Plant Mol Biol (2008) 68:239–250
DOI 10.1007/s11103-008-9365-x

ROLLED LEAF 9, encoding a GARP protein, regulates the leaf abaxial cell fate in rice

Song Yan · Chang-Jie Yan · Xiu-Hong Zeng · Ya-Chun Yang · Yu-Wei Fang · Chun-Yan Tian · Ya-Wei Sun · Zhu-Kuan Cheng · Ming-Hong Gu

Received: 31 January 2008 / Accepted: 15 June 2008 / Published online: 2 July 2008

Abstract Leaves, the collective organ produced by the shoot apical meristem (SAM), are polarized along their adaxial–abaxial axis. In this study, we characterized two rice (*Oryza sativa*) allelic rolled-leaf mutants, *rolled leaf 9-1* (*rl9-1*) and *rl9-2*, which display very similar phenotypes with completely adaxialized leaves and malformed spikelets. We cloned the *RL9* gene by way of a map-based cloning strategy. Molecular studies have revealed that *RL9* encodes a GARP protein, an orthologue of *Arabidopsis KANADIs*. *RL9* is mainly expressed in roots, leaves, and flowers. The transient expression of a *RL9*–GFP (green fluorescent protein) fusion protein has indicated that *RL9* protein is localized in the nucleus, suggesting that *RL9* acts as a putative transcription factor.

Keywords *Oryza Sativa* L. · Rolled leaf · KANADI · RL9

Song Yan and Chang-Jie Yan contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-008-9365-x) contains supplementary material, which is available to authorized users.

S. Yan · C.-J. Yan (✉) · X.-H. Zeng · Y.-C. Yang · Y.-W. Fang · C.-Y. Tian · Y.-W. Sun · M.-H. Gu (✉)
The Key Laboratory of Plant Functional Genomics, Ministry of Education of China, Jiangsu Key Laboratory of Crop Genetics and Physiology, Agricultural College of Yangzhou University, 12 East Wenhui Road, Jiangsu 225009, People's Republic of China
e-mail: yichuan@yzu.edu.cn

M.-H. Gu
e-mail: gumh@yzu.edu.cn

Z.-K. Cheng
Institute of Genetics and development biology, Chinese Academy of Science, Beijing 100101, China

Introduction

Plant leaves derive from primordia, which form in the peripheral zone of the shoot apical meristem (SAM). After emerging from the SAM flanks, the leaf primordia establishes polarity along three axes: the adaxial–abaxial, proximodistal, and mediolateral. Among these three axes, the adaxial–abaxial axis is fundamental for the subsequent asymmetric growth of the leaf and lamina expansion (Sussex 1955; Waites et al. 1995; McConnel and Barton 1998). The genetic mechanism behind leaf adaxial–abaxial polarity formation has been uncovered through a variety of mutants in *Arabidopsis thaliana*, *Antirrhinum majus*, and *Zea mays*, all of which exhibit abnormalities in leaf polarity. Morphological and molecular analyses of these mutants have led to the identification of genes that play important roles in leaf polarity establishment (for a review, see Xu et al. 2007).

In *Arabidopsis*, previous studies of abaxial–adaxial polarity establishment have indicated that transcription factors appear to be central, among all the components identified during leaf adaxial–abaxial polarity formation. Five functional categories of transcription factors were identified as being involved in the leaf adaxial–abaxial polarity formation. The first one is genes of the *HD-ZIP III* family, which are encoding members of a homeodomain/leucine-zipper (*HD-ZIP*) family of proteins, including *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*) genes. These genes have been shown to function in promoting leaf adaxial fate (Talbert et al. 1995; McConnell and Barton 1998; McConnell et al. 2001). Although carrying a recessive mutation in one of these three genes does not result in a plant having leaf defects in adaxial–abaxial patterning, plants lacking functional copies of all three genes exhibit a dramatic phenotype with an abaxialized, needle-like cotyledon distal to the hypocotyls

(Emery et al. 2003; Prigge et al. 2005). Two miRNAs, miR165/166, were found to negatively regulate *PHB/PHV/REV* by mediating transcript cleavage and degradation (Emery et al. 2003; Reinhart et al. 2002; Rhoades et al. 2002; Juarez et al. 2004a, b; Bao et al. 2004).

The second category consists of *ASYMETRIC LEAVES 1* and *2* (*AS1* and *AS2*). *AS1* encodes a putative R2–R3 MYB domain transcription factor (Bynre et al. 2000; Sun et al. 2002), while *AS2* encodes a LATERAL ORGAN BOUNDARIES (LOB) domain protein containing a leucine-zipper motif (Lin et al. 2003; Xu et al. 2003; Iwakawa et al. 2002). *AS1* and *AS2* can bind each other to form a complex that functions within the adaxial leaf domain (Xu et al. 2003), suggesting that *AS1* and *AS2* participate in the determinant of adaxial cell fate. The results of overexpression of *AS1* and *AS2* indicate that the normally spatially restricted *AS2* may be critical in determining the activity of the *AS1–AS2* complex, and the role of *AS1* in leaf polarity formation is dependent on its interaction with *AS2*.

The third category is *KANADIs*, which encodes putative GARP family transcription factors, including *KAN1*, *KAN2*, and *KAN3*. These three genes are expressed in a domain complementary to *PHB/PHV/REV* in multiple tissues (Emery et al. 2003; Eshed et al. 1999, 2001; Kerstetter et al. 2001; Hawker and Bowman 2004). Mutual suppression between *KAN1/2/3* and *PHB/PHV/REV* was found, which might be very important to leaf adaxial–abaxial polarity formation.

The fourth group consists of *AUXIN RESPONSE FACTORS 3* and *4* (*ARF3* and *ARF4*), encoding a plant-specific transcription factors. *ARF3* and *ARF4* were shown to be required for specifying leaf abaxial identity (Pekker et al. 2005). *ARF3/4* transcripts are targets of a *TAS3*-derived *trans-acting* short interfering RNA, tasiR-ARF, which guides the cleavage of *ARF3/4* mRNAs (Allen et al. 2005; Williams et al. 2005).

The fifth and final group is *FILAMENTOUS FLOWER (FIL)* and *YABBY3 (YAB3). FIL* and *YAB3* belong to the *YABBY* transcription factors family, and contain a conserved zinc-finger domain and an HMG-like *YAB* domain (Sawa et al. 1999; Siegfried et al. 1999). *FIL* and *YAB3* are each expressed in the abaxial leaf domain, and thus promote abaxial cell fate (Siegfried et al. 1999).

In addition, 26S proteasome was found to target a regulator that promotes leaf abaxial identity through a characterization of an *as1/as2* enhancer mutation, *ae3* (Huang et al. 2006). Therefore, five groups of transcription factors, two types of small RNAs, and the 26S proteasome protein degradation machinery have been shown to modulate the establishment of adaxial–abaxial polarity in *Arabidopsis*. More recently, numerous genetic factors—including transcription factors and small RNAs—have been identified as being responsible for leaf adaxial–abaxial polarity formation; however, a great amount of evidence has shown that their functions vary in different species (for a review, see Kidner and Timmermans 2007).

Although many genes have been identified and characterized in eudicot *Arabidopsis*, little progress has been achieved on studies into leaf adaxial–abaxial polarity formation in rice, a model plant of cereal crops. Leaves, the lateral outgrowths from the stem, are the major collective organ where photosynthesis takes place, and they thus play an important role in natural and agricultural productivity. Therefore, the shape of the rice leaf has been considered a critical factor in rice plant-type breeding (Yuan 1997). Many studies have demonstrated that leaf-rolling, to some degree, would benefit the plant in keeping it erect, as it would consequently optimize canopy light transmission and increase the photosynthesis rate in crops (Shen 1983; Chen et al. 2001, 2002; Lang et al. 2004a, b). Recently, *OsAGO7*, an orthologue of *Arabidopsis AGO7*, was isolated and characterized as being involved in leaf-curling (Shi et al. 2007). Apart from that, several genes (e.g., *RL1–RL9*) responsible for the rice leaf-rolling characteristic have been identified in recent years, through conventional genetic approaches. Among them, *RL7*, *RL8*, and *RL9* have been assigned to their corresponding chromosomes with molecular markers (Yan et al. 2006). However, to date, none of these rolled-leaf genes have been fully characterized in rice.

Previously, we isolated the *rl9-1* mutant from the M_2 generation of a *japonica* variety Zhonghua 11 via ^{60}Co $\gamma \sim$ ray radiation and then anchored the gene within a 42-kb region on chromosome 9 (Yan et al. 2006). In this work, we further isolated the *RL9* gene by way of a map-based cloning strategy. A sequence analysis revealed that the *RL9* gene encodes a GARP domain protein, an orthologue of *Arabidopsis KANADIs*, acting as a putative transcription factor. The *RL9* gene is mainly expressed in roots, leaves, and flowers, and the *RL9*-encoded protein is localized in nuclei.

Materials and methods

Transmission electron microscopy (TEM) analysis

Leaf samples of wild-type and *rl9-1* mutant rice were harvested from two-month-old plants. Leaf sections were fixed in primary fixation solution (2% paraformaldehyde and 2% glutaraldehyde). After washing with 0.05 M sodium cacodylate buffer (pH 7.2), they were post-fixed with 1% osmium tetroxide. The samples were then bloc-stained and dehydrated in a gradient alcohol series before transitions and infiltrations were processed. The

polymerization reaction was carried out overnight at 70°C. The sections were sliced to 60 nm with ultra-microtome (MT-X, RMC, USA), stained with 2% uranyl acetate and Reynolds' lead citrate, and observed under transmission electron microscopy (JEM-1010, JEOL, Japan).

Fine mapping of the *RL9* gene

The *rl9-1* mutant was isolated in the M_2 population of a *japonica* variety Zhonghua 11 radiated by ^{60}Co $\gamma \sim$ ray, and the rolled-leaf phenotype was found to be inherently stable (Yan et al. 2006). The *rl9-2* mutant was derived from the *japonica* variety Nipponbare during tissue culture. We crossed the *rl9-1* mutant with an *indica* variety, Dular, and then generated F_2 and F_3 populations for fine-mapping and gene-cloning. All these plants, as well as the derived mapping populations, were grown in the Experimental Farm of Yangzhou University, China, in 2006. Field management followed essentially the normal agricultural practice described by Yan et al. (2007).

The *RL9* gene has previously been restricted to the 42-kb region of AP005904 (Yan et al. 2006). A total of 1,267 rolled-leaf plants selected from F_3 were used for further fine-mapping of *RL9*. Genomic DNA was extracted from rice leaves using the CTAB method. STS and CAPS markers were developed, based on the diversity between the genomic DNA sequence of Nipponebare and 93-11 within the 42-kb region, and the sequences of the STS and CAPS primers (Supplemental Table 1) were designed using Vector NTI 9.0 software. A contig was constructed according to the fine-mapping results.

Sequence analysis of candidate genomic region

Gene prediction was performed using FGENESH (Salamov and Solovyev 2000), and intron/exon structures were verified by RT-PCR. The genomic DNA fragments of candidate genes from mutants and corresponding wild-type plants were amplified and sequenced. The sequencing reaction was performed by Shanghai Sangon Inc. (Shanghai, China).

RT-PCR

Total RNA was extracted from seedling leaves of wild-type plants (Zhonghua 11) and different tissues of wild-type plants at the heading stage, using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). For semi-quantitative RT-PCR analysis, total RNA (2 μg) was treated with RNase-free DNase, and first-strand cDNA was synthesized through reverse transcription by an oligo (dT) primer (TaKaRa). Subsequently, the first-strand cDNA was used as a template for semi-quantitative PCR analysis after being normalized with a rice *Actin* gene (*Act*). Amplification of the rice *Act* gene was performed with the forward primer 5′-GGAACTGGTATGGTCAAG GC-3′ and the reverse primer 5′-AGTCTCATGGATA CCCGCAG-3′. The PCR reaction for *RL9* was performed by using the specific primer r49, which defines a 341-bp fragment of the *RL9* cDNA. The PCR procedure is as follows: 94°C for 4 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, finishing with an elongation step at 72°C for 5 min. The PCR products were analyzed on 2% agarose gels.

In the quantitative RT-PCR experiments, total RNA was extracted from mature leaf tissue of the wild-type *rl9-1* and *rl9-2*. A total of 1 μg of total RNA was used for cDNA synthesis with an oligo (dT) primer and PrimeScript RT Enzyme (TaKaRa). The reaction mixture was diluted to 50 μl, whereupon 1 μl was used for real-time PCR reaction. The primers r69f/r69r for *RL9* were 5′-ATTCTT GCAACATGGACGCC-3′ and 5′-CATTAGCCTCTGTGA TTGCC-3′, which were designed to cross an intron in order to eliminate the possibility of genomic DNA amplification. Formation of the expected PCR product was confirmed by agarose gel electrophoresis (2%). We used *Act* as an internal control, by using the following primers: 5′-CTTCA TAGGAATGGAAGCTGCGGGTA-3′ and 5′-CGACCACC TTGATCTTCATGCTGCTA-3′. Amplification reactions were prepared with the SYBR PrimeScript RT-PCR Kit (TaKaRa), according to the manufacturer's instructions, with 0.4 μM of primers and 1 μl of cDNA per reaction. Each reaction was performed in triplicate. The threshold cycle (Ct) was determined by using the maximum-second-derivative function of the software. Error bars represent the standard error calculated on experiment repetitions.

Identification of the *RL9* coding sequence (CDS)

The CDS of *RL9* was determined by RT-PCR, which was performed using gene-specific primers in a total volume of 20 μl, including 1.0 μl of the cDNA, 2 μl (0.25 μM) gene-specific primers, 3.2 μl (2.5 μM) dNTP, 10 μl 2 × GC I buffer, 0.1 μl LA Taq DNA Polymerase (Takara), and 3.7 μl ddH_2O. PCR reactions were performed by denaturation at 94°C for 5 min, followed by 32 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. PCR products were separated on 1% agarose gel and stained with ethidium bromide. Four overlapping *RL9* transcript fragments, covering the entire coding sequence, were amplified after three rounds of PCR: in the first round, primers r62, r67, r49, and r59 were used on the first-strand cDNA; in the second round, r62f67r and r49f59r were used on the products of round I; and finally, in the third round, r62f59r was used on the products of round II (Fig. 1). PCR products were sequenced after ligation with pMD18-T

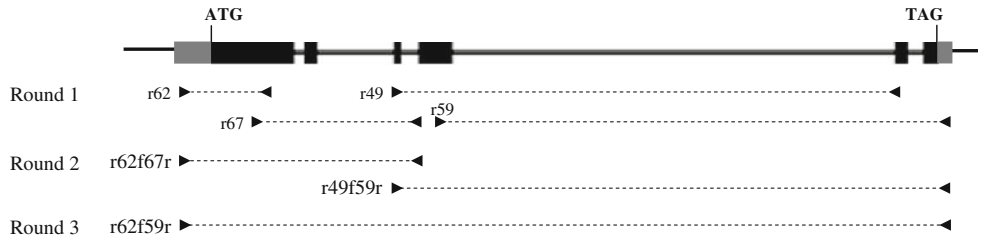

Fig. 1 Flow chart of identification of *RL9* coding sequences. Coding sequences were amplified after three rounds of PCR, as follows: first round, primers r62, r67, r49, and r59 were used on the first-strand cDNA; in the second round, r62f67r and r49f59r were used on the products of round I; and finally, in the third round, r62f59r was used on the products of round II

Simple vector (Takara). An in-frame stop codon was present at the end of the obtained cDNA, indicating that the CDS was complete.

Complementation test

First, two primers—RLEcoF, 5′-GCCGAATTCTACATC TACGACACCTTG-3′ and RLXbaR, 5′-GCCTCTAGAA GTCTTCACCTTTTCTCT-3′—were used to amplify a 550-bp DNA fragment from –6644 to –5987 upstream of the start code containing one *Apa* I clone site (Fig. 4c). Then, the amplified fragment was inserted into the binary vector pCAMBIA1300, after digesting with *Eco*R I and *Xba* I to generate an intermediate vector pCF. At the same time, a 15,366-bp DNA fragment containing a full-length genomic *RL9* gene was obtained by digesting the BAC clone, OSJNBa0083J10 (provided by National Center for Gene Research, CAS), by *Apa* I and *Xba* I. The digested fragment was then subcloned into plasmid pCF to form pCFRL, which was introduced into the *rl9-1* mutant for complementation testing by an *Agrobacterium tumefaciens*-mediated transformation method.

Phylogenetic analysis

Amino acid sequences of *RL9* homologues were obtained through a search of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). For the GARP superfamily phylogenetic analysis, 10 KAN amino acid sequences from *Physcomitrella patens subsp. patens*, *Arabidosis*, *Zea mays*, *Ipomoea nil*, and *Zinnia elegans*; AtPHR1; AtAPL, AtMYR1 from *Arabidosis*; Psr1 from *Chlamydomonas reinhardtii*; eight type-B ARRs from *Arabidosis*; two type-B ARRs from *Oryza sativa*; six *GLK*s; and the *RL9* were used. The alignment of amino acid sequences was carried out by using ClustalX 2.0 (Jeanmougin et al. 1998), and a neighbor-joining tree was generated by using MEGA 4.0 software (Tamura et al. 2007). The accession number of each amino acid sequence is shown in Supplemental Table 2.

Cellular localization assays with green fluorescent protein (GFP) fusion proteins

The green fluorescent protein (GFP) expression vector pJIT163-hGFP (gift of Prof. Zhukuang Cheng, IGDB, Beijing, China) is a recombinant derivative of pJIT163/hGFP. Two primers—RGHindf, 5′-TGAAGCTTGCTGATGGCGC CGATGATGC-3′ and RGBamr, 5′-CAGGATCCATCAT GATCTGCACCGTGCCAGTC-3′—were used to amplify the 1.1-kb full-length coding sequence of *RL9* not containing the stop codon from the aforementioned r62f59r PCR products. *RL9*–GFP fusion was then performed by in-frame fusion of the 1.1-kb sequence with pJIT163-hGFP. The *RL9*–GFP fusion and GFP alone (as a control) were introduced into onion skin epidermal cells with the Bio-Rad PDS-1000/He device (Bio-Rad, Hercules, CA, USA). After bombardment, tissues were incubated for 14 h at 25°C in the dark. Images were captured under the Olympus BX61 fluorescence microscope conjunct with a microCCD camera. Grayscale images were captured for each color channel and then merged using the software of IPlab.

Results

Characterization of the two *rl9* mutants

The leaves of both mutants rolled inward slightly at the seedling stage, and very much like a cylinder at the mature stage. The characteristics of the spikelets of the two mutants were distinct from those of the wild-type plants, with malformed spikelets resulting in low seed sets (Fig. 2a–c); furthermore, the *rl9-2* spikelets were almost complete sterile. The heading date of *rl9-2* was about 12 days later than that of the wild-type counterpart. In addition, previous studies into the morphology of leaves showed that in the *rl9-1* mutant, the mesophyll cells covered the vascular bundles on the abaxial side of the leaf and were filled with sclerenchyma cells in wild-type (Yan et al. 2006). Further studies with transmission electron microscopic (TEM) observation revealed that the arrangement of

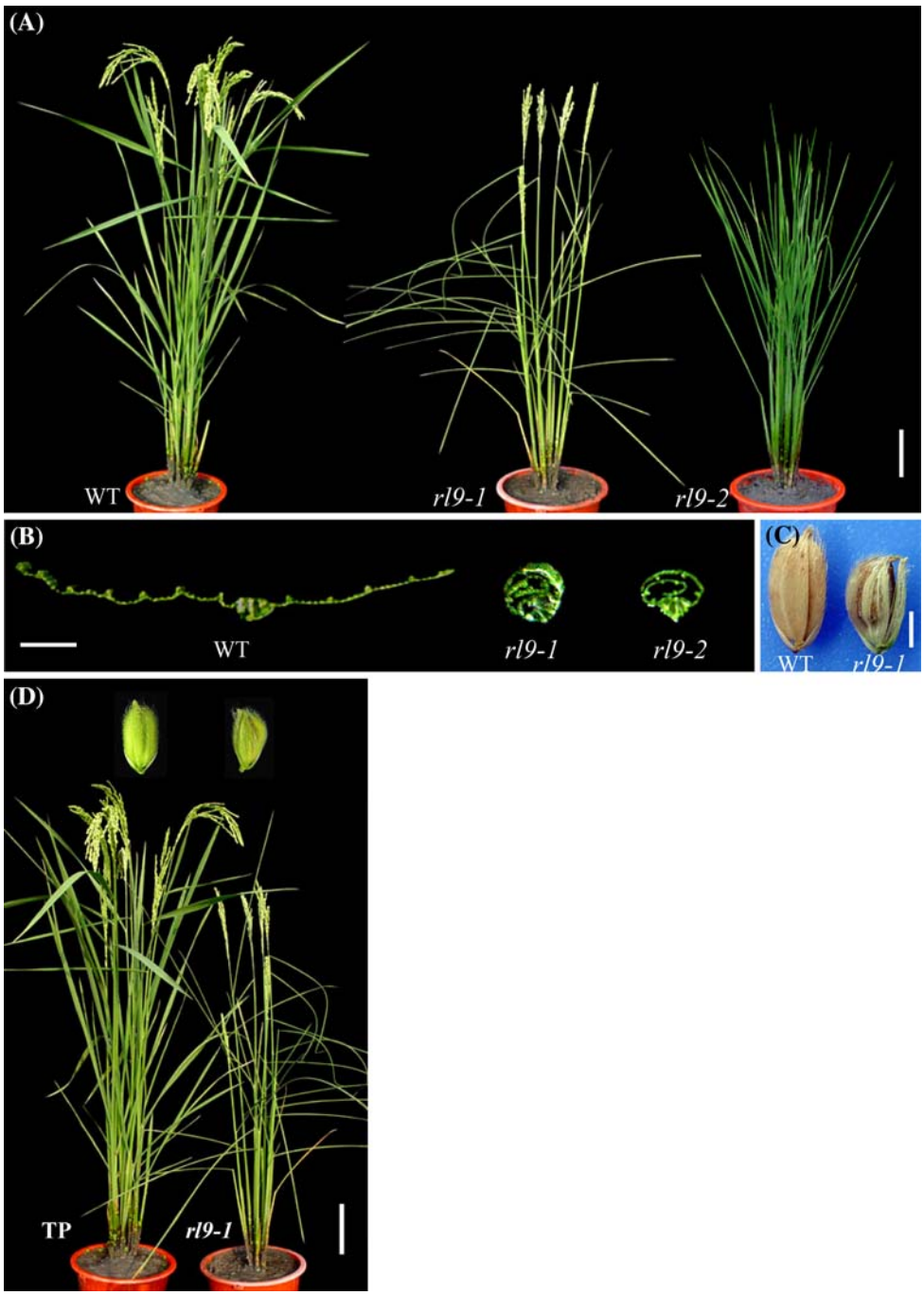

Fig. 2 Phenotypes of wild-type and *rl9* mutants. (**a**) Wild-type, *rl9-1*, and *rl9-2* plants. Bar = 10 cm. (**b**) Cross-sections of wild-type, *rl9-1*and *rl9-2* mature leaves. Bar = 2 mm. (**c**) Seeds of wild-type and *rl9-1* mutant. Bar = 2 mm. (**d**) Transgenic plant on the left; *rl9-1* mutant on the right, as the control. Bar = 10 cm

chloroplast grana lamellae were disordered and irregular in *rl9-1*mutant leaves (Fig. 3b, d), whereas the grana lamellae were parallel to the long axis of the chloroplast in wild-type leaves (Fig. 3a, c).

Cloning of the *RL9* gene

The *RL9* gene was previously mapped onto a 42-kb region of chromosome 9 (Fig. 4a, b). In this study, by using 1267 F_3 *rl9-1* mutant plants, the gene was further narrowed down to a 22-kb genomic region between CAPS marker c42 and polymorphic insertion/deletion marker c28, and co-segregated with c26 on AP005904. Within the 22-kb region, there was only one annotated gene located (Fig. 4c). The identity of this annotated gene as *RL9* was initially confirmed by the sequencing results with a 5-bp deletion in exon 1, which occurred in *rl9-1*, and a substitution of A (wild-type) with G at

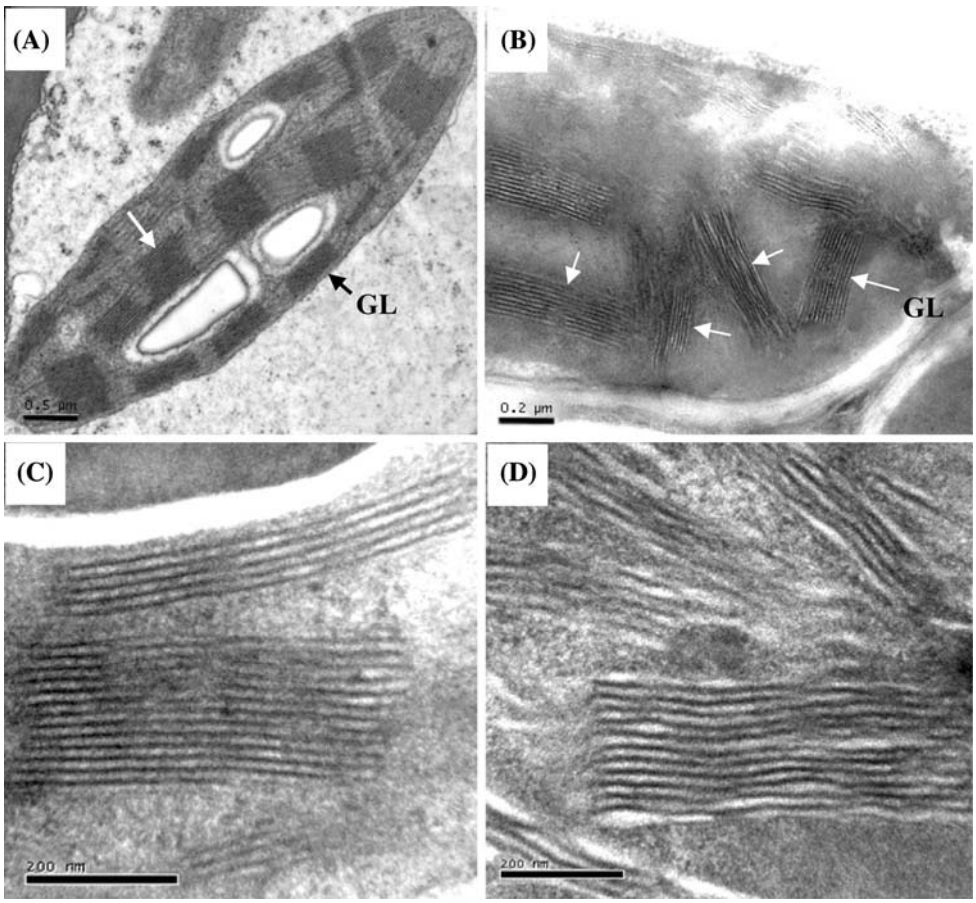

Fig. 3 Transmission electron microscope analysis of wild-type and *rl9-1*mutant. (**a**) A chloroplast of wild-type. (**b**) A part of one chloroplast of *rl9-1* mutant. (**c**) Chloroplast grana lamellae of wild-type. (**d**) Chloroplast grana lamellae of *rl9-1* mutant. GL, grana lamellae

the splice site of intron 1/exon 2 occurred in *rl9-2* (Fig. 4d).

The candidate gene was further confirmed by a complementation test. A 15.5-kb wild-type genomic DNA fragment harboring the entire candidate gene was cloned into pCAMBIA1300 and introduced into *rl9-1* mutant embryogenic callus via *Agrobacterium tumefaciens*-mediated transformation. Five independent transgenic lines were obtained, and they showed a complementation of the *rl9-1* phenotype (Fig. 2d). The ratio of wild-type-looking and mutant plants in the T_1 generation fitted 3:1 (Supplemental Table 3). Moreover, the *Hygromycin Phosphotransferase* (*HPT*) gene was detected in all wild-type-looking plants, but not in mutant plants, by PCR.

Identification of the *RL9* CDS

The identification of CDSs is an important step in the functional analysis of genes. Unfortunately, we found no available EST/cDNA in the database that supported any predicted gene model for *RL9*. In addition, we searched recently published rice whole-genome transcription profiling microarray data, and found 13 probe sets that also could not cover any predicted gene model. We therefore redefined the *RL9* CDS by means of PCR on the first strand cDNA of wild-type seedling leaves; however, the predicted *RL9* gene structures differ in the TIGR database, the rice genome annotation database, and the FGENESH software, among others. Primers for RT-PCR were designed according to the CDS predicted by FGENESH, which was the longest of all predicted gene models. Due to the 3′ end structures being the same in all predicted gene models—which were validated by RT-PCR results—we tried a 5′ RACE (rapid amplification of cDNA ends) only, but failed for the GC-rich template of the 5′ end region (~75%). Therefore, integration reactions were performed by three rounds of RT-PCR, to achieve the full-length CDS of *RL9* (Fig. 1). The last integrated sequence was submitted to align with a genome DNA sequence with the Basic Local Alignment Search Tool (BLAST), to determine the introns; the reasonable genomic DNA sequence was selected for further gene structure prediction by FGENESH. As a result, we concluded that the *RL9* gene consists of six exons and five

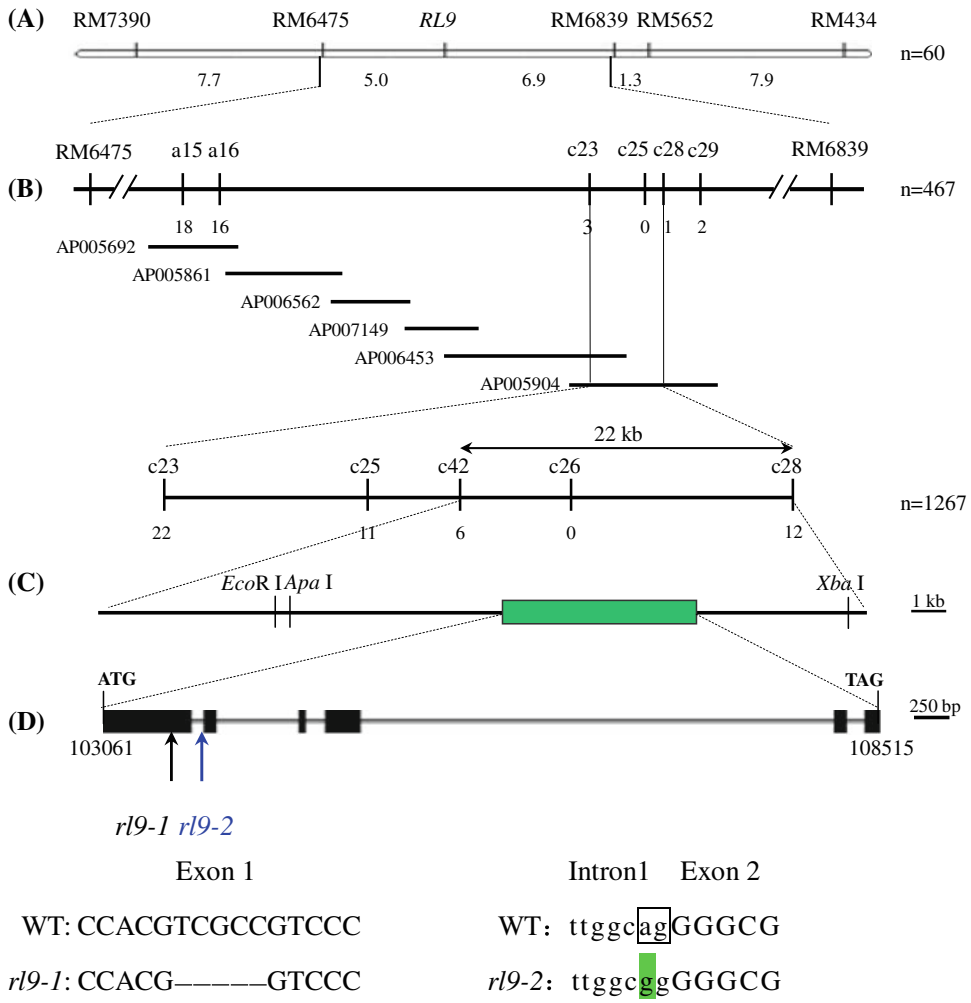

Fig. 4 Physical map of the *RL9* locus and mutation sites in two allelic mutants. (**a**) Linkage map of the gene *RL9* on chromosome 9. Vertical lines represent the positions of molecular markers; genetic distances (centimorgan, cM) between adjacent markers are shown below the linkage map. (**b**) Fine-mapping of the *RL9* locus. The numbers beside the physical map indicate the number of recombinants identified from F_2 and F_3 plants. The physical map of the *RL9* locus was constructed using six PAC clones; the *RL9* locus was further narrowed to a 22-kb genome region between markers c42 and c28, and it was co-segregated with marker c26 based on 1,267 F_3 recessive plants. (**c**) Only one candidate gene existed within the 22-kb restricted region. Three related enzymes used for functional complementation plasmid construction are marked. (**d**) The *RL9* gene structure and mutation positions in two mutants. Black boxes indicate exons. The *RL9* gene consists of six exons and five introns. The mutated DNA sequences of *rl9-1* and *rl9-2* are shown at the bottom. *rl9-1* has a five-base deletion in exon 1. *rl9-2* has a base substitution at the splicing site (framed), between intron 1 and exon 2. Numbers at right of the map indicate the size of the progeny mapping population

introns—a 1,134-bp CDS, encoding 377 amino acids (Fig. 4d and Supplemental Fig. 1).

RL9 encodes a GARP protein

A BLAST search with the RL9 protein and the predicated protein sequence revealed that the RL9 protein contains a 58-amino-acid domain (residues 167–224) that shares 50–100% amino-acid identity with more than 100 predicted genes in the rice genome, including many putative transcription factors. This domain has been named GARP (Riechmann et al. 2000) (Supplemental Fig. 1). However, the overall identity among these proteins is less than 33.5%.

RL9 gene is an orthologue of *Arobidopsis KANADI*

Orthologues of *RL9* were found in the NCBI database, from *Ipomoea nil*, *Arobidopsis*, and *Zea mays*. RL9 has 31.6–40.8% amino acid sequence identity with the three KANADI proteins, which all matched very well within the GARP domain. Furthermore, the C-terminal region is conserved among RL9 and these KAN proteins (Fig. 6). Based on the role of KANs in *Arabidopsis*, we deduce that RL9 may also function in regulating abaxial identity during leaf development in rice, and the loss of function of *RL9* is consistent with the *rl9* mutant phenotypes.

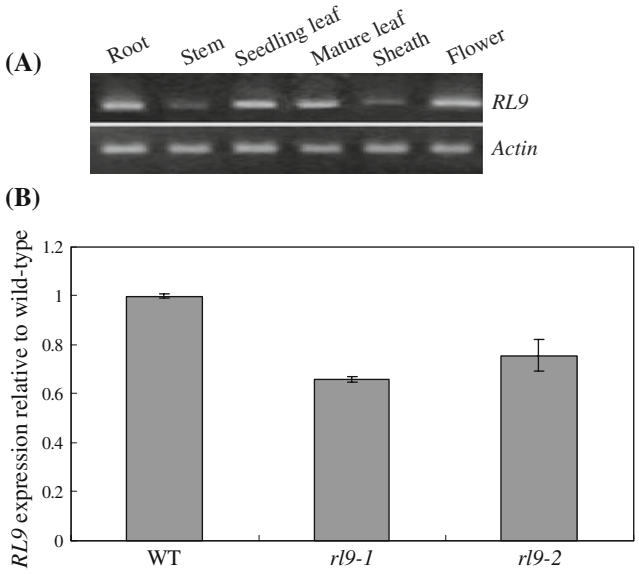

Fig. 5 *RL9* expression. (**a**) Semi-quantitative RT-PCR of *RL9* in organs, including root, stem, seedling leaf, mature leaf, sheath, and flower; the transcript of *Actin* was used as a control. (**b**) Real-time quantitative PCR analysis in the mature leaves of wild-type (WT), and *rl9-1* and *rl9-2* mutants. The expression levels were normalized to *Actin* transcript levels and are shown relative to wild-type levels. Error bars represent standard deviations

Phylogenetic analysis of RL9

To determine the evolutionary relationship between RL9 and GARP super-family members from the *Arabidopsis, Zea mays*, *Oryza sativa*, *Chlamydomonas reinhardtii*, and other representative identified GARP members, an unrooted phylogenetic tree was built using the neighbor-joining method, based on full-length protein sequences (Fig. 7). The result indicates that all the GARP protein sequences are divided into four subfamilies: subA [RL9, (*Physcomitrella patens*) PpKAN1, PpKAN2, PpKAN3, (*Zea mays*) ZmKAN, (*Ipomoea nil*) InKAN, (*Zinnia elegans*) ZeKAN, (*Arabidopsis thaliana*) AtKAN1, AtKAN2, AtKAN3, and AtKAN4], subB [(*Chlamydomonas reinhardtii*) CrPsr1, AtPHR1, AtAPL, and AtMYR1], subC [AtARR1, AtARR2, AtARR10, AtARR11, AtARR12, AtARR14, AtARR18, AtAPRR4, (*Oryza sativa*) OsARR10, and OsARR14], and subD (AtGLK1, AtGLK2, ZmGLK1, Zm GLK2, OsGLK1, and OsGLK2).

RL9 is classified into subA; with 40.8% identity, it is the most homologous to maize KAN1 (ZmKAN1). The next most homologous protein of the 10 KAN proteins is At-KAN1, which is 32.7% identical to RL9. Of the 11 members of subA, three KANs (AtKAN1-3) in *Arabidopsis*

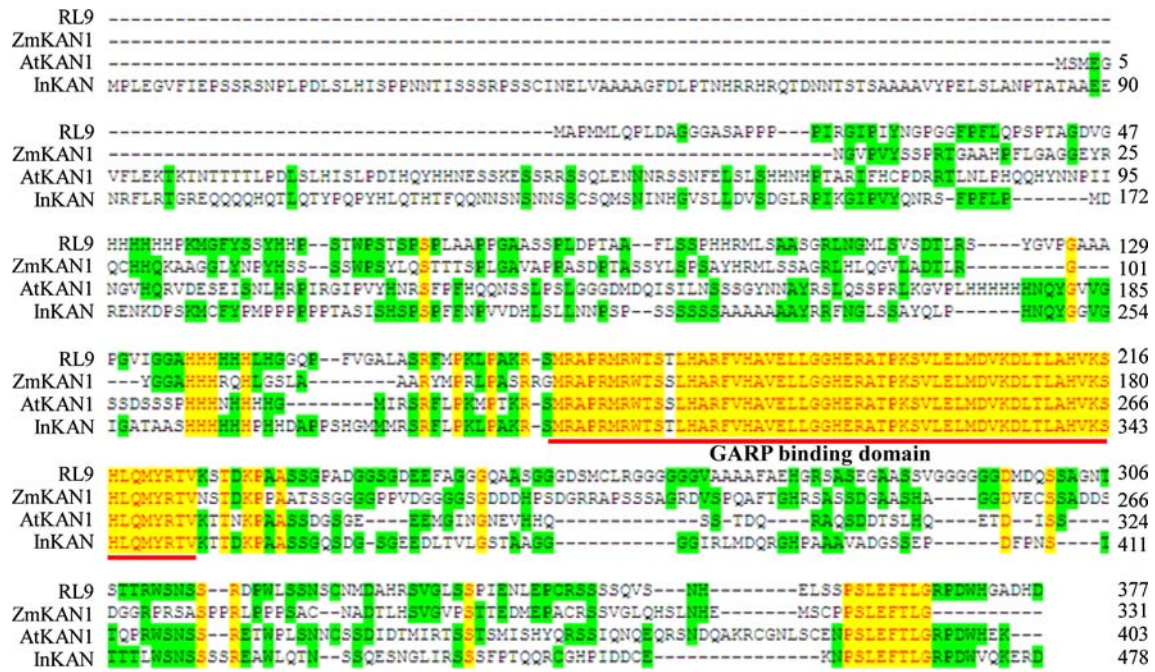

Fig. 6 Alignment of the *RL9* with KAN proteins from *Zea mays* (ZmKAN1, accession number ABB89932), *Arobidopsis thalina* (AtKAN1, accession number NP_568334), and *Ipomoea nil* (InKAN, accession number BAE73188). The sequences were aligned by the AlignX program of Vector NTI 9.0 software. Dashes indicate gaps introduced for maximal alignment. Yellow-on-red letters represent identical amino-acids. Green-on-black letters represent conservative amino-acids

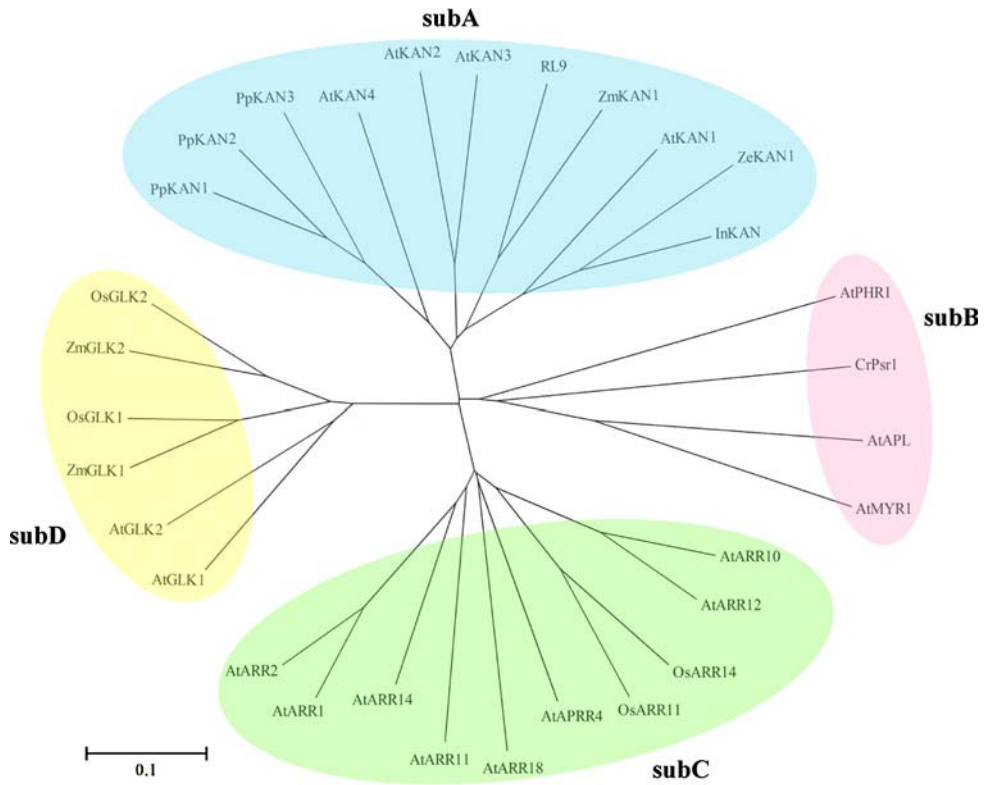

Fig. 7 Phylogenic analysis of *RL9*. An unrooted phylogenetic tree was generated with the full-length amino-acid sequences of *RL9* and GARP genes, from *Arabidopsis* and other eukaryotes. The subA family includes *Oryza sativa RL9*, *Physcomitrella patens* PpKAN1, PpKAN2, PpKAN3, *Zea mays* ZmKAN, *Ipomoea nil* InKAN, *Zinnia elegans* ZeKAN, *Arabidopsis thaliana* AtKAN1, AtKAN2, AtKAN3, and AtKAN4. The subB family includes *Chlamydomonas reinhardtii* CrPsr1, AtPHR1, AtAPL, and At MYR1. The subC family includes AtARR1, AtARR2, AtARR10, AtARR11, AtARR12, AtARR14, AtARR18, AtAPRR4, *Oryza sativa* OsARR10, and OsARR14. The subD family includes AtGLK1, AtGLK2, ZmGLK1, Zm GLK2, OsGLK1, and OsGLK2. The bar represents genetic distance in the phylogenetic tree. The accession numbers of each protein are provided in Table 2

were reported as having a function in regulating abaxial identity during leaf development (Eshed et al. 2001, 2004), and AtKAN4 has a role in the specification of polarity in *Arabidopsis* ovule integuments (McAbee et al. 2006). The other KAN members in subA have not been characterized yet. SubB includes four members; of those, *Chlamydomonas reinhardtii Psr1* regulates phosphorus metabolism in vascular plants (Wykoff et al. 1999) and *AtPHR1* has a role in repairing ultraviolet (UV)-damaged DNA (Sakamoto et al. 1998). The functions of the other two members, AtAPL and AtMYR1, remain to be elucidated. SubC consists of 10 type-B ARR proteins that are involved in His-to-Asp phospho-relay signal transduction systems in *Arabidopsis*, and function as a transcriptional activator for a type-A ARR, presumably in AHK-mediated cytokinin signaling (Hwang and Sheen 2001; Sakai et al. 2001). SubD comprises six *GLK* members from *Arabidopsis*, maize, and rice. *GLK* genes regulate chloroplast development in these plant species (Fitter et al. 2002).

It is therefore suggested that, with the exception of subB, GARP proteins in each subfamily are involved in similar processes within a plant. In this report, both of the *rl9* mutations cause complete adaxialization of leaves; as *RL9* belongs to subA, this indicates that *RL9* has a similar function with *AtKANs* in specifying the abaxial fate of leaves.

RL9 expression pattern

To study the expression pattern of *RL9*, a semi-quantitative RT-PCR was performed with wild-type seedling leaves and different tissues in the heading stage. The results showed that *RL9* is expressed in all organs of wild-type plants; is higher in roots, young leaves, mature leaves, and flowers; and is lower in stems and leaf sheaths (Fig. 5a). It is apparent that the expression of *RL9* is correlated with the rolled-leaf trait, and the high level of *RL9* expression in the flowers can explain the phenotype of abnormal seeds in the two *rl9* mutants examined.

We further compared the levels of gene expression in the mature leaves of two mutants and wild-type Zhonghua 11, using quantitative real-time PCR. The *RL9* transcripts of the two mutants were downregulated, but not

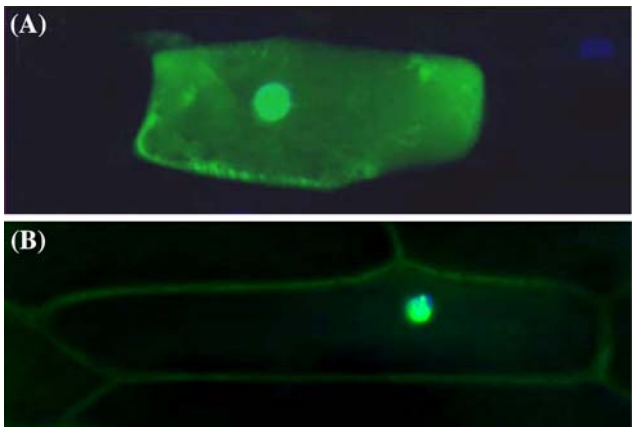

Fig. 8 Subcellular location of *RL9* protein. (**a**) GFP alone was introduced as a control for transient bombardment assays. (**b**) Nuclear localization of the *RL9*–GFP fusion protein

significantly (Fig. 5b); this indicates that mutations in the coding sequence and the 3′-splicing site of the first intron of *RL9* led to a slightly decreased level of *rl9* mRNA.

RL9 is a nuclear protein

Previous studies indicated that the *Arabidopsis* KAN1 protein and GARP domain-containing proteins are localized in the nucleus (Kerstetter et al. 2001; Hosoda et al. 2002). We hypothesize that RL9 is a transcription factor in rice, for it contains a GARP domain that may possess a nuclear localization signal. In order to determine whether *RL9* encodes a nuclear protein, a full-length *RL9* CDS was introduced into an expression vector pJIT163-hGFP. Vectors expressing the *RL9*–GFP fusion protein or GFP alone were introduced into onion epidermal cells by particle bombardment. In the case of GFP alone, fluorescence was observed in both the nuclei and cytoplasm, whereas in the case of *RL9*–GFP, fluorescence was localized solely in the nucleus (Fig. 8). This result suggests that *RL9* is a putative transcription factor.

Discussion

In this study, we cloned the *RL9* gene by way of a map-based cloning strategy, using single-recessive mutants exhibiting rolled leaves. Nucleotide sequencing results revealed that *rl9-1* had a 5-bp deletion in exon 1 and *rl9-2* had a single nucleotide substitution at the splice site of intron 1/exon 2—both of which resulted in the failure to produce functional *RL9* (Fig. 4d). The complementation of the *rl9-1* phenotype indicated that the rolled-leaf mutant is due to the 5-bp deletion of the *RL9* gene, and the results of a semi-quantitative RT-PCR were consistent with the phenotype of the two *rl9* mutants.

Our previous studies found a cause of unambiguous adaxial to abaxial transformations, that the *rl9-1* mutant produces mesophyll cells on the abaxial side of vascular bundles instead of sclerenchyma cells seen in the wild-type species (Yan et al. 2006). Further studies in this report demonstrated that the mutations in *RL9* not only affect the biosynthesis of sclerenchyma cells of the vascular bundles on the abaxial side of leaves, but also affect chloroplast development—especially the arrangement of chloroplast grana lamellae (Fig. 3).

Sequence analysis revealed that the *RL9* encodes a GARP DNA-binding protein. GARP was named after maize GOLDEN2, the ARR B-class proteins from *Arabidopsis*, and *Chlamydomonas* Psr1 (Riechmann et al. 2000). Phylogenetic and comparative genetic analyses indicated that the *RL9* gene is an orthologue of *AtKANs*. In the phylogenetic tree, the most homologous gene to *RL9* is *ZmKAN1* (40.8% identity, at the protein level), which is publicized in the NCBI database but not yet characterized. The next most homologous gene to *RL9* is *AtKAN1* (32.7% identity, at the protein level) (Figs. 6 and 7). In addition, the collected GARP proteins are divided into four subfamilies; members of each subfamily, except those of subB, appear to be involved in similar processes within different plant species. The fact that *RL9* belongs to subA indicates that the *RL9* gene may function in a fashion similar to *KANs*.

In *Arabidopsis*, members of the *KAN* family of GARP transcriptional regulators have been found to play essential roles in the specification of abaxial fate in leaf development. A combined loss of function mutations in *KAN* family members leads to progressive loss of abaxial identity. Either *kan1* or *kan2* mutants alone have very limited or no morphological alterations, respectively, and double *kan1kan2* mutants have dramatically reduced leaf expansion and form ectopic leaf-blade outgrowths on the abaxial leaf surface. Triple *kan1kan2kan3* mutants further reduce blade expansion and display more complete adaxialization (Eshed et al. 1999, 2001, 2004; Kerstetter et al. 2001; Emery et al. 2003). However, in the present study, we found that the loss of function of RL9 in the two mutants results in not only the rolled-leaf phenotype, but also malformed spikelets, suggesting that RL9 functions similar to *Arabidopsis* KANs, with some differences. Single *rl9* mutant leaves display complete adaxialization in rice, whereas triple mutants do in *Arabidopsis*, indicating that the *KAN* gene may exercise greater effects in rice than in dicots. However, it was found that, besides the role of *KAN* genes in specifying the abaxial fate in *Arabidopsis*, they also have some roles in ovule development (Eshed et al. 2001)—especially the *Arabidopsis aberrant testa shape* (*ats*) mutant, which produces a single integument instead of the two integuments seen in wild-type ovules; *ATS*

(referred to as *KAN4*) encoding a member of the KAN family has a limited expression pattern in the ovules (McAbee et al. 2006). It is suggested that the seeds of *rl9* mutants displaying aberrant hulls can also be explained by the loss of function in KAN.

In *Arabidopsis* asymmetric leaf development, KAN proteins have a mutually antagonistic relationship with HD-ZIP III proteins (Emery et al. 2003), and *KAN* genes are necessary for *YABBY* (*YAB*) expression (Eshed et al. 2004). However, no *HD-ZP III* genes have yet been characterized in rice, and four characterized *YAB* genes from rice, i.e. *DROOPING LEAF*, *YAB1*, *YAB3*, and *OsYAB4*, have been proved not to determine the abaxial cell fate, unlike their functions in dicot *Arabidopsis* (Jang et al. 2004; Yamaguchi et al. 2004; Dai et al. 2007a, b; Liu et al. 2007). The relationships among *HD-ZP III*, *YAB* genes, and *RL9* in rice leaf development remain to be elucidated. The availability of knowledge vis-à-vis *RL9* will facilitate an understanding of the mechanism of rice leaf polarity formation.

Acknowledgments We are grateful to Dr. Liu Qiaoquan (Agricultural College of Yangzhou University) for kindly providing the *rl9*-2 mutant. This research was supported in part by grants from the Ministry of Science and Technology of China (No. 2005CB120804), the Jiangsu Innovation Talent Project (No. BK2006506) and the Postgraduate Science Innovation Plan of Jiangsu Province.

References

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) MicroRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. Cell 121:207–221. doi:10.1016/j.cell.2005.04.004

Bao N, Lye KW, Barton MK (2004) MicroRNA binding sites in *Arabidopsis* Class III HD-ZIP mRNAs are required for methylation of the template chromosome. Dev Cell 7:653–662. doi:10.1016/j.devcel.2004.10.003

Byrne ME, Barley R, Curtis M, Arroyo JM, Dunham M, Hudson A et al (2000) *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. Nature 408:967–971. doi:10.1038/35050091

Chen ZX, Pan XB, Hu J (2001) Relationship between rolled leaf and ideal plant type of rice (*Oryza sativa* L.). J Jiangsu Agric Res 22:88–91

Chen ZX, Chen G, Pan XB (2002) Genetic expression and effects of rolled leaf gene *RL(t)* in hybrid rice (*Oryza sativa* L.). Acta Agronomica Sin 6:847–851

Dai MQ, Hu YF, Zhao Y, Liu HF, Zhou DX (2007a) A WUSCHEL-LIKE HOMEOBOX gene represse a YABBY gene expression required for rice leaf development. Plant Physiol 144:380–390. doi:10.1104/pp.107.095737

Dai MQ, Zhao Y, Ma Q, Hu YF, Hedden P, Zhang QF (2007b) The rice *YABBY1* gene is involved in the feedback regulation of Gibberellin metabolism. Plant Physiol 144:121–133. doi:10.1104/pp.107.096586

Emery JF, Floyd SK, Alvarez J, Eshed Y, Hawker NP, Izhaki A et al (2003) Radial patterning of *Arabidopsis* shoots by class III HD-ZIP and *KANADI* genes. Curr Biol 13:1768–1774. doi:10.1016/j.cub.2003.09.035

Eshed Y, Baum SF, Bowman JL (1999) Distinct mechanisms promote polarity establishment in carpels of *Arabidopsis*. Cell 99:199–209. doi:10.1016/S0092-8674(00)81651-7

Eshed Y, Baum SF, Perea JV, Bowman JL (2001) Establishment of polarity in lateral organs of plants. Curr Biol 11:1251–1260. doi:10.1016/S0960-9822(01)00392-X

Eshed Y, Izhaki A, Baum SF, Floyd SK, Bowman JL (2004) Asymmetric leaf development and blade expansion in *Arabidopsis* are mediated by KANADI and YABBY activities. Development 131:2997–3006. doi:10.1242/dev.01186

Fitter DW, Martin DJ, Copley MJ, Scotland RW, Langdale JA (2002) *GLK* gene pairs regulate chloroplast development in diverse plant species. Plant J 31:713–727. doi:10.1046/j.1365-313X.2002.01390.x

Hawker NP, Bowman JL (2004) Roles for Class III *HD-Zip* and *KANADI* genes in *Arabidopsis* root development. Plant Physiol 135:1–10. doi:10.1104/pp.104.040196

Hosoda K, Imamura A, Katoh E, Hatta T, Tachiki M, Yamada H et al (2002) Molecular structure of the GARP family of plant Myb-related DNA binding motifs of the *Arabidopsis* response regulators. Plant Cell 14:2015–2029. doi:10.1105/tpc.002733

Huang W, Pi L, Liang W, Xu B, Wang H, Cai R et al (2006) The proteolytic function of the *Arabidopsis* 26S proteasome is required for specifying leaf adaxial identity. Plant Cell 18:2479–2492. doi:10.1105/tpc.106.045013

Hwang I, Sheen J (2001) Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature 413:383–389. doi:10.1038/35096500

Iwakawa H, Ueno Y, Semiarti E, Onouchi H, Kojima S, Tsukaya H et al (2002) The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana*, required for formation of a symmetric flat leaf lamina, encodes a member of a novel family of proteins characterized by cysteine repeats and a leucine zipper. Plant Cell Physiol 43:5467–5478. doi:10.1093/pcp/pcf077

Jang S, Hur J, Kim SJ, Han MJ, Kim SR, An G (2004) Ectopic expression of *OsYAB1* causes extra stamens and carpels in rice. Plant Mol Biol 56:133–143. doi:10.1007/s11103-004-2648-y

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trends Biochem Sci 23:403–405. doi:10.1016/S0968-0004(98)01285-7

Juarez MT, Kui JS, Thomas J, Heller BA, Timmermans MC (2004a) MicroRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. Nature 428:84–88. doi:10.1038/nature02363

Juarez MT, Twigg RW, Timmermans MC (2004b) Specification of adaxial cell fate during maize leaf development. Development 131:4533–4544. doi:10.1242/dev.01328

Kerstetter RA, Bollman K, Taylor RA, Bomblies K, Poethig RS (2001) *KANADI* regulates organ polarity in *Arabidopsis*. Nature 411:706–709. doi:10.1038/35079629

Kidner CA, Timmermans MCP (2007) Mixing and matching pathways in leaf polarity. Curr Opin Plant Biol 10:13–20. doi:10.1016/j.pbi.2006.11.013

Lang YZ, Zhang ZJ, Gu XY, Yang JC, Zhu QS (2004a) Physiological and ecological effects of crimpy leaf character in rice (*Oryza sativa* L.) I Leaf orientation, canopy structure and light distribution. Acta Agronomica Sin 30:806–810

Lang YZ, Zhang ZJ, Gu XY, Yang JC, Zhu QS (2004b) Physiological and ecological effects of crimpy leaf character in rice (*Oryza sativa* L.) II Photosynthetic character, dry mass production and yield forming. Acta Agronomica Sin 30:883–887

Lin WC, Shuai B, Springer PS (2003) The *Arabidopsis LATERAL ORGAN BOUNDARIES*-domain gene *ASYMMETRIC LEAVES2* functions in the repression of *KNOX* gene expression and in adaxial–abaxial patterning. Plant Cell 15:2241–2252. doi:10.1105/tpc.014969

Liu HL, Xu YY, Xu ZH, Chong K (2007) A rice YABBY gene, *OsYABBY4*, preferentially expresses in developing vascular tissue. Dev Genes Evol 217:629–637. doi:10.1007/s00427-007-0173-0

McAbee JM, Hill TA, Skinner DJ, Izhaki A, Hauser BA, Meister RJ et al (2006) *ABERRANT TESTA SHAPE* encodes a KANADI family member, linking polarity determination to separation and growth of *Arabidopsis* ovule integuments. Plant J 46:522–531. doi:10.1111/j.1365-313X.2006.02717.x

McConnell JR, Barton MK (1998) Leaf polarity and meristem formation in *Arabidopsis*. Development 125:2935–2942

McConnell JR, Emery J, Eshed Y, Bao N, Bowman J, Barton MK (2001) Role of *PHABULOSA* and *PHAVOLUTA* in determining radial patterning in shoots. Nature 411:709–713. doi:10.1038/35079635

Pekker I, Alvarez JP, Eshed Y (2005) Auxin response factors mediate *Arabidopsis* organ asymmetry via modulation of KANADI activity. Plant Cell 17:2899–2910. doi:10.1105/tpc.105.034876

Prigge MJ, Otsuga D, Alonso JM, Ecker JR, Drews GN, Clark SE (2005) Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in *Arabidopsis* development. Plant Cell 17:61–76. doi:10.1105/tpc.104.026161

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. Genes Dev 16:1616–1626. doi:10.1101/gad.1004402

Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP (2002) Prediction of plant microRNA targets. Cell 110:513–520. doi:10.1016/S0092-8674(02)00863-2

Riechmann JL, Heard J, Martin G, Reuber L, Jiang CZ, Keddie J et al (2000) *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. Science 290:2105–2110. doi:10.1126/science.290.5499.2105

Sakai H, Honma T, Aoyama T, Sato S, Kato T, Tabata S et al (2001) ARR1, a transcription factor for genes immediately responsive to cytokinins. Science 294:1519–1521. doi:10.1126/science.1065201

Sakamoto A, Tanaka A, Watanabe H, Tano S (1998) Molecular cloning of *Arabidopsis* photolyase gene (*PHR1*) and characterization of its promoter region. DNA Seq 9:335–340. doi:10.3109/10425179809008473

Salamov AA, Solovyev VV (2000) *Ab initio* gene finding in *Drosophila* genomic DNA. Genome Res 10:516–522. doi:10.1101/gr.10.4.516

Sawa S, Watanabe K, Goto K, Liu YG, Shibata D, Kanaya E et al (1999) *FILAMENTOUS FLOWER*, a meristem and organ identity gene of *Arabidopsis*, encodes a protein with a zinc finger and HMG-related domains. Genes Dev 13:1079–1088. doi:10.1101/gad.13.9.1079

Shen FC (1983) Several opinions on how to use rolled leaf character of rice in breeding. Guizhou Agric Sci 5:6–8

Shi ZY, Wang J, Wan XS, Shen GZ, Wang XQ, Zhang JL (2007) Over-expression of rice *OsAGO7* gene induces upward curling of the leaf blade that enhanced erect-leaf habit. Planta 226:99–108. doi:10.1007/s00425-006-0472-0

Siegfried KR, Eshed Y, Baum SF, Otsuga D, Drews GN, Bowman JL (1999) Members of the *YABBY* gene family specify abaxial cell fate in *Arabidopsis*. Development 126:4117–4128

Sun Y, Zhou Q, Zhang W, Fu Y, Huang H (2002) *ASYMMETRIC LEAVES1*, an *Arabidopsis* gene that is involved in the control of cell differentiation in leaves. Planta 214:694–702. doi:10.1007/s004250100673

Sussex IM (1955) Morphogenesis in *Solanum tuberosum* L.: experimental investigation of leaf dorsiventrality and orientation in the juvenile shoot. Phytomorphology 5:286–300

Talbert PB, Adler HT, Parks DW, Comai L (1995) The *REVOLUTA* gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of *Arabidopsis thaliana*. Development 121:2723–2735

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599. doi:10.1093/molbev/msm092

Waites R, Hudson A (1995) *phantastica*: a gene required for dorsoventrality of leaves in *Antirrhinum majus*. Development 121:2143–2154

Williams L, Carles CC, Osmont KS, Fletcher JC (2005) A database analysis method identifies an endogenous *trans*-acting short-interfering RNA that targets the *Arabidopsis ARF2, ARF3*, and *ARF4* genes. Proc Natl Acad Sci USA 102:9703–9708. doi:10.1073/pnas.0504029102

Wykoff DD, Grossman AR, Weeks DP, Usuda H, Shimogawara K (1999) Psr1, a nuclear localized protein that regulates phosphorus metabolism in *Chlamydomonas*. Proc Natl Acad Sci USA 96:15336–15341. doi:10.1073/pnas.96.26.15336

Xu L, Xu Y, Dong A, Sun Y, Pi L, Xu Y et al (2003) Novel *as1* and *as2* defects in leaf adaxial–abaxial polarity reveal the requirement for *ASYMMETRIC LEAVES1* and *2* and *ERECTA* functions in specifying leaf adaxial identity. Development 130:4097–4107. doi:10.1242/dev.00622

Xu L, Yang L, Huang H (2007) Transcriptional, post-transcriptional and post-translational regulations of gene expression during leaf polarity formation. Cell Res 17:512–519. doi:10.1038/cr.2007.45

Yamaguchi T, Nagasawa N, Kawasaki S, Matsuoka M, Nagato Y, Hirano HY (2004) The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in *Oryza sativa*. Plant Cell 16:500–509. doi:10.1105/tpc.018044

Yan CJ, Yan S, Zhang ZQ, Liang GH, Lu JF, Gu MH (2006) Genetic analysis and gene fine mapping for a rice novel mutant ($rl9_{(t)}$) with rolling leaf character. Chin Sci Bull 51:163–169

Yan CJ, Zhou JH, Yan S, Chen F, Yeboah M, Tang SZ et al (2007) Identification and characterization of a major QTL responsible for erect panicle trait in *japonica* rice (*Oryza sativa* L.). Theor Appl Genet 115:1093–1100. doi:10.1007/s00122-007-0635-9

Yuan LP (1997) Super-high yield hybrid rice breeding. Hybrid Rice 12:1–6