results

Soybean transformation with a Gateway-based RNAi vector

Physical introduction of genes of interest into somatic embryos has become an established and efficient protocol for soybean transformation (Finer and Mcmullen 1991; Khalafalla et al. 2005). Stable transformants of somatic embryos can be obtained by hygromycin selection within 7 weeks of the transformation procedure. Transgenic plants are then regenerated from the hygromycin resistant somatic embryos. In the present study, we modified a vector used for particle bombardment–mediated transformation of soybean. The pUHR vector is based on pUC and contains the hygromycin phosphotransferase gene (*hpt*) for antibiotic selection and the red fluorescent protein gene (*DsRed2*) as a visual reporter to facilitate detection of stable transformants (Nishizawa et al. 2006). The multicloning site of pUHR was replaced by inverted Gateway sites derived from the pH7GWIWG2(I) plasmid (Karimi et al. 2002), and the seed-specific promoter of the soybean A2B1a proglycinin gene (*gy2*) was included for temporal and spatial regulation of the expression of hpRNA from the resultant vector pUHR:P11S-IR (Figure 1A).

To validate the feasibility of genetic manipulation of soybean with the Gateway-based RNAi vector, we examined its ability to induce gene silencing in both somatic embryos and transgenic seeds. We selected β-conglycinin as a target for RNAi for the following reasons: (i) β-conglycinin is a seed storage protein. (ii) It is composed of at least three subunits (α, α', and β) that share substantial sequence identity (92, 78, and 78% at the cDNA level for α and α', α and β, and α' and β, respectively) (Doyle et al. 1986; Harada et al. 1989; Sebastiani et al. 1990). (iii) At least six distinct β-conglycinin genes are present in the entire soybean genome (Glyma1.0) (Schmutz et al. 2010). (iv) β-Conglycinin accumulates in both mature somatic embryos and seeds (Nishizawa and Ishimoto 2009). And (v) β-conglycinin subunits are readily detected, allowing for evaluation of RNAi effects on their expression. Three DNA fragments (93, 103, and 300 bp) of the coding and 3′ untranslated regions (3′-UTR) of the gene for the α' subunit of β-conglycinin (Doyle et al. 1986) were introduced individually into pUHR:P11S-IR in both antisense and sense orientations to yield the corresponding pUHR:α'X-RNAi vectors (Figure 1B). The targeted region of the α' subunit cDNA shares 93

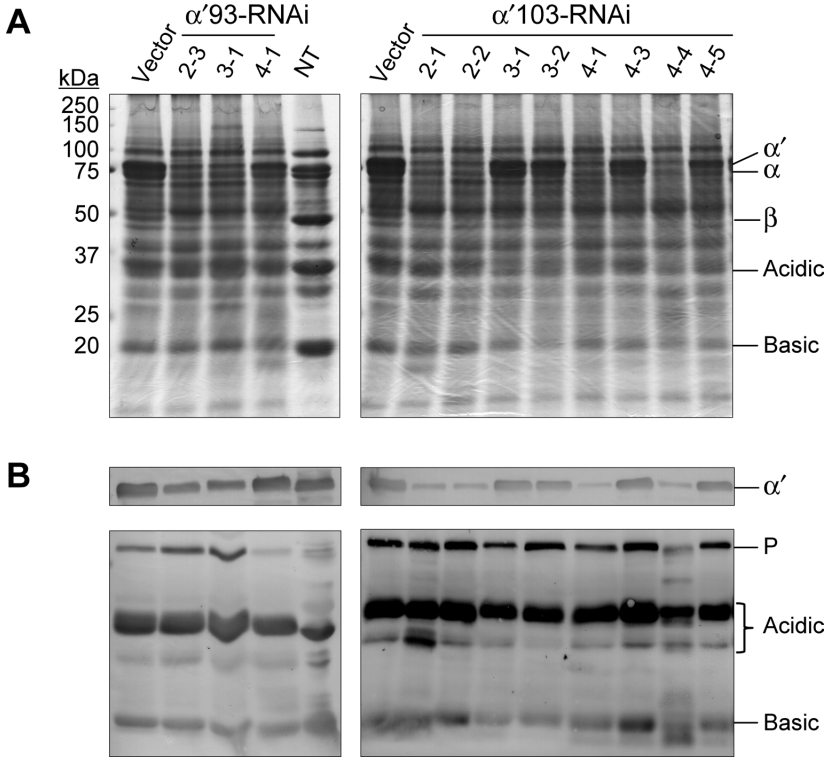

Figure 2. Suppression of the accumulation of the α' subunit of β-conglycinin in mature somatic embryos transformed with pUHR:α'93-RNAi or pUHR:α'103-RNAi. Protein extracts prepared from cotyledonary embryos of the indicated transgenic lines (2-3 to 4-1 for α'93-RNAi; 2-1 to 4-5 for α'103-RNAi) 25 days after the induction of maturation were subjected to SDS-PAGE (30 μg of protein per lane) (A) or to immunoblot analysis (21 μg of protein per lane) with antisera specific for the α' subunit of β-conglycinin (top panel) or for glycinin (bottom panel, internal control) (B). Somatic embryos transformed with the empty vector, pUHR, were also examined (Vector), as were nontransformed dry seeds (NT). The positions of the α', α, and β subunits of β-conglycinin as well as of the proprotein (P) and acidic and basic chains of glycinin are shown on the right.

and 81% sequence identity with the corresponding regions of the α and β subunit cDNAs, respectively (Figure 1C). We introduced each pUHR:α′X-RNAi vector into immature globular somatic embryos by particle bombardment. In a total of six independent trials for each RNAi vector, we obtained 3, 8, and 10 hygromycin-tolerant transgenic somatic embryos (α′93-RNAi, α′103-RNAi and α′300-RNAi) by the introduction of pUHR:α′93-RNAi, pUHR:α′103-RNAi, and pUHR:α′300-RNAi, respectively.

Suppression of β-conglycinin accumulation in somatic embryos

Hygromycin-tolerant somatic embryos were transferred to maturation medium (FNL0S3S3 liquid medium) to initiate embryo development. After 25 days, the abundance of the α′ subunit of β-conglycinin in the mature somatic embryos was examined by SDS-PAGE and immunoblot analysis (Figures 2, 3). In somatic embryos transformed with pUHR as a vector control, all the subunits of β-conglycinin (α, α′, and β) and glycinin were found to accumulate, with the extent of accumulation of the β subunit of β-conglycinin being the least pronounced. In contrast, the amount of the α′ subunit of β-conglycinin was markedly reduced in some transgenic lines, whereas the amount of glycinin was not altered. Glycinin accumulates in somatic embryos at a later stage of maturation than do the α and α′ subunits of β-conglycinin (Nishizawa and Ishimoto 2009). The reduction in the abundance of the α′ subunit of β-conglycinin in these transgenic lines was therefore not likely the result of delayed maturation of the somatic embryos.

In the transgenic lines α′93-RNAi 2-3 and 3-1, the reduction in the amount of the α′ subunit of β-conglycinin was readily detected by CBB staining, although a substantial signal for the α′ subunit was still apparent on immunoblot analysis (Figure 2). The transgenic lines α′103-RNAi 2-1, 2-2, 4-1, and 4-4 exhibited a greater depletion of the α′ subunit on CBB staining than did the α′93-RNAi lines, although faint signals were still apparent on immunoblot analysis (Figure 2). Expression of the α′ subunit was almost completely suppressed in the transgenic lines α′300-RNAi 1-2, 2-1, 2-3, 3-1, 4-3, and 4-5, as revealed by CBB staining and immunoblot analysis (Figure 3). These

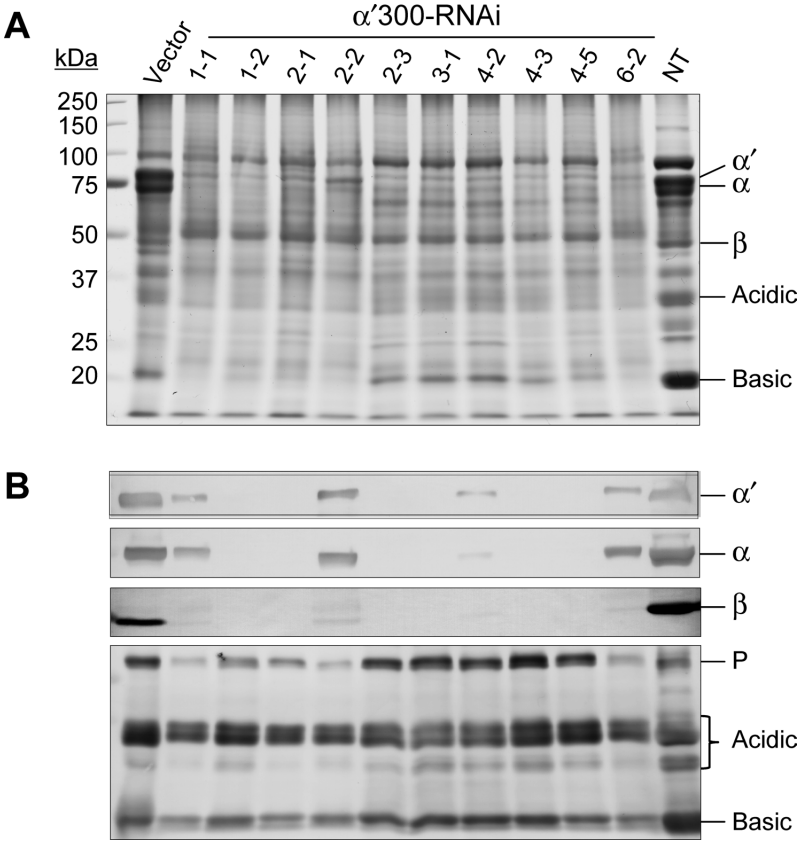

Figure 3. Suppression of the accumulation of β-conglycinin in mature somatic embryos transformed with pUHR:α′300-RNAi. Protein extracts prepared from somatic embryos of the indicated transgenic lines (1-1 to 6-2) 25 days after the induction of maturation were subjected to SDS-PAGE (30 μg of protein per lane) (A) or to immunoblot analysis (21 μg of protein per lane) with antisera specific for each subunit of β-conglycinin (top panels) or for glycinin (bottom panel, internal control) (B). Somatic embryos transformed with the empty vector, pUHR, were also examined (Vector), as were nontransformed dry seeds (NT). The positions of the α′, α, and β subunits of β-conglycinin as well as of the proprotein (P) and acidic and basic chains of glycinin are shown on the right.

results thus indicated that the extent of depletion of the α′ subunit of β-conglycinin differed according to the size of the gene fragment present in the RNAi vector. The abundance of the α and β subunits of β-conglycinin was also reduced in the lines showing depletion of the α′ subunit (Figure 3B), likely as a result of the high sequence homology of the corresponding genes to the α′ subunit gene.

We next examined whether the reduction in the amount of the α′ subunit of β-conglycinin in transgenic somatic embryos was the result of suppression of gene expression by analyzing three representative transgenic lines, α′300-RNAi 1-2, 3-1, and 4-2. Quantitative RT-PCR analysis (Figure 4A) revealed that the amount of α′ subunit mRNA in mature somatic embryos of α′300-RNAi 1-2 and 3-1 lines was <5% of that in those transformed with the empty plasmid (pUHR). The amount of the α′ subunit mRNA in the α′300-RNAi 4-2 line was ~40% of that in the pUHR transformant (Figure 4A), consistent with the results of the analysis of protein composition (Figure 3B). We detected siRNAs in mature somatic embryos of α′300-RNAi 1-2 and 3-1 lines by hybridization with a DNA probe corresponding to the targeted 300-bp fragment of the α′ subunit gene (Figure 1B); such siRNAs were not detected in the pUHR transformant (Figure 4B). The amount of such siRNAs was also smaller in the α′300-RNAi 4-2 line than in α′300-RNAi 1-2 or 3-1. Gene expression of the α′, α and β subunits in the transgenic somatic embryos were then examined by RT-PCR (Figure 4C). Gene expression of these subunits was detected well in the mature somatic embryos transformed with the empty vector. On the other hand, in α′300-RNAi 1-2 and 3-1 lines, gene expression of all the subunits was low, and it was moderate in α′300-RNAi 4-2 line. This result indicated that the reduction of α and β subunits of β-conglycinin as well as α′ subunit (Figure 3) was caused by down-regulation of the gene expression. Southern blot analysis

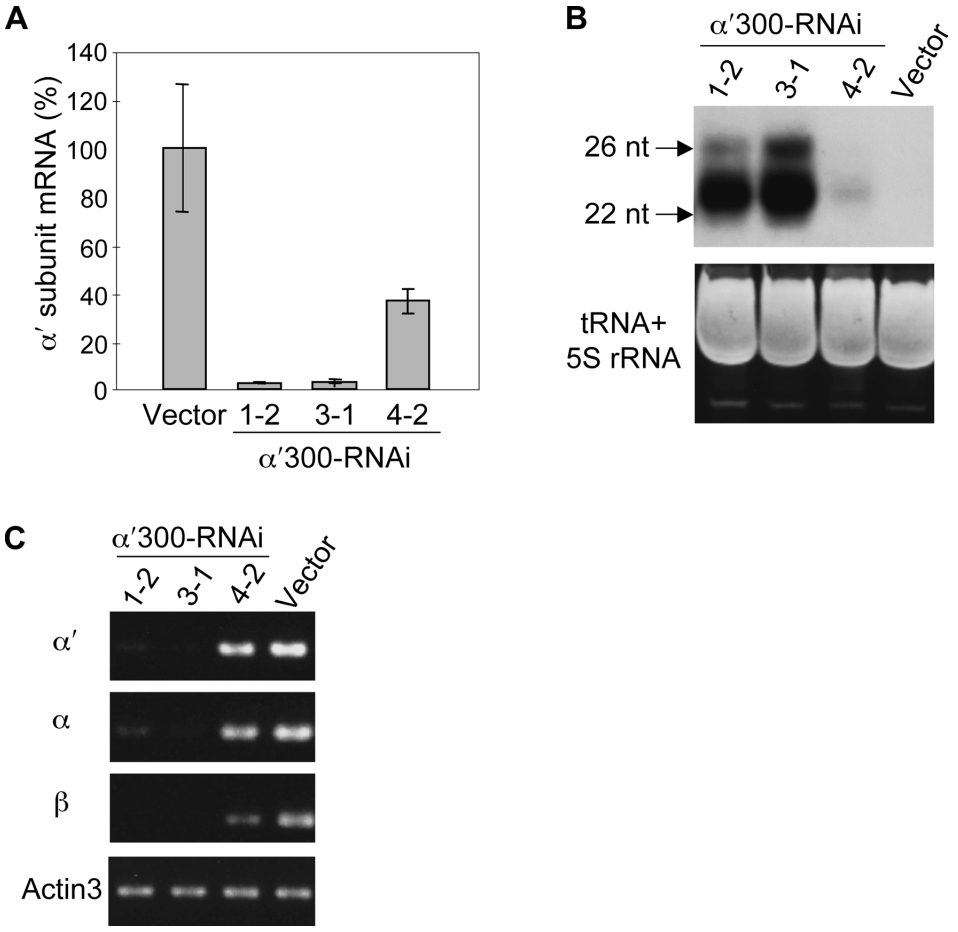

Figure 4. Silencing of the gene for the α′ subunit of β-conglycinin in mature somatic embryos transformed with pUHR:α′300-RNAi. (A) The amount of mRNA for the α′ subunit was measured by quantitative RT-PCR analysis in mature somatic embryos of the indicated transgenic lines. Data were normalized by the corresponding amount of 18S rRNA and expressed as a percentage of the normalized value for somatic embryos transformed with the empty vector, pUHR (Vector). Data are means±SD of triplicate samples. (B) Small-RNA fractions isolated from mature somatic embryos of the indicated transgenic lines were subjected to hybridization (top panel) for the detection of siRNAs with the RNAi insert as a DNA probe (Figure 1B). Both 22- and 26-nucleotide DNA oligomers were used as size markers. The bottom panel shows ethidium bromide staining of tRNA and 5S rRNA as a loading control. (C) Gene expression of α, α′ and β subunits of β-conglycinin in the indicated transgenic lines was analyzed by RT-PCR. Gene expression of actin3 was also confirmed as an internal control.

of genomic DNA from regenerated plants showed the presence of transgenes with different copy numbers in these transgenic lines (Figure 5). The banding patterns of the α′300-RNAi 1-2 and 3-1 lines, which exhibited pronounced depletion of β-conglycinin, were relatively simple, whereas α′300-RNAi 4-2, which exhibited moderate reduction of β-conglycinin, manifested multicopy integration of the RNAi construct. Transformation via particle bombardment frequently results in the introduction of high and rearranged copies of transgenes into genomic DNA (Dai et al. 2001; Kohli et al. 1998; Kohli et al. 1999; Shou et al. 2004). Formation of hpRNA might be impaired due to rearrangement of transgenes in the α′300-RNAi 4-2 line. The results of qRT-PCR, siRNA detection, RT-PCR and Southern blot analysis indicated that the depletion of β-conglycinin in the transgenic somatic embryo lines was attributable to various extents of RNAi-mediated gene silencing induced by pUHR:α′300-RNAi.

Suppression of β-conglycinin accumulation in transgenic seeds

Mature somatic embryos of all transgenic lines were allowed to regenerate plants. All the three lines transformed with the α′93-RNAi construct differentiated into plants, whereas the recovery of whole transgenic plants was limited for the α′103-RNAi and α′300-RNAi lines. Three of the α′103-RNAi lines and five of the α′300-RNAi lines thus produced regenerated plants. No abnormal growth or sterility of the resulting plants was observed. Transgenic plants produced seeds (T_1 generation) that manifested down-regulation of the α′ subunit of β-conglycinin to various extents (Figure 6). The amounts of the α and β subunits of β-conglycinin were also decreased in accordance with the reduction in that of the α′ subunit. The extent of the reduction in the abundance of β-conglycinin in transgenic seeds was consistent with that in transgenic somatic embryos. The three subunits of β-conglycinin were almost completely absent from the seeds of the 1-2, 3-1, and 4-5 α′300-RNAi lines. Germination, growth, and fertility of the T_1 plants were virtually normal, and the next generations lacking β-conglycinin were produced from the 1-2, 3-1, and 4-5 α′300-RNAi lines.

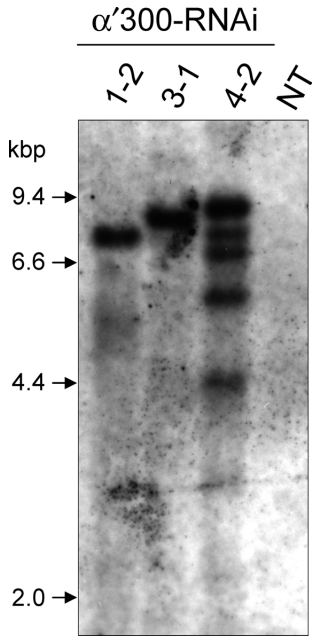

Figure 5. Southern blot analysis of genomic DNA from transgenic plants (T_0 generation) derived from somatic embryos transformed with pUHR:α′300-RNAi. Total genomic DNA (10 μg) isolated from plants of the indicated transgenic lines or from a nontransformant (NT) was digested with *Hin*dIII and subjected to Southern hybridization with a DNA probe corresponding to the sequence of the 35S terminator (Figure 1B). The positions of DNA size markers are indicated on the left.

Discussion

One of the major goals of functional genomics in soybean is to characterize the process of seed development, given that soybean seeds are an important source of protein, oil, and secondary metabolites (Stacey et al. 2004). Genetic transformation is an essential technique for verification of gene function, and genetic transformation of soybean has become routine with well-established methods mediated by *Agrobacterium* or particle bombardment (Finer and Mcmullen 1991; Kita et al. 2007; Olhoft et al. 2003; Sato S. et al. 1993; Zeng et al. 2004). It remains impractical, however, to verify the function of the large number of genes involved in determination of seed components with the use of transgenic soybean seeds, since it takes 3 to 4 months to generate transgenic plants, with transformation efficiencies being relatively low, and it takes several more months to obtain T_1 seeds (Sato H. et al. 2007). In the present study, we generated a sufficient mass of mature transgenic somatic embryos within 4 months of transformation, whereas we required an additional 6 months to obtain transgenic seeds. Furthermore, all transgenic explants do not yield regenerated plants, and some regenerated plants fail to produce viable seeds (Nunes et al. 2006). Indeed, we obtained a total of 21 transgenic somatic embryogenic lines in the present study, from which we recovered only 11 transgenic plant lines. Transgenic somatic embryos thus allow rapid and efficient analysis of gene function in soybean.

In the present study, we modified a Gateway-based vector, pH7GWIWG2(I) (Karimi et al. 2002), for particle bombardment–mediated transformation of soybean. Gateway technology facilitates cloning of PCR products and is therefore often used to insert target DNA fragments in opposite orientations into two regions joined by a linker sequence (Miki and Shimamoto 2004; Wesley et al. 2001). We generated three different-sized

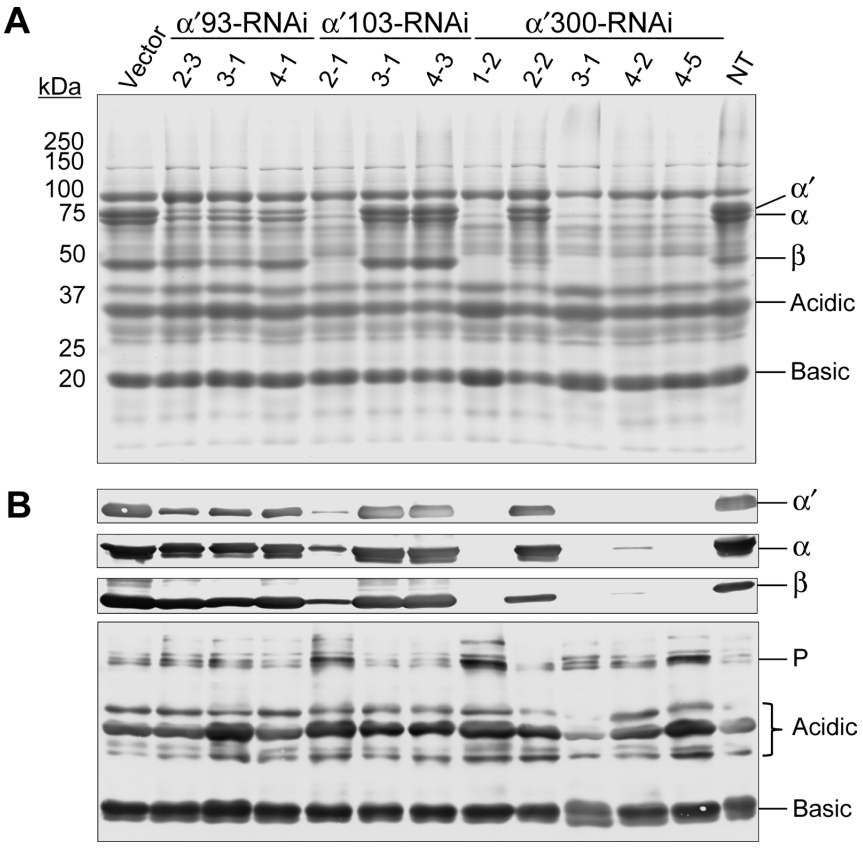

Figure 6. Suppression of the accumulation of β-conglycinin in T_1 seeds transformed with the three RNAi constructs. Protein extracts of representative T_1 seeds harvested from a regenerated plant of each of the 11 indicated transgenic somatic embryo lines (obtained with the RNAi constructs α′93-RNAi, α′103-RNAi, or α′300-RNAi) were subjected to SDS-PAGE (30 μg of protein per lane) (A) or to immunoblot analysis with antisera specific for β-conglycinin subunits (21 μg of protein per lane) or for glycinin (6 μg of protein per lane) (B). Transgenic seeds transformed with the empty vector, pUHR, were also examined (Vector), as were nontransformed seeds (NT). The positions of the α′, α, and β subunits of β-conglycinin as well as of the proprotein (P) and acidic and basic chains of glycinin are shown on the right.

(93, 103, and 300 bp) fragments including the 3′-UTR of the gene for the α′ subunit of β-conglycinin by PCR and then transferred them into the pUHR:P11S-IR vector by recombination. All of the three RNAi constructs yielded transgenic somatic embryos with various extents of depletion of the α′ subunit of β-conglycinin. Relatively short RNA sequences including those corresponding to the 3′-UTR have been shown to be effective for RNAi (Ishihara et al. 2005; Wesley et al. 2001). However, we found that the 93- and 103-bp sequences were less effective than the 300-bp sequence in suppressing β-conglycinin accumulation in both somatic embryos and seeds. The silencing ability of RNAi constructs appears to be affected by the structure of components including spacer sequences (Hirai et al. 2007).

Many attempts to eliminate β-conglycinin from soybean have been undertaken. However, it has proved difficult to remove this storage protein, in large part because of the redundancy of β subunit genes in the soybean genome (Harada et al. 1989). An induced mutant lacking all subunits of β-conglycinin was isolated after mutagenesis by γ-irradiation (Kitagawa et al. 1991). The mutant trait was found to be controlled by a single recessive gene, *cgdef*. Only seeds homozygous for the recessive mutant allele thus manifested loss of β-conglycinin, and the resulting plants were not able to grow normally and died before setting seeds (Hayashi et al. 1998; Kitagawa et al. 1991). In contrast, the T_1 generation lacking β-conglycinin in the present study was able to grow normally and to set T_2 seeds, suggesting that the suppression of β-conglycinin accumulation by RNAi was specific and seemed not to affect other genes required for growth and reproduction. A wild soybean line lacking all subunits of β-conglycinin (QT2) has also been described (Hajika et al. 1996). This line was found to grow normally and to produce successive generations. The deficiency of β-conglycinin in QT2 is controlled by a single dominant gene, *Scg-1* (Hajika et al. 1998), and has been suggested to result from the silencing of multicopy genes, given that the *Scg-1* locus resides in the vicinity of the genes for the α and β subunits of β-conglycinin and that this region is highly methylated in the mutant (Teraishi et al. 2001). The QT2 line is considered a suitable gene source

for breeding soybean varieties that lack β-conglycinin. However, conventional breeding is dependent on genetic resources, takes many years, and may be hindered by linkage drag in some instances (Boerma and Walker 2005). In contrast, RNAi-mediated gene silencing has been applied for modification of soybean seed components including enzymes and other proteins that are encoded by multiple gene copies (Chen et al. 2006; Flores et al. 2008; Nunes et al. 2006; Schmidt and Herman 2008; Shi et al. 2007; Wang and Xu 2008). RNAi, together with overexpression of exogenous or endogenous genes, is thus a powerful strategy for soybean improvement.

The recent sequencing of the soybean genome revealed that ~75% of the predicted genes are present in multiple copies as a result of two duplication events (Schmutz et al. 2010). RNAi-mediated gene silencing is therefore a potentially effective tool for characterization of gene function in soybean because of its ability to overcome functional redundancy of duplicated genes (Lawrence and Pikaard 2003; Miki et al. 2005; Travella et al. 2006). The high efficiency of *Agrobacterium rhizogenes*-mediated transformation has resulted in the widespread application of this approach to high-throughput analysis of cellular and biochemical processes in roots by RNAi-mediated silencing (Graham et al. 2007; Lee et al. 2005; Li et al. 2010; Subramanian et al. 2005). Although hairy roots are not suitable for the study of seed-specific traits because whole plants or embryos cannot be recovered from them, suppression of the isoflavone synthase gene by this approach was apparent not only in the emerged transgenic hairy roots but also in cotyledons at a position distal to the site of RNAi transformation by *A. rhizogenes* (Subramanian et al. 2005). Gene silencing in the cotyledons was transient, however, so only a limited number of genes might be amenable to analysis by this method. In addition, it is important to clarify whether cotyledons cut for infection with *A. rhizogenes* maintain the characteristics of developing seeds.

Virus-induced gene silencing has also been developed as an efficient transient system for functional analysis of plant genes (Baulcombe 1999; Sato F. 2005) and has been adapted to soybean plants. Since this approach is dependent on the host range of the virus vector, several types of vector have been developed and applied to different genotypes and developmental stages of soybean. Bean pod mottle virus-based vectors are an efficient and well-established tool for analysis of gene function in soybean, but such vectors were found not to induce gene silencing in seeds (Kachroo et al. 2008; Zhang and Ghabrial 2006). In contrast, cucumber mosaic virus- and apple latent spherical virus-based vectors induced gene silencing in both the vegetative and reproductive stages in soybean and were shown to affect metabolite concentrations in soybean seeds (Liu et al. 2010; Nagamatsu et al. 2007; Yamagishi and Yoshikawa 2009). However, the efficacy of induced gene silencing and the extent of induced symptoms varied with the specific combinations of each of these three types of viral vector and soybean cultivars. In addition, given that stage- or organ-specific silencing is difficult to achieve by virus-induced gene silencing, changes in seed composition might appear as a side effect of gene silencing in vegetative organs. In contrast, in the present study, the abundance of three subunits of β-conglycinin, which share high sequence similarity, was reduced in transgenic somatic embryos, and the extent of this reduction was similar to that observed in transgenic seeds obtained from the corresponding transgenic line. Furthermore, adverse effects of gene silencing in vegetative organs can be eliminated by the use of transgenic somatic embryos. Our results thus demonstrate that somatic embryos are an efficient and reliable option for functional studies of genes that contribute to the biosynthesis of soybean seed components by RNAi-mediated gene silencing. This approach is less applicable, however, to studies of seed components that are synthesized at the late stage of maturation, given that the similarity of somatic embryos to developing seeds at this stage is limited (Nishizawa and Ishimoto 2009). Improvement in medium for maturation of somatic embryos might overcome this limitation.

Acknowledgements

We thank S. Utsumi (Kyoto University, Japan) for providing antisera to the α, α′, and β subunits of β-conglycinin as well as C. Masuta, K. Goto, and Y. Kogure (Hokkaido University, Japan) for advice on siRNA detection. This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and, in part, by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation, GMZ-1004) and the Fuji Foundation for Protein Research (Osaka, Japan).

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