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Research Article

Brassica rapa Sec14-like protein gene *BrPATL4* determines the genetic architecture of seed size and shape

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Abstract Seed size traits are controlled by multiple genes in crops and determine grain yield, quality and appearance. However, the molecular mechanisms controlling the size of plant seeds remain unclear. We performed functional analysis of *BrPATL4* encoding Sec14-like protein to determine the genetic architecture of seed size, shape and their association analyses. We used 60 T_3 transgenic rice lines to evaluate seed length, seed width and seed height as seed size traits, and the ratios of these values as seed shape traits. Pleiotropic effects on general architecture included small seed size, erect panicles, decreased grain weight, reduced plant height and increased sterility, which are common to other mutants deficient in gibberellic acid (GA) biosynthesis. To test whether *BrPATL4* overexpression is deleterious for GA signal transduction, we compared the relative expression of GA related gene and the growth rate of second leaf sheath supplied with exogenous GA_3. Overexpression of *BrPATL4* did not affect GA biosynthesis or signaling pathway, with the same response shown under GA treatment compared to the wild type. However, the causal genes for the small seed phenotype (*D1*, *SRS1*, and *SRS5*) and the erection of panicles showed significantly decreased levels in mRNA accumulation compared to the wild type. These results suggest that the overexpression of *BrPATL4* can control seed size through the suppression of those genes related to seed size regulation. Although the molecular function of *BrPATL4* is not clear for small seed and erect panicles of *BrPALT4* overexpression line, this study provides some clues about the genetic engineering of rice seed architecture.

Keywords *Brassica rapa*, *BrPATL4*, Sec14-like protein, small seed, erect panicle

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Introduction

In crop plants, high yield is an important agronomic trait as well as biotic/abiotic stress resistance and grain quality. To date, many studies were done to improve the agronomic traits using various approaches (reviewed by Takeda and Matsuoka 2008). Traditional plant breeding is very effective to make an improved crop variety without any engineered modification in the genome. Additionally, the new elite cultivars originated from the classical breeding method for introducing selected trait can be directly released to the market without further investigation on the possible effects in humans and the environment. However, because of the recessive nature of most genes, the fixation of desired traits of most of breeding lines derived from the crosses between parental lines requires a long period of time. Recently, the introduction of foreign genes originated from intra-species into the interested organisms which are agronomically important crops have been made to improve morphological characteristics and this approach can introduce new agronomic traits which was never been done before. Also, it is possible to select faster with lower cost because the introduction of foreign gene generally uses strong constitutive expression promoter or enhancers which makes the dominant gain-of-function mutant. The main purpose of

crop plants is to supply food, thus, the improvement of crop yield is widely used as a goal of researchers. There are many factors determining crop productivity such as grain yield, cultivation techniques, and environmental condition. Of these, the grain yield is mainly determined by number of panicles, number of grains per panicle, and grain weight (Takeda and Matsuoka 2008; Xing and Zhang 2010). Among them, number of panicles and number of grains per panicle are dominated by the differentiation of lateral organs during floral transition such as tillers and branches except grain weight which is determined by grain size. The increase of grain weight is proportional to length, width, and thickness of grain (Xing and Zhang 2010). To date, there have been several reports about causal genes regulating the seed elongation. Gibberellin (GA) insensitive mutant, *Dwarf1* (*d1*) has mutation in the α-subunit of the heterotrimeric G protein affecting GA signaling, leading to the reduction of cell length in longitudinal direction in seeds (Ashikari et al. 1999; Ueguchi-Tanaka et al. 2000). *DWARF11* (*D11*) encodes a novel cytochrome P450 (CYP724B1) which is involved in brassinosteroid (BR) and shows reduced seed length (Tanabe et al. 2005). *dense and erect panicle 2*/ *SMALL AND ROUND SEED 1* (*DEP2*/*SRS1*) encoding a plant specific protein without any known functional domain showed wider and shorter grain than the wild type (Abe et al. 2010; Li et al. 2010). *SRS3* and *SRS5* encode a kinesin 13 protein (Kitagawa et al. 2010) and an α-tubulin in rice, respectively (Segami et al. 2012). In contrast, the constitutive overexpression of *OsmiR397* induces the increase of grain size through the posttranscriptional regulation of *OsLAC*, while most of causal genes related in seed elongation led to the reduction of seed length (Zhang et al. 2013).

In this study, we isolated a single mutant, *Br322* bearing erect panicles and small seeds from the screening of *B. rapa* Full-length cDNA Over-eXpressor (FOX) gene hunting library (Abdula et al. 2013). *Br322* encodes a *B. rapa PATLLIN 4* (*BrPATL4*) and showed pleiotropic phenotypes including small and round seed, erect panicles, and dwarfism. Although, it has been reported that *PATL*s have functions during plant cytokinesis or viral protein movement (Peterman et al. 2004; Peiro et al. 2014), there is no report yet about its regulation of seed size. This study will provide some clues about the genetic engineering of rice seed architecture.

Materials and Methods

Plant Materials and Growth Condition

Rice variety Gopum was used as a wild type in the generation of transgenic rice. Regenerated plants were transplanted in soil (50% compost and 50% earth soil) under greenhouse condition and acclimatized for two weeks. Young leaf samples were collected for genomic DNA analysis with *BrPATL4*-specific primers. Subsequently, plants confirmed to contain the gene insert were harvested, and the T1 seeds were used in the next planting season in the field. Transgenic rice plants together with the wild type were sown and grown up to T_3 generation (Sun et al. 2011). At vegetative stage, the young leaf tissue of five plants in each line was collected for DNA extraction and analysis. All transgenic plants used in the experiments were from T_3 generation.

Isolation of gene, vector construction and plant transformation

From the large FOX rice lines we previously developed and screened for abiotic stresses tolerant line (Abdula et al. 2013). In this study, *BrPATL4* was selected showing small seed, erect panicle, and dwarfism. The *BrPATL4* full-length complementary DNA (cDNA) was ligated into the *pSB11* vector (Komari et al. 1996). The coding sequence including untranslational regions (UTR) of both 5' and 3' of *BrPATL4* gene was constructed under the control of *ubiquitin-1* promoter and NOS terminator for constitutive expression (Fig. 5A) and transformed into rice by *Agrobacterium*-mediated method with modification (Lee et al. 2011).

Southern blot analysis of transgenic plants

Southern blot analysis was conducted to determine the copy number of transgene. *HPT* probe was synthesized using PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, USA) according to the manufacturer's instructions. The forward/reverse primers used for the probe synthesis were 5'-GGATT TCGGCTCCAATGTCCTGACGGA-3' and 5'-CTTCTACA CAGCCATCGGTCCAGA-3'. Aliquots of DNA (10 μg) were digested overnight individually with *Eco*RI, *Bam*HI, *Hind*III, and *Sac*I at 37°C, fractioned by 1% agarose gel electrophoresis and transferred to a positively charged nylon membrane (Amersham Biosciences, USA). DIG-labeled probe was then added (2 μL of probe/mL of buffer) and hybridization was carried out overnight at 42°C. The DIG-labeled DNA was detected based on manufacturer's manual (DIG Nucleic Acid Detection Kit; Roche Molecular Biochemicals, USA).

qRT-PCR analysis

Total RNA was extracted from the leaf tissues of wild type and transgenic plant by using RNeasy Plant Mini Kit (QIAGEN, USA), The relative purity and concentration of RNA was estimated using NanoDrop-1000 spectrophotometer (NanoDrop

Table 1 Phenotypic traits of wild type and *Br322* mutant

	Wild type	*Br322*
Seed length (mm)	4.83±0.19	2.85±0.12**
Seed width (mm)	3.88±0.15	3.00±0.19**
1000 grain weight (g)	24.25±0.31	19.74±0.09**
Plant height (cm)	118.12±46.7	95.46±61.2**
Culm length (cm)	79.74±90.2	64.28±15.9**
Panicle length (cm)	21.16±13.3	15.58±4.3**
Leaf length (cm)	69.5±4.7	50.9±1.3**
Leaf angle (cm)	5.0±1.2	3.2±0.4*
Ligule length (cm)	1.36±0.05	0.62±0.08**

Data are average of 10 plants (±SD). Gopum used as the wild type. Asterisk indicates significant different by LSD at 5% (*) and 1% (**) relative to Gopum.

Technologies, Inc. USA), and stored at -80°C. The first-strand cDNAs were synthesized using Oligo $(dT)_{20}$ primer and SuperScript™ III Reverse Transcriptase (Invitrogene, USA). The specific sequences of primer pairs used in quantitative real-time PCR (qRT-PCR) were described in Supplementary Table 1.

GA induction in shoot elongation

To estimate the effect of GA in second leaf sheath elongation, ten rice seeds were sterilized and incubated at 30°C for 1 day and then washed 4 times with sterilized water. Seeds were allowed to imbibe water for one more day and plated on agar containing various concentrations of GA_3 under continuous light at 30°C. After six days, the lengths of the second leaf sheaths were measured.

Evaluation of agronomic traits

At maturity, ten plants for each transgenic line and wild type were evaluated for 1000-grain weight, culm length, panicle length, leaf length, leaf angle, and ligule length. Evaluation was similar to that described in Cho et al. (2007).

Statistical analysis

Data requiring statistical analysis were computed using the Statistix version 8. Significant *P*-value was further analyzed using the two-sided Dunnett's multiple comparisons or the least significant difference (LSD) with the wild type as control.

Results

Characterization of a *BrPATL4* gene

The *PATELLIN* gene expressed in transgenic rice plant, *Br322,* encodes a cDNA (LOC103840314) consisting of 1,695 bp with 67 bp of 5' UTR, 1,488 bp of coding region and 140 bp of 3' UTR. The open reading frame encodes a polypeptide of 495 amino acids with a calculated mass of 55.7 kDa. Analysis with InterProScan (http://www.ebi.ac.uk/Inter ProScan/) showed that the *BrPATL4* has 16 phospholipid binding pockets and two salt bridges in Sec14 domain for binding with phospholipid that either bind or transfer phosphatidylinositol (PtdIin), respectively (Fig. 5A). These two motifs were well conserved in both *AtPATL4* in *A. thaliana* and *BrPATL4* in *B. rapa* (Fig. 5C). Also, Sec14 lipid binding domain has three essential domains, Glu_{207}, Lys_{239}, and Gly_{266} for transferring PtdIns. These domains are well conserved in all six *AtPATL*s (*AtPATL1* to *AtPATL6*) and *BrPATL4* (Fig. 5B). This result suggests that *BrPATL4* could have PtdIns binding activity like *AtPATL4* that was previously reported (Peterman et al. 2004). Phylogenetic tree analysis using the deduced amino acid of this gene showed that it is closely related to Patellin like proteins in *B. napus* and *B. oleracea* (Fig. 5C).

BrPATL4 gene expression and generation of transgenic plants

To investigate the expression pattern of *BrPATL4* in different tissues, qRT-PCR analyses were carried out on different tissues of *Brassica rapa*. The mRNA transcript of *Brassica rapa* was detected in seven tissues (leaf, stem, root, sepal, petal, stamen, and pistil), with the highest levels seen in the pistil, but not detected or barely detected in sepal, petal, and stamen (Fig. 2). The recombinant vector carrying *BrPATL4* was constructed under the control of *Ubiquitin-1* promoter and NOS terminator and transformed into rice using *Agrobacterium*-mediated transformation method. A total of six regenerated plants were analyzed by PCR. Confirmed plants were used to determine the expression pattern of *BrPATL4* gene in transgenic rice and wild type. mRNA transcript analysis of *Ubi-1::BrPATL4* plants showed an enhanced expression of the *BrPATL4* gene

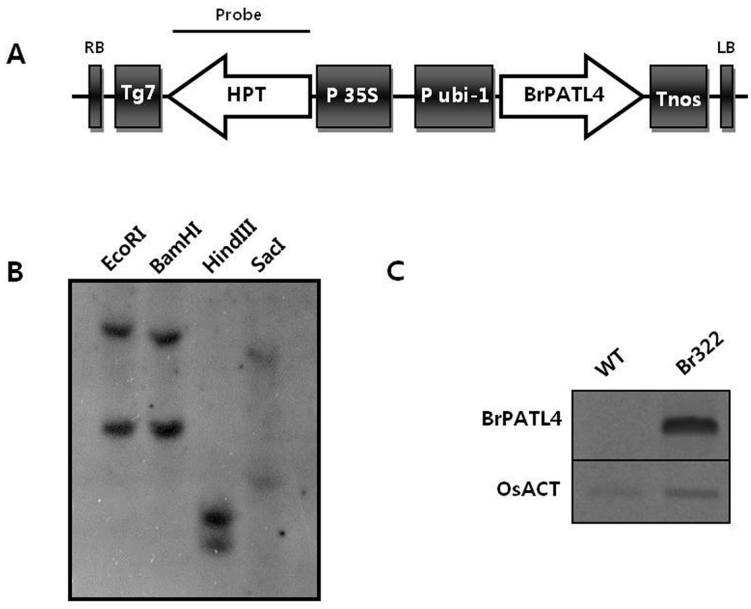

Fig. 1 Confirmation of transgene in *Br322*. (A) Schematic diagram of binary vector containing *BrPATL4*. Abbreviations are: P35S, CaMV 35S promoter; pUbi-1, maize *Ubiquitin-1* promoter; Tg7 and Tnos, polyadenylation signals from gene 7 and nopalin synthase (nos) gene; LB, left border; RB, right border. (B) Southern blot analysis. *HPT* gene was used as the probe and selected enzyme for genomic DNA digestion were indicated in the upper region of blot. (C) Relative expression of *BrPATL4* in wild type and *Br322* by qRT-PCR. *OsACT* was used as internal control

compared to that of the wild type (Fig. 1C).

Character of *Br322* mutant

In this study, we report a gain-of-function rice mutant, *Br322* that affects plant morphology especially in seed, panicle, and plant height. The *BrPATL4* gene introduced in a rice mutant, *Br322* was originally isolated from *Brassica rapa* by a FOX gene hunting library screening described as dwarf mutant with a defect in seed morphology (Fig. 3) (Abdula et al. 2013). The overall phenotypes of *Br322* are dwarf tillers, short dark green leaves, compact panicle, and small round grains (Fig. 3). To have a better understanding of the anomalous phenotypes in *Br322*, the evaluation of agronomic traits was performed at the fully developed stage in both wild type and *Br322* mutant (Table 1).

The overall lengths of all the organs in *Br322* mutant were shorter than those of wild type which were also used for generation of Fox hunting library (Fig. 3). The plant height has reduced by 80.8% in *Br322* mutant compared to the wild type. The culm length of wild type is longer than the culm length of *Br322*. Panicle and leaf blade length have also reduced by 73.6% and 73.2% in *Br322* compared to those of wild type, respectively (Table 1). When the grains of wild type and *Br322* were compared, the length and width of grains were significantly shorter than those of wild type (Fig. 3 and Table 1). The grain length and width in *Br322* were 59% and 77.3%

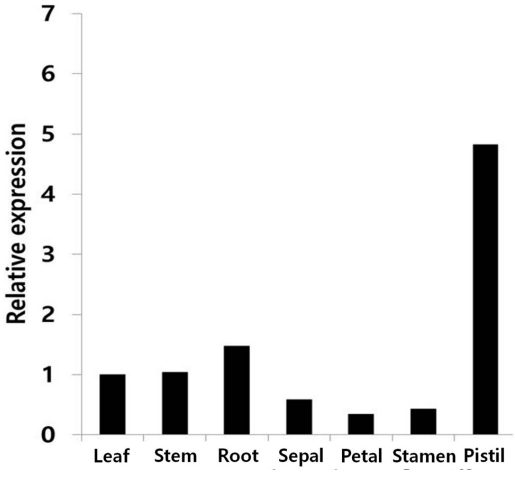

Fig. 2 Transcriptional expression pattern of *BrPATL4* as shown with q-RT PCR using different organs of *Brassica rapa*

of those of wild type, respectively (Table 1). Grain weight has also reduced in *Br322* mutant which was consistent with the reduction of length and grain width. 1,000-grain weight of wild type is higher than *Br322*.

Aside from the reduction of overall size, the leaf and panicle of *Br322* showed erect phenotypes, even after the grains were fully matured (data not shown). The average leaf angle of *Br322* was lower than that of the wild type (Fig. 3). There were no changes of morphologies in leaf except for reduction of size. We tried to find the reason for dense and erect panicle phenotypes of *Br322*. No distinct differences in

Fig. 3 Morphological phenotype of the *Br322* mutant. (A) The gross morphology of wild type (left) and *Br322* (right) during the filling stage. Gopum, which is a background variety of *Br322*, was used as the wild type plant. (B) Comparison of young florets of wild type (left) and *Br322* (right 3 plants). (C) Grain size of wild type (upper) and *Br322* (lower) (D) Panicle size and architecture of wild type (left) and *Br322* (E) Comparison of the panicle branches of wild type (left) and *Br322*. All plants were grown under field condition until ripening stage

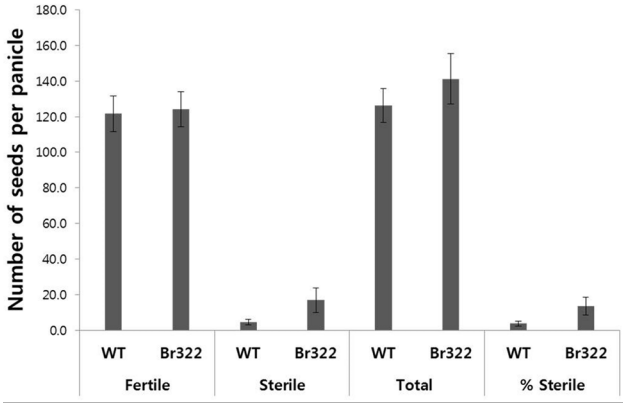

Fig. 4 Sterility of *Br322* transgenic line. Fertile and sterile grains were measured by counting the number of grains per panicle. Each value was the average of 10 panicles (±SD). Gopum was used as the wild type

the length of pedicels and the number of spikelet between wild type and *Br322* were observed. Also, the grain numbers per panicle were not different in both wild type (121.8±10) and *Br322* (124.3±9.8) (Fig. 4). However, grain sterility was higher in *Br322* than in wild type (Fig. 4). These results suggest that the dense and upright phenotypes of leaf and panicle in *Br322* were caused by the reduced length of panicle, while they have almost the same number of grains per panicle (Table 1 and Fig. 4).

Copy numbers and expression of the transgene

From the sequencing and expression studies of transgene used for the generation of *Br322* line, we found that *Br322* is a gain of function mutant of *PATELLIN* (*PATL*) of *Brassica rapa* (Fig. 1) which contains the conserved Sec14 lipid binding domain and Golgi dynamics (GOLD) domain as described previously (Allen-Baume et al. 2002; Bankaitis et al. 1990; Bankaitis et al. 1989; Peterman et al. 2004). The schematic representation of construct bearing *BrPATL4* was shown in Figure 1A. *Br322* transgenic lines showed double bands in all examined enzymes and this result showed that *Br322* has two copies of T-DNA (Fig. 1). Because *Br322* mutant was constructed using constitutive overexpression promoter, we analyzed the expression level of *BrPATL4* using RT-PCR. Total RNA was extracted from seedling stage of wild type and *Br322* and then amplified using *BrPATL4* specific primer set (Supplementary Table 1). Because *BrPATL4* is not a rice gene, no band was detected in wild type. However, strong expression of *BrPATL4* was observed in *Br322* line suggesting that the pleotropic phenotypes of *Br322* were caused by the overexpression of *Br322* (Fig. 1).

GA response of *Br322*, overexpression line of *BrPATL4*

The pleiotropic phenotypes of *Br322* are similarly shown with the mutants such as *Dwarf 1* (*d1*), *SMALL AND ROUNG SEED 1* (*SRS1*), and *SMALL AND ROUNG SEED 5* (*SRS5*) (Ashikari et al. 1999; Segami et al. 2012; Ueguchi-Tanaka et al. 2000). Of these, *d1*, which has a mutation in the α-subunit of the heterotrimeric G protein (Ueguchi-Tanaka et al. 2000) has the most similar phenotype with *Br322* especially in seed morphology and dwarfism. It has been reported that *d1* mutant was classified as GA-insensitive group leading a defect in gibberellin signal transduction (Ashikari et al. 1999). To determine whether pleiotropic phenotypes including broad, dark green leaves, compact panicles and short, round grains were caused by the defect of GA signal transduction, the length of second leaf sheath was estimated to find the reason on the pleiotropic phenotypes of *Br322*. As shown in Figure 6, the length of second sheath was not fully recovered in response to GA_3 treatment compared to wild type, the second leaf sheath of wild type and *Br322* have longer length according to the dose of GA_3 and showed similar response to exogenous GA_3 treatment. These results suggest that *Br322* still responds to GA_3 and the pleotropic phenotypes of *Br322* were not caused

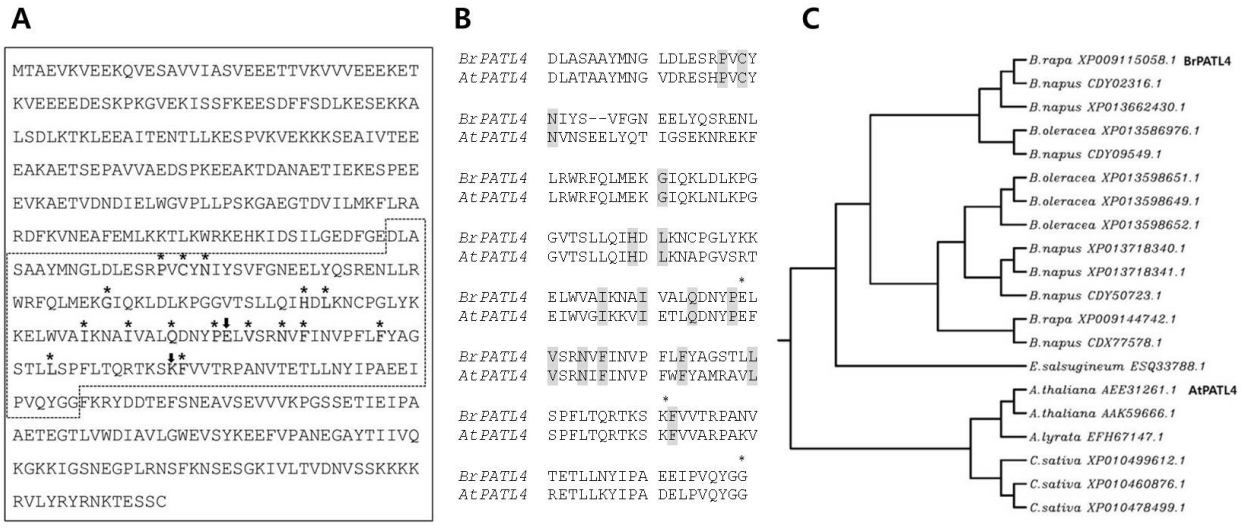

Fig. 5 Deduced amino acid sequence and phylogenetic analysis of *BrPATL4*. (A) Deduced amino acid sequence of *BrPATL4*. The dotted box indicates Sec14 domain (D219 to G376). Asterisks and arrows indicate phospholipid binding pockets and salt bridges, respectively. (B) Comparison of essential motifs of Sec14 domain. The three essential motif of Sec14 domain were marked with asterisk. The gray boxed amino acids indicate the phospholipid binding pocket of Sec14 domain. (C) Phylogenetic analysis of *BrPATL4*

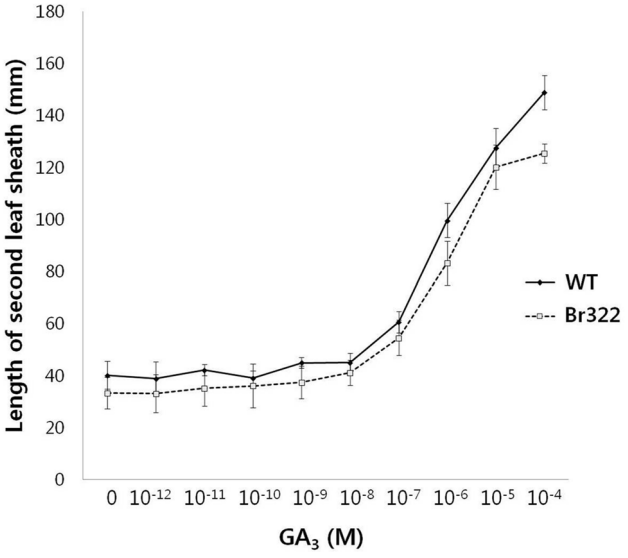

Fig. 6 Elongation of the second leaf sheath in response to GA treatment in wild type and *Br322*. The rice seeds were incubated on agar containing various concentrations of GA_3 (10^{-12} to 10^{-4} M) under continuous light at 30°C. The lengths of the second leaf sheaths were measured

by the defect of GA signal transduction (Fig. 6). Also these results were supported by the relative expression of *Ramy1A* gene (Supplementary Fig. 1) which encodes the GA induced α-amylase (Jacobsen and Beach, 1985). The accumulation of *Ramy1A* mRNA did not change in both wild type and *Br322*. In addition, we tested the phenotypic analysis under dark condition using wild type and *Br322* to find the relationship between the pleotropic phenotypes and Brassinosteroid (BR). There were no differences in growth pattern including mesocotyl and internode elongation in dark condition (Supplementary Fig. 2).

Relative expression of genes related to the small and round type seeds

To get a better understanding of the abnormal phenotypes of *Br322*, the relative expression of *D1*, *SRS1*, and *SRS5* which are causal genes related in seed morphology were examined (Ashikari et al. 1999; Segami et al. 2012; Ueguchi-Tanaka et al. 2000). *D1* (*RGA1*) encodes a hetrotrimeric G protein α-subunit which regulates cell number (Ashikari et al. 1999; Izawa et al. 2010). *SRS1* encodes a novel protein with no known functional domain which regulates cell length (Abe et al. 2010), and *SRS5* encodes α-tubulin and regulates seed cell elongation in rice (Segami et al. 2012). As most overall phenotypes of these mutants are similar to those of *Br322*, we investigated the transcriptional changes of these genes to find the reason of morphological defects in *Br322*. *D1*, *SRS1*, and *SRS5* in *Br322* showed lower level of mRNA accumulation in rice seed than in the wild type (Fig. 7). *D1* showed lower level of expression of about 30 folds compared to wild type and *SRS1* and *SRS5* showed 17 and 10 folds decreased levels compared with the wild type respectively. Although, the molecular mechanism of transcriptional regulation of these genes through the overexpression of *BrPATL4* in rice still remains to be elucidated, the pleiotropic phenotypes of *Br322* such as small and round seed and dwarfism were caused by the downregulation of these genes (Fig. 7).

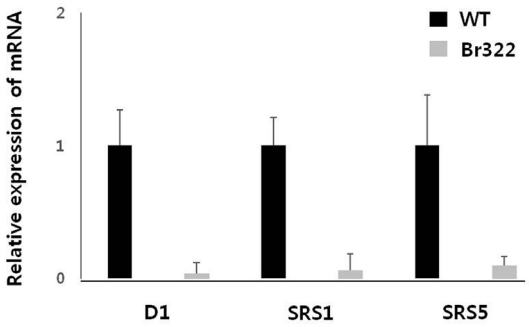

Fig. 7 Relative expression of *D1*, *SRS1*, and *SRS5* in both wild type and *Br322*. Total RNA was extracted from the seeds and analyzed by real-time PCR

Discussion

PATELLIN (*PATL*) is one of the small families in *Arabidopsis* and consists of six *PATL*s (*AtPATL1* to *AtPATL6*) (Peterman et al. 2004). *PATLs* contain a variable N-terminal, followed by a Sec14 lipid-binding domain and a Golgi dynamics (GOLD) domain. Sec14 is known as the major phosphatidylinositol/phosphatidylcholine transfer proteins (PITPs) of *S. cerevisiae* and has an important role in protein movement from the *trans*-Golgi network (Bankaitis et al. 1990). The first molecular function of *PATL*s has been reported in *Arabidopsis* by studying *AtPATL1* which is a novel cell-plate associated protein and has a role in membrane trafficking events associated with cell-plate expansion or maturation (Peterman et al. 2004). It was reported that another family members, *AtPATL3* and *AtPATL6* were able to interact with *Alfalfa mosaic virus* (*AMV*) MP for interfering viral movement, leading eventually to diminish viral cell-to-cell movement by interfering with MP targeting to plasmodemata (Peiro et al. 2014). However, there were no reports about functional analysis of *PATL*s other than these of *A. thaliana* so far.

From the screening of *B. rapa* FOX gene hunting library, we have generated 1,150 FOX rice lines (Abdula et al. 2013). Among them, we have isolated a dominant gain-of-function mutant, *Br322* which has a cDNA encoding *BrPATL4* showing pleiotropic phenotypes including small and round seed, erect panicles, and dwarfism (Fig. 3). The purpose of this study is to find the candidate gene using the genetic source of intra-species causing the improvement of agronomic trait in rice. However, other various phenotypes observed in *Br322* (Fig. 3) are not suitable to find a candidate gene from intra-species source. Seed size, number of panicles and number of seeds are known as three of the most important factors in grain yield (Takeda and Matsuoka 2008). Among the phenotypic changes in *Br322,* we focused on the study of seed size to understand its regulation in *Br322*.

It was reported that a number of causal genes confer the plant architecture especially the size regulation of seed in rice (Abe et al. 2010; Ashikari et al. 1999; Fujisawa et al. 1999; Kitagawa et al. 2010; Segami et al. 2012; Tanabe et al. 2005; Zhang et al. 2013). Most of these genes are involved in reduction of grain size except overexpression of *OsmiR397* which positively induces the grain size through the posttranscriptional regulation of *OsLAC* (Zhang et al. 2013). When compared to previously reported mutants with seed size defects (Segami et al. 2012), *Br322* showed most severe defects in seed morphology. In *Br322*, the grain length and width were 59% and 77.3% of those of wild type, respectively (Table 1). It was proposed that seed elongation in rice was mainly dominated by cell division and cell elongation which are regulated by GA and brassinosteroid (BR), respectively (Segami et al. 2012). To further investigate whether the pleiotropic phenotypes are caused by overexpression of *BrPATL4*, we analyzed the GA response and dark condition grown phenotypes of *Br322*. To confirm the GA response, the length of second leaf sheath was estimated by exogenous treatment of GA_3 in various concentrations. Results showed the same growth patterns in response to GA_3, while the length of second leaf sheath was not fully recovered by treatment of GA_3 (Fig. 6). This result suggests that *Br322* is not a mutant related in, at least, GA signal transduction. Also, to find the possibility that *Br322* is involved in BR signaling pathway, dark grown phenotypes were estimated (Supplementary Fig. 2). In dark condition, *Br322* did not show any differences at mesocotyl and internode elongation compared to those of wild type (Supplementary Fig. 2). Taken together, these results suggest that *Br322* might be independent of GA and BR signaling pathway.

In this study, we showed the possibility that the ectopic expression of *BrPATL4* could suppress the accumulation of genes, *D1*, *SRS1*, and *SRS5*, which are involved in seed elongation including regulation of cell number or cell length (Ashikari et al. 1999; Segami et al. 2012; Ueguchi-Tanaka et al. 2000). When the transcript levels of these genes in the seeds of wild type and *Br322* were compared, each gene showed significantly reduced levels in *Br322* (Fig. 7). These results explain the severe phenotype of *Br322* including shorter length of seed and other pleiotropic phenotypes. However, a mechanism behind such downregulation of these genes via ectopic expression of *Br322* remains to be investigated.

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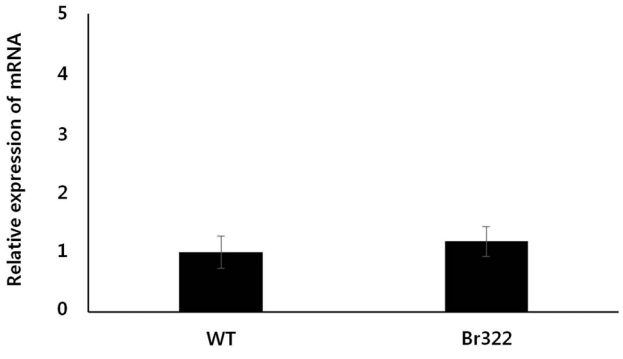
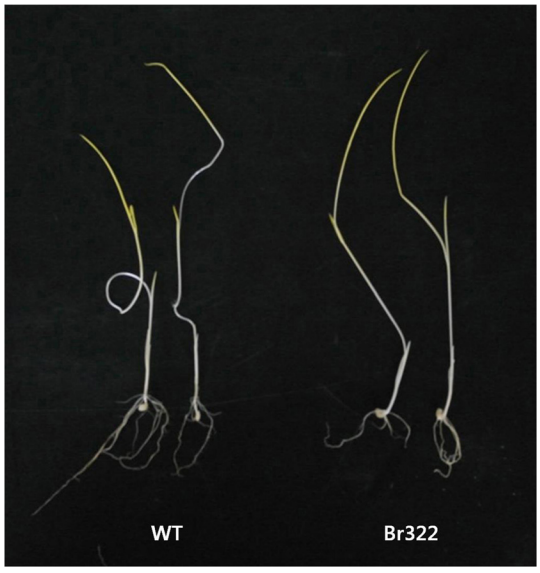
Supplementary Table 1 Primer sequences used in this study

Name	Orientation	Sequences (5' → 3')
D1	Forward	AGGTTATGGCAAGACCCAGC
D1	Reverse	CAACCTCCTGTTTCCCAGGT
SRS1	Forward	ACCTCACCATCCCCCTTATC
SRS1	Reverse	AATTAGCACGTGGGTTGGAG
SRS5	Forward	CCATTGGCAAGGAGATTGTT
SRS5	Reverse	CAGGTTGGTTTGGAACTCGT
Ramy1A	Forward	CAACGAGGCGCAGCTCAAGT
Ramy1A	Reverse	TCGTAGTTCGGCTTGCCGTC
BrPATL4	Forward	TGGAGAAGACTTTGGGGAGG
BrPATL4	Reverse	GTTCCGGGTAATTTTCCTGC
ACTIN	Forward	TGTATGCCAGTGGTCGTACC
ACTIN	Reverse	CCAGCAAGGTCGAGACGAA



Supplementary Fig. 1 Relative expression of *Ramy1A* in wild type and *Br322*. The transcript levels of each *Ramy1A* gene in the seeds were estimated by qRT-PCR. The accumulation of *Ramy1A* mRNA did not change in both wild type and *Br322*



Supplementary Fig. 2 Skotomorphogenesis of the *Br322* mutant. Morphologies of 9-day-old seedlings grown in darkness. Representative seedlings are the wild-type (left) and *Br322* mutant (right). Seedlings were germinated and grown in the dark condition for 9 days at 30°C