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Transcript profiling reveals diverse roles of auxin-responsive genes during reproductive development and abiotic stress in rice

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Auxin influences growth and development in plants by altering gene expression. Many auxin-responsive genes have been characterized in *Arabidopsis* in detail, but not in crop plants. Earlier, we reported the identification and characterization of the members of the *GH3*, *Aux/IAA* and *SAUR* gene families in rice. In this study, whole genome microarray analysis of auxin-responsive genes in rice was performed, with the aim of gaining some insight into the mechanism of auxin action. A comparison of expression profiles of untreated and auxin-treated rice seedlings identified 315 probe sets representing 298 (225 upregulated and 73 downregulated) unique genes as auxin-responsive. Functional categorization revealed that genes involved in various biological processes, including metabolism, transcription, signal transduction, and transport, are regulated by auxin. The expression profiles of auxin-responsive genes identified in this study and those of the members of the *GH3*, *Aux/IAA*, *SAUR* and *ARF* gene families were analyzed during various stages of vegetative and reproductive (panicle and seed) development by employing microarray analysis. Many of these genes are, indeed, expressed in a tissue-specific or developmental stage-specific manner, and the expression profiles of some of the representative genes were confirmed by real-time PCR. The differential expression of auxin-responsive genes during various stages of panicle and seed development implies their involvement in diverse developmental processes. Moreover, several auxin-responsive genes were differentially expressed under various abiotic stress conditions, indicating crosstalk between auxin and abiotic stress signaling.

The phytohormone auxin plays a central role in almost every aspect of growth and development in plants. Several recent discoveries in auxin biology, including the identification of F-box proteins as auxin receptors, have contributed to our understanding of the molecular mechanisms underlying auxin-regulated processes [1–4]. Auxin induces the very rapid accumulation of transcripts of a large number of genes, termed as primary auxin response genes, which are categorized in three major classes: auxin/indole-3-acetic acid (*Aux/IAA*), *GH3*, and small auxin-up RNA (*SAUR*) [5]. Auxin-responsive elements (AuxREs) have been identified in the promoters of several auxin-responsive genes [5–7]. The DNA-binding domains of auxin response factors (ARFs) bind to AuxREs of auxin-responsive genes and regulate their expression [8–10].

Abbreviations
ABA, abscisic acid; ARF, auxin response factor; AuxRE, auxin-responsive element; dap, days after pollination; GCRMA, GENECHIP robust multiarray average; IAA, indole 3-acetic acid; SAM, shoot apical meristem.

Several molecular genetic and biochemical findings have suggested a central role of *Aux/IAA* genes in auxin signaling [11,12]. The *Aux/IAA* genes encode short-lived nuclear proteins, which act as repressors of auxin-regulated transcriptional activation [12,13]. Although Aux/IAA proteins do not bind to AuxREs directly, they regulate auxin-mediated gene expression by controlling the activity of ARFs [9,10]. The developmental specificity of auxin response is determined by the interacting pairs of ARFs and Aux/IAAs [14]. The members of the *GH3* gene family encode enzymes that adenylate indole 3-acetic acid (IAA) to form amino acid conjugates, thereby preventing the accumulation of excessive free auxin, and are involved in auxin homeostasis [15]. In addition, GH3 enzymes also catalyze amido conjugation to salicylic acid and jasmonic acid [16]. The *SAUR* genes encode short-lived proteins that may play a role in auxin-mediated cell elongation [6,17].

The auxin signal transduction pathway has been largely unraveled through molecular genetic analysis of *Arabidopsis* mutants, but little work has been carried out in other plants. The recent advances in genomics provide opportunities to investigate these pathways in crop plants. To gain insights into the molecular mechanism of auxin action in rice, to begin with, we had earlier reported a genome-wide analysis of the early auxin-responsive, *GH3*, *Aux/IAA* and *SAUR* gene families in rice [7,18,19]. This work has now been extended further, and we have performed whole genome microarray analysis to identify auxin-responsive genes in rice. A comprehensive expression analysis of auxin-responsive genes identified from microarray analysis and members of the *GH3*, *Aux/IAA*, *SAUR* and *ARF* gene families during various stages of development and abiotic stress conditions was performed. The results provide evidence for a probable role of auxin-responsive genes in reproductive development and abiotic stress signaling in rice.

Results and Discussion

Identification and overview of auxin-responsive genes

Previously, we identified and characterized members of the early auxin-responsive gene families, including *GH3*, *Aux/IAA*, and *SAUR*, in rice [7,18,19]. In this study, we aimed to identify early auxin-responsive genes at the whole genome level in rice. Consequently, the microarray analysis of the RNA isolated from rice seedlings treated with IAA was carried out using the Affymetrix rice whole genome array. In an earlier study from our laboratory, the rice coleoptile segments depleted of endogenous auxin and floated in buffer containing various concentrations of IAA (0–50 μM) for 24 h showed maximum elongation with 30 μM IAA [20]. In this study, however, we used a higher concentration of IAA (50 μM), because the treatment was given to whole rice seedlings hydroponically and for a short duration (up to 3 h). Differential gene expression analysis between IAA-treated rice seedlings and mock-treated control seedlings was performed after normalization with the GENECHIP robust multiarray average (GCRMA) method and log transformation of the data. The probe sets showing at least two-fold increase or decrease in expression with a P-value ≤ 0.05 as compared with control were defined as differentially expressed auxin-responsive genes. After data analysis, a total of 315 probe sets showed significant differences in expression between control and hormone treatment. A hierarchical cluster display of average log signal values of these probe sets in control and IAA-treated

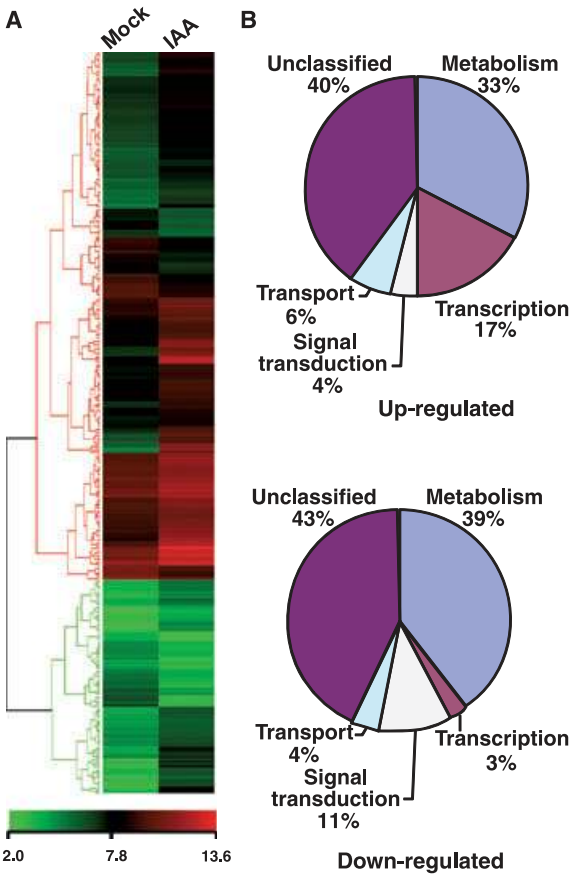

Fig. 1. Overview of early auxin-responsive genes in rice. (A) Cluster display of genes regulated by auxin. (B) Functional categorization of upregulated and downregulated genes.

samples is shown in Fig. 1A. These probe sets were mapped to the annotation available at the Rice Genome Annotation Project database (release 6) and rice full-length cDNAs to identify the corresponding genes. In total, 239 probe sets representing 225 unique genes were found to be upregulated by IAA (termed auxin-induced hereafter), and 76 probe sets representing 73 unique genes were found to be downregulated by IAA (termed auxin-repressed hereafter). A complete list of auxin-induced and auxin-repressed probe sets is provided in Table S1.

To investigate the functions of identified auxin-responsive genes, their annotations in the Rice Genome Annotation Project database and functional category were explored. Several members of the *GH3* and *Aux/IAA* gene families, which are well known to be induced rapidly in the presence of exogenous auxin [18,19], were represented in this list. This result confirms the reliability of the microarray experiment. Other families that were overrepresented in auxin-responsive genes include those encoding glutathione *S*-transferase, homeobox, cytochrome P450 and LOB domain proteins (Table S1). Although a large proportion of auxin-responsive genes are annotated as unknown and expressed proteins, putative functions have been assigned to other auxin-responsive genes. The functional categorization showed that the identified auxin-responsive genes are involved in various cellular processes, including metabolism, transcription, signal transduction, and transport (Fig. 1B), indicating that auxin-responsive genes perform crucial functions in various aspects of plant growth and development.

In addition to the *Aux/IAA*, *GH3* and *SAUR* families, several other genes are also induced by auxin [21]. These genes include those encoding cell wall synthesis enzymes, cell wall-modifying agents, cell wall component proteins, the ethylene biosynthetic enzyme (1-aminocyclopropane-1-carboxylate synthase), cell cycle regulatory proteins, and many other genes that still await characterization. The regulation of tissue elongation and/or cell expansion is an important function of auxin, but the molecular mechanisms underlying it are poorly understood. Our study shows that several genes, such as xylosyl transferase, glucanases, peroxidases and those involved in cell wall organization (cell wall synthesis, cell wall-modifying agents, and cell wall component proteins) are regulated by auxin. Several studies in *Arabidopsis* found crosstalk between auxin and other plant hormones [21–24]. Our study also shows that genes involved in cytokinin (e.g. cytokinin-*O*-glucosyltransferase, cytokinin dehydrogenase, and response regulators), ethylene (e.g. ethylene-responsive transcription factor, 1-aminocyclopropane-1-carboxylate oxidase, and 1-aminocyclopropane-1-carboxylate synthase) and gibberellin (e.g. gibberellin receptor, gibberellin-20-oxidase, and gibberellin-2β-dioxygenase) pathways are regulated by auxin. In addition, many cytochrome P450 genes, which are involved in brassinosteroid biosynthesis and catabolism, are upregulated by auxin [25]. These findings provide clues to unravel complex phytohormone signaling networks.

Expression profiles of auxin-responsive genes during reproductive development

Expression profiling can provide information about the functional diversification of different members of a gene family. In previous studies, we examined the expression profiles of all the members of the *GH3* and *Aux/IAA* gene families and a few members of the *SAUR* gene family in five different tissue samples (etiolated and green shoot, root, flower, and callus) by real-time PCR analysis, and showed their specific and overlapping expression patterns [7,18,19]. The expression patterns of members of *ARF* gene families have also been examined [26]. However, these studies revealed the expression profiles in only few tissue samples. To obtain greater insights, we performed comprehensive expression profiling of auxin-responsive genes in a large number of tissues/organs and developmental stages in this study.

To achieve gene expression profiling of auxin-responsive genes identified in this study and the members of *Aux/IAA*, *GH3*, *SAUR* and *ARF* gene families during various stages of development in rice, microarray analysis was carried out using Affymetrix GeneChip Rice Genome arrays as described previously [27]. The developmental stages of rice used for microarray analysis include seedling, root, mature leaf, Y-leaf [leaf subtending the shoot apical meristem (SAM)], SAM, and various developmental stages of panicle (P1-I–P1-III and P1–P6) and seed (S1–S5). Various stages of rice panicle and seed development have been categorized according to panicle length and days after pollination (dap), respectively, on the basis of the landmark developmental event(s) as described by Itoh *et al.* [28] (Table S2). The average log signal values of auxin-responsive genes (identified from microarray) and the members of the *Aux/IAA*, *GH3*, *SAUR* and *ARF* gene families in three biological replicates of each tissue/developmental stage sample are given in Tables S3 and S4, respectively. A hierarchical cluster display of average log signal values of auxin-responsive genes and members of the *GH3*, *Aux/IAA*, *SAUR* and *ARF* gene families is presented in Figs 2 and 3, respectively. The signal values revealed that most of the

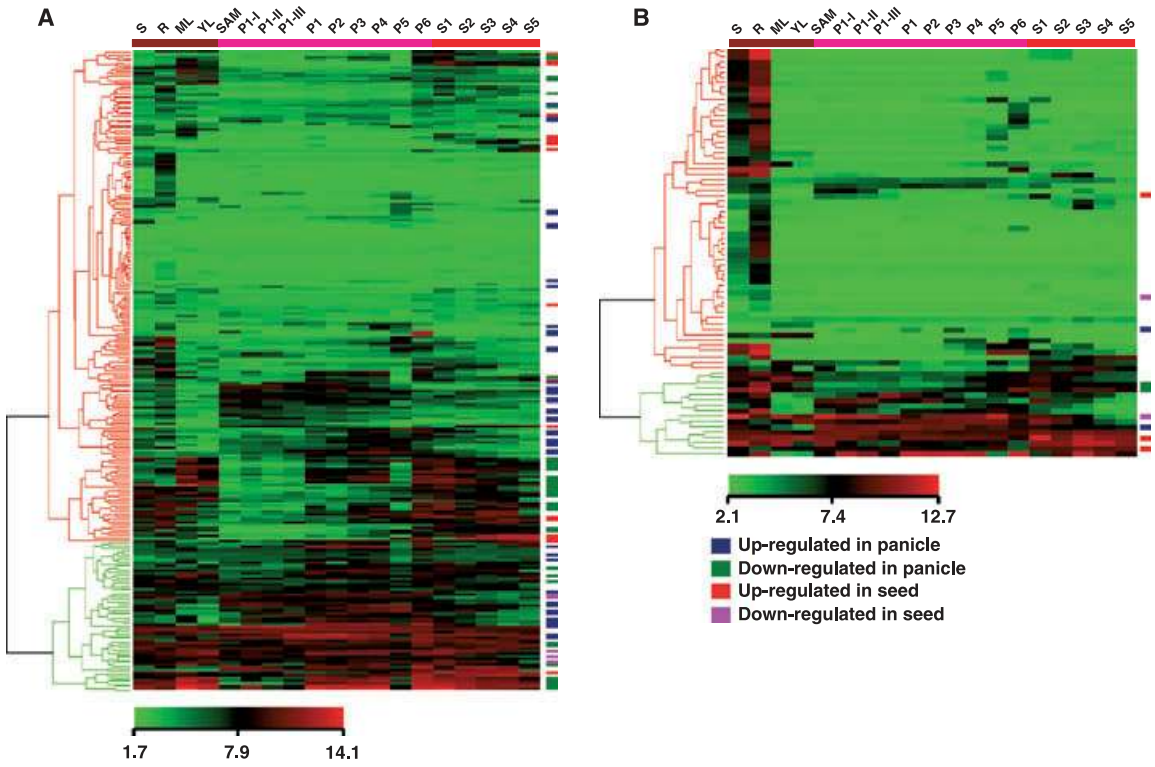

Fig. 2. Expression profiles of auxin-responsive genes in various tissues/organs and developmental stages of rice. A heatmap representing hierarchical clustering of average log signal values of auxin-induced (A) and auxin-repressed (B) genes in various tissues/organs and developmental stages (mentioned at the top of each lane) is shown. The color scale representing average log signal values is shown at the bottom of the heatmap. The genes significantly (at least two-fold, with *P*-value ≤ 0.05) upregulated and downregulated in at least one of the panicle and seed developmental stages are marked with color bars on the right. S, seedling; R, root; ML, mature leaf; YL, Y-leaf; P1-I–P1-III and P1–P6, stages of panicle development; S1–S5, stages of seed development. The average log signal values are given in Table S3. Enlarged versions of (A) and (B) are available as Figs S1 and S2, respectively.

auxin-responsive genes are expressed in at least one of the developmental stages analyzed. However, the expression patterns of auxin-responsive genes varied greatly with tissue and developmental stage.

Differential gene expression analysis was performed to identify auxin-responsive genes with preferential expression during panicle and seed development stage(s). This analysis revealed that at least nine *GH3*, 13 *Aux/IAA*, 18 *ARF* and 17 *SAUR* genes were significantly differentially expressed (more than two-fold) in at least one of the stages of panicle or seed development as compared with vegetative development stages. Furthermore, the genes expressed differentially at any stage of panicle development as compared with seed developmental stages and vice versa were identified. This analysis revealed that 37 genes, including six *GH3*, six *Aux/IAA*, 13 *ARF* and 12 *SAUR* genes, were differentially expressed in at least one stage of panicle development, and 10 genes, including one *GH3* gene, five *Aux/IAA* genes, three *ARF* genes and one *SAUR* gene were differentially expressed in at least one stage of seed development. A similar analysis performed for auxin-responsive genes revealed that, among a total of 84 genes that were differentially expressed, 48 (44 auxin-induced and four auxin-repressed) genes were upregulated and 36 (all of them auxin-induced) genes were downregulated during at least one stage of panicle development. Likewise, among a total of 28 genes that were differentially expressed, 23 (18 auxin-induced and five auxin-repressed) genes were upregulated and five (all of them auxin-induced) genes were downregulated during at least one stage of seed development. Real-time PCR analysis was employed to validate the differential expression of some of the representative genes deduced from microarray data analysis (Fig. 4). The results showed that the expression patterns obtained by Affymetrix rice whole genome array showed good correlation with those obtained by real-time PCR.

Several studies have suggested the importance of auxin during reproductive development in plants

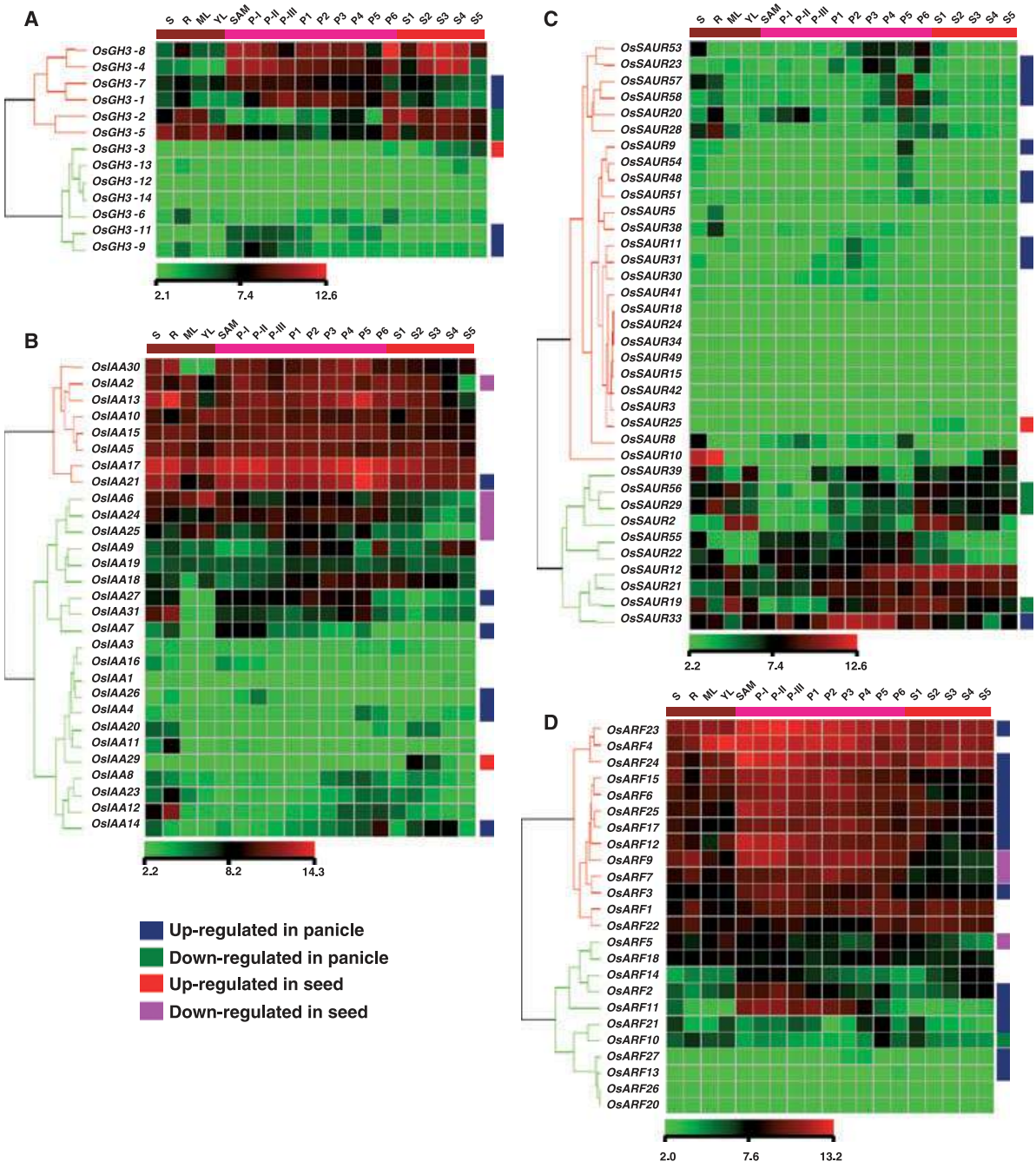

Fig. 3. Expression profiles of *GH3*, *Aux/IAA*, *SAUR* and *ARF* gene family members in various tissues/organs and developmental stages of rice. A heatmap representing hierarchical clustering of average log signal values of *GH3* (A), *Aux/IAA* (B), *SAUR* (C) and *ARF* (D) gene family members in various tissues/organs and developmental stages (mentioned at the top of each lane) is shown. The color scale representing average log signal values is shown at the bottom of the heatmap. The genes significantly (at least two-fold, with *P*-value ≤ 0.05) upregulated and downregulated in at least one of the panicle and seed developmental stages are marked with color bars on the right. S, seedling; R, root; ML, mature leaf; YL, Y-leaf; P1-I–P1-III and P1–P6, stages of panicle development; S1–S5, stages of seed development. The average log signal values are given in Table S4.

[29–34]. Plants genetically or chemically impaired in their ability to transport auxin fail to form floral primordia [29]. Live imaging of the *Arabidopsis* inflorescence meristem showed that auxin transport influences differentiation events that occur during flower primordium formation, including organ polarity

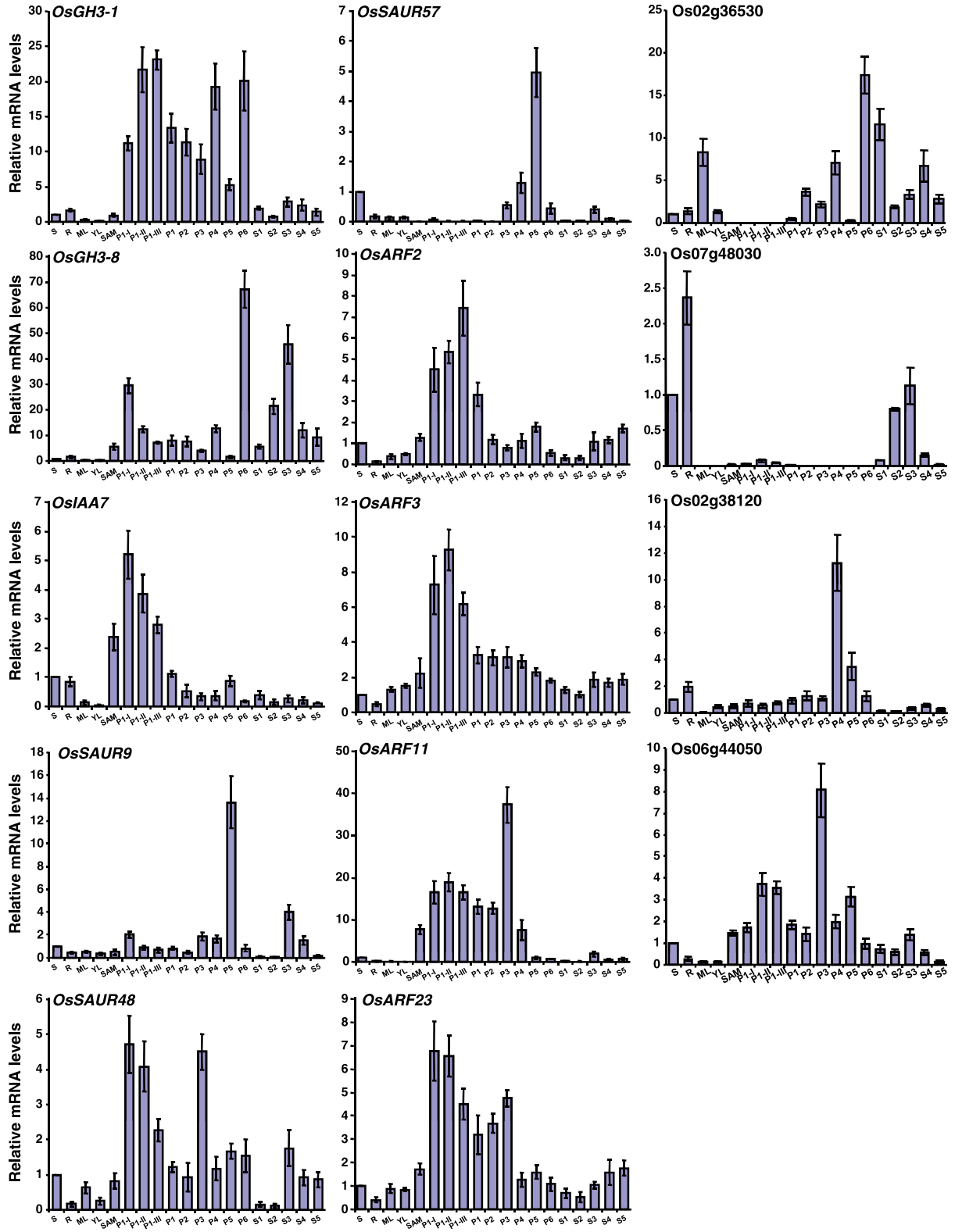

Fig. 4. Real-time PCR analysis of selected genes to validate their expression profiles during various stages of development. The mRNA levels for each gene in different tissue samples were calculated relative to its expression in seedlings. S, seedling; R, root; ML, mature leaf; YL, Y-leaf; P1-I–P1-III and P1–P6, stages of panicle development; S1–S5, stages of seed development.

and floral meristem initiation [35]. The biosynthesis of auxin by *YUCCA* family genes, which encode flavin monooxygenases, controls the formation of floral organs [36]. At least one member of the *GH3* gene family (designated *OsGH3-8* in [19]) has been reported as the downstream target of OsMADS1, a rice MADS transcription factor, involved in patterning of inner whorl floral organs [37]. We also found several *GH3* genes, including *OsGH3-8*, to be preferentially expressed during various stages of reproductive development. *OsGH3-1*, *OsGH3-4* and *OsGH3-8* showed relatively high expression in all stages of panicle and seed development, with some quantitative differences. *GH3-7* and *GH3-9* were expressed predominantly during stages of early panicle development. *OsGH3-3* was expressed at relatively higher levels during seed development stages. The mutation in the *MONOPTEROS* gene, which encodes ARF5, fails to initiate floral buds in mutant plants [38]. The mutation in the *ETTIN* gene, which also encodes an ARF, affects the development of floral meristem and floral organs [39,40]. Other members of the *ARF* gene family in *Arabidopsis* have also been implicated in various aspects of reproductive development [41–44]. Likewise, at least 13 *ARF* genes were found to be expressed differentially during panicle development in rice in this study. A very high level of expression of *OsARF11*, a putative ortholog of *MONOPTEROS*, during early panicle development, representing the stages of floral transition, floral organ differentiation and development, indicates their functional similarity. It has been demonstrated that anthers are the major sites of high concentrations of free auxin that retard the development of neighboring floral organs to synchronize flower development [33]. Recently, it has been suggested that auxin plays a major role in coordinating anther dehiscence, pollen maturation and preanthesis filament elongation in *Arabidopsis* [45]. In genome-wide gene expression profiling, auxin-related genes, including *ARF*, *SAUR*s, and *GH3*, were found to be preferentially expressed in stigma in rice [46]. Our data are consistent with these observations showing preferential expression of several members of the *GH3*, *Aux/IAA*, *ARF* and *SAUR* gene families, in addition to other auxin-responsive genes, during the P2–P6 stages of panicle development (Figs 2, 3 and S2), which represent the stages of male and female gametophyte development (Table S2). Our data indicate that most of the auxin-responsive genes exhibit differential expression during more than one stage of reproductive development; however, a few of these could be associated with a specific developmental stage as well. For example, *OsSAUR9* and *OsSAUR57* are specifically expressed during the P5 stage, and LOC_Os05g06670 (encoding a putative gibberellin 2-oxidase) and LOC_Os06g44470 (encoding a putative pollen allergen precursor) during the P6 stage. These genes might play specific roles during these developmental stages. Furthermore, the auxin-responsive genes that are involved in other plant hormone pathways showed differential expression during various stages of reproductive development as well (Table S3), indicating the coordinated regulation of these developmental events by different plant hormones. Taken together, the preferential expression of a significantly large number of auxin-responsive genes during various stages of reproductive development, including floral transition, floral organ development, male and female gametophyte development, and endosperm development, supports the idea that auxin is crucial for reproductive development.

Expression profiles of auxin-responsive genes under abiotic stress conditions

Plants counteract adverse environmental conditions by eliciting various physiological, biochemical and molecular responses, leading to changes in gene expression. A range of stress signaling pathways have been elucidated through molecular genetic studies. Plant growth hormones, such as abscisic acid (ABA), ethylene, salicylic acid, and jasmonic acid, mediate various abiotic and biotic stress responses. Although auxins have been implicated primarily in many developmental processes in plants, some recent studies suggest that auxin is also involved in stress or defense responses. It has been reported that the endogenous IAA level increases substantially upon pathogen infection [47], and the expression of some auxin-regulated genes is altered in infected plants [48]. Recently, it has been demonstrated that microRNA-mediated repression of auxin signaling enhances antibacterial resistance [49]. On the basis of expression profiling and mutant analysis, it has been hypothesized that repression of the auxin pathway is an important aspect of the defense response [50]. It has been shown that genes that are positively responsive to auxin signaling pathway are downregulated by wounding [51]. The expression of *Aux/IAA* and *ARF* gene family members is altered during cold acclimation in *Arabidopsis* [52]. Molecular genetic analysis of the auxin and ABA response pathways provided evidence for auxin–ABA interaction [53,54]. The role of IBR5, a dual-specificity phosphatase-like protein, supported the link between auxin and ABA signaling pathways [55].

To address whether auxin-responsive genes are also involved in stress responses in rice, their expression

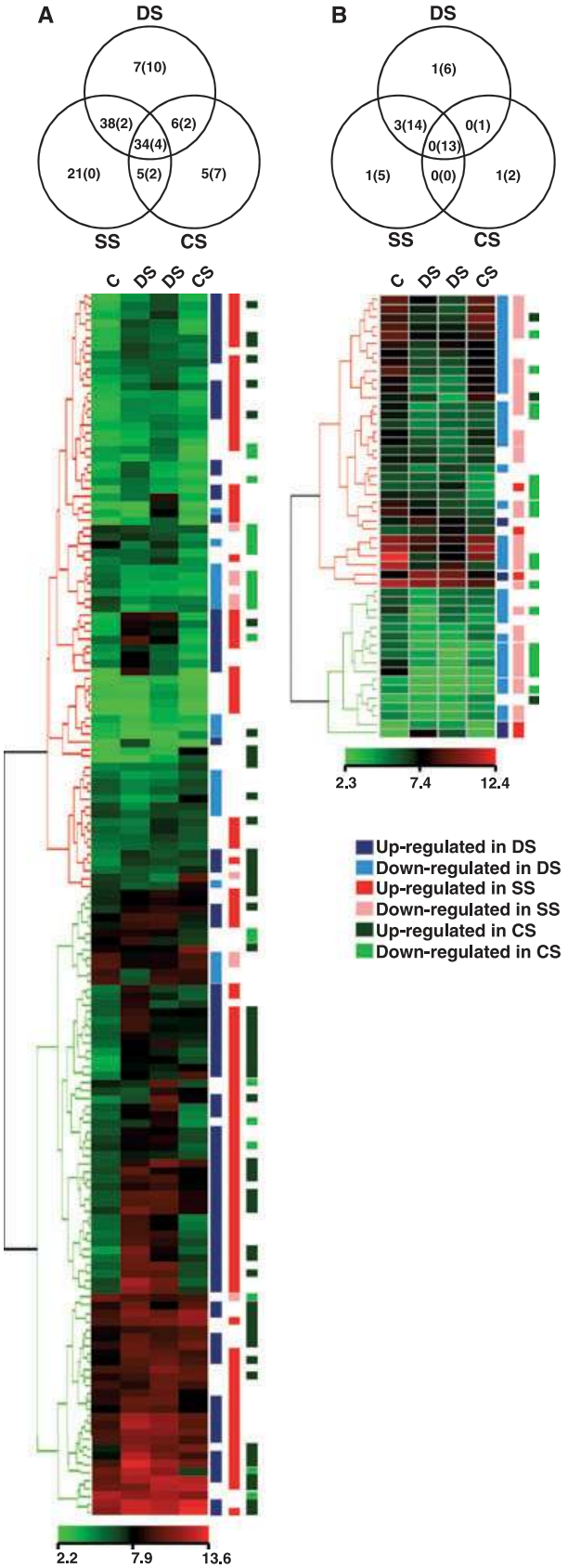

Fig. 5. Overview and expression profiles of auxin-induced (A) and auxin-repressed (B) genes differentially expressed under various abiotic stress conditions. The 7-day-old seedlings were either kept in water (as control) or subjected to desiccation (between folds of tissue paper), salt (200 mM NaCl) and cold (4 ± 1 °C) treatments, for 3 h each. The Venn diagram represents the numbers of genes upregulated and downregulated (in parentheses) under different stress conditions. The numbers of genes upregulated under one or more stress condition(s) and downregulated under other stress condition(s) are not shown in the Venn diagram. The average log signal values under control and various stress conditions (mentioned at the top of each lane) are presented as heatmaps. Only those genes that exhibited two-fold or more differential expression with a *P*-value < 0.05, under any of the given abiotic stress conditions, are shown and are distinguished with color bars on the right. The color scale representing average log signal values is shown at the bottom of the heatmap. C, control; DS, desiccation stress; SS, salt stress; CS, cold stress. The fold change value, *P*-value and regulation (up/down) are given in Table S5. An enlarged version of heatmaps from this figure is available as Fig. S3.

profile was analyzed by microarray analysis under abiotic stress conditions, including desiccation, salt, and cold. At least 154 auxin-induced and 50 auxin-repressed probe sets were identified that are differentially expressed, under one or more of the stress conditions analyzed (Fig. 5). Among the 154 auxin-induced genes, 116 and 27 genes were upregulated and downregulated, respectively, under one or more of the abiotic stress conditions analyzed (Fig. 5A). However, the remaining 11 genes were upregulated under one or more stress condition(s) and downregulated under other stress condition(s). Similarly, among the 50 auxin-repressed genes, six and 41 genes were upregulated and downregulated, respectively, under one or more of the abiotic stress conditions analyzed (Fig. 5B). However, three other genes were upregulated under one or more stress condition(s) and downregulated under other stress condition(s) (Table S5). Similarly, 41 members of auxin-related gene families were found to be differentially expressed under at least one abiotic stresss condition (Fig. 6). Among these, 18 (two *GH3*, seven *Aux/IAA*, seven *SAUR*, and two *ARF*) were upregulated and 18 (one *GH3*, five *Aux/IAA*, eight *SAUR*, and four *ARF*) were downregulated under one or more abiotic stress conditions (Fig. 6; Table S6). However, another five genes (*OsGH3-2*, *OsIAA4*, *OsSAUR22*, *OsSAUR48*, and *OsSAUR54*) were upregulated under one or more abiotic stress condition(s) and downregulated under other stress condition(s) (Table S6). Interestingly, among the 206 auxin-responsive (154 auxin-induced and 50 auxin-repressed) genes and 41 members of auxin-related gene families that were differentially expressed under at least one abiotic

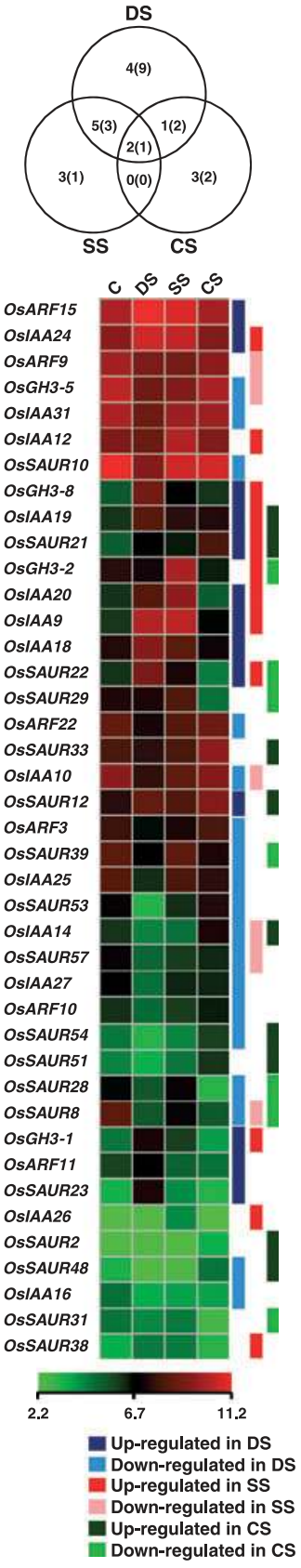

Fig. 6. Overview and expression profiles of *GH3*, *Aux/IAA*, *SAUR* and *ARF* gene family members differentially expressed under various abiotic stress conditions. The 7-day-old seedlings were either kept in water (as control) or subjected to desiccation (between folds of tissue paper), salt (200 mM NaCl) and cold (4 ± 1 °C) treatments, for 3 h each. The Venn diagram represents the numbers of genes upregulated and downregulated (in parentheses) under different stress conditions. The numbers of genes upregulated under one or more stress condition(s) and downregulated under other stress condition(s) are not shown in the Venn diagram. The average log signal values under control and various stress conditions (mentioned at the top of each lane) are presented as heatmaps. Only those genes that exhibited two-fold or more differential expression with a *P*-value of < 0.05, under any of the given abiotic stress conditions, are shown and are distinguished with color bars on the right. The color scale representing average log signal values is shown at the bottom of heatmap. C, control; DS, desiccation stress; SS, salt stress; CS, cold stress. The fold change value, *P*-value and regulation (up/down) are given in Table S6.

stress condition, only 51 and three genes, respectively, were differentially expressed under all three stress conditions (Figs 5 and 6). However, other genes exhibited differential expression under any two stress conditions or a specific stress condition. The real-time PCR analysis validated the differential expression of some representative genes under abiotic stress condition(s) as seen from the microarray data (Fig. 7).

Furthermore, the promoters (1 kb upstream sequence from the start codon) of all the auxin-responsive genes and members of auxin-related gene families differentially expressed under various abiotic stress conditions identified above were analyzed using the signal search program PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) to identify *cis*-acting regulatory elements linked to specific abiotic stress conditions. Although no specific *cis*-acting regulatory elements could be linked to a specific stress condition analyzed, several ABA and other stress-responsive elements were identified (data not shown). The presence of these elements further confirms the stress responsiveness of auxin-responsive genes. These results indicate the existence of a complex system, including several auxin-responsive genes, that is operative during stress signaling in rice. Although functional validation of these genes will provide more definitive clues about their specific roles in one or more abiotic stress conditions, it is obvious from these data that a larger number of auxin-responsive genes are involved in abiotic stress signaling than exprected. In *Arabidopsis*, the microarray data (available in public databases) analysis showed that a large number of auxin-responsive genes are involved in various abiotic stress responses as well (our unpublished results). The results of the

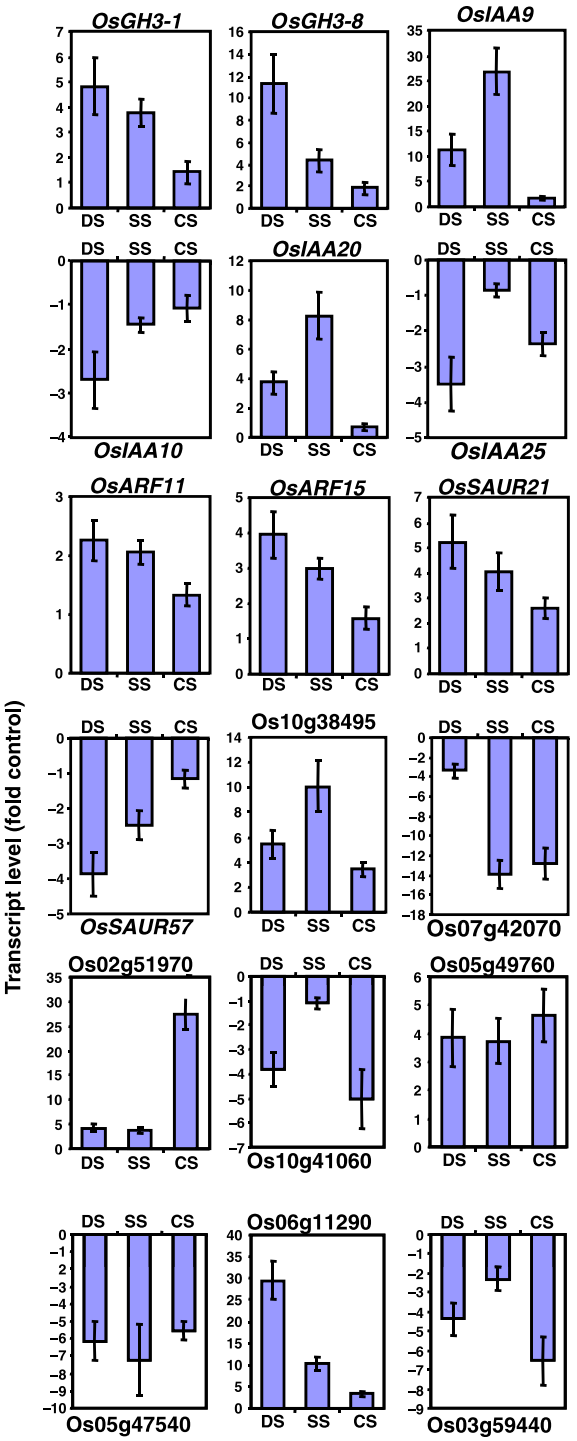

Fig. 7. Real-time PCR analysis of selected genes to validate their expression profiles under various abiotic stress conditions. The 7-day-old seedlings were either kept in water (as control) or subjected to desiccation (between folds of tissue paper), salt (200 mM NaCl) and cold (4 ± 1 °C) treatments, for 3 h each. The mRNA levels for each gene in different tissue samples were calculated relative to its expression in control seedlings. C, control; DS, desiccation stress; SS, salt stress; CS, cold stress.

present study suggest that auxin could also act as a stress hormone, directly or indirectly, that alters the expression of several stress-responsive genes, such as that encoding ABA, although validation of this assumption requires further experimentation.

The *Arabidopsis* seedlings subjected to oxidative stress exhibited various phenotypic effects consistent with alterations in auxin levels and/or distribution [56]. A wide variety of abiotic stresses have an impact on various aspects of auxin homeostasis, including altered auxin distribution and metabolism. Two possible molecular mechanisms have been suggested for altered distribution of auxin: first, altered expression of *PIN* genes, which mediate polar auxin transport; and second, inhibition of polar auxin transport by phenolic compounds accumulated in response to stress exposure [57]. On the other hand, auxin metabolism is modulated by oxidative degradation of IAA catalyzed by peroxidases [58], which in turn are induced by different stress conditions. Furthermore, it has been shown that reactive oxygen species generated in response to various environmental stresses may influence the auxin response [59,60]. Although these observations provide some clues, the exact mechanism of auxin-mediated stress responses still remains to be elucidated.

In earlier studies, crosstalk between various developmental processes and stress responses was detected [27,61,62]. Consistently, many auxin-responsive genes were related to both reproductive development and abiotic stress responses. Twenty (17 upregulated and three downregulated) genes were commonly regulated during various stages of panicle development and abiotic stress conditions, and 16 (all upregulated) genes were commonly regulated during various stages of seed development and abiotic stress conditions (Fig. S4; Table S5). Likewise, nine (seven upregulated and two downregulated) members of auxin-related gene families were commonly regulated during panicle development stages and abiotic stress conditions, and two (both downregulated) members were commonly regulated during seed development stages and abiotic stress conditions (Fig. S4; Table S6). These commonly regulated genes may act as mediators of plant growth response to various abiotic stress conditions during various developmental stages.

In conclusion, the expression profiles of auxin-responsive genes during various stages of vegetative and reproductive development of rice suggest that the components of auxin signaling are involved in many developmental processes throughout the plant life cycle. In addition, a significant number of auxin-responsive genes have been implicated in abiotic stress

responses, which indicates crosstalk between stress and auxin signaling. In the recent past, the identification of F-box proteins (TIR1/AFBs) as auxin receptors has been a milestone in our understanding of the molecular mechanisms of auxin signaling pathways. These F-box proteins are components of E3 ligase and target Aux/IAAs, in particular for degradation through the 26S proteasome, and allow ARFs to positively regulate the expression of downstream genes involved in auxin signaling. The differential and overlapping expression patterns of individual members of these gene families in rice offer an amazingly vast regulatory potential. Furthermore, it has been demonstrated that the gene expression of ARFs and TIR1/AFBs is regulated at the post-transcriptional level as well by microRNAs, which adds another layer of regulation to the auxin signal transduction pathway. The auxin signal transduction pathway is thus rapidly emerging as a complex network with the ability to regulate a wide variety of developmental processes and responses to environmental cues. The results reported in this study will be helpful in understanding the transcriptional network regulated by auxin and functional validation of selected auxin-responsive genes involved in developmental processes of both fundamental and agronomic importance.

Experimental procedures

Plant material

Plant tissue samples for various tissues/organs and developmental stages, including mature leaf, Y-leaf (youngest leaf subtending the SAM) and different stages of panicle (SAM, up to 0.5 mm; P1-I, 0.5–2 mm; P1-II, 2–5 mm; P1-III, 5–10 mm; P1, 0–3 cm; P2, 3–5 cm; P3, 5–10 cm; P4, 10–15 cm; P5, 15–22 cm; and P6, 22–30 cm) and seed (S1, 0–2 dap; S2, 3–4 dap; S3, 5–10 dap; S4, 11–20 dap; and S5, 21–29 dap) development, were harvested from rice (*Oryza sativa* L. ssp. *indica* var. IR64) plants grown under greenhouse or field conditions as previously described [27]. Roots were harvested from 7-day-old seedlings grown in water. The description of different developmental stages used for microarray analysis is given in Table S2.

Auxin and abiotic stress treatments

For auxin treatment, 7-day-old light-grown rice seedlings were transferred to a beaker containing a 50 μM solution of IAA. Seedlings mock-treated with dimethylsulfoxide (final concentration 0.1%) served as the control. The seedlings were harvested after 1 and 3 h of treatment, frozen immediately in liquid nitrogen, and stored at −80 °C until RNA isolation. Equal amounts of tissue samples of the 1 and 3 h time points for each treatment were pooled at the time of RNA isolation. The desiccation (between folds of tissue paper), salt (200 mM NaCl solution) and cold (4 ± 1 °C) stress treatments were given to 7-day-old light-grown rice seedlings for 3 h each, as previously described [27]. Seedlings kept in water for 3 h, at 28 ± 1 °C, served as control.

Microarray experiments

The Affymetrix GeneChip Rice Genome Arrays (Affymetrix, Santa Clara, CA, USA) representing 49 824 transcripts (48 564 of *O. sativa* spp. *japonica* and 1260 of *O. sativa* spp. *indica*) were used for microarray experiments. The microarray experiments for auxin treatment, various stages of vegetative and reproductive development and stress treatments were performed as described earlier [27]. Three independent biological replicates of samples of various developmental stages and stress treatments and two biological replicates of samples of auxin treatment with an overall correlation coefficient value of more than 0.94 were selected for final analysis. The microarray data for auxin treatment, various developmental stages and abiotic stress treatments in rice are available in the Gene Expression Omnibus database under the series accession numbers GSE5167, GSE6893, and GSE6901, respectively.

Microarray data analysis

For data analysis, the CEL files generated by GENECHIP OPERATING SOFTWARE were imported into ARRAYASSIST (version 5.0) software (Stratagene, La Jolla, CA, USA). The normalization and probe summarization were performed by the GCRMA method. To identify differentially expressed genes after auxin treatment, Student's *t*-test was performed on the log-transformed data. The genes that are upregulated or downregulated two-fold or more with a *P*-value ≤ 0.05 were considered to be significantly differentially expressed. For annotation of identified differentially expressed genes, the information provided on the rice multiplatform microarray search (http://www.ricearray.org/matrix.search.shtml) page of the NSF rice oligonucleotide array project (http://www.ricearray.org/) was used. The oligonucleotide sequences of the probes represented on the Affymetrix rice genome array have been mapped to the Rice Genome Annotation Project (release 6, http://rice.plantbiology.msu.edu/) cDNAs, rice full-length cDNAs, TIGR plant transcript assemblies or the Rice Genome Annotation Project pseudomolecules with the entire set of 11 probes (8–10 in some cases) present on the array aligned with 100% identity at 100% coverage.

To study the expression profiles of *GH3*, *Aux/IAA*, *SAUR* and *ARF* gene family members during various stages of development and abiotic stress conditions, the probe sets

representing these genes on the Affymetrix rice genome array were identified as previously described [27]. Probe sets with the entire set of 11 probes (8–10 in some cases) present on the array aligned with 100% identity over the entire length with the corresponding gene were considered to be significant. The probe sets for 13 *GH3*, 29 *Aux/IAA*, 24 *ARF* and 36 *SAUR* genes could be identified that were represented on the Affymetrix rice genome array (probe set IDs are given in Table S4). Following normalization by GCRMA and log transformation of data for all the rice genes present on the chip, the log signal intensity values for rice probe sets corresponding to the members of the *GH3*, *Aux/IAA*, *SAUR* and *ARF* gene families and auxin-responsive genes identified above were extracted as individual subsets, and differential gene expression analysis was performed. The genes that are upregulated or downregulated by two-fold or more were considered to be significantly differentially expressed. Hierarchical clustering was performed using the Euclidean distance metric and complete linkage rule.

Real-time PCR analysis

The validation of expression patterns of representative genes obtained by microarray analysis was performed by real-time PCR analysis, using gene-specific primers as described earlier [63]. The primer sequences are listed in Table S7. At least two independent biological replicates of each sample and three technical replicates of each biological replicate were used for real-time PCR analysis. The C_T (cycle threshold) values for all genes in different RNA samples were normalized to the C_T value of an internal control gene, *UBQ5*. The relative mRNA levels for each candidate gene in different tissue samples were calculated using the $\Delta\Delta C_T$ method (Applied Biosystems, Foster City, CA, USA). For every data point, the C_T value was the average of C_T values obtained from the two biological replicates, each with triplicate PCR analyses. Error bars in the figures indicate standard deviations.

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Supporting information

The following supplementary material is available:
Fig. S1. Expression profiles of auxin-induced genes in various tissues/organs and developmental stages of rice.
Fig. S2. Expression profiles of auxin-repressed genes in various tissues/organs and developmental stages of rice.
Fig. S3. Expression profiles of auxin-induced (A) and auxin-repressed (B) genes differentially expressed under various abiotic stress conditions.
Fig. S4. Venn diagram to represent the genes commonly regulated during reproductive (panicle and seed) development stages and abiotic stress conditions.
Table S1. Genes differentially expressed in the presence of auxin.
Table S2. Developmental stages of rice used for microarray analysis.
Table S3. Average log signal values of auxin-responsive genes in various rice tissues/organs and developmental stages.
Table S4. Average log signal values of members of the *GH3*, *Aux/IAA*, *SAUR* and *ARF* gene families in various rice tissues/organs and developmental stages.
Table S5. Auxin-responsive genes differentially expressed under various abiotic stress conditions.

Table S6. Members of the *GH3*, *Aux/IAA*, *SAUR* and *ARF* gene families differentially expressed under various abiotic stress conditions.

Table S7. Primer sequences used for real-time PCR analysis.

This supplementary material can be found in the online version of this article.

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