Tissue-Specific Transcriptome Analysis Reveals Cell Wall Metabolism, Flavonol Biosynthesis and Defense Responses are Activated in the Endosperm of Germinating *Arabidopsis thaliana* Seeds

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Seed germination is a result of the competition of embryonic growth potential and mechanical constraint by surrounding tissues such as the endosperm. To understand the processes occurring in the endosperm during germination, we analyzed tiling array expression data on dissected endosperm and embryo from 6 and 24 h-imbibed Arabidopsis seeds. The genes preferentially expressed in the endosperm of both 6 and 24 h-imbibed seeds were enriched for those related to cell wall biosynthesis/modifications, flavonol biosynthesis, defense responses and cellular transport. Loss of function of *AtXTH31/XTR8*, an endosperm-specific gene for a putative xyloglucan endotransglycosylase/hydrolase, led to faster germination. This suggests that *AtXTH31/XTR8* is involved in the reinforcement of the cell wall of the endosperm during germination. In vivo flavonol staining by diphenyl boric acid aminoethyl ester (DPBA) showed flavonols accumulated in the endosperm of both dormant and non-dormant seeds, suggesting that this event is independent of germination. Notably, DPBA fluorescence was also intense in the embryo, but the fluorescent region was diminished around the radicle and lower half of the hypocotyl during germination. DPBA fluorescence was localized in the vacuoles during germination. Vacuolation was not seen in imbibed dormant seeds, suggesting that vacuolation is associated with germination. A gene for δVPE (vacuolar processing enzyme), a caspase-1-like cysteine proteinase involved in cell death, is expressed specifically in endosperms of 24 h-imbibed seeds. The *δvpe* mutant showed retardation of vacuolation, but this mutation did not affect the kinetics of germination. This suggests that vacuolation is a consequence, and not a trigger, of germination.

Introduction

Seed germination begins when non-dormant seeds are imbibed in a desirable condition (Finch-Savage and Leubner-Metzger 2006). This process involves the activation of embryonic

growth potential and protrusion of the radicle through surrounding tissues (Bewley 1997). The germination processes are divided into three phases based on the nature of water uptake. The first phase is characterized by rapid water uptake (Phase I). The second phase follows with relatively no water uptake (Phase II). Lastly, the third phase follows, with the outgrowth of the embryo marking the end of germination. Resumption of gene expression after seed imbibition occurs in Phase I, and stored mRNAs and proteins play a significant role in this stage (Rajjou et al. 2004, Preston et al. 2009, Kimura and Nambara 2010). Phase II is thought to be a critical period for the control of germination (Cadman et al. 2006, Carrera et al. 2007). The surrounding tissues block the embryonic growth by acting as a mechanical barrier, or inhibiting the uptake of oxygen and nutrients. Thus, coat-imposed dormancy contributes remarkably to seed dormancy in nature (Finch-Savage and Leubner-Metzger 2006).

The structure and function of the endosperm are diverse in angiosperms. Some plant species contain thick endosperm layers, whereas others have a thin or no endosperm in mature seeds. For example, the endosperm of tomato seeds is hard, thus, weakening of this tissue is a key process to complete seed germination (Nonogaki et al. 2000). On the other hand, some dicotyledonous plants such as soybean produce exalbuminous seeds, in which cellular endosperms are consumed during seed development and little or no structural endosperm remains in the mature seeds. The structure of the remaining soybean endosperms is diverse even in the same plant species (Lackey 2010). Despite reports on the anatomical, physiological and metabolic characteristics of seeds, little is known about the molecular mechanisms that regulate these differences. In this regard, genomics research on this tissue will have a great impact on elucidating how structural and functional diversities of the seeds have been established.

The Arabidopsis embryo is enveloped in the endosperm and seed coat. The seed coat is composed of the testa and tegmen, which are derived from the outer and inner ovule integuments, respectively. The Arabidopsis seed coat is composed of five cell layers at the early stage after fertilization, two of which are compressed during seed development to form four apparent layers in the mature seed (Debeaujon et al. 2000, Nakaune et al. 2005). These accumulate polysaccharides and polyphenols such as mucilage and seed pigments, respectively, during seed development and are dead cells at maturity. The transparent testa (tt) mutants have defects in flavonoid synthesis that cause abnormalities in the structure and function of the seed coat. Many tt mutants have thinner seed coats and the non-dormant phenotype is associated with the maternal genotype (Debeaujon et al. 2000). On the other hand, endosperm in mature dry seeds forms a single cell layer that is composed of living cells even during seed germination. The endosperm accumulates seed reserves such as lipids and proteins, which are degraded during and after germination to serve as a source of nutrients for both germination and early seedling growth (Penfield et al. 2004). Bethke et al. (2007) reported that

Arabidopsis endosperm is effective in inhibiting germination even though the embryo itself is non-dormant. A comparative study between Arabidopsis and cress suggests that endosperm weakening occurs in these species during germination (Müller et al. 2006). The endosperm in germinating seeds is active in gene expression and is very sensitive to ABA and gibberellins (Ogawa et al. 2003, Liu et al. 2005, Penfield et al. 2006, Bethke et al. 2007). Dissected endosperm responds to exogenous ABA and gibberellins and regulates the aggregation of protein storage vacuoles (PSVs), a cellular marker for the progression of germination (Bethke et al. 2007). In addition, genes for ABSCISIC ACID-INSENSITIVE3 (ABI3), ABI4 and ABI5, transcription factors responsible for the seed ABA responsiveness, are expressed differently between the endosperm and embryo, suggesting that this differential expression contributes to the difference in ABA responsiveness of these tissues (Penfield et al. 2006). It is known that gene expression in the endosperm responds to different environmental factors such as water, and low and high temperature (Yamauchi et al. 2004, Okamoto et al. 2006, Toh et al. 2008, reviewed in Nambara et al. 2010). Despite their dynamic responses to environmental conditions, it remains unknown whether endosperm cells perceive environmental changes in a cell-autonomous fashion or whether it is a systemic response requiring the embryo.

Transcriptome analysis enables us to take a high-resolution snapshot of the cellular status by providing comprehensive mRNA profiles. These publicly available data are often utilized as a reference in seed biology to make hypotheses, to evaluate physiological results and to identify novel gene functions. Penfield et al. (2006) reported microarray-based expression analysis in the endosperm of germinating Arabidopsis seeds at 24 h after stratification. They found that gene expression in the endosperm responds to ABA and gibberellins differently from that in the embryo when the intact seeds were treated with ABA or paclobutrazol, a gibberellin biosynthesis inhibitor. Furthermore, genes that are expressed abundantly in the endosperm are shown to be enriched for those related to seed reserve mobilization, cell wall modifications, carbon metabolism and the transport of metabolites and metals (Penfield et al. 2006). Linkies et al. (2009) performed microarray analysis on the micropylar and non-micropylar endosperms of Lepidium sativum, a close relative of Arabidopsis. This work demonstrated that the micropylar endosperm is a major target for ethylene action, by counteracting ABA. To date, we have a certain amount of knowledge on hormonal regulation and a large number of marker genes in both the embryo and endosperm; however, it remains unclear as to how these components affect the downstream cellular processes. The aim of this study is to characterize the cellular processes in the endosperm, and to link the molecular markers to downstream events. To this end, tissue-specific tiling array experiments were performed. Our analysis reveals that over-represented gene ontology (GO) categories in the genes preferentially expressed in the endosperm include those for flavonol biosynthesis, defense response and cell wall metabolism. The function of the endosperm in germination is discussed.

Results and Discussion

Our previous analysis indicated that imbibition (Phase I) of Arabidopsis seeds takes place 3–6 h after the start of seed imbibition under our growth conditions. The initial changes in gene expression and plant hormone levels occur during seed imbibition (Preston et al. 2009). Phase II is a critical checkpoint of seed germination. In this study, we performed tiling array-based expression analysis on dissected endosperm/seed coat and embryo in 6 h-imbibed seeds (the early stage of Phase II) and 24 h-imbibed seeds (the established stage of Phase II). We consider that expression data from endosperm/seed coat samples reflect the expression in the endosperm because Arabidopsis seed coat cells are dead prior to seed maturity.

Tiling array expression analysis on dissected embryos and endosperms from germinating seeds

We performed triplicate expression analysis on dissected endosperms and embryos from 6 and 24 h-imbibed seeds using Affymetrix tiling arrays [the data on transcriptomes of endosperms and embryos from 24 h-imbibed seeds were publicly released prior to this paper through OmicBrowse (http://omicspace.riken.jp/gps/group/psca5), and these data were used for the principal component analysis of seed transcriptomes in Okamoto et al. (2010)]. We analyzed expression data of 26,682 protein-coding genes based on the TAIR8 gene model (**Supplementary Table S1A**). In the present study, genes that are preferentially expressed in the endosperm are defined by ratios of expression intensities in the endosperm vs. embryo >5 with a false discovery rate (FDR) <0.05. In addition, the expression of genes with ratios of expression intensities in the endosperm vs. embryo >50 is defined as endosperm specific (FDR <0.05).

Arabidopsis *Expansin2* (*EXP2*; At5g05290) and *Proline-rich extensin-like1* (*EPR1*; At2g27380) have been used as marker genes for endosperm-specific expression in 24 h-imbibed Arabidopsis seeds (Dubreucq et al. 2000, Ogawa et al. 2003, Penfield et al. 2006). The ratios of expression intensities of *EXP2* and *EPR1* in 24 h endosperm vs. 24 h embryo, are 104- and 76-fold in our tiling array. In addition, the embryo- and endosperm-specific expression patterns are consistent with previously published reverse transcription–PCR (RT–PCR) and microarray expression analyses (Okamoto et al. 2006, Penfield et al. 2006, Bethke et al. 2007, Tatematsu et al. 2008; **Supplementary Table S1B**), suggesting that negligible human errors occurred during manual dissection of endosperm/seed coat.

Temporal expression patterns of genes that are preferentially expressed in the endosperm

Six and 24 h time points represent the initiation and established stages of Phase II, respectively (Nakabayashi et al. 2005, Preston et al. 2009, Kimura and Nambara 2010). In 6 h-imbibed seeds, 12 genes show >50-fold expression intensities in the endosperm compared with the embryo, and 199 genes show >5-fold expression intensities (**Table 1**, **Supplementary Table S2**). In 24 h-imbibed seeds, 59 genes and 584 genes are expressed abundantly in the endosperm with 50-fold and 5-fold differences, respectively, relative to the embryo (**Table 2**, **Supplementary Table S3**). Sixty-five percent (122 out of 199 genes) of genes preferentially expressed in the endosperm at 6 h are also included in the list of those at 24 h (**Supplementary Fig. S1A**). Seeds imbibed for 24 h have three times more such genes than those imbibed for 6 h, suggesting that the endosperm acquires its specialized functions during Phase II.

Temporal expression patterns of these genes were found in the previously reported transcriptome data from whole seeds (Nakabayashi et al. 2005). These are categorized into three different types, designated as Group A, B and C (**Supplementary Fig. S1B**). Group A genes show low abundance in transcripts in dry seeds, and their expression is induced by imbibition. Group B genes show little change in expression intensities during germination. Group C genes show abundant mRNA accumulation in dry seeds, which declines after imbibition. The expression patterns of Group B and C genes are not associated with germination. To support this, mRNAs of these genes are present abundantly in the dry seeds of both Col and Cvi (Kimura and Nambara 2010). The down-regulation found in Group C genes is largely due to embryonic processes, and expression intensities in the endosperm do not change remarkably between 6 and 24 h-imbibed seeds (**Supplementary Tables S2, S3**).

Gene ontology in genes preferentially expressed in the endosperm

Over-represented GO categories of genes with 5-fold greater expression intensities in the endosperm than in the embryo (199 genes for 6 h, 584 genes for 24 h) were examined in comparison with the frequency of GO categories at genome scale at the MIPS Functional Category site (http://mips.helmholtz-muenchen.de/proj/funcatDB/search_main_frame.html; **Supplementary Table S4**).

Genes preferentially expressed in the endosperm are over-represented for those related to cellular transport ($P = 4.9E-04$), cell wall ($P = 2.3E-03$), systemic interaction with the environment ($P = 7.5E-04$), interaction with the environment ($P = 9.4E-04$), cell rescue/defense and virulence ($P = 4.2E-03$) and metabolism ($P = 9.3E-03$). The enriched cellular transport processes include the transport of sugars ($P = 9.3E-04$), nitrate ($P = 2.0E-03$) and carbohydrates ($P = 2.0E-03$). This suggests that one primary function of the endosperm is to uptake and export nutrients from the external environment to the embryo.

Genes preferentially expressed in the endosperm at 24 h are enriched for those related to metabolism ($P = 3.4E-11$), cell rescue/defense and virulence ($P = 1.2E-04$), systemic interaction with the environment ($P = 2.5E-05$), cellular transport ($P = 1.8E-04$), interaction with the environment ($P = 5.3E-04$) and cell wall ($P = 6.1E-04$). The endosperm-specific metabolism

Table 1 Endosperm-specific genes in 6 h-imbibed seeds

ID	6h Endo/Emb	24h Endo/Emb	FDR	Endo 6h	Emb 6h	Endo 24h	Emb 24h	Annotation
AT2G43580	100	16	3.E-02	10420	104	1616	101	Chitinase, putative
AT2G43590	92	94	2.E-02	18378	199	9729	104	Chitinase, putative
AT4G20860	89	24	3.E-02	22247	249	1991	82	FAD-binding domain-containing protein
AT4G15380	82	62	4.E-02	11569	141	5754	92	CYP705A4
AT4G08290	76	156	1.E-02	8649	114	20412	131	Nodulin MtN21 family protein
AT5G55410	68	38	7.E-03	35147	517	24511	647	Protease inhibitor/seed storage/lipid transfer protein
AT5G17090	67	59	3.E-03	5852	87	6103	104	Similar to unknown protein
AT2G39420	63	427	2.E-02	11924	189	49322	115	Esterase/lipase/thioesterase family protein
AT1G22500	61	40	1.E-02	58952	959	24018	604	ATL1C; C3HC4 RING finger family protein
AT1G79520	61	235	9.E-03	7192	119	15135	64	Cation efflux family protein
AT1G80460	58	8	2.E-02	25280	435	31809	3880	NHO1; carbohydrate kinase
AT1G70840	53	149	3.E-03	36709	693	24537	165	MLP31 (MLP-LIKE PROTEIN 31)

Endo/Emb: >300, >100, >30, >10

Expression Intensity: > 10,000, >5,000, >1,000, >500, >100, >100

The table indicates genes with 50-fold more mRNA in the endosperm than in the embryo (FDR <0.05).

genes include those for the metabolism of phenylpropanoids, glucosinolates and sulfur assimilation. Defense genes in the endosperm include those for oxidative stress responses. The genes for systemic interaction with the environment are enriched for auxin response and fungal-specific systemic sensing.

In the present study, we focused on GO categories enriched in the endosperm. We note that, as reported by Penfield et al. (2006) and Morris et al. (2011), most of the genes showed active expression in both the endosperm and embryo tissues. It is also true for the GO categories in which many categories are over-represented in both tissues when we examined gene lists made based on the expression intensities in each tissue rather than the ratios. Therefore, the majority of basic cellular functions might be similar between these tissues. For example, a primary role for the endosperm of germinating seeds is to mobilize seed reserves such as lipids to provide nutrients to the embryos (Penfield et al. 2004). Such genes are expressed abundantly in the endosperm of germinating seeds, although they are not selected in our gene lists.

Cell wall biosynthesis and modification: the role of AtXTH31/XTR8 in reinforcing the cell wall of the endosperm

One characteristic GO category found in the 24 h gene list is cell wall biosynthesis and modifications. Over-representation of cell wall-related genes in the endosperm transcriptomes was also well described in *L. sativum* (Morris et al. 2011). In Arabidopsis, genes for α-expansins (*AtEXPA2*, *AtEXPA8* and *AtEXPA9*), β-expansin (*AtEXPB1*), expansin-like protein (*AtEXPL1*), cellulose synthase-like proteins (*CSLA2* and *CSLC4*), xyloglucan endotransglycosylases (*AtXTH31/XTR8*, *AtXTH11*, *AtXTH18*, *AtXTH23/XTR6*, *AtXTH17*, *AtXTH33* and *AtXTH24/SEN4/MERI-5*) and mannanase (*AtMAN7*) are abundantly expressed in endosperm at 24 h. It was reported that among the eight *AtMAN* genes, *AtMAN7* is highly expressed in the endosperm and vascular elements of the radicle in germinating Arabidopsis

seeds (Iglesias-Fernández et al. 2011). Importantly, the mutant defective in *AtMAN7* (and also mutants for *AtMAN5* and *AtMAN6*) show delayed germination.

AtXTH31/XTR8 was chosen for functional analysis because of its mRNA abundance and endosperm-specific expression (**Fig. 1A, Table 2**). AtXTH31/XTR8 belongs to Group 3 xyloglucan transglycosylase/hydrolases (XTHs) (Rose et al. 2002), and its expression was shown to correlate with salicylic acid (SA)-dependent cell growth (Miura et al. 2010). Two independent T-DNA insertion mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). The T-DNA insertion of the *xtr8-1* mutant is located at the junction between the first intron and second exon, whereas the *xtr8-2* mutant contains a T-DNA in the second exon (**Fig. 1B**). The *xtr8-1* and *xtr8-2* mutants displayed faster germination (**Fig. 1C**). In addition, an ABA responsiveness assay showed that both *xtr8* mutants are less sensitive to ABA (**Fig. 1D**). The *xtr8* mutants produce visibly normal seeds in terms of size and shape, which is different from the *atbx3* mutants. The *atbx3* mutants have defects in a seed-specific arabinan hydrolase that is expressed in the endosperm at an early stage of seed development (Minic et al. 2006). This is consistent with our hypothesis that XTR8 functions in germination.

Xyloglucan is the most abundant hemicellulose of the dicotyledonous cell walls (Hayashi 1989, Fry 2005). Xyloglucan endotransglycosylase (XET) cleaves a xyloglucan polymer and transfer the cleaved end to another xyloglucan chain. XETs are encoded by a family of XET-related (XTR) or XTH genes. The *Arabidopsis thaliana* genome possesses 33 *AtXTH/XTR* genes (Yokoyama and Nishitani 2001, Rose et al. 2002), and seven of them are preferentially expressed in the endosperm of 24 h-imbibed seeds (i.e. the ratio of expression intensity in endosperm/embryo is >5). XTH does not have the ability to loosen the cell wall in vitro (McQueen-Mason et al. 1993) and is postulated to be involved in cell wall biogenesis to reinforce the wall (Campbell and Braam 1999). Indeed, PttXET16A, a poplar XTH, was shown to function in the formation of
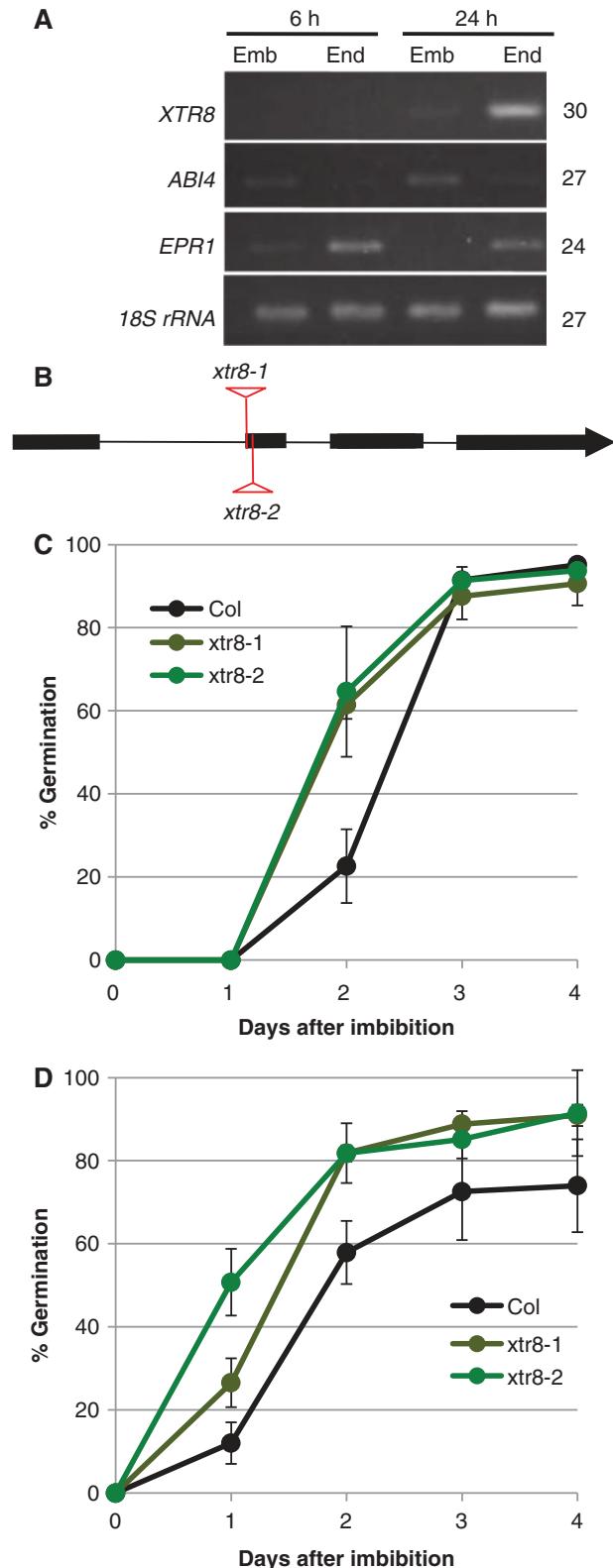
secondary cell wall of vascular tissues to reinforce the connection between primary and secondary wall layers (Bourquin et al. 2002). The phenotype of *xtr8* mutants indicates that AtXTH31/XTR8 is involved in slowing down the kinetics of germination probably by reinforcing endospermic cell walls. This is opposite to the role of AtMAN7 that is involved in endosperm weakening during germination (Iglesias-Fernábdez et al. 2011).

It is worth noting that public microarray data showed that expression of *AtXTH31/XTR8* is tightly associated with germination and that this gene is not induced under non-germinating conditions such as in dormant Cvi seeds, and non-germinating seeds whose germination is blocked by either mutations or chemicals (Cadman et al. 2006, Finch-Savage et al. 2007, Bassel et al. 2008). This suggests that AtXTH31/XTR8 plays a primary role not in inhibiting germination, but in slowing down germination processes. Interestingly, the expression pattern of *AtXTH11*, an *XTH* preferentially expressed in the endosperm of both 6 and 24 h-imbided seeds, is highly expressed in dormant seeds or non-germinating seeds (Cadman et al. 2006, Finch-Savage et al. 2007, Bassel et al. 2008), suggesting its involvement in maintaining dormancy by reinforcing endospermic cell walls. Cell wall biogenesis and modification requires many different types of enzymes, and systematic analysis will be needed to understand its regulations fully.

Flavonol biosynthesis in the endosperm during germination

Another enriched GO category in the genes preferentially expressed in the endosperm of 24 h-imbided seeds is secondary metabolism, especially for the flavonoid biosynthesis pathway. The endosperm-preferential expression of *Chalcone synthase* (*CHS*) and *Flavonol synthase1* (*AtFLS1*) was confirmed by RT–PCR (**Fig. 2A**). Genes for CHS, chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H) and flavonoid 3′-hydroxylase (F3′H) are expressed most abundantly in the endosperm at 24 h (**Fig. 2B**). Dihydroquercetin or dihydrokaempferol is the common intermediate for anthocyanidins and flavonols, and dihydroflavonol 4-reductase (DFR) and flavonol synthase compete for the same substrate to drive the metabolism either to anthocyanidin biosynthesis or to flavonol biosynthesis, respectively. Our tiling array data showed that *AtFLS1*, but not *DFR*,

Fig. 1 Continued

expression, respectively. *18S rRNA* is a control for ubiquitous expression. The number on the right represents PCR cycles for each expression analysis. (B) The position of T-DNA insertions in the *XTR8* gene. Bold and thin lines indicate exons and introns, respectively. Red marks indicate the positions of T-DNA insertions. (C). The *xtr8-1* and *xtr8-2* mutants germinated faster than the wild type. Freshly harvested seeds were sown on 0.8% agar plates and germination was counted daily. Three biological replicates were examined, and an average with a standard deviation is shown. (D) The *xtr8* mutants are less sensitive to ABA. Seed were sown on 1/2 MS–0.8% agar plates supplemented with 1 µM ABA, stratified for 2 d, and transferred to light conditions at room temperature. Germination was counted daily. An average with a standard deviation is shown.

Fig. 1 Expression of *XTR8* and germination of the *xtr8* mutants. (A) RT–PCR analysis of *XTR8* expression in dissected embryo and endosperm. Dissected embryo (Emb) and endosperm (End) of 6 and 24 h-imbided seeds were used for RT–PCR expression analysis. *ABI4* and *EPR1* are controls for embryo-specific and endosperm-specific

Table 2 Endosperm-specific genes in 24 h-imbibed seeds

ID	6h Endo/Emb	24h Endo/Emb	FDR	Endo 6h	Emb 6h	Endo 24h	Emb 24h	Annotation
AT2G39420	63.1	427	2.E-04	11924	189	49322	115	Esterase/lipase/thioesterase family protein
AT1G73220	2.5	293	8.E-04	222	90	40663	139	AtOCT1 (organic cation/carnitin transporter1)
AT3G25290	12.4	245	4.E-03	2022	163	35700	146	Auxin-responsive family protein
AT3G45010	1.9	237	2.E-03	142	73	34037	143	SCPL48 (serine carboxypeptidase-like 48)
AT1G79520	60.7	235	4.E-04	7192	119	15135	64	Cation efflux family protein
AT3G44990	0.8	222	3.E-03	80	96	41745	188	XTR8 (xyloglucan:xyloglucosyl transferase 8)
AT1G14940	15.0	205	3.E-04	93767	6237	44755	219	Major latex protein-related / MLP-related
AT1G14950	17.6	178	3.E-03	78787	4481	25141	142	Major latex protein-related / MLP-related
AT4G01450	9.6	178	8.E-04	2469	256	33163	187	Nodulin MtN21 family protein
AT3G20210	1.9	166	4.E-02	130	70	12663	76	Delta-VPE (delta vacuolar processing enzyme)
AT1G14930	10.2	157	6.E-04	94007	9246	41200	262	Major latex protein-related / MLP-related
AT4G08290	75.9	156	3.E-03	8649	114	20412	131	Nodulin MtN21 family protein
AT5G39580	34.9	154	9.E-03	12739	365	14839	96	PER62 (peroxidase62), putative
AT1G70840	53.0	149	1.E-03	36709	693	24537	165	MLP31 (MLP-like protein 31)
AT4G21490	2.0	143	4.E-02	124	61	9374	66	NDB3; NADH dehydrogenase
AT2G23620	1.5	131	6.E-04	4340	2971	16234	124	Esterase, putative
AT2G02930	34.7	131	3.E-03	51271	1478	24768	190	GST16 (glutathione S-transferase 16)
AT1G02900	1.0	128	2.E-03	136	135	14700	115	RALFL1 (RALF-like 1)
AT1G74670	4.9	124	4.E-03	368	75	91739	738	Gibberellin-responsive protein
AT2G17710	5.5	124	2.E-03	1391	254	16938	137	Similar to unnamed protein product
AT1G47980	23.3	123	1.E-03	30818	1323	33190	269	Similar to unknown protein
AT1G09380	1.8	117	2.E-03	164	91	10159	87	Nodulin MtN21-related
AT4G31330	2.2	115	9.E-05	224	100	24042	208	Similar to unknown protein
AT3G21380	7.8	114	1.E-02	8641	1105	11787	103	Similar to MBP1 (myrosinase-binding protein1)
AT5G05290	12.1	104	2.E-03	1727	142	93441	898	EXP2

Legend — Endo/Emb: >300, >100, >30, >10. Expression Intensity: >10,000, >5,000, >1,000, >500, >100, >100.

This table indicates genes with specific expression in the endosperm compared with the embryo (FDR <0.05). Due to the lack of space, only genes with >100-fold more mRNA in the endosperm vs. the embryo are shown.

was expressed preferentially in the endosperm, suggesting that the flavonol pathway is active in the endosperm of germinating seeds.

Flavonol accumulation was examined by an in vivo staining method with diphenyl boric acid 2-aminoethyl ester (DPBA). We performed DPBA staining on the seeds of Col and Cvi accessions in order to examine the pattern of flavonol accumulation in these accessions. The DPBA fluorescence was detected in both the embryo and endosperm in 6 h-imbibed Col seeds (**Fig. 3A, B**). This suggests that flavonols accumulated in both the embryo and endosperm at earlier stages, probably during seed development. The DPBA fluorescence was observed in the endosperm, but not in the seed coat (**Fig. 3A**). Lepiniec et al. (2006) showed that flavonols accumulated in the seed coat of immature seeds. It is possible that flavonols existing in the seed coat were converted to a structure undetectable by DPBA staining during seed maturation. Alternatively, flavonols accumulated in the seed coat of immature seeds may be degraded or leached during seed maturation and/or seed imbibition. DPBA staining was seen throughout the entire embryos, except for the region around the radicle. The region lacking DPBA staining resembles that which expands during the germination of Col seeds (Sliwinska et al. 2009). The DPBA staining pattern in Cvi was similar to that in Col, although the region lacking DPBA staining was narrower than in Col seeds and did not expand after imbibition (**Supplementary Fig. S2**). The fluorescence was not detected in the DPBA-treated *tt4* mutant (**Supplementary Fig. S3**) and non-treated wild-type control (**Supplementary Fig. S4**).

The subcellular localization of DPBA fluorescence in the endosperm was examined. Bethke et al. (2007) reported that the endosperm cells of 6 h-imbibed Col seeds were fully filled by PSVs, which were fused to make large lytic vacuoles within 24 h of imbibition, a process called vacuolation. The structure of seed vacuoles is complex and dynamic, having distinct compartments embedded inside the PSVs (Gillespie et al. 2005, Bolte et al. 2011). We also observed similar dynamics of vacuoles in the endosperm of 6 and 24 h-imbibed Col seeds (**Fig. 3C**). At 6 h, DPBA fluorescence was seen in the entire vacuoles of the endosperm in Col seeds (**Fig. 3C**). Concomitant with vacuolation, DPBA fluorescence became localized in the particular compartments within the vacuoles of 24 h-imbibed Col seeds (**Fig. 3C**). On the other hand, vacuoles of 24 h Cvi endosperm did not show vacuolation, and localization of the DPBA signals was similar between 6 and 24 h (**Supplementary Fig. S2**). It remains unclear if vacuole-localized flavonols have any physiological function during germination. It is also possible that undetectable flavonols outside the vacuole have some physiological functions.
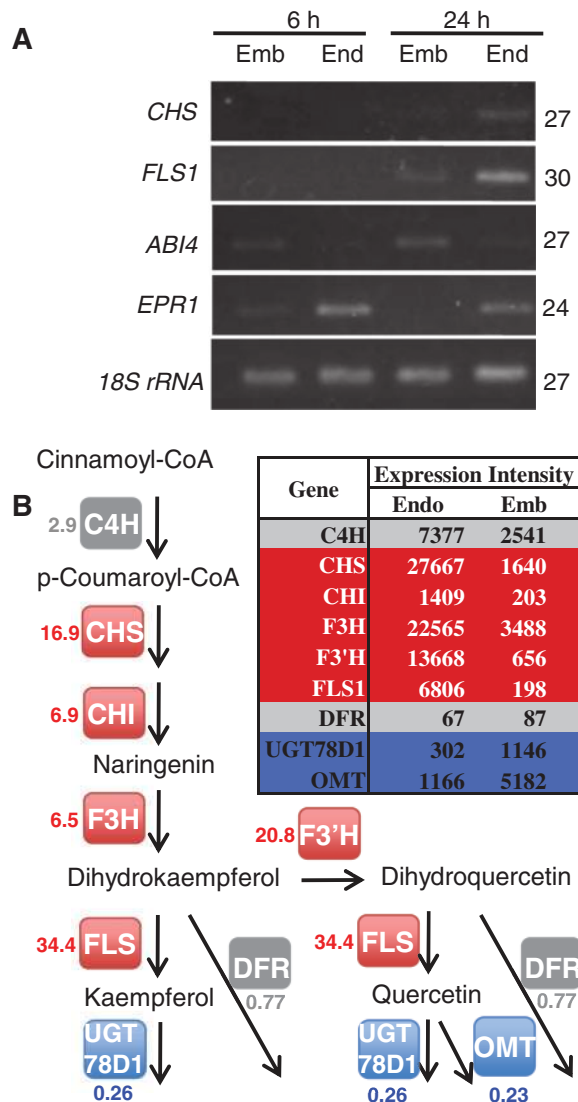
Fig. 2 Localized expression of flavonol biosynthesis genes in the endosperm of germinating seeds. (A) RT–PCR analysis of *CHS* and *FLS1* expression in dissected embryo and endosperm. Dissected embryo (Emb) and endosperm (End) of 6 and 24 h-imbibed seeds were used for RT–PCR expression analysis. *ABI4* and *EPR1* are controls for embryo-specific and endosperm-specific expression, respectively. *18S rRNA* is a control for ubiquitous expression. The number on the right represents PCR cycles for each expression analysis. (B) The flavonol biosynthesis pathway and an enzyme involved in each step are shown. The ratio of expression intensities in the endosperm vs. the embryo at 24 h is shown with the corresponding enzyme. Expression intensity is listed in the right top corner. The genes highlighted by red and blue indicate preferential expression in the endosperm and embryo, respectively. C4H (At2g30490), cinnamate 4-hydroxylase; CHS (At5g13930), chalcone synthase; CHI (At3g55120), chalcone isomerase; F3H (At3g51240), flavanone 3-hydroxylase; F3′H (At5g07990), flavonoid 3′-hydroxylase; FLS (At5g08640), flavonol synthase; DFR (At5g42800), dihydroflavonol 4-reductase; UGT78D1 (At1g30530), flavonol 3-*O*-rhamnosyltransferase; OMT1 (At5g54160), quercetin 3′-*O*-methyltransferase.

Routaboul et al. (2006) reported that Arabidopsis seeds accumulate quercetin derivatives as primary flavonoids, in contrast to kaempferol derivatives that are the primary forms in the leaves. More than 90% of quercetin-3-*O*-rhamnoside, a major flavonoid in Arabidopsis seeds, accumulated in the endosperm/seed coat of mature seeds. Our study shows that a DPBA-derived fluorescent signal was detected in the endosperm, but not in the testa (**Fig. 3A**). The fluorescence in the endosperm was enhanced during germination. This suggests that the endosperm is a primary site for the synthesis and accumulation of flavonols during germination. We note that intense DPBA fluorescence was also found in the embryo (**Fig. 3B**) as reported by Carrera et al. (2007). The embryonic pattern of DPBA fluorescence was characteristic in terms of lacking fluorescence signals around the radicle. The *tt* mutants (Lepiniec et al. 2006) are flavonoid deficient, and many of them have defects in the seed coat, in that they have thinner seed coats with increased permeability for compounds in the germination media (Debeaujon et al. 2000). Many *tt* mutants also display a non-dormancy phenotype (Debeaujon et al. 2000). The physiological role of flavonols in the endosperm is still unknown, although such flavonol (derivatives) possibly protect the embryo or control seed germination either by regulating auxin transport (Murphy et al. 2000) or by acting as scavengers. It is also worth pointing out that *comatose* mutants fail to accumulate flavonols in the embryo of imbibed seeds and show poor germination (Carrera et al. 2007). This is different from abundant flavonol accumulation in the dormant Cvi seeds. The simplest hypothesis is that the failed accumulation of flavonols in *comatose* mutants is a pleiotropic response acting as a feedback response of the germination defect. Further analysis will be needed on other germination mutants to examine their capacity with respect to flavonol accumulation.

Activation of biotic defense-like processes in the endosperm: δVPE plays a role in vacuolation in germinating endosperm

The third class of enriched processes in the endosperm of germinating seeds is defense responses. The genes related to detoxification are over-represented in the genes preferentially expressed in the endosperm. These include seven glutathione *S*-transferases, eight peroxidases, two glutaredoxin family proteins and a thioredoxin, which is consistent with previous reports that show the active synthesis of reactive oxygen species (ROS) in the endosperm of germinating seeds (Bailly 2006, Penfield et al. 2006). In addition, the genes related to plant–fungus interactions are over-represented in the genes preferentially expressed in the endosperm. These include a gene encoding SID2/ICS1 isochorismate synthase 1, an enzyme responsible for SA biosynthesis, and four genes coding for indole-3-acetic acid amido synthetases (At2g14960/GH3.1, At4g27260/GH3.5, At4g37390 and At5g54510/GH3.6). Members of the GH3 family encode enzymes that catalyze adenylation of both IAA and SA
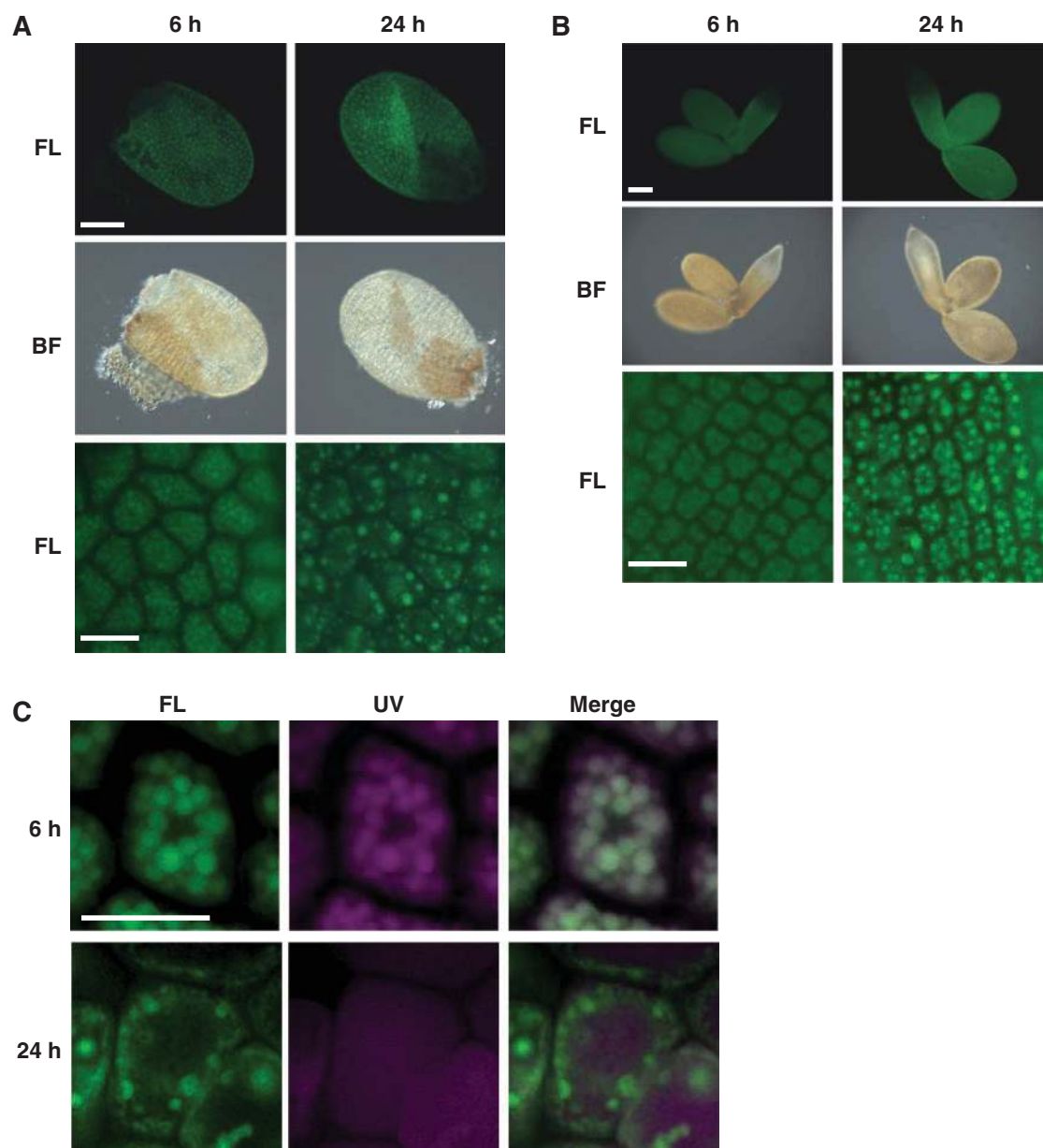
Fig. 3 Flavonol accumulation in Col seeds. Dissected endosperm (A) and embryo (B). Left, 6 h-imbibed seed; right, 24 h-imbibed seed. From top to bottom, DPBA fluorescence (FL), bright field (BF) and DPBA fluorescence (FL) with a higher magnification. Bars in the first and third lines indicate 300 and 50 μm, respectively. (C) Subcellular localization of flavonols in the endosperm. DPBA-stained endosperm cells were visualized under a fluorescent microscope. FL and UV indicate DPBA fluorescence and UV autofluorescence of vacuoles, respectively. The merge shows an overlay of FL and UV images. Top, 6 h-imbibed seeds; bottom, 24 h-imbibed seeds. A bar indicates 50 μm.

(Staswick et al. 2002). Overexpression of GH3 induces disease resistance (Jagadeeswaran et al. 2007, Nobuta et al. 2007). Overall, we conclude that the transcriptome in the endosperm of germinating seeds resembles that of biotic stress responses. The seeds are exposed to phytopathogens once they are dispersed from the mother plant. It is possible that abiotic defense responses are activated developmentally independent of exposure to phytopathogens during germination.

One defense-related gene with endosperm-specific expression is a seed-type *vacuolar processing enzyme*, δVPE (**Table 2**). δVPE is a caspase-1-like cysteine proteinase involved in cell death in seed coat formation during early seed development. The δ*vpe* mutants show delayed shrinkage of the testa cells (Nakaune et al. 2005). The endosperm-specific expression of δ*VPE* was confirmed by RT–PCR analysis (**Fig. 4A**). We examined the endosperm of the δ*vpe* mutant during germination. The formation of large lytic vacuoles is a typical morphological feature of plant vacuolar cell death (van Doorn et al. 2011). The DPBA staining pattern in the endosperm of the δ*vpe* mutant was similar to that in the wild type at 6 h (**Fig. 4B**). However, at

A

B
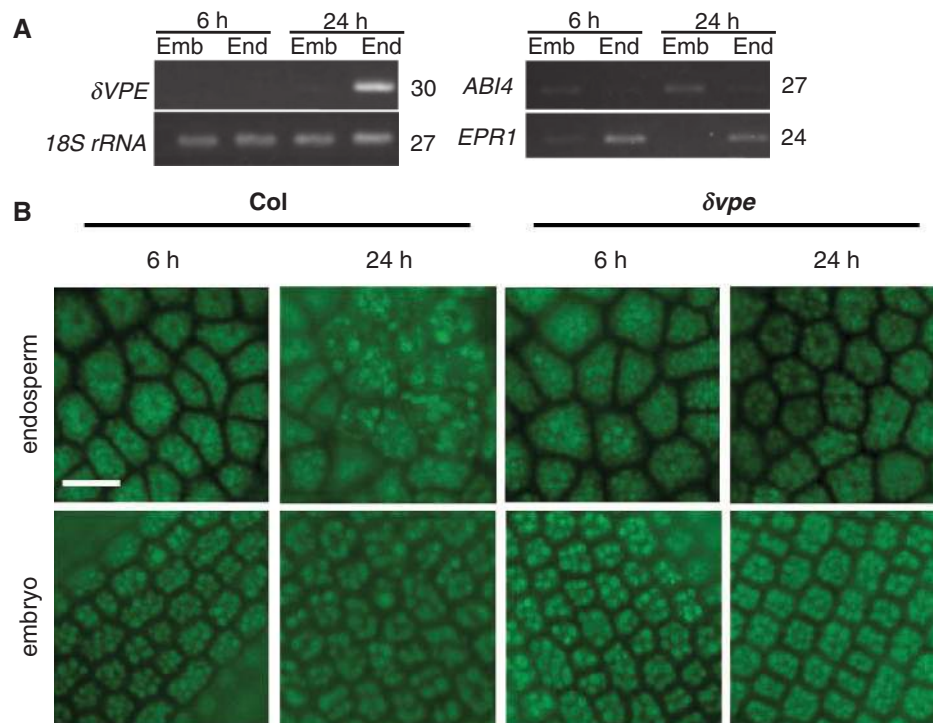
Fig. 4 DPBA fluorescence in the δ*vpe* mutant. (A) RT–PCR analysis of δ*VPE* expression in dissected embryo and endosperm. Dissected embryo (Emb) and endosperm (End) of 6 and 24 h-imbibed seeds were used for RT–PCR expression analysis. *ABI4* and *EPR1* are controls for embryo- and endosperm-specific expression, respectively. *18S rRNA* is a control for ubiquitous expression. The number on the right represents PCR cycles for each expression analysis. (B) DPBA fluorescence in 6 and 24 h-imbibed Col wild-type and δ*vpe* mutant seeds was visualized by DPBA. Left, wild type; right, δ*vpe* mutant. Top, endosperm; bottom, embryo. A bar indicates 50 μm.

24 h, the change in DPBA fluorescence observed in the wild type was not seen in the endosperm of the δ*vpe* mutant (**Fig. 4B**). The PSV was still fully packed in most of the endosperm cells of the δ*vpe* mutant at 24 h (**Fig. 4B**). The pattern of DPBA fluorescence of the δ*vpe* endosperm was similar to that of the wild type at 48 h (data not shown). Interestingly, vacuolation of the δ*vpe* mutant was also delayed in the embryo (**Fig. 4B**); however, germination of the δ*vpe* mutant showed similar kinetics when compared with wild-type seeds (data not shown).

Cell biology-based approaches in both monocots and dicots have shown that the endosperm of germinating seeds induces vacuolation, which is often associated with vacuolar cell death (van Doorn et al. 2011). Our tissue-specific transcriptome analysis indicates that δ*VPE* is highly expressed in an endosperm-specific manner during germination. This gene was previously shown to be involved in cell death during early testa development (Nakaune et al. 2006). The endosperm of the δ*vpe* mutant showed delayed vacuolation (**Fig. 4B**), indicating that δ*VPE* plays an important role in the vacuolation of the endosperm during germination. Vacuolation in the endosperm of the δ*vpe* mutant was apparent in 48 h-imbibed seeds (data not shown). This endosperm vacuolation phenotype is consistent with cell death observed in the seed coat where the δ*vpe* mutant showed delayed cell

death during seed development, but produced a normal seed coat at maturation. Arabidopsis seems to have another mechanism to compensate for the defect in δVPE on cell death in the seed coat during seed development and in the endosperm during germination. One of our findings is that the delay of vacuolation in the endosperm did not alter the kinetics of germination, at least in this condition. This suggests that vacuolation is probably not the trigger for germination, but rather it occurs as a consequence of germination. Another interesting finding is that not only the endosperm, but also the embryo displayed delayed vacuolation in the mutant (**Fig. 4B**). This indicates that the defect in the endosperm influences cellular processes in the embryo. Since endospermic and embryonic processes interact, signal communications between these tissues might be active and bidirectional. Elucidation of the mechanisms underlying this communication will be a challenge for future research.

Materials and Methods

Plant materials and growth condition

Arabidopsis thaliana Columbia and Cvi accessions were used in this study. The *xtr8-1* (SALK_046167C), *xtr8-2* (SALK_129686), δ*vpe* (SALK_093074) and *tt4* (SALK_020583) mutants were

obtained from the ABRC (Alonso et al. 2003). Seeds were harvested from plants grown in pots at 22°C under continuous light. The tiling array experiments were performed using the Col seeds harvested at the RIKEN Plant Science Center, and other experiments were performed using seeds harvested at the University of Toronto.

For tiling array analysis, wild-type seeds stored for 2 months (after-ripened) were imbibed on filter papers moistened with water. The embryos and endosperm/seed coat were dissected manually at 6 and 24 h after the start of imbibition. The dissected tissues were placed on wet filter papers, frozen by liquid nitrogen within 10 min after dissection, and stored at −80°C until use. In total, approximately 30,000 seeds were manually dissected to perform triplicate experiments at the indicated time points: i.e. ~5,000 seeds×2 time points×3 replicates.

For flavonol staining, Col and Cvi seeds were harvested at the same time, stored for 1–3 months at room temperature, and used for analysis. The Col seeds, but not Cvi seeds, germinated on the filter papers moistened with water under continuous light conditions. For mutant analysis, Col and mutant seeds were harvested at the same time, stored for 3 d to 2 weeks at room temperature, and used for analysis.

Germination test

Seeds stored at room temperature for 2 weeks were used for the germination test. Approximately 50 seeds were washed with ethanol, dried by a speedvac, sown on 0.8% agar plates and incubated at room temperature (24°C) under continuous light conditions as described in Kimura and Nambara (2010). For the ABA responsiveness assay, seeds stored for 4 weeks were used. Approximately 50 seeds were washed with ethanol, dried, and sown on 0.8% agar plates supplemented with 1/2 MS salts with 1 μM (+/−)-ABA (Sigma-Aldrich). The seeds were stratified for 2 d at 4°C in darkness and transferred to continuous light conditions at room temperature. Germination was recorded daily, with endosperm rupture as a criterion for completion of germination. Three independent seed batches were used for each experiment.

Extraction of total RNA

Total RNA was isolated using an RNAqueous column with Plant RNA Isolation Aid (Ambion) as described previously (Okamoto et al. 2006).

Whole-genome tiling array analysis

The GeneChip Arabidopsis tiling array sets (1.0 F array and 1.0 R array, Affymetrix) were used in this study (Zhang et al. 2006). Procedures for the probe synthesis, hybridization and tiling array analysis were described previously (Matsui et al. 2008, Okamoto et al. 2010). The tiling array data from 24 h-imbibed embryo and endosperm were used previously for principal component analysis to characterize other seed transcriptomes (Okamoto et al. 2010).

Pre-processing of tiling array data

The locations of perfect match (pm) probes in genome tiling arrays were re-examined in the Arabidopsis genome sequences (from TAIR, version 8) by our perl scripts. We used only the pm probes which are found as a unique genomic locus. After removing the spatial effect within a single array with the NMPP program (Wang and He 2006), the differential intensity between pm and mm (mismatch) was calculated in each position with the MAS5 (Affymetrix Microarray Analysis Suite v5.0) algorithm (Li and Wong 2001) in the R software environment (http://www.r-project.org). Tukey bi-weight values required as background correction in this procedure were calculated in each chromosome with the Bioconductor (http://www.bioconductor.org) affy package. The normalized quantiles function was used for between-array normalization by the NMPP program.

When several splicing patterns exist in a gene locus, the gene locus yields several gene models. In such cases, the gene model with the longest amino acid sequence was used as the representative model at a gene locus. For the annotated gene set, we used 27,235 loci of coding genes in TAIR, version 8. The expression intensity in each of the coding genes was defined to be the Tukey bi-weight value (statistically robust median) among normalized differential intensities of probes spotted in a coding gene. Expression intensities in each pair of various experimental conditions were compared by LIMMA (Wettenhall et al. 2004).

RT–PCR expression analysis

Procedures for cDNA synthesis and RT–PCR analysis were described elsewhere (Endo et al. 2008). Total RNA from dissected embryo and endosperm was used for cDNA synthesis, and RT–PCR was performed. The PCR products were size-fractionated by agarose gel electrophoresis, stained with ethidium bromide, and visualized by a transilluminator. Primers used are shown in **Supplementary Table S5**. Two biological replicates for 24 h samples (embryo and endosperm/seed coat) and one for 6 h samples were used for RT–PCR analysis (these are different from those used in the tiling array).

Flavonol staining

Dry seeds were sown on wet filter papers (7 cm diameter) placed in a plastic Petri dish (9 cm diameter, 1.5 cm height) and incubated for 6 or 24 h under illumination at room temperature (22–24°C). Imbibed seeds were divided into endosperm and embryo at each time point. The divided samples were washed with water briefly and DPBA staining was performed as described in Murphy et al. (2000). Samples were observed with an Zeiss Axio Imager M1 epifluorescent microscope equipped with a green fluorescent protein (GFP) filter unit (excitation 450–490 nm, emission 500–550 nm) for DPBA fluorescence or a 4′,6-diamidino-2-phenylindole (DAPI) filter unit (excitation 325–375 nm, emission 435–485 nm) for vacuole visualization. DPBA fluorescence was not seen in the *tt4* mutant that is defective in the *CHS* gene (see **Supplementary**

Fig. S3). To verify that fluorescence was from DPBA-stained flavonols, samples with or without DPBA staining were used for configuring microscopic parameters (see **Supplementary Fig. S4**).

Supplementary data

Supplementary data are available at PCP online.

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