THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Genetic Mapping of Rolled Leaf and Spotted Leaf Mutants in Rice

BY
HYERIM LEE

AUGUST, 2015

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRAUDUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

Genetic Mapping of Rolled Leaf and Spotted Leaf Mutants in Rice

HYERIM LEE

ABSTRACTS

Leaves are the organ for photosynthesis, respiration and transpiration, and have a major effect on growth and development related to crop yield. Therefore, morphological characteristics of leaf such as structure, shape and color are important agronomic traits in rice (*Oryza sativa L.*) breeding for ideal plant type.

To understand the genes related to leaf and those of regulations, we obtained two new leaf-related mutants, rolled leaf mutant and spotted leaf mutant, from Ilpum (*Oryza sativa japonica*) by the treatment of ethyl methane sulfonate (EMS). The rolled leaf mutant was observed to have increased bulliform cell number and size, and led to the outcurved leaf rolling. The spotted leaf mutant displayed brown and white spots from early vegetative stage.

The phenotypes of the F_1 plants derived from the cross between these mutants and wild-type plants were normal. In each F_2 population, segregation ratio between the

wild-type and the mutant type was 3:1. This genetic analysis indicated that each mutant phenotype was controlled by single recessive gene.

Bulked segregant analysis (BSA) and genetic mapping were conducted using F_2 population developed from the cross between each of mutants and Milyang23 (*Oryza sativa indica*). According to the results, the rolled leaf gene was located on the long arm of chromosome 2 between the flanking markers 2125f and 2126b (122kb) and the spotted leaf gene was located on the long arm of chromosome 5 between the flanking markers S05105 and S05112 (2.2Mb).

In order to search for candidate genes and causal SNP positions rapidly, whole-genome sequencing data of Ilpum and bulked F_2 progenies displaying the mutant phenotype were used. Through the combination of the BSA and SNP analysis, two candidate genes of rolled leaf mutant and a few candidate SNP positions of spotted leaf mutant were identified. This study will contribute to the further study of leaf-related mechanism in rice, and confirmation of each candidate genes is in progress.

Keywords: Rolled leaf, Bulliform cell, Spotted leaf, ROS staining, Genetic mapping, Next generation sequencing.

Student number: 2013-23228

CONTENTS

LIST OF ABBREVIATIONS

BSA	Bulked segregant analysis
dCAPS	Derived cleaved amplified polumorphic sequence
EMS	Ethyl methane sulfonate
INDEL	INsertions and DELetions
lv	Large vascular
M23	Milyang23
mut	Mutant
NGS	Next generation sequencing
NICS	National Institute of Crop Science
PCR	Polymerase Chain Reaction
RDA	Rural Development Administration
rl	Rolled leaf mutant
SNP	Single nucleotide polymorphism
spl	Spotted leaf mutant
STS	Sequence-tagged Site
sv	Small vascular
WGS	Whole genome sequencing
WT	Wild-type

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Rice is one of the most important food crops in the world and the model plant of monocotyledons. Many kinds of *Oryza sativa* cultivars are grown in more than 100 countries and an estimated 3.5 billion people worldwide, approximately half of the world population, considers that rice (*Oryza sativa L.*) is the staple food[1]. Production and consumption per capita of rice are rising steadily in many counties (such as sub-Saharan Africa, Caribbean and Latin America regions) and rice is the major source of nutrient especially for about 520 million people living in Asia[1]. Furthermore, the genome sequencing project of rice (http://www.rgp.dna.affrc.go.jp/) was conducted firstly among field crops as monocotyledonous model plant that have greatly contributed to genetic study and breeding. The Green Revolution had influenced on increasing the yield of rice. Nevertheless, researches about ideal plant type still need to meet the demand of rice in growing continuously.

Leaf characteristics are important agronomic traits because leaf is the organ of photosynthesis, transpiration, respiration. Leaves play a crucial role in growth and development of plant. To understand the regulatory mechanism of leaf properties, such as shape, development, color, and senescence, many researches were conducted in rice.

Above all, appropriate leaf morphology is an important for high yield in breeding of rice. Moderate leaf rolling contributes to the erectness of leaves, which can increase the light transmission rate and leads to higher photosynthetic efficiency[2-4]. Therefore, the study of rolling leaf and isolation of the genes that controls its phenotype will be a help to elucidate the mechanism of leaf rolling and to breed with ideal type plant with ideal type of plant.

According to the previous study, development progresses of leaf including determination of polarity and cell differentiation are supposed to major reasons of rolling leaf[3-7]. Furthermore, various types of leaf rolling were reported with direction of rolled leaves as inward or outward and degree of rolling; slightly, moderately, cylindrically rolled leaves. Some mutants and genes related to leaf rolling have been reported in rice. However, only a few genes were cloned and studied those function.

The genes of rolled leaf mutants, *rl1*, *rl2*, *rl3*, *rl4*, *rl5*, *rl6* were located on chromosomes 1, 4, 12, 1, 3, and 7 through conventional genetic analysis (http://www.gramene.org/) [8], and *rl7- rl14* were mapped on molecular map with chromosome 5 (*rl7* [9], *rl8* [10]), 9 (*rl9* [11,12], *rl10* [13]), 7(*rl11* [14]), 10 (*rl12* [15], *rl14* [7]) in rice. Most of them were assumed single recessive genes that controlled the traits. However, some mutants were controlled by quantitative trait loci (QTLs) and *rl(t)* located on chromosome 2 was an incomplete recessive gene[16,17].

In rice, *SHALLOT-LIKE1* (*SLL1*) encodes a SHAQKYF class MYB family transcription factor belonging to the KANADI family[4]. Mature *sll1* mutants showed extremely incurved leaves due to the regulation of the development for

abaxial side cells in leaf. Mutational *SLL1* results in defective programmed cell death of abaxial mesophyll cells and suppresses bulliform cell and sclerenchyma development on abaxial side. The *rl14* mutant had incurved leaves and shrunken bulliform cells on the adaxial side[7]. Expression of *RL14*, encoding a 2OG-Fe (II) oxygenase, affected the composition of the secondary cell wall. *SEMI-ROLLED LEAF1* (*SRL1*) which encodes a putative glycosylphosphatidylinositol-anchored protein, regulates the expression of genes encoding vacuolar H^+-ATPase subunits and H^+-pyrophosphatase. A defect of *SRL1* characterized the increased number of bulliform cells[18]. Defect of *NARROW AND ROLLED LEAF1* (*NAR1*), encoding the cellulose synthase-like protein D4 (OsCslD4), results in semi-rolled leaves by decreasing of bulliform cells size[19]. Furthermore, overexpression of the rice *OsAGO7* gene, orthologous to the Arabidopsis *ZIP/Ago7* gene, induces upward adaxial curling of the leaf blade[20].

Several abaxially rolled leaf mutants and the corresponding genes have been identified and analyzed. *Rice outermost cell-specific gene5* (*Roc5*) encodes a homeodomain (HD) leucine zipper class IV homeobox transcription factor. The *oul1* mutant, defect of *Roc5*, are showing abaxially rolled leaf with increased number and size of bulliform cells, whereas the transgenic plants overexpressing *Roc5* present adaxially rolled leaves[21]. Defective rice *leaf inclination2* (*LC2*), a VIN3-like protein, is also observed reversely rolled leaves[22]. Overexpression of *Abaxially Curled Leaf1* (*ACL1*) appeared abaxial curling of leaf blades due to increased bulliform cell number and size, and *ACL2* is its homolog in rice[23]. *ADAXIALIZED LEAF1* (*ADL1*) encodes a calpain-like cysteine proteinase which is associated with the maintenance of axis information in leaf[6]. The *adl1* mutant has

abaxially rolled leaves because of the ectopical formation of bulliform-like cells at the abaxial side. Therefore, histological properties including the size and number of bulliform cells could play an important role in leaf rolling.

In addition, Environmental effects were also involved in leaf rolling. *CONSTITUTIVELY WILTED 1* (*COW1*) encodes a member of the rice YUCCA gene family, and leaves of oscow1 mutant plants rolled upwardly[24].

In rice, various morphological changes of leaf are commonly observed in varieties and mutants induced by mutagens[25]. Leaf color is another important traits influenced on growth, development and yield of plant through photosynthesis[14, 26]. Therefore, researches about leaf color including spot, stripe, albino, chlorine were performed and then that will be a help to identify its genes regulating leaf color. Among these variations, spotted leaf mutants were reported considerably.

The lesions of the spotted leaf mutants usually appear at seedling to tillering stage firstly whereas some of them observed in other growth stage[27,28]. The spots are usually brown color, such as reddish brown and dark brown. The spotted leaf mutants generated lesions without abiotic and biotic stresses, however the lesions are similar to those caused by pest damage or pathogen infection. Therefore, these mutants are often termed lesion mimic or lesion simulating disease mutants[29,30]. Usually, cell necrosis of the spotted leaf mutants is similar to that resulted from hypersensitive response (HR). HR is a symptom of programmed cell deaths (PCD) induced by pathogen invasion[31], but cell death of spotted leaf mutants showed

without pathogen infection. To study PCD mechanisms and analyze defense gene expression, lesion mimic mutants are useful.

To date, 17 rice *spl* mutants (*spl1–11* and *bl1–6*) have been registered in the Rice Genetics Cooperative, and the genetic controls of 51 rice spotted-leaf mutants have been descripted[27,32-35]. Among the 51 *spl* mutants, most of them (42 mutants) are controlled by single recessive genes, one controlled by two recessive genes, and 8 mutants are regulated by single dominant genes (reviewed by Huang et al., 2010 [35]).

Many kinds of lesion mimic mutant were reported, but only several genes were cloned and most of them were remained unmapped. The *spl7* was the first spotted leaf gene to be cloned and encodes a heat stress transcription factor[36]. *Spl11* is a negative regulator of plant cell death defense due to encoding a U-Box/Armadillo repeat protein and manipulating flowering[37,38]. Furthermore, *spl11*, *OsPti1a*, *OsSRT1*, and *OsACRD1* are found to be responsible for spot initiation[39-41]. *Spl18* is related to seedling regeneration[42], and then *spl28* is involved in senescence[43]. Other *spl* genes encode various proteins and enzymes; zinc finger proteins[39,44], heat stress transcription proteins[36], membrane-associated proteins [45], ion channel regulators [46] and components in biochemical pathways responsible for metabolism of porphyrins [47,48], fatty-acids/lipids [49-51], phenolics[52], ubiquitination[53]. Those studies of the *spl* mutants and gene in rice were signified that multiple genetic mechanisms involved in lesion mimic phenotypes.

In the present study, a new rolled leaf mutant and spotted leaf mutant induced by chemical mutagen, ethyl methane sulfonate (EMS), was characterized. The objectives of this study were phenotypic characterization, genetic analysis and

mapping of these leaf-related mutants and identification of the candidate genes for leaf rolling and spotting.

MATERIALS AND METHODS

Plant materials

A rolled leaf mutant and spotted leaf mutant were induced by the treatment of ethyl methane sulfonate (EMS) from Ilpum, *japonica* cultivar. Each of the mutants was selected from M_2 generation and fixed in pure line by repetitive selfing. The pedigree of rolled leaf mutant is Ilpum E-B-572-1-B-2-B-B, and spotted leaf mutant is Ilpum E-B-319-1-2-2-1-1-1.

For genetic analysis and whole genome sequencing, mutants were crossed with its wild-type plant and F_2 populations were developed from these F_1. Mapping populations were derived from the cross between mutants and Milyang23, *indica* cultivar, and were used for BSA and genetic mapping.

All mutants and populations were grown in the experimental field and green house of Seoul National University in Suwon.

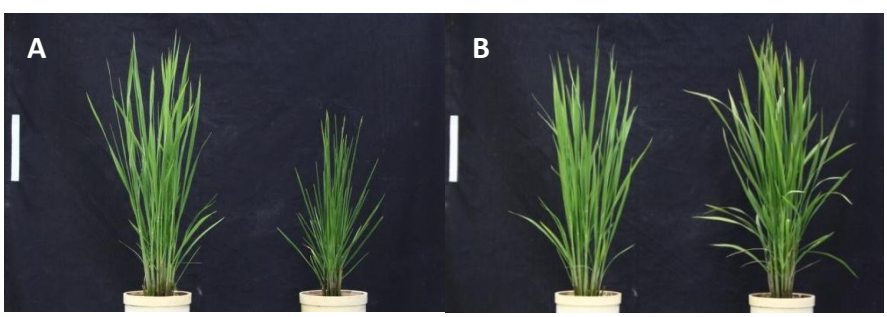


Figure 1. Phenotype of wild-type and mutants at vegetative stage.
(A) Plants of wild-type (left) and *rl* (right). (bar = 20cm)

(B) Plants of wild-type (left) and *spl* (right). (bar = 20cm)

Paraffin section for histological analysis of rolled leaf

Paraffin-embedded section was done according to the method of Ji et al. (2006)[54]with slight modifications. Leaf samples from the middle portion of leaves were collected at late vegetative stage and fixed in FAA solution(ethanol 50%, acetic acid 5%, formaldehyde 3.7%) and stored at 4℃ for overnight after vacuumed (3cycles of 5min each) using IR concentrator (Micro-Cenvac, NB-503CIR, N-Biotek).

The fixed leaves were dehydrated by soaking in a graded ethanol series (60%, 70%, 85%, 95%) each for 2 hours and incubated in 100% ethanol overnight. The samples were cleared by soaking for 2 hours in the clearing solution series consisting of ethanol : histoclear 3:1, 1:1, 1:3 in order and then soaked in 100% histoclear overnight. For paraffin infiltration, the leaf samples were soaked in the solution series of histoclear : paraffin 3:1, 1:1, 1:3 for 2 hours in order and 100% paraffin at 55℃ overnignt.

The infiltrated samples were embedded in a paraffin block and then cut into 8~20 ㎛ sections using a microtome (MICROM Lab, Walldorf, Germany). The sections were mounted on a Superfrost-plus glass slides (Fisher Scientific, Pittsburgh, PA, USA) coated with egg albumin solution (sodium salicylate 1g, egg white 50ml, glycerol 50ml)and dried at 42℃ for 1day. The sections were deparaffinized with 100% xylene for 1 hour followed by hydration by soaking in xylene : ethanol 1:1, 100% ethanol, and sterile water for 2min each. The sections were stained with 0.1% toluidine blue solution for ~30sec and washed with sterile water. For destaining, the slides with sections were soaked in 30%, 50%, 70%, 85%,

95% ethanol for 2 min in order. Finally, the slides were soaked in 100% xylene for 10 min and mounted in Canada balsam. The cross sections of leaf were observed and photographed at 40~300X magnification to measure the bulliform cell number and size.

Measurement of Leaf Rolling Index (LRI) and Leaf Erection Index (LEI) of rolled leaf

To determine the Leaf Rolling Index (LRI), the widths of the flag and second leaves at the heading stage were measured under either the natural (Ln) or unfolding state (Lw). The LRI was calculated as $LRI(\%) = (Lw - Ln)/Lw \times 100$.

The length of the leaf blade between the lamina joint and the tip in the natural (Lnl) and straightened situation (Lsl) were measured, and the LEI was calculated as $LEI(\%) = Lnl/Lsl \times 100$. The data of Lnl and Lsl were collected from flag and second leaves at heading stage, too.

Measurement of soil plant analysis development (SPAD) value of spotted leaf

To verify the physiological characteristics of the spotted leaf mutant, the amount of chlorophyll present in plant leaves was measured using Minolta Chlorophyll Meter SPAD-502 (Minolta Camera Co., Japan) and was designated the SPAD value.

SPAD value was measured on the day of flowering, and then again every 5 days until 20 day after flowering in three spots (near the tip, basal half and near the basal) of flag and second leaves from five plant replicates.

Detection of ROS accumulation of spotted leaf

ROS (Reactive Oxygen species) accumulation was determined by staining method described previously[55,56] with some modifications. Upper three leaves, from top to bottom, were used in staining.

For superoxide anion(O_2^-) detection, leaf samples were vacuumed (three cycles of 5 min each) and infiltrated in 0.5 mg/ml nitro blue tetrazolium (NBT) in 10mM potassium phosphate buffer (pH 7.8) for 20 h in the dark. For H_2O_2 determination, leaf samples were vacuum-infiltrated (three cycles of 5 min each) in 1 mg/ml 3,3′-diaminobenzidine (DAB) containing 10mM MES (pH 6.5) for 22h in the dark. Both reactions were performed until chlorophyll was completely removed and were stopped by transfer to 90% ethanol at 70℃. Following incubation period, the cleared leaves were stored and photographed in 70% glycerol.

Genetic mapping

Two F_2 mapping populations from the cross between rolled leaf mutant and Milyang23, spotted leaf mutant and Milyang23 were used.

For genetic mapping, BSA strategy[57] was done first. 10 mutant type and 10 wild-type plants based on their phenotype were selected from each of F_2 mapping

populations. Genomic DNA were extracted from young leaves by CTAB method. Equal concentration of DNA from individual plants which showed same phenotype were pooled into two bulks.

Total 92 STS markers of known chromosomal position throughout all chromosomes, which were designed in Crop Molecular Breeding Laboratory, Seoul National University, were tested and then the co-segregation markers were identified with these bulks. After BSA, phenotype and genotype of F_2 mapping population were used to determine gene position on chromosome and to conduct fine mapping. Some contiguous STS markers and additionally designed STS markers, based on the differences in sequences between the *japonica* and *indica* rice subspecies, were used.

PCR was performed in a total reaction volume of 20 ㎕ containing 100ng of template DNA, 10pM of each forward and reverse primer, 250μM of dNTP, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin and 0.5U of Taq DNA polymerase (made by laboratory). Amplication were performed in a PCT100 96U Termocycle (MJ Resaerch, Reno, NV, USA) in the following sequence : 5min at 95℃, followed by 35cycles of 30sec at 95℃, 30sec at 56℃, 30sec at 72℃, 10min at 72℃ for final extension and 10min at 10℃. PCR products were separated in 3.0% agarose/0.5X TBE gels and visualized by ethidium bromide staining.

Whole genome sequencing

Genomic DNA of F_2 population derived from the cross between each of mutants and wild-type was extracted from young leaves by CTAB method, and then it was pooled from several individuals (n=20, > 5 ㎍) showing the clear mutant phenotype among the progeny by equal ratio. For SNP analysis, the bulked DNA was sequenced using an Illumina HiSeq2500, and raw data of whole-genome sequence for Ilpum from NICS of RDA was also used.

DNA libraries for Illumina HiSeq2500 sequencing were prepared using the kits (Truseq Nano DNA LT sample preparation kit, FC-121-4001). The qPCR was conducted using these libraries, and then amplified clonal clusters were generated and performed paired-end sequencing using Illumina HiSeq2500 (250 cycles). Base calling was carried out by the instrument control software's Real Time Analysis (RTA).

Alignment of short reads to reference sequences and SNP and InDel calling

Whole genome sequencing and SNP analysis were performed to identify the mutation point generated by mutagen. 76 million and 83 million paired-end short reads from F_2 bulks DNA was obtained and these data preprocessing were carried out using btrim 0.2.0 (http://graphics.med.yale.edu/trim/). The filtering options of low-quality bases were cut off phred-score 20 and minimum read length 50. Processed short reads were mapped and aligned to public Nipponbare genome sequence, Build 5 ver. (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html)

with clc_ref_assemble (V 4.21.104315). whole-genome sequence for Ilpum from NICS of RDA was also filtered and mapped to Nipponbare in the same way. Subsequently, SNP detection and SNP calling were performed by find_variations (V 4.21.104315). SNP filtering options are read depth of the site ≥ 5, variant frequency of major base ≥ 0.9.

After mapping and SNPs calling, causal SNPs and InDels involved in phenotype of mutants were detected by following, modified MutMap method[58]. ⅰ) Remove the same SNPs and InDels in wild-type and mutant. ⅱ) Remove the common SNPs and InDels shared by at least two mutants lines. ⅲ) Calculate SNP index. ⅳ) Pick homozygous SNPs and InDels (SNP index=1) located in annotated gene region. SNP index is the ratio between the number of reads of a mutant SNP and the total number of reads at the each position.

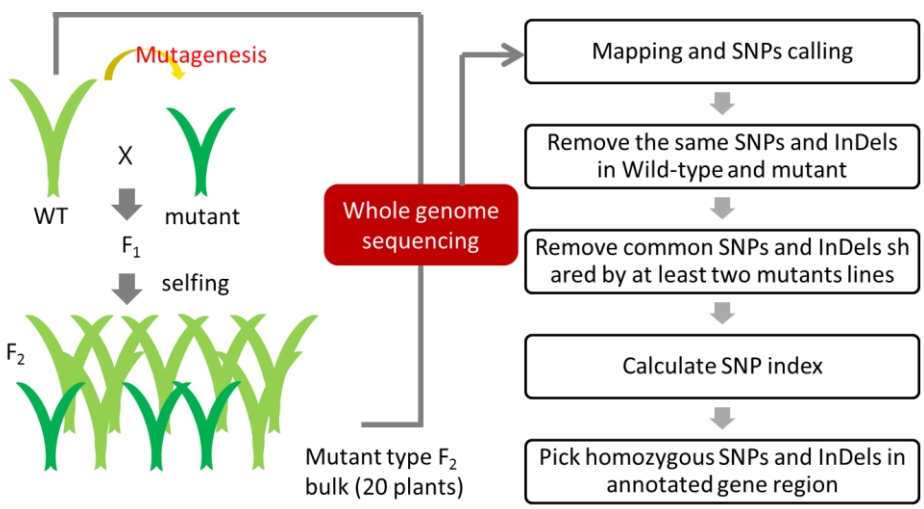


Figure 2. SNPs and InDels calling work-flow from whole genome sequencing data.

RESULTS

1. Rolled leaf mutant

Characterization of rolled leaf mutant in rice

The rolled leaf mutant plants appeared spiral shaped leaves at the seedling stage (Fig. 4 A). Distinction between mutant and wild-type plants in rolling of leaves became clearly evident as they grew. After the tillering stage, the leaves of the mutant plants showed outward rolling and cylindrical shape (Fig.4 B,C).

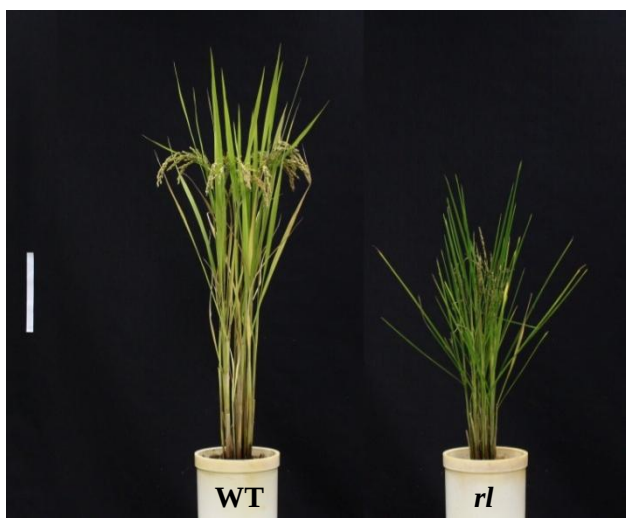


Figure 3. Phenotype of wild-type and rolled leaf mutant at ripening stage. (bar = 20cm)

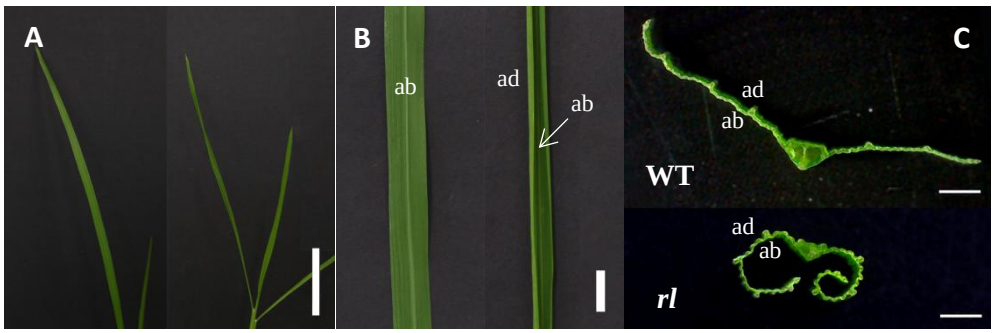


Figure 4. Leaf morphological characteristics of wild-type and rolled leaf mutant.

(A) *rl* (right) leaves showed spiral but WT (left) leaves were flat at early vegetative stage. (bar = 5cm) (B) *rl* (right) leaves rolled abaxially at late vegetative stage (bar = 1cm) and (C) showed cylinder-like shape in mature plants. (bar = 1mm) ab, abaxial side; ad, adaxial side.

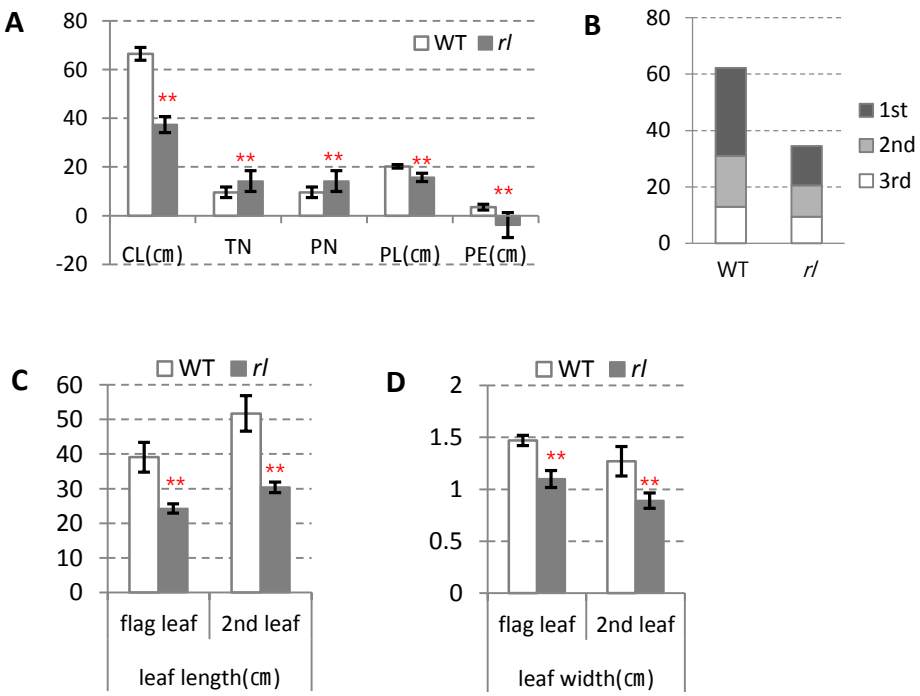


Figure 5. Agronomic traits of wild-type and rolled leaf mutant.

Comparison of the (A) culm length(CL), tiller number(TN), panicle number(PN), panicle length(PL), panicle exsertion(PE), (B) inter node length, (C) leaf length and (D) leaf width of flag and second leaves between wild-type and *rl*. Error bar represents SD (n>10) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01).

Agronomic traits were compared between the mutant and wild-type. The results showed that the mutant was deficient in most of them. The abaxially rolled leaf mutant showed smaller culm length, leaf length and leaf width than Ilpum, its wild type. Panicle length and exsertion were also reduced in case of rolled leaf mutant compared to the wild-type (Fig. 5). Grain length, width, fertility and 1000 grains weight of rolled leaf mutant were decreased, whereas tiller number and panicle number of the mutant were increased compared to the wild-type (Fig. 6, Table 1).

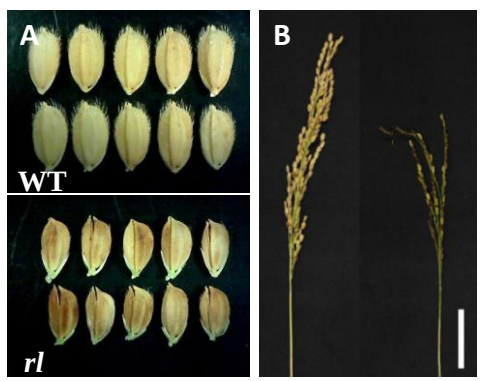


Figure 6. Grains and panicle of wild-type and rolled leaf mutant.
(A) Grain shape of wild-type and *rl*. (B) Panicle of wild-type (left) and *rl* (right). (bar = 5cm)

Table 1. Comparison of agronomic traits between wild-type and rolled leaf mutant.

	HD	GL(mm)	GW(mm)	1000 grain weight(g)	Grain fertility(%)
Wild-type	24-Aug	5.53±0.18	3.33±0.15	25.97±0.09	82.15±7.98
Mutant	28-Aug	4.68±0.15	2.83±0.13	11.56±0.26	2.93±2.45
Comparison	-	**	**	**	**

Values are mean ± standard deviation. Comparison of the heading date in 2014(HD), grain length(GL), grain width(GW), 1000 grain weight, grain fertility of wild-type and *rl*. Data show means and SD of biological replicates (n>10, n>5) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01, NS: not statically significant).

Histological analysis

According to previous studies, the leaf rolling, especially orientation of that, was influenced by alterations of bulliform cell. Paraffin-embedding sectioning of the leaves was carried out to investigate the change about cell differentiation.

In cross section analysis from mature 9th leaves, significant differences in number and size of bulliform cell were observed between the mutant and the wild-type (Fig. 8). The rolled leaf mutant had 7.31 bulliform cells between two vascular bundle ridges, whereas the wild-type bulliform cells were 4.49 cells (Fig. 7 A). Moreover, the average of bulliform cell area of the mutant (4595.90μm^2) was larger than that of the wild-type (2457.06μm^2) (Fig. 7 B). Bulliform cell number and area were increased in the rolled leaf mutant. Therefore, it was assumed that the abaxially rolled leaf phenotype of the mutant was resulted from altered bulliform cell number and area.

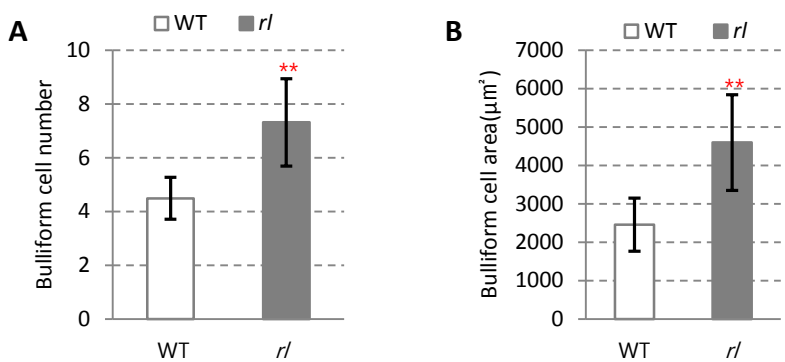


Figure 7. Rolled leaf mutant has increased bulliform cell number and area compare to wild-type.
9th leaf blades of *rl* show significantly increased (A) bulliform cell number and (B) area. Error bar represents SD (n>35) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01).

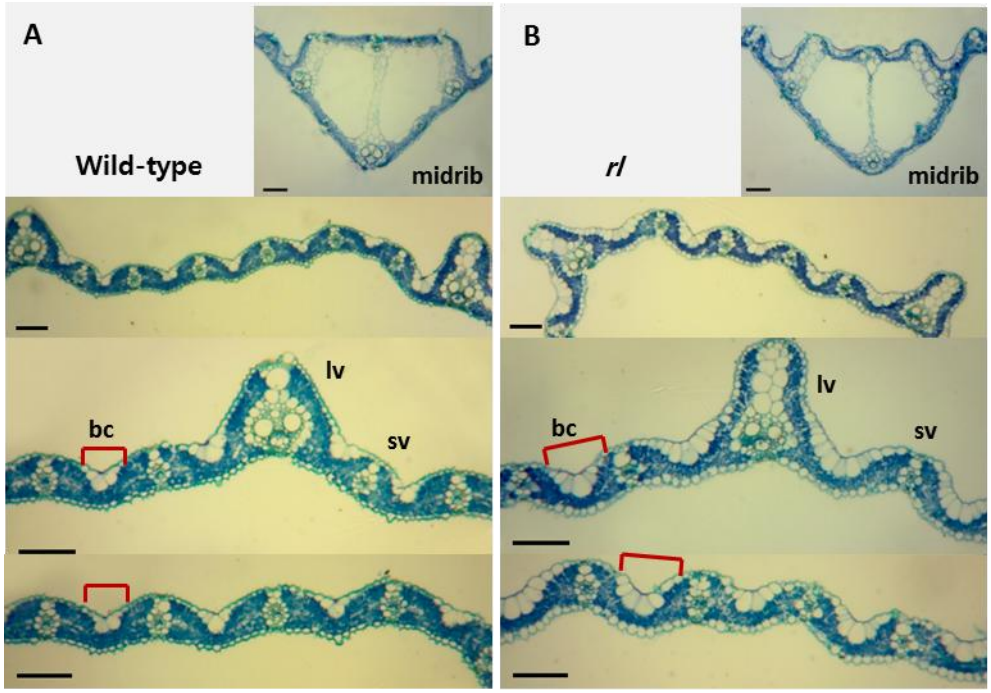


Figure 8. Leaf cross-section of wild-type and rolled leaf mutant.
Bulliform cell number and area between vascular bundle ridges were significantly increased in *rl*. Red lines indicate the bulliform cells. (bar = 100μm). bc, bulliform cells; lv, large vascular; sv, small vascular.

Leaf Rolling Index (LRI) and Leaf Erection Index (LEI)

LRI analysis showed that the mutant have evidently high LRI value compared to the wild-type. At the late vegetative stage, the LRIs of the flag and second leaves of the rolled leaf mutant reached to approximately 47% and 60%, whereas wild-type leaves were almost flat (Fig. 9 A).

On the other hand, the LEIs of the same leaves of the mutant were observed similar to that of wild-type (Fig. 9 B). This result was due to the erectness of flag and second leaves at the heading stage. Moreover, Ilpum had erected leaves so LEIs

were not different or slightly different between the rolled leaf mutant and the wild-type.

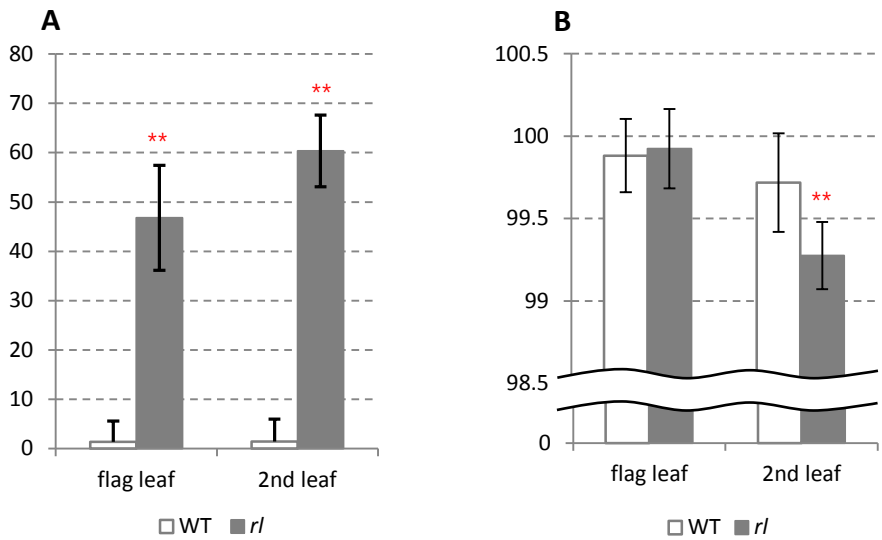


Figure 9. Comparison of the LRI and LEI of wild-type and rolled leaf mutant. (A) LRI and (B) LEI of wild-type and *rl*. Error bar represents SD (n>10) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01).

Genetic analysis of rolled leaf mutant in rice

The phenotypes of the F_1 plants derived from the cross between the mutant and Ilpum were normal. In F_2 population, segregation ratio between the wild-type and the mutant-type was 3:1 (Table 2). This genetic analysis indicated that leaf rolling is controlled by single recessive gene.

Table 2. Segregation ratio of F_2 population developed from the cross between the rolled leaf mutant and Ilpum.

Total	Normal	Mutant	X^2 (3:1) ($p=0.05$, $\chi^2=3.841$)
185	149	36	3.029

Genetic mapping of rolled leaf mutant in rice

Bulked segregant analysis (BSA) and genetic mapping were conducted using F_2 population derived from the cross between mutant and Milyang23 (Oryza sativa indica). According to the BSA results, the gene was located on the long arm of rice chromosome 2 between two flanking markers, S02109 and S02126 (Fig. 10).

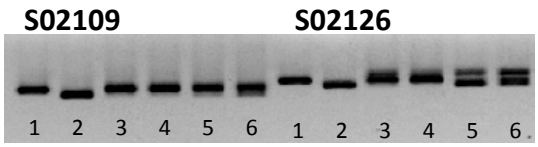
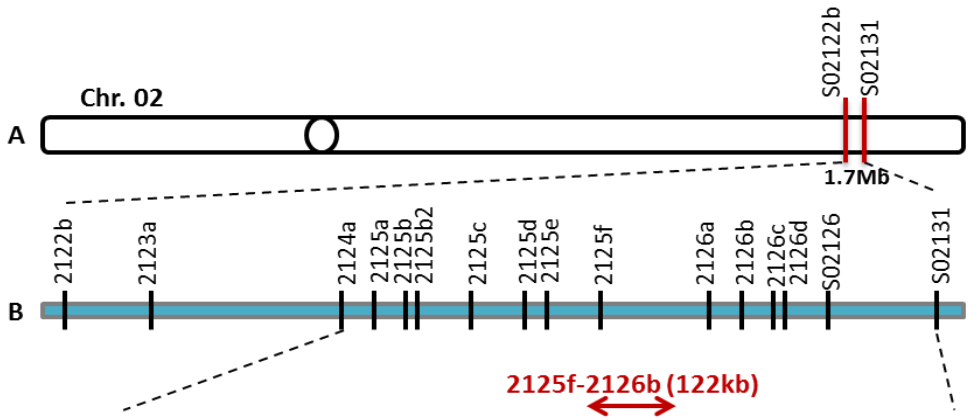


Figure 10. Bulked Segregant Analysis (BSA) of wild-type and rolled leaf mutant bulks from F_2 population.
1, *rl*; 2, Milyang 23; 3,4, Mutant bulks; 5,6, Wild-type bulks.

Additional 13 STS markers (Table 3), which were designed based on the variation of genome sequence from database (http://www.ncbi.nih.gov/ and http://www.rgp.dna.affrc.go.jp/), were located between two flanking markers and used in narrowing down the candidate region for rolled leaf gene. Fine- mapping results showed that 2125f and 2126b markers were closely linked to locus of rolled leaf gene and physical distance of them were estimated to be 122kb (Fig. 11). In this candidate region, total of 25 genes were located.

Table 3. The PCR-based additionally designed molecular markers for genetic mapping of the rolled leaf mutant.

STS marker	Forward primer (5′-3′)	Reverse primer (5′-3′)
2123a	ATTCGAGGGAGAAGGGAATC	AACTTGCAGGAGGAGACCAA
2124a	ATTTGTTTGACGGCCAAAAG	TGAAGTCGAAGCCTTTGAGG
2125a	TGCGTGTCGATTCCTTATTTT	AACCCGCCAGCCTATTACTC
2125b	CCTGCTCTTAAGGTGGTGGA	GATGAGCTAGCTGGCCTTGT
2125b2	CCTCAGCTTTGTCATTTCCAG	AGTGGCTCACATTGCTGATG
2125c	AGGCCCCGATTTAGCTTAGA	CTTCCAATTTGGCGAGGTAA
2125d	CAGGTCCTAAAAGGACAGCA	CTGGCCGTGAGGTAATTGTT
2125e	TTCAATTCGTTGTGCGTTGT	GGAGCCAGTTAGAAGGAAAGAA
2125f	AATCCGTCGATTTGTGTGTG	AACGTGCAATCATCCCATTC
2126a	TGCATGTGTGATGAGGACTG	CGGTGACACCCTTTAGAAGTC
2126b	TGGTTAGGTTTTTGACGATGAA	TAAATGCATGCGCTCTCAAG
2126c	TAGGGTGCAGTGGAATAGCC	AGGAACTGGGAAGCTGAAGG
2126d	TCCCGCTTGTACCGAATTAC	CGAAGGGTCACAGGACTCAC



	2124a	2125a	2125b	2125b2	2125c	2125d	2125e	2125f	2126a	2126b	2126c	2126d	S02126	S02131
W	H	H	H	H	H	H	H	H	H	M	M	M	M	M
M	H	H	H	H	M	M	M	M	M	M	M	M	M	M
W	M	M	M	M	M	H	H	H	H	H	H	H	H	H
M	H	H	H	H	H	H	H	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	M	M	M	M	M	H	H
M	H	H	H	H	H	H	H	H	M	M	M	M	M	M
M	H	H	M	M	M	M	M	M	M	M	M	M	M	M
M	H	H	H	M	M	M	M	M	M	M	M	M	M	M
W	M	M	M	M	M	M	M	H	H	H	H	H	H	H
W	M	M	M	M	M	H	H	H	H	H	H	H	H	H

Figure 11. Genetic and physical maps of the rolled leaf mutant.
(A) Locus of new rolled leaf gene was on the long arm of chromosome 2 between S02122b and S02131. (B) Candidate region was narrowed down using additional STS marker. (C) Graphical genotype results of the fine mapping. W, Wild type; M, Mutant type; H, hetero.

SNP analysis and searching for mutation point

To identify the candidate gene and causal SNP position rapidly, the SNP analysis was performed with whole-genome sequencing data of Ilpum and bulked F_2 progeny displaying the mutant phenotype. This analysis and genetic mapping results were analyzed synthetically.

Several SNP positions were founded in candidate region. Among them, only three homozygous SNPs which existed in genic region based on Rice Genome

Annotation Project database (http://rice.plantbiology.msu.edu/). One of the SNPs located on LOC_Os02g48570 and others located on LOC_Os02g48590 (Table 4).

Every SNP was confirmed by manual sequencing, and then co-segregation analysis was performed with F_2 population to validate the association between those SNPs and the rolled leaf phenotype of the mutant. dCAPS markers were designed and PCR was conducted in 10 mutant type and 10 wild type plants selected from F_2 population, derived from the cross between rolled leaf mutant and Ilpum.

After digestion with specific restriction enzyme, only mutant or wild type PCR product was cut depending on the markers. Each of clear co-segregations observed between mutant type and wild type plants from F_2 population (Fig 12 A,B).

According to in silico genome annotation, one of the SNPs, named SNP3, is likely to relate with phenotype of mutant. This mutation point found at the 5´ splice site of first intron. In eukaryotes, introns of pre-mRNA are spliced off before translation, mRNA to amino acid. Introns have specific sequences, which bind spliceosome assisted splicing, GU at its 5´ end and AG at its 3´ end for RNA processing. In this mutant, this G of 5´ has been mutated to A, which binds spliceosome. Therefore, this single nucleotide transition might lead to the splicing error (Fig.12 C).

Table 4. Homozygous SNPs position in candidate region for rolled leaf.

	Position	Status	Reference	Query	Gene ID		Description
SNP1	30596325	Difference	G	A	Os02g0716800	LOC_Os02g48570	TGF-beta receptor, type I/II extracellular region family protein.
SNP2	30601566	Difference	G	A	Os02g0717100	LOC_Os02g48590	Esterase/lipase/thioesterase domain containing protein.
SNP3	30602023	Difference	G	A			

SNPs were named SNP1, SNP2, SNP3 and position of SNPs was based on the Nipponbare genome sequence, Build 5 ver. (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html).

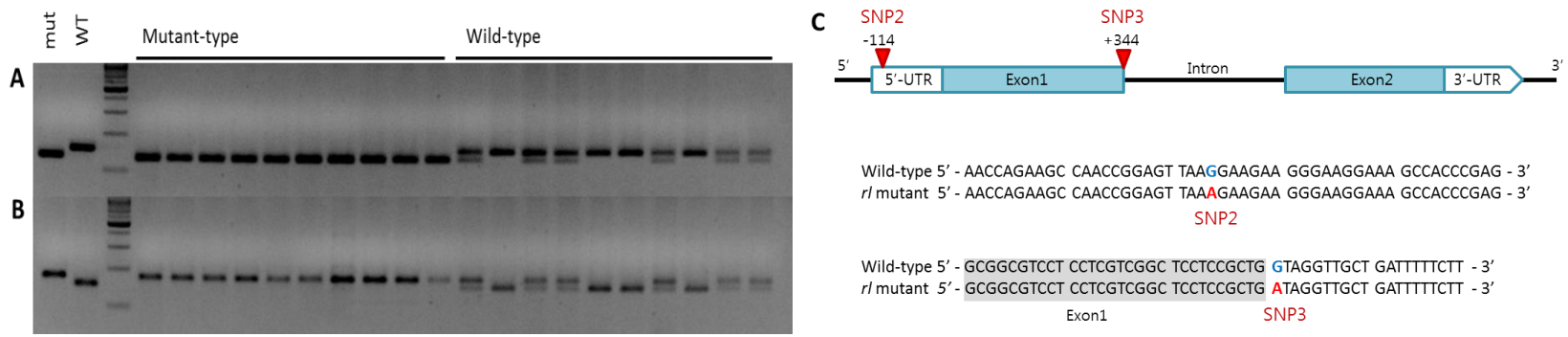


Figure 12. SNP2 and SNP3.

(A) and (B) are dCAPS marker genotype of F_2 plants from the cross between the rolled leaf mutant and Ilpum, classified by phenotype. (A) After digestion with restriction enzyme MseI of SNP2. (B) After digestion with restriction enzyme RsaI of SNP3. (C) Position of SNP2 and SNP3 in LOC_Os02g48590.

1. Spotted leaf mutant

Characterization of spotted leaf mutant in rice

Brown spots appeared on the leaves near the tip of the mutants after 5th leaf stage and the spots increased from the tip to the basal part of the leaf with growth. At later tillering stage, white spots were observed with the brown spots. As growing, both spots showed from the bottom to top of the plant (Fig. 14).

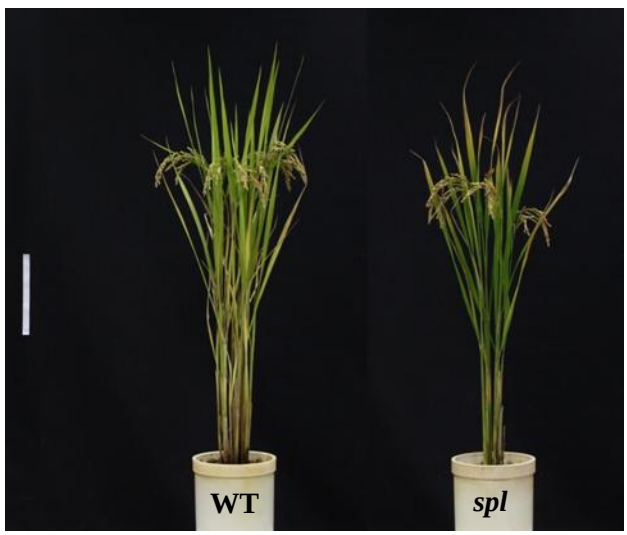


Figure 13. Phenotype of wild-type and spotted leaf mutant at ripening stage. (bar = 20cm)

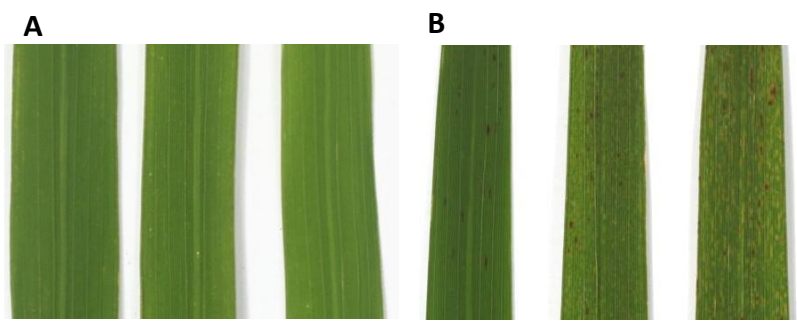


Figure 14. Lesion mimic phenotype of spotted leaf mutant.
First (left), second (middle), third(right) leaves from the top of (A) wild-type and (B) spotted leaf mutant at late vegetative stage.

To analyze the characteristics of the spotted leaf mutant, various agronomic traits of the mutant were compared with those of the wild-type. Most of the agronomic traits of mutant were similar to those of wild-type.

The spotted leaf mutant showed smaller culm length, tiller number and panicle number than Ilpum, its wild type. However, panicle length and exsertion, leaf length and width were not severely reduced in case of spotted leaf mutant compared to the wild-type (Fig. 15). 1000 grains weight of spotted leaf mutant was decreased, whereas grain length, width, and fertility of the mutant were similar to those of wild-type (Fig. 16).

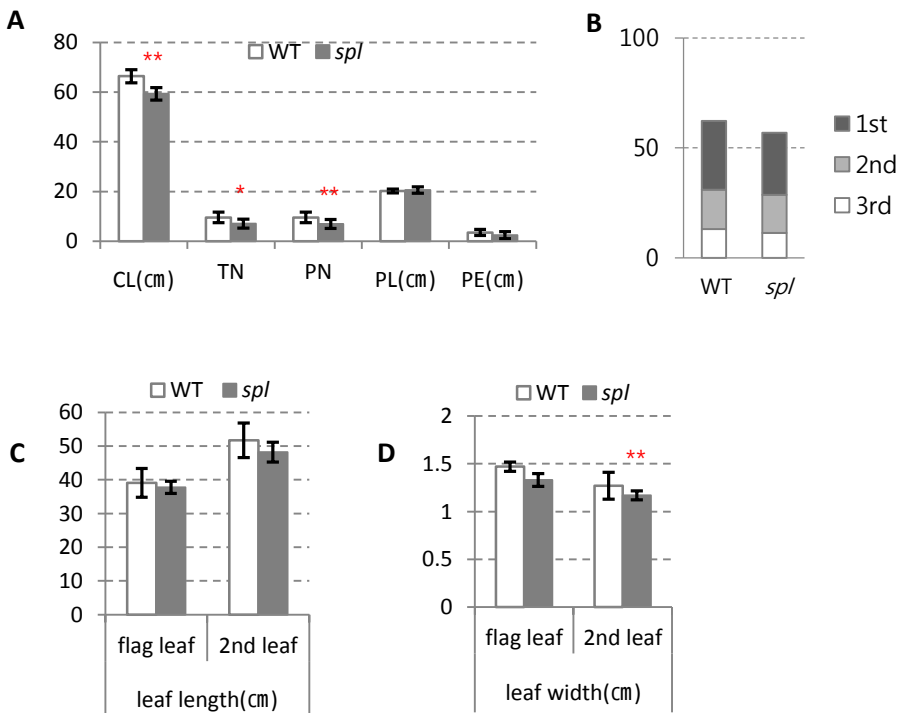


Figure 15. Agronomic traits of wild-type and spotted leaf mutant.
Comparison of the (A) culm length(CL), tiller number(TN), panicle number(PN), panicle length(PL), panicle exsertion(PE), (B) inter node length, (C) leaf length and (D) leaf width of flag and second leaves of wild-type and *spl* . Error bar represents SD (n>10) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01).

Figure 16. Grains and panicle of wild-type and spotted leaf mutant.
(A) Grain shape of wild-type and *spl*. (B) Panicle of wild-type (left) and *spl* (right). (bar = 5cm)

Table 5. Comparison of agronomic traits between wild-type and spotted leaf mutant.

	HD	GL(mm)	GW(mm)	1000 grain weight(g)	Grain fertility(%)
Wild-type	24-Aug	5.53±0.18	3.33±0.15	25.97±0.09	82.15±7.98
Mutant	20-Aug	5.58±0.10	3.37±0.05	21.12±0.32	85.64±5.46
Comparison	-	NS	NS	**	NS

Values are mean ± standard deviation. Comparison of the heading date in 2014(HD), grain length(GL), grain width(GW), 1000 grain weight, grain fertility of wild-type and *spl*. Data show means and SD of biological replicates (n>10, n>5) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01, NS: not statically significant).

Analysis of chlorophyll contents by SPAD value

To establish the connection between spot and degree of chlorophyll content, SPAD values were measured in mutants and wild-type plants. SPAD value, indirect

indicator of chlorophyll content in leaf, of the mutant plant was slightly higher than wild-type that means spotted leaf mutant naturally have much chlorophyll content compared to wild-type.

Although the lesions expanded over the leaf, SPAD values were slightly decreased. The result indicates that destruction of chlorophyll was not accelerated by the emergence of lesions (Fig. 17).

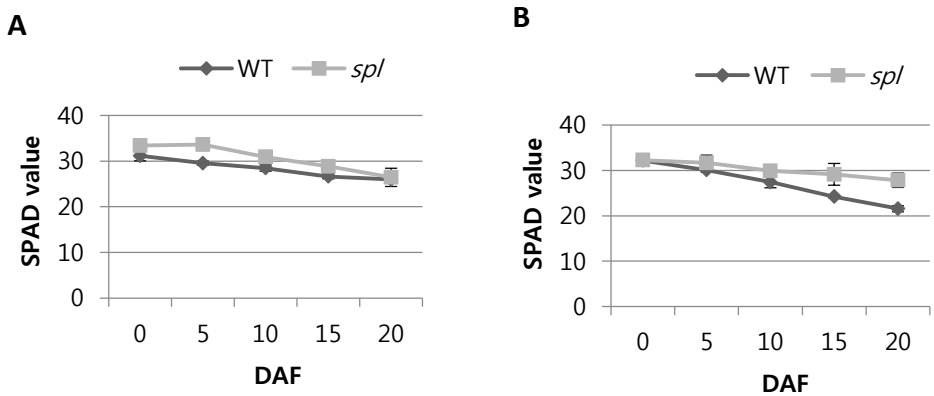


Figure 17. Comparison of the chlorophyll contents.
Chlorophyll contents in (A) flag leaf and (B) second leaf of wild-type and *spl*. SPAD value were measured at the indicated times after flowering (DAF, days after flowering). Data show means and SD of biological replicates (n>5).

Staining for ROS detection

The Result of NBT staining, displaying the blue color precipitates, indicate the superoxide anion(O_2^-) accumulation, Among the upper three leaves, first leaf of the mutant was quite distinct from those wild-type, and other leaves of the mutant were stained more than wild-type (Fig. 18).

Furthermore, to observe the H_2O_2 accumulation, DAB staining was also performed . DAB staining showed brown staining that detects areas of H_2O_2 accumulation. DAB staining results were similar to that of NBT staining.

This indicated that lesion formation in the mutant leaves was correlated with ROS accumulation.

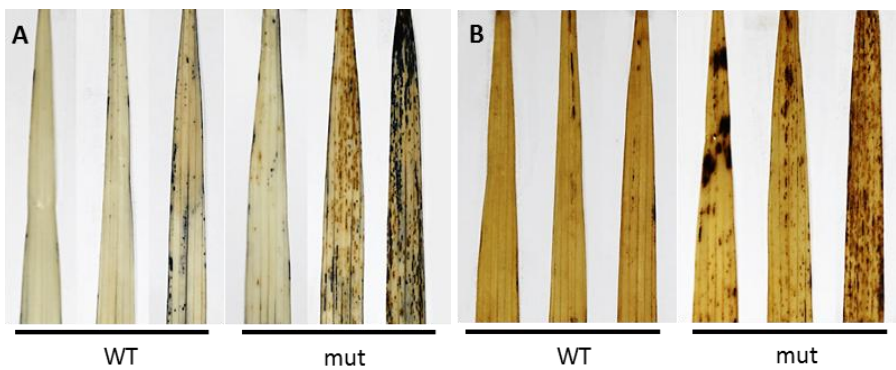


Figure 18. ROS staining.
ROS staining using first, second, third leaves from the top of wild-type and *spl* at heading stage. (A) NBT staining, (B) DAB staining.

Genetic analysis of spotted leaf mutant in rice

F_1 plants derived from the cross between the mutant and Ilpum showed the normal phenotype. In F_2 population, segregation ratio between the wild-type and the mutant-type was 3:1. According to this genetic analysis, spotted leaf gene which controls the emergence of lesion is single recessive gene.

Table 6. Segregation ratio of F_2 population developed from the cross between the spotted leaf mutant and Ilpum.

Total	Normal	Mutant	X^2 (3:1) ($p=0.05$, $\chi^2=3.841$)
190	148	42	0.085

Genetic mapping of spotted leaf mutant in rice

Bulked segregant analysis (BSA) was performed using F_2 population derived from the cross between mutant and Milyang23 (*Oryza sativa indica*). This analysis detected two flanking markers (S05080a, S05112) on the long arm of rice chromosome 5 (Fig. 19).

Additional 6 STS markers, which were located between two flanking markers were designed based on variation of genome sequence from available database (http://www.ncbi.nih.gov/ and http://www.rgp.dna.affrc.go.jp/). Fine- mapping was conducted, and then the candidate region was narrowed down between S05105 and S05112 markers (physical distance 2,2Mb).

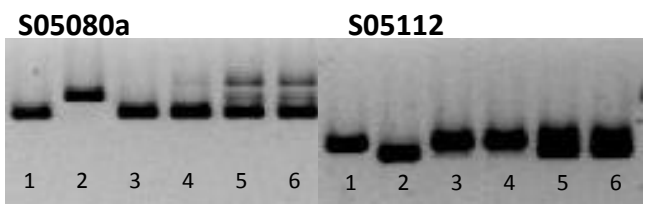
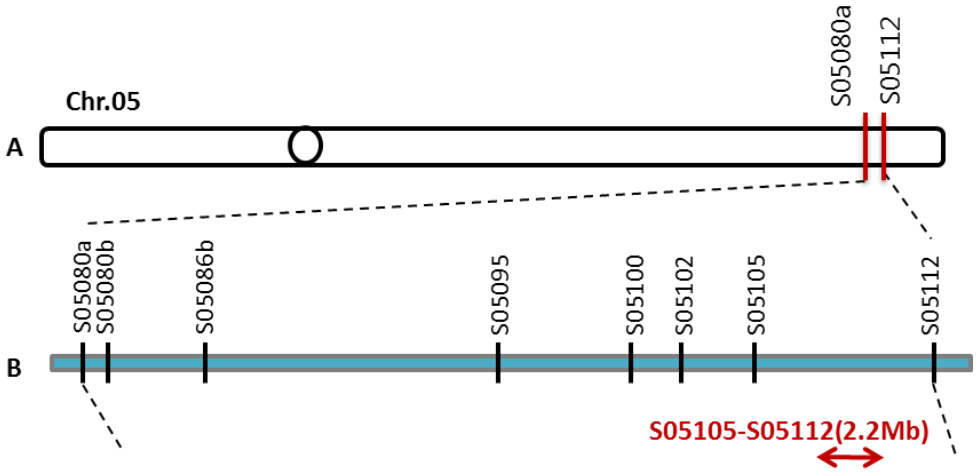


Figure 19. Bulked Segregant Analysis (BSA) of wild-type and spotted leaf mutant bulks from F_2 population.
1, *spl*; 2, Milyang 23; 3,4, Mutant bulks; 5,6, Wild-type bulks.



C

	S05080A	S05080b	S05086b	S05095	S05100	S05102	S05105	S05112
M	M	M	M	M	M	M	M	H
M	M	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	H
W	M	M	M	M	M	M	H	H
W	W	W	W	W	W	H	W	W
W	M	M	M	M	H	H	H	H
W	M	M	M	H	H	H	H	H
W	H	H	H	H	H	H	W	W
W	M	M	M	M	M	M	M	H
W	H	H	H	H	W	W	W	W
W	M	M	M	M	M	M	M	H

Figure 20. Genetic and physical maps of the spotted leaf mutant.
(A) Locus of spotted leaf gene was on the long arm of chromosome 5 between S05080a and S05112. (B) Candidate region was narrowed down using additional STS marker. (C) Graphical genotype results of the fine mapping. W, Wild type; M, Mutant type; H, hetero.

SNP analysis and searching for mutation point

Both rolled leaf mutant and spotted leaf mutant were derived from Ilpum. Therefore, similar strategies were applied to search for the candidate gene and causal SNP position.

The SNP analysis was performed with whole-genome sequencing data of Ilpum and bulked F_2 progeny displaying the mutant phenotype. This analysis and genetic mapping results were analyzed synthetically. In flanking region, 8 homozygous SNPs existed in genic region and several homozygous SNPs in intergenic region based on Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/).

Table 7. Homozygous SNPs position in candidate region for spotted leaf.

Position of SNPs was based on the Nipponbare genome sequence, Build 5 ver. (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html).

Position	Status	Reference.	Query	Gene ID		Description
26012356	Difference	C	T			
26154389	Difference	C	T			
26297755	Difference	C	T			
26373075	Difference	C	T			
26495547	Difference	C	T			
26814370	Difference	C	T			
26977732	Difference	C	G	Os05g0541400	LOC_Os05g46370	Similar to Transcription factor LAX PANICLE.
26977757	Insert	-	G			Similar to Transcription factor LAX PANICLE.
26977758	Difference	C	G			Similar to Transcription factor LAX PANICLE.
26977762	Deletion	C	-			Similar to Transcription factor LAX PANICLE.
26989827	Difference	T	G	Os05g0541700	LOC_Os05g46395	Similar to predicted protein.
26989832	Deletion	T	-			Similar to predicted protein.
26989850	Difference	A	C			Similar to predicted protein.
26989857	Difference	A	G			Similar to predicted protein.
27369645	Difference	C	T			
27604871	Difference	C	T			

DISCUSSION

As a major organ of photosynthesis, properties of the leaf are important agronomic traits and taken interest through previous studies.

Moderate rolling leaf could enhance the light capture efficiency through maintaining the erection of leaf. Thus, leaf rolling is related to crop yield. In rice, many kinds of rolled leaf mutants and genes were identified but the entire mechanism regulated leaf rolling was not provided yet. In this study, we identified the mutant induced by EMS treatment from Ilpum. It is a new rolled leaf, especially abaxially rolling, mutant. Among the previous reported rolling leaf mutants, abaxial rolling types were less than adaxialy rolled types. Therefore, it can be helpful to elucidate the mechanism involved in leaf rolling and to understand the rolling pattern. Even though the mutant has defective phenotypes about yield, it could be a role in improving yield later in the future.

This rolled leaf mutant shows higher LRI compared with the wild-type and has significantly increased bulliform cell number and area than wild-type. Alteration of bulliform cell was supposed to be involved in the outward leaf rolling phenotype. Bulliform cells exist specifically in the adaxial side of leaves in gramineous plants[59] and located between two vascular bundle ridges. They can be easily observed with the cross sections of leaf. Bulliform cells modulate the leaf rolling depending on water stress[60]. Under water stress, leaves are rolling because bulliform cells lose turgor. Otherwise, bulliform cells absorb water and swell up again when water stress is relieved. The functions of bulliform cells in leaf rolling

are some doubts[61] and remains to be elucidated. However, this study could explore the role of leaf bulliform cells with previous studies.

F_1 plants derived from crossing between the mutant and Ilpum showed normal phenotype which reveals the rolled leaf mutant is controlled by a recessive gene. To identify the gene, BSA, genetic mapping and SNP analysis through NGS were conducted. SNP calling results and genetic mapping results were analyzed synthetically, and we could rapidly search more reliable mutation point. Three filtered homozygous SNPs were detected in candidate region. One of them found at splice site, named SNP3, was assumed more powerful mutation. The gene including SNP3 mutation point might be the candidate for this rolled leaf gene.

Spotted leaf mutants were studied to observe the effect of leaf color on growth, development and yield of plant by photosynthesis[14,26]. Moreover, to understand mechanisms of hypersensitive response, a large number of spotted leaf mutants were identified. The lesions of these mutants resemble the disease symptoms but were formed in absence of pathogens in rice[27,40].

Hypersensitive response is a type of programmed cell death (PCD). Therefore, this new spotted leaf mutant can be useful to identify the mechanism of PCD. In addition, this spotted leaf mutant has two kinds of spot together, which is unusual phenotype.

Some rice lesion mimic mutants have resistance of disease, like bacterial blight or blast disease, and show defense response gene expression. Moreover, three of these

cell death and resistance (cdr) mutants were observed to have elevated levels of the phytoalexin momilactone A and highly activated expression of two defense response genes, PBZ1 and PR1, in leaves showing lesions[35]. This mutant has the possibility of resistance to rice blast and bacterial blight disease.

F_1 plants derived from crossing between the mutant and Ilpum showed wild-type phenotype. This result indicates that the spotted leaf mutant is controlled by a recessive gene, likewise rolled leaf mutant. BSA, genetic mapping and SNP analysis through NGS were conducted to identify the gene. The spotted leaf gene was mapped on the long arm of chromosome 5 between two STS markers (S05105 and S05112, physical distance 2,2Mb). In flanking region, it was difficult in fine-mapping since there are a few polymorphism of sequence between Ilpum and Milyang23, parents of mapping population. Several homozygous SNP were detected in flanking region, however we cannot confirm which one is causal SNP due to broad flanking region and error of sequencing.

In this study, to search mutational point, F_2 bulk sequencing data and genetic mapping result were used. SNP analysis was conducted on the basis of MutMap method[58] slightly modified. Original MutMap pipeline is performed with various program and many step to pick up the causal SNP. However, we can easily and rapidly analyze the SNP data of the whole genome sequence and obtain the candidate mutation point, through only examining the SNP in candidate region.

This characterization and genetic mapping about leaf-related genes will help expanding the knowledge of rice leaf properties. Moreover, there are possibilities of cloning a new rolled leaf and spotted leaf genes. Further study including cloning and the functional study of these genes need to be performed, and then may reveal the mechanisms of the leaf rolling and spot.

REFERENCE

1. IRRI. International Rice Research Institute, World Rice Statistics 2013. (2013).
2. Duncan, W.G. Leaf Angles, Leaf Area, and Canopy Photosynthesis. *Crop Science* **11**, 482-485 (1971).
3. Lang, Y., Zhang, Z., Gu, X., Yang, J. & Zhu, Q. Physiological and ecological effects of crimpy leaf character in rice (Oryza sativa L.)II.Photosynthetic character,dry mass production and yield forming. *Zuo wu xue bao* **30**, 883-887 (2004).
4. Zhang, G.H., Xu, Q., Zhu, X.D., Qian, Q. & Xue, H.W. SHALLOT-LIKE1 is a KANADI transcription factor that modulates rice leaf rolling by regulating leaf abaxial cell development. *Plant Cell* **21**, 719-35 (2009).
5. Micol, J.L. & Hake, S. The development of plant leaves. *Plant Physiol* **131**, 389-94 (2003).
6. Hibara, K. *et al.* The ADAXIALIZED LEAF1 gene functions in leaf and embryonic pattern formation in rice. *Dev Biol* **334**, 345-54 (2009).
7. Fang, L. *et al.* Rolling-leaf14 is a 2OG-Fe (II) oxygenase family protein that modulates rice leaf rolling by affecting secondary cell wall formation in leaves. *Plant Biotechnol J* **10**, 524-32 (2012).
8. Khush, G.S. & Kinoshita, T. Rice karyotype, marker genes, and linkage groups. 83-108 (CAB International, Wallingford, 1991).
9. Li, S. *et al.* Genetic analysis and mapping the flag leaf roll in rice (Oryza sativaL.). *Journal of Sichuan Agricultural University* **16**, 391-393 (1998).
10. Shao, Y.-J. *et al.* [One major QTL mapping and physical map construction for rolled leaf in rice]. *Yi chuan xue bao = Acta genetica Sinica* **32**, 501-506 (2005).
11. Yan, C. *et al.* Genetic analysis and gene fine mapping for a rice novel mutant (rl 9(t)) with rolling leaf character. *Chinese Science Bulletin* **51**, 63-69 (2006).
12. Yan, S. *et al.* ROLLED LEAF 9, encoding a GARP protein, regulates the leaf abaxial cell fate in rice. *Plant Mol Biol* **68**, 239-50 (2008).
13. Luo, Z. *et al.* Genetic analysis and fine mapping of a dynamic rolled leaf gene, RL10(t), in rice (Oryza sativa L.). *Genome* **50**, 811-7 (2007).
14. Shi, Y. *et al.* Genetic analysis and gene mapping of a new rolled-leaf mutant in rice (Oryza sativa L.). *Science in China Series C: Life Sciences* **52**, 885-890 (2009).
15. Luo, Y.-Z. *et al.* Genetic Analysis and Gene Mapping of a Novel Rolled-Leaf Mutant rl12(t) in Rice. *Acta Agronomica Sinica* **35**, 1967-1972 (2009).
16. Shao, Y. Fine mapping of an incom-plete recessive gene for leaf rolling in rice (Oryza sativa L.). *Chinese Science Bulletin* **50**, 2466 (2005).

17. Li, L. *et al.* Isolation and characterization of rl (t), a gene that controls leaf rolling in rice. *Chinese Science Bulletin* **59**, 3142-3152 (2014).
18. Xiang, J.J., Zhang, G.H., Qian, Q. & Xue, H.W. Semi-rolled leaf1 encodes a putative glycosylphosphatidylinositol-anchored protein and modulates rice leaf rolling by regulating the formation of bulliform cells. *Plant Physiol* **159**, 1488-500 (2012).
19. Hu, J. *et al.* Identification and characterization of NARROW AND ROLLED LEAF 1, a novel gene regulating leaf morphology and plant architecture in rice. *Plant Mol Biol* **73**, 283-92 (2010).
20. Shi, Z. *et al.* Over-expression of rice OsAGO7 gene induces upward curling of the leaf blade that enhanced erect-leaf habit. *Planta* **226**, 99-108 (2007).
21. Zou, L.P. *et al.* Leaf rolling controlled by the homeodomain leucine zipper class IV gene Roc5 in rice. *Plant Physiol* **156**, 1589-602 (2011).
22. Zhao, S.Q., Hu, J., Guo, L.B., Qian, Q. & Xue, H.W. Rice leaf inclination2, a VIN3-like protein, regulates leaf angle through modulating cell division of the collar. *Cell Res* **20**, 935-47 (2010).
23. Li, L. *et al.* Overexpression of ACL1 (abaxially curled leaf 1) increased Bulliform cells and induced Abaxial curling of leaf blades in rice. *Mol Plant* **3**, 807-17 (2010).
24. Woo, Y.M. *et al.* Constitutively wilted 1, a member of the rice YUCCA gene family, is required for maintaining water homeostasis and an appropriate root to shoot ratio. *Plant Mol Biol* **65**, 125-36 (2007).
25. Wu, J.L. *et al.* Chemical- and irradiation-induced mutants of indica rice IR64 for forward and reverse genetics. *Plant Mol Biol* **59**, 85-97 (2005).
26. Zhang, S. *et al.* The interactions among DWARF10, auxin and cytokinin underlie lateral bud outgrowth in rice. *J Integr Plant Biol* **52**, 626-38 (2010).
27. Wu, C. *et al.* Rice lesion mimic mutants with enhanced resistance to diseases. *Mol Genet Genomics* **279**, 605-19 (2008).
28. Huang, L. *et al.* Down-Regulation of a SILENT INFORMATION REGULATOR2-Related Histone Deacetylase Gene, OsSRT1, Induces DNA Fragmentation and Cell Death in Rice. *Plant Physiology* **144**, 1508-1519 (2007).
29. Dietrich, R.A. *et al.* Arabidopsis mutants simulating disease resistance response. *Cell* **77**, 565-577 (1994).
30. Lorrain, S. Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? *Trends in Plant Science* **8**, 263-271 (2003).
31. Walbot, V., Hoisington, D. & Neuffer, M.G. Disease Lesion Mimic Mutations. in *Genetic Engineering of Plants*, Vol. 26 (eds. Kosuge, T., Meredith, C., Hollaender, A. & Wilson, C.) 431-442 (Springer US, 1983).

32. KIYOSAWA, S. Inheritance of a particular sensitivity of the rice variety, Sekiguchi Asahi, to pathogens and chemicals, and linkage relationship with blast resistance genes. *Nogyo Gijutsu Kenkyusho Hokoku = Bull. Nat. Inst. agric. Sci.* **21**, 61-72 (1970).
33. Yoshimura, A., Ideta, O. & Iwata, N. Linkage map of phenotype and RFLP markers in rice. *Plant Mol Biol* **35**, 49-60 (1997).
34. Yin, Z. *et al.* Characterizing Rice Lesion Mimic Mutants and Identifying a Mutant with Broad-Spectrum Resistance to Rice Blast and Bacterial Blight. *Molecular Plant-Microbe Interactions* **13**, 869-876 (2000).
35. Huang, Q.-n., Yang, Y., Shi, Y.-f., Chen, J. & Wu, J.-l. Spotted-Leaf Mutants of Rice (Oryza sativa). *Rice Science* **17**, 247-256 (2010).
36. Yamanouchi, U., Yano, M., Lin, H., Ashikari, M. & Yamada, K. A rice spotted leaf gene, Spl7, encodes a heat stress transcription factor protein. *Proc Natl Acad Sci U S A* **99**, 7530-5 (2002).
37. Zeng, L., Yin, Z., Chen, J., Leung, H. & Wang, G.L. Fine genetic mapping and physical delimitation of the lesion mimic gene Spl11 to a 160-kb DNA segment of the rice genome. *Mol Genet Genomics* **268**, 253-61 (2002).
38. Vega-Sanchez, M.E., Zeng, L., Chen, S., Leung, H. & Wang, G.L. SPIN1, a K homology domain protein negatively regulated and ubiquitinated by the E3 ubiquitin ligase SPL11, is involved in flowering time control in rice. *Plant Cell* **20**, 1456-69 (2008).
39. Wang, L., Pei, Z., Tian, Y. & He, C. OsLSD1, a Rice Zinc Finger Protein, Regulates Programmed Cell Death and Callus Differentiation. *Molecular Plant-Microbe Interactions* **18**, 375-384 (2005).
40. Takahashi, A. *et al.* Rice Pti1a negatively regulates RAR1-dependent defense responses. *Plant Cell* **19**, 2940-51 (2007).
41. Kim, J.A. *et al.* Rice OsACDR1 (Oryza sativa accelerated cell death and resistance 1) is a potential positive regulator of fungal disease resistance. *Mol Cells* **28**, 431-9 (2009).
42. Mori, M. *et al.* Isolation and molecular characterization of a Spotted leaf 18 mutant by modified activation-tagging in rice. *Plant Mol Biol* **63**, 847-60 (2007).
43. Qiao, Y. *et al.* SPL28 encodes a clathrin-associated adaptor protein complex 1, medium subunit micro 1 (AP1M1) and is responsible for spotted leaf and early senescence in rice (Oryza sativa). *New Phytol* **185**, 258-74 (2010).
44. Dietrich, R.A., Richberg, M.H., Schmidt, R., Dean, C. & Dangl, J.L. A novel zinc finger protein is encoded by the Arabidopsis LSD1 gene and functions as a negative regulator of plant cell death. *Cell* **88**, 685-94 (1997).

45. Lorrain, S. *et al.* Vascular associated death1, a novel GRAM domain-containing protein, is a regulator of cell death and defense responses in vascular tissues. *Plant Cell* **16**, 2217-32 (2004).
46. Balague, C. *et al.* HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. *Plant Cell* **15**, 365-79 (2003).
47. Hu, G., Yalpani, N., Briggs, S.P. & Johal, G.S. A porphyrin pathway impairment is responsible for the phenotype of a dominant disease lesion mimic mutant of maize. *Plant Cell* **10**, 1095-1105 (1998).
48. Ishikawa, A., Okamoto, H., Iwasaki, Y. & Asahi, T. A deficiency of coproporphyrinogen Ⅲ oxidase causes lesion formation in Arabidopsis. *Plant J* **27**, 89-99 (2001).
49. Mou, Z., He, Y., Dai, Y., Liu, X. & Li, J. Deficiency in fatty acid synthase leads to premature cell death and dramatic alterations in plant morphology. *Plant Cell* **12**, 405-18 (2000).
50. Brodersen, P. *et al.* Knockout of Arabidopsis accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes Dev* **16**, 490-502 (2002).
51. Kachroo, A. *et al.* Oleic acid levels regulated by glycerolipid metabolism modulate defense gene expression in Arabidopsis. *Proc Natl Acad Sci U S A* **101**, 5152-7 (2004).
52. Gray, J., Close, P.S., Briggs, S.P. & Johal, G.S. A novel suppressor of cell death in plants encoded by the Lls1 gene of maize. *Cell* **89**, 25-31 (1997).
53. Zeng, L.R. *et al.* Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-box/armadillo repeat protein endowed with E3 ubiquitin ligase activity. *Plant Cell* **16**, 2795-808 (2004).
54. Ji, H.S. *et al.* Characterization and mapping of a shattering mutant in rice that corresponds to a block of domestication genes. *Genetics* **173**, 995-1005 (2006).
55. Kariola, T., Brader, G., Li, J. & Palva, E.T. Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. *Plant Cell* **17**, 282-94 (2005).
56. Mahalingam, R. *et al.* Analysis of oxidative signalling induced by ozone in Arabidopsis thaliana. *Plant Cell Environ* **29**, 1357-71 (2006).
57. Michelmore, R.W., Paran, I. & Kesseli, R.V. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc Natl Acad Sci U S A* **88**, 9828-32 (1991).

58. Abe, A. *et al.* Genome sequencing reveals agronomically important loci in rice using MutMap. *Nat Biotechnol* **30**, 174-8 (2012).
59. Swallen, J.R. Anatomy of Monocotyledons. vol. 1, Gramineae. Charles Russell Metcalfe. Oxford University Press, New York, 1960. lxi + 731 pp. Illus. $13.45. *Science* **133**, 1817-1818 (1961).
60. Price, A.H. & Tomos, A.D. Genetic dissection of root growth in rice (Oryza sativa L.). *Theoretical and Applied Genetics* **95**, 143-152 (1997).
61. Ristic, Z. & Cass, D.D. Morphological Characteristics of Leaf Epidermal Cells in Lines of Maize that Differ in Endogenous Levels of Abscisic Acid and Drought Resistance. *Botanical Gazette* **152**, 439-445 (1991).

초 록

벼에서 잎이 말리는(rolled leaf) 돌연변이와 잎에 반점이 생기는(spotted leaf) 돌연변이의 유전자 지도 작성

잎은 광합성, 호흡, 증발 등 작물이 생육하는 데에 필요한 여러 가지 역할들을 하는 기관이다. 따라서, 잎의 형질에 관련된 유전자를 찾고, 그 유전자의 작용 원리를 밝히는 것은 이상적인 작물을 육종하는데 도움이 될 수 있다.

본 연구에서는 일품벼에 EMS 를 처리 하여 얻은 잎에 관련된 두 종류의 돌연변이체를 실험 재료로 사용하였다. 그리고 이들의 표현형 특성을 검정하고, 유전자 지도를 작성 하는 것을 목표로 하였다.

잎이 말리는 돌연변이는 기동세포의 수와 그 영역이 증가되어 있었다. 또한, 잎에 반점이 생기는 돌연변이는 생육 초기부터 갈색반점과 흰색반점이 같이 관찰되는 특징을 보였다.

F_2 집단의 분리비가 3:1 로 나타나는 것으로 보아 돌연변이의 표현형은 단일열성유전자에 의해 조절되는 것을 알 수 있었으며, STS marker 를 이용해서 BSA 를 수행한 결과 각 돌연변이체를 조절하는 유전자는 염색체 2 번과 5 번에 위치하고 있는 것으로 확인되었다.

유전자의 정확한 위치를 찾기 위해서 Fine-mapping 을 수행하였다. 그리고 NGS(차세대 염기서열 분석)를 통해 얻어진 Whole-genome sequence 정보를 이용해서 SNP 분석을 수행하였다. Fine-mapping 결과와 SNP 분석 결과를 종합하여 후보유전자를 찾을 수 있었다.

해당 실험의 결과는 차후에 잎이 말리는 과정과 잎에 반점이 생기는 과정을 연구 하는데 있어서 도움이 될 것으로 생각한다.

주요어 : 잎 말림 돌연변이, 잎 반점 돌연변이, BSA, 기동세포, 차세대 염기서열 분석, 유전자 지도 작성

학번 : 2013-23228

THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Genetic Mapping of Rolled Leaf and Spotted Leaf Mutants in Rice

BY
HYERIM LEE

AUGUST, 2015

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRAUDUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

Genetic Mapping of Rolled Leaf and Spotted Leaf Mutants in Rice

HYERIM LEE

ABSTRACTS

Leaves are the organ for photosynthesis, respiration and transpiration, and have a major effect on growth and development related to crop yield. Therefore, morphological characteristics of leaf such as structure, shape and color are important agronomic traits in rice (*Oryza sativa L.*) breeding for ideal plant type.

To understand the genes related to leaf and those of regulations, we obtained two new leaf-related mutants, rolled leaf mutant and spotted leaf mutant, from Ilpum (*Oryza sativa japonica*) by the treatment of ethyl methane sulfonate (EMS). The rolled leaf mutant was observed to have increased bulliform cell number and size, and led to the outcurved leaf rolling. The spotted leaf mutant displayed brown and white spots from early vegetative stage.

The phenotypes of the F_1 plants derived from the cross between these mutants and wild-type plants were normal. In each F_2 population, segregation ratio between the

wild-type and the mutant type was 3:1. This genetic analysis indicated that each mutant phenotype was controlled by single recessive gene.

Bulked segregant analysis (BSA) and genetic mapping were conducted using F_2 population developed from the cross between each of mutants and Milyang23 (*Oryza sativa indica*). According to the results, the rolled leaf gene was located on the long arm of chromosome 2 between the flanking markers 2125f and 2126b (122kb) and the spotted leaf gene was located on the long arm of chromosome 5 between the flanking markers S05105 and S05112 (2.2Mb).

In order to search for candidate genes and causal SNP positions rapidly, whole-genome sequencing data of Ilpum and bulked F_2 progenies displaying the mutant phenotype were used. Through the combination of the BSA and SNP analysis, two candidate genes of rolled leaf mutant and a few candidate SNP positions of spotted leaf mutant were identified. This study will contribute to the further study of leaf-related mechanism in rice, and confirmation of each candidate genes is in progress.

Keywords: Rolled leaf, Bulliform cell, Spotted leaf, ROS staining, Genetic mapping, Next generation sequencing.

Student number: 2013-23228

CONTENTS

LIST OF ABBREVIATIONS

BSA	Bulked segregant analysis
dCAPS	Derived cleaved amplified polumorphic sequence
EMS	Ethyl methane sulfonate
INDEL	INsertions and DELetions
lv	Large vascular
M23	Milyang23
mut	Mutant
NGS	Next generation sequencing
NICS	National Institute of Crop Science
PCR	Polymerase Chain Reaction
RDA	Rural Development Administration
rl	Rolled leaf mutant
SNP	Single nucleotide polymorphism
spl	Spotted leaf mutant
STS	Sequence-tagged Site
sv	Small vascular
WGS	Whole genome sequencing
WT	Wild-type

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Rice is one of the most important food crops in the world and the model plant of monocotyledons. Many kinds of *Oryza sativa* cultivars are grown in more than 100 countries and an estimated 3.5 billion people worldwide, approximately half of the world population, considers that rice (*Oryza sativa L.*) is the staple food[1]. Production and consumption per capita of rice are rising steadily in many counties (such as sub-Saharan Africa, Caribbean and Latin America regions) and rice is the major source of nutrient especially for about 520 million people living in Asia[1]. Furthermore, the genome sequencing project of rice (http://www.rgp.dna.affrc.go.jp/) was conducted firstly among field crops as monocotyledonous model plant that have greatly contributed to genetic study and breeding. The Green Revolution had influenced on increasing the yield of rice. Nevertheless, researches about ideal plant type still need to meet the demand of rice in growing continuously.

Leaf characteristics are important agronomic traits because leaf is the organ of photosynthesis, transpiration, respiration. Leaves play a crucial role in growth and development of plant. To understand the regulatory mechanism of leaf properties, such as shape, development, color, and senescence, many researches were conducted in rice.

Above all, appropriate leaf morphology is an important for high yield in breeding of rice. Moderate leaf rolling contributes to the erectness of leaves, which can increase the light transmission rate and leads to higher photosynthetic efficiency[2-4]. Therefore, the study of rolling leaf and isolation of the genes that controls its phenotype will be a help to elucidate the mechanism of leaf rolling and to breed with ideal type plant with ideal type of plant.

According to the previous study, development progresses of leaf including determination of polarity and cell differentiation are supposed to major reasons of rolling leaf[3-7]. Furthermore, various types of leaf rolling were reported with direction of rolled leaves as inward or outward and degree of rolling; slightly, moderately, cylindrically rolled leaves. Some mutants and genes related to leaf rolling have been reported in rice. However, only a few genes were cloned and studied those function.

The genes of rolled leaf mutants, *rl1*, *rl2*, *rl3*, *rl4*, *rl5*, *rl6* were located on chromosomes 1, 4, 12, 1, 3, and 7 through conventional genetic analysis (http://www.gramene.org/) [8], and *rl7- rl14* were mapped on molecular map with chromosome 5 (*rl7* [9], *rl8* [10]), 9 (*rl9* [11,12], *rl10* [13]), 7(*rl11* [14]), 10 (*rl12* [15], *rl14* [7]) in rice. Most of them were assumed single recessive genes that controlled the traits. However, some mutants were controlled by quantitative trait loci (QTLs) and *rl(t)* located on chromosome 2 was an incomplete recessive gene[16,17].

In rice, *SHALLOT-LIKE1* (*SLL1*) encodes a SHAQKYF class MYB family transcription factor belonging to the KANADI family[4]. Mature *sll1* mutants showed extremely incurved leaves due to the regulation of the development for

abaxial side cells in leaf. Mutational *SLL1* results in defective programmed cell death of abaxial mesophyll cells and suppresses bulliform cell and sclerenchyma development on abaxial side. The *rl14* mutant had incurved leaves and shrunken bulliform cells on the adaxial side[7]. Expression of *RL14*, encoding a 2OG-Fe (II) oxygenase, affected the composition of the secondary cell wall. *SEMI-ROLLED LEAF1* (*SRL1*) which encodes a putative glycosylphosphatidylinositol-anchored protein, regulates the expression of genes encoding vacuolar H^+-ATPase subunits and H^+-pyrophosphatase. A defect of *SRL1* characterized the increased number of bulliform cells[18]. Defect of *NARROW AND ROLLED LEAF1* (*NAR1*), encoding the cellulose synthase-like protein D4 (OsCslD4), results in semi-rolled leaves by decreasing of bulliform cells size[19]. Furthermore, overexpression of the rice *OsAGO7* gene, orthologous to the Arabidopsis *ZIP/Ago7* gene, induces upward adaxial curling of the leaf blade[20].

Several abaxially rolled leaf mutants and the corresponding genes have been identified and analyzed. *Rice outermost cell-specific gene5* (*Roc5*) encodes a homeodomain (HD) leucine zipper class IV homeobox transcription factor. The *oul1* mutant, defect of *Roc5*, are showing abaxially rolled leaf with increased number and size of bulliform cells, whereas the transgenic plants overexpressing *Roc5* present adaxially rolled leaves[21]. Defective rice *leaf inclination2* (*LC2*), a VIN3-like protein, is also observed reversely rolled leaves[22]. Overexpression of *Abaxially Curled Leaf1* (*ACL1*) appeared abaxial curling of leaf blades due to increased bulliform cell number and size, and *ACL2* is its homolog in rice[23]. *ADAXIALIZED LEAF1* (*ADL1*) encodes a calpain-like cysteine proteinase which is associated with the maintenance of axis information in leaf[6]. The *adl1* mutant has

abaxially rolled leaves because of the ectopical formation of bulliform-like cells at the abaxial side. Therefore, histological properties including the size and number of bulliform cells could play an important role in leaf rolling.

In addition, Environmental effects were also involved in leaf rolling. *CONSTITUTIVELY WILTED 1* (*COW1*) encodes a member of the rice YUCCA gene family, and leaves of oscow1 mutant plants rolled upwardly[24].

In rice, various morphological changes of leaf are commonly observed in varieties and mutants induced by mutagens[25]. Leaf color is another important traits influenced on growth, development and yield of plant through photosynthesis[14, 26]. Therefore, researches about leaf color including spot, stripe, albino, chlorine were performed and then that will be a help to identify its genes regulating leaf color. Among these variations, spotted leaf mutants were reported considerably.

The lesions of the spotted leaf mutants usually appear at seedling to tillering stage firstly whereas some of them observed in other growth stage[27,28]. The spots are usually brown color, such as reddish brown and dark brown. The spotted leaf mutants generated lesions without abiotic and biotic stresses, however the lesions are similar to those caused by pest damage or pathogen infection. Therefore, these mutants are often termed lesion mimic or lesion simulating disease mutants[29,30]. Usually, cell necrosis of the spotted leaf mutants is similar to that resulted from hypersensitive response (HR). HR is a symptom of programmed cell deaths (PCD) induced by pathogen invasion[31], but cell death of spotted leaf mutants showed

without pathogen infection. To study PCD mechanisms and analyze defense gene expression, lesion mimic mutants are useful.

To date, 17 rice *spl* mutants (*spl1–11* and *bl1–6*) have been registered in the Rice Genetics Cooperative, and the genetic controls of 51 rice spotted-leaf mutants have been descripted[27,32-35]. Among the 51 *spl* mutants, most of them (42 mutants) are controlled by single recessive genes, one controlled by two recessive genes, and 8 mutants are regulated by single dominant genes (reviewed by Huang et al., 2010 [35]).

Many kinds of lesion mimic mutant were reported, but only several genes were cloned and most of them were remained unmapped. The *spl7* was the first spotted leaf gene to be cloned and encodes a heat stress transcription factor[36]. *Spl11* is a negative regulator of plant cell death defense due to encoding a U-Box/Armadillo repeat protein and manipulating flowering[37,38]. Furthermore, *spl11*, *OsPti1a*, *OsSRT1*, and *OsACRD1* are found to be responsible for spot initiation[39-41]. *Spl18* is related to seedling regeneration[42], and then *spl28* is involved in senescence[43]. Other *spl* genes encode various proteins and enzymes; zinc finger proteins[39,44], heat stress transcription proteins[36], membrane-associated proteins [45], ion channel regulators [46] and components in biochemical pathways responsible for metabolism of porphyrins [47,48], fatty-acids/lipids [49-51], phenolics[52], ubiquitination[53]. Those studies of the *spl* mutants and gene in rice were signified that multiple genetic mechanisms involved in lesion mimic phenotypes.

In the present study, a new rolled leaf mutant and spotted leaf mutant induced by chemical mutagen, ethyl methane sulfonate (EMS), was characterized. The objectives of this study were phenotypic characterization, genetic analysis and

mapping of these leaf-related mutants and identification of the candidate genes for leaf rolling and spotting.

MATERIALS AND METHODS

Plant materials

A rolled leaf mutant and spotted leaf mutant were induced by the treatment of ethyl methane sulfonate (EMS) from Ilpum, *japonica* cultivar. Each of the mutants was selected from M_2 generation and fixed in pure line by repetitive selfing. The pedigree of rolled leaf mutant is Ilpum E-B-572-1-B-2-B-B, and spotted leaf mutant is Ilpum E-B-319-1-2-2-1-1-1.

For genetic analysis and whole genome sequencing, mutants were crossed with its wild-type plant and F_2 populations were developed from these F_1. Mapping populations were derived from the cross between mutants and Milyang23, *indica* cultivar, and were used for BSA and genetic mapping.

All mutants and populations were grown in the experimental field and green house of Seoul National University in Suwon.

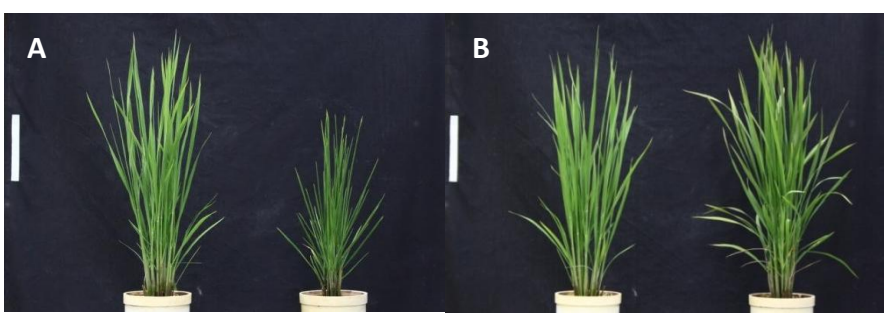


Figure 1. Phenotype of wild-type and mutants at vegetative stage.
(A) Plants of wild-type (left) and *rl* (right). (bar = 20cm)

(B) Plants of wild-type (left) and *spl* (right). (bar = 20cm)

Paraffin section for histological analysis of rolled leaf

Paraffin-embedded section was done according to the method of Ji et al. (2006)[54]with slight modifications. Leaf samples from the middle portion of leaves were collected at late vegetative stage and fixed in FAA solution(ethanol 50%, acetic acid 5%, formaldehyde 3.7%) and stored at 4℃ for overnight after vacuumed (3cycles of 5min each) using IR concentrator (Micro-Cenvac, NB-503CIR, N-Biotek).

The fixed leaves were dehydrated by soaking in a graded ethanol series (60%, 70%, 85%, 95%) each for 2 hours and incubated in 100% ethanol overnight. The samples were cleared by soaking for 2 hours in the clearing solution series consisting of ethanol : histoclear 3:1, 1:1, 1:3 in order and then soaked in 100% histoclear overnight. For paraffin infiltration, the leaf samples were soaked in the solution series of histoclear : paraffin 3:1, 1:1, 1:3 for 2 hours in order and 100% paraffin at 55℃ overnignt.

The infiltrated samples were embedded in a paraffin block and then cut into 8~20 ㎛ sections using a microtome (MICROM Lab, Walldorf, Germany). The sections were mounted on a Superfrost-plus glass slides (Fisher Scientific, Pittsburgh, PA, USA) coated with egg albumin solution (sodium salicylate 1g, egg white 50ml, glycerol 50ml)and dried at 42℃ for 1day. The sections were deparaffinized with 100% xylene for 1 hour followed by hydration by soaking in xylene : ethanol 1:1, 100% ethanol, and sterile water for 2min each. The sections were stained with 0.1% toluidine blue solution for ~30sec and washed with sterile water. For destaining, the slides with sections were soaked in 30%, 50%, 70%, 85%,

95% ethanol for 2 min in order. Finally, the slides were soaked in 100% xylene for 10 min and mounted in Canada balsam. The cross sections of leaf were observed and photographed at 40~300X magnification to measure the bulliform cell number and size.

Measurement of Leaf Rolling Index (LRI) and Leaf Erection Index (LEI) of rolled leaf

To determine the Leaf Rolling Index (LRI), the widths of the flag and second leaves at the heading stage were measured under either the natural (Ln) or unfolding state (Lw). The LRI was calculated as $LRI(\%) = (Lw - Ln)/Lw \times 100$.

The length of the leaf blade between the lamina joint and the tip in the natural (Lnl) and straightened situation (Lsl) were measured, and the LEI was calculated as $LEI(\%) = Lnl/Lsl \times 100$. The data of Lnl and Lsl were collected from flag and second leaves at heading stage, too.

Measurement of soil plant analysis development (SPAD) value of spotted leaf

To verify the physiological characteristics of the spotted leaf mutant, the amount of chlorophyll present in plant leaves was measured using Minolta Chlorophyll Meter SPAD-502 (Minolta Camera Co., Japan) and was designated the SPAD value.

SPAD value was measured on the day of flowering, and then again every 5 days until 20 day after flowering in three spots (near the tip, basal half and near the basal) of flag and second leaves from five plant replicates.

Detection of ROS accumulation of spotted leaf

ROS (Reactive Oxygen species) accumulation was determined by staining method described previously[55,56] with some modifications. Upper three leaves, from top to bottom, were used in staining.

For superoxide anion(O_2^-) detection, leaf samples were vacuumed (three cycles of 5 min each) and infiltrated in 0.5 mg/ml nitro blue tetrazolium (NBT) in 10mM potassium phosphate buffer (pH 7.8) for 20 h in the dark. For H_2O_2 determination, leaf samples were vacuum-infiltrated (three cycles of 5 min each) in 1 mg/ml 3,3′-diaminobenzidine (DAB) containing 10mM MES (pH 6.5) for 22h in the dark. Both reactions were performed until chlorophyll was completely removed and were stopped by transfer to 90% ethanol at 70℃. Following incubation period, the cleared leaves were stored and photographed in 70% glycerol.

Genetic mapping

Two F_2 mapping populations from the cross between rolled leaf mutant and Milyang23, spotted leaf mutant and Milyang23 were used.

For genetic mapping, BSA strategy[57] was done first. 10 mutant type and 10 wild-type plants based on their phenotype were selected from each of F_2 mapping

populations. Genomic DNA were extracted from young leaves by CTAB method. Equal concentration of DNA from individual plants which showed same phenotype were pooled into two bulks.

Total 92 STS markers of known chromosomal position throughout all chromosomes, which were designed in Crop Molecular Breeding Laboratory, Seoul National University, were tested and then the co-segregation markers were identified with these bulks. After BSA, phenotype and genotype of F_2 mapping population were used to determine gene position on chromosome and to conduct fine mapping. Some contiguous STS markers and additionally designed STS markers, based on the differences in sequences between the *japonica* and *indica* rice subspecies, were used.

PCR was performed in a total reaction volume of 20 ㎕ containing 100ng of template DNA, 10pM of each forward and reverse primer, 250μM of dNTP, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin and 0.5U of Taq DNA polymerase (made by laboratory). Amplication were performed in a PCT100 96U Termocycle (MJ Resaerch, Reno, NV, USA) in the following sequence : 5min at 95℃, followed by 35cycles of 30sec at 95℃, 30sec at 56℃, 30sec at 72℃, 10min at 72℃ for final extension and 10min at 10℃. PCR products were separated in 3.0% agarose/0.5X TBE gels and visualized by ethidium bromide staining.

Whole genome sequencing

Genomic DNA of F_2 population derived from the cross between each of mutants and wild-type was extracted from young leaves by CTAB method, and then it was pooled from several individuals (n=20, > 5 ㎍) showing the clear mutant phenotype among the progeny by equal ratio. For SNP analysis, the bulked DNA was sequenced using an Illumina HiSeq2500, and raw data of whole-genome sequence for Ilpum from NICS of RDA was also used.

DNA libraries for Illumina HiSeq2500 sequencing were prepared using the kits (Truseq Nano DNA LT sample preparation kit, FC-121-4001). The qPCR was conducted using these libraries, and then amplified clonal clusters were generated and performed paired-end sequencing using Illumina HiSeq2500 (250 cycles). Base calling was carried out by the instrument control software's Real Time Analysis (RTA).

Alignment of short reads to reference sequences and SNP and InDel calling

Whole genome sequencing and SNP analysis were performed to identify the mutation point generated by mutagen. 76 million and 83 million paired-end short reads from F_2 bulks DNA was obtained and these data preprocessing were carried out using btrim 0.2.0 (http://graphics.med.yale.edu/trim/). The filtering options of low-quality bases were cut off phred-score 20 and minimum read length 50. Processed short reads were mapped and aligned to public Nipponbare genome sequence, Build 5 ver. (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html)

with clc_ref_assemble (V 4.21.104315). whole-genome sequence for Ilpum from NICS of RDA was also filtered and mapped to Nipponbare in the same way. Subsequently, SNP detection and SNP calling were performed by find_variations (V 4.21.104315). SNP filtering options are read depth of the site ≥ 5, variant frequency of major base ≥ 0.9.

After mapping and SNPs calling, causal SNPs and InDels involved in phenotype of mutants were detected by following, modified MutMap method[58]. ⅰ) Remove the same SNPs and InDels in wild-type and mutant. ⅱ) Remove the common SNPs and InDels shared by at least two mutants lines. ⅲ) Calculate SNP index. ⅳ) Pick homozygous SNPs and InDels (SNP index=1) located in annotated gene region. SNP index is the ratio between the number of reads of a mutant SNP and the total number of reads at the each position.

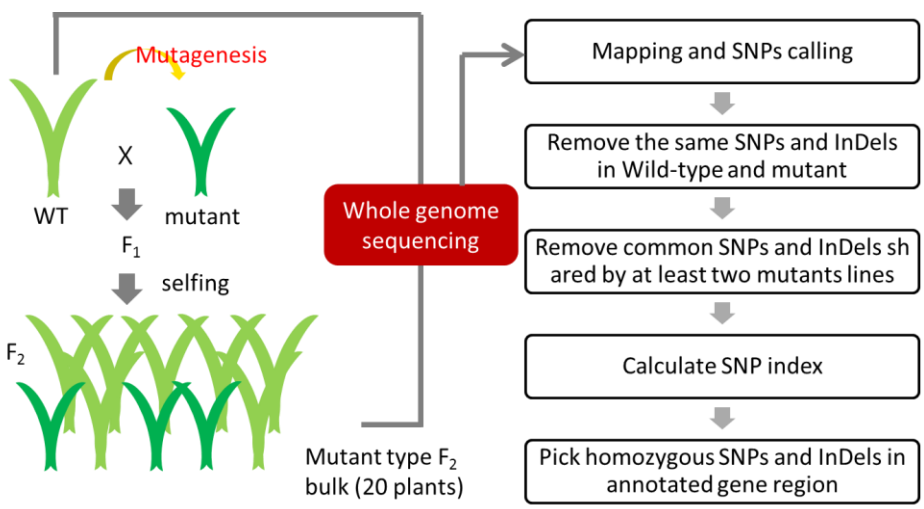


Figure 2. SNPs and InDels calling work-flow from whole genome sequencing data.

RESULTS

1. Rolled leaf mutant

Characterization of rolled leaf mutant in rice

The rolled leaf mutant plants appeared spiral shaped leaves at the seedling stage (Fig. 4 A). Distinction between mutant and wild-type plants in rolling of leaves became clearly evident as they grew. After the tillering stage, the leaves of the mutant plants showed outward rolling and cylindrical shape (Fig.4 B,C).

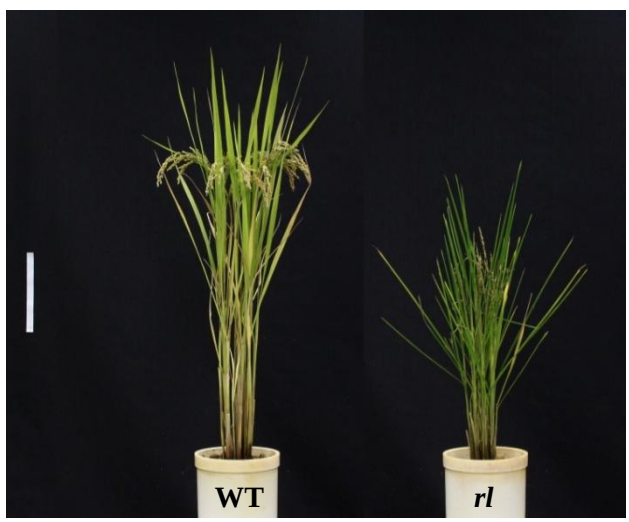


Figure 3. Phenotype of wild-type and rolled leaf mutant at ripening stage. (bar = 20cm)

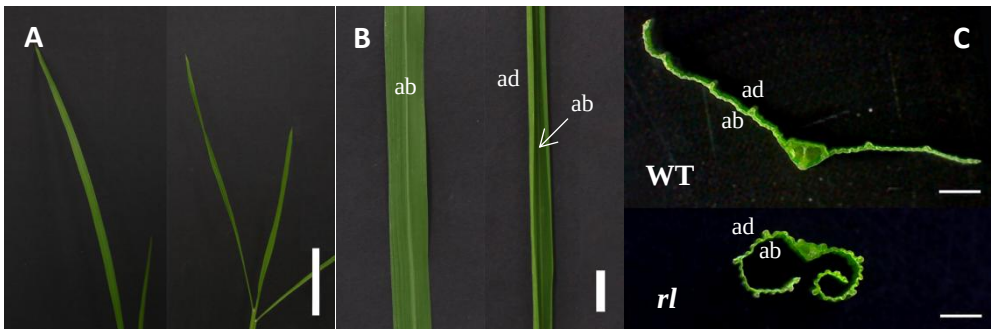


Figure 4. Leaf morphological characteristics of wild-type and rolled leaf mutant.

(A) *rl* (right) leaves showed spiral but WT (left) leaves were flat at early vegetative stage. (bar = 5cm) (B) *rl* (right) leaves rolled abaxially at late vegetative stage (bar = 1cm) and (C) showed cylinder-like shape in mature plants. (bar = 1mm) ab, abaxial side; ad, adaxial side.

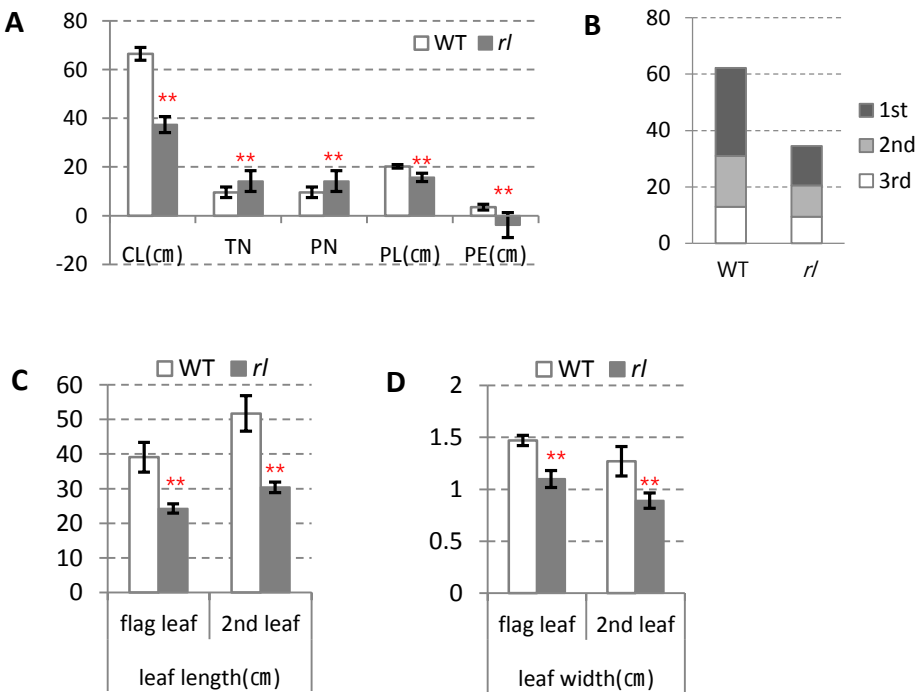


Figure 5. Agronomic traits of wild-type and rolled leaf mutant.

Comparison of the (A) culm length(CL), tiller number(TN), panicle number(PN), panicle length(PL), panicle exsertion(PE), (B) inter node length, (C) leaf length and (D) leaf width of flag and second leaves between wild-type and *rl*. Error bar represents SD (n>10) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01).

Agronomic traits were compared between the mutant and wild-type. The results showed that the mutant was deficient in most of them. The abaxially rolled leaf mutant showed smaller culm length, leaf length and leaf width than Ilpum, its wild type. Panicle length and exsertion were also reduced in case of rolled leaf mutant compared to the wild-type (Fig. 5). Grain length, width, fertility and 1000 grains weight of rolled leaf mutant were decreased, whereas tiller number and panicle number of the mutant were increased compared to the wild-type (Fig. 6, Table 1).

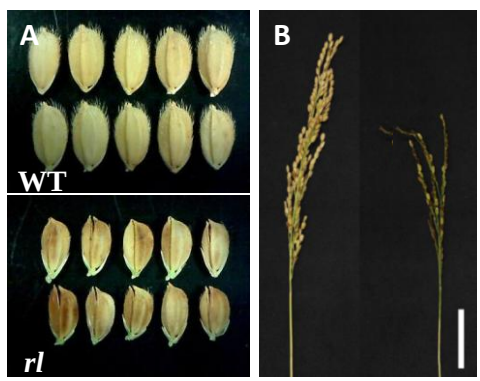


Figure 6. Grains and panicle of wild-type and rolled leaf mutant.
(A) Grain shape of wild-type and *rl*. (B) Panicle of wild-type (left) and *rl* (right). (bar = 5cm)

Table 1. Comparison of agronomic traits between wild-type and rolled leaf mutant.

	HD	GL(mm)	GW(mm)	1000 grain weight(g)	Grain fertility(%)
Wild-type	24-Aug	5.53±0.18	3.33±0.15	25.97±0.09	82.15±7.98
Mutant	28-Aug	4.68±0.15	2.83±0.13	11.56±0.26	2.93±2.45
Comparison	-	**	**	**	**

Values are mean ± standard deviation. Comparison of the heading date in 2014(HD), grain length(GL), grain width(GW), 1000 grain weight, grain fertility of wild-type and *rl*. Data show means and SD of biological replicates (n>10, n>5) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01, NS: not statically significant).

Histological analysis

According to previous studies, the leaf rolling, especially orientation of that, was influenced by alterations of bulliform cell. Paraffin-embedding sectioning of the leaves was carried out to investigate the change about cell differentiation.

In cross section analysis from mature 9th leaves, significant differences in number and size of bulliform cell were observed between the mutant and the wild-type (Fig. 8). The rolled leaf mutant had 7.31 bulliform cells between two vascular bundle ridges, whereas the wild-type bulliform cells were 4.49 cells (Fig. 7 A). Moreover, the average of bulliform cell area of the mutant (4595.90μm^2) was larger than that of the wild-type (2457.06μm^2) (Fig. 7 B). Bulliform cell number and area were increased in the rolled leaf mutant. Therefore, it was assumed that the abaxially rolled leaf phenotype of the mutant was resulted from altered bulliform cell number and area.

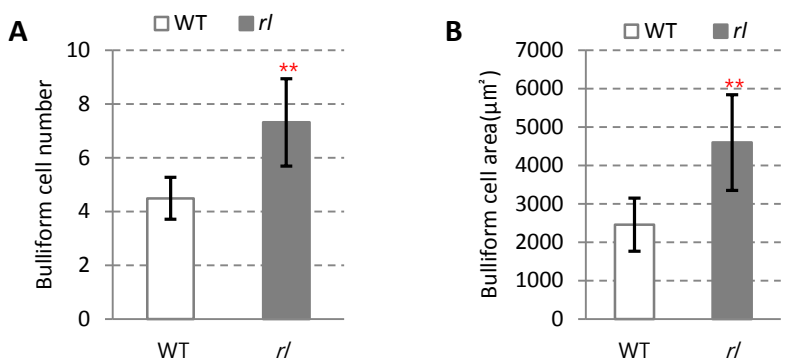


Figure 7. Rolled leaf mutant has increased bulliform cell number and area compare to wild-type.
9th leaf blades of *rl* show significantly increased (A) bulliform cell number and (B) area. Error bar represents SD (n>35) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01).

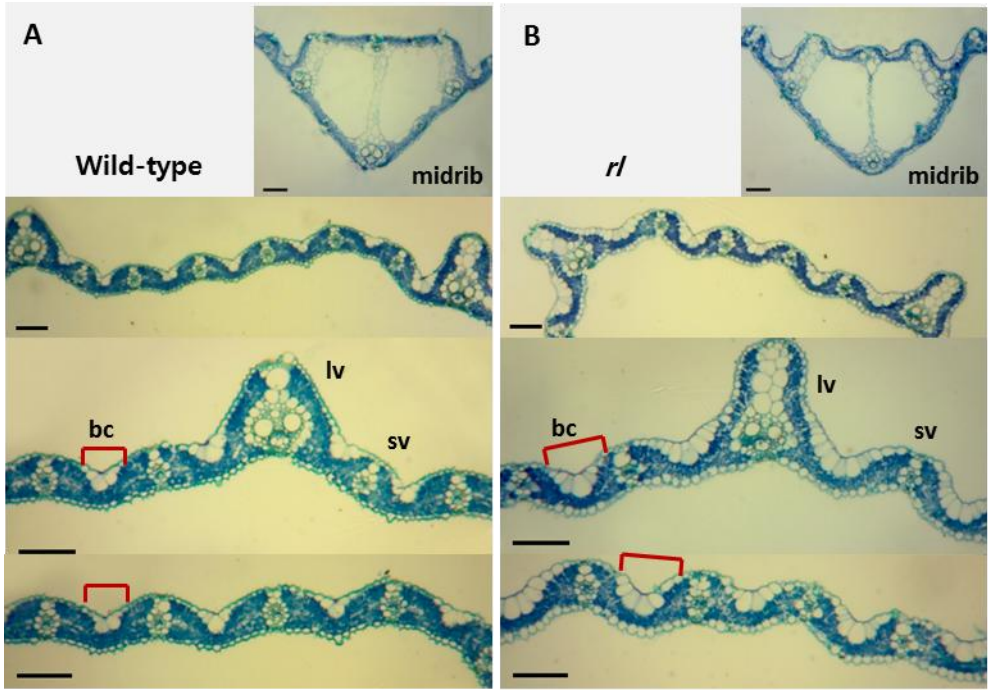


Figure 8. Leaf cross-section of wild-type and rolled leaf mutant.
Bulliform cell number and area between vascular bundle ridges were significantly increased in *rl*. Red lines indicate the bulliform cells. (bar = 100μm). bc, bulliform cells; lv, large vascular; sv, small vascular.

Leaf Rolling Index (LRI) and Leaf Erection Index (LEI)

LRI analysis showed that the mutant have evidently high LRI value compared to the wild-type. At the late vegetative stage, the LRIs of the flag and second leaves of the rolled leaf mutant reached to approximately 47% and 60%, whereas wild-type leaves were almost flat (Fig. 9 A).

On the other hand, the LEIs of the same leaves of the mutant were observed similar to that of wild-type (Fig. 9 B). This result was due to the erectness of flag and second leaves at the heading stage. Moreover, Ilpum had erected leaves so LEIs

were not different or slightly different between the rolled leaf mutant and the wild-type.

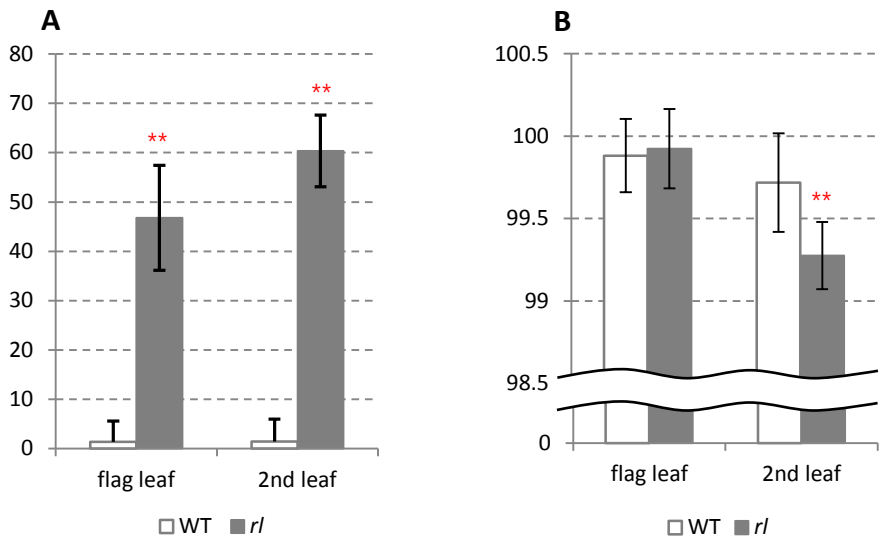


Figure 9. Comparison of the LRI and LEI of wild-type and rolled leaf mutant. (A) LRI and (B) LEI of wild-type and *rl*. Error bar represents SD (n>10) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01).

Genetic analysis of rolled leaf mutant in rice

The phenotypes of the F_1 plants derived from the cross between the mutant and Ilpum were normal. In F_2 population, segregation ratio between the wild-type and the mutant-type was 3:1 (Table 2). This genetic analysis indicated that leaf rolling is controlled by single recessive gene.

Table 2. Segregation ratio of F_2 population developed from the cross between the rolled leaf mutant and Ilpum.

Total	Normal	Mutant	X^2 (3:1) ($p=0.05$, $\chi^2=3.841$)
185	149	36	3.029

Genetic mapping of rolled leaf mutant in rice

Bulked segregant analysis (BSA) and genetic mapping were conducted using F_2 population derived from the cross between mutant and Milyang23 (Oryza sativa indica). According to the BSA results, the gene was located on the long arm of rice chromosome 2 between two flanking markers, S02109 and S02126 (Fig. 10).

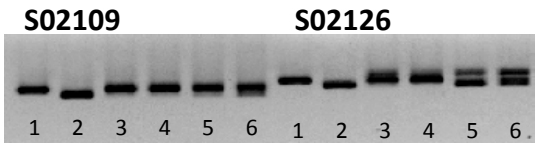
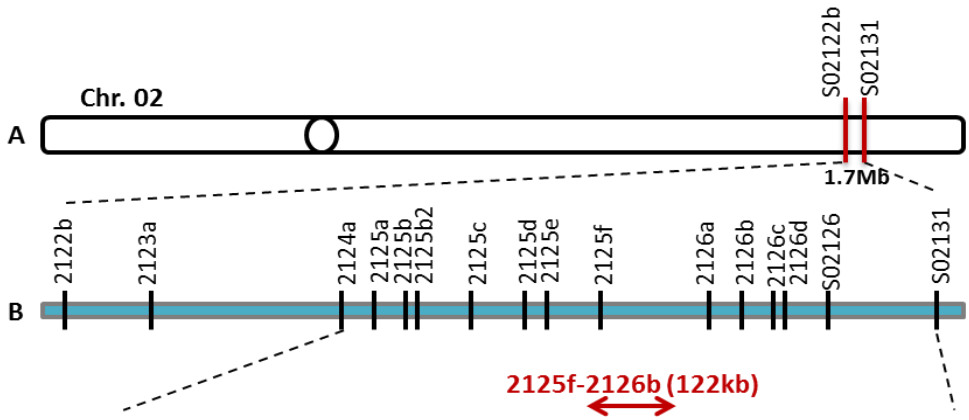


Figure 10. Bulked Segregant Analysis (BSA) of wild-type and rolled leaf mutant bulks from F_2 population.
1, *rl*; 2, Milyang 23; 3,4, Mutant bulks; 5,6, Wild-type bulks.

Additional 13 STS markers (Table 3), which were designed based on the variation of genome sequence from database (http://www.ncbi.nih.gov/ and http://www.rgp.dna.affrc.go.jp/), were located between two flanking markers and used in narrowing down the candidate region for rolled leaf gene. Fine- mapping results showed that 2125f and 2126b markers were closely linked to locus of rolled leaf gene and physical distance of them were estimated to be 122kb (Fig. 11). In this candidate region, total of 25 genes were located.

Table 3. The PCR-based additionally designed molecular markers for genetic mapping of the rolled leaf mutant.

STS marker	Forward primer (5′-3′)	Reverse primer (5′-3′)
2123a	ATTCGAGGGAGAAGGGAATC	AACTTGCAGGAGGAGACCAA
2124a	ATTTGTTTGACGGCCAAAAG	TGAAGTCGAAGCCTTTGAGG
2125a	TGCGTGTCGATTCCTTATTTT	AACCCGCCAGCCTATTACTC
2125b	CCTGCTCTTAAGGTGGTGGA	GATGAGCTAGCTGGCCTTGT
2125b2	CCTCAGCTTTGTCATTTCCAG	AGTGGCTCACATTGCTGATG
2125c	AGGCCCCGATTTAGCTTAGA	CTTCCAATTTGGCGAGGTAA
2125d	CAGGTCCTAAAAGGACAGCA	CTGGCCGTGAGGTAATTGTT
2125e	TTCAATTCGTTGTGCGTTGT	GGAGCCAGTTAGAAGGAAAGAA
2125f	AATCCGTCGATTTGTGTGTG	AACGTGCAATCATCCCATTC
2126a	TGCATGTGTGATGAGGACTG	CGGTGACACCCTTTAGAAGTC
2126b	TGGTTAGGTTTTTGACGATGAA	TAAATGCATGCGCTCTCAAG
2126c	TAGGGTGCAGTGGAATAGCC	AGGAACTGGGAAGCTGAAGG
2126d	TCCCGCTTGTACCGAATTAC	CGAAGGGTCACAGGACTCAC



	2124a	2125a	2125b	2125b2	2125c	2125d	2125e	2125f	2126a	2126b	2126c	2126d	S02126	S02131
W	H	H	H	H	H	H	H	H	H	M	M	M	M	M
M	H	H	H	H	M	M	M	M	M	M	M	M	M	M
W	M	M	M	M	M	H	H	H	H	H	H	H	H	H
M	H	H	H	H	H	H	H	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	M	M	M	M	M	H	H
M	H	H	H	H	H	H	H	H	M	M	M	M	M	M
M	H	H	M	M	M	M	M	M	M	M	M	M	M	M
M	H	H	H	M	M	M	M	M	M	M	M	M	M	M
W	M	M	M	M	M	M	M	H	H	H	H	H	H	H
W	M	M	M	M	M	H	H	H	H	H	H	H	H	H

Figure 11. Genetic and physical maps of the rolled leaf mutant.
(A) Locus of new rolled leaf gene was on the long arm of chromosome 2 between S02122b and S02131. (B) Candidate region was narrowed down using additional STS marker. (C) Graphical genotype results of the fine mapping. W, Wild type; M, Mutant type; H, hetero.

SNP analysis and searching for mutation point

To identify the candidate gene and causal SNP position rapidly, the SNP analysis was performed with whole-genome sequencing data of Ilpum and bulked F_2 progeny displaying the mutant phenotype. This analysis and genetic mapping results were analyzed synthetically.

Several SNP positions were founded in candidate region. Among them, only three homozygous SNPs which existed in genic region based on Rice Genome

Annotation Project database (http://rice.plantbiology.msu.edu/). One of the SNPs located on LOC_Os02g48570 and others located on LOC_Os02g48590 (Table 4).

Every SNP was confirmed by manual sequencing, and then co-segregation analysis was performed with F_2 population to validate the association between those SNPs and the rolled leaf phenotype of the mutant. dCAPS markers were designed and PCR was conducted in 10 mutant type and 10 wild type plants selected from F_2 population, derived from the cross between rolled leaf mutant and Ilpum.

After digestion with specific restriction enzyme, only mutant or wild type PCR product was cut depending on the markers. Each of clear co-segregations observed between mutant type and wild type plants from F_2 population (Fig 12 A,B).

According to in silico genome annotation, one of the SNPs, named SNP3, is likely to relate with phenotype of mutant. This mutation point found at the 5´ splice site of first intron. In eukaryotes, introns of pre-mRNA are spliced off before translation, mRNA to amino acid. Introns have specific sequences, which bind spliceosome assisted splicing, GU at its 5´ end and AG at its 3´ end for RNA processing. In this mutant, this G of 5´ has been mutated to A, which binds spliceosome. Therefore, this single nucleotide transition might lead to the splicing error (Fig.12 C).

Table 4. Homozygous SNPs position in candidate region for rolled leaf.

	Position	Status	Reference	Query	Gene ID		Description
SNP1	30596325	Difference	G	A	Os02g0716800	LOC_Os02g48570	TGF-beta receptor, type I/II extracellular region family protein.
SNP2	30601566	Difference	G	A	Os02g0717100	LOC_Os02g48590	Esterase/lipase/thioesterase domain containing protein.
SNP3	30602023	Difference	G	A			

SNPs were named SNP1, SNP2, SNP3 and position of SNPs was based on the Nipponbare genome sequence, Build 5 ver. (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html).

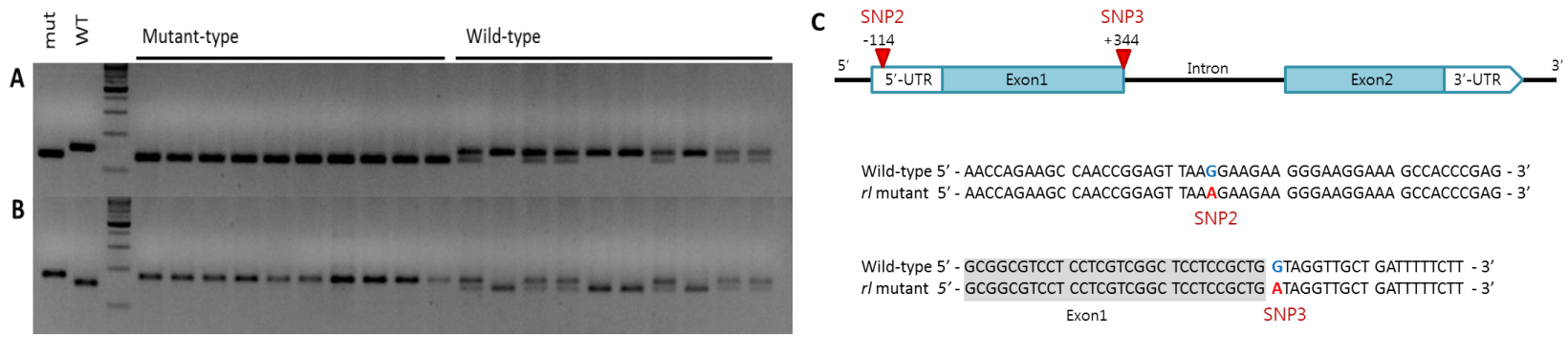


Figure 12. SNP2 and SNP3.

(A) and (B) are dCAPS marker genotype of F_2 plants from the cross between the rolled leaf mutant and Ilpum, classified by phenotype. (A) After digestion with restriction enzyme MseI of SNP2. (B) After digestion with restriction enzyme RsaI of SNP3. (C) Position of SNP2 and SNP3 in LOC_Os02g48590.

1. Spotted leaf mutant

Characterization of spotted leaf mutant in rice

Brown spots appeared on the leaves near the tip of the mutants after 5th leaf stage and the spots increased from the tip to the basal part of the leaf with growth. At later tillering stage, white spots were observed with the brown spots. As growing, both spots showed from the bottom to top of the plant (Fig. 14).

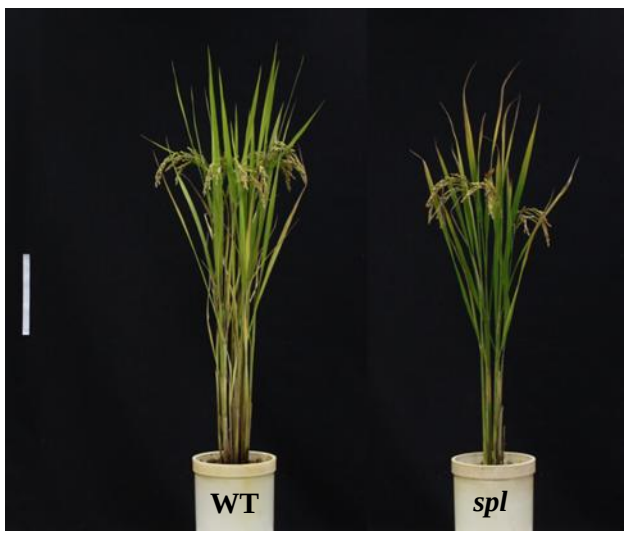


Figure 13. Phenotype of wild-type and spotted leaf mutant at ripening stage. (bar = 20cm)

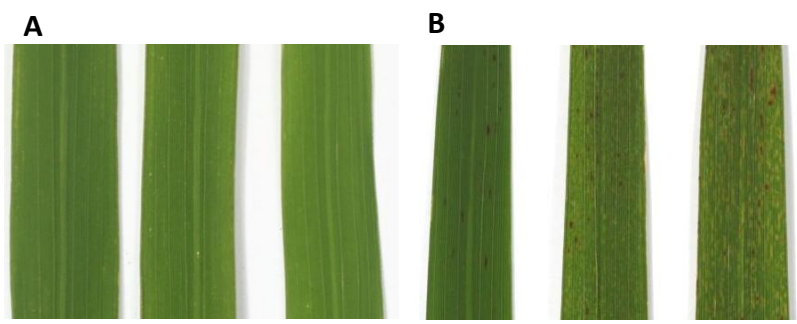


Figure 14. Lesion mimic phenotype of spotted leaf mutant.
First (left), second (middle), third(right) leaves from the top of (A) wild-type and (B) spotted leaf mutant at late vegetative stage.

To analyze the characteristics of the spotted leaf mutant, various agronomic traits of the mutant were compared with those of the wild-type. Most of the agronomic traits of mutant were similar to those of wild-type.

The spotted leaf mutant showed smaller culm length, tiller number and panicle number than Ilpum, its wild type. However, panicle length and exsertion, leaf length and width were not severely reduced in case of spotted leaf mutant compared to the wild-type (Fig. 15). 1000 grains weight of spotted leaf mutant was decreased, whereas grain length, width, and fertility of the mutant were similar to those of wild-type (Fig. 16).

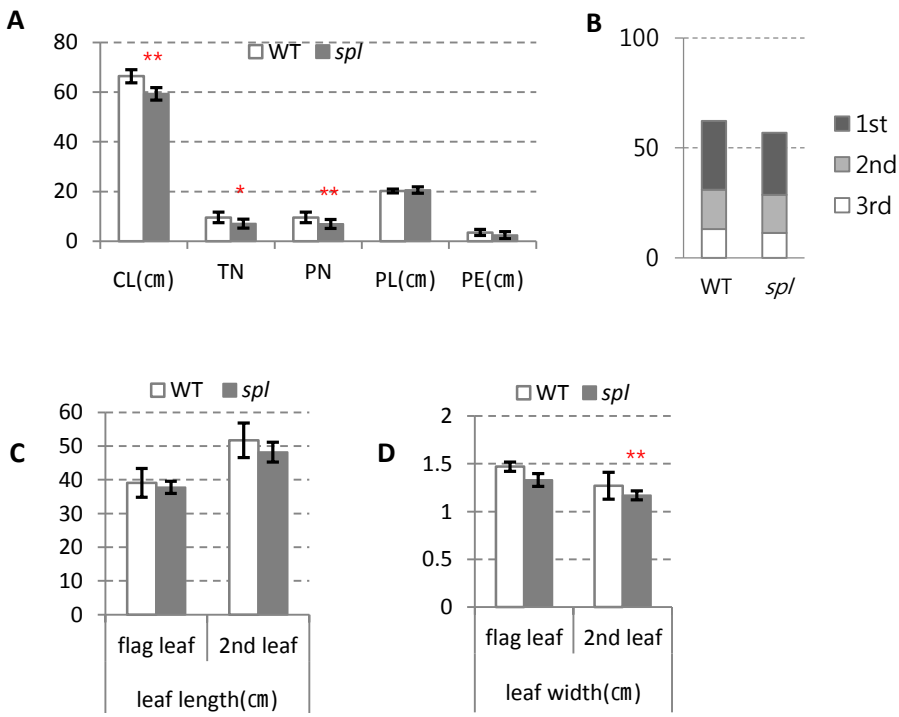


Figure 15. Agronomic traits of wild-type and spotted leaf mutant.
Comparison of the (A) culm length(CL), tiller number(TN), panicle number(PN), panicle length(PL), panicle exsertion(PE), (B) inter node length, (C) leaf length and (D) leaf width of flag and second leaves of wild-type and *spl* . Error bar represents SD (n>10) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01).

Figure 16. Grains and panicle of wild-type and spotted leaf mutant.
(A) Grain shape of wild-type and *spl*. (B) Panicle of wild-type (left) and *spl* (right). (bar = 5cm)

Table 5. Comparison of agronomic traits between wild-type and spotted leaf mutant.

	HD	GL(mm)	GW(mm)	1000 grain weight(g)	Grain fertility(%)
Wild-type	24-Aug	5.53±0.18	3.33±0.15	25.97±0.09	82.15±7.98
Mutant	20-Aug	5.58±0.10	3.37±0.05	21.12±0.32	85.64±5.46
Comparison	-	NS	NS	**	NS

Values are mean ± standard deviation. Comparison of the heading date in 2014(HD), grain length(GL), grain width(GW), 1000 grain weight, grain fertility of wild-type and *spl*. Data show means and SD of biological replicates (n>10, n>5) and statistically calculated by Student's t-test (* P<0.05, ** P<0.01, NS: not statically significant).

Analysis of chlorophyll contents by SPAD value

To establish the connection between spot and degree of chlorophyll content, SPAD values were measured in mutants and wild-type plants. SPAD value, indirect

indicator of chlorophyll content in leaf, of the mutant plant was slightly higher than wild-type that means spotted leaf mutant naturally have much chlorophyll content compared to wild-type.

Although the lesions expanded over the leaf, SPAD values were slightly decreased. The result indicates that destruction of chlorophyll was not accelerated by the emergence of lesions (Fig. 17).

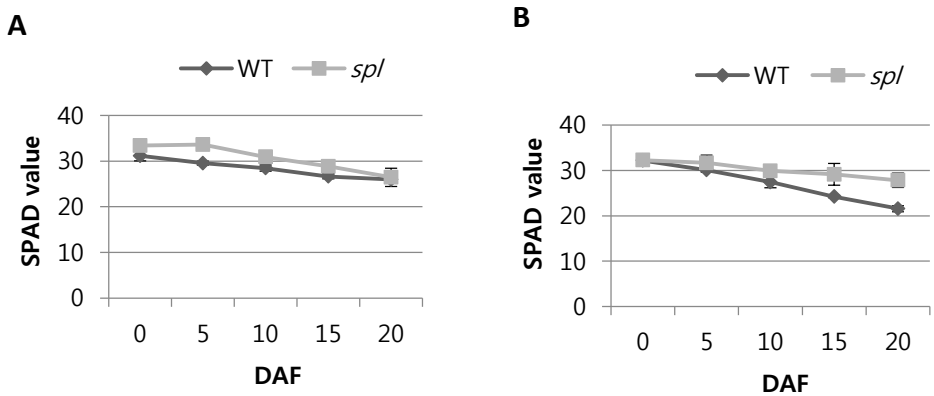


Figure 17. Comparison of the chlorophyll contents.
Chlorophyll contents in (A) flag leaf and (B) second leaf of wild-type and *spl*. SPAD value were measured at the indicated times after flowering (DAF, days after flowering). Data show means and SD of biological replicates (n>5).

Staining for ROS detection

The Result of NBT staining, displaying the blue color precipitates, indicate the superoxide anion(O_2^-) accumulation, Among the upper three leaves, first leaf of the mutant was quite distinct from those wild-type, and other leaves of the mutant were stained more than wild-type (Fig. 18).

Furthermore, to observe the H_2O_2 accumulation, DAB staining was also performed . DAB staining showed brown staining that detects areas of H_2O_2 accumulation. DAB staining results were similar to that of NBT staining.

This indicated that lesion formation in the mutant leaves was correlated with ROS accumulation.

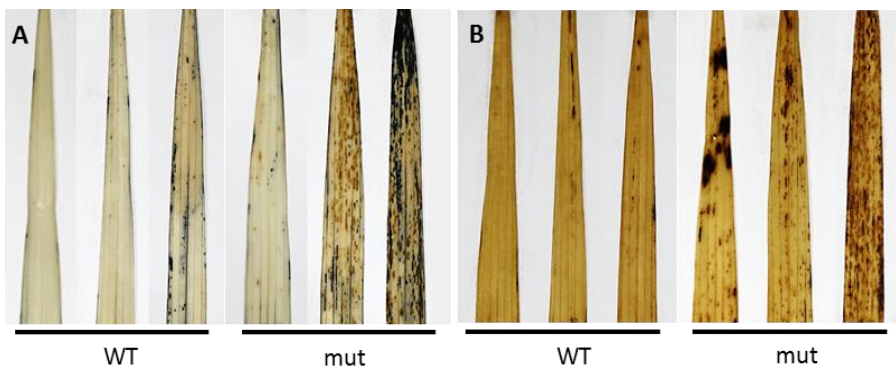


Figure 18. ROS staining.
ROS staining using first, second, third leaves from the top of wild-type and *spl* at heading stage. (A) NBT staining, (B) DAB staining.

Genetic analysis of spotted leaf mutant in rice

F_1 plants derived from the cross between the mutant and Ilpum showed the normal phenotype. In F_2 population, segregation ratio between the wild-type and the mutant-type was 3:1. According to this genetic analysis, spotted leaf gene which controls the emergence of lesion is single recessive gene.

Table 6. Segregation ratio of F_2 population developed from the cross between the spotted leaf mutant and Ilpum.

Total	Normal	Mutant	X^2 (3:1) ($p=0.05$, $\chi^2=3.841$)
190	148	42	0.085

Genetic mapping of spotted leaf mutant in rice

Bulked segregant analysis (BSA) was performed using F_2 population derived from the cross between mutant and Milyang23 (*Oryza sativa indica*). This analysis detected two flanking markers (S05080a, S05112) on the long arm of rice chromosome 5 (Fig. 19).

Additional 6 STS markers, which were located between two flanking markers were designed based on variation of genome sequence from available database (http://www.ncbi.nih.gov/ and http://www.rgp.dna.affrc.go.jp/). Fine- mapping was conducted, and then the candidate region was narrowed down between S05105 and S05112 markers (physical distance 2,2Mb).

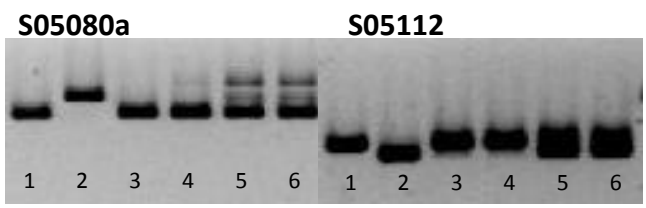
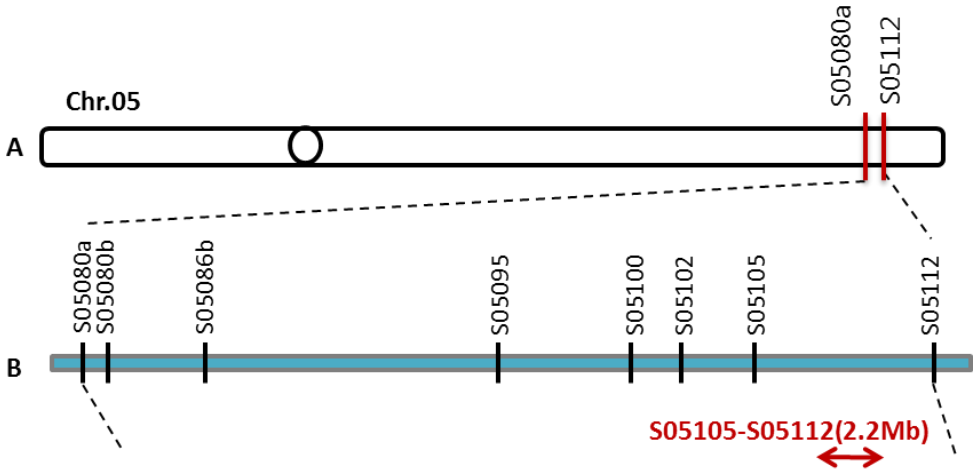


Figure 19. Bulked Segregant Analysis (BSA) of wild-type and spotted leaf mutant bulks from F_2 population.
1, *spl*; 2, Milyang 23; 3,4, Mutant bulks; 5,6, Wild-type bulks.



C

	S05080A	S05080b	S05086b	S05095	S05100	S05102	S05105	S05112
M	M	M	M	M	M	M	M	H
M	M	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	M
M	M	M	M	M	M	M	M	H
W	M	M	M	M	M	M	H	H
W	W	W	W	W	W	H	W	W
W	M	M	M	M	H	H	H	H
W	M	M	M	H	H	H	H	H
W	H	H	H	H	H	H	W	W
W	M	M	M	M	M	M	M	H
W	H	H	H	H	W	W	W	W
W	M	M	M	M	M	M	M	H

Figure 20. Genetic and physical maps of the spotted leaf mutant.
(A) Locus of spotted leaf gene was on the long arm of chromosome 5 between S05080a and S05112. (B) Candidate region was narrowed down using additional STS marker. (C) Graphical genotype results of the fine mapping. W, Wild type; M, Mutant type; H, hetero.

SNP analysis and searching for mutation point

Both rolled leaf mutant and spotted leaf mutant were derived from Ilpum. Therefore, similar strategies were applied to search for the candidate gene and causal SNP position.

The SNP analysis was performed with whole-genome sequencing data of Ilpum and bulked F_2 progeny displaying the mutant phenotype. This analysis and genetic mapping results were analyzed synthetically. In flanking region, 8 homozygous SNPs existed in genic region and several homozygous SNPs in intergenic region based on Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/).

Table 7. Homozygous SNPs position in candidate region for spotted leaf.

Position of SNPs was based on the Nipponbare genome sequence, Build 5 ver. (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html).

Position	Status	Reference.	Query	Gene ID		Description
26012356	Difference	C	T			
26154389	Difference	C	T			
26297755	Difference	C	T			
26373075	Difference	C	T			
26495547	Difference	C	T			
26814370	Difference	C	T			
26977732	Difference	C	G	Os05g0541400	LOC_Os05g46370	Similar to Transcription factor LAX PANICLE.
26977757	Insert	-	G			Similar to Transcription factor LAX PANICLE.
26977758	Difference	C	G			Similar to Transcription factor LAX PANICLE.
26977762	Deletion	C	-			Similar to Transcription factor LAX PANICLE.
26989827	Difference	T	G	Os05g0541700	LOC_Os05g46395	Similar to predicted protein.
26989832	Deletion	T	-			Similar to predicted protein.
26989850	Difference	A	C			Similar to predicted protein.
26989857	Difference	A	G			Similar to predicted protein.
27369645	Difference	C	T			
27604871	Difference	C	T			

DISCUSSION

As a major organ of photosynthesis, properties of the leaf are important agronomic traits and taken interest through previous studies.

Moderate rolling leaf could enhance the light capture efficiency through maintaining the erection of leaf. Thus, leaf rolling is related to crop yield. In rice, many kinds of rolled leaf mutants and genes were identified but the entire mechanism regulated leaf rolling was not provided yet. In this study, we identified the mutant induced by EMS treatment from Ilpum. It is a new rolled leaf, especially abaxially rolling, mutant. Among the previous reported rolling leaf mutants, abaxial rolling types were less than adaxialy rolled types. Therefore, it can be helpful to elucidate the mechanism involved in leaf rolling and to understand the rolling pattern. Even though the mutant has defective phenotypes about yield, it could be a role in improving yield later in the future.

This rolled leaf mutant shows higher LRI compared with the wild-type and has significantly increased bulliform cell number and area than wild-type. Alteration of bulliform cell was supposed to be involved in the outward leaf rolling phenotype. Bulliform cells exist specifically in the adaxial side of leaves in gramineous plants[59] and located between two vascular bundle ridges. They can be easily observed with the cross sections of leaf. Bulliform cells modulate the leaf rolling depending on water stress[60]. Under water stress, leaves are rolling because bulliform cells lose turgor. Otherwise, bulliform cells absorb water and swell up again when water stress is relieved. The functions of bulliform cells in leaf rolling

are some doubts[61] and remains to be elucidated. However, this study could explore the role of leaf bulliform cells with previous studies.

F_1 plants derived from crossing between the mutant and Ilpum showed normal phenotype which reveals the rolled leaf mutant is controlled by a recessive gene. To identify the gene, BSA, genetic mapping and SNP analysis through NGS were conducted. SNP calling results and genetic mapping results were analyzed synthetically, and we could rapidly search more reliable mutation point. Three filtered homozygous SNPs were detected in candidate region. One of them found at splice site, named SNP3, was assumed more powerful mutation. The gene including SNP3 mutation point might be the candidate for this rolled leaf gene.

Spotted leaf mutants were studied to observe the effect of leaf color on growth, development and yield of plant by photosynthesis[14,26]. Moreover, to understand mechanisms of hypersensitive response, a large number of spotted leaf mutants were identified. The lesions of these mutants resemble the disease symptoms but were formed in absence of pathogens in rice[27,40].

Hypersensitive response is a type of programmed cell death (PCD). Therefore, this new spotted leaf mutant can be useful to identify the mechanism of PCD. In addition, this spotted leaf mutant has two kinds of spot together, which is unusual phenotype.

Some rice lesion mimic mutants have resistance of disease, like bacterial blight or blast disease, and show defense response gene expression. Moreover, three of these

cell death and resistance (cdr) mutants were observed to have elevated levels of the phytoalexin momilactone A and highly activated expression of two defense response genes, PBZ1 and PR1, in leaves showing lesions[35]. This mutant has the possibility of resistance to rice blast and bacterial blight disease.

F_1 plants derived from crossing between the mutant and Ilpum showed wild-type phenotype. This result indicates that the spotted leaf mutant is controlled by a recessive gene, likewise rolled leaf mutant. BSA, genetic mapping and SNP analysis through NGS were conducted to identify the gene. The spotted leaf gene was mapped on the long arm of chromosome 5 between two STS markers (S05105 and S05112, physical distance 2,2Mb). In flanking region, it was difficult in fine-mapping since there are a few polymorphism of sequence between Ilpum and Milyang23, parents of mapping population. Several homozygous SNP were detected in flanking region, however we cannot confirm which one is causal SNP due to broad flanking region and error of sequencing.

In this study, to search mutational point, F_2 bulk sequencing data and genetic mapping result were used. SNP analysis was conducted on the basis of MutMap method[58] slightly modified. Original MutMap pipeline is performed with various program and many step to pick up the causal SNP. However, we can easily and rapidly analyze the SNP data of the whole genome sequence and obtain the candidate mutation point, through only examining the SNP in candidate region.

This characterization and genetic mapping about leaf-related genes will help expanding the knowledge of rice leaf properties. Moreover, there are possibilities of cloning a new rolled leaf and spotted leaf genes. Further study including cloning and the functional study of these genes need to be performed, and then may reveal the mechanisms of the leaf rolling and spot.

REFERENCE

1. IRRI. International Rice Research Institute, World Rice Statistics 2013. (2013).
2. Duncan, W.G. Leaf Angles, Leaf Area, and Canopy Photosynthesis. *Crop Science* **11**, 482-485 (1971).
3. Lang, Y., Zhang, Z., Gu, X., Yang, J. & Zhu, Q. Physiological and ecological effects of crimpy leaf character in rice (Oryza sativa L.)II.Photosynthetic character,dry mass production and yield forming. *Zuo wu xue bao* **30**, 883-887 (2004).
4. Zhang, G.H., Xu, Q., Zhu, X.D., Qian, Q. & Xue, H.W. SHALLOT-LIKE1 is a KANADI transcription factor that modulates rice leaf rolling by regulating leaf abaxial cell development. *Plant Cell* **21**, 719-35 (2009).
5. Micol, J.L. & Hake, S. The development of plant leaves. *Plant Physiol* **131**, 389-94 (2003).
6. Hibara, K. *et al.* The ADAXIALIZED LEAF1 gene functions in leaf and embryonic pattern formation in rice. *Dev Biol* **334**, 345-54 (2009).
7. Fang, L. *et al.* Rolling-leaf14 is a 2OG-Fe (II) oxygenase family protein that modulates rice leaf rolling by affecting secondary cell wall formation in leaves. *Plant Biotechnol J* **10**, 524-32 (2012).
8. Khush, G.S. & Kinoshita, T. Rice karyotype, marker genes, and linkage groups. 83-108 (CAB International, Wallingford, 1991).
9. Li, S. *et al.* Genetic analysis and mapping the flag leaf roll in rice (Oryza sativaL.). *Journal of Sichuan Agricultural University* **16**, 391-393 (1998).
10. Shao, Y.-J. *et al.* [One major QTL mapping and physical map construction for rolled leaf in rice]. *Yi chuan xue bao = Acta genetica Sinica* **32**, 501-506 (2005).
11. Yan, C. *et al.* Genetic analysis and gene fine mapping for a rice novel mutant (rl 9(t)) with rolling leaf character. *Chinese Science Bulletin* **51**, 63-69 (2006).
12. Yan, S. *et al.* ROLLED LEAF 9, encoding a GARP protein, regulates the leaf abaxial cell fate in rice. *Plant Mol Biol* **68**, 239-50 (2008).
13. Luo, Z. *et al.* Genetic analysis and fine mapping of a dynamic rolled leaf gene, RL10(t), in rice (Oryza sativa L.). *Genome* **50**, 811-7 (2007).
14. Shi, Y. *et al.* Genetic analysis and gene mapping of a new rolled-leaf mutant in rice (Oryza sativa L.). *Science in China Series C: Life Sciences* **52**, 885-890 (2009).
15. Luo, Y.-Z. *et al.* Genetic Analysis and Gene Mapping of a Novel Rolled-Leaf Mutant rl12(t) in Rice. *Acta Agronomica Sinica* **35**, 1967-1972 (2009).
16. Shao, Y. Fine mapping of an incom-plete recessive gene for leaf rolling in rice (Oryza sativa L.). *Chinese Science Bulletin* **50**, 2466 (2005).

17. Li, L. *et al.* Isolation and characterization of rl (t), a gene that controls leaf rolling in rice. *Chinese Science Bulletin* **59**, 3142-3152 (2014).
18. Xiang, J.J., Zhang, G.H., Qian, Q. & Xue, H.W. Semi-rolled leaf1 encodes a putative glycosylphosphatidylinositol-anchored protein and modulates rice leaf rolling by regulating the formation of bulliform cells. *Plant Physiol* **159**, 1488-500 (2012).
19. Hu, J. *et al.* Identification and characterization of NARROW AND ROLLED LEAF 1, a novel gene regulating leaf morphology and plant architecture in rice. *Plant Mol Biol* **73**, 283-92 (2010).
20. Shi, Z. *et al.* Over-expression of rice OsAGO7 gene induces upward curling of the leaf blade that enhanced erect-leaf habit. *Planta* **226**, 99-108 (2007).
21. Zou, L.P. *et al.* Leaf rolling controlled by the homeodomain leucine zipper class IV gene Roc5 in rice. *Plant Physiol* **156**, 1589-602 (2011).
22. Zhao, S.Q., Hu, J., Guo, L.B., Qian, Q. & Xue, H.W. Rice leaf inclination2, a VIN3-like protein, regulates leaf angle through modulating cell division of the collar. *Cell Res* **20**, 935-47 (2010).
23. Li, L. *et al.* Overexpression of ACL1 (abaxially curled leaf 1) increased Bulliform cells and induced Abaxial curling of leaf blades in rice. *Mol Plant* **3**, 807-17 (2010).
24. Woo, Y.M. *et al.* Constitutively wilted 1, a member of the rice YUCCA gene family, is required for maintaining water homeostasis and an appropriate root to shoot ratio. *Plant Mol Biol* **65**, 125-36 (2007).
25. Wu, J.L. *et al.* Chemical- and irradiation-induced mutants of indica rice IR64 for forward and reverse genetics. *Plant Mol Biol* **59**, 85-97 (2005).
26. Zhang, S. *et al.* The interactions among DWARF10, auxin and cytokinin underlie lateral bud outgrowth in rice. *J Integr Plant Biol* **52**, 626-38 (2010).
27. Wu, C. *et al.* Rice lesion mimic mutants with enhanced resistance to diseases. *Mol Genet Genomics* **279**, 605-19 (2008).
28. Huang, L. *et al.* Down-Regulation of a SILENT INFORMATION REGULATOR2-Related Histone Deacetylase Gene, OsSRT1, Induces DNA Fragmentation and Cell Death in Rice. *Plant Physiology* **144**, 1508-1519 (2007).
29. Dietrich, R.A. *et al.* Arabidopsis mutants simulating disease resistance response. *Cell* **77**, 565-577 (1994).
30. Lorrain, S. Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? *Trends in Plant Science* **8**, 263-271 (2003).
31. Walbot, V., Hoisington, D. & Neuffer, M.G. Disease Lesion Mimic Mutations. in *Genetic Engineering of Plants*, Vol. 26 (eds. Kosuge, T., Meredith, C., Hollaender, A. & Wilson, C.) 431-442 (Springer US, 1983).

32. KIYOSAWA, S. Inheritance of a particular sensitivity of the rice variety, Sekiguchi Asahi, to pathogens and chemicals, and linkage relationship with blast resistance genes. *Nogyo Gijutsu Kenkyusho Hokoku = Bull. Nat. Inst. agric. Sci.* **21**, 61-72 (1970).
33. Yoshimura, A., Ideta, O. & Iwata, N. Linkage map of phenotype and RFLP markers in rice. *Plant Mol Biol* **35**, 49-60 (1997).
34. Yin, Z. *et al.* Characterizing Rice Lesion Mimic Mutants and Identifying a Mutant with Broad-Spectrum Resistance to Rice Blast and Bacterial Blight. *Molecular Plant-Microbe Interactions* **13**, 869-876 (2000).
35. Huang, Q.-n., Yang, Y., Shi, Y.-f., Chen, J. & Wu, J.-l. Spotted-Leaf Mutants of Rice (Oryza sativa). *Rice Science* **17**, 247-256 (2010).
36. Yamanouchi, U., Yano, M., Lin, H., Ashikari, M. & Yamada, K. A rice spotted leaf gene, Spl7, encodes a heat stress transcription factor protein. *Proc Natl Acad Sci U S A* **99**, 7530-5 (2002).
37. Zeng, L., Yin, Z., Chen, J., Leung, H. & Wang, G.L. Fine genetic mapping and physical delimitation of the lesion mimic gene Spl11 to a 160-kb DNA segment of the rice genome. *Mol Genet Genomics* **268**, 253-61 (2002).
38. Vega-Sanchez, M.E., Zeng, L., Chen, S., Leung, H. & Wang, G.L. SPIN1, a K homology domain protein negatively regulated and ubiquitinated by the E3 ubiquitin ligase SPL11, is involved in flowering time control in rice. *Plant Cell* **20**, 1456-69 (2008).
39. Wang, L., Pei, Z., Tian, Y. & He, C. OsLSD1, a Rice Zinc Finger Protein, Regulates Programmed Cell Death and Callus Differentiation. *Molecular Plant-Microbe Interactions* **18**, 375-384 (2005).
40. Takahashi, A. *et al.* Rice Pti1a negatively regulates RAR1-dependent defense responses. *Plant Cell* **19**, 2940-51 (2007).
41. Kim, J.A. *et al.* Rice OsACDR1 (Oryza sativa accelerated cell death and resistance 1) is a potential positive regulator of fungal disease resistance. *Mol Cells* **28**, 431-9 (2009).
42. Mori, M. *et al.* Isolation and molecular characterization of a Spotted leaf 18 mutant by modified activation-tagging in rice. *Plant Mol Biol* **63**, 847-60 (2007).
43. Qiao, Y. *et al.* SPL28 encodes a clathrin-associated adaptor protein complex 1, medium subunit micro 1 (AP1M1) and is responsible for spotted leaf and early senescence in rice (Oryza sativa). *New Phytol* **185**, 258-74 (2010).
44. Dietrich, R.A., Richberg, M.H., Schmidt, R., Dean, C. & Dangl, J.L. A novel zinc finger protein is encoded by the Arabidopsis LSD1 gene and functions as a negative regulator of plant cell death. *Cell* **88**, 685-94 (1997).

45. Lorrain, S. *et al.* Vascular associated death1, a novel GRAM domain-containing protein, is a regulator of cell death and defense responses in vascular tissues. *Plant Cell* **16**, 2217-32 (2004).
46. Balague, C. *et al.* HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. *Plant Cell* **15**, 365-79 (2003).
47. Hu, G., Yalpani, N., Briggs, S.P. & Johal, G.S. A porphyrin pathway impairment is responsible for the phenotype of a dominant disease lesion mimic mutant of maize. *Plant Cell* **10**, 1095-1105 (1998).
48. Ishikawa, A., Okamoto, H., Iwasaki, Y. & Asahi, T. A deficiency of coproporphyrinogen Ⅲ oxidase causes lesion formation in Arabidopsis. *Plant J* **27**, 89-99 (2001).
49. Mou, Z., He, Y., Dai, Y., Liu, X. & Li, J. Deficiency in fatty acid synthase leads to premature cell death and dramatic alterations in plant morphology. *Plant Cell* **12**, 405-18 (2000).
50. Brodersen, P. *et al.* Knockout of Arabidopsis accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes Dev* **16**, 490-502 (2002).
51. Kachroo, A. *et al.* Oleic acid levels regulated by glycerolipid metabolism modulate defense gene expression in Arabidopsis. *Proc Natl Acad Sci U S A* **101**, 5152-7 (2004).
52. Gray, J., Close, P.S., Briggs, S.P. & Johal, G.S. A novel suppressor of cell death in plants encoded by the Lls1 gene of maize. *Cell* **89**, 25-31 (1997).
53. Zeng, L.R. *et al.* Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-box/armadillo repeat protein endowed with E3 ubiquitin ligase activity. *Plant Cell* **16**, 2795-808 (2004).
54. Ji, H.S. *et al.* Characterization and mapping of a shattering mutant in rice that corresponds to a block of domestication genes. *Genetics* **173**, 995-1005 (2006).
55. Kariola, T., Brader, G., Li, J. & Palva, E.T. Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. *Plant Cell* **17**, 282-94 (2005).
56. Mahalingam, R. *et al.* Analysis of oxidative signalling induced by ozone in Arabidopsis thaliana. *Plant Cell Environ* **29**, 1357-71 (2006).
57. Michelmore, R.W., Paran, I. & Kesseli, R.V. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc Natl Acad Sci U S A* **88**, 9828-32 (1991).

58. Abe, A. *et al.* Genome sequencing reveals agronomically important loci in rice using MutMap. *Nat Biotechnol* **30**, 174-8 (2012).
59. Swallen, J.R. Anatomy of Monocotyledons. vol. 1, Gramineae. Charles Russell Metcalfe. Oxford University Press, New York, 1960. lxi + 731 pp. Illus. $13.45. *Science* **133**, 1817-1818 (1961).
60. Price, A.H. & Tomos, A.D. Genetic dissection of root growth in rice (Oryza sativa L.). *Theoretical and Applied Genetics* **95**, 143-152 (1997).
61. Ristic, Z. & Cass, D.D. Morphological Characteristics of Leaf Epidermal Cells in Lines of Maize that Differ in Endogenous Levels of Abscisic Acid and Drought Resistance. *Botanical Gazette* **152**, 439-445 (1991).

초 록

벼에서 잎이 말리는(rolled leaf) 돌연변이와 잎에 반점이 생기는(spotted leaf) 돌연변이의 유전자 지도 작성

잎은 광합성, 호흡, 증발 등 작물이 생육하는 데에 필요한 여러 가지 역할들을 하는 기관이다. 따라서, 잎의 형질에 관련된 유전자를 찾고, 그 유전자의 작용 원리를 밝히는 것은 이상적인 작물을 육종하는데 도움이 될 수 있다.

본 연구에서는 일품벼에 EMS 를 처리 하여 얻은 잎에 관련된 두 종류의 돌연변이체를 실험 재료로 사용하였다. 그리고 이들의 표현형 특성을 검정하고, 유전자 지도를 작성 하는 것을 목표로 하였다.

잎이 말리는 돌연변이는 기동세포의 수와 그 영역이 증가되어 있었다. 또한, 잎에 반점이 생기는 돌연변이는 생육 초기부터 갈색반점과 흰색반점이 같이 관찰되는 특징을 보였다.

F_2 집단의 분리비가 3:1 로 나타나는 것으로 보아 돌연변이의 표현형은 단일열성유전자에 의해 조절되는 것을 알 수 있었으며, STS marker 를 이용해서 BSA 를 수행한 결과 각 돌연변이체를 조절하는 유전자는 염색체 2 번과 5 번에 위치하고 있는 것으로 확인되었다.

유전자의 정확한 위치를 찾기 위해서 Fine-mapping 을 수행하였다. 그리고 NGS(차세대 염기서열 분석)를 통해 얻어진 Whole-genome sequence 정보를 이용해서 SNP 분석을 수행하였다. Fine-mapping 결과와 SNP 분석 결과를 종합하여 후보유전자를 찾을 수 있었다.

해당 실험의 결과는 차후에 잎이 말리는 과정과 잎에 반점이 생기는 과정을 연구 하는데 있어서 도움이 될 것으로 생각한다.

주요어 : 잎 말림 돌연변이, 잎 반점 돌연변이, BSA, 기동세포, 차세대 염기서열 분석, 유전자 지도 작성

학번 : 2013-23228