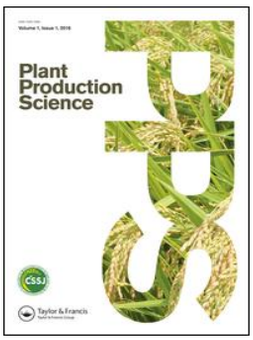

Plant Production Science

ISSN: 1343-943X (Print) 1349-1008 (Online) Journal homepage: https://www.tandfonline.com/loi/tpps20

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To cite this article: Toshiaki Mitsui, Hiromoto Yamakawa & Tohru Kobata (2016) Molecular physiological aspects of chalking mechanism in rice grains under high-temperature stress, Plant Production Science, 19:1, 22-29, DOI: 10.1080/1343943X.2015.1128112

To link to this article: https://doi.org/10.1080/1343943X.2015.1128112

PLANT PRODUCTION SCIENCE, 2016
VOL. 19, NO. 1, 22–29
http://dx.doi.org/10.1080/1343943X.2015.1128112

Molecular physiological aspects of chalking mechanism in rice grains under high-temperature stress

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ABSTRACT

High-temperature stress during grain filling hastens the growth rate of endosperm and causes grain chalkiness. Scanning microscopy of chalky areas reveals loosely packed, rounded starch granules with occasional small pits. Intensive investigation of the transcriptome, proteome, and metabolome in developing caryopses under high-temperature stress revealed the downregulation of starch synthesis enzymes and the upregulation of α-amylases. High-temperature ripening may unbalance the synthesis and degradation of starch in the developing endosperm cells. In addition to starches, storage proteins are synthesized, assembled, and stored in developing seeds. Several lines of evidence suggest that redox regulation affects seed maturation, including the accumulation of storage starches and proteins, and thus grain quality. A heat-tolerant cultivar of rice shows a characteristic high expression of superoxide dismutase (SOD). H_2O_2 produced by SOD under high-temperature stress possibly acts as a signal that rapidly can promote the expression of stress-response proteins. Herein, we will discuss the possible molecular physiology of grain chalking under high-temperature stress.

ARTICLE HISTORY
Received 17 October 2015
Revised 16 November 2015
Accepted 20 November 2015

KEYWORDS
Chalky grain; grain filling; *Oryza sativa*; starch synthesis and degradation; stress tolerance; reactive oxygen species

High temperatures in the rice growing season reduce rice quality and yield in most areas of Japan (Morita, 2008). The average temperature has increased by 0.011 °C yr^{-1} for the last century, but since 1940, the average temperature in August, the main grain-filling period, has increased by 0.013 °C yr^{-1} in Matsue, western Japan, and by 0.027 °C yr^{-1} in Niigata, central Japan (JMA, 2015). If global warming continues at projected rates, the average temperature in August in Japan is estimated to exceed 28 °C in 2050 (JMA, 2015).

After anthesis, drastic morphological changes occur in rice grains. The number of endosperm cells increases until about 10 days after anthesis, and then the cells begin to accumulate starch (Hoshikawa, 1967a). The endosperm achieves its maximum length and width by 2 weeks, and its maximum thickness by 6 weeks (Hoshikawa, 1967a, 1967b). The whole grain weight increases exponentially till around 10–20 days, and reaches its maximum by 5 weeks. High temperatures increase the grain-filling rate and shorten the grain-filling period (Egli, 1998; Sato & Inaba, 1973). The main reason for the decrease in grain yield and quality caused by high temperatures in rice (Inaba & Sato, 1976), wheat (Hawker & Jenner, 1993), and barley (Wallwork et al., 1998) has been thought to be the disappearance or disturbance of enzyme activity related to starch synthesis in the grains. However, the rate of assimilate supply to the grain plays an important role in the achievement of grain weight and quality under high temperatures during the grain-filling period (Kobata & Uemuki, 2004; Kobata et al., 2004). The grain-filling rate is higher in a high temperature sensitive cultivar such as 'Hatsuboshi,' which frequently shows milky white kernels (mostly constituting chalky grains), than in an insensitive cultivar such as 'Koshijiwase' (Kobata et al., 2011) (Figure 1). These facts suggest that the occurrence of milky white kernels caused by high-temperature stress is the result of an assimilate supply limit during the active grain-filling period, not a reduction of sink capacity. However, even if assimilate supply is increased after heading, the percentage of milky white kernels increases with an increase in temperature over 27 °C (Kobata et al., 2004). In addition, the activities of starch synthesis enzymes are affected by the supply of assimilate from source organs (Sasaki et al., 2005). Needless to say, further research will be necessary to identify and characterize the mechanisms and key factors involved in the negative response of rice grains to high temperature conditions.

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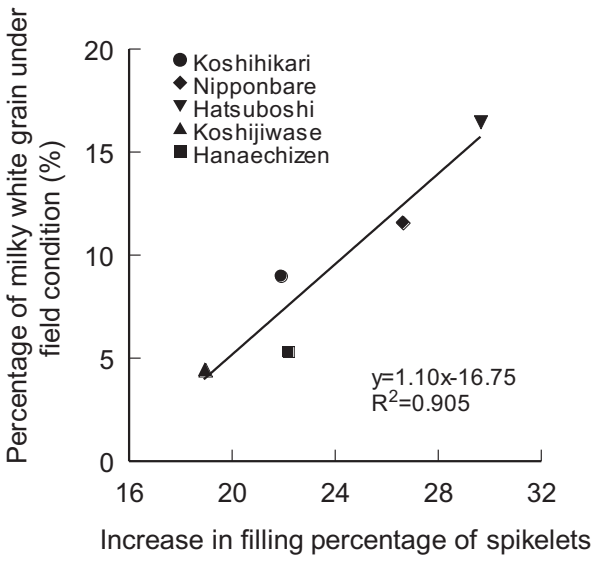

Figure 1. Increase in filling percentage of spikelets during solution culture of detached ears for one week at 28 °C and milky-white grain percentage observed under field conditions. 'Hatsuboshi' is sensitive and 'Koshijiwase' is resistant (Kobata et al., 2011).

Effect of high temperatures on metabolism in ripening grains

To reveal the metabolic activities in rice plants exposed to high temperatures, 'omics'-based approaches have been used in the past decade. Growth chamber experiments in which panicles and the rest of the plant (vegetative organs) were separately exposed to different temperatures revealed that exposure of panicles to high temperatures is sufficient to induce chalky grains, while exposure of the other parts has little effect, clearly indicating that developing caryopses are the most heat-sensitive organ in terms of grain chalkiness (Morita et al., 2004). This result prompted investigations of the effects of high temperature on the transcriptome, proteome, and metabolome of the developing caryopses.

When rice plants are exposed to temperatures above 26 °C during the early to middle phase of grain filling (from around 5 to 20 days after anthesis), severe grain chalkiness results and reductions in grain weight may occur. The chalky areas of the endosperm are composed of round starch granules with numerous air spaces between them (Zakaria et al., 2002), in clear contrast to normal translucent grains filled tightly with polygonal granules (Figure 2). This difference suggests that starch accumulation in the filling endosperm is restricted at high temperatures. Transcriptomic analyses by microarray revealed that high temperatures repress the expression of starch biosynthesis–related genes during ripening (Yamakawa et al., 2007). Among them, genes for granule-bound starch synthase (GBSSI) and starch branching enzyme (BEIIb), key enzymes for the synthesis of amylose and amylopectin, respectively, were downregulated at high temperatures, leading to low contents of amylose and long-chain-enriched amylopectin in mature grains. A proteome analysis confirmed the decrease in GBSSI protein (Lin et al., 2005), and the activity of GBSSI and BEIIb, the latter of which is labile at moderately high temperatures such as 30 °C (Ohdan et al., 2011), was reduced at elevated temperatures (Jiang et al., 2003; Umemoto & Terashima, 2002). Furthermore, high temperatures (33/28 °C, day/night) suppressed genes related to the synthesis of ADP-glucose, the substrate for starch biosynthesis: namely, ADP-glucose pyrophosphorylase (AGPS2b, AGPS1, and AGPL2), ADP-glucose translocator (BT1-2), and sucrose synthase (SuSy2); and those encoding a seed storage protein, 13-kD prolamin (Yamakawa et al., 2007). Conversely, the expression of a group of genes encoding starch-hydrolyzing α-amylase (Amy1A, Amy1C, Amy3A, Amy3D, and Amy3E), as well as their enzyme activity, were upregulated by high temperatures, suggesting that starch degradation occurs at high temperatures (Hakata et al., 2012; Yamakawa & Hakata, 2010; Yamakawa et al., 2007). Small pits observed on the surface of starch granules in the chalky endosperm indicated starch degradation consistent with the induced expression of the starch-lytic enzyme (Tsutsui et al., 2013). It was long time ago that starch-lytic α-amylase activity was detected in the starch-accumulating endosperm of ripening grains (Baun et al., 1970), implying that starch accumulation occurs through the turnover of starch by biosynthesis and degradation. These lines of evidence imply that high temperatures impair the accumulation of starch through a combination of decreased biosynthesis and increased degradation.

Metabolomic approaches also elucidated the effect of high temperature on metabolism. After exposure of developing caryopses to elevated temperatures, the contents of sugar phosphates related to glycolysis and of carboxylic acids of the tricarboxylic acid (TCA) cycle decreased, while those of amino acids increased (Yamakawa & Hakata, 2010). In accordance with the decreased expression of mitochondrial H^+-transporting ATPase genes (Yamakawa & Hakata, 2010), the ATP content in caryopses developing at high temperature was lower than that at ambient temperature (She et al., 2010). Since ATP is required for the synthesis of ADP-glucose, the precursor of starch biosynthesis, such changes in the central metabolism imply the impairment of starch biosynthesis due to the reduced ATP production. In addition, the activation of mitochondrial alternative oxidase genes and the accumulation of 4-aminobutyrate (GABA) in heat-stressed endosperm suggest that the redox status was perturbed (Yamakawa & Hakata, 2010).

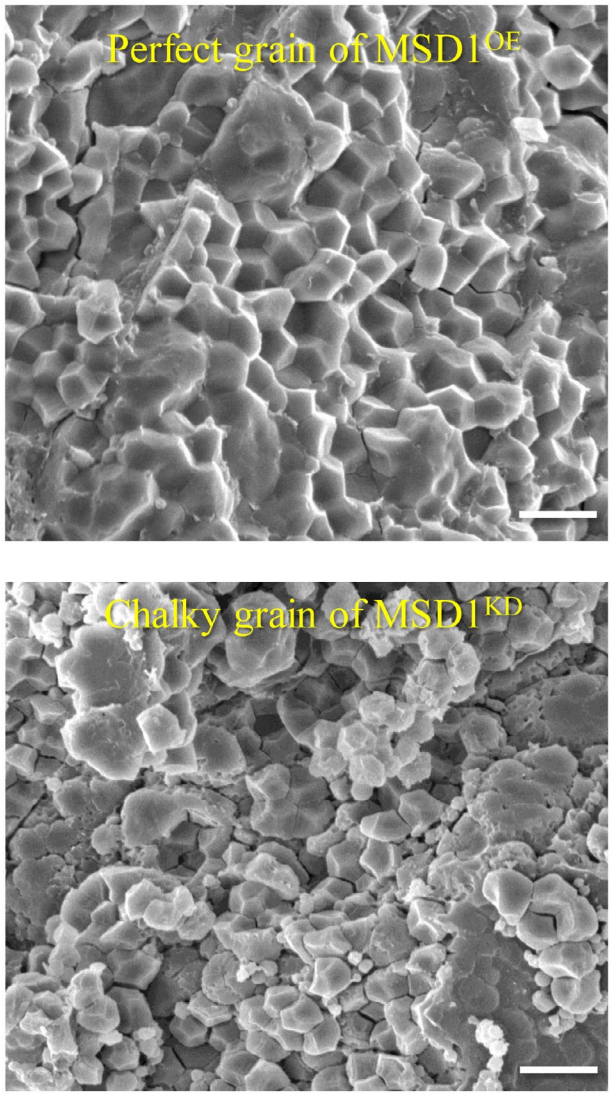

Figure 2. Scanning electron microscope images of perfect grain of MSD1 overexpressor (upper panel) and chalky grain of MSD1 knockdown (lower panel) plants. Bar, 10 µm.

Starch degradation is a significant factor in grain chalkiness

Considering the metabolic changes which occur at high temperatures, several strategies were undertaken to produce 'premium grain quality' rice plants with decreased chalkiness. Since the chalky endosperm is due to imperfectly filled starch granules, the accumulation of starch in the developing endosperm could be improved by the enhancement of starch biosynthesis or the suppression of starch degradation, when the importation of carbohydrate resources from source organs is sufficient. Starch biosynthesis is regulated by the combination of a series of enzymes, including starch synthase and ADP-glucose pyrophosphorylase, as well as by the efficiency of the transport of ADP-glucose into plastids (Nakamura 2002; Smith 1999). Given the coordinated expression of such starch biosynthesis genes in cereal endosperm, modification of the transcriptional network by the manipulation of transcriptional regulators might be feasible. For example, a deficiency of Rice Starch Regulator 1 (RSR1), an AP2/EREBP family transcription factor, led to the enhanced expression of starch biosynthesis genes in developing seeds, although it produced large grains with a chalky core (Fu & Xue, 2010).

The reduction of starch degradation was attempted by the suppression of the expression of α-amylase genes. The rice genome has at least eight α-amylase genes, five of which are upregulated by heat stress during ripening (Hakata et al., 2012). Simultaneous suppression of those genes in the developing endosperm by RNAi of their conserved sequences reduced the number of chalky grains, even at high ripening temperature (Hakata et al., 2012), and the phenotype was inherited in the following generations. Thus, grain quality could be improved by the suppression of individual enzymes. It is evident that starch degradation is a significant factor in grain chalking.

The TILLING (targeting induced local lesions in genomes) method (Colbert et al., 2001) allows high-throughput screening for chemically induced point mutations. By applying TILLING to mutant rice populations (Suzuki et al., 2008), it is possible to select mutant lines deficient in specific α-amylase genes. Since the activation of α-amylase is critical for seed germination, mutants lacking certain α-amylase genes might have impaired germinability and/or seedling vigor. However, α-amylases have been detected in the center of mature rice endosperm (Tsuyukubo et al., 2012), and detailed analysis of the localization of expression of their genes is in progress. Modifying the expression of these genes through mutations would improve resistance to chalkiness without the need for transgenic technology, that is highly acceptable to consumers.

Other factors involved in grain chalking

Factors other than starch metabolism are also involved in the regulation of grain size and starch quality (Fukuda et al., 2013; Ren et al., 2014). Both *GLUTELIN PRECURSOR MUTANT 6* (*GLUP6*) and *GLUTELIN PRECURSOR ACCUMULATION 3* (*GAP3*) are related to the accumulation and formation of protein storage organelles. GLUP6 is a guanine nucleotide exchange factor involved in intracellular transport from the Golgi apparatus to the protein storage vacuole, and glup6 mutants accumulate an abnormally large amount of proglutelin (Fukuda et al., 2013). GAP3 is involved in post-Golgi vesicular traffic for vacuolar protein sorting (Ren et al., 2014). As the mechanism of grain chalkiness caused by high-temperature stress may be highly complex, these factors also require detailed analysis.

Rice grain is sensitive to heat stress at an early stage of development (Nagata et al., 2004; Satake & Yoshida, 1978).

A comparative proteomic analysis of developing seeds of heat-tolerant 'Yukinkomai' and susceptible 'Todorokiwase' at 4 days after flowering with or without heat stress by two-dimensional polyacrylamide gel electrophoresis showed changes in the production of stress-responsive proteins, including heat shock proteins 70 and 16.9, 20S proteasome αF, abscisic acid – inducible protein R40g2, alcohol dehydrogenase, and Mn superoxide dismutase (SOD) (Shiraya et al., 2015). The Mn SOD was characteristically expressed in developing seeds of 'Yukinkomai' in both treatments, and was upregulated in 'Todorokiwase' under heat stress, and not under normal condition. The RiceXPro microarray database (http://ricexpro.dna.affrc.go.jp/) shows that the Mn SOD gene, OsMSD1, is actively expressed throughout the rice plant, particularly in the embryo and endosperm of developing seeds. MSD1 is reported to be a mitochondrial enzyme in both monocots and dicots (del Río et al., 2003; Kliebenstein et al., 1998; White & Scandalios, 1988; Wu et al., 1999). However, predictions by PSORT (http://psort.hgc.jp/form.html) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) showed that the rice MSD1 precursor's N-terminal peptide potentially acts as the signal sequence for translocation into the lumen of the endoplasmic reticulum (ER) (Sakamoto et al., 1993). In fact, the distribution of fluorescent MSD1 coincided with chloroplast autofluorescence. In addition, the fluorescence co-localized with fluorescently labeled trans-Golgi vesicles in onion cells. Thus, MSD1 is localized to multiple organelles, including plastids and Golgi apparatus (Shiraya et al., 2015).

Rice transformed with yeast mitochondrial Mn SOD fused to the transit peptide of glutamine synthase gained resistance to salt stress (Tanaka et al., 1999). In addition, rice transformed with pea mitochondrial Mn SOD fused to the transit peptide of pea Cu/Zn SOD under the control of an oxidative stress – inducible promoter was more resistant to oxidative stress (Wang et al., 2005). To test the possible stress-adapting function of MSD1, transgenic overexpressor (OE) plants with the maize *Ubiquitin-1* promoter fused to MSD1 ($MSD1^{OE}$) were generated and characterized. The results showed that hydrogen peroxide (H_2O_2) increased in the developing seeds and young seedlings of $MSD1^{OE}$ plants. Following incubation at normal or high temperatures after heading, the grain quality of $MSD1^{OE}$ was significantly greater than in wild type under heat stress. Moreover, two transgenic knockdown lines transformed with the promoter of *Wx* fused to MSD1 RNAi, designated Nipponbare $MSD1^{KD}$ and Yukinkomai $MSD1^{KD}$, were generated and grown under high temperature conditions. The expression of MSD1 mRNA in developing seeds of both lines was markedly decreased and the proportion of perfect grains was also reduced significantly (Shiraya et al., 2015). It is thus conceivable that, the constitutive high expression of MSD1 is involved in maintaining the quality of rice grains under high-temperature stress during ripening (Figure 2). H_2O_2 is one of the most abundant reactive oxygen species (ROS) in aerobic organisms, including plants, being a highly reactive and very toxic ROS in cells. However, in eukaryotes, H_2O_2 also has an important role as a signaling molecule in the regulation of a variety of biological processes and can activate the MAPK cascade (Apel & Hirt, 2004; Neill et al., 2002). Several studies have suggested that H_2O_2 can induce ascorbate peroxidase in embryos of germinating rice (Morita et al., 1999), in *Arabidopsis* (Karpinski et al., 1999), and in tobacco leaves (Gupta et al., 1993). Co-expression of SOD and ascorbate peroxidase improved tolerance to drought and salt stresses in tobacco and plums, respectively (Diaz-Vivancos et al., 2013; Faize et al., 2011). Furthermore, heat stress-induced H_2O_2 was involved in the early stage of activation of heat shock factor in *Arabidopsis* cell culture (Volkov et al., 2006). In rice leaves, H_2O_2 treatment induced the production of a chloroplastic small heat shock protein (Lee et al., 2000). Thus, H_2O_2 formed by MSD1 would be a key factor in the heat tolerance of $MSD1^{OE}$. Our preliminary results showed that H_2O_2 priming treatment during the heading period increased the ratio of perfect grains under heat stress during grain filling, suggesting that the timely enhancement of H_2O_2 level is important for improving high-temperature tolerance during ripening (Kaneko et al. unpublished data).

Perspective

As mentioned above, the chalkiness formation in rice deteriorates with elevation of α-amylase function in ripening seeds at high temperatures, and the decrease in starch biosynthesis ability at high temperatures alters the molecular structure and granule formation of rice (Inouchi et al., 2000; Ohdan et al., 2011; Tanaka et al., 2004; Umemoto & Terashima, 2002). However, grain chalkiness occurred at temperatures at which there were no significant differences in the chain-length distribution of starch (Tsutsui et al., 2013). Microscopic observation showed that chalking occurs at the belly, back, base, or center parts of the grains. Although highly effective quantitative trait loci (qWB6, qWB9) associated with the occurrence of white–back kernels have been identified (Kobayashi et al., 2013), the precise mechanism controlling the localization of chalking remains unclear. The imbalance of starch biosynthesis and degradation might be generated by the spatial expression of the genes responsible.

In addition to starches, storage proteins also are synthesized, assembled, and stored in developing seeds. Some types of proteins, such as glutelins, are synthesized in the ER and transported via the Golgi apparatus to the protein storage vacuoles (Ren et al., 2014; Washida et al., 2012).

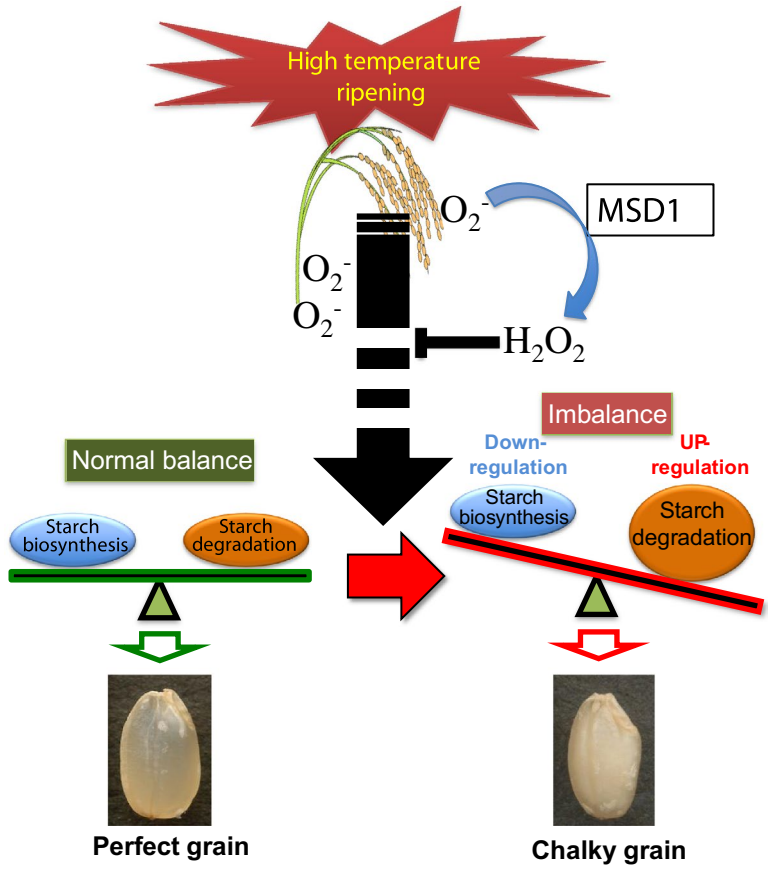

Figure 3. A hypothetical model for grain chalking under high-temperature stress.

The production of H_2O_2 from O_2 resulting from the maturation of glutelins in the endomembrane system (Onda et al., 2009) strongly suggests the existence of a mechanism that controls ROS in the compartment. Moreover, a comparative shotgun proteomic analysis revealed the upregulation of proteins involved in starch accumulation and downregulation of ER proteins (protein disulfide isomerase, PDIL 2-3; and a molecular chaperone, BiP) in the chalky tissue of a notched-belly mutant regardless of the environmental stress used (Lin et al., 2014). Thus, redox regulation may affect seed maturation, including the accumulation of both starch and storage proteins, and thereby grain quality (Onda & Kawagoe, 2011; Onda et al., 2011). In the proposed model of grain chalking (Figure 3), high-temperature ripening can cause an imbalance in the synthesis and degradation of starch in the endosperm tissue cells in rice plants, interfere with starch accumulation and granule formation, and could be considered the main cause for chalkiness formation (Figure 3). H_2O_2 produced by MSD1 under heat stress may act as a molecular signal at high-temperature, and this rapid induction can promote stress responsive proteins, which redress the imbalance. Therefore, it is highly conceivable that the molecular physiology of rice chalkiness involves a variety of factors with complex interrelations. Although the details are still largely unknown, the problem of rice chalkiness is due to malformation of the starch granule structure.

Abbreviation

H_2O_2 hydrogen peroxide;

MSD1 Mn superoxide dismutase 1;

SOD superoxide dismutase;

TCA tricarboxylic acid;

GABA 4-aminobutyrate;

RSR1 Rice Starch Regulator 1;

TILLING targeting induced local lesions in genomes;

GLUP6 Glutelin Precursor mutant 6;

GAP3 Glutelin Precursor Accumulation 3

Acknowledgment

We thank Dr. Marouane Baslam (Niigata University) for his critical reading of the manuscript and helpful comments.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was partially supported by Grants-in-Aid for Scientific Research (A) [15H02486D], (B) [15H04444D], (C) [15K07279] from the Ministry of Education, Culture, Sports, Science and Technology of Japan to T. M., T. K. and H.Y., respectively; a grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan [Genomics-based Technology for Agricultural Improvement, IVG3001] to H.Y.

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