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Stress memory in plants: a negative regulation of stomatal response and transient induction of *rd22* gene to light in abscisic acid-entrained *Arabidopsis* plants

Chang-Hyo Goh[1,*], Hong Gil Nam[1,2] and Yu Shin Park[3]

[1]*Division of Molecular and Life Science, Pohang University of Science and Technology, San 31, Hyoja-Dong, Nam-Gu, Pohang, Kyungbuk 790-784, Korea,*

[2]*Department of Life Science, Pohang University of Science and Technology, San 31, Hyoja-Dong, Nam-Gu, Pohang, Kyungbuk 790-784, Korea, and*

[3]*Biotechnology Research Center, Pohang University of Science and Technology, San 31, Hyoja-Dong, Nam-Gu, Pohang, Kyungbuk 790-784, Korea*

Received 11 May 2003; revised 17 July 2003; accepted 25 July 2003.
*For correspondence (fax +82 54 279 2199; e-mail gohunse@postech.ac.kr).

Summary

All organisms, including plants, perceive environmental stress, and they use this information to modify their behavior or development. Here, we demonstrate that *Arabidopsis* plants have memory functions related to repeated exposure to stressful concentrations of the phytohormone abscisic acid (ABA), which acts as a chemical signal. Repeated exposure of plants to ABA (40 μM for 2 h) impaired light-induced stomatal opening or inhibited the response to a light stimulus after ABA-entrainment under both dark/light cycle and continuous light. Moreover, there were transient expressions of the *rd22* gene during the same periods under both the growing conditions. Such acquired information in ABA-entrained plants produced a long-term sensitization. When the time of light application was changed, a transient induction of the *rd22* gene in plants after ABA-entrainment indicated that these were light-associated responses. These transient effects were also observed in *kin1*, *rab18*, and *rd29B*. The transient expression of *AtNCED3*, causing the accumulation of endogenous ABA, indicated a possible regulation by ABA-dependent pathways in ABA-entrained plants. An ABA immunoassay supported this hypothesis: ABA-entrained plants showed a transient increase in endogenous ABA level from 220 to 250 pmol g^{-1} fresh mass at 1–2 h of the training period, whereas ABA-deficient (*aba2*) mutants did not. Taking into account these results, we propose that plants have the ability to memorize stressful environmental experiences, and discuss the molecular events in ABA-entrained plants.

Keywords: abscisic acid (ABA), adaptation, gene expression, memory, stomatal behavior, stress.

Introduction

Plants experience a wide array of environmental stimuli, some of which are harmful. Unlike animals, plants are unable to move away from a damaging environment. Any unfavorable condition or substance that affects or blocks a plant's metabolism, growth, or development is considered to be a stress (Lichtenthaler, 1998; Vierling and Kimpel, 1992). Plants use various regulatory mechanisms to adapt to these environmental perturbations as a way to protect themselves against stress. The early events by which plants adapt to two or more environmental hazards include sensing of stress and subsequent signal transduction events that activate various physiological and metabolic responses. Such responses are mediated via numerous physiological alterations, including regulation of gene expression, alteration of plasma membrane composition, changes in phytohormone levels, and changes in leaf-water status (Bohnert and Sheveleva, 1998; Lichtenthaler, 1998; Matters and Scandalios, 1986; Vierling and Kimpel, 1992). They have been clearly correlated with the adaptation strategies of plants that were largely tolerant to these stresses (Mckersie and Leshem, 1994).

The protective effects of adapting to stressful stimuli in plants have been suggested to be mediated by both pre-existing and induced defenses (Alvarez *et al.*, 1998; Frye *et al.*, 2001), although the precise cellular and molecular mechanisms involved have not yet been defined.

Interestingly, recent physiological and biochemical studies have described 'memory' effects for stress in plants. When plants are exposed to repeated and aggressive biotic and abiotic agents, they can recognize the initial stressor and respond with an appropriate alarm signal. Examples of this include development of immunological memory of induced nicotine accumulation in tobacco (Baldwin and Schmelz, 1996); cold memory in *Arabidopsis* acclimation in response to pre-treatment with cold stress (Knight *et al*., 1996, 1998); and memory effects in conifers in response to ozone damage (Langebartels *et al*., 1998). Such memory effects have also been observed during phototropism to blue-light pulses and in gravitropism in maize coleoptiles (Nick and Schafer, 1988, 1991; Nick *et al*., 1990) and root (Leopold and Wettlaufer, 1989). However, the details about how these types of memory functions operate in plants remain unknown.

As it has been suggested that the adaptation process of animal cells by memory functions represents 'classical conditioning', which is known as associative learning (the recognition of predictive events within an individual's environment; Abrams and Kandel, 1988; Goelet *et al*., 1986), we selected two different stimuli, ABA and light, which are antagonistic to each other in controlling stomatal guard cell movement, so that by recognizing one stimulus, the plant is expected to predict the presence of the other – just like associative learning. ABA plays a central role in the long-distance signaling process in plants subjected to stresses, such as drought and salinity (Davies and Zhang, 1991; Gowing *et al*., 1993). The physiological and metabolic changes in plants in response to the environmental factors are typically initiated by changes in gene expression (Matters and Scandalios, 1986). Upregulation of certain plant mRNAs by ABA may depend on $[Ca^{2+}]_{cyt}$ (Napier *et al*., 1989), although the water-stress signal might be transduced directly by ABA (Espelund *et al*., 1992). On the other hand, ABA has been shown to positively regulate approximately 70 different genes during the vegetative growth of plants, largely in a time- and dose-dependent manner (Chandler and Robertson, 1994). Therefore, the study of gene expression on memory functions of plants in response to two distinct and well-known physiological stimuli, ABA and light, is valuable for understanding the molecular and cellular mechanisms by which plants adapt to chronic environmental signals.

In this study, we found novel evidence suggesting that specific induction of the *rd22* gene, which is well mediated by ABA (Yamaguchi-Shinozaki *et al*., 1992), may have physiological and molecular significance for processes underlying memory functions of plants in response to ABA and light pulses. Our results demonstrate that this type of memory functions act at the molecular level, in response to ABA stimulus in ABA-entrained plants.

Results

ABA response in intact plants: stomata closing and gene expression

In order to investigate memory functions in *Arabidopsis thaliana*, we first established the optimal experimental conditions to measure the ABA response in intact plants. When plants were exposed to ABA for 2 h of light, the stomata closed in a dose-dependent manner (Figure 1a), exhibiting a 43% decrease in aperture size in response to 40 μM ABA. Our results predicted that the ABA inhibition of stomata to light was evoked by the increase in endogenous ABA concentrations in ABA-treated plants, as suggested in many experimental reports in the literature. Figure 1(b) shows the values of ABA content determined by immunoassay. The endogenous ABA content in plants was increased to 497.7 pmol g^{-1} fresh mass in response to exogenous ABA (40 μM) treatment, consistent with the response in *Arabidopsis* plants, as reported earlier (Franks and Farquhar, 2001; Goh *et al*., 1996; Xiong *et al*., 1999). The elevated ABA levels then dropped dramatically to 40.2 pmol g^{-1} fresh mass 2 h after washing, but progressively declined over time in non-washed plants. The control plants showed ABA levels of only about 8–17 pmol g^{-1} fresh mass. Figure 1(c) shows a time course of this decrease in stomatal aperture in response to ABA in light. The stomata were able to largely recover from this stress when ABA was removed by washing; stomatal apertures recovered 85% of their initial values 12 h after washing, even after the 40-μM treatment. At higher doses of ABA (80 μM), although the stomata recovered well, recovery took much longer, which proved unsuitable for performing the present investigation. The reversibility of the stomatal response to light was consistent with the previous reports studying the effects of ABA (100 μM) on the epidermal strips and intact leaves of *Commelina communis* (Willmer *et al*., 1978). These results imply that the inhibition of stomatal response by ABA treatment resulted from the increase of endogenous ABA concentration. The concentration of ABA used in this study was, therefore, 40 μM, which induced a reversible response.

On the basis of stomatal responses, as shown in Figure 1, we investigated the expression of the ABA-inducible gene, *rd22*, that may be associated with the elucidation of memory responses under the conditions when the plants were treated with ABA under separate growth conditions (Figure 2a,b for an 8-h dark/16-h light cycle and for continuous light, respectively). The *rd22* gene (Yamaguchi-Shinozaki *et al*., 1992) responded well to ABA stimulus in both the growth conditions. The level of *rd22* expression was elevated 2 h after the plants were exposed to ABA. In plants exposed to ABA during the dark/light cycle, washing returned the expression to near-control levels, although the

transcript showed a sustained accumulation. Under conditions of continuous light, the *rd22* gene returned to near-control levels after washing, as in the dark/light cycle. At 2 h of the treatment, we estimated the expressed level by Image analysis to be 65% in comparison with that of a dark/light cycle. Meanwhile, we investigated the expression patterns of *cab*, a light-regulated gene, for assessing the experimental growth conditions; the expression of the gene was not significantly changed by ABA either under a dark/light cycle or under continuous light. Taken together, these results suggest that ABA stimulation (40 μM for 2 h) plays in the physiological limits during the treatment: it induces molecular changes that may contribute to the physiological memory response.

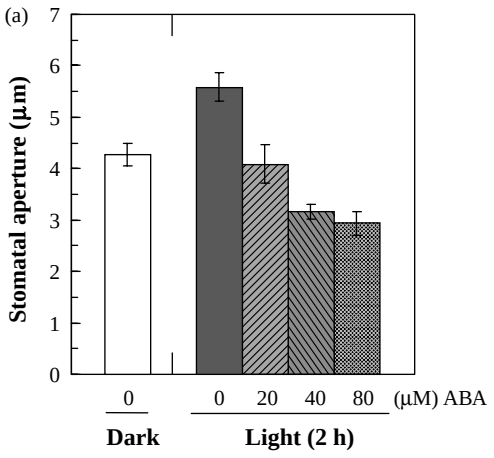

Figure 1. Effects of ABA on stomatal aperture of intact plants.
(a) Concentration-dependent ABA response of stomata. Plants were exposed to various concentrations of ABA for 2 h directly after exposure to light, as described. The data represent the mean ± SEM of five replicates ($160 \leq$ stomata ≤ 276, $P < 0.001$ by Dunnett's method) on separate days. The final concentration of DMSO was 0.02–0.08%.
(b) Accumulation of ABA concentration in ABA-treated plants. Plants were washed (□) or not washed (○) after the treatment with ABA (40 μM for 2 h). Control (▲) treatment was carried out as indicated in (a). The data represent the mean ± SEM of three replicates ($P = 0.002$ by Dunnett's method). ABA denotes free abscisic acid.
(c) ABA inhibition and recovery of stomatal opening in light. ABA (40 μM for 2 h) was applied as indicated in (a) and washed. The data represent the mean ± SEM of five replicates ($163 \leq$ stomata ≤ 350, $P = 0.018$ by Dunnett's method) on separate days. Open arrow in *X*-axis indicates a simultaneous application of ABA directly after exposure to light.

Transient induction of the rd22 *gene and decreased responsiveness of stomata to light in the absence of ABA after ABA-entrainment under an 8-h dark/16-h light cycle*

A population of plants was exposed to two different stimuli, ABA (40 μM for 2 h) as the unconditioned stimulus and light as the conditioned stimulus, which normally exhibit opposing effects on stomatal movement (Assmann and Shimazaki, 1999; Goh *et al.*, 1996). In order to first identify the adaptation capacity of memory functions in plants, we analyzed the expression patterns of the *rd22* gene in plants after ABA-entrainment for 1–4 days, respectively (Figure 3). Figure 3(a) shows a typical expression of the *rd22* in plants after ABA-entrainment for 3 days. From the image analysis of the expressed levels in time, we observed a dramatic and transient increase of the *rd22* gene (Figure 3b). There were two distinctive phenomena, a rapid response to light and a progressive increase in the *rd22* expression levels dependent upon the exposed frequency, during ABA-entrainment, although expression levels were observed to be substantially reduced in plants after ABA-entrainment for 4 days. This lower gene expression appears to be the result of a physiological disruption, such as plant senescence induced by ABA (Woo *et al.*, 2002). In our preliminary experiments, the plants did not grow well when they were placed under long-term exposure to ABA. Therefore, we limited the number of times of ABA treatments to three times during the entrainment.

We analyzed the responses of stomata to light in plants that were repeatedly exposed to ABA for 3 days (Figure 4b). Stomata exhibited impaired opening and closing responses to light, indicating a downregulation of this signal. During the training period of 1 h, in the absence of exogenous ABA, the stomata of ABA-entrained plants showed closing response to light. The stomata of the entrained plants

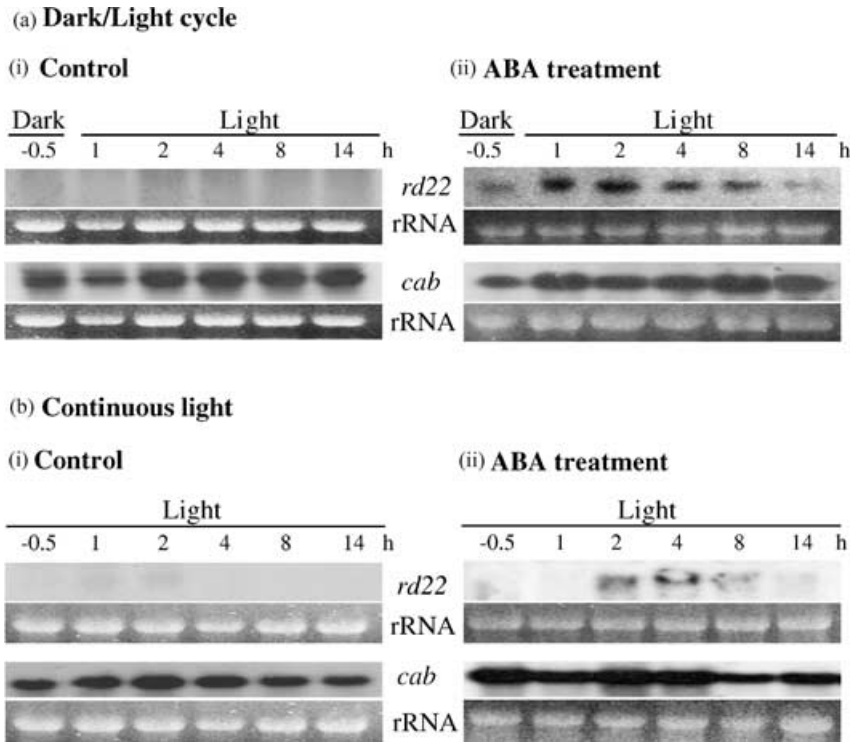

Figure 2. Expression patterns of *rd22* transcripts by ABA treatment in the plants under either a dark/light cycle (a) or continuous light (b).
Plants were treated with ABA (40 μM for 2 h), as indicated in Figure 1(c). Total RNA was extracted from whole plants at times specified by the number above each lane. Loading was monitored by hybridizing rRNA with a labeled rDNA. Each lane contains 30 μg RNA for *rd22* and 15 μg RNA for *cab* genes. −0.5 h indicates the time prior to the beginning of ABA treatment. Other experimental conditions used were as in Figure 1(c). Representative data are shown.

(229 $\leq$ stomata $\leq$ 318 in $n = 6$) exhibited unusual behavior in response to light exposure, with the aperture size decreasing by 36.8%. In contrast, stomata of non-entrained plants (247 $\leq$ stomata $\leq$ 308 in $n = 6$) showed a 12.9% increase in aperture size in response to light exposure. This explained that repeated treatment of the plants with ABA in presence of light led to a sudden decrease in stomatal opening in response to light in the absence of exogenous ABA. The stomatal aperture in ABA-entrained plants thereafter showed a gradual increase in size up to 151.9% at 14 h, suggesting a high recovery potential of stomatal guard cells to light. In the dark (−0.5 h), the smaller stomatal aperture in the ABA-entrained plants might result from a higher concentration of endogenous ABA in cells (see in Figure 10), implying incomplete degradation of ABA even by washing, as shown in Figure 1(b). Figure 4(c) shows typical stomatal behavior in ABA-entrained plants, with a reaction potential to light after the entrainment. In response to light, stomata, which are entrained by ABA, significantly close 1 h after light exposure (panel (ii)) in comparison with the control (panel (i)). This abnormal behavior of stomata suggests that components of the light-signaling pathway have been downregulated in response to repeated ABA exposure.

The molecular effect of ABA-entrainment on *rd22* expression was evaluated. As shown in Figure 3(a), the *rd22* gene was expressed in plants after ABA-entrainment, with a transient peak obtained 1 h after light stimulation (Figure 4e), but this was not detected in control plants (Figure 4d). The temporally expressed level decreased in time, but persisted for at least 8 h after exposure to lights. We further investigated the level of the *rd22* gene in ABA-insensitive mutants, *abi1* plants, after ABA-entrainment. They did not show any expression of the *rd22* gene over time (Figure 4g). ABA-entrained plants (wild type, Landsberg *erecta* (L*er*)) showed a remarkable expression of the *rd22* gene at 1 h after exposure to light as in Figure 4(e) (Figure 9b). The *abi1* mutant did not induce this gene in response to exogenous ABA as well (data not shown). This implies that the repeated stimulus of ABA played a role for the transient expression of the *rd22* gene in ABA-entrained plants. The level of *cab* expression after the entrainment was similar in both ABA-entrained and control plants. In both ABA-entrained and control plants, the *cab* gene was significantly induced 1 h after the light exposure, suggesting that the gene is regulated by both lights-on and lights-off signals in *Arabidopsis* seedlings (Millar and Kay, 1996). The molecular event of *rd22* expression in ABA-entrained *Arabidopsis* plants in the absence of an ABA stimulus showed periodic and temporal induction pattern, indicating an accomplishment by a transient negative regulation to light.

Transient decrease of stomatal response to light and transient induction of the rd22 *gene in ABA-entrained plants under continuous light*

Abscisic acid-entrained plants under conditions of continuous light were also analyzed, which would be expected to eliminate the light-regulated circadian effects (Johnson,

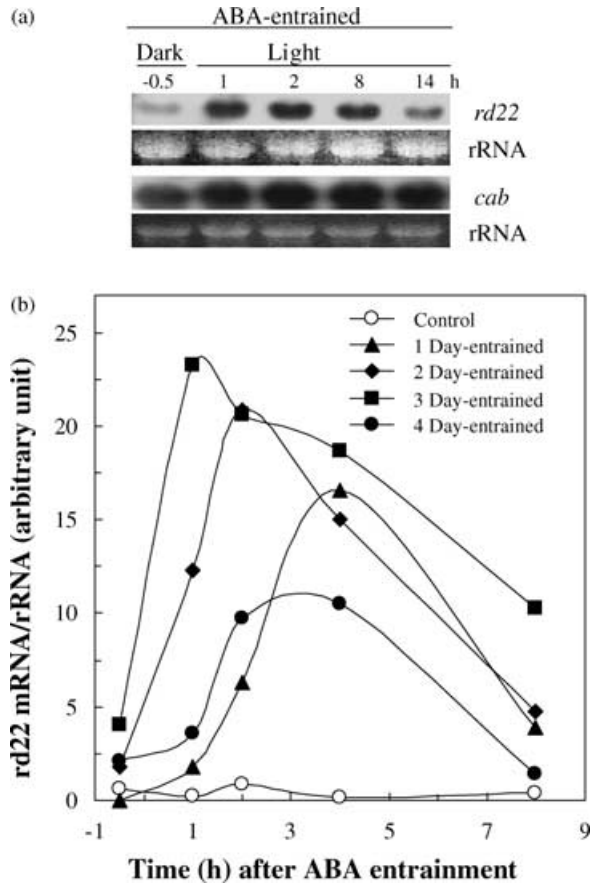

Figure 3. Different time course of *rd22* expression by the exposed frequency during ABA-entrainment.
(a) RNA blot analysis of *rd22* expression after ABA-entrainment for three times under a dark/light cycle. Each lane contains 42 μg RNA.
(b) Quantification of *rd22* expression. Microcal Origin 5.0 program was used to quantify the intensity of each band by surface area from the Scion image program (http://www.scioncorp.com). Each point is the relative intensity of *rd22* calculated using the intensity of *rd22* divided by that of rDNA. Symbol description in box indicates the number of times for ABA treatments to plants during the entrainment. Control (○) is a typical expression pattern of *rd22* after the entrainment with no ABA treatment for four times. Other experimental conditions used were as in Figure 2.

2001; Millar and Kay, 1996). When the plants were repeatedly exposed to ABA for 3 days (Figure 5a), stomata clearly exhibited impaired opening and closing responses to light in the absence of exogenous ABA treatment (Figure 5b). In control plants, stomata ($235 \leq$ stomata ≤ 318 in $n = 5$) showed a constant opening pattern in response to light, ranging from 5.0 to 5.4 μm in the aperture. However, the stomatal aperture of ABA-entrained plants ($189 \leq$ stomata ≤ 229 in $n = 5$) was decreased by 20.2% 2 h after ABA-entrainment. The recovery potential was increased with time, showing 131.2% recovery after 14 h. This suggests that an acquisition mechanism of the ABA signal exists in ABA-entrained plants.

In relation to this stomatal physiology, we also found a transient induction of the *rd22* gene in ABA-entrained plants (Figure 5d). In the absence of exogenous ABA, the *rd22* gene was expressed 1 h after the training period. The gene expression was long-lived, but eventually declined over a 14-h period. While this gene was being induced, the expression of *cab* gene was not significantly altered in response to ABA-entrainment. Under control conditions, the *rd22* gene level was undetectable (Figure 5c). As in Figure 4, this gene was transiently expressed in the same manner during the training period in plants after ABA-entrainment, even in the absence of ABA stimulation. Together, these results strongly suggest an operation of mechanism(s) by which the information is stored and retrieved during the training period in ABA-entrained plants, implying the habituated response.

The transient expression of the rd22 *gene is strongly associated with light exposure after ABA-entrainment*

Our results have suggested that this habitual response to ABA stimulus may be a result of acquisition of information about the repeated stimulus. However, we should consider a possibility that ABA stimulus itself was coordinated as circadian system during the training period. In order to identify whether light can trigger the habitual response, we characterized gene expression levels of *rd22* during controlled dark periods in plants after ABA-entrainment in an 8-h dark/ 16-h light cycle (Figure 6). Plants were first placed in a further dark incubation of 5.5 h prior to the beginning of light application, after the entrainment of ABA in an 8-h dark/16-h light cycle for 3 days (Figure 6a,b). The level of *rd22* expression increased within 1 h in response to light stimulation, and remained elevated for at least 7 h afterwards (Figure 6b). Under control conditions, *rd22* expression was not detectable (Figure 6a). *cab* expression was significantly reduced in the dark between −5.5 and −0.5 h in control plants. However, it did not show the expression to the same extent in ABA-entrained plants, rather an intense expression in the same dark period (Figure 6b), reflecting the circadian effects. As at the transcriptional level entrainment of *cab* expression is operated by a light-regulated circadian rhythm (Kreps and Kay, 1997; Park *et al.*, 1999), it might result in turning on of this gene by different signaling pathways in ABA-entrained plants when the period of circadian light pulse was changed. Further investigations about these are needed. In another set of control experiments, plants were subjected to either continuous darkness (Figure 6c) or continuous light (Figure 6d) after ABA-entrainment in an 8-h dark/16-h light cycle for 3 days. Under these conditions, the *rd22* gene was not expressed. These results suggest that the habitual response of *rd22* expression was associated with light, the conditioned stimulus when it delivered.

In another set of experiments to investigate the light-associated expression of *rd22* in plants after ABA-entrainment,

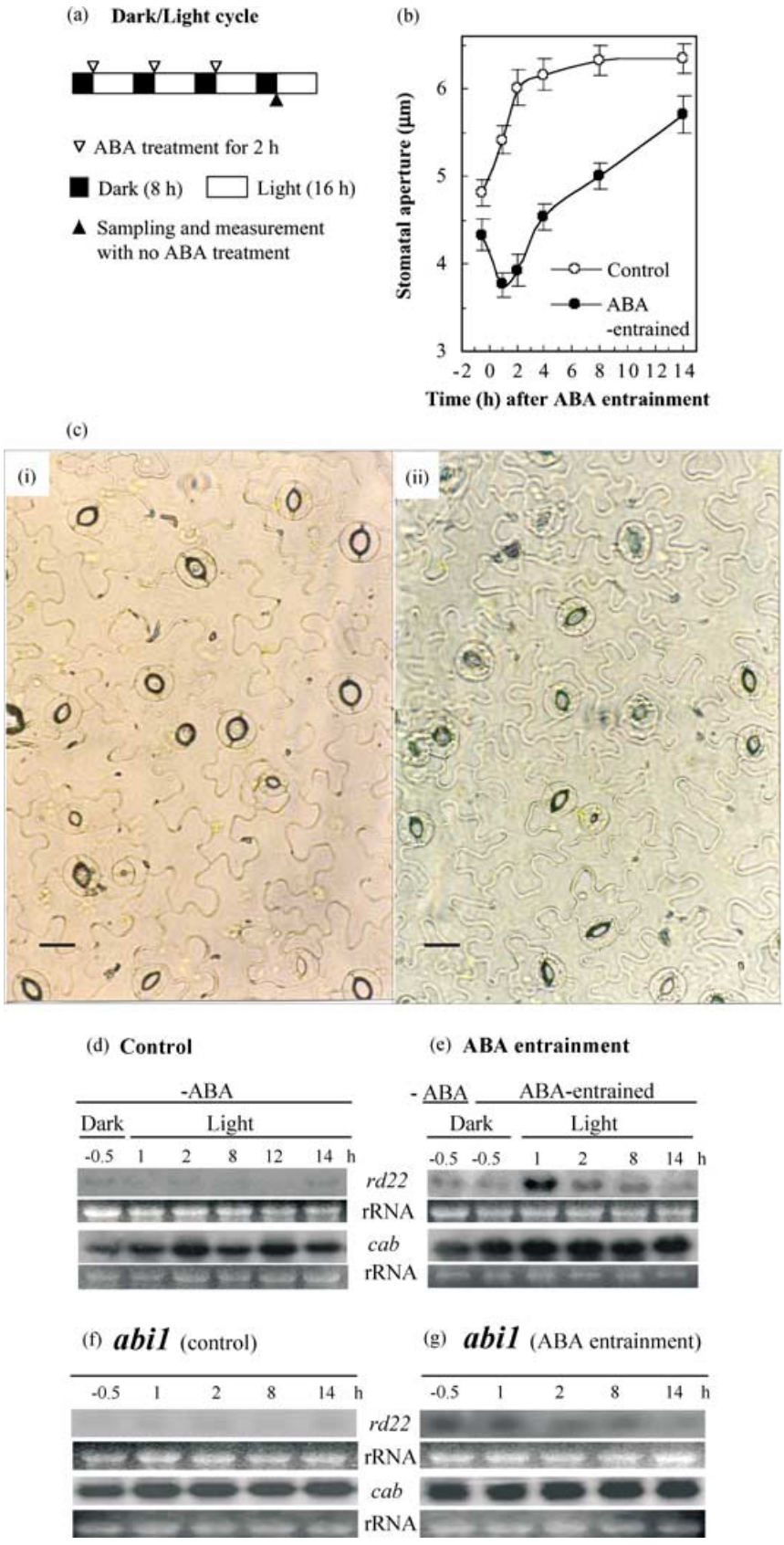

Figure 4. Stomatal behavior and expression pattern of the *rd22* gene after ABA-entrainment in plants under a dark/light cycle.
(a) Schematic summary of the photoperiodic time and ABA exposure in these experiments. The plants were repeatedly exposed to ABA for 2 h with a light application for 3 days.
(b) Light response of stomata in ABA-entrained plants. The data represent the mean ± SEM of six replicates ($247 \leq$ stomata ≤ 316 for control and $229 \leq$ stomata ≤ 308 for ABA-entrained plants, $P = 0.002$ by Dunnett's method) on separate days.
(c) Bright-field images showing the typical stomata from (b). Panels (i) and (ii) show stomata at 1 h after exposure to light in control and at 1 h in ABA-entrained plants, respectively. Bars: 13.5 μm.
(d) Control and (e) transient expression of the *rd22* gene in plants (Col) after ABA-entrainment.
(f) Control and (g) transient expression of the *rd22* gene in *abi1* mutant plants after ABA-entrainment. The samples corresponded to each time points shown in (b). Other experimental conditions were generated as in Figure 2(a). –ABA indicates non-entrained plants. −0.5 h indicates dark and the time prior to the beginning of training.

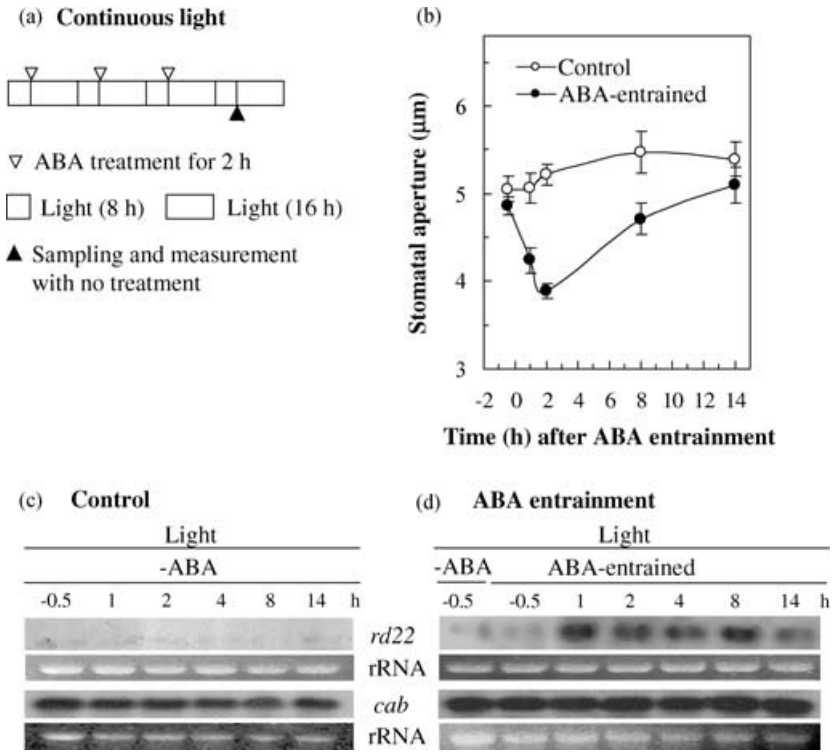

Figure 5. Stomatal behavior and expression patterns of the *rd22* gene after ABA-entrainment in plants under continuous light.
(a) Schematic summary of the photoperiodic time and ABA exposure in these experiments. The plants were repeatedly exposed to ABA for 2 h over 3 days.
(b) Response patterns of stomata to light in ABA-entrained plants. The data represent the mean ± SEM of five replicates ($235 \leq$ stomata ≤ 318 for control and $189 \leq$ stomata ≤ 229 for ABA-entrained plants, $P = 0.012$ by Dunnett's method) on separate days.
(c) Control and (d) transient expression of the *rd22* gene after ABA-entrainment. The samples corresponded to each time points shown in (a). Other experimental conditions were generated as in Figure 2. –ABA indicates the non-entrained plants. −0.5 h indicates the time prior to the beginning of training.

plants were exposed to a light period following an even shorter dark period (in this case 6 h) after ABA-entrainment in an 8-h dark/16-h light cycle for 3 days (Figure 6e,f). We found that *rd22* was significantly expressed in response to light exposure, and that the levels remained constant for 4 h and then progressively declined (Figure 6f). The non-entrained (control) plants did not express the *rd22* gene (Figure 6e). Taken together, these results further support that the habitual response of the *rd22* gene in ABA-entrained plants was switched on by exposure to light, the conditioned stimulus. Therefore, these light-evoked effects can be distinguished from circadian effects or after-effects of repeated ABA stimulation in ABA-entrained plants.

We further investigated how long the sensitivity of transient response can be maintained in ABA-entrained plants (Figure 7). Plants were grown either under an 8-h dark/16-h light cycle (Figure 7a) or under conditions of continuous light (Figure 7b). The levels of *rd22* expression remained elevated even after 3 days of ABA-entrainment and started decreasing in a time-dependent manner under both the growth conditions. Figure 7(c) shows a rhythmic accumulation of *rd22* mRNA in plants after ABA-entrainment under an 8-h dark/16-h light cycle. The quantified level of *rd22* expression was highly detected at 1 day after the entrainment and significantly decreased thereafter. This indicates that such an acquired character in plants after ABA-entrainment produced a long-term sensitization, which could persist for days.

Expression of other ABA-responsive genes in ABA-entrained plants

The molecular effect of ABA-entrainment on *rd22* expression was evaluated in the involvement of other ABA-responsive gene expressions for investigating the regulation (Figure 8). The induction of *rd22* is mediated by ABA and requires protein biosynthesis for ABA-dependent gene expression: rd22BP1 and ATMYB2 binding to the MYC and MYB sites of the *rd22* promoter (Abe *et al.*, 1997). In contrast, *kin1* (Ishitani *et al.*, 1998; Knight *et al.*, 1996) and *rab18* (Knight *et al.*, 1998; Lång and Palva, 1992) contain both dehydration-responsive elements (DRE) and ABA-responsive element (ABRE) as *cis*-acting elements in their promoter sequences, and their expressions are controlled by both ABA-dependent and -independent pathways. The expression of *kin1* and *rab18* in plants after ABA-entrainment in a dark/light cycle was not substantially different from that of *rd22* (Figure 8b), although *kin1* was accumulated 2 h after exposure to light. None of these genes were expressed in control plants under the given sets of ABA-entrained conditions (Figure 8a). This shows a possibility that the expression is controlled by the same signaling factors, such as ABA. We further examined the level of *rd29B* (Yamaguchi-Shinozaki and Shinozaki, 1994) in ABA-entrained plants (Figure 8d, above). In contrast to *kin1* and *rab18* at the transcriptional level, *rd29B* has only ABRE in the promoter and is controlled by ABA. The expression of *rd29B* was also significantly detected in a similar

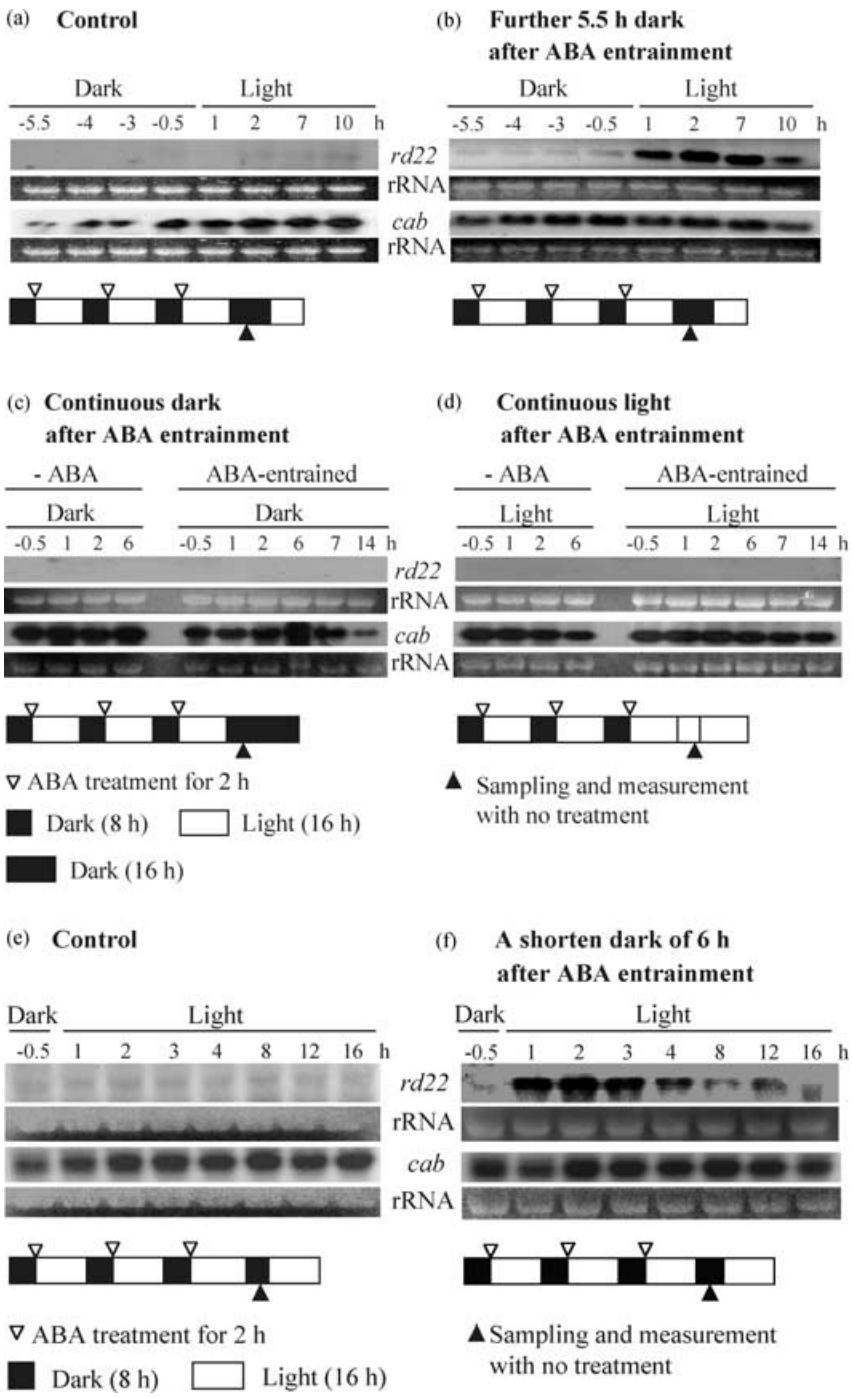

Figure 6. Light association of the transient induction of the *rd22* gene when the time of light application was changed after ABA-entrainment in an 8-h dark/16-h light cycle.
(a) Control (non-entrained plants), (b) light application in further prolonged darkness of 5.5 h after ABA-entrainment, (c) continuous darkness after ABA-entrainment, and (d) continuous light after ABA-entrainment. The time period between −5.5 and −0.5 h (dark) indicates the time prior to the beginning of light application. –ABA indicates the non-entrained plants. (e) Control (non-entrained plants) and (f) light application in a short period of dark of 6 h after ABA-entrainment. Other conditions were as in Figure 2. The open and closed bars beneath the blots give a schematic summary of the photoperiodic time and ABA exposure in these experiments.

manner with *rd22*, *kin1*, and *rab18* in ABA-entrained plants, but there was no such observation in control plants (Figure 8c, above). These results strongly suggest that the transient expression of ABA-responsive genes in plants after ABA-entrainment was governed by the same internal factor (possibly ABA) in the signal transduction pathways, although ABRE did not require protein synthesis.

To test this hypothesis, we examined the expression of *AtNCED3* (Iuchi *et al.*, 2001), which played a key role in the regulation of ABA synthesis (Figure 8c,d, below). The expression pattern of *AtNCED3* in plants after ABA-entrainment was not substantially different from that of *kin1*, *rab18*, and *rd22*, although the expression of the endogenous *AtNCED3* gene was also observed with a considerable intensity in the dark (−0.5 h) (Figure 8d, below). The expression was long-lived with a high level, but there was a progressive decrease in time. In control plants, *AtNCED3* induction was not observed (Figure 8c, below). This

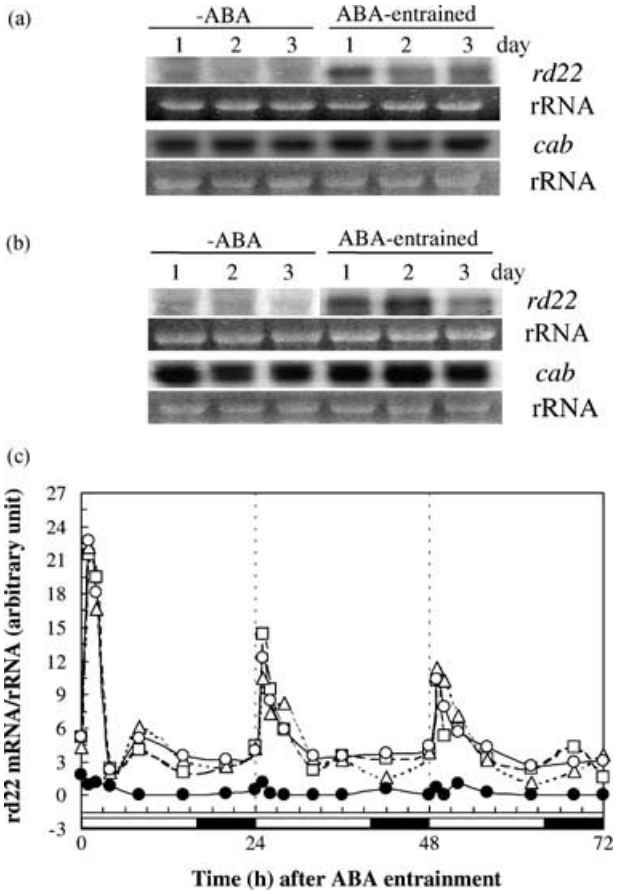

Figure 7. Maintenance of the acquired character in ABA-entrained plants under (a) a dark/light cycle and (b) continuous light.
The *rd22* and *cab* mRNA levels were measured 1 h after ABA treatment on consecutive days after ABA-entrainment. The typical data are shown. –ABA indicates the non-entrained plants. (c) Rhythmic accumulation of *rd22* mRNA in ABA-entrained plants after the entrainment under a dark/light cycle. Quantification of *rd22* expression was performed as in Figure 3. Data are representative of three experiments (mean ± SEM, $P = 0.033$ by Dunnett's method) for ABA-entrained plants and one for control. Open box on time axis above, light period; solid box, dark period.

suggests that the expression of *AtNCED3* produced the endogenous ABA concentrations at the beginning of training in ABA-entrained plants. This possible explanation is based on the fact that the expression of *AtNCED3* upregulated the endogenous ABA level (Iuchi *et al.*, 2001). Together, these indicate that the transient induction of *rd22, kin1, rab18*, and *rd29B* in ABA-entrained plants was thereby regulated by ABA-dependent pathway coupled with ABA biosynthesis.

The expression of rd22 *gene and ABA content in ABA-deficient (*aba2*) and ABA-insensitive mutants (*abi1*)*

In relation to ABA biosynthesis, the molecular effect of ABA-entrainment on *rd22* expression was evaluated in *aba2* mutant in 8-h dark/16-h light cycle. The *rd22* gene was strongly expressed with a transient peak at 1–2 h after the light stimulation (Figure 9b), which, however, was not detected in *aba2* mutant plants (Figure 9d). The levels of *cab* expression were similar in both ABA-entrained and control plants during ABA-entrainment. Only observation in ABA content was in the wild type (L*er*) of ABA-entrained plants, showing an increase of 219 pmol g^{-1} fresh mass at 2 h of the training period (Figure 9e). On the other hand, ABA-entrained *abi1* mutant plants also did not show any significant increase in endogenous ABA concentrations while transiently increasing the content at 1 h after the entrainment in the wild type (Figure 10). These results indicate a strong correlation of endogenous ABA content for the transient expression of the *rd22* gene in ABA-entrained *Arabidopsis* plants.

Discussion

We have analyzed the physiological and molecular properties of 'memory' functions in plants that were repeatedly exposed to ABA for a short and definite period. In relation to stress physiology in cultivated plants, it has been known that the low-stress events can be partially compensated for by acclimation, adaptation, and repair mechanisms, whereas strong-stress events cause considerable damage that may eventually lead to cell and plant death (Mckersie and Leshem, 1994). However, very little information is available about this type of adaptation to the environmental stresses.

In general, stomatal guard cells represent specific target cells for ABA with well-characterized physiological responses, showing the rapid effects of ABA that is suppressed by light (Assmann and Shimazaki, 1999; Goh *et al.*, 1996; Leung and Giraudat, 1998; Schroeder *et al.*, 2001; Zeiger *et al.*, 1987). In relation to this, Trejo *et al.* (1995) showed that stomata in epidermal strips of *C. communis* that had been substantially closed by applying a 30-min pulse of 10 μM ABA returned to initial apertures within 3 h. This led to the conclusion that short-term effects of elevated ABA concentrations were reversible (Franks and Farquhar, 2001). We therefore examined the effect of range of concentrations of ABA on the viability and physical appearance of the intact plants (Figure 1). Application of exogenous ABA to intact plants caused stomatal closure in a concentration-dependent manner (Figure 1a). At a 40-μM ABA treatment delivered over 2 h, ABA uptake was considerably accomplished and showed a large decline by washing, suggesting no permanent effect on stomatal function (Figure 1b). However, non-washed plants had a large amount of ABA with an incomplete degradation of ABA even after 12 h. Figure 1(c) showed that stomata inhibited by ABA were largely reversible in response to light after washing. This ABA inhibition of stomata indicates a down-regulation of stomata in response to light. Northern blot

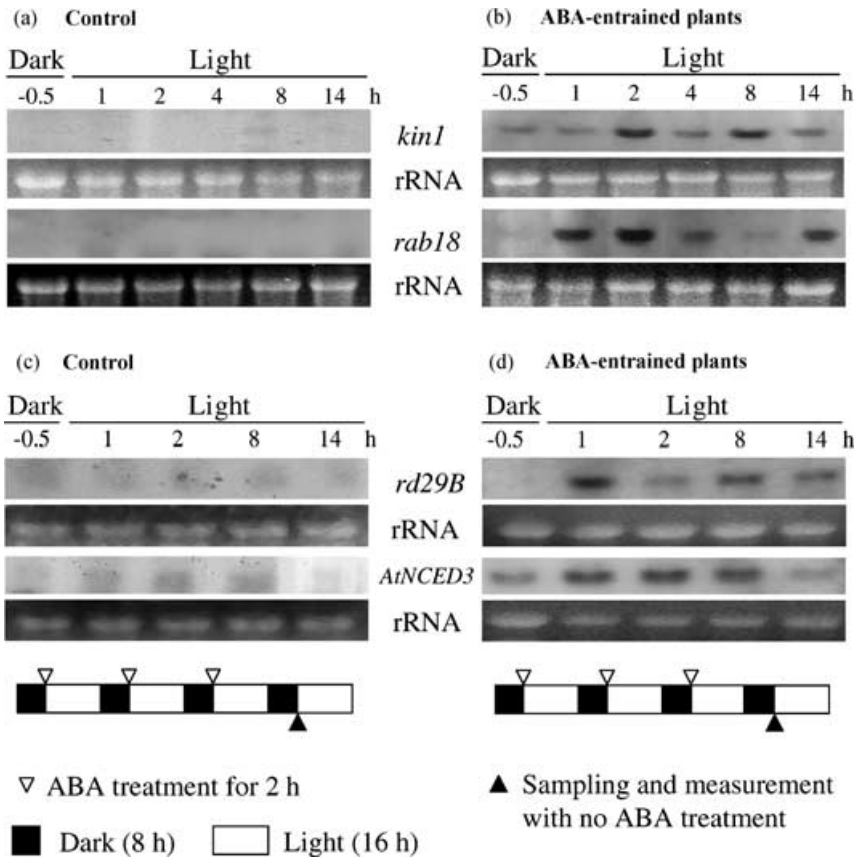

Figure 8. RNA blot analysis of induction of other ABA-responsive genes in plants after ABA-entrainment under a dark/light cycle.
(a) and (c) Controls (non-entrained plants); (b) and (d) ABA-entrained plants. The number above each lane indicates the time (h) after the entrainment. The *kin1, rab18, rd29B, AtNCED3,* and rDNA indicate the RNAs hybridizing with their respective probes. Other conditions were as in Figure 2. –ABA indicates non-entrained plants. −0.5 h indicates dark and the time prior to the beginning of training. The open and closed bars beneath the blots give a schematic summary of the photoperiodic time and ABA exposure in these experiments. The plants were repeatedly exposed to ABA for 2 h with a light application for 3 days.

analysis further supported the reversibility of the effects of exogenous ABA (Figure 2). These results suggest that ABA stimulus of 40 μM for 2 h can operate in the physiological limits.

In acquisition of information for adaptation in plants after ABA-entrainment, how many trials do they require in the training process? We have analyzed the expression patterns of the *rd22* gene when the plants were exposed to ABA under an 8-h dark/16-h light cycle for 1–4 days (Figure 3). Interestingly, they transiently increased the expression level of *rd22* even after one exposure to ABA. After a series of such trials, we found that the expression levels were dependent on the exposed frequency in the training process; they increased the expression levels in magnitude and sensitivity in response to the repeated stimulation. When plants were repeatedly exposed to ABA for 3 days under an 8-h dark/16-h light cycle, a light-induced stomatal opening was transiently impaired in the training period (Figure 4b), suggesting that ABA negatively regulated light signaling. Under conditions of continuous light, a similar phenomenon was observed in the stomatal behavior of ABA-entrained plants (Figure 5b). On the other hand, the smaller stomatal aperture observed in the dark (−0.5 h) of ABA-entrained plants might result from a higher level of endogenous ABA in cells (Figure 10), resulting from incomplete degradation of ABA even after washing (Figure 1b). Another possibility would be that it is a result of the difference in humidity in growth conditions – 50–53% for light and 68–72% for dark growth conditions (see Experimental procedures). A high humidity in growth conditions rather enhanced inward-rectifying K^+ channel current magnitudes even in guard cells of the *Arabidopsis* mutant *abh1*, which showed ABA-hypersensitive regulation of stomatal closing (Hugouvieux *et al.*, 2002). Therefore, the light responsiveness of stomata was negatively regulated in ABA-entrained plants.

Regarding the molecular properties of ABA-entrained plants, we analyzed mRNA levels of the *rd22* gene (Abe *et al.*, 1997; Yamaguchi-Shinozaki *et al.*, 1992). Here, we found a transient expression of the *rd22* gene in plants after ABA-entrainment under all experimental conditions (Figures 4e and 5d). The *rd22* gene was expressed maximally at 1 h after light stimulation in the absence of exogenous ABA, and progressively decreased over time. On the other hand, the ABA-insensitive mutant, *abi1*, did not induce the expression of *rd22* in plants after ABA-entrainment (Figure 4g). This could be because of inhibition of ABA signal transduction, as the mutation had a defect in protein phosphatase type 2C (Leung *et al.*, 1997). Stomata of *abi1*, therefore, did not close in response to exogenous

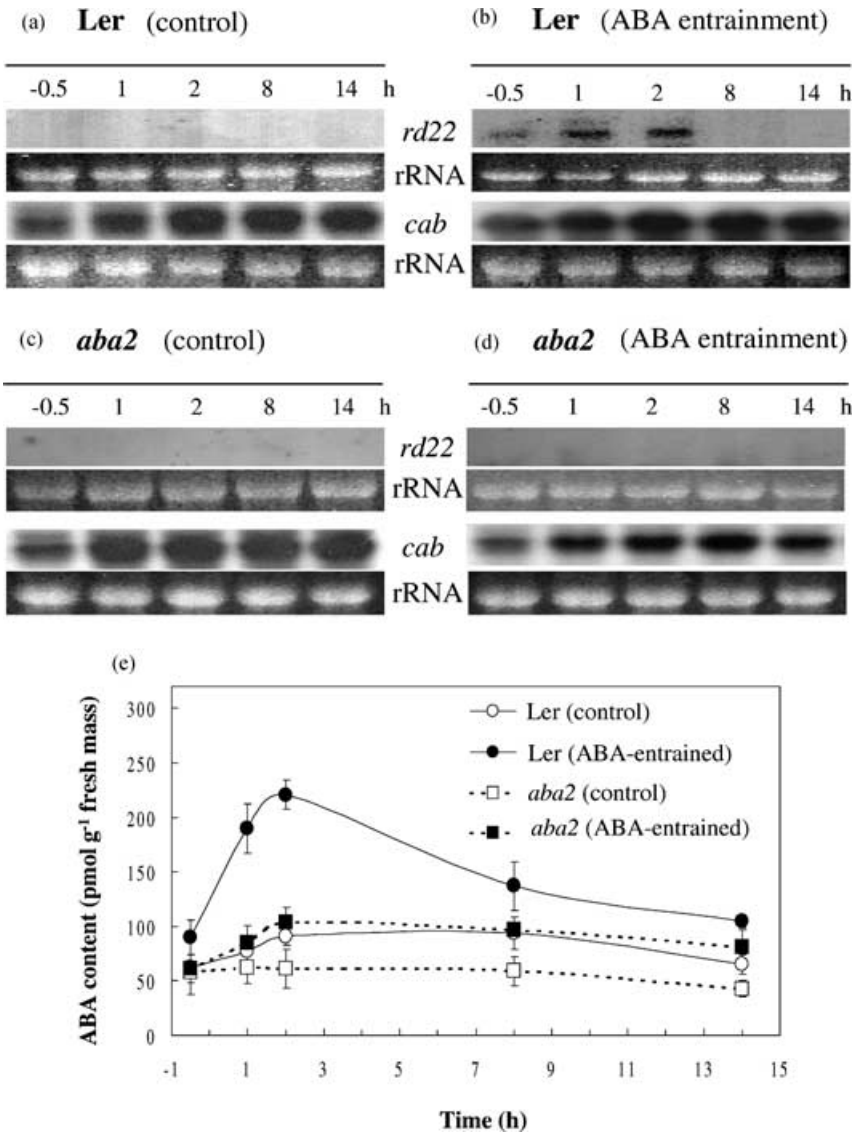

Figure 9. The expression patterns of the *rd22* gene and ABA content in *aba2* plants after ABA-entrainment under an 8-h dark/16-h light cycle. (a) Control (non-entrained plants) and (b) expression of the *rd22* gene after ABA-entrainment in the wild type (L*er*). (c) Control (non-entrained plants) and (d) expression of the *rd22* gene after ABA-entrainment in *aba2* mutant plants. Other conditions were as in Figure 2. −0.5 h indicates dark and the time prior to the beginning of training. (e) Endogenous ABA concentration in ABA-entrained *aba2* plants. The samples corresponded to each time points shown in (a–d). The data represent the mean ± SEM of three replicates ($P = 0.003$ by Dunnett's method). Other conditions were as in Figures 1(b) and 4. ABA denotes free abscisic acid.

ABA (Allen *et al.*, 1999). This supports that the repeated stimulus of ABA played a role in the transient expression of the *rd22* gene in the wild type. Therefore, the transient modification of gene expression in ABA-entrained plants may result from the habitual response to ABA stimulus by the process in which the information is stored and retrieved. We propose that *Arabidopsis* plants exhibit memory functions by which they memorize the effects of ABA stimulation.

In these training processes, ABA treatment was followed immediately by exposure of plants to a light pulse (Figure 4a). To determine whether the transient negative effects in ABA-entrained plants are significantly enhanced by the light stimulus after a series of such trials, we changed the dark periods to light after ABA-entrainment. Northern blots showed that light induced increases in the levels of *rd22* expression in ABA-entrained plants, which is consistent with the hypothesis that light is associated with memory responses, and that the achievement of memory function is strongly associated with the conditioned stimulus (Figure 6b,f). The effect of light on *rd22* expression varied considerably in both magnitude and duration. However, no expression of the *rd22* gene was observed in non-treated plants (Figure 6a,e) and ABA-entrained plants subjected to either continuous darkness or continuous light after the entrainment in an 8-h dark/16-h light cycle (Figure 6c,d). The transient responses were therefore considered to be a function of long-term memory (Figure 7). Such a light dependency in transient gene expression should not be correlated with an after-effect of ABA, as reported previously by Fischer *et al.* (1970). Furthermore, as *rd22* expression did not exhibit diurnal changes (Figure 6), it also distinguished the transient effects of the ABA-entrained plants from the circadian effects, as reviewed

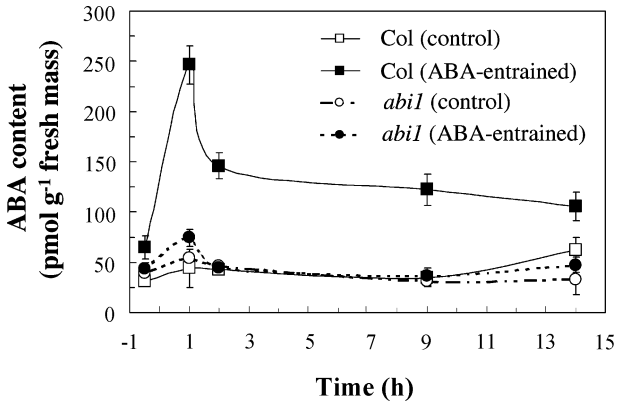

Figure 10. Endogenous ABA content in *abi1* plants after ABA-entrainment under an 8-h dark/16-h light cycle.
The data represent the mean ± SEM of three replicates ($P < 0.001$ by Dunnett's method). −0.5 h indicates dark and the time prior to the beginning of training. Other conditions were as in Figures 1(b) and 4. Control means non-entrained plants. ABA denotes free abscisic acid.

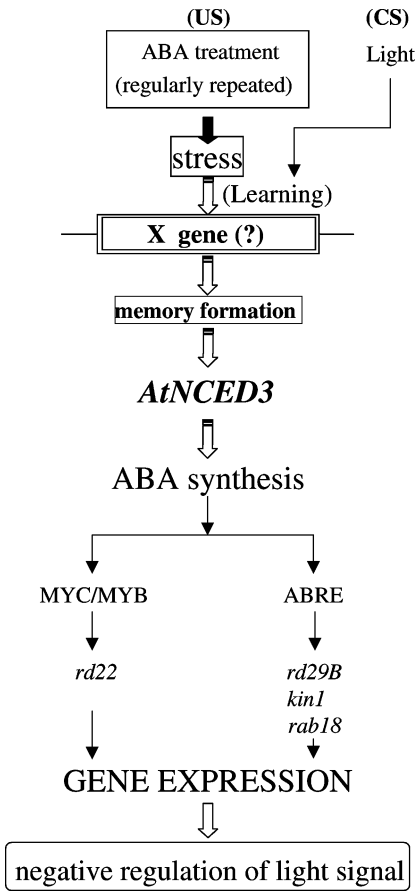

Figure 11. A working model for memory response in *Arabidopsis* plants by a repeated ABA stimulation.
The signal is stored as the biological information within plants when they are repeatedly exposed to ABA stimulation. This process might represent a kind of cellular learning, which builds up the memory in plants to the environmental stresses. X gene describes a not-yet-identified factor being capable for acquisition of information about the environment – in this case light. The activation of *AtNCED3* gene triggers off ABA biosynthesis and thereby induces the expression of the *rd22*, *rd29B*, *kin1*, and *rab18* genes by different signal transduction pathways, respectively. US, the unconditioned stimulus; CS, the conditioned stimulus.

previously by Kreps and Kay (1997). From these results, it is speculated that the transient responses in plants after ABA-entrainment are strongly connected to the molecular regulation and are dependent on light switch, implying that the habituated response of *rd22* and stomata was associated with light, a conditioned stimulus when it was given.

To obtain direct insight into the molecular mechanisms of light-induced stomatal opening and temporal activation of the *rd22* gene in ABA-entrained plants, we analyzed the association of other ABA-responsive genes in these experimental conditions. The expression of *rd22* is activated by the MYC and MYB regulation of the *rd22* promoter and is dependent on ABA concentrations (Abe *et al.*, 1997). In contrast, *kin1* and *rab18* contain both DRE and ABRE as *cis*-acting elements in their promoter sequences so that they are regulated by another transcriptional level of *rd22* gene. Figure 8(b) showed that *kin1* and *rab18* expressions were in patterns quite similar to those of *rd22*. This suggests a possible regulation of the gene expression in the ABA-dependent signal transduction pathways. On the other hand, the expression of *rab18* is also regulated by osmotic stress-induced cytosolic Ca^{2+} increases (Knight *et al.*, 1998), implying an ABA-independent osmotic stress response.

We further examined in plants after ABA-entrainment for the expression of endogenous *AtNCED3* gene, which caused the accumulation of endogenous ABA (Iuchi *et al.*, 2001). The expression pattern of *AtNCED3* was not substantially different from those of *kin1*, *rab18*, and *rd22* in ABA-entrained plants. We considered that this altered expression of *AtNCED3* affected levels of endogenous ABA in plants after ABA-entrainment, as overexpression of *AtNCED3* cDNA upregulated the endogenous ABA level in transgenics (Iuchi *et al.*, 2001). According to this, we investigated it in the phenotype of the ABA-biosynthetic mutant *aba2*, which indicated its function; for example, failure of stomata closure leads to the wilty phenotype (Léon-Kloosterziel *et al.*, 1996; Merlot *et al.*, 2002). However, the mutant plants did not show any transient expression of *rd22* or change in ABA content (Figure 9). It confirms that endogenous ABA plays a prominent role in controlling the transient induction of *rd22* as well as *kin1* and *rab18* in plants after ABA-entrainment. Moreover, the expression of *rd29B* (Yamaguchi-Shinozaki and Shinozaki, 1994), which had only ABRE in the promoter and is controlled by ABA, was also significantly detected in a similar manner with *rd22* in ABA-entrained plants (Figure 8d). These results strongly suggest that the transient gene expressions in ABA-entrained plants could be accomplished by

ABA-dependent regulation, although they were expressed via different ABA signaling pathways.

Next, we examined whether altered expression of *AtNCED3* upregulated the endogenous ABA levels in plants after ABA-entrainment. ABA levels were rapidly and transiently increased up to approximately 220–250 pmol g^{-1} fresh mass in response to light under an 8-h dark/16-h light cycle in both wild types of ABA-entrained plants (Col and L*er*) (Figures 9e and 10). This timing of ABA accumulation is considered as a consequence of the expression of *AtNCED3* gene, as indicated in a previous work by Iuchi *et al*. (2001). Such an elevation of ABA level was not significantly observed in ABA-entrained *aba2* (ABA-deficient mutant) (Figure 9b). There was also no elevation in the case of the ABA-sensitive mutant *abi1* (Figure 10). This resulted in a defect in ABA signal transduction, as mentioned above. This indicates the regulation of ABA biosynthesis by the activation of *AtNCED3* gene in ABA-entrained plants. A recent report showed that ABA content of leaves by exogenous ABA treatment had no influence on the accumulation of *NCED* mRNA (Thompson *et al*., 2000), implying that *AtNCED3* mRNA was not regulated by ABA. Therefore, the biochemical pathways, emphasizing on memory functions, probably accomplish the transient increase in ABA in ABA-entrained plants, although the mechanisms are still in investigation. This is clearly distinguishable from that of ABA-treated plants (Figure 1b), in which there is an increase in ABA content by ABA uptake (Goh *et al*., 1996). In field-grown sunflowers subjected to drought, ABA concentrations in xylem sap were dramatically increased with the onset of the daylight, and this was associated with the changes in gene expression (Cellier *et al*., 2000; Ouvrard *et al*., 1996), indicating that the metabolic events of ABA production within the plants could be regulated by light. Our results therefore suggest that ABA metabolism in ABA-entrained plants could be modulated by light. On the other hand, Zhang *et al*. (2001) observed endogenous ABA levels of 65 pmol g^{-1} fresh mass in control and 193 pmol g^{-1} fresh mass in water-stressed *Vicia* plants. Under these conditions, guard cells had about 1.3 fg ABA per guard-cell pair in control plants and 4.2 fg ABA per guard-cell pair in water-stressed plants, with a 64% decrease in leaf conductance. Comparing these results to our own, it seems possible that the transient elevation of endogenous ABA levels is a component of transient down-regulation of light responsiveness in stomata and transient induction of *rd22* in ABA-entrained plants. These findings indicate that the transient increase in endogenous ABA levels in ABA-entrained plants is responsible for the physiological and molecular changes in whole plants, thereby inducing memory responses.

Basing on our results, we propose a working model for memory response in plants after ABA-entrainment, as summarized in Figure 11. We have provided novel evidence for light-associative forms of memory functions in *Arabidopsis* plants in response to ABA stimulation. When externally applied ABA repeatedly acts as a short-term and low-stress stimulus for plant growth, it is likely that one or more of the genes that function in this capacity regulate the memory response in a context of learning for ABA biosynthesis, although the biochemical and molecular regulation mechanism(s) underlying memory formation in ABA-entrained plants are obscure. Further investigations are needed in this field. This may be done by replenishing cellular elements that mediate transient elevation of endogenous ABA levels and by integrating information at the level of transcription, such as MYC/MYB for *rd22* and ABRE for *rd29B*, *kin1*, and *rab18*. It is clear that careful scrutiny of learning and memory-related gene expression will be needed to understand the complex processes by which plants adapt to environmental hazards. In conclusion, we propose that *Arabidopsis* plants have the potential to encode memory of chronic stressful experiences. In principle, this memory capacity in plants will facilitate their growth and development by maintaining proper temporal responses to harmful stressors, such as hormonal, physical, and chemical stress signals that enable the plants to better withstand future stressors.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana (ecotype Columbia) was grown on agar plates with 0.5× B5 medium (pH 5.7) in Petri dishes for this study unless otherwise stated. Seeds of the *aba2* (ABA-deficient) and *abi1* (ABA-insensitive) mutants (L*er* background) were provided by Youn-il Park, Department of Biology, Chungnam National University, Korea. Seedlings were 2 weeks old at the beginning of treatment with ABA. The cultures were maintained at 20 ± 2°C in growth rooms under either 8-h dark/16-h light cycle (110 μmol m^{-2} sec^{-1}) or under continuous light illumination (90 μmol m^{-2} sec^{-1}). The humidity was maintained at 50–53% for light and 68–72% for dark growth conditions.

ABA ((+/−)-*cis, trans*-abscisic acid) was obtained from Sigma (St Louis, MO, USA).

ABA pre-treatment

The pre-treatment of ABA was performed by spraying uniformly on leaves of plants a solution of 40 μM in 0.1 mM MES-NaOH (pH 6.1) directly after beginning of a light stimulation unless otherwise stated. Control treatments were made by spraying a solution of 0.1 mM MES-NaOH (pH 6.1). In the training process, the plants were repeatedly exposed to 40 μM ABA for a short and definite interval – in this case 2 h each day over a period of 3 days unless otherwise stated. At the end of the pre-treatment period, the plants were thoroughly rinsed with sterile distilled water to allow recovery. In conditioning to light, the conditioned stimulus (light) preceded the unconditioned stimulus (ABA). After a series of such trials, the plants were harvested and analyzed. ABA was dissolved

in dimethylsulfoxide (DMSO) to make a 100 mM stock solution, which was then stored at −20°C.

The pre-treatment conditions were selected by testing the effect of a range of concentrations of ABA on the viability and physical appearance of the seedlings (Figures 1–3).

Measurements of stomatal aperture

Stomatal apertures of unstained epidermal strips were measured with a light microscope under bright-field illumination (Goh *et al.*, 1999). Epidermal strips were prepared from the underside of rosette leaves of mature plants, both ABA-stressed and unstressed (control). The detached epidermal strips were immediately pooled in sterile distilled water. Stomatal apertures were photographed within 3 min under 10 × 20 magnification and then compared with an eyepiece graticule that was calibrated with a 100 μm × 10 μm slide micrometer scale. Stomata varied considerably in size. Small stomata with a stomatal pore length of less than 7.5 μm were therefore not included while measuring stomatal aperture.

Northern blot analysis

Total RNA was isolated from plants, and either 15 μg (for *cab2*) or 30 μg (for *rd22*) of RNA was loaded in each lane of agarose-formaldehyde gels unless otherwise stated. RNA was then transferred to a nylon membrane (Hybond-N, Amersham, Aylesburgh, UK), as suggested by the manufacturer. Transcripts from *rd22* were detected by hybridization to cDNA clones. The cDNA was synthesized from 2 μg of poly(A)+ RNA of whole plants (*A. thaliana* ecotype Columbia) (Park *et al.*, 1993) using a cDNA synthesis kit with oligo (dT) as a primer (Pharmacia P-L Biochemicals, Piscataway, NJ, USA), according to the manufacturer's instructions. DNA probes for *rd22* (Yamaguchi-Shinozaki *et al.*, 1992), *kin1* (Kurkela and Borg-Franck, 1992), and *rab18* (Lång and Palva, 1992) were cloned from cDNA prepared in this study by polymerase chain reaction using the following primer pairs: for *rd22* (GenBank Accession number D10703), sense primer (5′-ATGGCGATTCGGCTTCCTCT-3′) and antisense primer (5′-CACACAACATGAGTCTCCGG-3′); for *kin1* (GenBank Accession number X51474), sense primer (5′-ATGTCAGAGACCAACAAGAAT-3′) and antisense primer (5′-CTACTTGTTCAGGCCGGTCTT-3′); and for *rab18* (GenBank Accession number X68042), sense primer (5′-ATGGCGTCTTACCAGAACCGT-3′) and antisense primer (5′-TTAACGGCCACCACCGGGAAG-3′). The full-length *AtNCED3* (GenBank Accession number AAL07104) (Iuchi *et al.*, 2001) and *rd29B* (GenBank Accession number D13044) (Yamaguchi-Shinozaki and Shinozaki, 1994) cDNA clones were obtained by PCR with the following oligonucleotide primers: for *AtNCED3*, 5′-ATGGCTTCTTTCACGGCAACGGCTGCG-3′ (sense) and 5′-TCACACGACCTGCTTCGCCAAATCATC-3′ (antisense) and for rd29B, 5′-TGGTCAACGGAAAGTCAACGTCGAGACG-3′ (sense) and 5′-AACATTACGTGTAACAGCAAGGACGAGG-3′ (antisense). The *cab2* cDNA was excised from the pUC19 vector by digestion with *Eco*RI and used for controls of experimental growing conditions of light.

Immunoassay for endogenous ABA content

Whole plants were frozen and stored at −70°C until the extraction of ABA. Fresh frozen or stored samples were ground in liquid N_2 and homogenized successively in 90% (v/v) aqueous methanol that contained 200 mg l^{-1} of diethyldithiocarbamic acid-Na with slight modifications (Zhang *et al.*, 2001). The complete homogenate was incubated overnight in a covered, silanized borosilicate tube in darkness at 4°C. Following a low-speed centrifugation, the supernatant was evaporated. The residue was re-dissolved in 5 ml of methanolic Tris buffered saline, which included 10% (v/v) methanol in 50 mM Tris (pH 8.1), 1 mM $MgCl_2$, and 150 mM NaCl. An aliquot of this solution (100 μl) was used in the ABA assay. All extractions were carried out in dim, indirect light. The competitive ELISA was used for the quantitative determination of ABA, with a Phytodetek immunoassay kit (Agdia Inc., IN, USA), according to the manufacturer's instructions.

Statistical analyses

We used SIGMASTAT from SPSS (Chicago; http://www.spss.com/software/science) to analyze the data from stomatal apertures. The *t*-test and ANOVA test were used to determine differences between the ABA-treated and non-treated plants, where a probability level of $P < 0.05$ was considered to be statistically significant.

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