Diverse Roles and Mechanisms of Gene Regulation by the Arabidopsis Seed Maturation Master Regulator FUS3 Revealed by Microarray Analysis

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The FUSCA3 (FUS3) transcription factor is considered a master regulator of seed maturation because a wide range of seed maturation events are impaired in its defective mutant. To identify comprehensively genes under the control of FUS3, two types of microarray experiments were performed. First, transgenic plants in which FUS3 expression could be induced by the application of estrogen (ESTR) were used to identify any genes up-regulated in young seedlings of Arabidopsis in response to the ectopic expression of FUS3. Secondly, the transcriptomes of the fus3 mutant and wild-type developing seeds were compared. The combined results of these experiments identified genes under the relatively immediate and robust control of FUS3 during seed development. The analysis has extended the range of identified gene types under the control of FUS3. The genes positively controlled by FUS3 are not confined to previously known seed maturation-related genes and include those involved in the production of secondary metabolites, such as glucosinolates, phenylpropanoids and flavonoids, and those involved in primary metabolism, such as photosynthesis and fatty acid biosynthesis. Furthermore, several different patterns were identified in the manner of ectopic activation by FUS3 with respect to the induction kinetics and ABA requirement of downstream gene induction depending on the nature of developmental regulation, suggesting mechanistic diversity of gene regulation by FUS3.

Keywords: ABA • Arabidopsis thaliana • B3 transcription factor • Estrogen • fusca3 (fus3) • Microarray • Seed development.

Abbreviations: Cy3, cyanine 3; Cy5, cyanine 5; DAF, days after flowering; ESTR, estrogen; FDR, false discovery rate; FUS3AC, FUS3-activated; fus3DR, fus3-down-regulated; GO, gene ontology; LEA, late embryogenesis abundant; QT, clustering using 13 developmental data sets; QT-C, clustering using 36 developmental data sets; QT-F, clustering of FUS3AC genes.

Introduction

The production of seeds in flowering plants is one of the vital strategies for the survival and propagation of their offspring. The embryo acquires resistance to extreme environmental conditions of temperature and water status during seed development and survives in a desiccated quiescent or dormant state until favorable conditions for further development and growth are secured (Bewley 1997). In addition to the acquisition of resistance to adverse external conditions, the embryo accumulates nutrient reserves such as storage lipids and proteins to insure the expected resumption of development, namely germination and early seedling growth (Santos-Mendoza et al. 2008). The seed maturation refers to the acquisition of embryonic characteristics that are essential for the seed to function after the basic body pattern is established; the maturation phase is, therefore, a highly specialized period of plant development (Santos-Mendoza et al. 2008). The seed maturation phase appears to be controlled by a highly organized global genetic program because several regulatory genes whose mutations broadly affect the maturation events have been reported. These are considered master regulators of seed maturation (Vicente-Carbajosa and Carbonero 2005) and include FUSCA3 (FUS3), LEAFY COTYLEDON1 (LEC1), LEAFY COTYLEDON2 (LEC2) and ABSCISIC ACID INSENSITIVE3 (ABI3) (Giraudat et al. 1992, Meinke 1992, Meinke et al. 1994, Keith et al. 1994, West et al. 1994, Lotan et al. 1998, Luerssen et al. 1998, Stone et al. 2001). These genes, except for LEC1, encode transcription factors constituting a closely related subfamily of the plant-specific B3 DNA-binding domain protein family (Giraudat et al. 1992, Lotan et al. 1998, Luerssen et al. 1998, Stone et al. 2001). The B3 domain of these transcription factors has been demonstrated to recognize a canonical sequence element designated the RY repeat or Sph element, which is preferentially associated with seed-specific genes (Suzuki et al. 1997, Reidt et al. 2000, Kroj et al. 2003, Mönke et al. 2004). Although the exact sequence requirement for the RY repeat
as a cis-acting element for FUS3, LEC2 or ABI3 has not been determined, CATGCA is widely recognized as the minimal consensus sequence in these studies. In addition to directly binding these B3 factors, ABI3 is known to function by another mode of action as a transcription factor (Hobo et al. 1999, Nakamura et al. 2001). LEC1 encodes a paralog of the NF-YB subunit of the trimeric CCAAT-binding protein that is widely conserved in eukaryotes (Lotan et al. 1998). However, it has been demonstrated that LEC1 can regulate transcription by a mechanism other than acting as a subunit of CCAAT-binding factor (Yamamoto et al. 2009). To date, a number of genes, including genes for seed storage protein, lipid biosynthesis genes and oil body-targeted protein genes, have been identified to be under the control of the B3 master regulators (Santos-Mendoza et al. 2008). Most of the genes reported to be under the control of the master regulators are directly related to known seed maturation events; however, the roles of some other genes in seed development are not yet resolved. Evidence demonstrating that these genes are controlled by the B3 regulators varies; reports include decreased levels of gene expression in the corresponding mutant (Finkelstein and Somerville 1990, Finkelstein 1993, Kirik et al. 1996, Parcy et al. 1997, Haslekås et al. 1998; Kirik et al. 1998a, Kirik et al. 1998b, Nambara et al. 2000, Vicent et al. 2000, Wehmeyer and Vierling 2000, Brocard-Gifford et al. 2003, Curaba et al. 2004, To et al. 2006, Yamamoto et al. 2009), activation of respective genes or their promoter by the ectopically or heterologously expressed B3 regulator (either transiently or stably) (Hattori et al. 1994, Busk et al. 1999, Crowe et al. 2000, Tamminen et al. 2001, Haslekås et al. 2003, Suzuki et al. 2003, Gazzarrini et al. 2004, Kagaya et al. 2005a, Wang et al. 2007), dependence of such activation on the RY repeat cis-element (Ezzurra et al. 2000, Reidt et al. 2000) and combinations of some of these experiments (Parcy et al. 1994, Kirik et al. 2003, Mönke et al. 2004, Kagaya et al. 2005b, Braybrook et al. 2006, Nakashima et al. 2006, Baud et al. 2007, Kotak et al. 2007, Stone et al. 2008, Baud et al. 2009a, Che et al. 2009, Roschützardtz et al. 2009). Depending on the lines of evidence, some genes, such as the At2S seed storage protein genes (Kirik et al. 2003, Stone et al. 2008), can be definitely considered the direct targets of the B3 regulators, but indirect regulation has not been excluded for other downstream genes. Chromatin immunoprecipitation (ChIP) is one of the best methods currently available to identify direct targets of transcription factors. However, this technique has only been applied for the ectopic activation of some target genes of LEC2, because of apparent technical limitations (Stone et al. 2008).

The modes of action of the B3 regulators as transcription factors, i.e. the binding of the RY repeat to the cis-element and the intrinsic transcriptional activation function, are quite well established; yet, high degrees of complexity exist in the gene regulation mechanisms mediated by the B3 maturation regulators. First, the master regulators direct the complex networks comprising hierarchical and combinatorial regulation they are integrated in. The genes known to be under the control of the B3 regulators and/or LEC1 also include transcription factors expressed preferentially in seeds (Brocard-Gifford et al. 2003, Braybrook et al. 2006, To et al. 2006, Baud et al. 2007, Kotak et al. 2007, Yamamoto et al. 2009). Some of them, e.g. L1L and bZIP67, appear to act not only as intermediate transcription factors in a transcription cascade but also as partners of combinatorial regulation with B3 and/or LEC1 (Yamamoto et al. 2009). The second level of complexity results from the functional differentiation and redundancy of the three B3 maturational regulators, FUS3, LEC2 and ABI3. Because these three B3 regulators all have highly conserved DNA-binding domains, they could function redundantly (Giraudat et al. 1992, Luerssen et al. 1998, Stone et al. 2001). However, the mutants of each regulator exhibit partially common but distinct effects on seed maturation. An extensive genetic study using mono-, di- and trigenic mutants, combined with reporter genes under the control of the promoters of these regulators and their target genes, revealed partially redundant molecular functions of these factors, which are controlled by complex regulatory networks consisting of self- and mutual regulation, as well as spatial regulation (To et al. 2006). Although the redundancy of molecular function among the B3 factors is evident, there are distinctions (Kroj et al. 2003). As mentioned above, ABI3 functions not only by direct DNA binding via the B3 domain but also by interacting with another transcription factor (Hobo et al. 1999, Nakamura et al. 2001). FUS3 and LEC2 may also function in this way by interacting with different factors. While the function of FUS3 and LEC2 as transcription activators with an intrinsic activation capacity has been demonstrated, there exists a two-step mechanism of gene activation in which ABI3 or FUS3 induce chromatin remodeling to depress target genes; the latter are subsequently activated by other signals such as ABA (Li et al. 1999, W-K Ng and Hall 2008). Therefore, the mode of action of these B3 factors is rather complex.

Mutant embryos defective in both the B3 factors and LEC1 not only fail to undergo maturation, but they also express germinative or post-germinative characters; thus they are characterized as heterochromatic mutants (Keith et al. 1994, Gazzarrini et al. 2004). However, the mechanisms regarding how these regulators repress genes that should not be expressed during embryogenesis are largely unknown. The decreased levels of gene expression in the mutant embryos may, therefore, not be readily taken as defects in positive regulation; either a direct or indirect mechanism could involve cascades of transcriptional regulation, but this could be interpreted as a manifestation of post-embryonic character.

For a more comprehensive understanding of the complex regulatory system of seed development governed by the B3 regulators, genome-wide information about the genes under the control of the B3 regulators is essential, even though a number of such genes are already known. Transcriptomic analyses using microarray have been reported for the genes induced by the ectopic expression of LEC2 and of VP1, the maize ortholog of ABI3 (Suzuki et al. 2003, Braybrook et al. 2006). However, the effects of the mutations of these B3 factors on the transcriptome of developing seeds have not
been investigated. Here, we characterized the impact of the fus3 mutation and the ectopic induction of FUS3 on the transcriptome of developing seeds and seedlings, respectively. The analysis robustly identified novel genes controlled by Fus3 and revealed extended ranges of functional categories of the downstream genes and diverse mechanisms of gene regulation by Fus3.

Results and Discussion

Overview of microarray experiments

To identify the genes under the control of Fus3, two different types of microarray experiments were performed using the Agilent 22K Arabidopsis oligo DNA microarray by the cyanine 3/cyanine 5 (Cy3/Cy5) two-color method. First, ER-FUS3-DH transgenic plants (Kagaya et al. 2005a), in which Fus3 expression could be induced by the application of estrogen (ESTR), were used to identify the genes up-regulated in young Arabidopsis seedlings in response to the ectopic expression of Fus3. This analysis was expected to identify genes that were under the relatively immediate control of Fus3. These new genes might include those that require a limited number of intermediate factors for their activation; some of these new genes could also be under the direct control of Fus3. Secondly, transcriptomes of wild-type (Col-0) and fus3-3 mutant developing seeds were compared to identify differentially expressed genes. In the first analysis, certain genes induced by the ectopic expression of Fus3 may be artifacts and may not necessarily represent the control of Fus3 in the developing seeds. In contrast, the second comparative transcriptome analysis should identify genes under the control of Fus3 during seed development in a broad sense. However, the differential expression of certain genes between the wild type and mutant may reflect the indirect effects of the mutation due to the heterochronic developmental defect (Keith et al. 1994, Gazzarrini et al. 2004). Thus, combining these two complementary analyses should strengthen the genome-wide view of Fus3-regulated genes.

Identification of genes affected by the fus3 mutation during seed development

To identify genes whose expression is affected by fus3 mutation, microarray analyses were performed with RNA from developing seeds of wild-type and fus3-3 plants at 8 days after flowering (DAF; bent cotyledon stage) and at 12 DAF (green cotyledon stage); these sampling points roughly corresponded to the onset and active stage of seed storage protein mRNA synthesis, respectively. Three biological replicates of the wild type and fus3 sample pairs were analyzed by two-color microarrays. For each comparison, the average of the fus3/wild type signal ratio for each probe from dye-swapped hybridizations was used as the data set. Genes that were differentially expressed between the wild type and mutant were assessed by the rank product method (Breitling et al. 2004) using a false discovery rate (FDR)-corrected probability value of $q<0.05$. Among the 21,500 probes on the array, the analysis determined that the expression levels of 785 genes were decreased and 644 probes were increased in the mutant at either 8 or 12 DAF (Supplementary Table S1). The 644 genes up-regulated in fus3-3 developing seeds, though not discussed here in depth, may mainly represent the operation of the post-germinative program due to the heterochronic nature of the mutant. In addition, they may include genes related to the pleiotropic effects conditioned by the truncation gene product of the fus3-3 allele (Tiedemann et al. 2008). The genes with decreased levels of expression in the fus3 mutant ($q<0.05$) are defined here as fus3-down-regulated (fus3DR) genes and were mainly analyzed in this study. Certain genes showing differential expression at $P<0.05$ (rank product) without FDR correction were also considered for the extended interpretation of the results in some cases.

Genes induced by the ectopic expression of Fus3

One of the ESTR-inducible Fus3 expression lines (ER-FUS3-DH), which we previously referenced in the study of Fus3 regulation of seed storage protein genes, was used here (Kagaya et al. 2005a). Seven-day-old seedlings of the ER-FUS3-DH line were treated with ESTR, ABA, or both at time zero and cultured for 3, 6, 12, 24 or 48 h. RNA was prepared from samples taken at each time point of each treatment and used to prepare Cy5-labeled cRNA. The labeled RNA was subsequently probed by a 22K Arabidopsis oligo DNA array with the reference Cy3-labeled cRNA prepared from the time zero untreated seedlings. The ABA treatment was included in the analysis because the induction of 12S seed storage protein (Crucliferin C; CRC) by Fus3 was previously reported as being strongly dependent on the presence of ABA (Kagaya et al. 2005a). The effects of Fus3 induction by ESTR, ABA or combined ESTR/ABA treatments were determined by comparing the relative signals (i.e. Cy5/Cy3 signal ratios) between the treatments at each time point. Gene activation by Fus3 at each time point was evaluated by the ratios of the relative signals of each gene probe between the ESTR alone (E) and the control treatment (N) or between ESTR plus ABA (D) and ABA alone (A). The genes whose probes showed a relative signal or ratio of E/N >1.5 at (at least) two consecutive time points, with a maximum E/N ratio >2 in two independent experiments, were defined as those induced by Fus3 alone. The same criteria were used for genes induced by Fus3 in the presence of ABA by using the D/A ratio instead of E/N. Similarly, the A/N ratio was used to define ABA-inducible genes. Using these criteria, 374 and 332 genes were identified as those that were induced by Fus3 alone and those that were induced by Fus3 in the presence of ABA, respectively (Supplementary Fig. S1). The genes activated by Fus3 only in the presence of ABA, which totaled 186, were considered as demonstrating Fus3 induction in an ABA-dependent manner (Supplementary Fig. S1). Thus, a total of 560 genes were determined to be induced by the ectopic expression of Fus3 in either the absence or presence of ABA; they were designated Fus3-activated (Fus3AC) genes. Among the fus3DR genes,

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Microarray analysis of gene regulation by Fus3

**Supplementary Fig. S1**

**Supplementary Table S1**
Developmental expression patterns of \textit{fus3}DR genes

Because FUS3 is one of the major regulators of seed maturation, one would expect that \textit{fus3}DR genes are mostly seed specific or genes preferentially expressed in seeds. However, little information about the ranges of developmental regulation of genes that are affected by \textit{fus3} mutation is available. To investigate the developmental regulation of the \textit{fus3}DR genes and to assess if any common features of developmental regulation are shared by the \textit{fus3}DR genes, clustering analysis was performed using the AtGenExpress developmental array data set (Schmid et al. 2005). First, the 727 \textit{fus3}DR genes included in the Affymetrix ATH1 probe set were subjected to QT clustering using data sets of 36 developmental samples selected from the AtGenExpress developmental array data. This clustering analysis is denoted ‘QTC-D36’ hereafter. Seventeen clusters (clusters I–XVII) and an unassigned group were resolved by this analysis (Supplementary Fig. S2). The largest cluster, cluster I, represented about 40% of \textit{fus3}DR genes showing clear seed-specific or seed-preferential expression patterns. However, each of the clusters other than cluster I included genes that are expressed not only in developing seeds or siliques but also in other tissues; these genes were expressed at comparable or higher levels in an organ-dependent or developmentally regulated manner. Interestingly, none of them exhibited constitutive or relatively flat expression profiles. These results indicated that the \textit{fus3} mutation down-regulates not only seed-specific/preferential genes but also other developmentally regulated genes. Notably, the genes in the second largest cluster, cluster II, representing 11% of the \textit{fus3}DR genes, are expressed at substantial levels in above-ground organs such as cotyledons, leaves, shoot apex and flowers, but at very low levels in roots, in addition to the expression detected at early stages of seed development (stages 3–7). While the \textit{FUS3}AC genes were significantly enriched in cluster I, they were apparently depleted in cluster II (Supplementary Fig. S2 and Table S2). The down-regulation of these non-\textit{FUS3}AC genes could result from indirect effects of \textit{fus3} mutation, or from the heterochronic or abnormal embryo development of the mutant. Alternatively, the high basal expression levels in the seedling tissues made the effect of the ectopically expressed \textit{FUS3} unobservable although they are positively regulated by FUS3 in a direct or relatively immediate manner. The next largest cluster, cluster III, exhibited significant expression in the floral organs and the shoot apex in addition to stages 3–7 of the developing seed. The \textit{FUS3}AC genes were slightly more enriched in cluster III than average, unlike the genes in cluster II (Supplementary Fig. S2 and Table S2).

To emphasize the expression patterns of the \textit{fus3}DR genes during seed development, QT clustering was performed using AtGenExpress data for three siliques, five seed and 7-day-old seedling organ samples (cotyledons, root, hypocotyl, true leaf and shoot apex; Fig. 1). We denoted this clustering analysis ‘QTC-D13’ hereafter; it classified 406 \textit{fus3}DR genes into 11 clusters (clusters A–K), each of which had a similar expression profile; also, 321 genes were classified as unassigned. Except for cluster D, all the clusters represented the genes with more or less preferential expression in developing seed/silique relative to the 7-day-old seedling organs. This indicates that the clustering was mainly based on differential expression patterns during seed development. It means that the genes with a range of different temporal patterns during seed development, which is not confined to the maturation period, were down-regulated in \textit{fus3} compared with the wild type. For example, the expression levels of the genes in cluster K were high at the early stages (stages 4–6), declined at stage 7 and became very low at the later stages; in contrast, gene expression levels in cluster B started to increase at stage 7 and were high at later stages. Half of the genes in the largest cluster, cluster A (126 genes), were already expressed at substantial levels at stage 4 and at even higher levels thereafter (until stage 10). The remaining cluster A genes were delayed in the onset and increase in the expression levels but showed sustained high-level expression, like the other half of the cluster A genes. The cluster A genes, particularly those of the former group, were highly enriched in \textit{FUS3}AC genes (like cluster I of QTC-D36) and in those having RY elements in the promoter, indicating that these genes are more likely to be under the direct control of FUS3.

The cluster B genes were clearly scarce in \textit{FUS3}AC genes compared with cluster A. Cluster B contained late embryogenesis abundant (LEA) protein genes, represented by \textit{AtEm1}. It also contained \textit{ABIs}, a gene which encodes a bZIP transcription factor involved in LEA gene regulation. This may indicate that the \textit{fus3} mutation down-regulates late embryogenesis genes less directly in comparison with cluster A genes. Considering the heterochronic nature of the \textit{fus3} mutant, the down-regulation may reflect the bypassing of the late embryogenesis program in the mutant embryo. When compared with the list of the genes reported to be highly activated (>10-fold) in the vegetative tissues of the transgenic Arabidopsis constitutively expressing VP1, the maize ortholog of ABI3 (Suzuki et al. 2003), most of them (22/28) were \textit{FUS3}AC and belonged mostly (19/22) to cluster A (of the remainder, two belonged to cluster C and one was unassigned) (Supplementary Table S3). Interestingly, however, five of the six non-\textit{FUS3}AC genes among the VP1 highly activated genes belonged to cluster B. Only one cluster A gene in this group appeared to be activated by FUS3, although it did not satisfy the \textit{FUS3}AC criterion. This indicates that ABI3 has a direct role in the control of the late embryogenesis program, and this is in line with the view that
Fig. 1 QT clustering of fus3DR genes using data from 13 selected developmental samples (QT-D13) from AtGenExpress developmental arrays. QT clustering (diameter, 0.15; minimum cluster size, 10) was performed with the MeV software using the median values of GCRMA-normalized data of the AtGenExpress developmental array data. Each cluster is presented by a heat map after hierarchical clustering by Pearson’s correlation. The FUS3 AC (ectopic activation) genes are depicted in green. Genes with an RY element (CATGCA) in the 0.5 and 1 kb upstream region are depicted in blue and red, respectively.
the effect(s) of the fus3 mutation on late embryogenesis resulted indirectly from the developmental defect.

Mechanistic consideration of FUS3-regulated gene expression

Using the time course data sets of the two replicate experiments, QT clustering analysis of the 190 genes that are both FUS3AC and fus3DR revealed that the pair of expression patterns for each gene were co-clustered with significant frequency, although some data pairs were classified into different, though similar, clusters; this confirmed the reproducibility of the two experiments (Fig. 2 and Supplementary Table S4). This clustering analysis is denoted ‘QTC-FAC’ hereafter. Because high sensitivity conditions were used, about half of the data sets were classified into an unassigned group. Further clustering, using a different method (K-means clustering), of the unassigned group also resulted in some groups with similar patterns to and others distinct from those of the first clustering (Supplementary Fig. S3). These analyses showed that FUS3 activates fus3DR genes with varying kinetics and distinct ABA dependence (Fig. 2A, C). Genes in clusters 1, 3 and 5 exhibited a strong ABA dependence of the FUS3 activation with different kinetics. In contrast, cluster 2 and 4 showed a much smaller ABA dependence, if any. Generally, the FUS3 activation of cluster 2 and 4 genes was not large in their fold changes, but was relatively rapid in their induction kinetics compared with cluster 1, 3 and 5 genes. Cluster 6 genes could be induced either by ABA or by FUS3 alone, but were synergistically activated by both. The activation of cluster 4 genes was often detectable at 3 or 6 h of ESTR treatment. Their expression levels mostly peaked at 24 h of the ESTR treatment and declined thereafter in the absence of ABA. In some cases, ABA enhanced the FUS3 activation slightly at the peak time point and appeared to delay or suppress the decline. These induction patterns and the effect of ABA resembled those of the FUS3 transcript, although the kinetics of FUS3 induction were faster (Fig. 2B). This suggests that cluster 4 genes are regulated by FUS3 in a more direct and simple manner compared with genes included in the other clusters. The induction pattern and ABA dependence of cluster 2 genes were relatively similar to those of cluster 4 genes except that the cluster 2 genes may require the prior activation of genes to provide intermediate factors. Compared with the relatively fast and ABA-independent increases and subsequent declines of the cluster 2 and 4 gene transcripts, which appeared to follow temporally that of FUS3, the strongly ABA-dependent and slower activation of cluster 1, 3 and 5 genes suggests the involvement of more complex processes in their ectopic activation by FUS3. The cluster 5 genes, as well as many cluster 1 and 3 genes, did not exhibit a decline in their transcript levels even after 36 h, when the FUS3 transcript level was decreased substantially in experiment 1. This suggests that FUS3 is not the main factor supporting the high levels of sustained expression of these genes.

It should be noted that clear differences in the developmental regulation between cluster 2/4 and cluster 1/3/5 genes were found. Cluster 1/3/5 genes were mostly (79%) found and significantly enriched in cluster I of QT-D36 of fus3DR (Supplementary Fig. S2 and Table S5); the latter represented most seed-preferentially expressed genes. In contrast, cluster 2/4 genes were slightly depleted in cluster I. Thus, cluster 1/3/5 genes were mostly seed specific or preferential genes, while cluster 2/4 genes were expressed not only in seeds but also in other tissues. Further hierarchical clustering of cluster 1 genes and of selected seed-specific genes strengthened this conclusion (Fig. 3). In addition, the latter analysis revealed that the vast majority of seed-specific genes were fus3DR genes and that most of the seed-specific FUS3AC genes (cluster 1/3/5 genes) are expressed during longer ranges of seed development compared with non-FUS3AC genes (Fig. 3, node 2-1); the expression of non-FUS3AC genes was confined to either earlier or later development (Fig. 3, node 1 or node 2-2, respectively).

The strong ABA dependence cluster 1/3/5 gene activation by FUS3 may partly reflect the two-step mechanism involving the derepression associated with changes in the chromatin states and the true activation required by ABA signaling (as originally proposed for the regulation of the 7S seed storage protein promoter of Vicia faba by ABI3 and by FUS3), although the detailed mechanisms may be different for ABI3 and FUS3 (Li et al. 1999, W-K Ng and Hall 2008). The relatively fast activation kinetics of cluster 2/4 genes may be due to the non-seed-specific nature of these genes because it would not require the derepression step.

The chromatin of seed-specific genes has been reported to be associated with repressive epigenetic markers such as histone H3-K27 trimethylation and like-heterochromatin protein (LHP) in vegetative tissues with a high frequency (Zhang et al. 2007a, Zhang et al. 2007b); thus, FUS3 may trigger the removal of such markers, and the relatively slow kinetic activation of cluster 1/3/5 genes could be partly due to the need to liberate them from the repressive chromatin states. The true activation of cluster 1/3/5 genes could be achieved either by transcription factors already present in vegetative tissues or by those ectopically induced by FUS3. For example, we previously showed that the seed storage protein gene CRC can be activated in an ABA-dependent manner by a combination of transcription factors L1L and bZIP67, both of which are fus3DR and FUS3AC genes (Yamamoto et al. 2009). The differential kinetics of gene activation by the ectopically expressed FUS3 may depend on the complexities of the mechanism for the induction of transcription factors or on signals required for true activation. Alternatively, different mechanisms may be required for the release from the repressive states of chromatin. A portion of the fus3DR genes, which could not be ectopically activated by FUS3, may require signals other than ABA and/or transcription factors that could not be induced by FUS3. Alternatively, those fus3DR genes could not be liberated from the repressive chromatin states by FUS3 alone.
Fig. 2 QT clustering of FUS3AC genes including fus3DR genes (QTC-FAC). QT clustering (diameter, 0.1; minimum cluster size, 10) of the 190 FUS3AC genes was performed with the MeV software using the microarray data sets of the FUS3 ectopic induction time course experiments. The data from experiments 1 and 2 were combined and analyzed. (A) Time course expression profiles of the resulting six clusters and an unassigned group. Black, control treatment; red, treatment with ABA; blue, treatment with ESTR; green, treatment with ABA and ESTR. (B, C) Time course expression profiles of FUS3 (B), and typical genes in each cluster (C). Signal ratio relative to time 0 (fold) is plotted against time in hours. The color coding of the treatments is the same as that in A.
The over-representation in the fus3DR gene set ($P = 3.4 \times 10^{-3}$); genes in the related lower level categories such as ‘phenylpropanoid metabolic process’ (GO:0010876) were also significantly enriched. In addition to the genes related to phenylpropanoid metabolism, the fus3DR genes categorized in ‘secondary metabolic process’ included genes involved in the biosynthesis of flavonoids, several plant hormones and glucosinolates.

**Regulation of genes involved in fatty acid biosynthesis**

Analysis of the GO categories for the fus3DR genes identified genes that were not only those involved in secondary metabolisms but also those participating in primary metabolism such as photosynthesis and lipid metabolism (Table 1). The over-representation of genes in the category ‘lipid biosynthetic process’ was an expected result of GO analysis because lipids accumulate as a storage nutrient together with storage proteins, which are the major events of seed maturation; this finding was also expected because of the known effects of the fus3 mutation on the seed lipid accumulation (Keith et al. 1994, Meinke et al. 1994, Mu et al. 2008). The fus3DR genes in this category included WRI1 (WRI1), encoding a transcription factor that regulates fatty acid biosynthesis genes (Focks and Benning 1998, Ruuska et al. 2002, Baud et al. 2009b, Mao et al. 2009), and fatty acid biosynthesis genes such as those encoding acyl carrier proteins (ACP1 and ACP5; AT5G27200 and AT3G05020, respectively), omega-3 fatty acid desaturase (FAD3; AT2G29980), fatty acid elongases (KCS17 and KCS18/FAE1; AT4G34510 and AT4G34520, respectively), long-chain fatty acid-CoA ligase (LACS4; AT4G23850) and acyl-[acyl-carrier-protein] desaturase (SSI2; AT2G43710). Furthermore, manual inspection of a literature-based list of 47 genes involved in plastidial fatty acid biosynthesis and the production of the acetyl-CoA substrate (late glycolytic pathway; downstream of phosphoglucomutase) revealed that eight of them were fus3DR genes (Supplementary Table S6). Furthermore, if a $P<0.05$ cut-off without FDR correction was applied, the number of down-regulated genes in the fus3 mutant seed increased to as many as 34 (Supplementary Table S6). Thus, it is likely that most of the genes encoding enzymes for the fatty acid biosynthetic pathway are under the control of FUS3. Six of the eight fus3DR genes (among the plastidial fatty acid biosynthesis genes) belonged to cluster III and cluster E in QTC-D36 (Supplementary Fig. S2) and QTC-D13 (Fig. 1), respectively (Supplementary Table S6). The cluster III genes are expressed in floral organs and the shoot apex, in addition to the expression in the developing seed mainly up to stage 7, which declines thereafter (Supplementary Fig. S2). In accordance with these observations, several authors have described that during the course of seed development, the expression of fatty acid biosynthesis genes starts earlier than that of seed storage protein genes and declines when the storage protein gene expression peaks (Ruuska et al. 2002, Wang et al. 2007, 2009).

**Gene ontology (GO) of fus3DR genes**

To characterize the functional nature of the fus3DR genes, their GO categories were analyzed using the web-based GOToolBox program (Martin et al. 2004; http://genome.crg.es/GOToolBox/) by referring to the TAIR version of the GO database (Table 1). The genes in the GO category ‘secondary metabolic process’ (GO:0019748) were significantly over-represented in the fus3DR gene set ($P = 3.4 \times 10^{-3}$); genes in the related lower level categories such as ‘phenylpropanoid metabolic process’ (GO:0010876) were also significantly enriched. In addition to the genes related to phenylpropanoid metabolism, the fus3DR genes categorized in ‘secondary metabolic process’ included genes involved in the biosynthesis of flavonoids, several plant hormones and glucosinolates.

![Hierarchical clustering of seed-specific genes and distribution of the fus3DR and FUS3AC genes](https://academic.oup.com/pcp/article-abstract/51/12/2031/1944122/Downloaded from https://academic.oup.com/pcp/article-abstract/51/12/2031/1944122). The GCRMA-normalized signal values of 13 developmental samples of AtGenExpress selected in QTC-FAC were used to select seed-specific genes under the following conditions: maximum signal value of non-seed/silique samples $<10$; ratio of maximum signal value of seed/silique samples to that of non-seed samples $>10$; ratio of the maximum signal value of seed/silique samples to the average signal value of seed/silique samples $>10$. By applying these conditions, a total of 164 genes were selected. Assignments to the QTC-FAC clusters of the fus3DR ($q<0.05$) and likely fus3DR ($P<0.05$) genes are marked with color blocks. Hierarchical clustering was performed with the MeV software by the Euclidian distance method.
Baud and Lepiniec (2009). In addition, good correlations in temporal expression patterns of these genes and that of FUS3 have been demonstrated (Wang et al. 2007). Baud and Lepiniec (2009) described the highly coordinated expression of 20 fatty acid biosynthesis genes during seed development. When a $P<0.05$ cut-off was applied, all 20 genes were considered down-regulated in the fus3 mutant seeds. Among the nine fus3 DR fatty acid biosynthesis genes, six were found to be FUS3 AC genes (Fig. 4). Similarly, 14 of the 34 fatty acid biosynthesis genes, which were suspected to be down-regulated in the mutant when applying the $P<0.05$ criterion, were FUS3 AC genes (Supplementary Fig. S4). After manual inspection of the expression data, more of the latter genes were likely to be activated by the ectopic expression of FUS3, although they did not meet the FUS3AC criteria (Supplementary Fig. S5). Interestingly, many of these FUS3AC and likely FUS3AC genes showed very similar activation kinetics and response to ABA of the cluster 2/4 type. Their expression levels started to increase at 3 or 6 h after FUS3 induction, mostly peaked at 24 h and declined thereafter. The WR11 transcript levels followed similar kinetics and ABA responses to those of FUS3, although the onset of the activation may have been slightly earlier than the biosynthesis genes (Fig. 4). In addition, many of these genes were identified as genes downstream of WR11 or target genes. (Baud et al. 2007, Baud et al. 2009b, Maeo et al. 2009) Thus, the FUS3 regulation of the array of fatty biosynthesis genes is probably mediated by the regulation of WR11, as proposed for their regulation by LEC2 (Baud et al. 2007); FUS3 is suspected to regulate WR11 directly just like LEC2, considering the proposed functional redundancy of LEC2 and FUS3 (Kroj et al. 2003, To et al. 2006).

### Regulation of photosynthetic genes by FUS3

The over-representation of photosynthetic genes in the fus3DR genes was somewhat unexpected. The positive regulation of photosynthetic genes by FUS3 and other seed maturation regulators has not been previously described. The photosynthetic fus3DR genes were mostly categorized into cluster II (QTC-D36)
or cluster D (QTC-D13). After manual inspection of cluster II, 28 genes related to photosynthesis, both the light and dark reactions, were identified (Supplementary Table S7). However, three of them were classified as FUS3AC; of these, only one (AT2G40100; light-harvesting chlorophyll a/b-binding protein) was demonstrated to be simply and clearly induced by FUS3 (Supplementary Fig. S6). It should be noted that the down-regulation of photosynthetic gene expression in fus3 mutant

![Fig. 4 Time course expression profiles of the six FUS3-activated (FUS3AC) fus3DR fatty acid biosynthesis genes and WRI1 in the FUS3 induction experiments. The numbers in parentheses refer to the fus3/wild type signal ratio at 8 and 12 DAF, respectively. All other details of presentation are the same as those given in Fig. 2B and C.](https://academic.oup.com/pcp/article-abstract/51/12/2031/1944122)
seeds was observed only at 8 DAF; three genes were exceptions, two of which were classified as FUS3AC. Some of them were rather up-regulated at 12 DAF. When statistical analyses using P<0.05 without the FDR correction were performed, more than half (16) of them were considered up-regulated in fus3 at 12 DAF (Supplementary Table S7). This is consistent with the fact that during seed development the expression of cluster II or cluster D genes was confined to earlier stages. In addition, based on Murray's germination microarray data (Masubelele et al. 2005), about a half of the photosynthetic fus3DR genes are germination induced (Supplementary Table S7). Therefore, the up-regulation of these genes at 12 DAF could be considered the heterochronic expression of post-germinative genes. The fact that most of the fus3DR genes involved in photosynthetic processes were non-FUS3AC genes may indicate that the down-regulation of these genes at 8 DAF could be an indirect effect of fus3 resulting from abnormal development. However, considering the existence of an obvious FUS3AC photosynthetic gene and that the effect of a developmental defect of fus3 mutation on these genes was rather seen at 12 DAF as heterochronic expression, FUS3 may have an active role in positively regulating the expression of photosynthetic genes during early seed development.

**Regulation of plant hormone biosynthesis genes by FUS3**

In the GO analysis of the fus3DR genes, one of the over-represented categories, ‘secondary metabolic process’, included genes involved in the biosynthesis of several plant hormones. By manual inspection, we discovered that more genes involved in plant hormone biosynthesis were included in the fus3DR genes. These included two ABA, three gibberellin, two brassinos-teroid, two cytokinin, two auxin, one ethylene and one jas-mionate biosynthesis genes as summarized in Table 2. Although complex interplay among these plant hormones is suspected to occur during embryogenesis, it is premature to discuss the significance of their regulation by FUS3, because of the limited information available about the roles of these plant hormones in seed development, the exception being the well-described functions of ABA. However, the regulation of auxin and gibberel-lin biosynthesis genes may need to be referenced with respect to previous studies (Curaba et al. 2004, Gazzarrini et al. 2004, Stone et al. 2008). YUCCA4 (YUC4), encoding a flavin monooxygenase required for IAA biosynthesis (Cheng et al. 2006), has been reported to be ectopically activated by LEC2 in seedlings (Stone et al. 2008). This LEC2 regulation of YUC4 is proposed to occur via the direct binding of LEC2 to the YUC4 promoter, and to contribute to the ability of LEC2 to induce somatic embryogenesis when it is ectopically expressed (Stone et al. 2008). In this study, FUS3 was also found to activate the expression of YUC4 ectopically (Supplementary Fig. S7), although YUC4 was not included in the fus3DR genes (Table 2). However, the average expression signal ratio (fus3/wild type) was 0.58 and 0.5 at 8 and 12 DAF, respectively; these values were significant when the FDR correction was not applied (P=0.006 and 0.019, respectively). In addition, YUC10, which is thought to encode an enzyme redundant with that encoded by YUC4 together with other YUC genes (Cheng et al. 2006), was also significantly down-regulated in fus3, although a clear ectopic activation by FUS3 was not observed (Table 2). Therefore, not only LEC2 but also FUS3 is likely to regulate auxin biosynthesis positively via YUC4 and YUC10 during seed development. It should be noted, however, that FUS3 is not able to induce somatic embryo development, like LEC2 (Stone et al. 2001, Gaj et al. 2005, Braybrook et al. 2006, Kurczyńska et al. 2007, Stone et al. 2008). The positive regulation by LEC2 of a MADS box transcription factor AGL15, which itself can also induce somatic embryogenesis, is also proposed to contribute LEC2’s capacity to induce somatic embryo development (Braybrook et al. 2006). However, we also found AGL15 to be a FUS3AC and fus3DR gene here. Therefore, some key genes, other than YUC4 and AGL15, under the control of LEC2 but not of FUS3 may exist and should account for the differential activities of somatic embryo induction observed between FUS3 and LEC2.

It has been previously reported that FUS3 positively and negatively regulates the levels of ABA and gibberellin, respectively (Curaba et al. 2004, Gazzarrini et al. 2004). Furthermore, the respective positive and negative roles of ABA and gibberel-lin in the determination of cotyledon identity during embryo development have been proposed (Gazzarrini et al. 2004). The increased levels of gibberellin in the developing seeds of the fus3 mutant have been attributed to the up-regulation of GA3ox2 in the mutant (Curaba et al. 2004). These observations apparently contradict our results, which report that several gibberellin biosynthesis genes, including ent-kaurenoate oxidase (CYP86A3), GA20ox2, GA20ox3 and GA3ox4, were down-regulated in the mutant (Table 2). The down-regulation of GA20ox3 and GA3ox4 in the mutant was probably not due to negative feedback regulation of gibberellin biosynthesis because the former was clearly activated by the ectopic induction of FUS3 (Supplementary Fig. S7), and the latter is highly seed specific (assignment to cluster I of QTC-D36; Table 2). Therefore, gibberelin synthesis is probably positively regulated by FUS3 during normal seed development. Rather, the increased levels of gibberelin and the GA3ox2 transcript could be due to the heterochronic development of the mutant embryo. Therefore, gibberelin may play roles at earlier stages and may be required for embryo growth.

**Conclusion**

The fus3DR and FUS3AC genes were not confined to genes that are seed specific or preferentially regulated in seeds. Genes involved in metabolic processes such as phenylpropanoid/ flavonoid and fatty acid biosynthesis were fus3DR and FUS3AC but not seed specific/preferential. Non-seed-specific FUS3AC genes were activated in a less ABA-dependent manner, had relatively rapid kinetics and showed temporal profiles in their transcript levels more similar, though slightly delayed, to that of induced FUS3, whereas seed-specific/preferential genes were generally ectopically activated by FUS3 in an ABA-dependent manner and to contribute to the ability of LEC2 to induce somatic embryogenesis when it is ectopically expressed (Stone et al. 2008).
Table 2: Down-regulated plant hormone biosynthesis genes in the *fus3* mutant seeds

<table>
<thead>
<tr>
<th>Hormone</th>
<th>AGI code</th>
<th>TAIR9 short description</th>
<th>FUS3 activation</th>
<th>Expression ratio (<em>fus3/wild type</em>)</th>
<th>QTC-D36 cluster</th>
<th>QTC-D13 cluster</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>QTC-D36 cluster</td>
<td>8 DAF Mean</td>
<td>q-value (down)</td>
<td>12 DAF Mean</td>
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<tr>
<td>ABA</td>
<td>AT1G78390</td>
<td>NCED9 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 9); 9-cis-epoxycarotenoid dioxygenase</td>
<td>No</td>
<td>0.68</td>
<td>3.3E-01</td>
<td>0.19</td>
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<td>AT3G24220</td>
<td>NCED6 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 6); 9-cis-epoxycarotenoid dioxygenase</td>
<td>No</td>
<td>0.72</td>
<td>3.9E-01</td>
<td>0.29</td>
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<tr>
<td></td>
<td>AT1G53940</td>
<td>ABA2 (ABA DEFICIENT 2); alcohol dehydrogenase/xoxidoreductase/shtoxin dehydrogenase</td>
<td>No</td>
<td>0.43</td>
<td>3.6E-02</td>
<td>0.55</td>
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<tr>
<td>Gibberellin</td>
<td>AT1G05160</td>
<td>CYP88A3 (CYTOCHROME P450 88 A3); ent-kaurenoate oxidase/oxygen binding</td>
<td>No</td>
<td>0.40</td>
<td>2.2E-02</td>
<td>0.23</td>
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<td></td>
<td>AT5G07200</td>
<td>YAP169; gibberellin 20-oxidase (GA20ox3)</td>
<td>Yes</td>
<td>0.82</td>
<td>7.8E-01</td>
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<td>AT5G18110</td>
<td>GA20ox2 (GIBBERELLIN 20 OXIDASE 2); gibberellin 20-oxidase</td>
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<td>0.91</td>
<td>1.1E+00</td>
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<td>AT1G80330</td>
<td>GA3ox4 (GIBBERELLIN 3-OXIDASE 4); gibberellin 3-beta-dioxygenase</td>
<td>No</td>
<td>0.34</td>
<td>1.2E-02</td>
<td>0.34</td>
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<tr>
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<td>AT5G36380</td>
<td>ROT3 (ROTUNDIFOLIA 3); oxygen binding/steroid hydroxylase</td>
<td>No</td>
<td>0.69</td>
<td>3.2E-01</td>
<td>0.18</td>
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<td>AT1G30180</td>
<td>BR6ox2 (BRASSINOSTEROID-6-OXIDASE 2); monooxygenase/oxygen binding</td>
<td>Yes</td>
<td>1.16</td>
<td>1.2E+00</td>
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<td>AT1G68460</td>
<td>ATIPT1 (isopentenyltransferase 1); a denylate dimethylallyltransferase</td>
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<td>AT1G25410</td>
<td>ATIPT6; ATP binding/xdenylate dimethylallyltransferase/tRNA isopentenyltransferase</td>
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<td>2.2E-03</td>
<td>0.10</td>
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<td></td>
<td>AT1G44300</td>
<td>NIT2 (nitrilase 2); indole-3-acetonitrile nitrilase/indole-3-acetonitrile nitrile hydratase/indole-3-acetonitrile nitrile hydratase</td>
<td>No</td>
<td>0.46</td>
<td>4.9E-02</td>
<td>0.43</td>
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<td>AT1G89910</td>
<td>YUC10; FAD binding/monooxygenase/oxidoreductase</td>
<td>No</td>
<td>0.29</td>
<td>6.3E-03</td>
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<tr>
<td></td>
<td>AT5G20960</td>
<td>AAO1 (ARABIDOPSIS ALDEHYDE OXIDASE 1); aldehyde oxidase/indole-3-acetaldehyde oxidase</td>
<td>No</td>
<td>1.96</td>
<td>1.0E+00</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>AT1G01480</td>
<td>ACS2; 1-aminocyclopropane-1-carboxylate synthase</td>
<td>No</td>
<td>0.89</td>
<td>9.2E-01</td>
<td>0.08</td>
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<td></td>
<td>AT1G15640</td>
<td>JMT (JASMONIC ACID CARBOXYL METHYLTRANSFERASE); jasmonate O-methyltransferase</td>
<td>No</td>
<td>0.41</td>
<td>2.5E-02</td>
<td>0.96</td>
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</tbody>
</table>
manner within different ranges of and with relatively slower kinetics. Thus, the activation of genes not specific to seeds by FUS3 appeared to involve less complex processes compared with that of seed-specific genes. This does not necessarily mean that non-seed-specific FUS3AC genes are directly activated; as suggested above, the activation of fatty acid biosynthesis genes is likely to be mediated by the activation of the transcription factor WR1. In contrast, the activation of seed-specific FUS3AC appeared to depend more on time-requiring processes for their ectopic activation by FUS3. This is highly likely to be the case even when FUS3 directly binds to their promoters because the promoters of seed-specific FUS3AC genes are highly enriched in FUS3-binding RY elements.

Seed-specific fus3DR genes expressed during longer ranges of seed development stages were highly enriched in FUS3AC genes. In contrast, seed-specific fus3DR genes, whose expression was confined to either earlier stages or later stages, were relatively scarce in FUS3AC genes. These results indicated that even among the seed-specific fus3DR genes, the mechanisms of FUS3 action were different and were dependent on their developmental regulation. The down-regulation of these genes in fus3 seeds could be accounted for by the heterochronic nature of fus3; fus3 seeds failed to express the late embryogenesis program but executed the post-germinative program instead. Alternatively, it is possible that an additional layer of repression, which cannot be liberated by FUS3, is operating for these genes in the seedlings.

In conclusion, the two types of microarray analyses performed in this study revealed diversities in the mechanisms for gene regulation by FUS3 and ranges of downstream genes under the control of FUS3. Although the mechanistic diversity suggested here from the ectopic action of FUS3 may depend on the chromatin and cellular states in seedling tissues, it should reflect distinctions in gene regulation during seed development; thus, it should be informative for the further dissection of molecular processes, as well as the comprehensive understanding of regulatory networks for seed development controlled by FUS3 and other master regulators of seed maturation.

Materials and Methods

Plant materials and growth

Plants [Arabidopsis thaliana, ecotype Columbia (Col-0) and fus3-3 mutant (Col-0 background; Keith et al. 1994)] were grown under long-day conditions (16 h light/8 h dark) under white fluorescent light (40 µmol m⁻² s⁻¹) on Rock Fiber (Nittobo, http://www.nittobo.co.jp) with 1/5th strength Murashige and Skoog (MS) salt mixture supplemented with 8% vitamins (pH 5.7), after establishing the seedlings on agar medium [half-strength MS salt mixture, pH 5.7, 8% vitamins, 1% (w/v) sucrose and 0.8% agar]. Three batches of plants were grown to provide independent biological replicates. Seeds were dissected from siliques marked on the day of flowering and used for RNA preparation.

Seeds of the ER-FUS3-DH transgenic line (Kagaya et al. 2005a) were plated on 0.6% agar plates with the same composition as above, kept for 3 d at 4 °C, then shifted to 22 °C under the light conditions described above, and cultured for 4 d. Transgenic plant seedlings with expanded cotyledons were transferred from the plates to 10 ml of liquid medium [half-strength MS salt mixture, pH 5.7, B5 vitamins and 1% (w/v) sucrose] in a 100 ml Erlenmeyer flask (100 seedlings per flask) and cultured for an additional 3 d. The 7-day-old (4 d on plates and 3 d in liquid culture) transgenic seedlings in the liquid culture were supplemented with either ABA at a final concentration of 100 µM or ESTR at a final concentration of 4 µM, or both for 3, 5, 12, 24, 36 or 48 h. Next, the seedlings were collected and frozen in liquid N₂ for RNA extraction. Control seedlings without treatments were collected at the time of treatment and were used as time zero samples. The experiments were independently repeated twice.

RNA preparation

Extraction of RNA from seedlings and seeds was performed by using the RNeasy Mini Kit (Qiagen) and Concert Plant RNA Reagent (Invitrogen) after grinding the frozen sample in liquid N₂ into a fine powder with a mortar and pestle. The total RNA was further purified using the RNeasy Mini Kit.

Microarray analysis

The RNA samples prepared as above were labeled by converting to cRNA in the presence of Cy3-CTP or Cy5-CTP using the Low RNA Input Linear Amplification & Labeling Kit (Agilent Technologies) according to the manufacturer’s protocol. The labeled cRNA was hybridized to the Arabidopsis2 Oligo DNA Microarray (Agilent Technology) using the In situ Hybridization Kit Plus (Agilent Technologies) according to the manufacturer’s protocol. The hybridized microarrays were scanned and analyzed with an Agilent Microarray Scanner (Agilent Technologies) and the Feature Extraction software (version A.6.1.1, A.7.1.1 or A.7.5.1; Agilent Technologies), respectively. The microarray data were deposited in the public NCBI Gene Expression Omnibus database under the GEO accession number GSE23974.

Rank product (Breitling et al. 2004) and clustering analyses were performed using the MeV software package version 4.5e (Saeed et al. 2003). The graphic presentation of time course data was performed using the R statistical software package (R Development Core Team 2008). Other statistical analyses and data handling were performed using Microsoft Excel 2003 (Microsoft Corporation).

Supplementary data

Supplementary data are available at PCP online.

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References


