Transcript Profiling of an Arabidopsis PSEUDO RESPONSE REGULATOR Arrhythmic Triple Mutant Reveals a Role for the Circadian Clock in Cold Stress Response

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Arabidopsis PSEUDO RESPONSE REGULATOR (PRR) genes are components of the circadian clock mechanism. In order to understand the scope of genome-wide transcriptional regulation by PRR genes, a comparison survey of gene expression in wild-type Arabidopsis and a prr9-11 prr7-10 prr5-10 triple mutant (d975) using mRNA collected during late daytime was conducted using an Affymetrix ATH-1 GeneChip®. The expression of ‘night genes’ increased and the expression of ‘day genes’ decreased toward the end of the diurnal light phase, but expression of these genes was essentially constant in d975. The expression levels of ‘night genes’ were lower, whereas the expression of ‘day genes’ was higher in d975 than in the wild type. Bioinformatics approaches have indicated that the set of up-regulated genes in d975 and the set of cold-responsive genes have significant overlap. We found that d975 is more tolerant to cold, high salinity and drought stresses than the wild type. In addition, dehydration-responsive element B1/C-repeat-binding factor (DREB1/CFB) is expressed around mid-day, is more highly expressed in d975. Raffinose and l-proline accumulated at higher levels in d975 even when plants were grown under normal conditions. These results suggest that PRR9, PRR7 and PRR5 are involved in a mechanism that anticipates diurnal cold stress and which initiates a stress response by mediating cyclic expression of stress response genes, including DREB1/CFB.

Keywords: Arabidopsis thaliana • Circadian rhythm • Cold stress • Microarray • PRR.

Abbreviations: CBF, C-repeat-binding factor; CCA1, CIRCADIAN CLOCK-ASSOCIATED 1; CCG, clock-controlled gene; DD, constant darkness; DREB1, dehydration-responsive element B1; ELF4, EARLY FLOWERING 4; FDR, false discovery rate; GC-TOF/MS, gas chromatography–time of flight/mass spectrometry; GI, GIGANTEA; GO, Gene Ontology; LD, 12 h light/12 h dark conditions; LHY, LATE ELONGATED HYPOCHOTYL; LL, constant light; LUX, LUX ARRHYTHMO; MS, Murashige and Skoog; PCL1, PHYTOCLOCK 1; PRR, PSEUDO-RESPONSE REGULATOR; RMA, robust multichip average; TOC1, TIMING OF CAB EXPRESSION 1; ZT, Zeitgeber time.

Introduction

The circadian clock is an endogenous 24 h pacemaker that allows plants to anticipate daily changes in environmental conditions (Bunning 1967). It also provides plants with selective advantages, including increased photosynthesis, growth and survival (Dodd et al. 2005). A wide variety of processes under the control of the circadian clock in Arabidopsis have been reported (McClung 2006). For example, Arabidopsis plants make rhythmic cotyledon and leaf movements, and stem elongation rates are dependent on circadian rhythms (Dowson-Day and Millar 1999, McClung 2006, Nozue et al. 2007). Microarray experiments detailing steady-state mRNA abundance indicate that about 6% of Arabidopsis genes are regulated by the circadian clock (Harmer et al. 2000), but 35% of enhancer trap lines display circadian-regulated transcription (Michael and McClung 2003). In addition, about
90% of Arabidopsis transcripts cycle in at least one condition of six diurnal and five circadian conditions (Michael et al. 2008). Recent microarray analyses suggest that large numbers of physiological events were under circadian clock control, including secondary metabolite biosynthesis, cold stress response and hormone responses (Harmer et al. 2000, Covington and Harmer 2007, Bieniawska et al. 2008, Covington et al. 2008, Michael et al. 2008, Mizuno and Yamashino 2008). Transcription of clock-controlled genes (CCGs) peaks at various times of the subjective day and night, indicating that a complex network regulates these genes throughout the various phases, but a majority of CCG expression occurs around either dawn or dusk (Blasing et al. 2005, Michael et al. 2008).

An interlocking transcriptional/translational 24 h cycle feedback loop of clock-associated genes comprises the circadian clock (Mizuno and Nakamichi 2005, Locke et al. 2006, McClung 2006, Zeilinger et al. 2006). CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYOCOTYL (LHY), the closest paralogs of MYB-like transcription factors, are thought to form a feedback loop (Millar et al. 1995, Schaffer et al. 1998, Wang and Tobin 1998, Makino et al. 2000, Strayer et al. 2000, Alabadi et al. 2001, Mizoguchi et al. 2002, Doyle et al. 2002, Farre et al. 2005, Hazen et al. 2005, Mizoguchi et al. 2005, Nakamichi et al. 2005, Onai and Ishiura 2005, Salome and McClung 2005, McClung 2006). We have demonstrated that the prr5-11 prr7-11 prr9-10 triple mutant (d975) arrhythmically expresses several clock-associated genes under constant light (LL) conditions (Nakamichi et al. 2005). CCA1 and LHY are expressed at dawn in wild-type plants, but expression is largely constitutive at a higher overall level in the d975 mutant line. In addition, the d975 line has pleiotropic phenotypes. For example, d975 plants flower late due to attenuation of the CONSTANS-mediated photoperiodic flowering pathway, and produce long hypocotyls under constant red light conditions (Ito et al. 2007a, Nakamichi et al. 2007). These phenotypes are apparently caused by the defective circadian expression of d975.

Despite the large number of CCGs which have been identified by array analyses, little direct evidence for their biological importance as being under circadian clock control has been produced by using clock mutants. Therefore, genome-wide transcriptional analyses combined with phenotypic analysis of the arrhythmic mutant d975 would probably provide a catalog of the biological processes controlled by the circadian clock.

In this study, we analyzed gene expression during subjective late afternoon (Zeitgeber time, ZT8–ZT12) in d975 using an Affymetrix ATH-1 GeneChip®. Our data showed that the loss of PRR9, PRR7 and PRR5 abolished the rhythmic expression of clock-associated genes and of most CCGs. In addition, these array data combined with a published array showing diurnal rhythm suggested that ‘day genes’ are up-regulated in d975, whereas ‘night genes’ were down-regulated. Informatics-based approaches indicated that up-regulated genes in d975 have significant overlap with cold stress-inducible genes. We also found that d975 tolerates cold, high salinity and drought, and has elevated expression of genes associated with cold stress responses, including DREB1.

Results

d975 is an arrhythmic mutant

Based on RNA samples taken at 3 h intervals, CCA1, TOC1 and GI are arrhythmically expressed in d975 plants (Nakamichi et al. 2005). To determine the phenotype of d975 more precisely, we introduced a luciferase reporter gene driven by the CCA1 promoter (CCA1::LUC) into d975, and measured the bioluminescence of individual plants every 30 min. Bioluminescence in the wild type oscillated with a peak at subjective dawn under both LL and constant dark (DD) conditions after growth under entrainment conditions of 12 h light and 12 h dark (LD, Fig. 1), but was constant in d975 under both LL and DD conditions. Similar results were obtained from two independent experiments. These results confirmed that d975 plants are arrhythmic under constant conditions.

Global expression profile during ZT8–ZT12 in d975 plants

The majority of CCG peaks appear from subjective dawn to mid-day (‘day genes’ is used as a convenient shorthand to simplify the description of peak expression time) or subjective dusk to night (‘night genes’) (Blasing et al. 2005, Michael et al. 2008). We reasoned that microarray analysis with mRNA collected from 18-day-old plants during the late afternoon (ZT8–ZT12) would detect any increases in the expression profile of ‘night genes’ and decreases in the profile of ‘day genes’ (Fig. 2A). Three independent RNA samples were used for array analyses.

To identify cyclic genes, genes whose expression increased or decreased with statistical significance during ZT8–ZT12 were noted. In the wild type, 152 genes had increased expression between ZT8 and ZT12, and 232 genes decreased using the false discovery rate (FDR) criteria stipulated in Materials and Methods (Fig. 2B, E; the lists of 152 ‘night genes’ and 232 ‘day genes’ are shown in Supplementary Tables S1 and S2, respectively). The expression levels of three genes were significantly different between ZT8 and ZT10, and no genes increased or decreased significantly between ZT10 and ZT12. The average number of detectable, ‘present’ genes in the array was between 14,200 and 15,100 (Supplementary Table S3).
Microarray analysis for an arrhythmic mutant

Forty-cluster (b). About wild-type and expression patterns of ‘day genes’. microarray analyses could detect the characteristic decreasing Blasing et al. 2005, Michael et al. 2008), indicating that microarray analyses using mRNA (tern, with maxima around dusk or during the night time maximum measured values for wild type#1, CCA1::LUC cycles (LD cycles) and transferred to LL (A) or DD conditions (B).

and 31,560 counts s–1, respectively. type#2, wild type, in ‘total genes’ (Fig. 3, Supplementary Table S4).

Most of the 152 ‘night genes’ show a diurnal–cyclic pattern, with maxima around dusk or during the night time (Supplementary Fig. S1A; Blasing et al. 2005, Michael et al. 2008), indicating that microarray analyses using mRNA collected during the late daytime could be used to detect the increasing expression of some ‘night genes’. In contrast, most of the 232 ‘day genes’ had a diurnal–cyclic pattern with maxima around dawn or later (Supplementary Fig. S1B; Blasing et al. 2005, Michael et al. 2008), indicating that microarray analyses could detect the characteristic decreasing expression patterns of ‘day genes’.

No genes in d975 met the criteria for significant changes in expression between ZT8 and ZT12, suggesting that gene expression was unaffected by the circadian clock, at least from ZT8 to ZT12. The 152 ‘night’ and 232 ‘day genes’ were with few exceptions constitutively expressed in d975 (Fig. 2C, F).

These data also indicated that CCG expression levels varied from the wild-type levels in the d975 mutant line. Nineteen (12.5%) of the 152 ‘night genes’ and 218 (94.0%) of the 232 ‘day genes’ were up-regulated in d975 relative to the wild type at ZT12 (Fig. 2D, G), implying that CCG expression levels in d975 are stalled at wild-type daytime levels.

Hierarchical clustering analysis of up-regulated genes in d975

The abnormal CCG expression profiles (i.e. constant patterns but changing levels) suggest that a number of biological processes are altered in d975. To investigate how disordered CCG expression influences the broader physiology of the plant, 445 up-regulated genes in d975 were compared with the wild type at ZT12 by Gene Ontology (GO) classification (q < 0.02, and 2-fold up-regulated in d975 compared with the wild type, in ‘total genes’ (Fig. 3, Supplementary Table S4). GO cellular component analysis suggested that 107 genes encode proteins localized in chloroplasts (Fig. 3A). Forty-eight genes encode proteins potentially localized in plastids, 41 encode proteins localized in nuclei and 38 genes encode proteins associated with the plasma membrane. There are fewer genes encoding proteins localized in other components than in the above four categories. GO cellular component analysis also suggested that PRR9, PRR7 and PRR5 affect the production of many proteins in chloroplasts, nuclei and plasma membranes. Another GO analysis indicated that the molecular functions of proteins encoded by 445 genes are highly diverse (Fig. 3B). For example, 72 proteins have putative transcription factor activity, 58 have transporter activity, 50 have transferase activity, 35 have hydrolase activity and 31 have kinase activity.

This analysis also suggested that 128 proteins are associated with responses to abiotic or biotic stimuli, 95 with a response to stress, 62 with transcription, 51 with protein metabolism, 39 with transport, 38 with developmental processes, 25 with signal transduction, nine with electron transport or energy pathways and seven with cell organization and biogenesis (Fig. 3C). GO analyses thus suggest that PRR9, PRR7 and PRR5 primarily affect genes which respond to abiotic or biotic stimuli, or to stress.

To explore the 445 up-regulated genes in d975 which respond to abiotic or biotic stimuli, or to stress, a cluster analysis for 445 genes was carried out using 10 DNA microarray data sets under stress-treated conditions (Kilian et al. 2007, Goda et al. 2008, Fig. 4). Expression of many of the 445 up-regulated genes was affected by cold, salt, osmotic or UV-B stresses, but not by heat, oxidative, wound, ABA or genotoxic stresses (Fig. 4). Cold stress causes up-regulation of 20–30% of the 445 genes [Fig. 4; cluster (b)]. About 10–20% of the genes are either up- or down-regulated by salt or osmotic stress treatments [cluster (c)], and UV-B stress...
Fig. 2 Global CCG expression pattern from ZT8 to ZT12. (A) Sampling schedule for microarray analysis. Microarray analysis was conducted with mRNA extracted during time points ZT8, ZT10 and ZT12 to detect changes in night genes (dashed line) and day genes (solid line). (B) The 152 ‘night genes’ detected during the period from ZT8 to ZT12 in the wild type, and (C) the same genes in d975 compared with the wild type. In both panels, the relative signal level of the wild type at ZT12 was set to 1.0. (D) The heatmap for the log2 ratio (d975/wild type) of 152 genes. The signal intensities resulting from the RMA algorithm were transformed to log2 ratios. Red indicates high expression and blue indicates low expression in d975. (E) The 232 ‘day gene’ expression patterns in the wild type from ZT8 to ZT12. (F) The same genes in d975 compared with the wild type. To facilitate comparison with the wild type, the genes having signal intensities >3.0 were excluded. For each gene in E and F, the signal level in the wild type at ZT12 was set to 1.0. (G) The heatmap for the log2 ratio (d975/wild type) of 232 genes. The lists of ‘night genes’ and ‘day genes’ are shown in Supplementary Tables S1, and S2, respectively.
d975 displays stress-tolerant phenotypes

Some stress-responsive genes encode proteins which protect cells from stress, such as chaperones, anti-freeze proteins, detoxification enzymes, key biosynthetic enzymes for osmo-lytes, including raffinose, and regulatory proteins involved in stress response signal transduction (Yamaguchi-Shinozaki and Shinozaki 2006). If up-regulated genes in d975 were to contribute significantly to stress responses, d975 may have a tolerant phenotype to a number of environmental stresses. Therefore, several stress tests were performed to evaluate

Microarray analysis for an arrhythmic mutant

Fig. 4 Conditional clustering analysis of 445 up-regulated genes in d975. The probe sets of 445 up-regulated genes in d975 were clustered using 10 microarray data sets of stress treatment tests. The expression levels of log2 ratios of d975/wild type, and stress-treated sample/control were used for the cluster analysis. Red indicates high expression and blue indicates low expression. The arrow (a) indicates signals of probe sets for d975/wild type. Cluster (b) indicates cold stress conditions. Cluster (c) indicates osmotic or salt stress conditions. Cluster (d) indicates UV-B stress of shoots. The tree on the right is conditional.

among the genes downstream of AREB1 in d975, suggesting that AREB signaling is about the same in the wild type and d975. In contrast to DREB2A- or AREB1-dependent genes, the expression levels of DREB1A-dependent genes were significantly higher in d975 (Fig. 6C). DREB1A and DREB1C expression levels were higher in d975 than in the wild type. The relative expression 40 DREB1A downstream genes was also examined in our macroarray data. No probes were
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![Figure 5](https://academic.oup.com/pcp/article-abstract/50/3/447/1842792)

**Fig. 5** Cold, salt and drought stress tests. (A) Wild-type and d975 plants were subjected to cold stress (−5°C) for 1 d. The plants were returned to 22°C, photographed and assessed for survival 3 d later. Averages from three tests were plotted. The error bar indicates the SD. (B) Wild-type and d975 seedlings were subjected to high salinity conditions for 6 d, after which bleaching rates were calculated and seedlings were photographed. The tests were performed three times, and average data were plotted. The error bar indicates the SD. (C and D) Wild-type and d975 plants were subjected to drought stress conditions. The growth stages were different in C and D. Averages from three tests were plotted. The error bar indicates the SD.

available for three of the genes on the ATH-1 GeneChip, and two of the gene signals were not detected (data not shown). Among the 35 detectable genes, 16 were significantly up-regulated in d975 at q < 0.05, five were up-regulated at q < 0.005 and four were down-regulated at q < 0.05. These data strongly suggest that the up-regulated DREB1A gene in d975 further up-regulates at least some of the DREB1A-dependent genes.

We quantified the expression levels of three DREB1 genes under LD cycles in d975 compared with the wild type. The expression of three DREB1 genes had a diurnal cycle pattern with a peak around ZT9 in the wild type (Fig. 7A), and these patterns were consistent with previous data (Harmer et al. 2000, Fowler et al. 2005). The expression levels of three DREB1 genes were higher in d975 than in the wild type at each time point throughout the day, except for DREB1B at two time points. The target genes of DREB1A (Gols3, ADS2 and LTI30) were also regulated by the circadian clock, and up-regulated in d975 (Supplementary Figs. S2, S3). Similar results were obtained from two independent experiments. We also quantified downstream metabolites of DREB1, because overexpression of DREB1 has been shown to increase the accumulation of raffinose and l-proline in Arabidopsis and rice (Gilmour et al. 2000, Ito et al. 2006). Gas chromatography-time of flight/mass spectrometry (GC-TOF/MS) analysis (using 3–14 independent plant samples per data point for proline, and 15 independent plants for other metabolites) revealed that raffinose concentrations were substantially higher in d975 than in the wild type from ZT9 to ZT17 (Fig. 7B). l-Glutamic acid concentrations were slightly higher in d975 than in the wild type, and l-proline concentrations were much higher in d975 (Fig. 7C).

**Gate effect on cold stress inducing DREB1 expression in d975**

The circadian clock regulates DREB1 expression in two distinct ways: by circadian regulation under non-stress conditions, and by time-dependent gating for induced responses to cold stress (Harmer et al. 2000, Fowler et al. 2005, Bieniawska et al. 2008). To address whether PRR genes are involved in this gating effect, we measured DREB1 induction by cold stress at several time points under LD conditions (Fig. 8). The induction of DREB1 by cold stress was gated by the circadian clock, as described previously (Fowler et al. 2005). Transferring plants to cold stress at ZT4 resulted in much greater accumulation of DREB1 transcripts than did transfer at other time points. The induction of DREB1 was almost the same at each of the four time points in d975, suggesting that PRR9, PRR7 and PRR5 are also essential to the gating effect of DREB1 induction. Similar results were obtained from two independent experiments. It should be noted that the induction of the DREB1 genes in d975 was nearly the same as the maximum level in the wild type when stressed at ZT4. That is, DREB1A and DREB1B are expressed...
Fig. 6 The heatmap for the log2 ratio (d975/wild type) of genes expressed downstream of DREB2A, AREB1 or DREB1A. (A) The expression levels of 20 of 23 (no available probes for one gene in the ATH-1 GeneChip, and two gene signals were not detected) genes downstream of DREB2A (Sakuma et al. 2006) were quantified in the array data. Group (a) indicates genes activated by both DREB1A and DREB2A, and group (b) indicates genes activated only by DREB2A. The log2 ratio of expression levels in d975 was compared with that in the wild type at ZT12. Double (**) and single (*) asterisks indicate q-values <0.005 and <0.05, respectively. (B) The expression levels of 23 of 34 (no available probes for three genes, and eight gene signals were not detected) genes downstream of AREB1 (Fujita et al. 2005) were quantified at ZT12. Note that the AREB1 probe was not available in the ATH1 GeneChip. (C) Genes up-regulated in DREB1A-overexpressing plants described by Maruyama et al. (2004) were chosen for the analysis.
at the same level, and DREB1C was 2–3 times higher in d975.
PRR9, PRR7 and PRR5 may thus repress DREB1 expression under non-stress conditions, but also via the gating mechanism.

Discussion

PRR9, PRR7 and PRR5 are physiologically active during the daytime

CCA1::LUC bioluminescence in d975 was arrhythmic under both LL and DD conditions (Fig. 1), but understanding the circadian clock mechanism requires knowing when PRR9, PRR7 and PRR5 are physiologically active. Release assays can be used to define arrest time points of clock mutants, and can provide some evidence regarding when clock genes are active (McWatters et al. 2000, Ding et al. 2007), but the arrhythmicity of d975 prevents the use of this assay because it requires measurable circadian oscillation. However, GeneChip data can provide a rough estimate of when PRR9, PRR7 and PRR5 are active. For example, in the case of d975, expression of ‘day genes’ does not decrease toward ZT12, thus the expression levels of ‘day genes’ tend to remain high in late daytime (Fig. 2G). The expression of ‘night genes’ tends to remain at a relatively constant level in d975, and does not increase toward ZT12 (Fig. 2D). These observations indicate that PRR9, PRR7 and PRR5 are active during the daytime so that they can directly or indirectly repress ‘day genes’ and activate ‘night genes’ (Fig. 9). One may wonder if PRR9, PRR7 and PRR5 are involved in biological processes other

Fig. 7 Cold stress-inducible genes and metabolites. (A) Relative expression levels of DREB1A, DREB1B and DREB1C. Bold and dashed lines indicate expression levels in the wild type and d975, respectively. (B) Relative galactinol and raffinose contents. (C) Relative glutamic acid and L-proline contents. For B and C, metabolites were quantified by GC-TOF/MS analysis. The data are presented as the relative normalized response where the value at any time period is divided by the mean of the normalized response of the wild type. Error bars represent the SE (n = 3–14 for L-proline, n = 15 for the other metabolites). The asterisk indicates P < 0.02 by t-test.
than the circadian clock, but about 90% of the 445 up-regulated genes and 70% of the 197 down-regulated genes in d975 are expressed in a clear diurnal cyclic pattern in LDHH_ST of the 'Diurnal' database (correlation value between the experiment and the model, >0.8, http://diurnal.cgrb.oregon-state.edu, Supplementary Tables S4, S7). There are few genes which are expressed in a non-cyclic pattern in this database. Therefore, the idea that PRR9, PRR7 and PRR5 act in biological processes other than the circadian clock could not be excluded. Collectively, however, our observations may suggest that PRR9, PRR7 and PRR5 act mainly as clock-associated genes, at least from the transcriptome point of view.

To test this hypothesis further, we evaluated the circadian disorder of d975 by re-examining the expression profiles of 85 CCGs, which were identified as cycling genes, under all six diurnal and five circadian conditions (Michael et al. 2008, Supplementary Table S6). Expression of 63 of the 85 genes (74.1%) was significantly altered between ZT8 and ZT12 in the wild type, but none of the genes was affected in d975 (q < 0.1). This observation supports the contention that circadian gene expression is generally disordered in d975 at least from ZT8 to ZT12. There was also a relationship between expression levels in d975 and the phase of expression maxima (Supplementary Fig. S4). That is, genes with expression peaks from ZT0 to ZT8 tended to be up-regulated, and those with expression peaks from ZT9 to ZT20 tended to be down-regulated. The expression profiles of these 85 genes in d975 thus support the idea that PRR9, PRR7 and PRR5 tend to repress ‘day genes’ and to activate ‘night genes’ during the daytime.
**Supplementary Fig. S5**

Supplementary time-dependent induction of genes and cold stress-responsive genes more effectively than lar mechanisms underlying cyclic clock mechanism (Fowler et al. 2005, Bieniawska et al. 2008). This suggests that genes are regulated by the circadian clock mechanism (Fowler et al. 2005). However, the molecular mechanisms underlying cyclic DREB1 expression, and the gating effect on cold-responsible genes are not known (Bieniawska et al. 2008). In this study, we propose that the circadian clock-associated genes PRR9, PRR7, and PRR5 repress DREB1 under non-stress conditions (Fig. 7A), and also in the gating mechanism (Fig. 8). In addition, genes and metabolites downstream of DREB1A tend to be up-regulated in d975 (Fig. 7, Supplementary Fig. S5). Recently, Achard et al. demonstrated that overexpression of DREB1B (CBF1) also causes a dwarf phenotype by up-regulating one of the DELLA genes, RGL3 (Liu et al. 1998, Achard et al. 2008). We checked RGL3 transcription under LD conditions (Supplementary Fig. S6A), and under cold stress (Supplementary Fig. S6B). RGL3 expression levels were almost the same in the wild type and d975 under LD conditions. However, cold stress induced RGL3 gene expression in a time-dependent manner in the wild type, with an induction maximum at ZT4 (Supplementary Fig. S6B), which was not the case in d975. Induction of RGL3 in d975 at all time points was almost equal to the maximum level in the wild type when stressed at ZT4, suggesting that PRR genes could repress cold stress-dependent induction of RGL3 at least by affecting the gating mechanism, probably by mediating changes in the DREB1 signaling pathway. DREB1 expression levels may explain why RGL3 expression was higher in d975 than in the wild type under cold stress conditions, but not under non-stress LD conditions. That is, DREB1 expression was higher in d975 under cold stress than under non-stress conditions (Fig. 8). There might be a certain DREB1 level that acts as a threshold for RGL3 induction. d975 plants grew long hypocotyl petioles rather than expressing a dwarf phenotype under non-stress conditions (Nakamichi et al. 2005, Ito et al. 2007a). Expression of PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and PIF5, which encode transcription factors involved in diurnal hypocotyl elongation (Fujimori et al. 2004, Nozue et al., 2007), were significantly up-regulated in d975 (Fig. 9). This might explain the long hypocotyl phenotype of d975. Interestingly, PIF4 protein is degraded by binding to DELLA proteins such as RGL3 (de Lucas et al. 2008). Therefore, if RGL3 proteins are up-regulated in d975, higher concentrations of PIF proteins may mask RGL3 function in cell elongation.

Two hypotheses can be put forward to explain how PRR genes repress DREB1. One is that the circadian disorder in d975 results in increased DREB1 expression, and the other is that PRR9, PRR7 and PRR5 directly repress DREB1. At present, there is no clear evidence to support one of the two possibilities over the other. However, we suspect that there is some sort of time-dependent activator or repressor which acts directly or indirectly on DREB1 expression. In addition, the effect of the three mutations in d975 on DREB1 expression appears to be dependent on cold stress, because the effect of the PRR mutations on DREB1 expression was less than the effect of cold (Fig. 8), and these mutations have no apparent additive effect on the DREB1 maximal induction level. It is also noted that ICE1 expression levels are almost...
same in d975 and the wild type during ZT8–ZT12 (data not shown).

GO classification of the 197 down-regulated genes in d975 (q < 0.02, and 2-fold down-regulated in d975 compared with the wild type, Supplementary Table S7) suggests that this gene set also contains many stress-responsive genes or genes that are downstream of stress-responsive regulators, but clustering analysis with 10 published DNA microarray data sets for stress-treated plants did not indicate clustering in any particular stress category (data not shown).

There are two reports indicating that clock mutants (cca1/lhy, Kant et al. 2008; gi-3, Cao et al. 2005) are sensitive to certain stresses, further suggesting that the clock mechanism has an effect on the stress response. Microarray analyses designed particularly for clock mutants may be of considerable benefit for understanding how clock components modulate abiotic stress responses.

Biological significance of diurnal DREB1 expression

DREB1 plays a significant role in Arabidopsis responses to cold stress (Liu et al. 1998, Gilmour et al. 2000). The circadian clock regulates DREB1 expression in two distinct ways: by circadian regulation under non-stress conditions; and by time-dependent gating for induced responses to cold stress (Harmer et al. 2000, Fowler et al. 2005, Bieniawska et al. 2008). DREB1 expression is higher in d975 under non-stress conditions (Fig. 7A), and d975 is more tolerant than the wild type to cold stress (Fig. 5). Therefore, a higher DREB1 expression level may provide a certain level of pre-formed stress tolerance to Arabidopsis and other temperate zone plants. Arabidopsis plants also have a higher capacity for tolerance to cold stress if they are acclimated with non-damaging low temperatures. ‘Cold acclimation’ treatment induces DREB1 expression and the production of l-proline and raffinose (Gilmour et al. 2000). D975 mutants mimic cold acclimation within a subset of cold stress response genes and metabolite profile. In addition, ablation of the gating effect on DREB1 expression in d975 may also contribute to stress tolerance.

Raffinose and l-proline act as osmoprotectants (Nanjo et al. 1999, Taji et al. 2002). We could not be certain, however, that the increased cold, saline and drought stress tolerance of d975 was limited to higher raffinose and l-proline concentrations, since other events under DREB1A control, including the altered expression of some COR and LTI proteins, could also increase stress tolerance in d975 (Fig. 6C). In either case, the slight activation of DREB1I in d975 prior to the incipient stress, and the abolished gating effect may be two of the reasons for increased stress tolerance of d975.

The wild-type expression maximum of DREB1 is around noon (Harmer et al. 2000, Fig. 7A, Supplementary Fig. S2), so DREB1A-induced genes are probably induced in the early afternoon, which would provide Arabidopsis with cold stress tolerance during the night-time hours when frosts are more likely to occur. This prediction mechanism for anticipating changes in daily temperature may be a common circadian clock function in some plants. Rice homologs of DREB1 exhibit a diurnal expression pattern (http://diurnal.cgrb. oregonstate.edu). In addition, tolerance to chilling stress in cotton seedlings is also under circadian control, but there have not yet been any genetic studies detailing why this should be (Rikin et al. 1993).

Conclusions

We detected the expression pattern of CCGs in an arrhythmic mutant plant, d975, using an Affimetrix ATH1 GeneChip® which indicates that PRR9, PRR7 and PRR5 repress ‘day genes’ and activate ‘night genes’ (Fig. 9). In addition, an informatics-based approach suggested that a large number of up-regulated genes in d975 overlap the set of cold stress response genes, including DREB1. The cold-tolerant mutant d975 accumulates more raffinose and l-proline, the synthesis pathways of which are downstream of DREB1. These data confirm that the circadian clock mechanism has a predictive role for adapting to diurnal cold stress, and suggest that PRR9, PRR7 and PRR5 repress some cold stress-responsive genes mediating DREB1 expression.

Materials and Methods

Plant materials and growth conditions

The Arabidopsis thaliana lines used in this study were derivatives of accession Columbia (Col-0). The triple mutant prr9-10 prr7-11 prr5-11 was described previously (Nakamichi et al. 2005). Plants were grown on MS medium (pH 5.7) containing 2% sucrose under LL or LD conditions at 22°C for 18 d. For cold and drought stress experiments, plants were germinated and grown on a mixture of peat soil (KUREHA, Tokyo, Japan) and vermiculite (6:4) with 1/5,000 hypoxen under 16 h light/8 h dark conditions at 90–100 µmol m⁻² s⁻¹ of white light. The gating experiment was described previously (Fowler et al. 2005).

Bioluminescence assays

CCA1::LUC construction was described previously (Nakamichi et al. 2004, Nakamichi et al. 2005), prr9-10 prr5-11/CCA1::LUC and prr7-11 prr5-11/CCA1::LUC were crossed to obtain prr9-10 prr7-11 prr5-11/CCA1::LUC, and then four independent prr9-10 prr7-11 prr5-11/CCA1::LUC plants were obtained from the F₂ generation. Growth conditions were as described above and bioluminescence assays were done as previously published (Kondo et al. 1993, Nakamichi et al. 2004, Nakamichi et al. 2005).
Preparation of RNA
RNA was purified with an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Each sample contained RNA extracted from 8–10 plants.

Quantitative real-time PCR
Real-time PCR was carried out as described previously (Nakamichi et al. 2007). The primer sets used in this study were, for DREB1A, S'-CATGAAGTCGCAGCGATG-3' and S'-CTCGTTTCAGTTTTACAAAACTAATG-3'; for DREB1B, S'-GAATCTACCTGAAAAAGAAAAAGAG-3' and S'-GAG GCTCGTAATCGGAG-3'; for DREB1C, S'-GGXGGTGA TGACGTGTC-3' and S'-CTGCACTCAAAACACTTTGCA TTTG-3'; for AtGolS3, S'-GTCCCTCAACTCTAAAC TTCC-3' and S'-CAATCTTGACTAGGGC-3'; for AD52, S'-CTTATTTGCCCCCTTCGGC-3' and S'-GGCAAGTCAT AGATCCAAAG-3'; for LTI30/XERO2, S'-CCGGAGTGCC AAAAGAAGG-3' and S'-GCTGCTCCATAACTTTTCCG-3'; and for CCA1, S'-GGTGGACGAGAAGAAC-3' and S'-GGAGAAAATTTCTGCAGGTAC-3'. The primer sets used for APX3, CI and RGL3 were described previously (Hazan et al. 2005, Nakamichi et al. 2007, Achard et al. 2008).

DNA microarray analysis
Microarray analyses were performed using an ATH1 GeneChip® (Affymetrix, Santa Clara, CA, USA). A 5 µg aliquot of total RNA was reverse-transcribed and labeled using a One-Cycle Target Labeling and Control Reagents kit (Affymetrix) according to the supplier’s protocol. A 15 µg aliquot of fragmented cRNA was hybridized to the chip at 45°C for 16 h. Affymetrix eukaryotic hybridization controls were added to the sample prior to hybridization as per the manufacturer’s instructions. Hybridization was controlled with a GeneChip™ Eukaryotic Hybridization Control Kit (Affymetrix). Washing and staining were performed in a Fluidics Station 450 (Affymetrix) using the protocol EukGE-WS2v4 and scanned in an Affymetrix GeneChip scanner. Relative expression values and detection (genes are tagged as 'present, P', 'marginal, M' or 'absent, A') were generated for each transcript using the MAS5.0 algorithm of the GeneChip® Operating Software (GCOS, Affymetrix). Generated data files (CEL) were the input used to calculate robust multichip average (RMA) to normalize and estimate signal intensities for each transcript using the MAS5.0 algorithm of the GeneChip® (Affymetrix, Santa Clara, CA, USA). A 5 µg aliquot of fragmented cRNA was hybridized to the chip at 45°C for 16 h. Affymetrix eukaryotic hybridization controls were added to the sample prior to hybridization as per the manufacturer’s instructions. Hybridization was controlled with a GeneChip™ Eukaryotic Hybridization Control Kit (Affymetrix). Washing and staining were performed in a Fluidics Station 450 (Affymetrix) using the protocol EukGE-WS2v4 and scanned in an Affymetrix GeneChip scanner. Relative expression values and detection (genes are tagged as 'present, P', 'marginal, M' or 'absent, A') were generated for each transcript using the MAS5.0 algorithm of the GeneChip® Operating Software (GCOS, Affymetrix). Generated data files (CEL) were the input used to calculate robust multichip average (RMA) to normalize and estimate signal intensities for each transcript using the MAS5.0 algorithm of the GeneChip® (Affymetrix, Santa Clara, CA, USA).

Statistical analysis for GeneChip® data
To identify which genes increased or decreased between two time points, CEL files were evaluated using RMA. The genes whose FDR-controlled q-values were <0.01 were defined as having increased or decreased (QVALUE; http://genomine.org/qvalue/). A total of 445 genes whose q-values were <0.02 between d975 and the wild type, and whose signal intensities in d975 were >2 times higher than in the wild type, were identified as up-regulated genes in d975 plants. To create a ‘heat’ map, expression levels in the wild type and d975 were transformed to a log2 scale and compared. The compared signal intensities were then analyzed using R (http://www.r-project.org/).

GO classification
GO Slim terms for genes (probes) were obtained from the TIGR database site (http://www.arabidopsis.org/tools/bulk/go/index.jsp).

Cluster analysis for DNA microarray data sets
The 445 up-regulated genes in d975 at ZT12 were extracted (q < 0.02, Supplementary Table S4) and compared against the AtGenExpress consortium database. Abiotic stress GeneChip experiments of ME00325 (cold stress), ME00326 (genotoxic stress), ME00327 (osmotic stress), ME00328 (salt stress), ME00329 (UV-B stress), ME00330 (wound stress), ME00333 (ABA treatment), ME00338 (drought stress), ME00339 (heat stress, except for data from cultured cells) and ME00340 (oxidative stress) (Kilian et al. 2007, Goda et al. 2008, http://www.arabidopsis.org/info/expression/ATGenExpress.jsp) were chosen. All array data were normalized and interpreted using RMA. The data set of log2 ratios for each of the 445 up-regulated genes in d975 was compared with each data set from the log2 ratio of stress-treated samples to control samples. Hierarchical clustering was performed using Cluster 3.0 software (de Hoon et al. 2004).

Metabolite profiling with GC-TOF/MS
Extraction, derivatization and GC-TOF/MS analyses were performed as described previously (Kusano et al. 2007) with minor modifications. Briefly, in the extraction step, each sample was extracted with 40 mg FW ml⁻¹ extraction medium containing 10 stable isotope reference compounds. Each isotopic compound was adjusted to a final concentration of 15 ng µl⁻¹ per 1 µl injection (Gullberg et al. 2004, Jonsson et al., 2004). After centrifugation, extracts were concentrated by evaporation of 250 µl of the initial volume. Samples were derivatized by methoxymation with 20 µl of methoxyamine hydrochloride (20 mg ml⁻¹) in pyridine for 21 h at room temperature. Samples were then trimethylsilylated for 1 h with 20 µl of N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) at 37°C with shaking. A 20 µl aliquot of n-heptane was added after silylation, and the derivatized samples were analyzed in an HP 6890 GC and a LECO Pegasus III time-of-flight mass spectrometer (LECO, St. Joseph, MI, USA) as described previously (Kusano et al. 2007).
Cold stress tests
Seeds were imbibed directly on soil supplemented with 1/5,000 Hyponex (Hyponex-Japan, Osaka, Japan). Plants were grown on soil at 22°C under 16 h light/8 h dark for 18 d. Plants were chilled to –5°C in the dark for 1 d, then returned to 22°C. Mean survival rates were assessed after 3 d using data from three independent experiments.

Salt stress tests
Wild-type and d975 plants were germinated on MS medium and grown under 16 h light/8 h dark photoperiods for 6 d. Vigorous seedlings were transferred to MS medium or to MS medium containing 0.25 M NaCl, grown for 6 d, and assessed for bleaching. Mean bleaching rates were determined from three independent examinations.

Accession numbers
Microarray data have been deposited in the Nottingham Arabidopsis Stock Center’s microarray database (NASCARRAY) under accession number NASCARRAYS-421. Sequence data for the genes described in this paper can be found in the Arabidopsis Genome Initiative and GenBank/DDBJ/EMBL data libraries under the following accession numbers: PRR9 (At2g46790), PRR7 (At5g02810), PRRS (At5g24470), CCA1 (At2g46830), GI (At1g22770), DREB1A/CBF3 (At4g25480), DREB1B/CBF1 (At4g25490), DREB1C/CBF2 (At4g25470), AtGolS3 (At1G09350), DELTA9 DESATURASE 2 (ADS2) (At2G31360), LOW TEMPERATURE-INDUCED 30/DEHYDRIN XERO 2 (LT130/XERO2) (AT3G50970) and RGL3 (At5g17490).

Supplementary data
Supplementary data are available at PCP online.

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References


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