THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Genetic Analysis of a Leaf Angle Mutant in Rice

BY

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FEBRUARY, 2014

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DEPARTMENT OF PLANT SCIENCE

THE GRAUDUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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UNDER THE DERECTION OF PROFESSOR HEE-JONG KOH
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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APPROVED AS A QUALIFIED THESIS OF AERI HAN
FOR THE DEGREE OF MASTER OF SCIENCE
BY THE COMMITTEE MEMBERS

FEBRUARY, 2014

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ABSTRACT

The leaf angle defined as the degree between leaf blade and stem is an important factor of crop yield. The most crucial part of leaf angle regulation in rice is a collar (lamina joint). If adaxial side of the collar is elongated, the leaf would be reversely rolled, otherwise, the plant would be erect type.

To figure out leaf angle regulation related genes and their mode of action, we identified a rice large leaf angle mutant obtained by EMS (ethyl methane sulfonate) treatment to Ilpumbyeo, *ilpum large leaf angle* (*illa*), which results from elongated cell length of adaxial side at the lamina joint.

The phenotypic characterization of *illa* mutant and genetic mapping were performed. Additionally, we carried out leaf inclination test to elucidate between Brassinosteroid (BR) and leaf angle regulation of *illa*.

Map based cloning using 30 STS (Sequence-Tagged Sites) and 57 SS-STS (Sub-species Specific Sequence-Tagged Site) markers showed that the *illa* gene seems to be located on the short arm of chromosome 11.

Each whole genome of Ilpumbyeo, *illa* mutant, and bulked F_2 plants showing mutant phenotype was sequenced using NGS method to find exact mutation point. After all SNPs and indels were called, the candidate gene was considered to be located on chromosome 1, 10 or 11. Further identification of each candidate genes is progressing.

Keywords : Leaf angle, collar, lamina joint, mapping, whole genome sequencing, Brassinosteroid

Student number : 2011-23485

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LIST OF ABBREVIATIONS

BL	Brassinolide
BR	Brassinosteroid
BSA	Bulked segregant analysis
EMS	Ethyl methane sulfonate
illa	Ilpum large leaf angle
M23	Milyang 23
mut	Mutant
NICS	National Institute of Crop Science
RDA	Rural Development Administration
SNP	Single nucleotide polymorphism
STS	Sequence-Tagged Sites
SS-STS	Sub-species Specific Sequence-Tagged Site
USDA	United States Department of Agriculture
WGS	Whole genome sequencing
WT	Wild type

INTRODUCTION

The demand for food product is rising due to increasing global population and changing eating habits. But because of the limit to broadening farmland, it would be very important to improve crop productivity within limited arable land. Rice (*Oryza sativa. L.*) is one of the most important food crop especially in Asia. International rice production occupies more than 20 percent of total food crop and it is staple food for almost 40 percent of the people in the general population [1].

Plant architecture means plant shape classified by traits such as leaf, stem, height and leaf angle. It is the best tool of identifying a plant species and affects to farming and harvesting efficiency [2]. In addition, plant architecture plays a key role for crop yield including lodging resistance and dense planting. For example, Green Revolution successfully increased crop productivity by modification of plant architecture [3].

The leaf angle, the degree between leaf blade and stem (Figure 1), is an important trait of crop yield in monocotyledonous plants as it affects on caption of sunlight and nitrogen storage. When the field space is limited, the rice with narrower leaf angle can get more sunlight and be more suitable for dense planting. Meanwhile, the rice

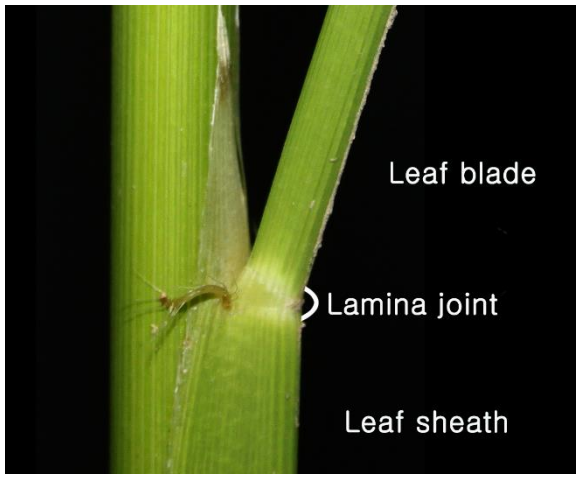

Figure 1 Lamina joint structure of rice

with broader leaf angle is hard to get sunlight because of shading in dense planting, it can get more yield only in wispy planting [4, 5].

Due to these importance, many previous studies about leaf angle regulation have been done. Some of the leaf angle related genes or QTLs are already known– the major detected QTL for leaf angle is *tiller angle 1 (Ta1)* [6, 7]– but its regulatory process is still unclear.

Leaf angle–altering genes are also related with plant phytohormones, especially Brassinosteroid (BR). Not only leaf angle was enlarged with concentration–dependent manner within a range of BR concentration [8, 9], but also most of leaf angle mutants are involved in the BR biosynthesis or signaling. The BR synthesis related OsDWARF4 deficient mutants showed smaller leaf angles and other BR biosynthesis–defective mutants [4], *ebisu dwarf* (*d2,* deficiency of CYD90D2/D2) [10] and *BR–deficient dwarf1* (*brd1,* deficiency of OsDWARF)[11] was also similar. Furthermore, the

mutant having imperfect rice *BRASSINOSTEROID INSENSITIVE1 (OsBRI1)* or *OsBZR1*, the BR signaling related genes, also displayed erect leaves [12-14]. On the other hand, sterol C-22 hydroxylase in charge of BR biosynthesis overexpressing plants showed inclined leaf angle [15, 16]. It means that biosynthesis and signaling of BR is important to control leaf angle regulation.

There are also some other phytohormones related with leaf angle. Ethylene is involved in BR-induced leaf inclination. And *leaf inclinatnion1 (lc1)* encoding an IAA-amido synthetase deficient mutants showed enlarged leaf angle. It was insensitive to IAA and hypersensitive to exogenous BR [8, 17]. Besides, SPINDLY, a GA signaling negative regulator, suppressed plants showed enlarged leaf angle [18].

A lot of transcription factors are involved in leaf angle regulation. Ectopic expression of *LAX PANICLE (LAX)* encoding a basic helix-loop-helix transcription factor resulted in enlarged leaf angle [19]. *BU1* encoding a helix-loop-helix transcription factor participates in BR signaling pathway resulting in positive regulation of leaf angle enlargement [20]. OsLIGULELESS1 (OsLG1), SQUAMOSA promoter binding protein domain containing transcription factor, defective mutants were defective in ligule, auricles and lamina joint [21]. As a result of genetic analysis of a T-DNA inserted mutant corresponding with leaf angle mutant, *large leaf angles* (*lla*),

OsWRKY11 encoding a WRKY transcription factor came out to be involved in leaf angle regulation [22].

In addition, *OsHDAC1* encoding a histone deacetylase overexpressed mutant and *lc2 (leaf inclination2)* encoding a VIN3-like protein deficient mutant also showed enlarged leaf angle [23, 24].

The most crucial part of plant for leaf angle regulation is a collar which is weak green part located between leaf blade and leaf sheath (Figure 1), also called lamina joint. If the collar is elongated, the leaf would be reversely rolled [17, 25], otherwise the leaf would be erect [26].

There are many factors and intricate regulatory networks about leaf angle regulation. To figure out related genes and their mode of action, we obtained the EMS (ethyl-methylsulfonate) treated Ilpumbyeo mutant showing severely broad angle between stem and leaf, thus we performed phenotypic characterization and genetic mapping. These results will play a part in elucidation of plant leaf architecture.

MATERIALS AND METHODS

Plant material

The *ilpum large leaf angle* (*illa*) mutant (Figure 2) was obtained by treating Korean *japonica* cultivar Ilpumbyeo with EMS (Ethyl-methane sulfonate) chemical mutagen. The mutant was selected in M2 generation and was fixed through selfing in successive generations. The pedigree record of *illa* mutant is Ilpum E-B-11-1-1-2-B-B. To genetically map the mutated gene, mapping population was developed by crossing the mutant (*illa* ; *japonica* background) with Milyang23 (Tongil-type *indica* cultivar). F_2 population were grown in the field and green house and these

Figure 2 Wild-type (Ilpumbyeo) and *illa* mutant at ripening stage (bar=20cm)

populations were used to BSA. The mutant was also crossed with its wild type plant (Ilpumbyeo) and F_2 population from this cross was grown in the green house and used for whole genome sequencing.

Paraffin sectioning of lamina joint and leaf

For paraffin sectioning, samples of rice lamina joint and leaf blade at the heading stage were fixed in formalin : acetic acid : 50% ethanol (FAA) at 2:1:17 and stored at 4° C overnight, and vacuumed using silicon cap and syringe. And it were dehydrated through a graded ethanol series from 30% to 95% each for 2 hours and incubated in 95% ethanol overnight. The samples were soaked in 100% ethanol for 2 hours. To clear out, samples were soaked in ethanol : histoclear 3:1, 1:1, 1:3 for 2hours in order and then soaked in 100% histoclear overnight. For paraffin infiltration, samples were soaked in histoclear : ethanol 3:1, 1:1, 1:3 for 2 to 3 hours in order and then, soaked in 100% paraffin at 55° C overnight.

The infiltrated sample was embedded in a paraffin block. After hardened, the sample was cut into 8 to 12 μm long sections using a microtome (MICROM Lab, Walldorf, Germany) and mounted on a Sperfrost-plus glass slides (Fisher Scientific, Pittsburgh, PA, USA) and dried at 42° C for 1day. For deparaffinization, the slides with sections were dipped into 100% Xylen for 1hour followed by soaking into ethanol : Xylen 1:1, 100% ethanol and sterile water in order each

for 2min for hydration. The sample was stained with 1% toluidine blue solution for 30sec and washed with sterile water. And slides were soaked in 50, 70 and 95% ethanol in order for 2min for destaining. As a last step, the slides were soaked in 100% Xylen for 10min for clearing and mounted in Canada balsam. Sections were microscopically examined at 100~300X magnification and photographed to measure the cell layers and cell lengths.

Lamina inclination test

Because BR is closely related with leaf angle regulation, we performed lamina inclination test to elucidate the relationship between BR and leaf inclination regulation of *illa* [27].

Rice seeds soaked in tap water for 48 hours were cultivated on soil in darkness at 25° C. After 7 days, ethiolated uniform rice seedlings were selected. The second lamina and sheath were cut into approximate 4cm long centering around lamina joint. To exclude internal hormonal effect, this segments were dipped into distilled water for 1 day and uniformly bent segments were selected. Ten of the segment were incubated in a magenta box containing 100ml distilled water with or without 0.1mM Brassinolide as upright state for 48 hours in darkness. At last, the angle between leaf sheath and blade was measured.

Genetic mapping

ILLA was mapped with 30 STS and 57 SS-STS markers, using 167 F_2 plants from the cross between *illa* mutant and Milyang23. We selected 10 mutant and 10 wild-type F_2 plants and pooled into each 2 bulks in same concentration for BSA. Using the CTAB method [28], genomic DNA were extracted from rice leaves.

PCR was performed in a reaction volume of 20 μl containing 100ng of template DNA, 10pM of each forward and reverse primer, 250 μM of dNTP, 100mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin and 0.5U of Taq DNA polymerase (homemade). Amplifications were performed in a PTC100 96U Thermocycler (MJ Research, Reno, NV, USA) in the following sequence : 5min at 94° C, followed by 35 cycles of 30sec at 94° C, 40sec at 56° C, 30sec at 72° C, 5min at 72° C for final extension and 10 min at 10° C. PCR products were separated in 2.5% agarose/0.5X TBE gels and visualized by EtBr staining.

Whole genome sequencing

Each whole genome of *illa* mutant and bulked F_2 plants (n=15) showing mutant phenotype derived from the cross between *illa* mutant and Ilpumbyeo was sequenced using NGS method with Illumina HiSeq2500. We also used wild type sequence raw data from NICS of RDA .

Genomic DNA was extracted from fresh rice leaves using the CTAB method. DNA from F_2 individuals showing mutant phenotype was mixed in an equal ratio (bulked DNA). Pure mutant DNA and mixed F_2 DNA (5μg) were used for preparation of libraries for Illumina sequencing according to the protocol for the TruSeq DNA Sample Preparation kits (Illumina). Using these libraries, cluster was generated on a flow cell and sequenced for total 101 cycles on an Illumina HiSeq2500. Base calling was performed by the instrument control software's Real Time Analysis (RTA) and then, the bcl conversion and sequence alignment were carried out by CASAVA (Illumina Pipeline). The filtering of low-quality bases were done using SOAPfilter 2.0 (Quality score 30 over Minimum length 90) ([29], Figure 3).

Alignment of short reads to reference sequences and SNP and indel calling.

To identify mutation site resulting from EMS treatment, we used a reference sequence of publicly available Nipponbare rice genome sequence and Ilpum wild type genome sequence (110 million paired-end short reads) from from RDA. As a result we got 67million of mutant (*illa*) and 73 million of F_2 bulk DNA paired end sequences. These short reads were aligned to the reference sequence with CLC Reference mapper (Version 4.06beta.67189) and applied to Illumina

Pipeline (CASAVA) v1.8.2 for identification of reliable SNPs designed to maximize true SNP detection and minimize false SNP calling. The filtering rule was that (i) minimum insert size of paired-end read should be 101bp, (ii) leave the SNPs in genomic regions and be covered by a minimum of eight reads for homozygous SNPs and a maximum of three-fold of average read depth over the genome, (iii) minimum averaged Illumina phred-like quality score should be 30.

After Paired-end sequence reads of wild type, mutant and bulked DNA of mutant F_2 progeny, these data were aligned to the public Nipponbare reference sequence, respectively, and SNPs and indels were detected through following steps (i) pick the SNPs and indels located in coding sequence, (ii) pick the SNPs and indels in bulked F_2 sequence showing equal or larger SNP index than 0.9 (SNP index$\geq$0.9). SNP indices of each position were calculated by the formula which is the the number of reads showing SNP of mutant sequence divided by total read number, (iii)pick the SNPs and indels shared by mutant type F_2 bulk and *illa,* (iv) remove the SNPs and indels shared by wild type and *illa*, (v) remove the low read SNPs and indels, (vi) remove silent (synonymous) mutations ([29], Figure 3).

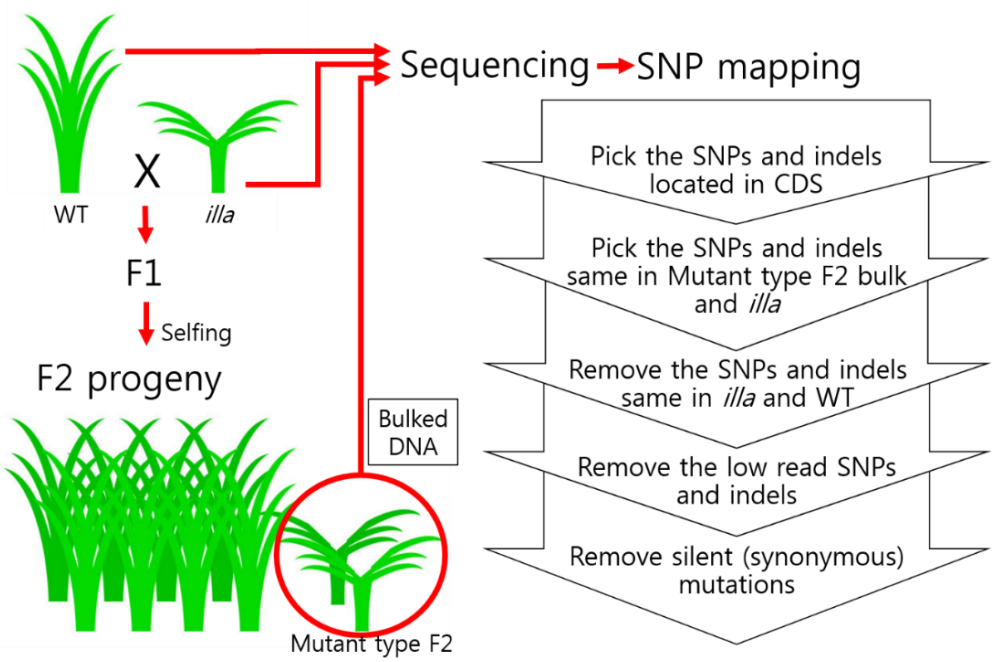

Figure 3 SNP and indel calling mechanism of whole genome sequencing of wild type (Ilpumbyeo), mutant and bulked F_2 plants

RESULTS

1. Characterization of the *illa* mutant

1) Leaf angle

At heading stage, leaf angles of *illa* mutant were much larger compared to the wild-type (WT), which is reaching to ~83° in *illa* mutant compared to 12° in WT. The calculation result about leaf angles observed at four stages (tillering, panicle formation, heading and ripening) to find out the exact point of leaf bending showed that the 1st leaf angle of mutant started to enlarge at panicle formation stage (Figure 4, 5).

2) Agronomic characteristics

Plant height, leaf length, and leaf width of *illa* mutant were smaller than WT. On the other hand, leaf length/plant height ratio of *illa* mutant was larger than WT (Figure 5B). To classify the dwarf type of *illa* mutant, we measured internode length and it revealed *illa* mutant is similar to DM type. Moreover, the panicle length of mutant (20.9cm) was also shorter than wild type (23.2cm, Figure 5C).

Grain length, width and 1000 grain weight were considerably reduced in case of mutant compared to wild type (Figure 5D). And

grain fertility was also reduced more than 10% in mutant (Figure 5E).

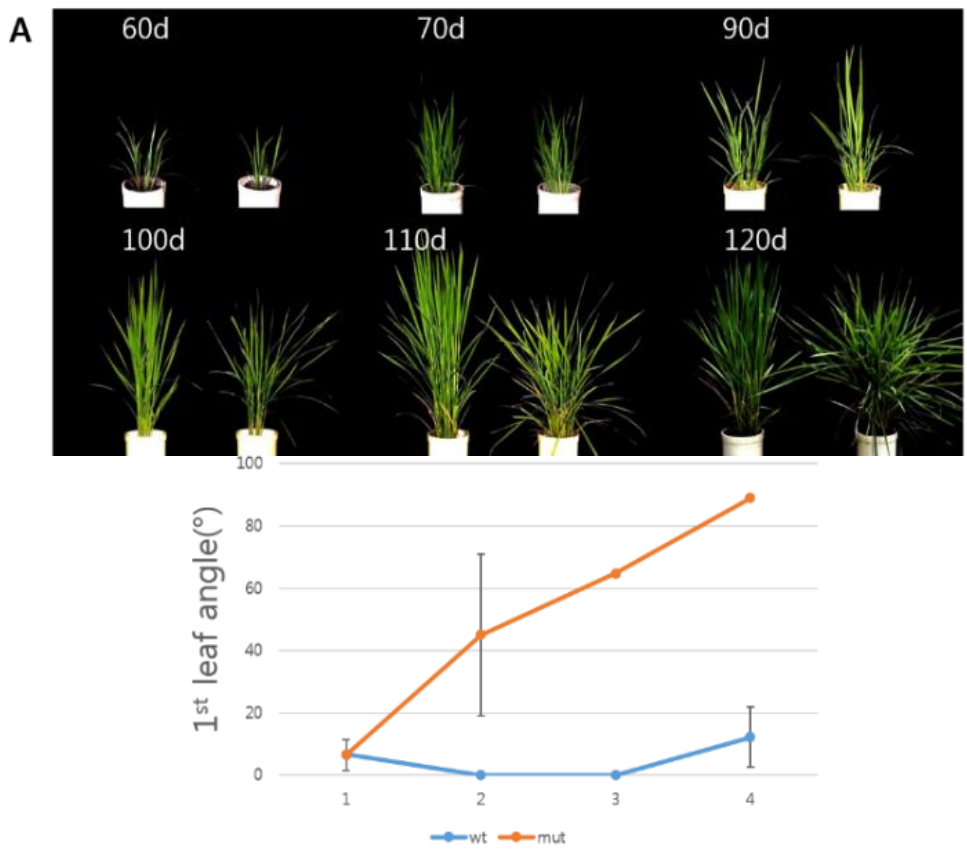

Figure 4 Serial change of wild-type and *illa* mutant (A) at 60, 70, 90, 100, 110 and 120 days after sowing. (B) 1. tillering, 2. Panicle formation, 3. Heading, 4. ripening stage.

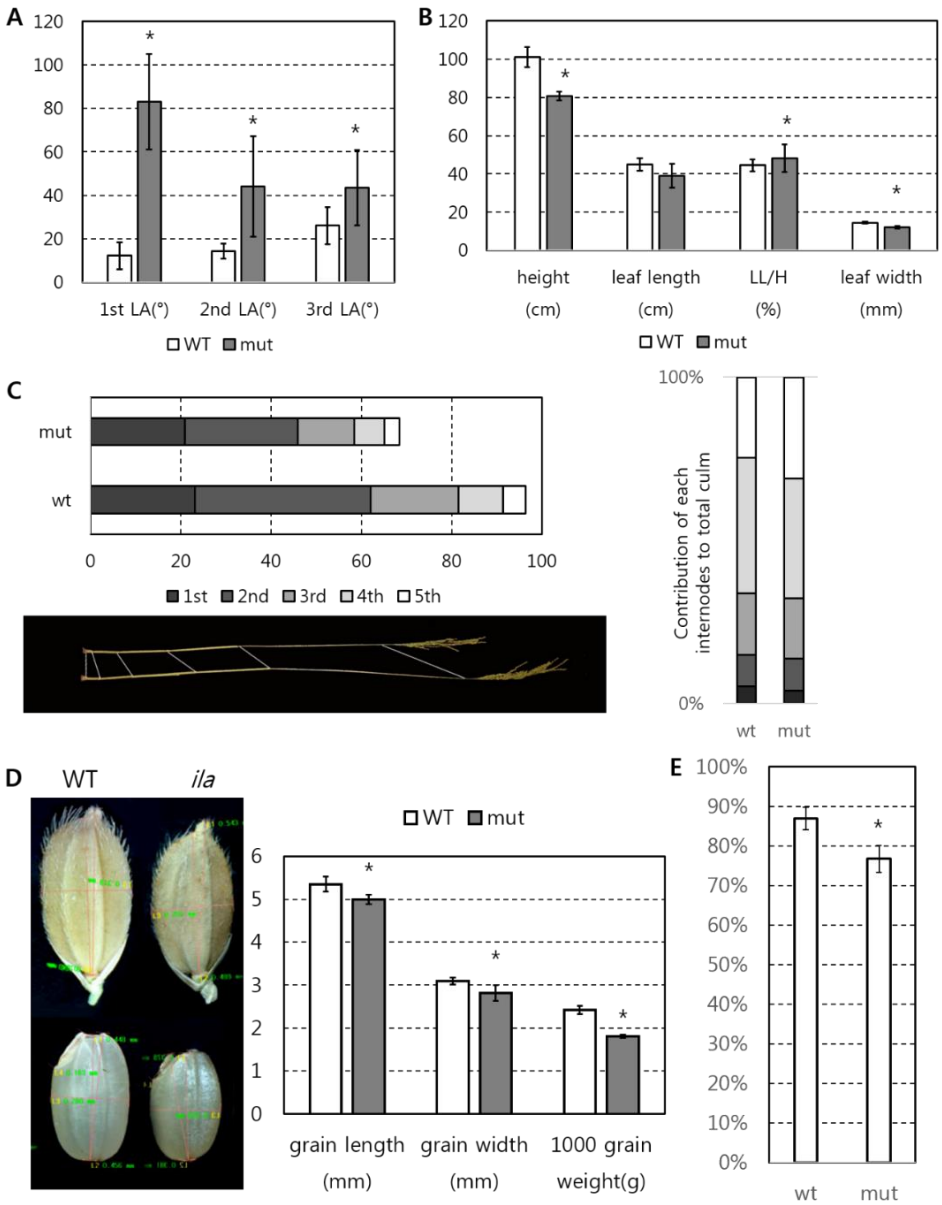

Figure 5 Comparison of the (A) leaf angle and (B) plant height, leaf length, leaf length/height ratio and leaf width of wild-type and *illa* mutant at heading stage. (C) absolute and relative internode length. (D) grain length, width, and weight of wild-type and *illa* mutant. (E) grain fertility. Error bar represents SE (n>10) and was statistically calculated by Student′ s t-test (*P<0.01)

3) Histological analysis–paraffin sectioning of lamina joint

Because previous studies showed that the lamina joint considerably contributes to the leaf angle formation and enlarged leaf angles may reflect alterations of lamina joint development [12, 17], we performed paraffin sectioning of lamina joint. As a result of cross and longitudinal sections, *illa* mutant is shown to have not only more cell layers but also longer cell length at adaxial side of lamina joint. However, there are no visible difference in leaf thickness and the number of cell layers (Figure 6).

4) Lamina inclination test

As a result of lamina inclination test, the leaf angle of *illa* mutant was enlarged much more than wild type under the Brassinolide treatment. Therefore, that shows *illa* is more sensitively responsive to BL (Figure 7).

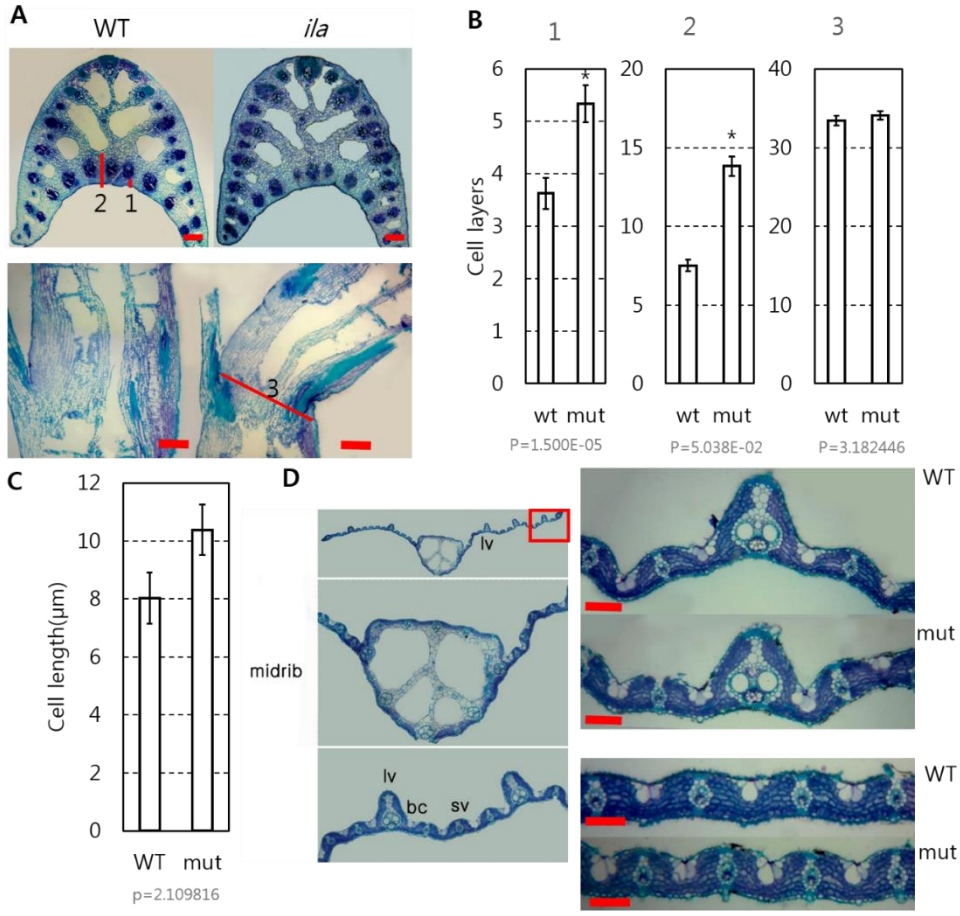

Figure 6 (A) Cross and longitudinal sections through the lamina joint of flag leaves of wild-type and *illa* mutant. (Bar = 200 μm) (B) cell number. (C) Adaxial side cell length (Bar = 200 μm). (D) Leaf cross section (Bar = 100 μm). Error bar represents SE (n>100) and was statistically calculated by Student's t-test (*P<0.01)

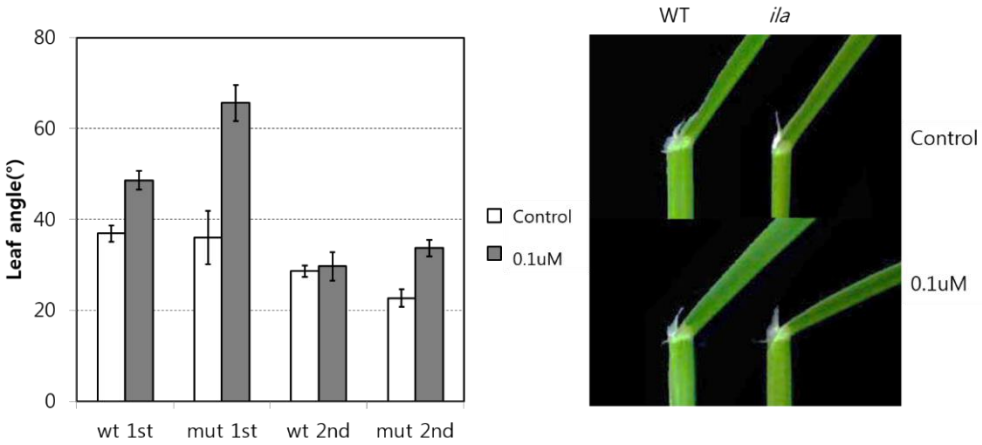

Figure 7 Comparison of Brassinolide response between wild-type and *illa* mutant. Error bar represents SE (n>10) and was statistically calculated by Student′ s t-test (*P<0.01)

2. Genetic control of mutant trait

Two kinds of F_1 phenotype derived from each *illa* mutant crossing with wild type (Ilpum) and Milyang23 showed that the mutated gene is recessive, but there was problem to distinguish the phenotype of some F_2 plants induced by crossing between *illa* and Milyang23 (a Tongil-type indica rice). So, we performed strict phenotyping and the segregation ratio was approximately 15:1 (wild type : mutant type, P=0.255). It means that *ILLA* is recessively inherited by 2 genes but it depends on severity level of phenotyping.

3. BSA and candidate gene analysis

According to BSA result, we assumed that the mutation related gene may be located on the short arm of chromosome 11 (Figure 8). As a

result of sequence comparison, it was confirmed that already known-leaf angle regulatory genes (*LC2, OsLIC* and *SGL)* showing considerably similar phenotypes are different from *illa*.

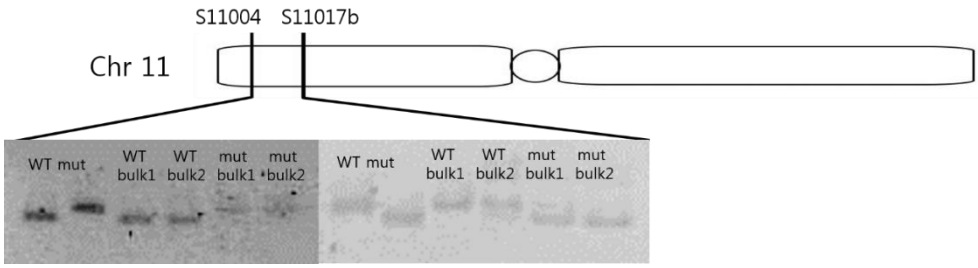

Figure 8 Bulked segregant analysis between wild-type and *illa* mutant showed the candidate region and flanking markers (S11004 and S11017b) of target gene. WT : wild type, mut : mutant.

4. Whole genome sequencing

To figure out the exact mutation point in the whole genome of wild type, *illa* mutant and bulked F_2 plants showing mutant phenotype derived from the cross between *illa* mutant and Ilpumbyeo was sequenced using NGS (next generation sequencing) method. As a result, chromosome 1, 10 and 11 were shown to have relatively plentiful SNPs.

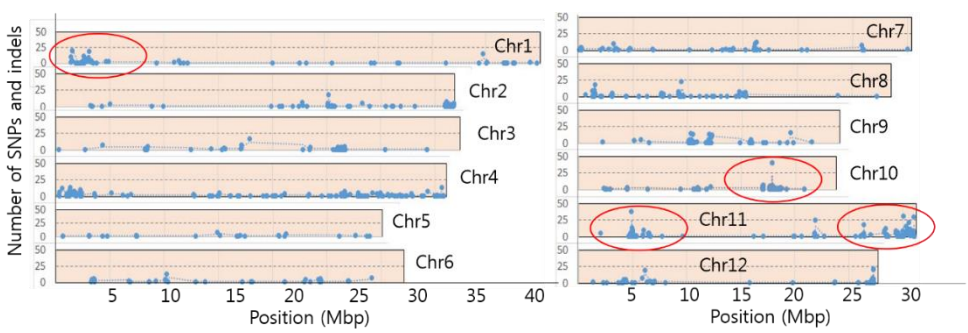

Figure 9 The number of SNPs and indels per 10kbp in each chromosome locations derived from whole genome sequencing of wild type, *illa* mutant and bulked F_2 plants showing mutant phenotype derived from the cross between *illa* mutant and Ilpumbyeo. The region with concentrated SNPs and indels marked with red circles.

DISCUSSION

When the field space is limited, the rice having large leaf angle can get more sunlight in upper side, whereas other leaves in lower side get less sunlight. Likewise, *illa* mutant was shown to get smaller and less grain compared to wild type (Figure 5). To elucidate the effect of planting density, the response of *illa* mutant to planting density should be additionally observed.

The alteration of lamina joint in various leaf angle mutants means that lamina joint plays a key role in regulating leaf angle. Therefore, developmental alteration of lamina joint results in leaf angle alteration. As we expected, *illa* mutant showed more cell layers and longer cell length at adaxial side of lamina joint. At a guess, it would be the reason why leaf angle of *illa* mutant was enlarged.

F_1 plants induced by crossing between *illa* and Milyang23 showed wild type phenotype which reveals *illa* is a recessive gene and the segregation ratio in the F_2 population was 15:1 by strict phenotyping. But when we scored the phenotype, many of the plants showed unclear phenotype. Therefore, if we didn't strict phenotyping, the segregation ratio could be changed.

As we faced to the trouble in fine-mapping the responsible gene, we sequenced whole genome of wild type, mutant, and bulked F_2 plants showing induced by crossing between *illa* and ilpum using NGS

method. At first, we tried to apply MutMap method. By MutMap method, a graph was drawn with SNP indices and it was followed by the seeking for the region showing the peak which has equal or higher SNP index than 0.9 (homo SNPs) [29]. Unfortunately, our data didn't show that peak and there were plenty of homo SNPs even after strict filtering. So we changed the strategy based on the hypothesis. EMS can induce the mutation at a rate of $5x10^{-4}$ to $5x10^{-2}$ base pair per gene evenly. And the F_2 plants showing mutant phenotype would be have SNPs randomly in almost region but intensively in phenotype linked region. According to the hypothesis, we sought the problem in the region having abundant SNPs and found 4 candidate region. Finally, when we synthetically analyzed the sequencing and BSA results, the candidate gene was considered to be located on the short arm of chromosome 11. When

Interestingly, one of the candidate genes is histone deacetylase located on the region. Histone deacetylase regulate gene expression by removing acetyl group from lysine of histone followed by tight binding of histone and DNA finally repress gene expression. Especially, one of the previous study showed that OsHDAC1 overexpressing mutant showed altered plant architecture including enlarged leaf angle. And in a mice study, defective HDAC1 showed specific subset genes were deregulated [30].

Previous studies for histone deacetylase considered, *illa* would probably be related with this gene. But it is still unlear. To clarify that, further study including co-segregation test should be followed.

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초 록

벼 엽각 돌연변이의 유전 분석

엽신과 줄기가 이루는 각도인 엽각은 작물의 수량에 중요한 요소이다. 그런데 벼에서 엽각 조절을 하는 데 가장 중요한 부분은 엽절이다. 엽절의 향축면이 신장되면 엽각이 벌어지고, 반대의 경우 똑바로 선 형태를 이룬다.

엽각 조절과 관련된 유전자와 그 유전자의 작용 기작을 알아보기 위해 벼 품종 일품에 EMS를 처리하여 얻은 엽각이 큰 돌연변이체인 illa를 동정하였고, 이를 통해 유전자 지도 작성과 표현형적 특성 관찰을 목표로 실험을 진행하였다.

엽각조절유전자는 식물호르몬 중에서도 특히 브라시노스테로이드와 관련이 있었다. 따라서 브라시노스테로이드와 illa 에서 엽각 조절과의 관계를 알아보기 위해 Leaf inclination test 를 수행하였다.

30 개의 STS marker 와 57 개의 SS-STS marker 를 이용한 map based cloning 결과를 바탕으로 돌연변이 유전자가 11 번 염색체의 short arm 에 존재할 것으로 추정하였다.

정확한 돌연변이 위치를 찾기 위해 야생형과 illa 그리고 돌연변이 표현형을 가진 F_2 bulk 의 전체 유전체를 NGS 방법으로 서열분석하였다.

SNP 와 indel 을 분석한 결과 후보유전자는 아마도 1 번, 10 번 혹은 11 번 염색체에 존재할 것으로 보인다.

주요어 : 엽각, 엽절, 유전자 지도, 전체 유전체 분석, 브라시노스테로이드

학번 : 2011-23485