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Stored and neosynthesized mRNA in Arabidopsis seeds: effects of cycloheximide and controlled deterioration treatment on the resumption of transcription during imbibition

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Abstract Dry seeds accumulate translatable mRNAs as well as functional proteins for transcription and translation. They are possibly involved in early physiological responses after imbibition, however, their functions remain poorly understood. The aim of this study is to investigate the function of seed stored transcriptional machinery in resumption of gene expression after the onset of imbibition in *Arabidopsis thaliana*. First, we examined the characters of stored mRNAs in *A. thaliana* dry seeds using microarray data from non-dormant Columbia (Col) and dormant Cape Verde Islands (Cvi) accessions. Transcriptomes of Col and Cvi dry seeds resembled one another, suggesting that patterns of stored mRNA do not reflect either the degree of dormancy or germination potential, but rather reflect the developmental context, such as seed maturation. Upon imbibition, changes in mRNA abundance of many genes were initiated between 1- and 2-h after the onset of imbibition. RT–PCR expression analysis of imbibition-responsive genes indicates that early induction was not altered by treatment of cycloheximide. This suggests that de novo protein synthesis is not required for gene expression during early imbibition stages. Moreover, controlled deterioration treatment (CDT), which causes artificial damages on dry seeds, disrupted gene expression specifically during the first 3 h after the start of imbibition, suggesting that seed stored transcription factors play a pivotal role in gene expression during early imbibition periods. Furthermore, the negligible effect of CDT on germination indicates that early imbibition response is dispensable and de novo synthesized proteins compensate for the function of stored proteins for germination.

Keywords Arabidopsis · Seed · Stored mRNA · Imbibition · Cycloheximide · Controlled deterioration treatment · Dormancy · Germination

Abbreviations

ABA	Abscisic acid
ABI	Abscisic acid-insensitive
ABRE	Abscisic acid-responsive element
BME3	Blue micropylar end 3
CDT	Controlled deterioration treatment
CHX	Cycloheximide
Col	Columbia
Cvi	Cape Verde Islands
GSTU22	Glutathione S-transferase tau 22
GO	Gene ontology
HSP	Heat shock protein
LEA	Late embryogenesis abundant
RT–PCR	Reverse transcription-PCR
TT	Transparent testa

Introduction

Stored macro-molecules in dry seeds are required for the resumption of genetic programs after the start of imbibition.

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Dry seeds accumulate a large amount of translatable mRNAs as well as functional proteins for metabolism, transcription and translation (Marcus and Feeley 1964; Dure and Waters 1965; Bewley and Black 1994). They are produced during seed development, retain their functions during the dry storage, and are used upon imbibition. These stored macro-molecules are damaged gradually during the period of dry storage, which reduces seed vigor and viability (Oracz et al. 2007). It is also known that storage of dry seeds, a period called after-ripening, reduces the degree of dormancy. Therefore, the functions of stored macro-molecules in dry seeds are essential for seed vigor and longevity, as well as maintenance of seed dormancy.

Dry seeds accumulate various mRNAs, called stored mRNA or long-lived RNA. Stored mRNAs were first identified in cotton seeds (Dure and Waters 1965), and are now known to be present in the dry seeds of all angiosperms examined. The stored mRNAs are thought to be translated after the onset of imbibition and to function during the early stage of imbibition (Comai et al. 1989; Hughes and Galau 1989, 1991). Microarray analysis revealed that more than 10,000 different mRNA species were present in Arabidopsis dry seeds of the Columbia (Col) accession, which include the genes for all functional categories (Nakabayashi et al. 2005). Accumulation of stored mRNAs in dry seeds is regulated by endogenous and environmental signals. Abscisic acid (ABA) plays an important role in seed development, maturation and germination (Nambara and Marion-Poll 2005) and is involved in the accumulation of stored mRNA in seeds (Nakabayashi et al. 2005). ACGT-containing ABA-responsive elements (ABREs) are highly over-represented in the promoters of the abundant stored mRNA genes. CE and RY elements are also co-located with ABREs in their promoters. ACGT-containing ABREs, CE, and RY elements are the targets of ABSCISIC ACID-INSENSITIVE5 (ABI5), ABSCISIC ACID-INSENSITIVE4 (ABI4), and ABSCISIC ACID-INSENSITIVE3 (ABI3) transcription factors, respectively (Giraudat et al. 1992; Finkelstein et al. 1998; Finkelstein and Lynch 2000; Lopez-Molina and Chua 2000). This indicates that ABA-mediated transcription plays a key role in determining the patterns of the abundant class of stored mRNAs. Environmental conditions of maternal plants are known to affect the accumulation pattern of a subset of stored mRNAs (Matakiadis et al. 2009).

It was reported that dry seeds contain the functional proteins for translation (Marcus and Feeley 1964). Rajjou et al. (2004) reported that germination of *Arabidopsis thaliana* seeds was inhibited by the cycloheximide (CHX) treatment, an inhibitor of protein synthesis, but not by α-amanitin that blocks PolII-mediated transcription (Rajjou et al. 2004). The authors concluded that de novo transcription is dispensable, but translatable mRNAs and proteins stored in dry seeds are sufficient for Arabidopsis germination. Recent progresses in proteome analysis have revealed overall components of stored proteins in dry and imbibed seeds (Gallardo et al. 2001, 2002). The major seed proteins in Arabidopsis are 12S globulins (cruciferins), 2S albumin (napins), late embryogenesis abundant (LEA) proteins, and stress inducible proteins, such as heat shock proteins (HSPs). Seed proteins abundantly accumulated in dry seeds also include enzymes for primary metabolism, such as respiration and gluconeogenesis. In rice seeds, similar proteins are also accumulated (Yang et al. 2007). Although it has been studied for a long period, it remains unclear how seed stored proteins are important for processes in seed imbibition, germination and dormancy.

Dry seeds contain a number of proteins that function after imbibition. The competence of transcription of dry seeds has been reported by several researchers. Nuclei isolated from dry seeds of rapeseed had transcriptional activity (Comai and Harada 1990) and mRNA for the β-1,3-Glucanase gene was increased in tobacco dry seeds during storage periods (Leubner-Metzger 2005). Rajjou et al. (2007) reported that the translational capacity of dry seeds is critical for seed viability and vigor. Recently, we reported a kinetics study on gene expression analysis during early imbibition of non-dormant Col and dormant Cape Verde Islands (Cvi) seeds (Preston et al. 2009). This analysis indicated that resumption of gene expression was initiated 1 h after the start of imbibition regardless of accessions. The aim of this work is to examine the function of seed stored components (i.e., stored mRNA and proteins). To do this, we conducted: (1) a comparison of stored mRNA transcriptomes between Col and Cvi, which was not analyzed in Preston et al. (2009), and (2) gene expression analysis during seed imbibition to examine how the transcriptional capacity of dry seeds contributes to this process. Our dry seed transcriptome data of Col and Cvi were obtained from seeds harvested at the same time from plants grown under the same growth conditions. This data set is suitable for comparative transcriptomes because comparison of multiple transcriptome data often provides a difference of growth conditions rather than experimental treatment (Hirai et al. 2003). We outlined stored mRNAs that are abundantly present in both Col and Cvi dry seeds. Moreover, the function of seed proteins stored in dry seeds on gene expression during early stages of seed imbibition was investigated after the following treatments: seed imbibition in the presence of CHX, an inhibitor of translation, and controlled deterioration treatment (CDT), which causes damage on seed stored components like proteins. These analyses suggest that transcription factors that are stored in dry seeds play a pivotal role in the resumption and

maintenance of gene expression during early stages of imbibition.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana wild type and *tt8* mutants (SALK_030966 and SALK_048673) used in this study were of Col accession (Alonso et al. 2003). The *tt8* mutants were donated by Professor Naoto Kawakami (Meiji University, Japan). Surface sterilized seeds were sown on 0.8% agar plates supplemented with 1/2 Murashige and Skoog salt mixture in xx-cm2 Petri dishes. A 2 week-old seedlings were transferred to soil. Plants were grown on soil under a 16 h white light/8 h dark cycle at 22°C. Plants were watered every 2–3 days until they failed to produce new flowers (approximately 2–3 months). Plants were stayed for 1 week without irrigation until harvest. Seeds were harvested and stored in darkness at room temperature.

Controlled Deterioration Test

Controlled deterioration test was performed as described (Delouche and Baskin 1973). Briefly, dry seeds were placed in a sealed plastic box with saturated vapor by adding 100 ml of water at 40°C in darkness for indicated periods. After this treatment, seeds were desiccated on filter paper at room temperature for 1 day.

Microarray data analysis

Triplicate microarray analysis of Col and Cvi dry seeds were performed using Affymetrix ATH1 arrays as described in Preston et al. (2009). The data is available from NASCArrays (http://affy.arabidopsis.info/), reference number: NASCARRAYS-499. Expression data of organelle-coded genes were omitted prior to analysis. Scatterplot was made by Microsoft Excel. Ontological analysis was performed using MIPS Arabidopsis thaliana Database (MAtDB, http://mips.helmholtz-muenchen.de/proj/funcatDB/search_main_frame.html) as described elsewhere (Tatematsu et al. 2008; Yamagishi et al. 2009).

Germination test

Germination tests were performed using 50 seeds per experiment. Triplicate experiments were performed using independent seed batches. A 2–4 week- stored seeds were surface-sterilized, imbibed on agar medium under a continuous white light condition (20 μm/m2/s) at 25 ± 1°C. Visible radicle protrusion was used as the criterion for germination and was scored daily. For CHX treatment, dry seeds were imbibed on 0.8% agar medium supplemented with 100 μM CHX under a continuous white light condition at 25 ± 1°C. One hundred mM of CHX solution in ethanol was added to media at a dilution of 1:1,000 (final concentration was 100 μM), whereas ethanol was added to control media at the same dilution ratio.

RNA isolation and semi-quantitative RT–PCR

Seeds were surface-sterilized and imbibed on filter papers moistened with distilled water under a continuous white light condition at 25°C. Total RNA was prepared from 20 mg of dry and imbibed seeds as described elsewhere (Oñate-Sánchez and Vicente-Carbajosa 2008). cDNA was synthesized using 600 ng of total RNA as a template with First Strand cDNA Synthesis Kit (Fermentas Canada Inc.) following the manufacturer's protocol. The obtained cDNA were used for PCR analysis. PCR products were subjected to electrophoresis on a 2% agarose gel. The primers used in this study were shown in Table S1.

Results and discussion

Stored mRNA in Arabidopsis dry seeds

Stored mRNA transcriptome in Col and Cvi

Stored mRNAs accumulated in dry seeds of Col and Cvi accessions were analyzed. Triplicate microarray data on dry seeds were used for this analysis. Figure 1 shows a scatterplot of transcript abundance in Col and Cvi dry seeds. These seeds displayed different degrees of dormancy and germination potential, however, the overall feature of dry seed transcriptomes of Col and Cvi resembled one another. Okamoto et al. (2010) reported a comparative transcriptome analysis on Col wild type, ABA-deficient (non-dormant) *aba2* and ABA-overaccumulating (hyper dormant) *cyp707a1a2a3* triple mutants. Transcriptomes in 24 h-imbibed seeds of wild type and these mutants are largely different from one another, however only subtle differences are observed in dry seeds. Taken together, we conclude that transcriptomes in dry seeds reflect largely on developmental context rather than genetic background or physiological conditions of seeds. Chibani et al. (2006) reported that the dry seed proteomes of Cvi and Landsberg *erecta* (L*er*) showed high conservation.

We also note that a subset of less abundant stored mRNA classes showed the accumulation pattern specific to either Col or Cvi (cut-off value, threefold). Biased gene ontology (GO) categories were searched on the MIPS website (http://mips.helmholtz-muenchen.de/proj/funcatDB/search_main_frame.html).

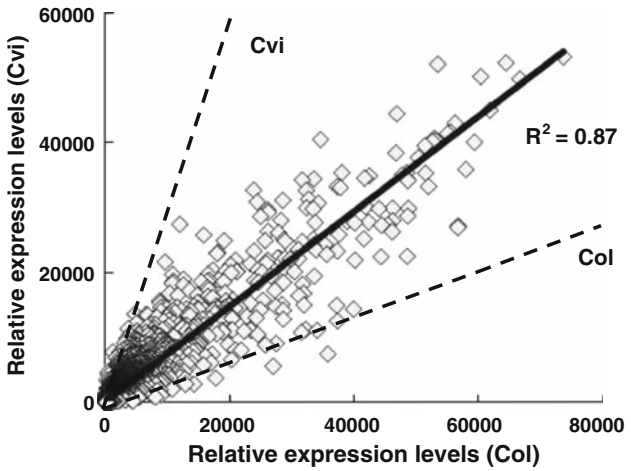

Fig. 1 A scatterplot of stored mRNA abundance in Col and Cvi dry seeds. Bold line indicates the regression line ($R^2 = 0.87$). X- and Y-axes indicate relative abundance of stored mRNA in Col and Cvi seeds, respectively. Dotted lines indicate the cut-off (threefold) for Col-specific and Cvi-specific mRNAs

Col-specific mRNAs were over-represented for small *Heat Shock Proteins* (*HSP*) genes ($P = 1.18e-8$). Expression of small *HSP* genes is ABI3-dependent (Kotak et al. 2007) and is associated with the dormant state of Cvi seeds (Cadman et al. 2006). Proteome analysis indicates that these small HSPs were induced specifically by osmopriming (Gallardo et al. 2001), suggesting that these are not correlated with germination. Another remarkable feature of Col-specific stored mRNAs are the over-representation of those related to reactive oxygen species (ROS). Those include superoxide metabolism ($P = 4.16e-4$), oxidative stress response ($P = 1.56e-3$), and glutathione conjugation reaction ($P = 3.37e-3$). These suggest that Col seeds are more active in either ROS production or signaling than the Cvi.

Cvi-specific mRNAs were highly over-represented for phosphate metabolism ($P = 2.39e-07$), suggesting that a subset of phosphate metabolism genes is regulated in an accession-specific manner. Also, Cvi-specific stored mRNAs are over-represented for those related to tubulin dependent transport ($P = 5.53e-06$), microtubule cytoskelton ($P = 3.65e-04$), actin cytoskelton ($P = 3.84e-03$), DNA topology ($P = 3.53e-02$), and lipid, fatty acid and isoprenoid metabolism ($P = 4.46e-02$). The fatty acid metabolism genes include those for 3-ketoacyl-CoA synthase1 (KCS1) (Todd et al. 1999), fatty acid elongase 1 (FAE1) (James et al. 1995), acyltransferase (FDH) (Yephremov et al. 1999), and acyl-CoA carboxylase (ACC2) (Baud et al. 2003). It is notable that all enriched Cvi-specific stored mRNAs are involved in lipid biosynthesis but none were found for those related to fatty acid breakdown.

Stored mRNAs for amino acid metabolisms are differentially accumulated in accessions. Col-specific stored mRNAs were over-represented for those related to metabolisms of valine, leucine and isoleucine. On the other hand, Cvi-specific stored mRNAs enriched for biosynthesis of glutamate and phenylalanine or for metabolisms of glycine, proline, threonine, and cysteine. It is unknown what do these biased GO categories mean, but these unique patterns of stored mRNAs might be useful fingerprints for characterizing the nature of stored mRNAs.

The abundant class of stored mRNAs

Microarray analysis reveals stored mRNAs from 13,202 Col genes and from 14,096 Cvi genes judged as "Present" by the analytical software MAS 5.0 at least once in triplicate experiments. We selected 100 genes whose mRNA levels were the highest in these stored mRNA genes in either Col or Cvi dry seeds (Table S2 and S3). These genes are similar to one another, and 79 genes are shared. In this study, we define these 79 genes as the abundant class of stored mRNA. This class represents less than 1% of genes in > 10,000 stored mRNA genes. This abundant mRNA class included many known genes for seed proteins.

Stored mRNA for LEA and seed storage proteins

The majority of the abundant class of stored mRNAs are the *LEA* genes: 17 genes are included in the abundant class of stored mRNAs among 21 genes in Col and 19 genes in Cvi (Table 1). *LEA* mRNAs accumulate in the late stage of seed development. The Arabidopsis genome contains 51 *LEA* genes that are structurally classified into nine groups (Wise 2003; Hundertmark and Hincha 2008). A subset of *LEA* mRNAs accumulate in dry seeds but some are also induced in plants under low temperature, drought, high light and salt stresses (Seki et al. 2002; Kimura et al. 2003; Hundertmark and Hincha 2008). The physiological and biochemical function of LEA proteins have not been fully elucidated, however, they are known to be involved in the stress tolerance response of plant cells (Brini et al. 2007; Dalal et al. 2009). The other major family of abundant class of stored mRNAs is those for seed storage proteins. The mRNAs for three cruciferins (12S globulins) and two napins (2S albumins) accumulate in dry seeds of both Col and Cvi (Table 1). There are three cruciferin genes and five napin genes in the Arabidopsis Col genome (Fujiwara et al. 2002). In addition, another abundant class of stored mRNAs includes those for stress proteins, such as HSPs (3 in Col and 4 in Cvi).

Stored mRNAs related to metabolism

The abundant class of stored mRNA contains those for lipid storage and mobilization (Table 1). Oil seeds

Table 1 A selected list of the abundant class of stored mRNAs in dry seeds

Affymetrix#	Locus ID	mRNA levels in Col	Rank in Col	mRNA levels in Cvi	Rank in Cvi	Description
Late embryogenesis abundant (LEA) proteins						
247095_AT	AT5G66400	73,597	1	53,200	1	Dehydrin RAB18
258224_AT	AT3G15670	66,605	2	49,803	5	LEA domain-containing
263753_AT	AT2G21490	59,280	6	40,080	15	Dehydrin family
250648_AT	AT5G06760	57,885	7	35,888	19	LEA group 1 domain-containing
256464_AT	AT1G32560	56,608	9	26,934	59	LEA group 1 domain-containing
263385_AT	AT2G40170	56,155	10	43,003	8	Em-like protein GEA6/AtEm6
262128_AT	AT1G52690	50,243	20	37,727	18	Similar to LEA from B. napus
266544_AT	AT2G35300	46,047	32	26,300	61	LEA group 1 domain-containing
256938_AT	AT3G22500	44,316	34	27,310	57	Seed maturation protein
258347_AT	AT3G17520	42,359	37	34,905	24	AtECP31
266392_AT	AT2G41280	42,247	38	27,792	52	LEA domain-containing protein
252137_AT	AT3G50980	35,239	55	24,311	73	LEA protein M10
252019_AT	AT3G53040	33,423	63	34,425	27	Dehydrin Xero1
246299_AT	AT3G51810	32,197	69	32,637	34	LEA domain-containing protein
246242_AT	AT4G36600	30,986	78	22,118	88	AtEm1
254440_AT	AT4G21020	29,659	82	22,069	89	LEA domain-containing protein
263492_AT	AT2G42560	28,453	90	31,125	36	LEA domain-containing protein
Seed storage proteins						
249082_AT	AT5G44120	54,948	12	41,429	10	CRA1 (CRUCIFERINA)
265095_AT	AT1G03880	53,352	13	52,083	3	CRU2 (CRUCIFERIN 2)
253767_AT	AT4G28520	46,787	28	44,457	7	CRU3 (CRUCIFERIN 3)
253902_AT	AT4G27170	46,107	30	29,909	41	2S albumin 4
253895_AT	AT4G27160	37,708	47	33,071	32	2S albumin 3
Heat shock proteins						
252515_A	AT3G46230	56,653	8	27,199	58	AtHSP17.4
250351_AT	AT5G12030	51,403	18	35,315	22	AT-HSP17.6A
261838_AT	AT1G16030	33,658	61	27,722	53	HSP70B (heat shock protein 70B)
Metabolism						
254095_A	At4G25140	55,456	11	41,596	9	OLEO1 (OLEOSIN1)
248520_AT	AT5G50700; AT5G50600	52,846	15	40,650	11	SDR family protein
256354_AT	AT1G54870	51,309	19	39,534	16	Oxidoreductase
249353_AT	At5G40420	48,659	21	29,876	42	OLEO2 (OLEOSIN 2)
258240_AT	At3G27660	46,649	29	38,417	17	OLEO4 (OLEOSIN4)
255905_AT	AT1G17810	31,556	75	24,804	71	β-TIP (aquaporin) intrinsic 1
257947_AT	AT3G21720	37,192	50	33,251	31	Isocitrate lyase
250868_AT	AT5G03860	32,625	67	31,306	35	Malate synthase
245168_ATT	At2G33150	28,143	92	28,045	49	PED1

We designated 79 mRNAs as the "abundant class of stored mRNAs" which belong to top the 100 mRNAs in both Col and Cvi dry seeds. This table includes selected genes as discussed in the text. mRNA levels in Col and Cvi dry seeds are obtained from microarray data. Rank indicates the order of mRNA abundance from ~22,000 Arabidopsis genes

including Arabidopsis seeds store triacylglycerol (TAG) in the oil bodies, and TAG is converted to sucrose during and after germination (Graham 2008). Recent proteome analysis on oil bodies revealed a set of protein components for the oil bodies (Jolivet et al. 2004; Katavic et al. 2006). Oleosins are the abundant proteins in oil bodies and are necessary for stabilization of oil bodies (Siloto et al. 2006). Oleosin genes compose a multigene family but only four isoforms are abundantly present in the Arabidopsis seeds (Jolivet et al. 2004). The abundant class of stored mRNA includes three out of four oleosin mRNAs for seeds. In addition, proteome analysis identified several other

components for oil bodies, including 11-β-hydroxysteroid dehydrogenase, short-chain dehydrogenase, embryo-specific protein, β-glucosidase, predicted GPI-anchored protein, aquaporin, myrosinase and its associated proteins. The abundant stored mRNAs in Col and Cvi dry seeds included those for three oleosins, 11-β-hydroxysteroid dehydrogenase (At5g50700/At5g50600), β-TIP (aquaporin; At1g17810), and short-chain dehydrogenase (At1g54870; Table S5). Moreover, stored mRNAs for the predicted GPI-anchored protein (At1g54860) and myrosinase-binding protein (At3g21380) are found in the abundant class in Col.

Fatty acids are converted to acetyl-CoA by β-oxidation and then to sucrose by glyoxylate cycle and gluconeogenesis. The abundant stored mRNAs include those for the glyoxylate cycle enzymes, malate synthase and isocitrate lyase, that accumulate abundantly in both Col and Cvi. In addition, *PED1/KAT2* is also listed in the abundant class. PED1/KAT2 encodes a 3-keto-acyl-CoA thiolase that catalyzes the conversion of 3-keto-acyl-CoA to acyl-CoA in glyoxysomes (Hayashi et al. 1998; Germain et al. 2001). Other mRNAs for lipid biosynthesis and mobilization did not belong to the abundant class, but their mRNAs were stored moderately in dry seeds. Some of these mRNAs tended to be differentially accumulated between accessions (see below).

Stored mRNAs for transcription factors

The Arabidopsis genome contains at least 1,834 genes for transcription factors, which are divided into 50 families based on the nature of DNA-binding domains (http://arabidopsis.med.ohio-state.edu/AtTFDB/). ATH1 arrays cover expression of 1,467 of 1,834 TF genes. The abundant classes of 79 stored mRNAs contain only one encoding a transcription factor (TF); ATAF1, a membrane-bound NAC domain transcription factor (Kim et al. 2006). ATAF1 has been implicated in the roles of stress response (Lu et al. 2007) and ABA signaling (Jensen et al. 2008). Interestingly, this family was recently shown to be involved in multiple responses to DNA damage (Yoshiyama et al. 2009).

Although ATAF1 is the only TF gene in the abundant class of 79 stored mRNAs, analytical software MAS5.0 indicates 741 Col and 818 Cvi TF mRNAs are called "Present" in the stored mRNA transcriptomes. Table 2 depicts the top 20 TF mRNAs in Col and Cvi dry seeds. Several families of TFs accumulated their mRNAs as the stored mRNA form relatively abundantly: NAC, AP2-EREBP, and zinc-finger proteins. Also, stored mRNAs for TFs that function in the regulation of ABA signaling and stress responses accumulated in dry seeds. RD26 encodes a NAC transcription factor involved in ABA signaling and stress responses (Fujita et al. 2004). The members of RAP2 transcription factors (RAP2.3, and 2.4) as well as DREB2A are the abundant class of AP2 TFs. ABI5 encodes a bZIP-type transcription factor (Finkelstein and Lynch 2000), and is a key regulator for ABA-responsive element (ABRE)-mediated transcription in seeds (Bensmihen et al. 2002; Carles et al. 2002; Kim et al. 2002). ABI5 mRNA was dramatically decreased after imbibition and was induced by drought, salt and ABA (Lopez-Molina et al. 2001; Nakashima et al. 2006). Enrichment of TFs involved in ABA signaling in the stored mRNA may be related to the previous report that ABREs were highly over-represented in the promoters of genes for stored mRNA (Nakabayashi et al. 2005).

Germination of seeds treated with CHX and CDT

Effects of CHX and CDT on germination

Seeds of some *transparent testa* (*tt*) mutants have increased permeability (Debeaujon et al. 2000). We used the *tt8* mutants of the Col accession (Gonzalez et al. 2009) to examine the effect of CHX on gene expression during imbibition. The treatment of 100 μM CHX inhibited germination of both Col and *tt8* seeds (data not shown). We also tested the effect of CDT on germination of Col and *tt8* seeds (Fig. 2). Dry seeds were subjected to saturated vapor at 40°C for various periods (Delouche and Baskin 1973). The *tt8* mutant seeds did not alter germination kinetics after the CDT for 1 day, but % germination and velocity of germination were affected after the CDT for 3 days (Fig. 2a). The mutant seeds failed to germinate when the seeds were subjected to the CDT for more than 5 days. The negative effects of CDT on Col germination were enhanced gradually when the period was prolonged to 7 days (Fig. 2b), in contrast to the abrupt decline of the *tt8* mutant after 5 days of the treatment. This is in agreement with a previous report that Arabidopsis testa mutants reduced both seed longevity and dormancy (Debeaujon et al. 2000).

Effects of CHX on mRNA abundance during imbibition

We investigated the effect of CHX on these imbibition-regulated genes in *tt8* mutant seeds to examine if early induction of these genes requires de novo synthesized proteins after imbibition. Down-regulated and up-regulated genes by imbibition were chosen based on clear changes in mRNA abundance from the gene list obtained from microarray data in Preston et al. (2009).

We chose the following five down-regulated genes: *RD29B* (Nakashima et al. 2006), At2g42560 (Hundertmark and Hincha 2008), *ABI2* (Leung et al. 1997), *ABI5* (Finkelstein and Lynch 2000; Lopez-Molina and Chua 2000), and *CP29* (Andersson et al. 2001). The decline of mRNA

Table 2 Top 20 of highly expressed TF mRNAs in Col and Cvi dry seeds

Affymetrix#	Locus ID	Description	Family	mRNA levels in Col	Rank in TFs (in total)	mRNA levels in Cvi	Rank in TFs (in total)
261564_AT	AT1G01720	ATAF1/ANAC002	NAC	30,553	1 (80)	22,205	1 (86)
262977_AT	AT1G75490	DNA binding/TF	AP2-EREBP	23,240	2 (122)	15,259	3 (142)
258434_AT	AT3G16770	ATEBP/RAP2.3	AP2-EREBP	22,642	3 (123)	13,231	5 (185)
250781_AT	AT5G05410	DREB2A	AP2-EREBP	20,283	4 (135)	12,945	6 (190)
250010_AT	AT5G18450	AP2 domain TF	AP2-EREBP	16,329	5 (175)	1,7680	2 (123)
263907_AT	AT2G36270	ABI5	bZIP	13,173	6 (217)	13,480	4 (180)
249944_AT	AT5G22290	ANAC089	NAC	12,803	7 (224)		
259705_AT	AT1G77450	ANAC032	NAC	12,726	8 (225)		
259932_AT	AT1G34370	STOP1	C2H2	11,700	9 (242)	11,960	7 (203)
248188_AT	AT5G54070	AT-HSFA9	HSF	11,349	10 (248)	7,401	14 (372)
261265_AT	AT1G26800	Zinc finger	C3H	8,087			
245207_AT	AT5G12310	Zinc finger	C3H	7,905	11 (359)	6,767	16 (421)
260229_AT	AT1G74370	Zinc finger	C3H	7,706	12 (378)	8,130	11 (329)
255926_AT	AT1G22190	RAP2.4	AP2-EREBP	7,618	13 (386)	10,675	8 (231)
248665_AT	AT5G48655	Zinc finger	C3H	7,071	14 (389)		
250119_AT	AT5G16470	Zinc finger	C2H2	6,985	15 (425)		
261648_AT	AT1G27730	STZ	C2H2	6,910	16 (431)		
253872_AT	AT4G27410	RD26/ANAC072	NAC	66,46	17 (437)		
257981_AT	AT3G20770	EIN3	EIL	5,758	18 (466)	7,620	12 (356)
257967_AT	AT3G19910	Zinc finger	C3H	5,576	19 (538)	6,438	17 (448)
261062_AT	AT1G07530	SCL14/AtGRAS2	GRAS		20 (562)	9,430	9 (265)
262590_AT	AT1G15100	RHA2A	C3H			8,286	10 (317)
247054_AT	AT5G66730	Zinc finger	C2H2			7,471	13 (365)
260540_AT	AT2G43500	AtNLP8	NLP			7,015	15 (405)
262197_AT	AT1G53910	RAP2.12	AP2-EREBP			6,339	18 (453)
267026_AT	AT2G38340	AP2 domain TF	AP2-EREBP			6,290	19 (456)
250849_AT	AT5G04410	ANAC078/NAC2	NAC			6,147	20 (468)

Twenty stored mRNAs whose levels are highest in Col and Cvi dry seeds. Rank indicates the order of mRNA levels from 1,467 TF genes and rank in parenthesis indicates the order of mRNA levels from 22,000 Arabidopsis genes. Genes highlighted blue and orange indicate the top 20 TF genes in Col and in Cvi, respectively

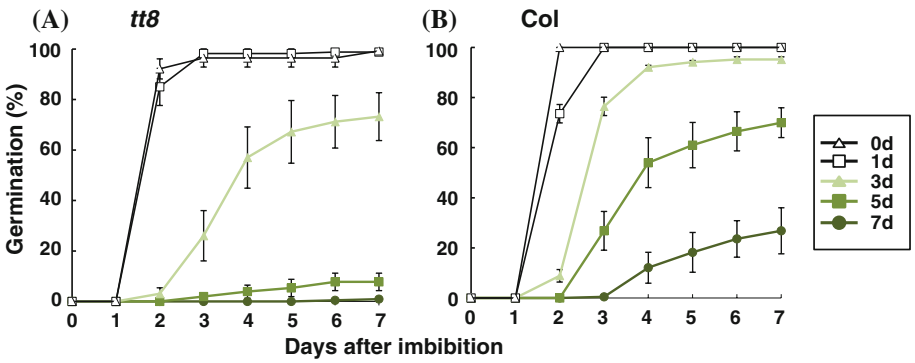

Fig. 2 Germination of seeds after controlled deterioration treatment Col (A) and *tt8* (B) seeds were subjected to controlled deterioration treatment and sown on agar medium. Germination was scored daily and triplicate experiments were performed using independent seed batches. A bar indicates the standard error

abundance of these genes in the control was first observed 2 h after the onset of imbibition (Fig. 3a). The down-regulation of all these genes was partially alleviated by CHX application (Fig. 3a). It is noteworthy that mRNA abundance of most of these genes was higher in CHX-treated seeds relative to the control at 1 h. The increase of mRNA by CHX treatment suggests that inhibition of translation precociously activated transcription possibly using stored transcriptional machinery. This suggests that, under our conditions, resumption of gene expression has a 1-h lag

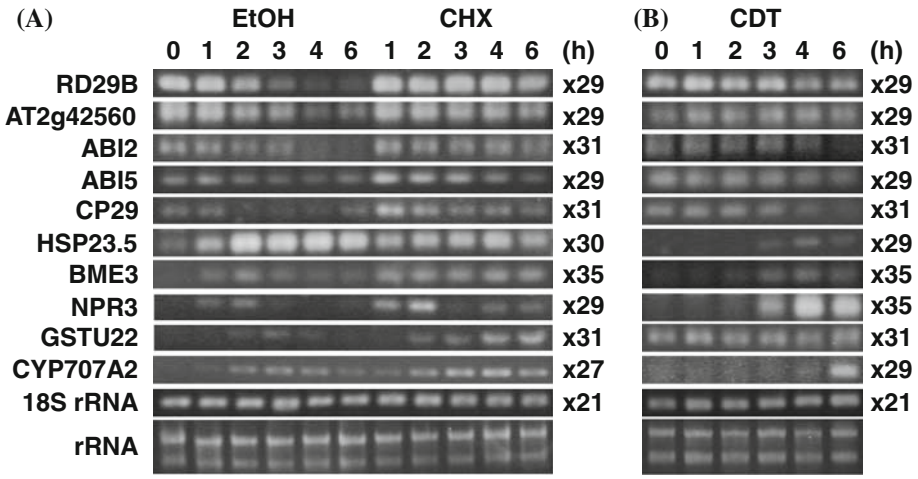

Fig. 3 Effects of cycloheximide and controlled deterioration treatment on gene expression during imbibition. (A) Expression patterns during imbibition under control (EtOH) and 100 μM cycloheximide (CHX). RT-PCR analysis was carried out by using total RNA extracted from *tt8* seeds. Two independent experiments were performed using different *tt8* alleles SALK_030966 and SALK_048673 (Figure S2). Consistent results were obtained from these experiments and data from SALK_030966 is shown. The number of PCR cycles is shown on the right. (B) Expression patterns during imbibition in damaged seeds. Seeds were exposed to saturated vapor at 40°C (controlled deterioration treatment) for 1 day, dried on filter paper under normal humidity conditions at room temperature for 1 day, followed by a germination test. Two independent experiments were performed using different *tt8* alleles, SALK_030966 and SALK_048673. Consistent results were obtained from these experiments and data from SALK_030966 is shown. The number of PCR cycles is shown on the right

period after the start of imbibition but can be precociously induced by CHX treatment. It is also possible that inhibition of translation enhanced mRNA protection more prominently than in control seeds. Rajjou et al. (2004) reported that α-amanitin-treated seeds enhanced translation of stored mRNA, suggestive of two parallel mechanisms that may be explained the effect of CHX on expression patterns of the down-regulated genes.

The following five genes were chosen to examine the effect of CHX on imbibition induced gene expression: *HSP23.5* (Waters et al. 2008), *BME3* (Liu et al. 2005), *NPR3* (Zhang et al. 2006), *GSTU22* (Edwards et al. 2000; Wagner et al. 2002), and *CYP707A2* (Kushiro et al. 2004; Okamoto et al. 2006). The transcript abundance of *HSP23.5* was increased 1 h after imbibition and its transcript abundance continued to increase thereafter. Induction of *BME3, NRP3, GSTU22* and *CYP707A2* were induced either 1 or 2 h after the start of imbibition, but their induction was transient and declined thereafter (Fig. 3a). CHX application did not affect the induction of *HSP23.5* at 1 h, but inhibited further induction at 2 h after the onset of imbibition (Fig. 3a). This suggests that induction of this gene after 2 h requires de novo protein synthesis. Moreover, induction of *BME3, NPR3, GSTU22* and *CYP707A2* at 2 h was also unaffected by CHX (Fig. 3a) suggesting that induction of these genes at this time point does not require de novo protein synthesis. It was recently shown that *CYP707A2* mRNA is translated during the first 6 h after the start of imbibition (Liu et al. 2009). We also note that induction of these genes in the CHX treatment was not transient, suggesting that down-regulation at the latter time points require de novo protein synthesis.

Effects of CDT on mRNA abundance during imbibition

Experiments using CHX indicated that stored proteins in dry seeds are involved in gene expression during early imbibition stages. To confirm the importance of stored macro-molecules in dry seeds, we performed gene expression analysis on seeds with CDT (Fig. 3b; Table S7). This treatment is expected to cause damage on stored macro-molecules in dry seeds, including stored components for transcription and translation.

The *tt8* mutant seeds were subjected to CDT for 1 day, which did not affect the velocity of germination (Fig. 2a). RT–PCR expression analysis showed gene expression patterns in CDT-treated seeds were largely altered during imbibition even though germination was visibly normal. Down-regulation of five genes was alleviated when compared with that of control seeds (Fig. 3a, EtOH *vs.* b). This suggests that efficient down-regulation of these genes requires factors stored in dry seeds. On the other hand, up-regulation of four of five genes was severely delayed compared with that of control seeds (Fig. 3a *vs.* b). This supports the notion that early induction of these genes requires factors stored in dry seeds. The expression pattern of *GSTU22* was different from those of four other up-regulated genes. Its mRNA level was high in dry seeds after CDT (Fig. 3b), suggestive of its induction during CDT treatment.

Moreover, the abundance of *GSTU22* mRNA was maintained high during imbibition. Rajjou et al. (2008) reported that CDT enhanced protein oxidation presumably due to accumulation of reactive oxygen species (ROS). It is possible that GSTU22 was induced by ROS during CDT.

In conclusion, 1-d-CDT had little effect on velocity of germination but did largely affect the expression patterns of genes that are regulated early in seed imbibition. In combination with CHX experiments, we propose that these data reflect the importance of seed stored factors in resumption of transcription and gene expression during seed imbibition. Furthermore, regardless of the severe effect on gene expression during imbibition, 1-d-CDT did not alter the velocity of germination. This suggests transcriptional factors in dry seeds may be dispensable and are probably compensated by neosynthesized proteins, consistent with that translational capacity is correlated with seed vigor and viability (Rajjou et al. 2007).

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