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Golgi/plastid-type manganese superoxide dismutase involved in heat-stress tolerance during grain filling of rice

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Summary

Superoxide dismutase (SOD) is widely assumed to play a role in the detoxification of reactive oxygen species caused by environmental stresses. We found a characteristic expression of manganese SOD 1 (*MSD1*) in a heat-stress-tolerant cultivar of rice (*Oryza sativa*). The deduced amino acid sequence contains a signal sequence and an *N*-glycosylation site. Confocal imaging analysis of rice and onion cells transiently expressing *MSD1-YFP* showed MSD1-YFP in the Golgi apparatus and plastids, indicating that MSD1 is a unique Golgi/plastid-type SOD. To evaluate the involvement of MSD1 in heat-stress tolerance, we generated transgenic rice plants with either constitutive high expression or suppression of *MSD1*. The grain quality of rice with constitutive high expression of *MSD1* grown at 33/28 °C, 12/12 h, was significantly better than that of the wild type. In contrast, *MSD1*-knock-down rice was markedly susceptible to heat stress. Quantitative shotgun proteomic analysis indicated that the overexpression of *MSD1* up-regulated reactive oxygen scavenging, chaperone and quality control systems in rice grains under heat stress. We propose that the Golgi/plastid MSD1 plays an important role in adaptation to heat stress.

Introduction

Impairment of rice (*Oryza sativa* L.) grain filling under global warming is a major threat facing Asian countries. Daily mean temperatures above 26 °C during the early ripening period of *japonica* rice compromises yields through decreases in grain size and quality (Morita *et al.*, 2004; Peng *et al.*, 2004; Tashiro and Wardlaw, 1991). Perfect grains are fully rounded, transparent and filled with normal starch granules. A chalky appearance reduces commercial value because of increased cracking during polishing (Fitzgerald *et al.*, 2009) and poorer cooking quality (Singh *et al.*, 2003; Tsutsui *et al.*, 2013). Scanning microscope images of chalky areas of grain ripened under heat stress show loosely packed rounded starch granules (Evers and Juliano, 1976; Ishimaru *et al.*, 2009; Tashiro and Wardlaw, 1991). The air spaces among these abnormal starch granules refract light, making the grain appear white. Occasional small pits on the surface of the starch granules suggest attack by starch-degrading enzymes (Iwasawa *et al.*, 2009; Zakaria *et al.*, 2002); the suppression of α-amylase genes improved the quality of rice grains ripened under heat stress (Hakata *et al.*, 2012). It is widely recognized that heat stress lowers the activity of starch synthesis enzymes (Jiang *et al.*, 2003; Umemoto and Terashima, 2002; Yamakawa *et al.*, 2007). Mutants deficient in genes for starch synthesis enzymes exhibited dramatic changes in grain phenotype, including shape and chalkiness (Fujita *et al.*, 2011; Kubo *et al.*, 1999; Nishi *et al.*, 2001; Tanaka *et al.*, 2004). Furthermore, novel factors such as FLOURY ENDOSPERM2 (FLO2), GLUTELIN PRECURSOR MUTANT6 (GLUP6) and GLUTELIN PRECURSOR ACCUMULATION3 (GAP3) have been shown to be involved in the regulation of rice grain size and starch quality (Fukuda *et al.*, 2013; Ren *et al.*, 2014; She *et al.*, 2010). FLO2 contains a tetratricopeptide repeat motif that interacts with late-embryogenesis and basic helix-loop-helix proteins (She *et al.*, 2010). GLUP6 is a guanine nucleotide exchange factor involved in intracellular transport from the Golgi apparatus to the protein storage vacuole, and the *glup6* mutant accumulates 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). In addition, redox regulation may affect seed maturation and quality (Onda and Kawagoe, 2011; Onda *et al.*, 2011). Thus, the mechanism of grain chalkiness caused by heat stress may be highly complex.

Abiotic stresses, including high light, drought, salinity and heat, lead to the accumulation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2; Apel and Hirt, 2004). ROS damage multiple cellular components, interfering with lipid peroxidation (Niki *et al.*, 2005), breaking DNA strands (Brawn and Fridovich, 1981) and inactivating enzymes (Fucci *et al.*, 1983). On the other hand, they also serve as signalling molecules, regulating processes including pathogen defence, programmed cell death and stomatal behaviour (Apel and Hirt, 2004). Although ROS are produced predominantly and continuously in chloroplasts, mitochondria and peroxisomes, the production and scavenging of ROS must be strictly controlled in the absence of stress. Enzymatic ROS

scavenging mechanisms involve superoxide dismutase (SOD), ascorbate peroxidase, glutathione peroxidase and catalase (Apel and Hirt, 2004).

Superoxide dismutase catalyses the conversion of O_2^- to H_2O_2; it is responsible primarily for defence against oxidative stress. There are three classes of SODs categorized by their metal cofactor: Fe SOD, Mn SOD and Cu/Zn SOD (Fridovich, 1975). Plant SODs have different subcellular localizations. Typically, Mn SOD is localized to the mitochondria, Fe SOD to the plastids and Cu/Zn SOD to the plastids and cytosol (Bowler *et al.*, 1992; Kliebenstein *et al.*, 1998). Peroxisomal and extracellular Cu/Zn SODs also exist (Bueno *et al.*, 1995; Streller and Wingsle, 1994). Numerous attempts have been made to enhance stress tolerance in plants by modifying the production of SOD enzymes. Ectopic production of cytosolic Cu/Zn SOD improved stress tolerance in tobacco (Faize *et al.*, 2011), potato (Perl *et al.*, 1993), sugar beet (Tertivanidis *et al.*, 2004) and plum (Diaz-Vivancos *et al.*, 2013). Overproduction of chloroplastic Cu/Zn SOD, Fe SOD and Mn SOD (fused to a chloroplast transit peptide) also increased stress resistance in tobacco (Badawi *et al.*, 2004; van Camp *et al.*, 1994, 1996; Sen Gupta *et al.*, 1993; Slooten *et al.*, 1995), potato (Perl *et al.*, 1993), sugar beet (Tertivanidis *et al.*, 2004), cotton (Payton *et al.*, 2001) and alfalfa (McKersie *et al.*, 2000). Transgenic rice overproducing cytosolic Cu/Zn SOD from mangrove (*Avicennia marina*) tolerated drought stress better than untransformed plants (Prashanth *et al.*, 2008). Rice transformed with a yeast mitochondrial Mn SOD fused to the transit peptide of glutamine synthase conferred resistance to salt stress (Tanaka *et al.*, 1999). Furthermore, rice transformed with pea (*Pisum sativum*) 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 induced by methyl viologen or polyethylene glycol (Wang *et al.*, 2005).

We have been searching for candidate genes involved in heat-stress tolerance during seed development to improve the formation of normal rice grains under a warming climate. In proteomic analysis, we detected a characteristic expression behaviour of Mn SOD in developing seeds of the heat-resistant cultivar Yukinkomai. This Mn SOD exhibited a unique subcellular localization that has never previously been described in the literature. Here, we report that control of the Golgi/plastid-type Mn SOD1 (MSD1) expression regulates tolerance to heat stress during grain filling of rice.

Results

Identification of Golgi/plastid-type Mn SOD (MSD1)

We examined the heat susceptibilities of three rice cultivars, Yukinkomai, Yukinosei and Todorokiwase, during seed development from 2004 to 2008. The plants were grown in paddy fields with irrigation water at either ambient temperature or 35 °C during the heading, ripening and maturity stages. The daily mean temperature at around the panicles in the warm-water field was 1.4–1.9 °C higher than that in the ambient-water field (25.4 °C). The percentage of damaged grains in Yukinkomai was about 22% in both treatments (Figure 1), indicating that Yukinkomai is tolerant to high temperatures during development. In contrast, that of Todorokiwase increased from 35% to 44%. Yukinosei was intermediate (Figure 1). To search for genes involved in the heat tolerance of Yukinkomai, we used a proteomic approach. As rice is sensitive to heat stress at an early stage of seed development (Nagata *et al.*, 2004; Satake and Yoshida, 1978), we separated grain proteins of Yukinkomai, Yukinosei and Todorokiwase at 4 days after flowering (DAF) by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The separation profiles showed changes in the production of stress-responsive proteins, including heat shock proteins 70 (HSP70) and 16.9 (HSP16.9), 20S proteasome αF, ABA-inducible protein (R40g2), alcohol dehydrogenase (ADH) and MSD1 (Figure 2). In the heat-tolerant Yukinkomai, 20S proteasome αF, ADH and HSP16.9 were up-regulated and R40g2 were down-regulated under heat stress (Figure 2a,b). In the susceptible Todorokiwase, in contrast, HSP70, HSP16.9 and MSD1 were up-regulated (Figure 2e,f). Those in Yukinosei were intermediate (Figure 2c,d). We focused on MSD1, which was characteristically and highly expressed in developing seeds of Yukinkomai in both treatments (Figure 2a,b).

The RiceXPro public microarray database (http://ricexpro.dna.affrc.go.jp/) shows that the *MSD1* gene (*OsMSD1*) is actively expressed throughout the rice plant, particularly in the embryo and endosperm of developing seeds. Our gel-based proteomic analysis of developing seeds supports the view that MSD1 is a major constituent in the seed proteome (Figure 2a,b). *OsMSD1* is located in the centre of chromosome 5 (Figure 3a). The cDNA is 901 bp in length, encoding 231 amino acid residues that form a 24.9-kDa precursor protein (Figure 3b). MSD1 is mitochondrial enzyme in both monocots and dicots (Kliebenstein *et al.*, 1998; del Río *et al.*, 2003; White and Scandalios, 1988; Wu *et al.*, 1999). Analyses by the PSORT algorithm (http://psort.hgc.jp/form.html) predicted an N-terminal mitochondrion-targeting sequence in the precursor proteins of MSD1 of *Arabidopsis*, maize, wheat and pea (Figure 3b). Indeed, pea MSD1 is localized chiefly in mitochondria (del Río *et al.*, 2003). However, the prediction by PSORT and signalP (http://www.cbs.dtu.dk/services/SignalP/) showed that the rice MSD1 precursor's N-terminal sequence potentially acts as signal to the endoplasmic reticulum (ER; Figure 3b; Sakamoto *et al.*, 1993).

To determine the subcellular localization of rice MSD1, we analysed the transient expression of *OsMSD1* fused with a gene for yellow fluorescent protein (*YFP*) in rice and onion epidermal cells, using particle bombardment. In rice cells, confocal laser scanning microscopy showed that the distribution of MSD1-YFP

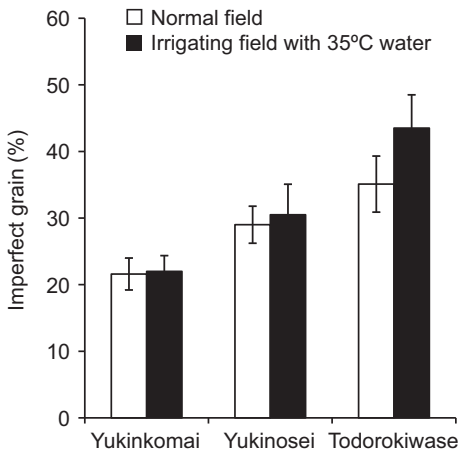

Figure 1 Proportions of imperfect grains of rice cultivars Yukinkomai, Yukinosei and Todorokiwase irrigated with water at ambient temperature (□) or 35 °C (■) from heading to maturity.

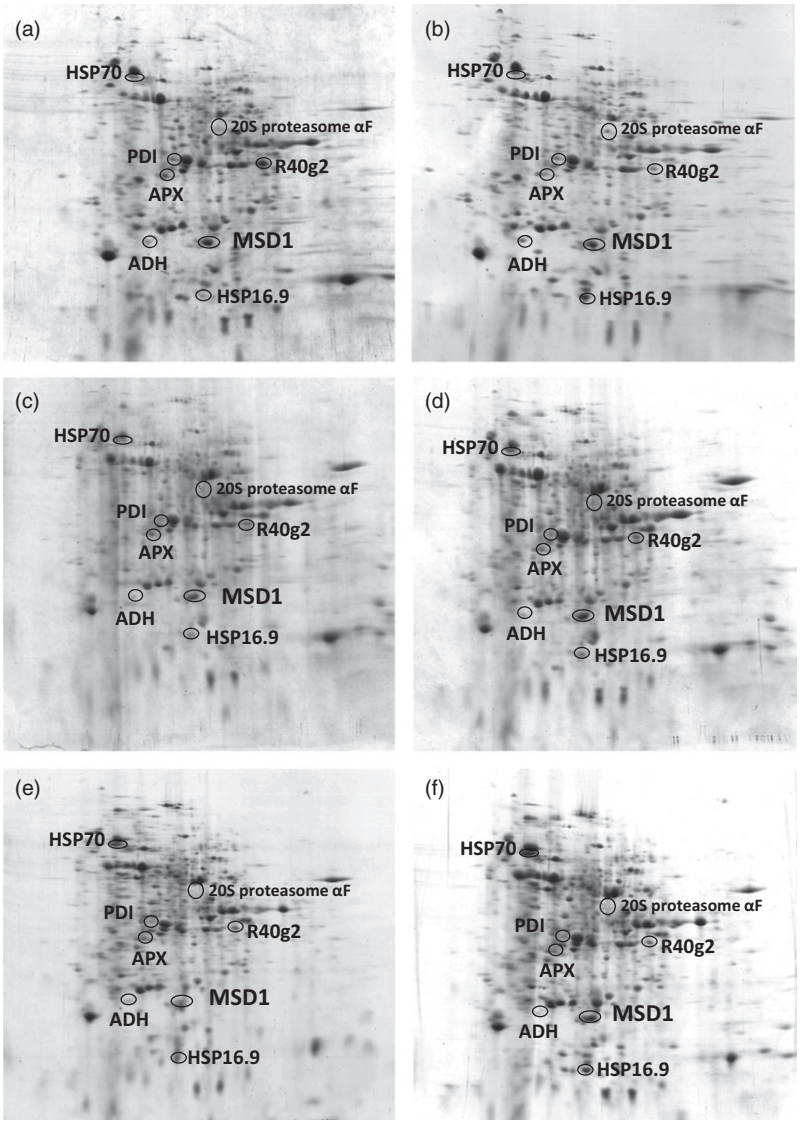

Figure 2 2D-PAGE separation profiles of proteins extracted from 4 days after flowering (DAF) grains of (a, b) Yukinkomai, (c, d) Yukinosei and (e, f) Todorokiwase grown under (a, c, e) normal or (b, d, f) heat stress treatment. Protein extracts were separated by isoelectric focusing followed by SDS-PAGE. Proteins identified included heat shock proteins 70 (HSP70; 66 kDa, pI 4.8), 20S proteasome αF (46 kDa, pI 5.7), protein disulphide isomerase (PDI; 40 kDa, pI 5.3), ABA-inducible protein (R40g2; 38 kDa, pI 6.8), ascorbate peroxidase (APX; 36 kDa, pI 5.2), alcohol dehydrogenase (ADH; 28 kDa, pI 5.0), Mn superoxide dismutase 1 (MSD1; 28 kDa, pI 5.6), HSP16.9 (18 kDa, pI 5.6) were identified in the 2D-gels.

coincided well with the autofluorescence of chloroplasts (Figure 4). In onion cells, MSD1-YFP revealed numerous particulate structures (Figure 5a,c). When *MSD1-YFP* was cobombarded with a sequence encoding a trans-Golgi marker (sialyltransferase, ST) fused at the transmembrane domain to monomeric red fluorescent protein (*ST-mRFP*) into onion cells, MSD1-YFP fluorescence overlapped well with the ST-mRFP-labelled trans-Golgi vesicles (Figure 5a). The GTPases ARF1 and SAR1 are essential for membrane trafficking between the ER and the Golgi apparatus in higher plant cells. Expression of dominant-negative ARF1 or constitutively active SAR1 mutant proteins, which are defective in GTPase cycling, prevents the ER-to-Golgi traffic (Takeuchi *et al.*, 2000, 2002). Golgi-resident proteins and secretory and vacuolar proteins are therefore retained in the ER with such mutants (Takeuchi *et al.*, 2000, 2002). We examined the effects of dominant-negative and constitutive-active mutants of ARF1 and SAR1 on the subcellular distribution of MSD1-YFP. We simultaneously expressed MSD1-YFP, the trans-Golgi marker ST-mRFP, and either AtARF1(T31N), AtARF1(Q71L), or AtSAR1(H74L) in onion cells. Both MSD1-YFP- and ST-mRFP-labelled vesicles were rearranged and remerged into tubular structures, which are probably part of the ER network, in cells expressing the mutants (Figure 5b).

Recent investigations have revealed the dual targeting of proteins to Golgi apparatus and plastids in *Arabidopsis* (Villarejo *et al.*, 2005), rice (Asatsuma *et al.*, 2005; Chen *et al.*, 2004; Kaneko *et al.*, 2011, 2014; Kitajima *et al.*, 2009; Nanjo *et al.*, 2006) and photosynthetic micro-organisms (van Dooren *et al.*, 2001; Nowack and Grossman, 2012; Sláviková *et al.*, 2006). To test the possibility of plastid-targeting of MSD1, we cobombarded *MSD1-YFP* with a sequence encoding a plastid marker, the transit peptide of Waxy (Klösgen and Weil, 1991) fused to red fluorescent protein from *Discosoma* sp. (*WxTP–DsRed*), into onion cells. MSD1-YFP was notably colocalized with the plastids visualized by WxTP–DsRed (Figure 5c). Simultaneous expression of MSD1-YFP, the plastid marker WxTP–DsRed, and either AtARF1(T31N), AtARF1(Q71L), or AtSAR1(H74L) indicated that the plastid targeting of MSD1-YFP was inhibited in cells expressing the ARF1 and SAR1 mutant proteins (Figure 5d,f). The overall results clearly indicate that MSD1 is a multilocalizing protein that is targeted to the interior of plastids from the Golgi apparatus via the secretory pathway.

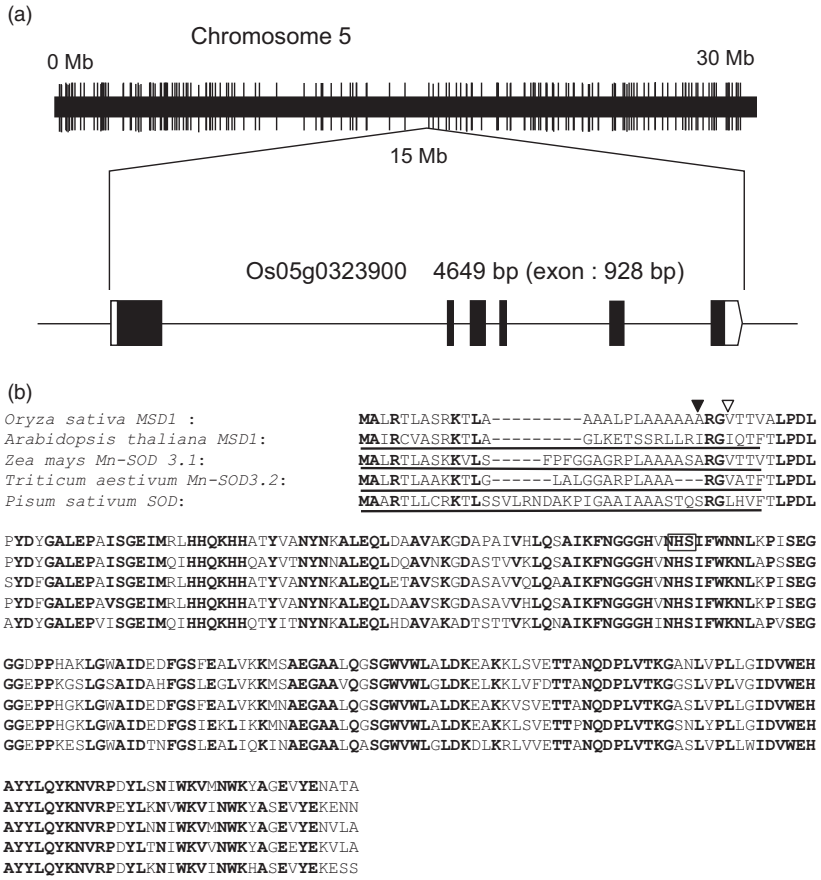

Figure 3 *OsMSD1*. (a) Structure and position of *MSD1* (Os05g0323900) on chromosome 5. Black boxes indicate exons. (b) Alignments of predicted amino acid sequences of the deduced MSD1 proteins of possible orthologous genes from *Oryza sativa*, *Arabidopsis thaliana*, *Zea mays*, *Triticum aestivum* and *Pisum sativum*. Conserved amino acids are boldfaced. Underlines represent mitochondrion-targeting sequence predicted by PSORT. In the rice MSD1 sequence, arrowheads show possible cleavage sites of the signal peptide predicted by (▽) PSORT and (▼) signalP. An *N*-glycosylation site is boxed.

Overexpression and suppression of *MSD1* affect the grain quality of rice ripened under heat stress

To determine the possible stress-adapting function of *MSD1* in ripening seeds of rice, we generated transgenic overexpressor (OE) plants with the maize *Ubiquitin-1* promoter (P*Ubi1*) fused to *MSD1* (MSD1^{OE}) by *Agrobacterium*-mediated transformation. It was reported that P*Ubi1*-controlled genes are highly expressed in various rice tissues (Cornejo *et al.*, 1993). The expression profiles of *MSD1* mRNA in leaves, roots and developing seeds of Nipponbare wild type (WT) and MSD1^{OE} revealed a constitutive high expression of *MSD1* in MSD1^{OE} plants (Figure 6a–c). Furthermore, H_2O_2 increased in the developing seeds and young seedlings of MSD1^{OE} (Figure 7). The ratio of H_2O_2 content between MSD1^{OE} and WT seedlings under hot condition revealed that H_2O_2 formation increased in MSD1^{OE} under heat stress (Figure 7b). When plants were incubated at normal or high temperatures after heading, the ratios of perfect grains harvested were 78% (WT) and 83% (MSD1^{OE}) at 28/23 °C, 77% (WT) and 88% (MSD1^{OE}) at 30/23 °C, and 26% (WT) and 60% (MSD1^{OE}) at 33/28 °C (Figure 6d–f). Under heat stress, the grain quality of MSD1^{OE} was significantly greater than that of WT (Figures 6f and S1). To suppress the expression of *MSD1* in developing seeds, we used a 696-bp fragment of *MSD1* cDNA which contains no sequence of more than 21 nucleotides conserved with other rice SODs to construct RNA interference (RNAi) binary vectors under the control of the promoter of the developing endosperm-specific *Waxy* (P*Wx*) by arranging two identical fragments derived from *MSD1* in a tail-to-tail manner, yielding a vector generating artificial hairpin-structure transcripts (Figure S2). We generated two transgenic knock-down (KD) rice plants transformed with P*Wx* fused to *MSD1* RNAi, designated Nipponbare MSD1^{KD} and Yukinkomai MSD1^{KD}. Both transformants were grown under heat stress after heading. The expression of *MSD1* mRNA in developing seeds decreased to 18% of WT in Nipponbare MSD1^{KD} and 53% in Yukinkomai MSD1^{KD} (Figure 6g,i), along with significant decreases in the proportion of perfect grains (to 12% and 71%, respectively; Figure 6h,j). The overall results indicate that the constitutive high expression of *MSD1* was involved in maintaining the quality of rice grains produced under heat stress during ripening.

Proteomic characterization of developing seeds of MSD1^{OE} under heat stress

To clarify how constitutive high expression of *MSD1* leads to adaptation to heat stress, we carried out quantitative shotgun proteomic analysis of ripening seeds. Proteins extracted from ripening seeds of Nipponbare WT and MSD1^{OE} grown under control (28/23 °C) and heat stress (33/28 °C) conditions at 4 and 10 DAF were labelled by iTRAQ (isobaric tag for relative and absolute quantitation), followed by tandem mass spectrometry (MS/MS) analysis. Under heat stress, 79 proteins (~6% of all identified proteins), including storage and allergen proteins, were down-regulated and 219 (~16%) were up-regulated in the ripening seeds of MSD1^{OE} relative to WT (Table S1). Under the control condition, however, the characteristic response of MSD1^{OE} did not appear. Under high temperature, scavengers of reactive oxygen species (ROS), including Cu/Zn SOD, peroxiredoxins, thioredoxin, peptide methionine sulfoxide reductase, ascorbate peroxidases, monodehydroascorbate reductase and NADH-ubiquinone oxido-reductase, were markedly up-regulated in MSD1^{OE} relative to WT

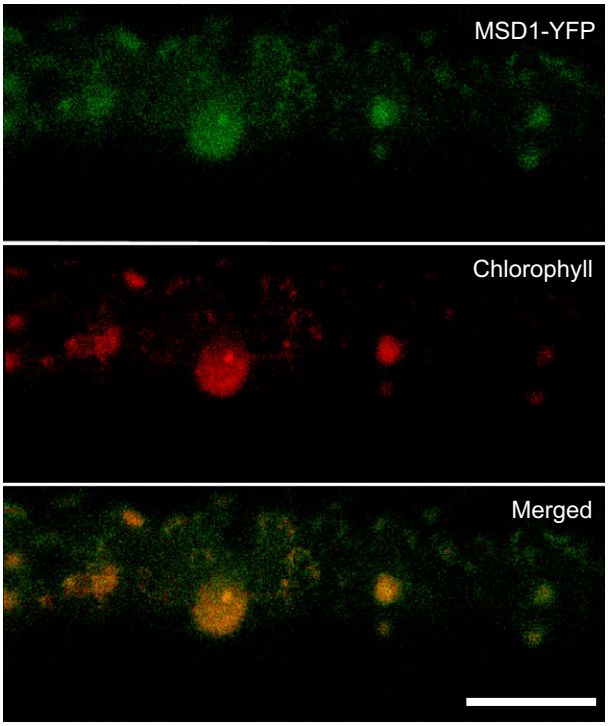

Figure 4 Expression and localization of MSD1-YFP in rice cells. Rice cells bombarded with *MSD1-YFP* were observed by laser scanning microscopy. Top: MSD1-YFP; middle: chlorophyll autofluorescence; bottom: merged. Panels are stacks of 30 images per cell, acquired from the top to the middle of the cell, every 1–2 μm. MSD1-YFP colocalized with chlorophyll autofluorescence. Bar = 10 μm.

(Figure 8 upper panel). Under the control condition, however, changes were minor. Several HSPs, chaperones, chaperonins, calreticulin, proteasome components and S-phase kinase-associated protein 1 were also up-regulated in MSD1^{OE} under heat stress (Figure 8 lower panel), but glutelin, prolamin and allergen family proteins were down-regulated (Figure S3).

Discussion

Identification of Golgi/plastid-type Mn SOD

Generally, Mn SODs are known as mitochondrial enzymes in both monocots and dicots (Kliebenstein *et al.*, 1998; del Río *et al.*, 2003; White and Scandalios, 1988; Wu *et al.*, 1999) and in eukaryotic algae (Kitayama *et al.*, 1999; Wolfe-Simon *et al.*, 2005). However, Mn SOD was localized in the chloroplasts of a marine diatom, *Thalassiosira pseudonana* (Wolfe-Simon *et al.*, 2006). The chloroplastic Mn SOD controlled by the nuclear-encoded *sodA* gene must have plastid/ER transit peptides, but typical transit peptides have not been identified (Wolfe-Simon *et al.*, 2006). As shown in Figure 3b, the deduced amino acid sequence predicted that MSD1 is an extracellular glycoprotein with an *N*-linked oligosaccharide chain. Confocal fluorescent microscopy revealed that rice MSD1 localized in multiple plastids and Golgi apparatus (Figures 4 and 5). Furthermore, the dominant-negative and constitutive-active mutants of ARF1 and SAR1 GTPases arrested the plastid-targeting of MSD1-YFP (Figure 5d), and the MSD1-YFP fluorescence was rearranged into an ER tubular network (Figure 5b). This indicates that MSD1 is transported from the Golgi apparatus via the secretory pathway to the plastid, as are *Arabidopsis* CAH1 (Burén *et al.*, 2011; Villarejo *et al.*, 2005), *O. sativa* Amyl-1 (Asatsuma *et al.*, 2005; Kitajima *et al.*, 2009) and NPP1 (Kaneko *et al.*, 2011, 2014; Nanjo *et al.*, 2006). This is the first report of the Golgi-to-plastid traffic of noncarbohydrate metabolism-related enzyme.

The electron transport chain in chloroplasts contains several auto-oxidizable enzymes. Ferredoxin in the reduced state can react with oxygen, releasing O_2^- (Asada and Takahashi, 1987) and the aprotic interior of thylakoid membranes also produces O_2^- (Takahashi *et al.*, 1988). The outer layer tissues of developing rice seeds, namely the pericarp and the endosperm, contain chloroplasts during grain filling. Large amounts of starch molecules are synthesized and accumulated in the amyloplasts of endosperm cells. Thus, there is a need for ROS scavenging in the plastids of developing rice seeds. In addition to starches, proteins are also actively synthesized, assembled and stored in developing seeds. Storage 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). The production of H_2O_2 from O_2 resulting from the maturation of glutelin in the endomembrane system (Onda *et al.*, 2009) suggests the existence of an endomembranous ROS scavenging system.

Overproduction of MSD1 improves quality grain ripened under heat stress

Ectopic production of Golgi/plastid-type MSD1 significantly improved the quality of rice grain ripened under heat stress (Figure 6f). On the other hand, suppression of MSD1 reduced the normal formation of rice grains (Figure 6h,j). These results indicate that the constitutive high expression of Golgi/plastid-type MSD1 is effective for maintaining the formation of perfect grains under heat stress during grain filling. The introduction of yeast *MnSOD* and pea mitochondrial *MnSOD* into chloroplasts of rice conferred tolerance to salt and oxidative stress (Tanaka *et al.*, 1999; Wang *et al.*, 2005). In addition, transgenic rice transformed with mangrove cytosolic *Cu/ZnSOD* showed better tolerance to drought (Prashanth *et al.*, 2008). We found that enhancement of *OsMSD1* conferred significant tolerance to high temperatures during rice grain filling.

OsMSD1 is located in the centre of chromosome 5 (Figure 3a). Quantitative trait loci (QTLs) controlling grain appearance quality have been identified in populations derived from crosses between *japonica* cultivars (Ebitani *et al.*, 2005; Kobayashi *et al.*, 2007; Tabata *et al.*, 2007), between *japonica* and *indica* cultivars (He *et al.*, 1999; Wan *et al.*, 2005) and between *O. sativa* and *Oryza glaberrima* (Li *et al.*, 2004). The identification of a grain chalkiness QTL (qAPG5-1, Ebitani *et al.*, 2008) close to the position of *MSD1* (Ebitani *et al.*, 2005; Yamakawa *et al.*, 2008) suggests that MSD1 is a determinant of chalkiness.

Proteomic characterization of developing seeds of MSD1^{OE} under heat stress

Quantitative proteomic analysis of ripening seeds of MSD1^{OE} and WT grown in normal and heat-stress conditions at 4 and 10 DAF revealed that 79 proteins were down-regulated and 219 were up-regulated in MSD1^{OE} under heat stress in comparison with WT (Table S1). The ROS scavenging system, molecular chaperones, chaperonins, calreticulin and proteasome components were markedly up-regulated in MSD1^{OE} under high temperature (Figure 8). In contrast, glutelin, prolamin and allergen family proteins were strongly down-regulated (Figure S3). We detected an increase in APX 1, 2 and 4 in the developing seeds of MSD1^{OE}. Monodehydroascorbate reductase, which regenerates ascorbate

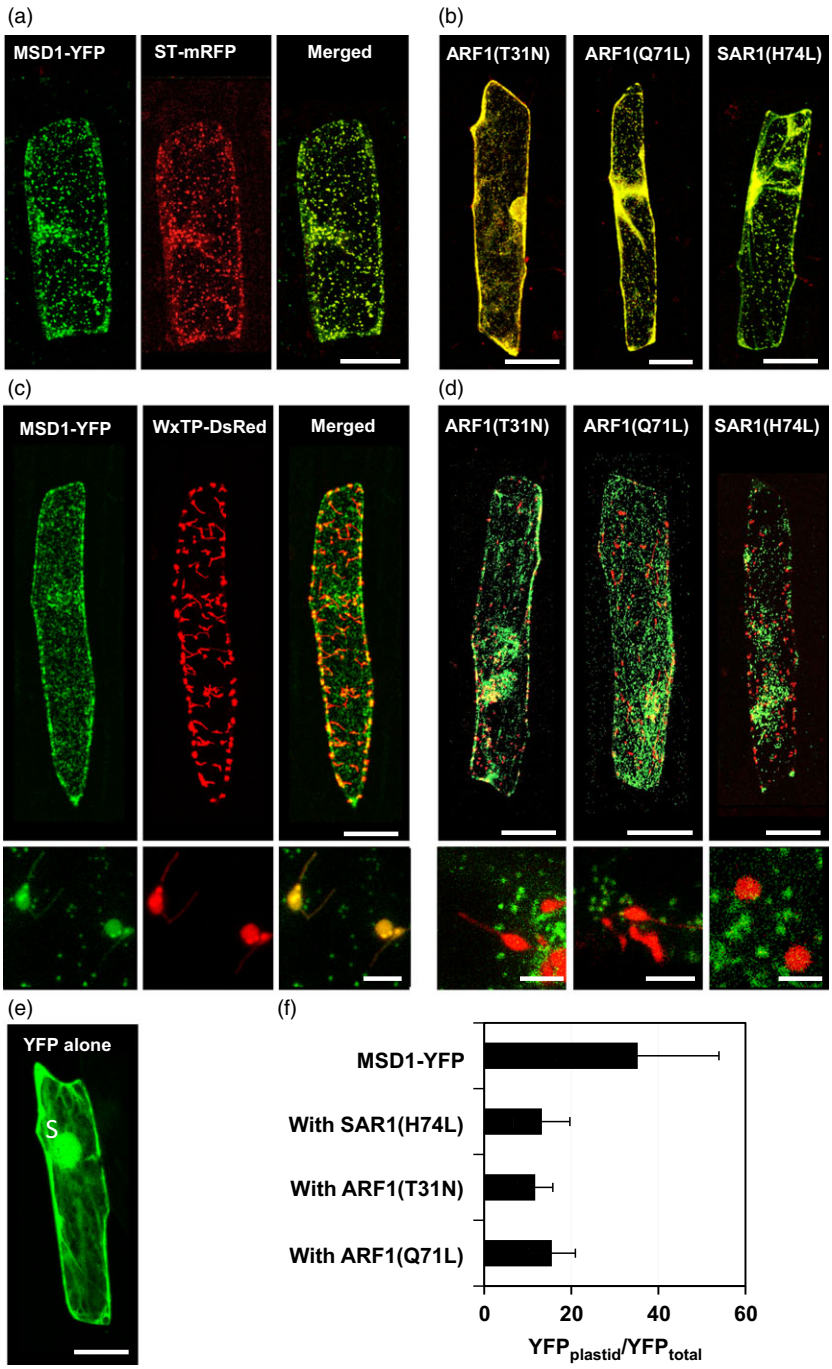

Figure 5 Expression and localization of MSD1-YFP in onion epidermal cells. (a) Onion cells expressing MSD1-YFP and ST-mRFP. Left: MSD1-YFP (green); middle: ST-mRFP (red); right: merged. Panels are stacks of 30 images per cell, acquired from the top to the middle of the cell, every 1–2 μm. MSD1-YFP colocalized with Golgi marker ST-mRFP. Bar = 100 μm. (b) Effects of AtARF1 (T31N), AtARF1(Q71L) and AtSAR1(H74L) on the distribution of MSD1-YFP and ST-mRFP. All images merge YFP with mRFP. Both MSD1-YFP and ST-mRFP were rearranged and remerged into ER tubular structures. (c) Onion cells expressing MSD1-YFP and WxTP-DsRed. Left: MSD1-YFP (green); middle: WxTP-DsRed (red); right: merged. MSD1-YFP overlapped with the plastid marker WxTP-DsRed. Bar = 100 μm. Bottom panels show close-up views of plastids; bar = 5 μm. (d) Effects of AtARF1(T31N), AtARF1(Q71L) and AtSAR1 (H74L) on the distribution of MSD1-YFP and WxTP-DsRed. All images merge YFP with DsRed. MSD1-YFP and WxTP-DsRed were distributed separately in cells. Bars = 100 μm (top) and 5 μm (bottom). (e) Onion cell expressing YFP alone. (f) Proportion of plastid localization of MSD1-YFP in the presence of AtSAR1(H74L), AtARF1(T31N) or AtARF1(Q71L). Values are means ± SD (n = 8–11) of ratios of the fluorescence intensity of YFP in the plastid to YFP in the whole cell ($YFP_{plastid}$/YFP_{total}, %).

from monodehydroascorbate, was also up-regulated (Figure 8 upper panel). The enhancement of APX production in rice (Lu *et al.*, 2007; Tanaka *et al.*, 1999) and other plants (Diaz-Vivancos *et al.*, 2013; Faize *et al.*, 2011) confers abiotic stress tolerance. In addition, a series of peroxiredoxins (thioredoxin peroxidases), including 2-Cys peroxiredoxin, were up-regulated in $MSD1^{OE}$ (Figure 8 upper panel). Yeast transformed with *O. sativa* 2-Cys peroxiredoxin showed increased stress tolerance and fermentation capacity (Kim *et al.*, 2013). Moreover, an HSP was increased in $MSD1^{OE}$ under heat stress (Figure 8 lower panel). In rice (Sato and Yokoya, 2008) and *Arabidopsis* (Mu *et al.*, 2013), overexpression of small HSPs enhanced tolerance to drought, salt and heat. Overall, these proteomic results and the literature strongly support the conclusion that $MSD1^{OE}$ rice showed improved adaptability to heat stress.

How is *MSD1* involved in the adaptation of $MSD1^{OE}$ to heat stress? We considered that the constitutive high expression of *MSD1* immediately converts O_2^- to H_2O_2 under heat stress, and H_2O_2 probably serves as a trigger for enhancing the expression of the ROS scavenging system and HSP genes, as the level of H_2O_2 was higher in $MSD1^{OE}$ than in WT (Figure 7). H_2O_2 is one of the most abundant ROS and is both highly reactive and toxic. However, H_2O_2 also functions as a signalling molecule and activates the MAPK cascade (Apel and Hirt, 2004; Neill *et al.*, 2002). For example, H_2O_2 induced ascorbate peroxidase in embryos of germinating rice (Morita *et al.*, 1999), in *Arabidopsis*

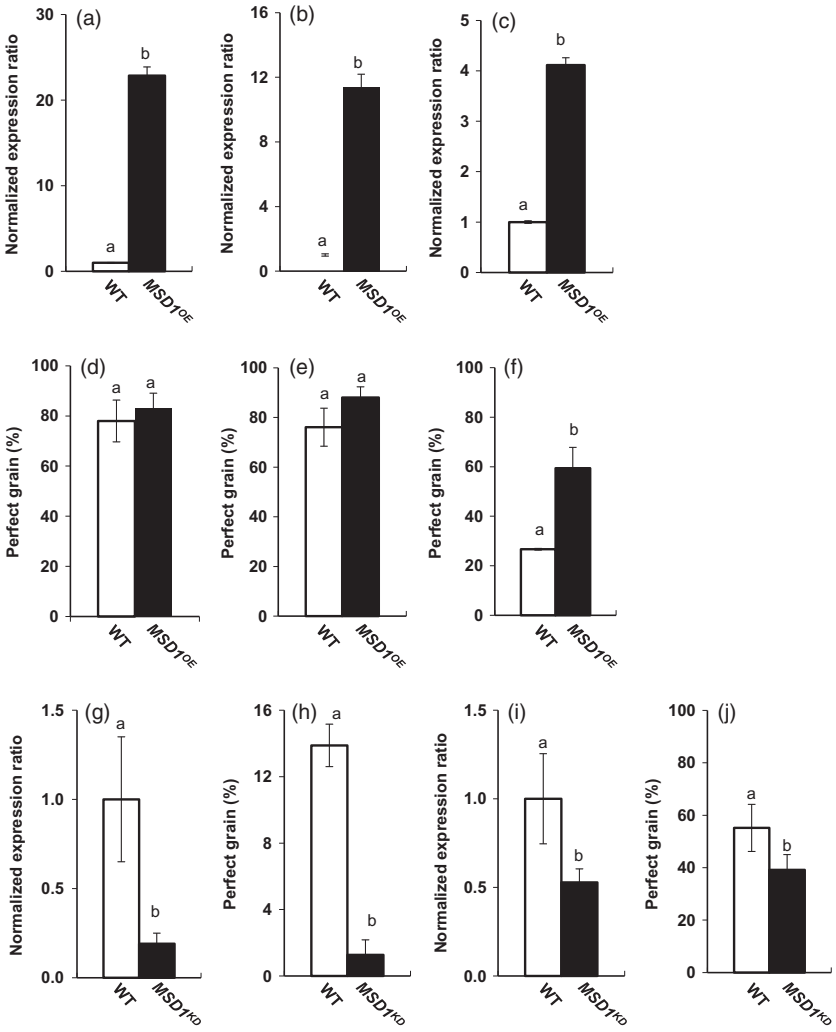

Figure 6 Evaluation of heat-stress tolerance during grain filling of rice with overexpression of *MSD1* (MSD1^{OE}) or developing endosperm-specific suppression of *MSD1* (MSD1^{KD}). (a–c) Expression profiles of *MSD1* mRNA in different organs of Nipponbare wild type (WT) and MSD1^{OE}. (a) Leaf blades and (b) roots at vegetative stage and (c) developing grains at 5 days after flowering (DAF) were harvested and used for fluorescence-based quantitative real-time PCR. Values are means ± SD (n = 3). (d–f) Nipponbare WT and MSD1^{OE} plants were incubated under (d) 28/23 °C (12/12 h), (e) 30/23 °C (12/12 h) or (f) 33/28 °C (12/12 h) after heading, and the appearance quality of harvested grains was evaluated. Values are means ± SD (n = 3–7) of proportions of perfect grains. (g, h) Nipponbare WT and MSD1^{KD} plants were incubated under 33/28 °C after heading; *MSD1* mRNA in developing grains at 5 DAF was quantified and appearance quality was evaluated (n = 5). (i, j) Yukinkomai WT and MSD1^{KD} plants were incubated under 33/28 °C after heading; *MSD1* mRNA in developing grains at 5 DAF was quantified, and appearance quality was evaluated (n = 3–7). The ratio of *MSD1* mRNA to 18S rRNA in each WT was set to 1. Columns with the same letter are not significantly different ($P < 0.05$, Student's t-test).

leaves (Karpinski *et al.*, 1999) and in tobacco leaves (Gupta *et al.*, 1993) and induced peroxiredoxin in mammalian thyroid cells (Kim *et al.*, 2000). Therefore, induced peroxiredoxin and ascorbate peroxidase likely work as the main regulators of intracellular H_2O_2 concentrations in MSD1^{OE}. Furthermore, heat-stress-induced H_2O_2 was involved in the early stage of activation of heat shock factor (HSF) in *Arabidopsis* cell culture (Volkov *et al.*, 2006). In rice leaves, H_2O_2 treatment induced the production of a chloroplastic small HSP (Lee *et al.*, 2000). Thus, H_2O_2 formed by Golgi/plastid-type MSD1 is the key factor that confers heat tolerance on MSD1^{OE}.

Storage and allergen family proteins were down-regulated in the early developing seeds of MSD1^{OE} under heat stress (Figure S3). The formation of protein bodies in developing seed cells of heat-susceptible Todorokiwase was brought forward by higher temperature (T. M., unpublished data). We infer that the constitutive high expression of Golgi/plastid-type MSD1 controls the redox state in the endomembrane system, leading to the normal programmed formation of protein bodies. Further studies will be needed to confirm this hypothesis.

In conclusion, we found a novel Golgi/plastid-type Mn SOD in developing rice seeds. The ectopic expression of *MSD1* dramatically induced the expression of ROS scavengers, molecular chaperones and the quality control system in developing seeds under heat stress. We consider that the constitutive high expression of *MSD1* maintains normal grain filling and the production of perfect grains of rice under heat stress.

Experimental procedures

Plasmids

The plasmids used in this study and references describing how they were constructed are listed in Table S2.

Plant materials and growth conditions

Seeds of rice cultivars Yukinkomai, Yukinosei, Todorokiwase and Nipponbare (a model cultivar used for transformant experiments) were obtained from the Niigata Agricultural Research Institute Crop Research Center (Nagaoka city, Niigata, Japan). Transgenic lines of rice (cv. Nipponbare) overexpressing *MSD1* under the control of maize *Ubiquitin-1* constitutive promoter (MSD1^{OE}) were obtained from the full-length cDNA overexpressor (FOX) lines of rice (Nakamura *et al.*, 2007).

Transgenic plants with suppression of the *MSD1* gene in developing seeds were generated as follows: *MSD1* cDNA (bp 1–696) which contains no sequence of more than 21 nucleotides conserved with other rice SODs was amplified by PCR from pOsMSD1 (accession no. AK104160) with a primer set (Table S2) and introduced into pESWA (Islam *et al.*, 2005) to construct the RNAi vector pWX-WB-MSD1-RNAi in combination with the *Wx*

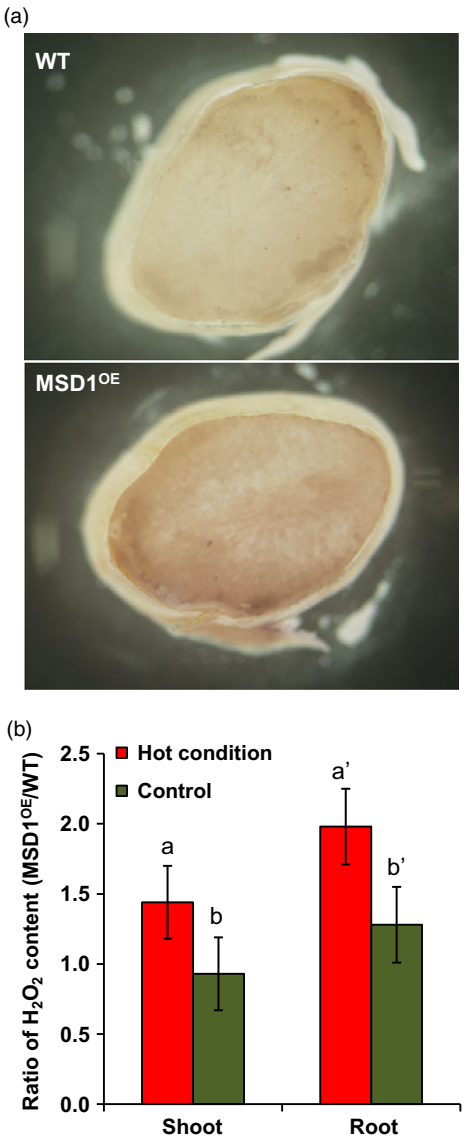

Figure 7 Increase of H_2O_2 formation in developing and germinating seeds of MSD1^{OE}. (a) Developing seeds of Nipponbare WT and MSD1^{OE} at 10 days after flowering (DAF) were stained with diaminobenzidine. (b) H_2O_2 contents in the shoots and roots of WT and MSD1^{OE} seedlings at 7 days after imbibition. Hot condition = 33/28 °C (12/12 h); control condition = 28/23 °C (12/12 h). Values are means ± SD (n = 3–4). Columns with the same letter are not significantly different ($P < 0.05$, Student's t-test).

promoter (Figure S2) using a pENTR Directional TOPO Cloning Kit and the Gateway LR Clonase Enzyme mix (Thermo Fisher Scientific, Waltham, MA). The binary RNAi vector was transformed into *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 1986), and *Agrobacterium*-mediated transformation of rice plants was performed as described by Hiei *et al.* (1994). We generated two transgenic knock-down (KD) rice lines transformed with pWX-WB-MSD1-RNAi, designated Nipponbare MSD1^{KD} and Yukinkomai MSD1^{KD}.

Yukinkomai, Yukinosei and Todorokiwase plants were grown in paddy fields of the Crop Research Center from 2004 to 2008 with ambient temperature or warm water. During the heading, ripening and maturity stages, the warm water was supplied at 35 °C at a flow rate of 80 L/min, making the daily mean temperature at around the ear 1.4–1.9 °C higher than that of the ambient temperature field (25.4 °C).

Transgenic and wild-type (Nipponbare) plants were grown under 28/23 °C (12 h at 20 000 lx/12 h dark) in a growth chamber (CFH-415; Tomy Seiko, Tokyo, Japan). Grain quality (chalky or translucent) was determined with a rice grain grader (RGQI20A; Satake, Hiroshima, Japan).

Microscopy studies

Yellow fluorescent protein (YFP) is a genetic mutant of green fluorescent protein from *Aequorea victoria*. We constructed pH35GY-OsMSD1-YFP to determine the subcellular localization of rice MSD1. We PCR-amplified *MSD1* from pOsMSD1 (primers in Table S2) and introduced it into pH35GY (Funakoshi Corp, Tokyo, Japan; Kubo *et al.*, 2005) to create pH35GY-OsMSD1-YFP. To obtain pH35GY-(AAGCTT)-YFP (YFP vector alone), we PCR-amplified an *OsEMP70* fragment (bp 1083–1584) from pOsEMP70 (primers in Table S2) and introduced it into pH35GY. The pH35GY-ΔOsEMP70-YFP construct was digested with *Hin*dIII to remove the *OsEMP70* fragment and ligated with a Mighty Mix DNA ligation kit (Takara Bio, Ohtsu, Japan).

Construction of pWxTP-DsRed (red fluorescent protein from *Discosoma* sp.; Kitajima *et al.*, 2009), pST-mRFP (monomeric red fluorescent protein; Matsuura-Tokita *et al.*, 2006), pMT121-ARF1 T31N, pMT121-ARF1 Q71L and pMT121-SAR1 H74L (Takeuchi *et al.*, 2000, 2002) were described elsewhere.

To introduce plasmid DNA into rice and onion (*Allium cepa*) epidermal cells, we used the particle bombardment method, using a helium-driven particle accelerator, as described previously (Kitajima *et al.*, 2009). Confocal laser-scanning microscopes (FV300 and FV1000; Olympus, Tokyo, Japan) were used for imaging YFP, DsRed and chlorophyll autofluorescence in rice and onion cells (Kitajima *et al.*, 2009). The FV300 uses an Ar laser at 488 nm to excite YFP and a green He/Ne laser at 543 nm to excite DsRed and chlorophyll. Fluorescence was detected at 510–530 nm through BA510IF and BA530RIF emission filters with an SDM570 emission dichroic mirror (YFP) and at >565 nm through a BA565IF emission filter (DsRed and chlorophyll). The FV1000 uses an Ar laser at 488 nm to excite YFP, and at 559 nm to excite DsRed and chlorophyll. Fluorescence was detected at 510 nm through an SDM560 emission dichroic mirror (YFP) and at 581 nm with an emission dichroic mirror (DsRed and chlorophyll). Images were observed through 40× air-objective (UApo/340, NA 0.90; Olympus) and 100× oil-objective lenses (UPlanSApo, NA 1.40 Oil; Olympus). The fluorescence intensity in plastids and in whole cells was determined using Lumina Vision imaging software. The background was always set at the maximum fluorescence intensity of an area in which no structural image was present. Areas identified by either chlorophyll autofluorescence or WxTP–DsRed were defined as plastids. To evaluate the plastid-targeting abilities of YFP-labelled proteins, we determined the ratio of the fluorescent intensity of YFP in the plastid to that in the whole cell ($YFP_{plastid}/YFP_{total}$; Kitajima *et al.*, 2009).

Assay and diaminobenzidine staining for H_2O_2

H_2O_2 assays were carried out according to the procedure of Rao *et al.* (2000) and Xiong *et al.* (2007). Shoots and roots from rice seedlings at 7 days after imbibition were frozen and ground to a powder. Each sample (100 mg) was suspended in 0.5 mL of

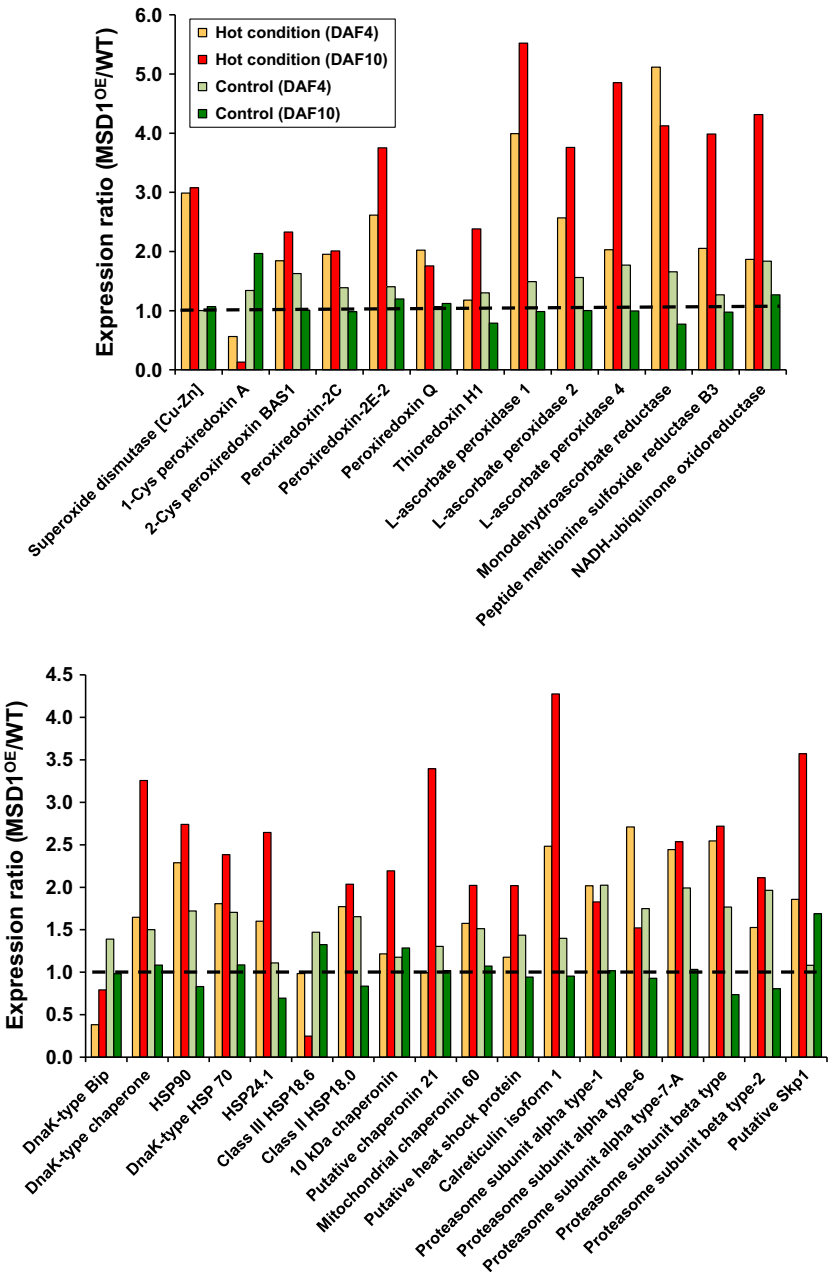

Figure 8 Increase in ROS scavenger, chaperone, quality control and programmed proteolysis systems in the developing seeds of MSD1^{OE} under heat stress. Developing seeds at 4 and 10 days after flowering (DAF) were analysed by quantitative shotgun proteomic analysis with iTRAQ labelling. WT, Nipponbare wild type; MSD1^{OE}, Nipponbare *MSD1* overexpressor. Hot condition = 33/28 °C (12/12 h); control condition = 28/23 °C (12/12 h).

0.2 M $HClO_4$, incubated on ice for 5 min and then centrifuged at 14 000 ***g*** for 10 min at 4 °C. The supernatant was neutralized with 0.2 M NH_4OH (pH 9.5) and centrifuged at 3000 ***g*** for 2 min. The neutralized extracts were passed through Sep-Pak Light Accell Plus QMA Carbonate columns (Nihon Waters, Tokyo, Japan) and were eluted with 0.5 mL water. H_2O_2 in the extracts was quantified using an Amplex Red Hydrogen Peroxide–Peroxidase Assay kit (Life Technology Japan, Tokyo, Japan) following the manufacturer's directions. Fluorescence was measured with an RF-5300PC spectrofluorophotometer (Shimadzu Corp., Kyoto, Japan) using excitation at 530 nm and fluorescence detection at 590 nm. For histochemical analysis, developing seeds (14 DAF) were sliced into 1-mm transverse sections and immersed in 20 mM Tris·HCl buffer (pH 6.5) containing 1% (w/v) 3,3′-diaminobenzidine. After vacuum infiltration for 30 min, the samples were incubated at room temperature for 20 h in the dark to develop a dark-brown colour of diaminobenzidine oxidized by H_2O_2.

Gel-based proteomics

Grains of Yukinkomai, Yukinosei and Todorokiwase (100 mg) at 4 DAF were extracted with 8 M urea, 1% (w/v) CHAPS detergent, 10 mM ethylene diamine tetraacetic acid (EDTA) and 5 mM phenylmethylsulfonyl fluoride and centrifuged at 10 000 ***g*** for 10 min at 4 °C. The supernatants were precipitated with 10% (w/v) trichloroacetic acid and resolved with 9 M urea, 3% (w/v) IGEPAL detergent, and 2% (v/v) 2-mercaptoethanol and then used for gel-based proteomics. The procedures of 2D polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) were essentially identical to the previous reports (Kaneko *et al.*, 2011; Nanjo *et al.*, 2004). In 2D-PAGE, the 1st dimension used

isoelectric focusing with ampholine (pH 3.5–10) and the 2nd dimension used sodium dodecyl sulphate (SDS)-PAGE with 16% separating gel. The 2D gels were stained with Coomassie brilliant blue R-250 (Nanjo *et al.*, 2004). The protein spots excised from the gels were digested by trypsin using standard procedures (Awang *et al.*, 2010). MALDI-TOF-MS was carried out with a matrix of α-cyano-4-hyrdoxycinnamic acid in an AXIMA-CFR mass spectrometer (Shimadzu Corp.) and an Autoflex III TOF/TOF mass spectrometer (Bruker BioSpin, Yokohama, Japan; Kaneko *et al.*, 2011).

Quantitative shotgun proteomics

At 4 and 10 DAF, developing seeds of WT and MSD1^{OE} grown under hot (33/28 °C, 12/12 h) or control conditions (28/23 °C, 12/12 h) were used in quantitative shotgun proteomic analysis with iTRAQ labelling. The seeds (0.2 g) were ground in liquid nitrogen to a fine powder and suspended in extraction buffer consisting of 100 mM Tris·HCl (pH 8.0), 20% (w/v) glycerol, 2% (w/v) Triton X-100, 20 mM dithiothreitol, 3 M urea, 2 M thiourea and 3% (w/v) CHAPS. The homogenates were centrifuged at 10 000 ***g*** at 4 °C for 5 min. The supernatant was collected and centrifuged again. The supernatants were mixed with 1/10 volume of 100% (w/v) trichloroacetic acid, incubated on ice for 15 min and centrifuged at 10 000 ***g*** at 4 °C for 15 min. The resulting protein precipitates were washed 3 times in ice-cold acetone and resuspended in 0.5 M triethylammonium bicarbonate buffer (pH 8.5) containing 0.1% SDS. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. Proteins (50 μg) were reduced with tris-(2-carboxyethyl) phosphine at 37 °C for 60 min and then alkalized with methylmethanethiosulfonate at 25 °C for 60 min. Samples were digested in 10 μL of trypsin (1 μg/μL) at 37 °C for 16 h and labelled with 4-plex iTRAQ tags (Thermo Fisher Scientific) according to Fukao *et al.* (2011), and the resultant 4 iTRAQ-labelled peptide samples were mixed.

For quantitative proteomics, we used a combined KYA DiNA-A (KYA Tech., Tokyo, Japan) and LTQ-Orbitrap XL (Thermo Fisher Scientific) liquid chromatography-MS/MS system. The ionization voltage and capillary transfer temperature at the electrospray ionization nano-stage were set to 1.7–2.5 kV and 200 °C. iTRAQ-labelled peptides were separated in a HiQ sil C18W column (75 μm i.d. ×50 mm, 3 μm particle size; KYA Tech.), using buffers A (0.1% [v/v] acetic acid and 2% [v/v] acetonitrile in water) and B (0.1% [v/v] acetic acid and 80% [v/v] acetonitrile in water). A linear gradient from 0% to 33% B for 240 min, 33% to 100% B for 10 min and back to 0% B over 15 min was applied, and peptides eluted from the column were introduced directly into an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) at a flow rate of 300 nL min^{-1} and a spray voltage of 4.5 kV.

Liquid chromatography-MS/MS data were acquired in data-dependent acquisition mode using Xcalibur 2.0 software (Thermo Fisher Scientific). The mass range selected for MS scan was set to 350–1600 m/z, and the top three peaks were subjected to MS/MS analysis. Full MS scan was detected in the Orbitrap, while the MS/MS scans were detected in the linear ion trap and Orbitrap. The normalized collision energy for MS/MS was set to 35 eV for collision-induced dissociation (CID) and 45 eV for higher energy C-trap dissociation (HCD). The resolution of the mass spectrometer (FTMS) was set to 60 000. Divalent or trivalent ions were subjected to MS/MS analysis in dynamic exclusion mode, and proteins were identified with Proteome Discoverer v. 1.1 software and the SEQUEST search tool (Thermo Fisher Scientific) using the UniProt (http://www.uniprot.org/) *O. sativa* subsp. *japonica* database (63 535 proteins) with the following parameters: enzyme, trypsin; maximum missed cleavages site, 2; peptide charge, 2+ or 3+; MS tolerance, 10 ppm; MS/MS tolerance, ±0.8 Da; dynamic modification; carboxymethylation (C); oxidation (H, M, W); iTRAQ 4-plex (K, Y, N-terminus). False discovery rates were <5%.

mRNA analysis

Sample tissues (0.1 g) were ground in liquid nitrogen to fine powder and suspended in an extraction buffer consisting of 2% (w/v) cetyl trimethyl ammonium bromide, 100 mM Tris·HCl (pH 8.0), 20 mM EDTA and 1.4 M NaCl. The homogenates were mixed with 1/2 volumes of phenol and chloroform/isoamyl alcohol (25:24:1, v/v), centrifuged at 10 000 g at 4 °C for 5 min, and total RNA was extracted with RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Ten ng of total RNA was applied to a real-time quantitative reverse transcription PCR using SsoFast Eva Green Supermix (Bio-Rad) and CFX96 real time PCR system/C1000TM Thermal Cycler (Bio-Rad) with the PCR primer sets listed in Table S3. The mRNA contents in each sample were normalized against those of constitutive 18S rRNA gene (Accession no. AK059783).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Appearance quality of grains harvested from Nippon-bare WT and MSD1^{OE} treated at 33/28 °C (12/12 h) after heading.

Figure S2 Map of pWX-WB-MSD1-RNAi.

Figure S3 Changes in the amounts of storage and allergen proteins in the developing seeds of MSD1^{OE} under heat stress.

Table S1 Quantitative shotgun proteomic analysis of developing seeds of MSD1^{OE} and WT at 4 and 10 days after flowering (DAF) under hot and control conditions.

Table S2 Plasmids used in this study.

Table S3 Real-time PCR primer sets.

