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# Genetic analysis and fine mapping of the pubescence gene *GL6* in rice (*Oryza sativa* L.)

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The pubescence of the leaf blade surface is an important agronomic characteristic for rice morphology and significantly influences rice growth as well as physiological characteristics. This characteristic was analyzed in $F_1$ and $F_2$ plants derived by crossing cultivar 75-1-127 with the *indica* cultivar Minghui 63, as well as the glabrous cultivar Lemont and *indica* cultivar 9311. Results indicated that the pubescence of the leaf blade surface was a dominant trait and controlled by a single gene. The *GL6* gene was primarily mapped on rice chromosome 6 with recessive $F_2$ population derived from 75-1-127/Minghui 63 by combining bulked segregation analysis and recessive class analysis using the Mapmaker3.0/MapDraw software. The genetic distances between the simple sequence repeat markers RM20491 and RM20547 were 7.2 and 2.2 cM, respectively. The *GL6* gene was fine mapped in the interval between InDel-106 and InDel-115 at genetic distances of 0.3 and 0.1 cM, respectively. The large, recessive $F_2$ population was derived from 75-1-127/Minghui 63. A high-resolution genetic and physical map of *GL6* was constructed. Derived from the map-based sequences published by the International Rice Genome Sequencing Project, the *GL6* gene was localized at an interval of 79 (*japonica*) and 116.82 kb (9311) bracketed by InDel-106 and InDel-115 within the BAC accession numbers AP008403 and AP005760. Seven annotated genes (*japonica*) and eight annotated genes (9311) were present. The basis was further set for *GL6* cloning and function analysis.

**rice (*Oryza sativa* L.), pubescence gene *GL6*, genetic analysis, fine mapping**

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Glabrous rice, also known as "Nuda" rice, has hairless stems, leaves, leaf sheath, veins, and grains [1,2]. Glabrous rice is suitable for the mechanization of production and operation by agricultural workers because no dust is produced during its harvest and processing, causing no damage to human skin [3] and preventing environmental pollution. Therefore, glabrous rice is also called "green rice" and mainly distributed in the United States, Africa, and Yunnan-Guizhou Plateau in China, among others. Glabrous rice is the main type of cultivar in the United States, Africa, and most countries [4]. Glabrous rice has generally been introduced around the world and is studied and used by most countries because of its properties and features such as thick leaves; light adaptation ability [5]; relatively short plant structure; strong lodging resistance [6]; large panicle; high spike rate; fine grain quality [7]; conduciveness for production, machining, and storage [3,8]; as well as good affinity with *japonica* and *indica* [7,8]. The glabrous rice in most common cultivars exhibit properties similar to those of Java rice and *japonica*, which have not undergone differentiation at subspecies level [6]. The geographic distribution and genetic differentiation of this glabrous rice are more complex, and the affinity to *japonica* and *indica* rice is excellent. Therefore, by utilizing the properties of glabrous rice, new

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hybrid varieties of *japonica* and *indica* subspecies can be developed. High-yield hybrid rice with the properties of glabrous rice varieties and strong adaptability can be bred in our country [8].

Pubescence is characterized by trichomes gathering on the plant surface, consisting of unicellular or multicellular exogenous substances on leaves, stems, and root surfaces. Pubescence may be related to the development of glandular cells and thus are called glandular hairs. Glandular hairs can accumulate and secrete alkaloids such as nicotine and terpenoids as well as some chemical substances against injurious insects. Most plants also have non-glandular hairs that can strengthen resistance to abiotic stress such as extremely high or low temperature, drought, and ultraviolet irradiation [9] to promote normal growth. Despite reports regarding the mechanism of the growth and development of pubescence, most of these studies are focused on *Arabidopsis*, as well as wheat, and tobacco.

Pubescence in *Arabidopsis* is distributed over the sepals, leaves, and stems. The pubescence on the blade is mainly distributed on the abaxial side. No pubescence is observed on cotyledons. Pubescence only forms when the second pair of leaves begin to develop [10]. In *Arabidopsis*, pubescence is differentiated from single cells, and its growth and development provides a classic model of plant cell differentiation [11]. A series of *Arabidopsis* genes that control growth and development of pubescence has been identified or cloned: genes that promote pubescence growth, including *TTG1*, *GL1* [12], *GL3* [13], *EGL3*, *GIS*, *GIS2*, and *ZFP8* [14] as well as genes that inhibit pubescence growth, including *TRY*, *CPC*, *CPC1* (ETC1) *ETC2*, *ETC3*, *TCL1*, and *SPL* [15]. *TTG1* and *GL1* are two key genes for the growth process of pubescence in *Arabidopsis*. Mutation of these genes can affect pubescence growth. *TTG1* encodes a WD40 protein and plays a pivotal role during differentiation. Pubescence is not present in *ttg* mutants. Furthermore, *ttg* mutants affect the synthesis of anthocyanins, seed coat mucus, as well as root hair growth of these mutants are affected. [16]. *GL1* has a full-length gDNA of 4.5 kb and encodes an MYB transcription factor. The expression level of *GL1* during the initial stage of pubescence is particularly high, indicating the importance of this gene in pubescence growth [17]. Unlike the ttg mutant, its mutant only affects pubescence growth [18]. Pubescence growth is also reportedly induced by gibberellin (GA) and cytokinin (CTK) [10,11,15,18]. *TRY*, *CPC*, *CPC1* (ETC1), *ETC2*, *ETC3*, *TCL1* form a set of genes that encode the MYB transcription factor and inhibit pubescence growth in *Arabidopsis* [11]. These transcription factors are transferred from the pubescence to the neighboring cells and compete for binding sites with *GL1* and *GL3*, preventing pubescence growth. Wheat genes *Hl2* and *Hl2Aesp* control pubescence growth and are located on chromosomes *4BL* and *7BS* [19]. A set of *GLOSSY* genes in corn has been cloned, which affects pubescence growth as well as morphology and is related to *cuticular* wax synthesis. In tobacco, overexpression of *MIXTA* results in abundant growth of pubescence in cotyledons, leaves, and stems. Overexpression of *CotMYBA* can also induce pubescence growth on tobacco cotyledons.

Pubescence in rice is mainly distributed on the leaves and glumes. Rice leaves have two types of pubescences, namely, large pubescence and small pubescence. Large pubescence are mainly distributed in silicon cells on the thin vascular bundle, whereas small pubescences are mainly distributed along stomatal cells or found adjacent to motor cells [20]. Zhu et al. [21] reported that pubescence on the leaves and chaff of *japonica* is mainly and regularly distributed in the junction of the light green stripes and the dark green stripes of the leaves. In addition, the pubescences are lanceolate in shape with a swollen base and slender tip. Chaff pubescence shows an erratic distribution and varying lengths. The genes in rice that affect pubescence growth are identified as *gl1*, *gl2*, *Hl1*, *Hl2*, and *Hg*. The genes *gl1* and *gl2* is a pair of tandem repeats [9,22–24], whereas the blade woolly genes HL-A and Hl-b are complementary genes [9,14,23–25]. Yu indicated that the glabrous gene (*gl1*) of rice leaf and glume is located on chromosome 5. The genetic distances from RG182, which are marked RFLP and RG403, were 14.3 ± 7.4 and 20.9 ± 8.3 cM [15]. Wang et al. [9] found that the linkage simple sequence repeat (SSR) markers of *gl1* are RM1024, RM1200, GL8, and GL311. The gene *gl1* is located in the 230-kb region between GL8 and GL311. Li et al. [26] reported that gl1 is located between RM1200/RM17786 and RM2010/RM17801, with genetic distances of 1.0 and 1.0 cM, respectively. Fine mapping the gene between the InDel markers ID33 and ID44 evaluated the physical distance at 54 kb. Bioinformatics analysis shows that this region contains 10 predicted genes. Os05g119000, one of the predicted genes, was selected as a candidate genes by experimental analysis. However, further research indicated that Os05g119000 is associated with aluminum tolerance.

In the present study, SSR and InDel molecular markers were used for bulked segregation analysis (BSA) and recessive class analysis (RCA). Genetic analysis and fine mapping of *GL6*, which is a gene for pubescence growth and development in rice, was conducted. This study aims to develop molecular markers that are closely linked to the glabrous gene *GL6* for further cloning and functional analysis of the gene *GL6*.

## 1 Materials and methods

### 1.1 Test parents and mapping population

The cultivar 75-1-127, which exhibits pubescence on leaves; Minghui 63, without pubescence; glabrous rice Lemont, 9311, and $F_1$ by hybridization; as well as segregating population $F_2$ by inbred of $F_1$ were used in the present study.

### 1.2 Survey of pubescence traits on the rice leaf surface

Pubescence on the rice leaf surface is a typical agronomic trait and can be easily observed. This trait can be directly observed in the field by the hand-touch method to determine the presence of pubescence on leaves. In addition, leaf surface pubescence can reflect sunlight; thus, leaf pubescence is easily observed under the sun.

### 1.3 DNA extraction and construction of pubescent and glabrous gene pools

The extraction of genomic DNA was conducted by a modified cetyltrimethylammonium bromide method [27]. Optical densities were measured using a DU800 ultraviolet spectrophotometer. The DNA concentrations of the individual plants were diluted into 50 ng $\mu L^{-1}$. Ten plants were randomly selected from the pubescent and glabrous individuals in the $F_2$ segregating population. The diluted DNA of 10 pubescent individuals and 10 glabrous individuals were mixed to create a pubescent gene pool and a glabrous gene pool [28].

### 1.4 Primary mapping of pubescence genes in rice

The SSR markers used were obtained from the rice bioinformatics Web site (www.gramene.org) and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The polymerase chain reaction (PCR) employed a reaction system (10 μL) consisting of 1 μL of 10× PCR buffer ($Mg^{2+}$-free), 0.6 μL of 25 μmol $L^{-1}$ $MgCl_2$, 0.4 μL of 10 μmol $L^{-1}$ dNTP mixture, 0.6 μL of 10 μmol $L^{-1}$ primer, and 0.2 μL of 5 U $\mu L^{-1}$ r*Taq* enzyme. The PCR reactions were conducted using a Bio-Rad DNA Engine Dyad PCR instrument. The reaction program was as follows: denaturation for 5 min at 94°C; 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; extension for 7 min at 72°C; and storage at 4°C. After the PCR amplification reaction, the amplification product was separated and detected using an 8.0% non-denaturing polyacrylamide gel [29].

### 1.5 Fine mapping of pubescence genes in rice

The primary mapping interval of the whole-genome sequence fragments in rice was downloaded from the biological information science web site. The new SSR-bit point of this area was determined using the SSRIT Software provided by the Web site. Generally, SSR-bit points with more than 10 repeats were used and developed. Specific primers for SSR points were designed using the primer design software Primer Premier 5.0. The ratio of the interval difference in the genomic fragment sequences between the two subspecies, Nipponbare (*japonica*) and 9311 (*indica*) was determined. The insertion–deletion database was used to obtain the different loci. The locus with the higher distribution density was determined for the insertion–deletion of InDel markers. These markers were then used for fine mapping the pubescence gene *GL6*. Primer synthesis, PCR reaction, and electrophoresis were conducted under the same conditions in above.

### 1.6 Genetic mapping of the pubescence gene *GL6*

Linkage analysis for each SSR marker and the pubescence gene GL6 was performed using the software Mapmaker/Exp3.0 [30]. The recombination rate was transformed into the genetic distance using the Kosambi's function. The chain results entered into Excel. A genetic linkage map was constructed using the mapping software MapDraw.

## 2 Results and discussion

### 2.1 Genetic analysis of pubescence characteristics in rice

To obtain $F_1$ plants, the hybridized pubescent cultivar 75-1-127 was crossed with the *indica* cultivar Minghui 63, and the glabrous cultivar Lemont was crossed with the *indica* cultivar 9311 in the summer of 2009 at the Xiamao experimental base, Shaxian County, Fujian Academy of Agricultural Sciences. In the winter of 2009, the $F_1$ generations were cultivated at the Hainan breeding base of Fujian Province. Upon maturity, $F_2$ seeds were obtained after self-seeding of $F_1$ plants. In the summer of 2010, the $F_2$ seeds were sowed according to the segregating populations of different genes. The leaf morphologies of the $F_1$ plants and $F_2$ populations were observed during the tillering stage. The statistical results showed that all $F_1$ plants were pubescent. The segregation ratio of pubescent plants to glabrous plants in the $F_2$ populations were 3:1 ($\chi^2$ = 1.801, 0.261, 0.61), which is in accordance with the Mendelian ratio of 3:1 (Table 1). Therefore, the pubescence characteristics were controlled by a dominant gene, with the allele coding

**Table 1** Segregation of pubescence characteristics between 75-1-127 and other varieties

| Combinations | Phenotype | | | | $\chi^2$ | $\chi^2_{0.05}$ |
|---|---|---|---|---|---|---|
| | Population | Dominant | Recessive | Dominant: Recessive | | |
| 75-1-127/Minghui 63 | 2744 | 2089 | 655 | 2089:655 | 1.801 | 3.84 |
| Lemont/75-1-127 | 1002 | 744 | 258 | 124:43 | 0.261 | 3.84 |
| 9311/75-1-127 | 2883 | 2156 | 727 | 2156:727 | 0.61 | 3.84 |

for pubescence being dominant over the allele for glabrousness. To further characterize the phenotype, the upper and lower epidermis of leaves in 75-1-127, Minghui 63, and $F_1$ were observed under an electron microscope, and apparent differences were observed (Figures 1 and 2).

### 2.2 Primary mapping of *GL6*

With the development of rice genome research and the completion of *japonica* and *indica* sequencing, more than 8000 pairs of SSR markers distributed on 12 chromosomes have been developed [31]. To map the *GL6* locus, 136 pairs of SSR markers on the 12 chromosomes were used to search for polymorphic markers in the parents ($F_1$ and glabrous gene pool). SSR marker RM30 on chromosome 6 was polymorphic between the $F_1$ and $F_2$ gene pools. Linkage analysis of glabrous plants from the $F_2$ population shows that RM30 was linked to the *GL6* locus.

In addition, 319 pairs of SSR markers on rice chromosome 6 were obtained from the bioinformatics Web site (www.gramene.org). Further analysis shows that RM20491, RM20517, RM20539, RM20566, RM20547, and RM5509 were linked to the *GL6* locus. Combined BSA and RCA were conducted to analyze all glabrous plants from the $F_2$ population and quantify the single-exchange plants. The results show that the exchange rate of RM20491 was the highest and that 84 of 655 glabrous plants from the $F_2$ population were exchanged. RM20547 was exchanged in seven plants. However, the two SSR markers were located on different sides in the *GL6* locus. Detection of mark RM20517 shows that no plants exchanged. In addition, RM20517 co-segregated with *GL6*. Mark RM20566 and RM5509 were mapped on the side of the *GL6* focus consistent with RM20547. However, more exchanged plants were observed. Therefore, RM20566 and RM5509 were located farther from the target gene. Chain relationship analysis of all SSR markers and *GL6* as well as genetic distances showed that, the *GL6* gene was mapped on rice chromosome 6. The gene was located between RM20491 and RM20547, with genetic distances of 7.2 and 2.2 cM, respectively (Figure 3).

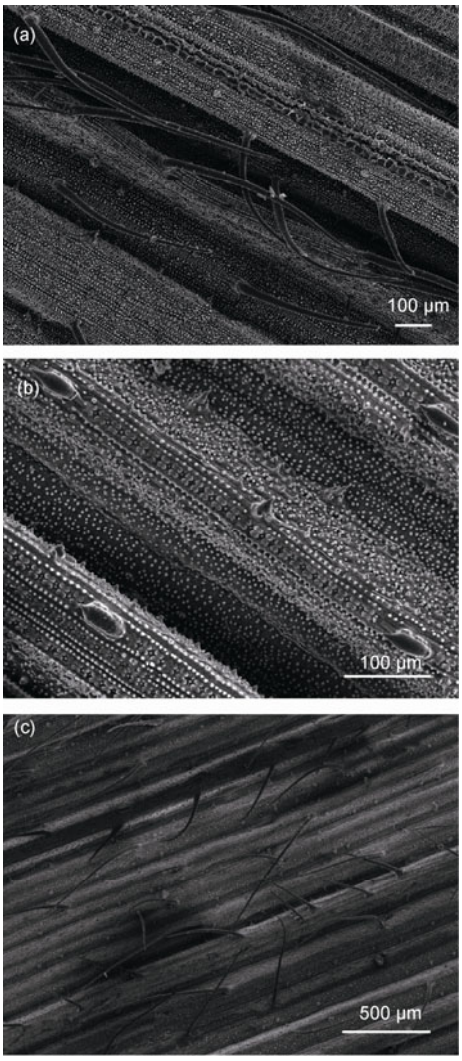


**Figure 1** Scanning electron micrographs showing the morphology of the forward epidermis of leaf in (a) 75-1-127, (b) Minghui 63, and (c) $F_1$.

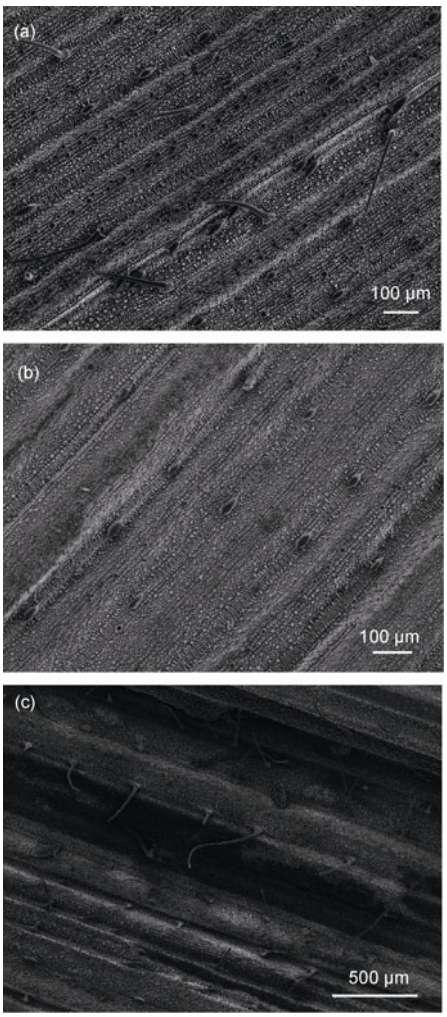


**Figure 2** Scanning electron micrographs showing the morphology of the reverse epidermis of leaf in (a) 75-1-127, (b) Minghui 63, and (c) $F_1$.

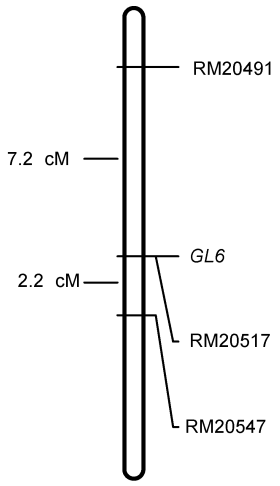


**Figure 3** Primary genetic linkage map of the pubescence gene *GL6*.

### 2.3 Fine mapping of the *GL6* locus

Fine mapping of the *GL6* locus was conducted in the summer of 2010 with pubescent 75-1-127 and hairless rice Minghui 63 as parents. The hybridized combinations were mated at the Xiamao (Shaxian) experimental base of Fujian Academy of Agricultural Sciences. In the winter of 2010, the hybridized $F_1$ seeds were sowed at the Hainan South breeding base of Fujian province. The $F_2$ seeds from each plant were harvested. In the summer of 2011, the $F_2$ segregating populations were sowed at the Xiamao (Shaxian) experimental base of Fujian Academy of Agricultural Sciences. Two populations, 720 and 670 hairless plants, were obtained. To determine for a marker linkage to the pubescence gene *GL6*, the mapped fragments of Nipponbare (*japonica*) and 9311 (*indica*) were analyzed based on InDel data. A total of 120 InDel loci were found. Specific InDel markers were subsequently developed and designed. After the polymorphic primer screening of the pubescent variety 75-1-127, glabrous variety Minghui 63, and gene pool, six pairs of stable polymorphism markers were found, including InDel-97, InDel-105, InDel-102, InDel-106, InDel-115, and InDel-116. Analysis of the recessive glabrous individuals of $F_2$ confirmed that these markers were tightly linked to the pubescence gene *GL6*. The new marked InDel sequences and amplified fragments are shown in Table 2.

The analysis 720 recessive glabrous individual groups showed no exchanged individual found by using the InDel-106, InDel-115, and InDel-116 markers. These markers cosegregated with the pubescence gene *GL6*.

The analysis of the 670 glabrous individuals (recessive class) shows that the exchange position of markers RM20491, InDel-97, InDel-102, InDel-105, and InDel-106 were the same and located on the same side of the pubescence gene *GL6*. RM20491 had the highest exchange frequency (72 exchanged individuals). This marker is the farthest and located in the upper end of the pubescence gene *GL6*. For InDel-97, 17 exchanged individuals were found. For InDel-102, InDel-105, and InDel-106, 6, 6 and 4 exchanged individuals were found, respectively. Markers InDel-115, RM20517, RM20539, and RM20547 were located on the other side of the pubescence gene. For InDel-115, RM20517, RM20539, and RM20547, 1, 2, 9, and 16 exchanged plants were found, respectively.

No marker co-segregated with the pubescence gene GL6 in 670 glabrous individuals according to the RCA analysis, suggesting that no marker was suitable for the fine mapping of the pubescence gene *GL6*. By using the Mapmaker/Exp3.0 software, the pubescence gene GL6 was fine-mapped between the markers InDel-106 and InDel-115, with genetic distances of 0.3 and 0.1 cM, respectively. The physical distance of this area for Nipponbare (*japonica*) and 9311 (*indica*) were 79 and 116.82 kb, respectively. Seven (Nipponbare) and eight (9311) predicted genes were observed.

The other genetic distances between the markers and the pubescence gene GL6 are shown in Figure 4. The position of each marker on the chromosome corresponded to their actual distribution position on the chromosome. A genetic linkage map was finally constructed using the mapping software MapDraw.

### 2.4 Construction of a physical map of the pubescence gene *GL6*

The BAC clone sequences of Nipponbare (*japonica*) were anchored to the corresponding positions of each marker linked to the pubescence genes to construct a physical map (Figure 5). The figure above each marker denotes the number

**Table 2** InDel primer sequence and amplified fragment size

| Name | Fragment size (bp) | | Primer sequence | |
|---|---|---|---|---|
| | Nipponbare | 9311 | Sense (5′→3′) | Anti-sense (5′→3′) |
| InDel-97 | 279 | 295 | TTGGGGTTTGAATTCTTTGC | ATAGCATCGCGGAGAGAAAA |
| InDel-102 | 312 | 325 | TCACGGTTACCATCACTATTTCTT | GCAATAACTGTAGGATTCGTCGTA |
| InDel-105 | 276 | 295 | GAACTCCCTGCTCCGACAA | TGAGAGGGTAGGTGGGGTAATA |
| InDel-106 | 172 | 148 | GAATAAGACGAACGGTCAAACA | ACGCACGGAAGAAGAAAATG |
| InDel-115 | 339 | 349 | AAACATCCTTCGGTGCTTCAA | CTCTTGGCTCTCGCTCTTCAG |
| InDel-116 | 137 | 193 | TTGTCGCATCGTTGTTGAGC | TTGAAGCACCGAAGGATGTTT |

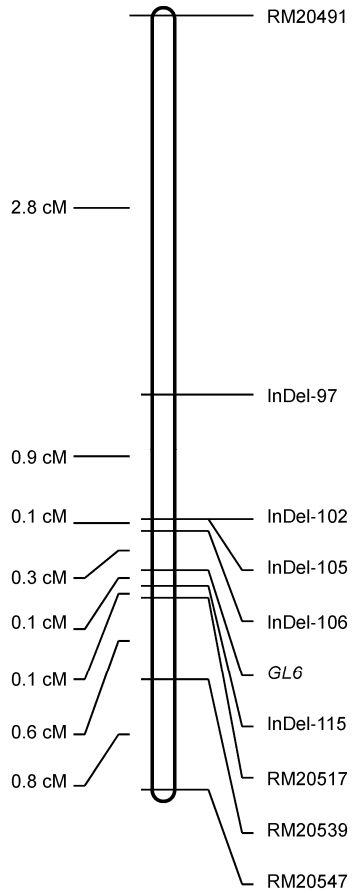


**Figure 4** Fine genetic linkage map of the pubescence gene *GL6.*

of exchanged individuals according to the RCA analysis of 670 glabrous individuals. In this study, the gene for pubescence growth and development, *GL6*, was fine mapped on chromosome 6. This gene was located between the markers InDel-106 and InDel-115, with genetic distances of 0.3 and 0.1 cM, respectively. The physical distance of this area for Nipponbare (*japonica*) was 79 kb (Figure 5), and seven predicted genes were found based on www.gramene.org. The physical distance for 9311 (*indica*) was 116.82 kb, with eight predicted genes. Comparison and analysis of these two subspecies revealed that the sequences of all predicted genes of *japonica* and *indica* were similar. Among the predicted genes of Nipponbare (*japonica*), Loc_Os06g44750 corresponds to an AP2 domain-containing protein. Loc_Os06g44820 is a pentatricopeptide repeat (PPR) domain-containing protein. The functions of the remaining proteins are unknown.

## 3 Discussion

The gene for the pubescence growth and development, *GL6,* was fine mapped on chromosome 6. This gene was located between the markers InDel-106 and InDel-115, with genetic distances of 0.3 and 0.1 cM, respectively. The physical distance of this area for Nipponbare (*japonica*) was 79 kb, with seven predicted genes based on www.gramene.org. The physical distance for 9311 (*indica*) was 116.82 kb, with eight predicted genes. The sequences of all predicted genes of *japonica* and *indica* were the same based on the comparison and analysis of these two subspecies.

Among the predicted genes of the Nipponbare (*japonica*), Loc_Os06g44750 corresponds to an AP2 domain-containing protein, which is a class of transcription factors. The AP2 domain is a DNA-binding region in plants. The core region of the 68 amino acids can form an amphiprotic α-helix, which influences the regulation mechanism during DNA binding. The AP2 family of transcription factors can be broadly divided into five subfamilies. The AP2-like class promotes the differentiation and development of flowers

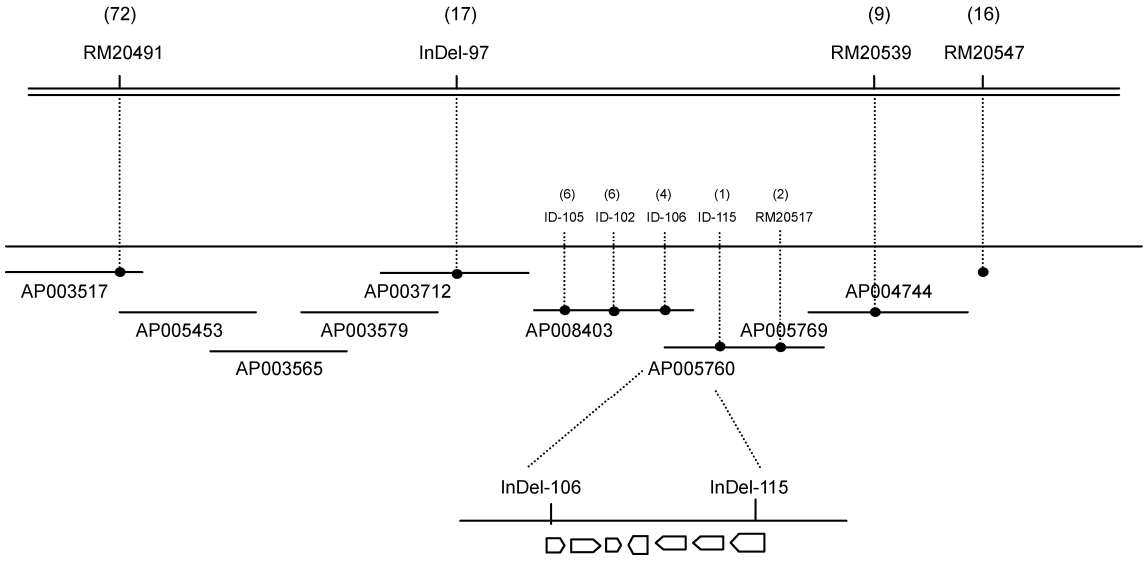


**Figure 5** Physical map of the pubescence gene *GL6.*

and seeds, and the ethylene-responsive element binding protein-like class affects plant resilience [32]. Loc_Os06g44820 is a PPR domain-containing protein. The PPR family has a tandem repeated with a 35 amino-acid sequence unit. More than 466 and 600 genes belong to the PPR family in *Arabidopsis* and rice, respectively. The targets of most of these proteins are mitochondria or chloroplasts. These proteins bind RNA in these organelles. The RNA is modified and processed after transcription. The 5′ and 3′ end fractures of RNA, cis- and trans-splicing, the stabilization and destabilization of the transcription product, and others [33]. The functions of the PPR protein include plant growth and development, organelle formation [34–39], fertility restoration of cytoplasmic male sterility [40–42], RNA editing and processing [43–45], reverse signal transmission between the nucleus and organelles [46], and adversity defense [47–52]. The remaining predicted genetic code were annotated as proteins of unknown function.

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