

Rapid paper

***Arabidopsis* Transcriptome Analysis under Drought, Cold, High-Salinity and ABA Treatment Conditions using a Tiling Array**

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Plants respond and adapt to drought, cold and high-salinity stresses in order to survive. In this study, we applied *Arabidopsis* Affymetrix tiling arrays to study the whole genome transcriptome under drought, cold, high-salinity and ABA treatment conditions. The bioinformatic analysis using the tiling array data showed that 7,719 non-AGI transcriptional units (TUs) exist in the unannotated “intergenic” regions of *Arabidopsis* genome. These include 1,275 and 181 TUs that are induced and downregulated, respectively, by the stress or ABA treatments. Most of the non-AGI TUs are hypothetical non-protein-coding RNAs. About 80% of the non-AGI TUs belong to pairs of the fully overlapping sense-antisense transcripts (fSATs). Significant linear correlation between the expression ratios (treated/untreated) of the sense TUs and the ratios of the antisense TUs was observed in the SATs of AGI code/non-AGI TU. We studied the biogenesis mechanisms of the stress- or ABA-inducible antisense RNAs and found that the expression of sense TUs is necessary for the stress- or ABA-inducible expression of the antisense TUs in the fSATs (AGI code/non-AGI TU).

Keywords: Abiotic stress — Antisense RNA — *Arabidopsis* — Non-protein-coding RNA — Tiling array.

Abbreviations: ARTADE, *Arabidopsis* tiling array-based detection of exons; fSAT, fully-overlapping sense-antisense transcript; HLE, Hodges-Lehmann estimator; PASR, promoter-associated short RNA; pSAT, partially-overlapping sense-antisense transcript; *RD*, responsive to dehydration; SAT, sense-antisense transcript; TAIR,

the *Arabidopsis* information resource; TASR, terminus-associated short RNA; TU, transcriptional unit.

Arabidopsis tiling microarray design and expression profiling data under drought-, cold-, high-salinity-stress or ABA treatments are available in GEO (<http://www.ncbi.nlm.nih.gov/geo/info/linking.html>) under the accession number GSE9646. The tiling array data can also be viewed along with additional information including small RNAs at <http://omicspace.riken.jp/gps/group/psca1>. The cDNA sequences of the non-protein-coding RNAs on the antisense strand have been submitted to DDBJ database with the accession numbers AB428729, AB428730 and AB428731. The supplementary data are available in the online version of this article and are available online at <http://pfgweb.gsc.riken.jp/supplements/matsui001/>.

Introduction

Plant growth is greatly affected by environmental abiotic stresses, such as drought, cold and high salinity. Plants must adapt to these stresses in order to survive. These stresses induce various biochemical and physiological responses in plants. Several hundred genes have been identified as the genes that respond to these stresses at the transcriptional level (Kreps et al. 2002, Seki et al. 2002b, Xiong et al. 2002, Zhu 2002, Shinozaki et al. 2003, Lee et al. 2005). It is important to analyze the functions of stress-inducible genes not only for understanding the molecular

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mechanisms of stress tolerance and responses of higher plants but also for improving the stress tolerance of crops by gene manipulation. Stress-inducible genes have been used to improve the stress tolerance of transgenic plants (Thomashow 1999, Hasegawa et al. 2000, Zhang 2003, Umezawa et al. 2006).

Drought stress triggers the production of the phytohormone ABA, which causes stomatal closure and induces the expression of drought stress-related genes (Finkelstein et al. 2002, Nambara and Marion-Poll 2005). Many drought-inducible genes are induced by exogenous ABA treatment (Seki et al. 2002a). Molecular analyses have demonstrated the existence of both ABA-dependent and ABA-independent regulatory systems in the transcriptional regulatory networks under drought stress (Yamaguchi-Shinozaki and Shinozaki 2005, 2006).

Several studies have revealed that small RNAs and antisense RNAs have functions in the responses to the abiotic stresses and ABA. Several small RNAs are regulated by the abiotic stresses (Sunkar and Zhu 2004, Borsani et al. 2005). Borsani et al. (2005) reported that the overlapping gene pair of Δ^1 -pyrroline-5-carboxylate dehydrogenase (*P5CDH*) (sense), a stress-related gene, and *SRO5* (antisense), a gene of unknown function, generates two types of siRNAs, 24-nt siRNA and 21-nt siRNAs. When the *SRO5* expression is induced by salt treatment, a 24-nt siRNA is formed by the natural antisense transcript (nat)-siRNA biogenesis pathway. Initial cleavage of the *P5CDH* transcript guided by the 24-nt siRNA establishes a phase for the subsequent generation of 21-nt siRNAs and further cleavage of *P5CDH* transcript, resulting in the tolerance to high-salinity stress due to higher proline accumulation.

Several genes involved in RNA processing (Lu and Fedoroff 2000, Hugouvieux et al. 2001, Xiong et al. 2001, Papp et al. 2004, Nishimura et al. 2005) and chromatin regulation (Sridha and Wu 2006) have been identified as components of the drought and salt stress signal transduction. The mutants of several components functioning in RNA processing, such as *abh1* (Hugouvieux et al. 2001), *ahg2* (Nishimura et al. 2005), *cbp20* (Papp et al. 2004), *hyl1* (Lu and Fedoroff 2000), and *sad1* (Xiong et al. 2001), exhibit ABA hypersensitivity. ABH1, AHG2, CBP20, HYL1 and SAD1 encode mRNA cap-binding protein, poly(A)-specific ribonuclease, mRNA cap-binding protein, nuclear-localized double-stranded RNA-binding protein, and Sm-like snRNP protein, respectively. These results suggest a functional link between mRNA processing and modulation of early ABA signal transduction and the existence of the novel functional non-coding RNAs involved in the ABA signal transduction.

Recently, tiling array technology has become a useful tool for the analysis of whole-genome transcriptome,

such as (i) mapping of transcripts (Yamada et al. 2003, Stolc et al. 2005, Li et al. 2006, Hanada et al. 2007), (ii) identification of alternative splice sites (Clark et al. 2002), (iii) identification of binding sites for the proteins (Katou et al. 2003, Thibaud-Nissen et al. 2006, Turck et al. 2007), (iv) comparative genomic hybridization (Bignell et al. 2004, Ishkanian et al. 2004), (v) mapping of DNA methylation sites (Martienssen et al. 2005, Zhang et al. 2006, Zilberman et al. 2007) and (vi) mapping of histone modification sites (Zhang et al. 2007, Li et al. 2008). Using tiling array technology, many novel transcripts including putative non-protein-coding RNAs have been identified in plants (Zhang et al. 2006). However, few reports on the stress-responsive non-coding RNAs except for the small RNAs have been published in plants.

Here, we describe the *Arabidopsis* whole-genome expression profiling studies using the tiling array in responses to drought, cold, high-salinity stress and ABA. In this study, to enable a global description of the *Arabidopsis* transcriptome, we used the term transcriptional unit (TU) to describe a segment of the genome from which transcripts are generated, in addition to annotated *Arabidopsis* Genome Initiative (AGI) code genes whose information is available from the *Arabidopsis* Information Resource (TAIR) (<http://www.arabidopsis.org/>). Note that most of the AGI code genes are thought to be protein-coding ones. A TU is defined by the identification of a cluster of transcripts containing a common core of genetic information (in some cases, protein-coding region) and a non-AGI TU is defined as the TU identified in unannotated intergenic regions by the bioinformatic analysis.

We identified many stress-responsive genes and TUs in both annotated AGI code regions and non-annotated ones and found significant linear correlation between the expression ratio (treated/untreated) of sense TUs and the ratio of antisense TUs in most of the sense-antisense transcripts (SATs). So far, the biogenesis mechanisms of the stress- or ABA-inducible antisense RNAs in the SATs have not been well understood. We studied the biogenesis mechanisms of the stress- or ABA-inducible antisense RNAs and found that the expression of sense TUs is necessary for the stress- or ABA-inducible expression of the antisense TUs in the fSATs (AGI code/non-AGI TU).

Results

Drought, cold, high-salinity stress and ABA-responsive AGI code genes

Using the *Arabidopsis* whole-genome tiling array, we identified a total of 16,876, 17,920, 17,458, 17,388 and 18,126 AGI code genes as the expressed genes under the drought-, cold-, high-salinity-, ABA-treatment and no treatment (Table 1 and Supplementary Table S2-1). In this analysis,

Table 1 Number of AGI code genes upregulated, downregulated and expressed under drought, cold, high-salinity stress, ABA treatment and no treatment

Treatment		No. of up-regulated AGI code genes ^a	No. of down-regulated AGI code genes ^b	No. of expressed AGI code genes ^c
Drought	2 h	1,188	217	16,744
	10 h	2,059	2,075	15,815
Cold	2 h	427	14	17,270
	10 h	740	396	16,349
High-salinity	2 h	1,471	495	16,697
	10 h	2,693	2,038	15,920
ABA	2 h	1,678	1,581	16,241
	10 h	3,179	2,220	16,181
Untreatment				18,126
Total ^d		5,303	3,974	20,264

^a We regarded the following AGI code genes as drought, cold, high-salinity and ABA upregulated ones: (i) Genes identified by Mann-Whitney U-test (FDR $\alpha=0.01$). (ii) Expression ratios (treated/untreated) >1.8. (iii) Genes identified as the expressed genes under each stress or ABA treatment (P-initial <10⁻⁸).

^b We regarded the following genes as drought, cold, high-salinity, and ABA downregulated AGI code genes: (i) Genes identified by Mann-Whitney U-test (FDR $\alpha=0.01$). (ii) Expression ratios (treated/untreated) <5/9. (iii) Genes identified as the expressed genes under untreatment (P-initial <10⁻⁸).

^c The genes were identified under each treatment by ARTADE-based method (P-initial <10⁻⁸).

^d These represent the total number of non-redundant AGI code genes identified as the upregulated, downregulated and expressed ones.

we used TAIR6 gene model (ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR6_genome_release) as of May 9, 2006.

Among them, 2,421, 996, 2,903 and 3,623 AGI code genes have been identified as ones upregulated by the treatments of drought-, cold-, high-salinity-stress or ABA, respectively (Supplementary Fig. S1; Table 1; Supplementary Tables S2-2). These genes included many reported drought-, cold-, high-salinity-stress- or ABA-upregulated genes, such as *RD29A/COR78/LTI78* [At5g52310, Yamaguchi-Shinozaki and Shinozaki 1994, *responsive to dehydration (RD) 29A/cold-regulated (COR) 78/low-temperature-induced (LTI) 78*], *RD29B* (At5g52300, Yamaguchi-Shinozaki and Shinozaki 1994), *RD20* (At2g33380, Takahashi et al. 2000), *DREB1A/CBF3* (At4g25480, Liu et al., 1998, *dehydration-responsive element (DRE)-binding protein 1A/C-repeat (CRT)-binding factor 3*, *DREB2A* (At5g05410, Liu et al. 1998, Sakuma et al. 2006, *DRE-binding protein 2A*) and *AtMYC2* (At1g32640, Abe et al. 2003) (Supplementary Tables S1, S2-2), which indicates that our tiling array analysis functions properly to find drought-, cold-, high-salinity- stress- or ABA-upregulated genes.

The stress- or ABA-inducible gene products can be classified into two groups, functional proteins and regulatory proteins, as shown in the previous reports (Fowler and Thomashow 2002, Kreps et al. 2002, Seki et al. 2002a, 2002b, Lee et al. 2005, Oono et al. 2006). The functional

proteins function in stress tolerance. They contain late embryogenesis-abundant (LEA) proteins, osmoprotectant biosynthesis-related proteins, ABA metabolism-related proteins, transporters and detoxification enzymes (Supplementary Table S2-2). The regulatory proteins function in further regulation of signal transduction and gene expression that probably function in the stress responses. They contain various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules (Supplementary Table S2-2).

We also identified 2,097, 402, 2,063 and 2,729 AGI code genes as genes downregulated by the treatments of drought, cold, high-salinity or ABA, respectively (Supplementary Fig. S1; Table 1; Supplementary Table S2-3). They contain many photosynthesis-related genes. These results are consistent with the previous results (Seki et al. 2002a, 2002b).

Venn diagram analysis indicates the existence of greater crosstalk between drought and high-salinity stress signaling processes than those between cold and high-salinity stress signaling processes, and greater crosstalk between ABA, drought and high-salinity stress signaling processes than those between ABA- and cold-stress signaling processes (Supplementary Fig. S1). These results are also consistent with our previous studies (Seki et al. 2002a, 2002b, Yamaguchi-Shinozaki and Shinozaki 2005).

Drought, cold, high-salinity stress and ABA-responsive non-AGI TUs

We identified 7,719 independent groups of non-AGI TUs (Table 2 and Supplementary Tables S3-1, S3-2) in the unannotated “intergenic” regions of *Arabidopsis* genome using the *Arabidopsis* Tiling Array-based Detection of Exons (ARTADE)-based method (Toyoda and Shinozaki 2005). A homology search using the BLASTX program against the registered protein sequence data sets in National Institutes of Health (NIH) nr database showed that about 94% (7,328/7,719) of the non-AGI TUs do not have sequence similarity with the protein sequence data sets (Supplementary Tables S3-1, S4), suggesting that these non-AGI TUs might function as the non-protein-coding RNAs. About 14% (1,079/7,719) of the non-AGI TUs overlaps with the *Arabidopsis* community full-length cDNAs including RIKEN *Arabidopsis* full-length (RAFL) cDNAs (Seki et al. 2002c). Note that 60 non-AGI TUs were identified by previous tiling array studies (Stolc et al. 2005, Zhang et al. 2006) and 4,153 of non-AGI TUs were hit with the “significant” and “reliable” signatures obtained by massively parallel signature sequencing (MPSS) of *Arabidopsis* transcripts (Meyers et al. 2004, Supplementary Tables S3-2,

S4). These results also support that the non-AGI TUs are really expressed in some conditions in *Arabidopsis*. About 6% (446) of non-AGI TUs identified in this study were registered as novel AGI gene models in the recently released TAIR 8 (ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR8_genome_release) as of April 28, 2008. Interestingly, about 84% (6,504/7,719) of the non-AGI TUs are novel transcripts without full-length cDNA support and AGI code gene in TAIR 8 gene model (Supplementary Tables S3-2, S4), indicating that the tiling array analysis is a powerful method to identify the novel genes and TUs.

Among the 7,719 non-AGI TUs, 1,275 and 181 non-AGI TUs were identified as ones upregulated and downregulated, respectively, by the drought, cold, high-salinity stress or ABA (Table 2 and Supplementary Table S3-2). BLASTX search showed that among them, 1,179 upregulated non-AGI TUs and 115 downregulated non-AGI TUs might function as the non-protein-coding RNAs. We confirmed the expression of the several stress- or ABA-inducible non-AGI TUs by Northern and real-time RT-PCR analyses (data not shown). The length of the 7,719 non-AGI TUs was from 50- to 12,800-nt and

Table 2 Number of non-AGI TUs upregulated, downregulated and expressed under drought, cold, high-salinity stress and ABA treatments and under no treatment

Treatment		No. of upregulated Non-AGI TUs ^a	No. of downregulated Non-AGI TUs ^b	No. of Non-AGI TUs ^c
Drought	2 h	115	7	2,727
	10 h	497	78	3,050
Cold	2 h	10	0	2,688
	10 h	44	9	2,203
High-salinity	2 h	290	24	3,150
	10 h	596	85	2,985
ABA	2 h	398	47	3,415
	10 h	577	99	3,087
Untreatment				4,047
Total ^d		1,275	181	7,719

^a We regarded the following TUs as drought, cold, high-salinity and ABA upregulated non-AGI TUs: (i) TUs identified in non-AGI code regions as the expressed TUs under the drought, cold, high-salinity and ABA treatments by ARTADE-based method (P-initial $<10^{-8}$). (ii) TUs identified as drought, cold, high-salinity and ABA upregulated by Mann-Whitney U-test (FDR $\alpha=0.01$). (iii) Expression ratios (treated/untreated) >1.8 .

^b We regarded the following TUs as drought, cold, high-salinity and ABA downregulated non-AGI TUs: (i) TUs identified in non-AGI code regions as expressed TUs under no treatment by ARTADE-based method (P-initial $<10^{-8}$). (ii) TUs identified as drought, cold, high-salinity and ABA downregulated by Mann-Whitney U-test (FDR $\alpha=0.01$). (iii) Expression ratios (treated/untreated) $<5/9$.

^c The non-AGI TUs were identified in non-AGI code regions under each condition by ARTADE-based method (P-initial $<10^{-8}$). The non-AGI TUs were clustered into 7,719 independent groups (Supplementary Tables S3-1, S3-2). Note that among the same groups, the length of the non-AGI TUs identified by each treatment was different.

^d These represent the total number of non-redundant TUs identified as upregulated, downregulated and expressed. When the TUs overlapped each other by more than one base, they were classified into the same group.

the average length was about 500 nt (Supplementary Fig. S2). The median of the signal intensity [the values of Hodges-Lehmann estimator (HLE), see Supplementary methods] for the non-AGI TUs and the AGI code genes was 683 and 1,338, respectively. The expression level of the non-AGI TUs was generally lower than that of the AGI code genes in the conditions studied (Supplementary Fig. S4).

We identified many non-AGI TUs on the 5'-upstream and 3'-downstream of the AGI code genes (Supplementary Fig. S7). Most of them are mapped within 100-base upstream or within 300-base downstream of the AGI code genes (Supplementary Fig. S5). Interestingly, 27 promoter-associated short RNA (PASR)-like TUs (Kapranov et al. 2007) and 27 termini-associated short RNA (TASR)-like TUs (Kapranov et al. 2007), that are supported by the full-length cDNAs, have been identified in the tiling array analysis (Supplementary Fig. S7 and Supplementary Tables S6-3, S6-4). Eight PASR-like TUs and ten TASR-like TUs that are supported by the full-length cDNAs show the ABA- or stress-responsive gene expression. The full-length-cDNA-unsupported 295 PASR-like TUs and 189 TASR-like TUs have been identified (Supplementary Fig. S7 and Supplementary Tables S6-5, S6-6).

The tiling array expression data indicated that the 5'- and 3'-end regions are short in TAIR 6 gene model as of May 9, 2006 for the 67 and 34 AGI code genes, respectively, (Supplementary Fig. S7 and Supplementary Tables S6-1, S6-2). They are supported by the full-length cDNAs and the gene models are corrected in several cases for the TAIR 8 version. Some of the non-AGI TUs mapped within 0.5-kb distance from the nearby AGI code genes showed

significant correlative expression with its neighboring genes (Supplementary Fig. S6). These results showed that the tiling array analysis is useful for improvement of the genome annotation. Similar application to the improvement of the gene models has been reported in previous tiling array studies (Yamada et al. 2003, Li et al. 2006).

Identification of sense-antisense transcripts

We identified 7,805 SATs with overlapping sequences greater than 20-nt (Table 3 and Supplementary Tables S3-1, S3-2, S5-1, S5-2). Such large-scale antisense transcripts have been found in previous tiling array analysis (Yamada et al. 2003, Stolc et al. 2005).

The SATs were classified into three groups, that is, pairs of AGI code genes and AGI code genes (AGI code/AGI code), pairs of AGI code genes and non-AGI TUs (AGI code/non-AGI TU) and pairs of non-AGI TUs and non-AGI TUs (non-AGI TU/non-AGI TU). Most of the SATs were pairs of AGI code/non-AGI TU in all conditions (Table 3).

The SATs were also classified into two groups based on the overlapping lengths and its coverage: the fully overlapping SATs (fSATs) in which the sequence of one TU covers more than 80% of the other TU sequence on the antisense strand and the partially overlapping SATs (pSATs) in which the overlapping length is greater than 20 nt and the coverage of one TU is less than 80% of the other TU (Fig. 1). About 90% (6,040/6,858) of SATs (AGI/non-AGI) was fSATs, supporting that most of the non-AGI TUs in the SATs (AGI/non-AGI) do not encode proteins (Table 3). On the other hand, about 90% (765/832) of SATs

Table 3 Classification of the SATs into the pairs of AGI code/AGI code, AGI code/non-AGI TU and non-AGI TU/non-AGI TU^a

Treatment		No. of SATs ^b (AGI/AGI)	No. of SATs ^b (AGI/Non-AGI)	No. of SATs ^b (Non-AGI/Non-AGI)
Drought	2 h	689 (53)	2,448 (2,118)	20 (14)
	10 h	665 (51)	2,798 (2,405)	18 (10)
Cold	2 h	724 (55)	2,368 (2,045)	20 (11)
	10 h	657 (52)	1,842 (1,599)	15 (9)
High-salinity	2 h	696 (53)	2,861 (2,500)	16 (9)
	10 h	657 (49)	2,709 (2,343)	36 (20)
ABA	2 h	676 (53)	3,195 (2,791)	24 (14)
	10 h	682 (54)	2,804 (2,433)	25 (20)
Untreatment		757 (58)	3,620 (2,987)	38 (19)
Total ^c		832 (67)	6,858 (6,040)	115 (67)

^a For identification of the SATs, the sequences of AGI code genes and non-AGI TUs identified as the expressed ones were used (ARTADE program: P-initial <10⁻⁸).

^b These represent the numbers of the SATs in each category. Numbers in parentheses represent the numbers of the fSATs in each category.

^c These represent the total number of non-redundant SATs. Numbers in parentheses represent the total numbers of non-redundant fSATs.

(AGI/AGI) was pSATs. Among the pSATs (AGI/AGI), a previously reported salt-stress-tolerance-related nat-siRNA-generating SAT pair of *P5CDH* gene (At5g62530), and a salt-inducible *SRO5* gene (At5g62520) (Borsani et al. 2005) was included (Supplementary S5-2). Only less than 10% of the SATs (AGI/AGI) was fSATs. Perhaps this is because pair of protein-coding genes constituting fSATs (AGI/AGI) would constrain each other's possible codons, preventing frequent evolution of such pair of the protein-coding genes.

The 7,719 non-AGI TUs were also classified into the following three groups based on the SATs: (i) the non-AGI TU of the fSATs, (ii) the non-AGI TU of the pSATs and (iii) the non-AGI TU in the intergenic regions (Supplementary Tables S3-2, S4). About 73% (5,651/7,719) and 8% (595/7,719) of the non-AGI TUs belong to ones of

Type 1: Fully-overlapping sense-antisense transcripts (fSATs)



Type 2: Partially-overlapping sense-antisense transcripts (pSATs)



Fig. 1 Classification of SATs. We mapped the sequence data of both AGI code genes and non-AGI TUs identified as “expressed ones” by ARTADE-based method to *Arabidopsis* genome sequence (TAIR 6) for identification of the SATs. The SATs were classified into 2 types. Type 1: The fully overlapping SATs (fSATs) in which the sequence of one TU covers more than 80% of the other TU sequence mapped on the antisense strand. Type 2: The partially overlapping SATs (pSATs) in which the overlapping length is longer than 20 nt and the sequence of one TU covers less than 80% of the other TU sequence mapped on the antisense strand.

the fSATs and pSATs, respectively (Supplementary Tables S3-2, S4). The ratios (no. of the non-AGI TUs with the mapped cDNAs/total no. of the non-AGI TUs) in the non-AGI TUs in the intergenic regions, the non-AGI TUs of the pSATs and the non-AGI TUs of the fSATs were about 27% (397/1,473), 16% (95/595) and 10% (587/5,651), respectively (Supplementary Table S4).

Significant linear correlation of the expression ratio (treated/untreated) in the SATs

A significant linear correlation between the expression ratios (treated/untreated) of the sense transcripts and the ratios of the antisense transcripts under drought, cold, high-salinity stress, and ABA treatments was observed in the SATs of AGI/non-AGI ($r=0.66$, $P<0.01$) and non-AGI/non-AGI ($r=0.68$, $P<0.01$). However, the significant linear correlation was rarely observed in the SATs of AGI/AGI ($r=0.07$, $P<0.01$) (Fig. 2 and Supplementary Fig. S3).

Fig. 3A shows an fSAT of the drought-inducible gene, *RD29A* and the novel drought-inducible antisense TUs. Although the AGI code is not annotated and the cDNAs have not been isolated on the antisense strand, tiling array analysis indicated that novel drought-inducible TUs exist on the antisense strand in this region. The presence of novel drought-inducible TUs on the antisense strand was confirmed by real-time RT-PCR and Northern analyses using strand-specific RNA probes (Figs. 3B, 3C). The tiling array expression data showed that the expression level of the antisense TUs is about one tenth that of the sense TU (Supplementary Tables S2-1, S3-1). In the Northern analysis against the antisense TUs, bands of short size were detected (Fig. 3B). The cDNAs for the antisense TUs were isolated (Fig. 3D, see supplementary data).

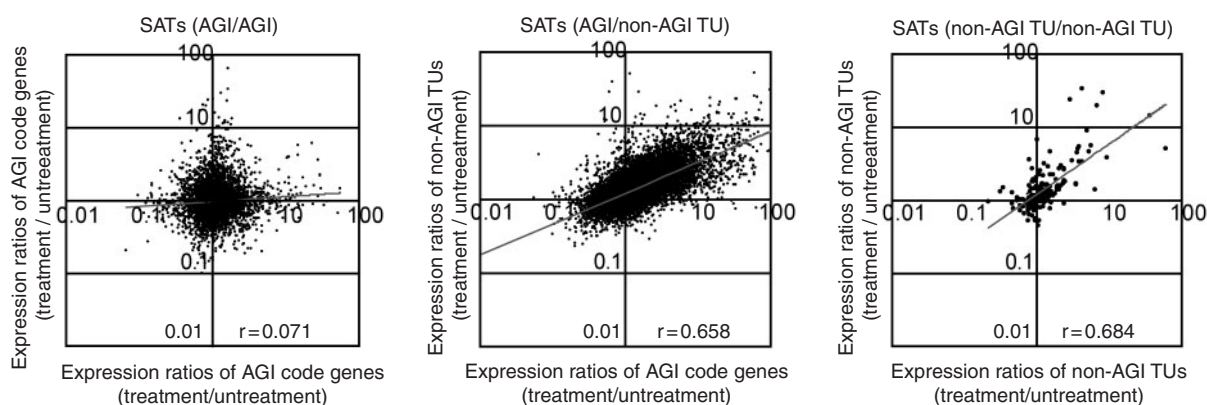


Fig. 2 The correlation of expression ratio (treated/untreated) between sense and antisense TUs of the SATs under drought, cold, high-salinity stress and ABA treatments. The log values for the expression ratios (stress and ABA treated/untreated) of sense and antisense TUs of the SATs were plotted. The left, middle and right panels represent the expression ratio in the SATs of AGI code/AGI code, AGI code/non-AGI TUs and non-AGI TUs/non-AGI TUs, respectively. In the left and middle panels, the X and Y axes represent the expression ratio of the AGI code genes (sense TUs) and the expression ratio of the TUs on the antisense strand, respectively. In the right panel, the X and Y axes represent the expression ratio of the non-AGI TUs (sense TUs) and the expression ratio of the TUs on the antisense strand, respectively. In each panel, the correlation coefficients are indicated.

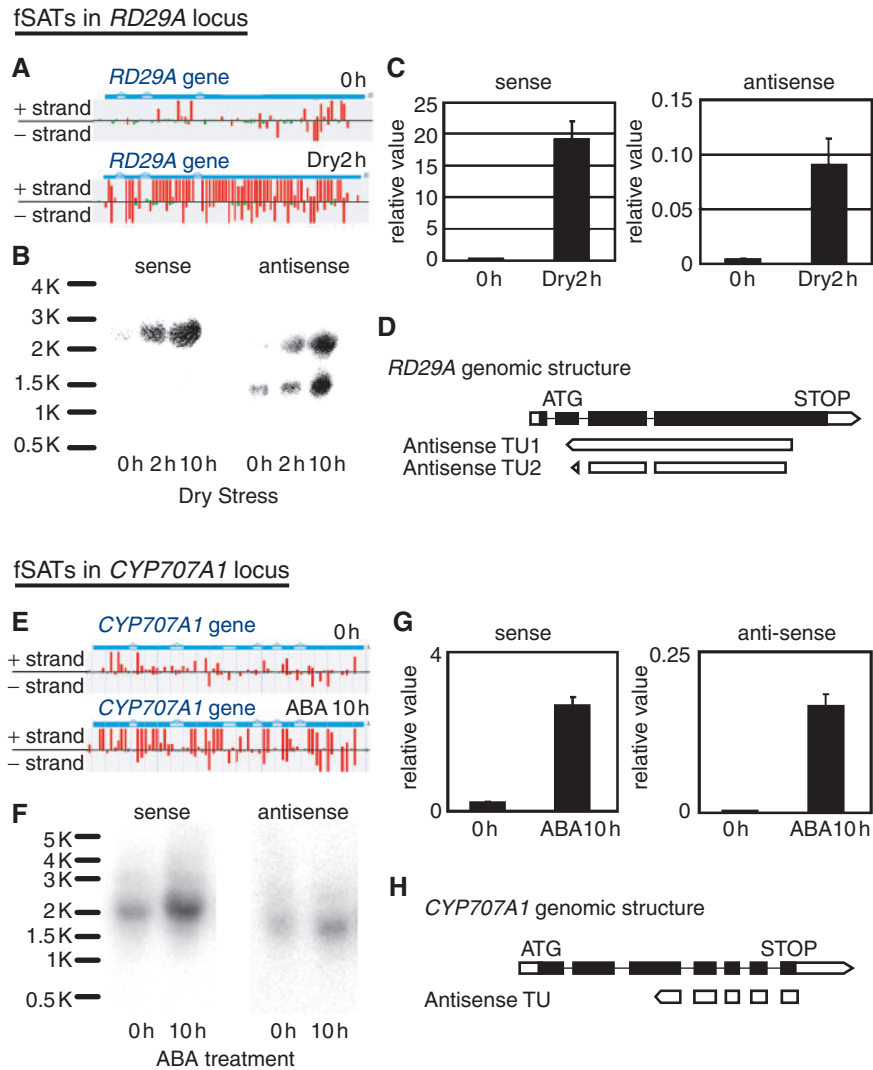


Fig. 3 Detection of the stress- or ABA-inducible sense and antisense TUs in the stress-inducible gene loci, *RD29A* and *CYP707A1*, by Northern and real-time RT-PCR analyses. The sense and antisense TUs in *RD29A* and *CYP707A1* loci were detected. The expression profiles using tiling array in *RD29A* locus for untreated and 2-h drought-treated conditions (A) and *CYP707A1* for untreated and 10-h ABA-treated conditions (E). The blue horizontal bars indicate gene model of *RD29A* and *CYP707A1* genes in the TAIR 6. The upward red or green bars indicate the signal intensity of probes on the “+” (Watson) strand. The downward red or green bars indicate the signal intensity of probes on the “-” (Crick) strand. When the signal intensity of the probes is higher than 400 and lower than 400, the intensity of the probes is shown in red and green, respectively. The Northern analysis for sense TU (mRNA) or antisense TU of *RD29A* locus without treatment, 2- and 10-h drought treated (B) and *CYP707A1* locus without treatment and 10-h ABA treated (F). For Northern blots, 10 µg of total RNA was loaded onto each lane and hybridized with the strand-specific RNA probes. The exposure time for the antisense TUs was three times longer than that for the sense TUs. The semi-quantitative real-time RT-PCR results for the sense and antisense TUs in *RD29A* locus under untreated and 2-h drought-treated conditions (C) and *CYP707A1* locus under untreated and 10-h ABA-treated conditions (G). The bar graphs show the results of densitometry of gene expression relative to *ACT2* and the error bars show the standard deviation of three experiments. Structural organization of the genome (upper) and the cDNAs from the antisense RNAs in the *RD29A* (D) and *CYP707A1* (H) gene regions. White boxes in the lower diagram represent the elements cloned from the antisense TUs.

We isolated the following two types of cDNAs named Antisense TU1 and 2. Antisense TU1 is a 1.9-kb cDNA for the transcript without poly (A). The sequence is complementary to the genomic sequence, that is, they contain both the exon and the intron sequences. Antisense TU2 is a 1.6-kb cDNA for the poly (A) tail-containing transcript.

The antisense sequence corresponding to the intron sequence in the sense strand was not contained in the antisense TU2 cDNA. The exon–intron junctions are conserved between the *RD29A* sense cDNA and the antisense TU2 cDNA, and only the *RD29A* sense cDNA conforms to the consensus sequence (GU...AG dinucleotides) for

splicing, suggesting the possibility that the antisense TU2 was derived from the *RD29A* sense mRNA. Sequence analysis of the four RACE PCR products showed that the number of the added (A) for the poly(A) tail-containing antisense TU was 5, 24, 25 and 51 nt and the poly(A) signal AAUAAA do not occur within 50 nt from the 3'-end.

Fig. 3E shows a fSAT of an ABA-inducible gene, *CYP707A1* (At4g19230) encoding ABA 8'-hydroxylase (Kushiro et al. 2004, Saito et al. 2004) and the novel ABA-inducible antisense TUs. Although AGI code is not annotated and the cDNAs have not been isolated on the antisense strand, our tiling array analysis indicated that novel ABA-inducible antisense TU was confirmed by real-time RT-PCR and Northern analyses using strand-specific RNA probes (Figs. 3F, 3G). The 0.8-kb cDNA for the transcript with the poly (A) was isolated (Fig. 3H). The exon-intron junction sequences were conserved between the *CYP707A1* sense cDNA and the antisense TU cDNA, and only the *CYP707A1* sense cDNA conforms to the consensus sequence (GU...AG dinucleotides) for splicing, suggesting the possibility that the antisense TU was derived from the *CYP707A1* sense mRNA.

We also identified ABA-inducible expression from both sense and antisense strands in the regions of several ABA- and drought-inducible genes, *AtMYC2*, *DREB2A* and *RD29B* by Northern analyses using the strand-specific RNA probes (Fig. 4). In the Northern analysis of the antisense TUs of the *AtMYC2* and *RD20*, hybridization smears were observed. Similar phenomena have been observed in analyses of the SATs that are produced from the same locus on mouse chromosomes (Kiyosawa et al. 2005). The constitutively expressed antisense TUs were detected at the opposite strand of the constitutively expressed genes, *GAE4* (At2g45310, UDP-D-glucuronate 4-epimerase 4) and an unknown protein-coding gene (At3g01345) (Fig. 4).

Biogenesis of the ABA- or stress-inducible antisense RNAs

In real-time RT-PCR analyses using 5'-end region of the *CYP707A1* gene (Fig. 5A), ABA-inducible expression was observed on both sense and antisense strands of all lines (Fig. 5B). In the line 3 (*cyp707a1-3*) where T-DNA inserted on the 3'-UTR, ABA-inducible TU accumulated on the antisense strand (Fig. 5B), showing that this ABA-inducible antisense TU is not produced by its upstream promoter.

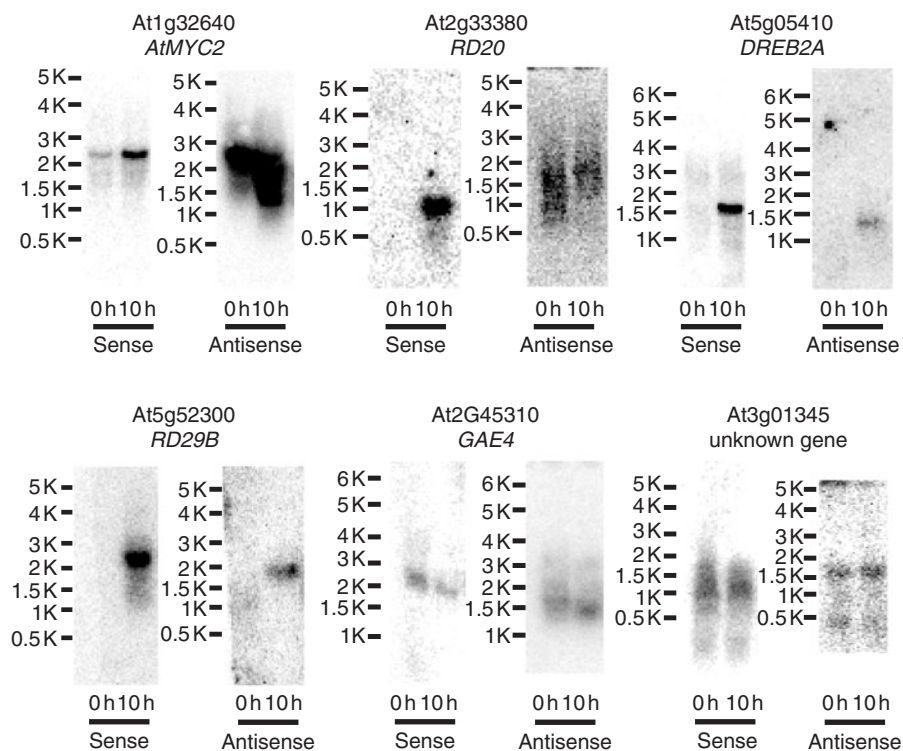


Fig. 4 Northern analysis of the sense and antisense transcripts in several ABA- and drought-inducible gene loci, and non-inducible gene loci. For Northern blots, 10 µg of total RNA from the wild-type plants under untreated and 10-h ABA-treated conditions was loaded into each lane and hybridized with the strand-specific RNA probes for the ABA- and drought-inducible genes, *AtMYC2*, *RD20*, *DREB2A* and *RD29B*, and the non-ABA-inducible genes, *GAE4* and an unknown protein-coding gene (At3g01345). The exposure time for the antisense transcripts was 5–10 times longer than that for the sense transcripts.

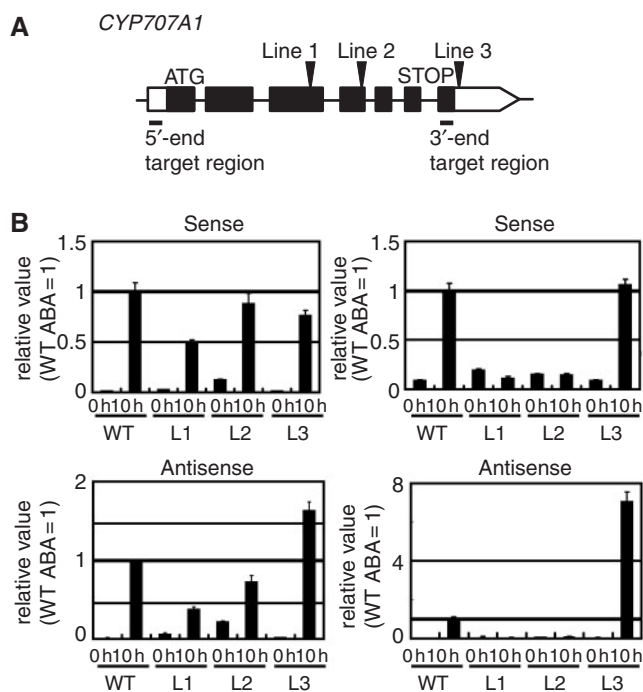


Fig. 5 Expression profile of an ABA-upregulated *CYP707A1* gene and novel ABA-inducible TUs mapped on the antisense strand. (A) Genomic structure of an ABA-upregulated *CYP707A1* gene. The black boxes correspond to exons. The positions of start and stop codons are indicated. Positions of T-DNA insertion in Lines 1 (*cyp707a1-1*, Okamoto et al. 2006), 2 (*cyp707a1-2*, Okamoto et al. 2006) and 3 (*cyp707a1-3*, SALK_002069) are indicated as arrows. (B) Semi-quantitative real-time RT-PCR analysis of *CYP707A1* gene in the Lines 1–3 and wild-type during 10-h ABA treatment. The data of real-time RT-PCR analysis using the 5'-end and 3'-end regions shown in (A) as the targets are shown in the left and right, respectively. The upper and the lower panels represent the expression data of the sense and antisense transcripts, respectively, of the *CYP707A1* locus. For the RT-PCR of the 5'-end regions, the primers, CYP707A1F-RT and CYP707A1R-RT (Supplementary Table S9) were used. For the RT-PCR of the 3'-end regions, the primers, CYP707A1F-RT2 and CYP707A1R-RT2 were used.

An ABA-responsive *cis*-acting element (ABRE, PyACGTGGC) (Yamaguchi-Shinozaki and Shinozaki 2005) was not observed in the 1.5-kb promoter regions of the antisense *CYP707A1* TU. When the ABA-inducible sense TU accumulates, ABA-inducible antisense TU also accumulates (Fig. 5B). On the other hand, when ABA-inducible sense TU does not accumulate, ABA-inducible antisense TU also does not accumulate. The sequence of the *CYP707A1* antisense TU is complementary to that of the *CYP707A1* sense TU (Fig. 3H). These results indicate that expression of the sense TU is necessary for the expression of the ABA-inducible antisense TU in the *CYP707A1* locus.

Next, we studied the possibility that this ABA-inducible antisense TU is produced by RDR enzymes (Xie et al. 2004). *Arabidopsis* genome has six *RDR* genes.

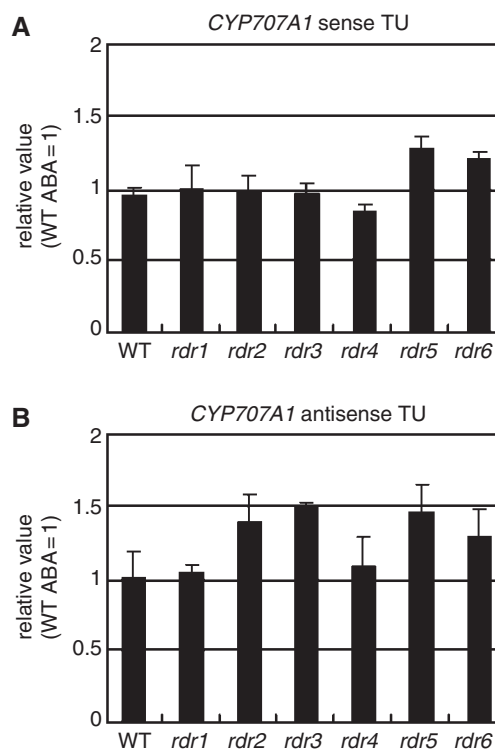


Fig. 6 Expression profile of the sense and antisense TUs in *CYP707A1* locus in *rdr* mutants. The expression of the sense (A) and antisense TUs (B) in *CYP707A1* locus was studied in *rdr1*, *rdr2*, *rdr3*, *rdr4*, *rdr5*, *rdr6* mutants and wild-type plants by real time RT-PCR analysis under 10-h ABA treatment. The bars represent the relative expression compared with wild-type plants. The error bars represent the standard deviation.

Therefore, we examined expression of the ABA-inducible antisense TU in mutants of six *RDR* genes. Real-time RT-PCR analyses showed that there are few differences of antisense TU expression between *rdr* mutants and wild type (Fig. 6). However, these results might not exclude that the ABA-inducible antisense TUs are produced by RDR enzymes, because six RDR enzymes might function redundantly each other for the biogenesis of the antisense RNAs and the sequence of the *CYP707A1* antisense TU is complementary to that of the *CYP707A1* sense TU (Fig. 3H).

Discussion

In this tiling array analysis, we identified a large number of drought, cold, high-salinity stress and ABA-responsive genes and TUs except for the poly(A)⁻ RNAs, small RNAs and small ORFs. The data presented here display the comprehensive landscapes of the transcriptome under drought, cold, high-salinity stress and ABA treatments in a model plant, *Arabidopsis*, and should be useful

for our understanding of the molecular mechanisms in the plant stress responses.

Generally, antisense RNAs were believed to control the expression of the sense transcripts negatively in plants (Borsani et al. 2005, Katiyar-Agarwal et al. 2006). We found a large number of non-protein coding transcripts belong to the transcripts of the SATs and the significant linear correlation between the expression ratios (treated/untreated) of the sense transcripts and the ratios of the antisense transcripts, indicating that many non-protein-coding antisense transcripts do not control the accumulation of the sense transcripts negatively. The information obtained in this study suggests the novel roles of the antisense RNAs in plants.

Many novel stress- or ABA-inducible non-protein-coding RNAs were identified

In this study, 1,275 stress- or ABA-inducible non-AGI TUs have been identified. BLASTX search indicated that most of them do not encode proteins and might function as non-protein-coding RNA molecules involved in the stress or ABA responses. Although the function of the mRNA-like non-protein-coding RNAs, such as human HOTAIR (Rinn et al. 2007) and *Arabidopsis INDUCED BY PHOSPHATE STARVATION 1 (IPSI)* (Franco-Zorrilla et al. 2007) have been revealed, the function of the stress- and hormone-responsive mRNA-like non-protein-coding RNAs is still poorly understood.

We also identified many novel TUs in the 5'- and 3'-UTR regions (Supplementary Fig. S7). Some of them show the ABA- or stress-responsive gene expression. Kapranov et al. (2007) reported the PASRs and the TASRs around 5' and 3' termini, respectively, in human. Although the functions of the PASRs and TASRs are largely unknown, the presence of the transcriptionally active regions in the UTR regions might be conserved between the plants and animals. Common sequence characteristics of the PASRs and TASRs seem to be missing. Martianov et al. (2007) demonstrated that a non-protein-coding transcript upstream of the human *dihydrofolate reductase (DHFR)* gene has a critical function in transcriptional repression of the *DHFR* gene. Some novel PASR-like TUs in the 5'-UTR regions might act as negative regulators of the downstream main TUs.

Most of non-protein-coding transcripts belong to pairs of the SATs

We also identified 7,805 SATs in this tiling array analysis. Recently, large-scale antisense activities were found in previous tiling array analysis (Yamada et al. 2003, Stolc et al. 2005) and large-scale cDNA analyses (Seki et al. 2002c, 2004, Jen et al. 2005, Wang et al. 2005) in *Arabidopsis*. Similar widespread existence of antisense

transcripts was also reported in several model organisms including yeast (David et al. 2006), rice (Osato et al. 2003), human (Lehner et al. 2002, Yelin et al. 2003, Chen et al. 2005), mouse (Kiyosawa et al. 2003, Katayama et al. 2005) and fly (Sun et al. 2006). The percentage of TUs involved in an overlap, ranges from 5% to 30% (Lapidot and Pilpel 2006).

About 90% (6,973/7,805) of the SATs contained non-AGI TUs as at least one transcript (Table 3). Previous large-scale cDNA analyses also revealed that more than half of the SATs contained non-annotated genes as one transcript (Seki et al. 2004, Wang et al. 2005). Most of the non-AGI TUs do not seem to encode proteins. These results suggest that many non-protein-coding RNAs exist in the non-AGI regions and might function in the regulation of the other TU's expression.

Expression ratios (treated/untreated) of the antisense TUs have significant linear correlation with the ratios of the sense TUs in most of the fSATs

Significant linear correlation between the expression ratios (stress or ABA treated/untreated) of the sense TUs and the ratios of the antisense TUs was observed in the SATs of AGI/non-AGI and non-AGI/non-AGI. About 90% of the SATs (AGI/non-AGI) was fSATs. The highest correlation of the expression ratio (stress or ABA treated/untreated) was observed in the fSATs (AGI/non-AGI). Most of the previous studies revealed that the expression of the sense and antisense transcripts is inversely regulated (Borsani et al. 2005, Katiyar-Agarwal et al. 2006). Previous studies using the Affymetrix mouse genome array found that the antisense transcriptomes are tissue specific (Werner et al. 2007). Similarly, *Arabidopsis* tiling array studies also indicated that the expression of the antisense RNAs was tissue specific for many genes (Yamada et al. 2003). However, the detailed list of the antisense RNAs is not available and detailed expression data of the sense and antisense transcripts in various plant tissues is not well understood.

Recently, Perocchi et al. (2007) pointed out the antisense artifacts in the microarray analysis. However, such antisense artifacts are not likely to occur in our tiling array experiments for the following reasons: (i) Our protocol for probe synthesis and hybridization in the array analysis is different from that of Perocchi et al. (2007). Perocchi et al. (2007) used the random primer for the first-strand cDNA synthesis and hybridized the first-strand cDNAs end-labeled using terminal transferase to the arrays directly. On the other hand, we used the oligo(dT) primer for the first-strand cDNA synthesis and hybridized the biotin-labeled cRNAs generated after the synthesis of the first-strand and second-strand cDNAs, in vitro transcription (IVT) amplification, and biotin labeling,

to the arrays. (ii) We also confirmed the expression of several antisense transcripts by Northern analysis using the strand-specific RNA probes (Figs. 3, 4). Previous human tiling array studies by the Affymetrix groups using a direct RNA end-labeling method also showed similar amounts of antisense transcription (Kampa et al. 2004), suggesting that large-scale stress- or ABA-inducible antisense transcripts are really generated in *Arabidopsis*.

We also studied the possibility of a data analysis artifact. For the fSATs, significant linear correlation between the expression (HLE values) of the expressed AGI code genes and the values of the antisense TUs identified by ARTADE was observed (Supplementary Fig. S8A). Positive correlation between the expression values of the expressed AGI code genes in the fSATs and the values of its corresponding antisense genomic sequences was also observed (Supplementary Fig. S8B), showing that the observed positive correlation is independent of the bias of the TUs identified by the ARTADE program. A positive correlation between the values of the 5'-half of the expressed AGI code genes in the fSATs and the values of the 5'-half of its corresponding genomic sequences on the antisense strand, was also observed (Supplementary Fig. S8C). However, the significant linear correlation was not observed between the values of the promoter regions of all AGI code genes and the values of its corresponding genomic sequences on the antisense strand (Supplementary Fig. S8D). These results show that the observed significant linear correlation is not due to the probe sequences used by chance and that significant linear correlation between the expression of the expressed AGI code genes and the expression of the antisense TUs really exists. The expression ratio (HLE values of the sense TUs/HLE values of the antisense TUs) is variable between the genes (Supplementary Fig. S8A), showing that the highly expressed antisense TUs do not always exist on the opposite strand of the highly expressed sense TUs. A similar tendency was also obtained by the Northern analyses (Figs. 3, 4).

Expression of sense transcripts is necessary for the stress- or ABA-inducible expression of antisense transcripts in the fSAT (CYP707A1/non-AGI TU)

We suggest the following three models for biogenesis of the stress- or ABA-inducible antisense RNAs: (i) Antisense RNAs are produced by the promoters upstream of the transcriptional start site of antisense TUs. (ii) Antisense RNAs are produced by RNA-dependent RNA polymerases (RDRs). (iii) Antisense RNAs are produced by some enzymes using the sense DNA as a template after transcription of the sense RNAs.

Nat-siRNA biogenesis pathways reported by Borsani et al. (2005) fit the first model. So far, three reports on the nat-siRNA pathways have been published in plants

(Borsani et al. 2005, Katiyar-Agarwal et al. 2006, Zubko and Meyer 2007). In these reports, expression profiles of the sense transcripts are different from the expression profiles of the antisense transcripts and the sense and antisense transcripts are partially overlapped. On the other hand, the sense and antisense transcripts that we found in this study are fully overlapped and the expression of both sense and antisense transcripts are stress or ABA inducible (Figs. 2, 3; Table 3; Supplementary Fig. S3).

Real-time RT-PCR analyses for *CYP707A1* locus indicated that ABA-inducible antisense RNA is not generated from its upstream promoter in the fSAT (*CYP707A1*/non-AGI TU). Although ABA- or abiotic-stress-responses-related *cis*-acting elements, such as ABRE and dehydration-responsive element (DRE, Yamaguchi-Shinozaki and Shinozaki 2005), were observed in the 1.5-kb promoter regions of many ABA- or stress-inducible AGI code genes (sense transcripts) [P-value for ABRE (ABREATCONSENSUS in the PLACE database, (C/T)ACGTGGC) and DRE (DRECRTCOREAT in the PLACE database, (A/G)CCGAC): 4.66×10^{-53} and 2.46×10^{-9} , respectively, see Supplementary methods)], such *cis*-elements were not observed in the 1.5-kb promoter regions of many ABA- or stress-inducible antisense non-AGI TUs (P-value for ABREATCONSENSUS and DRECRTCOREAT: 4.00×10^{-3} and 4.72×10^{-3} , respectively) for the fSATs (AGI/non-AGI). These results indicate that the ABA- or stress-inducible antisense RNAs are not generally generated from its upstream promoters in the fSATs (AGI/non-AGI).

We isolated the cDNAs for the stress- and ABA-inducible antisense RNAs. The cDNAs for RNAs with and without poly (A) have been isolated for the stress- and ABA-inducible antisense RNAs in the *RD29A* gene regions. Previously reported *cis*-acting elements, such as DRE and ABRE, do not exist in the 1.5-kb promoter regions upstream of the antisense TUs, suggesting that the stress- and ABA-inducible *RD29A* antisense RNAs are not produced by its upstream promoters. We assume that two mechanisms for the biogenesis of the antisense RNAs exist. One mechanism is antisense transcripts are generated by RDR enzymes from RNA templates, and the other one is antisense transcripts are generated from a DNA template. Because the exon-intron junctions are conserved between the poly(A) tail-containing antisense transcript and the sense transcript, the poly(A) tail-containing antisense transcript might be produced by RDR enzymes. The *CYP707A1* antisense RNAs might also be generated by RDR enzymes, because its exon-junctions are also conserved. The number of the added (A) for the poly(A)-containing antisense RNAs was shorter than that of the mRNAs translated into the proteins. In yeast, the cytoplasmic poly(A) polymerase which adenylates aberrant

rRNAs, snoRNAs and other nuclear unstable transcripts, function in the nuclear quality control pathway and lead to rapid 3' to 5' degradation (Read et al. 2002, Saitoh et al. 2002, Wyers et al. 2005). The poly(A)-containing antisense RNAs might be degraded by the nuclear quality control pathway. For the antisense RNAs without poly(A), they might be produced by some enzymes using the sense DNA as a template after transcription of the sense RNAs.

Several studies revealed that small RNAs such as siRNAs are generated from the overlapping regions of the SATs (Borsani et al. 2005, Katiyar-Agarwal et al. 2006, Zubko and Meyer 2007) and involved in the degradation of the mRNA on one strand. However, we could not detect the ABA- or stress-regulated siRNAs by Northern analyses in the overlapping regions for the fSATs of the *RD29A* and *CYP707A1* loci. Mapping of the *Arabidopsis* small RNAs collected previously also indicated that density of the small RNA loci per 1-kb genomic sequence was similar between the fSATs (AGI/non-AGI) and all expressed AGI code genes (Supplementary Table S7). These results suggest that the small RNA-mediated silencing is not involved in the antisense regulation for the fSATs.

Possible functions of the stress- or hormone-responsive antisense RNAs

Recent studies have shown that antisense RNAs participate in a broad range of types of regulation, such as gene silencing, RNA stability, alternative splicing, RNA editing, RNA masking and methylation (Hastings et al. 1997, Prescott and Proudfoot 2002, Tufarelli et al. 2003, Nishimura et al. 2004, Borsani et al. 2005, Enerly et al. 2005, Jen et al. 2005, Katiyar-Agarwal et al. 2006, Zubko and Meyer 2007, Matsui et al. 2008). The antisense RNAs might be degraded by the nuclear quality control pathway. Although there are extensive studies in various organisms for the functions of the antisense RNAs, understanding of the functions and regulation of the stress- and hormone-responsive antisense RNAs is at an early stage.

There are several lines of evidence for the clustering of co-expressed genes, such as chromatin-mediated transcriptional co-regulation (Boutanaev et al. 2002), operons and duplicate genes (Lercher et al. 2003) and bi-directional promoters (Trinklein et al. 2004). By providing genome-wide co-regulation of the sense and antisense transcripts under the stress and hormone treatment conditions, this study also expands our understanding of the coordinated gene regulation in higher eukaryotes. It might be expected that there is a balance between the expression of the sense genes and the expression of antisense ones under various conditions, which would be modulated by antisense regulation to adapt to different environmental stress conditions and to developmental stages.

Materials and Methods

Plant materials, stress treatments and RNA isolation

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown essentially as described previously (Oono et al. 2003). Dehydration, cold, high-salinity and ABA treatments were carried out essentially as reported previously (Seki et al. 2002a, 2002b, Oono et al. 2006). Total RNA was prepared using ISOGEN (Nippon Gene) according to the instructions. Detailed descriptions are included in the supplementary data.

Probe synthesis for whole genome tiling array analysis and microarray hybridization

Eight to fifteen μg of total RNA per sample was used for synthesis of double-stranded cDNA by the GeneChip One-Cycle cDNA Synthesis Kit (Affymetrix) using an oligo(dT) primer containing the T7 RNA polymerase promoter. Biotin-labeled cRNA was generated from the cDNA by in vitro transcription using the GeneChip IVT Labeling Kit (Affymetrix), fragmented, and about 10 μg of cRNA was hybridized with the GeneChip *Arabidopsis* tiling array set (1.0F Array and 1.0R Array, Affymetrix, see Supplementary data online) using the Hybridization Control Kit (Affymetrix). Microarray hybridization, staining and washing were performed according to the Eukaryotic Sample Protocol (Affymetrix). Scanning was performed at 0.7 μm resolution using a GeneChip Scanner 3000 7G (Affymetrix). Three independent biological replicates were performed for each strand array.

Computational analyses of RNA expression

Detailed descriptions are included in the supplementary data.

Detection of transcriptional activity in AGI code genes and non-AGI TUs

We used "the ARTADE-based method (P-initial value $<10^{-8}$) (Toyoda and Shinozaki 2005)" to detect the expressed genes and non-AGI TUs from the expression data. Detailed descriptions are included in the supplementary data.

Identification of the non-protein-coding RNAs

Homology search of the non-AGI TUs against the registered protein sequence data sets (NIH nr database) was done using the BLASTX program. When the score of homology search is higher than e^{-20} and less than e^{-20} , we regarded the TUs as the hypothetical non-protein-coding RNAs and as hypothetical proteins, respectively.

Identification of the stress-responsive AGI code genes and novel TUs

We used the intensity values (PM-MM) of all probes (2.9 million PM and 2.9 million MM probes) for this analysis and identified the stress- or ABA-responsive genes and TUs by Mann-Whitney U-test (FDR $\alpha = 0.01$) (Mann and Whitney 1947, Storey and Tibshirani 2003). The genes or TUs were further selected using the following criteria: (1) The expression ratios (treated/untreated) are greater than 1.8-fold and less than 5/9 for the stress- or ABA-inducible genes and for the stress- or ABA-downregulated genes, respectively. (2) The genes or TUs are identified as the expressed ones under the drought, cold, high-salinity and ABA treatments by ARTADE-based method (P-initial $<10^{-8}$) for the stress- or ABA-inducible genes. The genes or TUs are identified as the expressed ones without treatment by ARTADE-based method

(P-initial $<10^{-8}$) for the stress- or ABA-downregulated genes. Detailed descriptions are included in the supplementary data.

Identification of the sense-antisense transcripts

We used the sequence data of both AGI code genes and non-AGI TUs identified as “expressed genes and TUs” by “the ARTADE-based method” for the identification of the SATs.

Mapping of cDNAs and small RNAs to genomic sequence

Detailed descriptions are included in the supplementary data.

Real-time RT-PCR analysis and Northern analysis

Detailed descriptions are included in the supplementary data.

Cloning of the antisense transcripts of the RD29A and CYP707A1 genes

Detailed descriptions are included in the supplementary data.

Detection and evaluation of cis-elements

Detailed descriptions are included in the supplementary data.

Supplementary data

Supplementary data mentioned in the article is available at *Plant and Cell Physiology* online and our web site (<http://pfgweb.gsc.riken.jp/supplements/matsui001/>).

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