

# The Submergence Tolerance Regulator *Sub1A* Mediates Stress-Responsive Expression of AP2/ERF Transcription Factors<sup>1[C][W][OA]</sup>

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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 isoline 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 Voisenek, 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).

A phenotype directly linked to the presence of *Sub1A-1* is limited underwater elongation of the shoot and leaves (Fukao et al., 2006; Xu et al., 2006). The elongation of aerial organs during submergence of

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semiaquatic plants is closely tied to phytohormones (Bailey-Serres and Voesenek, 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 Voesenek, 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 Voesenek, 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, phylogeny, 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 embryo 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

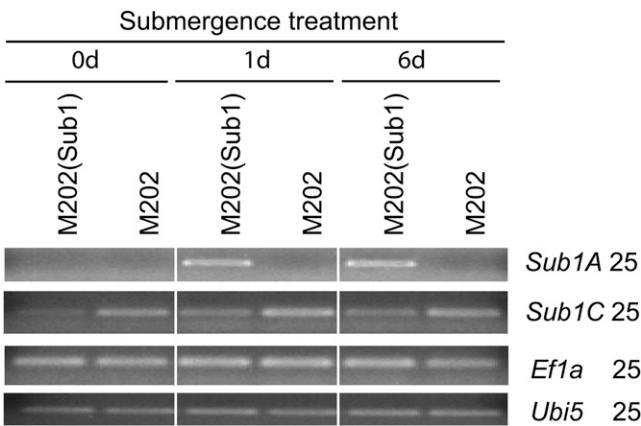
Phenotypic Characterization of the Submergence-Tolerant and -Intolerant Lines

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

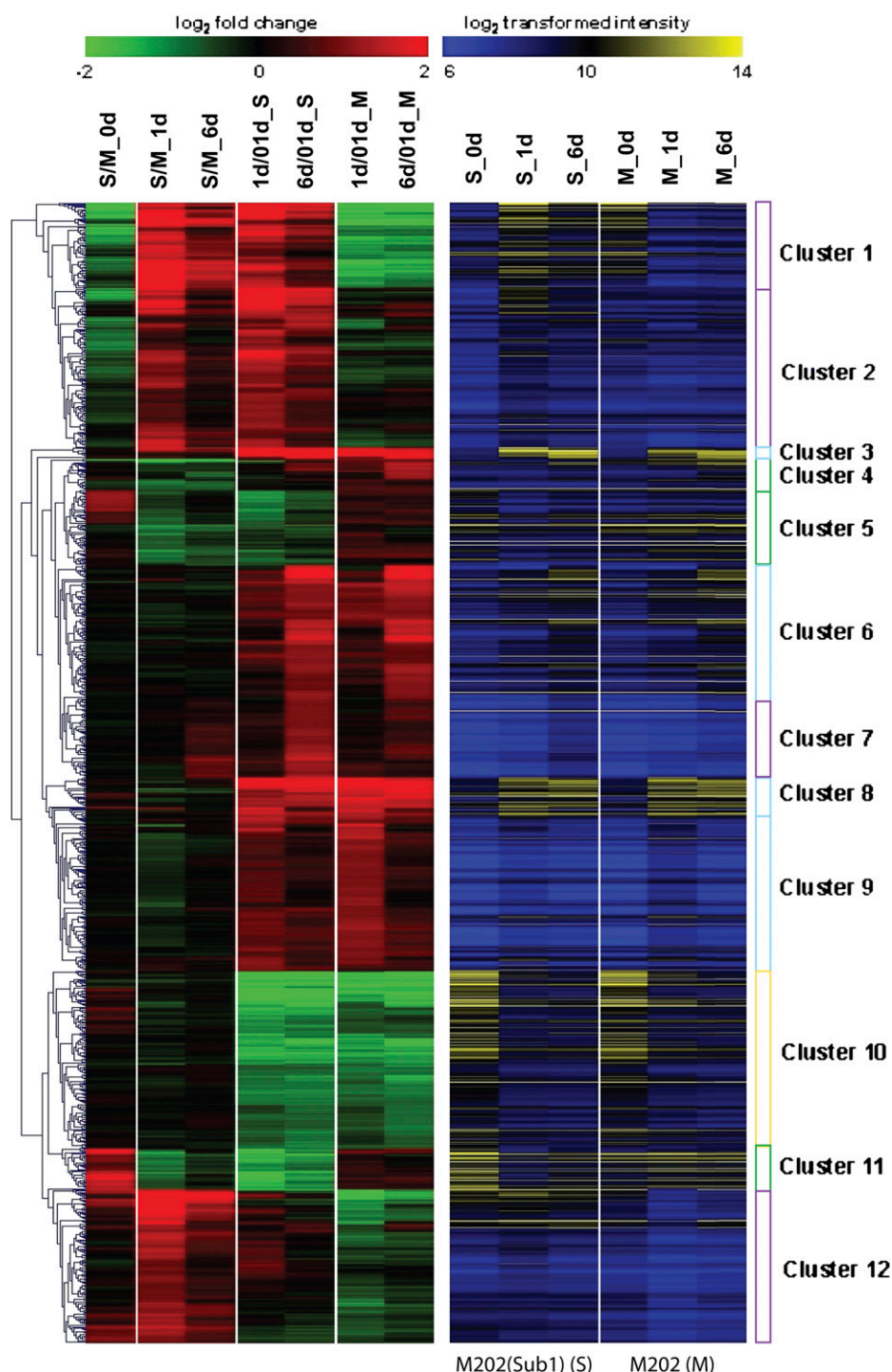
We used the NSF45K array to compare the transcript abundance of M202(*Sub1*) and M202 by hybridization



**Figure 1.** Quality test of samples used for the microarray experiment. Expression patterns are for marker genes for microarray experiments. *Ubiquitin5* (*Ubi5*; *Os01g22490.1*) and *Elongation factor1a* (*Ef1a*; *Os03g08010.1*) genes were used as internal controls for RT-PCR. RNA was extracted from M202(*Sub1*) and M202 at 0, 1, and 6 d after submergence.

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<sub>2</sub> 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<sub>2</sub> ratio ≥ 1) or down-regulation (log<sub>2</sub> ratio ≤ -1) in at least one of the seven comparisons with *t* test *P* ≤ 0.05 (see “Materials and Methods”). The average log<sub>2</sub> 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



**Figure 2.** HCL analysis of candidate genes showing significant differential expression patterns after submergence. The left panel displays  $\log_2$  fold ratios from seven comparisons, and the right panel represents  $\log_2$ -transformed normalized intensity in six samples used for this microarray experiment of each probe. Red indicates up-regulation in M202(*Sub1*) compared with M202 after submergence, in M202(*Sub1*) after submergence compared with before submergence, and in M202 after submergence compared with before submergence, and green indicates down-regulation for the same comparisons. Blue indicates background level of expression, and yellow indicates high level of expression. Various colored boxes indicate clusters showing similar tendencies of gene expression. Later, clusters 1, 2, 3, 6, 7, 8, and 9 were applied for Figure 6 and Supplemental Figure S3, and clusters 4, 5, 10, and 11 were applied for Supplemental Figures S5 and S6. S/M\_0 d,  $\log_2$  ratio of M202 (*Sub1*) compared with M202 before submergence; S/M\_1 d,  $\log_2$  ratio of M202(*Sub1*) compared with M202 at 1 d after submergence; S/M\_6 d,  $\log_2$  ratio of M202(*Sub1*) compared with M202 at 6 d after submergence; 1 d/0 d\_S,  $\log_2$  ratio of M202(*Sub1*) at 1 d after submergence compared with before submergence; 6 d/0 d\_S,  $\log_2$  ratio of M202(*Sub1*) at 6 d after submergence compared with before submergence; 1 d/0 d\_M,  $\log_2$  ratio of M202 at 1 d after submergence compared with before submergence; 6 d/0 d\_M,  $\log_2$  ratio of M202 at 6 d after submergence compared with before submergence. The blue-yellow bar indicates  $\log_2$ -transformed normalized expression levels of Affymetrix and NSF45K array data, and the range is from 14 to 6. The green-red bar indicates fold change values of seven comparisons in NSF45K array data, and the range is from  $-2$  to  $2$ . [See online article for color version of this figure.]

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 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 isolate 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 log<sub>2</sub> 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 GStat *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*)

| GO ID <sup>a</sup> | GO Name  | Total <sup>b</sup> | Sub1/M202_1<br>d_Up <sup>c</sup> |                         |                                | Sub1 1<br>d/0 d_Up <sup>d</sup> |                         |                                | M202 1<br>d/0 d_Down <sup>e</sup> |                         |                                |
|--------------------|--|--------------------|----------------------------------|-------------------------|--------------------------------|---------------------------------|-------------------------|--------------------------------|-----------------------------------|-------------------------|--------------------------------|
|                    |  |                    | OB<br>No. <sup>f</sup>           | EX.<br>No. <sup>g</sup> | GStat<br><i>P</i> <sup>h</sup> | OB<br>No. <sup>f</sup>          | EX.<br>No. <sup>g</sup> | GStat<br><i>P</i> <sup>h</sup> | OB<br>No. <sup>f</sup>            | EX.<br>No. <sup>g</sup> | GStat<br><i>P</i> <sup>h</sup> |
|                    |  |                    | OK <sup>i</sup>                  |                         |                                | OK <sup>i</sup>                 |                         |                                | OK <sup>i</sup>                   |                         |                                |
| GO:0010200         | Response to chitin   | 42                 | 7                                | 0.9                     | 2.21E-05                       | 5                               | 1.4                     | 0.010412                       | 7                                 | 0.8                     | 1.61E-05                       |
| GO:0009618         | Response to pathogenic bacteria                            | 77                 | 9                                | 1.6                     | 2.84E-05                       | 9                               | 2.6                     | 0.000872                       | 5                                 | 1.5                     | 0.01447                        |
| GO:0009611         | Response to wounding                                       | 235                | 17                               | 4.9                     | 7.97E-06                       | 25                              | 7.9                     | 3.31E-07                       | 12                                | 4.7                     | 0.00188                        |
| GO:0009414         | Response to water deprivation                              | 188                | 13                               | 3.9                     | 0.00013                        | 11                              | 6.3                     | 0.028463                       | 9                                 | 3.7                     | 0.00882                        |
| GO:0009873         | Ethylene-mediated signaling pathway                        | 81                 | 13                               | 1.7                     | 1.19E-08                       | 14                              | 2.7                     | 4.26E-07                       | 4                                 | 1.6                     | 0.05559                        |
| GO:0010105         | Negative regulation of ethylene-mediated signaling pathway | 53                 | 6                                | 1.1                     | 0.0007                         | 7                               | 1.8                     | 0.001567                       | 5                                 | 1.1                     | 0.0034                         |
| GO:0009723         | Response to ethylene stimulus                              | 136                | 9                                | 2.8                     | 0.00172                        | 15                              | 4.6                     | 4.32E-05                       | 6                                 | 2.7                     | 0.03577                        |
| GO:0009737         | Response to ABA stimulus                                   | 330                | 21                               | 6.9                     | 5.34E-06                       | 18                              | 11                      | 0.014838                       | 15                                | 6.6                     | 0.00172                        |
| GO:0009411         | Response to UV light                                       | 99                 | 6                                | 2.1                     | 0.01309                        | 9                               | 3.3                     | 0.004417                       | 5                                 | 2                       | 0.03374                        |
| GO:0008219         | Cell death   | 92                 | 5                                | 1.9                     | 0.0313                         | 15                              | 3.1                     | 3.64E-07                       | 5                                 | 1.8                     | 0.02669                        |
| GO:0006355         | Regulation of transcription, DNA dependent                 | 802                | 36                               | 17                      | 1.03E-05                       | 38                              | 27                      | 0.008219                       | 25                                | 16                      | 0.00829                        |
| GO:0006979         | Response to oxidative stress                               | 119                | 5                                | 2.5                     | 0.06583                        | 9                               | 4                       | 0.012471                       | 6                                 | 2.4                     | 0.02219                        |
| GO:0016049         | Cell growth  | 42                 | 4                                | 0.9                     | 0.00958                        | 1                               | 1.4                     | 0.34704                        | 5                                 | 0.8                     | 0.00125                        |
| GO:0009686         | Gibberellic acid biosynthesis                              | 56                 | 5                                | 1.2                     | 0.00518                        | 2                               | 1.9                     | 0.275034                       | 5                                 | 1.1                     | 0.00426                        |
| GO:0009631         | Cold acclimation   | 24                 | 6                                | 0.5                     | 7.59E-06                       | 2                               | 0.8                     | 0.147771                       | 6                                 | 0.2                     | 5.73E-06                       |
| GO:0009409         | Response to cold   | 193                | 14                               | 4                       | 4.60E-05                       | 9                               | 6.5                     | 0.087028                       | 9                                 | 3.8                     | 0.010164                       |
| GO:0009735         | Response to cytokinin stimulus                             | 122                | 7                                | 2.6                     | 0.010259                       | 15                              | 4.1                     | 1.24E-05                       | 3                                 | 2.4                     | 0.213479                       |

<sup>a</sup>GOSlim identifier. <sup>b</sup>Number of each GOSlim term in the whole genome. <sup>c</sup>Genes at least 2-fold up-regulated with *P* < 0.05 in M202(*Sub1*) relative to M202 at 1 d after submergence. <sup>d</sup>Genes at least 2-fold up-regulated with *P* < 0.05 in M202(*Sub1*) at 1 d after submergence relative to untreated control. <sup>e</sup>Genes at least 2-fold down-regulated with *P* < 0.05 in M202 at 1 d after submergence relative to untreated control. <sup>f</sup>Number of observed repeats for a GOSlim term in one of the gene lists. <sup>g</sup>Number of expected repeats for a GOSlim term in one of the gene lists. <sup>h</sup>Significance of selected GO terms with *P* values generated using the GStat tool. <sup>i</sup>Satisfaction 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<sub>1</sub> (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. OsDREB1s, 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*-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*

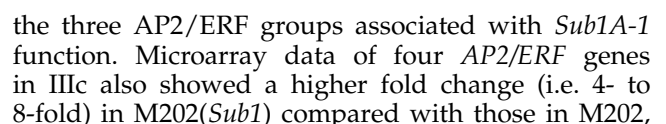
#### Construction of Phylogenomic Digital Gene Expression Data of the AP2/ERF Superfamily

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<sub>2</sub> 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), trans-zeatin 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. IIIc, VII, and VIIa) 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





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).

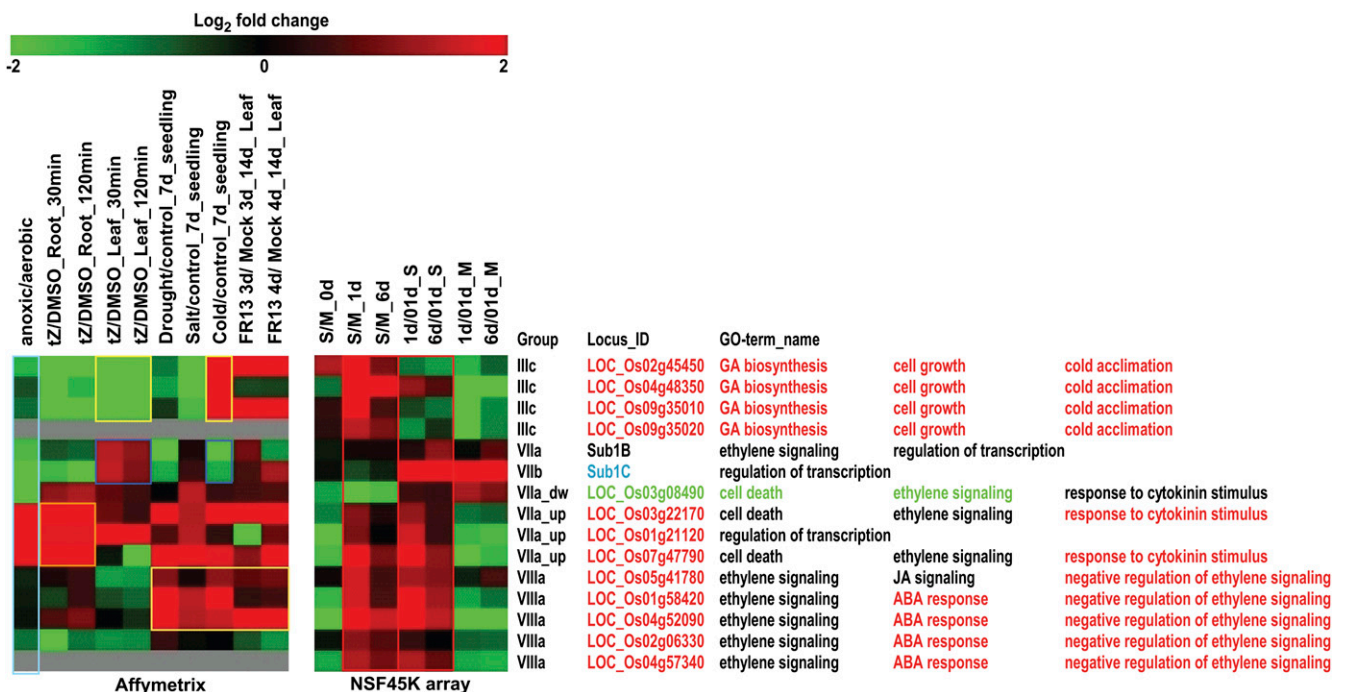
In contrast, *Os03g08490* in group VIIa was involved in negative association with *Sub1A-1* function, because this gene was more than 4-fold down-regulated in M202(*Sub1*) compared with M202 at 1 d after submergence (Fig. 3; Supplemental Table S2). Eleven AP2/ERF genes including *Sub1C* (*Os09g11460*) 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



**Figure 4.** Functional assignment of three AP2/ERF groups for *Sub1A-1* function with integrating GO terms and publicly available gene expression data. The left panel displays  $\log_2$  fold changes from gene expression data to compare anoxic versus aerobic coleoptiles, trans-zeatin (tZ) versus dimethyl sulfoxide (DMSO) in root and leaf (GSE6737), abiotic stresses (i.e. drought, salt, and cold) versus untreated control (GSE6901), *M. grisea* (FR13) versus mock treatment (GSE7256; Hirose et al., 2007; Jain et al., 2007), and seven comparisons used in Figure 2 from NSF45K array data. The right panel provides the names of the AP2/ERF groups, locus identifiers (ID) based on Osa1 version 5.0, and names of GO terms assigned to each AP2/ERF gene. See Figure 2 legend for the heat map. [See online article for color version of this figure.]



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 VIIa

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, *Os05g41780*, *Os01g58420*, and *Os04g52090*, may regulate a general stress tolerance response including submergence because these genes were induced by various stresses (Fig. 4). *Os04g52090* 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 regula-

tion 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 *SLRL1* 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 VIIa) 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 VIIIa, 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 (MEME) 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 MEME 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).

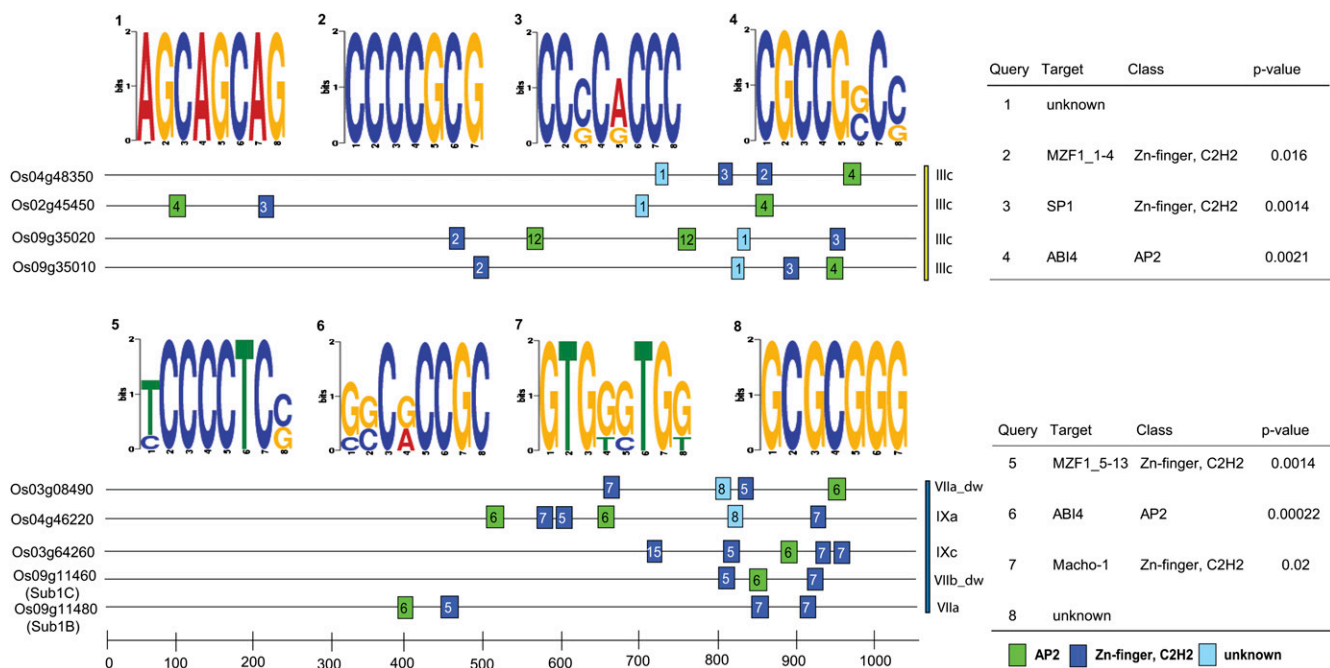
We identified four cis-acting elements for each *AP2/ERF* group. The most conserved six- to eight-nucleotide sequences in promoter regions of each group are presented in Figure 5 and Supplemental Figure S2. As restriction of shoot elongation during submergence plays a key role in the tolerance response of M202 (*Sub1*), we focus on the promoter analysis of four *AP2/ERF* genes in group IIIc. A DNA-binding sequence (AGCAGCAG) 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 [CCCCGCG and CC(C/G)C(A/G)CCC] attributed to SP1 and ABI4, respectively, are conserved in the pro-

moter regions of this group (Fig. 5). DNA-binding sequences [CGCCG and (G/C)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 colocalized 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\_5-13 encoding a C2H2-type zinc finger protein, Macho-1 encoding a C2H2-type zinc finger protein, and ABI4 (Fig. 5). Of these, the binding sequences [(G/C)(G/C)C(G/A)CCGC] to ABI4 were identified in promoter regions of all *AP2/ERF* genes in this group (Fig. 5). We hypothesize that *AP2/ERFs* in group VIIIa 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 VIIIa) may not bind to the promoter region of *Sub1C*. Instead, *AP2/ERF* genes in VIIIa are more likely to bind the ABI4-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 (GCGCGGG) 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

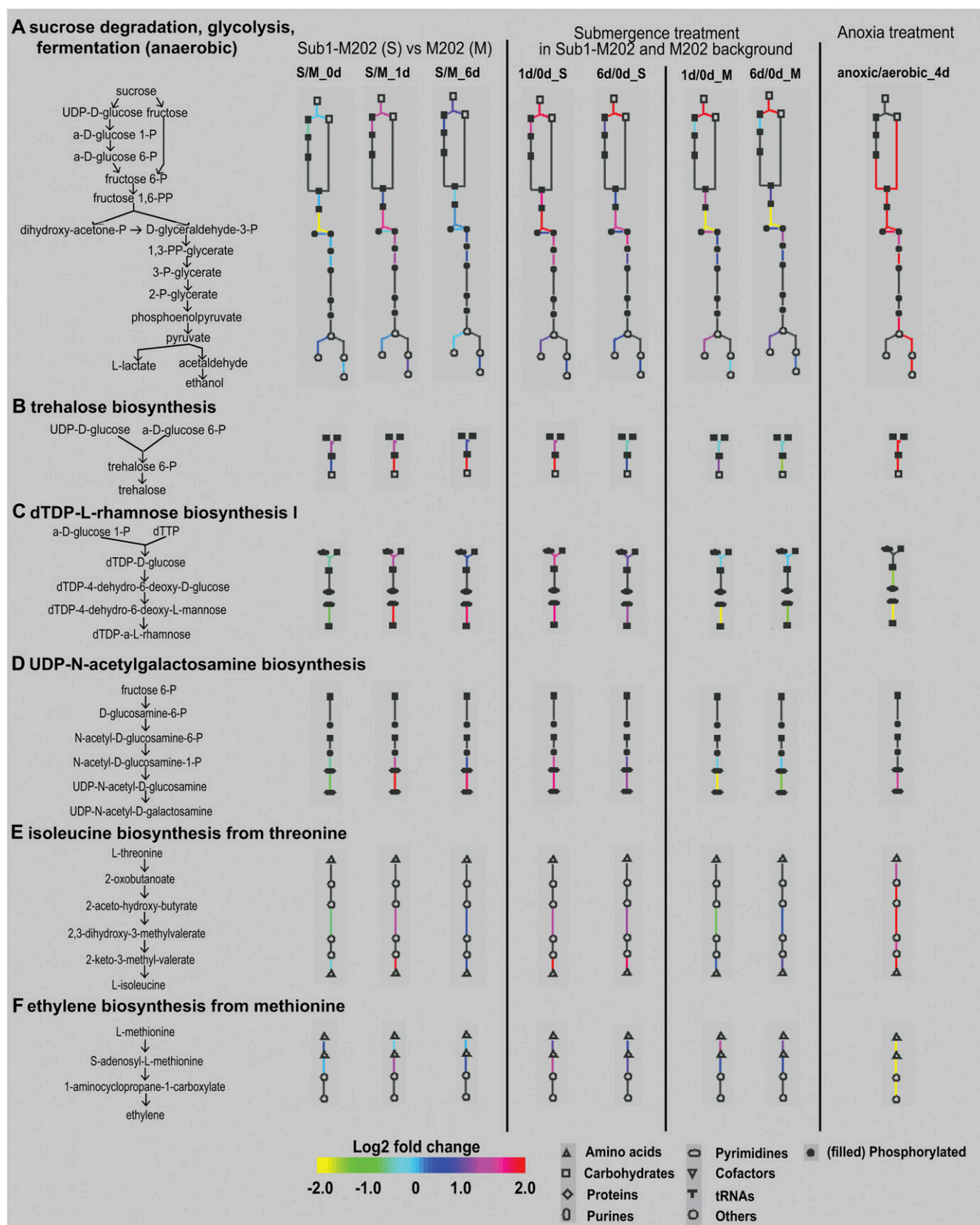


**Figure 5.** Identification of conserved cis-acting elements in significant AP2/ERF genes for *Sub1A-1* function. We used the MEME tool to find consensus cis-acting elements in the promoter regions of AP2/ERF genes in each of four groups. For each group, we provide four putative cis-acting elements with lowest E-value calculated via the Web server (<http://meme.nbcr.net>). Numerals 1 to 4 resulted from MEME searches for group IIIc; 5 to 8 resulted from MEME searches for a group consisting of *Sub1B*, *Sub1C*, *Os03g08490*, *Os04g46220*, and *Os03g64260* in negative association with *Sub1A-1* function except *Sub1B*. Analyses for VIIa and VIIa are presented in Supplemental Figure S2. In total, eight consensus sequence LOGOs are represented here and an additional eight for VIIa and VIIa are represented in Supplemental Figure S2, and the MEME tool automatically produced the LOGO. Query indicates the sequence LOGO used to search for matches to known TF-binding motifs; Target is the name of the putative TF binding to the sequence LOGO resulting from MAST searches; Class is the name of the gene family including the target; and P value represents the probability of obtaining a result and target identified with  $P < 0.05$ . Unknown, Absence of target identified with  $P < 0.05$  as a result of MAST. Green boxes indicate LOGOs binding to AP2/ERF TF; blue boxes indicate C2H2-type zinc finger protein; and light blue boxes indicate unknown. Numerals at bottom indicate the positions of nucleotides starting from ATG. [See online article for color version of this figure.]

Viewer (<http://www.gramene.org/pathway/ricecyc.html>) curated by Gramene. RiceCyc consists of 8,868 enzymes, covering 20% of the current 50,960 non-transposable element gene models (<http://rice.plantbiology.msu.edu/pseudomolecules/info.shtml>). 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 ( $P \leq 0.05$  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

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*; *Os01g55240*)-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 monooxygenase (*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 (Wohlgemuth 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

### Figure 6. (Continued.)

(C), UDP-*N*-acetylgalactosamine biosynthesis (D), and Ile biosynthesis from Thr (E) pathways are coexpressed and show positive association with *Sub1A-1* function and up-regulation in anoxic coleoptiles. Ethylene biosynthesis from the Met pathway (F) is up-regulated in M202(*Sub1*) and M202 after submergence but repressed in anoxic coleoptiles. For this figure, we used log<sub>2</sub> fold ratios of seven comparisons in Figure 2 and comparison of anoxic versus aerobic coleoptiles with the Affymetrix array from a public microarray database (NCBI GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and integrated the data to the RiceCyc Omics Viewer (<http://pathway.gemane.org/RICE/expression.html>). Expression patterns of candidate genes are marked as colored lines. See Figure 2 legend for a description of the seven comparisons. [See online article for color version of this figure.]



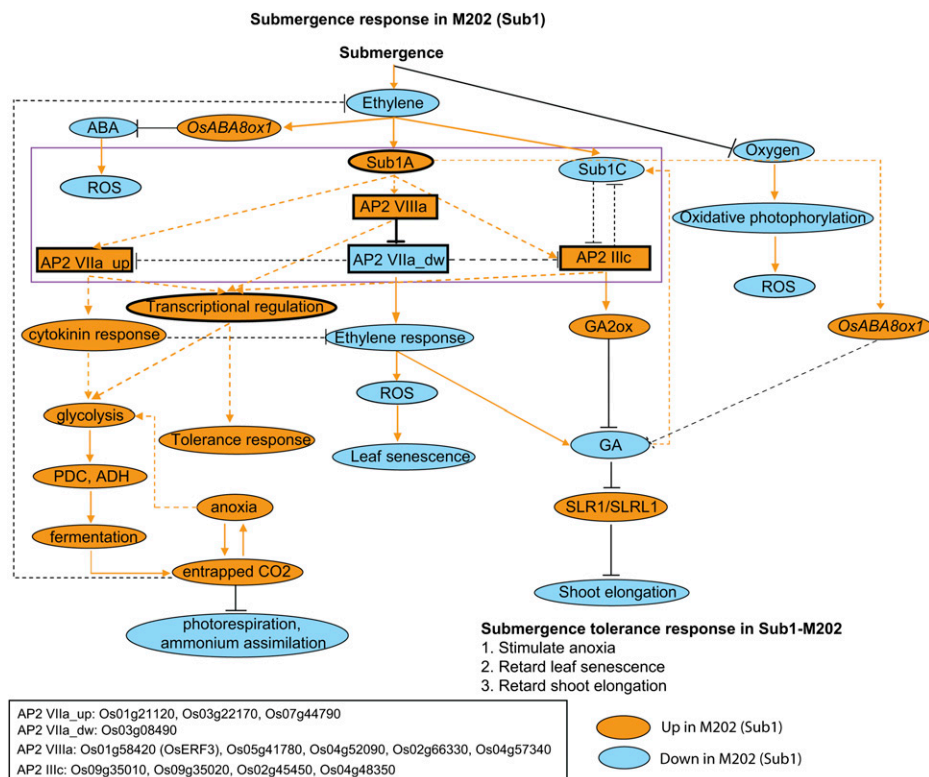
generally known to cause morphological and physiological changes, such as increased shoot elongation, stimulated anaerobic metabolic processes and starch/Suc degradation, activation of antioxidant systems, and induction of leaf or shoot senescence (Jackson and Ram, 2003; Fukao et al., 2006; Kawano et 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 VIIa; 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 VIIa 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 VIIa 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 VIIa, 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 VIIa (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 VIIa (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/ERFs 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 *SLRL1* (*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 *SLRL1*, because these AP2/ERF genes up-regulate the GA 2-oxidase catabolism 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 *SLRL1*; 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 Fukao 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*), the tolerant *japonica* cultivar near-isogenic line containing the *Sub1* locus from the *indica* FR13A, and the intolerant *japonica* cultivar M202 (Fukao et al., 2006) were germinated for 2 to 4 d underwater at 28°C and grown in soil in the greenhouse for 2 weeks. Forty-five plants of both genotypes were divided into three 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 P.M.

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  $A_{260}$  and  $A_{280}$  using a Nanodrop ND-1000. In addition, the level of protein contamination in the RNA was determined based on the  $A_{260}$ – $A_{280}$  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 are 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  $\log_2$ -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  $\log_2$  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).

In addition, we extracted 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 more 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 in 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  $\log_2$  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 sequences from 182 gene models (164 loci) identified from a rice TF database developed in PlnTFDB (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 GPL2025, 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-Kudahettige et al., 2007). A detailed method for the analysis of these data was described in our previous study (Jung et al., 2008a). The rice Multiplatform 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  $\log_2$  ratios of seven comparisons: M202(*Sub1*) over M202 at 0 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 over before submergence in M202(*Sub1*) (1 d/0 d\_S), 6 d after submergence over before submergence in M202(*Sub1*) (6 d/0 d\_S), 1 d after submergence over before submergence in M202 (1 d/0 d\_M), and 6 d after submergence over 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., 1998).

### 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 P.M. 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.plantbiology.msu.edu/pub/data/Eukaryotic\\_Projects/o\\_sativa/annotation\\_dbs/pseudomolecules/version\\_6.0/all.dir/](ftp://ftp.plantbiology.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*) after submergence and *Sub1B*. We performed MEME searches with promoter sequences of AP2/ERF genes in each group in the FASTA format via the Web server hosted by the National Biomedical Computation Resource (<http://meme.nbcr.net>; Bailey et al., 2006). We looked for up to four cis-acting elements with eight maximum motif widths. With the Motif Alignment and Search Tool (MAST) tool, we then searched DNA sequences for matches to the putative TF-binding site motifs found by MEME in a set of promoter sequences. The results are displayed in Figure 5 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.

## 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 log<sub>2</sub>-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 Figures S3, S5, and S6. Data used in Figure 2 are displayed in Supplemental Table S2.

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

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 (Zat1, for Arabidopsis zinc finger protein 1), and At3g49930 (Zat13) have orthology with Os03g32230 (<http://greenphyl.cirad.fr/cgi-bin/sequence.cgi?search=Os03g32230.1>) and At3g46070 (Zat16) and At2g28710 (Zat17) have orthology with Os03g60560 (<http://greenphyl.cirad.fr/cgi-bin/sequence.cgi?search=Os03g60560.1>).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Phylogenomic expression analysis of the AP2/ERF superfamily.

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

**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, photorespiration, 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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## LITERATURE CITED

- Ahn IP (2008) Glufosinate ammonium-induced pathogen inhibition and defense responses culminate in disease protection in bar-transgenic rice. *Plant Physiol* **146**: 213–227
- Almeida AM, Vriezen WH, Van der Straeten D (2003) Molecular and physiological mechanisms of flooding avoidance and tolerance in rice. *Russ J Plant Physiol* **50**: 743–751
- Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science* **301**: 653–657
- Bailey TL, Williams N, Misleh C, Li WW (2006) MEME: discovering and analyzing DNA and protein sequence motifs. *Nucleic Acids Res* **34**: W369–W373
- Bailey-Serres J, Voesenek LA (2008) Flooding stress: acclimations and genetic diversity. *Annu Rev Plant Biol* **59**: 313–339
- Balibrea Lara ME, Gonzalez Garcia MC, Fatima T, Ehness R, Lee TK, Proels R, Tanner W, Roitsch T (2004) Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. *Plant Cell* **16**: 1276–1287
- Barrett T, Troup DB, Wilhite SE, Ledoux P, Rudnev D, Evangelista C, Kim IF, Soboleva A, Tomashevsky M, Edgar R (2007) NCBI GEO: mining tens of millions of expression profiles. Database and tools update. *Nucleic Acids Res* **35**: D760–D765
- Berger JA, Hautaniemi S, Jarvinen AK, Edgren H, Mitra SK, Astola J (2004) Optimized LOWESS normalization parameter selection for DNA microarray data. *BMC Bioinformatics* **5**: 194
- Boutillier K, Offringa R, Sharma VK, Kieft H, Ouellet T, Zhang L, Hattori J, Liu CM, van Lammeren AA, Miki BL, et al (2002) Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *Plant Cell* **14**: 1737–1749
- Cao PJ, Bartley LE, Jung KH, Ronald CR (2008) Construction of a rice glycosyltransferase phylogenomic database and identification of rice-diverged glycosyltransferases. *Mol Plant* **1**: 858–877
- Dardick C, Chen J, Richter T, Ouyang S, Ronald P (2007) The rice kinase database: a phylogenomic database for the rice kinome. *Plant Physiol* **143**: 579–586
- Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in

- drought-, high-salt- and cold-responsive gene expression. *Plant J* **33**: 751–763
- Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* **30**: 207–210
- Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* **95**: 14863–14868
- Elliott RC, Betzner AS, Huttner E, Oakes MP, Tucker WQ, Gerentes D, Perez P, Smyth DR (1996) AINTEGUMENTA, an APETALA2-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth. *Plant Cell* **8**: 155–168
- Falcon S, Gentleman R (2007) Using GOstats to test gene lists for GO term association. *Bioinformatics* **23**: 257–258
- Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, et al (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422**: 442–446
- Fukao T, Bailey-Serres J (2008) Submergence tolerance conferred by *Sub1A* is mediated by SLR1 and SLRL1 restriction of gibberellin responses in rice. *Proc Natl Acad Sci USA* **105**: 16814–16819
- Fukao T, Xu K, Ronald PC, Bailey-Serres J (2006) A variable cluster of ethylene response factor-like genes regulates metabolic and developmental acclimation responses to submergence in rice. *Plant Cell* **18**: 2021–2034
- Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol* **124**: 1854–1865
- Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant J* **16**: 433–442
- Gomez-Cadenas A, Zentella R, Walker-Simmons MK, Ho TH (2001) Gibberellin/abscisic acid antagonism in barley aleurone cells: site of action of the protein kinase PKABA1 in relation to gibberellin signaling molecules. *Plant Cell* **13**: 667–679
- Gutha LR, Reddy AR (2008) Rice DREB1B promoter shows distinct stress-specific responses, and the overexpression of cDNA in tobacco confers improved abiotic and biotic stress tolerance. *Plant Mol Biol* **68**: 533–555
- Haake V, Cook D, Riechmann JL, Pineda O, Thomashow MF, Zhang JZ (2002) Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. *Plant Physiol* **130**: 639–648
- Hao D, Ohme-Takagi M, Sarai A (1998) Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. *J Biol Chem* **273**: 26857–26861
- Hattori Y, Nagai K, Furukawa S, Song XJ, Kawano R, Sakakibara H, Wu J, Matsumoto T, Yoshimura A, Kitano H, et al (2009) The ethylene response factors SNORKEL1 and SNORKEL2 allow rice to adapt to deep water. *Nature* **460**: 1026–1030
- Hirai MY, Sugiyama K, Sawada Y, Tohge T, Obayashi T, Suzuki A, Araki R, Sakurai N, Suzuki H, Aoki K, et al (2007) Omics-based identification of *Arabidopsis* Myb transcription factors regulating aliphatic glucosinolate biosynthesis. *Proc Natl Acad Sci USA* **104**: 6478–6483
- Hirose N, Makita N, Kojima M, Kamada-Nobusada T, Sakakibara H (2007) Overexpression of a type-A response regulator alters rice morphology and cytokinin metabolism. *Plant Cell Physiol* **48**: 523–539
- Hu YX, Wang YX, Liu XF, Li JY (2004) *Arabidopsis* RAV1 is down-regulated by brassinosteroid and may act as a negative regulator during plant development. *Cell Res* **14**: 8–15
- Ito Y, Katsura K, Maruyama K, Tajiri T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol* **47**: 141–153
- Jackson MB, Ram PC (2003) Physiological and molecular basis of susceptibility and tolerance of rice plants to complete submergence. *Ann Bot (Lond)* (Spec No) **91**: 227–241
- Jain M, Nijhawan A, Arora R, Agarwal P, Ray S, Sharma P, Kapoor S, Tyagi AK, Khurana JP (2007) F-box proteins in rice: genome-wide analysis, classification, temporal and spatial gene expression during panicle and seed development, and regulation by light and abiotic stress. *Plant Physiol* **143**: 1467–1483
- Jiao Y, Ma L, Strickland E, Deng XW (2005) Conservation and divergence of light-regulated genome expression patterns during seedling development in rice and *Arabidopsis*. *Plant Cell* **17**: 3239–3256
- Johnson PR, Ecker JR (1998) The ethylene gas signal transduction pathway: a molecular perspective. *Annu Rev Genet* **32**: 227–254
- Jung KH, Dardick C, Bartley LE, Cao P, Phetsom J, Canlas P, Seo YS, Shultz M, Ouyang S, Yuan Q, et al (2008a) Refinement of light-responsive transcript lists using rice oligonucleotide arrays: evaluation of gene-redundancy. *PLoS One* **3**: e3337
- Jung KH, Han MJ, Lee DY, Lee YS, Schreiber L, Franke R, Faust A, Yephremov A, Saedler H, Kim YW, et al (2006) Wax-deficient anther1 is involved in cuticle and wax production in rice anther walls and is required for pollen development. *Plant Cell* **18**: 3015–3032
- Jung KH, Lee J, Dardick C, Seo YS, Cao P, Canlas P, Phetsom J, Xu X, Ouyang S, An K, et al (2008b) Identification and functional analysis of light-responsive unique genes and gene family members in rice. *PLoS Genet* **4**: e1000164
- Kawano N, Ito O, Sakagami JI (2009) Morphological and physiological responses of rice seedlings to complete submergence (flash flooding). *Ann Bot (Lond)* **103**: 161–169
- Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**: 150–163
- Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JD, Schroeder JI (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in *Arabidopsis*. *EMBO J* **22**: 2623–2633
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948
- Lasanthi-Kudahettige R, Magneschi L, Loreti E, Gonzali S, Licausi F, Novi G, Beretta O, Vitulli F, Alpi A, Perata P (2007) Transcript profiling of the anoxic rice coleoptile. *Plant Physiol* **144**: 218–231
- Lee KW, Chen PW, Lu CA, Chen S, Ho TH, Yu SM (2009) Coordinated responses to oxygen and sugar deficiency allow rice seedlings to tolerate flooding. *Sci Signal* **2**: ra61
- Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* **10**: 1391–1406
- Magneschi L, Perata P (2009) Rice germination and seedling growth in the absence of oxygen. *Ann Bot (Lond)* **103**: 181–196
- Magome H, Yamaguchi S, Hanada A, Kamiya Y, Oda K (2008) The DDF1 transcriptional activator upregulates expression of a gibberellin-deactivating gene, GA2ox7, under high-salinity stress in *Arabidopsis*. *Plant J* **56**: 613–626
- Nagano H, Onishi K, Ogasawara M, Horiuchi Y, Sano Y (2005) Genealogy of the “Green Revolution” gene in rice. *Genes Genet Syst* **80**: 351–356
- Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiol* **140**: 411–432
- Novillo F, Alonso JM, Ecker JR, Salinas J (2004) CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in *Arabidopsis*. *Proc Natl Acad Sci USA* **101**: 3985–3990
- Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* **13**: 1959–1968
- Perata P, Voesenek LA (2007) Submergence tolerance in rice requires *Sub1A*, an ethylene-response-factor-like gene. *Trends Plant Sci* **12**: 43–46
- Ram PC, Singh BB, Singh AK, Ram P, Singh PN, Singh HP, Boamfa I, Harren E, Santosa E, Jackson MB, et al (2002) Submergence tolerance in rainfed lowland rice: physiological basis and prospects for cultivar improvement through marker-aided breeding. *Field Crops Res* **76**: 131–152
- Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2006) A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. *Proc Natl Acad Sci USA* **103**: 11081–11085
- Riano-Pachon DM, Ruzicic S, Dreyer I, Mueller-Roeber B (2007) PlnTFDB: an integrative plant transcription factor database. *BMC Bioinformatics* **8**: 42



- Rieu I, Eriksson S, Powers SJ, Gong F, Griffiths J, Woolley L, Benlloch R, Nilsson O, Thomas SG, Hedden P, et al (2008) Genetic analysis reveals that C19-GA 2-oxidation is a major gibberellin inactivation pathway in *Arabidopsis*. *Plant Cell* **20**: 2420–2436
- Rocke DM (2004) Design and analysis of experiments with high throughput biological assay data. *Semin Cell Dev Biol* **15**: 703–713
- Saika H, Okamoto M, Miyoshi K, Kushi T, Shinoda S, Jikumaru Y, Fujimoto M, Arikawa T, Takahashi H, Ando M, et al (2007) Ethylene promotes submergence-induced expression of OsABA8ox1, a gene that encodes ABA 8'-hydroxylase in rice. *Plant Cell Physiol* **48**: 287–298
- Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem Biophys Res Commun* **290**: 998–1009
- Santosa IE, Ram PC, Boamfa EI, Laarhoven LJ, Reuss J, Jackson MB, Harren FJ (2007) Patterns of peroxidative ethane emission from submerged rice seedlings indicate that damage from reactive oxygen species takes place during submergence and is not necessarily a post-anoxic phenomenon. *Planta* **226**: 193–202
- Shimada A, Ueguchi-Tanaka M, Sakamoto T, Fujioka S, Takatsuto S, Yoshida S, Sazuka T, Ashikari M, Matsuoka M (2006) The rice SPINDLY gene functions as a negative regulator of gibberellin signaling by controlling the suppressive function of the DELLA protein, SLR1, and modulating brassinosteroid synthesis. *Plant J* **48**: 390–402
- Steffens B, Sauter M (2005) Epidermal cell death in rice is regulated by ethylene, gibberellin, and abscisic acid. *Plant Physiol* **139**: 713–721
- Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98**: 5116–5121
- Wang Z, Triezenberg SJ, Thomashow MF, Stockinger EJ (2005) Multiple hydrophobic motifs in Arabidopsis CBF1 COOH-terminus provide functional redundancy in trans-activation. *Plant Mol Biol* **58**: 543–559
- White CN, Rivin CJ (2000) Gibberellins and seed development in maize. II. Gibberellin synthesis inhibition enhances abscisic acid signaling in cultured embryos. *Plant Physiol* **122**: 1089–1097
- Wohlgemuth H, Mittelstrass K, Kschieschan S, Bender J, Weigel HJ, Overmyer K, Kangasjarvi J, Sandermann H, Langebartels C (2002) Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. *Plant Cell Environ* **25**: 717–726
- Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailey-Serres J, Ronald PC, Mackill DJ (2006) *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature* **442**: 705–708
- Xu K, Xu X, Ronald PC, Mackill DJ (2000) A high-resolution linkage map of the vicinity of the rice submergence tolerance locus *Sub1*. *Mol Genet* **263**: 681–689
- Zhu Y, Cai XL, Wang ZY, Hong MM (2003) An interaction between a MYC protein and an EREBP protein is involved in transcriptional regulation of the rice Wx gene. *J Biol Chem* **278**: 47803–47811
- Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, et al (2006) ELONGATED UPPERMOST INTERNODE encodes a cytochrome P450 monooxygenase that epoxidizes gibberellins in a novel deactivation reaction in rice. *Plant Cell* **18**: 442–456