Specific expression of the gibberellin 3 β -hydroxylase gene, *HvGA3ox2*, in the epithelium is important for *Amy1* expression in germinating barley seeds

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Abstract We have cloned gibberellin (GA) 3 β -hydroxylase cDNA, (*HvGA3ox*), a homolog of the rice gene *OsGA3ox* from germinating barley seeds. *HvGA3ox* cDNA was expressed in *Escherichia coli* as a fusion protein capable of oxidizing GA₉ to GA₄ and GA₂₀ to GA₁. Deduced amino acid sequence analysis showed that *HvGA3ox* was identical to *HvGA3ox2*, which shares a high homology with the *OsGA3ox2* isoform that plays an important role for induction of α -amylase in germinating rice seeds. Northern blot analysis showed that *HvGA3ox2* gene was expressed only in germinating seeds, not in other organs. Typical feedback regulation was not observed in seedling treated with either biologically active GA (GA₃) or uniconazole, an inhibitor of GA biosynthesis. *HvGA3ox2* mRNA was detected from 12 h after imbibition just before expression of α -amylase gene, *Amy1*. *In situ* hybridization of germinating seeds revealed that *HvGA3ox2* mRNA was localized in the epithelium up to at least 3 days after imbibition. Our results suggest that GA biosynthesis in epithelium is important for expression of α -amylase in germinating barley seeds, and that *HvGA3ox2* encodes the key enzyme of this event.

Key words: α -amylase, epithelium, gibberellin, *Hordeum vulgare*.

Gibberellins (GAs) are diterpene compounds biosynthesized from mevalonic acid that act as regulators of plant growth and development. Kasahara et al. (2002) demonstrated that GAs are predominantly synthesized from the MEP (methylerythritol phosphate) pathway in Arabidopsis seedlings. In higher plants GA promotes germination, stem elongation, seed development and flowering (Olszewski et al. 2002). Of the 126 GAs that have been identified in higher plants, fungi and bacteria, only a small number are biologically active (Hedden and Phillips 2000; Olszewski et al. 2002).

It is widely held that active GAs are biosynthesized in the embryo and released to aleurone cells in germinating cereal grain. Active GA triggers the expression of hydrolytic enzymes that convert the starchy endosperm of the seed into nutrients for the growing seedling in aleuron cells (Fincher and Stone 1993). There are many reports about the relationship between GAs and hydrolases, especially α -amylase (Paleg 1960; Yomo 1960; Chrispeels and Varner 1967; Stuart et al. 1986). It was demonstrated that α -amylase is induced by GA produced from a stored GA-precursor lying later in the pathway than *ent*-kaurenoic acid, but *de novo* GA

biosynthesis has been found to be essential for shoot elongation in germinating barley seeds by using an inhibitor of *ent*-kaurene oxidation (Großelindemann et al. 1991). Based on biochemical and molecular studies, it was suggested that scutellum tissue may be important for *de novo* GA biosynthesis, and can initiate aleurone cell death and culmination of the developmental program (Appleford and Lenton 1997).

A series of genes encoding the enzymes involved in the GA biosynthetic pathway has been cloned and analyzed from a variety of species (Hedden and Phillips 2000). GA 3 β -hydroxylase (GA3ox) catalyses the final step of active GA biosynthesis that converts both GA₂₀ to GA₁ and GA₉ to GA₄ (Hedden and Kamiya 1997). This enzyme is also known as 2-oxoglutarate-dependent dioxygenase (2-ODD), and belongs to a large class of Fe-containing enzymes found in plants and fungi that share a common reaction mechanism (Hedden and Phillips 2000; Olszewski et al. 2002). Expression of GA3ox genes is likely to play a key regulatory role in controlling the appropriate level of active GAs during plant growth and tissue development, including seed germination, by feedback regulation (Lester et al. 1997; Cowling et al.

Abbreviations: DIG, dioxigenin; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA end.

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1998; Yamaguchi et al. 1998; Yamaguchi et al. 2001; Itoh et al. 2001).

Itoh et al. (2001) cloned and analyzed two GA3ox genes (*OsGA3ox1* and *OsGA3ox2*) from rice. Their study indicated that *OsGA3ox1* was preferentially expressed in unopened flower and *OsGA3ox2* in elongating leaves. Kaneko et al. (2002) reported that *OsGA3ox2* production in epithelium cells was important for α -amylase induction in early stages of germinating rice seeds. On the other hand, little is known about the molecular mechanism of GA biosynthesis in germinating barley seeds. Recently, Spielmeier et al. (2004) reported isolation and chromosome mapping of two genes encoding GA 3 β -hydroxylase, *HvGA3ox1* and *HvGA3ox2*, from barley. However, there is no information about the expression pattern of these genes, and their role in α -amylase expression during germination remains to be clarified. The research described here predicts that *HvGA3ox2* plays an important role in GA biosynthesis for the induction of hydrolytic enzymes, including α -amylase, in germinating barley seeds.

Materials and methods

Plant materials

Barley seeds (*Hordeum vulgare* cv. Harrington) were surface-sterilized in 1% NaClO for 10 min and rinsed in distilled water. The seeds were imbibed in distilled water for 8 h, plated on petri dishes with wetted filter paper, and grown in a culture chamber at 20°C in the dark. Seedling samples were collected from 0 (immediately following imbibition) to 4 days after imbibition. Seeds were treated with biologically active GA, GA₃, and uniconazole (10⁻⁵ M) 24 h after imbibition for 72 h. Germinating seeds were planted on soil and grown in greenhouse at 20°C for a month, at which time leaves, roots, stem, spikes before heading and spikes after heading were collected. Germinating seeds were used for isolation of mRNA to produce cDNA, and all samples were used for Northern blot analysis.

Chemicals

[17,17-²H₂] GA₁₉, [17,17-²H₂] GA₂₀, [17,17-²H₂] GA₂₉, [17,17-²H₂] GA₄₄ and [17,17-²H₂] GA₅₃ were purchased from L. Mander, Australian National University (Canberra). [15,17,17-²H₃] GA₉ was synthesized from GA₉-norketone and (methyl-d₃) triphenylphosphonium-Br by Wittig reaction. All GAs used in this study were analyzed by full-scan GC-MS to determine purity. GA₃, biologically active GA isolated from fungi, and uniconazole, an inhibitor for GA biosynthesis, were purchased from Wako Pure Chemicals (Osaka, JAPAN).

RNA isolation and cDNA synthesis

Total RNA was prepared using the phenol/SDS method described by Karrer et al. (Karrer et al. 1991). Poly(A)⁺ RNA was separated by using oligotex-dT30 (Super) according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Complementary DNA was synthesized from 1 μ g of poly (A)⁺ RNA prepared from germinating seeds 3 d after imbibition by the ThermoScript RT-PCR system according to manufacturer's instructions (Invitrogen Corp., Carlsbad, CA USA).

Isolation of GA 3 β -hydroxylase cDNA clones

Reverse transcription PCR was carried out using cDNA prepared from germinating seeds 3 days after imbibition and degenerate primers. The primers were designed based on the amino-acid sequence of GA3ox from rice, Arabidopsis, pea and pumpkin. One of the primer pairs, TO-313 (5'-TCCTTCTTCTCCAAGCTCATGTGGT-3') and TO-314 (5'-GGAAGAGGTCGCCGACGTTRACNAC-3'), gave a DNA fragment of expected size (approximately 0.5 kbp), and it was cloned into pCR2.1-TOPO (Invitrogen Corp., Carlsbad, CA, USA), and sequenced. The resulting construct was designated as pHvGA3ox-p. To identify full-length *HvGA3ox* cDNA, 5'- and 3'-RACE reactions were performed with SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). We used poly (A)⁺ RNA from germinating seeds 3 days after imbibition as the template and the primers, TO-347: 5'-AAGAAGCCCGAGTCCGTGTGC-3' for 5' RACE, and TO-346: 5'-CCAACCTCCGCTCCGACCTCC-3' for 3' RACE.

Products from the 5'- and 3'-RACE (approximately 0.9 kbp for 5'-RACE and 0.79 kbp for 3'-RACE) were cloned into pCR2.1-TOPO to generate pHvGA3oxU and pHvGA3oxD, respectively. The fragments of *EcoRI* (fill in)-*KpnI* from pHvGA3oxU and *ApaI*-*KpnI* from pHvGA3oxD were inserted into *ApaI* and *NotI* (fill in) in the multicloning site of pUC119 to generate full-length *HvGA3ox* cDNA, designated pUC-HvGA3oxF. The 1.6 kbp fragment digested with *MluI* and *SmaI* from pUC-HvGA3oxF was ligated with the vector fragment digested with *EcoRI* (fill in) and *MluI* of pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ, USA) to generate pGEX-HvGA3ox.

Heterogenous expression of HvGA3ox in *Escherichia coli* and enzyme assay

Expression vector pGEX-HvGA3ox was transformed into *E. coli* strain BL21. Overnight culture of *E. coli* (100 ml) was used to inoculate 1 liter of LB medium containing ampicillin (0.05 mg ml⁻¹), and incubated for 18 h. Isopropyl β -D-thiogalactopyranoside (IPTG) was added at a concentration of 10 μ M, and the culture was incubated overnight at 30°C. The cells were harvested,

Table 1. Identification of the metabolites from GAs incubated with recombinant barley GA 3 β -hydroxylases by full-scan GC-MS and Kovats retention indices (KRI).

Recombinant Protein	Substrate	Metabolite	KRI	MS data: m/z (% of relative intensity)
HvGA3ox	² H ₂ -GA ₂₀	GA ₁	2,680	508(51), 450(13), 378(13), 209(100)
	² H ₃ -GA ₉	GA ₄	2,528	421(5), 389(4), 331(6), 287(35), 227(100)

homogenized by sonication for 20 min with an output of 200 W, and centrifuged to collect a crude extract. The presence of *HvGA3ox* recombinant protein in the crude extract was confirmed by SDS/PAGE. The crude extract was concentrated by affinity column with Glutathion Sepharose 4B (Amersham Pharmacia Biotech.), and eluted from the column for enzyme assays. The reaction conditions for GA 3 β -hydroxylation and product analysis by GC-MS were as described by Itoh *et al.* (2001).

Northern blot and RT-PCR analysis

Total RNA (10 μ g per lane) was separated by 1% agarose-2.2 M formaldehyde gels and blotted onto Hybond NX (Amersham Bioscience). The fragment of the GA 3 β -hydroxylase gene amplified by PCR using TO-347 and TO-368 (5'-TAACCGGAATTCATGCA GACACTCACAGCGTCC-3') as the primers was ³²P-labeled using the Megaprime labelling system (Amersham Bioscience) and used as a hybridization probe.

First strand cDNA synthesis was carried out using 2.5 μ g of total RNA treated with DNaseI (Worthington, Lakewood, USA) by using ThermoScript Reverse Transcriptase (RT) (Invitrogen, San Diego, USA). Primers for the detection of *HvGA3ox1*, *HvGA3ox2*, *Amy1*, and *Actin* expression were as follows: *HvGA3ox1* (5'-GGTACCCCAAGTGCCCTGAC-3' and 5'-TGCGT CCTTTGCCGAAGTC-3'); *HvGA3ox2* (5'-TGATCGC GCACACGGACTC-3' and 5'-GAGAGGACGTCGGG GAGGAC-3'); *Amy1* (5'-AACTCGGAGTCGTGGAA GC-3' and 5'-CTGGGTGCGTGAGGATGTA-3'); and *Actin* (5'-CCCCATGCCATTCTTCGTTT-3' and 5'-CTG GGCCAGACTCGTCGTA-3').

In situ hybridization

Germinating seeds were fixed in PBS containing 4% (w/v) paraformaldehyde at 4°C overnight, then in PBS containing 10%, 15% and 20% sucrose for 4 h each. The fixed material was embedded in O.C.T. compound (Sakura Fine Tech, Tokyo Japan), and flash frozen in liquid nitrogen. Microtome sections (10 μ m thick) were mounted on siliconized glass slides (Matsunami Glass, Osaka, Japan), and stored at -80°C.

In situ hybridization was performed using digoxigenin-labeled sense or antisense RNA probes prepared from pHvGA3oxU with DIG-RNA labeling kit (Roche, Germany) according to manufacturer's instructions. Hybridization was performed according to

the method described by Noji (2001) with slight modification. Glass slides were dehydrated with a graded ethanol series, (100%, 90%, 80%, 70% and 50% in PBS), then washed once in PBS containing 2% Triton X-100 for 5 min, and twice in PBS for 5 min. Glass slides were treated with Proteinase K (1 μ g ml⁻¹) for 5 min, washed in PBS containing 0.2% glycine and then in PBS for 5 min. Glass slides were fixed again in PBS containing 4% (w/v) paraformaldehyde for 5 min. After 5 min wash in PBS, they were soaked in TEA buffer (1% triethanolamine, 0.5% acetic anhydride and 0.175% hydrochloric acid) for 5 min for acetylation. Hybridization was carried out at 65°C overnight.

Results

Isolation of genes encoding GA3 β -hydroxylase from Barley

Using barley seedling cDNA as a template, we amplified a DNA fragment with homology to the 3 β -hydroxylase genes cloned from rice (Itoh *et al.* 2001) using degenerate primers. Based on the nucleotide sequence of the amplified fragment, rapid amplification of cDNA end (RACE)-PCR was performed to construct full-length cDNA clones. Deduced amino acid sequence was completely identical to that of *HvGA3ox2* (GenBank accession no. AY551431).

Functional Analysis

To demonstrate the substrate specificity in detail, we subcloned the putative coding sequence of *HvGA3ox2*-cDNA in pGEX-4T-1 to produce a GST fusion protein in *E. coli*. Results of the functional assay for 3 β -hydroxylase activity are summarized in Table 1. The recombinant protein converted GA₉ and GA₂₀ to GA₄ and GA₁, respectively (Table 1). However, the extract did not convert the substrates to GA₅ and GA₃ or GA₇ under our experimental conditions.

Gene Expression

The expression of *HvGA3ox2* was examined in several barley organs using RNA gel blot analysis with a gene-specific hybridization probe. The expression of *HvGA3ox2* was observed only in germinating seeds, but not in other organs (Figure 1). The expression of *HvGA3ox2* was observed continuously from 1 to 4 days after imbibition, but not immediately after imbibition (day 0) (Figure 2A).

Figure 1. Northern blot analysis of *HvGA3ox* in barley organs. Total RNA was isolated from upper stems, lower stems, spikes before heading, spikes after heading, mature leaves, young leaves, seedlings and roots. Ten μ g of total RNA from each sample was loaded onto the gel. rRNA was used as loading control.

Figure 2. Northern blot analysis of *HvGA3ox* in germinating seeds. Total RNA was isolated from several seedling stages. (A) Numbers indicate the day(s) after imbibition. (B) Effects of GA₃ and uniconazole on the expression of *HvGA3ox* in germinating seeds. Numbers indicate the days after imbibition. Lanes +Uni and +GA₃ indicate treatment with 10 μ M uniconazol or 10 μ M GA₃ 1 day after imbibition.

It has been reported that the expression of GA3ox genes are under feedback regulation, in pea (Lester et al. 1997), *Arabidopsis* (Yamaguchi et al. 1998, 2001) and rice (Itoh et al. 2001) for controlling the amount of bioactive GAs in plants. We used germinating seeds to examine the expression of *HvGA3ox2* in the presence of GA₃ or uniconazole, the GA biosynthetic inhibitor. The expression of *HvGA3ox2* was not drastically affected by treatment with GA₃ or uniconazole (Figure 2B), suggesting that *HvGA3ox2* was not regulated by feedback inhibition.

We compared the expression pattern of the *HvGA3ox1*, *HvGA3ox2* and α -amylase gene, *Amy1*, in early stage of germinating barley seeds with RT-PCR analysis to investigate the contribution of these GA3oxs

Figure 3. RT-PCR analysis of *HvGA3ox1*, *HvGA3ox2* (A), *Amy1* and *Actin* for internal control (B). Number indicates the hours after imbibition. All the PCR were performed for 25 cycles.

for α -amylase induction. The expression of *HvGA3ox2* was detected from 12 h after imbibition (HAI), though not detected just after imbibition (0 HAI) (Figure 3A). The expression of *Amy1* was faintly detected at 12 HAI and clearly detected from 24 HAI (Figure 3B). *HvGA3ox1* mRNA was constitutively detected to 48 HAI including seeds 0 HAI (Figure 3A). *HvGA3ox1* mRNA was also detected in dry seeds at a similar level to at 0 HAI (data not shown).

In situ hybridization

To identify the expression site of *HvGA3ox2* in seedlings, *in situ* hybridization was performed with digoxigenin-labeled RNA probes synthesized from a cDNA clone of *HvGA3ox2* in both sense and antisense orientations. Blue staining by an antisense probe for *HvGA3ox2* was observed in epithelium beginning 1 day after imbibition (Figure 4A, B), and continuing until at least 3 days after imbibition (data not shown). No staining was observed with sense-strand probes (Figure 4C, D) with either probe immediately after imbibition (Figure 4E, F). The timing of *HvGA3ox2* induction *in situ* corresponded with the result of the RNA gel blot analysis (Figures 2A, 3A).

Discussion

We isolated a cDNA clone encoding GA 3 β -hydroxylase (*HvGA3ox*) from germinating barley seeds. The deduced amino acid sequence of *HvGA3ox* was identical to *HvGA3ox2* recently reported by Spielmeyer et al. (2004) which also has high homology to *OsGA3ox2*, one of the isozymes of rice GA3ox (Itoh et al. 2001). The product of heterologous expression in *E. coli* of *HvGA3ox2* was capable of catalyzing the conversion of GA₂₀ to GA₁ as described by Spielmeyer et al. (2004) and also of GA₉ to GA₄, but not GA₂₀ to GA₅ or GA₃ which are 3 β -

Figure 4. *In situ* localization of *HvGA3ox* mRNA during germination. (A, B, C and D); primordial seedling tissues 1 day after imbibition. (E and F); samples immediately following imbibition. Section of a seedling hybridized with antisense (A, B and E) or sense probe (C, D and F). Abbreviations: Ep, Epithelium cells; Sc, Scutella cells; Sa, Shoot apex cells; R, Roots.

hydroxylation products with rearrangement of a double bond. This enzymatic activity is similar to *OsGA3ox2*.

The expression of *HvGA3ox2* gene was detected from 1 days after imbibition (Figure 2A) by Northern analysis. This corresponded well with the timing of an increase in the level of GA₁, which is the major active GA for α -amylase induction in germinating barley seeds (Kobayashi *et al.* 1995) and *de novo* GA biosynthesis of barley caryopsis (Grobelindemann *et al.* 1991).

Spielmeyer *et al.* (2004) reported that there was another active GA3ox gene (*HvGA3ox1*) in barley. By detailed expression analysis in early stage of germinating barley seeds, *HvGA3ox2* mRNA was detected from 12 h after imbibition (HAI), but not in seeds just after imbibition (Figure 3A), indicating that *HvGA3ox2* was newly expressed in early stage of germination. On the other hand *HvGA3ox1* mRNA was constitutively detected until 48 HAI including in dry seeds (Figure 3A), as was the case for *OsGA3ox1* in germinating rice seeds. The expression of *Amy1* was weakly detected 12 HAI and strongly detected from 24 HAI (Figure 3B), confirming the report by Sugimura *et al.* (Sugimoto *et al.* 1998), and corresponded with the expression of *HvGA3ox2*. These results strongly suggested that newly expressed *HvGA3ox2* contributes for the GA biosynthesis following to the induction of *Amy1* expression.

Akazawa and Hara-Nishimura (1985) reported the predominant formation of α -amylase in the scutellar epithelium of germinating cereal seeds. Moreover, proteinase and RNase also appeared to be secreted from the epithelial layer of barley seeds (Okamoto *et al.* 1980). It is reported that the scutellar tissue might be important for *de novo* GA biosynthesis in germinating wheat (Appleford and Lenton 1997) and rice (Sugimoto *et al.* 1998) seeds. Kaneko *et al.* (2002) found that expression of *OsGA3ox2* in epithelium was important for induction of α -amylase expression in the endosperm during rice seed germination by using mutants defective in shoot and epithelium formation. *In situ* hybridization analysis revealed that *HvGA3ox2* transcript was localized to epithelial cells (Figure 4) during 3 days after imbibition, though *OsGA3ox2* transcripts were not only localized to epithelial cells but were also found in the shoot apical region in rice (Kaneko *et al.* 2002). It has also been reported that expression of α -amylase gene (*Amy1*) initiated in the scutellar epithelium and continued in the aluerone layer by possible diffusion of GAs synthesized in embryo in germinating barley seeds. Expression of *Amy1* in epithelium was first detected 16 h after imbibition (HAI) and was clearly evident at 28–64 HAI (Sugimoto *et al.* 1998). Epithelial expression of *HvGA3ox2* was observed continuously from 1 to at least 3 days after imbibition (Figure 4), correlating well with the epithelial expression of *Amy1*. These results strongly suggest that the epithelium itself is the site of active GA biosynthesis for induction of α -amylase in germinating barley seeds and *HvGA3ox2* plays a central role for this event. The expression of *HvGA3ox2* was detected only in germinating seeds, not in other organs (Figure 2), in contrast to the expression of the *OsGA3ox2* in rice that was expressed in several organs (Itoh *et al.* 2001). Typical feedback regulation of *HvGA3ox* expression was not observed in the presence of active GA, GA₃, and the GA biosynthesis inhibitor, uniconazol (Figure 3B), a result similar to *AtGA3ox2* regulation (Yamaguchi *et al.* 2001), and in contrast to the regulation seen with *OsGA3ox2* (Itoh *et al.* 2001). These results indicate that there might be some functional differences between *HvGA3ox2* and *OsGA3ox2* in spite of amino acid sequence similarity and chromosomal synteny (Spielmeyer *et al.* 2004).

Base on the present results, it must be concluded that *HvGA3ox2* regulates and stimulates germination through the production of active GAs that induce hydrolytic enzymes including α -amylase in barley seeds. The expression pattern of *HvGA3ox2* is similar with *AtGA3ox2*, the Arabidopsis GA 3-oxidase gene that is expressed specifically during germination (Yamaguchi *et al.* 1998, 2001).

Grobelindemann *et al.* (1992) reported production of GA₃ from GA₂₀ *via* GA₅ in cell-free system from 2 days

old germinating embryos of barley. Kobayashi et al. (1995) demonstrated that GA₃ is accumulated at slightly lower levels than GA₁ in late stage of germination. *HvGA3ox2* expressed in *E. coli* did not catalyze the conversion of GA₂₀ to GA₅ nor GA₃ (Table 1). It has been reported that another GA3ox gene, *HvGA3ox1*, whose mRNA was detected in germinating barley seeds (Figure 3A), has the ability to convert GA₂₀ to GA₁ (Spielmeyer et al. 2004), but it is not clear whether it can convert GA₂₀ to GA₅ and GA₃, meaning that some other GA3ox(s) might be expressed in late stage of germination. Moreover active GA should be biosynthesized in other growing stages beyond germination, so GA3ox isozyme(s) expressed in other organs should exist in barley. Further research is necessary to confirm the contribution of *HvGA3ox1* and hypothetical *HvGA3ox* in germination but also in other GA dependent processes, such as stem elongation, flower development and seed development.

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