Anatomical and Transcriptomic Studies of the Coleorhiza
Reveal the Importance of This Tissue in Regulating
Dormancy in Barley1[W]

José M. Barrero, Mark J. Talbot, Rosemary G. White, John V. Jacobsen, and Frank Gubler*

Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra, Australian Capital Territory 2601, Australia

The decay of seed dormancy during after-ripening is not well understood, but elucidation of the mechanisms involved may be important for developing strategies for modifying dormancy in crop species and, for example, addressing the problem of preharvest sprouting in cereals. We have studied the germination characteristics of barley (Hordeum vulgare ‘Betzes’) embryos, including a description of anatomical changes in the coleorhiza and the enclosed seminal roots. The changes that occur correlate with abscisic acid (ABA) contents of embryo tissues. To understand the molecular mechanisms involved in dormancy loss, we compared the transcriptome of dormant and after-ripened barley embryos using a tissue-specific microarray approach. Our results indicate that in the coleorhiza, ABA catabolism is promoted and ABA sensitivity is reduced and that this is associated with differential regulation by after-ripening of ABA 8'-hydroxylase and of the LIPID PHOSPHATE PHOSPHATASE gene family and ABI3-INTERACTING PROTEIN2, respectively. We also identified other processes, including jasmonate responses, cell wall modification, nitrate and nitrite reduction, mRNA stability, and blue light sensitivity, that were affected by after-ripening in the coleorhiza that may be downstream of ABA signaling. Based on these results, we propose that the coleorhiza plays a major role in causing dormancy by acting as a barrier to root emergence and that after-ripening potentiates molecular changes related to ABA metabolism and sensitivity that ultimately lead to degradation of the coleorhiza, root emergence, and germination.

The seeds of many plants show dormancy at harvest, a property that blocks their germination even under favorable conditions. Dormancy can be affected by several treatments involving hormones, light quality, temperature, or nutrition, and it disappears with time in a process called after-ripening (for review, see Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008). The grains of barley (Hordeum vulgare) and wheat (Triticum aestivum) have low dormancy as a result of human selection for rapid germination during breeding. Because of this, and depending on the environmental conditions, mature grains can germinate in the ear before harvest (preharvest sprouting). In some years, this can substantially reduce crop value (Gubler et al., 2005). Although it has been extensively studied, there is very little known about the molecular mechanisms that control dormancy onset, maintenance, and release during after-ripening. Understanding these premises is crucial for developing new strategies for solving the problem.

Studies in Arabidopsis (Arabidopsis thaliana), maize (Zea mays), and barley support a principal role for abscisic acid (ABA) as a dormancy promoter. It is well established that mutants with impaired ABA synthesis or perception produce seeds with low dormancy, while mutants or transgenic lines with ABA overproduction or hypersensitivity produce seeds with high dormancy. Both dormant (D) and after-ripened (AR) seeds show an initial decline in ABA content during imbibition, but this reduction is only transient in D seeds, and ABA content increases again after 24 h of imbibition. In agreement with this, exogenous application of ABA retards the germination of AR seeds (for review, see Gubler et al., 2005; Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008).

Because of the importance of ABA in dormancy, the metabolism and signaling of this hormone are likely to be important components in dormancy control. For example, it has been reported recently that blue light, which promotes dormancy in barley, stimulates ABA synthesis (Gubler et al., 2008). Also, it has been reported that changes in ABA catabolism, in particular ABA conversion to phaseic acid by the enzyme ABA 8'-hydroxylase (ABA8' OH; Kushiro et al., 2004; Millar et al., 2006; Okamoto et al., 2006), can modify dormancy. Loss of function of the gene encoding this enzyme increases the ABA content in seeds in both Arabidopsis and barley and results in increased dor-
mancy (Kushiro et al., 2004; Millar et al., 2006; Gubler et al., 2008). Interestingly, the spatial expression pattern of these genes in Arabidopsis seeds and barley grains suggests that ABA breakdown occurs primarily in the endosperm and in the coleorhiza, respectively (Millar et al., 2006; Okamoto et al., 2006).

The endosperm in Arabidopsis is an embryonic tissue that surrounds the embryo and consists of a single cell layer in mature seeds. This tissue appears to be a strong barrier to germination, since removing the testa in D seeds is not sufficient to break dormancy (Bethke et al., 2007). If the seeds are AR, the endosperm weakens following imbibition, allowing the protrusion of the root through the micropylar endosperm. ABA application seems to specifically block endosperm weakening, preventing germination in Arabidopsis and *Lepidium* (Müller et al., 2006). In barley, *HvABA8*′OH-1 is found principally in the coleorhiza during germination of AR grains (Millar et al., 2006). The coleorhiza is a nonvascularized multicellular embryonic tissue that covers the seminal roots of grass seeds and elongates in the early stages of imbibition before root emergence (Bradbury et al., 1956; Gould and Shaw, 1983). The coleorhiza has been thought to have a role in protecting the emerging roots (Sargent and Osborne, 1980), but it has not been associated with seed dormancy.

Although the coleorhiza is involved in germination and successful establishment of all of the major monocot crop seeds, our knowledge of its morphology, anatomy, and function is very sparse. In this work, we analyze the anatomical changes occurring in the coleorhiza during germination and the distribution of ABA in the different parts of the embryo. Also, we use tissue-specific microarray analyses to study coleorhiza function. Global gene expression analyses have been done in Arabidopsis to find genes related to dormancy and after-ripening (Nakabayashi et al., 2005; Cadman et al., 2006; Finch-Savage et al., 2007; for review, see Holdsworth et al., 2008a, 2008b; Barrero et al., 2009). In barley, transcriptomic approaches have been used to study grain development and germination (Sreenivasulu et al., 2008), but they have not been used to study dormancy and after-ripening. We focus on changes in function that occur during after-ripening that could be involved in dormancy release. In addition to previously reported changes in ABA metabolism, we demonstrate that a decrease in ABA sensitivity occurs during after-ripening and that this change happens in the coleorhiza.

Our studies indicate that the ability to germinate may depend on reduction of the ABA level and also of the sensitivity to that hormone in the coleorhiza, which could lead to cell elongation and separation, thus permitting penetration by the seminal roots (germination). We propose a major role for the coleorhiza in controlling cereal dormancy.

RESULTS

Embryo Growth

We imbibed D and AR grains for various times over 30 h and measured the length of the scutellum, the coleorhiza, and the coleoptile (Fig. 1). The scutellum length remained constant during the time course in both D and AR embryos (Fig. 1A), as did its width (data not shown). Elongation of the coleorhiza in AR embryos began 12 h after imbibition, but there was no elongation in D seeds (Fig. 1B). Coleoptile elongation began after 24 h in AR embryos (Fig. 1C), and there was no elongation in D grains. This experiment shows that elongation of the coleorhiza and enclosed seminal roots precedes elongation of the coleoptile and that it occurs soon after the ABA levels have substantially decreased at around 8 h after hydration has commenced, as reported by Millar et al. (2006).

ABA Quantification

In AR grains, the reduction of ABA content of the embryo is associated with expression of *HvABA8*′OH-1.

---

**Figure 1.** Measurements of embryo growth after hydration. D and AR seeds were imbibed for different times. Embryos were isolated every 5 h and photographed. The lengths of the scutellum (A) and the elongation of coleorhiza (B) and coleoptile (C) were measured on the photographs. In C, the length of the coleoptile decreases, because we measured the distance between the tip of the coleorhiza and the margin of the scutellum. As the coleorhiza elongates, that distance is reduced. Root emerged after 30 h. Each point represents the average of three replicates. Error bars represent se.
in the coleorhiza (Millar et al., 2006). To determine how these results relate to the embryo-wide ABA distribution, we determined ABA content in three embryo components after hydration. At 8 and 24 h after commencement of hydration of D and AR grains, we isolated coleorhiza, root, and rest-of-the-embryo (mesocotyl, scutellum, and shoot) components and assayed ABA in each fraction. After 8 h of hydration, there was a significant difference between D and AR coleorhiza but no difference in the other components (Fig. 2A). After 24 h, differences were detected in all fractions (Fig. 2B and C). At this time, ABA levels in all fractions of D embryos were similar (approximately 450 ng g⁻¹), and similarly, all fractions from AR grains contained approximately 225 ng g⁻¹, a 50% decrease in ABA content compared with D embryos.

Coleorhiza Anatomy

We examined structural changes in the coleorhiza during hydration and germination of intact grains. There were no obvious morphological or anatomical differences between D and AR grains before hydration (Fig. 3A; Supplemental Fig. S1, A and B). Longitudinal sections of D and AR dry grains revealed a radial gradient in coleorhiza cell length from short epidermal cells to longer cells closest to the embryo root (Fig. 3E; Supplemental Fig. S1, E and F). Cryo-scanning electron microscopy (cryo-SEM) showed that these elongated, internal cells were much more loosely packed than the outer cells (Fig. 3I). There were large intercellular spaces, and cells appeared partially separated from their neighbors except at their ends. The outer epidermal cell walls appeared wrinkled in cryo-SEM images of dry grains; nevertheless, distinct intercellular spaces could be seen as holes at cell junctions (Fig. 3G).

The coleorhiza in AR hydrated grains commenced elongation after 12 h and ceased just prior to root emergence (Fig. 1). No elongation was seen in D grains, although the coleorhiza cells enlarged radially as they rehydrated (Supplemental Fig. S1, C and G). During this time, the innermost cells of the AR coleorhiza elongated and separated further, often remaining attached to adjacent cells only by their end walls (Fig. 3F and J). Cell separation spread radially outward to cells just under the coleorhiza epidermis (Supplemental Fig. S1, I and J), and the air spaces between epidermal cells also enlarged (Fig. 3H). No cell division was detected in coleorhiza cells.

While elongation was occurring in the coleorhiza flank cells, there was relatively little change in the cells of the coleorhiza tip adjacent to the root cap. These cells were originally (even in dry seeds) isodiametrical, with only small intercellular spaces (Fig. 3C; Supplemental Fig. S1K). They expanded isodiametrically during hydration and did not become separated (Supplemental Fig. S1L). When the primary root breaks through the coleorhiza, it does so through longitudinal cracks, rarely through the coleorhiza tip (Fig. 3D), further evidence that the cells within the coleorhiza tip continue to adhere to each other more tightly than the elongating coleorhiza cells.

Transcriptome Analysis of Coleorhiza and Root

Our observations presented the possibility that the coleorhiza could control dormancy and germination and that it may be strongly affected by after-ripening. In order to investigate this possibility, we did a global transcriptome comparison of coleorhiza and roots, from D and AR barley embryos, using the Affymetrix Barley1 microarray (Close et al., 2004). We were interested in changes in gene expression occurring very early during hydration, before any coleorhiza elongation can be detected, and also in changes that occur later during imbibition, after elongation has begun. Thus, we dissected coleorhiza and root fractions from D and AR embryos that were imbibed for 8 and 18 h (designated A8C, A18C, A8R, A18R, D8C, D18C, D8R, and D18R; see legend for Fig. 4). RNA was obtained from three biological replicates for each of these eight

Figure 2. ABA quantification in barley embryo tissues. D and AR barley grains were imbibed for 8 and 24 h. Embryos were then isolated and the coleorhiza (A), root (B), and rest-of-the-embryo (C) components were dissected. ABA was isolated from the different fractions. Twenty embryos were dissected for each replicate. Three biological replicates were performed. Error bars represent se. FW, Fresh weight.
samples and hybridized to microarray slides for gene expression analysis. After the microarray data quality was assessed and expression was normalized (see "Materials and Methods"; Supplemental Table S1), we performed a principal component analysis in order to assess the consistency between replicates and treat-

Figure 3. Coleorhiza anatomy in AR barley embryos during hydration. A, C, E, G, and I, Sections and images of dry embryos. B, D, F, H, and J, Images of embryos after 24 h of hydration (a 36-h-hydrated seed is shown in D). A, Confocal image of a longitudinal median section of a dry embryo showing the coleorhiza enclosing the largest embryonic root. c, Coleorhiza; ce, coleoptile; e, epiblast; m, mesocotyl; r, root; s, scutellum; sh, embryo shoot. B, Longitudinal section of an embryo following 24 h of hydration, showing elongated coleorhiza and root. The scale is the same for A and B. C, Cryo-SEM of the dry coleorhiza (indicated by the dotted line). D, Cryo-SEM of roots emerging through longitudinal cracks in the coleorhiza of a 36-h-hydrated seed. The scale is the same for C and D. E, Confocal image of coleorhiza and root cells of a hydrated seed showing elongated, separated coleorhiza cells and short meristematic root cells. The scale is the same for E and F. G, Higher magnification cryo-SEM of epidermis from the coleorhiza flank of a dry seed showing small air spaces (arrowheads) between cells. H, Higher magnification cryo-SEM of a 24-h-hydrated seed showing enlarged air spaces (arrowheads) between coleorhiza epidermal cells (similar area to that shown in G). The scale is the same for G and H. I, Cryo-SEM image showing partial separation of the innermost cells of the coleorhiza in a dry seed. J, Cryo-SEM of the innermost coleorhiza cells in a hydrated seed, showing separation of the cells along their side walls, occasionally remaining attached at their end walls (arrowhead). The scale is the same for I and J.
Global gene expression changes. Differentially expressed genes (from Supplemental Tables 2–5) at 8 or 18 h after hydration were grouped according to tissue (A and B). The same genes were also grouped according to the imbibition time (C and D). The number of genes in each group is shown in parentheses.

ABA-Related Changes during After-Ripening

In this study, we have found ABA-related genes that were strongly affected by after-ripening during the early hydration of the embryos, which can explain at the molecular level the changes in ABA content and sensitivity occurring during after-ripening. Such changes are specific to the coleorhiza or at least higher sensitivity occurring during after-ripening. Such changes are specific to the coleorhiza or at least higher sensitivity occurring during after-ripening. Such changes are specific to the coleorhiza or at least higher sensitivity occurring during after-ripening.

Probes for several ABA metabolic genes were present in the barley1 microarray. The first gene in the biosynthetic pathway, a putative barley ABA DEFICIENT (HvABA1; Marin et al., 1996), was up-regulated in AR coleorhiza after 18 h of hydration (Fig. 6B). On the contrary, the last gene in the pathway, a putative ABIDOPSIS ALDEHYDE OXIDASE1 (HvAAO1; Seo et al., 2000), was up-regulated in D coleorhiza at 8 h (Fig. 6C). Other probes with similarity to other AAO family members were not differentially expressed. An important regulatory step in the pathway is catalyzed by the NINE-CIS-EPOXYCAROTENOID DIOXYGENASE (NCED) family of genes (Qin and Zeevaart, 1999). However, we did not find any change in the expression of the two described NCED barley genes (HvNCED1 [Contig4988; Fig. 6D] and HvNCED2 [HT11N18r_s_at; Supplemental Table S1]), and expression was low in all cases.

ABA signaling is also affected by after-ripening in the coleorhiza. A putative ortholog of the Arabidopsis ABI3-INTERACTING PROTEIN2 (HvAIP2; Kurup et al., 2000) is up-regulated in coleorhiza at 8 h and in coleorhiza and root at 18 h (Fig. 6E).

One branch of the protein phosphatase 2C (PP2C) family is well known in Arabidopsis for containing negative regulators of ABA signaling, such as ABA INSENSITIVE1 (ABI1), ABI2, HOMOLOGY TO ABI1 (HAB1), HAB2, ABA-HYPERSENSITIVE GERMINATION1 (AHG1), and AHG3 (Nishimura et al., 2007). In barley, there are several PP2C genes that are related to the Arabidopsis group, and some of them are expressed highly in the coleorhiza and less so in the root.

ABA signaling is also affected by after-ripening in the coleorhiza. A putative ortholog of the Arabidopsis ABI3-INTERACTING PROTEIN2 (HvAIP2; Kurup et al., 2000) is up-regulated in coleorhiza at 8 h and in coleorhiza and root at 18 h (Fig. 6E).

One branch of the protein phosphatase 2C (PP2C) family is well known in Arabidopsis for containing negative regulators of ABA signaling, such as ABA INSENSITIVE1 (ABI1), ABI2, HOMOLOGY TO ABI1 (HAB1), HAB2, ABA-HYPERSENSITIVE GERMINATION1 (AHG1), and AHG3 (Nishimura et al., 2007). In barley, there are several PP2C genes that are related to the Arabidopsis group, and some of them are expressed highly in the coleorhiza and less so in the root.

ABA signaling is also affected by after-ripening in the coleorhiza. A putative ortholog of the Arabidopsis ABI3-INTERACTING PROTEIN2 (HvAIP2; Kurup et al., 2000) is up-regulated in coleorhiza at 8 h and in coleorhiza and root at 18 h (Fig. 6E).

One branch of the protein phosphatase 2C (PP2C) family is well known in Arabidopsis for containing negative regulators of ABA signaling, such as ABA INSENSITIVE1 (ABI1), ABI2, HOMOLOGY TO ABI1 (HAB1), HAB2, ABA-HYPERSENSITIVE GERMINATION1 (AHG1), and AHG3 (Nishimura et al., 2007). In barley, there are several PP2C genes that are related to the Arabidopsis group, and some of them are expressed highly in the coleorhiza and less so in the root.

ABA signaling is also affected by after-ripening in the coleorhiza. A putative ortholog of the Arabidopsis ABI3-INTERACTING PROTEIN2 (HvAIP2; Kurup et al., 2000) is up-regulated in coleorhiza at 8 h and in coleorhiza and root at 18 h (Fig. 6E).

One branch of the protein phosphatase 2C (PP2C) family is well known in Arabidopsis for containing negative regulators of ABA signaling, such as ABA INSENSITIVE1 (ABI1), ABI2, HOMOLOGY TO ABI1 (HAB1), HAB2, ABA-HYPERSENSITIVE GERMINATION1 (AHG1), and AHG3 (Nishimura et al., 2007). In barley, there are several PP2C genes that are related to the Arabidopsis group, and some of them are expressed highly in the coleorhiza and less so in the root.

ABA signaling is also affected by after-ripening in the coleorhiza. A putative ortholog of the Arabidopsis ABI3-INTERACTING PROTEIN2 (HvAIP2; Kurup et al., 2000) is up-regulated in coleorhiza at 8 h and in coleorhiza and root at 18 h (Fig. 6E).
especially in D grains (e.g. Contig11720_at; Fig. 6F). The expression of these genes has been found to increase in response to ABA (Yoshida et al., 2006). Other ABA-induced genes were down-regulated in AR coleorhiza and root, including HVA22 (Guo and Ho, 2008) and genes encoding late embryogenesis-abundant proteins (Fig. 6, I and J).

Our studies yielded additional evidence for changes in ABA sensitivity during after-ripening. Two members of the LIPID PHOSPHATE PHOSPHATASE (LPP) family (Katagiri et al., 2005) were clearly induced by after-ripening in the coleorhiza (Fig. 6, G and H). In Arabidopsis, LPP2 was found to be up-regulated by after-ripening (Carrera et al., 2008), so we decided to focus on this family and study the relation of these genes to dormancy level and its role in the after-ripening process in barley.

In the Barley1 microarray, we have identified three putative HvLPP genes (HvLPP1–HvLPP3), two of which have been described previously (Racagni et al., 2008). Using real-time PCR, we have validated the expression of these genes at 8 h (Fig. 7A), demonstrating that HvLPP1 and HvLPP2 were up-regulated in AR coleorhiza and that HvLPP3 was not. Also, we have measured the expression of the HvLPP genes in dry seeds and during hydration using whole embryos (Fig. 7, B–D). This experiment shows that the up-regulation of HvLPP1 and HvLPP2 by after-ripening was already detectable in dry seeds. HvLPP3 expression was low and was not regulated by after-ripening.

**Sensitivity to ABA**

Looking for direct evidence of a change in sensitivity to ABA in this tissue, we scored the elongation of the coleorhiza of D and AR seeds in the presence of ABA. This experiment was performed in the dark in order to alleviate the dormancy of the seeds and promote the germination of D grains (Supplemental Fig. S3). Figure 8 shows a germination time course of D and AR grains on water and on ABA. After 5 d of hydration on water, all AR grains and 60% of D grains had elongated coleorhiza. In the presence of ABA, more than 90% of AR grains showed elongated coleorhiza after 5 d. In D grains on ABA, less than 5% were elongated. These results indicated that in AR grains,
inhibition caused by ABA is about 1%, while in D grains, the inhibition is about 80%.

Gibberellin Metabolism

While ABA acts to promote dormancy, gibberellin commonly promotes germination. We looked for gibberellin-related genes in our data. Although we detected expression of genes related to gibberellin metabolism and signaling, their expression did not correlate with germination. BarleyKAURENOIC ACID OXIDASE1(HvKAO1; Supplemental Fig. S2A) is involved in gibberellin synthesis and was up-regulated only in D coleorhiza after 18 h. On the other hand, the expression of GIBBERELLIN 2-OXIDASE1(HvGA2ox1; Supplemental Fig. S2B), which is involved in gibberellin inactivation, was up-regulated in AR coleorhiza after 18 h. In relation to signaling, we found that the putative barley GA-INSENSITIVE DWARF1 gibberellin receptor(HvGIDI; Supplemental Fig. S2C) was slightly induced in D coleorhiza. The changes that we have found in gibberellin-related genes appeared only after 18 h of imbibition, while the ABA-related changes were detected much earlier. These results provide no support for a role for gibberellins in dormancy release in coleorhiza, supporting previous studies in which a similar conclusion was reached (Jacobsen et al., 2002).

Light-Related Changes during After-Ripening

Blue light has a major effect in promoting dormancy in barley (Gubler et al., 2008). In this study, freshly harvested barley grains displayed a deep dormancy when hydrated in blue or white light, while dormancy was partially alleviated if the hydration was done in the dark (Supplemental Fig. S3). In AR grains, the effect of light on dormancy disappeared and all grains germinated equally well in the dark and in the light. Several light-related genes were strongly affected by after-ripening, especially in the coleorhiza. Because of this, we investigated the relation between after-ripening and light perception in order to understand if the ability to perceive blue light could be involved in dormancy release. Figure 9 shows the light-related genes that were up- or down-regulated by after-ripening in the coleorhiza or root. Genes encoding Early Light-Inducible Proteins (ELIPs; Grimm and Kloppstech, 1987) were strongly suppressed in the coleorhiza by after-ripening (Fig. 9, A and B). ELIPs are induced in barley after exposure to high light, and in our germination conditions (under blue light), they were highly expressed in D but not in AR coleorhiza.

The barley ELONGATED HYOCOTYLM (HvHY5; Contig15369_at), a basic Leu zipper transcription factor acting in the light signaling pathway (Oyama et al., 1997), was strongly up-regulated in D coleorhiza at 8 and 18 h after imbibition (Fig. 9C). Also, the putative barley Really Interesting New Gene (RING) COP1-INTERACTING PROTEIN8(HvCIP8; Torii et al., 1999; Contig18584_at) was up-regulated in AR coleorhiza (Fig. 9D).

Another gene involved with light sensitivity that was repressed in AR coleorhiza and root was PHOTO-TROPIN1 (PHOT1; Contig11167_at; Fig. 9E), a putative homolog of PHOT1 in Arabidopsis. Phototropins are
blue light receptors in plants (Briggs and Huala, 1999). Also, Contig8698_s_at (PHOT2; Supplemental Table S1) was down-regulated in AR coleorhiza. Probes for the cryptochromes (CRY), another class of blue light receptors, did not show a significant difference between D and AR samples (CRY1; Contig5800_at; Supplemental Table S1) or were up-regulated in AR coleorhiza (CRY2; Contig14562_at; Supplemental Table S1).

Other Significant Changes during After-Ripening

We also identified genes that are involved in other processes that could be important in dormancy release and germination. Some of them have previously been associated with dormancy and/or germination. Several jasmonate-related genes were induced in coleorhiza. Different probes identified as JASMONATE 12-OXOPHYTODIENOIC ACID REDUCTASE (HvOPR; e.g. Contig6194_s_at), which is involved in jasmonate synthesis, were up-regulated in AR coleorhiza (Fig. 10A). The ALLENE OXIDE SYNTHASE (HvAOS; Fig. 10B) is also involved in jasmonate synthesis and was also up-regulated in AR coleorhiza. The putative barley CORONATINE INSENSITIVE1 (HeCOI1; Xie et al., 1998; Fig. 10D) is a positive regulator of jasmonate signaling, and it was up-regulated at 8 h in AR coleorhiza. On the contrary, the first gene in the biosynthetic pathway, LIP0XYGENASE (HvLOX; Fig. 10C), was up-regulated in D coleorhiza.

Genes related to cell wall modification were strongly affected by after-ripening. For example, several glucan endo-1,3-β-glucosidases (e.g. Contig6967_at), which are involved in cell wall degradation during endosperm and testa rupture and are inhibited by ABA (for review, see Leubner-Metzger, 2003, 2005), were highly expressed in AR coleorhiza (Fig. 10E). Other genes encoding proteins that could be related to cell wall formation, like several xyloglucan endotransglycosylases (XETs; e.g. Contig2670_x_at; Bourquin et al., 2002; Fig. 10F), were up-regulated in D coleorhiza. Genes related to cell elongation, like expansins, were strongly expressed in AR coleorhiza after 18 h of hydration (e.g. Contig2878_at; Fig. 10G).

Genes related to the metabolism of oxides of nitrogen. We found that a nitrate reductase (Fig. 10H) and a nitrite reductase (Fig. 10I) were highly expressed in AR coleorhiza at 8 h.

mRNA stability has been related to dormancy (Holdsworth et al., 2008b). We found two genes involved in mRNA stability that were after-ripening regulated. A polyadenylation factor subunit-like gene (Fig. 10J) was strongly induced at 18 h in D coleorhiza. An mRNA cap methyltransferase-like gene (Fig. 10K) was highly expressed in D tissues and down-regulated in AR tissues. Its expression was stronger in roots.

DISCUSSION

Role of the Coleorhiza

It is significant that in seeds of distantly related species, ABA catabolism occurs in tissues that surround the root in the seed, coleorhiza in barley and endosperm in Arabidopsis (Millar et al., 2006; Okamoto et al., 2006), in spite of their totally different developmental origins. Our results indicate that the amount of ABA in the coleorhiza is a key factor in controlling dormancy and germination. We suggest that the coleorhiza in barley and other grasses and the aleurone layer in Arabidopsis and Lepidium are functionally related tissues, acting as a barrier to germination. This role has been previously proposed for the Brassicaceae endosperm (aleurone) layer (for review, see Finch-Savage and Leubner-Metzger, 2006; Müller et al., 2006; Bethke et al., 2007), which is able to block germination in D seeds. In AR seeds, it becomes weak, allowing the protrusion of the root. The coleorhiza in the barley embryo may have the same function as the endosperm of dicotyledonous seeds, permitting germination when its cells elongate and separate.
Earlier studies have proposed that the coleorhiza protects the seminal roots during development and germination, ensuring that the root tip and meristem are protected until they become self-supported (Sargent and Osborne, 1980). The coleorhiza has also been thought to act in conjunction with its hairs in water and nutrient uptake, as a water reserve during dehydration, as a food store (starch and lipid) to support embryo growth before endosperm reserves are mobilized, and in seed anchoring (Nishimura, 1922; Howarth, 1927; Walne et al., 1975; Debaene-Gill et al., 1994). We propose here a new role for the coleorhiza, in which it acts as the key tissue preventing root emergence (germination) in D seeds. However, we acknowledge that root emergence may not depend only on weakening the coleorhiza and that the expansive force of the root may be important to assist in penetration of the coleorhiza.

Transcriptome Analysis

Our results show that a large number of genes were differentially expressed between D and AR tissues. The Barley1 Affymetrix chip (Close et al., 2004) contains probes for 22,795 putative genes, and more than 15% of them are significantly differentially expressed (more than 2-fold) between D and AR embryos after just 8 h of imbibition. This indicates that the biology that occurs during the imbibition of seeds is very complex. Another observation is that 23% of the genes are differentially regulated in the coleorhiza, while only 16% are differentially regulated in the root, indicating a more active role for the coleorhiza, at least during the early hours of hydration. This may reflect a repressive role for the coleorhiza, inhibiting the growth of the root. At 18 h of hydration, the number of genes differentially regulated increased in root but not in coleorhiza. This may reflect a more active role of the root during late imbibition, once elongation has started.

ABA Sensitivity in the Coleorhiza Changes during After-Ripening

Analysis of the transcriptome in coleorhiza and root from D and AR embryos clearly indicates changes in the expression of many genes related to ABA metab-
olism and sensitivity. These changes happen principally in the coleorhiza during the first 8 h of hydration. We have found that biosynthetic genes (HvABA1 and HvAAO1; Fig. 6, B and C) and the catabolic HvABA-8′OH-1 gene (Fig. 6A) are affected by after-ripening. While the biosynthetic changes are difficult to correlate with the behavior of D and AR seeds (e.g. HvABA1 is up-regulated in AR coleorhiza), the catabolic changes can explain the reduction of ABA content occurring during germination. This supports our previous work indicating a role for HvABA8′OH-1 in controlling dormancy in the coleorhiza (Millar et al., 2006). Biosynthetic genes have been shown to be induced in many species by environmental factors like light, cold, and salt stress (Marin et al., 1996; Xiong et al., 2002; Barrero et al., 2006; Seo et al., 2006). In barley, HvNCED1 is regulated by blue light during imbibition and is not affected by after-ripening (Gubler et al., 2008). This is in agreement with our microarray results. Also, HvNCED1 is induced by incubating barley grains at 30°C, a condition that stops the germination of D grains promoting secondary dormancy (Leymarie et al., 2008). These results indicate that HvNCED1 is involved in preventing germination in nonoptimal conditions (i.e. in the light or in high temperatures), but in fully AR grains, changes affecting ABA catabolism and sensitivity overcome the effect on HvNCED1.

Our results also indicate that ABA signaling in the coleorhiza is affected by after-ripening. A decrease in ABA sensitivity occurring during after-ripening has been reported previously (Walker-Simmons, 1987; Wang et al., 1995; Corbineau et al., 2000; Benech-Arnold et al., 2006; Gubler et al., 2008). In Arabidopsis, ABI3 is one of the transcription factors in the ABA signaling cascade, and its mutation produces ABA-insensitive seeds with very low dormancy. Its ortholog in monocots, VIVIPAROLISI (VP1; McCarty et al., 1991), generates the same phenotype when mutated, producing seeds prone to preharvest sprouting (McCarty et al., 1989). The ABI3 gene is regulated at the protein level and is ubiquitin targeted for degradation during imbibition by a RING finger E3 enzyme called AIP2 (Zhang et al., 2005). The mutation of AIP2 mimics the effect of ABI3 overexpression, and its overexpression mimics the abi3 mutants. Alternative splicing of VP1 has been described in wheat as a regulatory system (McKibbin et al., 2002), but no information is available about regulation at the protein level. Our microarray experiment does not show any significant change in the expression of the probes with similarity to VP1 (data not shown). Very interestingly, the putative AIP2 ortholog in barley (Contig8948_at) is induced in AR tissues, first only in coleorhiza but also in root at 18 h, suggesting that its action is initiated in the coleorhiza and then spreads to the root. The expression pattern of this gene correlates with the decrease in ABA sensitivity found in AR grains and suggests a posttranslational regulation of VP1 during after-ripening in barley.

Phospholipids have been shown to be important in ABA signaling. In particular, phosphatidic acid is a lipid signaling molecule that triggers early signal transduction events, leading to responses to ABA during seed germination in barley and in Arabidopsis. In Arabidopsis, phosphatidic acid acts upstream of ABI4, another transcription factor involved in ABA signaling during germination (Katagiri et al., 2005). Phosphatidic acid is synthesized from diacylglycerol pyrophosphate (which is a lipid messenger as well) by the enzyme LPP. The role of this enzyme may be very important, since it targets two molecules that are ABA signaling components. In Arabidopsis, there are four LPP genes, and three of them are expressed during germination. Only LPP2 has been functionally analyzed, and its mutation produces ABA hypersensitivity during germination (Katagiri et al., 2005). Interestingly, this gene is induced by after-ripening and its expression is very low in D imbibed seeds (Carrera et al., 2008). In barley, two LPP genes, HvLPP1 and HvLPP2, have been described (Racagni et al., 2008). In our microarray data, we found three probes annotated as LPP genes, and two of them, Contig11040_at (HvLPP1) and Contig8594_at (HvLPP2), were induced in coleorhiza by after-ripening. The third one, Contig17837_at (designated HvLPP3), was only expressed at low levels and was not regulated by AR. After studying the expression of these genes in a time course (comparing their expression between D and AR whole embryos), we discovered that HvLPP1 and HvLPP2 were induced in dry AR embryos. These results highlight the importance of LPP genes in dormancy release and point to a mechanism for reducing ABA sensitivity before hydration. Also, the higher expression of some PP2C and other ABI3-induced genes in D coleorhiza could be related to the higher ABA sensitivity of D embryos. Maybe the change in ABA sensitivity in AR grains will lead to a reduction of the expression of ABA-induced genes during hydration. For example, the expression of HVA22 has been related to stopping programmed cell death in aleurone cells (Guo and Ho, 2008), and its down-regulation in AR coleorhiza could be related to the degradation of this tissue after 18 h of hydration.

We have previously reported that barley transgenic lines containing more ABA (and showing increased initial dormancy) needed the same after-ripening period as the wild type for dormancy to decay, even though their ABA content was still very high (Gubler et al., 2008). This indicates that the decrease in ABA sensitivity must be a crucial part of the after-ripening mechanism. Our results provide evidence of the molecular changes that can explain this phenomenon.

Other Processes Affected by After-Ripening

We have identified other after-ripening-induced changes that, unlike those discussed above in relation to HvLPP ABA sensitivity, are only evident after hydration but not in dry embryos (e.g. HvHY5, HV90,
and HV58 genes; data not shown). Such changes could be downstream of the initial reduction in ABA sensitivity found in dry seeds, but we cannot rule out the possibility of ABA-independent events. Furthermore, this second group of genes could be involved in germination rather than dormancy.

The gene expression changes related to jasmonate that we have detected implicate this hormone in promoting germination or dormancy decay in coleorhiza. Three genes (two biosynthetic genes and one signaling gene) were induced in AR tissues. The expression of LOX apparently contradicts this idea, but its expression could be related to a feedback mechanism (Wasternack and Hause, 2002). The role of jasmonate in germination has been described previously (Carrera et al., 2007; Footitt et al., 2007), but we may consider germination and dormancy release as separate events. The comatose mutant in Arabidopsis is not able to germinate, because it is affected in one step necessary for jasmonate biosynthesis and is jasmonate deficient (Theodoulou et al., 2005). It has also been described that jasmonate stimulated the germination of dormant seeds in Acer tataricum, Douglas fir (Pseudotsuga menziesii), and apple (Malus domestica; Berestetzy et al., 1991; Jarvis et al., 1997; Yildiz et al., 2007). The mechanism linking jasmonate and germination is unknown. Interestingly, the exogenous application of jasmonate induces the production of nitric oxide in Taxus cell cultures (Wang and Wu, 2005). There are reports of nitrate, nitrite, and especially nitric oxide breaking dormancy of seeds (Bethke et al., 2005, 2006). We found that two genes related to nitrate reduction (encoding nitrate and nitrite reductase) were up-regulated in AR coleorhiza after 8 h of hydration but not after 18 h. Thus, jasmonate may cause dormancy breakage through nitric oxide.

Cell wall synthesis, degradation, and modification are processes that are associated with germination (for review, see Kucera et al., 2005). We have found a number of genes related to cell wall degradation (e.g. several glucan endo-1,3-β-glucosidases) that are strongly expressed in AR coleorhiza hydrated for both 8 and 18 h. This indicates that coleorhiza cell walls are undergoing modification prior to and during cell elongation. We also found expansins to be induced in coleorhiza at 18 h after imbibition, when elongation had already begun. On the other hand, we found genes encoding enzymes involved in cell wall construction and elongation, like XETs, to be very highly expressed in D coleorhiza, suggesting that in these cells the cell wall is still undergoing construction or turnover. We are currently analyzing several of these cell wall-related genes and their roles in dormancy and germination.

Posttranscriptional RNA processing, maturation, and metabolism appear to be related to dormancy, because many mutants affected in these RNA processes, like ABA hypersensitive1 (Hugouvieux et al. 2001) and hyponastic leaves1 (Lu and Fedoroff, 2000), have altered dormancy. Also AHG2, which encodes a poly(A)-specific ribonuclease, produces ABA hypersensitivity when mutated, suggesting that mRNA stability is important for proper ABA responses (Hirayama and Shinozaki, 2007). Supporting this, we have found two genes, one encoding a polyadenylation factor subunit and the other encoding an mRNA cap methyltransferase-like protein, that are induced in D tissues. These results indicate that the stability of mRNA stored in seeds may be important in dormancy.

Light Sensitivity and After-Ripening

After-ripening-regulated genes associated with light were also identified in this study. We have previously demonstrated that light promotes dormancy in barley by maintaining high levels of ABA during hydration and that this is affected by blue light, while red and far-red light have no effect (Gubler et al., 2008). Blue light induces the expression of the ABA biosynthetic gene HvNCED1 but does not affect HvABA8′OH-1. This explains why the ABA content is high in D seeds under blue light. After-ripening increases the expression of HvABA-8′OH-1 and reduces ABA sensitivity, but it has no effect on the expression of HvNCED1. Our transcriptome analysis shows that several light-related genes are regulated by after-ripening, and these changes can explain the reduction of light sensitivity in AR embryos. A putative phototropin blue light receptor (PHOT1) is up-regulated in D tissues. Not only this receptor but also HvHY5, which is a major transcription factor in light signaling (Oyama et al., 1997), is also up-regulated in D coleorhiza, and HvCIP8, which in Arabidopsis is involved in HY5 degradation (Karniol and Chamovitz, 2000), is induced in AR tissues. In Arabidopsis, this signaling pathway regulates the expression of genes encoding ELIPs (Harari-Steinberg et al., 2001), quickly induced by light in many plants. In barley, two ELIPs have been characterized, Hv58 and Hv90 (Grimm and Kloppstech, 1987), and both of them are strongly expressed in D coleorhiza but not in roots and AR coleorhiza. These proteins have been associated with protection and modification of the photosynthetic apparatus in green tissues and with the development of photosynthetic units in the first hours of the greening process. They are also produced in response to desiccation in fern spores (Raghaban and Kamalay, 1993) and in resurrection plants like Craterostigma plantagineum and Tortula ruralis (Bartels et al., 1992; Zeng et al., 2002). Interestingly, in these species, ELIP expression is induced by ABA but only in the presence of light. Blue light is also more effective at inducing ELIPs in pea (Pisum sativum) plants and Arabidopsis (Adamska et al., 1992; Harari-Steinberg et al., 2001). Another link between ELIP and ABA is found in the alga Dunaliella bardawil, where there is an ELIP-like protein involved in carotene biogenesis (Lers et al., 1991), the first precursor for ABA biosynthesis. However, no relation between ELIPs and dormancy or germination has been reported. ELIPs have also been
shown to be induced by heat shock in Arabidopsis (Harari-Steinberg et al., 2001) and to be repressed by jasmonates in barley in a light-independent manner (Wierstra and Kloppstech, 2000). The light-regulated pathway that controls the expression of ELIPs must be different from the pathway that regulates \( \text{HoNCED1} \), since \( \text{HoNCED1} \) is not regulated by after-ripening. Because of strong light regulation of ELIPs and also because of their relation to ABA, high temperatures, and jasmonate, these proteins may play a role in dormancy and germination, but further experiments are needed in order to determine if they have roles in the coleorhiza.

### Coleorhiza Anatomy and Behavior during Germination

As in other cereals and grasses, the barley coleorhiza is a nonvascularized tissue, consisting primarily of parenchymatous cells with the seminal roots embedded in these cells (detailed in Brown and Morris, 1890; Merry, 1941). It merges with the scutellum on one side of the embryo and with the epiblast on the other side and is continuous with the mesocotyl. The gross morphology of the coleorhiza in quiescent, and germinating seeds have been described previously for a number of species, including barley (Davidson, 1979), wheat (Avery, 1930; Bradbury et al., 1956; Foard and Haber, 1962; Symons et al., 1984), Setaria lutescens (Rost, 1975), perennial ryegrass (Lolium species; Debaene-Gill et al., 1994), Festuca species (Howarth, 1927), and many other grasses (Brown and Morris, 1890; Yung, 1938; Rost and Lersten, 1973). In AR barley seeds, with reduced ABA levels, the coleorhiza and enclosed roots are able to commence elongation within 12 h of imbibition, and these tissues continue to elongate in parallel for a further 12 to 18 h. When coleorhiza growth ceases, the primary root breaks through it and then commences very rapid elongation. At the same time, the coleorhiza epidermis produces epidermal hairs, seen also in wheat and other species (Noda and Hayashi, 1960; Foard and Haber, 1962; Oziasakins and Vasili, 1983), in a time course similar to that of root hair development, which also commences as the root epidermis ceases elongation. The coleorhiza is thought to protect the embryo root during desiccation and dispersal, it is the first organ of the embryo to come into contact with water as the seed hydrates (Price and Ey, 1970), and it is likely the be the first in perceiving other environmental factors like light.

There are few reports describing the cellular and subcellular details of the coleorhiza or changes initiated during imbibition and germination, with the exception of germination in rye (Secale cereale; Hallam et al., 1972; Sargent and Osborne, 1980). Several features of the coleorhiza are particularly relevant to dormancy and germination. The first of these is that in order for the roots, particularly the first seminal root, to emerge, coleorhiza cells need to separate. This process appears to start during seed maturation, before the seeds become completely desiccated, as the innermost cells in dry seeds are already partially separated (Fig. 3I). Indeed, enlargement of the primary seminal root is also interrupted by desiccation, since root cells occasionally show incomplete cell walls, as if arrested during cytokinesis. Coleorhiza cells closest to the root are most detached from their neighbors, and they are also somewhat more elongated compared with cells farther from the root (Fig. 3E). This suggests that there may be a gradient in a root-derived signal that promotes cell separation and elongation in the coleorhiza but not in the roots themselves. Cell separation, producing intercellular spaces, extends out to the coleorhiza epidermis, with small air spaces seen at intersections of neighboring epidermal cells even in the dry seed. This may aid the rapid entry of gases and water to facilitate embryo hydration and germination. By contrast, the epidermis of the embryonic or mature root is always tightly sealed.

Upon imbibition, these cell elongation and separation processes continue throughout the coleorhiza of AR seeds. As seen in other cereals, including barley (Sargent and Osborne, 1980), there is no cell division that, together with the pronounced vacuolation of these cells, indicates that this is a terminally differentiated tissue with no potential for further meristematic activity, although the epidermal cells are capable of producing hairs. As well as elongating to allow growth of the enclosed primary seminal root, the coleorhiza cells must separate further to allow enlargement of the...
secondary seminal roots, which are initially much smaller in the mature, desiccated embryo.

A Model for Dormancy/Germination in Coleorhiza

The results of this study lead us to believe that the coleorhiza plays a major role in regulating dormancy and germination in barley. In this apparently simple grass-specific tissue, we have detected gene expression changes in response to after-ripening that, we believe, help us to understand how dormancy is regulated. The model in Figure 11 depicts mechanisms involving ABA synthesis, catabolism, and signaling that are present in the coleorhiza and that can explain the transition from dormancy to germination during after-ripening. The model shows that ABA synthesis can be affected by environmental conditions (blue light and high temperature) but not by after-ripening. On the other hand, ABA catabolism and signaling are strongly affected by after-ripening (catabolism is induced and signaling is reduced), while light and temperature have no effect. This model explains how different scenarios could affect ABA levels and sensitivity, which ultimately determine whether germination will occur or not.

We have also described other possible mechanisms that are affected by after-ripening in coleorhiza. These include reduction in light sensitivity, nitrate and nitrite reduction, mRNA stability, cell wall modification, and jasmonate action. More work is needed in order to understand the roles of these other mechanisms and whether their action is ABA dependent or independent.

We propose that the coleorhiza in grasses is functionally related to the mature endosperm of dicots, acting as a barrier to root elongation and thus germination. The facts that the coleorhiza is a seed-specific tissue and that it is dispensable after germination make it a perfect target for developing strategies to modify dormancy and germination in cereals without affecting other phases of plant growth and development and without introducing pleiotropic effects.

MATERIALS AND METHODS

Plant Material and Germination Assays

Barley (Hordeum vulgare ‘Betzes’) plants were grown in naturally lit phytotron glasshouses with air temperature set at 17°C/9°C for the day/night cycle as described previously (Jacobsen et al., 2002; Millar et al., 2006). Seeds were harvested as described by Cubler et al. (2008). Half of the grains was stored at −20°C to preserve dormancy, and the other half was incubated at 37°C for 4 months to after-ripen.

For imbition experiments, replicate sets of 20 D and AR grains were placed on 9-cm plastic petri dishes containing two 9-cm Whatman number 1 filter papers and 5 mL of distilled water. The plates were sealed with Parafilm and incubated at 20°C under continuous blue light at 30 μmol m⁻² s⁻¹ as described by Cubler et al. (2008) or wrapped in two layers of aluminum foil for darkness. We had previously shown that blue light is a strong promoter of dormancy in barley. Grains were scored as germinated when the coleorhiza had emerged beyond the husk. For ABA sensitivity assay, triplicate sets of 20 grains were placed, in the same conditions as above, on water or in 10⁻⁴ M ABA and incubated in the dark.

Embryo Growth Measurements

To measure coleorhiza and coleoptile elongation, 10 embryos were isolated from grains hydrated for various lengths of time and fixed in 4% formaldehyde. The embryos were photographed, and various embryo dimensions were measured on the photographs. Coleorhiza elongation was monitored by measuring the distance the coleorhiza tip had grown past the scutellum edge at the coleorhizal end of the grain. Coleoptile growth was monitored by measuring the distance between the scutellum edge (at the scutellum end) and the tip of the coleoptile.

Embryo Anatomy

D or AR grains were hydrated under blue light on moist filter paper in sealed petri dishes as described above. For confocal microscopy of cell walls, barley grains were fixed, sectioned on a vibratome, stained with 0.05% aqueous calcofluor white for 5 to 10 min, and observed using 405-nm excitation. Images are composites of several optical sections. For light microscopy, dry or hydrated grains were fixed in 3% glutaraldehyde in 50 mM PIPES buffer, pH 6.8, for 2 h at room temperature with vacuum infiltration and then left overnight at 4°C. After buffer rinses, they were dehydrated in an ethanol series and then infiltrated with LR White resin (medium grade) over several days before embedding. Sections 1 to 2 μm thick were stained with calcofluor white as above. For cryo-SEM, grains (either whole or dissected to reveal coleorhiza cells) were frozen in liquid nitrogen, coated with 20-nm gold with an Oxford CT1500 cryo-transfer unit, and observed on a cold stage attached to a JEOL 6400 SEM apparatus. Images were processed (sharpened and brightness and contrast adjusted) and assembled using Adobe Photoshop CS2.

ABA Quantification

The ABA contents of dissected coleorhiza, roots, and rest-of-the-embryo fractions were measured using a Phytodetek Competitive ELISA kit (Agdia). Twenty-five embryos were dissected using the microscope after 8 or 18 h of hydration under the blue light conditions described by Cubler et al. (2008), and the samples were frozen on dry ice. ABA extractions were performed in 80% methanol as described previously (Cubler et al., 2008). ABA content was measured in the competitive ELISA following the manufacturer’s protocol. Three biological replicates were carried out.

RNA Extraction and Microarray Preparation

Samples used in the microarray experiment (coleorhiza and roots) were dissected from D and AR grains hydrated for 8 and 18 h under blue light as described above. The dissected tissues were frozen in liquid nitrogen, and RNA was prepared from isolated coleorhiza and roots using the hexadechltrimethylammonium procedure described by Chang et al. (1993). After isolation, the samples were treated with DNase, and RNA quality was assessed on an Agilent Bioanalyzer 2100 (Agilent Technologies). Probe synthesis, labeling, and hybridization to the barley1 gene chip (Close et al., 2004) were carried out at the Australian Genome Research Facilities following the manufacturer’s recommendations (Affymetrix). Microarray analyses were performed on three biological replicates of RNA.

Microarray Analysis

Data from the Australian Genome Research Facilities were loaded into R version 2.7.0 and analyzed using packages from Bioconductor (Gentleman et al., 2004; http://www.bioconductor.org/) using default settings. Microarray hybridization quality was assessed using the AffyPLM package (Methods for Fitting Probe-Level Models), and RNA degradation was assessed using the Affy package (Methods for Affymetrix Oligonucleotide Arrays). Principal component analysis based on conditions was performed using the Smida package (Wit and McClure, 2004). Normalization and identification of differentially expressed genes was conducted using the Limma package (Linear Models for Microarray Data; Smyth, 2005) following
Quantitative Real-Time PCR

RNA from whole embryos was isolated using the same protocol as described in the microarray section. The RNA was treated with Dnase on mini RNasey columns (Qiagen), and its quality was assessed on a NanoDrop 2000 Spectrophotometer (Thermo Scientific). A total of 2 μg of total RNA was then used to synthesize cDNA using SuperScript III (Invitrogen Life Sciences) following the supplier’s recommendations in 20-μL reactions. RNA extractions were performed on three biological replicates of 15 embryos isolated following the supplier’s recommendations in 20-μL PCR samples with Platinum Taq (Invitrogen Life Sciences) and SYBR Green (Invitrogen). Specific primers were designed for HvLPP1 (5'-CACCAT-GAGAGACCACCACCTT-3' and 5'-GATGAGAGATGCAATGAGGAT-3'), HvLPP2 (5'-ATATCTTCGCTCTCCCTG-5' and 5'-ATGGGCCTAAC-CTTTACGCTC-3'), and HvLPP3 (5'-GGGATTTCTAGAATAGACAA-3'). Reactions were run on a Rotorgene 3000A real-time PCR machine (Corbett Research), and data were analyzed with Rotor-gene software using the comparative quantitation method described in the microarray section. The RNA was treated with DNase on mini RNeasy columns (Qiagen), and its quality was assessed on a NanoDrop 2000 Spectrophotometer (Thermo Scientific). A total of 2 μg of total RNA was then used to synthesize cDNA using SuperScript III (Invitrogen Life Sciences) following the supplier’s recommendations in 20-μL reactions. RNA extractions were performed on three biological replicates of 15 embryos isolated from hydrated or dry grains. cDNA was diluted 50-fold, and 10 μL was used in 20-μL PCR samples with Platinum Taq (Invitrogen Life Sciences) and SYBR Green (Invitrogen). Specific primers were designed for HvLPP1 (5'-CACCAT-GAGAGACCACCACCTT-3' and 5'-GATGAGAGATGCAATGAGGAT-3'), HvLPP2 (5'-ATATCTTCGCTCTCCCTG-5' and 5'-ATGGGCCTAAC-CTTTACGCTC-3'), and HvLPP3 (5'-GGGATTTCTAGAATAGACAA-3'). Reactions were run on a Rotorgene 3000A real-time PCR machine (Corbett Research), and data were analyzed with Rotor-gene software using the comparative quantitation method. The expression of HvActin (At1G14545; 5'-GCCGCTGTTCTTCCCTG-3' and 5'-GCTCTCCTGATGCTGCTCTA-3'); Trevaskis et al., 2006) was used as an internal control to normalize gene expression. Three biological replicates were performed for each experiment. For validations, real-time PCR was performed using as a template cDNA synthesized from the same RNA used in the microarray experiment.

Supplemental Data

The following materials are available in the online version of this article.
Supplemental Figure S1. Details of coleorhiza anatomy.
Supplemental Figure S2. Gibberellin-related genes.
Supplemental Figure S3. Effects of blue and white light and dark on germination of D and AR barley grains during imbibition.
Supplemental Table S1. Normalized expression values of all genes in all conditions.
Supplemental Table S2. Differentially expressed genes between D and AR coleorhiza after 8 h of hydration.
Supplemental Table S3. Differentially expressed genes between D and AR coleorhiza after 18 h of hydration.
Supplemental Table S4. Differentially expressed genes between D and AR root after 8 h of hydration.
Supplemental Table S5. Differentially expressed genes between D and AR root after 18 h of hydration.

ACKNOWLEDGMENTS

We thank Ingrid Venables and Trijntje Hughes for their technical assistance and Drs. Yves Al-Ghazi and Sally Walford for their help with microarray analysis.
Received March 3, 2009; accepted April 17, 2009; published April 22, 2009.

LITERATURE CITED

Avery GS (1930) Comparative anatomy and morphology of embryos and seedlings of maize, oats and wheat. Bot Gaz 89: 1–39
Craterose late in phase II of germination. Plant Physiol 214–224
Plant Physiol. Vol. 150, 2009

The Coleorhiza Regulates Dormancy in Barley.
Barrero et al.


Leubner-Metzger G (2005) Beta-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. Plant J 41: 133–145


Nicotiana plumbaginifolia. JBiol Chem 231: 10203–10208

Nishimura M (1922) Comparative morphology and development of Poa pratensis. Curr Opin Plant Biol 41: 523–532


Triticum aestivum (wheat, Gramineae) embryos. Genes Dev 11: 55–65

Parthenium hysterophorus. Jpn J Bot 1: 55–65

Pratensis (Poa pratensis) and Aegilops lathyris. Jpn J Bot 50: 935–949


sequences during development and germination of spores of the sensitive fern, *Onclea sensibilis* L. Planta 189: 1–9


Yung CT (1938) Developmental anatomy of the seedling of the rice plant. Bot Gaz 99: 786–802


The Coleorhiza Regulates Dormancy in Barley