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Original Paper

Implications of the gene for F1–ATPase β subunit (*AtpB*) for the grain quality of rice matured in a high-temperature environment

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Abstract Rice grain filling is impaired by high-temperature stress during seed development. This event results in reduced grain yield and quality because of formation of smaller and chalky grains, and often causes problems in agriculture. High-temperature stress causes disorders in primary metabolism pathways including ATP accumulation in the developing seeds. To determine the effect on the genes for ATP biosynthesis, we created the transformant rice plants in which expression of an F1–ATPase β subunit gene (*AtpB*) was reduced by its RNAi gene, and elevated in developing seeds by its overexpression using the GlutelinB4 promoter. The transformants, which showed a high level of ATP accumulation as well as abundant *AtpB* transcript in the developing seeds, showed acquired tolerance to high-temperature stress during seed development because the ratio of perfect grains without any chalky part was significantly increased. In contrast, transformants harboring the RNAi gene showed reduction of grain quality with increase of ratio of white-core and white-berry grains when they were developed in the normal growth condition. Most of these seeds never germinated, and the RNAi transgene was poorly inherited by the progenies. These results suggest that *AtpB* plays important roles in rice seed development and that its gene expression is strongly influenced by high-temperature stress during the grain filling stage.

Key words: F1–ATPase beta subunit gene, grain filling, rice transformant, ripening under high-temperature condition.

The activity of grain production is sensitive to abiotic stresses. The quality of rice grains is reduced by applying high temperature to the developing panicles (Morita et al. 2004). The high temperature during seed development depresses the starch accumulation and causes a chalky texture in mature grains such as white-back, basal-white and milky white grains (Nagato and Ebata 1965). The chalky grain appearance was associated with the development of numerous air spaces between loosely packed starch granules and a change in light refraction (Tashiro and Wardlaw, 1991). The endosperm cells had many small amyloplasts containing small single starch granules (Zakaria et al. 2002). Rice yields decrease with a high nighttime temperature associated with global warming (Peng et al. 2004).

The marker-assisted selection has identified a QTL *qWB6*, and has allowed introduction of a high-temperature tolerant trait of Hanaechizen into a sensitive cultivar Niigatawase (Kobayashi et al. 2013). The QTL mapping has delimited a gene, named *Appearance quality of brown rice 1* (*Apq1*), within the 48-kb region of a locus containing five candidate genes, which is involved in improving grain quality without affecting other important agronomic traits (Murata et al. 2014). The japonica cultivar Koshijiwase has alleles at the four QTLs that decrease the incidence of white-back kernels in the recombinant inbred lines compared with the background cultivar Chiyonishiki with additive effects (Tabata et al. 2007).

It has been reported that several starch synthesis-related genes are downregulated by high temperature, whereas those for starch-consuming α-amylases are upregulated (Yamakawa et al. 2007). Starch deposition might be impaired by downregulation of sucrose import/degradation and starch biosynthesis, and/or up-regulation of starch degradation as well as inefficient ATP production by an inhibited cytochrome respiration chain, as indicated by the response of gene expression to high temperature (Yamakawa and Hakata 2010). In addition to the abnormal features of amyloplasts, the presence of small pits has been observed on the surface of the amyloplasts in the chalky part of high-temperature-injured grains (Iwasawa et al. 2009). It has been shown that the proportion of chalky grains decrease

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in correlation to the level of RNAi-mediated suppression of α-amylase genes, and that activation of α-amylase is a critical trigger for the grain chalkiness from high-temperature stress (Hakata et al. 2012). A heat-tolerant cultivar of rice shows a characteristic high expression of superoxide dismutase (SOD), and H_2O_2 produced by SOD possibly acts as a signal that rapidly can promote the expression of stress response proteins (Mitsui et al. 2016). A small heat shock protein OsHSP18.2 is an aging responsive protein, which functions as a molecular chaperone and possibly protect, and stabilizes the cellular proteins from irreversible damage (Kaur et al. 2015). We have reported that a shortage of ATP accumulation in immature seeds is correlated with the appearance of grain chalkiness (She et al. 2010a). Among the japonica rice cultivars, Norin-22 showed a high sensitivity to the high-temperature stress, and this allele may be inherited by the sensitive progenies, Nipponbare and Koshihikari (She et al. 2012).

We have identified a gene playing a role in accumulation of storage starch and proteins, named *FLO2* (Kawasaki et al. 1996; She et al. 2010b). The *flo2* mutant also shows a feature similar to chalky grains developed under high-temperature environment, which may be derived from lacking the expression of a heat-tolerant trait of the cultivar Kinmaze as well as the individual genes involved in production of storage starch and proteins in endosperm. In this mutant, the accumulation of ATP in immature seeds was significantly decreased, suggesting that the reduced rice grain production under high-temperature stress closely correlates with ATP shortage during seed development (She et al. 2010b). Here we report on the involvement of a gene for ATP synthesis in the appearance of chalky grains using transgenic plants in which the gene for β-subunit gene of F1ATPase (AtpB) was repressed or overexpressed.

Materials and methods

Plant materials and the growth condition

Oryza sativa Japonica cv. Nipponbare, as a wild type and the host of transgenic plants, was grown in a greenhouse. For the "high-temperature condition", we subjected the plants to high-temperature stress by transferring the plants into a growth chamber with settings of 33°C 12-h day and 28°C 12-h night temperature for 10 days from the 5th day after flowering. The mature seeds were harvested at around 40 days after flowering.

Generation of a transgenic rice plant containing a gene for expressing F1–ATPase beta subunit gene (AtpB) under the GluB4 promoter

The DNA fragment for the *AtpB* cDNA was amplified from the rice full-length cDNA clone (AK061681) by PCR using the primer set, 5′-ctatctagaatggcgactcgccgggc-3′ and 5′-ccccacgtgttatgaagccgactccttg-3′, and the fragment for the GlutelinB4 promoter (Qu and Takaiwa 2004) was amplified from rice genomic DNA, using the primer set, 5′-cccgaattctacagggttccttgcgtgaa-3′ and 5′-ccctctagaagctatttgaggatgttatt-3′, respectively. These amplified fragments were digested by restriction enzymes *Xba*I and *Pma*CI, and *Eco*RI and *Xba*I, respectively and inserted between the *Eco*RI and *Pma*CI sites of the pCAMBIA-1301 (AF234297). The resultant plasmid was used for transformation of rice cells by the Agrobacterium-mediated rice transformation procedure (Hiei et al. 1994). The regenerated rice plants (GluB4:AtpB lines) were grown in the greenhouse.

Generation of a transgenic plant containing an RNAi gene for reduced expression of AtpB

For the RNAi to the *AtpB* gene, a fragment covering the 170 bp region of the *AtpB* ORF was amplified using the cDNA by PCR using the primer set 5′-cacctactgatcttggaggacttcaa-3′ and 5′-aagctcagagatctgtcgtgacaa-3′. The amplified fragment was entered into the pANDA vector in which the ubiquitin1 promoter drove the RNAi expression (Miki and Shimamoto, 2004) by the standard Gateway technology using pENTR-D-topo kit (Invitrogen).

Analysis of the AtpB transcript

The expression level of F1-ATPase subunit genes was determined by real-time RT-PCR using the THUNDERBIRD kit (TOYOBO) with the following primer sets: For the measurement of the transcripts for genes of F1–ATPase α subunit (AK10646), β subunit (AK061681), γ subunit (AK072694), and ε subunit (AK243027), 5′-cgaagacgtgtcgaagtgaa-3′ and 5′-actctcatttgtgccccttg-3′, 5′-ctcgtggtgtccaaaaggtt-3′ and 5′-aacttcagccacatggaagg-3′, 5′-tcggaatctggtaaggttgg-3′ and 5′-gggtgagtcgatcaagcatt-3′, and 5′-gggatgacctacatcggcta-3′ and 5′-gacagtcggcttctcctgct-3′, respectively, were used. The amount of the actin1 (AK100267) transcript was measured as the internal control using primers, 5′-ccctcctgaaaggaagtacagtgt-3′ and 5′-gtccgaagaattagaagcatttcc-3′. Total RNA was prepared from immature seeds at 10 days after pollination that were developed under the normal and high-temperature conditions, and subjected to making cDNA using ReverTraAse kit (TOYOBO).

Phenotypes and genotypes of the progeny plants

White-core and other endosperm phenotypes were determined by illumination using a backlight. Phenotypes of the chalky seeds as well as perfect grains without any chalky part were judged according to the method of Hoshikawa (1993). Genotypes of the progeny plants of the transformants were determined by PCR using the genomic DNAs prepared from the leaves, and the primers, 5′-cgggtaaatagctgcgccgatggt-3′ and 5′-tgctggggcgtcggtttccactat-3′.

Analysis of the ATP content

ATP content in immature seeds was measured by the luciferase assay system coupled with the reaction from luciferin to

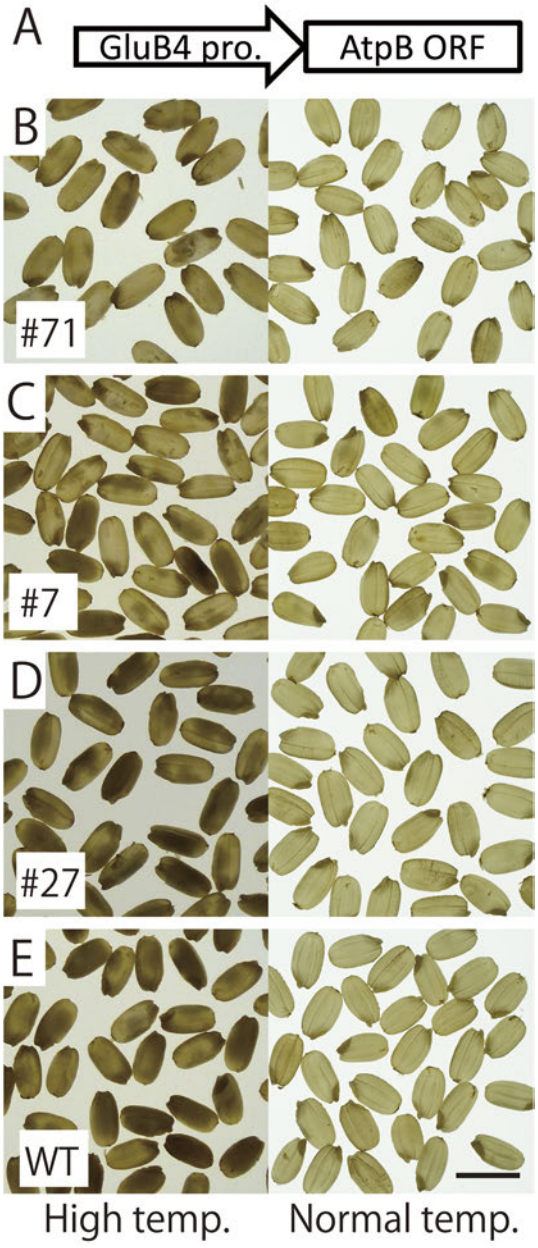

Figure 1. Shapes of the seeds developed in the high-temperature condition and in the normal condition. (A) Schematic representation of the transgene designed for overexpression of the *AtpB* gene. "GluB4 pro" and "AtpB ORF" indicate the rice *Glutelin-B4* promoter and the cDNA encoding the full-length F1–ATPase β subunit, respectively. (B-E) Features of the seeds harvested from the representative transgenic rice lines of pGluB4:AtpB #71, #7, and #27, and those from the wild-type plants (WT). The left and right panels indicate the shapes of the seeds developed in the high-temperature condition and the normal condition, respectively. The pictures were taken using transmission light, by which chalky grains were shown by muddy features. bar=5 mm.

oxyluciferin using a Lucifer250-plus kit (Kikkoman Co., Japan) (Kimmich et al. 1975), and the luminescent intensity of oxyluciferin was detected by ARVO Light luminometer (PerkinElmer, Inc., USA) (She et al. 2010a).

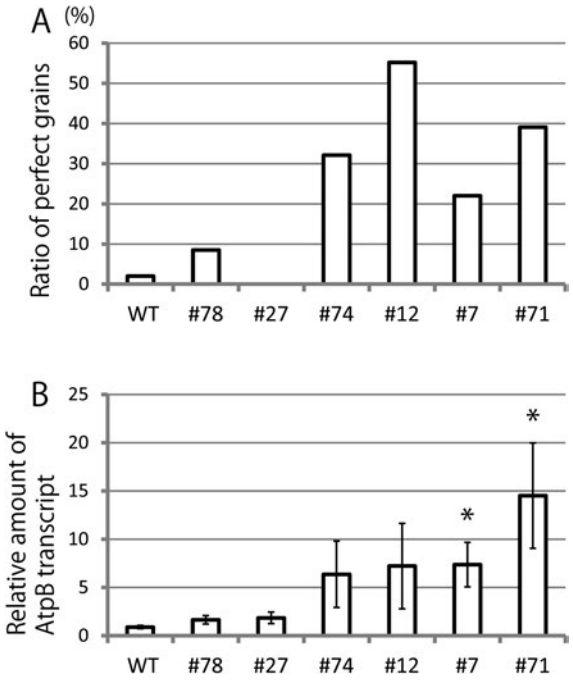

Figure 2. Correlation of the ratio of perfect grains and *AtpB* expression level. (A) Proportion of perfect grains in the harvested seeds of the pGluB4:AtpB transgenic lines that were developed in the high-temperature condition. To determine the quality of grains, 50 (WT), 47 (#78), 50 (#27), 28 (#74), 29 (#12), 50 (#7) and 23 (#71) seeds were analyzed. (B) The relative amount of the transcript of the *AtpB* gene in immature seeds harvested at 10 days after flowering is shown. Error bars represent standard errors of 3 seeds from each line. The value was normalized as the amount relative to those of the *Actin1* mRNA used as an internal control. An asterisk indicates a significant difference between the values of the transformants and WT at $p<0.05$.

Results

High-level expression of the gene for F1-ATPase β leads to an acquired tolerance to the high-temperature stresses in the transgenic rice plants

We created transgenic rice plants containing the gene for F1–ATPase β subunit (*AtpB*, acc. AK061681) driven under the GlutelinB4 promoter, and named GluB4::AtpB (Figure 1A). The GlutelinB4 promoter is strongly expressed in immature seeds during seed development (Qu and Takaiwa 2004). These transgenic plants grew normally, and produced seeds as well as the wild type did in a normal growth environment.

In the high-temperature condition, grains showed different features depending on the transgenic lines, and some lines exhibited improved quality of the grains (Figure 1B-E). The ratio of perfect grains was high in the seeds of GluB4::AtpB lines, #74, #12, #7, and #71, whereas many chalky grains were observed in the seeds of the lines, #78, #27, and the wild-type plant (Figure 2A). To determine the correlation between the expression level of the *AtpB* gene and frequency of appearance of the normal-shaped seeds, we analyzed the amount of the *AtpB* transcript in immature seeds. Real-time RT-PCR

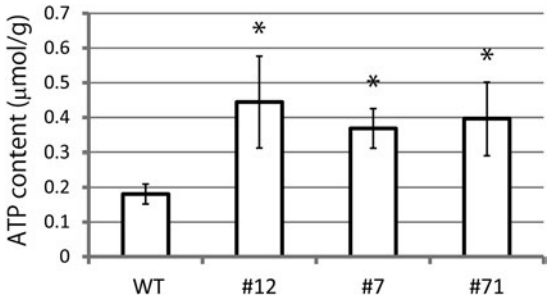

Figure 3. ATP content in the developing seeds of pGluB4:AtpB transgenic lines. Amounts of ATP were analyzed in an immature seed of the wild-type plant (WT), and transformants #12, #7, and #71, respectively, which were harvested at 10 days after flowering. Error bars represent standard errors of 4 seeds from each line. An asterisk indicates a significant difference between the values of the transformants and WT at $p<0.05$.

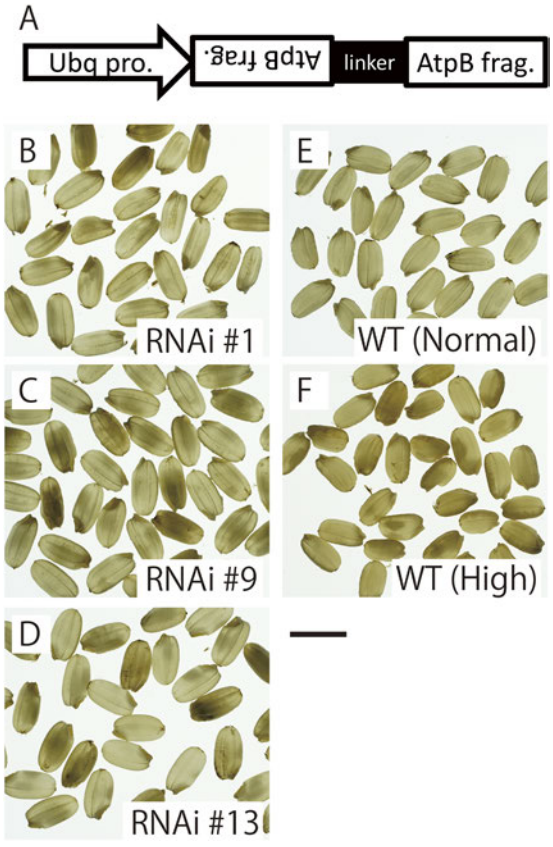

Figure 4. Shapes of the seeds harvested from RNAi-AtpB lines and wild-type plants. (A) Schematic representation of the transgene introduced into the RNAi-AtpB lines. "Ubq pro" indicates the maize *Ubiquitin1* promoter containing the first intron and 5′UTR region of the second exon. "AtpB frag". indicates the fragment of *AtpB* cDNA used as the trigger for the RNAi to *AtpB* gene. "linker" indicates the GUS linker contained in the pANDA vector (Miki and Shimamoto 2004). (B-D) RNAi #1, RNAi #9, RNAi #13, and WT (Normal) indicate features of the seeds of the RNAi-AtpB lines, #1, #9, and #13, and the wild-type plant, which were ripened in the normal growth condition. (F) Those of the wild-type plant that were developed in the high-temperature condition (WT (High)). Panels show the pictures of seeds taken using transmitting light (B-F). bar=5 mm.

detected that the *AtpB* mRNA existed more abundantly in the immature seeds of the transgenic lines #74, #12, #7, and #71 than those of #78, #27, and the wild-type plants. In the immature seeds of these lines, the amount of the *AtpB* transcript increased more than six times compared with those of the wild-type plants (Figure 2B). The transgene was not overexpressed in the lines #78 and #27, whose ratio of perfect grains was similar to that of the wild-type plants (Figure 2). These results indicated that there was some correlation between the *AtpB* gene overexpression and improved grain quality.

We analyzed the ATP content in the immature seeds of the transformants showing overexpression of the *AtpB* gene. The lines, #12, #7 and #71 showed a significantly higher level of ATP content than that of the wild type (Figure 3). The amount of ATP content in these lines was around double that of the wild type.

Decrease of AtpB gene expression results in reduction of grain quality

To clarify the effect of the *AtpB* gene expression on the quality of grains, we created 20 transgenic rice plants, in which an RNAi gene to the *AtpB* was introduced. Transgenic plants, designated RNAi-AtpB lines, were divided into different three types: plants showing normal growth (line #19), those showing slight reduction of the growth (lines #1, #9, and #13) (Figure 4), and those showing growth retardation without setting any seeds (other lines). Lines #1, #9, and #13 only set a small amount of their seeds. In addition, chalky grains, such as white-core and white-belly grains, formed a large proportion of the seeds from these transformants, even when they were developed in the normal growth condition (Figure 4 and 5). This feature resembled that of the wild-type plants when they were ripened in the high-

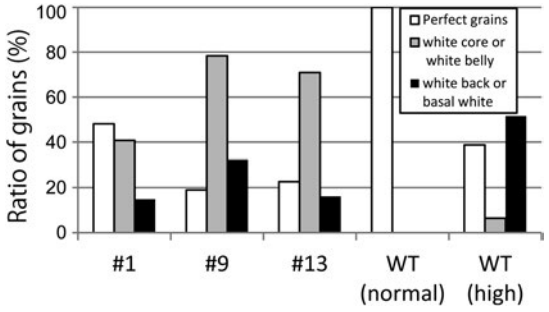

Figure 5. Proportion of the respective types of chalky grains harvested from the RNAi-AtpB lines and the wild-type plant. The ratio of perfect grains, white-core or white-belly grains, and white-back or basal-white grains are indicated by white bars, shaded bars, and filled bars, respectively. The transgenic lines #1, #9, and #13 of the RNAi-AtpB lines were analyzed and compared with the wild-type plant. WT (normal) and WT (high) indicate the results for grains of the wild-type plant that were developed in the normal condition and the high-temperature condition, respectively. The quality of grains was determined in 27 (#1), 37 (#9) 31 (#13), and 27 (WT normal), and 31 (WT high) seeds, respectively.

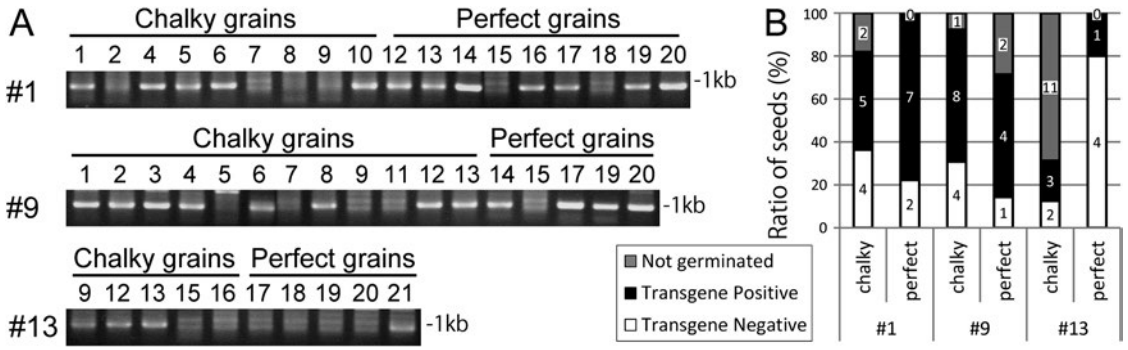

Figure 6. Inheritance of the RNAi transgene by the progeny seeds showing chalky and perfect grain features. (A) Detection of the RNAi transgenes in the leaves of the progenies germinated from the seeds of RNAi-AtpB lines #1, #9 and #13. "Chalky grains" and "Perfect grains" on the panel indicate the features of the seeds. Transgenes were detected by PCR. Numbers on the panels indicate individual progenies. The missing numbers correspond to the defective individuals that never germinated. (B) The ratio of segregants of the transgene in the chalky and perfect grains. The proportions of the individuals containing and lacking the transgene in the progenies are shown. This was analyzed independently in the chalky grains and the perfect grains. Open bars and filled bars indicate the ratio of the seeds that maintained the transgene or deleted it, respectively. The shaded bars indicate the ratio of seeds that never germinated.

temperature environment. Because most grains ripened in the high-temperature condition showed white-back and basal-white phenotypes, it seemed that there was some difference between them. These facts suggest that defective activity of RNAi-AtpB may have a lethal effect on the plant, and that lines #1, #9, and #13 retained an amount of *AtpB* beyond the minimum necessary to maintain their physiological functions.

The germination ratio of RNAi-AtpB lines, #1, #9, and #13 was 90%, 85%, and 48%, in 20, 20, and 21 seeds, respectively. To determine the inheritance of the RNAi gene by these progenies, the genotypes of these progenies were analyzed using seedlings generated from seeds of these plants. The transgene was detected in 12 individuals in 18 progenies of #1, 12 individuals in 17 progenies of #9, and 4 individuals in 10 progenies of #13, respectively (Figure 6). These results show that inheritance of the transgene was lower than that predicted by the Mendelian rule. The transgene was uniformly spread in the seedlings generated from the normal and chalky seeds.

Influence of high temperature stress to the expression of the F1-ATPase subunit genes

To determine the influence of high-temperature stress on expression of F1–ATPase subunit genes, we analyzed the alteration of their transcripts in immature seeds. A real-time RT-PCR analysis showed a significant reduction of the amount of these transcripts (Figure 7). These results suggest that the expression level of the genes for F1-ATPase subunits was uniformly decreased in the high-temperature condition.

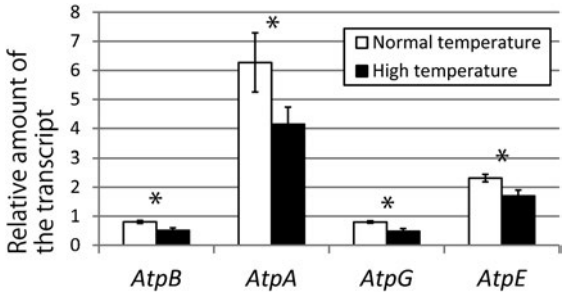

Figure 7. The relative amount of the transcript for F1–ATPase subunit genes. *AtpB*, *AtpA*, *AtpG*, and *AtpE* indicate F1–ATPase β, α, γ, and ε subunit genes, respectively, in the wild-type plant. The relative amount of these transcripts in immature seeds is shown. Open and filled bars indicate the values determined in the seeds developed in the normal growth condition and in the high-temperature condition, respectively. The values were normalized as the relative amounts to those of the Actin1 mRNA used as the internal control. Error bars represent standard errors of six spikelets. An asterisk indicates a significant difference between the values of the transformants and WT at $p<0.05$.

Discussion

We observed that the GluB4:AtpB lines, which showed overexpression of the gene for F1-ATPase β, acquired a tolerance to high-temperature stress during seed development, and resulted in improvement of the grain quality, with increased ratio of perfect grains. Conversely, the transformant containing an RNAi to the *AtpB* exhibited reduced grain quality, with production of a large proportion of chalky grains. Tolerance to the high-temperature stress occurred in concert with the elevated level of *AtpB* expression as well as ATP content in immature seeds. Consistent with our previous reports (She et al. 2010a, 2012), these results also suggest that the shortage of ATP accumulation occurred in immature seeds by high-temperature stress. This also indicates that ATP is an important factor and that a sufficient amount of ATP in immature seeds is needed to maintain good grain quality.

The appearance of chalky grains was frequently observed in the seeds of rice lines containing the RNAi

gene to *AtpB* (Figure 4 and 5). This phenomenon mimics an event in the injured seeds that were developed in the high-temperature environment, although an increase in the ratio of white-core and white-belly grains was observed in the RNAi-AtpB lines, whereas white-back or basal-white grains were abundant in seeds of the wild-type plants when they were ripened in the high-temperature condition. It is unclear why the RNAi transformants showed difference on their phenotypes although in both case significant reduction of grain quality was detected. There might be some additional factors other than ATP shortage.

Most of the transformants harboring the RNAi to *AtpB* never set some seeds. These facts suggest that *AtpB* is essential for rice plants to survive. We presume that a little amount of the activity of F1-ATPase β subunit still remained in lines #1, #9, and #13 to maintain their minimum physiological functions. Genotyping of RNAi-AtpB lines indicated that these lines were heterozygous for the transgene at a single allele. In both lines #1 and #9, the segregation ratio of the transgene was observed as approximately 2 : 1, and we obtained no homozygote plant in their progenies. These facts suggest that any homozygous progeny seed was lethal because of a strong inhibition of the *AtpB* gene expression by the RNAi genes in both alleles. It is also suggested that the defective seed formation may be dependent on the degree of inhibition by the RNAi. In line #13, the segregation ratio of the transgene was extremely low, suggesting that #13 showed a severer phenotype. Because many seeds from the RNAi-AtpB transformants lacked germination ability, it is suggested that the RNAi-AtpB gene also affected the viability of the seeds. We also observed chalky grain features in the grains in which the RNAi gene had been segregated. Because the RNAi was driven by Ubiquitin 1 promoter that was expressed ubiquitously, some maternal effect might occur on these grains during seed development.

It has been shown that the amount of ATP content is reduced in immature seeds by high-temperature stress (She et al. 2010a). High-temperature stress decreased the expression of the genes for the F1–ATPase subunits (Figure 7). Reduction of these gene expressions may cause the shortage of ATP in immature seeds by the high-temperature stress, resulting in poor grain quality. Our results suggest that the enhanced expression of *AtpB* leads to acquisition of improved grain filling ability in a high-temperature environment. A larger amount of ATP accumulation was found in the immature seeds of the transformants that showed high-level expression of the *AtpB* gene (Figure 2 and 3). These facts suggest that the amount of β subunit of F1-ATPase is a crucial factor for ATP production in developing rice seeds. The amount of the β subunit of F1-ATPase may be a bottleneck for the construction of the active ATPase. Probably, enhanced expression by the external *AtpB* gene may compensate for the shortage of the β subunit of F1–ATPase in a high-temperature environment. Our results may show a new strategy for creation of plants with an acquired tolerance by introduction of enriched *AtpB* expression, which may overcome damage from high-temperature stress.

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Author contributions

H.K. designed the work, performed the experiments, and wrote the manuscript. H.S. conducted the work and supervised the manuscript preparation. Y.A., J.N., M.Y. and K.S. performed the experiment. All authors reviewed the manuscript.

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