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-Suppresser1) and PREs (Paclobutrazol Resistance) play important roles in auxin, brassinosteroid and gibberel-lin 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, Il1 (increased leaf inclination) and OsIIBH (II1 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 rice.
addition to leaf bending and internode structure (Tanaka et al. 2009). Constitutive overexpression of *LAX PANICLE* (*LAX*) caused dwarfism, stem elongation, dark color, abnor-
mal 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 involvem ent of bHLH proteins in de-
termining 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 analysis 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-indepen-
dent pathway.

**Materials and Methods**

**Sequence analysis of bHLH proteins**

Whole amino acid sequences of atypical bHLHs in sub-
family 16 (Carretero-Paul 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.megassoftware.net/).

**Plant materials and observation of phenotypes**

*Rice* (*Oryza sativa* L.) cv Nipponbare was used for trans-
formation 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 was calculated 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′-ATGTCCAGCAGAAGGTTCGTC-3′ and RPGL2: 5′-TCAGGAGCGAGGATGCTG-3′). The rice actin gene was used (Act_F: 5′-CCCTCCTGAAAGGAAG TACAGTGT-3′ and Act_R: 5′-GTCCGAGAATTAGAAA CATTTC-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 chitin-
ase promoter (Heang and Sassa 2012, Takakura et al. 2000), was amplified from Nipponbare genomic DNA by PCR (FchiH: 5′-CCCAACGCTTGGTATGCCTTTGCTAT-3′ and RchiK: 5′-GGGGTACCTGCCGAGGAATGCTTTCTTGCTTAT-3′), fused to the 496 bp genomic sequence of *PGL2* (Os02g0747900) amplified by PCR (FPGL2k: 5′-GGGGTACCATCGTCGAGCAGAAGGTTCGTC-3′ and RPGL2h: 5′-CGGGATCTCAGGAGCGAGGATGCTG-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 ex-
pression 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′-GGGGTACCATCGTCGAGCAGAAGGTTCGTC-3′ and RPGL2h: 5′-CGGGATCTCAGGAGCGAGGATGCTG-3′) and sub-
cloned into the *phK7* vector (Harikrishna et al. 1996) at *KpnI/BamHI* and *HindIII/KpnI* to generate an expression vector for the thioredoxin-PGL2 fusion protein (Trx-PGL2). *BU1* was amplified from lemma/palea cDNA (FBU1e: 5′-GGAATTCTATGTCGAGCCGGAGGT CGTTC-3′ and RBUIxh: 5′-CCCTCGAGGCTAGCGGAGGA GGCTGCGGA-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′-GGAATTCTATGTCGAGCCGGAGGT CGTTC-3′ and RBUIxh: 5′-CCCTCGAGGCTAGCGGAGGA GGCTGCGGA-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 ana-
yzed 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 amyllose 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., et al. 1996). 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 an 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 Il1 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
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) 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 curved lines indicate the basic region, loop region and helix region, respectively. Asterisks (*) denote the conserved His at position 9, Gliu 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). 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.
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 examined 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 (EYF) fused to PGL2 (YC-PGL2) and N-terminal half of EYFP fused to APG (APG-YN) were

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

| Line       | 1000-grain weight (g) | Grain length (mm) | Grain width (μm) | Cell length (μm) | Cell width (μm) |
|------------|------------------------|-------------------|------------------|-----------------|---------------|
| WT         | 23.2 (100%)            | 6.9 ± 0.3         | 2.8 ± 0.2        | 102.2 ± 20.6    | 45.7 ± 6.8    |
| PGL2:OX Ci1| 24.3 (104%)            | 6.9 ± 0.2**       | 2.9 ± 0.2**      | 115.6 ± 28.1*** | 44.5 ± 4.9**  |
| PGL2:OX Ci3| 22.7 (97%)             | 6.9 ± 0.2**       | 2.8 ± 0.1**      | 116.0 ± 28.4*** | –             |
| PGL2:OX Ci4| 34.9 (149%)            | 8.3 ± 0.2***      | 3.4 ± 0.2*       | 165.0 ± 33.4*** | 46.5 ± 5.0*   |
| PGL2:OX Ci5| 30.0 (128%)            | 8.2 ± 0.2***      | 3.3 ± 0.2*       | 161.6 ± 28.4*** | 47.5 ± 6.4*** |
| PGL2:OX Ci6| 32.0 (137%)            | 8.6 ± 0.4***      | 3.2 ± 0.1**      | –               | –             |

* a,b data are the average for 10 samples (±sd).
* c,d data are the average for 250 samples (±sd).
* ns, not-significant; * p < 0.05; ** p < 0.01; *** p < 0.001.

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co-infiltrated 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. 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
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, PLG1–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 indicated that of APG 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.

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-YC, co-infiltration of N-EYFP alone and APG-C-EYFP (lower). (bar = 100 μm)

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.
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