Proteomic Analysis of Lettuce Seed Germination and Thermoinhibition by Sampling of Individual Seeds at Germination and Removal of Storage Proteins by Polyethylene Glycol Fractionation

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Germination and thermoinhibition in lettuce (Lactuca sativa ‘Jianyexianfeng No. 1’) seeds were investigated by a proteomic comparison among dry seeds, germinated seeds at 15°C, at 15°C after imbibition at 25°C for 48 h, or at 25°C in KNO3 (all sampled individually at germination), and ungerminated seeds at 25°C, a thermoinhibitory temperature. Before two-dimensional gel electrophoresis analysis, storage proteins (greater than 50% of total extractable protein) were removed by polyethylene glycol precipitation, which significantly improved the detection of less abundant proteins on two-dimensional gels. A total of 108 protein spots were identified to change more than 2-fold (P < 0.05) in abundance in at least one germination treatment. Nineteen proteins increasing and one protein decreasing in abundance during germination had higher abundance in ungerminated 25°C seeds than in ungerminated 25°C seeds. Gene expression of 12 of those proteins correlated well with the protein accumulation. Methionine metabolism, ethylene production, lipid mobilization, cell elongation, and detoxification of aldehydes were revealed to be potentially related to lettuce seed germination and thermoinhibition. Accumulation of three proteins and expression of five genes participating in the mevalonate (MVA) pathway of isoprenoid biosynthesis correlated positively with seed germinability. Inhibition of this pathway by lovastatin delayed seed germination and increased the sensitivity of germination to abscisic acid. MVA pathway-derived products, cytokinins, partially reversed the lovastatin inhibition of germination and released seed thermoinhibition at 25°C. We conclude that the MVA pathway for isoprenoid biosynthesis is involved in lettuce seed germination and thermoinhibition.

Seed germination is a complex process that is influenced by many environmental factors, such as light, temperature, and moisture (Bewley et al., 2013). Temperature is the most important environmental factor in regulating seed germination. For a given species, there is an optimal temperature or temperature range for seed germination, below and above which seed germination will be delayed or inhibited. The failure of a seed to germinate at supraoptimal temperatures is commonly called thermoinhibition (Reynolds and Thompson, 1971; Abeles, 1986). In nature, thermoinhibition of seed germination is an adaptive regeneration strategy for the seeds of winter annual plants, which prevents the seeds germinating during the summer when the conditions are not appropriate for seedling growth (Baskin and Baskin, 1998; Allen et al., 2007). However, thermoinhibition often creates a problem for crop production. It causes delayed and poor germination of crop species with low optimal temperatures for seed germination and thus limits the time and region for crop cultivation. In the case of lettuce (Lactuca sativa) seeds, which exhibit thermoinhibition at 25°C to 30°C depending on variety and production environment (Guzman et al., 1992; Kozarewa et al., 2006; Contreras et al., 2009; Huo et al., 2013), seeds will hardly germinate during the summer or in tropical areas, when or where the temperature usually exceeds the upper limit temperature for germination. Therefore, production of more thermotolerant seeds will be very useful. This requires a deeper understanding of the physiological and molecular mechanisms underlying seed germination and thermoinhibition.

1 This work was supported by the National Sciences and Technology Support Program (grant no. 2012BA01B05), the National Natural Sciences Foundation of China (grant nos. 31171624 and 81102757), and the Chinese Academy of Sciences (visiting professorship to I.M.M.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.15.00045
Phytohormones, such as abscisic acid (ABA), GA, and ethylene, play an essential role in seed germination (Kucera et al., 2005; Finkelstein et al., 2008; Linkies and Leubner-Metzger, 2012). A number of physiological and molecular studies have revealed a key role of ABA and GA and their interactions in modulating thermoinhibition (Yoshioka et al., 1998; Gonai et al., 2004; Tamura et al., 2006; Argyris et al., 2008; Toh et al., 2008; Huo et al., 2013; Lim et al., 2013). In lettuce seeds, the ABA content decreases rapidly when the seeds are germinated at optimal temperatures, but it is maintained at elevated levels at high temperatures (Yoshioka et al., 1998; Toh et al., 2008). Application of fluridone, an inhibitor of ABA biosynthesis, promotes the germination of lettuce seeds at high temperature (Yoshioka et al., 1998; Gonai et al., 2004). In many plant species, exogenous application of GA alleviates the thermoinhibition of seed germination (Madačak et al., 1993; Carter and Stevens, 1998; Gonai et al., 2004). Exogenous GA lowers the endogenous ABA content in Arabidopsis seeds by enhancing the catabolism of ABA and the export of the catabolites from intact seeds (Gonai et al., 2004). High temperature promotes the expression of genes involved in ABA biosynthesis (Argyris et al., 2008; Toh et al., 2008) but inhibits the expression of genes involved in ABA catabolism (Toh et al., 2008) and GA biosynthesis (Argyris et al., 2008; Toh et al., 2008). Recently, Huo et al. (2013) reported that the expression of an ABA biosynthesis gene, 9-cis-EPOXYCAROTENOID DIOXYGENASE4, is essential for the thermoinhibition of lettuce seed germination.

Ethylene is another important phytohormone determining the thermoinhibition of seed germination. Numerous studies have found that exogenous application of ethylene or its precursors alleviates, whereas ethylene biosynthesis or action inhibitors enhance, thermoinhibition (Saini et al., 1986; Nascimento et al., 2000, 2004; Kozarewa et al., 2006; Matilla and Matilla-Vázquez, 2008). When seed germination is inhibited at high temperatures, the expression of ethylene biosynthetic genes, such as 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE1 (ACS1) and 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE2 (ACO2), is inhibited or maintained at low levels (Argyris et al., 2008; Huo et al., 2013). Deng and Song (2012) found that the application of sodium nitroprusside, potassium ferricyanide, nitrite, or nitrate promotes lettuce seed germination at thermoinhibitory temperatures via a nitric oxide-dependent mechanism. At the physiological and genetic levels, these studies have clarified the way in which seeds sense the temperature to control germination. However, our understanding of thermoinhibition, especially at the translational level, is still incomplete.

Proteomics is a useful approach both to visualize and compare complex mixtures of proteins and to gain information about the individual proteins involved in specific biological responses and/or processes (van Wijk, 2001; Rajjou et al., 2011). De novo protein synthesis is an essential process for seed germination (Rajjou et al., 2012; Gallard et al., 2014). Seed germination will not occur when translation is inhibited by cycloheximide (Rajjou et al., 2004; Sano et al., 2012). In contrast, protrusion of the seed radicle can be observed in the presence of a transcriptional inhibitor, α-amanitin or actinomycin D (Rajjou et al., 2004; Sano et al., 2012). This suggests that seed germination has been programmed during maturation on the mother plant and that protein synthesis during the initial stages of germination relies mainly on the stored mRNA templates in mature dry seeds without de novo transcription (Rajjou et al., 2004; Sano et al., 2012). Therefore, proteomics can reveal key features of seed germination. Proteomics have been applied to investigate seed germination in many species, such as Arabidopsis (Arabidopsis thaliana; Gallardo et al., 2001, 2002; Gallard et al., 2014), rice (Oryza sativa; Yang et al., 2007), and pea (Pisum sativum; Wang et al., 2012b). These studies focused on seed germination at the optimal temperature under normal conditions or in response to chemical treatments, such as GA (Gallardo et al., 2002; Kim et al., 2008), ABA (Kim et al., 2008), and salicylic acid (Rajjou et al., 2006), and environmental stress, such as salinity (Yacoubi et al., 2011, 2013). To the best of our knowledge, no proteomic study has examined the effect of temperature on seed germination.

The dynamic resolution of proteomics is limited when large proteomes consisting of thousands of proteins are analyzed, and only the most abundant proteins can be detected (van Wijk, 2001). In seeds, storage proteins are highly abundant and account for as much as 60% of the total proteins (Miernyk and Hajduch, 2011). Abundant storage protein will significantly decrease the detection of low-abundance proteins, which are usually those we require (Miernyk and Hajduch, 2011). This problem also occurs in other plant tissues (e.g. about 50% of the leaf protein is Rubisco). To improve the detection of the lower abundance proteins in proteomic analyses, especially by two-dimensional gel electrophoresis (2-DE), approaches have been developed to deplete high-abundance proteins partly or completely by precipitation during sample preparation using chemical compounds such as CaCl2 (Krishnan et al., 2009), Ca2+ phytate (Krishnan and Natarajan, 2009), and polyethylene glycol (PEG; Kim et al., 2001; Xi et al., 2006; Acquadro et al., 2009). PEG is an excellent general reagent for differential precipitation of proteins, and PEG fractionation is simpler and more effective in removing high-abundance proteins (Juckes, 1971). This approach has been applied to effectively remove most of the Rubisco in globe artichoke (Cynara scolymus; Acquadro et al., 2009), tomato (Solanum lycopersicum; Ahsan et al., 2007), rice (Lee et al., 2007), and Arabidopsis (Xi et al., 2006) leaves. In addition, it increases the number of detectable protein spots and allows for the detection of many low-abundance proteins in these tissues. However, whether PEG fractionation can be used for preparing seed protein samples remains to be established.

In this study, we have investigated the effect of temperature on lettuce seed germination by comparing the proteomes of seeds germinated at optimal and
thermo inhibitory temperatures. We used PEG fractionation to remove highly abundant storage proteins from the seed extracts before analyzing the extracts using 2-DE. The expression of genes encoding candidate proteins involved in seed germination and thermoinhibition also was examined by quantitative reverse transcription (qRT)-PCR. The combined results demonstrate an involvement of the mevalonate (MVA) pathways for isoprenoid biosynthesis in the regulation of lettuce seed germination and thermoinhibition.

RESULTS

Seed Germination

The optimum temperature for germination is at 15°C to 21°C, and thermoinhibition of germination is observed at 23°C or higher in lettuce ‘Jianyexianfeng No. 1’ seeds (Deng and Song, 2012). At 15°C (15G), seed started to germinate after 24 h, and all seeds had germinated after 48 h of imbibition (HI; Fig. 1). At 25°C (25G), only 10% of seeds germinated within 48 HI (Fig. 1). However, when the ungerminated seeds at 25°C after 48 HI were transferred to 15°C (25/15G), they all germinated (Fig. 1), demonstrating the occurrence of thermoinhibition at 25°C. These seeds also exhibited an increasing rate of germination at 15°C (Fig. 1). Seeds imbibed at 25°C in 10 mM KNO₃ (25nG) germinated much more (about 60%) than 25G seeds but took longer to complete germination (about 72 h) than 15G and 25/15G seeds (Fig. 1).

PEG Precipitation of Abundant Storage Proteins

In order to improve the chance of detecting low-abundance proteins in lettuce seeds, a PEG fractionation method was developed to remove the highly abundant storage protein before 2-DE. 15G seeds that all germinated (see below) were sampled as materials, and a series of PEG concentrations were chosen to look for the optimal concentration for protein precipitation. The workflow of the PEG fractionation is depicted in Figure 2A.

Total extractable protein (TP) from the 15G seeds included a large amount of storage protein (Fig. 2, B and C; Supplemental Fig. S1; Supplemental Table S1). Adding 4% (w/v) PEG to the TP sample precipitated about 55% of proteins, most of which were storage proteins (Fig. 2B; Supplemental Table S2). This indicates that more than 50% of the proteins in lettuce seeds are storage proteins. Adding PEG to 8% further decreased the amount of storage protein. A further increase to 12% PEG had no obvious effect on the precipitation of these proteins, but it started to precipitate a wide range of other proteins (Fig. 2B). Therefore, 8% PEG was selected to be the optimum concentration for the precipitation and removal of storage proteins from lettuce seed extracts before 2-DE analysis.

Adding 8% PEG to TP samples greatly increased the numbers and resolution of the protein spots on two-dimensional (2-D) gels, especially where the storage proteins were highly dominant (Fig. 2, C and D, boxed areas, and E, a representative region of the 2-D gel; Supplemental Table S3). After Coomassie Brilliant Blue R-250 staining, about 668 reproducible protein spots were detected in three replicates of the TP samples, while this number increased to 801 in the samples after 8% (w/v) PEG precipitation (S8; Fig. 2, C and D; Supplemental Table S3). About 97% of the protein spots (647 spots) in the TP samples were also found in S8 samples (Supplemental Table S3), indicating an excellent reproducibility of PEG fractionation in preparing lettuce seed protein samples. A huge difference of spot numbers between TP and S8 samples was observed within the regions dominated by storage proteins (Fig. 2, C and D, boxed regions). Within region a, 193 reproducible spots were detected in TP samples, while this number increased to 266 in S8 samples (Fig. 2, C and D; Supplemental Table S3). Within region b, 46 and 67 reproducible spots were found in TP and S8 samples, respectively (Fig. 2, C and D; Supplemental Table S3).

Proteomic Analysis of Seed Germination and Thermoinhibition

During germination sensu stricto or during both germination and postgerminative growth, seeds are normally sampled in batches for a proteomic study. In this study, a distinct sampling strategy was developed, which ensures that all individuals in a given seed lot are sampled at precisely the same germination stage (Supplemental Fig. S2). For 15G, 25/15G, and 25nG seeds, germination was monitored every 2 h, and the
seeds that had germinated within the previous 2-h period were collected and immediately frozen in liquid N2. After all the seeds had germinated, the collected seeds were pooled for each treatment and stored at –70°C until used for proteomic analysis (Supplemental Fig. S2B). For 25G seeds, the germinated seeds (10%) were discarded after 48 HI, and only the ungerminated seeds (90%) were frozen and stored at –70°C (Supplemental Fig. S2C). The dry, germinated 15G, 25/15G, and 25nG, and ungerminated 25G seeds were used for protein extraction and 2-DE analysis (Supplemental Fig. S2). Before 2-DE, the total extractable protein for each treatment was precipitated by 8% PEG as described above. Total protein content and protein yield of PEG precipitation for each sample are shown in Supplemental Table S4.

A total of 127 protein spots showed significant changes of more than 2-fold (P < 0.05) in at least one treatment when the proteomes of dry seeds, germinated 15G, 25/15G, and 25nG seeds, and ungerminated 25G seeds were compared with each other (Fig. 3B; Supplemental Table S8). Proteins were identified in 113 spots by searching the peptide mass fingerprints against the Plant EST database, and four more were identified when the remaining 14 spots were subjected to search in the National Center for Biotechnology Information nonredundant (NCBI nr) database. This left 10 spots, for which no protein match could be found in any protein database (Supplemental Tables S5–S7). Among the identified protein spots, 108 were found to contain only one protein (Supplemental Table S6), and nine were matched to two or three proteins (Supplemental Table S7). Only the spots matched to one protein were considered in the following analyses.

The abundance of 36 and 35 spots increased and decreased significantly in 15G seeds, respectively (Fig. 3B; Supplemental Table S8), while the numbers were 22 and 34 in 25G seeds, respectively (Fig. 3B; Supplemental Table S9). At both temperatures, proteins involved in cell defense and rescue were the most numerous that increased in abundance during imbibition (Fig. 3B; Supplemental Tables S8 and S9). In 25/15G seeds, 36 and 11 protein spots increased and decreased in abundance, respectively (Fig. 3B; Supplemental Table S10). Metabolism-related proteins and storage protein dominated in the increased abundance pattern in 25/15G seeds (Fig. 3B; Supplemental Table S10). The number of protein spots increasing in abundance was higher in 25nG seeds than in 25G seeds, but the number of proteins decreasing in abundance was similar (Fig. 3B; Supplemental Table S11). Among the proteins of 25nG seeds exhibiting an increased abundance, proteins related to metabolism were the most numerous (Fig. 3B; Supplemental Table S11). Relatively few of the protein spots (11) decreased in abundance in 25/15G seeds compared with 15G, 25G, and 25nG seeds (Fig. 3B). Storage protein was the major group decreasing in abundance in all of the above germination treatments (Fig. 3B).
Protein spots increasing and decreasing in abundance in 15G, 25G, 25/15G, and 25nG seeds were compared with each other. Venn diagrams were plotted to display the similarity and difference among these treatments (Fig. 3C). Protein spots increasing in abundance were generally similar in 15G, 25nG, and 25/15G seeds. A large number of protein spots (19) increased consistently in abundance among these seeds (Fig. 3C). In contrast, proteins decreasing in abundance had much similarity in both germinated 15G and 25nG seeds and ungerminated 25G seeds, and 20 protein spots, more than half of the identified proteins in a given germination treatment, were found in all of these seeds (Fig. 3C). Only eight protein spots (five increased and three decreased) had a consistent change in all of the germination treatments (Fig. 3C).

It would be expected that the proteins whose abundance were higher or lower in germinated 15G, 25nG, and 25/15G seeds than in both dry and ungerminated 25G seeds may play important roles in regulating seed germination and thermoinhibition. A total of 22 protein spots belonging to 20 unique gene products followed this pattern (Table I). Out of these, 21 spots increased in abundance, while one spot (spot 1012) decreased in abundance in seeds during imbibition (Table I). These proteins participated in various biological processes, such as amino acid metabolism, lipid and sterol metabolism, establishment of cellular structure, and detoxification (Table I). Three spots containing S-ADENOSYL-METHIONINE SYNTHASE (SAM; spots 682, 707, and 713) were found to be at low or undetectable levels in ungerminated 25G and dry seeds but were increased significantly in abundance in germinated 15G, 25/15G, and 25nG seeds (Table I). CLAVAMINATE SYNTHASE-LIKE PROTEIN (CVS) with unknown function followed the same pattern, as did ISOCITRATE LYSASE (ICL; spot 290), ACTIN-DEPOLYMERIZING FACTOR2 (ADF2; spot 1431), ANNEXIN1 (ANN1; spot 981), and ACYLTRANSFERASE (ATF) homolog (spot 420; Table I).
Table I. Candidate proteins involved in lettuce seed germination and thermoinhibition

Protein expression levels are illustrated in dry (D), 25G, 15G, 25/15G, and 25nG seeds as the percentage of normalized volume × 100. The columns in the graphs are arranged in that order. ID, Identifier.

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<td>682</td>
<td>DW133353</td>
<td>S-Adenosyl-l-methionine synthetase</td>
<td>Amino acid metabolism</td>
<td><img src="image1" alt="Graph" /></td>
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<td>707</td>
<td>DW137691</td>
<td>S-Adenosyl-l-methionine synthetase</td>
<td>Amino acid metabolism</td>
<td><img src="image2" alt="Graph" /></td>
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<td>713</td>
<td>DW137090</td>
<td>S-Adenosyl-methionine synthase2</td>
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<td>126</td>
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<td>Lipid and sterol metabolism</td>
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<td>783</td>
<td>DY974670</td>
<td>Predicted: acetyl-CoA acetyltransferase, cytosolic1</td>
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<td>298</td>
<td>DW129004</td>
<td>2,3-Bisphosphoglycerate-independent phosphoglycerate mutase</td>
<td>Glycolysis</td>
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<td>290</td>
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<td>1414</td>
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<td>1070</td>
<td>DW064597</td>
<td>1-Aminocyclopropane-1-carboxylate oxidase1</td>
<td>Cell growth</td>
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(Table continues on following page.)
Expression of Genes Encoding the Candidate Proteins Involved in the Regulation of Seed Germination and Thermoinhibition

Except for storage protein (spots 1012, 1388, 1406, 1411, and 1414) and dehydrin (spot 1069), genes encoding the proteins potentially important for seed germination (Table I) were all selected for expression analysis in order to elucidate further their role in seed germination (Fig. 4). Because full-length clones of these genes have not been reported, we will here refer to them by the identified protein name. The best matched EST for a given protein spot was searched against the National Center for Biotechnology Information (NCBI) unigene database to acquire an assembled mRNA sequence, which was then used to design primers for qRT-PCR analysis. 15G, 25G, 25/15G, and 25nG seeds imbibed for 0, 6, 12, and 24 h, germinated 15G, 25/15G, and 25nG seeds (at 24 on the horizontal axis), and ungerminated 25G seeds at 48 HI (at 24 on the horizontal axis) were sampled for the qRT-PCR analysis (Fig. 4).

The differentially accumulated spots 682 and 707 were found to be homologous to the same SAM, but, in fact, they are encoded by two different genes (named SAM1a and SAM1b for spots 682 and 707, respectively), according to their best matched EST (Fig. 4). Among the three SAM-encoding genes, only the expression of SAM1b correlated with protein accumulation (Fig. 4B). SAM1a increased gradually in the mRNA level in 15G, 25G, and 25nG seeds and was expressed most in 25G seeds. Expression of this gene decreased in 25/15G seeds (Fig. 4A). The abundance of SAM2 transcripts decreased in 15G, 25/15G, and 25nG seeds and was maintained relatively constant in 25G seeds at the early stages and then increased 2-fold at 48 HI (Fig. 4C). The transcript levels of LIPOXYGENASE2 (LOX2; Fig. 4D) and ICL (Fig. 4G) genes increased continuously with time in all of the germination treatments, but they accumulated much higher in germinated 15G, 25/15G, and 25nG seeds than in ungerminated 25G seeds. The expression of genes encoding ACETYL-COENZYME A ACETYLTRANSFERASE (AACT1; Fig. 4E), 2,3-BISPHOSPHOGLYCERATE-INDEPENDENT PHOSPHOGLYCERATE MUTASE (BIPM; Fig. 4F), ALDEHYDE DEHYDROGENASE1 (ADH1; Fig. 4L), ANN1 (Fig. 4N), and CVS (Fig. 4P) were completely repressed or decreased slightly in 25G seeds but increased greatly in 15G, 25/15G, and 25nG seeds (Fig. 4L).

A Mascot search for the peptide mass fingerprint of spot 1070 did not find any matched EST in lettuce but

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<td>807</td>
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<td>DW134585</td>
<td>Actin-depolymerizing factor2</td>
<td>Cell structure</td>
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<td>424</td>
<td>DY960524</td>
<td>Aldehyde dehydrogenase1</td>
<td>Detoxification</td>
<td><img src="image3" alt="Graph" /></td>
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<td>898</td>
<td>DY984667</td>
<td>Predicted: probable aldo-keto reductase2-like</td>
<td>Stress response</td>
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<td>981</td>
<td>DW132409</td>
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<td>DY979528</td>
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<td>DW127960</td>
<td>Clavaminate synthase-like protein</td>
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found the best fit (DW064597) in the same genus, Lactuca saligna (Table I). The translated protein sequence for DW064597 was highly homologous to ACO1 in Morus notabilis (Table I). Therefore, this matched EST was used to design primers to amplify the potential ACO gene in lettuce (termed ACOx).

When the designed primer was BLASTed against the NCBI EST or nucleotide database using Lactuca as genus, no EST or gene sequences matched significantly to this primer. However, qRT-PCR results showed that a gene in lettuce had been amplified by this primer (Supplemental Fig. S3). This indicates that ACOx is a possible ACO gene in lettuce. The expression of ACOx increased greatly (more than 40-fold) in 15G, 25/15G, and 25nG seeds when germinated and increased initially and then decreased to a very low level at 48 HI in 25G seeds (Fig. 4I).

The abundances of mRNA for genes encoding the predicted α-1,4-GLUCAN-PROTEIN SYNTHASE2 (UDP forming; GPS2; Fig. 4J), ADF2 (Fig. 4K), and ATF (Fig. 4O) declined initially in 15G, 25G, and 25nG seeds and then were kept relatively constant in 25G seeds, but they increased in 15G and 25nG seeds after 12 HI. The expression of these genes increased continuously in 25/15G seeds with extended time (Fig. 4, J, K, and O). Transcripts of genes encoding CHLOROPLAST CHAPERONIN21 (CPN21; Fig. 4H) and predicted/probable ALDO-KETO REDUCTASE2-LIKE (AKR2; spot 898) showed no significant changes in both germinated and ungerminated seeds. The expression of many genes, including SAM1b (Fig. 4B), AACT1 (Fig. 4D), BIPM (Fig. 4F), GPS2 (Fig. 4J), ADF2 (Fig. 4K), ADH1 (Fig. 4L), ANNI (Fig. 4N), ATF (Fig. 4O), and CVS (Fig. 4P), was initiated at 6 or 12 HI in 25/15G seeds and much earlier than in the

Figure 4. Expression of genes encoding the potentially important proteins for seed germination and thermoinhibition. mRNA samples were extracted from dry seeds (0 h), seeds imbibed in water at 15˚C (15G) and at 25˚C (25G), at 15˚C after imbibition at 25˚C for 48 h (25/15G) or in 10 mM KNO3 at 25˚C (25nG) for 6, 12, and 24 h, in ungerminated 25G seeds at 48 h of imbibition (shown as >24 on the horizontal axes), and from germinated 15G, 25/15G, and 25nG seeds (shown as >24 on the horizontal axes). Selected candidate proteins (Table I) and their encoding genes are as follows: A to C, SAM (spot 682, SAM1a; spot 707, SAM1b; spot 713, SAM2); D, LOX2 (spot 126); E, predicted AACT1 (spot 783); F, BIPM (spot 298); G, ICL (spot 290); H, CPN21 (spot 1,243); I, ACOx (spot 1,070); J, predicted GPS2 (spot 807); K, ADF2 (spot 1,431); L, ADH1 (spot 424); M, predicted probable ALDO-KETO REDUCTASE 2-LIKE (AKR2; spot 898); N, ANNI (spot 981); O, ATF (spot 420); and P, CVS (spot 800). Note that different scales are used along the y axis in the different parts. Data are means ± se (n = 3).
15G and 25nG seeds. Among these genes, AACT1 (Fig. 4D), BIPM (Fig. 4F), GPS2 (Fig. 4J), and CVS (Fig. 4P) were also expressed much earlier in 15G seeds than in 25nG seeds. These results are consistent with the difference in germination rate among 25/15G, 15G, and 25nG seeds (Fig. 1).

In conclusion, the expression of 12 genes, SAM1b (Fig. 4B), LOX2 (Fig. 4D), AACT1 (Fig. 4D), BIPM (Fig. 4F), ICL (Fig. 4G), ACOx (Fig. 4I), GPS2 (Fig. 4J), ADF2 (Fig. 4K), ADH1 (Fig. 4L), ANN1 (Fig. 4N), ATF (Fig. 4O), and CVS (Fig. 4P), correlates well with their protein accumulation. Transcript levels of these genes were much higher in 15G, 25/15G, and 25nG seeds, all of which germinated well, than in 25G seeds, in which very poor germination was observed.

Expression of Genes Encoding Enzymes of the MVA Pathway

Besides AACT1, abundances of two other proteins, 3-HYDROXY-3-METHYLGLUTARYL-CoENZYME A SYNTHASE2 (HMGS2; spot 650) and MEVALONATE DECARBOXYLASE (MDPC2; spot 632), involved in the MVA pathway for isoprenoid biosynthesis, were significantly higher (more than 1.5-fold higher) in germinated 15G, 25/15G, and 25nG seeds than in both dry and ungerminated 25G seeds (Figure 5, C and H). Supplemental Table S5). These results suggest a potential role of the MVA pathway for isoprenoid biosynthesis in regulating seed germination and thermoinhibition. To elucidate this role further, we measured the expression of all the candidate genes encoding the enzymes of this pathway (Fig. 5). We could not find the full-length clones for those genes in lettuce. Therefore, we used the genes that have been reported in Arabidopsis (Vranová et al., 2013) to identify the homologous genes in lettuce. Alleles of a given gene were named numerically according to the numerical order of their unigene accession numbers except for the above-mentioned AACT1.

The initial reaction of the MVA pathway is the condensation of two molecules of acetyl-CoA to 3-hydroxy-3-methylglutaryl-Coenzyme A (HMGS-CoA). In lettuce, genes encoding this enzyme had two homologs, termed HMGS1 and HMGS2. There was a higher HMGS1 transcript level in 25G, 15G, and 25/15G seeds than in 25nG seeds at 24 HI, but a similar level in all of these seeds during the following inhibition (Fig. 5D). The mRNA level for HMGS2 was much higher in 15G and 25/15G seeds at both 24 HI and more than 24 HI and in 25nG seeds at more than 24 HI than in 25G seeds (Fig. 5D).

In the following reaction, HMGS converts the acetoacetyl-CoA to 3-hydroxy-3-methylglutaryl-coenzyme A (HMGC-CoA). In lettuce, genes encoding this enzyme had two homologs, termed HMGR1 and HMGR2. There was a higher HMGR1 transcript level in 25G, 15G, and 25/15G seeds than in 25nG seeds at 24 HI but a similar level in all of these seeds during the following inhibition (Fig. 5D). The mRNA level for HMGR2 was much higher in 15G and 25/15G seeds at both 24 HI and more than 24 HI and in 25nG seeds at more than 24 HI than in 25G seeds (Fig. 5D).

In the next reaction, HMG-CoA is converted to MVA by 3-HYDROXY-3-METHYLGLUTARYL REDUCTASE (HMGR). In Arabidopsis, genes encoding this enzyme have two alleles (AtHMGR1 and AtHMGR2; Vranová et al., 2013), while three genes in lettuce (named HMGRI–HMGR3) were highly homologous to these two alleles (Fig. 5). Transcripts of HMGR1 and HMGR2 accumulated abundantly in 25G seeds compared with 15G, 25/15G, and 25nG seeds, while the expression of HMGR3 was lowest in 25G seeds, higher in 15G seeds, and highest in 25/15G and 25nG seeds (Fig. 5E).

In the following reaction of the MVA pathway, MVA undergoes two phosphorylation reactions, catalyzed sequentially by MEVALONATE KINASE (MK) and PHOSPHO-MEVALONATE KINASE (PMK), to form MVA 5-pyrophosphate (Fig. 5). MK was identified to be encoded by one gene in lettuce. The amount of mRNA for MK was higher in 15G, 25/15G, and 25nG seeds at 24 HI and much higher (more than 4-fold) when germinated (more than 24 HI) than in 25G seeds (Fig. 5F). Genes of PMK in lettuce had two alleles (termed PMK1 and PMK2) homologous to those in Arabidopsis (Fig. 5). Transcript for PMK1 had little difference among all the germination treatments, while that for PMK2 was much higher in 15G, 25/15G, and 25nG seeds than in 25G seeds at both 24 HI and more than 24 HI (Fig. 5G).

In the final reaction of the MVA pathway, the MVA 5-pyrophosphate is decarboxylated to isopentenyl diphasphate, the universal precursor of all isoprenoids (Nagegowda, 2010), by MDPC (Fig. 5). Two genes (termed MDPC1 and MDPC2) homologous to Arabidopsis genes were identified in lettuce. In contrast to protein accumulation, expression of the MDPC genes was greater in 25G seeds than in 15G, 25/15G, and 25nG seeds (Fig. 5I).

Effect of Lovastatin on Seed Germination

Lovastatin is a highly potent competitive inhibitor of HMG not only in animals and microorganisms but also in plants (Alberts et al., 1980; Bach and Lichtenthaler, 1983). Inhibition of HMG-CoA reductase with this compound prevents sterol biosynthesis and the prenylation of proteins and reduces plant growth (Randall et al., 1993; Josekutty, 1998). We imbibed the lettuce seeds using a series of concentrations of lovastatin to examine its effect on seed germination (Fig. 6A). Lovastatin did not influence the final percentage of seed germination and seedling growth (data not shown) but delayed germination (Fig. 6A). The seed germination rate decreased as the concentration of lovastatin increased (Fig. 6, A and C). Lovastatin also increased the sensitivity of germination to ABA (Fig. 6B). Although 10 μM lovastatin itself did not affect the germination rate, it promoted the effect of ABA on seed germination (Fig. 6B). Lovastatin at 100 μM plus ABA strongly decreased the seed germination rate and delayed full germination by about 24 h (Fig. 6B).
The effect of a number of MVA pathway products was tested to see if they could reverse the lovastatin inhibition of seed germination and release the thermoinhibition of lettuce seed germination at 25°C. Three cytokinins, KT, 6-BA, and ZT, partially reversed the inhibition of germination by 100 μM lovastatin (Fig. 6C). KT, 6-BA, and ZT promoted seed germination at the thermoinhibitory temperature of 25°C, from about 10% to 78%, 90%, and 90%, respectively (Fig. 6D). Application of the cytokinin thidiazuron, three phytoestrogens, stigmasterol, β-sitosterol, and campesterol, and brassinolide, a brassinosteroid, had no effect on seed germination at 15°C in the presence of 100 μM lovastatin or at 25°C (data not shown).

**DISCUSSION**

**Seed Germination and Thermoinhibition**

Lettuce seeds are reported to exhibit thermoinhibition at 25°C to 30°C, depending on variety and cultivation environment (Guzman et al., 1992; Kozarewa et al., 2006; Contreras et al., 2009; Huo et al., 2013). In lettuce ‘Jianyexianfeng No. 1’, seed germination started to be inhibited at 23°C (Deng and Song, 2012), there was only 10% germination at 25°C (Fig. 1), and seeds were fully inhibited at 27°C (Deng and Song, 2012). This inhibition is not due to the death of the seeds or the induction of dormancy. When the ungerminated seeds were transferred to 15°C after imbibition...
When the seeds were imbibed in KNO₃ at 25°C, response to the application of 50 µM lovastatin (L10) or 100 µM lovastatin (L100) was not shown. D, Germination of seeds at 25°C in response to the application of 50 µM Kinetin (KT), 6-benzylaminopurine (6-BA), or zeatin (ZT). Fifty micromolar cytokinins is an optimum concentration for seed germination (other concentrations are not shown).

Germination conditions. Data are plotted as means ± SE (n = 3). Where the SE is not visible, it was smaller than the symbol.

Genetic Analyses
Sampling Seed Material for Proteomic and Genetic Analyses

It is very difficult to identify physiological changes in a single seed during germination, so seed batches are normally used to study germination. However, the physiological state differs between individual seeds in a population (e.g., germination always occurs over a period of time). Lettuce seeds incubated at 15°C started to germinate at 24 HI and finished at about 48 HI (Fig. 1). When the seeds were imbibed in KNO₃ at 25°C, this interval was extended to nearly 50 h (Fig. 1). Thus, if we had sampled the entire seed batch when the first seed had germinated, as is the standard for germination sensu stricto, we would have been studying seeds differing in developmental stage by 24 to 48 h. Instead, we removed and froze germinated seeds (15G, 25/15G, and 25nG) every 2 h and pooled them afterward for each batch. For the 25G seeds, which germinated very poorly, the few germinated seeds (10%) were discarded and only the ungerminated seeds were sampled. In this way, we ensured that all the seeds analyzed were at precisely the same germination stage: newly germinated sensu stricto.

PEG Precipitation Increases the Number of Detectable Protein Spots on 2-D Gels

Lettuce seeds contain many abundant storage proteins, accounting for more than 50% of total extractable protein (Fig. 2; Supplemental Table S2). Storage proteins play an essential role in providing nutrition and energy for seed germination and seedling growth (Rajjou et al., 2012; Tan-Wilson and Wilson, 2012). However, they can complicate proteomic studies, especially when the 2-DE approach is used. As shown in Figure 2, D and E, many proteins were masked in the region of the 2-D gel where the storage proteins dominated. This situation occurs also in other plant tissues, such as leaf, in which Rubisco is highly abundant (Ahsan et al., 2007; Acquadro et al., 2009). PEG fractionation is recognized as a convenient and reproducible method for the removal of Rubisco and to increase the resolution of low-abundance proteins in leaf extracts of many species (Xi et al., 2006; Ahsan et al., 2007; Lee et al., 2007; Acquadro et al., 2009). This method is clearly also applicable to seed samples (Fig. 2; Supplemental Tables S2 and S3). PEG precipitation increased the number of detectable protein spots on the 2-D gels (Fig. 2, C and D; Supplemental Table S3).

Because of this fractionation, we could identify several protein spots in the storage protein-dominated region, such as ANN1 (spot 981) and ACO1 (spot 1070), that were potentially important for seed germination (Figs. 2 and 3; Table I). Storage proteins are the most abundant proteins precipitated by PEG in lettuce seeds. It is a potential concern that differences in the amount of storage protein would lead to differential precipitation of protein between treatments and result in unreliable comparisons. We observed that TP from 100 seeds was constant in dry, germinated 15G, 25/15G, and 25nG, and ungerminated 25G seeds (Supplemental Table S4). The yield of PEG precipitation was also constant in all seed samples (Supplemental Table S4). Therefore, it seems reasonable to assume that the same percentage of storage protein was present in different seed samples and precipitated by the PEG. This was validated by 1-D gel comparisons of protein samples of all seed types before and after PEG precipitation (Supplemental Fig. S4). PEG precipitation cannot lead to false positives but only to false negatives (Supplemental Discussion S1).

Figure 6. Effect of lovastatin (an inhibitor of HMGR), lovastatin plus ABA or cytokinins, and cytokinins on seed germination. A, Germination of seeds at 15°C in response to increasing concentrations of lovastatin. B, Germination of seeds at 15°C in response to the application of 10 µM ABA (A10) and 10 µM ABA plus 10 µM lovastatin (A10+L10) or 100 µM lovastatin (A10+L100). C, Germination of seeds at 15°C in response to the application of 100 µM lovastatin plus 50 µM kinetin (KT), 6-benzylaminopurine (6-BA), or zeatin (ZT). Fifty micromolar cytokinins is an optimum concentration for seed germination (other concentrations are not shown). D, Germination of seeds at 25°C in response to the application of 50 µM KT, 6-BA, and ZT. The dotted lines in B and C show the time course of germination without the application of chemicals at 15°C. The insets in A to C show the germination rate (inverse of time to reach 50% germination [1/t50]) in different germination conditions. Data are plotted as means ± SE (n = 3). Where the SE is not visible, it was smaller than the symbol.

at 25°C for 48 h, all the seeds germinated (Fig. 1). Thermoinhibition of lettuce seed germination could be alleviated by exogenous application of KNO₃ (Fig. 1). Application of sodium nitroprusside, potassium ferricyanide, nitrite, and nitrate also promoted lettuce seed germination at the thermoinhibitory temperatures (Deng and Song, 2012). Nitrate is a natural product and is also an important source of nitrogen for plant growth. Therefore, its use in the alleviation of lettuce seed thermoinhibition will be important for crop production.
PEG fractionation significantly increased the number of spots detected on 2-D gels (Fig. 2; Supplemental Table S3) as well as allowed twice the loading of other proteins, both of which would allow the detection of more differentially accumulated nonstorage proteins. We cannot exclude the possibility that a few differentially accumulated proteins were lost as a result of PEG precipitation. However, on balance, we conclude that PEG precipitation is a reliable and useful approach in proteomic analyses of lettuce seed germination and thermoinhibition.

Proteins Increased in Abundance Differ Greatly between the Germinated and Ungerminated Seeds, But Proteins Decreased in Abundance Are Mostly Similar

Seed germination is a complex process and requires the activation of a series of physiological and metabolic processes, such as amino acid metabolism, energy production, protein synthesis, mobilization of storage reserves, enzymatic weakening of the surrounding structure, and cell and embryo expansion (Nonogaki et al., 2010; Weitbrecht et al., 2011; Bewley et al., 2013). Proteomic analysis of lettuce seed germination at the optimal temperature identified several proteins participating in these processes.

SAM (spots 682, 707, and 713) is involved in Met metabolism, LOX (spot 126) and ICL (spot 290) are both related to lipid degradation, and predicted peptidyl-prolyl cis-trans-isomerase CYP40-like isoform X1 (spot 848) and CPN21 (spot 1243) are both involved in protein folding and thus assist in protein synthesis (Fig. 3B; Supplemental Table S8). However, not all of these identified proteins are involved in seed germination. Some proteins, such as peptidyl-prolyl cis-trans-isomerase CYP40-like isoform (spot 848), predicted proteasome subunit β type-3-A-like (spot 1273), and β-hydroxyacyl-ACP dehydratase (spot 1395), accumulated differentially in seeds imbibed at both the optimal and the thermoinhibitory temperatures (Supplemental Tables S8 and S9).

It appears that a thermoinhibitory temperature regulates seed germination more by affecting protein synthesis and less by affecting degradation. The number of protein spots increasing in abundance during imbibition was lower in ungerminated 25G seeds than in germinated 15G and 25nG seeds, but the number decreased in abundance was approximately the same in the ungerminated and germinated seeds (Fig. 3B). In addition, when the ungerminated 25G seeds were transferred to 15°C, germination was initiated and many proteins increased in abundance but a few decreased (Fig. 3B). Most of the proteins whose abundance increased during imbibition were found in all germinated 15G, 25/15G, and 25nG seeds but not in ungerminated 25G seeds. In contrast, the majority of the proteins decreasing in abundance were found in both germinated 15G and 25nG and ungerminated 25G seeds (Fig. 3C). These results are consistent with the role of protein synthesis in seed germination that has been proposed in Arabidopsis (Rajjou et al., 2004; Galland et al., 2014) and rice (Sano et al., 2012).

The Abundance of 12 Proteins and Their mRNA Correlated Positively with the Ability of Lettuce Seeds to Germinate

Twenty-two protein spots belonging to 20 gene products were identified to play a potential role in regulating seed germination and thermoinhibition due to their higher or lower accumulation in germinated 15G, 25/15G, and 25nG seeds than in both dry and ungerminated 25G seeds. Four storage proteins following this accumulation pattern had an experimental mass lower than their theoretical mass (Supplemental Table S6). They may have been generated by the initiation of seed storage mobilization, which is a common phenomenon found during seed germination (Bewley et al., 2013; Wang et al., 2015). Similarly, DEHYDRIN2, with a range of different apparent sizes (21–34 kD) and pI (6.7–7.5), was found in lettuce seeds imbibed under different conditions (Supplemental Table S6), indicating that it had been modified by degradation or other posttranslational modifications. It is clear that the accumulation of storage proteins and dehydrin cannot simply be explained by gene expression, which, therefore, was not monitored in this study. Of 16 selected protein-encoding genes, 12 had expression levels consistent with the protein accumulation (Fig. 4). They may be particularly important for seed germination and thermoinhibition.

The abundances of three SAMs were low or undetectable in dry seeds and increased greatly in germinated 15G, 25/15G, and 25nG seeds, but they did not change, or even decreased, in ungerminated 25G seeds (Table I). Among the SAM family members, SAM1b might play the primary role in lettuce seed germination and thermoinhibition. Expression of SAM1b increased only in the germinated seeds, while it was constant in ungerminated seeds (Fig. 4B), which accords with the change in abundance of its encoded protein. SAM catalyzes the synthesis of S-adenosylmethionine (AdoMet) from Met and ATP. AdoMet supplies the methyl group for many specific transmethylation reactions (Hanson and Roje, 2001). Some AdoMet-dependent methyltransferases, such as protein t-isospartyl methyltransferase and the DNA methyltransferases, have been proposed to play roles in seed germination and seedling growth (Rajjou et al., 2012). In addition, AdoMet is a precursor for the biosynthesis of polyamines, ethylene, and biotin (Roje, 2006; Rajjou et al., 2012). Seed germination is blocked when 7-keto-8-aminopelargonic acid synthase, an enzyme in the early step of biotin biosynthesis, is inhibited by triphenyltin acetate, and this block is reversed by biotin (Hwang et al., 2010).

Ethylene is an important signal molecule involved in seed germination (Linkies and Leubner-Metzger, 2012). It promotes seed germination by inhibiting ABA signaling (Linkies and Leubner-Metzger, 2012).
Ethylene alleviates the thermoinhibition of lettuce seed germination especially when combined with GA or a cytokinin (Abeles, 1986; Saini et al., 1986; Khan and Prusinski, 1989), and ethylene production is reduced in seeds germinated at thermoinhibitory temperature (Prusinski and Khan, 1990; Nascimento et al., 2000). The expression of ACS1 and ACO2, two ethylene biosynthetic genes, is tightly related to the thermoinhibition of lettuce seed germination (Argyris et al., 2008; Huo et al., 2013). Here, we found that a potential ACO enzyme identified in L. saligna (ACOx; spot 1070) increased in protein abundance in 15G, 25/15G, and 25nG seeds but was constant in 25G seeds compared with the dry seeds (Table I). Transcripts of ACOx-encoding genes were more than 40-fold higher in abundance in 15G, 25/15G, and 25nG seeds at more than 24 HI than in 25G seeds (Fig. 4I). The accumulation of SAM and ACO at both the protein and gene expression level is consistent with the important role of ethylene in the modulation of seed germination and thermoinhibition. This role is further underlined by our finding that application of the ethylene producer ethephon promoted the germination of all seeds at 25°C and that application of an inhibitor of ethylene action (2,5-norbornadiene), but not of ethylene production (CoCl2), inhibited lettuce seed germination at 15°C (Supplemental Fig. S5).

Lipid is one of the storage reserves in lettuce seeds (Ouellette and Bewley, 1986). Mobilization of lipid provides the metabolic building blocks and energy for seed germination and seedling growth (Graham, 2001). Mutants of two ICL alleles, icl1 and icl2, showed no difference in germination and seedling establishment under continuous light, but they exhibited a gradual decrease in seedling establishment with decreasing light intensity compared with the wild type (Eastmond et al., 2000). The moment that seeds have completed germination sensu stricto is, by definition, the time that seedling growth commences. Therefore, it is not surprising to find that ICL amount and gene expression were highly abundant in the germinated lettuce seeds. However, whether ICL plays a role in lettuce seed germination or seedling growth or both requires further investigation.

In many species, embryo cell elongation, but not cell division, is thought to be essential for the completion of seed germination (Barróco et al., 2005; Gimeno-Gilles et al., 2009; Sliwinska et al., 2009). Several proteins involved in this process, including ADF2 (spot 1431), GPS2 (spot 807), and ANN1 (spot 981), increased greatly in abundance in germinated 15G, 25/15G, and 25nG seeds but much less in ungerminated 25G seeds (Table I). Expression of the genes encoding these proteins exhibited the same pattern (Fig. 4, J, K, and N). Actin is one of the central components of the cytoskeleton. The actin cytoskeleton constantly undergoes dynamic assembly and disassembly, which allows the cell to reorganize the cellular structure that rapidly responds to multiple cellular processes, such as cell motility, cell elongation, intracellular trafficking, and cytokinesis (Staiger, 2000; Pollard and Cooper, 2009; Staiger et al., 2010). ADF is one of the modulators of this process (Augustine et al., 2008; Ketelaar, 2013). In tomato (Sheoran et al., 2005) and pea (Wang et al., 2012b), this protein also increases in abundance during seed germination. One would expect cell elongation to require a dynamic change in the cytoskeleton, and ADF2 may have this role. In addition, during cell elongation, the cell wall extension may require newly synthesized cell wall components, and here, GPS2 could have a role, since it is an enzyme responsible for the biosynthesis of cellulose, one of the main components of the plant cell wall (Bewley et al., 2013).

Annexins are multifunctional lipid-binding proteins and play important roles in various biological processes (Mortimer et al., 2008; Laohavisit and Davies, 2011). Much evidence has pointed toward a role of annexins in cell elongation. The expression of annexins has been detected in the root elongation zone of maize (Zea mays; Carroll et al., 1998) and Arabidopsis (Clark et al., 2005a, 2005b) and is associated with cotton (Gossypium hirsutum) fiber elongation (Yang et al., 2008) and expansion of the tomato pericarp (Faurobert et al., 2007) and tobacco (Nicotiana tabacum) cells (Seals and Randall, 1997). Hayes et al. (2006) found that ANNEXIN2 interacting with actin plays a role in
regulating actin filament dynamics. Annexins have also been proposed to function in the Golgi-mediated secretion of cell wall and plasma membrane materials (Mortimer et al., 2008; Laohavisit and Davies, 2011). Other important functions of annexins include peroxidase activity, ion transport, and Ca^{2+} signaling (Mortimer et al., 2008; Laohavisit and Davies, 2011). In view of these roles, it seems reasonable that it is important for seed germination. Consistent with this view, Lee et al. (2004) reported that mutants of AtANN1 and AtANN4 display delayed germination and hypersensitivity to osmotic stress and ABA during germination.

ADH1 increased in abundance in germinated 15G, 25/15G, and 25nG seeds but not in ungerminated 25G seeds (Table I). The mRNA of ADH1 increased more than 30-fold in 15G, 25/15G, and 25nG seeds at more than 24 HI but was relatively constant in 25G seeds (Fig. 4). ADHs catalyze the irreversible oxidation of a wide range of reactive aldehydes to their corresponding carboxylic acids (Perozich et al., 1999). ADHs might play a pivotal role in detoxifying the aldehydes generated by reactive oxygen species (ROS), such as by lipid peroxidation during seed germination. ROS production is an unavoidable process during seed germination (Bailly, 2004). In plant cells, the mitochondrial electron transport chain is a major site of ROS production (Moller, 2001; Moller et al., 2007). During seed germination, the activation of mitochondrial respiration will result in the production of ROS (Bailly, 2004). ADHs may also have a role in the response to hypoxia during seed germination. After imbibition, the inside of the seed may become hypoxic. In pea seeds, hypoxia was detected after 7 h of imbibition due to both oxygen diffusion limitation caused by the seed-covering layers and an increase in the rate of respiration (oxygen consumption; Rolletschek et al., 2009). Fermentation occurring during hypoxia will lead to the production of toxic acetaldehyde, which is converted to acetic acid by ADHs. When the seeds germinated, the broken seed coat increases the oxygen diffusion into the inner seeds. Reoxygenation of the hypoxia tissues will induce the production of ROS and aldehydes (Magneschi and Perata, 2009). Therefore, the accumulation of ADH in the germinated seeds might protect the seeds from the damage caused by these toxic compounds.

Glycolysis is activated during seed germination in many species (Weitbrecht et al., 2011). In lettuce seeds, both protein accumulation and gene expression of a glycolytic enzyme, BIPM (spot 298), were more abundant in well-germinated 15G, 25/15G, and 25nG seeds than in dry and poorly germinated 25G seeds (Table I; Fig. 4F).

We observed that ATF and CVS, with unclear functions, varied greatly not only in protein accumulation but also in gene expression between the germinated and ungerminated seeds (Table I; Fig. 4, O and P). Therefore, it will be interesting to study the functions of these proteins and their roles in seed germination.

Involvement of the MVA Pathway for Isoprenoid Synthesis in Seed Germination

Isoprenoids represent the functionally and structurally most diverse group of metabolites, with over 50,000 natural products identified in extant organisms (Vranová et al., 2013). All isoprenoids are derived from a core biosynthetic precursor isopentenyl pyrophosphate, which is synthesized via two independent pathways: the MVA pathway and the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway. Isoprenoids derived from the MVA pathway are responsible for the production of cytokinins, sesquiterpenes, triterpenes, phytosterols, and brassinosteroids, which are involved in the regulation of many biological processes, such as membrane biogenesis and growth and development (Newman and Chappell, 1999; Vranová et al., 2013). The isoprenoids farnesyl diphosphate and geranylgeranyl diphosphate are also used for protein prenylation, a process that regulates several developmental processes in plants (Galichet and Gruissem, 2003; Crowell and Huizinga, 2009).

In lettuce seeds, proteomic, genetic, and physiological analyses all point toward an involvement of the MVA pathway for isoprenoid biosynthesis in seed germination. Three proteins (AACT1, HMGS2, and MDPC2) participating in this pathway were identified to have significant differences in abundance between the germinated and ungerminated seeds (Fig. 5). mRNA levels of AACT1 and HMGS2 consistently correlated with protein accumulation, and the expression of HMGR3, MK, and PMK2 involved in this pathway also had a positive correlation with the seed germination ability (Fig. 5). At the same time, lovastatin inhibition of isoprenoid biosynthesis via the MVA pathway delayed seed germination at 15°C, but it did not fully inhibit it (Fig. 6A). One possible explanation is that isoprenoids, or their derived products, are not absolutely required for seed germination. Another possibility is that isoprenoids from the MEP pathway compensate for the decreased biosynthesis via the MVA pathway, because there is cross talk between the MVA and MEP pathways of isoprenoid biosynthesis (Laule et al., 2003). It has been reported that the overexpression of Brassica juncea HMGS1 in Arabidopsis increases the seed germination rate (Wang et al., 2012a). These results suggest that isoprenoids from the MVA pathway may play a role in regulating the germination ability of lettuce seed at different temperatures. Cytokinins produced from this pathway are potentially responsible for lettuce seed germination ability in different conditions, as the cytokinins KT, 6-BA, and ZT partially reversed the lovastatin inhibition of seed germination (Fig. 6C) and released the thermoinhibition of lettuce seed germination at 25°C (Fig. 6D). Although brassinosteroids, one of the end products of the MVA pathway, have been reported to promote seed germination in many species, including Arabidopsis (Steber and McCourt, 2001) and tomato (Leubner-Metzger, 2001), we did not observe any effect
of this chemical on lettuce seed germination (data not shown).

Application of KT, 6-BA, or ZT did not fully reverse the inhibition of germination by lovastatin. It is possible that other products derived from the MVA pathway are also involved in lettuce seed germination. Several studies have shown that protein prenylation plays an essential role in seed germination (Cutler et al., 1996; Galichet and Gruissom, 2003; Crowell and Huizinga, 2009). Substrates for protein prenylation, including farnesyl diphosphate and geranylgeranyl diphosphate, are derived from the MVA pathway. Cutler et al. (1996) reported that knockout mutations in Arabidopsis ENHANCED RESPONSE TO ABSCISIC ACID1, a gene encoding the β-subunit of protein farnesyl transferase, cause a prolonged seed dormancy due to an enhanced response to ABA. They proposed that protein farnesylation may be essential for the negative regulation of ABA signaling in seeds (Cutler et al., 1996). Therefore, it was not unexpected to observe that lovastatin inhibition of the MVA pathway increased the sensitivity of germination to ABA (Fig. 6B). ABA plays a major role in preventing seeds germinating at the thermoinhibitory temperature (Gonai et al., 2004; Tamura et al., 2006; Argyris et al., 2008; Huo et al., 2013). In lettuce seeds, a thermoinhibitory temperature decreased the accumulation of proteins and expression of the genes in the MVA pathway (Fig. 5). This might decrease the production of isoprenoids and thus promote the effect of ABA on inhibiting seed germination.

CONCLUSION

PEG fractionation increased the number of protein spots detected on the 2-D gels, especially in areas where the storage proteins dominated. Many proteins increasing in abundance were found in all of the germinated seeds but not in the ungerminated seeds, while proteins decreasing in abundance were found in both the germinated and ungerminated seeds, indicating the necessity for protein synthesis in seed germination. The accumulation of 12 proteins and their mRNAs correlated positively with the ability of the seeds to germinate. The proteins are involved in Met metabolism, ethylene production, lipid mobilization, cell elongation, glycolysis, isoprenoid biosynthesis, and detoxification of aldehydes, which means that these processes are directly or indirectly involved in seed germination. The thermoinhibitory temperature repressed the activation of these processes in lettuce seeds during imbibition, which might prevent the seeds from germinating. Protein accumulation, gene expression, and the effect of lovastatin on seed germination all suggest an involvement of the MVA pathway for isoprenoid biosynthesis in seed germination and thermoinhibition. The production of cytokinins from this pathway could influence the germination ability of lettuce seeds. However, the MVA pathway can produce a large number of bioactive products, some of which, such as farnesyl diprophosphate and geranylgeranyl diprophosphate, may also play the role in seed germination. In addition, there is cross talk between the MVA and MEP pathways (Laule et al., 2003). Therefore, the role of the MVA pathway in seed germination is quite complex.

MATERIALS AND METHODS

Plant Material

Lettuce (Lactuca sativa 'Jianyexianfeng No. 1') seeds were kindly provided by Guangji Liu (Zhonggu Sichuan). Seeds were stored at −20°C in an aluminum foil bag until use for the following study.

Seed Germination

Three replicates of 50 seeds each were placed on two layers of filter paper in closed 90-mm-diameter petri dishes and incubated at 15°C (15G), 25°C (25G), or 15°C after imbibition at 25°C for 48 h (25/15G) in 4.5 mL of distilled water and at 25°C in 4.5 mL of 10 mM KNO3 (25nG) in 14 h of light (66.3 μmol m⁻² s⁻¹) and then continuous darkness. To examine the effect of Lovastatin (an inhibitor of HMGCR; National Standard Materials; 98% purity), ABA, and ABA plus lovastatin on seed germination, seeds were imbibed in 0, 10, 50, and 100 μM lovastatin, 10 μM ABA, or 10 μM ABA plus 10 or 100 μM Lovastatin at 15°C under the conditions described above. To examine whether MVA pathway-derived products could reverse the inhibition of germination by lovastatin, seeds were imbibed in 10 and 50 μM KT (Sigma), 6-BA (Sigma), ZT (Sigma), and thidiazuron (Sigma), 0.001, 0.01, and 0.1 μM stigmasterol (J&K Scientific), β-sitosterol (J&K Scientific), and campesterol (J&K Scientific), and 0.001, 0.01, and 0.1 μM brassinolide (Santa Cruz Biotechnology) plus 100 μMLovastatin at 15°C under the conditions described above. Seeds were also imbibed in the same concentration of the above MVA pathway-derived products at 25°C under the conditions described above. A seed was considered to be germinated when protrusion of the radicle from the seed coat was observed. The germinated seeds were sampled within 2 h of their germination as described in “Results” (Fig. 1; Supplemental Fig. S2). Germination rate was considered as the reverse time to reach 50% germination.

PEG Fractionation of Total Extractable Proteins

Extraction of Total Protein

Three replicates of 200 seeds each were ground in a prechilled mortar and homogenized in 3 mL of ice-cold protein extraction buffer containing 0.5 M Tris-HCl (pH 7.8), 2% (v/v) Nonidet P-40, 20 mM MgCl₂, 2% (v/v) β-mercaptoethanol, 1 mM phenylmethylsulfonfluoride, 1 mM EDTA, and 1% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 16,000g at 4°C for 15 min. After discarding the pellets, the supernatant was further centrifuged at 32,000g at 4°C for 15 min. The pellets were discarded, and the supernatant contained the total extractable protein.

PEG Fractionation

PEG fractionation was performed according to Acquadro et al. (2009). A flow chart of the PEG fractionation procedure is shown in Figure 2A. TP was added to PEG4000 up to 4% (w/v) concentration, and the mixture was shaken on ice for 30 min and then centrifuged at 16,000g at 4°C for 15 min. The protein (P4) was saved, PEG4000 up to 8% (w/v) was added to the supernatant (S4), and the mixture was shaken and centrifuged as above. The protein (P8) was saved, and the supernatant (S8) was treated like S4 to produce the 12% (P4) was saved, PEG4000 up to 8% (w/v) concentration, and the mixture was shaken on ice for 30 min and then centrifuged at 16,000g at 4°C for 15 min. The pellet (P4) was saved, PEG4000 up to 8% (w/v) was added to the supernatant (S4), and the mixture was shaken and centrifuged as above. The protein (P8) was saved, and the supernatant (S8) was treated like S4 to produce the 12% PEG fraction (P4 and S4) (Fig. 2A). Samples from TP, from supernatants S4, S6, and S8, and from pellets P4, P8, and P12 were precipitated with 5 volumes of cold 10% (w/v) TCA/acetone at −80°C and then lyophilized overnight at −20°C. All these samples were subjected to one-dimensional gel electrophoresis (1-DE), and samples TP and S8 were also
subjected to 2-DE analysis. The protein concentration in each sample was determined by the Bradford (1976) method using bovine serum albumin as the standard.

1-DE
For 1-DE analysis, each fraction, supernatant, and pellet was mixed with sample buffer (Laemmli 1970) and heated at 95°C for 4 min. Discontinuous SDS-polyacrylamide gels (12% separation gel, 5% stacking gel) were prepared and run according to Laemmli (1970) using a Mini-Protein II electrophoresis cell (Bio-Rad).

2-DE
Isoelectrofocusing was carried out using a PROTEAN i12 isoelectrofocusing system (Bio-Rad) and 17-cm Immobiline Dry Strips with a linear pH gradient of 5 to 8 (Bio-Rad). Sample (500 µg of protein) was loaded onto the gel strip. The second dimension was carried out on a 12% SDS-polyacrylamide gel. Electrophoresis was performed at 15°C in SDS electrophoresis buffer (pH 8.3) containing 25 mM Tris base, 192 mM Gly, and 1% (w/v) SDS.

Image Analysis, in-Gel Digestion with Trypsin, and Protein Identification by Matrix-Assisted Laser-Desorption Ionization-Time-of-Flight/Time-of-Flight Mass Spectrometry
Gels (1-D and 2-D) were stained overnight with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 5:14 (v/v) methanol/acetic acid-water and destained in 2:1:7 (v/v) methanol/acetic acid:water solution with three to five changes of the solution until a colorless background was achieved. The stained gel was scanned at 300 dots per inch resolution with a UMAX Power Look 2100XL scanner (Maximum Technology). Spot detection and gel comparisons were made with ImageMaster 2D Platinum, version 5.01 (GE Healthcare Bio-Science). After automated detection and matching, manual editing was carried out to correct the mismatched and unmatched spots.

Well-separated gels of the four independent biological replicates for each treatment were used for proteomic comparisons. Spots were considered reproducible when they were well resolved in at least three biological replicates. The normalized volume (based on the total spot volumes) of each spot was assumed to represent its protein abundance. When comparing spot size between groups, a difference was considered significant when the change was greater than 2-fold and P < 0.05 (Student’s t test). Differentially accumulated protein spots were excised from the stained gels. In-gel digestion and peptide extraction were performed as described by Shevchenko et al. (1996) with minor modifications (Wang et al., 2014). Excised gel pieces via destaining, reduction, and alkylation treatments were imbibed in 10 ng of trypsin in 50 mM NH4HCO3 for 45 min at 4°C. After imbibition, the excess buffer was discarded and the gel pieces were covered with 50 mM NH4HCO3 for overnight digestion at 37°C.

Prior to mass spectrometry (MS) analysis, peptide mixtures were desalted on in-house-made microcolumns packed with the reverse-phase material POROS 20R2 (Applied Biosystems). After equilibrium in 10 µL of 1% (v/v) trifluoroacetic acid, peptides were eluted from the column with 3 µL of matrix solution (5 µg µL−1 5-amino-4-hydroxycinnamic acid in 70% [v/v] acetonitrile and 0.1% [v/v] trifluoroacetic acid) directly onto the matrix-assisted laser-desorption ionization (MALDI) target plate (Opti-TOF 384 Well Insert; Applied Biosystems). MS and tandem mass spectrometry (MS/MS) spectra were acquired on an UltraFlexXtreme MALDI time-of-flight/time-of-flight mass spectrometer (Bruker Daltonics) by the use of FlexAnalysis 3.3 software. MS spectra in the range 700 to 3,500 mass-to-charge ratio were acquired automatically with external calibration to a standard β-lactoglobulin tryptic digest. Collision-induced dissociation was used for fragmentation of the 10 most intense precursor ions and was performed automatically with default calibration. Protein identification was performed using Biotools 3.2 (Bruker Daltonics) by searching the Plant EST or NCBI database with Mascot 2.2.04 (Matrix Science). The following search parameters were used: database, Plant EST (EST_118) and/or NCBI database (NCBI database 20140323); taxonomy, green plants (149,962,980 sequences in EST_118 and 1,749,148 sequences in NCBI database 20140323); maximum one missed cleavage; Cys carbamidomethylation as a fixed modification; Met oxidation and N-terminal acetylation as variable modifications; and mass tolerance of 100 ppm in MS mode and 0.6 D for MS/MS. Protein scores greater than 96 (in Plant EST) or 75 (in NCBI database) were significant (P < 0.05). The identified protein name, accession number, Mascot score, sequence coverage, number of sequenced peptides (the peptides matched by the MS/MS spectra [ion score > 20]) and of matched peptides (peptides matched by the peptide mass fingerprinting), and theoretical protein mass and pl found in the databases are shown in Supplemental Tables S6 and S7.

Gene Expression Analysis
The best matched EST for a given protein spot was searched against the NCBI unigene database to find a corresponding unigene, which was then used for primer design. For genes encoding enzymes of the MVA pathway, the candidate gene sequences corresponding to the best BLAST hit were identified within the NCBI Transcriptome Shotgun Assembly database through sequence homology to known genes in Arabidopsis (Vranová et al., 2013). Primer sequences were designed using Primer Premier 6 (Applied Biosystems) to amplify 80- to 250-bp PCR amplicons for qRT-PCR analyses. Genes and primers used for qRT-PCR analyses are shown in Supplemental Tables S12 and S13.

Total RNA was extracted from dry or imbibed lettuce seeds using the SV Total RNA Isolation System (Promega). RNA quality was assessed by gel analysis and 260-280 nm and 260-230 nm spectrophotometric absorbance ratios. Two micrograms of total RNA was reverse transcribed using random primers (TransScript First-Strand cDNA Synthesis SuperMix; TransGen Biotech; www.transgen.com.cn). Complementary DNAs from a housekeeping ubiquitin ligase gene and genes of interest were PCR amplified in the Agilent MX3000P Real-Time PCR System using KAPA SYBR FAST qPCR Kits (Kapa Biosystems; www.kapabiosystems.com). The relative quantification of the transcript levels was performed using the comparative cycle threshold method (Livak and Schmittgen, 2001). Three biological replications plus two technical replications were conducted for each sample.

Supplemental Data
The following supplemental materials are available.

Supplemental Figure S1. Protein bands on a 1-D gel and spots on a 2-D gel of 15G seeds subjected to identification by MALDI-time-of-flight/time-of-flight MS.

Supplemental Figure S2. Flow chart of proteomic analysis of lettuce seed germination and thermoinduction.

Supplemental Figure S3. Amplification plots, dissociation curve, and standard curve for qRT-PCR analysis of ACC oxidase gene expression.

Supplemental Figure S4. 1-D gel maps of total extractable protein and supernatant and pellet after 8% PEG precipitation.

Supplemental Figure S5. Effect of ethephon (A; 25°C), CoCl2 (B; 15°C), and 2,5-norbornadiene (C; 15°C) on germination of seeds.

Supplemental Table S1. Identification of storage proteins on the 1-D and 2-D (Fig. 1S) gels of 15G seeds.

Supplemental Table S2. Protein contents and yields of various PEG precipitation fractions.

Supplemental Table S3. Spot numbers on a 2-D gel before and after PEG precipitation.

Supplemental Table S4. Total protein content and protein yield after 8% (w/v) PEG fractionation of total protein extracted from seeds under different germination conditions.

Supplemental Table S5. Volume (%) of protein spots accumulated differentially under different germination conditions, and the ratio and associated P values of comparison.

Supplemental Table S6. Differentially accumulated protein spots identified to match one protein when searched in the Plant EST or NCBI database.

Supplemental Table S7. Protein spots identified to match more than one protein.

Supplemental Table S8. Proteome changes in seeds during germination at 15°C (germinated 15G seeds versus dry seeds).
Supplemental Table S9. Proteome changes in seeds germinated at 25°C (ungerminated 25G seeds versus dry seeds).

Supplemental Table S10. Proteome changes in seeds during germination at 15°C after imbibition at 25°C for 48 h (germinated 25/15G seeds versus ungerminated 25G seeds).

Supplemental Table S11. Proteome changes in seeds during germination at 25°C in KNO₃ (germinated 25mG seeds versus dry seeds).

Supplemental Table S12. Primer design for genes encoding candidate proteins involved in seed germination and thermoinhibition.

Supplemental Table S13. Primer design for genes encoding enzymes of the MVA pathway for isoprenoid biosynthesis.

Supplemental Discussion S1. PEG fractionation gives no false positives, only false negatives.

ACKNOWLEDGMENTS

We thank Dr. Zhuang Lu (Institute of Botany, Chinese Academy of Sciences) for doing the MS analyses.

Received January 13, 2015; accepted March 1, 2015; published March 3, 2015.

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