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Original Paper

Specific expression of the gibberellin 3β-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) 3β-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 GA_9 to GA_4 and GA_{20} to GA_1. 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 (GA_3) 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*-keurene 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 3β-hydroxylase (GA3ox) catalyses the final step of active GA biosynthesis that converts both GA_{20} to GA_1 and GA_9 to GA_4 (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.

The nucleotide sequence reported in this paper has been submitted to DDBJ/GenBank/EMBL nucleotide sequence database under the accession number AB189152.

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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, Spielmeyer et al. (2004) reported isolation and chromosome mapping of two genes encoding GA 3β-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. Geminating 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-$^2H_2$] GA_{19}, [17,17-$^2H_2$] GA_{20}, [17,17-$^2H_2$] GA_{29}, [17,17-$^2H_2$] GA_{44} and [17,17-$^2H_2$] GA_{53} were purchased from L. Mander, Australian National University (Canberra). [15,17,17-$^2H_3$] GA_9 was synthesized from GA_9-norketone and (methyl-d3) triphenylphosphonium-Br by Witting reaction. All GAs used in this study were analyzed by full-scan GC-MS to determine purity. GA_3, 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 μg 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 3β-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′-GGAAGAGGTCGCCGACGTTRACN AC-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′-AAGAAGCCCGAGTCC GTGTGC-3′ for 5′ RACE, and TO-346: 5′-CCAACCTC CGCTCCGACCTCC-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 *Eco*RI (fill in)-*Kpn*I from pHvGA3oxU and *Apa*I-*Kpn*I from pHvGA3oxD were inserted into *Apa*I and *Not*I (fill in) in the multicloning site of pUC119 to generate full-length *HvGA3ox* cDNA, designated pUC-HvGA3oxF. The 1.6 kbp fragment digested with *Mlu*I and *Sma*I from pUC-HvGA3oxF was ligated with the vector fragment digested with *Eco*RI (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 μM, 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 3β-hydroxylases by full-scan GC-MS and Kovats retention indices (KRI).

Recombinant Protein	Substrate	Metabolite	KRI	MS data: m/z (% of relative intensity)
HvGA3ox	$^2H_2$-GA_{20}	GA_1	2,680	508(51), 450(13), 378(13), 209(100)
	$^2H_3$-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 3β-hydroxylation and product analysis by GC-MS were as described by Itoh et al. (2001).

Northern blot and RT-PCR analysis

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

First strand cDNA synthesis was carried out using 2.5 μg of total RNA treated with DNaseI (Worthington, Lakewood, USA) by using ThermoScript Reverse Transcriptase (RT) (Invitorogen, San Diego, USA). Primers for the detection of *HvGA3ox1*, *HvGA3ox2*, *Amy1*, and *Actin* expression were as follows: *HvGA3ox1* (5′-GGTACCCCAAGTGCCCTGAC-3′ and 5′-TGCGT CCTTTGCCGAACTC-3′); HvGA3ox2 (5′-TGATCGC GCACACGGACTC-3′ and 5′-GAGAGGACGTCGGG GAGGAC-3′); *Amy*1 (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 μm thick) were mounted on siliconized glass slides (Matsunami Glass, Osaka, Japan), and stored at −80°C.

In situ hybridization was performed using digoxygenin-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 μg 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 3β-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 3β-hydroxylase activity are summarized in Table 1. The recombinant protein converted GA_9 and GA_{20} to GA_4 and GA_1, respectively (Table 1). However, the extract did not convert the substrates to GA_5 and GA_3 or GA_7 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).

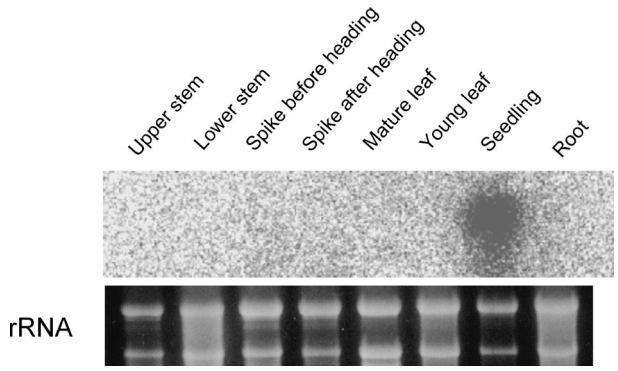

Figure 1. Northern blot analysis of *HvGA3ox* in barley organs. Total RNA was isolated from upper stems, lower stems, spikes before heading, spikes after heading, mature leaves, young leaves, seedlings and roots. Ten μg of total RNA from each sample was loaded onto the gel. rRNA was used as loading control.

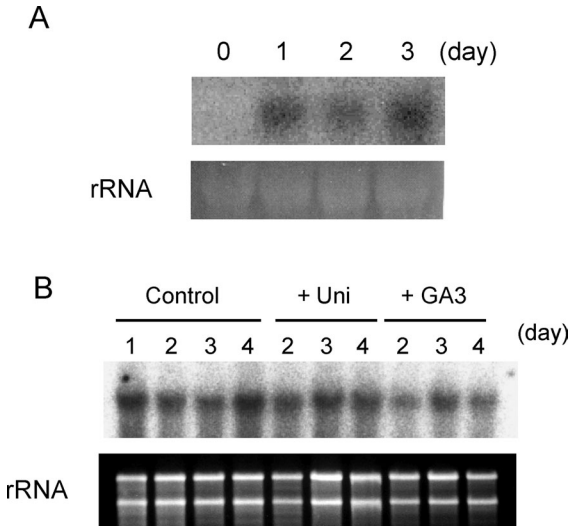

Figure 2. Northern blot analysis of *HvGA3ox* in germinating seeds. Total RNA was isolated from several seedling stages. (A) Numbers indicate the day(s) after imbibition. (B) Effects of GA_3 and uniconazole on the expression of *HvGA3ox* in germinating seeds. Numbers indicate the days after imbibition. Lanes +Uni and +GA3 indicate treatment with 10 μM uniconazol or 10 μM GA_3 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 GA_3 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

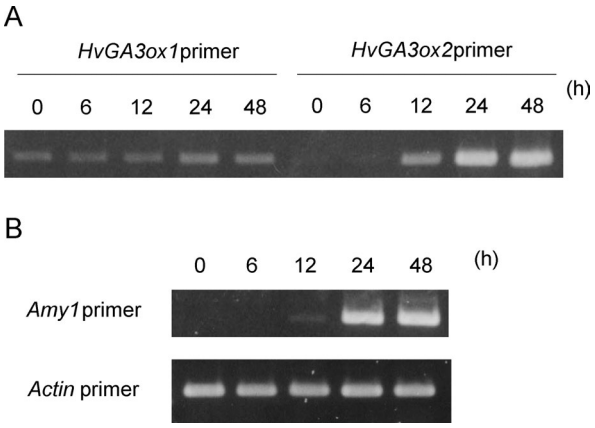

Figure 3. RT-PCR analysis of *HvGA3ox1*, *HvGA3ox2* (A), *Amy1* and *Actin* for internal control (B). Number indicates the hours after imbibition. All the PCR were performed for 25 cycles.

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 *Amy*1 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 digoxygenin-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 3β-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 GA_9 to GA_4, but not GA_{20} to GA_5 or GA_3 which are 3β-

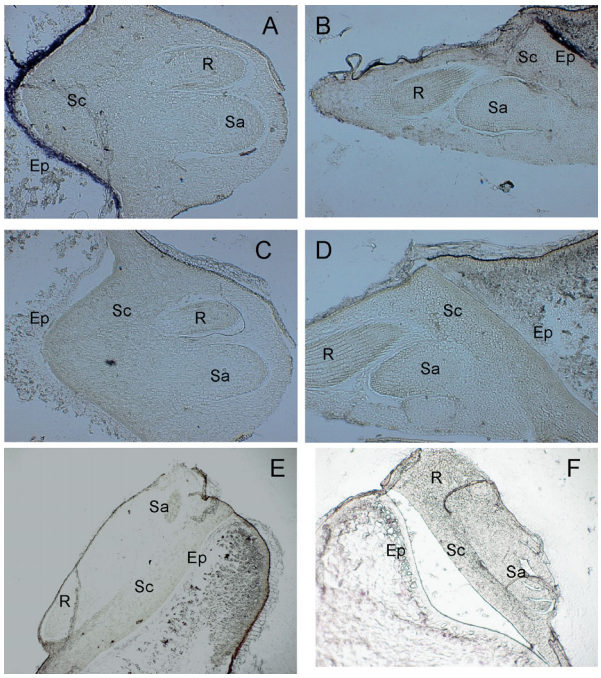

Figure 4. *In situ* localization of *HvGA3ox* mRNA during germination. (A, B, C and D); primordial seedling tissues 1 day after imbibition. (E and F); samples immediately following imbibition. Section of a seedling hybridized with antisense (A, B and E) or sense probe (C, D and F). Abbreviations: Ep, 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 (Großelindemann 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 *Amy*1 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 (*Amy*1) 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 *Amy*1 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 *Amy*1. 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, GA_3, 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).

Großelindemann et al. (1992) reported production of GA_3 from GA_{20} *via* GA_5 in cell-free system from 2 days

old germinating embryos of barley. Kobayashi et al. (1995) demonstrated that GA_3 is accumulated at slightly lower levels than GA_1 in late stage of germination. *HvGA3ox2* expressed in *E. coli*. did not catalyze the conversion of GA_{20} to GA_5 nor GA_3 (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 GA_{20} to GA_1 (Spielmeyer et al. 2004), but it is not clear whether it can convert GA_{20} to GA_5 and GA_3, 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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References

Akazawa T, Hara-Nishimura I (1985) Topographic aspects of biosynthesis, extracellular secretion, and intracellular storage of proteins in plant cells. *Annu Rev Plant Physiol* 36: 441–472

Appleford NEJ, Lenton JR (1997) Hormonal regulation of alfa-amylase gene expression in germinating wheat (*Triticum aestivum*) grains. *Physiol Plant* 100: 534–542

Chrispeels MJ, Varner JE (1967) Gibberellic acid-enhanced synthesis and release of alfa-amylase and ribonuclease by isolated barley aleurone layers. *Plant Physiol* 42: 398–406

Cowling RJ, Kamiya Y, Seto H, Harberd NP (1998) Gibberellin dose-response regulation of GA4 gene transcript levels in Arabidopsis. *Plant Physiol* 117: 1195–1203

Fincher GB, Stone BA (1993) Physiology and biochemistry of germination in barley. In: MacGregor AW, Bhatty RS (eds) *Barley: Chemistry and Technology.* St. Paul, MN, USA American Association of Cereal Chemists, pp 247–295

Großelindemann E, Graebe JE, Stöckl D, Hedden P (1991) *Ent*-Kaurene biosynthesis in germinating barley (*Hordeum vulgare* L., cv Himaraya) caryopses and its relation to α-amylase production. *Plant Physiol* 96: 1099–1104

Großelindemann E, Lewis MJ, Heddn P, Graebe JE (1992) Gibberellin biosynthesis from gibberellin A_{12}-aldehyde in a cell-free system from germinating barley (*Hordeum vulgare* L., cv Himaraya) embryos. *Planta* 188: 252–257

Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: Enzymes, genes and their regulation. *Annu Rev Plant Physiol Mol Biol* 48: 431–460

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. *Trends in Plant Science* 5: 523–530

Itoh H, Ueguchi-Tanaka M, Sentoku N, Kitano H, Matsuoka M, Koabayashi M (2001) Cloning and functional analysis of two gibberellin 3beta-hydroxylase genes that are differently expressed during the growth of rice. *Proc Natl Acad Sci USA* 98: 8909–8914

Kaneko M, Itoh H, Ueguchi-Tanaka M, Ashikari M, Matsuoka M (2002) The alfa-amylase induction in endosperm during rice seed germination is caused by gibberellin synthesized in epithelium. *Plant Physiol* 128: 1264–1270

Karrer EE, Litts JC, Rodriguez RL (1991) Differential expression of alfa-amylase genes in germination rice and barley seeds. *Plant Mol Biol* 16: 797–805

Kasahara H, Hanada A, Kuzuyama T, Takagi M, Kamiya Y, Yamaguchi S (2002) Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of gibberellins in Arabidopsis. *J Biol Chem* 277: 45188–45194

Kobayashi M, Gomi M, Agematsu J, Asami T, Yoshida S, Sakurai A (1995) Fluctuation of endogenous gibberellin and abscisic acid levels in germinating seeds of barley. *Biosci Biotech Biochem* 59: 1969–1970

Lester DR, Ross JJ, Davis PJ, Reid JB (1997) Mendel's stem length gene (Le) encodes a gibberellin 3beta-hydroxylase. *Plant Cell* 9: 1435–1443

Noji S, (2001) The state-of-art methods of in situ hybridization. In: Noji S (ed) *Immunostaining and In Situ Hybridisation in Post Genomic Research Era.* Yodosha, Tokyo, Japan, pp 37–42 (in Japanese).

Okamoto K, Kitano K, Akazawa T (1980) Biosynthesis and excreation of hydrolases in germinating cereal seeds. *Plant Cell Physiol* 21: 201–204

Olszewski N, Sun TP, Gubler F (2002) Gibberellin signaling: biosynthesis, catabolism, and response pathways. *Plant Cell Supplement* S61–S80

Paleg LG (1960) Physiological effects of gibberellic acid: I. On carbohydrate metabolism and amylase activity of barley endosperm. *Plant Physiol* 35: 293–299

Spielmeyer W, Ellis M, Robertson M, Ali S, Lenton JR, Chandler PM (2004) Isolation of gibberellin metabolic pathway genes from barley and comparative mapping in barley, wheat and rice. *Theor Appl Genet* 109: 847–855

Stuart IM, Loi L, Fincher GB (1986) Development of (1-3, 1-4)-beta-D-glucan endohydrolase isoenzymes in isolated scutella and aleurone layers of barley (Holdeum vulgare). *Plant Physiol* 94: 320–327

Sugimoto N, Takeda G, Nagato Y, Yamaguchi J (1998) Temporal and spatial expression of the α-amylase gene during seed germination in rice and barley. *Plant Cell Physiol* 39: 323–333

Yamaguchi S, Smith MW, Brown RGS, Kamiya Y, Sun TP (1998) Phytochrome regulation and differential expression of gibberellin 3beta-hydroxylase genes in germinating Arabidopsis seeds. *Plant Cell* 10: 2115–2126

Yamaguchi S, Kamiya Y, Sun TP (2001) Distinct cell-specific expression patterns of early and late gibberellin biosynthetic genes during Arabidopsis seed germination. *Plant J* 28: 443–453

Yomo H (1960) Studies on barley malt. The sterilisation of barley seed and amylase formation of separated embryos and endosperms. *Hakko Kyokaishi* 16: 444–448 (in Japanese).