The submergence tolerance regulator Sub1A mediates stress-responsive expression of AP2/ERF transcription factors.
The Submergence Tolerance Regulator Sub1A Mediates Stress-Responsive Expression of AP2/ERF Transcription Factors

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We previously characterized the rice (Oryza sativa) Submergence1 (Sub1) locus encoding three ethylene-responsive factor (ERF) transcriptional regulators. Genotypes carrying the Sub1A-1 allele are tolerant of prolonged submergence. To elucidate the mechanism of Sub1A-1-mediated tolerance, we performed transcriptome analyses comparing the temporal submergence response of Sub1A-1-containing tolerant M202(Sub1) with the intolerant isolate M202 lacking this gene. We identified 898 genes displaying Sub1A-1-dependent regulation. Integration of the expression data with publicly available metabolic pathway data identified submergence tolerance-associated pathways governing anaerobic respiration, hormone responses, and antioxidant systems. Of particular interest were a set of APETALA2 (AP2)/ERF family transcriptional regulators that are associated with the Sub1A-1-mediated response upon submergence. Visualization of expression patterns of the AP2/ERF superfamily members in a phylogenetic context resolved 12 submergence-regulated AP2/ERFs into three putative functional groups: (1) anaerobic respiration and cytokinin-mediated delay in senescence via ethylene accumulation during submergence (three ERFs); (2) negative regulation of ethylene-dependent gene expression (five ERFs); and (3) negative regulation of gibberellin-mediated shoot elongation (four ERFs). These results confirm that the presence of Sub1A-1 impacts multiple pathways of response to submergence.

Submergence of rice (Oryza sativa) during the monsoon flooding season seriously limits rice production in south and southeast Asia, causing annual losses of over one billion U.S. dollars (Xu et al., 2006). Complete submergence for 1 to 2 weeks leads to death of most rice cultivars (Xu et al., 2006; Perata and Voesenek, 2007). However, some cultivars, such as FR13A (an indica rice), can survive up to 2 weeks of complete submergence. Previous quantitative trait locus studies demonstrated that the Submergence1 (Sub1) locus on chromosome 9 is the major source of tolerance (Xu et al., 2000, 2006). The Sub1 locus consists of three ethylene-responsive factor (ERF) transcriptional regulators (Sub1A, Sub1B, and Sub1C). The introgression of the FR13A Sub1 locus (Sub1A-1, Sub1B-1, Sub1C-1) into a submergence-intolerant background (japonica cv M202; Sub1B-2, Sub1C-2) using marker-assisted selection led to the development of the M202(Sub1) submergence-tolerant near-isogenic line (Fukao et al., 2006). Submergence tolerance is strongly associated with the presence and pronounced expression of Sub1A-1 for up to 14 d following submergence (Fukao et al., 2006; Nakano et al., 2006; Xu et al., 2006). Sub1A-1 mRNA abundance is significantly up-regulated in M202(Sub1) in response to submergence (Fukao et al., 2006; Xu et al., 2006) and is constitutively expressed and not responsive to submergence in Sub1A-1 overexpression lines, although the transgene transcript progressively declines over prolonged submergence (Xu et al., 2006; Fukao and Bailey-Serres, 2008). In genotypes with Sub1A-1, submergence-induced accumulation of Sub1C-1 mRNA is reduced (Fukao et al., 2006; Xu et al., 2006).

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semiaquatic plants is closely tied to phytohormones (Bailey-Serres and Voeselek, 2008). Recently, we evaluated the role of the Sub1 quantitative trait locus in modulating ethylene and gibberellin (GA) and their signaling/synthesis during submergence (Fukao et al., 2006; Fukao and Bailey-Serres, 2008). Ethylene levels rapidly increase upon submergence and activate genes associated with acclimation responses such as carbohydrate metabolism, resulting in shoot elongation and leaf senescence (Steffens and Sauter, 2005; Fukao et al., 2006). Increased ethylene level upon submergence activates Sub1A-1 expression, which positively regulates the GRAS-domain transcription factors (TFs) Slender Rice1 (SLR1) and SLR1-Like1 (SLRL1), delaying shoot elongation by restricting GA-dependent signaling responses (Fukao et al., 2006; Shimada et al., 2006). Ethylene-induced Sub1A-1 stimulation restricts ethylene production (Fukao et al., 2006). Manipulation of leaf elongation during submergence by the application of a GA biosynthesis inhibitor, paclobutrazol, confirms the role of GA-mediated underwater shoot elongation (Ram et al., 2002; Steffens and Sauter, 2005). Sub1C has been proposed to function downstream of GA signaling and likely plays roles in shoot elongation in submergence-intolerant rice during submergence (Fukao and Bailey-Serres, 2008). Ethylene in part contributes to the reduction of abscisic acid (ABA) concentration in submerged rice by activating ABA 8′-hydroxylase genes (OsABA8ox1, Os02g47470) to inactivate ABA (Saika et al., 2007). Application of exogenous ABA in a dose-dependent manner suppresses shoot elongation of japonica rice (cv Nipponbare) during submergence (Saika et al., 2007), which lacks Sub1A-1 (Xu et al., 2006). The effect of ABA on shoot elongation is antagonistic to that of GA application during seed germination (White and Rivin, 2000; Gomez-Cadenas et al., 2001). It has also been reported that ABA effectively slowed the ethylene-induced and GA-enhanced cell death (Steffens and Sauter, 2005).

The intolerant rice cv M202 also shows age-dependent senescence of underwater leaves during prolonged submergence (Fukao et al., 2006; Perata and Voeselek, 2007). Based on these reports, another potentially important tolerance trait upon submergence could be the detoxification of reactive oxygen species (ROS), which cause cellular damage by oxidative stress. Excessive production of ROS is a component of many stresses, including oxygen deprivation (Bailey-Serres and Voeselek, 2008). When plants suffering from anoxia are exposed to air during desubmergence, they can be severely damaged by a burst of ROS (Jackson and Ram, 2003). To overcome this challenge, plants have developed diverse antioxidant defense systems consisting of a range of enzymes (e.g. superoxide dismutase, catalases, ascorbate peroxidase, and glutathione peroxidase) and reductants (e.g. ascorbate, glutathione, phenolic compounds, and tocopherols) that scavenge ROS (Jackson and Ram, 2003). Significantly high levels of activities of these antioxidant enzymes were only observed in submergence-tolerant cv FR13A but not in the intolerant cv IR42 and the submergence-avoiding cv CR 383-10 (Almeida et al., 2003). The obvious phenotypic difference under desubmergence after prolonged submergence between the tolerant and intolerant cultivars suggests that FR13A maintains a high antioxidant defense system but IR42 does not (Almeida et al., 2003). Despite the importance of protecting antioxidant systems during anoxia, there are few reports that explore the differential expression of enzymes associated with antioxidant defense in submergence-tolerant cultivars (Santosa et al., 2007). In addition, it has been reported that ROS is needed for cell elongation in Arabidopsis (Arabidopsis thaliana; Foreman et al., 2003). Genome-wide suggestions on the regulation of ROS in M202 (Sub1) rice plants may provide us clues to understand mechanisms to restrict shoot elongation under submergence.

The APETALA2 (AP2)/ERF superfamily of rice can be divided into three families based on sequence similarity and numbers of domains: AP2, ERF, and RAV (for related to ABI3/VP). Of these, the ERF family is classified into 14 groups (I–XIV) based on gene structures, phylogenetic, and conserved motifs (Nakano et al., 2006). As mentioned previously, the Sub1 locus contains a cluster of two or three genes carrying ERF domains. ERF family members control diverse biological functions in growth and development such as leaf epidermal cell density, flower development, and embryonic development (Elliott et al., 1996; Boutilier et al., 2002) as well as hormonal signaling mediated by ethylene, cytokinin (Rashotte et al., 2006), and brassinosteroid (Alonso et al., 2003; Hu et al., 2004). ERFs are also involved in responses to biotic and abiotic stimuli such as pathogen infection, drought, and freezing stresses (Hao et al., 1998; Liu et al., 1998; Gilmour et al., 2000). Functions of some of the members of the ERF group have been reported in Arabidopsis. For example, group VII is related to abiotic stress, group VIIIa is associated with hormonal responses, and group IXc mediates disease resistance (Nakano et al., 2006). Sub1A, Sub1B, and Sub1C in the Sub1 locus belong to ERF family group VII. We previously reported on the regulation of Sub1A, Sub1B, and Sub1C transcript accumulation. Here, we investigate the involvement of other AP2/ERF superfamily members and additional TFs in the submergence response.

To obtain molecular insight on how the M202(Sub1) plants trigger tolerance in response to a submergence challenge, we generated gene expression profiles to compare M202(Sub1) (tolerant) and M202 (intolerant) at 0, 1, and 6 d after submergence using the NSF45K array (Jung et al., 2008a, 2008b). We identified a set of genes displaying Sub1A-1-dependent tolerance responses. We then applied several analytical tools to categorize these genes. This work allowed us to identify three groups of AP2/ERF superfamily members that are associated with different acclimation processes for the Sub1A-mediated tolerance response. Based on these results, we propose a model for how Sub1A modulates AP2/
ERF gene family member expression to promote survival during prolonged submergence.

RESULTS
Phenotypic Characterization of the Submergence-Tolerant and -Intolerant Lines
We reconfirmed the phenotypes of M202(Sub1) (tolerant) and M202 (intolerant) lines before performing the transcriptome assays as we described in a previous study (Fukao et al., 2006) and also validated the expression of the two marker genes, namely Sub1A and Sub1C of the Sub1 locus (Fig. 1). We collected aboveground shoot tissue from M202(Sub1) and M202 that were grown for 2 weeks and then submerged for 0, 1, and 6 d. Consistent with previous reports (Fukao et al., 2006; Xu et al., 2006; Fukao and Bailey-Serres, 2008), the levels of Sub1A mRNA were highly induced in M202(Sub1) at 1 and 6 d after submergence. The transcript was not detected in M202, since it lacks the gene. Sub1C transcript induction was more pronounced in M202 compared with M202(Sub1) at 1 and 6 d after submergence, and Sub1B did not exhibit a significant differential expression between M202(Sub1) and M202 in any of the comparisons (Fig. 1).

To perform genome-wide transcriptome analysis, we used two independent biological replicates and one or two dye swaps for each treatment. The experimental plans are summarized in Supplemental Table S1.

Identification of Candidate Genes Showing Sub1A-Dependent Gene Expression Patterns through Clustering Analysis
We used the NSF45K array to compare the transcript abundance of M202(Sub1) and M202 by hybridization of labeled cDNA prepared for three durations of submergence [relative ratio of M202(Sub1) signal/M202 signal (S/M)_0 d, S/M_1 d, and S/M_6 d]. Using the normalized array data from these experiments, we monitored the transcriptional profiles from M202(Sub1) by comparing either 0 d versus 1 d or 0 d versus 6 d after submergence to identify differential gene expression in the same genetic background at two time points (1 d/0 d_S and 6 d/0 d_S). A similar approach was also applied to the M202 data at two time points (1 d/0 d_M and 6 d/0 d_M). We then generated seven log_2 fold change values: three from the direct comparisons of M202(Sub1) (S) versus M202 (M) at three time points (S/M_0 d, S/M_1 d, and S/M_6 d) and four from the indirect comparisons of 0 d versus 1 d or 0 d versus 6 d for each background after submergence (1 d/0 d_S, 6 d/0 d_S, 1 d/0 d_M, and 6 d/0 d_M; Fig. 2).

Using hierarchical clustering (HCL) analysis, we generated 12 clusters with distinct gene expression patterns (Fig. 2). The left panel in Figure 2 shows the result of HCL analysis of genes showing 2-fold or greater up-regulation (log_2 ratio ≥ 1) or down-regulation (log_2 ratio ≤ −1) in at least one of the seven comparisons with t test F ≤ 0.05 (see “Materials and Methods”). The average log_2 intensity corresponds to normalized expression levels in six samples of M202 (Sub1) and M202 at 0, 1, and 6 d after submergence. Of the 12 distinct clusters, 1, 2, and 12 (91 probes in cluster 1, 174 in cluster 2, and 168 in cluster 12) showed positive associations with the presence of Sub1A-1, as the cluster members were up-regulated in M202(Sub1) as compared with M202 at 1 and 6 d after submergence (Supplemental Table S2). In addition, these genes were either up-regulated or unchanged in M202(Sub1) at 1 and 6 d after submergence compared with the 0-d time point and down-regulated in M202 at 1 and 6 d after submergence compared with the 0-d sample of M202 (Fig. 2). Cluster 7 showed slight up-regulation in M202(Sub1) compared with M202 at 6 d after submergence. These genes averaged 2.3- and 1.4-fold up-regulation in M202(Sub1) and M202 at 6 d after submergence, respectively. Clusters 3, 6, 8, and 9 displayed up-regulation in both M202(Sub1) and M202 after submergence but did not show clear differential expression patterns: clusters 3 and 8 showed up-regulation in both M202(Sub1) and M202 at 1 and 6 d after submergence; and cluster 6 showed a more obvious up-regulation in M202(Sub1) and M202 at 6 d after submergence. Cluster 9 showed distinct up-regulation in M202(Sub1) and M202 at 1 d after submergence. Most genes in this cluster displayed slight down-regulation in M202(Sub1) compared with M202 at 1 d after submergence (Fig. 2).

The genes in clusters 3, 6, 8, and 9 may be involved in tolerance responses to submergence, because these genes are stimulated by the stress. Thus, we also included these clusters in the candidate gene list from clusters 1, 2, and 12 for further analyses to elucidate the Sub1A-mediated tolerance response. In total, we
identified genes represented by 898 probes positively involved in the Sub1A-mediated tolerance response via clustering analysis.

Clusters 4, 5, and 11 expression patterns exhibit a negative relationship with Sub1A expression, because these genes in these clusters are down-regulated in M202 (Sub1) compared with M202 after submergence. In the same genetic background, the expression patterns in these clusters showed down-regulation in M202(Sub1) (e.g. 1 d/0 d_S and 6 d/0 d_S) but up-regulation in M202 (e.g. 1 d/0 d_M and 6 d/0 d_M) in response to submergence (Fig. 2). In addition, members of cluster 10 were down-regulated in both M202(Sub1) and M202 after submergence. The genes in clusters 4, 5, 10, and 11 are represented by 333 probes that are down-regulated by submergence stress. Some of these (i.e. clusters 4, 5, and 11) are uniquely regulated by Sub1A and could be involved in the Sub1A-mediated tolerance response, including down-regulation of carbohydrate consumption and elongation growth.
To summarize, we identified 898 probes up-regulated and 333 probes down-regulated by submergence. A subset of these genes were regulated specifically in the isoline with Sub1A-1: genes represented by 433 probes in clusters 1, 2, and 12 were positively related to Sub1A-1, and those represented by 177 probes in clusters 4, 5, and 10 were negatively associated with Sub1A-1.

Identification of Gene Ontology Terms Associated with Biological Processes Enriched in Submerged M202(Sub1)

Gene Ontology (GO) enrichment analysis with genome-wide transcriptome data effectively identifies groups of genes categorized into biological processes, molecular functions, and cellular components. In this work, we identified the biological processes associated with the Sub1A-1-mediated tolerance response. We carried out GO enrichment analysis in biological process category with log2 ratios of M202(Sub1) versus M202 at 1 d after submergence, of M202(Sub1) at 1 d versus 0 d after submergence, and of M202 at 1 d versus 0 d after submergence (Table I). Our objective for this analysis was to identify enriched GO terms in genes showing significant up-regulation in M202(Sub1) compared with M202 at 1 d after submergence, in genes showing significant up-regulation in M202(Sub1) at 1 d after submergence compared with before submergence, and in genes showing down-regulation in M202 at 1 d after submergence compared with before submergence. We determined significant GO terms by considering both GOstat P values indicating statistical significance and GO enrichment values representing numeric enrichment of selected GO terms (see "Materials and Methods").

Twelve GO terms are listed in Table I. They are the most enriched biological processes associated with Sub1A-1 function because they satisfied all three criteria stated above. Of these GO terms, regulation of DNA-dependent transcription is the most abundant one in the enriched GO terms in association with Sub1A-1 function. In addition, enrichment of GO terms related to response to ABA stimulus, ethylene-mediated signaling pathway, negative regulation of ethylene-mediated signaling pathway, and response to ethylene stimulus indicates the involvement of hormone responses such as ethylene and ABA for the submergence tolerance response.

In Table I, we also present four GO terms enriched in the comparison of M202(Sub1) and M202 at 1 d after submergence [M202(Sub1) 1 d versus M202 1 d] and M202 at 1 d after submergence compared with before submergence (M202 1 d versus 0 d), implying that stimulation in M202(Sub1) compared with M202 at 1 d

### Table I. Identification of GO terms enriched in submerged M202(Sub1)

<table>
<thead>
<tr>
<th>GO IDa</th>
<th>GO Name</th>
<th>Totalb</th>
<th>Sub1/M202_1 d_Upc P</th>
<th>Sub1 1 d/0 d_Upc P</th>
<th>M202 1 d/0 d_Downc P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>OKi</td>
<td>OKi</td>
<td>OKi</td>
</tr>
<tr>
<td>GO:0010200</td>
<td>Response to chitin</td>
<td>42</td>
<td>7</td>
<td>0.9</td>
<td>2.10E-05</td>
</tr>
<tr>
<td>GO:0009618</td>
<td>Response to pathogenic bacteria</td>
<td>77</td>
<td>9</td>
<td>1.6</td>
<td>2.84E-05</td>
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<tr>
<td>GO:0009611</td>
<td>Response to wounding</td>
<td>235</td>
<td>17</td>
<td>4.9</td>
<td>7.97E-06</td>
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<tr>
<td>GO:0009414</td>
<td>Response to water deprivation</td>
<td>188</td>
<td>13</td>
<td>3.9</td>
<td>0.000011</td>
</tr>
<tr>
<td>GO:0009873</td>
<td>Ethylene-mediated signaling pathway</td>
<td>81</td>
<td>13</td>
<td>1.7</td>
<td>1.19E-08</td>
</tr>
<tr>
<td>GO:0010105</td>
<td>Negative regulation of ethylene-mediated signaling pathway</td>
<td>53</td>
<td>6</td>
<td>1.1</td>
<td>0.00007</td>
</tr>
<tr>
<td>GO:0009723</td>
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<td>136</td>
<td>9</td>
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<tr>
<td>GO:0009737</td>
<td>Response to ABA stimulus</td>
<td>330</td>
<td>21</td>
<td>6.9</td>
<td>5.34E-06</td>
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<tr>
<td>GO:0009411</td>
<td>Response to UV light</td>
<td>99</td>
<td>6</td>
<td>2.1</td>
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<tr>
<td>GO:0008219</td>
<td>Cell death</td>
<td>92</td>
<td>5</td>
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<tr>
<td>GO:0006355</td>
<td>Regulation of transcription, DNA dependent</td>
<td>802</td>
<td>36</td>
<td>17</td>
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<tr>
<td>GO:0006979</td>
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<td>119</td>
<td>5</td>
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<tr>
<td>GO:0016049</td>
<td>Cell growth</td>
<td>42</td>
<td>4</td>
<td>0.9</td>
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<tr>
<td>GO:0009686</td>
<td>Gibberellin acid biosynthesis</td>
<td>56</td>
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<td>GO:0009631</td>
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<td>GO:0009409</td>
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<td>14</td>
<td>4.60E-05</td>
<td>9.65</td>
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<tr>
<td>GO:0009735</td>
<td>Response to cytokinin stimulus</td>
<td>122</td>
<td>7</td>
<td>2.6</td>
<td>0.010259</td>
</tr>
</tbody>
</table>

*aGOslim identifier.  bNumber of each GOslim term in the whole genome.  cGenes at least 2-fold up-regulated with P < 0.05 in M202(Sub1) relative to M202 at 1 d after submergence.  dGenes at least 2-fold up-regulated with P < 0.05 in M202(Sub1) at 1 d after submergence relative to untreated control.  eGenes at least 2-fold down-regulated with P < 0.05 in M202 at 1 d after submergence relative to untreated control.  fNumber of observed repeats for a GOslim term in one of the gene lists.  gNumber of expected repeats for a GOslim term in one of the gene lists.  hSignificance of selected GO terms with P values generated using the GOstat tool.  iSatisfaction of any of the gene list criteria.

after submergence is mostly due to the repression in M202 at 1 d after submergence compared with before submergence. One salient phenotypic change in M202 after submergence is enhanced shoot elongation. In contrast, the increase in plant height of the tolerant line was the same under submergence and normal conditions (Fukao et al., 2006). Enrichment of GO terms related to cell growth and GA biosynthesis in the tolerant line at 1 d after submergence could potentially explain the differing phenotypes during prolonged submergence between the tolerant line and the intolerant line. There were four genes containing GO terms for cell growth and five for GA biosynthesis. Of five genes related to GA biosynthesis, four encode AP2/ERF TFs, and they all belong to the same AP2/ERF group (Nakano et al., 2006). They also have GO terms related to cell growth. The non-AP2/ERF gene related to GA metabolism is GA 2-oxidase1 (OsGA2ox1, Os02g41954), which inactivates bioactive GA$_1$ (Rieu et al., 2008). Therefore, we predict that these AP2/ERF TFs (Os04g48350, Os02g45450, Os09g35010, Os09g35020) promote the activation of GA2ox1 or genes encoding other repressors of cell growth and lead to the restriction of underwater shoot elongation in the tolerant lines. In addition, submergence challenge could conceivably be accompanied by low-temperature stress, as floodwater temperatures are likely lower than ambient air temperatures. Cold acclimation after submergence may be conferred by genes associated with responses to cold or cold acclimation. The identified four AP2/ERF TFs may additionally execute critical roles in cold acclimation in the tolerant lines. OsDREBs, known to be involved in cold-responsive gene expression, are in the same group with these AP2/ERF TFs (Ito et al., 2006).

Table I shows GO terms enriched in the first [M202 (Sub1) 1 d versus M202 1 d] and the second [M202 (Sub1) 1 d versus 0 d] gene lists, indicating induction in the tolerant lines compared with the intolerant lines. This category is enriched in cytokinin stimulus and is characteristic of the tolerant line. GO terms identified from the enrichment analysis revealed novel biological processes linked to the Sub1A-1-mediated tolerance response. Genes and the expression data subjected to GO terms listed in Table I are shown in Supplemental Table S3. This GO term enrichment analysis suggested that the Sub1A-1-mediated tolerance response is modulated by a complex orchestration of transcriptional regulation of hormonal responses.

Phylogenomic Gene Expression Analysis of the AP2/ERF Superfamily Reveals Three Groups under the Control of Sub1A-1

Construction of Phylogenomic Digital Gene Expression Data of the AP2/ERF Superfamily

Recently, we reported the construction of rice phylogenomic databases for kinases and glycosyltransferases (Dardick et al., 2007; Cao et al., 2008). In these studies, phylogenomic gene expression data of gene family members are built onto the frame of the kinase and glycosyltransferase phylogenetic trees (Cao et al., 2008). The integration of gene expression and the phylogenetic tree of a large gene family enabled us to visually identify groups with distinct expression patterns conserved across the gene family members (Cao et al., 2008). The GO enrichment analysis described earlier revealed that DNA-dependent transcriptional regulation is the most abundant GO term positively associated with Sub1A-1 function. There are 16 AP2/ERF genes out of 36 genes associated with the regulation of DNA-dependent transcription up-regulated in M202(Sub1) 1 d compared to M202 1 d (Supplemental Fig. S1). This suggests that a subset of AP2/ERF genes could potentially be playing a key role in Sub1A-1-mediated tolerance responses. The role of individual AP2/ERF genes in Sub1A-1 function remains to be determined and will require elaborate functional characterization as well as overcoming the issue of functional redundancy. However, significant insight can be gained by employing a phylogenomic gene expression panel of the AP2/ERF superfamily genes by integrating expression data related to submergence, anoxia, hormone, abiotic stress, and pathogen responses with the AP2/ERF phylogenetic tree. We used log$_2$ fold ratios in seven comparisons from the NSF45K data related to submergence for this analysis (Fig. 2). Other gene expression data are available in a public microarray database, the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/; Edgar et al., 2002); the comparisons of anoxic versus aerobic coleoptiles (NCBI GEO accession no. GSE6908), transzeatin versus mock dimethyl sulfoxide in root and leaf at 30 and 120 min after treatment (GSE6737), abiotic stresses (i.e. drought, salt, and cold) versus untreated control (GSE6901), and Magnaporthe grisea (FR13) versus mock treatment on third leaf at 3 or 4 d after treatment (GSE7256; Figs. 3 and 4; Supplemental Fig. S1; Hirose et al., 2007; Jain et al., 2007).

The tree consists of 182 AP/ERF TFs identified in the integrative Plant TF Database (PlnTFDB; http://plntfdb.bio.uni-potsdam.de; Riano-Pachon et al., 2007). We first divided this AP2/ERF superfamily into two subfamilies, single AP2/ERF and double AP2, based on previous characterization of this family (Sakuma et al., 2002; Nakano et al., 2006). The single AP2/ERF subfamily was further classified into ERF and DREB (Sakuma et al., 2002; Nakano et al., 2006). The nomenclature assigned to each group was consistent with previous work on the AP2/ERF superfamily in rice and Arabidopsis (Nagano et al., 2005). Based on our analysis, we found gene clusters representing three AP2/ERF groups (i.e. IIC, VII, and VIIIa) to be positively linked with the presence of Sub1A-1 (Supplemental Fig. S1). Selective phylogenomic gene expression data of this gene family including the three AP2/ERF groups is displayed in Figure 3A. We validated the gene expression patterns of 12 AP2/ERF genes in these...
groups by reverse transcriptase (RT)-PCR analysis (Fig. 3B). The differential expression patterns of AP2/ERF genes in group IIIc in comparison with M202 (Sub1) versus M202 after submergence were evident in the three AP2/ERF groups associated with Sub1A-1 function. Microarray data of four AP2/ERF genes in IIIc also showed a higher fold change (i.e. 4- to 8-fold) in M202(Sub1) compared with those in M202,

Figure 3. Phylogenomic digital expression analysis of the AP2/ERF family and validation of expression patterns of 12 downstream AP2/ERF genes of Sub1A-1. A, Phylogenomic digital expression analysis of 86 AP2/ERF TFs including three groups (i.e. VIIa, VIIIa, and IIIc) that are positively involved in Sub1A-1 function. Phylogenomic digital expression analysis of all AP2/ERF genes (182) is presented in Supplemental Figure S1. B, Validation of gene expression patterns of 11 AP2/ERF genes downstream of Sub1A-1. Gene models colored red in A were at least 2-fold up-regulated in M202(Sub1) compared with M202 at 1 d after submergence; a gene model colored green in A was more than 2-fold down-regulated in M202(Sub1) compared with M202 at 1 d after submergence; and gene models colored light blue in A were 1.4- to 2-fold down-regulated in M202(Sub1) compared with M202 at 1 d after submergence. See Figure 2 legend for the heat map. [See online article for color version of this figure.]
whereas a 2- to 4-fold higher induction in VIIa and VIIa was observed (Supplemental Table S2). In addition, Os10g41330, Os02g43790, Os03g09170, and Os04g46440 were present in higher transcript abundance in M202(Sub1) relative to M202 at 1 d after submergence (Supplemental Fig. S1).

In contrast, Os03g08490 in group VIIa was involved in negative association with Sub1A-1 function, because this gene was more than 4-fold down-regulated in M202(Sub1) compared with M202 at 1 d after submergence (Fig. 3; Supplemental Table S2). Eleven AP2 genes including Sub1C (Os09g11460) colored light blue in Figure 3 also showed similar expression patterns to that of Os03g08490, even though the differential expression levels were less dramatic than Os03g08490.

To gain further insight on three AP2/ERF groups, we examined the GO terms in the biological processes category as well as differential expression patterns after applying hormone, abiotic stress, and fungal pathogen for individual AP2/ERF genes in each group (Fig. 4).

Roles of VII

The Sub1 locus of submergence-tolerant FR13A encodes three TFs (Sub1A-1, Sub1B-1, and Sub1C-1) belonging to the AP2/ERF superfamily group VII (Nakano et al., 2006; Xu et al., 2006). Sub1A-1 and Sub1B-1 belong to group VIIa, and Sub1C-1, which lacks the conserved CMVII-1 domain in group VIIa, belongs to group VIIb (Nakano et al., 2006). Transcripts from three other AP2/ERF loci (Os01g21120, Os03g22170, and Os07g47790) in group VIIa were at least 2-fold up-regulated in M202(Sub1) compared with M202 at 1 d after submergence. These genes were induced in M202(Sub1) and repressed in M202 at 1 d after submergence relative to controls (Figs. 3A and 4). We also examined gene expression patterns of these genes from publicly available Affymetrix microarray data sets. Interestingly, the transcripts of Os01g21120, Os03g22170, and Os07g47790 are also up-regulated in anoxic coleoptiles of Nipponbare, which lacks Sub1A but exhibits vigorous anaerobic germination as compared with M202 and FR13A (Magneschi and Perata, 2009; Figs. 3A and 4). Based on these expression comparisons across experiments, we hypothesize that the identified AP2/ERF gene family members may regulate a component(s) that is expressed in both submergence tolerance and anaerobic germination responses. In fact, in anoxic coleoptiles, transcripts of Os01g21120 and Os07g47790 loci showed expression trajectories similar to Alcohol Dehydrogenase1 (ADH1; Os11g10480), a marker gene for low-oxygen stress. These results point to an important role for the identified AP2/ERF genes in the regulation of anaerobic...
metabolic processes in rice. Using the NSF45K data, we found that ADH1 expression is more abundant in M202 (Sub1) compared with M202 at 1 d after submergence, consistent with previous findings (Fukao et al., 2006). This result further supports roles of AP2/ERF genes in group VIIa in M202(Sub1), which stimulates the metabolic acclimation processes against oxygen deficiency such as sugar degradation, glycolysis, and alcohol fermentation (Supplemental Table S4). Recently, it was shown that calcineurin B-like-interacting protein kinase (CIPK15) plays a key role in anaerobic germination in rice (Lee et al., 2009). Therefore, this gene may regulate the expression or function of AP2/ERF genes in VIIa. In addition, three AP2/ERF genes in this group were commonly induced in roots by cytokinin and in seedling or leaves by salt and blast pathogen (Fig. 4).

It is pertinent to point out that Sub1C was down-regulated in anoxic coleoptiles and up-regulated in cytokinin-treated leaves but not in cytokinin-treated roots, unlike three AP2/ERF genes showing up-regulation in the roots. In addition, expression data of this gene in response to various stresses as mentioned above indicate that cytokinin treatment in leaves causes the activation of this gene, implying that Sub1C is responsive to cytokinin in the leaves, which can promote the production of ethylene and ultimately a GA response (Johnson and Ecker, 1998), shown previously to regulate Sub1C (Fukao et al., 2006; Fukao and Bailey-Serres, 2008; Fig. 4).

Roles of VIIIa

A common feature of AP2/ERF group VIIa in Arabidopsis and rice is the CMVIII-1 and CMVIII-2 motifs that are identical to the EAR motif that inhibits GCC box-mediated transcription (Nakano et al., 2006). In rice, eight AP2/ERF TFs were categorized to AP2/ERF group VIIa (B-1; Nakano et al., 2006). Five of them showed significant up-regulation in M202(Sub1) compared with M202 at 1 d after submergence. These genes are also up-regulated in M202(Sub1) but down-regulated in M202 at 1 d after submergence compared with untreated controls (Fig. 4). We also examined Affymetrix array data from various stress treatments for gene orthologs represented on the rice array. Expression of transcripts from these loci did not clearly show differential expression by anoxia treatment of coleoptiles (Figs. 3A and 4). GO terms in the biological process category associated with the five AP2/ERF TFs suggested a possible involvement with the ABA signaling pathway (GO:0009737; Table I; Supplemental Table S3).

Generally, the ABA signaling pathway is associated with various stress responses. In this context, three AP2/ERF TFs in group VIIa, Os09g35020, Os09g35010, and Os04g48350, may regulate a general stress tolerance response including submergence because these genes were induced by various stresses (Fig. 4). Os04g48350 is likely to be involved in the most broad-range stress response in VIIa. Other functions of VIIa members can be inferred from GO terms related to negative regulation of ethylene-mediated signaling (GO:0010105). All five genes selected in VIIa retain the same GO term (GO:0010105). Negative regulation of ethylene-mediated activation of GA responsiveness is critical for the survival of M202(Sub1) during prolonged submergence (Fukao and Bailey-Serres, 2008). This process involves SLR1 and SLR1L immediately upstream of the GA response, but factors that differentially regulate the transcript and protein abundance of these GRAS family proteins or other upstream factors have yet to be elucidated. Os01g58420 (OsERF3) in group VIIa is an active repressor of transcription (Ohta et al., 2001). AP2/ERF genes including OsERF3 in this group are expected to carry out negative regulation of the ethylene-mediated signaling pathway or ethylene-mediated activation of GA responsiveness. These AP2/ERF proteins may have a role in inhibiting leaf senescence and elongation.

Roles of IIIc

Genes belonging to group IIIc are known to play important role during cold, salt, and drought stress treatments in plants (Gilmour et al., 1998; Haake et al., 2002; Dubouzet et al., 2003; Nakano et al., 2006). Among these genes, the C-repeat-binding factor/dehydration-responsive element-binding factor1 (CBF/DREB1) genes encode a group of transcriptional activators that have been reported to play a key role in freezing tolerance and cold acclimation in Arabidopsis (Novillo et al., 2004). The C-terminal region of 98 amino acids of Arabidopsis CBF1/DREB1B was reported to function as a transactivation domain (Wang et al., 2005). Similarly, OsDREB1A and OsDREB1B, which are orthologous genes in rice for CBF/DREB1, are involved in increased tolerance to high salt and freezing (Ito et al., 2006). Our analysis indicates that four of the AP2/ERF genes in group IIIc were at least 4-fold induced in M202(Sub1) compared with M202 at 1 d after submergence (Fig. 4). Specifically, Os04g48350 has an expression pattern similar to the AP2/ERF members of groups VIIa and VIIa that positively associate with Sub1A-1 function (Figs. 3A and 4). Other AP2/ERF genes in group IIIc, Os09g35020, Os09g35010, and Os02g45450, are also up-regulated in M202(Sub1) compared with M202 at 1 d after submergence, like Os04g48350, but the expression patterns deviate from the AP2/ERF genes in the Sub1A-dependent groups (i.e. VIIa and VIIIa) and Os04g48350. These genes were down-regulated in both M202(Sub1) and M202 at 1 d after submergence compared with untreated controls, and the extent of down-regulation is relatively less in M202(Sub1) than M202 (Fig. 4).

GO terms associated with GA biosynthesis (GO:0009686) and cell growth (GO:0016049) are enriched in M202(Sub1) compared with M202 at 1 d after submergence. Four of the five genes related to GA biosynthesis belong to group IIIc (Table I). These genes were also down-regulated in anoxic coleoptiles and in leaves and roots treated with cytokinin (Fig. 4). How-
ever, these genes were up-regulated by cold treatment in 7-d-old seedlings (Fig. 4). As mentioned in the GO enrichment analysis, they are probable repressors of GA biosynthesis. Furthermore, four of the AP2/ERF genes in this group were members of GO terms related to GA biosynthesis, cell growth, and cold acclimation (Table I). Interestingly, the expression patterns of Os09g35020, Os09g35010, and Os02g45450 are in striking contrast to those of Os03g08490 and Sub1C in response to submergence in M202(Sub1) and M202. The difference was also observed in response to cytokinin and cold treatment. These results strongly suggest an antagonistic relationship between the two gene sets in response to multiple treatments.

In summary, we validated the gene expression patterns of three AP2/ERF genes in group VIIa, five in group VIIa, and three in group IIIc by RT-PCR (Fig. 3B). By combining phylogenetic analysis and GO annotation with our expression data, we have predicted putative functions for the 12 AP2/ERF genes in the context of the Sub1A-1-mediated tolerance response. Thus, these AP2/ERFs likely act downstream of Sub1A.

cis-Acting Element Analysis of Submergence-Responsive AP2/ERF Genes

Multiple EM for Motif Elicitation (MIME) is one of the most widely used tools for discovering conserved TF-binding sites in promoter sequences (Bailey et al., 2006). Based on the identification of 12 AP2/ERF TFs in three AP2/ERF groups with a putative function downstream in Sub1A-1-mediated submergence tolerance, we explored the possibility of conserved motifs in their promoter regions. To this end, we performed the MIME searches available through the National Biomedical Computation Resource (http://meme.nbcr.net) to find upstream transcriptional regulators for four AP2/ERF genes in group IIIc of three AP2/ERF groups using the Nipponbare sequence data (Fig. 5). We also attempted to identify cis-acting elements of three AP2/ERF genes (Os03g08490, Os04g46220, and Os03g64260) and Sub1C-2 that are negatively associated with Sub1A-1 function (Fig. 4; Fukao et al., 2006).

We identified four cis-acting elements for each AP2/ERF group. The most conserved six- to eight-nucleotide sequences in promoter regions of each group are presented in Figure 5 and Supplemental Figure S2. As restriction of shoot elongation during submergence plays a key role in the tolerance response of M202 (Sub1), we focus on the promoter analysis of four AP2/ERF genes in group IIIc. A DNA-binding sequence (AGCACGAC) was conserved in the promoter regions of AP2/ERF genes in group IIIc related to the repression of GA biosynthesis or the cold acclimation response (Fig. 5). Identification of the unknown protein that binds to this sequence may give us a potential upstream transcriptional regulator of this group that has not been identified. Also, binding sequences [CCCGCG and C(C/G)(A/G)CCC] attributed to SP1 and ABf4, respectively, are conserved in the promoter regions of this group (Fig. 5). DNA-binding sequences [CGCGCG and (G/C)(C/G)] attributed to MZF1_1-5 encoding C2H2-type zinc finger protein are conserved in promoter regions of three AP2/ERF genes besides Os02g45450 in this group (Fig. 2). We also examined the promoter region of the GA2ox1 gene containing the same GO terms (GA biosynthesis; GO:0009686) with AP2/ERF proteins in this group and found two GCCGCC boxes starting at 735 and 758 bp upstream of the ATG and two binding sites of barley (Hordeum vulgare) CBF1[(G/A)(C/T)CGAC] at 433 and 43 bp upstream of the ATG. AP2/ERF TFs in this group may bind to these sites in the promoter of GA2ox1 and activate the expression of this gene.

Because Sub1B shows similar expression patterns with Sub1C in many other stresses and is colocализed at the Sub1 locus on chromosome 9, it is possible that Sub1B may be functionally associated with Sub1C (Fig. 5; Xu et al., 2006). In the promoter regions of four down-regulated AP2/ERF genes and Sub1B, we identified DNA-binding sequences to MZF1_1-5 encoding a C2H2-type zinc finger protein, Macho-1 encoding a C2H2-type zinc finger protein, and ABf4 (Fig. 5). Of these, the binding sequences [(G/C)(G/C)(G/A)CCGC] to ABf4 were identified in promoter regions of all AP2/ERF genes in this group (Fig. 5). We hypothesize that AP2/ERFs in group VIIa or IIIc may bind to these sequences as repressor(s) of ethylene or GA responses. Interestingly, the promoter of Sub1C did not have the typical GCCGCC sequence (GCC box), which is present in the promoter regions of a large number of ethylene-inducible genes, because the last nucleotide (C) was replaced with G. Therefore, a repressor of ethylene response (AP2/ERF in VIIa) may not bind to the promoter region of Sub1C. Instead, AP2/ERF genes in VIIa are more likely to bind the ABf4-binding sequence in the promoter of Os03g08490 in VIIa, which has a typical GCC box (Fig. 5). The binding sequence in the promoter region of Sub1C may target AP2/ERFs in group IIIc. The AP2/ERF TFs bound to this sequence may repress Sub1C, which acts in stimulating shoot elongation (Fukao et al., 2006; Fukao and Bailey-Serres, 2008). In addition, expression patterns of three of the AP2/ERFs in group IIIc except Os04g48350 are more antagonistic to that of Sub1C (Fig. 4). The DNA-binding sequence (CCGCCGG) for the unknown target protein (s) was only conserved in Os03g08490 and Os04g46220. Identification of consensus cis-acting elements in an evolutionarily coregulated subset of genes will provide us with clues to discover new upstream target proteins. Promoter analyses of three AP2/ERF genes in group VIIa and five in group VIIIa are described in the Supplemental Description S1 (Supplemental Fig. S2).

Identification of Metabolic Pathways Positively Associated with the Sub1A-1-Dependent Tolerance Response

To further investigate the role of the Sub1A-1-regulated genes, we used the RiceCyc Pathway Tools Omics
We also analyzed the publicly available rice gene expression data (more than 1,000 array experiments; GEO [http://www.ncbi.nlm.nih.gov/geo/]; Barrett et al., 2007). These two high-throughput tools are useful for integrated diverse genomics data as shown in Arabidopsis (Hirai et al., 2007). The integrated analysis facilitated the identification of metabolic/biochemical pathways closely linked to Sub1A-1 genotype-dependent effects.

To discover metabolic/biochemical pathways differentially activated by Sub1A-1, we first integrated gene expression data from seven comparisons of 898 probes in clusters 1, 2, 3, 6, 7, 8, 9, and 12 into the RiceCyc Pathway Tool Omics Viewer (Fig. 6). Recently, the transcriptome data for rice coleoptiles grown under aerobic and anoxic conditions were reported (Lasanthi-Kudahettige et al., 2007). The rice cv Nipponbare used in this comparison is submergence intolerant (Xu et al., 2006) but exhibits vigorous germination under anoxic conditions (Magneschi and Perata, 2009). Although the low-oxygen systems (nitrogen gas versus deep water), tissue (coleoptiles versus the entire aerial tissue), and duration of the stress (4 d versus 1–6 d) are distinct in the anoxic coleoptiles and the submerged rice shoots, it can be an interesting reference to compare the two transcript profiles under oxygen deprivation in rice seedlings. Therefore, we performed the analysis using gene expression profiles represented by the 3,499 probes showing at least a 2-fold induction in anoxic coleoptiles ($P < 0.05$ by $t$ test). From this work, we identified 13 pathways showing a significant positive relationship with the presence of Sub1A-1. These included genes up-regulated in submerged M202(Sub1) plants as well as the down-regulation or the absence of up-regulation in submerged M202 plants. Among these pathways, activation of sugar degradation/glycolysis/ethanol fermentation, trehalose biosynthesis, UDP-N-acetylglactosamine biosynthesis, Ile biosynthesis,
Figure 6. Integration of gene expression data and metabolic pathways to identify pathways positively involved in Sub1A-1 function. Genes associated with Suc degradation/glycolysis/fermentation (A), trehalose biosynthesis (B), dTDP-L-Rha biosynthesis (C), UDP-N-acetylgalactosamine biosynthesis (D), isoleucine biosynthesis from threonine (E), and ethylene biosynthesis from methionine (F).
ascorbate biosynthesis, and glycerol and myoinositol biosynthesis pathways in response to oxygen deficiency are conserved in submerged M202(Sub1) and anoxic coleoptiles, suggesting that these pathways play important roles in overcoming the oxygen-deficient environment (Fig. 6; Supplemental Fig. S3).

Interestingly, genes involved in Rha biosynthesis, glutathione redox reaction, GA inactivation, glycerol degradation, and ABA inactivation pathways were activated or unchanged in submerged M202(Sub1) plants but repressed or largely unchanged in anoxic coleoptiles. For instance, genes associated with ethylene biosynthesis are up-regulated or unchanged in M202(Sub1) and M202 during submergence but repressed in anoxic coleoptiles, indicating that submerged rice seedlings activate ethylene biosynthesis during submergence but anoxic coleoptiles do not (Fig. 6). The GA2-β-dioxygenase (GA2ox; Os01g555240)-encoding enzyme, which causes degradation of bioactive GAs, is significantly up-regulated in M202(Sub1). Increases in GA2ox may restrict shoot elongation in M202(Sub1) during prolonged submergence by restricting active GA biosynthesis. OsABA8ox1 (Os02g47470), catalyzing the breakdown of ABA and involved in maintaining lower ABA levels in plants, was also up-regulated in M202(Sub1) after submergence (Supplemental Fig. S3). Activation of this gene may contribute to reducing ROS, which are known to be produced during ABA signaling in Arabidopsis (Kwak et al., 2003). Alternatively, OsABA8ox1 may have a role in restricting GA biosynthesis, as in the case of elongated uppermost internode (EUI; Zhu et al., 2006), because OsABA8ox1 encodes a cytochrome P450 monoxygenase (CYP707A5) similar to EUI (CYP714 d1; Os05g40384). The expression of EUI in our microarray data was not differentially regulated in the comparison of M202(Sub1) versus M202 after submergence (data not shown). Based on our collective pathway analysis and literature searches, we hypothesized that restriction of ROS production or removal of ROS during submergence and after desubmergence are critical factors for survival.

To test this hypothesis, we measured systemic hydrogen peroxide production in leaves using a chemical staining method (Wohlgermuth et al., 2002). We chose the second leaf for this analysis because we observed a severe decrease in the viability of second leaf in M202 at 7 d after submergence compared with M202(Sub1) (Fukao et al., 2006). We monitored hydrogen peroxide production corresponding to red-brown colors in M202(Sub1) and M202 at 0, 1, 6, and 14 d after submergence (Supplemental Fig. S4). Results of the hydrogen peroxide measurement assays after short-term submergence (1 d) were similar between M202 (Sub1) and M202 (Supplemental Fig. S4). However, the effect of prolonged submergence challenges (6 or 14 d) was detrimental to M202. Thus, hydrogen peroxide is higher in leaves of M202 at 6 d after submergence compared with M202(Sub1) (Supplemental Fig. S4). The effect is dependent on the duration of submergence (Supplemental Fig. S4). These observations correlated well with previous results, such as chlorophyll content differences between M202(Sub1) and M202 after prolonged submergence (Fukao et al., 2006; Xu et al., 2006). Gene expression data of genes associated with Rha biosynthesis, glutathione redox reaction, GA degradation, glycerol degradation, and ABA inactivation pathways identified from the integrative analysis are described in Supplemental Table S4 (Fig. 6; Supplemental Description S1). Identification of metabolic pathways negatively involved in the Sub1A-1-dependent tolerance response was analyzed using a similar approach to identify positively involved pathways. The results are presented in the Supplemental Description S1 (Supplemental Figs. S5 and S6; Supplemental Table S4).

**DISCUSSION**

The genome-wide transcriptome comparison between tolerant M202(Sub1) and sensitive M202 after submergence provides insight into the transcriptional regulation of the Sub1A-1-mediated tolerance response. To effectively utilize these data for elucidating the submergence tolerance response, we categorized the differentially expressed gene list by integrating multiple analyses that connected genome-wide data sets to several biological themes.

Integration of submergence gene expression data with clustering and metabolic pathway analysis suggested 13 pathways that are associated with Sub1A-1 function (Fig. 6; Supplemental Fig. S3). Of these pathways, we focused on the ones that had a higher frequency of members identified as differentially expressed (Fig. 6). These criteria enabled us to select pathways (i.e. eight comparisons used in Fig. 6 and Supplemental Fig. S3) that seem to be shared by submergence and anoxia or specific for submergence responses. Submergence of rice lacking Sub1A-1 is
generally known to cause morphological and physiological changes, such as increased shoot elongation, stimulated anaerobic metabolic processes and starch/Suc degradation, activation of antioxidant systems, and induction of leaf or shoot senescence (Jackson and Ram, 2003; Fukao et al., 2006; Kawano et al., 2009).

The most abundant GO term associated with the Sub1A-1-dependent tolerance response is the regulation of DNA-dependent transcription (GO:0006355), and there are 36 genes in this GO category (Supplemental Table S3). This indicates that M202(Sub1) likely employs well-organized transcriptional regulation to trigger submergence tolerance. Notably, there are 16 AP2/ERF genes that are in the same gene family with Sub1A. Of these, 10 AP2/ERF genes belong to the ERF subfamily and six to DREB (Supplemental Fig. S2). These results suggest that the Sub1A regulates the ethylene response using AP2/ERF genes in the ERF subfamily and the stress tolerance response with AP2/ERF genes in the DREB subfamily and validate previous reports suggesting these functions (Dubouzet et al., 2003; Zhu et al., 2003). Application of phylogenomic gene expression data for the AP2/ERF superfamily enabled us to distinguish 12 of the above 16 AP2/ERF genes into three groups (i.e. IIIc, VIIa, and VIIIa; Fig. 3). Gene expression data associated with each group proposed distinct roles for AP2/ERF genes in the three groups regulated by Sub1A (Figs. 4 and 7).

We identified three to five AP2/ERF genes in each group, suggesting that Sub1A-1 regulates tolerance by modulating multiple members in the same group. The expression of three AP2/ERF genes (Os01g21120, Os03g22170, and Os07g44790) in VIIa was up-regulated in M202(Sub1) but down-regulated in M202 at 1 d after submergence. These genes were also induced by cytokinin treatment, but only in roots (Hirose et al., 2007; Figs. 3A and 4). From these results, we hypothesize that an important component of the Sub1A-1-dependent response could be mediated by cytokinin. Cytokinins are known to delay senescence possibly via ethylene during submergence (Balibrea Lara et al., 2004). However, this is a speculative interpretation of the results. The direct evidence for a role of cytokinin in mediating Sub1A-1 action awaits further experimentation. In addition, it was reported that two AP2/ERF genes in VIIa, SNORKEL1 and SNORKEL2, trigger a deepwater response via ethylene signaling (Hattori et al., 2009). It is interesting that gene family members in the same subgroup conferred different functions related to shoot elongation: Sub1A restricts the shoot elongation during submergence, and SNORKEL genes stimulate internode elongation. Evolutionarily, japonica rice adapted to less flood-prone environments. This subspecies lacks Sub1A and SNORKEL genes (Fukao et al., 2006; Xu et al., 2006; Hattori et al., 2009). To recover the extremes in submergence response provided by these genes, their integration can be achieved by modern molecular breeding or activation of suppressed target gene expression by transgenic approaches (Xu et al., 2006; Hattori et al., 2009).

A group VIIa member, OsERF3, is known to repress GCC box-mediated transcription. Other AP2/ERF

**Figure 7.** Summary of Sub1A-1-mediated submergence tolerance responses revealed with integrating omics tools. Orange boxes indicate events up-regulated in M202(Sub1) after submergence, and light blue boxes indicate events down-regulated in M202(Sub1) after submergence. The orange arrow indicates activation of downstream events; black lines represent repression of downstream events; solid lines indicate validated events; and dotted lines indicate predicted events. The red open box designates the functional roles executed by AP2/ERF genes in three groups in positive association with Sub1A-1. PDC, Pyruvate decarboxylase. [See online article for color version of this figure.]
genes of the group may share this function with OsERF3. For example, AtERF4 in group VIIIa was shown to be a negative regulator of ethylene-responsive genes in Arabidopsis. Similarly, we can expect that some of the rice AP2/ERF gene(s) belonging to group VIIIa may have a repressive role on the expression of Os03g08490 in M202(Sub1) by binding the GCC box in the promoter of this gene (Fig. 7). Repression of Os03g08490 in M202(Sub1) after submergence may be related to augmentation of the expression of the Os01g21120, Os03g22170, and Os07g44790 members of group VIIa.

GO term enrichment analysis also suggested that the negative regulation of the ethylene-mediated signaling pathway could be an important component of Sub1A-1 function during submergence (Table I). This result is consistent with the previous report that Sub1A-1 limits ethylene production during submergence, possibly by feedback regulation (Fukao et al., 2006). Moreover, negative regulation of ethylene-mediated signaling is more closely associated with AP2/ERF TFs in group VIIIa, because four out of six genes with significant up-regulation in M202(Sub1) compared with M202 at 1 d after submergence were members of group VIIIa (Fig. 4). However, Sub1C may not be a direct target of transcriptional repression by this AP2/ERF group, because Sub1C does not have a GCC box in the promoter region. Instead, Sub1C contains cis-acting elements [(T/C)CCCCTC(C/G) and GTG(G/T)(G/C)TG(G/T)] associated with binding by C2H2-type zinc finger TFs. Zinc finger TFs of Arabidopsis can repress transcription (Ohta et al., 2001). In our microarray data, there are two zinc finger TFs (Os03g32230 and Os03g60560) that showed similar expression patterns to AP2/ERF genes in VIIIa (Supplemental Table S3). Accordingly, we hypothesize that the expression of Sub1C during submergence or ethylene may be repressed by zinc finger TFs binding to cis-acting elements (Supplemental Fig. S2).

AP2/ERF genes in group IIIc may play critical roles in M202(Sub1) for survival during prolonged submergence by restricting GA-mediated shoot elongation under water. Notably, four of the five genes assigned to the GO term related to GA biosynthesis in Table I were also catalogued as AP2/ERF group IIIc members (Figs. 3A and 4). One or more of these could be required to inhibit GA biosynthesis. Arabidopsis TF DREB1 in IIIc controls the expression of many stress-inducible genes. OsDREB1A and OsDREB1B, DREB1 orthologs in rice, are induced by cold stress, and the overexpression of OsDREB1 increased tolerance to high-salt and freezing stresses (Ito et al., 2006; Gutha and Reddy, 2008). These rice transgenic plants also showed growth retardation under normal conditions, implicating rice AP2/ERF TFs in group IIIc function as repressors of GA biosynthesis, although we cannot ignore the possibility that group IIIc regulates BR biosynthesis or response (Ito et al., 2006). Moreover, overexpression of the Arabidopsis DWARF AND DELAYED FLOWERING1 (DDF1) gene encoding an AP2/ERF TF in group IIIc causes dwarfism mainly by reducing levels of bioactive GA in transgenic Arabidopsis (Magome et al., 2008). Like DDF1, up-regulation of Os04g48350, Os09g35010, Os09g35020, and Os02g45450 in M202(Sub1) compared with M202 after submergence may trigger the expression of GA 2-oxidase (Os01g55240), encoding a GA-inactivating enzyme, which could be involved in the limitation of shoot elongation of M202(Sub1) during prolonged submergence (Fukao et al., 2006; Xu et al., 2006; Supplemental Fig. S7). Unfortunately, we have been unsuccessful in quantifying the low-abundance bioactive GA in these lines under control or submergence conditions to address whether differences in GA levels are responsible in part for the distinctions in underwater growth (T. Fukao and J. Bailey-Serres, unpublished data). Additionally, fold induction of these AP2/ERF genes in M202(Sub1) compared with M202 at 1 d after submergence identified by both RT-PCR and microarray is the highest in three AP2/ERF genes positively triggering the Sub1A-1-mediated tolerance response, further supporting the significance of this AP2/ERF group.

Recently, we suggested that Sub1A-1 might limit the response to GA because ectopically expressed Sub1A-1 displays a classical GA-insensitive phenotype (Fukao et al., 2006). Also, Sub1A-1 stimulated the accumulation of the GA signaling repressors SLR1 (Os03g49990) and SLR1 (Os03g51330) during submergence, resulting in retarding shoot elongation by GA response (Fukao and Bailey-Serres, 2008). Consistently, we found that both of these GRAS domain protein genes belong to cluster 2 (Fig. 2) and show an expression pattern associated with Sub1A-1-mediated tolerance (Supplemental Table S2). We propose that AP2/ERF genes in group IIIc repress GA production upstream of SLR1 or SLR1, because these AP2/ERF genes up-regulate the GA 2-oxidase catalysis of active GAs, as seen in Arabidopsis overexpressing rice DDF1. Moreover, a reduction in endogenous GA caused by GA 2-oxidases may result in the accumulation of these GRAS domain proteins (e.g. SLR1 and SLR1; Magome et al., 2008; Fig. 7). Expression patterns of Sub1C in M202(Sub1) and M202 were opposite those of the group IIIc AP2/ERFs Os09g35010, Os09g35020, and Os02g45450, because Sub1C was up-regulated in M202(Sub1) and M202 after submergence but the AP2/ERF genes were down-regulated in both genotypes. From these comparisons, we expect that Sub1C and these three AP2/ERF genes in group IIIc may function antagonistically to each other for shoot elongation during submergence. This result is also speculative, awaiting further experiments to be verified.

In conclusion, we have proposed several transcriptional programs associated with the submergence tolerance response mediated by Sub1A-1. This information has allowed us to construct a model of the transcriptional networks that modulate submergence tolerance.
MATERIALS AND METHODS

Sample Collection and Microarray Experiments

For microarray analysis, rice (*Oryza sativa*) plant culture and submergence treatment were performed as described by Fuku et al. (2006). Two biological replicates were prepared for three time points (0, 1, and 6 d after submergence). The following description is for the sampling that was used for validation. Seeds of M202(Sub1) were planted in polystyrene containers containing a waterlogged soil mixture in order to simulate submergence conditions. The rice plants were grown under greenhouse conditions. Forty-five plants of each genotype were divided into two groups. The aerial portion of 15 plants of the first group was harvested and pooled before submergence (untreated controls or 0 d after submergence); similarly, 15 plants were harvested 1 and 6 d after submergence. The plants were 25 to 28 cm tall before submergence and immersed in a 100-cm water column at 3±1°C. We isolated 500 μg to 1 mg of total RNA using Trizol reagent, and the total RNA was DNaseI treated for 15 min and purified using the RNeasy Midi Kit (Qiagen). The total RNA was then enriched for poly(A) RNA (1–2 μg) using the Oligotex mRNA Kit (Qiagen). All steps were performed according to the manufacturer’s instructions. The quantity of total RNA and mRNA was determined by measuring A260 and A280 using a Nanodrop ND-1000. In addition, the level of protein contamination in the RNA was determined based on the A260:A280 ratio. Only RNA samples with ratios of 2.0 to 2.2 were used for these experiments. Probe preparation, pretreatment, hybridization, washing, scanning, and spot-finding processes to perform the microarray experiment were well described in our previous study (Jung et al., 2008a, 2008b). We generated at least three replicates, including two biological replicates for each time point, and detailed plans of the hybridizations are given in Supplemental Table S1. The NSF45K array was deposited to the public microarray database NCBI GEO, and the data series accession number is GSE17704.

Processing of Microarray Data

We normalized replicated data to minimize the variations caused by experimental procedures using the Lowess normalization method in the LMGene Package in R (Berger et al., 2004; Rocke, 2004). We further normalized for signal intensity among different experiments using averages of all the gene signals obtained during individual experiments. To identify genes differentially expressed between M202(Sub1) (tolerant) and M202 (intolerant) at 0, 1, and 6 d after submergence, Student’s t test was conducted by comparing log2-transformed M202(Sub1) versus M202 expression values with two-sample hypothesis and equal variation assumptions. Then, we selected candidate genes showing at least 2-fold change with P < 0.05. We presented averages of the three log2 values for M202(Sub1) over M202 values (Supplemental Table S1). Genes selected by these criteria were considered to be differentially expressed in the analysis of microarray data (Jiao et al., 2005). We identified differentially expressed genes in M202(Sub1) and M202 at 1 or 6 d after submergence. These comparisons are indirect and, to do this, we normalized all slides in the same conditions to reduce variation by slide. The reason why we generate these comparisons is to clarify results from the direct comparison [M202(Sub1) versus M202] and lead to the identification of more significant genes. For example, there are five possibilities of up-regulation in M202(Sub1) compared with M202 after submergence: the first is the case of genes displaying induction in M202(Sub1) after submergence but reduction in M202; the second is the case that is induced in M202(Sub1) after submergence but not changed in M202; the third is the case that is induced in M202(Sub1) after submergence but less induced in M202 than those in M202(Sub1); the fourth is the case that is not changed in M202(Sub1) after submergence but reduced in M202; and the last is the case that is reduced in M202(Sub1) after submergence but less reduced in M202 than those in M202(Sub1). The most obvious Sub1A-1-dependent tolerance response is implicated from the induction in M202(Sub1) relative to M202 after submergence, the induction in M202(Sub1) after submergence, and reduction or no differential expression in M202.

HCL Analysis

HCL analysis was applied to all genes showing M202(Sub1) versus M202 differential expression at least one of seven comparisons that we generated (Fig. 2). Candidate genes showing significant differential expression as mentioned above were selected. We used The Institute for Genomic Research (TIGR) MultiExperiment Viewer (http://www.tm4.org/mev.html) to carry out clustering analyses of candidate genes (Eisen et al., 1998; Tusher et al., 2001). For HCL analysis, we based the analysis on the Euclidean distance, the difference in log2 ratios between two genes, which is the default metric distance used for HCL, and the complete linkage clustering algorithm was used (Eisen et al., 1998). As a result, we manually generated 12 clusters.

GO Enrichment Analysis

GO annotation was used for gene functional classification. We evaluated enrichment of GO terms for genes up-regulated in M202(Sub1) compared with M202 at 1 d after submergence, genes up-regulated in M202(Sub1) at 1 d after submergence compared with untreated controls, and genes down-regulated in M202 at 1 d after submergence compared with untreated controls in the biological process category. We calculated fold enrichment for each GO term for gene lists determined with at least 2-fold changes in each comparison and false discovery rate threshold values of 0.05 or less. For each GO term, we provide the numbers observed and expected in the gene list with total number of repeats in the whole genome. We used the hypergeometric distribution to evaluate the probability of randomly observing the enrichment for each GO term (Falcon and Gentleman, 2007; Jung et al., 2008a). Cutoff GOStat P values were less than 0.05, and cutoff GO enrichment values were at least 1.5.

Gene Expression Analysis in the Phylogenetic Context

We constructed a phylogenetic tree of the AP2 family with the domain developed in PinTFDB (Riano-Pachon et al., 2007). We aligned the domain sequences in these families using ClustalW version 2.0 with default options (Larkin et al., 2007). The unrooted phylogenetic tree was constructed with the neighbor-joining method executed in MEGA version 3.1 (Kumar et al., 2004). The raw data for the rice Affymetrix microarray experiment were downloaded from the NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/). The GEO accession number of this platform is GPL205, and the GEO accession number for transcript profiling of the anoxic versus aerobic rice coleoptiles (http://www.plantphysiol.org/cgi/content/full/144/1/218-FN2) is GSE6908 (Lasanthi-Kudathettige et al., 2007). A detailed method for the analysis of these data was described in our previous study (Jung et al., 2008a). The rice Multi-platform Microarray Search tool (http://www.ricearray.org/matrix.search.shtml) was used to assign the corresponding Affymetrix probe sets for rice AP2 (Jung et al., 2008a). For the NSF45K array data to compare M202(Sub1) and M202, we used log2 ratios of seven comparisons: M202(Sub1) over M202 at 1 d after submergence (S/M_0 d), M202(Sub1) over M202 at 1 d after submergence (S/M_1 d), M202(Sub1) over M202 at 6 d after submergence (S/M_6 d), 1 d after submergence versus before submergence in M202(Sub1) (1 d/0 d_S), 6 d after submergence versus before submergence in M202(Sub1) (6 d/0 d_S), 1 d after submergence versus before submergence in M202 (1 d/0 d_M), and 6 d after submergence versus before submergence in M202 (6 d/0 d_M). With the same order of gene models in the AP2/ERF phylogenetic tree, we aligned the heat map of gene expression patterns. The heat map was generated by the TIGR MultiExperiment Viewer version 4.1 (http://www.tm4.org/mev.html; Eisen et al., 1999).

Histochemical Detection of Hydrogen Peroxide Accumulation

The accumulation of hydrogen peroxide was examined in the submerged rice leaves at different times (0, 1, 6, and 14 d). The rice leaves were prepared in the same conditions for the microarray experiment. The detection of hydrogen peroxide was performed as described previously (Ahn, 2008). The collected leaves at 3:00 to 4:00 PM were stained with 0.1% (w/v) diminobenzidine (Sigma). The stained leaves were cleared with 96% (w/v) ethanol and preserved in 50% (w/v) ethanol. Diminobenzidine staining for hydrogen peroxide was observed as a red-brown color with the light microscope.

RT-PCR

We validated gene expression patterns of 12 AP2/ERF genes using RT-PCR analyses. RT-PCR with the same mRNA used for the microarray experiment was carried out as described in a previous study (Jung et al., 2006). In addition,
we prepared the validation data using RT-PCR for leaf samples after 6-d submergence (Supplemental Figure S8). The primer sequences are described in Supplemental Table S5.

Promoter Analysis of AP2/ERF Genes

To identify consensus cis-acting elements in the promoters of downstream AP2/ERF genes of Sub1A-1, we extracted 1-kb sequences upstream of ATG of 18 AP2/ERF genes from the TIGR/Michigan State University rice genome annotation project (ftp://ftp.planetbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_6.0/all.dir/). Then, those sequences were divided into four groups: the gene list in three of them is the same as the description in Figure 4, and the fourth group consists of AP2/ERF genes down-regulated in M202(Sub1)/M202 fold change values in Supplemental Figure S2, and known target proteins with known target proteins with P < 0.05 were selected. In cases in which we did not find any known target proteins with P < 0.05, we described that the queried motifs matched with an unknown target protein.

Integration of Gene Expression Data into the Metabolic Pathway Tool

The Rice Pathway Tools Expression Viewer based on Gramene RiceCyc spreads data values from the user’s high-throughput data onto the Cellular Overview diagram for rice (http://pathway.gramene.org/RICE/expression.html). For featured expression patterns in clusters 1, 2, 3, 6, 7, 8, and 9 or clusters 4, 5, 10, and 11, we uploaded log2-transformed fold change values in the seven comparisons of NSF45K array data mentioned above. We display several representative results from M202(Sub1)/M202 fold change values in Figure 6 and Supplemental Figure S2, and known target proteins with P < 0.05 were selected. In cases in which we did not find any known target proteins with P < 0.05, we described that the queried motifs matched with an unknown target protein.

Identification of Arabidopsis Orthologs of Two C2H2 Zinc Finger TFs Coregulated with AP2/ERF Genes in Group VIIIa

The GreenPhyl Web server has serviced phylogenomic analysis of rice and Arabidopsis (Arabidopsis thaliana) paralogous protein families including C2H2 zinc finger TFs. From this analysis, At1g49900, At5g67450 (Azf1, for Arabidopsis zinc finger protein 1), and At3g49930 (Zat13) have orthology with Os03g32230 (http://greenphyl.cirad.fr/cgi-bin/sequence.cgi?search=Os03g32230) and At3g46070 (Zat16) and At2g26710 (Zat17) have orthology with Os03g60560 (http://greenphyl.cirad.fr/cgi-bin/sequence.cgi?search=Os03g60560).

Supplemental Data

The following materials are available in the online version of this article. Supplemental Figure S1. Phylogenetic expression analysis of the AP2/ERF superfamily.

Supplemental Figure S2. Conserved cis-acting elements for groups VIIa and VIIIa.

Supplemental Figure S3. Identification of pathways positively involved in Sub1A-1 function not described in Figure 6.

Supplemental Figure S4. Effects of submergence on hydrogen peroxide accumulation.

Supplemental Figure S5. Gene expression patterns related to gluconeogenesis, photosynthesis, and ammonia assimilation cycle pathways.

Supplemental Figure S6. Gene expression patterns related to aerobic respiration, reduced tricarboxylic acid cycle, and Calvin cycle pathways.

Supplemental Figure S7. Expression patterns of two genes related to GA biosynthesis or GA inactivation.

Supplemental Figure S8. Validation of 11 AP2 genes after 6-d submergence using RT-PCR.

Supplemental Table S1. Scheme of microarray experiments used in this study.

Supplemental Table S2. Detailed gene expression data used for HCL analysis in Figure 2.

Supplemental Table S3. Gene expression data of genes related to GO terms enriched in Table I.

Supplemental Table S4. Detailed gene expression data used in Figure 6 and Supplemental Figures S3, S5, and S6.

Supplemental Table S5. Primer sequences used in Figures 1 and 3.

Supplemental Description S1.

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