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Regulation of EXLA1 Stability by E3 Ubiquitin Ligase GW2 in Rice

BY

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THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Grain Weight 2 (GW2), known as the E3 ubiquitin ligase, important role in controlling the size and weight of the grains. Such gene expression and regulation by GW2 has been studied for a long time. Nevertheless, the post-translational research of GW2 rarely progressed. Previous studies have confirmed *in vitro* that Grain Weight 2 (GW2) in rice could act as an E3 ubiquitin ligase for EXPANSIN LIKE 1 (EXLA1). Here, we showed that Grain Weight2 (GW2) could regulation EXLA1 stability. In this paper, particle bombardment experiments showed that GW2 and EXLA1 interacted in epidermal cells of onion. The yeast two-hybrid experiment also confirmed that the expansin family except EXLA1 did not bind to GW2. In addition, the expression of EXLA1 was found to be high in the *gw2* mutant and the expression of EXLA1 was found to be different when GA3, Cytokinin, and Auxin were treated to the WT Norin. We also found that BSG1, a candidate group other than EXLA1, binds to GW2 through yeast two-hybrid experiments and *in vitro* pull down experiments. Taken together, we confirmed that

the stability of EXLA1 was regulated by GW2, and confirmed that it binds to a candidate group other than EXLA1.

Keyword: E3 Ubiquitin ligase GW2, EXLA1, ubiquitination, expansin

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LIST OF ABBREVIATIONS

AD Activating Domain

BD Binding Domain

CFP Cyanin Fluorescent Protein

YFP Yellow Fluorescent Protein

Os *Oryza Sativa*

GA3 Gibberellic acid

MBP Maltose-binding protein

GST Glutathione S-transferase

IPTG Isopropyl-β-D-thiogalactoside

3-AT 3-amino-1, 2, 4-triazole

INTRODUCTION

Globally, population continues to increase but food shortages continue. In order to feed the population of the world's population, the productivity of crops must be increased. In 2009, 25 million people were undernourished, according to the Food and Agriculture Organization of the United Nations.

Among them, rice is a popular crop grain crop. Filling rate of grain is affected by biological and abiotic stress. But the weight of whole grains is known to be dominated by genetic factors. In particular, the size of seeds is known to have a great influence on the yield of seeds.

The yield of rice is determined by various factors including the size of seeds. Among these, the size of seed is an important factor in yield. The seed size of rice is determined by length, width and thickness. Studies of these factors have continued for decades. Many of the QTLs found are known to be related to the size of rice grains. The amount of grain and the volume of the grain determine the weight of the grain. The volume of these grains is known to be regulated by several genes.

One of them is GW2, which controls the length and width. GW2 is known as Ring type ubiquitin ligase. Previous studies have shown that the *gw2* mutant produces larger rice seeds and that GW2 negatively regulates the division of cells within rice seeds (Fan et al., 2006; Song et al., 2007). The size of rice seeds eventually results from an increase in the number or size of cells. To increase the size of a cell, resistance from the cell wall must be overcome. This cell expansion

occurs through the action of expansin, which breaks hydrogen bonds that link the hemicellulose and cellulose in the cell wall. expansins are small cell wall proteins with many subfamilies. These expansins affect most of the growth stages of plants (Cosgrove et al, 2000).

Post-transitional modification is an essential component in the process of protein production in cells. Of these post-transitional modifications, the ubiquitin pathway is essential for the cell to function normally. Ubiquitination is carried out by E1, E2 and E3. E1 and E2 carry ubiquitin. Here, E2 works specifically with E3, and E3 causes ubiquitin to be attached to specific lysine residues to cause Ubiquitination of the target protein. The ubiquitination of the target protein causes a variety of changes. In general, polyubiquitination degrades polyubiquitinated proteins by the 26S proteasome (Vierstra et al, 2009).

This ubiquitination is known to be a major process related to the yield of crops. Among them, GW2 is known as a RING type E3 ubiquitin ligase, and research on a target protein of GW2 is becoming active. Studies of these target proteins will greatly advance research into mechanisms related to seed size and yield by ubiquitination.

Previous experiments have confirmed the relationship between EXLA1 and GW2 to some extent. EXLA1 is ubiquitinated *in vitro* by GW2. And have been reported to play an important role in determining the size of the seed and expressed in GW2 and vastly different tissues. We then determined how GW2 and EXLA1 interacted on the epidermal cells of the onion. The difference in the expression level of EXLA1 by GW2 and the difference in expression level of EXLA1 by plant hormone were also confirmed. Other than EXLA1, GW2 also interacts with other

proteins. We suggest that the ubiquitination of EXLA1 by the E3 ubiquitin ligase, GW2, is very important in controlling the seed size.

MATERIALS AND METHODS

Yeast two hybrid assay

Yeast two-hybrid assay was performed using the GAL4-based two-hybrid system (Clontech) to test interaction between GW2 and EXLA1. Full-length *GW2* and EXLA1 cDNAs were cloned into pGAD424 and pGBT8 (Clontech). The resulting constructs *AD-GW2* and *BD-EXLA1* were transformed into yeast strain AH109 using the lithium acetate method. Yeast cells were grown on minimal medium (–Leu/–Trp). Transformants were plated onto minimal medium (–Leu/–Trp/–His/2 mM 3-AT (3-amino-1, 2, 4-triazole)) to test the interactions between GW2 and EXLA1. We also examined the interaction between GW2 and EXLA1 homologues by yeast two hybrid. Full length cDNAs encoding EXLA3, EXPA2, EXPA4, EXPB4, EXPB11, EXPB5 and EXPB15 were cloned into pGBT8 (Clontech), respectively. The resulting constructs *AD-GW2* and *BD-EXLA1* homologues were transformed into yeast strain AH109 and further analysis was performed as described in above. All constructs were verified by automatic DNA sequencing to ensure that no mutations were introduced.

Subcellular localization of GW2 and EXLA1

The YFP or CFP coding sequences were fused in-frame to the 5’-end of *GW2*, and the YFP coding sequence was fused in-frame to the 3’-end of *EXLA1*. Fusion genes

encoding CFP, CFP, and YFP were expressed from the 35S promoter. Three micrograms of each plasmid was bombarded into the epidermis of the inner surface of onion scales using a particle gun-mediated system (Bio-Rad). The bombarded tissues were analyzed by confocal microscopy for visualization of transient expression, after incubation for 24 hr in darkness.

Bacteria and plant materials and growth conditions

The *Escherichia coli* strain DH5α was used for cloning work and plasmid preparation. The strain BL21/DE3 pLysS (Invitrogen) was used to express recombinant proteins. The cells were grown on LB medium supplemented with the appropriate antibiotics (100 μg/ml ampicillin, 50 μg/ml kanamycin, 100 μg/ml spectinomycin and/or 34 μg/ml chloramphenicol) at 37℃. Rice plants including wild type, mutant and transgenic lines were grown under long day conditions (26℃, 16 hr light/8 hr dark) in greenhouse or field. The japonica natural *gw2* mutant Oochikara (Accession No. 54075) was obtained from National Institute of Agrobiological Sciences (NIAS), Japan and wild type Norin 22 was kindly provided by Dr. Hee Jong Koh, Seoul National University

Construction of recombinant plasmids

To produce maltose binding protein (MBP)-GW2 and glutathione S-transferase (GST)-BSG1, the cDNA sequences encoding full-length *GW2* and *BSG1* were

amplified by PCR with gene-specific primers and inserted into the pGEX4T-1 (Amersham Biosciences) and pMALc2 (New England Biolabs) vectors, respectively. All constructs were transformed into *E. coli* strain BL21 cells. The transformed cells were treated with isopropyl-β-D-thiogalactoside (IPTG) to induce fusion protein expression. All constructs were verified by DNA sequencing to ensure that no mutations were introduced.

Purification of recombinant proteins

All recombinant proteins were expressed in *E. coli* strain BL21 and purified according to the manufacturer's instructions. Briefly, for purification of MBP and MBP-GW2 purification, bacteria were lysed in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 2 mM PMSF containing proteinase inhibitor cocktail (Roche), and purified on amylose resins (New England BioLabs). For GST-EXLA1, GST-EXLA1m1 and GST-EXLA1m2 purification, bacteria were lysed in PBS buffer (pH 7.5) containing 1% Triton X-100, 2 mM PMSF, and proteinase inhibitor cocktail (Roche), and purified on glutathione resins (Pharmacia). Protein concentrations were determined by the Bradford assay (Bio-Rad).

***In vitro* pull-down assay**

To examine *in vitro* binding of MBP-GW2 to GST-BSG1, 2 μg of full-length MBP-

GW2 and 2 μg of full-length GST-BSG1 were added to 1 ml of binding buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 0.2% glycerol, 0.5 mM β-mercaptoethanol). After incubation at 25℃ for 1 hr, the reaction mixtures were incubated with an amylose resin for 1 hr before washing six times with buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100). Absorbed proteins were analyzed using 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and detected by western blotting with anti-GST antibody (0.4 $\mu g ml^{-1}$; Santa Cruz Biotechnology).

Phylogenetic analysis

The amino acid sequences of expansin family were aligned by CLUSTAL W2(https://www.ebi.ac.uk/Tools/msa/clustalw2/), and the phylogenetic tree was constructed by MEGA 7.0 using the neighbor-joining method. Bootstrap values are shown each node.

RESULTS

EXLA1 specifically interacts with GW2

Expansin is a representative protein that loosens the cell walls of plants. There are various expansins in the expansin family, except for EXLA1, which are somewhat functional. Therefore, we also examined whether there is an expansin family that binds to GW2. However, since the function of EXLA1 is not known, we have investigated whether other expansin families interact with GW2. First, a phylogenetic tree was drawn using the homologs of EXLA1 (Figure 1A). The phylogenetic tree confirmed the phylogeny of EXLA1 and other expansin families. In Figure 1A, we selected seven proteins: EXLA3, EXPA2, EXPA4, EXPB4, EXPB5, EXPB11 and EXPB15. We chose these because they are similar to EXLA1 in the phylogenetic tree. The sequence of the expansin family was identified and used in Rice Genome annotation.

We conducted a yeast two-hybrid experiment to confirm the interaction between the next selected expansin family and GW2. Their full length cDNA was used for the Yeast two hybrid experiment. These full length cDNAs were constructed from Norin cDNA. EXLA1 was found in minimal medium as in experiments, but the selected expansin family did not survive minimal medium. As a result, it was confirmed that other families except EXLA1 did not bind to GW2 (Figure 1B). This result indicating that EXLA1 specifically binds to GW2 unlike other selected expansin families. The precise function of EXLA1 is unknown, but

specific binding to GW2 is a cell expansion related protein, suggesting that GW2 affects EXLA1 and eventually is associated with cell expansion.

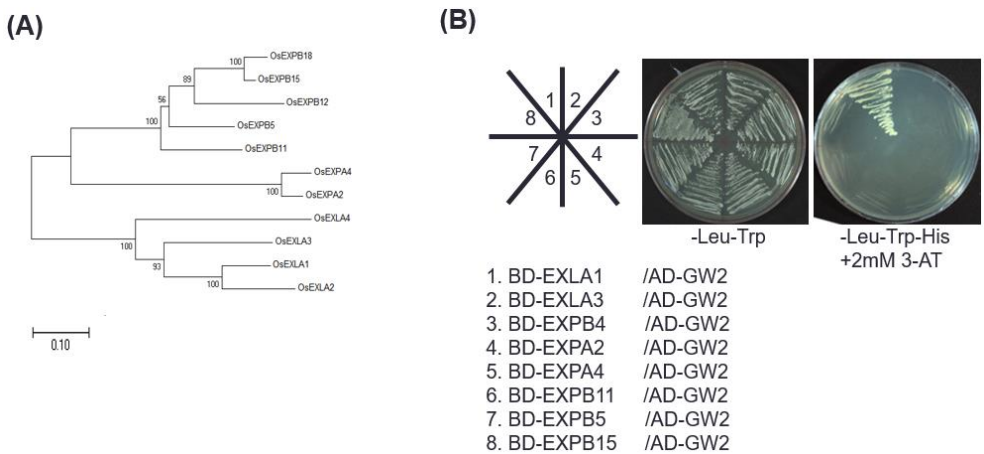

Figure 1. Yeast two hybrid assay of GW2 and expansin family. (A) Phylogenetic tree of expansin family. The amino acid sequences of expansin family were aligned by CLUSTAL W, and the phylogenetic tree was constructed by MEGA 7.0 using the neighbor-joining method. Bootstrap values are shown each node. (B) OsGW2 cDNA and expansin family were fused to sequences encoding Gal4 activation domain and the Gal4 DNA-binding domain respectively. Yeast two-hybrid analysis of the interaction between GW2 and expansin family. Minimal medium was supplemented with 2mM of 3-AT and cultured for 1 week.

GW2 and *EXLA1* colocalize in nucleus

We have previously confirmed that GW2 and EXLA1 bind through *in vitro* pull-down and yeast two-hybrid experiments. We then carried out an experiment to determine where these two proteins bind to the ubiquitination of the cells. As an experiment to confirm, we performed a colocalize experiment using particle bombardment on the epidermal cells of onion. CFP-GW2 and EXLA1-YFP were spotted on epidermal cells of onion. CFP-GW2 was localized on nucleus and YFP-EXLA1 was localized on cell wall (Figure 2A-B) when expressed individually. This means that GW2 is present in the nucleus and in the cell wall when EXL1 is expressed alone.

We then conducted an experiment to confirm the appearance of GW2 and EXLA1 simultaneously expressing in the epidermal cells of onion. CFP-GW2 and EXLA1-YFP were simultaneously expressed in onion epidermal cells. Previously, when each was tested on epidermal cells of onion, GW2 was nuclear and EXLA1 was found on cell wall, however co-expression of CFP-GW2 and EXLA1-YFP were localized in the nucleus (Figure 2C). This means that EXLA1 does not exist in the cell wall when EXLA1 and GW2 were simultaneously expressed in onion epidermal cells. It is also evidence that GW2 regulates the stability of EXLA1. This result corresponds to previous EXLA1 and GW2 *in vitro* ubiquitination experiments.

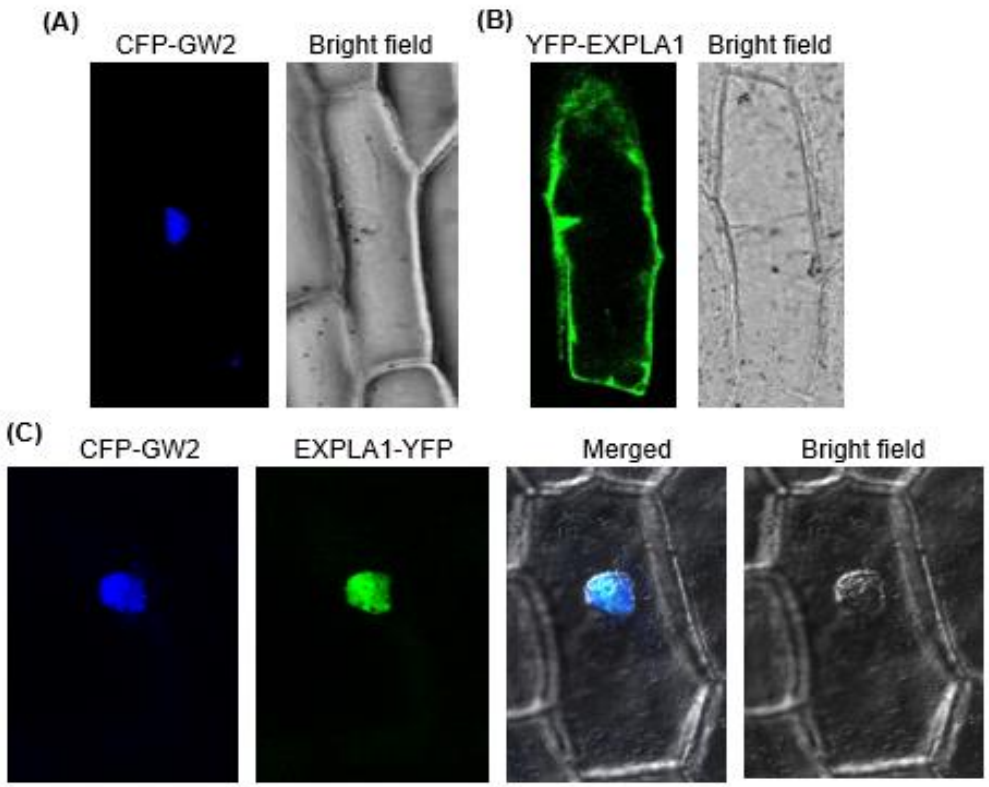

Figure 2. Co-Localization of CFP-tagged OsGW2 and YFP–tagged OsEXLA1 in onion epidermal cells. (A) Under dark-field for Cyanine blue fluorescence. (B) Under dark-field for green fluorescence. (C) Under dark-field and merged bright-field coexpression of CFP::OsGW2 and EXLA1::YFP. OsGW2 and OsEXLA1 were localized in nucleus. Onion epidermal peels were bombarded with DNA-coated tungsten particles and YFP and CFP expression was visualized 24 h later.

Transcriptional level of EXLA1 affected by GW2 and some plant hormones.

Previous experiments have confirmed that EXLA1 is affected by protein levels by GW2. Therefore, we conducted an experiment to determine whether GW2 independently regulates EXLA1 at the protein level, not at the gene. We tested *EXLA1* transcriptional level in *gw2* mutant and WT. To do this, we first preparation *gw2* mutant and WT RNA and made cDNA (Figure 3A). And then measure relative-transcriptional level. As a result, *gw2* mutant *EXLA1* transcriptional level is higher than WT about double. This means that the expression level of EXLA1 may vary depending on the degree of expression of GW2.

We also confirmed the effects of plant hormones because plant hormones were affected by GW2 and could affect the expression of EXLA1. Also we tested some plant hormones treatment in WT. GA3 treatment sample *EXLA1* transcriptional level is lower than WT sample (Figure 3B). Cytokinin treatment sample *EXLA1* transcriptional level is lower than WT sample (Figure 3C). Auxin treatment sample *EXLA1* transcriptional level is higher than WT sample (Figure 3D). These results indicate that expression levels of *EXLA1* can be regulated by plant hormones other than post-transitional modification. However, it was confirmed that this effect was less than the effect of post-transitional modification.

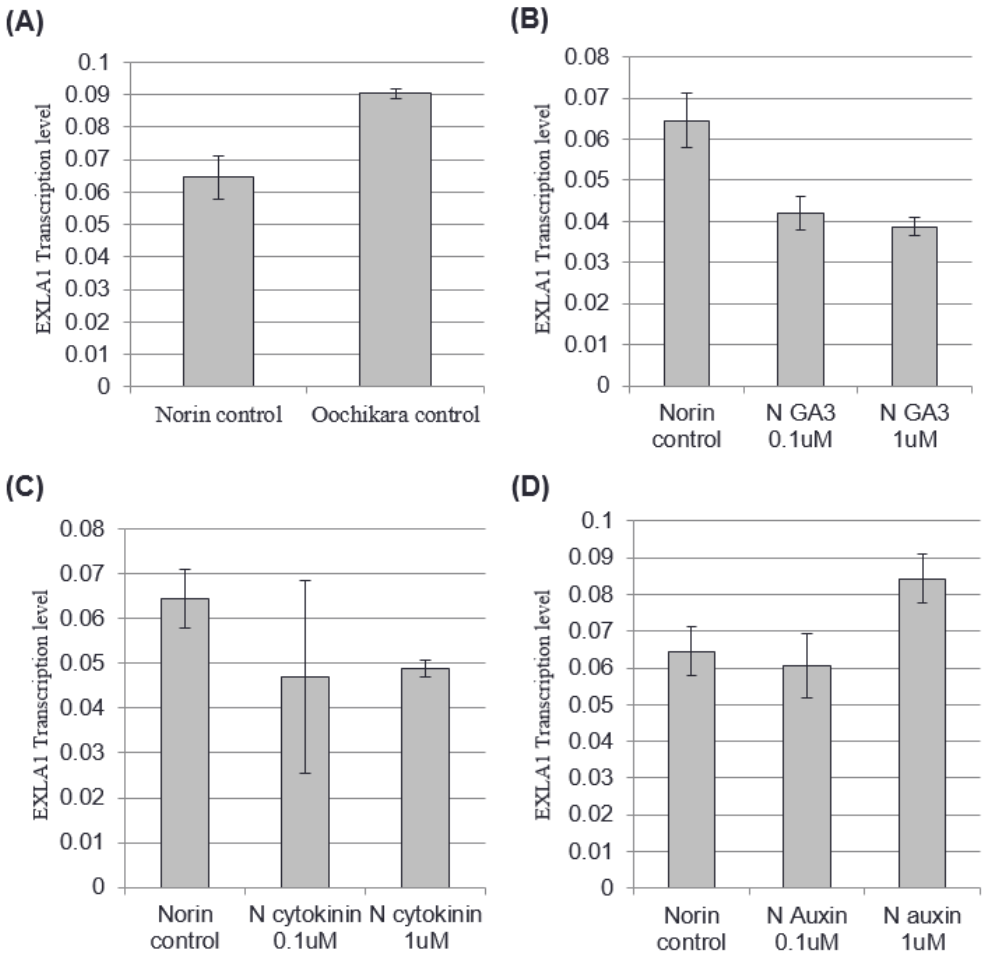

Figure 3. EXLA1 Transcriptional Expression in *gw2* mutant and after GA3, Cytokinin, Auxin treatment. (A) EXLA1 transcriptional expression in WT and *gw2* mutant. *gw2* mutant and WT were collected at the same time. (B) EXLA1 transcriptional expression after GA3 treatment. (C) EXLA1 transcriptional expression after Cytokinin treatment. (D) EXLA1 transcriptional expression after Auxin treatment. Each substance treated 0.1uM and 1uM over 48h. Each plant hormone was diluted in water and treated with liquid for 2 weeks in water.

BSG1 physically interacts with GW2

In previous experiments, EXLA1 was influenced by other than by GW2, and thought that there would be other proteins affected by GW2. Therefore, we have also confirmed that other proteins besides EXLA1 bind to GW2.

The *gw2* mutant differs from the WT in grain shape including lemma and palea. For this reason, we selected several genes and proteins related to grain shape, confirming that Beak-shaped grain 1 (BSG1) is related to GW2 and carried out subsequent experiments (Yan et al 2013). BSG1 is also a protein specifically expressed in grain lemma and palea (Yan et al 2013).

We thus chose BSG1 for further experiment. We examined the interaction between GW2 and BSG1 by yeast two hybrid assay using the full-length cDNAs of *GW2* and *BSG1*. cDNAs of BSG1 and GW2 were extracted from Norin, respectively, and each sequence was obtained from Rice genome annotation. The cDNAs were cloned into yeast expression vectors and the constructs were introduced into yeast strain AH109. As expected, BSG1 strongly interacted with EXLA1 (Figure 4A).

We confirmed that BSG1 binds strongly to GW2 in a yeast-two hybrid experiment, so we examined whether BSG1 and GW2 directly bind to protein-protein. *In vitro* pull-down assay was also performed to confirm protein-protein interaction. Two recombinant plasmids were constructed: MBP-tagged GW2 and GST-tagged BSG1. Over-expression of tagged proteins in *E. coli* was induced by IPTG treatment and proteins were purified with amylose and glutathione resins, respectively. After purification, we conducted *in vitro* pull-down assays for GST-BSG1 using MBP or MBP-GW2. Consistent with the results of the yeast two

hybrid assay, we observed a strong interaction between GW2 and BSG1 (Figure 4B). This result indicating that GW2, known as E3 ubiquitin ligase, is likely to interact with proteins that regulate or modify the size of other seeds in addition to EXLA1. In addition, it was confirmed that GW2 affects the stability of EXLA1 and may affect other proteins.

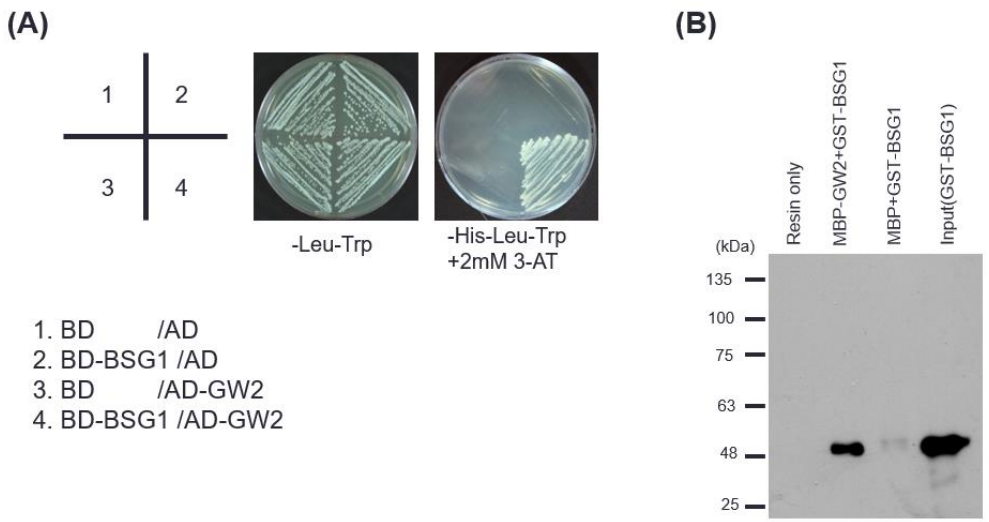

Figure 4. Interaction of BSG1 with GW2. (A) OsGW2 cDNA and OsBSG1 cDNA were fused to sequences encoding Gal4 activation domain and the Gal4 DNA-binding domain respectively. Investigation of binding between GW2 protein and BSG1 protein through Yeast two-hybrid assay. Minimal medium was supplemented with 2mM of 3-AT and cultured for 1 week. (B) *In vitro* pull down assay between MBP-GW2 and GST-BSG1. The MBP-GW2 protein was pulled down with GST-BSG1 protein, separated on 8% SDS-polyacrylamide gels, and analyzed by western blotting with an anti-GST antibody.

DISCUSSION

Efforts to increase the size of seeds have been ongoing for decades to increase crop yield and commercial viability. However, only a small number of genes involved in the regulation of seed size in rice have been commercialized and the exact mechanism has not yet been established. Therefore, understanding of the mechanism has to be understood because it is related to the increase of grain crop production. GW2 is known as the rice seed regulator. GW2 has been reported to regulate the size of seeds by increasing the size and number of cells (Fan et al., 2006; Song et al., 2007).

This GW2 is known as the RING type E3 ubiquitin ligase and the target protein for the corresponding ligase was not known. Previous studies on yeast two hybrid screening have reported that GW2 interacts with polyubiquitin. It was also consistent with screening in our laboratory. However, there has been no study of the functional relationship between the two proteins. Previous studies have confirmed that expansin, one of the interacting proteins of GW2, has cell wall expansion.

Expansin is known as a protein that acts to relax cell walls in growing cells. The role of expansin in controlling seed size has not yet been established. However, recent experiments have shown that expansin families are associated with grain yields (Lizana et al, 2010; Marowa et al 2016) and overexpression of the expansin gene in sweet potatoes in Arabidopsis has been shown to produce larger seeds (Bae et al 2014). Thus, EXLA1 was selected as the target protein of GW2, and *in vitro*

pulldown *in vitro* ubiquitination experiments revealed that GW2 was an E3 ubiquitin ligase for EXLA1. Experiments *in vivo* have not yet been proven, but these results suggest that ubiquitination may occur by combining two proteins somewhere *in vivo*. We also performed particle bombardment experiments on the epidermal cells of the onion to confirm this. GW2 and EXLA1 were present in nuclei and cell walls when expressed separately (Figure A and B).

However, when both proteins were expressed simultaneously, they were simultaneously expressed in the nucleus (figure C). These results suggest that EXLA1 is degraded by polyubiquitination due to the activation of GW2 in the nucleus. We now know that EXLA1 is degraded after ubiquitination. This means that unless EXLA1 is transformed into ubiquitin, it is stable and not degraded in plants.

In addition, EXLA1 has also been shown to increase its transcriptional level in *gw2* mutant (Figure 3A). This result confirms that the transcriptional level of EXLA1 also changes as GW2 disappears or decreases as well as post-transitional modification. The transcriptional level of EXLA1 was influenced by GA3, Cytokinin, and Auxin, which are plant hormones in addition to GW2 (Figure 3B-D). This was the result of confirming that the transcriptional level of EXLA1 could be changed by indirect effect other than directly by the transcriptional level of EXLA1 by GW2.

Target protein of GW2 has not been clearly identified, several candidate groups have been further tested to confirm that the BSG1 protein binds to GW2. The binding of GW2 and BSG1 was confirmed by yeast two-hybrid experiments (Figure 4A). In addition, each protein was purified and *in vitro* pull down

experiments were performed to confirm binding (Figure 4B). BSG1 is known to affect grain shape. It also appears to be related to cell expansion inside Grain (Yan et al., 2013; Peng et al 2017). These results indicate that GW2 binds to proteins other than EXLA1 and can change the size or shape of the grain.

In conclusion, it was found that GW2 is related to the protein responsible for cell expansion and grain shape. Our data also confirms that GW2 degrades the stability of EXLA1, a protein associated with cell expansion. Subsequent studies should examine whether EXLA1 is indeed degraded by the E3 ubiquitin role of GW2 in the plant, resulting in problems with cell expansion in the grain. These studies will be helpful in identifying the target protein of GW2 and understanding the mechanism of grain size change by GW2.

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ABSTRACT IN KOREAN

E3 ubiquitin ligase로 알려져 있는 Grain Weight 2(GW2)는 Grain의 크기와 무게를 조절하는데 있어 중요한 역할을 한다. 이러한 GW2에 의한 유전자 발현 및 조절에 대한 연구는 이전부터 많이 수행되어왔다. 하지만 GW2의 번역 후 단계에서의 연구는 거의 진행되지 않았다. 선행 연구에서 벼에서의 Grain Weight 2(GW2)가 EXLA1의 E3 ubiquitin ligase로써 작용할 수 있다는 것을 *in vitro* 상에서 확인하였다. 본 논문에서는 Particle bombardment 실험을 통하여 GW2와 EXLA1이 양파의 표피세포에서 상호작용한다는 것을 확인 할 수 있었다. 또한 Yeast two-Hybrid 실험을 통하여 EXLA1이외의 expansin family는 GW2와 결합하지 않음을 확인하였다. 또한 *gw2* 돌연변이 개체에서 EXLA1의 발현이 높음을 확인하였고 야생형인 Norin에 GA3, Cytokinin, Auxin 을 처리하였을 때 EXLA1의 발현이 달라짐을 확인하였다. 또한 EXLA1 이외의 다른 후보군인 BSG1이 GW2와 결합함을 Yeast two-hybrid 실험과 *in vitro* pull down 실험을 통하여 밝혔다. 이를 종합하여 볼 때, EXLA1이 GW2에 의해서 그 안정성이 조절됨을 확인할 수 있었고, EXLA1이외의 BSG1과도 결합함을 확인하였다.

색인어: E3 Ubiquitin ligase GW2, EXLA1, ubiquitination.

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