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Original Paper

Overexpression of a basic helix–loop–helix gene *Antagonist of PGL1* (*APG*) decreases grain length of rice

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Abstract Previously we reported that three basic helix–loop–helix (bHLH) protein dimers are involved in regulating grain length of rice. Two atypical bHLH proteins named Positive regulator of grain length 1 (PGL1) and PGL2 positively regulate grain length in transgenic rice when they are overexpressed in lemma/palea. Their interaction partner Antagonist of PGL1 (APG), a typical bHLH, is assumed to have opposite function. Suppression of *APG* expression level by RNAi increased grain length significantly. Here, we report further evidence that APG is a negative regulator of grain length in rice. Overexpression of *APG* resulted in short grain. Inner epidermal cell layer of lemma from *APG* overexpression lines showed reduced cell length. Taken together, the results illustrated the biological role of a typical bHLH APG as a negative regulator of rice grain length that controls cell elongation.

Key words: Basic helix–loop–helix, cell elongation, grain length, rice.

Like other cereal crops, rice yield partly depends on the grain size (Mao et al. 2010). At least four parameters are considered to contribute to the rice grain size: grain length, width, thickness and filling ability (Xing and Zhang 2010). Although these important traits are also major objectives of many breeding programs for yield improvement in rice, the genetic and molecular studies on rice grain size remain limited. Only a few genes have been characterized for grain length, width and grain filling to date (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). Among these studies, one of the research focus is lemma and palea covering the endosperm to compose a rice grain. The size of the grain have been demonstrated to be partly limited by the size of lemma and palea (Shomura et al. 2008; Song et al. 2007; Heang and Sassa 2012). Therefore, uncovering the molecular mechanism controlling lemma/palea size is expected to contribute the understanding of regulation of grain size in rice.

Basic helix–loop–helix (bHLH) protein is a large family of transcription factors in plant (Feller et al. 2011). 167 and 177 genes are predicted to encode the class of proteins in Arabidopsis and rice, respectively (Carretero-Paulet et al. 2010). The class of proteins contain approximately 60 conservative amino acids known as bHLH domain (Massari and Murre 2000). The N-terminal of bHLH domain is known as a basic region which functions as a DNA binding domain (Toledo-Ortiz et al. 2003). E-box motif (5′-CANNTG-3′) is recognized by the basic domain of the protein for binding, once Glu at position 13 and Arg at position 16 are presence (positions are referred to Toledo-Ortiz et al. 2003). When the central bases of E-box are specified to CG, the motif is called as G-box (5-CACGTG-3) to which His at position 9 of basic domain is additionally required for binding of bHLH proteins (Toledo-Ortiz et al. 2003). Because of the lack of the conserved amino acid residues, some proteins are considered to be unable to bind DNA, and grouped to atypical bHLH proteins (Li et al. 2006). HLH domain is required to form protein dimers. A typical bHLH protein retains both DNA binding and protein dimerization domains. Recent studies have revealed the involvement of the bHLH transcription factor in diverse plant growth and development processes such as, light signaling (Fairchild et al. 2000; Khanna et al. 2004), hormone signaling (Schlereth et al. 2010; Tanaka et al. 2009; Zhang et al. 2009), root development (Yi et al. 2010), fruit and flower development (Penfield et al. 2005; Szecsi et al. 2006) and drought response (Seo et al. 2010). More importantly, many of bHLH genes are reported to be involved in organ size and cell elongation (de Lucas et al. 2008; Fairchild et al. 2000; Mara et al. 2010; Varaud et al. 2011). However, involvement of bHLH protein family in controlling grain size is largely unknown.

Abbreviations: APG, Antagonist of PGL1; bHLH, basic helix–loop–helix; PGL1 and 2, Positive regulator of grain length 1 and 2.
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By using reverse genetics approaches, we have identified three bHLH genes involved in grain length and weight of rice (Heang and Sassa 2012a, 2012b). Two genes encoded atypical bHLH proteins and positively regulated the grain length and weight when overexpressed in lemma/palea of transgenic rice plants, named *Positive Regulator of Grain Length*$_1$ (*PGL1*) and *PGL2*. Their interaction partner Antagonist of PGL1 (APG), a typical bHLH, was identified, and its silencing by RNAi resulted in elongated grain length (Heang and Sassa 2012a, 2012b). Here, we report further evidence of *APG* in controlling grain length and weight by overexpression of *APG* under constitutive promoter of maize ubiquitin.

Materials and methods

Vector construction for plant transformation and phenotype observation

Genomic fragment of *APG* (*Antagonist of Pgl*$_1$) was amplified from *Oryza sativa* cv. Nipponbare by PCR with a primer pair (FAPGbam 5′-CGGGATCCATGCTACGCGGGAACGACAC-3′ and RAPGbam 5′-CGGGATCCTCACGCCTGCTTCACG GCGG-3′) and sub-cloned to pBI101-H-Ub binary vector at *Bam*HI restriction site (Figure 1A) (Yokoi et al. 1998). The binary vector construct was introduced to *Agrobacterium* strain EHA105. Plant transformation was carried out using Nipponbare calli as described (Hiei and Komari 2008). Transgenic T_0 plants were named as APG:Ox (Ox-#) lines. Ten fertile seeds from transgenics and wild types were chosen at random for measuring grain length and width with vernier calipers. Thousand seeds weight was calculated from the weight of 200 fully mature seeds.

Gene expression analysis by qPCR

Total RNA (2 μg) extracted from lemma/palea at the pre-anthesis stage using RNeasy plant mini kit (Qiagen) 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 (FAPGa 5′-GCGTCATGAACT TCACCTTCTTCTC-3′ and RAPGb 5′-GGGTGCTTCTCA GCCGGTTC-3′). The rice actin gene was used as a control (OsACT1U 5′-TCCATCTTGGCATCTCTCAG-3′ and OsACT1L 5′-GTACCCGCATCAGGCATCTG-3′). Data were collected using an ABI PRISM 7000 sequence detection system (Applied Biosystems) and analyzed according to the instruction manual.

Histological observation of lemma cell

Pre-anthesis lemma were randomly selected and subjected for cell structure observation. The inner epidermal layers were stained using 1 M Tris-HCl pH 9.0 with 0.1 mg/l of calcofluor (fluorescent brightener 28, Sigma-ALDRICH) and images were taken under a confocal microscope (Leica Microsystems,

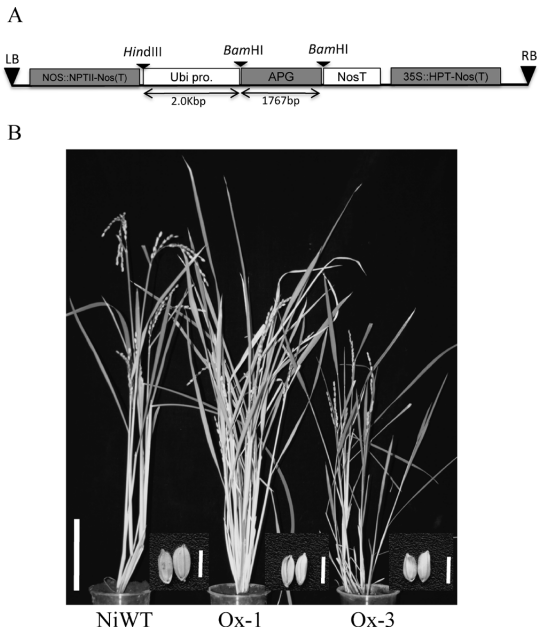

Figure 1. Overexpression of *APG* in rice. A) Structure of the expression cassette of *APG* gene under ubiquitin promoter. B) Phenotype of T_0 transgenic plants (Ox-#) compared with the wild type (WT) (bar=10 cm). Insets are grain phenotypes of wild type and transgenics (bar=5 mm).

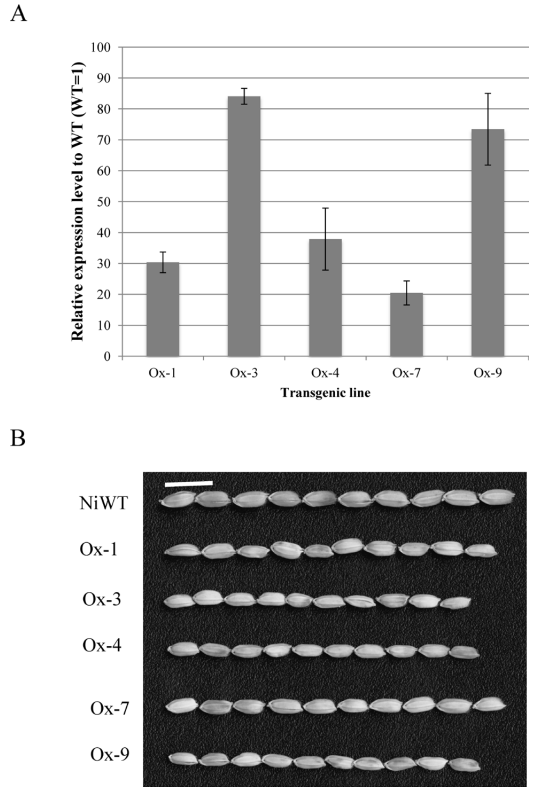

Figure 2. Overexpression of *APG* decreased grain size in rice. A) Quantitative PCR expression analysis of *APG* in the lemma/palea of T_0 plants compared with the wild type (WT=1) normalized by *OsActin*. Error bar indicates ±sd over three biological repeats. B) Grain phenotype of T_0 transgenic plants (Ox #) compared with the wild type (WT) (bar=1 cm)

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

Line	1000-grain weight (g)	Grain length[a] (mm)	Grain width[b] (mm)	Cell length[c] (μm)	Cell width[d] (μm)
WT	22.3 (100%)	6.9±0.34	2.8±0.21	102.3±20.6	43.7±6.8
Ox-1	19.7 (88%)	6.9±0.25^{ns}	2.8±0.16^{ns}	100.4±30.6***	43.4±6.0^{ns}
Ox-3	16.5 (74%)	6.4±0.15*	2.7±0.29^{ns}	90.8±35.6***	34.4±6.8***
Ox-4	17.9 (80%)	6.6±0.25^{ns}	2.7±0.15^{ns}	—	—
Ox-6	20.9 (94%)	7.0±0.29^{ns}	2.9±0.12^{ns}	—	—
Ox-7	20.6 (92%)	6.9±0.17^{ns}	2.9±0.10^{ns}	—	—
Ox-9	16.7 (75%)	6.5±0.17*	2.7±0.2^{ns}	95.8±17.0***	44.5±7.7*

a, b: data are the average of 10 samples (±sd). c, d: data are the average of 250 samples (±sd). ns, none-significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Heerbrugg, Germany). Cell length and width were measured using ImageJ software (http://rsb.info.nih.gov/ij/).

Results

Expression level of APG negatively correlates to the grain length in transgenic rice

We previously reported that suppression of *APG* by RNAi in lemma/palea increased rice grain length (Heang and Sassa 2012a). Therefore, we hypothesized that overexpression of *APG* would provide an opposite phenotype, short grain. To examine the hypothesis, we produced transgenic plants overexpressing *APG* under the control of maize ubiquitin promoter, and observed the phenotypes. The transgenic plants showed altered phenotypes in different aspects such as plant height and grain size (Figure 1B). We randomly selected five transgenic plants (Ox-1, Ox-3, Ox-4, Ox7 and Ox-9) for further analysis. qPCR analysis of APG expression level in lemma/palea revealed that Ox-3 line showed the highest expression level, ~80 fold higher than the wild type (Figure 2). The expression level of APG was negatively correlated to the grain length of transgenic T_0 plants, and the highest expression line Ox-3 produced the shortest grain length (Figure 2 and Table 1). The 1000-grain weight of transgenic lines showed up to 26% reduction probably because of shorter grains since grain width of transgenics were largely unaffected (Table 1).

Overexpression of APG decreases cell length in lemma

We previously reported that the elongated grain of APG RNAi transgenic plants are caused by enhancement of cell length in lemma/palea (Heang and Sassa 2012a). To test whether APG overexpression lines provide an opposite results, we selected three lines, Ox-1, Ox-3 and Ox-9, for inner epidermal cells examination. Confocal microscopic observation revealed that the short grain length of the transgenics is caused by decrease in cell length of lemma/palea while cell width is largely unaffected, although Ox-3 cell width were slightly narrower than wild type (Figure 3A–C). Together, our results demonstrated the biological function of *APG* as a negative regulator to cell elongation in lemma/palea.

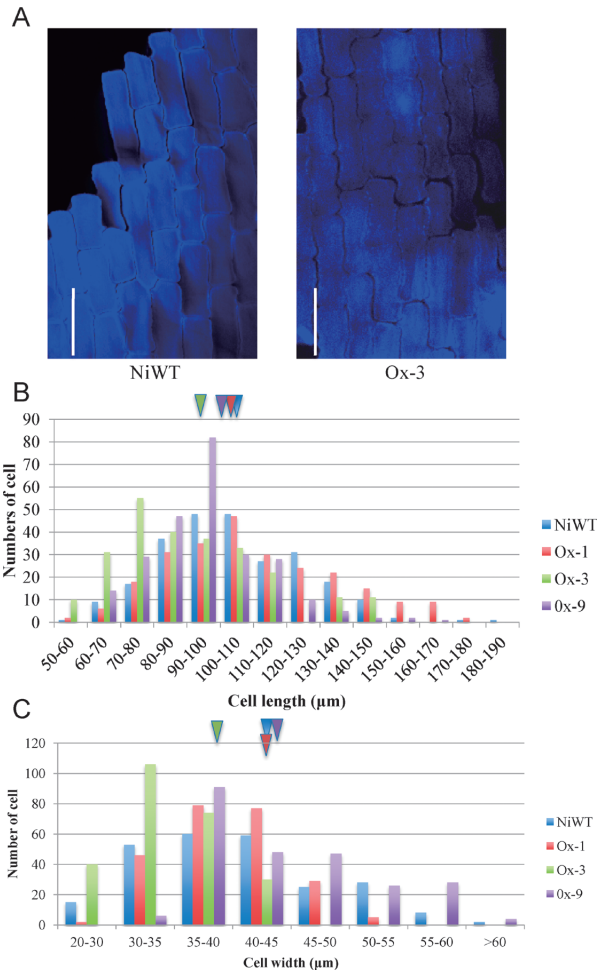

Figure 3. Inner epidermal cells observed by confocal microscopy. A) Lemma inner epidermal cells of WT and transgenic APG:OX (Ox-3), (bar=100 μm). B) Distribution of number of cells for cell lengths. C) Distribution of number of cells for cell widths; WT, wild type in cyan; T_0 transgenic APG:OX line Ox-1 in red and Ox-3 in green. Triangles represent average values of the respective lines.

Discussion

APG negatively controls rice grain length

APG encodes a typical bHLH protein that is predicted to bind to G-box motif (5′ CACGTG 3′) because it retains all conserved amino acids required for binding activity at assigned position (His at position 9, Glu 13

and Agr 16; Carretero-Paulet et al. 2010; Heang and Sassa 2012a). *In vivo* analysis using transient expression of GFP (green fluorescent protein)-APG in *Nicotiana* leaves revealed that the protein is localized in nuclei, being consistent with its expected function as a transcription factor (Heang and Sassa 2012a). A typical bHLH protein is often suppressed or abolished their DNA binding activity by atypical bHLH through protein dimerization (Massari and Murre 2000; Toledo-Ortiz et al. 2003). In our previous study, we have identified two atypical bHLH proteins, PGL1 and PGL2, that form dimers with APG *in vitro* and *in vivo*. The three protein dimers between the two atypical bHLH proteins and APG, PGL1-AGP, PGL2-APG and APG-APG dimers, are most likely to regulate rice grain length through APG as an integrator (Heang and Sassa 2012a, 2012b). Supporting with this, we have demonstrated that overexpression of *PGL1* and *PGL2* and suppression of *APG* by RNAi resulted in the grain elongation. Our present finding that overexpression of *APG* decreases grain length represents further support of the concept.

To achieve drastic decrease of grain length by overexpression of *APG*, however, high level of transgene expression was found to be required. For instance, Ox-1 and Ox-7 showed ~30 and ~20 folds overexpression, respectively, but the grain length of them were not significantly different from that of wild type (Figure 2 and Table 1). Ox-3 and Ox-9 accumulated ~70 and ~80 folds of APG transcript, respectively, while they showed ~7 and ~6% decrease of grain length, respectively. Given that greater increase in grain length has been observed by suppression of *APG* by RNAi or overexpression of *PGL1/2* (Heang and Sassa 2012a, 2012b), negative effect of *APG* on grain length may be strong enough in wild type, so that additional *APG* has subtle effect on grain length. Another possibility is that increase of *APG* transcript is not resulted in accumulation of huge amount of APG protein because of posttranslational regulation.

Genes involved in biosynthesis or signaling of phytohormones such as gibberellin and brassinosteroid (BR) have been implicated in regulation of rice grain size (Ashikari et al. 1999; Tanabe et al. 2005; Tanaka et al. 2009). Especially, alteration of BR-related genes affected grain length with leaving grain width largely unaffected, the phenotype similar to that observed by overexpression of *PGL1* and *PGL2*, and RNAi of *APG* (Heang and Sassa 2012a, 2012b; Tanabe et al. 2005; Tanaka et al. 2009). It is noteworthy that overexpression of *PGL1*, the interaction partner of *APG*, shows hypersensitivity to BR, suggesting that *PGL1* and *APG* are BR-signaling genes (Heang and Sassa 2012a). It is likely that APG and its interactors PGL1 and PGL2 are involved in regulation of rice grain size through BR-signaling pathway.

HLH/bHLH dimers have been shown to play crucial roles in controlling size of different organs and cells (Fairchild et al. 2000; Wang et al. 2009; Zhang et al. 2009). For instance, antagonistic HLH/bHLH protein pairs Ili1/OsIBH1 function to control cell length of laminar joints in rice (Zhang et al. 2009), ATBS1/AIF1 dimer controls leaf cell size through brassinosteroid signaling in *Arabidopsis* (Wang et al. 2009) and HFR1/PIFs interaction controls hypocotyls length through light signaling in *Arabidopsis* (Fairchild et al. 2000). In line with our previous finding of APG protein dimers in controlling rice grain length by elongating the cells of lemma/palea, here, our results further demonstrated the biological role of the typical bHLH protein, APG, as a negative regulator of rice grain length by controlling cell elongation. However, further investigations are required to uncover the genetic network in which APG regulates grain length, e.g., identification of target genes of APG, other suppressors of APG than PGL1 and PGL2. These findings would provide more detail of the molecular basis of bHLH protein family in controlling rice grain size and cell elongation.

Besides grain length, APG:OX lines showed other phenotypes such as short plant height, suggesting that overexpression of *APG* affected cell length in different organs. Overexpression of *PGL1* and *PGL2* also showed pleiotropic effects in addition to enhanced grain length (Heang and Sassa 2012a, 2012b). These findings suggest that genes controlled by the transcription factors *PGL1*, *PGL2* and *APG*, and genes encoding their interaction partners are expressed in different organs and involved in controlling cell length. Being consistent with this, expression of *APG*, *PGL1* and *PGL2* was observed in different organs such as root, shoot, leaf, panicle, and pistil in addition to lemma/palea (Heang and Sassa 2012a, 2012b). On the other hand, pistil length was not affected by overexpression of *PGL1*, suggesting that effect of these bHLH genes on cell length is different in respective organs. To understand the basis of the differential effect of these bHLH genes, identification and characterization of target genes regulated by these bHLH genes and interactors of them would be required.

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