Hordoindolines are Predominantly Expressed in the Aleurone Layer in Late Kernel Development in Barley

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In order to isolate grain-specific genes during kernel development, two differential screening methods—suppression subtractive hybridization (SSH) and differential hybridization (DH)—were applied. Two cDNAs encoding hordoindolines known to be related to grain hardness were isolated and designated as *HvIDa* and *HvIDb*. The cDNAs encoding *HvIDa* and *HvIDb* contained a 450 bp and a 444 bp open reading frame (ORF) that encoded the putative hordoindoline-a and -b precursors consisting of 150 and 148 amino acids, respectively. The deduced amino acid sequences of both *HvIDa* and *HvIDb* contained one tryptophan-rich domain and ten highly conserved cysteine residues. The expression of the *HvIDa* gene was high at 5 DAF, reached a peak at 8 DAF and decreased slightly until 20 DAF, while the expression of *HvIDb* began to be detectable at 8 DAF when it was higher than that of other developmental stages and decreased slightly until 20 DAF. The *HvIDa* and *HvIDb* genes were predominantly detected in the aleurone cell layers in the late part of kernel development, e.g. at 20 DAF. These findings may provide clues to the molecular mechanisms of kernel development and contribute to the determination of the grain texture in barley.

Key Words: aleurone layer, barley, hordoindolines, kernel development.

Introduction

Improvement of grain quality is a major breeding objective in most cereals. In particular, the structural composition of mature endosperm, including protein content and grain hardness, is an important factor in determining end-use quality, e.g. milling performance, malting quality etc. In wheat, the genetic basis of grain hardness has been well characterized. For instance, it is genetically recognized that wheat grain hardness is closely associated with mutations in the friabilin components, which are composed of puroindoline a and puroindoline b and were used as marker proteins for grain softness (Giroux and Morris 1998). In previous studies, the change from glycine to serine in puroindoline b was considered to be important for the grain hardness texture due to the alteration of the secondary and tertiary structures (Giroux and Morris 1997). However, the same researchers reported that the serine mutation of puroindoline b was not found in several hard wheat varieties, which did not express transcripts encoding puroindoline a, suggesting that these two proteins alone may act together to affect grain hardness (Giroux and Morris 1998).

Gautier et al. (2000) reported that puroindoline genes were present in diploid ancestors of wheat and its closely related species, such as barley, oats, and rye. They designated the barley orthologs of puroindolines as *hordoindolines* based on the latin generic name, *Hordeum*. Beecher et al. (2001) reported the existence of substantial allelic variations in *hordoindoline a* (*hinA*) and *hordoindoline b* (*hinB*) sequence types that were located at the extreme telomeric end of the short arm of chromosome 5 (5H). Based on quantitative trait locus (QTL) analysis using the “Steptoe” × “Morex” mapping population, the largest QTL corresponding to grain hardness was mapped in the *hinA/hinB/Gsp* region, which could explain 22% of the hardness differences (Beecher et al. 2002). Recently, the barley genomic sequences harboring the endosperm texture have been revealed for comparative sequence analysis, suggesting that two *hinB* copies, *hinb-1* and *hinb-2* indicating an additional copy duplicated, were closely linked to the *hinA* gene with the same orientation (Caldwell et al. 2004). However, the

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molecular expression of hordoinolines during kernel development in barley has not been fully elucidated although their physical location is well defined. Darlington et al. (2001) reported that the expression of hordoinoline b mRNA could be detected in the starch endosperm and aleurone layer of the developing seed.

Grain development is one of the major topics in agricultural studies due to the high economic value of grains as an edible source of carbohydrates. Despite numerous reports on cereal grain development, molecular mechanisms of barley kernel development have not been well defined. We previously reported the molecular characterization of two genes, HvCaBP1 and HvSec61a, induced during kernel development in barley using the suppression subtractive hybridization (SSH) method (Jang et al. 2003, Jang et al. 2005). Although both genes were induced early during kernel development with grain-specific expression, there was a slight difference in the spatial expression, namely one showed an embryo-specific expression, while the other an endosperm-specific expression. The expression of the kernel developmental genes tends to change by treatments with exogenous hormones or by abiotic stresses, especially drought stress. In the present study, we reported the existence of two other genes encoding putative hordoinoline-a and -b, using northern blotting and in situ hybridization.

Materials and Methods

Plant materials
A barley cv. “Karl” (Clho 15487) was grown in the research field of Korea University. Each spike was labeled at 1 day after fertilization (DAF), based on the emergence from the leaf sheath. The samples were harvested at 5, 8, 11, 14, 17 or 20 DAF, frozen immediately in liquid nitrogen and stored at −80°C until use. For avoiding variations in the flowering time, mainly middle florets in a spike should be adjusted manually. The tissues of grains, pericarp, leaves and stems were also collected from plants at 14 DAF.

Isolation of full-length cDNAs encoding hordoinoline a and b
Suppression subtractive hybridization (SSH) was carried out with a PCR-selected cDNA Subtractive kit according to the manufacturer’s protocol (Clontech Co. Mountain View, CA). Driver and tester RNAs were isolated from grains at 14 and 5 DAF, respectively, using Trizol, according to the manufacturer’s instructions. A cDNA library was constructed from grains at 14 DAF using the Uni-ZAP XR vector (Stratagene Co. La Jolla, CA). A SSH clone harboring the indoline homologue was labeled using digoxigenin and a random primer labeling kit (Roche Co. Basel, Switzerland). About 1 x 10⁶ plaques which were transferred to two positive nylon membranes (GE Osmonics Inc. Minnetonka, MN) were screened, according to the manufacturer’s protocol (Roche).

In order to isolate genes expressed specifically in grains, differential hybridization (DH) was performed using each of the probes prepared from grains and pericarp at 14 DAF. Five hundred randomly selected cDNA clones were blotted to each of two positive nylon membranes with 2 replications with a vacuum dot-blot apparatus (Invitrogen Co. Carlsbad, CA). Hybridization and detection were conducted as described by Jang et al. (2003). Plasmid DNAs were extracted using the QIAprep Spin Miniprep kit (Qiagen Co. Valencia, CA). The inserted cDNAs were amplified with both universal T3 and T7 primers using the Big Dye Terminator Cycle Sequencing Ready Kit (Applied Biosystems Co. Foster City, CA). Electrophoresis was performed using the ABI PRISM 3700 Genetic Analyzer (Applied Biosystems). DNA sequencing data were analyzed using the Internet network program, Compute pI/MW tool, (http://www.expasy.ch) for determining the molecular weight and theoretical isoelectric focusing point. The deduced amino acids were compared with the public database at NCBI using BLAST search.

Northern analysis and in situ hybridization
Total RNA samples from each kind of tissue were extracted with Trizol, as described in the commercial protocol (Invitrogen). Total RNA (10μg) was loaded into 1% formaldehyde agarose gel and transferred to the positive nylon membrane (GE Osmonics). Each of the full-length cDNAs encoding Hordeum vulgare indoline-g or -b (HvDa and b) was used as a template for labeling with Biotin (Invitrogen) by the PCR method. The membranes were pre-hybridized for 1 hr and then hybridized for at least 16 hrs at 68°C with the labeled probe in a dextran solution (1 mM EDTA, 7% SDS, 0.25 M disodium phosphate pH 7.2, and 5% dextran sulfate). Detection was performed using Southern-Light and Southern-Star systems (Applied Biosystems).

In situ hybridization was performed as described by Jang et al. (2002) with some modifications. The kernels were harvested at 5, 17 and 20 days and immediately vacuum-infiltrated for 1 hr with a fixative (30 mM sodium phosphate, 130 mM sodium chloride, 4% paraformaldehyde, and 0.1% Triton X-100) followed by 18 hrs at room temperature in a new fixative. Sense and anti-sense RNA probes were labeled using a DIG-RNA labeling kit (Roche), according to the manufacturer’s instructions. Hybridization was carried out in a hybridization buffer (50% formamide, 4X SSC, 150 μg/ml tRNA and 0.5% blocking buffer) for 18 hrs at 42°C. Detection was performed with a DIG luminescent detection kit (Roche).

Results
Isolation of cDNAs encoding the HvIDA and HvIDb genes
In order to isolate grain-specific genes expressed during kernel development, two screening methods, namely suppression subtractive hybridization (SSH) and differential hybridization (DH) were applied.

In previous studies, grains sampled at 14 DAF and 5
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DAF were used as a tester and a driver for SSH analysis, respectively (Jang et al. 2003, 2005). A total of 780 clones that were specifically expressed by the grains at 14 DAF were subjected to reverse northern blotting using the same RNAs of the tester (14 DAF) and the driver (5 DAF). A SSH clone matched the sequence of the hordoindoline-a gene that was reported by Gautier et al. (2000). To isolate full-length cDNA of putative hordoindoline-a, a cDNA library constructed from grains of the barley cv. “Karl” at 14 DAF was screened with the labeled SSH clone. One clone which was identified as a homologue to hordoindoline-a was designated as HvIDa (Hordeum vulgare indoline a).

DH analysis was conducted with randomly selected 500 cDNAs of barley plants at 14 DAF using grain tissues as a tester and pericarp tissues as a driver, respectively. Among the genes that were differentially expressed in grains, one clone which showed a high homology to hordoindoline-b (Gautier et al. 2000), was designated as HvIDb (Hordeum vulgare indoline b). Two full-length sequences were used for further studies to evaluate the molecular mechanism during kernel development.

The cDNAs encoding HvIDa and HvIDb contained a 450 bp and a 444 bp open reading frame (ORF) that encoded the putative hordoindoline-a and -b precursors consisting of 150 and 148 amino acids, respectively (Fig. 1). These ORFs contained a putative signal peptide consisting of 19 amino acids. The putative molecular weight of HvIDa and HvIDb was calculated to be 16.5 kDa and 16.1 kDa. Isoelectric focusing points of HvIDa and HvIDb were 8.72 and 8.84, respectively. The deduced amino acid sequences of both HvIDa and HvIDb contained one tryptophan-rich domain (WRWWRWWK) and ten highly conserved cysteine residues through amino acids of other plant indolines (Fig. 2).

Tissue-specific and developmental expression of HvIDa and HvIDb

Tissue-specific expression of the HvIDa and HvIDb genes was examined using different plant tissues, such as grains, pericarp, stems and leaves at 14 DAF. Transcripts of the HvIDa gene were highly expressed in grains, stems and leaves but absent in the pericarp. In contrast, the HvIDb gene was only highly expressed in grains, but not in the other tissues. To determine the developmental expression of both genes northern blot hybridization was also performed in
grains from 5 DAF to 20 DAF (Fig. 4). The expression of the HvIDa gene was high at 5 DAF, reached a peak at 8 DAF, and decreased slightly until 20 DAF, while the expression pattern of HvIDb began to be detected and was the highest among those at other stages at 8 DAF, and decreased slightly until 20 DAF.

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In order to examine the spatial patterns of both genes, *in situ* hybridization was performed on different developing kernels at 5, 17 or 20 DAF (Fig. 5). Transcripts of the HvIDa gene were detected in the vascular tissues of the lodicules and the glume but were absent in the grains at 5 DAF (Fig. 5A). It is interesting to note that in the kernel that at 17 DAF, HvIDa mRNA was expressed in the starchy endosperm near aleurone layer (Fig. 5B). Eventually, the HvIDa gene was predominantly detected in the aleurone layer in the kernel at 20 DAF (Fig. 5C). Spatial expression of the pattern of the HvIDb gene was similar to that of HvIDa at 17 DAF (Fig. 5F) and 20 DAF (Fig. 5G), but different from that detected at 5 DAF (Fig. 5E). No signals were detected using sense RNA probes of HvIDa and HvIDb as the negative controls (Fig. 5D and 5H).

Discussion

The structural composition of the mature cereal endosperm is an important factor for end-use quality, including...
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The milling performance of wheat and the malting quality of barley depend on the protein content of the endosperm and the texture of grain, which are determined during kernel development. The protein content and texture of grain are also affected by the puroindoline genes, which have been implicated in differences in endosperm texture in wheat (Sourdille et al. 1996, Giroux and Morris 1998). In addition, the wheat puroindoline genes have been found to affect the endosperm texture of transgenic rice when expressed as transgenes (Krishnamurthy and Giroux 2001). Puroindoline-like sequences have been found in other members of the Triticeae tribe (Gautier et al. 2000, Simone and Lafiandra 2005) and oats (Tanchak et al. 1998).

However, there are few studies on either the expression or allelic variation of puroindoline orthologs in species other than wheat. This appears to be a potentially rewarding area of investigation due to the similarity between barley and wheat.

In the present study, the clones HvIDa and HvIDb, closely matched to hordoindoline-a and hordoindoline-b, were isolated by using two differential screening methods. They consisted of 678 and 566 nucleotides including the coding sequences of hordoindoline-a (149 aa) and hordoindoline-b (147 aa) with the signal peptide consisting of 19 amino acids, respectively. Compared with puroindoline orthologs of other plants, a tryptophan-rich domain that also contained basic residues highly conserved was a conspicuous feature of the primary structure of HvIDa and HvIDb. Lillemo and Morris (2000) reported that additional two-point mutation in puroindoline b as well as the mutation previously described by Morris and co-worker could be considered as a “loss of function” (i.e. soft to hard wheat) and their structure analysis suggested a dramatically reduced effect on the lipid-binding ability. However, the deduced amino acid sequences of the HvIDb gene of cv. Karl did not show the three-point mutation of puroindoline b. This result was consistent with the finding that the deduced amino acid sequences of hordoindoline-b were identical in both soft- and hard-kernel barleys, except for the single amino acid substitutions/mutations which were not close to the putative starch-binding domain in a few cultivars (Darlington et al. 2001).

Expression patterns of the HvIDa and HvIDb genes were investigated by northern hybridization and in situ hybridization. It appeared that HvIDa and HvIDb were expressed at reasonably high levels at the proper location (i.e. developing grain) to influence the grain texture in barley. As observed in northern hybridization, the HvIDa and HvIDb genes accumulated at early stages of kernel development, although the HvIDb gene was not detected in the 5DAF grains. Darlington et al. (2001) reported that the hordoindoline-b mRNA levels which were already high in both the endosperm and aleurone at 14 days after anthesis (DAA) based on the determination from 14 DAA, showed a peak at 20 DAA, and decreased at 30 DAA. Our findings suggested hordoindolines might play some role(s) in grain development as well as grain hardness in early kernel development. In situ hybridization showed that HvIDa and HvIDb were predominantly expressed in the endosperm near the aleurone cells and aleurone layer at 17 and 20 DAF unlike in the embryo. Dubreil et al. (1998) reported that puroindolines were localized in the starchy endosperm and in the aleurone cells using monoclonal and polyclonal antibodies against puroindoline-a protein. Digeon et al. (1999) reported similar findings for the GUS activity integrated with the puroindoline-b promoter detected in the endosperm, aleurone cells and pericarp cell layers, but not in the embryo and other vegetative tissues such as leaves, stems and roots. Our findings showing that the HvIDa gene was expressed in vegetative tissues, namely stems and leaves, suggested the presence of another function of hordoindoline(s) in vegetative tissues; for example,
antifungal activity (Dubreil et al. 1998). However, further studies should be carried out to analyze the critical functions of hordoindolines in barley.

In our previous reports, SSH methods were applied for analyzing the molecular mechanism and for isolating grain-specific genes during kernel development in barley (Jang et al. 2003, Jang et al. 2005). All genes mined by the SSH method, e.g. HvCaBP1, HvSec61a, HvIDa and HvIDb, were conducted to be grain-specific genes due to their tissue-specific expression. Interestingly, spatial expression patterns in the grain of these genes varied with the duration of kernel development, e.g. embryo-specific expression (HvCaBP1), endosperm-specific expression (HvSec61a) and aleurone layer-specific expression (HvIDa and HvIDb). These genes could be used as suitable materials for analyzing the molecular mechanisms of other grain-specific genes for cell-specific targeting of foreign materials in grains through fusion of their promoters.

Unfortunately, we did not observe any correlation between grain hardness, which is an important factor for quality improvement in malting barley, and the expression of the HvIDa and HvIDb in barley grain. In the previous studies, it was concluded that there was no direct relationship between the presence of hordoindolines and gain hardness in barley (Darlington et al. 2001). Our objective was to enhance the characterization the hordoindoline genes of barley to use this information in barley improvement programs. Although we did not demonstrate that HvIDa and HvIDb are related to the grain texture in barley, these genes together with other plant indolines, i.e. puroindolines should give clues for the determination of the grain texture in barley.

Nucleotide sequences for HvIDa and HvIDb were deposited in GenBank database (AY959939 and AY959940).

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