Plant Mol Biol
DOI 10.1007/s11103-013-0056-x

The endoplasmic reticulum stress induced by highly expressed OsrAAT reduces seed size via pre-mature programmed cell death

Liping Zhang · Daiming Jiang · Jianlei Pang · Rong Chen · Xianghong Wang · Daichang Yang

Received: 22 February 2013 / Accepted: 3 April 2013

Abstract The high accumulation of a recombinant protein in rice endosperm causes endoplasmic reticulum (ER) stress and in turn dramatically affects endogenous storage protein expression, protein body morphology and seed phenotype. To elucidate the molecular mechanisms underlying these changes in transgenic rice seeds, we analyzed the expression profiles of endogenous storage proteins, ER stress-related and programmed cell death (PCD)-related genes in transgenic lines with different levels of *Oryza sativa* recombinant alpha antitrypsin (OsrAAT) expression. The results indicated that OsrAAT expression induced the ER stress and that the strength of the ER stress was dependent on OsrAAT expression levels. It in turn induced upregulation of the expression of the ER stress response genes and downregulation of the expression of the endogenous storage protein genes in rice endosperm. Further experiments showed that the ER stress response upregulated the expression of PCD-related genes to disturb the rice endosperm development and induced pre-mature PCD. As consequence, it resulted in decrease of grain weight and size. The mechanisms for the detriment seed phenotype in transgenic lines with high accumulation of the recombinant protein were elucidated.

Keywords Storage protein · ER stress · PCD · Transgenic grain · Recombinant protein

Electronic supplementary material The online version of this article (doi:10.1007/s11103-013-0056-x) contains supplementary material, which is available to authorized users.

L. Zhang · D. Jiang · J. Pang · R. Chen · X. Wang · D. Yang
State Key Laboratory of Hybrid Rice, Engineering Research Center for Plant Biotechnology and Germplasm Utilization, Ministry of Education, Wuhan, People's Republic of China

L. Zhang · D. Jiang · J. Pang · R. Chen · X. Wang · D. Yang (✉)
College of Life Sciences, Wuhan University, Wuhan, People's Republic of China
e-mail: dyang@whu.edu.cn

Introduction

Endoplasmic reticulum (ER) is the site of protein synthesis, where secretory, membrane-bound and some organelle-targeted proteins are synthesized and folded into the proper conformations before they are transported to their final destinations. However, biotic and abiotic stimuli perturb ER homeostasis to produce unfolded or misfolded proteins. The ER stress-activated signaling pathways, collectively called the unfolded protein response (UPR), help ameliorate the accumulation of unfolded or misfolding proteins. In mammalian cells, there are three major signaling pathways in the UPR, namely the inositol-requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) pathways. In the IRE1 pathway, IRE1 dimerizes under ER stress, followed by auto-transphosphorylation, which then triggers its RNase activity to process X-box binding protein (XBP1)/basic leucine zipper transcription factor (HAC1) to produce an active transcription factor, spliced-XBP1/HAC1(Ron and Walter 2007; Schroder et al. 2003). PERK, a type-I ER transmembrane protein, phosphorylates eukaryotic translation initiator factor 2α (eIF2α) to attenuate general protein synthesis (Harding et al. 2000). After the initiation of ER stress, ATF6 is translocated from the ER to the Golgi through an interaction with the coat protein II (COPII) complex, where it is digested by site-1 proteases (SP1) and site-2 proteases (SP2) to release a

Published online: 08 April 2013

cytosolic fragment (ATF6f); then, ATF6f, a transcription factor, enters the nucleus to upregulate XBP1 and the ER-associated degradation (ERAD) component genes (Calfon et al. 2002; Lee et al. 2002; Yoshida et al. 2001). Recently, we found that ER stress promoted the ERAD pathway in rice endosperm with high GM-CSF expression (Luo et al. 2008). Finally, programmed cell death (PCD) is triggered to eliminate damaged cells with irreversible ER stress in both plants and animals (Yang et al. 2012; Hetz 2012; Howell 2013). Until to now, there are only two ER stress sensors, IRE1 and bZIP 17/28, have been reported in plants (Iwata and Koizumi 2012; Howell 2013).

Cereal endosperm development involves four major stages: the syncytial, cellularization, differentiation and maturation stages (Brown et al. 1996; Olsen et al. 1995; Olsen et al. 1992). The aleurone cell and embryo maintained their intact cell structures and dormancy, while starchy endosperm cells underwent PCD during rice seed maturation. Previous studies indicated that, in maize endosperm cells, PCD occurred in the starchy endosperm at 16 day after pollination (DAP) (Young et al. 1997). In wheat seeds, the nucleus/pericarp first undergoes PCD at 4–6 DAP (Young and Gallie 1999). Recently, Kobayashi and coworkers elucidated the PCD process in the developing rice starchy endosperm (Kobayashi et al. 2013). However, until now, the connection between ER stress and PCD in plants has not been well studied (CACAS 2010).

The mechanisms of PCD in the context of irreversible ER damage are well understood in mammalian cells. The IRE1 signaling pathway regulates PCD through the activation of the IRE1α–JUN N-terminal kinase (JNK) and IRE1-dependent decay (RIDD) (Hetz 2012; Hetz et al. 2011). PERK signaling upregulates the Querypro-apoptotic transcription factor C/EBP-homologous protein (CHOP) to downregulate the anti-apoptotic protein, B cell lymphoma 2 (BCL 2), leading to apoptosis. Caspase 2 involves in the response to ER stress, mediating PCD via the cleavage of the BCL 2 homology 3 (BH3) protein and then of BH3-interacting domain death agonist (BID) to activate BH antagonist or killer (BAK) cells and the pro-apoptotic multi-domain BAX proteins (Hetz 2012; Woehlbier and Hetz 2011). To date, extensive studies of PCD have been performed in mammalian cells (Logue et al. 2013), but its investigation in plants has been limited. Nevertheless, PCD in plants is a crucial component of the reaction to biotic and abiotic stimuli and a central process during plant development (Olvera-Carrillo et al. 2012). Recent results have indicated that PCD is involved in leaf and floral organ senescence (Guiboileau et al. 2010; Lim et al. 2007; Van Doorn and Woltering 2008). Zuppini et al. (2004) reported that ER stress induced PCD in soybean suspension cells (Zuppini et al. 2004).

In this study, we systematically analyzed the seed phenotypes and storage protein expression levels in wild type rice (ZH11) and two transgenic lines, 132-10 and 132-17. The grain width (GW), grain length (GL), grain thickness (GT), and thousand-grain weight (TGW) decreased as the OsrAAT level increased. The decrease in the expression levels of endogenous storage protein genes depended on the OsrAAT accumulation level in the rice endosperm. Further studies indicated that OsrAAT accumulation in the rice endosperm caused the ER stress and perturbed rice endosperm development. TUNEL assays of immature seeds at 2, 4, and 6 DAP indicated that the ER stress caused PCD to occur 2 days prematurely in the rice endosperm, which disturbed the development of the starchy endosperm, leading in turn to decreases in grain weight and size.

Materials and methods

Plant materials and phenotype determination

Two transgenic lines, 132-10 and 132-17, and Zhonghua 11 (ZH11) were obtained as described by Zhang et al. (2013). The rice plants were grown at the Campus of Wuhan University in a natural environment. To determine the grain phenotypes, 20 grains for each line were randomly chosen and measured. The flowering date of the rice spikelet was recorded, and immature seeds were collected at 2, 3, 4, 6, and 9 DAP and stored at −70 °C for later use.

Quantitative PCR analysis

The immature seeds were harvested at 3, 6, and 9 DAP and ground in liquid nitrogen. Total RNA was isolated using RNA Plant Plus Reagent (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. cDNA synthesis was performed as described previously (Wang et al. 2011). The primers for this study are listed in supplemental Table 1. qPCR amplification was performed as described previously (Wang et al. 2011). The *Actin1* gene was used as reference for quantification. The forward primer 5′ TCGCCTCAGCTACGCGCTTCAGTGC 3′ and reverse primer 5′ TGCTTCGAGCCACCGGGTTTCGGTT 3′ were used for PCR amplification to detect the splicing product of *OsbZIP50* mRNA. The PCR program was as follows: 94 °C for 5 min, followed by 32 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The PCR products were resolved by 4 % agarose gel electrophoresis.

Western blot analysis

Crude protein was extracted with protein extraction buffer (66 mM Tris–HCl, pH 6.8, 2 % SDS, 1 mM DTT; 1:5 w/v)

at 4 °C for 1 h and then centrifuged at 10,620×*g* for 5 min. Approximately 20 μg of crude protein was fractionated by 15 % SDS–PAGE. The Western blots were prepared as described previously (Xie et al. 2008). The antibodies against globulin and glutelin were described in a previous study (Yang et al. 2003).

Microscopic observations

Immature seeds at 2, 6, and 9 DAP were harvested and fixed immediately in 4 % paraformaldehyde in PBS (pH 7.4) with 0.1 % Triton X-100 under a low vacuum for 20 min and then incubated in the same solution for 40 min at room temperature. After three washes with 1× PBS, the sections were incubated in an ethanol series with concentrations of 25, 50, 75, 95 and 100 % at RT for 60 min each. Then, the samples were immersed in 100 % ethanol and transferred to 37 °C. Subsequent steps were performed as described previously (Steedman 1957; Vitha et al. 1997). Finally, the sections were transferred onto poly-L-lysine coated glass slides (Lab-Scientific, Inc., NJ, NY USA) and stored at 4 °C until use. The slides were imaged with an Olympus IX51 fluorescence microscope. The micrographs were captured with a digital camera (Retiga 2000R) and pseudo-colored, merged and analyzed using *Image-Pro*$^{\hat{}}$*Plus* version 6.0.

TUNEL assay

The fragmentation of DNA in the endosperm cells was measured using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Results

OsrAAT overexpression in the rice endosperm caused changes in seed morphology

Two transgenic lines, 132-10 and 132-17, with expression levels of 0.11 and 2.24 mg/g OsrAAT, respectively, were produced in a previous study (Zhang et al. 2013). Obvious morphological differences were observed in the seeds of these lines. As shown in Fig. 1a, a semi-opaque seed phenotype in transgenic line 132-10 and opaque phenotype with floury features in 132-17 were observed, whereas wild type ZH11 displayed a transparent phenotype. Further characterization of the seed phenotype showed that the GW, GL, GT, and TGW were significantly decreased in the transgenic lines compared with the wild type (ZH11). The TGW, GW, GL and GT decreased by 9.5, 2.9, 3.8 and 7.9 % in 132-10 and 18.2, 6.9, 5.5 and 22.2 % in 132-17 comparison with ZH11 (Table 1). Further analysis indicated that the AAT mRNA expression levels in 132-10 and

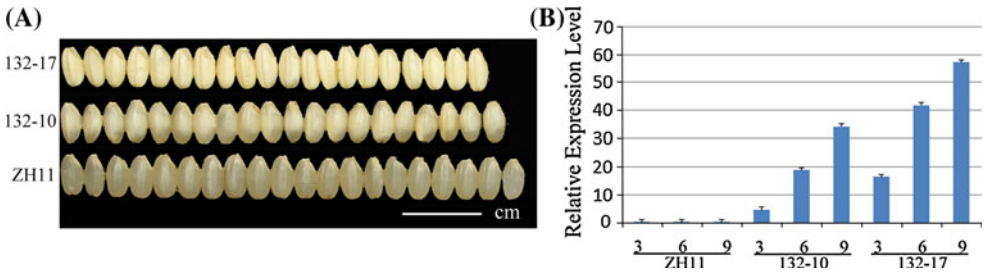

Fig. 1 The phenotypes of the transgenic and non-transgenic lines. **a** Photographs of the grains. The seed of ZH11 is transparent, 132-10 is semi-opaque and 132-17 is full opaque. **b** The expression profiles of OsrAAT in ZH11, 132-10 and 132-17 at *3*, *6*, and *9* DAP (*$P < 0.05$; **$P < 0.01$). OsrAAT mRNA expression levels in 132-17 were higher than that of 132-10 at *3*, *6*, and *9* DAP

Table 1 The grain phenotypes of the transgenic lines and non-transgenic line ZH11

Transgenic lines	Weight of 1,000 grains (g)		Grain width (mm)		Grain length (mm)		Grain thickness (mm)	
Decrease to WT	Average ± SE	%	Average ± SE	%	Average ± SE	%	Average ± SE	%
ZH11 (WT)	25.68 ± 0.36	100	3.03 ± 0.05	100	5.59 ± 0.1	100	2.16 ± 0.06	100
132-14	25.45 ± 0.41	−0.8	3.01 ± 0.03	−0.6	5.55 ± 0.2	−0.7	2.15 ± 0.2	0.4
132-10	23.24 ± 0.15	−9.5	2.94 ± 0.07	−2.9	5.38 ± 0.1	−3.8	1.99 ± 0.04	−7.9
132-18	22.74 ± 037	−11.4	2.89 ± 0.05	−4.6	5.33 ± 0.15	−4.6	1.85 ± 0.18	−14.3
132-17	20.99 ± 0.30	−18.2	2.82 ± 0.09	−6.9	5.28 ± 0.09	−5.5	1.68 ± 0.07	−22.2

WT wild type, *SE* standard error

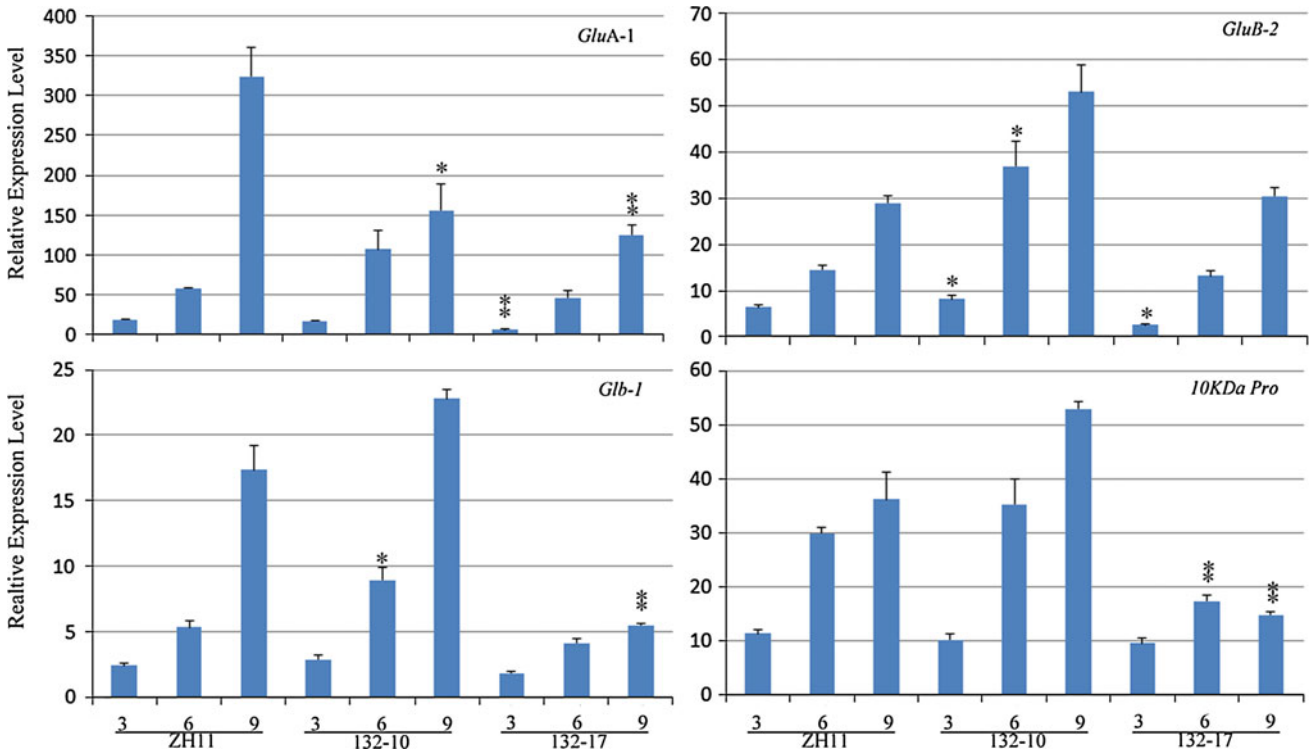

Fig. 2 The expression profiles of the storage protein genes in ZH11, 132-10 and 132-17 at *3*, *6*, and *9* DAP (*$P < 0.05$; **$P < 0.01$). *GluA-1, GluB-2, Glb-1and 10KDa pro* mRNA expression levels were monitored by Quantitative-PCR in 132-10 and 132-17 at *3*, *6*, and *9* DAP. Four genes were decreased expression in 132-17, but only *GluA-1* decreased in 132-10, other three genes expression increased in 132-10

132-17 were consistent with the OsrAAT protein expression levels (Fig. 1b), and they were also highly correlated to the seed size and other morphological changes. These results indicated that the seed size in transgenic plants was dependent on OsrAAT accumulation in the rice endosperm at both the transcriptional and protein levels.

The accumulation of high levels of OsrAAT in the rice endosperm decreased the expression of storage proteins

mRNA expression was increased in 132-10, suggesting that the overexpression of OsrAAT in the rice endosperm could significantly affect the expression of glutelin and prolamin at both the transcriptional and post-transcriptional levels, whereas globulin could be regulated at the post-transcriptional level (Figs. 2, 3). Taken together, these results indicated that the accumulation of high levels of OsrAAT in the rice endosperm seriously affected endogenous storage protein expression at either the transcriptional or post-transcriptional level.

The accumulation of OsrAAT in the rice endosperm caused ER stress

To further determine whether the expression of OsrAAT in the rice endosperm caused ER stress, the protein body morphology was examined by transmission electron microscopy (TEM). As shown in Fig. 4a, although protein body I (PBI) was not obviously different between ZH11 and the line with lower OsrAAT expression level (132-10), protein body II (PBII) increased in size in 132-10. However, the morphology of both PBI and PBII showed obvious changes in 132-17, i.e., PBI were smaller in size, and most of them were retarded in the ER to produce PBI-like bodies, while PBII were enlarged and had rough edges (Fig. 4c). These observations indicated that the ER stress in rice endosperm cells was severe in the presence of high OsrAAT. To further investigate whether the ER stress-related chaperones and proteins were upregulated, we examined the expression profiles of an ER chaperone, *Osbip1*, and the UPR-related proteins *Osbzip50* and *Osbzip60.* The results indicated that the levels of *Osbip1, Osbzip50* and *Osbzip60* were significantly upregulated at 3, 6 and 9 DAP in 132-10 and 132-17, indicating that ER stress occurred in both transgenic lines. We also found that *Osbzip50, a* homolog of the transcription factor XBP1 in mammals and of HAC1 in yeast (Hayashi et al. 2011, 2012), was spliced at 9 DAP in 132-17 but not in 132-10 and ZH11, indicating that the *OsIre1–Osbzip50* signaling pathway was activated when OsrAAT accumulated continuously during endosperm development. These results indicated that ER stress was induced by the accumulation

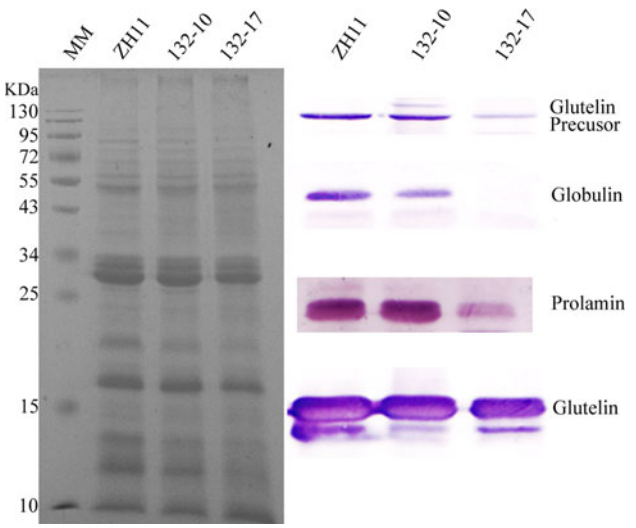

Fig. 3 SDS–PAGE (*left*) and Western blot (*right*) analysis of glutelin, globulin and prolamin in the rice endosperm. Glutelin, globulin and prolamin protein reduced in 132-17, but only globulin reduced in 132-10, other two-protein expression profiles were not affected in 132-10

of OsrAAT in endosperm cells of 132-10 and 132-17, and the strength of ER stress was dependent on the level of OsrAAT accumulation.

Rice endosperm development was affected by OsrAAT accumulation

The smaller seed size and lower TGW in the high OsrAAT expression line 132-17 prompted us to investigate whether ER stress also affected seed development. We observed the progression of rice endosperm development at 2, 4, and 6 DAP. As shown in Supplemental Fig. 2, there were no obvious differences in endosperm development between ZH11 and the transgenic lines at 2 DAP. However, the endosperm development at 4 and 6 DAP was obviously altered in 132-10 and 132-17 (Fig. 6). At 4 DAP, the nuclei were spherical in ZH11 but deformed and enlarged in 132-10 and 132-17. Furthermore, there were obvious differences in endosperm development among ZH11, 132-10 and 132-17 at 6 DAP. In general, the central point in the starchy endosperm is a landmark for endosperm cellularization and differentiation, which proceeds along a precisely orchestrated developmental program. We observed the central point in the endosperm of ZH11 and 132-10 at 6 DAP but not in that of 132-17, suggesting that the endosperm cellularization and differentiation in 132-17 have been disturbed and that the centripetal cell layer disappeared (Fig. 6). These results indicated that the accumulation of high levels of OsrAAT in the endosperm could affect normal endosperm development and seed phenotype.

The ER stress caused premature PCD in the transgenic endosperm cells

The observation of severe ER stress, activation of the *OsIre1–Osbzip50* signaling pathway and upregulation of ER stress response genes in response to the OsrAAT expression level implied that protein misfolding could be a major contributor to ER stress. *Osbip1* expression levels upregulated in both 132-10 and 132-17 at 3, 6 and 9 DAP. Thus, we assumed that *Osbip1*, which is associated with *stromal cell-derived factor 2-like 1* (*SDF2-L1*) and *ER-localized co-chaperone Hsp40 protein-like* (*ERdj3*), could be involved in the operation of the protein folding machinery to maintain cell homeostasis (Meunier et al. 2002; Jin et al. 2008; Howell 2013). The previous study has indicated that *SDF2-like 1*, *ERdj3*, *endoplasmic reticulum oxidoreductin* 1 (*Ero1*) and *profilin* upregulated in ER stress resulting in premature PCD (Yang et al. 2012). To verify premature PCD in endosperm cells of 132-10 and 132-17, we monitored the expression profiles of *SDF2-like 1*, *ERdj3,Ero1* and *profilin* and conducted TUNEL assays. As shown in Fig. 5, the expression levels of the *ERdi3B-like* and *profilin* genes were upregulated as development progressed in both 132-10 and 132-17 (Fig. 5a, c). The *SDF2-like1* and *Ero1* genes were upregulated in 132-17. These results suggested that precocious PCD of the rice endosperm cells could occur in both transgenic lines.

In general, PCD occurred first in the pericarp/nucellus and subsequently in the starchy endosperm cells. DNA degradation in the nucleus serves as a marker of PCD. We postulated that the decreases in grain weight and size in the seed with high OsrAAT accumulation could be caused by premature PCD. To further confirm whether PCD occurred prematurely in 132-17, TUNEL assays were performed. As shown in Supplemental Fig. 2, PCD initially occurred in the pericarp at 2 DAP in ZH11, 132-10 and 132-17. No PCD was observed in the starchy endosperm at 4 DAP in ZH11. However, obvious PCD was observed in the starchy endosperm cells, nucellus and pericarp of 132-10 and 132-17 at 4 DAP; in particular, strong PCD signals were found in the pericarp and starchy endosperm cells of 132-17 (Fig. 6). These results indicated that PCD occurred 2 days earlier in the transgenic lines than in ZH11. The starchy endosperm cell at 6 DAP underwent PCD in ZH11, 132-10 and 132-17, significantly more PCD was observed in 132-17 than in ZH11 and 132-10 (Fig. 6). Taken together, our results indicate that ER stress causes PCD, which results in premature rice endosperm development and, in turn, lower grain weight and size.

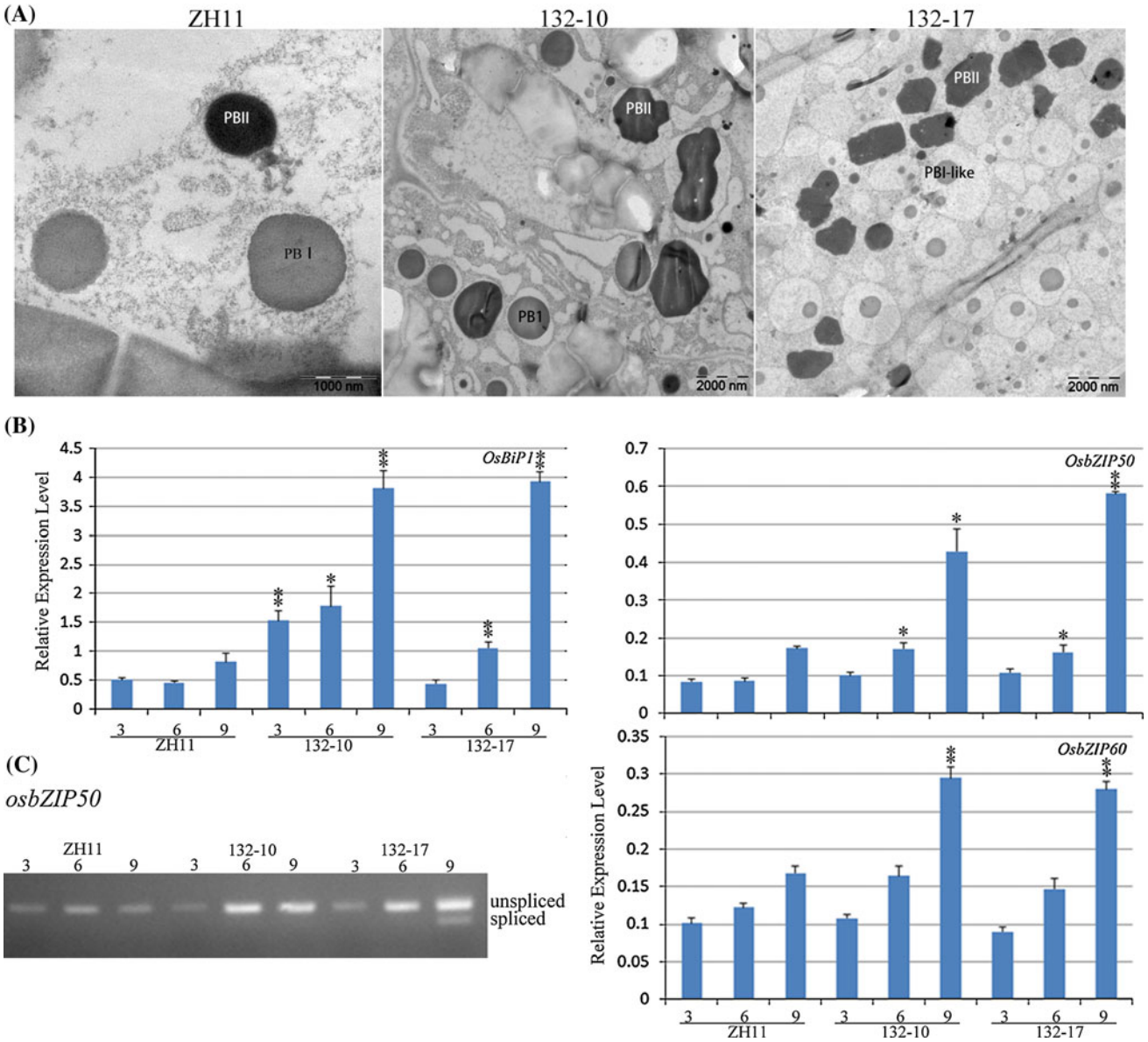

Fig. 4 Micrographs and the expression profiles of ER stress-related genes. **a** Transmission electron microscopic (TEM) observation of the developing endosperm from the ZH11, 132-10 and 132-17. **b** The expression profiles of *Osbip1*, *Osbzip50* and *Osrbzip60* at different stages of seed development. *Osbip1*, *Osbzip50* and *Osrbzip60* mRNA expression increased in both 132-10 and 132-17 at 3, 6, and 9 DAP. **c** The expression of the splicing product of *Osbzip50* at the different stages of seed development in ZH11, 132-10 and 132-17. Spliced-*Osbzip50* was observed in 132-17 at 9 DAP, but fail to observe in ZH11 and 132-10, indicating that severe ER stress happened in 132-17

Discussion

Aberrant phenotypes associated with the high expression of a recombinant protein in cereal crops have been reported previously (Wakasa et al. 2011; He et al. 2011). These phenotypes include opaque endosperm, abnormal vacuole morphology and the upregulation of molecular chaperones (Wakasa et al. 2011, 2012). Previous studies have indicated that severe ER stress occurred in the rice endosperm (Wakasa et al. 2010, 2012). However, the molecular mechanism connecting ER stress and these aberrant phenotypes remains to be determined. We observed that transgenic rice with high OsrAAT expression showed an opaque endosperm and reduced grain weight, length, width and thickness, but the lower OsrAAT expression in the endosperm of 132-10 indicated the almost normal seed phenotype with relative reduction of grain weight, length, width and thickness. These grain phenotypes were highly correlated to the level of OsrAAT expression in the rice endosperm. Further analysis revealed that ER stress and PCD during rice seed development were linked to the OsrAAT accumulation levels. Premature endosperm PCD in these transgenic lines, which

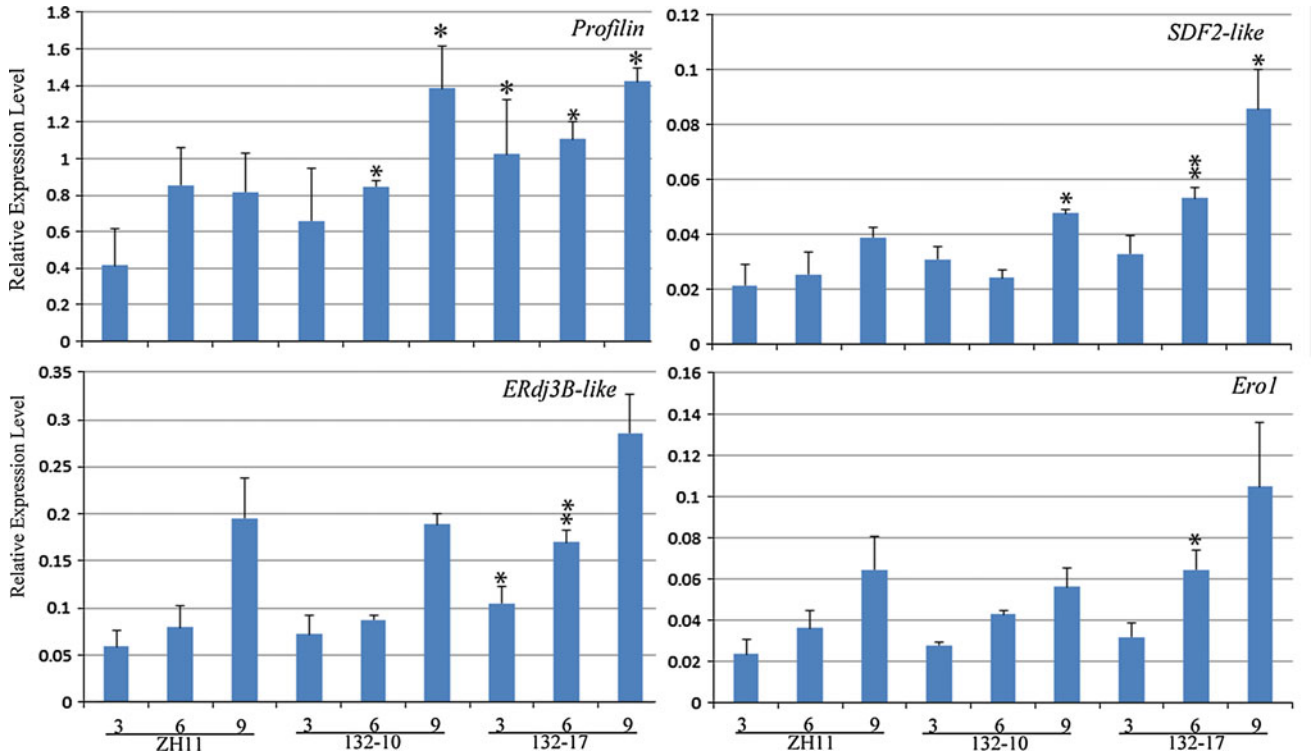

Fig. 5 The expression profiles of PCD-related genes at *3*, *6*, and *9* DAP seeds. *Profiin, SDF2-like, ERdj3B-like* and *Ero1* mRNA expression levels were monitored by quantitative-PCR in 132-10 and 132-17 at *3*, *6*, and *9* DAP (*$P < 0.05$; **$P < 0.01$). *Profiin* and *SDF2-like* mRNA expression increased in 132-10 and 132-17, but the expression of *ERdj3B-like* and *Ero1* only increased in 132-17

directly caused the decrease in grain weight, was confirmed by the TUNEL assay.

In general, the compensatory mechanism involves in hemostasis of the total protein contents in rice seeds for supplying the seed germination (Kawakatsu and Takaiwa 2010; Kawakatsu et al. 2010). In our case, when OsrAAT highly accumulated in rice endosperm, endogenous storage protein expression was significantly reduced. These changes could be explained by compensatory mechanisms to homeostatic total proteins contents in rice seed (Kawakatsu and Takaiwa 2010; Kawakatsu et al. 2010). The difference in the gene expression related to the ER stress in 132-10 and 132-17 reflected the different responding to different accumulating levels of OsrAAT in rice endosperm cell. To response the ER stress, the cells have evolved the complex network of signaling to activate the multiple cellular responses to alleviate ER stress (Hetz 2012). Among them, the two waves of cellular responses could influence the protein accumulation, including the activation of PERK to inhibit the general protein expression through phosphorylation of eIF2α and the selective degradation of mRNA encoding for certain ER-located proteins by regulated *IRE1*-dependent decay (RIDD).

The splicing of the *Osbzip50* gene is known to be induced by ER stress (Hayashi et al. 2011), which was always induced by treatment with ER-inducible agents, such as dithiothreitol (DTT) or tunicamycin (TM). We observed the splicing of the *Osbzip50* transcript in rice endosperm with high OsrAAT expression, suggesting that the *IRE1–Osbzip50* signaling pathway was activated by the ER stress in the rice endosperm cells. However, PCD induced by the ER stress was independent to the activation of the *IRE1–Osbzip50* signaling pathway. The morphological changes in the storage vacuoles indicated that the accumulation of recombinant protein in the rice endosperm cells generated a crowded environment in the ER lumen. Cells have evolved complex signaling networks to activate multiple cellular responses to alleviate ER stress (Hetz 2012). These cellular responses included the upregulation of the ER stress response genes; the decrease in endogenous storage protein expression and accumulation; the activation of PERK to inhibit general protein expression through the phosphorylation of eIF2α and the selective degradation of mRNAs encoding certain ER-localized proteins via the regulation of *IRE1*-dependent decay (RIDD).

Previous research indicated that PCD is involved in maize, wheat and rice endosperm development (Young et al. 1997; Berger 1999; Wei et al. 2002). As a consequence of PCD, the cellular structures in mature rice starchy endosperm completely disappear, leaving the protein bodies and starch granules. In transgenic lines with high OsrAAT expression, the grain size and weight were largely reduced,

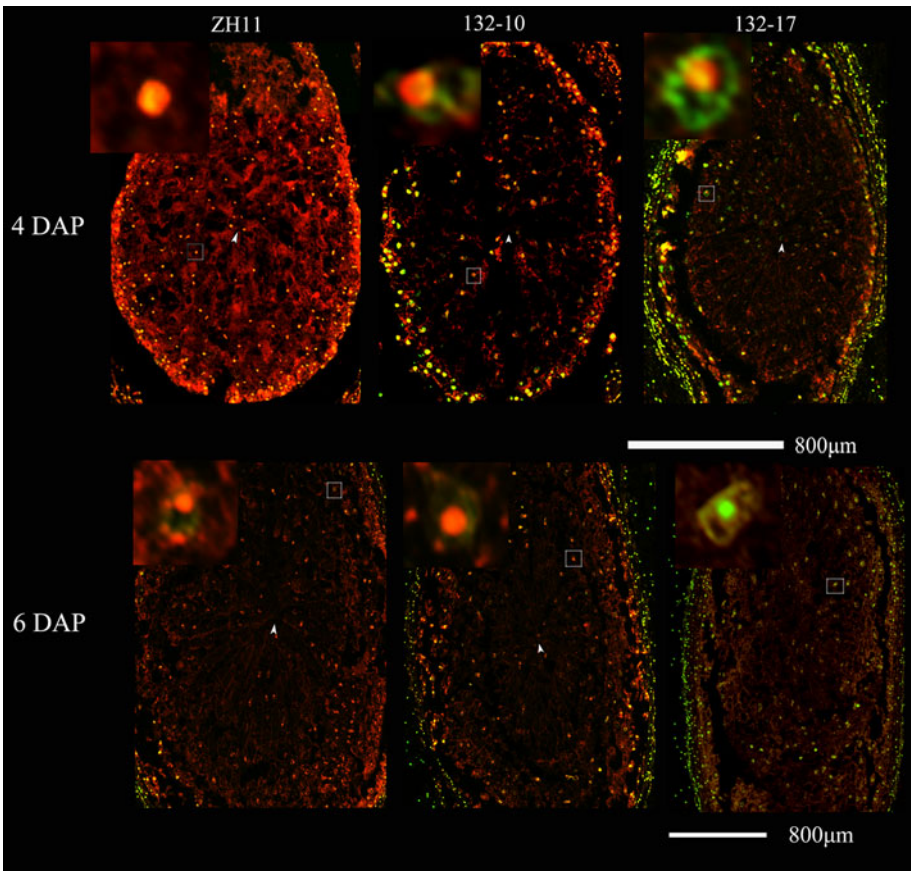

Fig. 6 PCD signals in rice seed development cells were monitored by TUNEL assay at *4* and *6* DAP. The *open arrows* indicate the central points. The *top panel* is a higher magnification image of the region corresponding to the *rectangular frame* in the *lower panel*. PCD signals in 132-17 were stronger than that of ZH11 and 132-10 at 2, 4 DAP. The *central points* were observed in ZH11 and 132-10, but disappeared in 132-17 at 6 DAP. The *red signal* indicates DNA, and the *green signal* shows DNA fragmentation

and an opaque endosperm developed. We found that the genes involved in PCD were significantly upregulated in these lines. Furthermore, the TUNEL assay indicated that PCD in the endosperm occurred earlier in the transgenic lines than in non-transgenic lines. The main consequence of pre-mature PCD was an earlier termination of endosperm development, leading to decreases in grain size and weight. This premature PCD has also been found to be involved in *indica–japonica* hybrid sterility (Yang et al. 2012). These results provided evidence supporting that ER stress induced pre-mature PCD in transgenic rice endosperm, which results in the detrimental seed phenotype.

Acknowledgments We are grateful to Dr. Masahiro Ogawa for kindly providing the prolamin antibody. This work was supported by the National Scientific Foundation (No. 30671286), the National High-tech R&D Program (863 Program) of China (No. 2011AA100604) and the Major Projects of Genetically Modified Crop of China (No. 2011ZX08001-006).

References

Berger F (1999) Endosperm development. Curr Opin Plant Biol 2(1):28–32

Brown RC, Lemmon BE, Olsen OA (1996) Development of the endosperm in rice (*Oryza sativa* L.): cellularization. J Plant Res 109(3):301–313

Cacas JL (2010) Devil inside: does plant programmed cell death involve the endomembrane system. Plant Cell Environ 33(9):1453–1473

Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 415(6867):92–96

Guiboileau A, Sormani R, Meyer C, Masclaux-Daubresse C (2010) Senescence and death of plant organs: nutrient recycling and developmental regulation. Comptes Rendus Biol 333(4):382–391

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 6(5):1099–1108

Hayashi S, Wakasa Y, Takahashi H, Kawakatsu T, Takaiwa F (2011) Signal transduction by IRE1-mediated splicing of bZIP50 and other stress sensors in the endoplasmic reticulum stress response of rice. Plant J 96:946–956

Hayashi S, Wakasa Y, Takaiwa F (2012) Functional integration between defence and IRE1-mediated ER stress response in rice. Sci Rep 2(670):1–5

He Y, Ning T, Xie T, Qiu Q, Zhang L, Sun Y, Jiang D, Fu K, Yin F, Zhang W, Shen L, Wang H, Li J, Lin Q, Sun Y, Li H, Zhu Y, Yang D (2011) Large-scale production of functional human serum albumin from transgenic rice seeds. Proc Natl Acad Sci USA 108(47):19078–19083

Hetz C, Martinon F, Rodriguez D, Glimcher LH (2011) The unfolded protein response: integrating stress signals through the stress sensor IRE1α. Physiol Rev 91(4):1219–1243

Hetz C (2012) The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 13(2):89–102

Howell SH (2013) Endoplasmic reticulum stress responses in plants. Annu Rev Plant Biol. doi:10.1146/annurev-arplant-050312-120053

Iwata Y, Koizumi N (2012) Plant transducers of the endoplasmic reticulum unfolded protein response. Trends Plant Sci 17(12):720–727

Jin Y, Zhuang M, Hendershot LM (2008) ERdj3, a luminal ER DnaJ homologue, binds directly to unfolded proteins in the mammalian ER: identification of critical residues†. Biochemistry 48(1):41–49

Kawakatsu T, Takaiwa F (2010) Differences in transcriptional regulatory mechanisms functioning for free lysine content and seed storage protein accumulation in rice grain. Plant Cell Physiol 51(12):1964–1974

Kawakatsu T, Hirose S, Yasuda H, Takaiwa F (2010) Reducing rice seed storage protein accumulation leads to changes in nutrient quality and storage organelle formation. Plant Physiol 154(4):1842–1854

Kobayashi H, Ikeda TM, Nagata K (2013) Spatial and temporal progress of programmed cell death in the developing starchy endosperm of rice. Planta. doi:10.1007/s00425-013-1854-8

Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, Yoshida H, Mori K, Kaufman RJ (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. Genes Dev 16(4):452–466

Lim DH, Kim TS, Joo SP, Kim SH (2007) Intracerebral hematoma caused by ruptured traumatic pseudoaneurysm of the middle meningeal artery: a case report. J Korean Neurosurg Soc 42(5):416–418

Logue SE, Cleary P, Saveljeva S, Samali A (2013) New directions in ER stress-induced cell death. Apoptosis. doi:10.1007/s10495-10013-10818-10496

Luo J, Ning T, Sun Y, Zhu J, Zhu Y, Lin Q, Yang D (2008) Proteomic analysis of rice endosperm cells in response to expression of hGM–CSF. J Proteome Res 8(2):829–837

Meunier L, Usherwood YK, Chung KT, Hendershot LM (2002) A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins. Mol Biol Cell 13(12):4456–4469

Olsen OA, Potter R, Kalla R (1992) Histo-differentiation and molecular biology of developing cereal endosperm. Seed Sci Res 2(03):117–131

Olsen OA, Brown R, Lemmon B (1995) Pattern and process of wall formation in developing endosperm. BioEssays 17(9):803–812

Olvera-Carrillo Y, Salanenka Y, Nowack MK (2012) Control of programmed cell death during plant reproductive development. Biocommun Plants 14(2012):171–196

Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 8(7):519–529

Schroder M, Clark R, Kaufman RJ (2003) IRE1- and HAC1-independent transcriptional regulation in the unfolded protein response of yeast. Mol Microbiol 49(3):591–606

Steedman HF (1957) Polyester wax: a new ribboning embedding medium for histology. Nature 179(4574):1345

van Doorn WG, Woltering EJ (2008) Physiology and molecular biology of petal senescence. J Exp Bot 59(3):453–480

Vitha S, Baluska F, Mews M, Volkmann D (1997) Immunofluorescence detection of F-actin on low melting point wax sections from plant tissues. J Histochem Cytochem 45(1):89–95

Wakasa Y, Yasuda H, Oono Y, Kawakatsu T, Hirose S, Takahashi H, Hayashi S, Yang L, Takaiwa F (2010) Expression of ER quality control-related genes in response to changes in BiP1 levels in developing rice endosperm. Plant J 65(5):675–689

Wakasa Y, Yasuda H, Oono Y, Kawakatsu T, Hirose S, Takahashi H, Hayashi S, Yang L, Takaiwa F (2011) Expression of ER quality control-related genes in response to changes in BiP1 levels in developing rice endosperm. Plant J 65(5):675–689

Wakasa Y, Yasuda H, Takaiwa F (2012) Secretory type of recombinant thioredoxin *h* induces ER stress in endosperm cells of transgenic rice. J Plant Physiol 170(2013):202–210

Wang W, Liu Z, Guo Z, Song G, Cheng Q, Jiang D, Zhu Y, Yang D (2011) Comparative transcriptomes profiling of photoperiod-sensitive male sterile rice Nongken 58S during the male sterility transition between short-day and long-day. BMC Genomics 12(1):462

Wei CX, Lan SY, Xu ZX (2002) Ultrastructural features of nucleus degradation during programmed cell death of starchy endosperm cells in rice. Acta Bot Sin Chin Ed 44(12):1396–1402

Woehlbier U, Hetz C (2011) Modulating stress responses by the UPRosome: a matter of life and death. Trends Biochem Sci 36(6):329–337

Xie T, Qiu Q, Zhang W, Ning T, Yang W, Zheng C, Wang C, Zhu Y, Yang D (2008) A biologically active rhIGF-1 fusion accumulated in transgenic rice seeds can reduce blood glucose in diabetic mice via oral delivery. Peptides 29(11):1862–1870. doi:10.1016/j.peptides.2008.07.014

Yang D, Guo F, Liu B, Huang N, Watkins SC (2003) Expression and localization of human lysozyme in the endosperm of transgenic rice. Planta 216(4):597–603

Yang J, Zhao X, Cheng K, Du H, Ouyang Y, Chen J, Qiu S, Huang J, Jiang Y, Jiang L (2012) A killer–protector system regulates both hybrid sterility and segregation distortion in rice. Science 337(6100):1336–1340

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107(7):881–892

Young TE, Gallie DR (1999) Analysis of programmed cell death in wheat endosperm reveals differences in endosperm development between cereals. Plant Mol Biol 39(5):915–926

Young TE, Gallie DR, DeMason DA (1997) Ethylene-mediated programmed cell death during maize endosperm development of wild-type and shrunken2 genotypes. Plant Physiol 115(2):737–751

Zhang L, Shi J, Jiang D, Stupak J, Ou J, Qiu Q, An N, Li J, Yang D (2013) Expression and characterization of recombinant human alpha-antitrypsin in transgenic rice seed. J Biotechnol. doi:10.1016/j.jbiotec.2013.01.008

Zuppini A, Navazio L, Mariani P (2004) Endoplasmic reticulum stress-induced programmed cell death in soybean cells. J Cell Sci 117(12):2591–2598