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ORIGINAL PAPER

Do rice suspension-cultured cells treated with abscisic acid mimic developing seeds?

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Abstract Starch synthesis is activated in the endosperm during seed development and also in rice suspension cells cultured with abscisic acid. In the anticipation that the mechanisms of starch synthesis are similar between the endosperm and the suspension cells cultured with abscisic acid, expression of genes involved in starch synthesis was evaluated in the suspension cells after abscisic acid treatment. However, it was found that the regulatory mechanism of starch synthesis in the suspension cells cultured with abscisic acid was different from that in developing seeds. Expression analyses of genes involved in oil bodies, which accumulate in the embryo and aleurone layer, and seed storage proteins, which accumulate mainly in the endosperm, showed that the former were activated in the suspension cells cultured with abscisic acid, but the latter were not. Master regulators for embryogenesis, *OsVP1* (homologue of *AtABI3*) and *OsLFL1* (homologue of *AtFUS3* or *AtLFL2*), were expressed in the suspension cells at levels comparable to those in the embryo. From these results, it is suggested that interactions between regulators and abscisic acid control the synthesis of phytic acid and oil bodies in the cultured cells and embryo. We suggest that the system of suspension cells cultured with abscisic acid helps to reveal the mechanisms of phytic acid and oil body synthesis in embryo.

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Introduction

Starch, proteins and lipids which are stored in plant seeds are used as nutrient and energy source for human beings. Phytic acid, which is a storage compound of phosphorus, is known as a source of phosphorus pollution in water environment. To control their amount, it is informative to understand regulatory mechanisms of their synthesis.

Starch accumulates in the starchy endosperm from approximately 5 days after flowering (DAF) in rice (Jeon et al. 2010; Zhu et al. 2011). The weight of starch increases in parallel with increasing grain weight and eventually accounts for approximately 75 % of grain weight (Zhu et al. 2011). Seed storage proteins accumulate from 4 DAF and are classified into insoluble glutelin, alcohol-soluble prolamin, salt-soluble globulin and water-soluble albumin (Yamagata et al. 1982; Luthe 1983). The protein content of milled rice is approximately 8 %, and most of it is glutelin in the starchy endosperm (Villareal and Juliano 1978; Shewry and Halford 2002; Agboola et al. 2005). Seed storage proteins also accumulate in the embryo and aleurone layer, but these proteins are different from endosperm proteins (Cagampang et al. 1966). Lipids are stored from approximately 5 DAF as oil bodies consisting of triacylglycerols (TAGs) and surface proteins, oleosin and caleosin, in the embryo and aleurone layer (Choudhury and Juliano 1980; Chuang et al. 1996; Frandsen et al. 2001; Ichihara et al. 2003; Chen et al. 2012). Phytic acid, *myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, is a storage form of phosphorus and accumulates in the embryo and aleurone layer (O'Dell et al. 1972; Ogawa et al. 1979; Iwai et al. 2012).

A previous study using an antibody to abscisic acid (ABA) for inactivating it showed that the synthesis of seed storage proteins and lipids during seed development is affected by ABA activity (Phillips et al. 1997). Moreover, ABA is involved in conferring desiccation tolerance, dormancy and suppression of viviparous phenotype in developing seeds and stress response in vegetative tissues (Finkelstein et al. 2002; Gutierrez et al. 2007; Nakashima and Yamaguchi-Shinozaki 2013). Interactions between ABA and *VP1/ABI3* or *FUS3* induce the expression of seed storage proteins in *Arabidopsis* (Suzuki et al. 2003; Kagaya et al. 2005). Accumulation of starch during seed development positively correlates with accumulation of ABA (Yang et al. 2006; Tang et al. 2009). The expression of sucrose synthase, which is considered as a key enzyme of starch synthesis in developing seeds, is regulated by ABA and sucrose (Tang et al. 2009).

Starch synthesis was activated in suspension cells of rice cultured with ABA and sucrose (Akihiro et al. 2005, 2006). Rice cells cultured with ABA also activate phytic acid synthesis similar to that in developing seeds as the expression of genes involved in phytic acid synthesis in developing seeds is also up-regulated in cells cultured with ABA (Matsuno and Fujimura 2014). These results suggest that the suspension cells react with ABA in the same manner as developing seeds.

In the present study, starch synthesis in the suspension cells was investigated in detail to test this hypothesis and showed that starch content in suspension-cultured cells increased with increased concentrations of ABA. The expression analysis of genes involved in starch synthesis in suspension cells cultured with ABA indicated that the regulation of starch synthesis in the suspension cells was different from that in the endosperm. The expression analysis of the gene involved in synthesis of lipids and storage proteins showed that the former was activated in the suspension cells cultured with ABA without activation of the latter. Higher expression levels of master regulators of embryogenesis was observed, suggesting interactions between the master regulators and ABA-activated phytic acid and storage oil synthesis.

Materials and methods

Cultured cells

Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) suspension cells were induced and cultured as described previously (Matsuno and Fujimura 2014). Rice seeds were sterilised by immersion in 70 % ethanol for 1 min and then in sodium hydrochloride (5 % available chloride) for 20 min. After five rinses with sterile deionised water, the seeds were placed on a solid N6 medium (Chu et al. 1975) supplemented with sucrose (30 g/L), proline (10 mM), casein hydrolysate (300 mg/L) and 2,4-dichlorophenoxyacetic acid (2 mg/L) and were incubated at 28 °C for 3 weeks. Friable calli formed were transferred to 15 mL of a liquid N6 medium in a 100-mL flask. The calli were cultured in the dark at 28 °C on a reciprocal shaker (115 strokes per min), and the resulting suspension-cultured cells were subcultured every 5 days.

Cells cultured were passed through a 1.5-mm mesh to remove larger cell masses and then cultured in a fresh medium for 2 days. They were then transferred to a fresh medium after another sieving through a 1.75-mm mesh and cultured for 24 h to minimise the transfer effect. ABA was then added to the medium, and the culture was harvested after various intervals by filtration under reduced pressure. The cells were desiccated at 80 °C to measure starch contents or frozen in liquid nitrogen and stored at −80 °C for gene expression analyses.

Plant materials

Rice plants were grown in the field at the Agricultural and Forestry Research Center (University of Tsukuba, Ibaraki, Japan) under natural environmental conditions from May to September, 2012. Seeds were harvested 7 DAF, immersed in RNAlater (Ambion®) and kept at 4 °C. Dehulled seeds were separated into three parts: embryo, starchy endosperm and aleurone layer. The embryo was first isolated with a pair of tweezers. The seed coat, its inner part rinsed with RNAlater, was harvested as the aleurone layer. The rinse containing the inner part of the seed coat was harvested as the starchy endosperm.

Another rice plant was cultivated hydroponically with Yoshida nutrient solution (Yoshida et al. 1976) in a growth chamber (16 h light at 27 °C). At the six-leaf stage, the fifth leaf was harvested and stored at −80 °C for gene expression analyses.

Quantitative and semi-quantitative RT-PCR

Total RNA was extracted from the suspension cells with the Isogen® reagent (Nippon Gene), from the embryo, the aleurone layer or fifth leaf with the RNeasy Plant Mini Kit (Qiagen) or from the endosperm with the Isogen® reagent and High salt precipitation solution (Nippon Gene). The RNAs were then reverse transcribed using the PrimeScript® RT Master Mix (Takara Bio) following the manufacturer's instructions after treatment with DNase I.

Expression levels of genes (Tables 1, 2) were quantified by real-time PCR (RT-PCR) using the SYBR® Premix Ex Taq™ II (Takara Bio) and the Thermal Cycler Dice® Real-Time System (Takara Bio). The standard curve method or the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was applied,

Table 1 Primers for qPCR of genes involved in starch synthesis

Enzyme	Gene	Acc. no.	Primer (5′ → 3′)	
			F	*R*
ADP-glucose pyrophosphorylase large subunit 1	*OsAGPL1*	AK100910	CGAAACGCCAAACGAAACGAG	AAGAAGGCAAACGGGAACGAG
ADP-glucose pyrophosphorylase large subunit 2	*OsAGPL2*	AK071497	CGGGACCGTCATATAAAAGC	ACGCTATCCGTCTTGAATCC
ADP-glucose pyrophosphorylase large subunit 3	*OsAGPL3*	AK069296	AAACAGCGGCATCAACAAGG	CTCGACGAATCCATCTCCAAAC
ADP-glucose pyrophosphorylase large subunit 4	*OsAGPL4*	AK121036	TCAATCCCTTCTGGTTTGTTGC	TCCCTCAGTTCCTAGCCTCATTC
ADP-glucose pyrophosphorylase small subunit 1	*OsAGPS1*	AK073146	TTCACCAAGCTCTGCATCAAC	CAGACACGGTTTTCGGACTTAC
ADP-glucose pyrophosphorylase small subunit 2a	*OsAGPS2a*	AK071826	ACTCCAAGAGCTCGCAGACC	GCCTGTAGTTGGCACCCAGA
ADP-glucose pyrophosphorylase small subunit 2b	*OsAGPS2b*	AK103906	ATCTTCCCTTCCCGCTCCAA	CAAGCACACTCTCATCGACAC
Starch debranching enzyme: isoamylase I	*OsISA1*	Os08g0520900	TCCGCTGGGACAAGAAAGAAG	ACCGTTGAGCTGTTGGGAAG
Starch debranching enzyme: Isoamylase II	*OsISA2*	AK288307	GGGCTTTCTCTTGTTGATCTGG	TTGCGTATCTGCCTTAGCCTTG
Starch debranching enzyme: isoamylase III	*OsISA3*	AK101554	AAATGTTGGAACCGTGTGGTG	TGCCTTGAGCAGAATGGAAGAG
Starch debranching enzyme: pullulanase	*OsPUL*	Os04g0164900	TGGGTGACAAGGTCTCTGATTG	CATCGTGGCATCTCTGCTTG
Starch phosphorylase H	*OsPHOH*	AK103367	AATGATGTTGGTGCTGTGGTG	TGCGGTACTGATGTGCTGTG
Starch phosphorylase L	*OsPHOL*	AK063766	TGGCAGGAAGGTTTCGCTTTG	AGAGGCTGCTACACATTACAGG
Granule-bound starch synthase I	*OsGBSSI*	AK070431	CTGCTTCACTTCTCTGCTTGTG	TGGTTGTCTAGCTGTTGCTGTG
Granule-bound starch synthase II	*OsGBSSII*	AK067654	ACCGGCAAAGCCACTATTTG	ACATGCAGAAGGAGCGAGAG
Starch branching enzyme I	*OsBEI*	AK119436	GCACTCACACAATGACACAAAAC	TCCCTCTCACCAATCACCAC
Starch branching enzyme IIa	*OsBEIIa*	Os04g0409200	AAGAGAAGGTGGCGTTGTGAG	GTTCCGCAGAGGACAGCAAG
Starch branching enzyme IIb	*OsBEIIb*	AK108535	AGCACTTCACTGCCGATTGTTC	TGCCTCTTGGTGTTCTCATTCC
Disproportionating enzyme I	*OsDPE1*	Os07g0627000	AATCCACACTTGCCCCATAACC	TTGCCACCAACCAATAACCG
Disproportionating enzyme II	*OsDPE2*	AK067082	ATCTCTGGCGTCAAAATGCG	TGCATAACAGGGTGAACACAGG
Starch synthase I	*OsSSI*	AK109458	ATACCACGCAAGCAACCTCTC	AGAACAGCACAGGCGACAAAG
Starch synthase IIa	*OsSSIIa*	AK101978	CGAGGCATGGTGGAGTTGAG	CGCATCAATGGACATAAC AAACAC
Starch synthase IIb	*OsSSIIb*	AK066446	TGCCGTTGCGAAGCAAAATG	TTCAGCCTTGGAAGCAGGAAC
Starch synthase IIc	*OsSSIIc*	AK072339	ACATTTATGGCGGAGACCGAAC	TAGCCACCACAAGGAACATACC
Starch synthase IIIa	*OsSSIIIa*	AK061604	GTGGGAGTATGTATCAGCTA TGTTTC	ATAGCCATAGTCCAGATGTAGCG
Starch synthase IIIb	*OsSSIIIb*	AK122098	CGGCATCCCAGTACATACCC	ACAACCGCAGGATAACGGAAAG
Starch synthase IVa	*OsSSIVa*	AK104176	CGGCAAGTGAGTGTGGATCTC	ACACAATCTGAACGAACGAAGC
Starch synthase IVb	*OsSSIVb*	AK067577	ACGGGAAATGGTCAGGAAAAGG	CTGCTGCTATGTGGATGATGTG

Genes in this list were obtained from Ohdan et al. (2005). Primers were designed with Primer3Plus (http://www.bioinformatics.nl/primer3plus)

and the rice actin 1 gene (*OsACT1*, AK100267) or ubiquitin 5 gene (*OsUBQ5*, AK061988) (Jain et al. 2006) was amplified with 5′-CCCAAGGCCAATCGTGAGAAG-3′ and 5′-ACCATCACCAGAGTCCAACACAA-3′ or 5′-AAGG AAGGAGGAGGAAATCG-3′ and 5′-GGGCATCACAATC TTCACAG-3′, respectively, and used as an internal control. Semi-quantitative RT-PCR was performed with Tks Gflex® DNA Polymerase (Takara Bio) for 35 cycles. *OsACT1* and *OsUBQ5* were used as internal controls.

Measurement of starch content

An aliquot of desiccated and ground cells was immersed in 80 % (v/v) ethanol at 82 °C for 5 min, and then the

Table 2 Primers for PCR and qPCR

Gene	Acc. no.	Primer (5′ → 3′)	
		F	*R*
OsVP1	Os01g0911700	AGTTGTGCATGTGAATCGTAGCG	CGACGACACACTACAGGGGTT
OsLFL1	Os01g0713600	ATGGACATGGCAAACACTCTGAAC	ACTTTGGGTTGGGAATCAAGGAATG
OsLEC1A	Os06g0285200	GGCCCCAACTATGAGTAAAAAC	TGCTAATCGATCGACAGACG
OsLEC1B	Os02g0725700	AGCATCCGTTCGGATACAAG	TGGTTCAAGTTAGCCAGTTGC
OsWRI1a	Os11g0129700	GCTTCTGTAGGCGTTCTGTTC	TGCCTTTGGGTTTCTAGGAG
OsWRI1b	Os12g0126300		
OsOLE18	Os03g0699000	CCAAGACATCCTCGTAAGCAC	ACTACGAAGCGAAGCGAAAC
OsOLE16	Os04g0546500	TCTGCTGAGCTCTGTAAATGC	AAACACGACATCCACCAGAC
OsCAL32	Os04g0510900	TGTGAGATCGAGCCTGTGAC	TTACAAGCAGACACGACACG
RPBF	Os02g0252400	TACTACGCGCCTCTCATCAC	ATGCTGCATCAGAGAACCAG
OsbZIP58	Os07g0182000	AGCTTCATGCACCAGGTTTC	GCCACCACAAGCTCTATTCTG
RSR1	Os05g0121600	TGGACCTGGGAGTTTTGTTC	AGATCACCTTTCCGTCCAAG
OsGluB-1a	Os02g0249800	ATGCGTATCGCATCTCAAGG	TCTCGCTTTCGGACTCATTC
OsGlb-1	Os05g0499100	TTAGCTGGCTTGCCTCATAG	TGTTTTGATCACTATCTCGTTGC

Primers were designed with Primer3Plus (http://www.bioinformatics.nl/primer3plus). The primer pair for *OsWRI1a* also amplified *OsWRI1b*

supernatant was discarded to remove soluble sugars. The treatment was repeated twice. The total starch content of the cells was determined with a total starch assay kit (Megazyme International Ireland Ltd.) by the KOH method following the manufacturer's instructions.

Results

The accumulation of starch in the suspension cells cultured with ABA

The suspension cells cultured without ABA contained smaller amounts of starch. The starch content increased with increasing concentrations of ABA 24 h after ABA treatment (Fig. 1) by 1.4-, 2.7- and 2.9-fold at 0.17, 17 and 50 μM ABA, respectively, compared with that without ABA. Given that starch accumulation differed little between 17 and 50 μM ABA, 17 μM ABA was used for subsequent experiments.

Starch contents in the suspension cells cultured with 17 μM ABA increased 3.1-fold from their initial value 24 h after adding ABA (Fig. 2a) and increased to 7.1-fold by 96 h (Fig. 2a) with a temporary decrease at 90 min (Fig. 2b).

Expression analysis of genes involved in starch synthesis after adding ABA

Expression levels of genes involved in starch synthesis (Table 1) reported by Ohdan et al. (2005) were evaluated

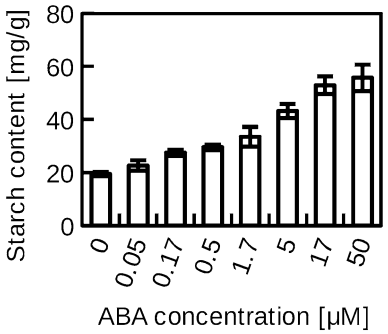

Fig. 1 Starch contents in rice cells cultured in a medium containing ABA. Cells were cultured with various concentrations of ABA (0–50 μM) for 24 h, and their starch content (mg/g dried cells) was measured. Values are means, and *error bars* represent standard deviations ($n = 4$)

in cells cultured with 17 μM ABA. *OsGBSSI* expression in the suspension cells was not detected by qPCR. Eighteen genes, Os*AGPL1*, Os*AGPL3*, Os*AGPS1*, Os*AGPS2a*, *OsAGPS2b*, Os*ISA2*, Os*ISA3*, Os*PUL*, Os*GBSSII*, Os*BEIIa*, Os*BEIIb*, Os*DPE1*, Os*DPE2*, Os*SSI*, Os*SSIIa*, *OsSSIIb, OsSSIIc* and Os*SSIVa* responded to the addition of ABA (Fig. 3). Ten of these, *OsAGPL1*, *OsAGPL3*, *OsAGPS1*, *OsAGPS2a*, *OsISA2*, *OsISA3*, *OsBEIIa*, *OsDPE1*, *OsSSI* and *OsSSIIa*, maintained higher expression levels than the control for 24 h after adding ABA, seven, *OsABS2b*, *OsPUL, OsGBSSII*, *OsBEIIb*, *OsDPE2*, *OsSSIIc* and *OsSSIVa*, increased temporarily and *OsSSIIb* decreased temporarily.

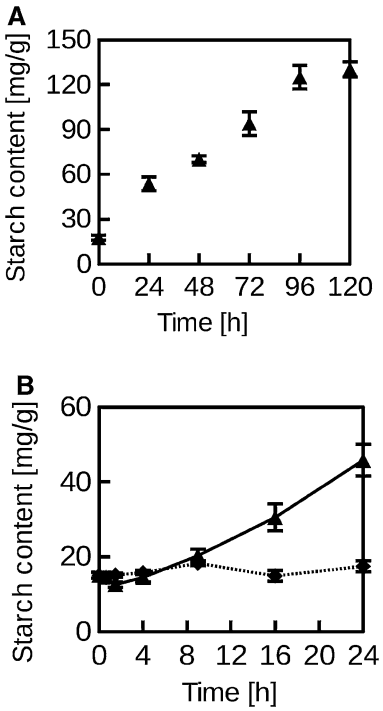

Fig. 2 Accumulation of starch in suspension-cultured cells after addition of ABA. Cells were cultured for 120 h (**a**) and 24 h (**b**) with 17 μM ABA (*solid line*) or without ABA (*dotted line*). The values are means, and *error bars* represent standard deviations; **a** $n = 4$, **b** $n = 3$

Comparison of the expression of genes involved in starch synthesis in rice suspension-cultured cells with ABA in the endosperm and in silico

Expression patterns of genes involved in starch synthesis were clustered based on the data set RXP_0001 (accession number GEO21396, Gene Expression Omnibus) and were analysed with RiceXPro (Sato et al. 2011, 2013) (NIAS, http://ricexpro.dna.affrc.go.jp/). *OsSSIIIa*, *OsBEIIb*, *OsBEI*, *OsAGPS2a/b*, *OsSSIIa*, *OsAGPL2*, *OsGBSSI*, *OsPUL* and *OsISA1* constituted a cluster, and the expression of these genes increased rapidly at 5 DAF in the ovary and occurred predominantly in the endosperm (Fig. 4, clade I). The expression pattern was spatially and temporally consistent with starch synthesis in the rice endosperm. Expression levels of those genes were evaluated with ad hoc qRT-PCR and proved higher in the endosperm than the embryo, except for *AGPS2a* (Fig. 5). Thus, it was inferred that they may play roles in starch synthesis in the endosperm. In the rice suspension cells, 10 genes, *OsAGPL1*, *OsAGPL3*, *OsAGPS1*, *OsAGPS2a*, *OsISA2*, *OsISA3*, *OsBEIIa*, *OsDPE1*, *OsSSI* and *OsSSIIa*, increased for 24 h after adding ABA (Fig. 3), suggesting that these genes play roles in activated starch synthesis in cells. Only two of these genes, *OsAGPS2a and OsSSIIa*, were included in clade I and the other eight were excluded from it. These results showed that the set of genes involved in starch synthesis activated in the cells cultured with ABA was different from the set of genes involved in starch synthesis in the endosperm.

RPBF (Kawakatsu et al. 2009), *OsbZIP58* (Wang et al. 2013) and *RSR1* (Fu and Xue 2010) have been reported as regulators of starch synthesis in the endosperm. To investigate the effects of these regulators on the activation of starch synthesis in the suspension cells cultured with ABA, their expression levels were evaluated (Fig. 6a). *OsbZIP58* was expressed at a low level in the cells, but no expression of *RPBF* was detected. *OsbZIP58* and *RPBF* were accordingly considered to be of little relevance to the activation of starch synthesis in the suspension cells. *RSR1*, which is a negative regulator, was expressed to some degree in the suspension cells, but the level decreased after ABA addition (Fig. 6a). This result raised the possibility that *RSR1* functions in the activation of starch synthesis in the suspension cells.

Expression of genes involved in seed storage proteins and lipids

As starch and phytic acid were accumulated in the suspension cells cultured with ABA, similar to developing seeds (Akihiro et al. 2005; Matsuno and Fujimura 2014), synthesis of seed storage proteins and lipids in the suspension cells was also expected to be activated by addition of ABA. However, the expression of *OsGlb-1* and *OsGluB-1a*, encoding globulin and glutelin, respectively, (Nakase et al. 1996; Kawakatsu et al. 2008) was not detected in the suspension cells with or without ABA (Fig. 6b). The expression of *OsWRI1a/b*, which is regarded as a regulator of fatty acid synthesis in rice, *OsOLE16, OsOLE18* and *OsCAL32*, encoding 16 and 18 kDa oleosins composing oil bodies (Chuang et al. 1996) and encoding 32 kDa caleosin (Chen et al. 2012), respectively, were also analysed (Figs. 6c, 7). *OsOLE16*, *OsOLE18* and *OsCAL32* were expressed clearly in the suspension cells but weakly in leaves (Fig. 7). After ABA treatment, their expression levels were strongly up-regulated in the suspension cells, and the expression of *OsWRI1a/b* was up-regulated threefold compared with that of the control.

Master regulators of seed development in the rice suspension cells

In *Arabidopsis*, four master regulators of embryogenesis, *LEC1*, *LEC2*, *FUS3* and *ABI3*, have been well investigated and are known to be involved in the synthesis of seed storage substances (Meinke et al. 1994; Gutierrez et al. 2007). To investigate the ability of master regulators to function in rice cells, expression levels of *OsLEC1A* (Xie et al. 2008), *OsLEC1B* (Xie et al. 2008), *OsLFL1* (Peng et al. 2007, 2008) and *OsVP1* (Hattori et al. 1994), homologues of *Arabidopsis* master regulators, were evaluated. The expression of

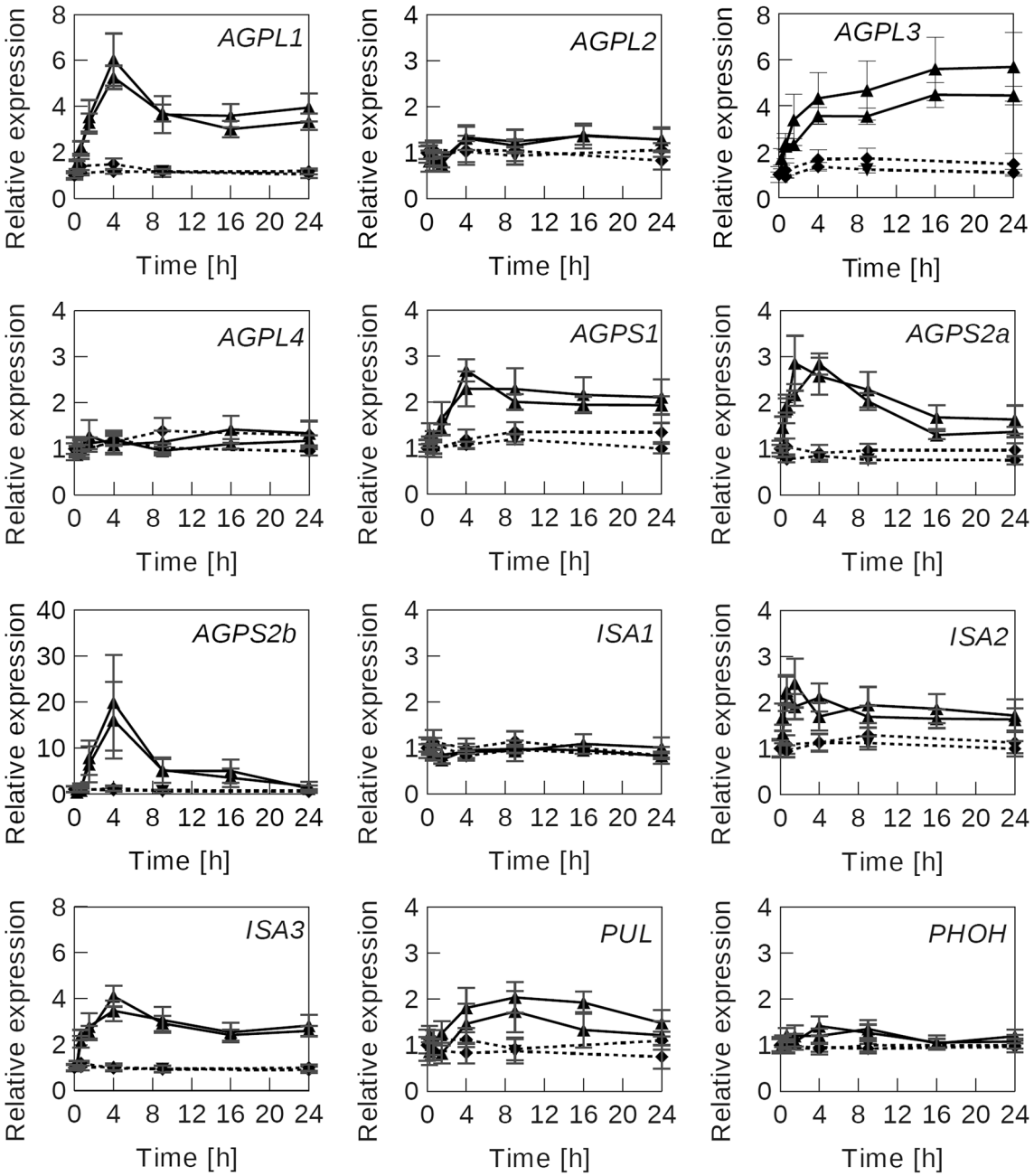

Fig. 3 Expression analysis of genes involved in starch synthesis in suspension-cultured cells after addition of ABA. The expression levels of genes were evaluated by qRT-PCR in the suspension cells cultured with 17 μM ABA (represented by *triangles* and *solid lines*) or without ABA (represented by *diamonds* and *dotted lines*). The expression levels were calculated by the standard curve method. *OsACT1* was used as an internal control, and the 0-h value was used as the reference value. Values shown are means and standard deviations of each qPCR ($n = 3$). Cultivation and sampling of cells were performed in two independent experiments for each treatment

OsLEC1A was not detected in the suspension cells (Fig. 6d). *OsLFL1* and *OsVP1* were expressed at levels comparable with those in the embryo at 7 DAF and were not altered by ABA addition in the suspension cells (Fig. 7). *OsLEC1B* was expressed at a higher level in the ABA-free medium than in the embryo, and its expression was reduced in the suspension cells by ABA addition (Fig. 7).

Discussion

In the study, starch synthesis in the suspension cells was activated by the addition of ABA as previously reported (Akihiro et al. 2005). A positive correlation between added ABA concentration and starch content was observed in the suspension cells (Fig. 1) as rice kernels matured

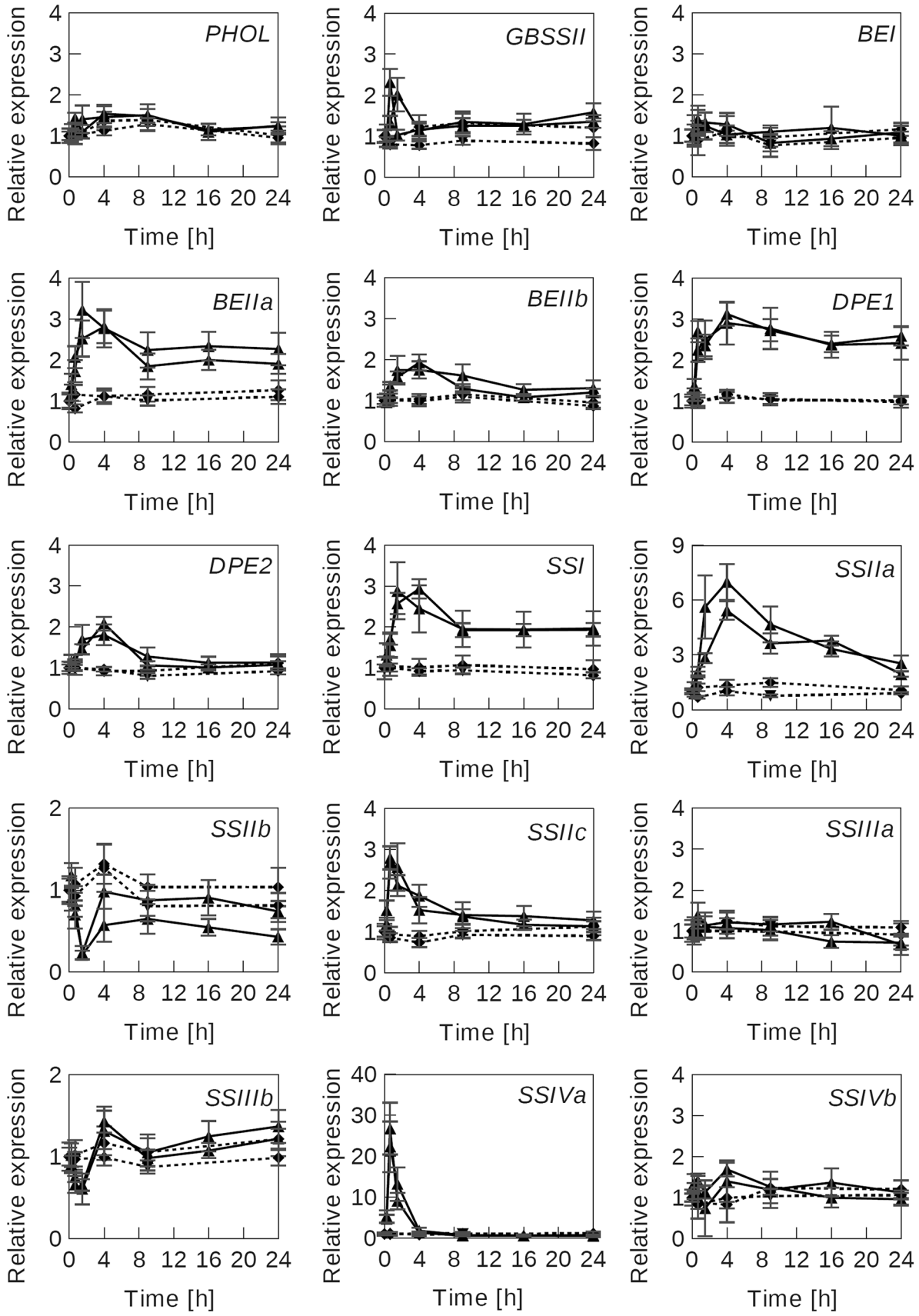

Fig. 3 continued

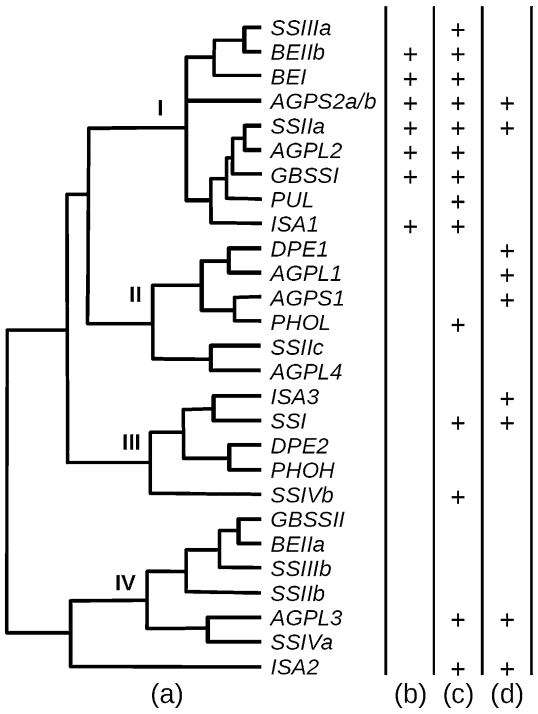

Fig. 4 Expression levels of genes involved in starch synthesis. Twenty-eight genes were subjected to clustering by RiceXPro (http://ricexpro.dna.affrc.go.jp/) based on dataset RXP_0001 to generate a dendrogram (*a*). Given that *OsAGPS2a* and *OsAGPS2b*, transcripts of the locus Os08g0345800, were not distinguishable by probes used in the microarray analysis, they are described as *OsAGPS2a/b*. Genes in clusters labelled I, II, III and IV were expressed mainly in the ovary and endosperm, ovary and embryo, embryo and leaf in vegetative stage, respectively. Genes with plus sign (+) in column (*b*) showed abnormal features in the endosperm when they were mutated (Sano et al. 1986; Kubo et al. 1999; Nishi et al. 2001; Umemoto et al. 2002; Satoh et al. 2003; Lee et al. 2007). Genes with plus sign (+) in column (*c*) were considered to function in starch synthesis in the endosperm (Ohdan et al. 2005), and genes with plus sign (+) in column (*d*) were up-regulated in the cultured cells by ABA treatment for 24 h

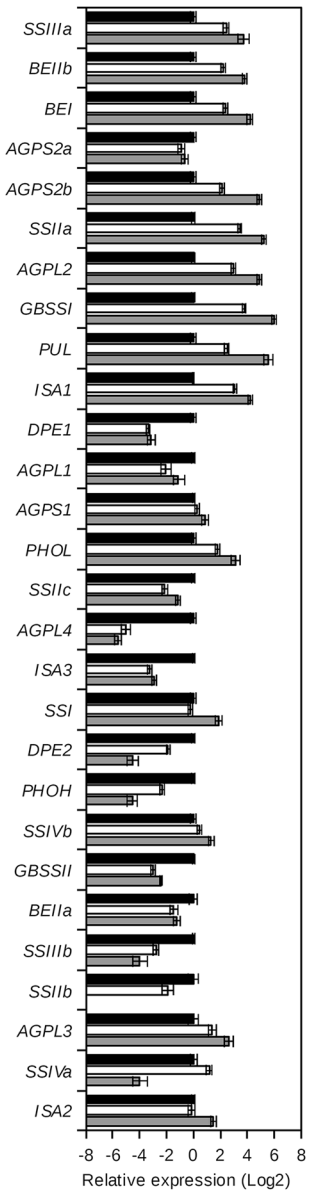

Fig. 5 qRT-PCR analysis of the expression of genes involved in starch synthesis. Immature seeds at 7 DAF were harvested and dissected into three parts: embryo (*black bars*), aleurone layer (*white bars*) and endosperm (*grey bars*). RNAs extracted were analysed by qRT-PCR with primer sets for the same genes as in Fig. 4. The expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with the references of the embryo tissue and the internal control of *OsUBQ5*. Values shown are means and standard deviations of each qPCR ($n = 3$)

(Yang et al. 2006; Tang et al. 2009). These results suggest that ABA participates in starch accumulation in both the endosperm and suspension cells.

Developing seeds begin starch accumulation rapidly from approximately 5 DAF in the endosperm and reach the maximum at approximately 21 DAF (Jeon et al. 2010; Zhu et al. 2011). *OsAGPL2* (Lee et al. 2007), *OsAGPS2b* (Lee et al. 2007), *OsSSIIa* (Umemoto et al. 2002), *OsBEI* (Satoh et al. 2003), *OsBEIIb* (Nishi et al. 2001), *OsISA1* (Kubo et al. 1999) and *OsGBSSI* (Sano et al. 1986), which are known to be involved in starch synthesis in the endosperm, constituted a gene cluster in the dendrogram (Fig. 4, cluster I). Genes in the cluster were expressed at higher levels in the endosperm than in the embryo, except for *AGPS2a* (Fig. 5), and these findings support the assumption that these genes function in starch synthesis in the endosperm (Ohdan et al. 2005). Thus, the genes in the cluster are considered to be also involved in starch synthesis in the endosperm. In the suspension cells cultured with ABA, *OsAGPL1*, *OsAGPL3*, *OsAGPS1*, *OsAGPS2a*, *OsISA2*, *OsISA3*, *OsBEIIa*, *OsDPE1*, *OsSSI* and *OsSSIIa* were expressed at higher levels than in the control during the 24 h after the addition of ABA (Fig. 3), suggesting that

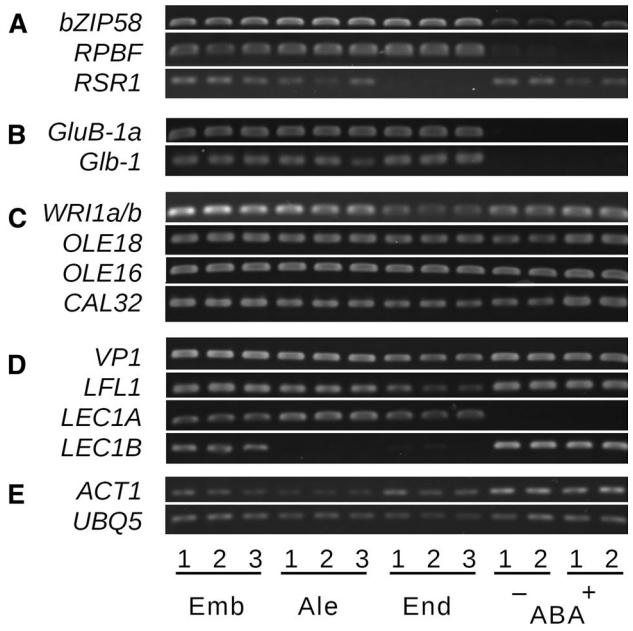

Fig. 6 Expression levels of regulatory genes in three parts of immature seeds and the suspension cells. Seeds at 7 DAF were harvested and dissected into three parts: embryo, aleurone layer and endosperm. RNAs were extracted from them and from the suspension cells cultured with (+) or without (−) 17 μM ABA for 12 h. Expression levels of regulators of starch synthesis (**a**), seed storage proteins (**b**), seed oil bodies (**c**), master regulators (**d**) and internal control (**e**) were evaluated by RT-PCR. Numbers of PCR cycles were 26 for *OsUBQ5*, 28 for *OsACT1* and 35 for others. Samples from seed parts were triplicated and from cells were duplicated

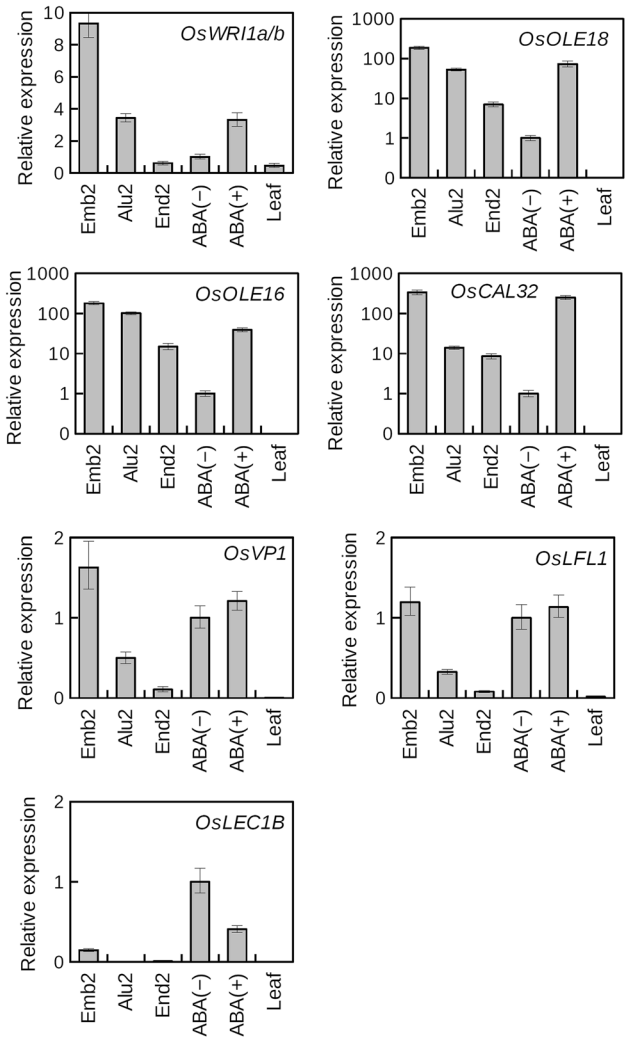

Fig. 7 Expression levels of genes in suspension-cultured cells. RNAs were extracted from three parts of immature seeds at 7 DAF: embryo, aleurone layer and endosperm, suspension cells cultured with (+) or without (−) 17 μM ABA for 12 h and the fifth leaf of young rice plants. Expression levels were evaluated by qRT-PCRs using the $2^{-\Delta\Delta Ct}$ method with the value of ABA (−) as reference. Values shown are means and standard deviations of each qPCR ($n = 3$)

these genes contributed to activated starch synthesis in the suspension cells cultured with ABA. *OsbZIP58*, which is known as a regulator of starch synthesis in the endosperm, controls gene expression involved in starch synthesis in the endosperm, and starch in the endosperm of an *Osbzip58* mutant is altered quantitatively and qualitatively (Wang et al. 2013). *OsbZIP58* is expressed specifically in the endosperm at maximum levels at 5–10 DAF (Sato et al. 2011; Wang et al. 2013). In the suspension cells, the expression of *OsbZIP58* was low and not altered by ABA treatment (Fig. 6a), suggesting that increased starch synthesis in the suspension cells was not regulated by *OsbZIP58*. Taking these results together, it is indicated that the regulatory mechanism of starch synthesis in the suspension cells cultured with ABA is different from that in the endosperm.

Most seed storage proteins are classified into glutelin and stored in the endosperm (Villareal and Juliano 1978; Yamagata et al. 1982). *OsGlb-1*, encoding 26 kDa α-globlin, and *OsGluB-1a*, encoding glutelin, are expressed specifically in the endosperm and are up-regulated by *RPBF* and *OsbZIP58* synergistically (Wu et al. 1998; Yamamoto et al. 2006; Kawakatsu et al. 2008). The expression analysis showed that the expression levels of *OsGluB-1a*, *OsGlb-1* and *RPBF* were undetected and that *OsbZIP58* expression was detected at a low level in the suspension cells (Fig. 6a, b), suggesting that the seed storage protein was not synthesised in the suspension cells.

Seed storage lipids are stored in the embryo and aleurone layer as oil bodies composed of TAGs surrounded by a single layer of phospholipids and proteins, oleosins and caleosin (Frandsen et al. 2001; Chen et al. 2012). A transcription factor, *AtWri1*, activates fatty acid synthesis and thereby activates TAG synthesis (Focks and Benning 1998; Cernac and Benning 2004; To et al. 2012). Two homologues of this factor are found in both rice and maize (Pouvreau et al. 2011). As *ZmWRI1* activates fatty

acid synthesis in maize (Shen et al. 2010), *OsWRI1a/b,* orthologue of *ZmWRI,* is presumed to activate fatty acid synthesis in rice. Two isoforms of oleosin, 16 and 18 kDa oleosins, have been detected in the embryo and aleurone layer, and 32 kDa caleosin has been detected in the embryo in rice (Chuang et al. 1996; Qu and Takaiwa 2004; Chen et al. 2012). Expression levels of *OsOLE18*, *OsOLE16* and *OsCAL32* were up-regulated by ABA treatment at levels comparable to those in the embryo (Figs. 6c, 7). As the expression of *OsWRI1a/b* was also up-regulated by ABA treatment (Figs. 6c, 7), oil body synthesis must have been activated in the suspension cells cultured with ABA.

In the rice seed, the accumulation of glutelin is mostly observed in the endosperm along with starch accumulation, and the accumulation of seed storage lipids is observed in the embryo and aleurone layer along with phytic acid accumulation. The regulation of phytic acid synthesis in the suspension cells appeared similar to its regulation in developing seeds (Matsuno and Fujimura 2014). In contrast, with respect to starch synthesis, regulatory mechanisms seemed to be different in the suspension cells and in developing seeds as mentioned above. The combined results suggest that the suspension cells mimic embryo-specific expression and ABA response (Fig. 8). The suggestion would imply the possibility that the epigenetic regulation in the suspension cells was influenced by its origin since the suspension cells was derived from embryo.

In the study, expression of *OsVP1*, a homologue of *AtABI3*, was observed at a level comparable to that in the embryo at 7 DAF (Fig. 7), a finding also reported by Nakagawa et al. (1996). VP1/ABI3 regulates gene expression and induces seed-specific ABA response (Suzuki et al. 2003). The expression of oleosin gene in the suspension cells of *Brassica napus* was activated by ABA treatment and overexpression of *ABI3* (Crowe et al. 2000). In the rice suspension cells, it is likely that highly expressed *OsVP1* (Fig. 7) enables the expression of oleosin genes as a seed-specific ABA response (Fig. 8). *OsLEC1A* and *OsLEC1B* are homologues of *AtLEC1* (Xie et al. 2008). Microarray data [RiceXPro (Sato et al. 2011)] have shown that *OsLEC1A* and *OsLEC1B* were expressed specifically in the endosperm and embryo, respectively. Our results also showed that *OsLEC1B* was expressed specifically in the embryo (Figs. 6d, 7). Given that *OsLEC1B* was expressed at high levels in the suspension cells but *OsLEC1A* was not, the suspension cells resemble the rice embryo in expression specificity (Figs. 6d, 7). *OsLFL1* has high similarity to *AtFUS3* and *AtLEC2* and is expressed in the embryo, callus, anther and pollen (Peng et al. 2007, 2008). Given that *AtLEC2* and *AtFUS3* regulates fatty acid metabolism via *AtWri1* (Baud et al. 2007; Wang and Perry 2013), *OsLFL1* also appears to regulate fatty acid metabolism through *OsWri1a/b*. The interaction of AtFUS3 with ABA regulates

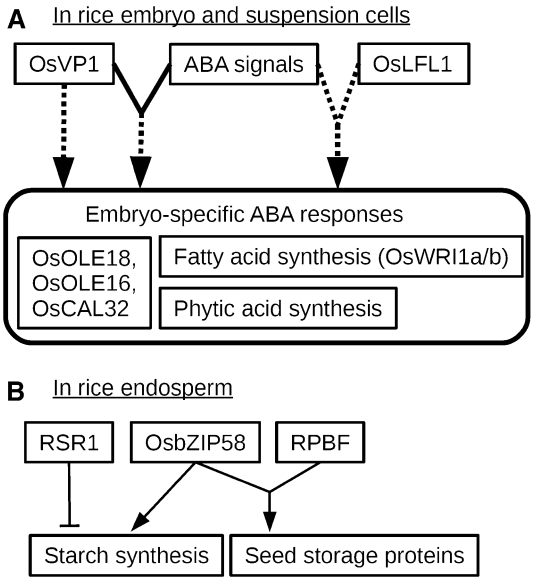

Fig. 8 Predicted regulation mechanisms for synthesis of storage substance in the immature rice seed and suspension cells. **a** Phytic acid and oil body synthesis in the rice embryo and suspension cells are presumed to be under the control of OsVP1 and OsLFL1. OsVP1 and OsLFL1 may interact with ABA signals and then regulate embryo-specific ABA responses including fatty acid synthesis and expression levels of oleosin and caleosin genes and phytic acid synthesis. **b** Starch and seed storage protein synthesis in the endosperm are under the control of RSR1, OsbZIP58 and RPBF. *Solid lines* represent demonstrated relationships (Suzuki et al. 2003; Yamamoto et al. 2006; Kawakatsu et al. 2008, 2009; Wang et al. 2013), and *dotted lines* have been assumed here

the expression of seed-specific or seed-preferential genes (Yamamoto et al. 2010). In addition to the interaction of OsVP1 and ABA, the interaction of OsLFL1 with ABA may enable the suspension cells to display a seed-specific ABA response (Fig. 8).

In the rice suspension cells, starch synthesis was activated by ABA treatment via regulatory mechanisms different from those in the endosperm. The synthesis of oil bodies was suggested to be activated by ABA treatment, but that of seed storage protein was not. The synthesis of phytic acid in the suspension cells has been reported to be activated by ABA (Matsuno and Fujimura 2014). *OsVP1* and *OsLFL1* were expressed at levels comparable to those in embryo at 7 DAF, and it was accordingly inferred that the suspension cells mimic embryos rather than the endosperm or aleurone layer in ABA response, including the synthesis of phytic acid and oil bodies. In developing seeds, a study focusing on the relationship between master regulators and synthesis of phytic acid or oil bodies is difficult because master regulators affect many aspects of early seed development including the process of embryogenesis, which is accompanied by morphological changes. Further studies using the suspension cells will clarify the regulatory

mechanism of *OsVP1* or *OsLFL1* in the synthesis of phytic acid or oil bodies.

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