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ORIGINAL PAPER

Nucleotide sequence variation of *GLABRA1* contributing to phenotypic variation of leaf hairiness in Brassicaceae vegetables

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Abstract *GLABRA1* (*GL1*) belongs to the group of R2R3-MYB transcription factors and is known to be essential for trichome initiation in Arabidopsis. In our previous study, we identified a *GL1* ortholog in *Brassica rapa* as a candidate for the gene controlling leaf hairiness by QTL analysis and suggested that a 5-bp deletion (B-allele) and a 2-bp deletion (D-allele) in the exon 3 of *BrGL1* and a non-synonymous SNP (C-allele) in the second nucleotide of exon 3 possibly cause leaf hairlessness. In this study, we transformed a *B. rapa* line having the B-allele with the A-allele (wild type) or the C-allele of *BrGL1* under the control of the CaMV 35S promoter. The transgenic plants with the A-allele showed dense coverage of seedling tissues including stems, young leaves and hypocotyls with trichomes, whereas the phenotypes of those with the C-allele were unchanged. In order to obtain more information about allelic variation of *GL1* in different plant lineages and its correlation with leaf hairiness, two *GL1* homologs, i.e., *RsGL1a* and *RsGL1b,* in *Raphanus sativus* were analyzed. Allelic variation of *RsGL1a* between a hairless line and a hairy line was completely associated with hairiness in their BC_1F_1 population. Comparison of the full-length of *RsGL1a* in the hairless and hairy lines showed great variation of nucleotides in the 3′ end, which might be essential for its function and expression.

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Introduction

Trichomes are hair-like structures that extend from the epidermis of aerial tissues of plants, and each trichome is comprised of either a single cell or several cells and can be secretory glandular or non-glandular (Esau 1977; Uphof 1962). They are involved in temperature control, water regulation, and protection against insect herbivores and UV irradiation (Johnson 1975; Mauricio and Rausher 1997). The leaf trichome in Arabidopsis is a single epidermal cell and an excellent model system to study plant differentiation at a single-cell level. A number of genes involved in trichome development have been identified and their regulatory network has been elucidated by genetic study of mutants and the study of protein interaction (Hülskamp 2004; Szymanski et al. 2000).

Trichome patterning and initiation in Arabidopsis utilize a lateral inhibition mechanism that depends on the interaction of several transcription factors. These transcription factors can be divided into two groups: positive regulators and negative regulators. Three classes of positive regulators, including the R2R3-MYB transcription factor GL1 (GLABRA1) (Oppenheimer et al. 1991), the basic helix-loop-helix (bHLH) proteins, i.e., GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Payne et al. 2000; Zhang et al. 2003), and the WD40 repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) (Walker

et al. 1999), are postulated to form a transcriptional activation complex. It has been proposed that this complex regulates the expression of two other positive factors, i.e., GL2 (GLABRA2), which is a HD-Zip (homeodomain-leucine Zip) factor (Rerie et al. 1994; Masucci et al. 1996), and TTG2, which is a WRKY transcription factor (Johnson et al. 2002). The trichome-specific differentiation genes are thought to be activated by GL2 (Rerie et al. 1994). Concurrent binding of GL1 and GL3 to the promoter of *GL2* via their own DNA-binding domains is probably required to activate *GL2* (Wang and Chen 2008). TTG2 is also considered to be involved in the regulation of *GL2* expression (Ishida et al. 2007). All the negative regulators, i.e., TRY (TRIPTYCHON) (Schellmann et al. 2002), CPC (CAPRICE) (Wada et al. 1997), and ETC1 and ETC2 (ENHANCER of TRY and CPC1 and 2) (Kirik et al. 2004), are single-repeat MYB proteins with no obvious transcriptional-activation domain. It is assumed that TRY, CPC, ETC1 and ETC2 compete with GL1 for binding of GL3, thereby repressing the formation of a transcriptional-activation complex for trichome initiation and patterning (Hülskamp 2004; Wada et al. 1997; Schellmann et al. 2002; Esch et al. 2003; Kirik et al. 2004).

Despite substantial progress in the study of the genetic mechanism controlling trichome development in *Arabidopsis thaliana* (Pesch and Hülskamp 2009), little is known about the genetics underlying striking variation for trichome density among natural populations. Symonds et al. (2005) have identified nine QTLs for trichome density from four recombinant inbred mapping populations of *A. thaliana*, and *TTG1, TTG2, GL1* and *GL3* are considered to be candidate genes. Hilscher et al. (2009) have revealed that a K-to-E change at amino acid 19 of ETC2 is responsible for a low-trichome-number phenotype in natural *A. thaliana* populations. Different mutations in the *GL1* genes of *A. thaliana* and *A. lyrata* can apparently explain the occurrence of glabrous plants in both species (Hauser et al. 2001; Kivimäki et al. 2007). In *Brassica rapa*, a *GL1* ortholog has been identified by QTL analysis as a candidate for the gene controlling leaf hairiness (Li et al. 2009). Subsequent study on nucleotide sequence variation of *BrGL1* in a natural population has suggested that a 5-bp deletion (B-allele) and a 2-bp deletion (D-allele) in exon 3 of *BrGL1* and a non-synonymous SNP (C-allele) in the second nucleotide of exon 3 likely cause leaf hairlessness (Li et al. 2011). To verify the above conclusion, we carried out transformation of a *B. rapa* line having the B-allele by A-allele (wild type) or the C-allele of *BrGL1*. In addition, to further clarify the allelic variation of *GL1* genes in different plant lineages and its correlation with leaf hairiness, a *GL1* ortholog in *Raphanus sativus* was analyzed and the contribution of its nucleotide sequence polymorphism between a hairless line and a hairy line to their hairiness was investigated.

Materials and methods

Plant materials and DNA extraction

Hairy line 'Yellow sarson' C634 and hairless line C139 of *B. rapa* (Tohoku University Brassica Seed Bank) were used for isolation of the A-allele and C-allele of *BrGL1* (Li et al. 2011), respectively. *B. rapa* 'Osome' with the B-allele of *BrGL1* was used as a material for plant transformation. In order to study a relationship between nucleotide sequence variation of a *GL1* gene in *R. sativus* and leaf hairiness, a line self-pollinated for three generations from 'Sayatori 26704' (hereafter 'Sayatori', National Institute of Vegetable and Tea Science, Japan) and a DH line, Ra38 (Takii Seed Co., Japan), derived from a cultivar of Aokubi type were used. 'Sayatori' has no hair except for a few hairs on the leaf edge, and is thus regarded as hairless, and Ra38 has many hairs on the abaxial and adaxial surfaces of leaves, thus being regarded as hairy. These two lines were crossed to generate F_1, which was then crossed with 'Sayatori' to produce a BC_1F_1 population of 189 individuals. A hairy line of *R. sativus* derived from a cultivar of an Aokubi type was used for Southern blot analysis of *GL1* homologs.

Genomic DNA was prepared from leaves by a modified Cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990).

Vector construction for plant transformation

A- and C-allele sequences of *BrGL1* were amplified from genomic DNAs of *B. rapa* lines C634 and C139 using the primers BrGL1-full-f1 (5′-ATACCCGGGCATAACGAAA TTCCATACCTC-3′) and BrGL1-full-r1 (5′-AACGAG CTCATTATCAAAACTAGAGGCAGTAGCC-3′), which were designed to add *Sac*I and *Xba*I restriction sites to the 5′ end of the GL1fullF and GL1fullR primers (Li et al. 2011), respectively. Polymerase chain reaction (PCR) was performed in a reaction mixture of 25 µl, consisting of 20 ng genomic DNA as a PCR template, 1 µM of primers, 0.5 U of KOD-FX DNA polymerase (Toyobo, Japan), 1× PCR buffer and 200 µM of dNTPs. The thermal cycle of PCR was set to be as follows: 2-min denaturation at 94 °C, 35 cycles of 10-s denaturation at 98 °C, 30-s annealing at 58 °C, 1.5-min extension at 68 °C and a final extension at 68 °C for 10 min. The amplification products were cloned into the pGEM-T® Easy Vector (Promega). Plasmid DNAs from single clones of the A-allele and C-allele of *BrGL1* were digested by both *Sac*I and *Xba*I (Takara Biomedicals,

Japan), followed by ligation with a binary vector pBI121, to yield pBI121-A-allele *BrGL1* and pBI121-C-allele-*BrGL1* constructs (Fig. 1).

Plant transformation

The constructs were introduced into *Agrobacterium tumefaciens* strain EHA105. Plant transformation was carried out following the method of Takasaki et al. (1997). After elongation of hypocotyls to 4–5 cm from aseptically sown seeds of *B. rapa* 'Osome', 5–10-mm sections were used as explants. The explants were inoculated with a 1/10 dilution of overnight-cultured suspension of *Agrobacterium*, followed by transfer to a co-cultivation medium containing MS salts (Murashige and Skoog 1962), 100 mg/l myo-inositol, 1.3 mg/l thiamine-HCl, 200 mg/l KH_2PO_4, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3.0 % (w/v) sucrose, 20 mg/l acetosyringone and 0.7 % agar. After co-cultivation for 3 days at 25 °C under dark conditions, the hypocotyls were placed on a B5 agar medium (Gamborg et al. 1968) containing 1 mg/l 2, 4-dichlorophenoxyacetic acid, 125 mg/l carbenicillin, 30 g/l sucrose and 0.7 % agar and cultured at 25 °C for 7 days. Then they were transferred to a B5 medium containing 3 mg/l 6-benzylaminopurine, 1 mg/l trans-zeatin, 125 mg/l carbenicillin, 10 mg/l kanamycin, 10 mg/l $AgNO_3$, 10 g/l sucrose and 0.7 % agar and cultured for 7 days, followed by three successive transfers to petri dishes containing the same medium without $AgNO_3$. Regenerated shoots were cultured on a B5 medium containing 125 mg/l carbenicillin, 10 mg/l kanamycin, 10 g/l sucrose and 0.7 % agar for root regeneration, and the regenerated plants were grown in a closed greenhouse for isolated cultivation.

The transgenic plants were confirmed by PCR using the primers 35S-f (5′-CAGCAGGTCTCATCAAGACG-3′) and GL1c-f (5′-CTTTGCCGTGAGTAAGGACA-3′) (Fig. 1), sequences of which were taken from the sequences of the 35S promoter and *BrGL1*, respectively.

Southern blot analysis

About 3 μg genomic DNA of a transgenic plant was digested with *Hin*dIII, and that of an *R. sativus* hairy line was digested with *Hin*dIII and *Eco*RI. The digested DNAs were electrophoresed on 1.0 % agarose gel, followed by transfer of DNA onto a nylon membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA). The membrane was hybridized with a probe of a partial genomic sequence of *GL1* exon 3 labeled with digoxigenin (Roche, Indianapolis, IN, USA) by PCR using a primer pair of GL1-5-F and GL1-5-R (Li et al. 2011) or RsGL1a1-f (5′-TCTTTCCAA TCCAGGTGGTC-3′) and RsGL1a1-r (5′-ACGACGTCGC TATGTGTCAA-3′). The membrane was washed twice with a solution of 0.1× SSC and 0.1 % SDS at 65 °C for 20 min.

RNA extraction and reverse-transcription PCR (RT-PCR)

Using the SV Total RNA Isolation System (Promega), total RNAs were extracted from 30 mg of mature leaves of *B. rapa* transgenic plants and from shoot apical meristems with young leaf primordia of hairy plants and hairless plants in the BC_1F_1 population of radish. First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcription using a First-Strand cDNA Synthesis Kit (GE Healthcare UK, Little Chalfont, Buckinghamshire, UK). qRT-PCR for quantifying transcripts of the A-allele or C-allele of *BrGL1* in transgenic plants was performed using SsoAdvanced™ SYBR® Green Supermix with primers BrGL1q-f (5′-CTCATTATTCGTCTCCACAA) and BrGL1q-r (5′-ACTTGGTTATCTGTCCTTCC). The thermal cycle of qRT-PCR was set at 95 °C for 3 min

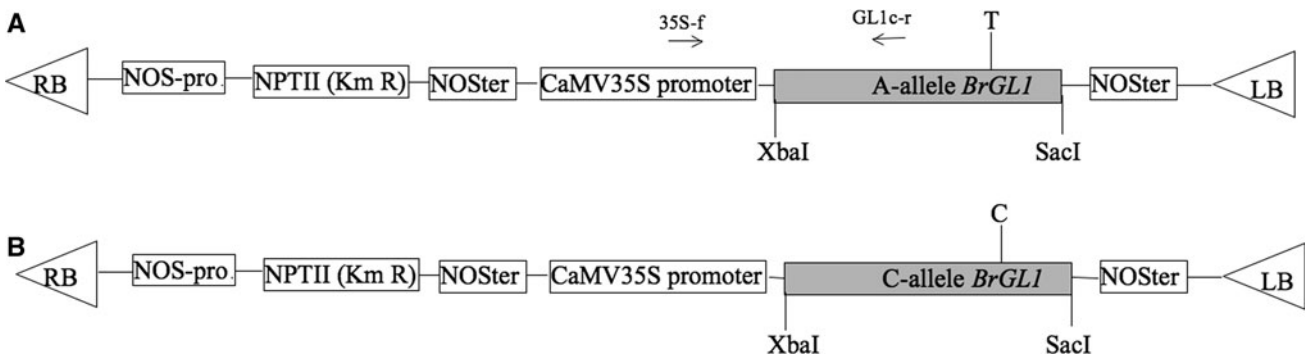

Fig. 1 T-DNA region of vector constructs for transformation of 'Osome'. **a** A construct of the A-allele *BrGL1* transgene. **b** A construct of the C-allele *BrGL1* transgene. *Gray boxes* indicate a *BrGL1* region. The A- and C-alleles differ in an SNP (T → C) at the second base of the third exon (Li et al. 2011). 35S-f and GL1c-r indicate primers for detection of the transgene in the transgenic plants by PCR. *LB* left border, *NOS pro* nopaline synthase gene promoter, *NPTII* neomycin phosphotransferase gene, *NOSter* nopaline synthase gene terminator, *CaMU35S promoter* cauliflower mosaic virus 35S promoter

denaturation, followed by 40 cycles of 95 °C for 1 s and 60 °C for 30 s. Following denaturation at 95 °C for 30 s and cooling to 65 °C for 30 s, a melting curve was generated by heating from 65 °C to 95 °C in 0.5 °C increments with a dwell time at each temperature of 2 s while continuously monitoring the fluorescence. Reverse-transcription PCR (RT-PCR) for detection of *RsGL1* transcripts was performed using RsGL1a2-f (5′-TATGGAATCGCA TTGTCAGG-3′) and RsGL1a2-r (5′-ACGACGTCGCTAT GTGTCAA-3′): 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s.

Analysis of *GL1* orthologs in *R. sativus*

Three primer pairs (Table 1) were designed using the nucleotide sequences of exons of *BrGL1*. PCR was performed in a reaction mixture of 20 µl containing 20 ng genomic DNA as a PCR template, 0.5 µM of primers, 1 U of *TaKaRa Ex Taq*® DNA polymerase (Takara Biomedicals, Japan), 1× Ex Taq buffer and 200 µM of dNTPs. The thermal cycle of PCR was set as follows: 1-min denaturation at 94 °C, 35 cycles of 30-s denaturation at 94 °C, 30-s annealing at 58 °C, and 1-min extension at 72 °C, and a final extension at 72 °C for 4 min.

The amplification products were purified by UltraCleanTM15 DNA Purification Kit (MO BIO, USA) and sequenced with a DNA sequencer (CEQ2000, Beckman Coulter). The sequences were aligned using SEQUENCHER version 4.7 (Gene Codes Corporation, MI, USA). When determined nucleotide sequences were ambiguous, the PCR product was cloned into the pGEM-T® Easy Vector (Promega) and sequences of six clones were determined. The determined sequence reads were assembled to form contigs and aligned with the *BrGL1* gene. Two homologous *GL1* genes of *R. sativus* were identified and designated as *RsGL1a* and *RsGL1b*.

Because the three above-mentioned primer pairs amplify only middle regions of *GL1* orthologs in *R. sativus*, suppression PCR (Siebert et al. 1995) was adopted to isolate both ends. Genomic DNA was digested with *Hin*dIII, *Dra*I, *Pvu*II and *Stu*I, and the digested products were ligated to adaptors AD-F and AD-R (Table 1), followed by the addition of ddGTP to the 3′ end of the ligation products to form four libraries. For the primary PCR, DNA of each library was used as a template and was annealed with Adaptor primer 1 (AP1, Table 1) and *RsGL1a- or RsGL1b*-specific primer 1 (RSPa-f1, RSPb-f1, RSPa-r1 or RSPb-r1, Table 1; Fig. 2). PCR was performed in a reaction mixture of 20 µl, containing 1 µl of 1/10 diluted DNA of each library, 1 µM of primers, 4 U of *TaKaRa Ex Taq*® DNA polymerase, 1× Ex Taq buffer, and 200 µM of dNTPs. The thermal cycle of PCR was set to be as follows: 1-min denaturation at 94 °C, 35 cycles of 1-min denaturation at 94 °C and 6-min annealing and extension at 68 °C and a final extension at 68 °C for 10 min. Subsequently, nested PCR was performed with a 1/10 dilution of the primary PCR products and a primer set of Adaptor primer 2 (AP2, Table 1) and the *RsGL1a*- or *RsGL1b*-specific primer 2 (RSPa-f2, RSPb-f2, RSPa-r2 or RSPb-r2; Table 1; Fig. 2) under the same PCR condition as the primary PCR.

Table 1 Sequences of primers used for isolation of *GL1* orthologs in *R. sativus*

Primer name	Primer sequence (5′–3′)
GL1-f1	CTCCACAAGCTACTTGGCAAC
GL1-r1	GAGGAATAATCCCCGACGA
GL1-f2	GCATCGTCAGGAAAACTGGT
GL1-r2	CGTCGTCATTAAACCAGAAAGC
GL1-f3	CGTCGTCATTAAACCAGAAAGC
GL1-r3	CCTGGAAGGACAGATAACCAA
RSPa-f1	CTTCCATTTGTAACCTCTCTCACA
RSPa-f2	CCGGAGAAGACAACTCTCCA
RSPa-r1	GTACCTGTTGCCGAGGAGTT
RSPa-r2	GCTTTTTCCACACCTCTTCAA
RSPb-f1	GGTGCCTGGAAGGACAGATA
RSPb-f2	GGATTATTCCTCCGCTGTCA
RSPb-r1	TGTACCTACACTGTGAGAGAAAGAGA
RSPb-r2	TTTACGTTTGCACACCATTTG
ADF	CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT
ADR	CCCGTCCA
AP1	GGATCCTAATACGACTCACTATAGGGC
AP2	AATAGGGCTCCGAGCGG

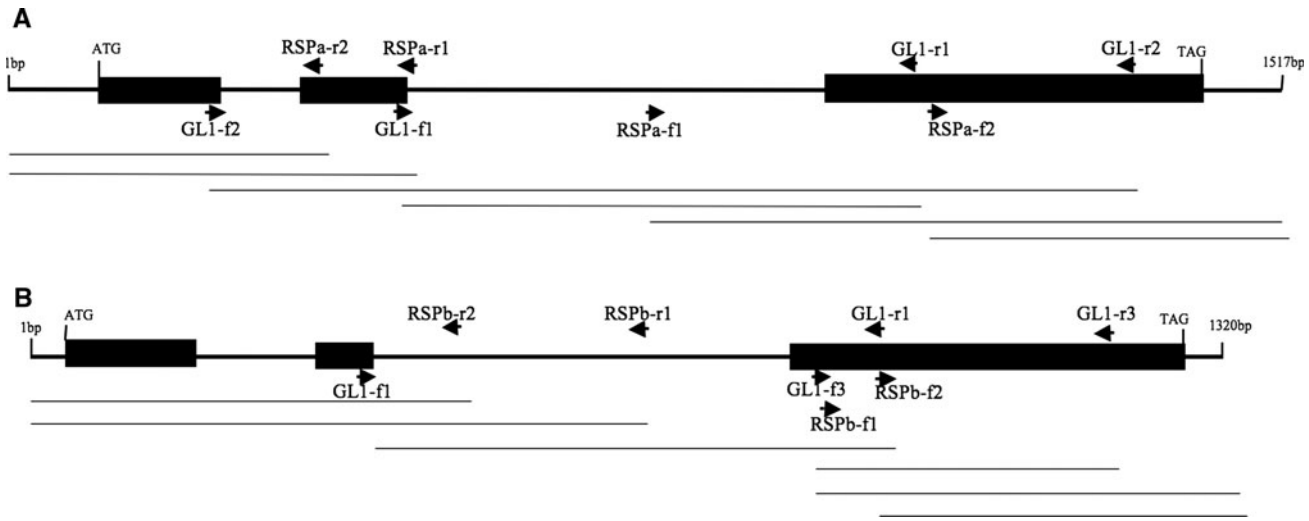

Fig. 2 Primer positions (*arrowheads*) in the *RsGL1* genes of *R. sativus*. **a** Primer RSPa-f1 or RSPa-r1 was annealed with the primer AP1, and primer RSPa-f2 or RSPa-r2 was annealed with the primer AP2 in suppression PCR to amplify the both ends of *RsGL1a*, respectively. **b** Primer RSPb-f1 or RSPb-r1 was annealed with the primer AP1, and primer RSPb-f2 or RSPb-r2 was annealed with the primer AP2 in suppression PCR to amplify the both ends of *RsGL1b*, respectively. ATG and TGA indicate start and stop codons, respectively. *Black boxes* indicate exon regions. *Solid lines* stand for introns. *Bars* indicate DNA fragments amplified by PCR

Analysis of dot-blot-SNP markers

Bridge probes (Shiokai et al. 2010) were designed to distinguish two alleles of *RsGL1a*. Amplicons from 189 individuals of the BC_1F_1 population, 'Sayatori', Ra38, and their F_1 by PCR using a primer pair of RsGLa3-F (5′-TCGTCAGGAAAACTGGTCTCT-3′) and RsGLa3-R (5′-CGTCTTCATTAAACCAGAAAGC-3′) were analyzed by dot-blot-SNP to determine *RsGL1a* genotypes of these plants. A probe for Ra38-allele was 5′-TAACTTAAATGA TGTAATATATTTACATTCGCAATTAAGAGGCTTCGT-3′ and that for 'Sayatori'-allele was 5′-TAACTTAACTG ATGTAATATATTCCCTCCGTCAGCGGATC-3′. Dot-blotted membranes were hybridized at 35 °C and washed twice in a solution containing 0.5 % SSC and 0.1 % SDS at 35 °C for 20 min.

Results

Phenotypic and molecular analyses of transformants

Eleven and four B-allele homozygous plants transformed with pBI121-A-allele *BrGL1* and pBI121-C-allele *BrGL1*, respectively, were obtained. Nine plants transformed with the A-allele showed dense hairs on adaxial and abaxial surface of leaves and petioles (Fig. 3a), and the other two with the A-allele were hairless, whereas all four plants with the C-allele were hairless like 'Osome' (Fig. 3b). After bolting, cauline leaves of the transformants with the A-allele were hairless like those with the C-allele and 'Osome' (Fig. 3d, e). Except for hairiness, no significant difference was observed between the transformants with different *BrGL1* alleles and 'Osome' during their developmental processes (Fig. 3c, d). Interestingly, trichomes were observed on hypocotyls of T_1 plants with the A-allele, whereas no trichomes appeared on hypocotyls of the hairy line 'Yellow sarson' C634 (Fig. 3e, f).

Four hairy transformants with the A-allele and two transformants with the C-allele in these transgenic plants were used for transgene detection by PCR with specific primers 35S-f and GL1c-r, all of them being positive. The copy numbers of the transgene in three transformants with the A-allele and one transformant with the C-allele were analyzed using Southern blot analysis. Except for the endogenous *BrGL1* gene of *B. rapa* 'Osome', the three transgenic plants with the A-allele showed a single transgene copy in the genome, whereas multiple insertion of the transgene was detected in the transgenic plant with the C-allele (Fig. 4a). In order to elucidate whether the hairlessness of the transformant with the C-allele was due to high transgene copies, four and seven T_1 plants derived from a T_0 plant with the A-allele and C-allele, respectively, were grown. Segregation of the transgene copies in the T_1 plants with the C-allele was revealed by Southern blot analysis (Fig. 4b), but all the T_1 plants showed a hairless phenotype.

Because the *BrGL1* gene is not expressed in mature leaves (Li et al. 2011), the mature leaves of T_1 plants were used for detection of *BrGL1* transcripts in the transformants. Real-time PCR showed that the transgenes were ectopically expressed in the mature leaves of all the T_1

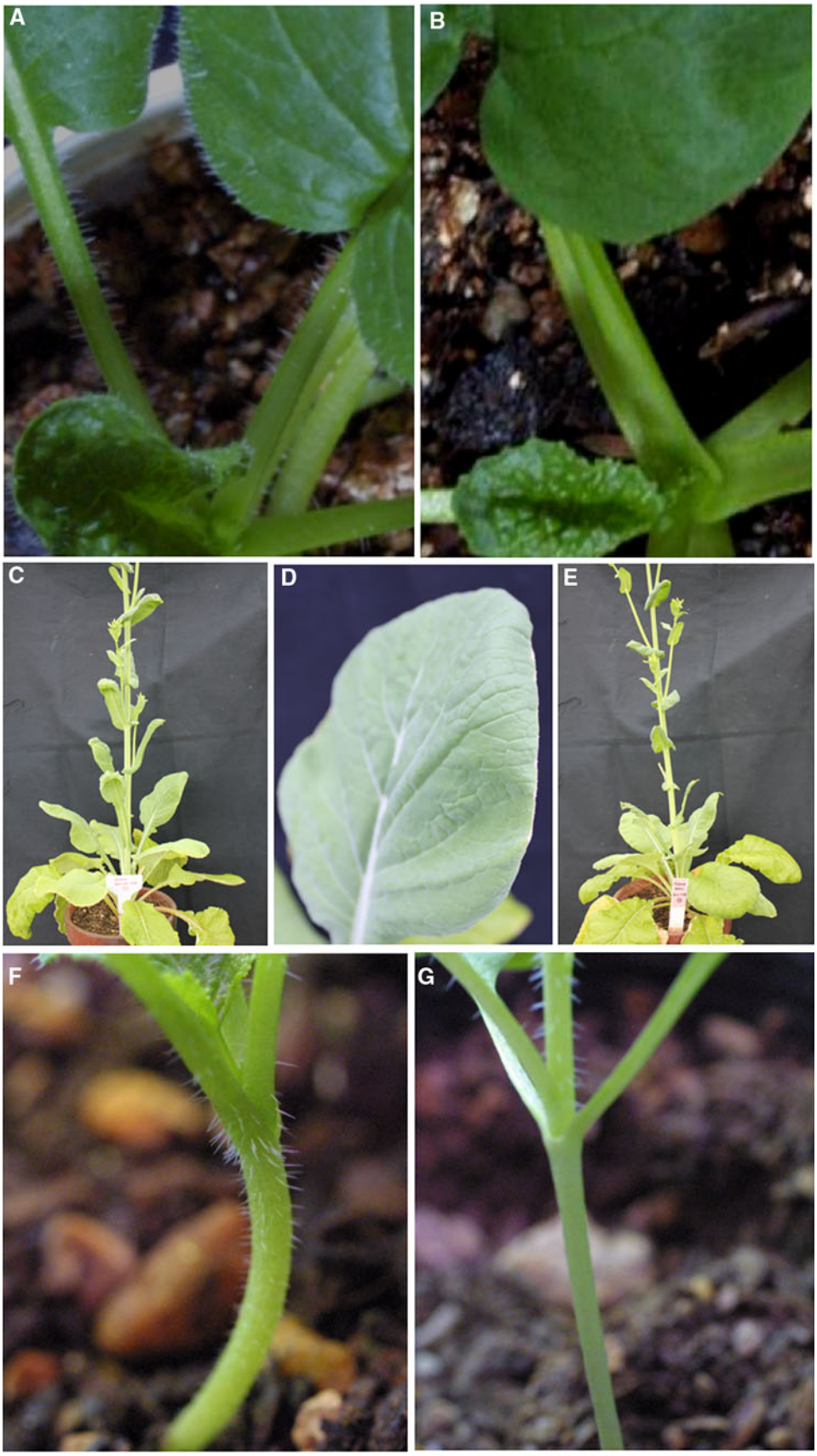

Fig. 3 Phenotype of transgenic plants. Leaf hairiness phenotypes observed in transgenic 'Osome' seedlings with the A-allele (**a**) and the C-allele of *BrGL1* (**b**). Overall morphology of transformants with the A-allele (**c**) and the C-allele of *BrGL1* (**e**). Phenotype of a cauline leaf in transformants with the A-allele (**d**). Trichome phenotypes of hypocotyls in a T_2 transgenic seedling with the A-allele (**f**) and a non-transgenic seedling of 'Yellow sarson' C634 (**g**)

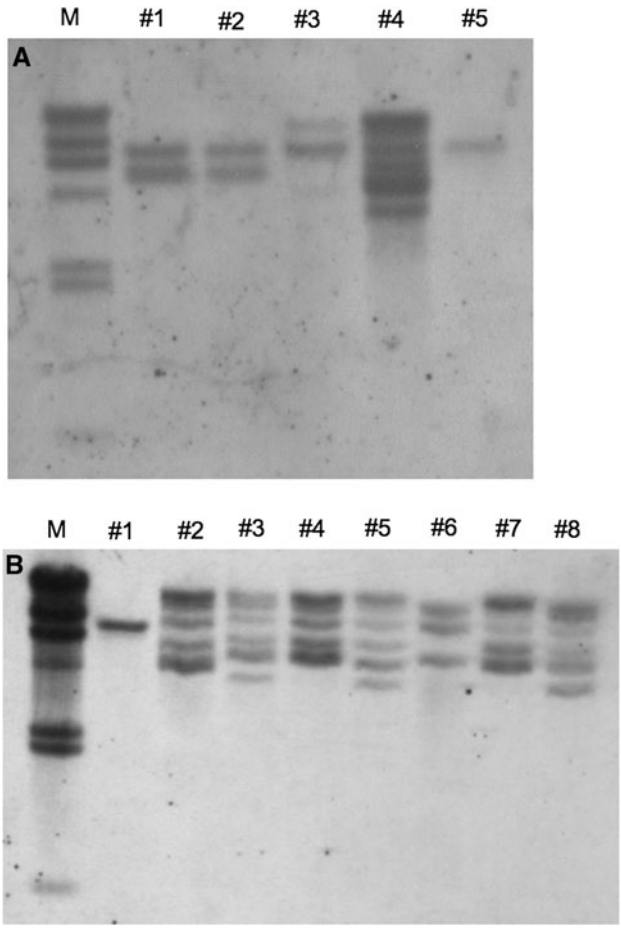

Fig. 4 Genomic Southern blot analysis of transgenic plants using a probe of the partial sequence of *BrGL1* exon 3. **a** *#1*, *#2*, *#3*, T_0 plants with the A-allele of *BrGL1*; *#4*, a T_0 plant with the C-allele of *BrGL1*; *#5*, non-transformed *B. rapa* 'Osome'. **b** #1, non-transformed *B. rapa* 'Osome'; *#2* to *#8*, T_1 plants derived from a T_0 plant with the C-allele of *BrGL1*. Genomic DNAs of transgenic plants were digested with *Hin*dIII. *M*: lambda/*Hin*dIII marker

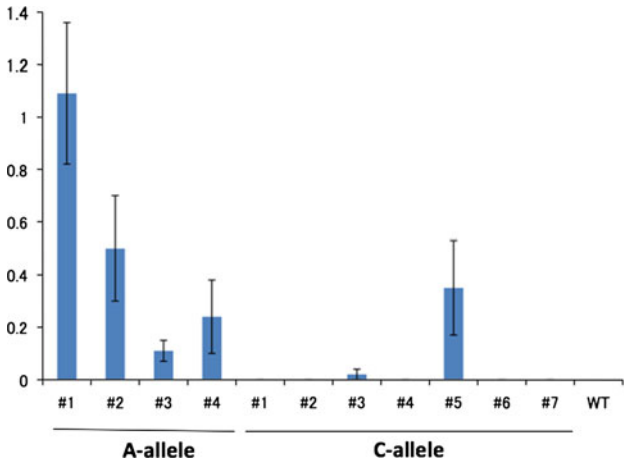

Fig. 5 Real-time RT–PCR analysis of *BrGL1* in mature leaves of the transgenic plants (mean ± SE, $n = 3$). Four and seven T_1 plants derived from a T_0 plant with the A- and C-allele of *BrGL1*, respectively, were used. WT was a non-transformed 'Osome'

plants with the A-allele, but not in non-transgenic 'Osome' and some of the T_1 plants with the C-allele (Fig. 5). The expression level of the *BrGL1* transgene in a T_1 plant with two copies of the C-allele, i.e., #5, which was hairless, was higher than those in the #3 and #4 plants with the A-allele, which were hairy.

Analysis of *GL1* genes in *R. sativus*

PCR products amplified with the primers GL1-f1 and GL1-r1 (Fig. 2) were directly sequenced, but the sequences were ambiguous. Therefore, the PCR product was cloned into the pGEM-T® Easy Vector. Sequencing of six clones revealed two homologs. Direct sequencing of PCR products amplified with a primer pair GL1-f2 and GL1-r2 and a primer pair GL1-f3 and GL1-r3 (Fig. 2) yielded clear sequences, which could be assembled with the sequences of DNA clones, respectively, to produce two independent contigs. Using a genome-walking method, these two contigs were extended to their flanking regions. These sequences were assembled to form two *GL1* homologous genes, *RsGL1a* and *RsGL1b,* which were deposited in GenBank with the accession numbers AB747346 and AB747347, respectively. Alignment of nucleotide sequences of *RsGL1a* and *RsGL1b* indicated that both genes have the identical structure, which contains three exons and two introns similar to *BrGL1* (Li et al. 2011). The most striking difference between these two homologous *RsGL1* genes is that the second exon of *RsGL1b* is 66-base shorter than that of *RsGL1a*, causing deletion of 22 amino acids in the DNA-binding domain of a putative RsGL1b protein and destruction of the first helix-turn-helix (HTH) motif (Supplemental Fig. 1).

To detect *GL1* homologs in the genome of *R. sativus*, Southern blot analysis was carried out using a 289-bp fragment of an *RsGL1* exon 3 sequence as a probe. Two hybridized bands were detected in *R. sativus* (Supplemental Fig. 2).

Correlation of allelic variation of the *GL1* homologs and leaf hairiness in *R. sativus*

The hairiness of the BC_1F_1 population derived from the hairless line 'Sayatori' and hairy line Ra38 was investigated. Two kinds of obviously different phenotypes of leaf hairiness similar to those of parents, i.e., hairy and hairless, were observed on the leaf surfaces of this population. Among the 189 individuals, 86 were hairy and 103 were hairless, in accordance with the expected Mendelian 1:1 ratio ($\chi^2 = 1.99$, $\chi^2 < \chi^2_{0.05,\ 1j}$), indicating that the hairiness is controlled by a single gene. A dot-blot SNP marker was

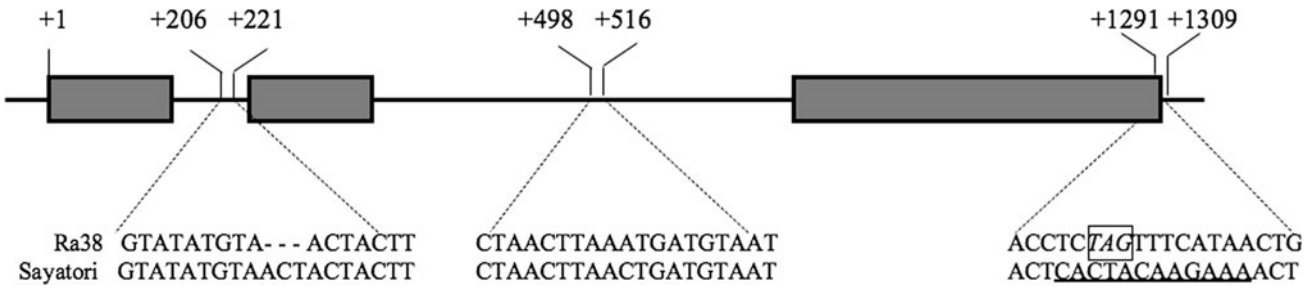

Fig. 6 Nucleotide polymorphisms of *RsGL1a* between Ra38 and 'Sayatori'. *Gray boxes* represent exons, and *numbers* above the boxes indicate regions containing nucleotide polymorphisms between Ra38 and 'Sayatori'. Nucleotides shown in *italics* with *open boxes* indicate a stop codon. An *underlined sequence* shows typical features of CACTA-family transposons. A dot-blot SNP marker for genotyping Ra38-allele and 'Sayatori'-allele of *RsGL1a* was designed based on the SNP in the second intron

developed according to an SNP in the second intron of *RsGL1a*, and was used for genotyping of the BC_1F_1 plants. The results showed that all the plants of 'Sayatori'-allele homozygote of *RsGL1a* were hairless and that all the 'Sayatori'/Ra38 heterozygotes were hairy, indicating *RsGL1a* to be responsible for the phenotypic difference of hairiness in *R. sativus*.

Alignment of the full sequences of *RsGL1a* in 'Sayatori' (accession number AB747348) and Ra38 revealed a 3-bp insertion in the first intron, the above-mentioned SNP, and a great sequence variation from the stop codon to the 3′ noncoding region of *RsGL1a* in 'Sayatori' (Fig. 6), leading to a loss of the stop codon and the presumed addition of 27 amino acids (Supplemental Fig. 3). RT-PCR analysis of *RsGL1a* transcripts from a shoot apical meristem with young leaf primordia was performed using three individuals of 'Sayatori'-allele homozygotes of *RsGL1a*, hairless, and heterozygotes, hairy. Transcripts of *RsGL1a* were not detected in the 'Sayatori'-allele homozygotes, whereas they were detected in the 'Sayatori'/Ra38-allele heterozygotes (Fig. 7).

Fig. 7 Expression of *RsGL1a* in shoot apical meristems with young leaves of hairy and hairless lines in the BC_1 population of *R. sativus*

Discussion

In this study, transformation of hairless *B. rapa* 'Osome' having the B-allele of *BrGL1* with the A-allele of *BrGL1* produced hairy plants. Contrastively, the four transgenic plants with the C-allele were all hairless, and, furthermore, a T_1 plant with relatively high expression of the C-allele transgene was also hairless, suggesting that the cause of the hairlessness of lines with the B-allele or the C-allele of *BrGL1* is a dysfunction of the *BrGL1* gene. *GL1* is a regulatory gene, which belongs to a group of R2R3-Myb transcription factors (Oppenheimer et al. 1991). The R2R3-MYB transcription factors are characterized by two repeat motifs, i.e., R2 and R3, each comprising three α-helices. The second and third helices form a helix-turn-helix structure for binding to specific DNA sequences. GL1 DNA-binding activity is required for a TTG1-bHLH complex to select target genes, such as *GL2, CPC, ETC1* and *TTG2* (Zhao et al. 2008). Both the frameshift at aa 68 causing translation termination at aa 93 in *GL1* of *A. thaliana* and that at aa 95 producing a stop codon at aa 105 in *GL1* of *A. lyrata* have been considered to be the cause of hairlessness (Hauser et al. 2001). The A-allele of *BrGL1* was regarded as a wild type, whereas the B-allele had a 5-bp deletion causing the frameshift at aa 110 resulting in translation termination at aa 154 (Supplemental Fig. 4). These deletions in natural accession or lines in *Arabidopsis* species or *B. rapa* change the whole or part of the R3 myb motif, and, therefore, the activities of their DNA-binding domains might be affected. In the present study, direct evidence for the importance of the DNA-binding domain of the *GL1* gene in trichome initiation was obtained. In *A. lyrata*, one non-synonymous substitution causing an amino acid change from alanine at position 95 to aspartate has been shown to be strongly associated with glabrousness (Kivimäki et al. 2007). Similarly, the replacement of amino acid tryptophan at position 92 with arginine (C-allele) was found to be associated with hairlessness in *B. rapa* in the present study. Both aa 92 and aa 95 are conserved in the R3 myb motif of GL1 (Supplemental Fig. 3 and Supplemental Fig. 4) and other myb genes (Grotewold et al. 2000), revealing their importance for the function of R2R3 myb genes. The present transformation experiment yielded the first evidence that an amino acid substitution in the myb motif causes dysfunction of the *GL1* gene.

In *Arabidopsis*, trichomes are distributed on rosette leaves, cauline leaves, stems, inflorescence and sepals, whereas trichomes grow only on leaf surfaces in *B. rapa* and *B. napus*. The *Arabidopsis gl1* mutant is glabrous, whereas functional complementation of *GL1* can restore hairiness of the whole plant (Oppenheimer et al. 1991). In the present study, overexpression of the *BrGL1* did not induce trichomes on the cauline leaves, inflorescence and sepals, while it did make hypocotyls hairy. Overexpression of *AtGL1* and *AtGL3* together in *B. napus* has been found to stimulate a "super-dense" leaf trichome phenotype on seedling tissues, while not on flower tissues (Gruber et al. 2006). These results suggest that genes involved in trichome initiation at the reproductive stage are not present in *B. rapa* and *B. napus*.

Two homologous *GL1*s, i.e., *RsGL1a* and *RsGL1b*, were isolated from *R. sativus* by an overlapping-PCR strategy. Comparative analysis of deduced amino acids of these two *RsGL1* genes revealed a defective R2 myb motif in *RsGL1b*. Because our transformation experiment revealed the importance of the R2 and R3 myb motifs, it can be inferred that *RsGL1b* has no function as a *GL1* gene. The association of *RsGL1a* genotype with leaf hairiness showed that the *RsGL1a* allele of 'Sayatori' is responsible for its hairlessness. Alignment of the full sequences of the *RsGL1a* alleles in 'Sayatori' and hairy Ra38 revealed a large sequence variation from the stop codon to the 3′ non-coding region, which is similar to the nucleotide polymorphism between glabrous and hairy lines in *Arabidopsis halleri* subsp. *gemmifera* (Kawagoe et al. 2011). Interestingly, a CACTA-family transposon ca. 8 kb in length has been identified in the *GL1* 3′ end of the glabrous lines of *A. halleri* subsp. *gemmifera* (Kawagoe et al. 2011), and we also identified a CACTA motif (5′-CACTACAAGAAA-3′) in the 'Sayatori' allele of *RsGL1a* (Fig. 7). Presumably, a CACTA-family transposon was inserted in the 3′ end of the *RsGL1a* 'Sayatori' allele. In *A. thaliana*, an enhancer in the 3′ non-coding region is essential for the function and normal expression of *GL1* in trichome cells (Larkin et al. 1993). That no *RsGL1a* transcript was detected in 'Sayatori' might be due to the great nucleotide variation in its 3′ end.

The correlation of allelic variation of *GL1* and phenotypic variation of hairiness was interestingly similar among *A. thaliana*, *A. lyrata*, *A. halleri, B. rapa* and *R. sativus* (Hauser et al. 2001; Kawagoe et al. 2011; Kivimäki et al. 2007). Similar cases have also been reported. For example, allelic variation at the *FRI* (*FRIGIDA*) and *FLC* (*FLOWERING LOCUS C*) loci are major determinants of flowering time in both *Arabidopsis* and *Brassica* species (Gazzani et al. 2003; Wang et al. 2011; Yuan et al. 2009). These results suggest that orthologous genes with a relatively simple inheritance or large effects might have contributed to parallel evolutionary changes in different plant lineages. Therefore, the genes underlying natural variation for plant development and growth in model plants might provide a starting point in the study of the genetic basis of phenotypic variation in other species.

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