Simultaneous site-directed mutagenesis for soybean ß-amyrin synthase genes via DNA-free CRISPR/Cas9 system using a single gRNA

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Running head: Site-directed mutagenesis of soybean ß-amyrin synthase genes

Key message: We generated soybean mutants related to two ß-amyrin synthase genes using DNA-free site-directed mutagenesis system. Our results suggested that one of the genes is predominant in the soyasaponin biosynthesis.

Keywords: *Glycine max*, ß-amyrin synthase, soyasaponins, shoot apical meristem, ribonucleoprotein, particle-bombardment

Abstract

Soyasaponins, which are triterpenoid saponins contained in soybean [*Glycine max* (L.) Merril], are responsible for the astringent aftertaste of soyfood, and their complete elimination from soybean seeds is a key challenge in the development of cultivars with improved taste. While the loss of function in the ß-amyrin synthase genes (*GmBAS1* and *GmBAS2*) has proven effective in reducing soyasaponin content in soybean seeds, the specific functional roles of these genes remain unclear. In this study, site-directed mutagenesis was performed on two *GmBAS* loci using a DNA-free clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonuclease 9 (Cas9) system. A complex of sgRNA targeting sequences conserved in the two loci and Cas9 protein was introduced into the shoot apical meristems of soybean embryonic axes via bombardment. Cleaved amplified polymorphic sequences (CAPS) analysis conducted one month post-bombardment revealed that 138 seedlings out of 1,467 screened exhibited mutations at one or both *GmBAS* loci. CAPS and sequencing analysis in the subsequent generation identified a total of 16 plants with inheritable mutations ranging from one to ten nucleotides. High-performance liquid chromatography (HPLC) analysis showed that site-directed mutagenesis in the *GmBAS1* locus resulted in the absence of soyasaponins in mature seeds, as well as in young roots, stems, and leaves. These findings demonstrate that *GmBAS1* is the predominant ß-amyrin synthase gene in soybean plants. Additionally, the DNA-free CRISPR/Cas9 system was shown to be highly efficient in inducing simultaneous mutagenesis at two target loci using a single gRNA.

Introduction

Soyasaponins are oleanane-type triterpenoid saponins contained in soybean [*Glycine max* (L.) Merril] and cause the astringent aftertaste, one of the undesirable taste, of soyfood (Okubo et al. 1992). Since the demand for soyfoods is expected to rise as a critical source of vegetable protein, it is becoming increasingly important to remove the undesirable taste from soyfood. Genetic improvement towards the depletion of soyasaponins in soybean seeds has been performed (Kato et al. 2007). Some studies have suggested that soyasaponins play a role in antioxidant activity (Tsujino et al. 1994; Yoshiki and Okubo 1995; Yoshiki et al. 2001), root growth stimulation (Tsurumi and Tsujino 1995), and rhizosphere formation (Fujimatsu et al. 2020). The exact impact on these functions in plants has not been thoroughly investigated. Consequently, targeted mutagenesis for lacking soyasaponins in the same genetic background is a significant

challenge in developing cultivars with improved flavor profiles and elucidating the exact biological role of soyasaponins.

The biosynthetic pathway of soyasaponins has been extensively studied. The first step in soyasaponin biosynthesis is the cyclization of 2,3-oxidosqualene, catalyzed by ß-amyrin synthase (Kushiro et al. 1998). Subsequent oxidation of ß-amyrin by cytochrome P450 enzymes produces soyasapogenol A and B, the aglycones for soyasaponins. Glycosylation of these aglycones further diversifies the structural and functional characteristics of soyasaponins (Seki et al. 2015). Several genes involved in soyasaponin biosynthesis have been identified in soybean, including those encoding ß-amyrin synthase (*BAS*) (Takagi et al. 2011), three cytochrome P450 enzymes (Shibuya et al. 2006; Yano et al. 2017), and eight uridine 5'-diphosphate-glycosyltransferases (Shibuya et al. 2010; Sayama et al. 2012; Yano et al. 2018; Takagi et al. 2018; Sundaramoorthy et al. 2019; Chung et al. 2020).

Since no naturally occurring soybean varieties completely lack soyasaponins, artificial modifications have been employed to target genes involved in soyasaponin biosynthesis. For example, knockdown of the *GmBAS* genes led to a significant reduction of soyasaponins in soybean seeds (Takagi et al. 2011). Additionally, soyasaponin-deficient mutants identified in a high-density soybean mutant library, generated through ethyl methanesulfonate treatment, were found to possess mutations in the *GmBAS1* locus (Krishnamurthy et al. 2019). These findings underscore that loss of function in the *GmBAS* genes is highly effective at reducing soyasaponin content in soybean seeds.

Two homologs coding for ß-amyrin synthase (*GmBAS1* and *GmBAS2*) were reported in a previous molecular biological study of soybeans (Takagi et al. 2011). These homologs share over 95% similarity in their amino acid sequences and likely retain key motifs essential for enzymatic function (Takagi et al. 2011). While the high similarity between their amino acid sequences suggests potential functional redundancy, their expression patterns differ across tissues. Expression of *GmBAS1* is significantly higher than *GmBAS2* in various tissues (Takagi et al. 2011). In contrast, *GmBAS2* exhibits low, specific expression in flowers and pods, as indicated by RNA-seq data retrieved from a gene expression database (Almeida-Silva et al. 2023, https://soyatlas.venanciogroup.uenf.br/). These differences may reflect the spatial and temporal regulation of soyasaponin biosynthesis in soybean tissues. However, the functional roles of these homologs remain unclear. Therefore, generating mutants that lack one or both functions of the BAS homologs in the same genetic background,

followed by their physiological characterization, will be essential for understanding the distinct roles of these genes.

Site-directed mutagenesis is an effective technique for generating mutations in target genes. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonuclease 9 (Cas9) system is one of the most widely used site-directed mutagenesis systems for higher plants. In soybeans, the CRISPR/Cas9 system is primarily applied through two transformation methods: *Agrobacterium*-mediated and particle bombardment (Sim et al. 2023). Both methods typically require the production of transgenic plants via regeneration from explants. However, the efficiency of plant regeneration is genotype-dependent, and soybean transformation is only successful in a limited range of genotypes (Yamada et al. 2012). This limitation also restricts the application of site-directed mutagenesis in soybean. Furthermore, the integration of the CRISPR/Cas9 expression module as foreign DNA into the soybean genome poses a significant challenge for commercial use, as the removal of foreign genes through genetic segregation is a time-consuming and labor-intensive process. Recently, Kuwabara et al. (2024) optimized the in planta bombardment-ribonucleoprotein (iPB-RNP) method, initially developed as a DNA-free site-directed mutagenesis system in wheat (*Triticum aestivum*) (Kumagai et al. 2022), for soybean. In the iPB-RNP system, a complex of sgRNA and Cas9 protein is directly introduced into the shoot apical meristems (SAMs) of soybean embryonic axes via particle bombardment (Kuwabara et al. 2024). This method circumvents the need for regeneration to obtain mutants, thus expanding the range of genotypes available for site-directed mutagenesis. Additionally, the direct delivery of the ribonucleoprotein (RNP) complex reduces the risk of off-target mutations due to probable rapid degradation of the RNP, and it eliminates the need for genetic segregation to remove foreign DNA.

In this study, as an advanced application of the iPB-RNP method, site-directed mutagenesis was conducted at each individual locus and both loci using a single RNP targeting a common sequence in two homologous *GmBAS* genes. Sequence analysis revealed heritable mutations, including deletions and/or insertions of bases, at one or both *BAS* loci in the next generation. Mutagenesis at the *GmBAS1* locus resulted in the absence of soyasaponins in mature seeds, as well as in young roots, stems, and leaves. These results demonstrate that *GmBAS1* is the predominant ß-amyrin synthase gene in soybean plants, as evidenced by simultaneous mutagenesis at the duplicated loci via the iPB-RNP system.

Materials and Methods

Preparation of soybean shoot apical meristem

Mature soybean seeds (*Glycine max* L. cv. GL3510) were sterilized using 1.5% sodium hypochlorite for 2 minutes and thoroughly rinsed. After being washed ten times with sterile distilled water, the seeds were soaked in sterile distilled water for 15–16 hours in the dark at 25°C. Embryonic axes were carefully excised from the cotyledons using a scalpel, and unifoliate leaves and primary stipules were delicately removed under a stereomicroscope. A total of 16 explants were arranged in a circular pattern, approximately 1 cm in diameter, with the shoot apical meristem regions oriented upward on a bombardment medium. This medium consisted of Murashige-Skoog (MS) medium (pH 5.7, Sigma-Aldrich), 20 g/L sucrose, 36.4 g/L mannitol, 36.4 g/L sorbitol (Wako), 0.5 mg/L MES (Dojindo Laboratories), 0.7 mg/L L-proline (Wako), 20 mg/L meropenem (Sumitomo Dainippon Pharma), and 4.1 g/L phytagel (Millipore Sigma).

Preparation of gold particles and particle bombardment

In this study, only crispr RNA (crRNA) and trans-activating crRNA (tracrRNA) were used as guide sequences for the Cas9 protein to perform DNA-free site-directed mutagenesis. Both the crRNA and tracrRNA were obtained from FASMAC Co., Ltd. and dissolved in nuclease-free water to a concentration of 1 μg/μL, stored at -80°C. To form a single guide RNA (sgRNA), guide crRNA and tracrRNA were mixed in equal amounts and incubated on ice for 10 minutes. Subsequently, *Streptococcus pyogenes* Cas9 (SpCas9) protein (NEB), 1x CutSmart buffer (NEB), and RNase inhibitor (Thermo Fisher Scientific) were added, and the mixture was incubated at room temperature for an additional 10 minutes. The sgRNA-SpCas9 complex solution was then combined with TransIT transfection reagent (TaKaRa) and incubated at room temperature for 5 minutes. Gold particles (1.0 μm; BioRad) were added to the mixture and incubated on ice for 10 minutes. The suspension was centrifuged at 1,200 *g* for 1 second, and the resulting pellet was resuspended in nuclease-free water. The sgRNA/SpCas9-coated gold particle suspension was distributed onto a hydrophilic membrane (Scotchtint, 3M) attached to a microcarrier and allowed to dry naturally prior to bombardment.

Bombardment was performed using a Biolistic PDS-1000/He Particle Delivery System (BioRad) according to a slightly modified version of the manufacturer's instructions. The distance between the target plantlets and the stopping screen was approximately 3.5 cm. Each plate containing the explants was bombarded at a helium pressure of 1,800 psi. Following bombardment, the explants were incubated at 25°C in darkness for 24 hours.

Caring of explants

Twenty-four hours after bombardment, the explants were transferred to MS medium (pH 5.7) supplemented with 30 g/L sucrose, 0.5 g/L MES, 20 mg/L meropenem, 3 mL/L Plant Preservative Mixture (Plant Cell Technology), 1.12 mg/L 6-Benzyladenine (Wako), 7 g/L charcoal (Nacalai Tesque), and 2.05 g/L phytagel. These explants were maintained at 25°C under an 18-hour light photoperiod. Approximately two weeks later, explants with successfully elongated shoots and roots were individually transferred to seedling trays filled with potting soil (Sumitomo Forestry Landscaping). To prevent desiccation of the young leaves, each tray was covered with a plastic sheet. The plantlets were kept at 25°C under a 16-hour light photoperiod. The E_0 plants were subsequently transferred to plastic pots filled with a mixture of potting soil and culture soil (Katakura & Co-op Agri Corp) in a 1:1 ratio. These plants were maintained in a closed greenhouse under a 12-hour photoperiod at 25°C until the E_1 seeds were collected.

Cleaved amplified polymorphic sequence (CAPS) analysis

To extract genomic DNA, leaf pieces were collected in bulk from bombarded seedlings, with 3–4 trifoliate leaf pieces obtained separately from each plant. Genomic DNA extraction from leaf pieces and mature seeds was conducted as described by Sugano et al. (2020). The target regions in the *GmBAS1* and *GmBAS2* loci were amplified by PCR using specific primers (Table S1). PCR conditions were as follows: 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The amplified products were digested with the EcoNI restriction enzyme (NEB) and separated by electrophoresis in 2.0% agarose gels. DNA fragments of the expected sizes, derived from the target region containing mutations, were identified as mutants.

DNA sequencing

Fragments amplified using specific primers for sequencing were purified using ExoSAP-IT reagent (Affymetrix) and sequenced directly or after cloning into the pGEM-T-Easy vector (Promega). Sequencing of the amplified products was performed on an ABI3130 sequencer (Applied Biosystems). This sequence analysis was carried out by the Instrumental Analysis Division at the Graduate School of Agriculture, Hokkaido University, and occasionally by FASMAC Co., Ltd. through their contract analysis service.

Expression analysis of the *GmBAS* genes by quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the leaves, stems, and roots of juvenile seedlings, as well as from immature seeds, using the LiCl precipitation procedure (Adachi et al. 2021). Each cDNA was synthesized from 500 ng of total RNA using the M-MLV reverse transcriptase system (Invitrogen) with random hexamer primers and an oligo(dT)20 primer, following the manufacturer's instructions.

The qRT-PCR reaction was carried out in a 20-μL volume containing 1 μL of cDNA, 0.8 μL of each primer (10 μM), and 10 μL of SYBR Premix ExTaq II (Tli RNaseH plus) (TaKaRa). Reactions were performed using a CFX 96 Real-Time System (BioRad) under the following conditions: 95°C for 4 minutes, followed by 40 cycles of 95°C for 10 seconds, 59.5°C for 20 seconds, 72°C for 20 seconds, and 78°C for 2 seconds. Fluorescence was quantified before and after incubation at 78°C to monitor primer dimer formation. The expression levels of *GmBAS1* (Glyma.07G001300) and *GmBAS2* (Glyma.08G225800) were normalized against the expression of the *Bic-C2* gene (Glyma.03G064800). The genome of Glycine max Wm82.a2.v1 (Phytozome genome ID: 275) were used as a reference. Amplification of a single DNA fragment for each transcript was confirmed by melting curve analysis. Averages and standard errors of relative expression levels were calculated from three independent plants. Primers used for expression analysis are listed in Table S1.

Extraction and HPLC analysis of soyasaponins

Soyasaponins were extracted from mature seeds, leaves, stems, and roots of juvenile seedlings. Dried seeds and lyophilized leaves, stems, and roots were ground using a Multi-Beads Shocker (MB1200; Yasui Kikai Co.). Thirteen mg of fine powder was thoroughly mixed with 260 μL of 70% aqueous ethanol containing 0.1% (v/v) acetic acid. The mixture was incubated at 4°C for 1 hour in darkness, with vortexing every 15 minutes. After centrifugation at 17,900 g for 10 minutes, 100 μL of the supernatant was transferred into a 0.2 μL tube. Leaf, stem, and root samples were filtered using 0.45 μm GL chromatodisk filters (GL Sciences). Ten μL of HCl was added to the samples, and the mixture was incubated at 85°C for 3 hours. The hydrolyzed solution was then transferred into an HPLC vial insert.

Analysis was performed using an HPLC system (Hitachi LaChrom Elite, Hitachi High-Technologies Corp.) with a Unison UK-C18 reverse-phase column (3.0 mm × 150 mm, Imtakt Corp.). The column temperature was maintained at 40°C, and separation was performed under gradient conditions. Solvent A was acetonitrile (0.1% acetic acid), and solvent B was water (0.1% acetic acid). The gradient program for mature seeds was as follows: linear 35–25% B from 0–12.5 minutes; isocratic at 25% B from 12.3–13 minutes;

isocratic at 0% B from 13.1–15.5 minutes; and isocratic at 35% B from 15.6–24 minutes. For leaves, stems, and roots of juvenile seedlings, the gradient was linear 50–46% B from 0–40 minutes; linear 46–0% B from 40–42 minutes; isocratic at 0% B from 42–50 minutes; linear 0–50% B from 50–52 minutes; and isocratic at 50% B from 52–60 minutes. The injection volume for each sample was 10 μL, and the flow rate was 0.44 mL/min. Soyasapogenols were detected at a wavelength of 210 nm. Soyasapogenol A and B, purchased from FUJIFILM WAKO Pure Chemicals, were dissolved in 70% aqueous ethanol. The content of soyasapogenols in mature seeds was determined based on the peak area ratio of the standards. In leaves, stems, and roots, the content was calculated based on the peak height ratio of the standards.

Statistical analysis

Tests for significance among the soyasapogenol contents were performed using Student's *t*-test. *P*-values < 0.05 were considered statistically significant. Test of significance among means of transcript abundances was carried out with Tukey-Kramer test. *P*-values < 0.01 were considered statistically significant.

Results

Induction of mutations at soybean ß-amyrin synthase (*GmBAS*) loci in the E_0 generation

GmBAS homologs are located on chromosomes 7 and 8 and consist of 16 exons (Figure 1a). To investigate the expression patterns of *GmBAS* homologs in GL3510, qRT-PCR was performed on juvenile seedlings and immature seeds using homolog-specific primer sets. Expression of *GmBAS* homologs was detected in all tissues examined, whereas the expression level of *GmBAS2* was lower than that of *GmBAS1* across the tissues (Figure 1b, c).

A gRNA targeting sequences conserved in both *GmBAS* homologs was designed to induce site-directed mutagenesis (Figure 2a). Mutation induction at the target regions was assessed by cleaved amplified polymorphic sequence (CAPS) analysis one month after bombardment. DNA fragments were classified as wild-type or mutant-type based on the expected fragment size (Figure 2b). Among the 1,467 seedlings screened, 80 showed mutations at the *GmBAS1* locus, 10 at the *GmBAS2* locus, and 48 at both loci (Table 1). In this study, the post-bombarded generation was designated as E_0. To obtain mutants at either the *GmBAS1* or *GmBAS2* loci, or both, in the E_1 generation, E_0 plants were analyzed by CAPS at the 3–4 trifoliate leaf development stage and in the E_1 seeds.

A total of 36 E_0 plants were selected for further analysis in the next generation (Table 1).

Detection of inheritable mutations to the E_1 generation

The inheritance of mutations to the next generation was evaluated by CAPS and sequencing analyses in the E_1 generation (Table S2). A total of 16 E_0 plants displayed inheritable mutations in the E_1 generation: seven at the *GmBAS1* locus, two at the *GmBAS2* locus, and seven at both loci (Table 1). E_1 seeds possessing homozygous mutant alleles or biallelic mutations, as confirmed by CAPS analysis, were subjected to sequencing. Sequencing analysis revealed a range of mutations, including single-nucleotide insertions and deletions of 1, 3, 4, 5, 6, or 7 nucleotides at the *GmBAS1* locus, and single-nucleotide insertions and deletions of 1, 2, 4, 5, 7, or 10 nucleotides at the *GmBAS2* locus (Figure 3 and Table S3). Based on predicted amino acid sequences, all mutant alleles—except those with deletions of 3 or 6 nucleotides—were likely to generate premature stop codons through frameshift mutations, resulting in nonfunctional mutant proteins (Figure S1). Moreover, no off-target mutations were detected in the E_1 generation (Table S4).

Determination of soyasaponin in mutant seeds and juvenile seedlings

To assess the impact of loss of function in each *GmBAS* homolog on soyasaponin accumulation in soybean seeds, the saponin content of mutant seeds was measured using high-performance liquid chromatography (HPLC). Mutant lines used in HPLC analysis are listed in Table 2. These lines were categorized into three haplotypes (*bas1*, *bas2*, and *double*) based on the genotypes of the *GmBAS* loci. Three lines (27-10-1, 50-3-2, and 104-4-2) were selected as representative *bas1* mutants. Another three lines (32-10-2-1, 52-11-2-13, and 99-3-2) were chosen as representative *bas2* mutants. The remaining four lines, 52-11-1-5, 113-7-4, 113-7-6, and 113-7-10 were classified as representative *double* mutants. Two plants, 52-11-2-13 and 52-11-1-5, were separated from the E_1 plants that were judged to be *double* mutants and were subsequently divided as *bas2* and *double* mutant haplotypes, respectively (Table 2).

Due to the structural diversity of soyasaponins, which vary in the number and composition of sugar moieties, identifying all soyasaponins via HPLC is challenging. Therefore, extracts from mature seeds were hydrolyzed to detect soyasaponin aglycons (soyasapogenols), and the total soyasapogenols content was estimated. In the extracts from wild-type seeds, peaks corresponding to soyasapogenol A and B were detected (Figure 4). The contents of soyasapogenol A and B were 88.9 ± 29.9 mg/100 gDW and

166.2 ± 42.0 mg/100 gDW, respectively (Table 3). In the extracts from mutant-type seeds, peaks corresponding to both soyasapogenols were detected only in the *bas2* mutant lines, but not in the *bas1* and *double* mutant seeds (Figure 4). The concentrations of soyasapogenol A and B in seeds were 92.6 ± 8.5 and 163.6 ± 18.4 mg/100 gDW in the 32-10-2-1 line, 75.4 ± 11.0 and 150.9 ± 20.2 mg/100 gDW in the 52-11-2-13 line, and 76.2 ± 6.2 and 155.1 ± 15.5 mg/100 gDW in the 99-3-2 line, respectively (Table 3). No statistically significant difference in soyasapogenols content was observed between the *bas2* mutants and control seeds.

To assess the effects of *GmBAS* mutations in other plant tissues, soyasapogenols content in the roots, stems, and leaves of juvenile seedlings was measured. In wild-type plant extracts, peaks for soyasapogenol A and B were detected in the roots and stems, while only soyasapogenol B was detected in the leaves (Figure 5). In wild-type roots, the concentrations of soyasapogenol A and B were 105.4 ± 16.5 and 377.4 ± 33.7 mg/100 gDW, respectively. In wild-type stems, the concentrations of soyasapogenol A and B were 14.3 ± 10.1 and 124.2 ± 41.1 mg/100 gDW, respectively. In wild-type leaves, the concentration of soyasapogenol B was 150.0 ± 30.0 mg/100 gDW (Table 4).

For the HPLC analysis in juvenile seedlings, two mutant lines from each haplotype were selected (Table 4). Peaks for soyasapogenols were detected in the roots, stems, and leaves of only the *bas2* mutant plants, and not in the *bas1* or *double* mutant plants. In the roots of the 32-10-2-1 line, the concentrations of soyasapogenol A and B were 92.4 ± 20.6 and 360.9 ± 61.1 mg/100 gDW, respectively, and in the 52-11-2-13 line, they were 102.8 ± 18.7 and 386.6 ± 42.7 mg/100 gDW (Table 4). In the stems, the concentrations of soyasapogenol A and B were 26.5 ± 3.2 and 245.1 ± 114.8 mg/100gDW in the 32-10-2-1 line, and 25.1 ± 15.7 and 278.9 ± 93.5 mg/100 gDW in the 52-11-2-13 line (Table 4). The concentrations of soyasapogenol B in leaves were 225.6 ± 46.4 mg/100 gDW in the 32-10-2-1 line, and 237.6 ± 33.2 mg/100 gDW in the 52-11-2-13 line (Table 4).

Discussion

Site-directed mutagenesis of *GmBAS* homologs involved in the first step of soyasaponin biosynthesis in soybeans was performed using the iPB-RNP system. In this study, the iPB-RNP method was advanced by executing simultaneous mutagenesis at two target loci using a single gRNA without off-target mutations. Although the conserved sequences were targeted to the *GmBAS1* and *GmBAS2* loci in this study, the mutagenesis frequency of the *GmBAS1* locus was higher than that of the *GmBAS2* locus

(Table 1). In Arabidopsis, heterochromatic features were strongly associated with the low mutagenesis frequencies using the CRISPR/Cas9 system (Weiss et al. 2022). Epigenetic resources may therefore influence the differences in site-directed mutagenesis frequencies between *GmBAS1* and *GmBAS2* loci.

Conventional site-directed mutagenesis through *Agrobacterium* transformation with a single gRNA has achieved simultaneous mutagenesis at multiple target regions in soybean (Kanazashi et al. 2018; Li et al. 2019; Wang et al. 2019; Jia et al. 2020; Shen et al. 2022; Fu et al. 2022). However, in the iPB-RNP method, opportunities for simultaneous mutagenesis at multiple loci are limited, as RNPs introduced into cells are rapidly degraded during cell division. In this study, mutant alleles at both target loci were detected in the E_1 seeds from seven E_0 plants (Table 1). These findings indicate that simultaneous mutagenesis was induced in a single cell where RNPs had been delivered. Furthermore, the introduction of RNPs into cells also generated different mutant alleles in one target locus. One- or five-nucleotide deletions at the *GmBAS1* locus, and two- or four-nucleotide deletions at the *GmBAS2* locus, were detected in the progeny of the 113-7 E_0 plant (Table 2). In conventional site-directed mutagenesis via *Agrobacterium* transformation, four mutant alleles were identified at the *Gly m Bd 30K* locus, one of the allergenic genes in soybean, in the progeny of one T_0 plant (Sugano et al. 2020). This suggests that the constant presence of the CRISPR/Cas9 module via exogenous genes may result in the production of multiple mutant alleles at the target locus. Interestingly, in addition to the two mutant alleles, a wild-type allele was detected at the *GmBAS1* locus in the progeny of the 113-7 E_0 plant (Figure S2), suggesting that three alleles were produced at this locus. In the iPB-RNP system, the delivery of RNPs to cells that will differentiate into germ cells is crucial for inducing inheritable mutations in subsequent generations (Kuwabara et al. 2024). Thus, the three alleles detected in the progeny of the 113-7 plant likely originated from two or more cells that later differentiated into germ cells. Enhancing the precision of RNP delivery to target cells in the iPB-RNP system could increase the likelihood of producing multiple mutant alleles at target loci.

In this study, deletion mutations ranging from one to ten nucleotides were observed in the target regions (Table S3). By contrast, deletion mutations exceeding ten nucleotides are sometimes found in conventional site-directed mutagenesis via *Agrobacterium* transformation in soybeans (Li et al. 2019; Shen et al. 2022). Although there are few reports of iPB-RNP system-based site-directed mutagenesis in soybeans, deletion mutations of up to 13 nucleotides have been observed in the *Gly m Bd 30K* gene, another allergenic gene, using the iPB-RNP system (Kuwabara et al. 2024). These

results indicate that the number of nucleotides removed by the iPB-RNP system is generally lower compared to conventional site-directed mutagenesis via transformation. In site-directed mutagenesis, nucleotide deletions primarily result from errors during the non-homologous end joining (NHEJ) repair process (Li et al. 2015). The availability of the CRISPR/Cas9 modules at target regions differs significantly between conventional site-directed mutagenesis and iPB-RNP systems. The iPB-RNP system provides these modules transiently, while conventional methods allow prolonged exposure. If large nucleotide deletions occur more frequently during certain stages of cell division due to NHEJ repair errors, the difference in CRISPR/Cas9 module availability may influence the occurrence of large deletions.

Frameshift mutations at the *GmBAS1* locus resulted in the deficiency of soyasaponins in mature seeds, roots, stems, and leaves of mutant plants (Figures 4, 5). These results suggest that *GmBAS1* is predominant in soyasaponin biosynthesis in these tissues. The amino acid sequence similarity between *GmBAS* homologs is 95.9% (Takagi et al. 2011), and their enzymatic activities are likely redundant, as they conserve the DCTAE and QW motifs, which are responsible for substrate binding and stabilization of carbocationic intermediates, respectively (Poralla et al. 1994; Abe and Prestwich 1995). Expression analysis in this study demonstrates that *GmBAS1* is highly expressed in immature seeds, roots, stems, and leaves, whereas *GmBAS2* expression is minimal in these tissues (Figures 1b, c). In *Glycyrrhiza uralensis*, the expression of the *BAS* gene was upregulated in response to methyl jasmonate, a compound associated with plant defense responses (Tamura et al. 2018). Although *GmBAS2* expression was low in this study, the expression of *GmBAS* genes may increase in response to biotic stress.

Quantitative analysis of soyasaponins revealed that soyasapogenol B levels were higher than those of soyasapogenol A in all tissues examined (Tables 3, 4). The *bas2* mutants tented to have higher soyasapogenol B content in stems and leaves than the wild type (Tables 3, 4). Comprehensive gene expression analysis of stems and leaves in the *bas2* mutants may reveal that soyasapogenol B biosynthesis is more activated than in the wild type. Previous studies have shown that 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) soyasaponins, which contain soyasapogenol B as an aglycon, preferentially accumulate in various soybean tissues (Tsuno et al. 2018; Ma et al. 2024). These findings indicate that the soyasapogenol B detected in this study is likely derived primarily from DDMP soyasaponins. The root growth of *Lactuca sativa* is enhanced under the presence of DDMP soyasaponins (Tsurumi and Tsujino 1995), suggesting that DDMP soyasaponins may play a physiological role in plant growth. Although no significant differences in morphological characteristics were observed between mutant

and control plants under the controlled growth conditions in this study (Figure S3), the relationship between soyasaponin deficiency and plant growth warrants further investigation. The soyasaponin content in roots was the highest among all tissues examined (Tables 3, 4). Various soyasaponin compounds accumulate in roots during the vegetative growth stage, and some of these compounds are secreted externally to regulate the rhizosphere environment (Tsuno et al. 2018). However, the physiological functions of soyasaponins remain unclear. The soyasaponin-deficient mutants generated in this study will provide valuable tools to investigate the biological and physiological roles of soyasaponins in soybeans.

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Author contributions

HA, KM, RS, TY, YM, and TY designed the experiments. HA and CK performed the site-directed mutagenesis. HA, CK, and TY analyzed the data. All authors approved the final manuscript and agreed to be accountable for the work presented.

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Data availability

The datasets generated or analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest

TY and AS were employed by Kanematsu Corporation. TY receives research support from Kanematsu Corporation. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Figure legends

Fig. 1 Structure and expression properties of *GmBAS* genes.
a Schematic diagrams of *GmBAS1* and *GmBAS2* genes. Light blue and grey boxes represent translated and untranslated regions, respectively. Bold lines represent introns.
b, c Expression analysis of *GmBAS* genes in soybean tissues. Relative expression levels were normalized to the internal control gene *Bic-C2* (Glyma.03G064800). Seed developmental stages were classified based on fresh seed weight (mg per seed). Leaf, stem, and root samples were collected from young seedlings. Error bars represent the standard deviation from three biological replicates. Different alphabets indicate significant differences based on Tukey-Kramer test, ($P<0.01$).

Fig. 2 Target regions of gRNA and detection of mutations by CAPS analysis.
a Target sequences of gRNA conserved between *GmBAS* genes. The gRNA was designed within conserved sequences between the two genes and is represented by vertical lines in the schematic diagrams. Bold sequences represent the target sequences of gRNA and the PAM region. Numbers in the box indicate exon numbers, and arrows show PCR products from each gene used in this study. Underlined sequences indicate the EcoNI recognition site.
b CAPS analysis of amplified products from the target loci. Solid and dotted square brackets beside the image represent amplified and digested products from *GmBAS1* and *GmBAS2*, respectively. Asterisks denote mutant-type fragments. Numbers above each lane indicate E_1 plant numbers. 'M' represents the 100 bp ladder marker. 'WT/-' indicates undigested products amplified from the control plant.

Fig. 3 Mutations detected within the target loci in E_1 seeds.
'WT' indicates the target sequences and flanking regions of *GmBAS1* and *GmBAS2* genes in the control plant. Numbers on the left side of the sequences correspond to E_1 and E_2 seed numbers. Bold sequences represent the gRNA-targeted sequences; underlined sequences denote the PAM region. Italic letters on the right side indicate mutation types, e.g., *d1* (a single-nucleotide deletion) and *i1* (a single-nucleotide insertion).

Fig. 4 HPLC analysis of soyasapogenols extracted from mutant and control seeds.
Hydrolyzed seed extracts were compared to authentic standards. 'WT' represents the chromatogram of a control seed. Numbers in the upper left of each panel represent mutant line numbers. SA and SB denote the peaks for soyasapogenol A and B, respectively.

Fig. 5 HPLC analysis of soyasapogenols extracted from mutant and control seedlings.

Hydrolyzed extracts from root (**a**), stem (**b**) and leaf (**c**) samples were compared to authentic standards. 'WT' represents the chromatogram of a control seedling. Numbers in the upper left of each panel represent mutant line numbers. SA and SB denote the peaks for soyasapogenol A and B, respectively.

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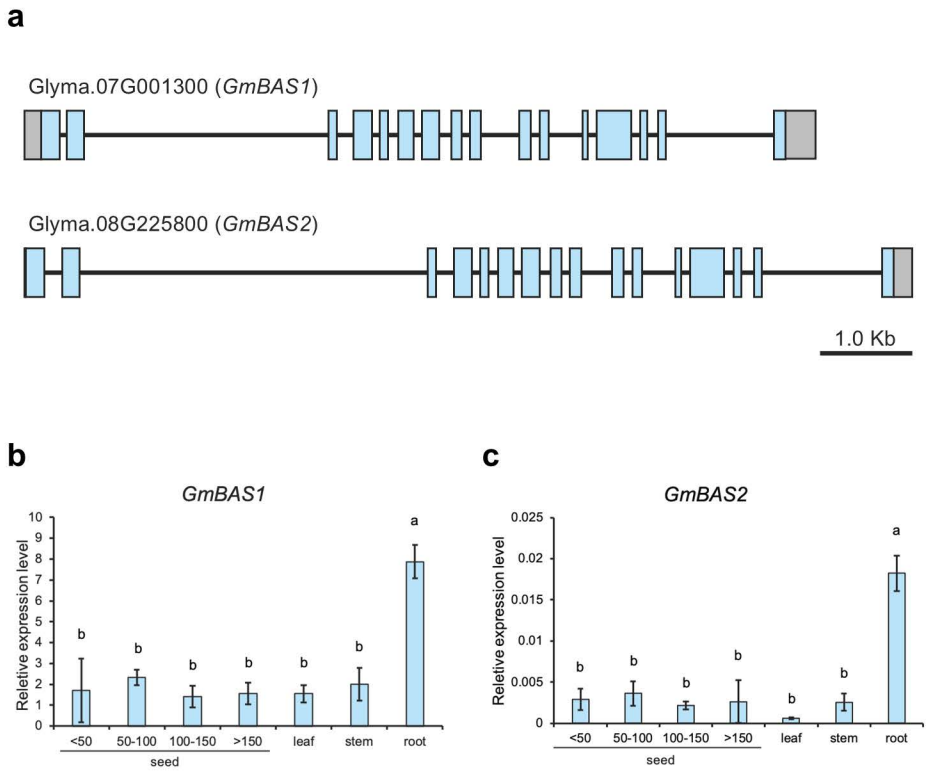
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8
7
6
5
4
3
2
1
0
b
b
b
b
b
b
a
<50
50-100
100-150
>150
leaf
stem
root
seed
c
GmBAS2
Reletive expression level
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0.02
0.015
0.01
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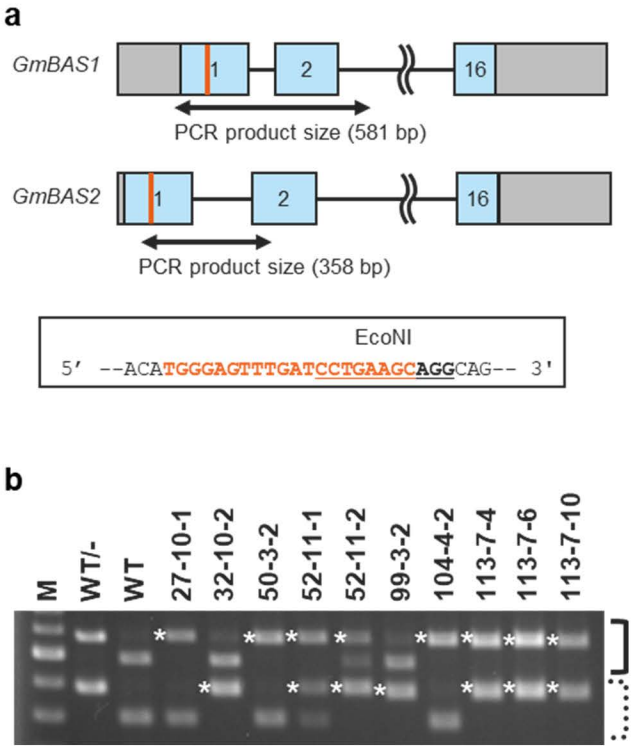
leaf
stem
root
seed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2
16
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1
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b
M
WT/-
WT
27-10-1
32-10-2
50-3-2
52-11-1
52-11-2
99-3-2
104-4-2
113-7-4
113-7-6
113-7-10

GmBAS1

WT	AGACA**TGGGAGTTTGATCCTGA-AGC**AGGCAGTC	
27-10-1	AGACA**TGGGAGTTTGATCCTGAAAGC**AGGCAGTC	*i1*
50-3-2	AGACA**TGGGAGTTTGAT------AGC**AGGCAGTC	*d5*
52-11-1-5	AGACA**TGGGAGTTTGATCCTGATAGC**AGGCAGTC	*i1*
104-4-2	AGACA**TGGGAGTTTGATCCTG--AGC**AGGCAGTC	*d1*
113-7-4	AGACA**TGGGAGTTTGAT------AGC**AGGCAGTC	*d5*
113-7-6	AGACA**TGGGAGTTTGATCCTG--AGC**AGGCAGTC	*d1*

GmBAS2

WT	AGACA**TGGGAGTTTGATCCTGA-AGC**AGGCAGTC	
32-10-2-1	AGACA**TGGGAGTTTGAT------AGC**AGGCAGTC	*d5*
52-11-1-5	AGACA**TGGGAGTTTGATCCTGATAGC**AGGCAGTC	*i1*
52-11-2-13	AGACA**TGGGAGTTTGATCCTGATAGC**AGGCAGTC	*i1*
99-3-2	AGACA**TGGGAGTTTG-------AGC**AGGCAGTC	*d7*
113-7-4	AGACA**TGGGAGTTTGATC-----AGC**AGGCAGTC	*d4*
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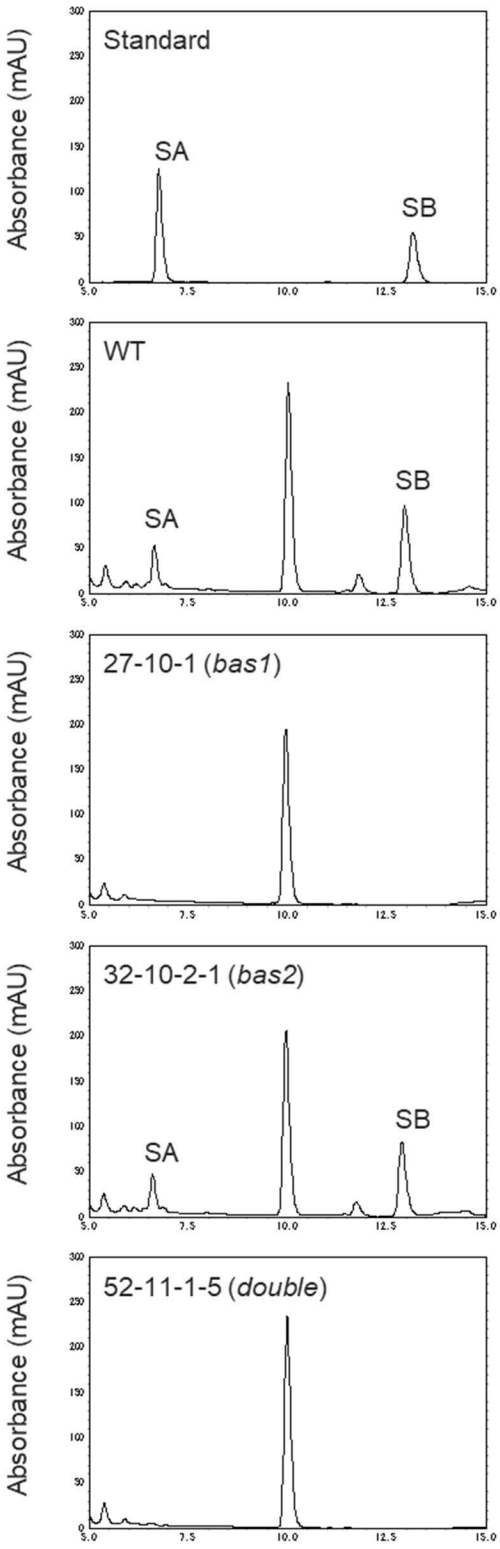
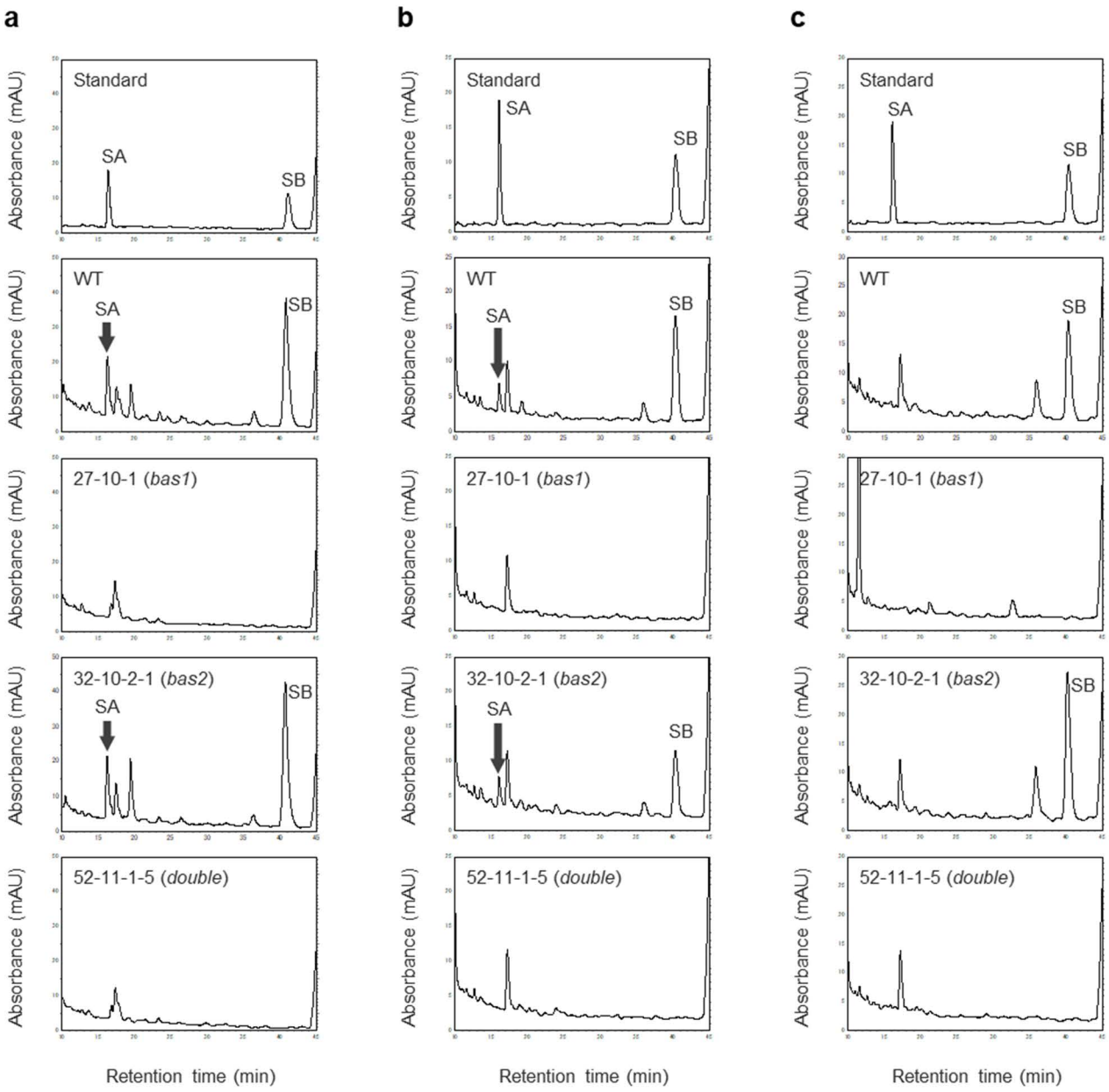

Standard
SA
SB
WT
SA
SB
27-10-1 (bas1)
32-10-2-1 (bas2)
SA
SB
52-11-1-5 (double)
Absorbance (mAU)
Retention time (min)


a
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Standard
SA
SB
WT
27-10-1 (bas1)
32-10-2-1 (bas2)
52-11-1-5 (double)
Absorbance (mAU)
Retention time (min)

Table 1. Mutation frequency at the target loci in E_0 and E_1 generations

No. of bombarded embryonic axes	No. of analyzed E_0 plants	No. of mutants in E_0 generation				No. of plants analyzed in E_1 generation	No. of mutants in E_1 generation			
		GmBAS1	*GmBAS2*	Both loci	Total		*GmBAS1*	*GmBAS2*	Both loci	Total
1697	1467	80	10	48	138	36	7	2	7	16

Table 2. Genotypes of representative mutants used in HPLC analysis

Line No.	Genotypes		Haplotype	Analyzed tissues in HPLC analysis
	GmBAS1 locus	*GmBAS2* locus		
WT	*wt*	*wt*	*wt*	seed, root, stem, leaf
27-10-1	*i1(A)*	*wt*	*bas1*	seed, root, stem, leaf
50-3-2	*d5*	*wt*	*bas1*	seed, root, stem, leaf
104-4-2	*d1*	*wt*	*bas1*	seed
32-10-2-1	*wt*	*d5*	*bas2*	seed, root, stem, leaf
52-11-2-13	*wt*	*i1(T)*	*bas2*	seed, root, stem, leaf
99-3-2	*wt*	*d7*	*bas2*	seed
52-11-1-5	*i1(T)*	*i1(T)*	*double*	seed, root, stem, leaf
113-7-4	*d5*	*d4*	*double*	seed
113-7-6	*d1*	*d2*	*double*	seed
113-7-10	*d5*	*d4*	*double*	root, stem, leaf

Italic letters denote mutation types: e. g., *d1* (a single-nucleotide deletion); *i1* (a single-nucleotide insertion); *(A)* (adenine insertion); *wt* (no mutation).

Table 3. Soyasapogenols content in mutant and control seeds

Line No.	Haplotype	SA content (mg/100 gDW)	SB content (mg/100 gDW)
WT	*wt*	88.9 ± 29.9	166.2 ± 42.0
27-10-1	*bas1*	nd	nd
50-3-2	*bas1*	nd	nd
104-4-2	*bas1*	nd	nd
32-10-2-1	*bas2*	92.6 ± 8.5	163.6 ± 18.4
52-11-2-13	*bas2*	75.4 ± 11.0	150.9 ± 20.2
99-3-2	*bas2*	76.2 ± 6.2	155.1 ± 15.5
52-11-1-5	*double*	nd	nd
113-7-4	*double*	nd	nd
113-7-6	*double*	nd	nd

SA and SB denote soyasapogenol A and B, respectively. Italic letters denote haplotype. Values represents the mean ± SD (n = 5). nd, not detected.

Table 4. Soyasapogenols content in mutant and control seedlings

Plant tissue	Line No.	Haplotype	SA content (mg/100 gDW)	SB content (mg/100 gDW)
root	WT	*wt*	105.4 ± 16.5	377.4 ± 33.7
	27-10-1	*bas1*	nd	nd
	50-3-2	*bas1*	nd	nd
	32-10-2-1	*bas2*	92.4 ± 20.6	360.9 ± 61.1
	52-11-2-13	*bas2*	102.8 ± 18.7	386.6 ± 42.7
	52-11-1-5	*double*	nd	nd
	113-7-10	*double*	nd	nd
stem	WT	*wt*	14.3 ± 10.1	124.2 ± 41.1
	27-10-1	*bas1*	nd	nd
	50-3-2	*bas1*	nd	nd
	32-10-2-1	*bas2*	26.5 ± 3.2	245.1 ± 114.8
	52-11-2-13	*bas2*	25.1 ± 15.7	278.9 ± 93.5*
	52-11-1-5	*double*	nd	nd
	113-7-10	*double*	nd	nd
leaf	WT	*wt*	nd	150.0 ± 30.0
	27-10-1	*bas1*	nd	nd
	50-3-2	*bas1*	nd	nd
	32-10-2-1	*bas2*	nd	225.6 ± 46.4**
	52-11-2-13	*bas2*	nd	237.6 ± 33.2**
	52-11-1-5	*double*	nd	nd
	113-7-10	*double*	nd	nd

SA and SB denote soyasapogenol A and B, respectively. Italic letters denote haplotype. Values are presented as mean ± SD (n = 4). "nd" denotes values that were not detected. Asterisks indicate significant differences between the mutant lines and the control plant, based on an unpaired two-tailed Student's *t*-test, (*P<0.05, **P<0.01).