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Genetic analysis and molecular mapping of a presenescing leaf gene *psl1* in rice (*Oryza sativa* L.)

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Abstract A rice *psl1* (presenescing leaf) mutant was obtained from a *japonica* variety Zhonghua 11 via radiation of ^{60}Co-γ in M2 generation. Every leaf of the mutant began to wither after it reached the biggest length, while the leaves of the wild variety could keep green for 25 — 35 d. In this study, genetic analysis and gene mapping were carried out for the mutant identified. The SSR marker analysis showed that the mutant was controlled by a single recessive gene (*psl1*) located on chromosome 2. Fine mapping of the *psl1* locus was conducted with 34 new STS markers developed around *psl1* anchored region based on the sequence diversity between Nipponbare and 93-11. The *psl1* was further mapped between two STS markers, STS2-19 and STS2-26, with genetic distances of 0.43 and 0.11 cM, respectively, while cosegregated with STS2-25. A BAC contig was found to span the *psl1* locus, the region being delimited to 48 kb. This result was very useful for cloning of the *psl1* gene.

Keywords: rice, simple sequence repeat (SSR) marker, sequence-tagged site (STS) marker, molecular mapping, BAC contig.

Crop biomass and economic yield are mainly resulting from photosynthesis, while the leaf is the most important organ where the photosynthesis takes place. The growth, development and senescence of crop leaf are tightly correlated to their yield. Leaf senescence is tightly controlled by the development to increase the fitness of the whole plant. But the early leaf senescence usually leads to a loss of the crop product, It is theoretically estimated that the grain product will increase by 2% if the leaf senescence is delayed by 1 d, while it will increase by 1% in practice in rice[1,2]. Crop yield will be increased and the storage life of some crop products will be prolonged if the leaf senescence is regulated. The investigation into the molecular mechanism of plant leaf senescence and the approaches to regulating the plant leaf senescence play a very important role in crop production.

Leaf senescence, the last stage of development that precedes death, is a genetically programmed process. Processes associated with leaf senescence include the disorganization of chlorplasts, shrinkage of cytoplasmic volume and decrease in cellular metabolic activities. Proteins are degraded to amino acids, RNA is broken down to low molecular weight nitrogen compounds, and membrane lipids are metabolized to sugar. The onset and progression of leaf senescence is accompanied by changes in expression of a large number of genes[1−6].

Biochemical and molecular studies over the last few decades have shown that senescence is an active process that requires the expression of novel genes and the synthesis of new proteins. Genetic studies on leaf senescence, in general, invoke two different approaches. (1) Genes with a role in leaf senescence can be identified by the isolation and characterization of mutants that are defective in some aspect of the senescence pathway. For example, the mutants that are insensitive to ethylene in *Arabidopsis* were used to investigate the synthesis pathway and signal transduction of ethylene and the roles it plays in the plant senescence. (2) Many research groups have used differential expression as a way of identifying senescence-enhanced genes via differential display, suppression subtractive hybridization and gene chip. Many senescence associated genes (often called SAGs or more accurately, SEN genes (senescence enhanced)) have been identified in *Arabidopsis*, rape, barly and corn[3,5−17].

Rice (*Oryza sativa*) is the staple food for more than half of the world's population and has emerged as a model plant for cereal genomics particularly because of its compact genome (389 Mb), the smallest among graminaceous crops, and the availability of vast genetic and molecular resources. The scientific value of rice is further enhanced with the elucidation of the genome sequence of the two major subspecies of cultivated rice, *Oryza sativa* ssp. *japonica* and ssp. *indica*. The sequence of the *japonica* cultivar Nipponbare was recently completed by a consortium of 10 countries. Moreover, many advances have been made in public

databases currently containing 386487 rice ESTs (dbEST release 031105). A collection of more than 32000 nonredundant rice full-length cDNA sequences is also publicly available. The development of rice genomics facilitates the cloning of important genes. More rice genes have been cloned and characterized with the availability of rice sequence recently[17−30].

Up to now, more than 10 rice leaf colour mutants have been mapped via morphologic or molecular markers, and three of them have been cloned and characterized[31,32](http://www.gramene.org/newsletters/rice-genetics/rgn12/v12p93.html). A premature plant mutant was reported and the *pse*(*t*) gene was anchored on a 220-kb region on chrosome 7[33]. But no presenescing leaf mutant is reported in rice as yet. The genetic analysis and molecular mapping of a presenescing leaf gene is presented in this paper.

1 Materials and methods

1.1 Plant materials

The presenescing leaf mutant was obtained from a *japonica* variety Zhonghua 11 via radiation of ^{60}Co-γ in M2 generation. The mutant was self-pollinated for several generations until genetically proven to be truly inherited. The varieties employed in the study also included two control *indica* varieties, i.e. Nanjing 11 (N11) and Nanjing 6 (N6) and a control japonica variety Zhonghua 11.

1.2 Construction of F_2 segregation population

In summer of 2004, the *psl1* mutant, as maternal parent, was crossed to N6 and N11 in the experimental farm of the Agricultural College, Yangzhou University, and the F_1s were planted in Hainan in the same year. In the spring of 2005, the seeds were harvested from F_1 plants, along with their parents in Hainan. In the summer of 2005, two large F_2 populations of *sl1* mutant/N11 were planted for the fine mapping experiments in Yangzhou.

1.3 DNA extraction and SSR analysis

Rice DNA was prepared from fresh-frozen leaves according to Liang *et al*.[34]. SSR primers were synthesized by Shanghai Shenggong Inc. The sequence of these primers was downloaded from http://www.gramene.org. DNA amplification was performed by PCR machine (MJ Corporation, USA), programmed for an initial 300s at 94℃, followed by 35 cycles of 60s at 94℃, 60s at 55℃, 90s at 72℃, and finally 600s at 72℃. Reactions were carried out in a volume of 25 μL containing 1 μmol/L of primers, 200 μmol/L of dNTP, 5 ng of DNA template, 2 mmol/L $MgCl_2$, 2.5 μL 10× buffer (supplied by Shenggong Inc. with *Tag* polymerase) and 1 U of *Tag* polymerase. Amplification products were analyzed on 4% agarose gels stained with ethidium bromide, and photographed with UVP system. If there was no poly-morphism to be detected on the agarose gel, the amplifica-tion products were further analyzed on 6% polyacrylamide gel stained with 0.1% silver nitrate.

1.4 *Rough mapping of psl1*

The bulked segregant analysis (BSA) method[35] was used to find the markers that could be linked to *psl1*. Two DNA pools (presenescing leaf and its wild type) were constructed based on the phenotype; each pool was composed of 10 individuals from the F_2 population of the presenescing leaf mutant and N11. SSR markers were employed to detect the polymorphism between two parents of segregation population, and the polymorphic markers were further used to detect the polymorphism between the two DNA pools. If the marker was polymorphic between the two DNA pools, it was thought to be putatively linked to the target gene, and then confirmed by the F_2 segregation population.

1.5 Development of new STS markers

The sequence of the *japonica* variety Nipppobare and the *indica* variety 9311 in the anchored region was compared. If a sequence difference which was bigger than 8 bp, it was used to develop STS markers with the soft primer premier 5.0.

1.6 Fine mapping of psl1

According to the rough mapping of *psl1*, 34 new STS markers were developed with the method mentioned above in the anchored region, and recessive presenescing leaf plants in the F_2 and their parents were used to finely map the *psl1* gene.

1.7 Linkage analysis

Linkage relationship and map distances (in cM) were estimated using the program MAPMAKER/3.0b[25].

2 Results

2.1 The performance of the presenescing leaf mutant

During the entire growing period, the significant

character of the mutant was that the former leaf began to become yellow when the cardiac lobe shooted out. All the leaves on each tiller of a plant had a similar performance (Fig. 1). While all leaves of the wild plant could keep green for more than 25 d.

Fig. 1. The seedling of the *psl1* mutant.

2.2 The inheritance of the presenescing leaf mutant

In order to investigate the presenescing leaf trait genetically, the *psl1* mutant was crossed to two wild types N11 and N6. In the F_1s, all the true hybrids had normal green leaves, suggesting that presenescing leaf trait was controlled by a recessive gene. In the F_2 populations, the phenotypes of all progenies from each population were observed (Table 1), and the recessive individuals were found to be similar to the mutant in terms of leaf colour. Also in the F_2 populations (*psl1* mutant/ N11 and *psl1* mutant/N6), the ratio of presenescing leaf to normal green fitted the 3∶1, indicating the monogenic inheritance from *psl1* mutant (Table 1).

2.3 Rough mapping of the psl1 gene

In order to find which chromosome the *psl1* gene might lie on, about 600 SSR markers distributed on 12 chromosomes were used to investigate the polymorphism between two parents and two pools. The results showed that 6 SSR markers on chromosome 2 were polymorphic between the two parents and between the two pools, suggesting that the *psl1* gene might lie on chromosome 2 in rice. The six polymorphic markers RM6, RM5303, RM5474, RM425, RM406 and RM166 were further employed to roughly map the *psl1* genes with 106 recessive individuals of the F_2 (*psl1* mutant/N11). The *psl1* was located between RM5472 and RM425 on chromosome 2, with a genetic distance of 8.02 and 3.78 cM, respectively (Fig. 2). The electrophoresis bands of some F2 plants with RM5472 and RM425 as primers are shown in Fig. 3.

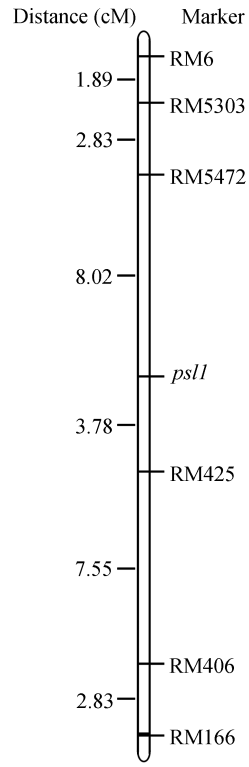

Fig. 2. The location of *psl1* on chromosome 2.

2.4 Fine mapping of the psl1 gene

Fine mapping of an interested gene is the key step in a map-based cloning approach. To finely map the *psl1* locus, a big F_2 population was constructed and 34 new STS were synthesized on the genome sequence between two SSR markers RM5472 and RM425. Nine of these STS markers were found to be polymorphic between *psl1* mutant and N11 as well as N6 (Table 2). These polymorphic markers were used to analyze plants in the F_2 populations. The *psl1* gene was further anchored between two STS markers STS2-19 and STS 2-26, with a genetic distance of 0.43 and 0.11 cM, respectively (Fig. 4). The electrophoresis bands of some F_2 plants with STS2-19 and STS 2-26 as primers are shown in Fig. 5.

2.5 Construction of the contig spanning the psl1 locus

Through fine mapping, five STS and SSR markers were found to be tightly linked to *psl1*. Based on the sequence data related to sequencing of rice chromosome 2 (http:// rgp.dna.affrc.go.jp), RM6535 was lo-

Table 1 Segregation of the presenescing leaf in F2 populations[a)]

Crosses	P_1 leaf colour	P_2 leaf colour	F_1 leaf colour	F_2				
				total	GL	PL	ratio	X^2 (3∶1)
psl1 mutant/N11	PL	GL	GL	431	325	106	3.066	0.02
psl1 mutant/N6	PL	GL	GL	891	671	220	3.05	0.03

a) PL, Presenescing leaf; GL, green leaf.

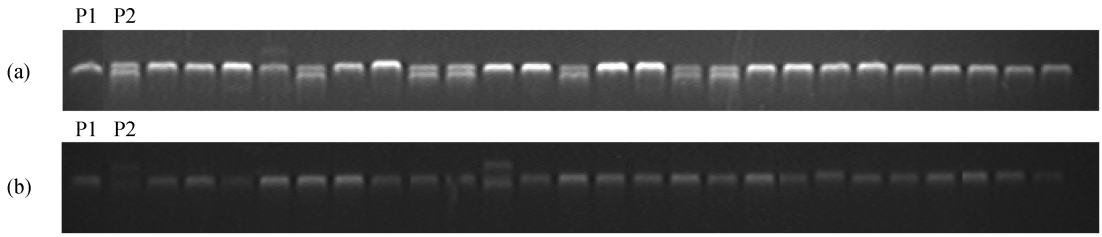

Fig. 3. Segregation of the SSR markers RM5472 and RM425 in the F_2 population of psl1 plants. (a) RM5472; (b) RM425. P1, *psl1* pool; P2, normal leaf pool.

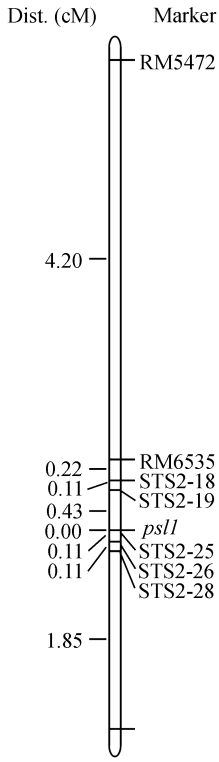

Fig. 4. The fine location of *psl1* on chromosome 2.

cated on the BAC clone AP005297; STS2-18, STS2-19 on the BAC clone AP004114, and STS2-25, STS2-26, STS2-28 on the BAC clone AP005733. According to the relationship between these clones, a contig covering *psl1* locus was constructed (Fig. 6). The tightly linked markers STS2-19 and STS2-25 all lay on the same clone AP005733. The *psl1* gene was delimited to a 48-kb region. This will help us to clone the gene.

3 Discussion

Arabidopsis and rice are both the model plants for plant genomics. The complete sequencing of the two plants was finished in 2002 and 2005, respectively. The extensive genomic resources available for *Arabidopsis* and rice make it very attractive for the identification and functional analysis of senescence-regulated genes. The growth stages of *Arabidopsis* and rice have been carefully defined, which allows an accurate sampling of materials for comparative analysis. However, *Arabidopsis* may not be the ideal plant for the study of senescence since the leaves have a very short lifetime and senescence seems to start as soon as full expansion is reached. Levels of the large subunit of RUBISCO have already been diminished as the leaf reaches its full size[7]. Moreover, the correlation between the senescence of the individual rosette leaves may not be closely linked to the developmental stage of the plant[13]. In contrast rice may be the ideal plant, for our study on senescence since all of the leaves in a plant have a rather long lifetime of at least 25 d, although the lifetime of leaves varies with variety, leaf age and cultivation The chlorophyll content of rice leaf keep invarint for a long time. The lifetime of rice leaves can be significantly prolonged when the ear is removed. Moreover, extensive genomic resources for rice leaf mutant are available.

Biochemical and molecular studies over the last few decades have shown that senescence is an active process that requires the expression of novel genes and the synthesis of new proteins. Genetic studies on leaf

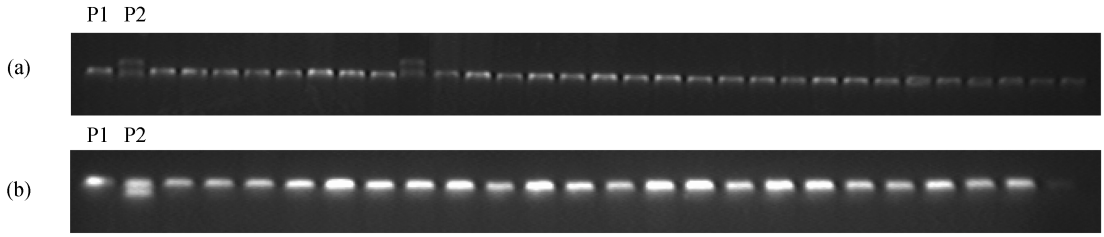

Fig. 5. Segregation of the SSR markers STS2-19 and STS2-25 in the F_2 population of psl1 plants. (a) STS2-19; (b) STS2-19. P1, *psl1* pool; P2, normal leaf pool.

Table 2 New developed polymorphic STS markers

Markers	Primer sequence(5′—3′)	Related BAC clones
STS2-5 F	ATTTGGTTTGATTTTATTCC	AP004159
R	TCTTGGTATGTGCTCACTTT	
STS2-6 F	TGCTGTGGTTGTTCCTGTC	AP004159
R	TTTAGGTTATGTTTACAAGAGGC	
STS2-9 F	GGGAACTAAACCAAGCCTAA	AP005002
R	CATCCGCATCGTAGTATTCA	
STS2-11 F	ATCTGATGGGCGAACAACCT	AP005848
R	TGGTCGTCGTCACTGTTGGA	
STS2-18 F	CAGAGCAGCCTACCTAGCAG	AP004114
R	TTCAGCCTTGACAAACCATC	
STS2-19 F	CTGCGACATCTTCTGGCTAA	AP004114
R	GCAGGCAGAAAGCGTACTAAC	
STS2-25 F	TGCGACATCTTCTGGCTAA	AP005733
R	CAGGCAGAAAGCGTACTAAC	
STS2-26 F	GATGGTGGGACTGGCTAGTGT	AP005733
R	TGTGGTGGTTATGTTGGGAAG	
STS2-28 F	TCTCCCGATACATTCTCAA	AP005733
R	ATACGGCATACAACCAAAC	

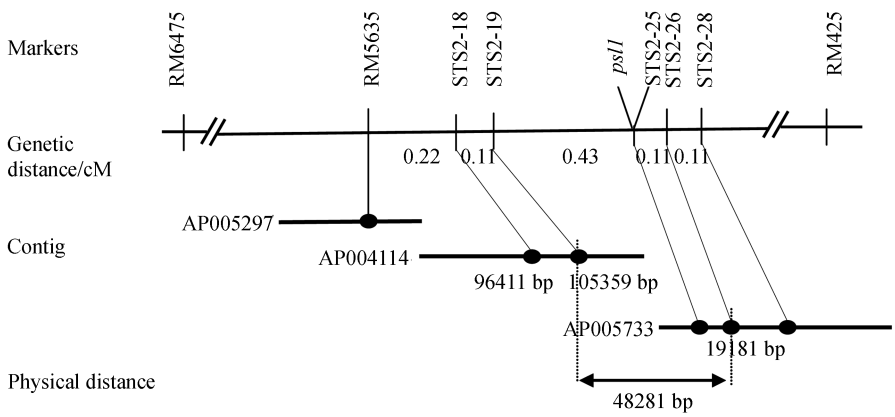

Fig. 6. The fine map encompassing *psl1* locus on chromosome 2 and BAC contig spanning *psl1* locus.

senescence are in general via two ways, the investigation into the related mutant and the identification of leaf senescence-related genes. In the studies of mutants, a lot of mutants on leaf senescence have been identified and characterized. The phenotype of the famous stay-green pea gene *H3* used by Mendel has been shown to be due to a mutation a pheophorbide *a* oxygenase gene[36]. In this mutant, the chlorophyll degradation pathway is affected, but much of the senescence proceeds normally. A number of different soybean

genotypes have been identified that show alterations in senescence. For example, homozygous *d1d1d2d2* lines show similarity to the *H3* mutant, in that chlorophyll and chlorophyll binding proteins are maintained but photosynthesis declines. The homologour gene was also cloned in other plant[37]. In *Arabidopsis*, a mutant that showed an early senescence phenotype, *hys1* (*hypersenescence1*) was recently identified. This mutant showed early loss of chlorophyll and expression of senescence enhanced genes and was found to be allelic with *cpr5* (constitutive expresser of pathogenesis related genes 5)[38]. Moreover, three delayed senescence mutants *ore1*, *ore3* and *ore9* were identified and *ore9* is an F-box protein involved in the regulation of leaf senescence[39]. In rice, a stay green gene was identified and mapped[40]. But no leaf senescence mutant has been reported until now. In the identification of leaf senescence-related genes, about 1400 genes were identified during the three stages of leaf senescence with gene chip and a signal transduction pathway was suggested in *Arabidopsis*[13]. Thousands of up- or down-regulated genes involved in dark-induced senescence were identified. An innovative biochemical pathway involving pyruvate orthophosphate dikinase in generating asparagine for nitrogen remobilization in dark-treated leaves was postulated[6].

The map-based cloning approach is a method to isolate the interested gene based on an intensive genetic and physical mapping. The *psl1* was first anchored in an interval between two molecular markers RM425 and RM5472 on chromosome 2. Further, fine mapping of *psl1* locus suggested that this locus was mapped between two STS markers STS2-19 and STS2-26, with a genetic distances of 0.43 and 0.11 cM, respectively. These two molecular markers provided a start point to construct the contig spanning the *psl1* locus. A two-BACs contig was found to span the *psl1* locus. The region containing *psl1* was delimited to 48 kb. The BAC contig will allow us to identify the *psl1* candidate gene in the near future. The cloning and functional analysis of the *psl1* gene will help us know more about molecular mechanism of rice leaf senescence.

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