Identification of Functional Single-Nucleotide Polymorphisms Affecting Leaf Hair Number in *Brassica rapa* 1[CC-BY]

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Leaf traits affect plant agronomic performance; for example, leaf hair number provides a morphological indicator of drought and insect resistance. *Brassica rapa* crops have diverse phenotypes, and many *B. rapa* single-nucleotide polymorphisms (SNPs) have been identified and used as molecular markers for plant breeding. However, which SNPs are functional for leaf hair traits and, therefore, effective for breeding purposes remains unknown. Here, we identify a set of SNPs in the *B. rapa* ssp. *pekinesis* candidate gene *BrpHairy Leaves1* (*BrpHL1*) and a number of SNPs of *BrpHL1* in a natural population of 210 *B. rapa* accessions that have hairy, margin-only hairy, and hairless leaves. *BrpHL1* genes and their orthologs and paralogs have many SNPs. By intensive mutagenesis and genetic sequencing, we selected the functional SNPs for leaf hairs by the exclusion of nonfunctional SNPs and the orthologous and paralogous genes. The residue tryptophan-92 of *BrpHL1a* was essential for direct interaction with GLABROUS3 and, thus, necessary for the formation of leaf hairs. The accessions with the functional SNP leading to substitution of the tryptophan-92 residue had hairless leaves. The orthologous *BrHL1b* from *B. rapa* ssp. *chinensis* regulates hair formation on leaf margins rather than leaf surfaces. The selected SNP for the hairy phenotype could be adopted as a molecular marker for insect resistance in *Brassica* spp. crops. Moreover, the procedures optimized here can be used to explain the molecular mechanisms of natural variation and to facilitate the molecular breeding of many crops.

The genus *Brassica* is one of the most important branches of the Brassicaceae family, including many varieties of common vegetable crops. By means of natural and artificial selection through time, many crops in *Brassica rapa* have evolved that show obvious differences in leaf traits, such as heading Chinese cabbage (*B. rapa* ssp. *pekinesis*), nonheading Chinese cabbage (*B. rapa* ssp. *chinensis*), turnip (*B. rapa* ssp. *rapifera*), and yellow sarson (*B. rapa* ssp. *trilocularis*). The Brassica Database Web site (http://brassicadb.org/brad/) has released the complete genome sequences of several *Brassica* spp. crops (Cheng et al., 2011). Traditionally, leaf shape, size, and curvature are the main traits in these crops that have been genetically selected for improved yield and quality. Hence, studying the diversity of the leaf traits in *Brassica* spp. could provide valuable information to help understand leaf development and leaf variation and how to genetically manipulate these vegetable crops in the future.

A leaf hair (trichome) is an epidermal hair that serves as a physical barrier on plant surfaces against biotic and abiotic stress, including insect herbivores, pathogenic microorganisms, UV light, excessive transpiration, freezing, etc. (Harada et al., 2010; Van Cutsem et al., 2011; Nafisi et al., 2015; Hegebarth et al., 2016). The adaptive significance of leaf hairs for arid land plants has been documented (Ehleringer and Mooney, 1978). The density and localization of leaf hairs vary with crops within *B. rapa*. In *Arabidopsis* (*Arabidopsis thaliana*), leaf hairs usually exist throughout the whole plant, except for the cotyledons and epicotyls. A range of mutants defining specific aspects of trichome development have been found in *Arabidopsis*. The genetic analysis of these mutants has revealed a number of key genes controlling this patterning process, and the mechanism by which trichome differentiation is triggered in individual cells has been best studied in *Arabidopsis* (Oppenheimer et al., 1991; Galway et al., 1994; Rerie et al., 1994; Wada et al., 1997; Payne et al., 2000).

As most biological traits are genetically complicated, mapping quantitative trait loci (QTLs) is a powerful tool to understand the genetic basis of the traits. Using genetic mapping and comparative genomics, QTLs governing leaf hair traits were mapped to the *BrpHL1* gene (Wada et al., 1997; Payne et al., 2000; Wang et al., 2005). However, the specific mechanism by which this gene regulates leaf hair number remains unknown. Recently, the first *Arabidopsis* gene involved in the regulation of trichome development was identified, which controls trichome number and size by regulating trichome patterning (Wada et al., 2005). In this study, we identified a set of SNPs in the *BrpHL1* gene that affect leaf hair traits in *Brassica* spp. crops.
means for estimating the framework of the genetic architecture for a trait and potentially identifying the genes responsible for a specific phenotype. Recombinant inbred lines (RILs) of two Arabidopsis ecotypes, hairy Columbia and less hairy Landsberg erecta, have been constructed to identify QTLs contributing to trichome number, and a major locus named REDUCED TRICHOME NUMBER has been confirmed (Larkin et al., 1996). In another study, four recombinant inbred mapping populations based on six Arabidopsis ecotypes have been used to reveal QTLs controlling trichome density, and nine QTLs have been identified as responsible for trichome initiation and development (Symonds et al., 2005).

Some studies report that the leaf hairs in Chinese cabbage are controlled by a single dominant gene, whereas others have shown that leaf hairs are a quantitative phenotype, controlled by several major QTLs (Song et al., 1995; Zhang et al., 2009). The mechanisms of trichome development in Brassica spp. crops and in Arabidopsis might be highly conserved (Nayidu et al., 2014; Alahakoon et al., 2016). A gene (Bra009770) located on chromosome A06 in B. rapa is homologous with TRANSPARENT TESTA GLABRA1 (TTG1) in Arabidopsis, and it controls trichome formation and seed coat color (Zhang et al., 2009). Moreover, nucleotide polymorphisms of four alleles in the GLABROUS1 (GL1) ortholog (BrGL1) are associated with hairless leaves (Li et al., 2011). In addition, a 5-bp deletion in Brtri1 (BrGL1) is related to a glabrous phenotype (Ye et al., 2016).

The molecular understanding of the functional consequences of genetic variation is critical for the application of single-nucleotide polymorphisms (SNPs) to plant breeding. Progress toward this goal has been mostly successful when the genetic variation falls within a coding region. Unfortunately, most SNPs identified in plants are located within large introns or are distal to coding regions.

SNPs have a wide distribution and can be found in any region of a gene, mRNA, or intergenic region. Although the identification of SNPs is an important first step in understanding the relationship between variation and phenotypes, a major challenge in the post-genome-wide association study (GWAS) era is to understand the functional significance of the identified SNPs and to apply these SNPs to plant breeding. Usually, GWAS SNPs (SNPs at QTLs) in the coding sequences of the candidate genes are used to design DNA markers. In practice, many GWAS SNPs are not effective for the selection of objective traits, and many breeders have experienced failures in the practice of molecular marker-aided selection with GWAS SNPs. Among these SNPs, some are functional for developmental events and traits because they affect the levels of gene expression or translation, splicing, efficiency to enhance or inhibit mRNA stability, and protein function. Many polyploid plants have multiple gene copies and many SNPs. Selection of the causal SNPs for certain traits largely depends on the exclusion of nonfunctional SNPs. Identifying functional SNPs for objective traits from a large number of SNPs presents a bottleneck in the process. Furthermore, successful molecular breeding of crops relies largely on the accuracy of functional SNPs.

Recently, many SNPs have been identified in B. rapa (Kim et al., 2016; Tanhuapanä et al., 2016; Yu et al., 2016). However, which SNPs are responsible for leaf hairs remains unknown. In this study, we took advantage of recent advances in genome resequencing to perform QTL mapping using 150 RILs derived from the cross between the hairy genotype Bre (B. rapa ssp. pekinensis) and the hairless genotype Wut (B. rapa ssp. chinensis) of Chinese cabbage (Yu et al., 2012). We identified functional SNPs from numerous SNPs in the candidate genes and their duplicated copies and optimized the procedures for the selection of functional SNPs for agronomic traits. The selected SNP for leaf hair is a molecular marker of insect resistance in Brassica spp. crops, and the optimized procedures can be used to explain the molecular mechanism of natural variation and to manipulate the molecular breeding of many crops.

RESULTS

Genetic Control of Leaf Hair Number

The species B. rapa includes heading Chinese cabbage (B. rapa ssp. pekinensis), nonheading Chinese cabbage (B. rapa ssp. chinensis), turnip (B. rapa ssp. rapa), and yellow sarson (B. rapa ssp. trilocularis). Nonheading Chinese cabbage consists of many crop types: baicai, caixin, caitai, purple caitai, taicai, and wutacai. These crops and crop types are characterized by their specialized product organs: curved leaves, leafy heads, fleshy petioles, fleshy stems, and fleshy roots.

To analyze the leaf variation of B. rapa, we collected 210 accessions of B. rapa. The leaf hairs on young leaves of these accessions were observed with a binocular stereomicroscope. There were three types of leaves with regard to leaf hairs (Table I). Most of the accessions belonged to the all-hairy leaves, in which the leaf hairs were visible on the surfaces and margins. Some accessions belonged to the margin-only leaves, in which the leaf hairs were visible only on the leaf margins, and the rest of the accessions had hairless leaves. Among the 210 accessions observed, 99 showed the all-hairy phenotype while 16 displayed the margin-only hairy phenotype (Table I). Bre is a representative of the all-hairy phenotype and Wut is a representative of the margin-only hairy phenotype: numerous hairs were detected on the leaf surface and leaf margins of Bre, whereas only a few leaf hairs were seen on the leaf margins of Wut (Fig. 1, A and B). Leaf hairs of all-hairy and margin-only hairy phenotypes were not branched, in contrast with the branched trichomes of Arabidopsis (Fig. 1, C and D). Most heading Chinese cabbage ac-
cessions showed the all-hairy phenotype (Table I; Fig. 1, E, F, I, and J), and most nonheading Chinese cabbage accessions displayed a hairless or margin-only hairy phenotype (Table I; Fig. 1, G, H, K, and L).

**Positional Cloning of the Hairy Leaves1 Locus**

In the course of previous studies, the inbred lines Bre and Wut were crossed to construct RIL populations for inheritance analysis and chromosomal mapping of the related QTLs. One major QTL, qTr, located on chromosome 6, was identified as a locus for hairy leaves, and Bra025311 in the reference genome Chiifu-401-42 was selected as a candidate gene (Yu et al., 2013). Bra025311 belongs to the MYB gene family (Supplemental Fig. S1) and is homologous to Arabidopsis GL1, with 59% amino acid identity (Supplemental Fig. S2A). GL1 encodes an R2R3-MYB transcription factor with a central function in the leaf hair patterning pathway and is involved in epidermal cell fate specification in leaves, promoting leaf hair formation and endoreplication (Szymanski et al., 1998).

We named the candidate gene Bra025311 in Bre and Wut as BrpHL1a (B. rapa ssp. pekinensis Hairy Leaves-1a) and BrcHL1a (B. rapa ssp. chinensis HL1a), respectively. The gene body (5′ untranslated region [UTR], exons, introns, and 3′ UTR) and cDNAs of Bre BrpHL1a and Wut BrcHL1a were cloned on the basis of genomic resequencing. Bre BrpHL1a showed 56 SNPs in the gene body compared with Bra025311 of Chiifu-401-42.

### Table I. Number of accessions with and without leaf hairs in a collection of 210 B. rapa accessions

| Crop Types                        | No. of Accessions | Total | All Hairy | Margin-Only Hairy | Hairless |
|-----------------------------------|-------------------|-------|-----------|-------------------|----------|
| Heading Chinese cabbage           |                   | 116   | 85        | 12                | 19       |
| Nonheading Chinese cabbage        |                   | 70    | 10        | 2                 | 58       |
| Baitai                            |                   | 5     | 1         | 0                 | 4        |
| Caixin                            |                   | 8     | 0         | 0                 | 8        |
| Taicai                            |                   | 2     | 0         | 0                 | 2        |
| Wutacai                           |                   | 4     | 0         | 1                 | 3        |
| Turnip                            |                   | 3     | 1         | 1                 | 1        |
| Yellow sarson                     |                   | 2     | 2         | 0                 | 0        |
| Total                             |                   | 210   | 99        | 16                | 95       |

Figure 1. Leaf hairs of different crop types in B. rapa. A and B, Plants of Bre (A) and Wut (B) at the seedling stage. C and D, Scanning electron microscopy showing the leaf hairs of Bre (C; bar = 500 µm) and Wut (D; bar = 200 µm). E and F, Hair distribution on adaxial surfaces of leaves in Da38 (E) and Da15 (F). G and H, Hair distribution on leaf margins in B26 (G) and W12 (H). I and J, Hair distribution on abaxial surfaces of leaves in Da102 (I) and Da203 (J). K and L, Leaf surface without hairs in B55 (K) and B61 (L).
SNP Analysis of BrpHL1a and BrpHL1b Alleles in B. rapa

Compared with Chifu-401-42 BrpHL1b, Bre BrpHL1b has an A/G SNP 85 bp prior to the start codon, causing a shifted reading frame and prolongation of the first exon. This gene also shows a 4.5-kb insertion in its second exon (Fig. 2B; Supplemental Fig. S5A), which suggests that BrpHL1b was not functional in Bre. In contrast, Wut BrpHL1b seemed to be functional, as the A/G SNP and the large insertion were not detected.

To find all the SNPs in B. rapa, we cloned and sequenced BrpHL1a and BrpHL1b genes of 13 representative genotypes (Supplemental Tables S1 and S2). A subset of SNPs was detected. In total, 169 nucleotides (9%) of Chifu-401-42 BrpHL1a and 27 nucleotides (2%) of Chifu-401-42 BrpHL1b were substituted by the other nucleotides of various BrpHL1a and BrpHL1b alleles, respectively. To confirm the accuracy of SNPs, we cloned the full-length cDNA sequences of BrpHL1a and BrpHL1b genes. Sequence analysis of these clones confirmed the accuracy of the genomic sequences of BrpHL1a and BrpHL1b genes.

We then analyzed the association between SNPs of BrpHL1 genes and the hairy phenotype. For BrpHL1 alleles, all the genotypes (Wut, Rippomisica, and Qinci) with SNP 274C showed the hairless phenotype (Supplemental Table S1), revealing an association between 274C and the hairless phenotype. Among 10 genotypes with 274T, seven showed the all-hairy phenotype. Two of seven genotypes with SNP 403G showed the hairless phenotype. Four of six genotypes with 403T showed the all-hairy phenotype. For BrpHL1b alleles, two of three genotypes with 256C/T showed the hairless phenotype (Supplemental Table S2). Therefore, we were not certain that these SNPs were associated with the all-hairy phenotype.

To clarify the relationship between SNPs and leaf hairs, we extended the SNP calling to a natural population of 210 B. rapa accessions. The resequencing of these accessions generated two paired-end libraries with 150-bp reads (Supplemental Table S3). According to the reference genome of B. rapa version 1.5 (Cheng et al., 2011), the sequencing depth of the parental lines was more than 10-fold in each accession, and the mapped depth was about nine to 20. Each SNP supported by fewer than four reads was filtered out, leading to 0.5 to 1.69 million high-quality SNPs (Supplemental Table S4). These SNPs include many nucleotide substitutions, insertions, and deletions.

Based on the genomic data in the Brassica Database, the SNPs were used to update the genomic sequences of the BrpHL1a alleles (Supplemental Table S5) and separately estimated by grouping the 210 accessions. Among 28 accessions with SNP 274C, 24 were hairless while three were margin-only hairy (Table II; Supplemental Table S6), thus showing the high association between SNP 274C and the hairless phenotype. Among 184 accessions with SNP 274T, 96 showed the all-hairy phenotype, revealing that nearly half of the accessions with SNP 274T failed to show the all-hairy phenotype. Surprisingly, the accessions with SNP 274T included a large proportion of hairless accessions and a small proportion of margin-only hairy accessions.

Expression Patterns of BrpHL1 Genes

Reverse transcription-quantitative PCR (RT-qPCR) and reverse transcription (RT)-PCR were used to examine the differences in expression of BrpHL1a/BrpHL1b between Bre and Wut using the same pair of primers. The expression level of BrpHL1a/BrpHL1b in Wut leaves was considerably higher than that in Bre leaves (Fig. 3A). A similar result was obtained using RT-qPCR (Fig. 3B). To investigate the expression patterns of BrpHL1a in the plants, we fused BrpHL1a and BrpHL1b with the GUS gene. In seedlings of the resultant transgenic plants, the GUS signals of BrpHL1a::GUS and BrpHL1b::GUS were visible in all organs, especially in cotyledons and rosette leaves (Fig. 3C). RT-PCR showed that BrpHL1a and BrpHL1b genes were expressed in the leaf, stem, cotyledon, and root of Bre and Wut (Fig. 3D). These results show that the temporal and spatial expression patterns of two BrHL1a genes in Bre and Wut are similar.

Functional Analysis of the SNPs in BrpHL1a Genes

To examine the GWAS SNPs of BrpHL1a alleles, we aligned gene bodies of BrpHL1a with Wut BrpHL1a identified by GWAS. There were 44 GWAS SNPs (Supplemental Fig. S3). Fifty-five SNPs were identified in Wut BrpHL1a (Supplemental Fig. S4). Compared with Chifu-401-42 BrpHL1a, 11 SNPs were detected in the exons of Bre BrpHL1a, causing seven nonsynonymous substitutions, and 13 SNPs were found in the Wut BrpHL1a gene, leading to nine nonsynonymous substitutions. Compared with Bre BrpHL1a, Wut BrpHL1a had two more SNPs in the exons: 274T/C (the 274th dTMP of the coding sequence was changed to dCMP) and 403T/G (403rd T to dGMP; Fig. 2A). SNPs 274T/C and 403T/G of Wut BrpHL1a caused two nonsynonymous substitutions, W92R and Y135D, while 256C/T (256th dCMP to dTMP) of Wut BrpHL1b leads to one nonsynonymous substitution, Y86H.

B. rapa is a mesohexaploid and has more duplicated genes than Arabidopsis. To check the other copies of the BrpHL1a gene, we searched for the genome sequences of a collection of B. rapa accessions. BrpHL1b and BrpHL1b were detected on chromosome 9 in Bre and Wut, respectively. The alignment shows that BrpHL1b and BrpHL1b are homologous with GL1 of Arabidopsis (Supplemental Fig. S2B). Thus, four members of the BrpHL1 gene family were related to the hairy phenotype of B. rapa. Our aim was to first discover which SNPs in the candidate BrpHL1a gene were functional or causal for all hairy or hairless phenotypes. Then, we aimed to determine whether and how the contributions of the other three members of the BrpHL1 gene family could be ruled out.
Figure 2. cDNA and amino acid sequences of BrpHL1a and BrpHL1b. A, SNPs of BrpHL1a and nonsynonymous substitutions of BrpHL1a between Bre, Wut, and Chiifu-401-42. B, SNPs of BrpHL1b and nonsynonymous substitutions of BrpHL1b between Bre, Wut, and Chiifu-401-42. SNPs are shown on a white background. Nonsynonymous substitutions are boxed. Black lines indicate 5′ UTR, exon, or 3′ UTR; red arrows show R2 and R3 domains of MYB transcription factors.
Table II. Correlation between the accessions with the SNPs 274T/C and 403T/G of BrpHL1a and hairy phenotypes

| Genotype | 274 Site | 403 Site | Total | All Hairy | Margin-Only Hairy | Hairless |
|----------|----------|----------|-------|-----------|-------------------|----------|
| T T      | 111      | 63       | 174   | 63        | 7                 | 41       |
| T G      | 67       | 31       | 98    | 31        | 5                 | 31       |
| C T      | 0        | 0        | 0     | 0         | 0                 | 0        |
| C G      | 28       | 1        | 29    | 1         | 3                 | 24       |
| T X      | 4        | 2        | 6     | 2         | 1                 | 1        |
| Total    | 210      | 97       | 16    | 97        |                   | 97       |

Figure 3. Temporal and spatial expression of BrpHL1a and BrpHL1b and the phenotypic rescue of Arabidopsis gl1 by BrpHL1a and BrpHL1b and their mutated versions. A and B, RT-PCR (A) and RT-qPCR (B) showing the expression of BrpHL1a/BrpHL1a in Bre and Wut. C and D, GUS fusion signals in Arabidopsis (C) and RT-PCR (D) in B. rapa showing the expression patterns of BrpHL1a and BrpHL1a in 20-d-old seedlings. Bars = 5 mm. E, Seedling phenotypes of the wild type (Columbia [Col]) and gl1 mutants of Arabidopsis. Bars = 10 mm. F and G, Seedling phenotypes of gl1 mutants transgenic for genomic BrpHL1a-g and BrpHL1a-g (F) and BrpHL1a-c and BrpHL1a-c (G) cDNAs under the control of the native promoters. Bars = 10 mm. H, RT-qPCR showing the expression levels of BrpHL1a/BrpHL1a in gl1 mutants and all transgenic Arabidopsis lines. Three biological replicates were used. Error bars indicate sd.
in BrpHL1a alleles (Supplemental Table S1), most of which were located in the introns. We found that the two SNPs, 274T/C and/or 403T/G, cause nonsynonymous substitutions. To determine SNPs functional for leaf hairs, we mutagenized BrpHL1a genomic DNA with 274C and/or 403G and BrpHL1a-g genomic DNA with 274T and/or 403G and constructed a series of binary vectors of the mutated genes under their control of the native promoters (Table III). We then transferred them into the null gl1 mutants of Arabidopsis that are deficient in trichome formation (Fig. 3, E and F). First, the genomic BrpHL1a-g completely rescued the phenotype in terms of trichome formation, whereas the genomic BrpHL1a-c was unable to rescue the phenotypes of the gl1 mutants (Table III), revealing that Wt BrpHL1a-g is deficient in the formation of leaf hairs. Second, the C274T mutagenesis in BrpHL1a-g did not rescue the gl1 phenotype, thus indicating that 274T/C is the functional SNP for leaf hair formation. It was unable to rescue the phenotypes of BrpHL1a-gl1 mutants, whereas the genomic BrpHL1a-c rescued the phenotypes of BrpHL1a-gl1 mutants by BrpHL1a-g and BrpHL1a-c, respectively (Fig. 3G). We conclude that the substitution of the 92nd Ser to Phe (S92F) amino acid substitution was within this conserved sequence. To further examine whether the Trp-92 mutation interferes with the interaction of BrpHL1a and GL3, we performed pull-down assays. The result showed that the interaction of Arabidopsis maltose-binding protein (MBP)-AtGL3 with GST-BrpHL1a and GST-BrpHL1aR92W (Fig. 4A) was strong, while that of MBP-AtGL3 with GST-BrpHL1a and GST-BrpHL1aR92W was weak. These results suggest that Trp-92 plays a critical role in the direct interaction between BrpHL1a and BrpGL3.

To further confirm the function of Trp-92, we performed a bimolecular fluorescence complementation (BiFC) assay based on enhanced yellow fluorescent protein (EYFP). The full-length coding sequences of AtGL3, BrpHL1a, BrpHL1aR92W, and BrpHL1aR92W were fused to the N- or C-terminal halves of EYFP. Both types of fusion proteins were transiently introduced into mesophyll protoplasts of Arabidopsis. The protein–protein interaction between the tester proteins resulted in the proper folding of EYFP, leading to its subsequent fluorescence in the coinfiltrated protoplasts. The strong EYFP signals between BrpHL1a and AtGL3 and between BrpHL1aR92W and AtGL3 were observed in the nucleus (Fig. 4B), whereas much weaker BiFC signals were observed between BrpHL1aR92W and AtGL3 and between BrpHL1a and AtGL3. This result reveals that the interaction between BrpHL1a and AtGL3 was weak, thus confirming the critical role of Trp-92 in the formation of leaf hairs.

### Activation of BrpGL2 by BrpHL1a

In Arabidopsis, GL2 is required for normal trichome development. GL2 expression is regulated by GL1 (Reirrie et al., 1994). GL1 and GL3 bind directly to the GL2 promoter (Wang and Chen, 2008). Dai et al. (2016) report that the substitution of the 92nd Ser to Phe (S92F)
Functional SNPs for Leaf Hair Number

in the R3 domain of Arabidopsis GL1 does not affect the interaction of GL1 and GL3 but affects the binding of GL1 to the promoter of GL2. In Bre BrpHL1a, the 92nd Trp corresponds to the 91st Trp rather than the 92nd Ser in Arabidopsis GL1. We supposed that the expression level of BrpGL2 in Wut would be reduced compared with that of BrpGL2 in Bre if W92R in BrpHL1a was responsible for the interaction between BrpHL1a and BrpGL3. To address this deduction, we performed RT-qPCR using the same pair of primers whose sequences are conserved in Bre and Wut. BrpGL2 expression was considerably lower than BrpGL2 expression in Bre (Fig. 5A).

To confirm the role of the 92nd Trp in the relevance of BrpHL1a to BrpGL2, we analyzed the expression levels of GL2 in the Arabidopsis gl1 mutants with exogenous BrpHL1a and BrpHL1a. GL2 expression was up-regulated in pBrpHL1a::BrpHL1a plants but not in pBrpHL1a::BrpHL1a plants (Fig. 5B), indicating that BrpHL1a was not able to activate GL2.

Analysis of BrpHL1b Gene Functions

Considering that BrpHL1a functions in the formation of leaf hairs in Bre while BrpHL1a does not in Wut, we wanted to test whether and how BrpHL1b and BrpHL1b function in the formation of leaf hairs. So we analyzed the sequences of these two genes and found that a 4.5-kb insertion was detected in the second exon of Bre BrpHL1b but not in Wut BrpHL1b. The RT-PCR results showed no expression of BrpHL1b in Bre (Fig. 5C), meaning that BrpHL1b is not functional. Also, BrpHL1a was the only functional gene of BrpHL1 genes in Bre. BrpHL1b of Wut did not contain any insertions/deletions (InDels) that interrupt the protein sequence. If BrpHL1b of Wut is functional for leaf hair, its hairless phenotype would be difficult to explain. To verify the function of Wut BrpHL1b, we cloned the BrpHL1b gene body, including the promoter from Wut plants, and transferred pBrpHL1b::BrpHL1b (under the control of the native promoter) and pAA6::BrpHL1b (under the control of the constitutive promoter AA6) constructs into gl1 mutants. Although no trichomes were observed on the leaf surface of pBrpHL1b::BrpHL1b plants, a few trichomes were seen on the leaf margin. pAA6::BrpHL1b plants also showed more trichomes on the leaf margin than pBrpHL1b::BrpHL1b plants (Table III; Fig. 5D). Altogether, these results indicated that BrpHL1b regulates hair formation on leaf margins rather than on the leaf surface.

**DISCUSSION**

Natural Variation at the BrpHL1 Locus Is Extensive

Genetic variation is brought about by mutation. Fundamentally, the numbers and density of SNPs in a genome reflect the extent of natural variation in this species. In *B. rapa*, our natural population of 210 accessions showed 0.5 to 1.69 million high-quality SNPs compared with the reference genome Chiifu-401.
BrpHL1a on chromosome 6 of Bre shows 169 nucleotides that are substituted by its alleles of 13 representative crop types, revealing that natural variation at the BrpHL1 locus is extensive. However, most of the SNPs are located in introns and may not be functional. The −85A/G substitution of BrpHL1b on chromosome 9 in some accessions should change the start codon at the 5′ side and, thus, could affect the function of BrpHL1b. On the other hand, BrpHL1b (the second copy of BrpHL1a) of Bre has a 4.5-kb insertion in the second exon compared with BrcHL1b of Wut. This insertion causes a frame shift and truncation of BrpHL1b in Bre. Although the SNP −85A/G and 4.5-kb insertion are not GWAS SNPs, they substantially affect the function of BrpHL1b in Bre.

The SNPs of BrcHL1a and BrcHL1b in Wut may be related to the hairless phenotype, as Wut leaves are hairless. Among these SNPs, 403T/G is not a causal element for leaf hair, since the T-to-C mutation in BrcHL1a is not able to rescue the gl1 mutant phenotype of Arabidopsis. Expression of BrcHL1b under the control of its native promoter causes marginal trichomes on the gl1 mutant, thus showing the margin-specific expression of BrcHL1b. In this way, the contribution of the SNPs to all hairy phenotypes is excluded, except for SNP 274T/C in the BrcHL1a allele.

274T/C Is a Functional SNP for Leaf Hairs

In recent years, with the rapid development of next-generation sequencing technologies and bioinformatics methods, crop breeding theory and technology have undergone major changes. Numerous studies on genetic map construction and marker-assisted selection have been carried out in Brassica spp. crops. Genomic resequencing is a method designed to sequence all regions of the genome aimed at simplifying genome complexity. Marker-assisted selection is an effective technology for obtaining large numbers of molecular markers and has been used widely for high-throughput SNP discovery and for genotyping in different organisms that are now widely used for large-scale high-throughput SNP genotyping, particularly for de novo SNP discovery. In addition to the advantages of high density and high throughput, our GWAS analysis of SNPs for leaf hairs in B. rapa was effective. One major advantage of using the RIL populations is that researchers can identify some QTLs for the specific traits using low-covered resequencing. Compared with GWAS in a natural population, GWAS in biparental cross populations is more efficient and accurate (Yu et al., 2013). The QTLs obtained in this way are suitable for the selection of candidate genes and GWAS SNPs relevant to leaf hairs and, thereby, establish the
relationship between SNPs and leaf hairs in a natural population.

The identification of causal SNPs is more difficult and should be combined with the exclusion of other SNPs and the relevant alleles. The four members of the BrpHL1 gene family are relevant to leaf hairs. We resequenced 210 accessions of B. rapa and identified an SNP, 274T/C, in the candidate BrpHL1a gene. The function of the candidate genes in two parents was identified by transgenic plants of suitable mutants. Through point mutagenesis of SNPs in BrpHL1a genes and functional analysis of these SNPs in the gl1 mutants of Arabidopsis, we excluded some SNPs in introns and nonsynonymous SNPs in coding sequences. This selection criterion is more advanced than that reported in many other previous studies. Nonetheless, a functional test in Arabidopsis is not necessarily a proof of function in B. rapa. Therefore, we suggest that the SNPs in the coding regions of B. rapa genes could be identified accurately by gene transfer into Brassica spp. crops.

The number of functional SNPs reported remains very limited, and some functional SNPs should be embedded in the resequencing data. Thus, a great deal of work is still needed to improve the SNP calling and QTL mapping accuracy by using high-throughput sequencing technologies and making full use of the reference Chinese cabbage genome.

Trp-92 Is Essential for Direct Interaction with GL3

In Arabidopsis, a network of three classes of proteins consisting of TTD1 (a WD40 repeat protein), GL3 (a bHLH factor), and GL1 (a MYB transcription factor) activates trichome initiation and patterning (Zhang et al., 2003). As positive regulators, these three proteins form an MBW activator complex. GL3 participates in physical interactions with GL1, TTG1, and itself, but GL1 and TTG1 do not interact with each other. We also found that the Trp-92 in BrpHL1a is critical for the interaction with GL3. The interaction would be disrupted as soon as the critical amino acids in BrpHL1a are mutated. In our BiFC experiments, the interactions between BrpHL1a and GL3 and between BrpHL1a<sup>Trp-92R</sup> and GL3 were hardly detectable, while the interactions between BrpHL1a and GL3 and between BrpHL1a<sup>Trp-92W</sup> and GL3 were strong. In pull-down assays, the relative interaction strengths were similar. The hairy phenotype of pBrpHL1a:<brpHL1a plants and hairless pBrpHL1a:<brpHL1a plants in the gl1 background demonstrated that Trp-92 of pBrpHL1a is essential for the formation of leaf hairs in B. rapa. Analyses of loss-of-function mutants reveal that single-repeat R3 MYB transcription factors act as negative regulators (Schnittger et al., 1999; Schellmann et al., 2002; Gan et al., 2011). In Arabidopsis, intron 1 and the 3' noncoding region of GL1 have been shown to be important for the expression of GL1 (Larkin et al., 1993; Wang et al., 2004). Chromatin immunoprecipitation results show that the single-repeat R3 MYB transcription factor TRICHOMELESS1 can be recruited to the cis-acting regulatory elements of GL1, negatively regulating trichome cell specification by directly suppressing the transcription of GL1 (Wang et al., 2007). The importance of Trp-92 for plant phenotype reveals that intron 1 and the 3' noncoding region of BrpHL1a are not as essential as Trp-92 for hair formation on the leaf surface.

The MYB (GL1)-bHLH (GL3/EGL3)-WDR (TTG1) proteins form a trimeric MBW complex that activates the expression of the homeodomain protein, GL2, which, in turn, induces trichome formation (Rerie et al., 1994). Here, we noticed that the expression of GL2 was reduced in Trp-92 mutant plants, suggesting that blocking the interaction between BrHL1a and GL3 affects hair formation on the leaf surface.

**BrpHL1a Regulates Hair Formation on Both the Leaf Surface and Leaf Margin, While BrcHL1b Functions Only on the Leaf Margin**

Usually, there are many SNPs in one gene, which makes it difficult for researchers to select functional SNPs. It is very important to exclude the nonfunctional GWAS SNPs, especially when duplicated genes are predicted to have biological functions. Although the exclusion of nonfunctional SNPs is time consuming and labor intensive, it is necessary for us to explain the complicated genetic process of agronomic traits. Bre is a representative hairy crop type, as the surface and margins of leaves are hairy. By contrast, Wut is regarded as a representative hairless crop type, as hair is not seen on the surface of leaves and only a few hairs are detected on leaf margins. The hairy phenotype in Bre corresponds to 274T and 403T in BrpHL1a and a shift in the reading frame and a large insertion in the second exon in BrpHL1b. In contrast, the hairless phenotype is concurrent with 274C and 403G in BrpHL1a and a normal reading frame in BrpHL1b. By genetic transformation, we confirmed that BrpHL1a regulates hair formation on both the leaf surface and leaf margin while BrpHL1b does not. The point mutation of BrpHL1a and BrpHL1a genes shows that 274T of BrpHL1a or Trp-92 of BrpHL1a is essential for hair formation while 403T or Tyr-135 is dispensable. Comparison of intronic and nonintronic BrpHL1a transgenes reveals that the first intron and 3' UTR of BrpHL1a is not essential for its function, in contrast with Arabidopsis GL1, whose first intron and 3' UTR play roles in trichome formation by the interaction of GL1 with GL2 and GL3, respectively (Larkin et al., 1993; Wang et al., 2004). Young developing gl3 leaves lack marginal trichomes, a phenotype further enhanced in the hgl3 double mutant, indicating that both TT8 and GL3 are essential for trichome development on leaf margins (Maes et al., 2008).

The function of BrpHL1b and BrpHL1b should be considered when that of BrpHL1a and BrpHL1a is clarified. BrpHL1b in Bre is not functional, due to the shift in the reading frame and the large insertion in the coding region. However, BrpHL1b in Wut is functional, because its exogenous expression in the gl1 mutant
rescues the trichome formation on leaf margins. Interestingly, the hair formation on leaf margins in *B. rapa* is not attributable to the promoter region of *BrcHL1b*, as its native and constitutive promoters generate leaf hairs on the same regions of leaf margins.

Functional SNPs Are Useful for Molecular Breeding by Design

Traditional breeding is based on phenotype and, therefore, depends primarily on breeders’ experience. Since many traits of crops, such as disease resistance and yield, cannot be observed easily, traditional breeding faces challenges and demands high-throughput genotyping platforms. Molecular breeding by design is considered the best option for breeders to improve their breeding efficiency. With the progress in functional genomics research, increasing numbers of genes and QTLs responsible for agriculturally important traits have been identified, which provide valuable genetic resources for molecular breeding. Resequencing and SNP genotyping are two key strategies used in GWAS research and the development of molecular markers to target agronomic traits. To be suitable for molecular breeding by design, we optimized the procedures for the selection of functional SNPs for agronomic traits (Fig. 6). The segregation populations, including RILs or doubled haploid lines, are suitable for QTL identification, in which the genomic resequencing of different lines generates the saturated SNPs. The SNPs located at the QTL are regarded as GWAS SNPs, because the candidate genes at the QTL locus are predicted according to GWAS analysis. On the other hand, natural populations include major cultivars, inbred lines, and mutants and, therefore, are very useful for variation analysis of many agronomic traits. SNP genotyping on the basis of genomic resequencing provides a strong tool for the detection of SNPs in the large accession collections. Through the comparison of GWAS SNPs from segregation and natural populations, the candidates of functional SNPs are selected. They may be from exon-intron junctures, DNA-RNA binding sites, protein-DNA binding sites, protein-protein interaction domains, and microRNAs and miRNA-target complementary sites. The functional identification of the candidate SNPs is important but time consuming. All the binary vectors dedicated for genetic transformation should be designed to exclude all of the nonfunctional SNPs. The null mutants of the genes examined should be chosen for phenotypic rescue. The molecular mechanism underlying the functional SNPs for agronomic traits could be clarified.

In rice (*Oryza sativa*), a high-density SNP array with 51,478 markers has been developed on the Illumina Infinium platform for use in functional genomics studies and molecular breeding (Chen et al., 2014). However, many molecular markers designed according to the GWAS SNPs are not effective in actual crop breeding, largely due to nonfunctional SNPs. Among our accessions with SNP 274T/C, a large proportion of accessions with 274T that are expected to have the all-hairy phenotype show the hairless phenotype. One explanation is that some genes downstream of *BrpHL1* genes are mutated. Second, some cis- and trans-elements exert effects on *BrpHL1a*. If 274T was used to design the

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**Figure 6.** Procedures optimized for the selection of functional SNPs. (1) Building of segregation populations and natural populations. (2) Phenotyping, DNA resequencing, SNP calling with segregation populations, and variation analysis of agronomic traits with natural populations. (3) QTL identification with segregation populations and DNA resequencing with natural populations. (4) Selection of GWAS SNPs with natural populations and SNP genotyping with natural populations. (5) Functional identification of the candidate genes and SNPs. (6) Prediction of functional SNPs on the basis of SNP mutagenesis and phenotype rescue. (7) Exclusion of nonfunctional SNPs. (8) Selection of functional SNPs. (9) Designing of effective markers. (10) Molecular breeding by design. DH, Doubled haploid lines; GWAS, genome-wide association study; QTL, Quantitative trait locus; SNP, Single nucleotide polymorphism; RIL, Recombinant inbred lines.
molecular marker for leaf hair, the high proportion of false breeding materials would be selected. In contrast, almost all accessions with 274C display the hairless phenotype. Therefore, 274C is a functional SNP for designing effective molecular markers to select the hairless breeding materials. The application of functional SNPs to designing molecular markers will facilitate the selection of germplasms, parents, and hybrids. Furthermore, the functional SNPs must be verified for their effectiveness in the breeding of new varieties with desirable traits.

MATERIALS AND METHODS

Plant Materials

A total of 210 accessions of Brassica rapa were used in this study to survey leaf hairs. They include many subspecies and varieties, such as B. rapa ssp. chinensis, B. rapa var. parachinensis, B. rapa var. purpurana, B. rapa ssp. oleifera, B. rapa ssp. nurinosa, B. rapa var. perevodis, and B. rapa ssp. nipposinica. The seeds of these crop types were sown in the field at the SIPPE Farm Station in Shanghai during August 20 to 25 of 2008, 2009, and 2010.

Arabidopsis (Arabidopsis thaliana) gti1 (SALK_039478) mutants were kindly provided by Xiaoya Chen (Wang et al., 2004). For phenotypic observation, seeds were sown in pots with peat soil, incubated at 4°C in darkness for 3 to 4 d, and then moved to a growth chamber with 22°C temperature and 16/8 h of light/dark.

Phenotyping of Leaf Hairs

The third leaves at seedling stages were fixed and observed, the leaf hairs were observed with an anatomical microscope, and the numbers of leaf hairs on the surfaces and margins of blades and petioles were observed. The mean of the numbers of leaf hairs per leaf in 10 plants was calculated. Plants with one or two hairs that were too short to be recognized were regarded as hairless plants, while plants with more than two hairs were considered hairy plants.

Sequencing Data and Alignment with the Reference Genome

The DNA samples were sent to Novogene for sequencing by the Illumina hiseqXTEN system, which produced the paired-end libraries with 2 × 150 bp read length. All data were submitted to The Sequence Read Archive with BioProject identifier PRJNA421038.

After cutting adapters, the mean of the quality scores and the GC proportion of raw reads were calculated. The first whole-genome sequence of the Brassica A genome species (B. rapa ssp. pekinensis Chiifu-401-42) was used as the reference (http://brassicadb.org). The raw paired-end libraries of the 210 accessions were aligned to the reference genome using SOAPaligner (SOAP2) software with the parameter -3.32 × 40 × 5 × 10 × 1000 × 2, as well as bwa/samtools with the default parameter. The effective depth of sequencing was calculated as follows: the total length of clean reads minus that of the filter reads that could not match to the reference genome, all divided by the length of the reference genome.

SNP Calling and Filtering

Based on the alignment file of SOAPaligner, the reads of genomic resequencing that aligned to the 10 different chromosomes were separated into 10 files and ordered according to the physical location of the chromosome. SAMtools (http://samtools.sourceforge.net) was used for SNP and InDel detection of each chromosome using Bayesian theory.

The true SNPs were selected based on the following criteria: (1) no second heterozygous base existed; (2) there was a quality score over 20; and (3) there were at least five supported reads. The genes containing SNPs and short InDel were selected by comparing the locations of SNPs and InDels with those of all Brassica spp. gene models version 1.5 (http://blast.ncbi.nlm.nih.gov). SNPs were further determined according to whether they were located in exon regions and whether they caused synonymous/nonsynonymous mutation, premature termination, or abnormal termination.

Gene Cloning and Genetic Transformation

The BrpHL1a promoter region (1,824 bp upstream of the translation start site), 3’ UTR (1,608 bp upstream of the translation termination site), and a full-length coding sequence (1,596 bp) were amplified from Bte. Meanwhile, the BrCH1a promoter region (1,886 bp upstream of the translation start site), 3’ UTR (1,622 bp upstream of the translation termination site), and a full-length coding sequence (1,224 bp) were amplified from Wut. The promoter, 3’ UTR, and a full-length coding sequence were cloned into pCAMBIA1301 binary vectors to obtain the pBpHL1a::BrpHL1a and pBCH1a::BrCH1a constructs, respectively.

To verify the function of the mutation site, site-directed mutagenesis was performed. The C274T nucleotide substitution of the BrCH1a coding sequence resulted in the mutated BrCH1aW1059S, while the T403G nucleotide substitution of the BrpHL1a coding sequence gave rise to the mutated BrpHL1aR92W. The primers used for PCR are listed in Supplemental Table S7.

The Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). For the selection of transgenic plants, the seeds were sterilized and germinated on agar medium containing 50 mg mL⁻¹ hygromycin. Seedlings conferring resistance to the hygromycin were transplanted in a greenhouse and grown at 22°C under an 8-h light regime.

RNA Analysis

For RT-qPCR, total RNA was extracted using Trizol (Invitrogen) and treated with DNase I (TaKaRa), followed by a phenol/chloroform extraction to remove contaminating DNA. Approximately 4 μg of purified RNA was used for first-strand cDNA synthesis using PrimeScript Reverse Transcriptase (TaKaRa) with oligo(dT) primers. RT-qPCR was performed using specific primer pairs (Supplemental Table S7) in the Myq2 two-color real-time PCR detection system (Bio-Rad). The comparative Ct method was used to determine relative transcript levels (Myq2 two-color real-time PCR detection system; Bio-Rad). Expression was normalized relative to that of ACTIN. Two developing leaves in one B. rapa seedling and 10 shoots of Arabidopsis seedlings were harvested for RNA sampling. Three biological replicates and three technical replicates were performed. Error bars indicate sd.

GUS Staining

GUS staining was performed on 14-d-old plants. Seedlings of the transgenic plants were placed in staining solution (50 mm Na3PO4, pH 7, 0.5 mm 5-bromo-4-chloro-3-indolyl glucuronide, and 20% [v/v] methanol), vacuum infiltrated, and incubated at 37°C overnight. After staining, tissues were fixed in alcohol for further analysis.

BiFC Assays

Paired cYFP-tagged and mYFP-tagged constructs were cotransformed into Arabidopsis protoplasts. After incubation at 22°C in darkness for 12 h, GFP and YFP fluorescence signals were excited with 488- or 514-nm argon laser lines, with emission bands of 495 to 540 nm for GFP detection, 520 to 560 nm for YFP detection, and 675 to 765 nm for chlorophyll autofluorescence by confocal microscopy.

In Vitro Pull-Down Assays

For MBP pull-down assays, MBP-tagged proteins were bound to amylose resin (New England Biolabs) in binding buffer containing 25 mm Tris, pH 7.4, 1 mm EDTA, 0.01% (w/w) Nonidet P-40, and 2 × NaCl and incubated with GST-tagged proteins overnight at 4°C. Then, the resin was washed 10 times in the binding buffer and eluted by boiling in SDS-PAGE loading dye. Aliquots of eluents (20 μL) were resolved on SDS-PAGE gels for immunoblotting with the GST antibody.

Accession Numbers

Accession numbers are as follows: Arabidopsis GL1, AT3G227920; GL2, AT1G79840; and GL3, AT5G13135; Brassica rapa BrpHL1a, Bra025311; BrpHL1b, Bra039065; and BrpGL2, Bra003535.
SUPPLEMENTAL DATA

The following supplemental materials are available.

**Supplemental Figure S1.** Alignment of BrpHL1a with homologs in other plant species.

**Supplemental Figure S2.** Alignment of BrpHL1a and BrpHL1b amino acid sequences with GL1.

**Supplemental Figure S3.** Alignment of cloned BrpHL1a with a reference gene sequence.

**Supplemental Figure S4.** Alignment of cloned BrpHL1a with a reference gene sequence.

**Supplemental Figure S5.** Schematic diagram of BrpHL1b on chromosome 9 in Bre and Wut.

**Supplemental Table S1.** Genomic sequences of the BrpHL1a alleles cloned from 13 B. rapa genotypes (inbred lines).

**Supplemental Table S2.** Genomic sequences of the BrpHL1b alleles cloned from 13 B. rapa genotypes (inbred lines).

**Supplemental Table S3.** Summary of genome resequencing and mapping data in 210 B. rapa accessions.

**Supplemental Table S4.** Summary of SNP calling data from 210 B. rapa accessions.

**Supplemental Table S5.** SNP genotyping of BrpHL1a alleles from genome resequencing of 210 B. rapa accessions.

**Supplemental Table S6.** Association between SNPs 274T/C and 403T/G of BrpHL1a with hairy phenotypes in 210 B. rapa accessions.

**Supplemental Table S7.** Primer sequences used in this study.

ACKNOWLEDGMENTS

The gtl mutants were obtained from Dr. S. Wang.

Received January 11, 2018; accepted April 9, 2018; published April 23, 2018.

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