

Global Patterns of Gene Expression in the Aleurone of Wild-Type and *dwarf1* Mutant Rice^{1[W]}

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The cereal aleurone layer is a model system for studying the regulation of transcription by gibberellin (GA) and abscisic acid (ABA). GA stimulates and ABA prevents the transcription of genes for α -amylases and other secreted hydrolytic enzymes, but how GA and ABA affect the transcription of other genes is largely unknown. We characterized gene expression in rice (*Oryza sativa*) aleurone using a half-genome rice microarray. Of the 23,000 probe sets on the chip, approximately 11,000 hybridized with RNA from rice aleurone treated with ABA, GA, or no hormone. As expected, GA regulated the expression of many genes, and 3 times as many genes were up-regulated by GA at 8 h than were down-regulated. Changes in gene expression resulting from ABA treatment were not consistent with the hypothesis that the role of ABA in this tissue is primarily to repress gene expression, and 10 times more genes were up-regulated by ABA at 8 h than were down-regulated by ABA. We also measured transcript abundance in aleurone of *dwarf1* (*d1*) mutant rice. The *d1* protein is the sole α -subunit of heterotrimeric G-proteins in rice. Genes up-regulated by GA or ABA had higher expression in wild type than in *d1* aleurone, and genes down-regulated by GA had lower expression in wild type relative to *d1* aleurone. The *d1* mutation did not result in a decrease in sensitivity to GA at the level of transcription. Rather, changes in transcript abundance were smaller in the *d1* mutant than in wild type.

The aleurone layer of small cereal grains, such as barley (*Hordeum vulgare*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), and wild oats (*Avena fatua*), has been used extensively to characterize the response of a plant tissue to GA and abscisic acid (ABA). The power of the aleurone layer for the study of gene expression lies in the simplicity of the tissue and the strength of the response. Aleurone layers contain a single cell type that is not photosynthetic and neither grows nor divides following grain maturation. Because of this uniformity, the cells in the aleurone layer respond nearly synchronously to exogenously applied GA and ABA. For some genes, these responses are dramatic. It has been estimated that transcripts for α -amylases may make up 20% of all newly transcribed mRNAs 24 h after GA addition, and α -amylase protein represents as much as 60% of all newly synthesized protein (Higgins et al., 1982). These rapid, large, hormone-dependent changes reflect the function of the aleurone layer, which is to produce a battery of hydrolytic

enzymes that breaks down the stored carbohydrate and protein reserves in the dead starchy endosperm. The resulting sugars, small peptides, and amino acids are used to support postgerminative growth of the seedling. In the intact grain, GA produced by the embryo triggers the production of these hydrolases (Jacobsen et al., 2002). In the event that environmental conditions are unfavorable for germination or continued seedling growth, ABA produced by the embryo and aleurone layer prevents the synthesis of hydrolases and the break down of stored endosperm reserves (Jacobsen and Chandler, 1987).

In parallel to studies on hormone-regulated transcription in cereal aleurone layers, other work has focused on identifying signal transduction elements in this tissue. Some of these elements, such as changes in the concentration of cytosolic Ca^{2+} , are not directly regulated at the level of transcription. A few, such as GAMYB, are transcriptionally regulated (Gubler et al., 1997). In rice, *dwarf1* (*d1*) is a hormone-signaling mutant that lacks the sole G_α -subunit of heterotrimeric G-proteins in the rice genome. Mutant plants are semi-dwarf and deep green. Aleurone layers from *d1* grain are less sensitive to GA than those from wild-type grain with regard to α -amylase mRNA accumulation, secreted α -amylase activity, and Ca^{2+} -ATPase mRNA accumulation (Ueguchi-Tanaka et al., 2000). Previous experiments with *d1* have led to the bypass model for GA signaling in which heterotrimeric G-proteins are important components of the GA signal transduction network at low concentrations of GA, but are much less important at high concentrations of GA when alternative signaling pathways are presumed to be paramount (Ueguchi-Tanaka et al., 2000; Iwasaki et al., 2003).

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With the advent of microarray technology, it is now possible to examine changes in transcript abundance for thousands of genes within a single experiment. Data from microarray experiments therefore have the potential to add substantial depth to our understanding of how genes are regulated in response to a defined signal. In particular, it is now possible to compare the responses of selected, individual genes to the responses of large groups of genes that have not been identified in advance. Global or large-scale changes in transcription have been determined for cereals on a few occasions (Potokina et al., 2002; Lin et al., 2003; Rabbani et al., 2003; Close et al., 2004; Schnable et al., 2004; Yang et al., 2004; Yazaki et al., 2004). In most cases, however, RNA was isolated from complex tissues or after relatively long treatment times. For example, RNA for hybridization with cDNA or oligonucleotide-based microarrays has been extracted from developing rice seedlings (Lin et al., 2003; Rabbani et al., 2003); rice shoots, leaves, or roots (Kawasaki et al., 2001; Ozturk et al., 2002; Yang et al., 2004); barley leaves (Close et al., 2004); or maize (*Zea mays*) embryos (Lee et al., 2002). Interpretation of the data for these experiments is complicated because different tissues and cell types are expected to show distinct, yet overlapping, patterns of gene expression. A small increase in the measured amount of a transcript may reflect a small increase in all cells and tissues, or a large increase in a tissue that makes up a small percentage of the total tissue mass. Likewise, the observed change in transcription of a gene within a small gene family will be reduced if different tissues or cells utilize different family members. An alternative has been to isolate RNA from rice callus (Yazaki et al., 2004), which eliminates some of the difficulties associated with multiple or complex tissues, but complicates attempts to compare the microarray data with transcriptional changes that occur in planta.

We have used an oligonucleotide microarray containing 23,780 probe sets representing approximately 21,000 rice genes (Zhu et al., 2003) to examine transcript profiles in rice aleurone. Approximately one-half of the genes in the rice genome are represented on this array (Goff et al., 2002). Our data confirm the gene expression data that have been published previously for the rice aleurone layer and reveal novel aspects of transcriptional control by GA and ABA. We have also used *d1* grain to determine how loss of G_a influences transcript abundance in tissue treated with GA or ABA and to test the bypass model of GA signaling. Finally, we have identified some of the genes in rice aleurone that are likely to be hormonally and/or temporally regulated.

RESULTS

Rice Half-Grains Contain Transcripts for Approximately One-Half of the Rice Genome

To learn more about hormonal control of gene expression in rice aleurone layers, we monitored the

transcriptome of imbibed, de-embryonated rice grains (half-grains) treated with 20 mM CaCl_2 alone (no hormone) for 0, 0.5, 1, 3, 6, or 8 h, or CaCl_2 and either 5 μM GA or 5 μM ABA for 0.5, 1, 3, 6, or 8 h. The data in Table I make it clear that rice half-grains contained transcripts for a large number of genes. Regardless of hormone treatment, 45% to 50% of the probe sets on the microarray detected transcripts from the sample. Approximately 9,762 probe sets, or 41.1% of the total number of probe sets, had present calls for all treatments at all times, and this percentage may be an estimate for the percentage of the genome required for proper function of the rice aleurone.

Expression Profiles of Rice Aleurone Genes Reveal Patterns of Gene Expression

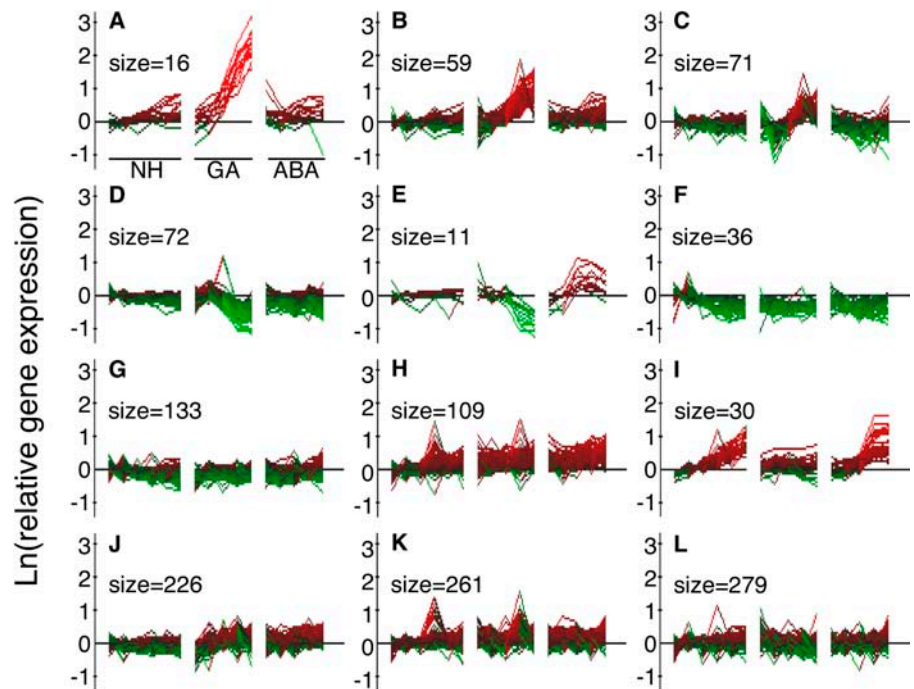
Hormone-dependent changes in the abundance of specific mRNAs are likely to be associated with responses of rice aleurone layers to GA or ABA and, hence, to aleurone layer function. We identified subsets of the expressed genes that were enriched for probe sets with hormone-responsive changes in abundance. As an initial step, we removed probe sets from the data for which there was little or no evidence for a hormone-dependent change in transcript abundance. To do this, we computed an *F* statistic by comparing the expression values for the five samples not treated with hormone with the five samples treated with ABA or GA at 0.5, 1, 3, 6, and 8 h. Probe sets with a *P* value >0.05 do not have a hormone-dependent change in variance and are unlikely therefore to have a statistically meaningful, hormone-dependent change in expression within 8 h of hormone treatment. Using this procedure, we obtained datasets containing approximately 1,300 probe sets that were enriched for those with GA-dependent differences in gene expression and 1,350 probe sets that were enriched for those with ABA-dependent differences in gene expression relative to controls that were not treated with hormone.

To visualize the nature of global changes in gene expression in rice aleurone, we used *k*-means clustering. The datasets enriched for GA- or ABA-responsive genes were sorted into 12 clusters based on their time- and hormone-dependent pattern of expression. The clusters for the probe sets enriched for GA-regulated genes are shown in Figure 1. For each cluster, the transformed probe set intensity is shown at each time of RNA extraction for tissue treated with no hormone (left six points), GA (middle five points), and ABA (right five points). Three clusters showed various degrees of up-regulation in GA-treated tissue. These

Table I. Number of probe sets called present when RNA samples from rice half-grains were hybridized to the rice oligonucleotide microarray

Treatment	Probe Sets Present	Percentage of Total Probe Sets
No hormone	11,506	48.4%
ABA (5 μM)	10,899	45.8%
GA (5 μM)	11,126	46.8%

Figure 1. Patterns of gene expression in a dataset containing 1,311 probe sets enriched for those that were GA responsive. The natural logarithm (\ln) of probe set intensity at each time relative to the average intensity with no hormone (NH) at 0, 0.5, and 1 h is shown for each of the 16 microarrays used in this experiment. Samples were extracted from rice half-grains treated with NH for 0, 0.5, 1, 3, 6, and 8 h; GA for 0.5, 1, 3, 6, and 8 h; and ABA for 0.5, 1, 3, 6, and 8 h. Red indicates an increase in transcript abundance, and green indicates a decrease. The number of probe sets in each k -means cluster is indicated as its size.



are seen in Figure 1, A to C, and the probe sets in each cluster are presented in Supplemental Table I, A to C. The most strongly up-regulated cluster (Fig. 1A) contains 10 secreted hydrolases and includes α -amylases and endo-1,4- β -xylanases, as expected. This cluster also includes two probe sets for K^+ transporters and one for a calcineurin B-like (CBL) protein. The clusters in Figure 1, B and C, show genes that are up-regulated by GA, but to a lesser extent than those in Figure 1A. Figure 1B has several probe sets for gene functions associated with the mobilization of stored reserves, including gluconeogenesis (lipase, acyl CoA oxidase), proteolysis (two carboxypeptidases, Cys proteinase), and phytate hydrolysis (acid phosphatases). Also in this cluster are two sugar transporters and a peptide transporter. Genes with functions in protein synthesis and posttranslational modification include BiP, calnexin, sec61B, a protein disulfide isomerase, and glucosyl-, acyl-, and xylosyl-transferases. Potential signaling elements include GAMyb, a CBL protein, and a protein phosphatase 2C. Cluster C in Figure 1 contains probe sets with similar putative functions, including Glc and peptide transporters, oryzain, vignain, acetyltransferase, and several cytochrome oxidase-related proteins (Supplemental Table IC). Two clusters show GA-dependent down-regulation of expression (Fig. 1, D and E; Supplemental Table I, D and E) with the data in Figure 1E also showing up-regulation by ABA. This cluster contains probe sets for dehydrin and late embryogenesis abundant (LEA) genes, among others. Genes down-regulated in all treatments relative to zero time (Fig. 1F), up-regulated in all treatments (Fig. 1H), or up-regulated with ABA or no hormone, but not strongly up-regulated by GA (Fig. 1I; Supplemental Table I, I),

are also represented by clusters of at least 30 probe sets. Figure 1, J to L, shows little obvious pattern of regulation, except for a trend toward an increase in expression with time in ABA-treated samples (Fig. 1J), an increase in expression with GA and ABA at intermediate time points (Fig. 1K), and a decrease in expression with time in ABA-treated samples (Fig. 1L).

The same analysis was used to visualize changes in expression for the probe sets contained in the gene list enriched for ABA-responsive genes. Genes up-regulated by ABA and down-regulated by GA are seen in Figure 2, A and B, and Supplemental Table II, A and B, and genes up-regulated by ABA and less strongly up-regulated by no hormone and GA are seen in Figure 2C. The genes represented by Figure 2, A and B, include several dehydrins and LEA proteins. Genes that are up-regulated in all treatments relative to time zero are represented by the clusters shown in Figure 2, D to F. Genes down-regulated in all treatments are contained in the clusters shown in Figure 2, G to I. Two clusters show little or no obvious patterns of expression except for an up-regulation of expression at late times with ABA (Fig. 2, J and K). There are no clusters that show down-regulation by ABA in the absence of a change with GA or no hormone.

Genes Specifically Regulated by GA or ABA Are Rare

The data in Figures 1 and 2 suggested to us that few genes were regulated specifically by GA or ABA relative to no-hormone controls. To quantify hormonally regulated changes in gene expression, we identified the probe sets that were called present and whose signal intensity increased or decreased 2-fold or 1.5-fold

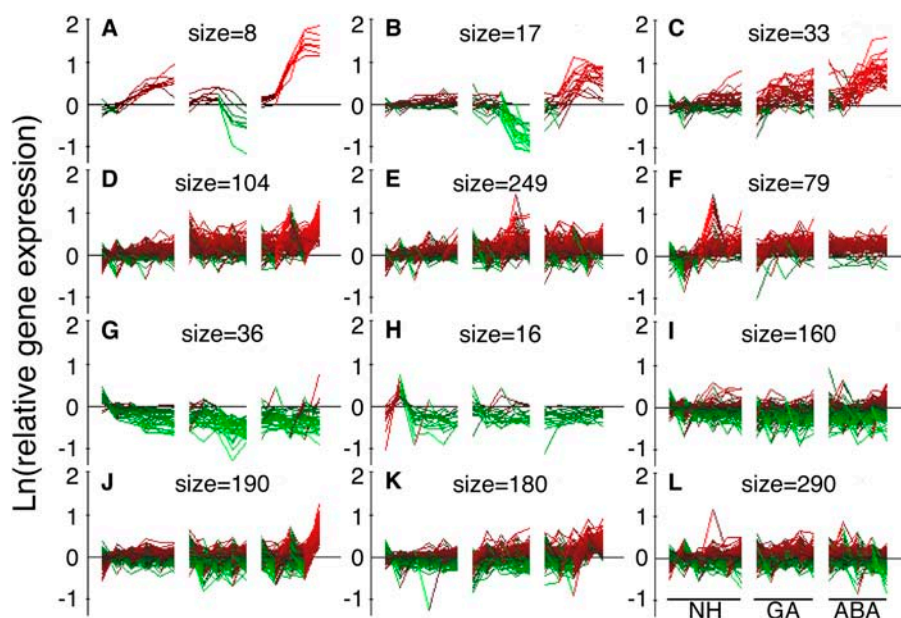


Figure 2. Patterns of gene expression in a dataset of 1,369 probe sets enriched for those that are ABA-responsive. The natural logarithm (ln) of probe set intensity relative to the average intensity with no hormone (NH) at 0, 0.5, and 1 h is shown for each of the 16 microarrays used in this experiment. Samples were extracted from rice half-grains treated with NH for 0, 0.5, 1, 3, 6, and 8 h; GA for 0.5, 1, 3, 6, and 8 h; and ABA for 0.5, 1, 3, 6, and 8 h. Red indicates an increase in transcript abundance, and green indicates a decrease. The number of probe sets in each k -means cluster is indicated as its size.

relative to no hormone. Previous work by others and our own analyses (Supplemental Fig. 1) indicate that the false positive rate for the rice array is 0.5% (Zhu et al., 2003) or less (0.37%; Supplemental Fig. 1), with a 2-fold cutoff and approximately 1.9% with a 1.5-fold cutoff (Supplemental Fig. 1). Regardless of the expression ratio used, the number of hormonally regulated genes was relatively small. As seen in Figure 3A, fewer than 60 genes were specifically up-regulated or down-regulated by either ABA or GA at any of the five time points relative to no-hormone controls when a 2-fold cutoff was used. Even with the less stringent criterion of 1.5-fold, fewer than 200 genes showed an increase or decrease in expression in response to hormone treatment. A comparison of the genes up- or down-regulated by GA or ABA at 8 h shows that there is little evidence to support the hypothesis that many genes up-regulated by one hormone are down-regulated by the other when the hormones are added individually (Fig. 3B). Only one gene was down 2-fold with ABA and up 2-fold with GA, and this gene did not have any hits against other genes with a known function. Three genes were up with ABA and down with GA: a LEA protein and two dehydrins. Nine genes were up 2-fold with both hormones. No genes were specifically down-regulated by both GA and ABA.

The data in Figure 3 illustrate three important findings related to the global nature of gene regulation by ABA and GA in rice aleurone layers. First, the number of genes up-regulated by ABA is comparable to the number of genes up-regulated by GA. Second, GA down-regulates about one-third as many genes as it up-regulates. For example, 8 h after hormone treatment, 59 genes were specifically up-regulated and 20 genes were specifically down-regulated by GA. Third, GA down-regulates many more genes than ABA down-regulates. Indeed, the maximal number of genes down-

regulated by ABA does not exceed the expected number of false positives.

The G_α -Protein in Rice Amplifies Transcriptional Changes

The mutated protein in *d1* rice is the sole G_α -subunit in rice, and a role for G_α in GA signaling has been

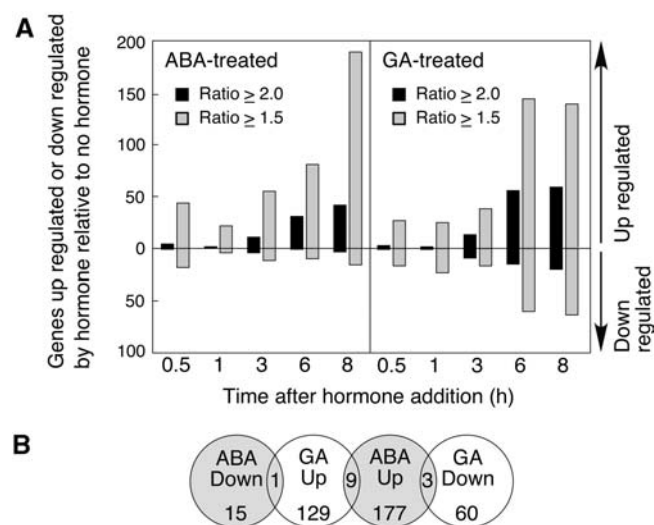


Figure 3. Number of genes specifically up- or down-regulated in rice half-grains by ABA or GA is small. A, RNA was extracted from rice half-grains treated with 5 μ M ABA, 5 μ M GA, or no hormone for 0.5, 1, 3, 6, or 8 h, and probe sets with a fold change in intensity >1.5 or 2 between hormone-treated and untreated were identified at each time point. B, Distribution of probe sets that were hormonally responsive by 1.5-fold or more at 8 h into categories of decreased in abundance with ABA (ABA down), increased in abundance with GA (GA up), increased in abundance with ABA (ABA up), and decreased in abundance with GA (GA down). The number of probe sets in two adjacent categories is indicated where the circles overlap.

demonstrated previously (Ueguchi-Tanaka et al., 2000). We used transcriptional profiling and *d1* grain to determine how loss of G_α function influences transcript abundance on a global scale. To show that the approach was suitable for this analysis, we compared transcript abundance for 13,818 probe sets called present in arrays hybridized with RNA from imbibed wild-type and *d1* half-grains. As shown in the scatter plot in Figure 4, there was an extremely tight correspondence ($r^2 = 0.962$) between probe set intensities in wild type and *d1* at time zero. Intensity values for only 0.17% of the probe sets differed by more than 2-fold when the data from wild-type half-grains were compared with the data from *d1* half-grains. This further confirms the reliability of the method as a tool for genome-scale comparisons and shows that there are small errors in the experimental system.

To assess the extent to which G_α influences transcript abundance, we compared expression in wild type with expression in *d1* rice half-grains 8 h after treatment with either 5 μM GA or 100 nM GA. We chose 100 nM GA for one treatment because previous experiments demonstrated a large difference between wild type and *d1* in the transcription of the α -amylase genes at this concentration (Ueguchi-Tanaka et al., 2000). We used 5 μM GA to facilitate comparisons with the time-course experiment and because there was a small difference between wild type and *d1* in the amount of transcript for the α -amylase genes at this concentration (Ueguchi-Tanaka et al., 2000). Systematic differences in transcription between wild type and mutant are easily seen when the probe sets are ordered from those whose signal intensity increased most to those whose signal intensity decreased most 8 h after treatment of half-grain with 5 μM GA. Probe sets were grouped into bins of 25 probe sets to facilitate statistical analysis. Only those groups of 25 probe sets whose average expression at 8 h was 1.5-fold higher or lower than that at 0 h were included in this analysis.

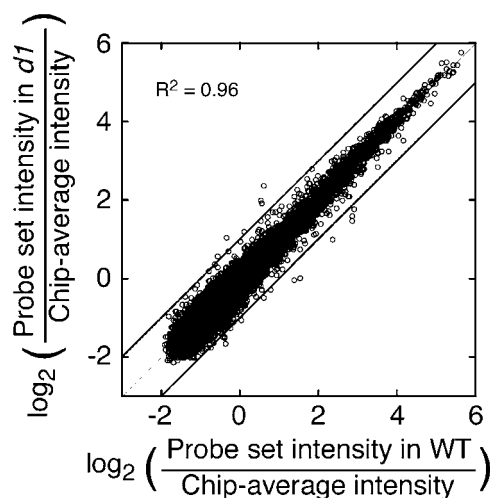


Figure 4. Scatter plot of probe set intensities (13,818 probe sets) for wild-type rice and *d1* mutant rice half-grains shows that few probe sets vary in intensity by more than 2-fold (diagonal lines) at time zero.

As seen in Figure 5, there is a clear, genome-wide effect of the G_α -subunit on transcript abundance. In this figure, ratios are plotted as \log_2 values so that relative changes in magnitude are easily compared and equal weight is given to comparable increases and decreases in intensity (e.g. a ratio of 2 and a ratio of 0.5). Genes that had the largest relative increase in expression in GA-treated samples (Fig. 5, data points farther to the right) had higher expression in wild-type half-grains relative to *d1* half-grains (i.e. \log_2 ratio of expression > 0). In samples treated with 5 μM GA, the 25 probe sets most strongly induced by GA (Fig. 5A, data point farthest to the right) had an average expression in wild type that was almost twice that in *d1* at 8 h. The ratio of wild-type to *d1* expression for the same set of genes in samples treated with 100 nM GA was 2.2 (Fig. 5B). Conversely, probe sets that decreased in intensity relative to time zero had lower ratios of wild-type to *d1* expression. Genes most strongly repressed with GA (Fig. 5, data points farther to the left) had an average expression in wild type that was as much as 30% less than that in *d1* half-grains when samples were treated with 100 nM GA (Fig. 5B). These changes in wild-type expression relative to *d1* expression were related to changes in mRNA abundance. For the data from half-grains treated with 5 μM GA, there was a good correlation ($r^2 = 0.83$) between gene expression at 8 h relative to 0 h and the ratio of wild-type to *d1* expression (Fig. 5A). For half-grains treated with 100 nM GA, this correlation was even better ($r^2 = 0.92$). It is particularly noteworthy that these correlations hold for genes that show small changes in mRNA abundance during the 8 h of the experiment, as well as for genes showing large changes in abundance. Thus, the G_α -protein amplifies transcriptional changes, but is not required for most transcriptional changes. These data also suggest that the G_α -protein influences the abundance of many transcripts in GA-treated samples, regardless of whether they are up- or down-regulated. The differences in mRNA abundance between wild type and *d1*, however, were small. Only those probe sets with strong up- or down-regulation with GA had ratios of expression between wild type and *d1* that were over 50%.

To show that these relatively small differences were statistically significant, we asked whether each group of 25 probe sets had a mean ratio of wild-type to *d1* expression that was different from 1.00. Groups of 25 genes in Figure 5 that have P values from one-tailed t tests < 0.001 are indicated by black data points. Those groups of 25 genes where $P > 0.001$ are indicated by white circles. Overall, for the 33 groups of 25 probe sets used in this analysis, 19 had a P value < 0.001 for samples treated with 5 μM GA and 22 had a P value < 0.001 for samples treated with 100 nM GA. In both cases, the observed number is much greater than the expected number, which for this P value is less than 1.

As a control for this analysis, we looked at the intensity values for the same groups of GA-regulated genes in ABA-treated samples. In this case, there was

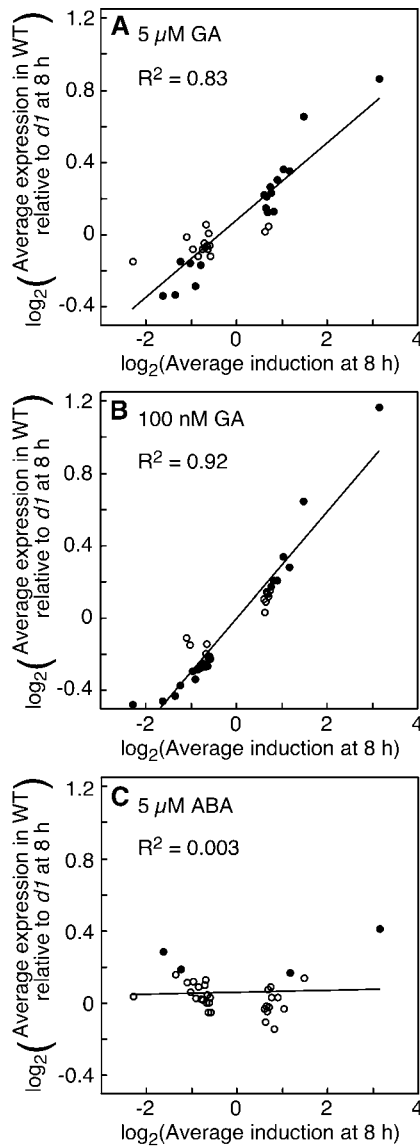


Figure 5. Changes in RNA abundance are reduced in *d1* half-grains compared to wild-type half-grains at both 5 μM GA (A) and 100 nM GA (B). Each point in A to C represents 25 probe sets, with the 25 probe sets having the highest induction with GA at 8 h represented by the rightmost point and the 25 probe sets with the highest repression with GA represented by the leftmost point. Black circles have an average expression ratio different from 1.0 at the level of $P = 0.001$. The *d1* mutation does not affect the expression of the same sets of probes in half-grains treated with ABA (C). Note that the graphs in A to C are plotted on a \log_2 - \log_2 scale.

very little difference in average expression between wild type and *d1* (Fig. 5C) and little correlation between the ratio of wild-type to *d1* expression and changes in gene expression with GA (Fig. 5C; $r^2 = 0.003$).

Effects of G_α -Protein on Transcription Are Not Specific for GA

To see whether G_α -protein affected transcription of ABA-regulated genes, we compared the signal inten-

sities of ABA-regulated genes in wild-type and *d1* half-grains. Using the same approach as that described above, genes from ABA-treated samples were sorted and grouped by expression 8 h after treatment of wild-type samples with 5 μM ABA relative to expression at 0 h. As seen in Figure 6A, there was a good correlation between wild-type to *d1* expression ratio and changes in intensity in samples treated with ABA ($r^2 = 0.65$). As for the GA-treated samples, probe sets with larger changes in expression at 8 h relative to 0 h had larger differences in expression in wild type relative to *d1*.

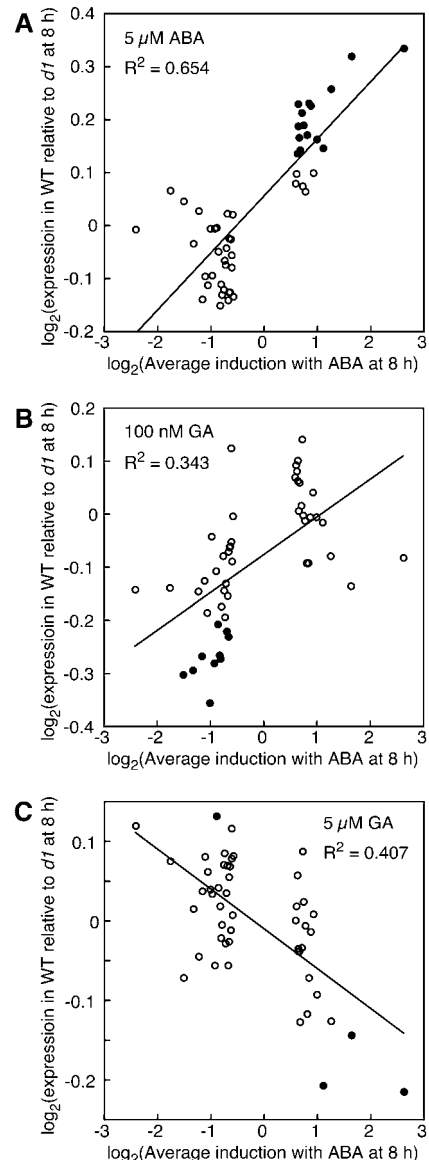


Figure 6. Changes in RNA abundance for many genes that are up- or down-regulated with ABA are reduced in *d1* mutant half-grains relative to wild type. Each point in A to C represents 25 probe sets, with the 25 probe sets having the highest induction with ABA at 8 h represented by the rightmost point and the 25 probe sets with the highest repression with ABA represented by the leftmost point. Black circles have an average expression ratio different from 1.0 at the level of $P = 0.001$. Note that the graphs in A to C are plotted on a \log_2 - \log_2 scale.

These differences in expression between wild-type and *d1* grains were small, but are likely to be statistically meaningful. Of the 50 groups of 25 probe sets used in this analysis, 15 (black circles) had a P value <0.001 when one-tailed t tests were used to compare the ratio of wild-type to *d1* expression for the 25 probe sets to probe sets having a ratio of 1.00 (Fig. 6A). When the analysis was extended to look at the same probe sets in GA-treated samples, there were still weak correlations between the ratio of expression in wild type to *d1* and changes in gene expression in ABA-treated tissue (Fig. 6, B and C). This most likely reflects the observation that many changes in gene expression are not hormone specific (see below).

Loss of G_{α} -Protein Does Not Decrease the Sensitivity to GA for Most Genes

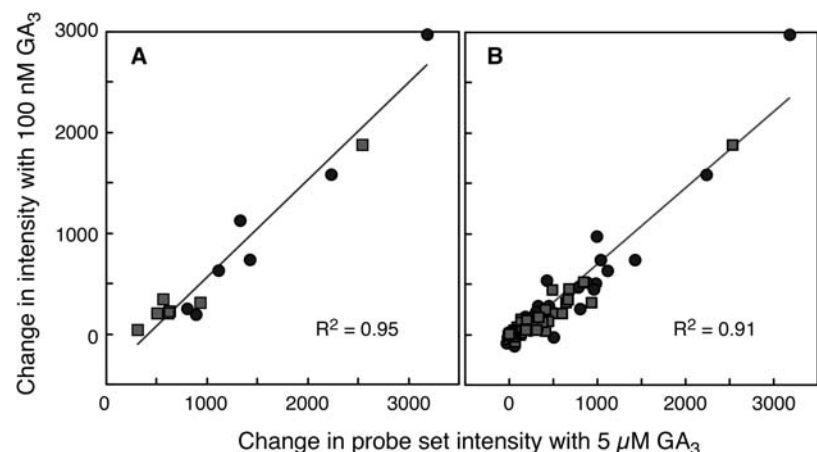
The *d1* mutation results in decreased sensitivity to GA with respect to internode elongation and secreted α -amylase activity (Ueguchi-Tanaka et al., 2000). These data gave rise to the bypass model. We wished to know whether there was also a decrease in sensitivity to GA at the level of transcription. For this analysis, we examined two sets of genes. First, we looked at the seven α -amylases that were strongly up-regulated by GA (Fig. 1A; Supplemental Table IA). Second, we looked at the probe sets corresponding to the 59 genes that were specifically up-regulated 2-fold with GA in the time-course experiment 8 h after GA treatment (Fig. 3). These 59 probe sets are listed in Supplemental Table III and include five α -amylases, four proteases, an endo- β -xylanase, a GAMyb, two CBL proteins, and an acyl CoA oxidase. To look for changes in sensitivity to GA, we plotted the change in probe set intensity between 0 and 8 h with 5 μM GA against the change in intensity with 100 nM GA for tissue from wild-type and *d1* mutant grains. If there is a difference in sensitivity between wild type and the *d1* mutant, the data for changes in gene expression in wild type should fall on one line and the data for changes in gene expression in the *d1* mutant should fall on a different

line with a lower slope. The data for α -amylases are shown in Figure 7A. What is clear from this figure is that the line relating changes in gene expression at 5 μM GA to changes at 100 nM GA is no different for RNA samples from *d1* mutants than for RNA samples from wild-type grains. Therefore, although the magnitude of the change observed with the *d1* mutant is less than the magnitude of the change observed with wild type, there was no decrease in sensitivity to GA. For both wild type and *d1* mutants, an α -amylase gene that had a large increase in transcript abundance with 5 μM GA had a large increase in transcript abundance with 100 nM GA. Conversely, for both wild type and *d1* mutants, an α -amylase gene that had a moderate increase in transcript abundance with 5 μM GA had a moderate increase in transcript abundance with 100 nM GA. The data for the 59 probe sets that are up-regulated by GA show a similar pattern and again provide no evidence to support the hypothesis that the loss of the G_{α} -subunit results in a decrease in the sensitivity to GA at the level of transcription (Fig. 7B).

Time-Dependent Changes in Gene Expression Occur in the Absence of Hormone Treatment

Time-dependent changes in gene expression may be as important as those that are hormone dependent. To identify genes in rice half-grains that have a temporal increase or decrease in mRNA, we compared signal intensity values for 9,762 probe sets called present 8 h after treating wild-type rice grains with GA or ABA with signal intensity values at 0 h. In the case of ABA-treated half-grains, the changes are very similar to those that occur in the absence of added hormone. As seen in Figure 8A, the differences in probe set signal intensity between 0 and 8 h are nearly equal for tissue treated with ABA and for tissue treated with no hormone, and the slope of the best-fit line is 1.0 ($r^2 = 0.71$). In some cases, these changes are very large. Differences in hybridization signal intensity of over 2,000 units were observed in these data, where the mean signal intensity is 80. For 80% of the dataset,

Figure 7. Sensitivity of transcription to GA is not reduced in the *d1* mutation. A, Changes in probe set intensity for the seven probe sets corresponding to α -amylase genes. B, Changes in probe set intensity for a group of GA-regulated genes. Data from wild-type half-grains in A and B are indicated by circles, and data from *d1* half-grains are indicated by squares.



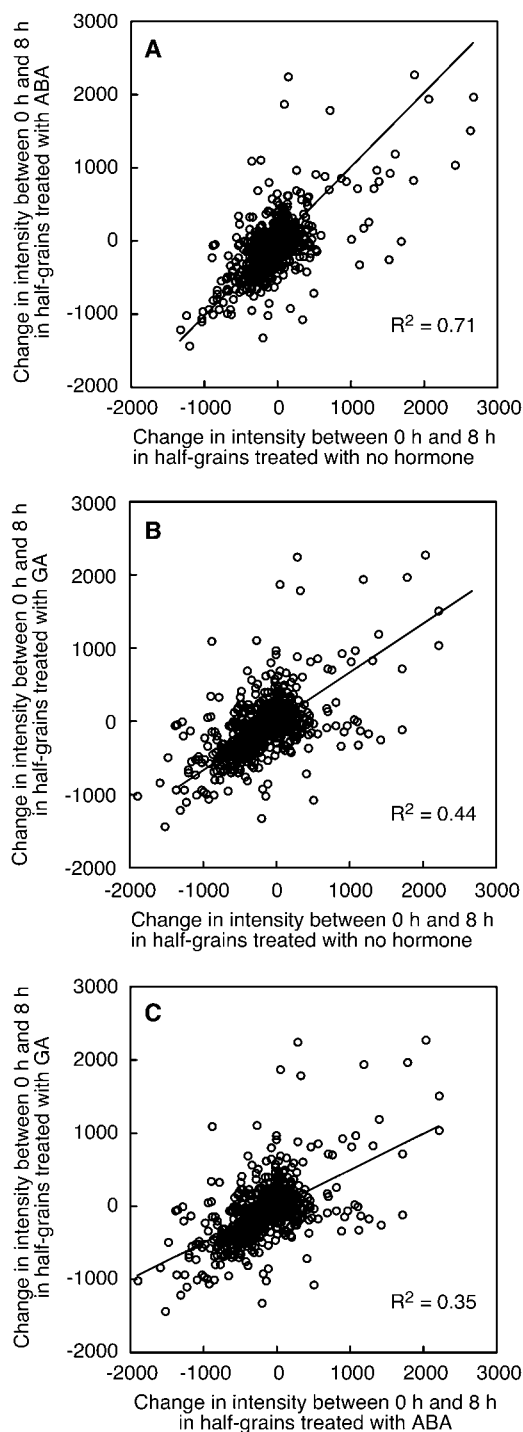


Figure 8. Many changes in gene expression are not dependent on hormone treatment. A to C, Changes in intensity between 8 and 0 h for 9,762 probe sets in rice half-grains treated with 5 μM ABA and no hormone (A), 5 μM GA and no hormone (B), and 5 μM GA and 5 μM ABA (C). Note that the average intensity value for all probe sets on each microarray is 80.

however, changes in transcription between 0 and 8 h are less than 25%. Likewise, changes in probe set signal intensity in GA-treated tissue mirror changes in tissue treated with no hormone (Fig. 8B). In this case, however, the correlation between changes in half-grains

not treated with hormone and half-grains treated with GA was fairly low ($r^2 = 0.44$) and this reflects the increases and decreases in mRNA abundance that occur with GA but do not occur in the absence of added hormone. The correlation between changes in probe set signal intensity for GA- and ABA-treated half-grains was lower ($r^2 = 0.35$), but there was still a general trend such that probe sets that increased in intensity in one treatment increased in intensity in the other (Fig. 8C).

Identification of Genes That Have Time-Dependent Changes in Transcription

To identify genes that were up-regulated or down-regulated with time, we found the probe sets whose signal intensities were up- or down-regulated in wild-type half-grains by 2-fold or more in both the rice time-course experiment and the *d1* experiment. Over the 8-h time course of the experiments, 37 genes showed a 2-fold increase in expression in GA-treated tissue between 0 and 8 h (these are listed in Table II). As expected, hydrolytic enzymes are prominent and the probe sets identified include those for amylases (four times), endo-1,4- β -xylanase, endo-1,3- β -glucosidase, and proteases and carboxypeptidases (four times). Several up-regulated probe sets corresponded to genes that are involved in gluconeogenesis, including a lipase and acyl CoA oxidase. Two probe sets for CBL genes are also included in the list of genes up-regulated in GA-treated half-grains.

The genes that we observed to be up-regulated in GA-treated tissue echo previous reports on aleurone cell biology. Less is known about genes down-regulated in GA-treated tissue. We therefore determined the probe sets that showed a decrease in expression between 0 and 8 h after GA treatment. Forty-seven genes were down-regulated more than 2-fold in both experiments. As seen in Table III, these include many genes for metabolic enzymes and genes associated with stress responses. The down-regulation of two Cys proteinase inhibitors is consistent with the biology of the aleurone cell because GA treatment increases the activity of secreted and vacuolar Cys proteinases. It is notable that few signaling molecules are present in this list. We did not identify any strong candidates for protein kinases, protein phosphatases, or transcription factors, with the possible exception of a gene having similarity to nucleic acid-binding proteins.

Genes specifically down-regulated by ABA are rare (Fig. 3), but many genes are down-regulated with time in half-grains treated with ABA (Fig. 8). We compared probe set signal intensities at 8 h with those at 0 h for wild-type half-grains treated with ABA in two separate experiments. Signal intensities for the 35 probe sets listed in Table IV were down-regulated by 2-fold or more in both experiments. These down-regulated genes have functions in basic cellular processes (e.g. pyruvate dehydrogenase, pyruvate kinase, and malate oxidoreductase) or are related to the mechanisms of

Table II. Probe sets up-regulated with time in GA-treated rice half-grains

Induction is the ratio of expression at 8 h relative to 0 h. Similar proteins are identified by accession numbers and a brief description.

Probe Set	Induction at 8 h in Time Course	Induction at 8 h in <i>d1</i> Experiment	Accession Nos.	Description
Hydrolases				
OS000411_i_at	30.4	6.5	gi 113766 sp P17654	AMY1_ORYSA α -amylase 1
OS000626_s_at	10.72	23.6	gi 113680 sp P27937	AM3B_ORYSA α -amylase isozyme 3B
OS022194_at	9.96	20.95	gi 113683 sp P27934	AM3E_ORYSA α -amylase isozyme 3E
OS000501_at	7.59	22.01	gi 113683 sp P27934	AM3E_ORYSA α -amylase isozyme 3E
OS023228_at	9.69	11.59	P14768	XYNA_PSEFL endo-1,4- β -xylanase A
OS000920_s_at	5.22	3.69	P15737	E13B_HORVU glucan endo-1,3- β -glucosidase GII
OS006213_at	2.6	5.03	gi 7262670 gb AAF43928.1 AC012188_5	Contains similarity to acid phosphatase and contains a sterol desaturase domain (Arabidopsis)
OS000590_f_at	4.57	2.27	Q06015	CHI3_ARAHY endochitinase 3
Proteolysis				
OS017015_s_at	2.88	2.66	gi 7671402 emb CAB89316.1	Carboxypeptidase (Arabidopsis)
OS020092.1_at	2.1	3.32	gi 3859606 gb AAC72872.1	Contains similarity to Cys proteases (Arabidopsis)
OS009235.1_at	2.93	2.34	P37891	CBP3_ORYSA Ser carboxypeptidase III
OS012349_at	2.21	2.36	gi 3859607 gb AAC72873.1	Contains similarity to Cys proteases (Arabidopsis)
OS000667.1_at	2.44	2.08	gi 7635921 emb CAB88392.1	Bowman-Birk trypsin inhibitor (rice)
Lipid mobilization				
OS008739.1_at	4.76	12.81	gi 4587543 gb AAD25774.1 AC006577_10	Lipase/acylhydrolase with GDSL-motif family (Arabidopsis)
OS001843_f_at	3.19	2.34	P10974	NLTB_RICCO nonspecific lipid-transfer protein B
OS012669.1_at	2.68	2.46	gi 3044212 gb AAC13497.1	Acyl-CoA oxidase (Arabidopsis)
Membrane transport				
OS006069.1_at	2.43	5.75	gi 7262678 gb AAF43936.1 AC012188_13	Contains similarity to UDP-galactose transporter (Arabidopsis)
OS005993.1_at	3.85	3.09	gi 5091611 gb AAD39600.1 AC007858_14 gb U43629	Integral membrane protein from <i>Beta vulgaris</i> and member of the sugar transporter family (rice)
OS019839_s_at	2.96	3.76	gi 4249409 gb AAD13706.1	Sugar transporter (Arabidopsis)
Signaling				
OS015683_at	5.66	3.2	gi 4938494 emb CAB43852.1	CBL protein (Arabidopsis)
OS009519_at	4.45	3.5	gi 1277092 gb AAB03774.1	<i>n</i> -Acetyltransferase hookless1
OS015049_i_at	4.28	2.13	gi 3309086 gb AAC26010.1	CBL protein 3 (Arabidopsis)
Other				
OS013253_at	2.4	7.69	gi 4584534 emb CAB40764.1	Cytochrome P450-like protein (Arabidopsis)
OS015512.1_at	2.73	2.92	gi 6863054 dbj BAA90487.1	Heat shock protein 90 (rice)
OS000907_s_at	2.44	3.1	gi 585960 sp P38389	S61B_ARATH protein transport protein sec61 β -subunit
OS011960.1_s_at	3.06	2.41	gi 2052381 gb AAC49696.1	Calreticulin
OS024781_at	9.56	11.63	gi 4972056 emb CAB43924.1	Putative protein (Arabidopsis)
OS006047_i_at	9.02	6.91	gi 3297809 emb CAA19867.1	Putative protein (Arabidopsis)
OS003244.1_s_at	3.54	6.67	gi 7378611 emb CAB83287.1	Putative protein (Arabidopsis)
OS_ORF013995_at	3.98	4.11		Open reading frame
OS019454_f_at	4.88	2.06		F21B7.3 (Arabidopsis)
OS010175.1_at	2.32	3.85	gi 7269851 emb CAB79710.1	Putative protein (Arabidopsis)
OS001396_at	3.27	2.75	gi 5123547 emb CAB45313.1	Putative protein (Arabidopsis)
OS_ORF015460_at	2.37	3.4		Open reading frame
OS006045.1_at	2.11	3.27	gi 7485404 pir T02648	Hypothetical protein F12C20.9 (Arabidopsis)
OS009650_at	2.22	2.77		F17L21.8 (Arabidopsis)
OS001908_i_at	2.01	2.65	gi 7430871 pir T00843	Basic blue protein (Arabidopsis)

Table III. Probe sets down-regulated with time in GA-treated rice half-grains

Induction is the ratio of expression at 8 h relative to 0 h. Similar proteins are identified by accession numbers and a brief description.

Probe Set	Induction at 8 h in Time Course	Induction at 8 h in <i>d1</i> Experiment	Accession Nos.	Description
Stress related				
OS000677.1_f_at	0.27	0.3	gi 3980385 gb AAC95188.1	Small heat shock protein (Arabidopsis)
OS000130_f_at	0.28	0.48	P27777	HS11_ORYSA 16.9-kD class I heat shock protein
OS000148.1_at	0.48	0.28	gi 971280 dbj BAA09947.1	RAB24 protein (rice)
OS000170_f_at	0.33	0.44	P31673	HS12_ORYSA 17.4-kD class I heat shock protein
OS009060_at	0.35	0.42	gi 2944088 gb AAC05216.1	Glutathione <i>S</i> -transferase (rice)
OS011595_r_at	0.48	0.3	gi 445133 prf 1908434C	Chilling tolerance-related protein (rice)
OS009441_f_at	0.45	0.35	gi 5080803 gb AAD39312.1 AC007258_1	Glutathione transferase (Arabidopsis)
Metabolism				
OS009718.1_at	0.27	0.13	gi 5103836 gb AAD39666.1 AC007591_31	Member of the glyoxalase family (Arabidopsis)
OS000253_at	0.38	0.19	gi 1778821 gb AAC05590.1	<i>S</i> -adenosyl-L-Met synthetase (rice)
OS001932.1_at	0.37	0.26	P43279	MAOC_ORYSA malate oxidoreductase, chloroplast
OS005778.1_at	0.41	0.34	P34799	URID_CANLI uricase II clone PCLNUO-02
OS014904_at	0.37	0.4	gi 294285 gb AAA33840.1	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase
OS002747_at	0.46	0.34	gi 322787 pir JC1481	Pyruvate kinase, cytosolic (<i>Solanum tuberosum</i>)
OS006190_at	0.35	0.45	gi 6729036 gb AAF27032.1 AC009177_22	Glucose and ribitol dehydrogenase homolog (Arabidopsis)
OS009600.1_at	0.49	0.39	gi 3738320 gb AAC63661.1	Cinnamoyl CoA reductase (Arabidopsis)
OS010021_at	0.49	0.45	gi 2739000 gb AAB94588.1	CYP71D10p (<i>Glycine max</i>)
Proteolysis				
OS000768_i_at	0.46	0.3	gi 2245006 emb CAB10426.1	Cys proteinase inhibitor-like protein (Arabidopsis)
OS009331.1_at	0.41	0.41	gi 2641619 gb AAC12662.1	Ubiquitin-conjugating enzyme protein E2 (maize)
OS004395.1_r_at	0.43	0.43	gi 4263720 gb AAD15406.1	Cys proteinase inhibitor C (Arabidopsis)
Oleosome				
OS006526_at	0.27	0.29	gi 944830 emb CAA43183.1	Soybean 24-kD oleosin isoform (<i>G. max</i>)
OS000217_s_at	0.26	0.3	gi 1171352 gb AAC02239.1	16-kD oleosin (rice)
OS005956_f_at	0.31	0.31	gi 4455257 emb CAB36756.1	Oleosin, 18.5K (Arabidopsis)
Membrane transport				
OS004773_at	0.33	0.33	gi 2655098 gb AAC32034.1	Peptide transporter (barley)
OS012445_at	0.48	0.46	gi 3319374 gb AAC28223.1	Chloroplast triose phosphate translocators (Arabidopsis)
Other				
OS004203.1_f_at	0.23	0.15	gi 2058502 gb AAC49884.1	Hemoglobin 2 (rice)
OS005002.1_f_at	0.24	0.21	gi 544880 gb AAB29661.1	Multicystatin; PMC (<i>S. tuberosum</i>)
OS012817.1_at	0.24	0.24	gi 224647 prf 1109273A	Nodulin 35 (<i>G. max</i>)
OS003629.1_at	0.3	0.21	gi 2894603 emb CAA17137.1	Putative protein (Arabidopsis)
OS001028.1_at	0.29	0.29		Hypothetical protein (Z97343; rice)
OS000786.1_at	0.38	0.27	gi 4105683 gb AAD02495.1	Unknown (rice)
OS_ORF002741_at	0.44	0.26		Open reading frame
OS015813_f_at	0.37	0.34	gi 6175183 gb AAF04909.1 AC011437_24	RNA-binding protein (Arabidopsis)
OS_ORF014778_at	0.36	0.36		Open reading frame
OS010428.1_f_at	0.44	0.3	P45592	COF1_RAT cofilin, non-muscle isoform
OS003584_at	0.38	0.39	gi 4263648 gb AAD15370.1	Hypothetical protein (Arabidopsis)
OS000702_at	0.32	0.47	gi 4056503 gb AAC98069.1	Unknown protein (Arabidopsis)
OS009202_at	0.45	0.37	gi 6223644 gb AAF05858.1 AC011698_9	Unknown protein (Arabidopsis)
OS011577_s_at	0.49	0.37	gi 4091080 gb AAC98962.1	Nucleic acid-binding protein (rice)
OS011620_at	0.49	0.38	gi 6815055 dbj BAA90342.1	Unknown protein (rice)
OS001718.1_i_at	0.49	0.38	gi 439273 emb CAA80984.1	54 amino acids (barley)
OS014974_s_at	0.44	0.44	gi 5281030 emb CAB45966.1	Putative protein (Arabidopsis)
OS011954_at	0.42	0.47	gi 2130118 pir S67993	Amylogenin (maize)
OS009626.1_at	0.44	0.47	gi 4454458 gb AAD20905.1	Unknown protein (Arabidopsis)
OS025228.1_at	0.45	0.46	gi 2262116 gb AAB63624.1	Cellulose synthase isolog
OS012207_f_at	0.43	0.49	gi 8843793 dbj BAA97341.1	Unknown protein (Arabidopsis)
OS010104.1_s_at	0.48	0.46	gi 5262762 emb CAB45910.1	Putative protein (Arabidopsis)

Table IV. Probe sets down-regulated with time in ABA-treated rice half-grains

Induction is the ratio of expression at 8 h relative to 0 h. Similar proteins are identified by accession numbers and a brief description.

Probe Set	Induction at 8 h in Time Course	Induction at 8 h in <i>d1</i> Experiment	Accession Nos.	Description
Stress related				
OS000677.1_f_at	0.25	0.4	gi 3980385 gb AAC95188.1	Small heat shock protein (Arabidopsis)
OS011595_r_at	0.43	0.27	gi 445133 prf 1908434C	Chilling tolerance-related protein (rice)
OS002696_at	0.27	0.5	gi 6746590 gb AAF27638.1 AF217458_1	Heat shock protein 70 (Arabidopsis)
OS009441_f_at	0.38	0.41	gi 5080803 gb AAD39312.1 AC007258_1	Glutathione transferase (Arabidopsis)
OS009060_at	0.45	0.39	gi 2944088 gb AAC05216.1	Glutathione S-transferase (rice)
OS010527_f_at	0.48	0.49	Q28514	GTP_MACMU glutathione S-transferase P
Redox related				
OS000156_at	0.24	0.46	gi 505136 dbj BAA06456.1	Ferredoxin (rice)
OS000729_s_at	0.42	0.35	gi 516839 dbj BAA05494.1	Catalase (rice)
Metabolism				
OS004308_at	0.14	0.14	gi 4894182 emb CAB43506.1	12-Oxophytodienoate reductase (<i>Lycopersicon esculentum</i>)
OS002747_at	0.36	0.23	gi 322787 pir JC1481	Pyruvate kinase cytosolic (<i>Solanum tuberosum</i>)
OS001932.1_at	0.35	0.25	P43279	MAOC_ORYSA malate oxidoreductase, chloroplast
OS022578_at	0.42	0.37	gi 2924784 gb AAC04913.1	Myrosinase-binding protein (Arabidopsis)
OS013868_at	0.48	0.45	P32588	Nuclear and cytoplasmic polyadenylated RNA-binding protein PUB1
OS004744_at	0.49	0.48	gi 3850999 gb AAC72192.1	Pyruvate dehydrogenase E1 β -subunit isoform 1 (maize)
Other				
OS004203.1_f_at	0.14	0.05	gi 2058502 gb AAC49884.1	Hemoglobin 2 (rice)
OS001666.1_at	0.36	0.11	gi 2058498 gb AAC49882.1	Hemoglobin 1 (rice)
OS000124_at	0.41	0.11	gi 7489465 pir T04144	DNA-binding protein (rice)
OS005499.1_at	0.33	0.24	gi 7076779 emb CAB75894.1	Glycoprotein-like (Arabidopsis)
OS000701_at	0.29	0.41	gi 1155263 gb AAA91170.1	Eukaryotic release factor 1 homolog
OS005611.1_at	0.44	0.29	gi 949980 emb CAA61258.1	Open reading frame (maize)
OS009331.1_at	0.46	0.28	gi 2641619 gb AAC12662.1	Ubiquitin-conjugating enzyme protein E2 (maize)
OS017078_at	0.32	0.45	gi 7658344 gb AAF66134.1	Unknown protein (Arabidopsis)
OS003018.1_at	0.37	0.46	gi 2760362 gb AAB95261.1	15.9-kD subunit of RNA polymerase II (Arabidopsis)
OS005587_at	0.41	0.47	gi 3169182 gb AAC17825.1	Unknown protein (Arabidopsis)
OS010228_at	0.44	0.45	gi 4803958 gb AAD29830.1 AC006202_8	Unknown protein (Arabidopsis)
OS012990.1_at	0.48	0.43	gi 7671481 emb CAB89322.1	Putative protein (Arabidopsis)
OS001505_at	0.48	0.46	gi 4836932 gb AAD30634.1 AC006085_7	Unknown protein (Arabidopsis)
OS003584_at	0.49	0.45	gi 4263648 gb AAD15370.1	Hypothetical protein (Arabidopsis)

transcription and translation, the proteasome, and cellular redox status. Three glutathione S-transferases were identified in the latter category. Genes for hemoglobin were identified twice. We did not identify transcription factors or signaling molecules in this analysis.

We used a similar approach to look at genes with increased expression in ABA-treated half-grains. The 26 probe sets listed in Table V had increased intensity in wild-type half-grains in both the time-course and *d1* experiments. Many of the ABA up-regulated genes that we identified fell into three general categories: seed maturation, ABA and stress related, and proteolysis. Dehydrins and LEA proteins are known to be ABA up-regulated in cereals, and genes corresponding

to several LEAs and dehydrins were up-regulated by ABA in these experiments. Fluorescence intensity from two probe sets for protease inhibitors and two tonoplast intrinsic proteins also increased more than 2-fold in ABA-treated half-grains.

The lists of temporally regulated genes contained in Tables II to V have little overlap. The only significant overlap was between the lists of probe sets down-regulated in ABA-treated tissue and down-regulated in GA-treated tissue. Nine probe sets were in common between these two lists and these include two glutathione S-transferases, hemoglobin, a small heat shock protein, a malic enzyme, a ubiquitin-conjugating enzyme, cofilin, a chilling tolerance-related protein, and an unknown protein. None of the probe sets that increased

Table V. Probe sets up-regulated with time in ABA-treated rice half-grains

Induction is the ratio of expression at 8 h relative to 0 h. Similar proteins are identified by accession numbers and a brief description.

Probe Set	Induction at 8 h in time Course	Induction at 8 h in <i>d1</i> experiment	Accession Nos.	Description
Seed maturation				
OS001162_at	2.58	14.5	P46518	LE14_GOSHI LEA protein LEA14-A
OS003541_at	5.89	5.22	Q00742	DH15_WHEAT dehydrin RAB 15
OS001970.1_r_at	2.78	6.43	gi 5802240 gb AAD51623.1 AF169020_1	Seed maturation protein PM35 (<i>Glycine max</i>)
OS003416.1_r_at	3.62	5.1	P28640	DHN2_PEA dehydrin DHN2
OS001415_at	2.25	6.08	gi 5802250 gb AAD51628.1 AF169025_1	Seed maturation protein PM41 (<i>G. max</i>)
OS003879.1_s_at	2.81	3.47	gi 1486503 gb AAC03364.1	LEA-like protein (rice)
OS001562.1_r_at	2.23	3.3	gi 1399913 gb AAB03330.1	Dehydrin (rice)
OS008919.1_f_at	2.05	3.35	gi 100706 pir JH0324	RAB 16b protein (rice)
OS004708_at	2.61	2.72	gi 1526424 dbj BAA11017.1	LEA protein in group 3 (Arabidopsis)
ABA and stress related				
OS018179_f_at	5.13	15.08	gi 440847 gb AAA21819.1	Cold acclimation-induced WCS66
OS016473_f_at	5.87	13.1	P46526	CS66_WHEAT cold shock protein CS66
OS000966_s_at	3.3	8.24	gi 1724112 gb AAB38504.1	ABA-induced plasma membrane protein PM 19 (wheat)
OS016003_f_at	2.46	2.3	gi 22460 emb CAA41854.1	ABA inducible (maize)
Proteolysis				
OS003094.1_at	3.49	2.15	gi 4678299 emb CAB41090.1	Cys proteinase (Arabidopsis)
OS008030.1_s_at	2.73	2.12	gi 7438264 pir S72492	Probable proteinase inhibitor (<i>L. esculentum</i>)
OS000988_f_at	2.25	2.04	gi 475253 emb CAA55588.1	Proteinase inhibitor (maize)
Membrane transport				
OS009470_f_at	4.17	4.55	gi 2244974 emb CAB10395.1	Pore protein homolog (Arabidopsis)
OS003998.1_at	3.94	3.2	P26587	TIPA_ARATH tonoplast intrinsic protein- α
OS007184_at	2.68	3.54	gi 2605714 gb AAB84183.1	β -Tonoplast intrinsic protein (Arabidopsis)
Other				
OS005119_at	8.78	2.47	gi 4105681 gb AAD02494.1	Unknown (rice)
OS000552_at	6.17	2.28	P33044	THHR_HORVU antifungal protein R
OS006670_at	2.6	5.06	gi 4432835 gb AAD20684.1	Unknown protein (Arabidopsis)
OS003185.1_f_at	2.22	5.2	gi 2253578 gb AAC69143.1	Hypothetical protein (Arabidopsis)
OS002033.1_at	2.08	4.63	gi 7268686 emb CAB78894.1	Hypothetical protein (Arabidopsis)
OS003245_i_at	2.6	2.95	gi 5262190 emb CAB45787.1	Inositol 1,3,4-trisphosphate 5/6-kinase-like protein (Arabidopsis)
OS_ORF003709_f_at	2.91	2.08		Open reading frame

in GA- or ABA-treated tissues appeared in any of the other lists.

DISCUSSION

The cereal aleurone layer is a model system for studying the regulation of transcription by GA and ABA in part because of the dramatic stimulation of transcription that these hormones bring about for the α -amylases and other secreted hydrolases. Although much has been learned about the signaling elements and trans-acting factors required for high rates of α -amylase transcription, the scope of previous studies has been restricted to examining the responses of a few, usually highly expressed genes. Here we have used an oligonucleotide microarray to characterize global

changes in transcription that occur in rice half-grains treated with GA, ABA, or no hormone. The data presented here include transcript profiles during an 8-h time course for approximately one-half of all rice genes. These data give us a much more complete picture of GA- and ABA-regulated gene expression in this tissue and have revealed heretofore unknown aspects of transcript regulation in rice aleurone layers. In particular, we have shown that the transcriptome in imbibed rice half-grains contains RNAs for approximately 11,000 of the 23,000 probe sets on the microarray (Table I). This extensive set of RNAs is dynamic, with large changes in transcript abundance occurring with time in the presence or absence of exogenous GA or ABA (Fig. 8). Despite this, genes specifically up- or down-regulated by GA or ABA alone are rare (Fig. 3). We have shown that the G_{α} -subunit of heterotrimeric G-proteins amplifies

changes in transcript abundance in GA- or ABA-treated half-grains, but that this effect is small for all but a handful of genes (Figs. 5 and 6). Finally, we have shown that loss of the G_α -subunit does not result in reduced sensitivity to GA at the level of transcription (Fig. 7).

Our microarray data include transcript profiles for several well-characterized groups of proteins. In all cases, the microarray data are entirely consistent with previously published data. For example, we see large, GA-stimulated increases in transcription of genes for α -amylases, endo-1,4- β -xylanases, and proteases (Fig. 1, A and B; Supplemental Tables I, A and B, and III). Similarly, dehydrin genes and LEA genes have been shown on numerous occasions to be induced by ABA, and the data presented here show this as well (Fig. 2, A and B; Supplemental Table II, A and B). The down-regulation of these same genes by GA is also illustrated in Figures 1E and 2, A and B.

The analysis presented in Figures 1 and 2 allowed us to visualize common patterns of gene expression in rice aleurone layers treated with no hormone, GA, or ABA. These patterns were based on a subset of the data that had been enriched for probe sets that were hormone responsive, with 16 data points for each probe set. As a result, the patterns shown in Figures 1 and 2 are relatively insensitive to random variation in intensity at any one point. Specific expression values for the individual genes contained in each cluster were omitted from Supplemental Tables I and II, however, in recognition of the fact that only one hybridization was done at each time point for each hormone treatment.

We have found that rice half-grains contain a surprisingly large number of mRNA species. At all time points in all treatments (i.e. in the data from 16 separate microarray hybridizations), 9,762 probe sets were called present out of 23,000 possible probe sets on the chip. This percentage of the probe sets hybridizing with RNA from the samples (41%) is similar to the percentage of the genome transcribed in a barley leaf (45%; Close et al., 2004), although the identity of the individual mRNAs is likely to be different. For example, genes for endo-1,4- β -xylanases and LEA proteins are highly expressed in rice aleurone, but are not expected to be abundant in leaves. Because the aleurone layer is the only living tissue in the rice half-grain, it is highly likely that these mRNAs have their origin in the aleurone layer, although a few preexisting mRNAs may be present in the dead starchy endosperm (Potokina et al., 2002).

Despite the large number of mRNAs present in rice half-grains, very few were hormonally responsive. We looked for changes in transcript abundance that were associated specifically with GA and ABA treatment by comparing transcript profiles of tissue treated with these hormones to profiles from tissue incubated for the same length of time without hormone (Figs. 1–3). At most, 59 genes were specifically up-regulated 2-fold in GA-treated tissue and 42 genes in ABA-treated tissue (Fig. 3). Regulation of transcription by GA or ABA has been examined by others using microarrays, and, in general, larger numbers of hormone-responsive

genes have been identified. In rice callus, 206 out of 20,500 genes were up-regulated with GA and 110 genes were up-regulated with ABA 3 d after hormone treatment (Yazaki et al., 2004). For 2-week-old rice seedlings, 43 of 1,700 cDNAs were induced by ABA (Rabbani et al., 2003). For rice shoots, 29 of 4,000 cDNAs were up with GA and 42 were down with GA 24 h after hormone treatment (Yang et al., 2004), and 30 of 6,144 cDNAs were up with ABA and seven down with ABA within 12 h of hormone treatment (Lin et al., 2003). Accounting for these differences is difficult, but with the exception of experiments with rice callus, the larger number of hormone-responsive genes may reflect the greater number of cell types involved.

Most previously published data on aleurone cells supported the view that GA up-regulated a large number of genes in this tissue. ABA antagonized this effect of GA for a few genes, and ABA had been shown to up-regulate several genes on its own. Our data add substantial breadth to this simple model for gene regulation in the aleurone layer. In particular, we showed that GA specifically down-regulated about one-third as many genes as it up-regulated (Fig. 3). Surprisingly, ABA up-regulated as many genes as did GA, and it is noteworthy that virtually no genes were specifically down-regulated by ABA (Fig. 3). With a few exceptions, transcripts that decreased in abundance in ABA-treated tissues also decreased in one of the other treatments to a similar extent (Figs. 1, 2, and 8). Because of this, we speculate that the promoters of genes that are strongly down-regulated in ABA-treated aleurone cells must contain regulatory elements for binding factors that are not dependent on ABA perception or concentration.

Many genes showed a temporal change in transcript abundance during the 8 h of the experiment (Fig. 8). In many cases, these changes were not dependent on hormone treatment (Fig. 8). We hypothesize that many of these changes result from imbibition of the half-grains and rehydration of the aleurone cells. We identified those genes that increased or decreased 2-fold or more 8 h after hormone treatment in two separate experiments (Tables II–V). Virtually none of the genes identified were obvious signal transduction components such as protein kinases or transcription factors. A notable exception was a CBL gene that we have characterized in detail (Hwang et al., 2005). This analysis includes northern blots for this CBL that confirm the expression pattern seen in the array data (Hwang et al., 2005). A GAMyb was among the 59 genes specifically up-regulated by GA (Supplemental Table III). Other previously identified signaling elements whose transcription is regulated by GA or ABA, such as some of the WRKY-domain transacting factors, are present on the microarray. The expression of these genes, however, does not change enough for them to be flagged by our analyses as hormonally or temporally regulated genes. Overall, our data suggest that aleurone cell function during this period utilizes preexisting signaling components or posttranslational modifications

rather than large changes in transcription for the regulation of cellular activity.

One of the GA-signaling mutants that has been characterized in rice is the *d1* mutant. We used *d1* to see how widespread the effect of the G_α -protein was on transcript abundance and to determine the effect that GA has on transcript accumulation at high (5 μM) and low (100 nM) concentrations of GA. Our data make it clear that transcript abundance for many genes is affected by G_α (Figs. 5–7) and that the relative difference is larger as the gene is more strongly up- or down-regulated with GA (Fig. 5). This is true for tissue treated with both high and low GA concentrations (Fig. 5, A and B). G_α also influenced transcript accumulation in ABA-treated tissue (Fig. 6). As for GA-regulated genes, the effect was larger for those groups of genes that were most strongly up-regulated with ABA.

We also tested the hypothesis that the G_α -protein affects the sensitivity of transcription to GA concentration. Previously published data show clearly that the G_α mutation reduces the sensitivity to GA of α -amylase secretion and internode elongation. Our data, however, show that the sensitivity of transcription to GA is no different in *d1* mutant half-grains than in wild-type half-grains. This was demonstrated for both the α -amylases (Fig. 7A) and the GA-regulated genes that had been identified in an independent experiment (Fig. 7B). In both cases, there was a linear relationship between changes in transcript abundance with 5 μM GA and changes in transcript abundance with 100 nM GA, and this relationship was the same for wild-type and *d1* mutant half-grains. It should be noted that our experiments looked at changes in expression that occurred within 8 h of treating rice half-grains with GA, whereas expression data after 4 d gave rise to the bypass model (Ueguchi-Tanaka et al., 2000). It is possible that differences in transcript accumulation that occur at later times are mediated by the G_α -protein and that these differences are more sensitive to GA concentration at later times than at earlier times.

Although the *d1* mutation affects transcript abundance for many genes, most of the changes were small (Figs. 5 and 6). Because of this, we propose that the G_α -protein is used to fine tune changes in transcript accumulation. Additional roles for G_α are also possible. The microarray data, however, raise fundamental questions about how the *d1* phenotype arises. In particular, does the phenotype result from the subtle changes in gene expression that we observed, or are most of these changes in gene expression a consequence of the dwarf phenotype?

MATERIALS AND METHODS

Plant Material

Wild-type and *d1* mutant rice (*Oryza sativa* L. sp. Japonica cv Nipponbare) grains were a gift from Makoto Matsuoka (Nagoya University). Some grains were used immediately. Others were germinated and seedlings were grown to maturity in a glass house in Berkeley, California, under natural light conditions. Grains were harvested from the primary inflorescence and tillers of

these plants as they ripened. The *d1* mutant is in the Nipponbare background, and grains from *d1* mutant plants grown in Berkeley were harvested from plants grown alongside wild-type plants.

Hormone Treatment of Grains

Grains were cut transversely to remove the embryo, surface sterilized with 1% sodium hypochlorite and 0.01% Tween X-20 for 10 min with shaking, and washed extensively with distilled water. Residual hypochlorite was removed by soaking the grains in 0.01 N HCl for 10 min followed by additional washes with distilled water. De-embryonated grains (half-grains) were placed in sterile, tall 10-cm-diameter petri dishes containing 15 mL of sterile distilled water at 28°C. After overnight (18 h) incubation designed to minimize changes in transcription caused by cutting, sterilizing, or shaking the grain, distilled water was replaced by 20 mL of 10 mM CaCl₂. Some petri dishes also received ABA or GA₃ to a final concentration of 5 μM and, in the case of the *d1* mutant experiment, 100 nM GA₃. Each petri dish was shaken gently at 100 rpm at 28°C for the indicated time prior to RNA extraction.

RNA Extraction and Hybridization

RNA extraction was performed by the method described by Hwang et al. (1999), followed by re-extraction using the Qiagen Plant RNeasy kit (Qiagen). For the rice time-course experiment, total RNA was extracted 0.5, 1, 3, 6, and 8 h after hormone addition for samples treated with ABA, GA, or no hormone. No-hormone samples were also isolated at the time of hormone addition (time zero). Typical extractions started with 70 half-grains. For the *d1* experiment, RNA was isolated 8 h after treatment of wild-type and mutant grains with 5 μM GA, 100 nM GA, or 5 μM ABA. RNA was also isolated from wild-type and *d1* grains that were not treated with either GA or ABA (no hormone) at 0 h. For each hybridization, RNA from two or more biological replicates was pooled in order to minimize variability in the data. For both the time-course experiment and the *d1* experiment, single hybridizations were done for each of the 21 combinations of genotype, hormone treatment, and time. The data reported here, therefore, come from 24 separate microarrays.

All microarray experiments were done using the custom-built Genechip described by Zhu et al. (2003). Preparation of cDNA and cRNA, fluorescent labeling, hybridization to the chip, and quality control steps were done exactly as described previously (Zhu et al., 2003). Briefly, double-strand cDNAs were synthesized from 5 μg total RNA using an oligo(dT)₂₄ primer containing a 5' T7 RNA polymerase promoter sequence and SuperScript II reverse transcriptase (Invitrogen). Biotinylated complementary RNAs were transcribed in vitro from synthesized cDNA by T7 RNA polymerase (ENZO Biochem). Labeled cRNAs were applied to the rice GeneChip microarray, and subsequent hybridization, washing, and staining were conducted according to the Affymetrix recommended protocol. The image files (*.DAT) and raw files (*.CEL) were acquired using Microarray Analysis Suite 5.0 (Affymetrix). The signal intensity of a probe set was computed using the Robust Multi-Array Average method (Irizarry et al., 2003). If the difference is greater than 2, a present call will be assigned; if the difference is less than 0, an absent call will be assigned; a marginal call will be assigned if the difference is between 0 and 2.

Data Analysis

k-means clustering was done using EPCLUST software from the European Bioinformatics Institute (<http://ep.ebi.ac.uk/EP/EPCLUST>). To better visualize hormone-dependent responses, the data from the complete rice time-course experiment were enriched for GA-responsive genes or for ABA-responsive genes. Probe sets that had minimum intensity values <5 as well as maximum intensity values <25 were removed from the initial dataset of 23,780 probe sets because these genes are unlikely to be expressed at any time in rice half-grains. From the remaining 18,144 probe sets, we removed those probe sets that had $P > 0.05$ for an *F* test that compared samples treated with no hormone at 0.5, 1, 3, 6, and 8 h with samples treated with either GA (for GA-responsive genes) or ABA (for ABA-responsive genes) at 0.5, 1, 3, 6, and 8 h. To reduce the effect of expression intensity on the final clusters, the data were transformed by taking the natural log of the intensity value at each time point divided by the average intensity of the 0-, 0.5-, and 1-h samples treated with no hormone. These first three time points rarely showed differences and were used to give a more accurate estimate of mRNA abundance at the start of the experiment. Euclidean distance was used to cluster the data, and individual probe sets that repeatedly showed up in single member clusters were

excluded from the final dataset. Other statistical analyses were done using Microsoft Excel. For one-tailed *t* tests, we compared intensity data for groups of 25 probe sets with the 1,000 probe sets near the middle of the dataset whose average ratio of wild-type to *d1* expression equaled 1.00.

For scatter plots comparing wild-type to *d1* expression ratios, average induction by GA or ABA was computed as follows. Induction for a probe set was the intensity value at 8 h in samples treated with 5 μ M GA (Fig. 5) or 5 μ M ABA (Fig. 6) divided by the intensity value at 0 h. For each probe set, two values of induction were computed, one for samples from wild-type half-grains and one for samples from *d1* mutant half-grains. The average induction for each group of 25 genes was computed as the mean of 50 individual induction values, 25 from wild-type and 25 from *d1* mutant half-grains. This allowed the probe sets to be sorted from most up-regulated with GA to most down-regulated with GA without a strong bias for either wild-type or *d1* mutant grains.

Public Access to Microarray Data

All of the *.chp files for all of the experiments described in this article will be made available through the Internet. Proprietary probe set sequences and gene annotations for all of the genes (included in Figs. 1, A–E, I, and 2, A and B; Tables II–V; and Supplemental Tables I, A–E, I, II, A and B, and III) are included as supplemental data. This list of approximately 895 probe sets contains all of the genes described in the “Results” section of this article.

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