An atypical bHLH protein encoded by *POSITIVE REGULATOR OF GRAIN LENGTH 2* is involved in controlling grain length and weight of rice through interaction with a typical bHLH protein APG

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Grain size is an important yield component in rice, however, genes controlling the trait remain poorly understood. Previously, we have shown that an antagonistic pair of basic helix-loop-helix (bHLH) proteins, *POSITIVE REGULATOR OF GRAIN LENGTH 1* (PGL1) and *ANTAGONIST OF PGL1* (APG), is involved in controlling rice grain length. Here, we report the involvement of another atypical bHLH protein gene, *POSITIVE REGULATOR OF GRAIN LENGTH 2* (PGL2), in the regulation of rice grain length. Overexpression of *PGL2* in the lemma/palea increased grain length and weight in correlation with the level of transgene expression. Observation of the inner epidermal cells of lemma of *PGL2*-overexpressing lines revealed that the long grain size is caused by an increase in cell length. *PGL2* interacts with a typical bHLH protein APG, a negative regulator of rice grain length and weight, *in vitro* and *in vivo*. It was reported that overexpression of *BU1* (*BRASSINOSTEROID UPREGULATED 1*), the closest homolog of *PGL2*, caused an increase in grain length. However, we detected no interaction between *BU1* and APG. These findings suggest that *PGL2* and *PGL1* redundantly suppress the function of APG by forming heterodimers to positively regulate the rice grain length, while the pathway through which *BU1*, the closest homolog of *PGL2*, controls grain length is independent of APG.

Key Words: basic helix-loop-helix, cell elongation, grain length, rice, transcription factor.

Introduction

The lemma and palea cover the endosperm to compose the grain of rice. The sizes of these organs have been considered to be one of the major parameters determining the grain size of rice (Shomura *et al.* 2008, Song *et al.* 2007). Studies on homeotic genes for rice florets have showed that a number of genes are required for normal development of the lemma and palea (Jeon *et al.* 2000, Jin *et al.* 2011, Ohmori *et al.* 2009, Sentoku *et al.* 2005, Wang *et al.* 2010, Yuan *et al.* 2009). However, the genes involved in controlling the size of these organs remain largely unclear.

Basic helix-loop-helix (bHLH) proteins are a large family of plant transcription factor (Carretero-Paulet *et al.* 2010, Feller *et al.* 2011, Pires and Dolan 2009) containing two adjacent regions, a basic region and a HLH region. A typical bHLH protein with both domains functions as a transcription factor by forming a homo/hetero dimer with another bHLH protein at the HLH domain and binds directly to DNA in the basic region (Massari and Murre 2000). Another class of bHLH, the atypical bHLH, is unable to bind DNA because of a lack of conserved amino acid residues, but retains the

ability to form a heterodimer (Massari and Murre 2000). Frequently, atypical bHLH proteins function as an inhibitor of typical bHLH protein through dimerization (Sun *et al.* 1991, Toledo-Ortiz *et al.* 2003).

Recent studies have revealed crucial roles for some atypical bHLH proteins in organ development in different species. In Arabidopsis, *ROOT HAIR DEFECTIVE 6 LIKE 4* (*RSL4*) encodes an atypical bHLH and functions to promote cell growth in root hairs and root length (Yi *et al.* 2010). Atypical bHLH genes such as *ATBS1* (*Activation-Tagged Bri1-Suppressor1*) and *PREs* (*Paclobutrazol Resistance*) play important roles in auxin, brassinosteroid and gibberellin signaling in Arabidopsis and rice (Schlereth *et al.* 2010, Wang *et al.* 2009, Zhang *et al.* 2009). Defective phenotypes including dwarfism and narrow leaves were observed in bHLH mutants which resulted from alterations of cell size in the respective organs (Clouse 2011, Wang *et al.* 2009, Zhang *et al.* 2009). The rice genome is predicted to contain 177 bHLH genes (Carretero-Paulet *et al.* 2010, Li *et al.* 2006), however, findings on the roles of these genes in organ development are limited. For instance, an antagonistic pair of atypical bHLH proteins, *Ili1* (increased leaf inclination) and *OsIBH* (*IL11* binding bHLH), acts together to control lamina joint cell length and leaf bending. Overexpression of *BRASSINOSTEROID UPREGULATED 1* (*BU1*) under the control of a constitutive promoter increased grain size in

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addition to leaf bending and internode structure (Tanaka *et al.* 2009). Constitutive overexpression of *LAX PANICLE* (*LAX*) caused dwarfism, stem elongation, dark color, abnormal leaves, leaf bending and severe sterility (Komatsu *et al.* 2003). These studies demonstrated the important roles of bHLH transcription factors on sizes of different organs in plants. However, the involvement of bHLH proteins in determining rice grain size is largely unknown.

Previously, we identified an antagonistic pair of bHLH proteins, the atypical bHLH protein POSITIVE REGULATOR OF GRAIN LENGTH 1 (PGL1) and typical bHLH protein ANTAGONIST OF PGL1 (APG), as involved in regulation of the grain length of rice (Heang and Sassa 2012). Here, we report the role of another atypical bHLH named POSITIVE REGULATOR OF GRAIN LENGTH 2 (PGL2) in the regulation of rice grain size. The phenotype of *PGL2*-overexpressing lines, increased grain length, was very similar to that of *APG* RNAi and *PGL1*-overexpressing lines (Heang and Sassa 2012). Histological observation of the inner epidermal cell layer of the lemma/palea revealed that the increase of grain length in transgenic lines is caused by elongation of the cells. We show that *PGL2* interacts with *APG*, a negative regulator of grain length and weight (Heang and Sassa 2012), while the closest homolog of *PGL2*, *BU1*, does not. These findings suggest that *PGL2* and *PGL1* inhibit the function of *APG* through heterodimerization to regulate grain length positively, while the closest homolog of *PGL2*, *BU1*, controls grain length through an *APG*-independent pathway.

Materials and Methods

Sequence analysis of bHLH proteins

Whole amino acid sequences of atypical bHLHs in subfamily 16 (Carretero-Paulet *et al.* 2010, Chen *et al.* 2007) and the bHLH domain of *APG* were aligned by CLUSTALW. Based on the alignment, a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) using MEGA v.5.0 (Tamura *et al.* 2011) (<http://www.megasoftware.net/>).

Plant materials and observation of phenotypes

Rice (*Oryza sativa* L.) cv Nipponbare was used for transformation as described previously (Hiei and Komari 2008). Ten fertile seeds from transgenic and wild type plants were chosen randomly for measuring grain length and width with vernier calipers. Thousand seeds weight was calculated from the weights of 200 fully fertile seeds after drying at 41°C for one week after harvest (Wu *et al.* 2008).

Gene expression analysis by qPCR

Lemma/palea and pistils at the pre-anthesis stage, leaves and roots of one-week old plants were separated and used for RNA extraction with a RNeasy plant mini kit (Qiagen). Extracted RNA was treated with DNase (Wako) followed by phenol chloroform purification and stored at -80°C until

used. Total RNA (2 µg) was used to synthesize first-strand cDNA with cDNA synthesis kit (Toyobo). Quantitative PCR (qPCR) for gene expression analysis was carried out with SYBR Thunderbird (Toyobo) using gene specific primers (FPGL2: 5'-ATGTCGAGCAGAAGGTCGTC-3' and RPGL2: 5'-TCAGGAGCGGAGGATGCTGC-3'). The rice actin gene was used (Act_F: 5'-CCCTCCTGAAAGGAAGTACAGTGT-3' and Act_R: 5'-GTCCGAAGAATTAGAA GCATTTCC-3') as a control (She *et al.* 2010). Data were collected using an ABI PRISM 7000 sequence detection system (Applied Biosystems) and analyzed according to the instructions manual.

Construction of plasmids

a) *chitinase::PGL2*: The 1685 bp upstream region of a rice chitinase gene (AB012855), hereafter referred to as the chitinase promoter (Heang and Sassa 2012, Takakura *et al.* 2000), was amplified from Nipponbare genomic DNA by PCR (FchiH: 5'-CCCAAGCTTGTTATGCTCGTTTGTCTAT-3' and RchiK: 5'-GGGGTACCGGCAAGATGCTTATTTCT-3'), fused to the 496 bp genomic sequence of *PGL2* (Os02g0747900) amplified by PCR (FPGL2k: 5'-GGGGTACCATGTCGAGCAGAAGGTCGTC-3' and RPGL2b: 5'-CGGGATCCTCAGGAGCGGAGGATGCTGC-3') and inserted into a binary vector pPZP2H-Lac (Fuse *et al.* 2001) to create chitinase::PGL2 (Fig. 3A). First, *PGL2* and chitinase promoter fragments were amplified separately and sub-cloned into the pHK7 vector (Harikrishna *et al.* 1996) at *KpnI/BamHI* and *HindIII/KpnI*, respectively. Then, the expression cassette was moved to the binary vector pPZP2H-Lac (Fuse *et al.* 2001) (Fig. 3A).

b) *protein expression*: The open reading frame of each gene was amplified from Nipponbare lemma/palea cDNA by gene specific primers. The coding sequence of the *PGL2* fragment was amplified by PCR (FPGL2k: 5'-GGGGTACCATGTCGAGCAGAAGGTCGTC-3' and RPGL2b: 5'-CGGGATCCTCAGGAGCGGAGGATGCTGC-3') and sub-cloned into pET32a (Novagen) at *KpnI/BamHI* to generate an expression vector for the thioredoxin-PGL2 fusion protein (Trx-PGL2). *BU1* was amplified from lemma/palea cDNA (FBU1e: 5'-GGAATTCATGTCGAGCCGGAGGTCGTC-3' and RBU1xh: 5'-CCCTCGAGCTAGCGGAGGAGGCTGCGGA-3') and sub-cloned into pGEX-4T-1 (GE, Healthcare) at *EcoRI/XhoI*. MBP-APG was generated as described in Heang and Sassa (2012).

Pull-down assay

Interaction between Trx-PGL2 and MBP-APG was analyzed as described in Heang and Sassa (2012). Trx-PGL1 and MBP-APG were expressed in *Escherichia coli* strain BL21(DE3 pLysS) (Novagen). MBP and MBP-APG were purified using amylose resin beads (New England BioLabs). The beads bound to MBP-APG or MBP were incubated with an equal amount of extracts of bacteria expressing Trx-PGL2 in binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM DTT) supplemented with

1 × final concentration of protease inhibitor complete (Roche). The mixture was rotated at 4°C for 2 hours and the beads were washed five times with washing buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.05% Tween-20, 1 mM EDTA and 1 mM DTT). The proteins were eluted from the beads by heating at 65°C for 5 min in 30 µl of 2 × SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 2% 2-mercaptoethanol and 20% glycerol). From each sample, 10 µl was loaded onto a 13% SDS-PAGE gel. Gel blots were reacted with anti-His antibody (Covance) or anti-GST antibody.

Bimolecular fluorescence complementation (BiFC) assay

In vivo interaction between PGL2 and APG was examined as described in Heang and Sassa (2012). Briefly, *PGL2* and *APG* were amplified from Nipponbare lemma/palea cDNA and inserted into binary pBiFC vectors (Niwa, M., Daimon, Y. and Araki, T. unpublished). All eight possible pairwise combinations of BiFC constructs were transformed into *A. tumefaciens* COR308 (Hamilton *et al.* 1996). A construct for expression of the p19 protein of tomato bushy stunt virus was used to suppress gene silencing (Voinnet *et al.* 2003). Agrobacteria harboring BiFC constructs were co-infiltrated with the p19 construct into four-week-old leaves of *Nicotiana benthamiana* at an OD₆₀₀ ratio of 0.7 : 0.7 : 1.0. The plants were kept for 48 hours after infiltration under continuous light at 26°C prior to observation. Yellow fluorescence protein (YFP) was visualized with a Leica DMR fluorescence microscope (Leica Microsystems, Heerbrugg, Germany).

Histological observation of lemma cells

Ten florets at pre-anthesis were fixed with FAA overnight, washed with ethanol and kept until use. The middle of each lemma was excised to obtain a uniform part for observation. 1 N NaOH was added to the excised sample and boiled in hot water for 5 min. The samples were transferred to a staining solution containing 1 M Tris-HCl pH 9.0 with 0.1 mg/L of calcofluor (Fluorescent Brightener 28, Sigma-ALDRICH) and kept in the dark for 12 hours before observation. The inner epidermal layers were peeled and images were taken with the Leica confocal microscope (Leica Microsystems, Heerbrugg, Germany). A total of 250 random cells from 10 lemma images were measured using ImageJ software (<http://rsb.info.nih.gov/ij/>) for cell length and width.

Results

PGL2 encodes an atypical bHLH protein of subfamily 16 and is expressed in various organs

Os02g0747900 (LOC_Os02g51320 in MSU Rice Genome Annotation Project) is an atypical bHLH of unknown function and was named *Os170* and belonging to subfamily 16 of bHLH proteins in a phylogenetic study (Carretero-Paulet *et al.* 2010). Phylogenetic analysis of amino acid

sequences of members of subfamily 16 revealed that the closest homolog of Os02g0747900 is a rice bHLH protein BRASSIONOSTEROID UPREGULATED 1 (BU1, Tanaka *et al.* 2009) with 90.8% amino acid identity (Fig. 1A, 1B). Many members of subfamily 16, such as rice Ili1 and BU1, Arabidopsis PRE1 and ATBS1 and tomato Style2.1, were reported to control cell elongation and expansion in specific organs probably through heterodimerization with other bHLH proteins (Wang *et al.* 2009, Zhang *et al.* 2009). In addition, we recently reported *PGL1* (Os03g0171300) as a positive regulator of the grain length and weight of rice (Heang and Sassa 2012). However, the function of Os02g0747900 has not been elucidated yet. We re-named Os02g0747900 as *PGL2* (*POSITIVE REGULATOR OF GRAIN LENGTH 2*) based on our functional analyses (see below). *PGL2* has 52.9% and 94.3% whole amino acid identity and similarity, respectively, to *PGL1* and 48.1% and 98.1% identity and similarity, respectively, in the HLH domain alone.

For the DNA-binding activity of the basic domain of bHLH, at least 2 amino acid residues are essential. Glu at position 13 plays an important role in binding to the E-box motif (5'-CANNTG-3') (Carretero-Paulet *et al.* 2010, Toledo-Ortiz *et al.* 2003), while Arg at position 16 stabilizes the binding activity (Toledo-Ortiz *et al.* 2003). The amino acid positions described herein refer to the same position as in Carretero-Paulet *et al.* (2010), Li *et al.* (2006), Toledo-Ortiz *et al.* (2003). The binding is specific to the G-box motif (5'-CACGTG-3') when His occurs at position 9 (indicated by *, Fig. 1B). Amino acid alignment with a typical bHLH, APG, shows that eight members of bHLH subfamily 16 including *PGL2* do not have the conserved amino acid residues required for binding DNA at the domain, suggesting they are incapable of binding DNA (Fig. 1B). However, they have Leu at position 27 and 73 (indicated by ♦, Fig. 1B) in the first and second helix domains, respectively, which is a conserved amino acid for all plant bHLH proteins and most probably required for protein dimerization (Brownlie *et al.* 1997).

Expression analysis of *PGL2* by RT-PCR showed that the gene is expressed in different organs including reproductive organs such as the young panicle, pistil and lemma/palea (Fig. 2).

Overexpression of PGL2 increases grain length and weight

To analyze the function of *PGL2*, we overexpressed the gene by using a rice chitinase promoter, which was reported to induce gene expression predominantly in rice florets (Heang and Sassa 2012, Takakura *et al.* 2000). Although Takakura *et al.* (2000) found weak GUS staining in lemma/palea of the transgenic rice expressing *GUS* gene under the chitinase promoter, we observed high level of expression of *PGL1* in lemma/palea by using the chitinase promoter (Heang and Sassa 2012). This may be because the length of the promoters and/or the cultivars from which the promoters were cloned were different between the two studies [1250 b

Fig. 1. Phylogenetic tree and amino acid alignment of atypical bHLH proteins. A) Phylogenetic tree of deduced whole amino acid sequences of PGL2 and its homologs belonging to subfamily 16 (Carretero-Paulet *et al.* 2010) generated using the neighbor-joining method. A typical bHLH protein APG (Heang and Sassa 2012) was used as an outgroup. Proteins from rice and tomato are shaded in black and gray, respectively. Arabidopsis proteins are not shaded. B) Amino acid alignment of PGL2 and its homologs using CLUSTALW. The dotted, solid and curve lines indicate the basic region, loop region and helix region, respectively. Asterisks (*) denote the conserved His at position 9, Glu at position 13 and Arg at position 16 in the basic domain of bHLH required for binding to G-box (5'-CACGTG-3') and black diamonds (◆) denote Leu at positions 27 and 73, the conserved amino acids for plant bHLH (Carretero-Paulet *et al.* 2010, Toledo-Ortiz *et al.* 2003). OsBP-5 and PIF3 are typical bHLH proteins from rice and Arabidopsis, respectively (Toledo-Ortiz *et al.* 2003, Zhu *et al.* 2003).

sequence from an *indica* cultivar ‘IR24’ in Takakura *et al.* (2000), 1679 b from a *japonica* cultivar ‘Nipponbare’ in Heang and Sassa (2012)]. In this study, we used the chitinase promoter that was used to overexpress *PGL1* (Heang and Sassa 2012) (Fig. 3A). Eight independent T₀ transgenic lines, PGL2 : OX, were produced. We observed that grain sizes of the T₀ lines are larger than those of wild types (Fig. 3B). We previously showed that overexpression of *PGL1*, a homolog of *PGL2*, in the lemma/palea increased grain length and weight in rice (Heang and Sassa 2012). Therefore, we analyzed the correlation between the expression levels of *PGL2* in lemma/palea and the grain size of transgenic plants. As expected, up-regulation of *PGL2* expression in lemma/palea was associated with the grain length and weight. Quantitative PCR (qPCR) revealed that

Fig. 2. Expression pattern of PGL2. Expression analysis of PGL2 by RT-PCR in different organs of rice. R = root of one-week-old plant, L = leaf of one-week-old plant, S = seedling of one-week-old plant, YP = young panicle ~5 cm, Pis = pistil at pre-anthesis stage, Le/P = lemma/palea at pre-anthesis stage and G = genomic DNA. PCR cycling numbers are in parentheses.

Fig. 3. Overexpression of *PGL2* increases grain size in rice. A) Structure of the overexpression cassette of *PGL2* in the pZP2H-lac binary vector. B) Grain phenotype of T₀ transgenic plants (Ci#) compared with wild type (NiWT) (bar = 1 cm). C) Quantitative RT-PCR analysis of *PGL2* in the lemma/palea of T₀ plants compared with the wild type (WT = 1) normalized by *OsActin*. Error bar indicates $\pm$ sd over three biological repeats. D) Correlation between accumulation of *PGL2* mRNA in the lemma/palea and grain length.

the *PGL2* : OX lines Ci4 and Ci6 (T₀) accumulating 11 and 18-fold more of the *PGL2* transcript compared to wild type in the lemma/palea, respectively, showed a 49% and 37% increase in thousand grains weight, respectively, while lines Ci1 and Ci3 with a 4 and 2-fold increase in *PGL2* expression, respectively, did not show a significant difference in grain weight (Fig. 3C and Table 1). The accumulation of *PGL2* mRNA in lemma/palea is well correlated to the grain length in transgenic T₀ plants (Fig. 3D).

Overexpression of *PGL2* increases cell length in lemma/palea

To test whether the enhanced grain length is caused by increased size or number of cells in lemma/palea, we exam-

ined the structure of inner epidermal cells. Three transgenic lines with different grain sizes were selected from *PGL2* : OX (Ci1, Ci4 and Ci5) to compare the inner epidermal cells of the lemma to those of the wild type. Confocal microscopic observations revealed that the longer grain is caused by extended cell length (Fig. 4A). Transgenic plants with longer grains produced more long cells than the wild type, while the width of cells was mostly unaffected (Fig. 4B). The results suggested that, like *PGL1*, *PGL2* controls grain length by elongating the cells in the lemma/palea probably through heterodimerization with a typical bHLH.

PGL2 but not *BU1* interacts with *APG* in vitro and in vivo

Atypical bHLH proteins are thought to function as inhibitors of typical bHLH proteins through protein dimerization (Sun *et al.* 1991, Toledo-Ortiz *et al.* 2003). We previously showed that a *PGL2* homolog, *PGL1*, interacts with the typical bHLH protein *APG*, a negative regulator of grain length and controls grain length in rice (Heang and Sassa 2012). Overexpression of *PGL2* and *PGL1* with the same rice chitinase promoter resulted in a very similar phenotype. Therefore, we reasoned that *PGL2* would also act as a repressor of *APG* through heterodimerization to control the grain length of rice. We performed pull-down assays using recombinant thioredoxin (Trx)-*PGL2* and maltose-binding protein (MBP)-*APG* and found that the two proteins were co-precipitated by amylose resin (Fig. 5A), indicating that *PGL2* and *APG* interact with each other *in vitro*. Previous phylogenetic analysis and amino acid alignment showed that the closest homolog of *PGL2* is *BU1*. Overexpression of *BU1* also resulted in long grain size (Tanaka *et al.* 2009). Thus, we examined if *BU1* also interacts with *APG*. Pull-down assay using recombinant glutathione-S-transferase (GST)-*BU1* and MBP-*APG* did not show co-precipitation of the two proteins (Fig. 5B), indicating that, unlike *PGL2*, *BU1* does not interact with *APG* *in vitro* regardless its high homology to *PGL2*.

To examine the interaction between *PGL2* and *APG* *in vivo*, we performed a bimolecular fluorescent complementation (BiFC) assay. *Agrobacterium* harboring constructs for expression of the C-terminal half of enhanced yellow fluorescent protein (EYFP) fused to *PGL2* (YC-*PGL2*) and N-terminal half of EYFP fused to *APG* (*APG*-YN) were

Table 1. Grain traits and lemma inner epidermis cells of wild type and *PGL2* : OX lines

Line	1000-grain weight (g)	Grain length ^a (mm)	Grain width ^b (mm)	Cell length ^c (μm)	Cell width ^d (μm)
WT	23.2 (100%)	6.9 ± 0.3	2.8 ± 0.2	102.2 ± 20.6	45.7 ± 6.8
<i>PGL2</i> : OX Ci1	24.3 (104%)	6.9 ± 0.2 ^{ns}	2.9 ± 0.2 ^{ns}	115.6 ± 28.1***	44.5 ± 4.9*
<i>PGL2</i> : OX Ci3	22.7 (97%)	6.9 ± 0.2 ^{ns}	2.8 ± 0.1 ^{ns}	—	—
<i>PGL2</i> : OX Ci4	34.9 (149%)	8.3 ± 0.2***	3.4 ± 0.2*	165.0 ± 33.4***	46.5 ± 5.0*
<i>PGL2</i> : OX Ci5	30.0 (128%)	8.2 ± 0.2***	3.3 ± 0.2*	161.6 ± 28.4***	47.5 ± 6.4***
<i>PGL2</i> : OX Ci6	32.0 (137%)	8.6 ± 0.4***	3.2 ± 0.1 ^{ns}	—	—

^{a,b} data are the average for 10 samples ($\pm$ sd).

^{c,d} data are the average for 250 samples ($\pm$ sd).

^{ns}, not-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 4. Inner epidermal cells observed by confocal microscopy. A) Lemma inner epidermal cells of NiWT and transgenic PGL2:OX (Ci4), (bar = 100 μm). B) Distribution of the number of cells of various lengths. Triangles represent average values of the respective lines. C) Distribution of the number of cells of various widths. Triangles represent average values of the respective lines.

co-infiltrated into *Nicotiana benthamiana* leaves. The YFP fluorescence was observed in the nucleus (Fig. 6), indicating that the two proteins interact *in vivo* and are localized in the nucleus. No YFP signals were observed for the combination of YC-PGL2 and N-EYFP (YN) or the combination of C-EYFP (YC) and APG-YN (Fig. 6), further suggesting that interaction between PGL2 and APG is necessary for reconstruction of the YFP protein.

Discussion

Findings on the molecular mechanism underlying rice grain size remain limited (Xing and Zhang 2010). A few genes involved in controlling grain length, width and filling have been isolated by a map-based cloning approach (Fan *et al.*

Fig. 5. Analysis of the interaction of PGL2 and BU1 with APG. A) Interaction between PGL2 and APG *in vitro* analyzed by pull-down assay. Amylose resin-bound MBP-APG or MBP was incubated with an equal amount of Trx-PGL2. Proteins co-precipitated with the amylose resin were detected by immunoblotting using anti-His antibody (upper). B) Interaction between BU1 and APG *in vitro* analyzed by pull-down assay. Amylose resin-bound MBP-APG or MBP was incubated with an equal amount of GST-BU1. No co-precipitation was detected by immunoblotting using anti-GST antibody (upper). Ponceau staining indicated comparative amounts of MBP (●) and MBP-APG (◆).

2006, Kitagawa *et al.* 2010, Li *et al.* 2011, Shomura *et al.* 2008, Song *et al.* 2007, Takano-Kai *et al.* 2009, Wang *et al.* 2008). In these previous studies, genetic mapping using different plant populations detected various QTLs responsible for the corresponding grain size-related traits and led to the identification of genes involved. However, none of these genes belonged to the bHLH gene family. Using the reverse genetics approach, we previously have shown that an antagonistic pair of bHLH proteins, PGL1 and APG, underlies grain length (Heang and Sassa 2012). In this study, we further identified another atypical bHLH protein, PGL2, as a positive regulator of grain length. Given that atypical bHLH

Fig. 6. Analysis of interaction between PGL2 and APG by BiFC assay. Light microscopic images of the interaction *in vivo* between PGL2 and APG revealed by a BiFC assay in the *N. benthamiana* leaf epidermis. BF, bright field image; YFP, yellow fluorescent protein; DAPI, 4',6-diamidino-2 phenylindole for nuclear staining; MERGE, merged view of the YFP and DAPI images. YC-PGL2 + APG-YN indicates *Agrobacterium* mediated co-infiltration of constructs encoding C-EYFP-PGL2 and APG-N-EYFP (upper), arrows indicate nucleus localization of YFP; YC-PGL2 + YN, co-infiltration of C-EYFP-PGL2 and C-EYFP alone (middle); YN+ APG-YN, co-infiltration of N-EYFP alone and APG-C-EYFP (lower). (bar = 100 μ m)

proteins such as PGL2 and PGL1 lack DNA-binding activity due to an absence of conserved amino acid residues in the basic region, identification of their interaction partner(s), such as typical bHLH proteins, is crucial to uncover the transcription regulatory network. This idea led us to identify the three bHLH protein dimers, PGL1-APG and APG-APG (Heang and Sassa 2012) and PGL2-APG, involved in controlling rice grain size. APG is a typical bHLH and functions as a negative regulator of rice grain length (Heang and Sassa 2012). In addition, phenotypes resulting from the overexpression of PGL2 and PGL1, and that of RNAi of APG included elongated grain length. Taken together, these results suggest that APG is an integrator of the two atypical bHLHs, PGL1 and PGL2, for the grain size controlling mechanism in rice (Fig. 7).

Three parameters have been conceptualized as important determinants of the specificity of transcription factors, i.e., differences in DNA binding, protein interactions and spatiotemporal expression patterns (Grove *et al.* 2009). A study on the dimerization network of bHLH proteins of *C. elegans* showed that most proteins interact with one partner, but some bind with many other proteins (Grove *et al.* 2009). APG retains the amino acid residues required for DNA binding, and is expressed in most organs analyzed (Heang and Sassa 2012). The current study and our previous study showed that APG interacts with PGL1, PGL2 and itself (Heang and Sassa 2012) and the genes of these proteins are expressed in different organs. These findings would suggest that the control of grain size is one of the diverse functions of these bHLH

Fig. 7. Model for regulation of grain length by bHLH proteins in rice. Typical bHLH proteins APG and a hypothetical protein, bHLHx, negatively regulate rice grain length. Atypical bHLH proteins PGL1 and PGL2 function as positive regulators of grain length and suppress the function of APG through heterodimerization, while the closest homolog of PGL2, BU1, does not. BU1 is assumed to be involved in regulation of grain length through heterodimerization with bHLHx to suppress its function. Dotted lines indicate a hypothetical pathway.

proteins. Being consistent with this, RNAi lines of *APG* and overexpression lines of *PGLs* also showed increased leaf bending (Heang and Sassa 2012, data not shown).

Although both PGL1 and PGL2 would positively regulate grain length through heterodimerization with APG, the strength of their effect seems to differ slightly. To reach a >40% increase of thousand grains weight, *PGL1* needed to be increased up to ~170 fold over the wild type (Heang and Sassa 2012), while a ~20 fold increase of transcripts was sufficient in *PGL2* overexpressing lines. The affinity for APG of PGL2 might be higher than that of PGL1. It is also possible that expression level of endogenous *PGL2* is higher than that of *PGL1*. Another possibility is that PGL2 also interacts with a negative regulator of grain length other than APG to control grain size. This is consistent with the findings that the closest homolog of PGL2, BU1, increased grain length when overexpressed by the maize ubiquitin promoter (Tanaka *et al.* 2009), but showed no interaction with APG. The grain length-controlling pathway in which BU1 is involved may be independent of, if related to, that of APG. This is supported by the differences in phenotypes for the overexpression of BU1 and PGL1. The driving of BU1 expression by the maize ubiquitin promoter resulted in sterility in most transgenics (Tanaka *et al.* 2009), while most plants were fertile in lines with the overexpression of PGL1 using the rice chitinase promoter (Heang and Sassa 2012) or by maize ubiquitin promoter (data not shown). Further identification of upstream genes that regulate these bHLH genes and downstream genes that are targeted by these bHLH proteins should uncover the relationships between the BU1-pathway and APG-pathway and grain length-controlling network in which these bHLH genes are involved.

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