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Characterization and Molecular Mapping of Rolled Leaf Mutant and Elongated Uppermost Internode Mutant in Rice

BY

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UNDER THE DIRECTION OF PROFESSOR HEE-JONG KOH
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
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ABSTRACT

To understand the various plant architecture and morphogenesis in rice (*Oryza sativa* L.), ethyl methanesulfonate treated Ilpum mutant with narrow and rolled leaf phenotype and Koshihikari mutant with elongated uppermost internode trait were identified. The leaf rolling trait is said to be more advantageous under high temperature and heat stress. Cytological analysis of the leaf suggested that smaller size and less number of the bulliform cells caused leaf rolling. Ilpum/*rl* crossed F_1 plants showed normal phenotype. Genetic analysis of its F_2 population suggested that the mutation was controlled by a single recessive gene with segregation ratio of 3:1. Using M23/*rl* F_2 mapping population, each chromosome was screened with STS markers by the BSA method. The candidate region was detected to a long arm of chromosome 1, near the centromeric region. Through whole genome sequencing

and its MutMap analysis, the causal SNP within the candidate region was identified. The result of RT PCR suggested that splicing error was occurred due to a base change from G to A at the beginning of 5th intron of *Loc_Os01g37837*, a gene which encode putative seryl-tRNA synthetase, and various forms of transcripts were presented. A novel gene that cause change of leaf morphology into narrow and rolled leaf was newly discovered. *Eui* mutant showed significantly increased first three internodes. The phenotype of F_2 progenies of Koshihikari/*eui* was determined by measuring the length of first and second internodes. The distribution of each measurement implied that the mutant phenotype was controlled by a single recessive gene. BSA result showed that the flanking region was set to a long arm of chromosome 5. Further narrow down was conducted into 984kb of the markers S0595e and S05101c, including *EUI1*. However, sequencing result suggested that the *eui* didn't have mutation correspond to *EUI1*. Another gene than EUI1 is thought to be related with internode elongation trait in chromosome 5.

Key words: rice, plant architecture, rolled leaf, narrow leaf, bulliform cell, elongated uppermost internode

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LIST OF ABBREVIATIONS

BSA	Bulked segregant analysis
CTAB	Cetyltrimethylammonium bromide
dCAPS	Derived cleaved amplified polymorphic sequence
DNA	Deoxyribonucleic acid
EMS	Ethyl methanesulfonate
eui	Elongated uppermost internode mutant
InDel	Insertions anddeletions
M23	Milyang23
PCR	Polymerase chain reaction
rl	Rolled leaf mutant
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
STS	Sequence tagged site

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important human food, eaten by more than half of the world's population every day. Food crisis had emerged as population growth reached to critical level, so that the importance of food security had never been more emphasized. Researches for increasing yield potential have been conducted all over the world, and the aim was first achieved by modification of plant architecture with desirable traits. The ideotype of rice is the one characterized with erect type, decreased tiller number, long panicle size, and semi dwarf type (Khush, 2001). Not only for the increase of yield potential, researches related to plant architecture aiming for other specific goals have been conducted as well.

The plant architecture determines the efficiency of its physiology including light capture, photosynthesis, and energy formation, so that the leaf shape has been considered as one of the important characteristics in plant breeding. The leaf is a major organ of photosynthesis in rice. Advanced scientific studies have revealed that the moderate leaf rolling trait could lead to the ideal plant architecture with erected leaves throughout the growth period (Lang et al., 2004). As a consequence, high-energy utilization efficiency, delayed leaf senescence, and even increased yield are anticipated as positive effects of the trait. The mutants showing

rolled leaf trait could be classified by the direction of rolling; incurved or abaxially rolled leaves and outcurved or adaxially rolled leaves. Studies have revealed that the trait is controlled by several factors, either of leaf polarity in the abaxial–adaxial epidermis, deformativebulliform cells, defective development of sclerenchymatous cells, and defective programmed cell death of mesophyll cells. There are some genes found, but only few among them have been characterized up to date.

Elongation of internode is not exactly the ideotype of rice mentioned since it could cause lodging, but EUI mutant has been importantly used in hybrid breeding. For hybrid breeding, the role of male sterile plant is critical. However, most of male sterile plants have panicle exsertion problem, so enormous amount of auxin to compromise was used. The use of auxin led to viviparous germination and caused severe environment issue. Since 1980's when the trait was first discovered by Rutger and Carnahan (1981), the panicle exsertion problem of male sterile plant was resolved. Other than that, use of male plant with EUI trait could ease the pollen shedding in usual cross breeding.

The objectives of this study were to characterize mutants, and to identify novel genes that are directly related to each trait by genetic analysis, mapping, and whole genome sequencing technology.

MATERIALS AND METHODS

1. Plant materials and growth conditions

The *rl* mutant was obtained from ethyl methanesulfonate (EMS) treated Korean *japonica* rice cultivar 'Ilpum', and *eui* mutant was generated from EMS treated Japanese *japonica* rice cultivar 'Koshihikari'. Each mutant was selected at M_2 generation and fixed into pure-line by repetitive selfing.

For genetic analysis and whole genome sequencing, F_2 population generated from the cross of each mutant and the wild type was used. For BSA and genetic mapping, F_2 population of the cross of each mutant and M23 was used.

All plant materials were grown in the paddy field of Seoul National University Experimental Farm with natural long day conditions at 37° N latitude, Suwon, Korea.

2. Measurement of agronomic traits

The plant height was measured from the surface of the ground to the leaf tip for 10 individual mature plants before heading for each of wild type, *rl*, and *eui*. Heading date, culm length panicle length, panicle number, spikelet per panicle, 1000-grain weight, and grain fertility were measured. For measuring 1000-grain weight, 500 seeds of each wild type and mutants were collected, the weight was

measured and the value was multiplied by 2. The process was repeated twice.

The observed measurements were analyzed for significance relation using Statistical Analysis System (SAS) program.

3. Histology and microscopy analysis

For paraffin section analysis of wild type and *rl*, Ji's paraffin embedding method (2006) with slight modifications was used. The widest part of second leaf was taken, and all leaf samples were collected at late vegetative stage. The 1cm length of leaf samples were separately fixed into a FAA solution (formaldehyde 3.7%: acetic acid 5% : ethanol 50%) for 1day at 4℃ and then dehydrated for 2 hours each in a graded ethanol solution series(70%, 85%, 95% and 100%). At the final step, the leaf samples were dehydrated for 1day. After that, dehydrated samples were cleared for 2hours each in a clearing solution series consisted of 75% ethanol/25% histo-clear, 50% ethanol/50% histo-clear, 25% ethanol/75% histo-clear, followed by clearing with 100% histo-clear for 1 day.

For paraffin infiltration, samples were soaked for 2 hours each in the histo-clear/paraffin solution series consisted of 75% histo-clear/25% paraffin, 50% histo-clear/50% paraffin, 25% histo-clear /75% paraffin, and 100% paraffin at 55℃ for 1day. The paraffin infiltrated samples were embedded in a paraffin block and then cut

into 8~12㎛ 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 using 100% xylene for 1 hour and hydrated 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 100X, and 300X magnification to measure the bulliform cell number and size.

4. Measurement of the Leaf Rolling Index

For measuring the Leaf Rolling Index (LRI) value of wild type and *rl*, the LRI values were calculated as described by Shi et al (2007). The flag leaf and second leaf of plants at heading stage were used in measuring the widths of leaves under the natural condition (Ln) and unfolded condition (Lw). The formula for calculating the LRI is as follows: LRI(%)=(Lw-Ln)/Lw×100.

5. DNA extraction and PCR amplification

Genomic DNAs were extracted from fresh young leaves using the modified CTAB method (Murray and Thompson 1980). PCR amplification was performed in a total of 20ul reaction mixture containing 2ul of genomic DNA (30ng/ul), 1ul of 10X buffer (Mg^{2+}), 1ul of each forward and reverse primer (10 pM/ul), 250 uM of dNTP, and 0.5U of Taq DNA polymerase. Amplification was performed in a PCT100 96U Thermocycle (MJ Research, USA). The PCR conditions were 5min at 95℃ and 35cycles of 30 sec at 95℃, 30sec at 56℃, 30sec at 72℃, followed by 10min at 72℃ for the final extension. For detection of polymorphisms, the PCR products were electrophoresed on 2.5% agarose gel in 0.5X TBE buffer, and visualized by ethidium bromide staining.

6. Primers

Primers were developed based on available rice genome sequence data (http://www.ncbi.nlm.nih.gov; http://www.gramene.org). CAPS and dCAPS markers were designed with dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) and STS markers were designed by *in silico* approach (Primer3 software version 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0/).

7. Genetic analysis and mapping

For genetic analysis, F_1 and F_2 populations derived from the cross of each mutants and its wild type were used. The phenotype of individuals was recorded, and the segregation ratio was calculated. Using Chi square test, the observed segregation ratio of each mutants were compared with the expected ratio.

For genetic mapping of *rl* and *eui* gene, F_2 population was developed from a cross between each mutants and M23. For *rl*, 405 F_2 plants were used, and 354 F_2 plants and 128 F_3 plants were used for mapping *eui*. The phenotype of *eui* mutant type in the population was determined by measuring the length of all internodes after heading.

For BSA, 12 mutant and 12 wild type plants were selected and pooled into 3 bulks each with the same concentration. Total of 104 STS markers with known chromosomal position throughout all chromosomes, which were previously designed from Crop Molecular Breeding Laboratory, Seoul National University, were tested and the co-segregation markers were identified using these bulks. After the flanking region was determined, the phenotypes and genotypes of the F_2 mapping population was screened to sort the recombinants. With the identified recombinants, fine mapping was conducted using newly designed InDel markers (Table 1).

8. WGS of bulked DNA and MutMap analysis

DNA samples were prepared by bulking DNA from leaves of *rl*/Ilpum F2 individuals using CTAB method. The library for sequencing was constructed from five micrograms of DNA samples using TruseqNano DNA LT sample preparation kit (FC-121-4001). The qPCR was conducted using these libraries. The libraries were used for cluster generation and sequenced for 250 cycles on an Illumina HiSeq2500.

To generate Ilpum 'reference sequence', 20.12 Gb of Ilpum wild type sequence reads obtained by Illumina sequencing (from NICS of RDA) were aligned to the Nipponbare reference genome (build five genome sequence; http://rapdblegacy.dna.affrc.go.jp/download/index.html) by BWA (Burrows-Wheeler Aligner) software (Li and Durbin, 2009). The Ilpum 'reference sequence' was constructed by replacing Nipponbare nucleotides with those of Ilpum at the 225,249 SNP positions that were identified between the two cultivars.

Short reads were aligned to Ilpum 'reference sequence' using BWA. Alignment files were converted to SAM/BAM files using SAM tools, and the aligned short reads were filtered by Coval (S. Kosugiet al., unpublished;http://sourceforge.net/projects/coval105/?source=directory) to improve SNP calling accuracy. The SNP index was calculated as described by Abe et al. (2012).

Sliding window analysis was applied with 4 Mb window size and

10 kb increment. The average SNP-index and average *P*-value in Fisher's exact test for the SNPs were calculated.

9. RNA extraction and cDNA synthesis

Total RNA was extracted from leaves of Ilpum, and *rl* mutant plants, using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

For RT-PCR analysis, total RNA (2 lg) was treated with RNase-free DNase, and firststrand cDNA was synthesized through reverse transcription by an oligo (dT) primer (TaKaRa). Subsequently, the firststrand cDNA was used as a template for RT PCR analysis after being normalized with a rice Actin gene (Act). Amplification of the rice Act gene was performed with the forward primer 5' -TGTCATGGTTGGAATGGGCCA-3' and the reverse primer 5' -AGGCAGTCAGTCAGATCACGA-3' .

The PCR reaction for rl was performed by using the specific primer rl(ex5-6), which defines a 481-bp fragment of the cDNA, the forward primer 5' -AGCAGGTGGAAGGGGTTACT-3' and the reverse primer 5' -GCTCGGATGTAGCGATGAGA-3' . The PCR procedure is as follows: 94° C for 5 min, followed by 30 cycles at 95° C for 30 s, 58° C for 30 s, and 72° C for 30 s, finishing with an elongation step at 72° C for 5 min. The PCR products were analyzed on 2% agarose gels.

Table 1. PCR-based molecular markers designed for fine mapping.

	Marker	Forward primer (5′→3′)	Reverse primer (5′→3′)
	S01054	GCGAAGCCTGCTTTTTGAT	CGGAGATTTTTCCCTAAAACAA
	S01073	AGCAGTTGGTCAGTTGGACT	AAGAAGATAGAGAGAAGAGCAGTGG
	S01078	GGGAACTGCATTTCGATTCA	TCGCTACAAGACATCACAAGAA
	S01081	CCTAGCCAGCTAGCCTTGTG	TGGAAAAGGTGCACAAACTG
	S01082G	TGCTACAGTAGAAGGGCGTGT	TTTCCTCTTGTTGGTAGGAATAGG
rl	DC1083C	CCAAGCAAGAACGAACTGAT	CACTCTCGATCTCTCCCACAG
	S01085	CACTCGTGCATCTCCATCAT	CTACGCGTCTGGTTGTGTGT
	S01086	TCGGCACACACATACCAGAT	GGGCTTGGATCAGAACAAAA
	S01087	GAGTGGGCCATCATTTCTGT	ATGTTCCTGATTGCGTAGGG
	S01091	CCACCATGTATTCAGGCCATA	GGCTCACATAATGCTTCTGCT
	S01100C	TTGAGCATCTGGCATAAGCA	CGACGGATGCATGTGAGTAG
	S05086B	CGTTTGATCGTTCAGTTTCG	GATTTTGCCGCTAGCTGACT
	S05095	AGGCAGCAAGAGCATACCAT	CACTTGACCCTTGCAGGAAT
	S05095C	TCATCATGCAGGACATTGATA	AGAGCAAGAACAACTTGTGGT
	S05095e	ACTCCACCCATGGACTACTA	GATGGTCATCTGTTGGTTG
	S05098b	CACATTTGACAATATGGGCC	TCAGATTTACGTGGGTGTTT
eui	S05099	TTCAGGGTTTACAGTCTCGT	CGCAATTATTAGTTCTGCAA
	S05101	TGGGTGGAAATTTTCTTCTCAG	GGGTACAAAGCTCATTCTTTGG
	S05101c	CTTCAGCTCGATTCATCACT	ATGCATATGAACCGAAGAAC
	S05102	GGGCTGATATGTTCCTCGAA	TCAAGAGGAGACCATAACCATTG
	S05105	TGGCGTCCAAGAAGTAGGTC	TCTCTTGAAATCAACCCATCAA

RESULTS

1. Rolled leaf

Characterization of rolled leaf mutant

EMS treated Ilpum mutant showed upward rolling of leaves. This distinctive phenotype was noticed as early as 28 days after sowing. The leaves were not only rolled, but also narrowed in width and the characteristic lasted until the end of the life cycle of the plant (Fig 1).

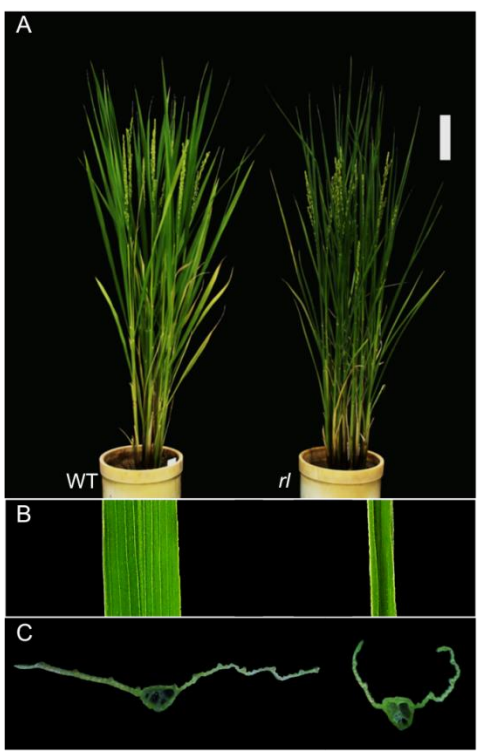


Figure 1. Morphology of wild type and *rl*.

(A) After heading. Bar = 10cm. WT: Ilpum, *rl*: *rl* mutant. (B) The leaf of Ilpum and *rl*. (C) The cross section of each leaf.

As shown in Figure 2, the lengths of flag leaf and second leaf were significantly higher in wild type plants (flag leaf and second leaf length of wild type were 42.2 cm and 52.5 cm, and that of *rl* were 30.5 cm and 46.8 cm). The leaf width of *rl* was significantly narrow in both flag and second leaf (1.42 cm, 1.19cm of wild type and 1.19 cm, 1.01 cm of *rl*, respectively). LRI value of *rl,* which expresses the severity of rolling, was 49.43% in flag leaf and 51.46% in second leaf. The result showed higher degree of leaf rolling in *rl.*

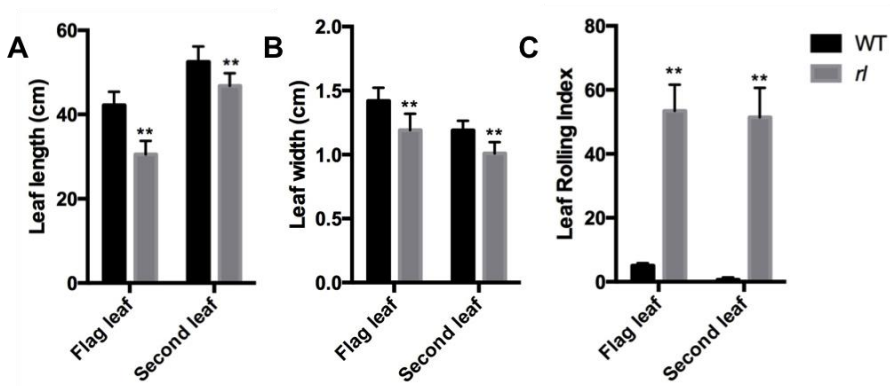


Figure 2. Comparison of the characteristics related to leaf of the wild type and *rl*.

To figure out other differences between wild type and *rl*, general agronomic traits were measured (Table 2). As shown in the figure below, most of the agronomic traits of *rl* were inferior compared to those of wild type plants. Especially, the differences were most prominent in plant height, culm length, spikelet per panicle, and 1000 grain weight, which are important in determining the yield.

Table 2. Comparison of agronomic traits of wild type and *rl*.

	HD (date)	PH** (cm)	CL** (cm)	PL* (cm)	PN^{ns} (No.)	SPP** (No.)	SF* (%)	TGW** (g)
WT	Aug. 20	85.2±3.8	68.7±2.7	22.13±1.1	12.6±1.9	149.9±13.5	90.3±4.9	25.4
rl	Aug. 8	79.9±2.8	54.4±3.1	20.69±0.9	12±2.8	101.8±8.9	86.4±2.0	19.0

HD=heading date, PH=plant height, CL=culm length, PL=panicle length, PN=panicle number per plant, SPP=spikelet per panicle, SF=spikelet fertility, TGW=thousand-grain weight.

Unpaired *t* test with Welch's correction was used to show significant difference (* $P < 0.05$, ** $P < 0.01$) compared with the wild type ($n>10$).

Histological analysis

As proved from many previous researches, cross section of leaf was conducted to figure out the consequence of histological changes on leaf morphology. Related studies revealed that the major factor controlling leaf-rolling trait is change in leaf polarity caused by the alteration of bulliform cells. Comparing the midrib of wild type (Fig. 3A) and *rl* (Fig. 3B), any kind of difference was not observed. However, the bulliform cells of *rl* located on the sides of large vascular bundle (Fig. 3D) and small vascular bundle (Fig. 3F) were deformed with significantly smaller size and less number (Fig. 4).

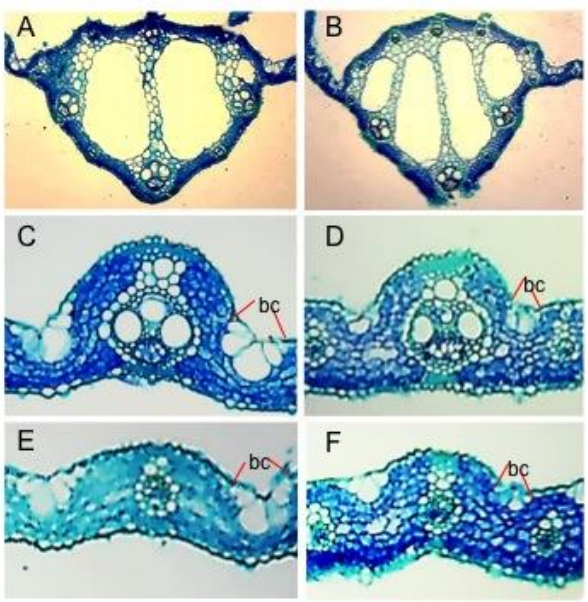


Figure 3. Leaf cross section of wild type and *rl*.

Deformation of bulliform cells (bc) led to leaf rolling. Midrib of wild type (A) and *rl*(B). Large vascular bundle (C and D) and small vascular bundle (E and F).

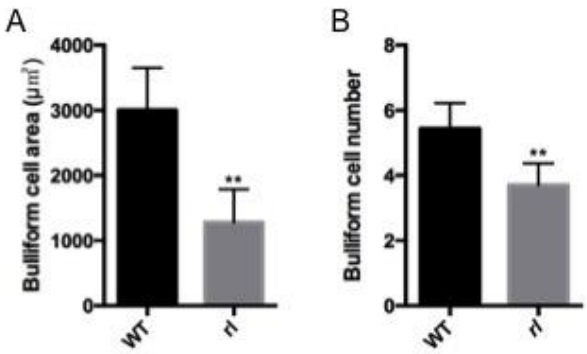


Figure 4. Comparison of the bulliform cell area and number of wild type and *rl*.

Genetic analysis

Using the F_1 and F_2 populations generated from the cross between Ilpum and *rl*, the phenotype was observed and recorded. The phenotypes of F_1progenies were all normal. In F_2 population, phenotypes were segregated into wild type and mutant type. The segregation ratio was calculated into 3:1, and the result of Chi square test (χ^2=0.213 <$\chi^2_{0.05(1)}$=3.841) suggested that the leaf rolling trait is controlled by a single recessive gene (Table 3).

Table 3. Segregation ratio of F_2 population ($\chi^2_{0.05(1)}$ = 3.841).

Total	Wild	*rl*	χ^2 (3:1)	P value (P=0.05)
189	139	50	0.213	0.6441

Genetic mapping of *rl* gene

Using 405 F_2 plants derived from the cross between *rl* and M23, mapping of *rl* gene was conducted. The result of BSA showed that from 104 STS markers used, the marker S01054 and S01100Clocated near the centromeric region of the long arm of chromosome 1 produced polymorphism between wild type and *rl* bulks (Fig. 5).

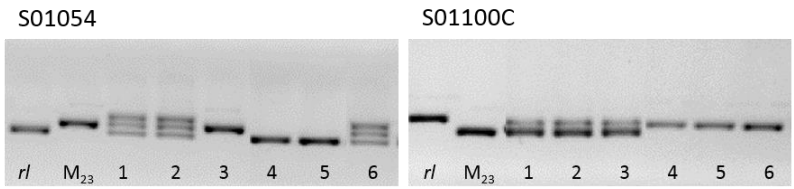
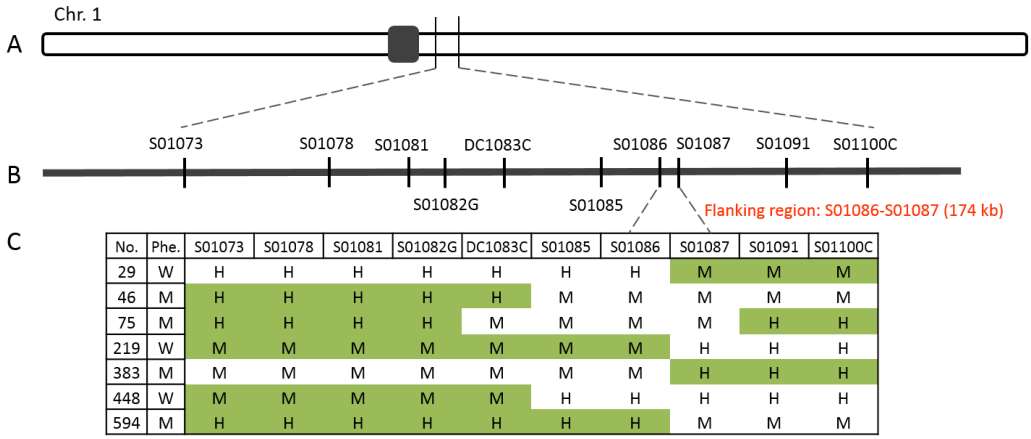


Figure 5. Bulked Segregant Analysis of wild type and *rl* bulks from *rl*/Milyang23 F_2 population.

rl: mutant, M23: Milyang23, 1−3: wild bulks, 4−6: *rl* bulks.

Using those two markers, F_2 population was genotyped into three types. To further narrow down the flanking region, InDel markers and dCAPS markers were newly designed and used. Out of 405 plants, there were 4 recombinants between S01086 and S01087, and the flanking region was narrowed down to 174kb (Fig. 6). As listed below in Table 4, 27 genes were identified within the region based on the database.



No.	Phe.	S01073	S01078	S01081	S01082G	DC1083C	S01085	S01086	S01087	S01091	S01100C
29	W	H	H	H	H	H	H	H	M	M	M
46	M	H	H	H	H	H	M	M	M	M	M
75	M	H	H	H	H	M	M	M	M	H	H
219	W	M	M	M	M	M	M	M	H	H	H
383	M	M	M	M	M	M	M	M	H	H	H
448	W	M	M	M	M	M	H	H	H	H	H
594	M	H	H	H	H	H	H	H	M	M	M

Figure 6. Genetic and physical maps of *rl*.

Rl was positioned on long arm of chromosome 1 near centromeric region flanked by the markers S01086 and S01087 (A). The candidate region was narrowed down into markers flanking from S01086 to S01087 (B). Genotype of closely linked recombinants. W=wild type, M=mutant type, H=hetero (C).

Table 4. The list of genes in the candidate region.

Locus Name	Gene Product
LOC_Os01g37590	peptide transporter PTR2, putative, expressed
LOC_Os01g37600	agenet domain containing protein, expressed
LOC_Os01g37610	retrotransposon protein, putative, unclassified, expressed
LOC_Os01g37630	esterase, putative, expressed
LOC_Os01g37650	esterase, putative, expressed
LOC_Os01g37660	hypothetical protein
LOC_Os01g37670	OsFBX15 - F-box domain containing protein, expressed
LOC_Os01g37690	sodium/calcium exchanger protein, putative, expressed
LOC_Os01g37700	expressed protein
LOC_Os01g37710	expressed protein
LOC_Os01g37720	retrotransposon protein, putative, unclassified
LOC_Os01g37740	expressed protein
LOC_Os01g37750	glutathione S-transferase, putative, expressed
LOC_Os01g37760	glutamate dehydrogenase protein, putative, expressed
LOC_Os01g37770	RWD domain containing protein, expressed
LOC_Os01g37780	expressed protein
LOC_Os01g37790	expressed protein
LOC_Os01g37800	ras-related protein, putative, expressed
LOC_Os01g37810	expressed protein
LOC_Os01g37820	oxidoreductase/ transition metal ion binding protein, putative, expressed
LOC_Os01g37825	M16 domain containing zinc peptidase, putative, expressed
LOC_Os01g37832	thioredoxin, putative, expressed
LOC_Os01g37837	seryl-tRNAsynthetase, putative, expressed
LOC_Os01g37842	expressed protein
LOC_Os01g37850	expressed protein
LOC_Os01g37860	retrotransposon protein, putative, unclassified, expressed
LOC_Os01g37870	PPR repeat domain containing protein, putative, expressed

Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu)

MutMap analysis

To locate the causal mutation and the gene responsible for leaf rolling trait, bulk of mutant phenotype DNA of 20 F_2 progenies derived from cross between Ilpum and *rl* were used for WGS and MutMap analysis. Using Illumina HiSeq 2500, paired-end sequencing was conducted and 16.59 Gb of sequence reads were obtained as a result (Table 5).

Table 5. Summary of Illumina sequencing and alignment data.

Sample	No. of mutant F_2 plants bulked	Total read (Gb)	Mapped alignment data (Gb)	Mean depth
Ilpum[a]	-	16.84	16.68	43.57
rl[b]	20	16.59	6.17	16.13

[a]The short reads of Ilpum were aligned to Nipponbare reference genome (IRGSP Build 5).

[b]The short reads from rl bulk were align to the Ilpum 'reference sequence'.

The sequence reads were aligned to Ilpum 'reference sequence' and total of 208,214 SNPs were identified through out 12 chromosomes. SNP index of each SNP positions were calculated and the SNP index plots were generated. Refer to Figure 7, the average SNP index peak from 20.59 Mb to 25.69 Mb on chromosome 1 was the most probable candidate region.

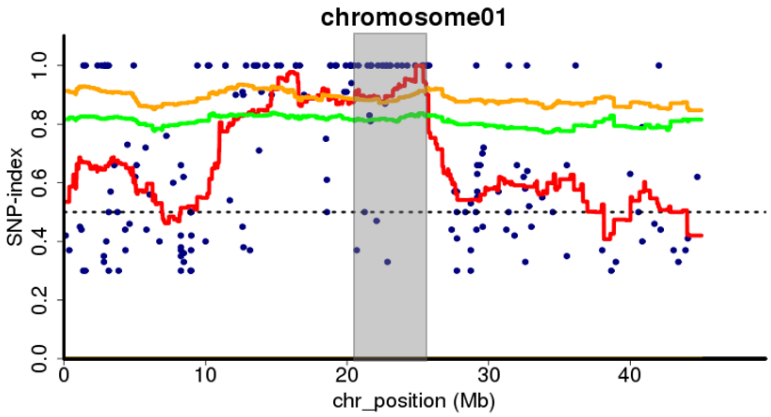


Figure 7. SNP index plot of chromosome 1 for identification of genomic regions harboring the causal mutation of *rl*.

Blue dot: SNP−index, red line: sliding window average of SNP−index, green line: sliding window average of 95%−confidence interval upper side, orange line: sliding window average of 99%−confidence interval upper side.

Considering the result of SNP index peak and genetic map together, the flanking region set by marker S01086 and S01087 was revealed to be a candidate region overall. Within this region, there were 10 SNPs with SNP index of 1. There was one *rl* specific SNP, physical position of 22,809,006 bp, located in genic region with point mutation from G to A (Table 6).The change of base pair at the position was confirmed by DNA sequencing as well. Using the SNP, dCAPS marker was designed for cosegregation test. After the enzyme treatment, corresponding bands of wild type and mutant type progenies were shown (Fig. 8).

Table 6. The candidate SNPs.

Chr.	Position	Ref.	Query	Depth	RAP locus ID	MSU locus ID
1	22764897	G	A	27	-	-
1	22765064	G	A	13	-	-
1	22779664	G	-	7	-	-
1	22787241	G	A	14	-	-
1	22788840	C	T	6	-	-
1	22803853	A	G	20	-	-
1	22809006	G	A	13	Os01g0559100	LOC_Os01g37837
1	22821193	T	C	13	-	-
1	22834459	C	T	19	-	-

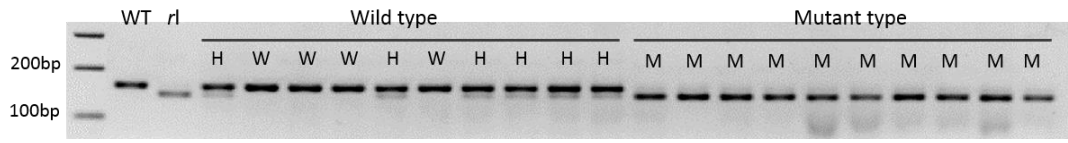


Figure 8. Genotyping of *rl*/Ilpum F_2 plants using designed dCAPS marker to confirm the causal SNP.

After restriction enzyme SpeI was digested for 2 hours.

Candidate gene study

According to the database, http://rapdb.dna.affrc.go.jp/, there were variations in gene structure, andSNP-22809006 was located on the splicing junction of Os01g0559100 (Fig. 9).

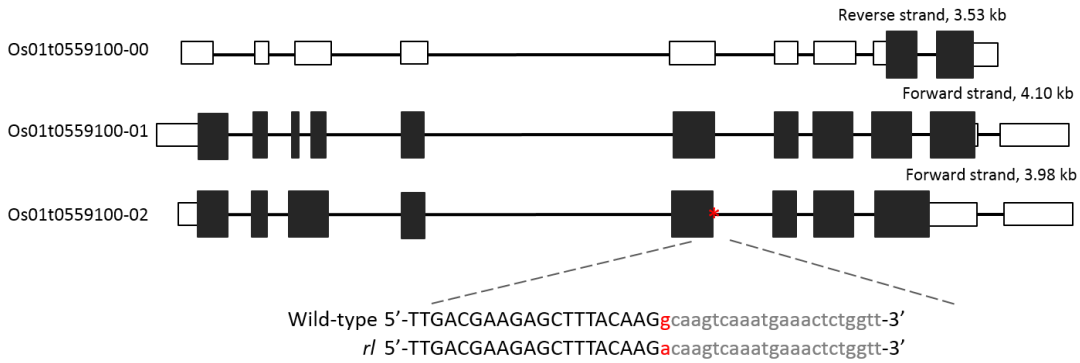


Figure 9. Variations in the gene structure of Os01g0559100.

The predicted gene structure is shown by white boxes (untranslated regions), black boxes (exons) and lines (introns).

The red asterisk mark on the beginning of 5th intron of Os01t0559100-2 indicates the mutation point.

Since the SNP was localized on a splicing recognition site, sequencing of cDNA was performed to prove the consequence of the mutation in splicing error.

Forward and reverse primers covering exon5, intron5, and exon6 were designed for RT-PCR. As presented in Figure 10B, there were 2 additional transcript forms from *rl*. The transcript with

length of 481 bp indicates that the splicing didn't occur and whole intron 5 part was transcribed. On the other hand, the transcript with 266bp size include 36 bp of intron 5, thus alternative splicing was occurred. There was also wild type transcript form, but the major forms were the alternative ones mentioned ahead.

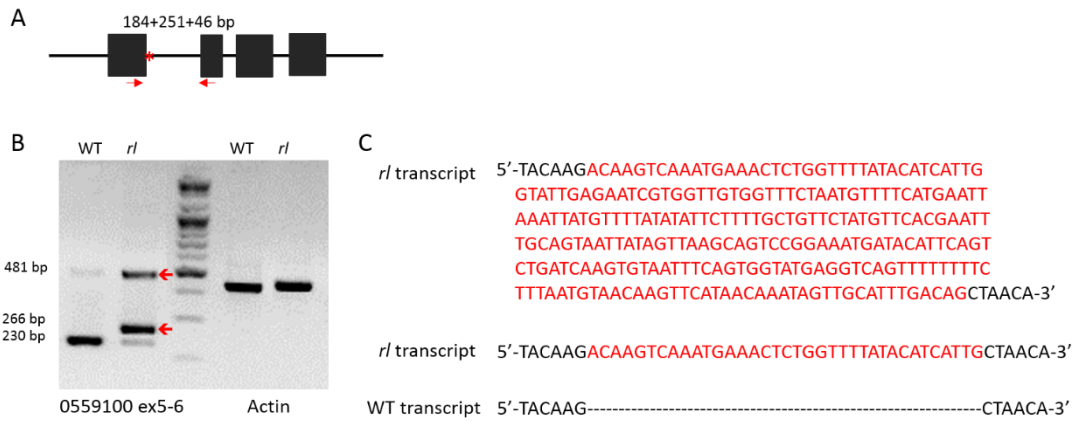


Figure 10. Confirmation of splicing error.

The partial structure of Os01g0559100 (A). Red asterisk mark indicates the mutation point, and red arrows indicates the forward and reverse primers designed for RT-PCR. The electrophoresed RT-PCR result (B). The sequence information of alternatively spliced products and the wild type (C).

2. Elongated uppermost internode

Characteristics of *eui* mutant

Among the EMS treated Koshihikari mutants, *eui* mutant with prominent elongation of upper internodes at heading stage was observed (Fig. 11). The general growth at vegetative stage was more superior in mutant. The longitudinal section of first internode suggested that the cell length was increased in *eui* (Fig. 12).As shown in Figure 13, increase in the length of first, second, and third internodes were significantly higher than that of wild type. Moreover, the rate of increase was highest in the second internode, 33.1 %, and followed by the first internode, 35.4 %.

Figure 11. Morphology of *eui* and wild type plants. (A) Before

flowering (B) After flowering (C) Comparison of the first, second, and third internodes of Koshihikari and *eui*.

As shown in Table 7, general agronomic traits were measured and compared between wild type and *eui*. Not only the internodes were elongated, but also plant height was increased. The plant growth in general was more superior in *eui*. *Eui* showed dominant growth over wild type in plant height, culm length, panicle length, and spikelet per panicle. Panicle number and spikelet fertility didn't show any significant differences,

Table 7. Comparison of agronomic traits of wild type and *eui*.

	HD (date)	PH** (cm)	CL** (cm)	PL** (cm)	PN^{ns} (No.)	SPP** (No.)	SF^{ns} (%)	TGW* (g)
WT	Aug. 10	97.4±5.5	86.2±3.0	18.8±1.1	12.1±2.5	106.8±9.5	94.4±4.2	25.8
eui	Aug. 9	114.2±4.3	114.6±11.3	22.2±1.8	10.4±2.8	125.4±14.9	94.9±2.3	24.6

HD=heading date, PH=plant height, CL=culm length, PL=panicle length, PN=panicle number per plant, SPP=spikelet per panicle, SF=spikelet fertility, TGW=thousand-grain weight.

Unpaired *t* test with Welch's correction was used to show significant difference (* $P < 0.05$, ** $P < 0.01$) compared with the wild type (*n*>

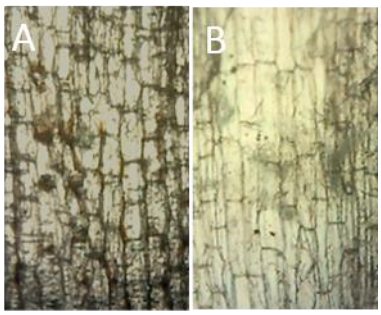


Figure 12. Longitudinal section of first internodes of wild type and *eui*. Wild type (A) and *eui* (B) under microscope (200x).

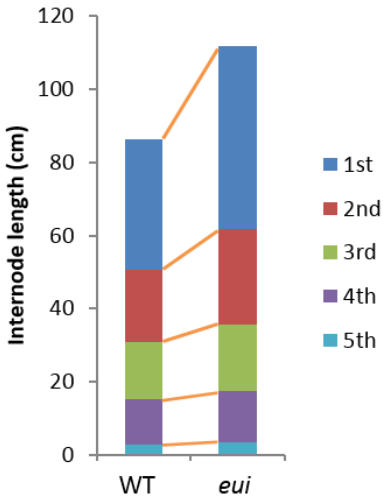


Figure 13. Comparison of the internode lengths. The rate of increase was highest in first and second internode (33.1% and 35.4%, respectively).

Genetic analysis

For the genetic analysis, the lengths of first and second internodes of F_1 and F_2 populations of Koshihikari/*eui* cross combination was

measured and recorded. The progenies of F_1 population showed all wild phenotype. Since the trait is qualitative, the distribution of each measurement of F_2 progenies was represented in Figure 14. As clearly shown, the graphs showed bimodal distribution along the average lengths of wild type and mutant type. Moreover, the frequency of peaks were segregated into 3 is to 1. Therefore, the trait is controlled by a single recessive gene.

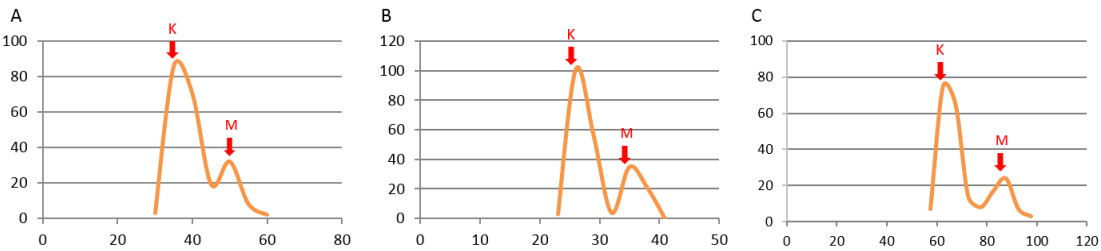


Figure 14. Distribution of the phenotype of F_2 population. The length of first internode (A) second internode (B), and the sum of first and second internodes (C). Arrow indicates the average first internode length of Koshihikari (35.5 cm) and *eui* (50.2 cm), and average second internode length of Koshihikari (24.9 cm) and *eui* (33.7 cm).

Genetic mapping of *eui* gene

Using the same method, BSA was performed using F_2 and F_3 progenies of M23/*eui* cross combination. Along the 12 chromosomes, the right segregation of genotype according to the phenotype was shown from marker S05086B and S05105 (Fig. 15).

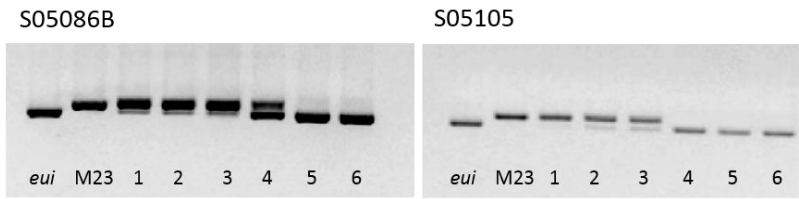
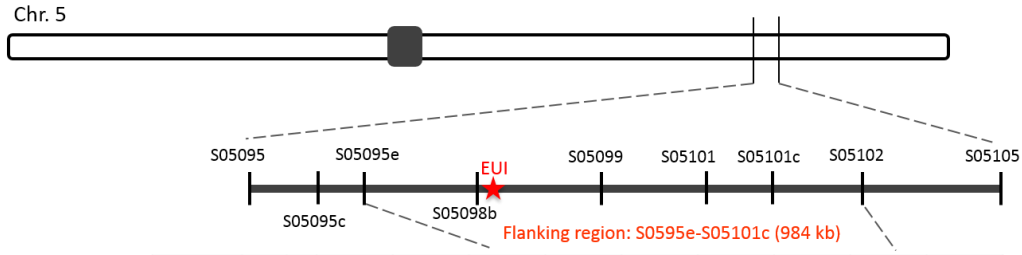


Figure 15. Bulked Segregant Analysis of wild type and *eui* bulks from *eui*/Milyang23 F_2 population.

eui: mutant, M23: Milyang23, 1−3: wild bulks, 4−6: mutant bulks

Setting the two markers as boundaries, InDel markers within the region was newly designed and used for the further narrow down (Fig. 16). As a result, *eui* was positioned on a long arm of chromosome 5 within 984 kb region flanked by the markers S05095e and S05101c, and cosegregated with the markers S05098b, S05099 and S05101. There was EUI1, previously found gene, located within the flanking region. However, it was confirmed that *eui* is different from EUI1 by manual sequencing of the genic region.



		P	S05095	S05095c	S05095e	S05098b	S05099	S05101	S05101c	S05102	S05105
F2	28	W	M	M	M	H	H	H	H	H	H
	40	W	H	H	H	H	H	H	H	H	M
	129	M	W	M	M	M	M	M	M	H	H
	399	M	H	H	H	M	M	M	M	M	M
	472	W	H	H	H	H	H	H	H	M	M
	493	M	M	M	M	M	M	M	M	M	H
F3	6	W	M	M	M	H	H	H	H	H	M
	37	W	M	M	M	W	W	W	W	W	W
	71	W	M	M	M	H	H	H	M	M	M
	86	M	M	M	M	M	M	M	M	M	H
	99	W	M	M	M	H	H	H	H	H	H

Figure 16. Physical mapping of *eui*.

*Eui*was positioned on long arm of chromosome 5 flanked by the markers S05095e and S05101c.W=wild type, M=mutant type, H=hetero.

DISCUSSION

The modification of plant architecture has been considered as important way of controlling potential yield of crops. There were countless genes found to be related with the architecture up to date. In this study, characterization and genetic mapping of *rl* and *eui* were conducted.

Rl showed upward curling and narrowed width of leaves as early as 28 days after sowing. Although other general agronomic traits are not favorable with low yield components, the leaf rolling trait is said to ease the light transmission in the canopy when crops are densely planted. According to the previously studied researches, bulliform cells on the surface of the leaves are one of the factors in leaf rolling, that change in bulliform cells could affect the leaf polarity and determine the direction of rolling. The result of paraffin cross section of *rl* revealed that deficient formation of bulliform cells with less number and size led to upward curling of leaves.

Based on the standard, F_2progenies of Ilpum/*rl* cross were differentiated into wild type and mutant type. The observed phenotypes were segregated into 3:1, and suggested that the trait was controlled by a single recessive gene. To locate the exact position of the gene, BSA was performed first. The flanking region was set to a centromeric region of long arm of chromosome 1.

Further narrow down was carried on using newly designed InDel markers and dCAPS markers. As a result, the candidate region was narrowed down into 174 kb, from S01086 to S01087. Bulked whole genome sequencing and MutMap analysis revealed the causal SNP within the flanking region. Based on a database, the SNP was located on a splicing recognition site of 5th intron of Os01g0559100. The splicing error is commonly generated by GT at the beginning and AG at the end of the intron. By a single base change from G to A, there were variations in splicing. There were two dominant transcript forms; 481 bp length including whole intron 5 that no splicing had occurred, and 266 bp length with alternative splicing on 37thbp of intron 5. The normal transcript form with 230 bp was also found in *rl*, but it is not a major transcript since the band intensity was faded.

The gene Os01g0559100 is known to encode putative seryl-tRNA synthetase, belongs to class-II aminoacyl-tRNA synthetase family. Aminoacyl-tRNA synthetases (ARSs) are a group of enzymes, which activate amino acids and transfer them to specific tRNA molecules as the first step in protein biosynthesis (Schimmel 1987). Although ARSs play key determinant role in transmission of genetic information and protein synthesis, the studies on ARSs had been limited to *E. coli*, yeast and some mammals (Ibba and Soll 2000). There are only confined number of reports on function and

regulation of the ARS genes and proteins in plants (Browning 1996). In *Arabidopsis*, a tDNA insertion mutant with defect in cytosolic and mitochondrial AlaRS caused alterations in patterns of cell division and differentiation, and showed an embryonic lethality (Ge et al. 1998). Moreover, *twn2* mutant was reported to have severe defects in early embryogenesis due to the arrested development of apical cells from altered expression of cytosolic ValRS (Zhang and Somerville 1997). Kim et al. (2005) revealed that gene silencing of the *N. benthamiana NbERS* and *NbSRS*, which encode organellar GluRS and SerRS, respectively, drastically reduced numbers and size of chloroplasts and chlorophyll content, resulted in severe leaf yellowing and abnormal leaf morphology. Further studies to connect the role of the protein in rolled leaf phenotype are required.

Eui showed significantly increased first, second and third internodes length compared to the wild type. Among five internodes, the second internode showed the highest rate of increase. Longitudinal section of first internode indicated that the cell length was increased, and led to an internode elongation. According to the study of Zhu et al. (2006), other mutant showed increase of cell length and cell number together. Genetic analysis was conducted by measuring the length of first and second internodes. The distribution of each measurement was compared. The figures showed bimodal distribution aligned with the average length of wild

type and *eui*. As anticipated, distribution of the sum of first and second internodes showed sharper form. The result suggested that internode elongation trait is controlled by a single recessive gene. BSA and fine mapping was conducted to locate the gene using the recombinants from F_2 and F_3 populations generated from a cross of M23 and *eui*. The flanking region was narrowed down into 984 kb region of long arm of chromosome 5. Although EUI1 discovered by Xu et al. (2004) is located within the flanking region, another gene is thought to be involved in controlling the trait within the region that any kind of mutation in EUI1 gene region was not found in *eui*. Further narrow down of the flanking region is required.

In conclusion, a novel gene associated with semi-narrow and adaxially rolled leaf trait was identified. The fine mapping of *eui* is currently conducted. This study could be helpful to generate rice plant architecture with various morphogenesis for specific purposes.

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초록

잎 말림 돌연변이 벼와 절간신장 돌연변이 벼의 형질조사와 유전자 지도 작성

식물의 초형에 대한 이해를 높이기 위하여 일품과 고시히카리 품종 각각에 EMS처리 하여 얻어진 잎 말림 돌연변이와 절간신장 돌연변이에 관한 연구가 진행 되었다. 잎 말림 형질은 식물체가 제한된 면적에 밀집되어 심어져 있을 때에 군락의 수광태세를 좋게 하고, 가뭄의 저항성 기작으로 작용하게 되면 에너지 이용효율에 큰 이점이 있다고 알려져 있다. 해당 유전자는 잎이 위로 말리는 형태를 보이며, 파라핀을 이용하여 잎을 횡단면으로 절단 후 관찰 한 결과 잎 표면적의 기동세포(bulliform cell)의 크기와 수가 현저하게 작아져 있음을 발견하였다. 일품/*rl* 교배집단의 표현형 조사를 실시하여 분리비를 살펴보았는데, F1의 경우 모두 정상개체의 표현형을 보였고, F2 집단의 경우 3:1의 분리비로 표현형이 나뉘었다. 카이제곱 검증으로 확인한 결과, single recessive gene에 의하여 표현형이 조절된다는 것을 알 수 있었다. 밀양23/*rl* 교배집단을 이용하여 bulked segregant analysis를 수행 한 결과 후보유전자가 1번 염색체의 long arm 상단부에 위치함을 확인하였고, 그 구간에서 InDel마커 제작을 통하여 narrow down 을 진행 한 결과 마커 S01086과 S01087사이의 174kb로 좁힐 수 있었다. 또한 차세대 염기서열 분석방법을 이용하여 해당 구간 내에서 돌연변이에 직접적으로 관여하는 SNP를 확인하였다. 분석에 따르면 유전자 Os01g0559100의 5번째 인트론

시작 부분에 G에서 A로 base change가 일어났고, 이는 splice site에 해당한다. 일품/*rl* F2 집단에 dCAPS 마커 분석을 실시 하여 변이를 확인하였다. RNA추출을 통한 cDNA 합성 및 RT PCR 결과, 변이로 인하여 다양한 형태의 alternative splicing이 일어나, 해당 유전자의 encoding protein에도 영향을 미쳤을 것으로 생각된다. 절간신장 돌연변이의 경우 정상개체에 비하여 이삭추출도 및 상위 세 번째까지 절간의 길이가 두드러지게 증가하였다. 이외의 다른 일반 농업형질에서도 전반적으로 우수한 생육을 보였다. 고시히카리/eui 교배집단의 표현형 조사는 상위 두번째 절간까지의 길이를 측정하였으며 이를 분산그래프로 나타내어 분석에 이용하였다. 그 결과, 그래프의 모양이 이정 곡선을 그리며 각 정점이 세배의 차이가 나는 것으로 보아 절간신장 형질 또한 single recessive gene에 의하여 조절되는 것을 알 수 있었다. 밀양23/eui 교배집단을 이용하여 BSA를 수행 한 결과, 후보유전자가 5번 염색체의 하단부에 위치 하는 것을 확인 하였다. Narrow down을 통하여 flanking region을 마커 S05095e와 S05101c의 984kb 사이로 좁힐 수 있었다. 해당 구간 내에는 전에 연구 된 *EUI1* 유전자가 위치 하지만, 시퀀싱 확인 결과 이 돌연변이에 관여하지 않음을 확인 하였고 5 번 크로모좀 내에 *EUI1* 유전자 외에 다른 유전자가 절간신장에 관여 할 것으로 생각된다.

주요어: 벼, 식물 초형, 잎 말림 형질, 기동세포, 유전자지도작성, 절간신장

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Characterization and Molecular Mapping of Rolled Leaf Mutant and Elongated Uppermost Internode Mutant in Rice

BY

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UNDER THE DIRECTION OF PROFESSOR HEE-JONG KOH
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
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YOON KYUNG LEE

ABSTRACT

To understand the various plant architecture and morphogenesis in rice (*Oryza sativa* L.), ethyl methanesulfonate treated Ilpum mutant with narrow and rolled leaf phenotype and Koshihikari mutant with elongated uppermost internode trait were identified. The leaf rolling trait is said to be more advantageous under high temperature and heat stress. Cytological analysis of the leaf suggested that smaller size and less number of the bulliform cells caused leaf rolling. Ilpum/*rl* crossed F_1 plants showed normal phenotype. Genetic analysis of its F_2 population suggested that the mutation was controlled by a single recessive gene with segregation ratio of 3:1. Using M23/*rl* F_2 mapping population, each chromosome was screened with STS markers by the BSA method. The candidate region was detected to a long arm of chromosome 1, near the centromeric region. Through whole genome sequencing

and its MutMap analysis, the causal SNP within the candidate region was identified. The result of RT PCR suggested that splicing error was occurred due to a base change from G to A at the beginning of 5th intron of *Loc_Os01g37837*, a gene which encode putative seryl-tRNA synthetase, and various forms of transcripts were presented. A novel gene that cause change of leaf morphology into narrow and rolled leaf was newly discovered. *Eui* mutant showed significantly increased first three internodes. The phenotype of F_2 progenies of Koshihikari/*eui* was determined by measuring the length of first and second internodes. The distribution of each measurement implied that the mutant phenotype was controlled by a single recessive gene. BSA result showed that the flanking region was set to a long arm of chromosome 5. Further narrow down was conducted into 984kb of the markers S0595e and S05101c, including *EUI1*. However, sequencing result suggested that the *eui* didn't have mutation correspond to *EUI1*. Another gene than EUI1 is thought to be related with internode elongation trait in chromosome 5.

Key words: rice, plant architecture, rolled leaf, narrow leaf, bulliform cell, elongated uppermost internode

Student number: 2014-20032

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LIST OF ABBREVIATIONS

BSA	Bulked segregant analysis
CTAB	Cetyltrimethylammonium bromide
dCAPS	Derived cleaved amplified polymorphic sequence
DNA	Deoxyribonucleic acid
EMS	Ethyl methanesulfonate
eui	Elongated uppermost internode mutant
InDel	Insertions anddeletions
M23	Milyang23
PCR	Polymerase chain reaction
rl	Rolled leaf mutant
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
STS	Sequence tagged site

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important human food, eaten by more than half of the world's population every day. Food crisis had emerged as population growth reached to critical level, so that the importance of food security had never been more emphasized. Researches for increasing yield potential have been conducted all over the world, and the aim was first achieved by modification of plant architecture with desirable traits. The ideotype of rice is the one characterized with erect type, decreased tiller number, long panicle size, and semi dwarf type (Khush, 2001). Not only for the increase of yield potential, researches related to plant architecture aiming for other specific goals have been conducted as well.

The plant architecture determines the efficiency of its physiology including light capture, photosynthesis, and energy formation, so that the leaf shape has been considered as one of the important characteristics in plant breeding. The leaf is a major organ of photosynthesis in rice. Advanced scientific studies have revealed that the moderate leaf rolling trait could lead to the ideal plant architecture with erected leaves throughout the growth period (Lang et al., 2004). As a consequence, high-energy utilization efficiency, delayed leaf senescence, and even increased yield are anticipated as positive effects of the trait. The mutants showing

rolled leaf trait could be classified by the direction of rolling; incurved or abaxially rolled leaves and outcurved or adaxially rolled leaves. Studies have revealed that the trait is controlled by several factors, either of leaf polarity in the abaxial–adaxial epidermis, deformativebulliform cells, defective development of sclerenchymatous cells, and defective programmed cell death of mesophyll cells. There are some genes found, but only few among them have been characterized up to date.

Elongation of internode is not exactly the ideotype of rice mentioned since it could cause lodging, but EUI mutant has been importantly used in hybrid breeding. For hybrid breeding, the role of male sterile plant is critical. However, most of male sterile plants have panicle exsertion problem, so enormous amount of auxin to compromise was used. The use of auxin led to viviparous germination and caused severe environment issue. Since 1980's when the trait was first discovered by Rutger and Carnahan (1981), the panicle exsertion problem of male sterile plant was resolved. Other than that, use of male plant with EUI trait could ease the pollen shedding in usual cross breeding.

The objectives of this study were to characterize mutants, and to identify novel genes that are directly related to each trait by genetic analysis, mapping, and whole genome sequencing technology.

MATERIALS AND METHODS

1. Plant materials and growth conditions

The *rl* mutant was obtained from ethyl methanesulfonate (EMS) treated Korean *japonica* rice cultivar 'Ilpum', and *eui* mutant was generated from EMS treated Japanese *japonica* rice cultivar 'Koshihikari'. Each mutant was selected at M_2 generation and fixed into pure-line by repetitive selfing.

For genetic analysis and whole genome sequencing, F_2 population generated from the cross of each mutant and the wild type was used. For BSA and genetic mapping, F_2 population of the cross of each mutant and M23 was used.

All plant materials were grown in the paddy field of Seoul National University Experimental Farm with natural long day conditions at 37° N latitude, Suwon, Korea.

2. Measurement of agronomic traits

The plant height was measured from the surface of the ground to the leaf tip for 10 individual mature plants before heading for each of wild type, *rl*, and *eui*. Heading date, culm length panicle length, panicle number, spikelet per panicle, 1000-grain weight, and grain fertility were measured. For measuring 1000-grain weight, 500 seeds of each wild type and mutants were collected, the weight was

measured and the value was multiplied by 2. The process was repeated twice.

The observed measurements were analyzed for significance relation using Statistical Analysis System (SAS) program.

3. Histology and microscopy analysis

For paraffin section analysis of wild type and *rl*, Ji's paraffin embedding method (2006) with slight modifications was used. The widest part of second leaf was taken, and all leaf samples were collected at late vegetative stage. The 1cm length of leaf samples were separately fixed into a FAA solution (formaldehyde 3.7%: acetic acid 5% : ethanol 50%) for 1day at 4℃ and then dehydrated for 2 hours each in a graded ethanol solution series(70%, 85%, 95% and 100%). At the final step, the leaf samples were dehydrated for 1day. After that, dehydrated samples were cleared for 2hours each in a clearing solution series consisted of 75% ethanol/25% histo-clear, 50% ethanol/50% histo-clear, 25% ethanol/75% histo-clear, followed by clearing with 100% histo-clear for 1 day.

For paraffin infiltration, samples were soaked for 2 hours each in the histo-clear/paraffin solution series consisted of 75% histo-clear/25% paraffin, 50% histo-clear/50% paraffin, 25% histo-clear /75% paraffin, and 100% paraffin at 55℃ for 1day. The paraffin infiltrated samples were embedded in a paraffin block and then cut

into 8~12㎛ 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 using 100% xylene for 1 hour and hydrated 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 100X, and 300X magnification to measure the bulliform cell number and size.

4. Measurement of the Leaf Rolling Index

For measuring the Leaf Rolling Index (LRI) value of wild type and *rl*, the LRI values were calculated as described by Shi et al (2007). The flag leaf and second leaf of plants at heading stage were used in measuring the widths of leaves under the natural condition (Ln) and unfolded condition (Lw). The formula for calculating the LRI is as follows: LRI(%)=(Lw-Ln)/Lw×100.

5. DNA extraction and PCR amplification

Genomic DNAs were extracted from fresh young leaves using the modified CTAB method (Murray and Thompson 1980). PCR amplification was performed in a total of 20ul reaction mixture containing 2ul of genomic DNA (30ng/ul), 1ul of 10X buffer (Mg^{2+}), 1ul of each forward and reverse primer (10 pM/ul), 250 uM of dNTP, and 0.5U of Taq DNA polymerase. Amplification was performed in a PCT100 96U Thermocycle (MJ Research, USA). The PCR conditions were 5min at 95℃ and 35cycles of 30 sec at 95℃, 30sec at 56℃, 30sec at 72℃, followed by 10min at 72℃ for the final extension. For detection of polymorphisms, the PCR products were electrophoresed on 2.5% agarose gel in 0.5X TBE buffer, and visualized by ethidium bromide staining.

6. Primers

Primers were developed based on available rice genome sequence data (http://www.ncbi.nlm.nih.gov; http://www.gramene.org). CAPS and dCAPS markers were designed with dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) and STS markers were designed by *in silico* approach (Primer3 software version 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0/).

7. Genetic analysis and mapping

For genetic analysis, F_1 and F_2 populations derived from the cross of each mutants and its wild type were used. The phenotype of individuals was recorded, and the segregation ratio was calculated. Using Chi square test, the observed segregation ratio of each mutants were compared with the expected ratio.

For genetic mapping of *rl* and *eui* gene, F_2 population was developed from a cross between each mutants and M23. For *rl*, 405 F_2 plants were used, and 354 F_2 plants and 128 F_3 plants were used for mapping *eui*. The phenotype of *eui* mutant type in the population was determined by measuring the length of all internodes after heading.

For BSA, 12 mutant and 12 wild type plants were selected and pooled into 3 bulks each with the same concentration. Total of 104 STS markers with known chromosomal position throughout all chromosomes, which were previously designed from Crop Molecular Breeding Laboratory, Seoul National University, were tested and the co-segregation markers were identified using these bulks. After the flanking region was determined, the phenotypes and genotypes of the F_2 mapping population was screened to sort the recombinants. With the identified recombinants, fine mapping was conducted using newly designed InDel markers (Table 1).

8. WGS of bulked DNA and MutMap analysis

DNA samples were prepared by bulking DNA from leaves of *rl*/Ilpum F2 individuals using CTAB method. The library for sequencing was constructed from five micrograms of DNA samples using TruseqNano DNA LT sample preparation kit (FC-121-4001). The qPCR was conducted using these libraries. The libraries were used for cluster generation and sequenced for 250 cycles on an Illumina HiSeq2500.

To generate Ilpum 'reference sequence', 20.12 Gb of Ilpum wild type sequence reads obtained by Illumina sequencing (from NICS of RDA) were aligned to the Nipponbare reference genome (build five genome sequence; http://rapdblegacy.dna.affrc.go.jp/download/index.html) by BWA (Burrows-Wheeler Aligner) software (Li and Durbin, 2009). The Ilpum 'reference sequence' was constructed by replacing Nipponbare nucleotides with those of Ilpum at the 225,249 SNP positions that were identified between the two cultivars.

Short reads were aligned to Ilpum 'reference sequence' using BWA. Alignment files were converted to SAM/BAM files using SAM tools, and the aligned short reads were filtered by Coval (S. Kosugiet al., unpublished;http://sourceforge.net/projects/coval105/?source=directory) to improve SNP calling accuracy. The SNP index was calculated as described by Abe et al. (2012).

Sliding window analysis was applied with 4 Mb window size and

10 kb increment. The average SNP-index and average *P*-value in Fisher's exact test for the SNPs were calculated.

9. RNA extraction and cDNA synthesis

Total RNA was extracted from leaves of Ilpum, and *rl* mutant plants, using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

For RT-PCR analysis, total RNA (2 lg) was treated with RNase-free DNase, and firststrand cDNA was synthesized through reverse transcription by an oligo (dT) primer (TaKaRa). Subsequently, the firststrand cDNA was used as a template for RT PCR analysis after being normalized with a rice Actin gene (Act). Amplification of the rice Act gene was performed with the forward primer 5' -TGTCATGGTTGGAATGGGCCA-3' and the reverse primer 5' -AGGCAGTCAGTCAGATCACGA-3' .

The PCR reaction for rl was performed by using the specific primer rl(ex5-6), which defines a 481-bp fragment of the cDNA, the forward primer 5' -AGCAGGTGGAAGGGGTTACT-3' and the reverse primer 5' -GCTCGGATGTAGCGATGAGA-3' . The PCR procedure is as follows: 94° C for 5 min, followed by 30 cycles at 95° C for 30 s, 58° C for 30 s, and 72° C for 30 s, finishing with an elongation step at 72° C for 5 min. The PCR products were analyzed on 2% agarose gels.

Table 1. PCR-based molecular markers designed for fine mapping.

	Marker	Forward primer (5′→3′)	Reverse primer (5′→3′)
	S01054	GCGAAGCCTGCTTTTTGAT	CGGAGATTTTTCCCTAAAACAA
	S01073	AGCAGTTGGTCAGTTGGACT	AAGAAGATAGAGAGAAGAGCAGTGG
	S01078	GGGAACTGCATTTCGATTCA	TCGCTACAAGACATCACAAGAA
	S01081	CCTAGCCAGCTAGCCTTGTG	TGGAAAAGGTGCACAAACTG
	S01082G	TGCTACAGTAGAAGGGCGTGT	TTTCCTCTTGTTGGTAGGAATAGG
rl	DC1083C	CCAAGCAAGAACGAACTGAT	CACTCTCGATCTCTCCCACAG
	S01085	CACTCGTGCATCTCCATCAT	CTACGCGTCTGGTTGTGTGT
	S01086	TCGGCACACACATACCAGAT	GGGCTTGGATCAGAACAAAA
	S01087	GAGTGGGCCATCATTTCTGT	ATGTTCCTGATTGCGTAGGG
	S01091	CCACCATGTATTCAGGCCATA	GGCTCACATAATGCTTCTGCT
	S01100C	TTGAGCATCTGGCATAAGCA	CGACGGATGCATGTGAGTAG
	S05086B	CGTTTGATCGTTCAGTTTCG	GATTTTGCCGCTAGCTGACT
	S05095	AGGCAGCAAGAGCATACCAT	CACTTGACCCTTGCAGGAAT
	S05095C	TCATCATGCAGGACATTGATA	AGAGCAAGAACAACTTGTGGT
	S05095e	ACTCCACCCATGGACTACTA	GATGGTCATCTGTTGGTTG
	S05098b	CACATTTGACAATATGGGCC	TCAGATTTACGTGGGTGTTT
eui	S05099	TTCAGGGTTTACAGTCTCGT	CGCAATTATTAGTTCTGCAA
	S05101	TGGGTGGAAATTTTCTTCTCAG	GGGTACAAAGCTCATTCTTTGG
	S05101c	CTTCAGCTCGATTCATCACT	ATGCATATGAACCGAAGAAC
	S05102	GGGCTGATATGTTCCTCGAA	TCAAGAGGAGACCATAACCATTG
	S05105	TGGCGTCCAAGAAGTAGGTC	TCTCTTGAAATCAACCCATCAA

RESULTS

1. Rolled leaf

Characterization of rolled leaf mutant

EMS treated Ilpum mutant showed upward rolling of leaves. This distinctive phenotype was noticed as early as 28 days after sowing. The leaves were not only rolled, but also narrowed in width and the characteristic lasted until the end of the life cycle of the plant (Fig 1).

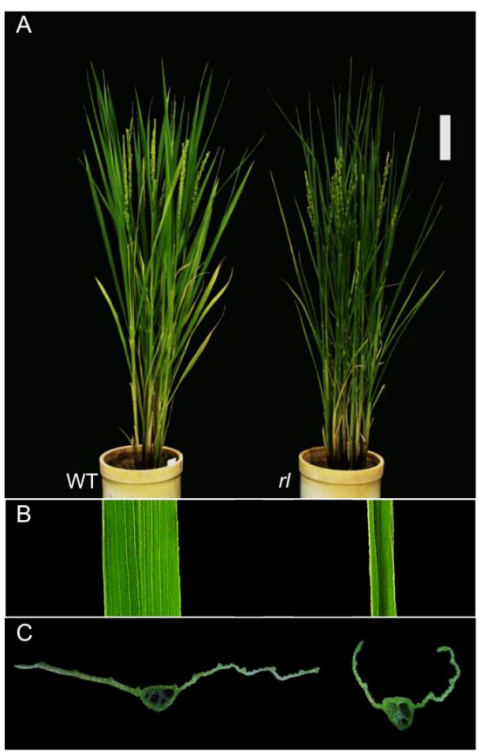


Figure 1. Morphology of wild type and *rl*.

(A) After heading. Bar = 10cm. WT: Ilpum, *rl*: *rl* mutant. (B) The leaf of Ilpum and *rl*. (C) The cross section of each leaf.

As shown in Figure 2, the lengths of flag leaf and second leaf were significantly higher in wild type plants (flag leaf and second leaf length of wild type were 42.2 cm and 52.5 cm, and that of *rl* were 30.5 cm and 46.8 cm). The leaf width of *rl* was significantly narrow in both flag and second leaf (1.42 cm, 1.19cm of wild type and 1.19 cm, 1.01 cm of *rl*, respectively). LRI value of *rl,* which expresses the severity of rolling, was 49.43% in flag leaf and 51.46% in second leaf. The result showed higher degree of leaf rolling in *rl.*

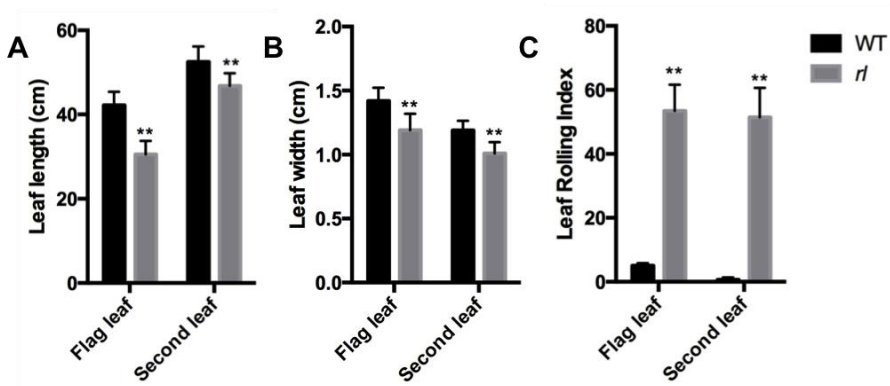


Figure 2. Comparison of the characteristics related to leaf of the wild type and *rl*.

To figure out other differences between wild type and *rl*, general agronomic traits were measured (Table 2). As shown in the figure below, most of the agronomic traits of *rl* were inferior compared to those of wild type plants. Especially, the differences were most prominent in plant height, culm length, spikelet per panicle, and 1000 grain weight, which are important in determining the yield.

Table 2. Comparison of agronomic traits of wild type and *rl*.

	HD (date)	PH** (cm)	CL** (cm)	PL* (cm)	PN^{ns} (No.)	SPP** (No.)	SF* (%)	TGW** (g)
WT	Aug. 20	85.2±3.8	68.7±2.7	22.13±1.1	12.6±1.9	149.9±13.5	90.3±4.9	25.4
rl	Aug. 8	79.9±2.8	54.4±3.1	20.69±0.9	12±2.8	101.8±8.9	86.4±2.0	19.0

HD=heading date, PH=plant height, CL=culm length, PL=panicle length, PN=panicle number per plant, SPP=spikelet per panicle, SF=spikelet fertility, TGW=thousand-grain weight.

Unpaired *t* test with Welch's correction was used to show significant difference (* $P < 0.05$, ** $P < 0.01$) compared with the wild type ($n>10$).

Histological analysis

As proved from many previous researches, cross section of leaf was conducted to figure out the consequence of histological changes on leaf morphology. Related studies revealed that the major factor controlling leaf-rolling trait is change in leaf polarity caused by the alteration of bulliform cells. Comparing the midrib of wild type (Fig. 3A) and *rl* (Fig. 3B), any kind of difference was not observed. However, the bulliform cells of *rl* located on the sides of large vascular bundle (Fig. 3D) and small vascular bundle (Fig. 3F) were deformed with significantly smaller size and less number (Fig. 4).

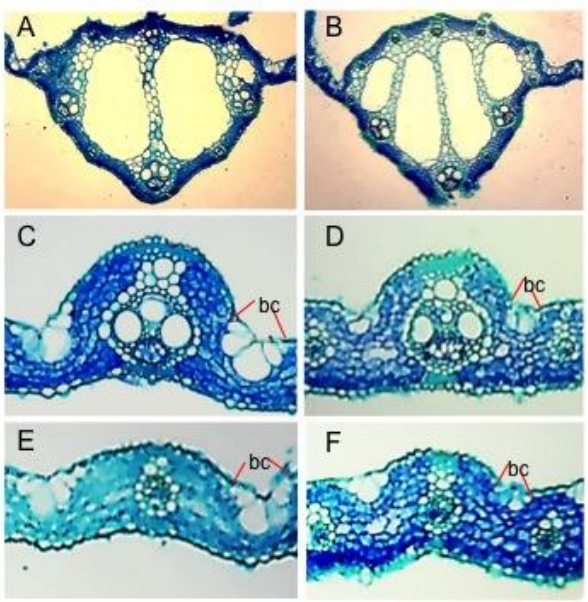


Figure 3. Leaf cross section of wild type and *rl*.

Deformation of bulliform cells (bc) led to leaf rolling. Midrib of wild type (A) and *rl*(B). Large vascular bundle (C and D) and small vascular bundle (E and F).

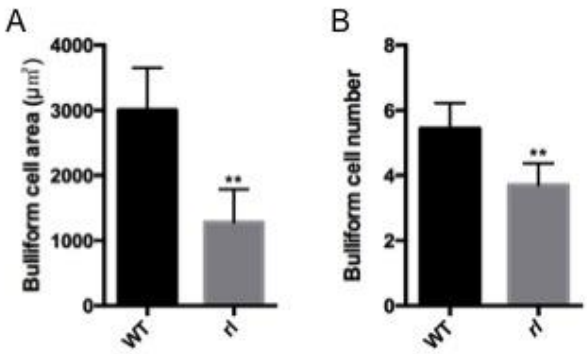


Figure 4. Comparison of the bulliform cell area and number of wild type and *rl*.

Genetic analysis

Using the F_1 and F_2 populations generated from the cross between Ilpum and *rl*, the phenotype was observed and recorded. The phenotypes of F_1progenies were all normal. In F_2 population, phenotypes were segregated into wild type and mutant type. The segregation ratio was calculated into 3:1, and the result of Chi square test (χ^2=0.213 <$\chi^2_{0.05(1)}$=3.841) suggested that the leaf rolling trait is controlled by a single recessive gene (Table 3).

Table 3. Segregation ratio of F_2 population ($\chi^2_{0.05(1)}$ = 3.841).

Total	Wild	*rl*	χ^2 (3:1)	P value (P=0.05)
189	139	50	0.213	0.6441

Genetic mapping of *rl* gene

Using 405 F_2 plants derived from the cross between *rl* and M23, mapping of *rl* gene was conducted. The result of BSA showed that from 104 STS markers used, the marker S01054 and S01100Clocated near the centromeric region of the long arm of chromosome 1 produced polymorphism between wild type and *rl* bulks (Fig. 5).

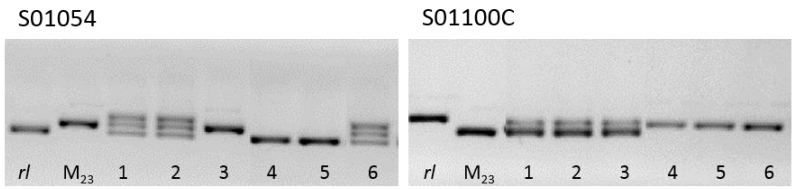
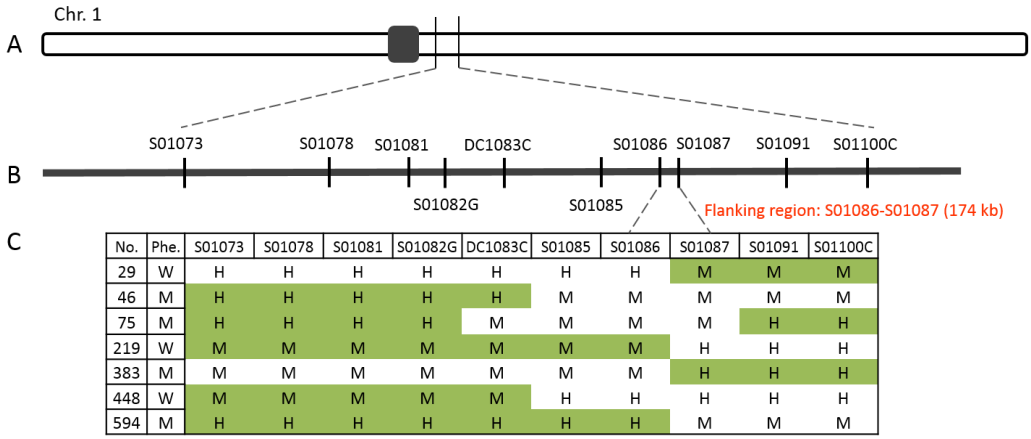


Figure 5. Bulked Segregant Analysis of wild type and *rl* bulks from *rl*/Milyang23 F_2 population.

rl: mutant, M23: Milyang23, 1−3: wild bulks, 4−6: *rl* bulks.

Using those two markers, F_2 population was genotyped into three types. To further narrow down the flanking region, InDel markers and dCAPS markers were newly designed and used. Out of 405 plants, there were 4 recombinants between S01086 and S01087, and the flanking region was narrowed down to 174kb (Fig. 6). As listed below in Table 4, 27 genes were identified within the region based on the database.



No.	Phe.	S01073	S01078	S01081	S01082G	DC1083C	S01085	S01086	S01087	S01091	S01100C
29	W	H	H	H	H	H	H	H	M	M	M
46	M	H	H	H	H	H	M	M	M	M	M
75	M	H	H	H	H	M	M	M	M	H	H
219	W	M	M	M	M	M	M	M	H	H	H
383	M	M	M	M	M	M	M	M	H	H	H
448	W	M	M	M	M	M	H	H	H	H	H
594	M	H	H	H	H	H	H	H	M	M	M

Figure 6. Genetic and physical maps of *rl*.

Rl was positioned on long arm of chromosome 1 near centromeric region flanked by the markers S01086 and S01087 (A). The candidate region was narrowed down into markers flanking from S01086 to S01087 (B). Genotype of closely linked recombinants. W=wild type, M=mutant type, H=hetero (C).

Table 4. The list of genes in the candidate region.

Locus Name	Gene Product
LOC_Os01g37590	peptide transporter PTR2, putative, expressed
LOC_Os01g37600	agenet domain containing protein, expressed
LOC_Os01g37610	retrotransposon protein, putative, unclassified, expressed
LOC_Os01g37630	esterase, putative, expressed
LOC_Os01g37650	esterase, putative, expressed
LOC_Os01g37660	hypothetical protein
LOC_Os01g37670	OsFBX15 - F-box domain containing protein, expressed
LOC_Os01g37690	sodium/calcium exchanger protein, putative, expressed
LOC_Os01g37700	expressed protein
LOC_Os01g37710	expressed protein
LOC_Os01g37720	retrotransposon protein, putative, unclassified
LOC_Os01g37740	expressed protein
LOC_Os01g37750	glutathione S-transferase, putative, expressed
LOC_Os01g37760	glutamate dehydrogenase protein, putative, expressed
LOC_Os01g37770	RWD domain containing protein, expressed
LOC_Os01g37780	expressed protein
LOC_Os01g37790	expressed protein
LOC_Os01g37800	ras-related protein, putative, expressed
LOC_Os01g37810	expressed protein
LOC_Os01g37820	oxidoreductase/ transition metal ion binding protein, putative, expressed
LOC_Os01g37825	M16 domain containing zinc peptidase, putative, expressed
LOC_Os01g37832	thioredoxin, putative, expressed
LOC_Os01g37837	seryl-tRNAsynthetase, putative, expressed
LOC_Os01g37842	expressed protein
LOC_Os01g37850	expressed protein
LOC_Os01g37860	retrotransposon protein, putative, unclassified, expressed
LOC_Os01g37870	PPR repeat domain containing protein, putative, expressed

Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu)

MutMap analysis

To locate the causal mutation and the gene responsible for leaf rolling trait, bulk of mutant phenotype DNA of 20 F_2 progenies derived from cross between Ilpum and *rl* were used for WGS and MutMap analysis. Using Illumina HiSeq 2500, paired-end sequencing was conducted and 16.59 Gb of sequence reads were obtained as a result (Table 5).

Table 5. Summary of Illumina sequencing and alignment data.

Sample	No. of mutant F_2 plants bulked	Total read (Gb)	Mapped alignment data (Gb)	Mean depth
Ilpum[a]	-	16.84	16.68	43.57
rl[b]	20	16.59	6.17	16.13

[a]The short reads of Ilpum were aligned to Nipponbare reference genome (IRGSP Build 5).

[b]The short reads from rl bulk were align to the Ilpum 'reference sequence'.

The sequence reads were aligned to Ilpum 'reference sequence' and total of 208,214 SNPs were identified through out 12 chromosomes. SNP index of each SNP positions were calculated and the SNP index plots were generated. Refer to Figure 7, the average SNP index peak from 20.59 Mb to 25.69 Mb on chromosome 1 was the most probable candidate region.

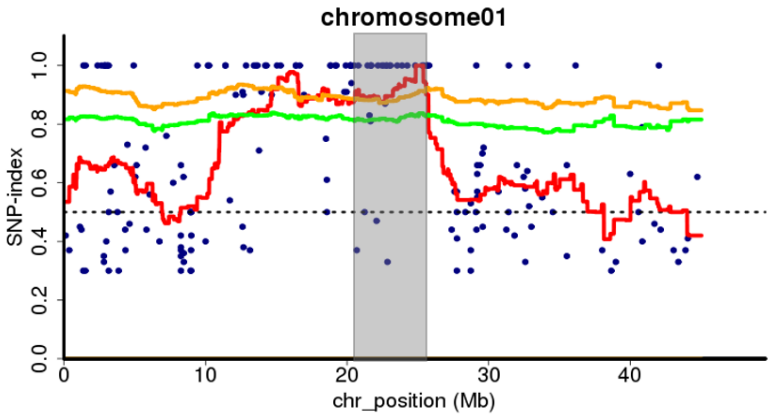


Figure 7. SNP index plot of chromosome 1 for identification of genomic regions harboring the causal mutation of *rl*.

Blue dot: SNP−index, red line: sliding window average of SNP−index, green line: sliding window average of 95%−confidence interval upper side, orange line: sliding window average of 99%−confidence interval upper side.

Considering the result of SNP index peak and genetic map together, the flanking region set by marker S01086 and S01087 was revealed to be a candidate region overall. Within this region, there were 10 SNPs with SNP index of 1. There was one *rl* specific SNP, physical position of 22,809,006 bp, located in genic region with point mutation from G to A (Table 6).The change of base pair at the position was confirmed by DNA sequencing as well. Using the SNP, dCAPS marker was designed for cosegregation test. After the enzyme treatment, corresponding bands of wild type and mutant type progenies were shown (Fig. 8).

Table 6. The candidate SNPs.

Chr.	Position	Ref.	Query	Depth	RAP locus ID	MSU locus ID
1	22764897	G	A	27	-	-
1	22765064	G	A	13	-	-
1	22779664	G	-	7	-	-
1	22787241	G	A	14	-	-
1	22788840	C	T	6	-	-
1	22803853	A	G	20	-	-
1	22809006	G	A	13	Os01g0559100	LOC_Os01g37837
1	22821193	T	C	13	-	-
1	22834459	C	T	19	-	-

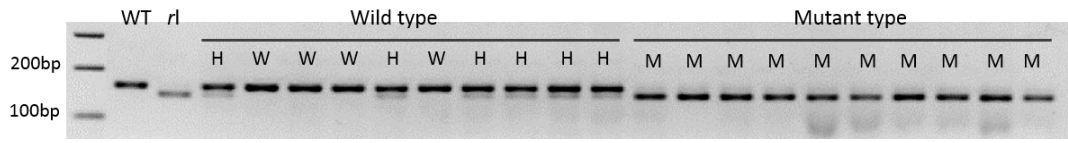


Figure 8. Genotyping of *rl*/Ilpum F_2 plants using designed dCAPS marker to confirm the causal SNP.

After restriction enzyme SpeI was digested for 2 hours.

Candidate gene study

According to the database, http://rapdb.dna.affrc.go.jp/, there were variations in gene structure, andSNP-22809006 was located on the splicing junction of Os01g0559100 (Fig. 9).

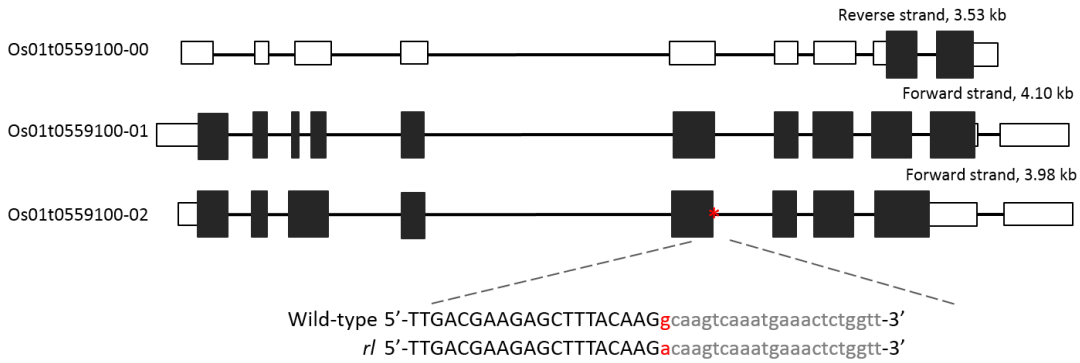


Figure 9. Variations in the gene structure of Os01g0559100.

The predicted gene structure is shown by white boxes (untranslated regions), black boxes (exons) and lines (introns).

The red asterisk mark on the beginning of 5th intron of Os01t0559100-2 indicates the mutation point.

Since the SNP was localized on a splicing recognition site, sequencing of cDNA was performed to prove the consequence of the mutation in splicing error.

Forward and reverse primers covering exon5, intron5, and exon6 were designed for RT-PCR. As presented in Figure 10B, there were 2 additional transcript forms from *rl*. The transcript with

length of 481 bp indicates that the splicing didn't occur and whole intron 5 part was transcribed. On the other hand, the transcript with 266bp size include 36 bp of intron 5, thus alternative splicing was occurred. There was also wild type transcript form, but the major forms were the alternative ones mentioned ahead.

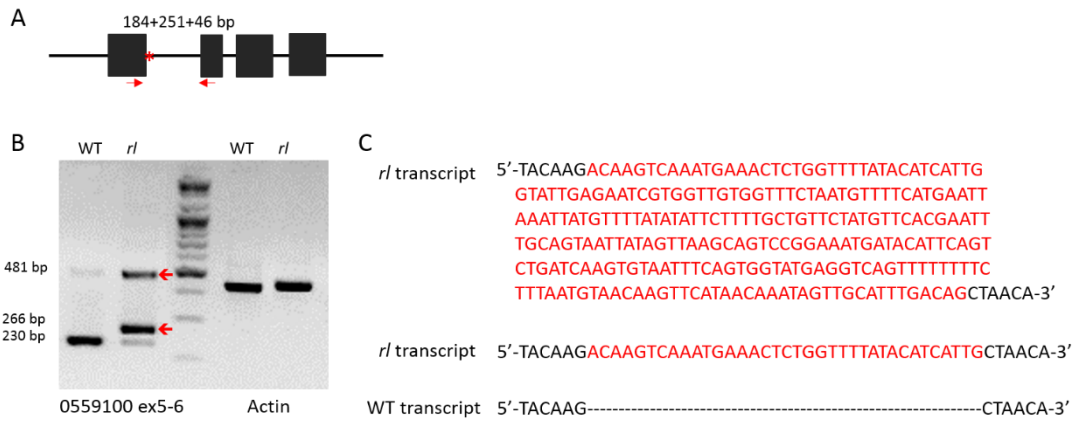


Figure 10. Confirmation of splicing error.

The partial structure of Os01g0559100 (A). Red asterisk mark indicates the mutation point, and red arrows indicates the forward and reverse primers designed for RT-PCR. The electrophoresed RT-PCR result (B). The sequence information of alternatively spliced products and the wild type (C).

2. Elongated uppermost internode

Characteristics of *eui* mutant

Among the EMS treated Koshihikari mutants, *eui* mutant with prominent elongation of upper internodes at heading stage was observed (Fig. 11). The general growth at vegetative stage was more superior in mutant. The longitudinal section of first internode suggested that the cell length was increased in *eui* (Fig. 12).As shown in Figure 13, increase in the length of first, second, and third internodes were significantly higher than that of wild type. Moreover, the rate of increase was highest in the second internode, 33.1 %, and followed by the first internode, 35.4 %.

Figure 11. Morphology of *eui* and wild type plants. (A) Before

flowering (B) After flowering (C) Comparison of the first, second, and third internodes of Koshihikari and *eui*.

As shown in Table 7, general agronomic traits were measured and compared between wild type and *eui*. Not only the internodes were elongated, but also plant height was increased. The plant growth in general was more superior in *eui*. *Eui* showed dominant growth over wild type in plant height, culm length, panicle length, and spikelet per panicle. Panicle number and spikelet fertility didn't show any significant differences,

Table 7. Comparison of agronomic traits of wild type and *eui*.

	HD (date)	PH** (cm)	CL** (cm)	PL** (cm)	PN^{ns} (No.)	SPP** (No.)	SF^{ns} (%)	TGW* (g)
WT	Aug. 10	97.4±5.5	86.2±3.0	18.8±1.1	12.1±2.5	106.8±9.5	94.4±4.2	25.8
eui	Aug. 9	114.2±4.3	114.6±11.3	22.2±1.8	10.4±2.8	125.4±14.9	94.9±2.3	24.6

HD=heading date, PH=plant height, CL=culm length, PL=panicle length, PN=panicle number per plant, SPP=spikelet per panicle, SF=spikelet fertility, TGW=thousand-grain weight.

Unpaired *t* test with Welch's correction was used to show significant difference (* $P < 0.05$, ** $P < 0.01$) compared with the wild type (*n*>

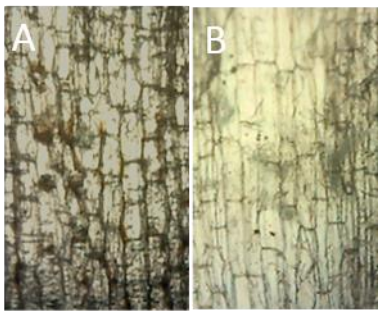


Figure 12. Longitudinal section of first internodes of wild type and *eui*. Wild type (A) and *eui* (B) under microscope (200x).

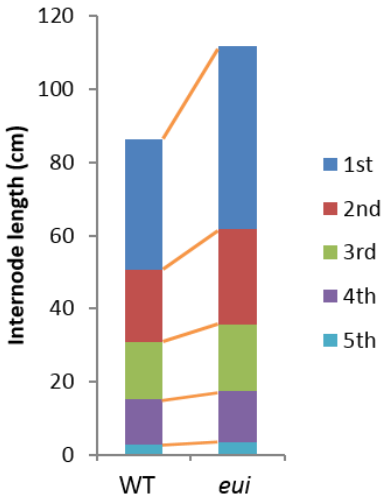


Figure 13. Comparison of the internode lengths. The rate of increase was highest in first and second internode (33.1% and 35.4%, respectively).

Genetic analysis

For the genetic analysis, the lengths of first and second internodes of F_1 and F_2 populations of Koshihikari/*eui* cross combination was

measured and recorded. The progenies of F_1 population showed all wild phenotype. Since the trait is qualitative, the distribution of each measurement of F_2 progenies was represented in Figure 14. As clearly shown, the graphs showed bimodal distribution along the average lengths of wild type and mutant type. Moreover, the frequency of peaks were segregated into 3 is to 1. Therefore, the trait is controlled by a single recessive gene.

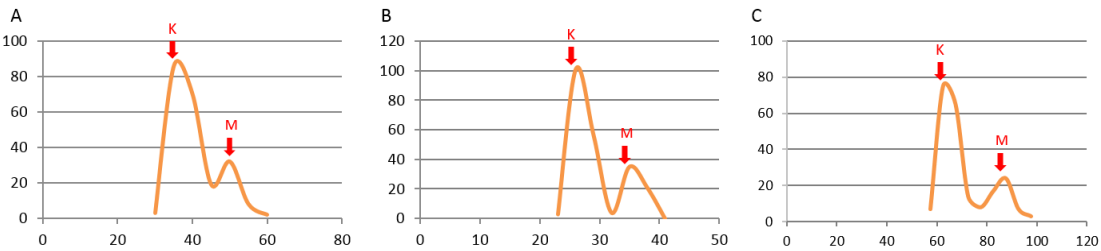


Figure 14. Distribution of the phenotype of F_2 population. The length of first internode (A) second internode (B), and the sum of first and second internodes (C). Arrow indicates the average first internode length of Koshihikari (35.5 cm) and *eui* (50.2 cm), and average second internode length of Koshihikari (24.9 cm) and *eui* (33.7 cm).

Genetic mapping of *eui* gene

Using the same method, BSA was performed using F_2 and F_3 progenies of M23/*eui* cross combination. Along the 12 chromosomes, the right segregation of genotype according to the phenotype was shown from marker S05086B and S05105 (Fig. 15).

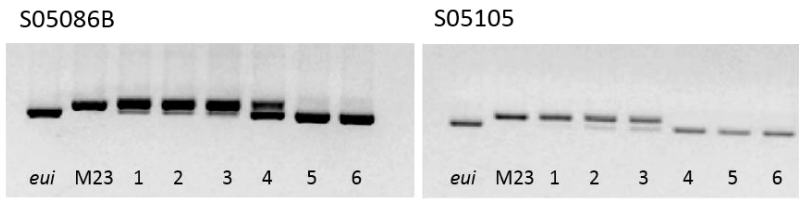
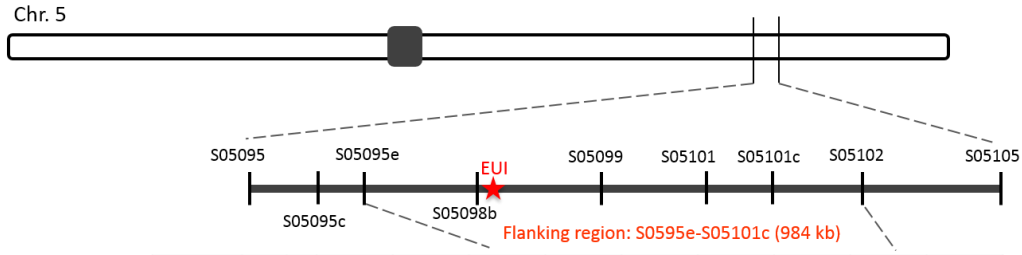


Figure 15. Bulked Segregant Analysis of wild type and *eui* bulks from *eui*/Milyang23 F_2 population.

eui: mutant, M23: Milyang23, 1−3: wild bulks, 4−6: mutant bulks

Setting the two markers as boundaries, InDel markers within the region was newly designed and used for the further narrow down (Fig. 16). As a result, *eui* was positioned on a long arm of chromosome 5 within 984 kb region flanked by the markers S05095e and S05101c, and cosegregated with the markers S05098b, S05099 and S05101. There was EUI1, previously found gene, located within the flanking region. However, it was confirmed that *eui* is different from EUI1 by manual sequencing of the genic region.



		P	S05095	S05095c	S05095e	S05098b	S05099	S05101	S05101c	S05102	S05105
F2	28	W	M	M	M	H	H	H	H	H	H
	40	W	H	H	H	H	H	H	H	H	M
	129	M	W	M	M	M	M	M	M	H	H
	399	M	H	H	H	M	M	M	M	M	M
	472	W	H	H	H	H	H	H	H	M	M
	493	M	M	M	M	M	M	M	M	M	H
F3	6	W	M	M	M	H	H	H	H	H	M
	37	W	M	M	M	W	W	W	W	W	W
	71	W	M	M	M	H	H	H	M	M	M
	86	M	M	M	M	M	M	M	M	M	H
	99	W	M	M	M	H	H	H	H	H	H

Figure 16. Physical mapping of *eui*.

*Eui*was positioned on long arm of chromosome 5 flanked by the markers S05095e and S05101c.W=wild type, M=mutant type, H=hetero.

DISCUSSION

The modification of plant architecture has been considered as important way of controlling potential yield of crops. There were countless genes found to be related with the architecture up to date. In this study, characterization and genetic mapping of *rl* and *eui* were conducted.

Rl showed upward curling and narrowed width of leaves as early as 28 days after sowing. Although other general agronomic traits are not favorable with low yield components, the leaf rolling trait is said to ease the light transmission in the canopy when crops are densely planted. According to the previously studied researches, bulliform cells on the surface of the leaves are one of the factors in leaf rolling, that change in bulliform cells could affect the leaf polarity and determine the direction of rolling. The result of paraffin cross section of *rl* revealed that deficient formation of bulliform cells with less number and size led to upward curling of leaves.

Based on the standard, F_2progenies of Ilpum/*rl* cross were differentiated into wild type and mutant type. The observed phenotypes were segregated into 3:1, and suggested that the trait was controlled by a single recessive gene. To locate the exact position of the gene, BSA was performed first. The flanking region was set to a centromeric region of long arm of chromosome 1.

Further narrow down was carried on using newly designed InDel markers and dCAPS markers. As a result, the candidate region was narrowed down into 174 kb, from S01086 to S01087. Bulked whole genome sequencing and MutMap analysis revealed the causal SNP within the flanking region. Based on a database, the SNP was located on a splicing recognition site of 5th intron of Os01g0559100. The splicing error is commonly generated by GT at the beginning and AG at the end of the intron. By a single base change from G to A, there were variations in splicing. There were two dominant transcript forms; 481 bp length including whole intron 5 that no splicing had occurred, and 266 bp length with alternative splicing on 37thbp of intron 5. The normal transcript form with 230 bp was also found in *rl*, but it is not a major transcript since the band intensity was faded.

The gene Os01g0559100 is known to encode putative seryl-tRNA synthetase, belongs to class-II aminoacyl-tRNA synthetase family. Aminoacyl-tRNA synthetases (ARSs) are a group of enzymes, which activate amino acids and transfer them to specific tRNA molecules as the first step in protein biosynthesis (Schimmel 1987). Although ARSs play key determinant role in transmission of genetic information and protein synthesis, the studies on ARSs had been limited to *E. coli*, yeast and some mammals (Ibba and Soll 2000). There are only confined number of reports on function and

regulation of the ARS genes and proteins in plants (Browning 1996). In *Arabidopsis*, a tDNA insertion mutant with defect in cytosolic and mitochondrial AlaRS caused alterations in patterns of cell division and differentiation, and showed an embryonic lethality (Ge et al. 1998). Moreover, *twn2* mutant was reported to have severe defects in early embryogenesis due to the arrested development of apical cells from altered expression of cytosolic ValRS (Zhang and Somerville 1997). Kim et al. (2005) revealed that gene silencing of the *N. benthamiana NbERS* and *NbSRS*, which encode organellar GluRS and SerRS, respectively, drastically reduced numbers and size of chloroplasts and chlorophyll content, resulted in severe leaf yellowing and abnormal leaf morphology. Further studies to connect the role of the protein in rolled leaf phenotype are required.

Eui showed significantly increased first, second and third internodes length compared to the wild type. Among five internodes, the second internode showed the highest rate of increase. Longitudinal section of first internode indicated that the cell length was increased, and led to an internode elongation. According to the study of Zhu et al. (2006), other mutant showed increase of cell length and cell number together. Genetic analysis was conducted by measuring the length of first and second internodes. The distribution of each measurement was compared. The figures showed bimodal distribution aligned with the average length of wild

type and *eui*. As anticipated, distribution of the sum of first and second internodes showed sharper form. The result suggested that internode elongation trait is controlled by a single recessive gene. BSA and fine mapping was conducted to locate the gene using the recombinants from F_2 and F_3 populations generated from a cross of M23 and *eui*. The flanking region was narrowed down into 984 kb region of long arm of chromosome 5. Although EUI1 discovered by Xu et al. (2004) is located within the flanking region, another gene is thought to be involved in controlling the trait within the region that any kind of mutation in EUI1 gene region was not found in *eui*. Further narrow down of the flanking region is required.

In conclusion, a novel gene associated with semi-narrow and adaxially rolled leaf trait was identified. The fine mapping of *eui* is currently conducted. This study could be helpful to generate rice plant architecture with various morphogenesis for specific purposes.

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초록

잎 말림 돌연변이 벼와 절간신장 돌연변이 벼의 형질조사와 유전자 지도 작성

식물의 초형에 대한 이해를 높이기 위하여 일품과 고시히카리 품종 각각에 EMS처리 하여 얻어진 잎 말림 돌연변이와 절간신장 돌연변이에 관한 연구가 진행 되었다. 잎 말림 형질은 식물체가 제한된 면적에 밀집되어 심어져 있을 때에 군락의 수광태세를 좋게 하고, 가뭄의 저항성 기작으로 작용하게 되면 에너지 이용효율에 큰 이점이 있다고 알려져 있다. 해당 유전자는 잎이 위로 말리는 형태를 보이며, 파라핀을 이용하여 잎을 횡단면으로 절단 후 관찰 한 결과 잎 표면적의 기동세포(bulliform cell)의 크기와 수가 현저하게 작아져 있음을 발견하였다. 일품/*rl* 교배집단의 표현형 조사를 실시하여 분리비를 살펴보았는데, F1의 경우 모두 정상개체의 표현형을 보였고, F2 집단의 경우 3:1의 분리비로 표현형이 나뉘었다. 카이제곱 검증으로 확인한 결과, single recessive gene에 의하여 표현형이 조절된다는 것을 알 수 있었다. 밀양23/*rl* 교배집단을 이용하여 bulked segregant analysis를 수행 한 결과 후보유전자가 1번 염색체의 long arm 상단부에 위치함을 확인하였고, 그 구간에서 InDel마커 제작을 통하여 narrow down 을 진행 한 결과 마커 S01086과 S01087사이의 174kb로 좁힐 수 있었다. 또한 차세대 염기서열 분석방법을 이용하여 해당 구간 내에서 돌연변이에 직접적으로 관여하는 SNP를 확인하였다. 분석에 따르면 유전자 Os01g0559100의 5번째 인트론

시작 부분에 G에서 A로 base change가 일어났고, 이는 splice site에 해당한다. 일품/*rl* F2 집단에 dCAPS 마커 분석을 실시 하여 변이를 확인하였다. RNA추출을 통한 cDNA 합성 및 RT PCR 결과, 변이로 인하여 다양한 형태의 alternative splicing이 일어나, 해당 유전자의 encoding protein에도 영향을 미쳤을 것으로 생각된다. 절간신장 돌연변이의 경우 정상개체에 비하여 이삭추출도 및 상위 세 번째까지 절간의 길이가 두드러지게 증가하였다. 이외의 다른 일반 농업형질에서도 전반적으로 우수한 생육을 보였다. 고시히카리/eui 교배집단의 표현형 조사는 상위 두번째 절간까지의 길이를 측정하였으며 이를 분산그래프로 나타내어 분석에 이용하였다. 그 결과, 그래프의 모양이 이정 곡선을 그리며 각 정점이 세배의 차이가 나는 것으로 보아 절간신장 형질 또한 single recessive gene에 의하여 조절되는 것을 알 수 있었다. 밀양23/eui 교배집단을 이용하여 BSA를 수행 한 결과, 후보유전자가 5번 염색체의 하단부에 위치 하는 것을 확인 하였다. Narrow down을 통하여 flanking region을 마커 S05095e와 S05101c의 984kb 사이로 좁힐 수 있었다. 해당 구간 내에는 전에 연구 된 *EUI1* 유전자가 위치 하지만, 시퀀싱 확인 결과 이 돌연변이에 관여하지 않음을 확인 하였고 5 번 크로모좀 내에 *EUI1* 유전자 외에 다른 유전자가 절간신장에 관여 할 것으로 생각된다.

주요어: 벼, 식물 초형, 잎 말림 형질, 기동세포, 유전자지도작성, 절간신장

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