Differential Expression of Acid Invertase Genes during Seed Germination in Arabidopsis thaliana

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In Arabidopsis thaliana (L.) Heynh ecotype Landsberg, levels of soluble acid invertase activity are closely related to the progress of seed germination. To study the mechanism(s) of the development of these enzymes, two cDNA clones that encode putative vacuolar acid invertases were isolated from germinating seeds and very young seedlings using reverse-transcription polymerase chain reactions with degenerate primers. These fragments corresponded to the genes Atβfruct3 and Atβfruct4 from the Columbia ecotype. An apoplastic invertase gene corresponding to Atβfruct1/ATCWINV1 was also isolated from these samples. Northern blot analyses showed that Atβfruct3 and Atβfruct4 are expressed concomitantly with germination and the subsequent seedling growth. In contrast, the Atβfruct1/ATCWINV1 mRNA is translated before germination. These expression patterns are regulated by phytochrome, which perceives red light and in turn triggers de novo synthesis of gibberellin, initiating Arabidopsis seed germination. To test the effects of gibberellin on the expression of these genes, seed were treated with a gibberellin biosynthesis inhibitor, uniconazole or prohexadione. These chemicals inhibited both seed germination and plant development. In contrast, the active gibberellic acid (GA) can mimic the effects of R irradiation,25) it has been proposed that the active form of phytochrome (Pfr) stimulates the synthesis of GAs in seeds, and the synthesized GA initiates the signal transduction pathway that leads to germination.26,27) In fact, genes that encode GA 3-oxidase (GA 3β-hydroxylase) which catalyze the formation of active GA, are stimulated by phytochrome in the germinating seed.25)

The Arabidopsis ecotype Columbia contains two genes that encode vacuolar acid invertases and six that encode apoplastic acid invertases, as predicted by the TAIR database (http://www.arabidopsis.org/). Complementary DNAs for these genes have been isolated,3,28–30) and their expression patterns have been analyzed by Northern blot hybridization or semi-quantitative RT-PCR.3,30,31) These invertase genes are expressed in both a developmental and tissue- or organ-specific manners. For example, the Atβfruct1/ATCWINV1 transcript, which

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Seed germination is a complex physiological event, the initiation of which is controlled by multiple environmental conditions, including water, light, or temperature. Sugars are thought to induce and be involved in many physiological events, and several sugar-sensing mechanisms have been demonstrated in plants.1–3) Some aspects of germination may be regulated by sugar, since a hexose-sensing mechanism has been shown to be involved in germination in Arabidopsis,1,5) rice,5,7) and barley.9) However, it is still unclear how sugars such as hexose are supplied within the germinating seed.

In higher plants invertase catalyzes hydrolysis of sucrose into hexoses. Acid invertases (β-D-fructofuranosidase, EC 3.2.1.26) are thought to have an important role in cell, tissue, and organ growth, as the supply hexoses that will be used as carbon sources or signal molecules.9–12) The hexoses produced by these enzymes also increase the osmotic potential within the cell.13–15) Although acid invertase genes are induced during seed germination, the roles of these enzymes during both seed germination and plant development are largely unknown.16–20) Arabidopsis thaliana seed are photoblastic, as their germination is mediated by the phytochrome photoreceptor,21–25) and their germination can be synchronized by red (R) light irradiation.21) Since exogenously applied active gibberellic acid (GA) can mimic the effects of R irradiation,25) it has been proposed that the active form of phytochrome (Pfr) stimulates the synthesis of GAs in seeds, and the synthesized GA initiates the signal transduction pathway that leads to germination.26,27)

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corresponds to an apoplastic enzyme, is present in cotyledons, mature leaves, stems, flowers, and developing seeds. In contrast, the At\textit{fruct2}/Atcw\textit{INV2} transcript, which encodes a different apoplastic enzyme, is specifically transcribed in flower organs and developing seeds. In cotyledons, there is a relatively high level of At\textit{fruct3} transcripts, which encode a vacuolar enzyme, but there are in low levels of this transcript in leaves, root, and flower buds. At\textit{fruct4}, which corresponds to another vacuolar enzyme, is expressed in leaves of very young plants but not in mature leaves. However, no information is available on the acid invertase genes that are expressed in the germinating seeds.

In this study, we isolated three cDNA fragments of acid invertase genes using RT-PCR with degenerate primers and poly (A)$^+$ RNA isolated from germinating Arabidopsis thaliana ecotype Landsberg erecta seeds. These fragments are closely related to the previously reported At\textit{fruct1}/Atcw\textit{INV1}, At\textit{fruct3}, and At\textit{fruct4} mRNAs from the Columbia ecotype. Northern blot analyses were done to determine the expression pattern of these genes during germination and young seedlings. The expression of both At\textit{fruct3} and At\textit{fruct4}, which encode vacuolar type invertases, is correlated with germination and early seedling growth. An apoplastic invertase gene, At\textit{fruct1}/Atcw\textit{INV1}, was transcribed before emergence of radicles from seeds. The expression of these genes during and after germination was depressed by treatment with different GA biosynthesis inhibitors, and this repression could be reversed by exogenously applied GA$_3$, which is an active GA in Arabidopsis. Therefore, the expression of genes encoding two vacuolar invertases and an apoplastic invertase could be separately controlled by de novo-synthesized active GA during or after seed germination.

Materials and Methods

Plant materials. Seeds of Arabidopsis thaliana (L.) Heynh ecotype Landsberg erecta were purchased from Lehle Seeds (Tucson, Arizona, USA) and stored at 4°C until use. Ten mg of dry seeds (about 500 seeds) were spread on a layer of filter paper moistened with 1.2 ml of distilled water or a test solution in a 4.5-cm-diameter Petri dish. After 2 h of imbibition in darkness at 25±1°C, the seeds were irradiated for 15 min with red light (R, 4.5 Wm$^{-2}$), far-red light (FR, 3.5 Wm$^{-2}$) or R followed by FR. The seeds were then incubated for 48 h in darkness at 25±1°C. Germination was defined as the emergence of a radicle from the seed.

Light source. Red light was supplied by fluorescent lamps (FL20S, RBF, Toshiba, Tokyo, Japan) filtered through an acrylic filter (Shinkolite A 102, Mitsubishi Rayon, Tokyo, Japan). For far red light, a Deraglass 102 acrylic filter (Asahikasei, Tokyo, Japan) was used. White light was supplied by FL40SS.W37 fluorescent tubes (National, Tokyo, Japan).

Preparation of crude enzyme solution. About 500 seeds or young seedlings were extracted with 1.5 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM PMSF, and 16% (v/v) glycerol, using a mortar and pestle. The homogenate was centrifuged at 30,000 $\times$ g for 20 min at 4°C, and the supernatant was put on a NAP-10 desalting column (Pharmacia, Upplands, Sweden) equilibrated with 25 mM MES-NaOH buffer, pH 5.5, containing 16% (v/v) glycerol. The eluate from the column was used as the crude enzyme solution.

Assay of soluble acid invertase activities. Reaction mixture (0.2 ml) containing 100 mM sucrose, 25 mM MES-NaOH buffer, pH 5.5, and appropriate amounts of crude enzyme solution were incubated at 30°C for 3 h. To stop the reactions, 0.25 ml of ice cold-Somogyi’s reagent was added. The amount of reducing sugars hydrolyzed from sucrose was measured using the method of Nelson. One unit of activity was defined as a 1.0-unit increase in absorbance at 660 nm/mg protein/h.

Reverse-transcription PCR (RT-PCR). Two degenerate primers were used for PCR: 5’-RARAAYTGGATNAAYGAYCC-3’ as the sense primer and 5’-ARRT-CNRSRCAYTCCCACAT as the antisense primer. The design of these primers was based on conserved amino acid sequences in acid invertases from A. thaliana ecotype Columbia. Total RNAs were prepared from dry seeds, germinating seeds, young seedlings, FR-light-treated ungerminating seeds, and mature green plants. Seeds harvested 1 h and 12 h after R treatment were combined to prepare mRNAs for the “early stage of germination” sample. Young seedlings harvested at 24 h, 30 h, and 46 h after R treatment were combined and used to prepare mRNAs for the “late stage of germination” sample. For the “young seedling” sample, mRNAs were isolated from 5-day-old seedlings after R treatment. In the “non-germinating seed” sample, mRNAs were isolated from seeds harvested 1 h, 24 h, and 46 h after FR treatment. Double-stranded cDNA was synthesized from mRNAs as described previously.

About 10–20 ng of each double-stranded cDNA was used as a template for PCR. The 50-μl reaction mixtures contained 200 μM of deoxyribonucleotide triphosphate, 1.5 mM MgCl$_2$, 1 μM of each primer, and 2.5 units of Expand HF (Boehringer Mannheim). Samples were heated to 95°C for 2 min and put through to 40 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min.

Cloning and sequence analysis of PCR products. PCR products were purified by agarose-gel electrophoresis and ligated into the pCRII vector using a TA cloning kit (Invitrogen, San Diego, CA, USA). The ligation products were introduced into Escherichia coli JM109, and recombinant clones were selected. The nucleotide sequence was determined by a DNA cycle-sequencing method (MWG, Madison, WI, USA).
sequence of each clone was analyzed using a Taq cycle sequencing kit (Dye Primer, Applied Biosystems) and a DNA sequencer (Model ABI 377, Applied Biosystems).

**PCR for full-length cDNAs.** The double-stranded cDNAs described in the RT-PCR section above were also used as templates for standard PCR. Reactions were done using the 3’ and 5’ end primers to amplify the coding regions. The PCR conditions used were as described above except that the annealing temperature was 65°C and the final extension was for 1 min.

**Sequence similarity searches and alignment of amino acid sequences.** Homology searches of databases were done using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments of amino acid sequences were done using the CLUSTAL W program (http://www.clustalw.genome.ac.jp/).

**Northern blot analyses.** Total RNA (10 μg/lane) was denatured and electrophoresed on a 1% (w/v) agarose + 2.2 M formaldehyde gel. The RNA was transferred onto a nylon membrane (Hybond N+, Amersham) using standard blotting techniques. 36) Membranes were pre-hybridized for 3 h at 68°C and then hybridized with a 32P-labeled full-length PCR fragment for 18 h at 68°C in a rapid-hybridization buffer (Amersham). The membrane was washed successively at 68°C with 2x SSC + 0.1% (w/v) SDS for 10 min, 1x SSC + 0.1% SDS for 1 h, and 0.2x SSC + 0.1% SDS for 1 h. Radioactive signals were recorded on an imaging plate (BAS-2000, Fujix, Tokyo, Japan). The Northern blot analysis was repeated three times with independent RNA preparations to confirm the reproducibility of the results.

**Results**

**Changes in soluble acid invertase activity during and after seed germination in Arabidopsis thaliana**

The emergence of a radicle from the seed was used as the criterion for seed germination. 33) Following R light irradiation, no germination was observed before 20 h, and about 50% and 70% of the seeds had germinated by 30 and 50 h, respectively (Fig. 1A, dotted line). An increase in soluble acid invertase activities was observed that roughly paralleled the timing of seed germination, although the pattern was slightly different from the change in the germination rate (Fig. 1A). The increase in these activities may promote radicle expansion. Dry seeds and imbibed and R-light-irradiated seeds treated for less than 24 h had very low acid invertase activity, but after 24 h, major soluble activities began to increase linearly until at least 48 h (Fig. 1A, solid line). When germination was prevented by irradiation with FR light, no increase in the major activities was observed (Fig. 1A, middle-dotted-line). In addition, FR light given after R-light treatment suppressed germination (data not shown) and the increase in the major invertase activities (Fig. 1B). This suppression was completely reversed by R-FR-R irradiation (Fig. 1B). These results indicate that increase in the major soluble acid invertase activities is probable regulated by phytochrome-related machinery.

**Isolation of cDNAs from germinating seeds that encode acid invertase mRNAs**

RT-PCR was used to isolate acid invertase genes that are expressed in germinating seeds and/or very young seedlings, using degenerate primers that were designed on the basis of conserved amino acid sequences in the active sites of Arabidopsis acid invertases. Messenger RNA templates were prepared from dry seeds, germinating seeds, young seedlings, and seeds in which the germination had been suppressed by FR-light treatment. Seeds harvested at 1 h and 12 h after R irradiation were combined to prepare mRNAs for the “early stage of germination” sample. Very young seedlings harvested at 24 h, 30 h and 46 h after R irradiation were mixed for
preparation of mRNAs for the “late stage of germination” sample. As the “young seedling” sample, mRNAs from 5-day-old seedlings were used. For the “non-germinating seed” sample, mRNAs were used from seeds harvested at 1 h, 24 h, and 46 h after FR treatment. Complementary DNAs synthesized from these poly (A)+ RNAs were used in RT-PCR. No fragments amplified from mRNAs prepared from dry seeds or the “early stage of germination” sample, but at least three different fragments were obtained from the “late stage of germination” sample, and one fragment was also amplified from “young seedling” mRNAs. No significant bands were amplified from “non-germinating seed” mRNAs.

Amplified cDNA bands of the expected sizes were sequenced. The three different clones obtained were identical to the \textit{Atfruct1}/At\textit{cwINV1}, \textit{Atfruct3}, and \textit{Atfruct4} sequences of \textit{A. thaliana} ecotype Columbia, except for a few small changes (data not shown). For preparation of full-length cDNAs, gene-specific primers were designed on the basis of the amino acid sequences of the Columbia enzymes. \textit{Atfruct3} and 4 are classified as vacuolar invertase and \textit{Atfruct1}/At\textit{cwINV1} is classified as an apoplastic invertase, based on the apoplastic-type-specific motif WECPD presen in \textit{Atfruct1}/At\textit{cwINV1}.\textsuperscript{37}

Expression analysis of the amplified invertase genes in germinating seeds and young seedlings

The expression of the invertase genes (\textit{Atfruct1}/At\textit{cwINV1}, \textit{Atfruct3} and \textit{Atfruct4}) in germinating seeds and young seedlings was analyzed by Northern blotting using full-length cDNAs as probes. No signals were observed in samples from dry seeds (data not shown) and FR-irradiated seeds (Fig. 2). The \textit{Atfruct1}/At\textit{cwINV1} transcript, which encodes an apoplastic enzyme, began to accumulate at 6 h after R treatment, gradually increasing during and after germination (Fig. 2). However, no significant signals were observed for the \textit{Atfruct3} and 4 mRNAs, until 24 h after treatment, at which point both mRNAs accumulated concomitantly with the increase in soluble acid invertase activities during germination and early seedling growth (Fig. 1A). The expression of the \textit{Atfruct1}/At\textit{cwINV1}, \textit{Atfruct3} and \textit{Atfruct4} genes was clearly phytoreversible, possibly through the action of phytochrome-related machinery. The expression that was induced by R irradiation could be completely suppressed by subsequent irradiation with FR, and a further irradiation with R restored the expression of these genes (data not shown).

Effects of exogenously applied gibberellic acid (GA) and/or inhibitors of its synthesis on the transcription of \textit{Atfruct1}/At\textit{cwINV1}, \textit{Atfruct3}, and \textit{Atfruct4}

As described earlier, active GA is involved in the induction of \textit{Arabidopsis} seed germination.\textsuperscript{25} Therefore, the effects of GA on the expression of \textit{Atfruct1}/

\textbf{Fig. 2.} Expression of the Acid Invertase Genes, \textit{Atfruct1}/At\textit{cwINV1} (protein accession number: S37212), \textit{Atfruct3} (nucleotide accession number: X99111, At1g62660), and \textit{Atfruct4} (nucleotide accession number: Y11559, At1g12240) in \textit{A. thaliana} Seeds and Young Seedlings Following R or FR Treatment. Imbibed seeds were irradiated with R or FR for 15 min, and then incubated in darkness for the times shown in the figure. The seeds were then harvested and stored at ~80°C until use. Total RNA (10 μg/lane) extracted from the seeds was used for Northern blot analysis. Equal loading of RNA was confirmed by ethidium bromide staining of the ribosomal RNAs on each gel and membrane (data not shown). Reproducibility of the data was confirmed in three independent experiments.

\textit{AtcwINV1}, \textit{Atfruct3}, and \textit{Atfruct4} genes were investigated by Northern blot analysis of seed treated with GA and/or GA biosynthetic inhibitors, uniconazole or prohexadione. At 60 h after R irradiation, transcription of all of the genes was apparent (Fig. 3). Uniconazole or prohexadione treatment of the seeds completely suppressed radicle emergence, and no increase in the transcripts for \textit{Atfruct1}/At\textit{cwINV1}, \textit{Atfruct3} and 4 was observed (Fig. 3, R treatment, lanes U and P). Exogenously applied GA\textsubscript{4} reversed the inhibition of seed germination and restored the transcription levels for these genes (R treatment, lanes U+G and P+G). GA\textsubscript{4} also reversed the suppression of germination and transcription by FR irradiation (Fig. 3, FR treatment, lane Cont and G). Treatment with GA\textsubscript{4} could even induce germination and the expression of these genes in FR-irradiated seeds treated with prohexadione or uniconazole (FR treatment, lanes U+G and P+G).

These results indicate that the expression of the three acid invertase genes is probably controlled by phytochrome-mediated \textit{de novo} synthesis of active GA.

Discussion

To clarify the relationship between the roles of acid invertases, light, and gibberellic acid (GA) in \textit{Arabidopsis} seed germination, we observed the expression patterns of invertase genes after treatment with different wavelengths of light, active GA (GA\textsubscript{4}), or GA biosynthesis inhibitors. The expression of gene encoding one apoplastic and two vacuolar acid invertase genes (\textit{Atfruct1}/At\textit{cwINV1}, \textit{Atfruct3} and \textit{Atfruct4}) isolated in this study, was induced by red (R) light irradiation and inhibited by far-red (FR) light irradiation or treat-
ment with GA biosynthesis inhibitors. Exogenously applied GA₄ mimicked the effect of red (R) light irradiation, as reflected in the expression of the acid invertase genes used in this study (Figs. 2 and 3).

Yamaguchi et al.25 demonstrated that two distinct GA 3-oxidase genes are expressed independently and sequentially in A. thaliana seed following R irradiation. Both of the genes were stimulated within 1 h after irradiation with red light. The expression of one of these genes peaked at 4 h before diminishing, but increased again beginning at around 24 h, at the time of the beginning of seed germination. Expression of the other gene peaked at 12 h after light irradiation, and large amounts of its mRNA remained until 36 h. The timing of the expression of these genes is very similar to the induction of the acid invertase genes examined in this study. If the expression level of these GA 3-oxidase genes is directly linked to the production of active GA, GA production in the seeds might peak twice, at points before and during germination. These putative first and second increase in GA production may correspond to the expression of Atbfrcut1/AtcwINV1, Atbfrcut3 and Atbfrcut4. To investigate these possibilities, we searched in the GenBank database (http://www.ncbi.nlm.nih.gov/entrez/) for similarities to the upstream sequences of these invertase genes. The upstream region of Atbfrcut1/AtcwINV1 contain two putative pyrimidine boxes and four GARE sequences (Gibberellic acid-responsive element),38 and the corresponding regions in Atbfrcut3 and Atbfrcut4 have one and two putative pyrimidine box(es), respectively, and one GARE sequence. The presence of multiple GARE sequences might be the cause of the early expression of the Atbfrcut1/AtcwINV1 gene. Further analysis of these promoters will be required to clarify the regulatory mechanisms for these genes during the early stage of germination.

However, it is still unclear whether other signals following germination induce the expression of these genes. More clear evidence for the GA contribution might be obtained from seeds whose germination is physically inhibited by an osmoticam such as mannitol or polyethylene glycol, when no disturbing of GA biosynthesis is revealed in those conditions. If there were mutant seeds which are prevented from germination with the normal profile of GA production, it will be a good material to clarify the role of GA. Therefore, development of these materials should be required for our future study.

The expression pattern has been examined for eight invertase genes encoding six apoplastic and two vacuolar invertases in A. thaliana ecotype Columbia,3,30,31 although the expression in germinating seeds was not investigated. Only the Atbfrcut3 transcripts were detected with in situ hybridization of mature seeds.31 However, we detected only faint signals on Northern blots for the Atbfrcut3 and Atbfrcut4 mRNAs in dry seeds (data not shown). No significant signal was observed for the Atbfrcut1 mRNAs in dry seeds. The Atbfrcut3 transcripts may be degraded during maturation and desiccation of these seeds. It is assumed that most of the active acid invertase is synthesized following R light irradiation.

Our results demonstrate that the expression pattern of Atbfrcut3 and Atbfrcut4 parallel the activities of soluble acid invertase (Figs. 1 and 2), which may trigger the expansion of the embryo. However, the accumulation pattern of the Atbfrcut1/AtcwINV1 mRNA did not parallel the germination events. What role does this enzyme have during the very early stage of seed germination? In the future, it will be interesting to
examine the cell-wall fraction for the presence of apoplastic invertase activities.

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