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Global classification of transcriptional responses to light stress in *Arabidopsis thaliana*

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Abstract

The transcriptional response of higher plants to light stress is mediated *via* hydrogen peroxide-dependent and –independent pathways. In order to analyze these two pathways at the genomic level, microarray analysis for expressional profiling was carried out with the aid of the whole genome GeneChip covering ca 23,000 genes of *Arabidopsis thaliana*. The analysis revealed that both of the hydrogen peroxide-dependent and –independent HL-activation constitute 40 % of the HL-inducible genes. One quarter of the former group responded to drought stress as well. Many heat shock-related genes including *HSP* and *HSF* were regulated by the hydrogen peroxide-dependent HL pathway. A group of genes regulated by the hydrogen peroxide-dependent pathway has been revealed to show transient expression with a peak after 1 h during high light irradiation. In addition, some high light-inducible genes have been found to be down-regulated by hydrogen peroxide. Our results suggest that hydrogen peroxide functions not only as a direct mediator of high light signaling but also as a modulator of expression patterns of some of the high light-responsive genes.

Introduction

In higher plants, most of the genes for the chloroplast proteins are encoded by the nuclear genome (Abdallah et al. 2000, Timmis et al. 2004). Therefore, gene regulation in the nucleus is a major determinant of protein composition in the chloroplast and thus of its activity. Nuclear gene expression for chloroplast regulation is controlled by environmental or developmental cues, e.g., light signals mediated by photoreceptors (Nagy and Schäfer 2002; Lin and Shalitin 2003) or light-independent signals during seedling development (Brusslan and Tobin 1992). In addition, nuclear gene expres-

sion is also known to reflect the status of the chloroplast, achieved by *inter*-organellar communication (Oelmüller 1989; Taylor 1989). This type of regulation has been observed in several situations, including deletion of chloroplast DNA, defect in the plastidic ribosome or metabolism, depletion of carotenoids, inhibition of plastidic transcription and translation and also inhibition of the photosynthetic electron transport system (Oelmüller 1989; Taylor 1989; Susek and Chory 1992; Susek et al. 1993; Pfannschmidt et al. 2001). The mechanism of this communication is largely unknown.

Irradiation of plants with excess light, or high light (HL), causes damage to chloroplasts. The light stress triggers an anti-stress program in the nucleus where many photosynthesis-related genes are shut down and anti-stress genes are activated (Rossel et al. 2002; Kimura et al. 2003b). Because recognition of high light occurs in the chloroplast (Kimura et al. 2001; Karpinski et al. 2003; Kimura et al. 2003a), high light signaling is considered the method of inter-organellar communication that establish a feedback regulation from the chloroplast to nucleus.

Under light stress, reactive oxygen species (ROS) evolve from the photosynthetic electron transport system at the thylakoid membrane, and one of these, hydrogen peroxide, turned out to be a signaling component for high light induction of an ascrobate peroxidase gene, *APX2* (Karpinski et al. 1997; Karpinski et al. 1999). Analyses of gene expression of another high light-inducible gene, *ELIP2*, have revealed that the gene is activated by high light in a hydrogen peroxide-independent manner (Kimura et al. 2001; Kimura et al. 2003a). These studies showed that gene activation by high light is achieved by at least two pathways: one hydrogen peroxide-dependent and the other hydrogen peroxide-independent. Both common and distinct features of these two pathways have been reported (Kimura et al. 2003a).

In this report, we analyze transcriptional responses of these two types of high light activation at the genomic level with the aid of the recently developed microarray technology. The results indicate that they show distinct expression profiles in regard to kinetic and dose responses. In addition, it is suggested that hydrogen peroxide is not only an indispensable mediator of the HL signal transduction, as reported previously (Karpinski et al. 1999), but also a modulator of the high light response for some genes by acting as a suppressor of their gene expression.

Material and Methods

Plant Growth and Treatments

Seeds of *Arabidopsis thaliana* ecotype Columbia were surface-sterilized, plated in Petri dishes containing agar medium, and grown under the continuous low light as reported elsewhere (Kimura et al. 2001). Ten day-old seedlings were illuminated with high light (Kimura et al. 2001), and harvested for RNA extraction (Yamamoto et al. 1998). For H_2O_2-treatments, H_2O_2 solution was sprayed onto low light-grown seedlings as described previously (Kimura et al. 2001). Water was sprayed onto high light-treated seedlings as well as control siblings before the irradiation (Kimura et al. 2001). Drought treatment was achieved by removing of the lid of the Petri dishes for 3 h. After the treatment, desiccation of the seedlings was visibly noticeable.

Microarray Analysis

For each treatment, experiments were duplicated and total RNA was extracted independently, and equal amounts of the duplicated samples were mixed for hybridization. Prepared total RNA was further purified and used for preparation of biotinylated cRNA probe (Masuda et al. 2003). The probe was hybridized to an Affimetrix Arabidopsis GeneChip covering ca 23,000 genes (ATH1) (Masuda et al. 2003). Data analysis was performed described previously (Kimura et al. 2003b). Consistent with the results of the cDNA microarray analysis (Kimura et al. 2003b), *OHP* (At5g02120) did not respond to high light under our experimental conditions (data not shown).

Mutagenesis and Mutant Screening

Four mutagenic populations were generated from a transgenic *Arabidopsis* (L*er*) line containing *ELIP2::LUC* (Kimura et al. 2001), using ethyl methane sulfonate (EMS), the *EnI* transposon (Arts et al. 1995), an enhancer tag (Hayashi et al. 1992), and heavy ion beam irradiation. EMS treatment was done by soaking the seeds in 0.1 % EMS solution overnight at room temperature (Lightner and Caspar 1998). For heavy ion beam treatment, a $^{12}C^{6+}$ and $^{20}Ne^{10+}$ ion beam was generated and accelerated by the ring cyclotron at the RIKEN Accelerator Research Facility (RARF) to give a LET (Linear Energy Transfer) of 22.5 and 61.5 keV/µm, respectively, and a velocity of 135 MeV/nucleon for both ion species (Yamamoto et al. 2004). Dry seeds at atmospheric pressure were irradiated with the $^{12}C^{6+}$ beam at a dosage of 100, 150, and 200 Gy, and with the $^{20}Ne^{10+}$ beam at a dosage of 150 Gy. Screening for mutants defective in transcriptional response to light stress was carried out by visualization of *in vivo* luciferase activity (Kimura et al. 2001). Eight to 10 day-old seedlings grown under low light (6 W/m^2) were screened for mutants showing higher luciferase activity. They were illuminated with high light (150 W/m^2) for 3 h, and screened for mutants with lower luciferase activity. Stress-sensitive mutants were isolated from one to two week-old seedlings grown under moderately high light (50 W/m^2) by visual inspection. The stress sensitive mutants were subjected to a second screening by assessing their luciferase activity (Yamamoto et al. 2004).

Results

Global Transcript Analysis

In order to examine the relationship of the high light response to with drought and hydrogen peroxide responses, seedlings were treated with high light (350 W/m^2, ca. 1,800 µE/m^2/s), hydrogen peroxide and desiccation. Subsequently total RNA was extracted for gene expression analysis. An Affimetrix GeneChip covering almost all the *Arabidopsis* genes were used for global expression analysis and a positive hybridization signal was detected for 14,900 genes in at least one of the treatments.

Crosstalk with Drought Response

Comparison of high light-, drought-, and hydrogen peroxide-activated genes, identified by having a threshold of a 3 fold-induction, revealed considerable overlaps (Fig. 1). Approximately 20 % of the high light-inducible genes were also activated by drought stress (Fig. 1, 49 out of 248). We previously discussed that genes activated by both high light and drought would be stimulated by the accumulation of ROS, such as hydrogen

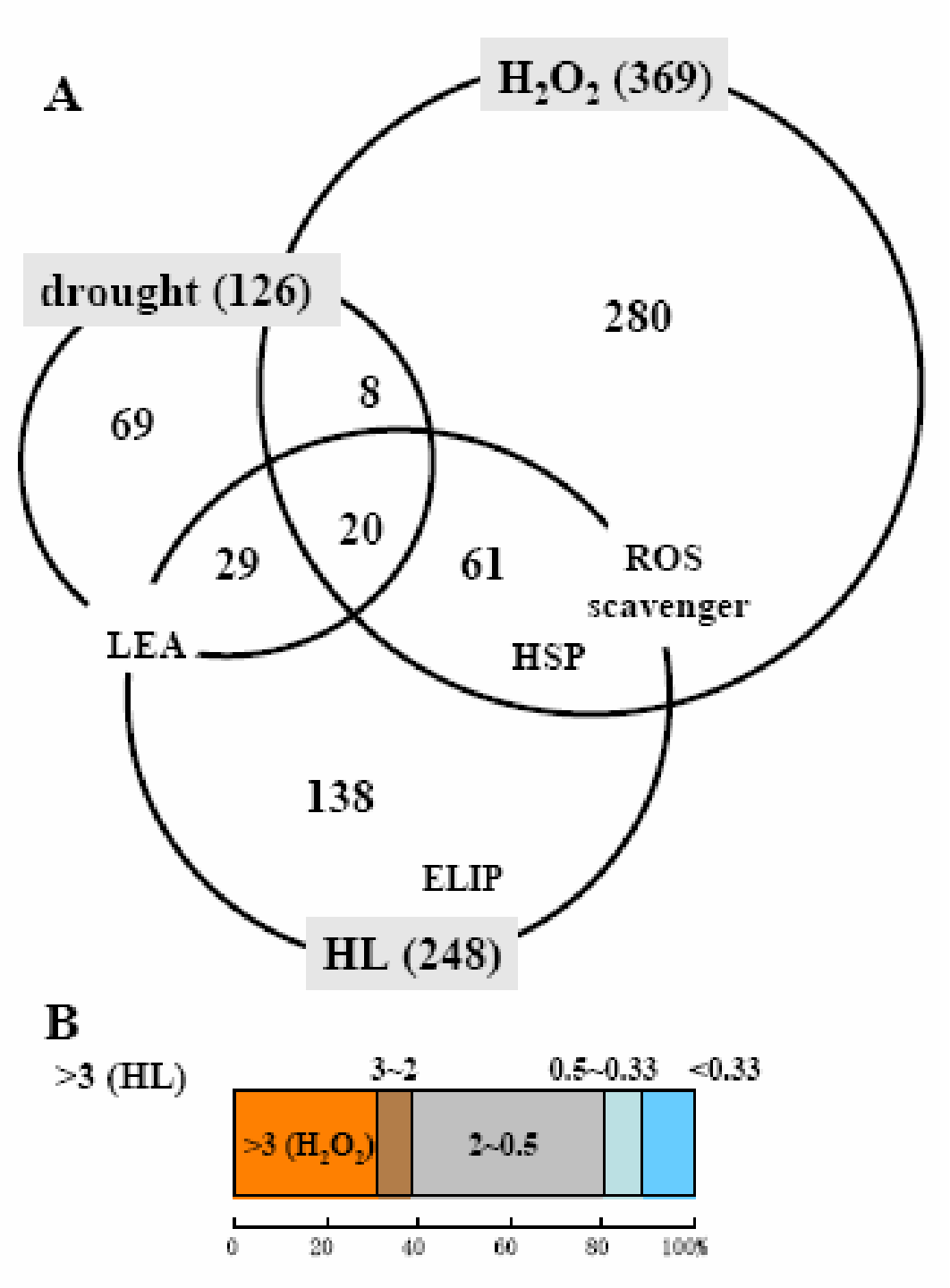

Figure 1: Relationship between HL-, H_2O_2-, and drought-inducible genes
A: Number of genes with response of over 3 fold is indicated. B: high light-inducible genes were divided according to the degree of response to H_2O_2.

peroxide, or an imbalance between light and dark reactions due to either an overdrive of the light reaction by high light or by CO_2 limitation caused by stomata closure under drought stress (Kimura et al. 2003b). Supporting the former hypothesis, 20 of the cross-induced genes were also activated

by the application of hydrogen peroxide. The other group of 29 genes might represent the latter situation, although there is no experimental evidence to support this.

Hydrogen Peroxide-Dependent and –Independent Pathways for High Light Signaling

In order to understand how many of the high light-regulated genes are mediated by hydrogen peroxide, we compared high light-inducible genes with hydrogen peroxide-inducible ones. As shown in Figure 1B, approximately 40 % of the high light-inducible genes were activated by application of hydrogen peroxide (the orange plus brown area). This group represents the HL-inducible genes with H_2O_2-mediation. In addition, about 40 % did not respond to H_2O_2 (gray in the bar graph), so their HL-activation is achieved independent of hydrogen peroxide. These analyses revealed that each group of genes regulated by the H_2O_2-dependent and –independent HL pathways, respectively, are both major constituents of the HL-responsive group. Some of the HL- and H_2O_2-activated genes are shown in Table 1.

Table 1 documents that a considerable number of the genes strongly induced by both stimuli were heat shock-related genes, including *HSP*s and also genes for heat shock transcription factors (*HSF*s) (see later).

Recently, Vandenabeele et al. have analyzed the effect of suppression of *CAT2,* a gene encoding peroxisomal catalase (Vandenabeele et al. 2004). Suppression of *CAT2* caused increased accumulation of hydrogen peroxide within leaves as expected from its catalase function. Microarray analysis using cDNA array has revealed genes that are up-regulated by *CAT2* suppressison during HL-illumination. Comparison of their data with ours reveals a striking overlap. As shown in Table 1 ("*CAT2* suppression"), almost all of the genes in the table and applicable to Vandenabeele's data were identified as genes up-regulated by *CAT2* suppression. This high consistency indicates that there are few false positives in Table 1.

In the *CAT2* suppression line, the cross-positive genes were activated by high light treatment for 3 h in a comparable manner to the wild type, and the effect of *CAT2* suppression was found to be clearer at 8 h (Fig. 2). This suggests that in wild type *CAT2*, possibly together with other *CAT* genes, reduces the accumulation of hydrogen peroxide mainly after 3 h.

Table 1: Top 33 genes that showed strong induction by both HL and H_2O_2

AGI Code	Protein	HL	H_2O_2	*CAT2* sup-pression[1]	
At1g53540		148.97	202.47	86.0	17.6 kDa heat shock protein
At3g46230		141.12	112.12	na	heat shock protein 17
At5g12030		104.55	173.89	27.7	heat shock protein 17.6A
At1g52560		86.60	214.86	3.7	chloroplast-localized small heat shock protein, putative
At4g25200		68.63	78.96	na	mitochondrion-localized small heat shock protein (AtHSP23.6-mito)
At2g29500		66.82	78.99	na	putative small heat shock protein
At5g48570		61.08	38.48	na	Peptidyl-prolyl isomerase
At1g74310		29.25	46.53	30.9	HSP101
At4g12400		26.99	32.91	na	stress-induced protein sti1-like protein
At1g72660		23.32	24.50	na	putative GTP-binding protein
At1g59860		15.20	20.77	na	heat shock protein, putative
At1g17870		14.87	4.00	na	hypothetical protein
At5g52640		11.37	16.11	16.6	heat-shock protein
At5g12110		10.91	4.62	na	elongation factor 1B alpha-subunit
At1g54050		10.23	20.09	na	heat-shock protein, putative
At3g24500		10.06	5.63	11.4	ethylene-responsive transcriptional coactivator, putative
At3g12580		9.88	7.97	0.7	heat shock protein 70
At5g25450		8.47	10.13	na	ubiquinol--cytochrome-c reductase-like protein
At2g26150		8.40	8.17	na	putative heat shock transcription factor
At5g51440		7.80	22.16	na	mitochondrial heat shock 22 kd protein-like
At3g09700		7.52	6.35	na	similar to a region of DNAJ domain-containing protein MCJ
At4g10250		7.31	7.73	na	heat shock protein 22.0
At2g32120		6.93	14.99	5.5	70kD heat shock protein
At5g64510		6.62	7.00	na	putative protein
At1g71000		6.09	6.21	na	heat shock protein DNAJ, putative
At3g53230		6.04	6.72	na	CDC48 - like protein transitional endoplasmic reticulum ATPase
At4g23493		5.89	5.33	na	expressed protein
At3g16050		5.82	9.84	2.1	putative ethylene-inducible protein
At1g21550		5.79	3.84	3.3	unknown protein
At2g19310		5.47	3.89	na	putative small heat shock protein
At2g46240		5.40	21.78	na	hypothetical protein
At1g07350		5.32	3.28	3.0	transformer-SR ribonucleoprotein, putative
At5g37670		5.07	14.00	na	low-molecular-weight heat shock protein - like

Among the genes up-regulated by both high light irradiation (350W/m^2 for 3 h) and H_2O_2 feeding, genes with strong induction by HL (>5-fold) are listed. [1]Effect of *CAT2*-suppression on the induction ration by 8

h-treatment of high light (1,600 µE/m^2/s), identified by cDNA microarray experiments (Vandenabeele, et al., 2004). “na”- no data available.

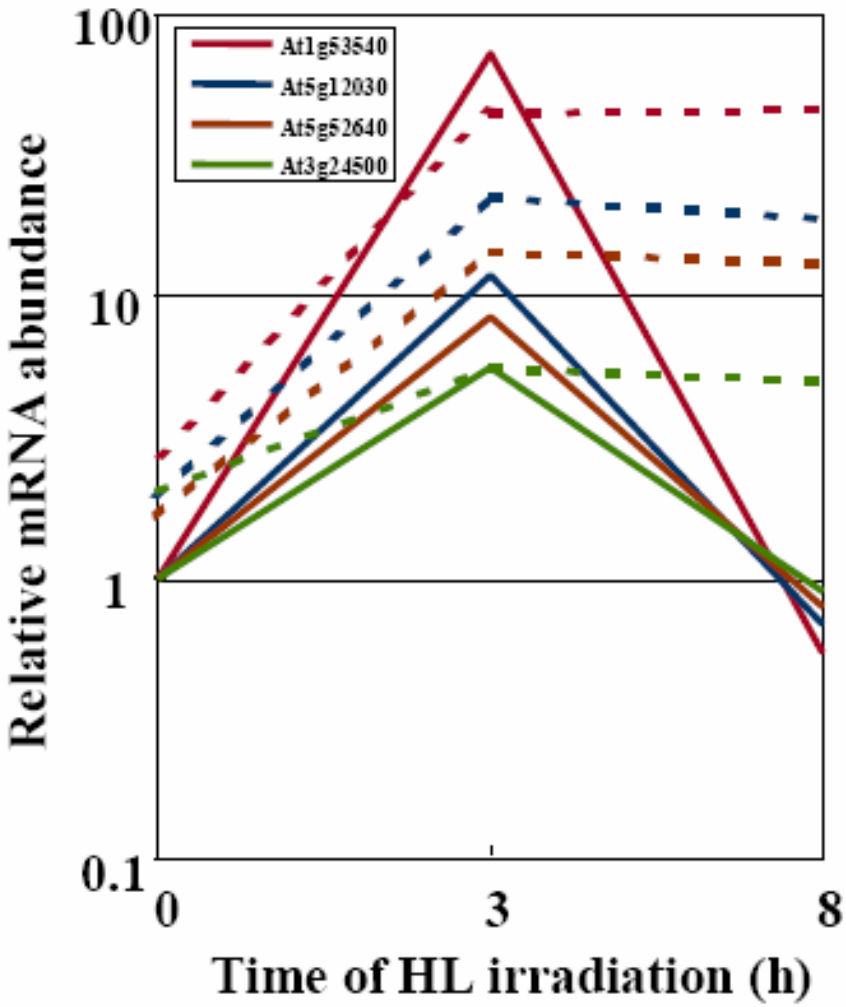

Figure 2: Effect of *CAT2* suppression on HL induction
Among the genes in Table 1, ones activated by high light more than 5 fold after irradiation for 3 h are shown. Lines and dotted lines on the graph are expression data of the wild type and a *CAT2* suppression line (CAT2HP1), respectively. For each gene, amount of mRNA relative to wild type is indicated. Data has been modified from Vandenabeele *et al.* (Vandenabeele, et al., 2004).

Heat Shock-Related Genes

The GeneChip used in this study contained 134 heat shock-related genes (*HSP*s and *HSF*s, data not shown). Of these, 107 gave positive signals in controls or in samples of any of the treatments carried out in this study, and 41 showed activation by HL or H_2O_2. Their classification revealed very strong positive correlation between high light and H_2O_2 responses, as shown in Figure 3. As such a strong correlation was not observed in any other functional gene group, it would appear to be an unique characteristic of heat shock-related genes.

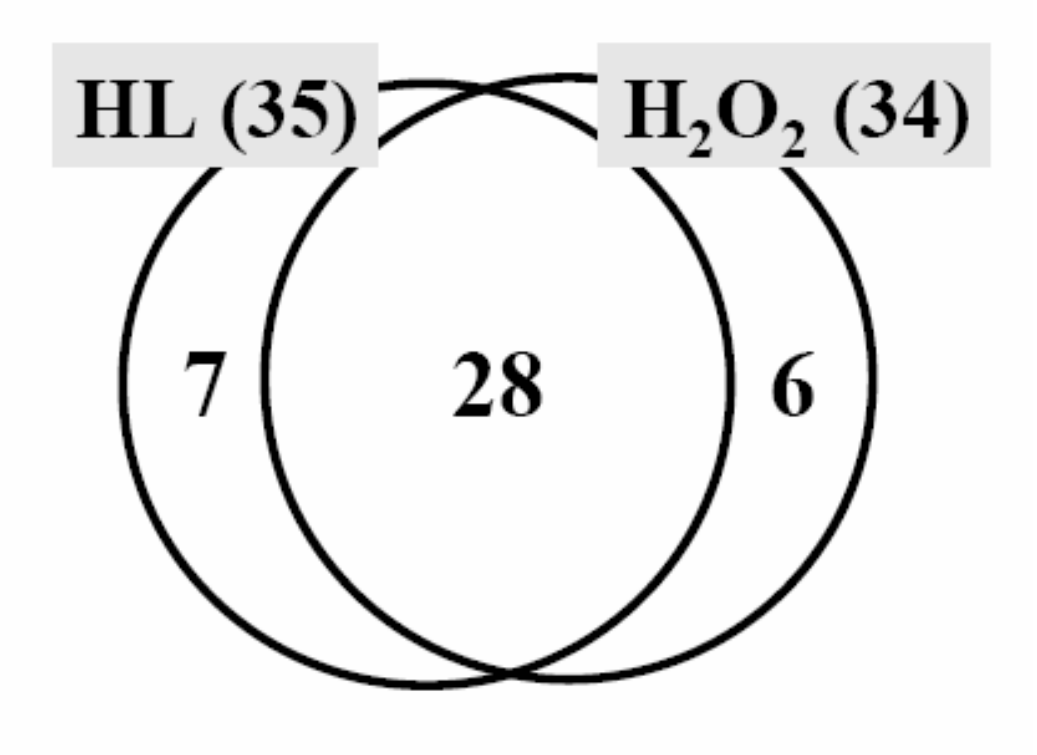

Figure 3: Relationship between the response to HL and H_2O_2 among heat shock-related genes
Genes with more than 2 fold induction were identified as HL (350 W/m^2 for 3 h) or H_2O_2-inducible genes. Strong positive correlation was observed

Profile of the H_2O_2-Dependent and –Independent HL Responses

In order to further characterize the high light response, we decide to analyze kinetic and dose responses. Seedlings were illuminated with high light (150 W/m^2) for 0, 1, and 3 h, and subjected to microarray analysis. In the kinetic assay, we classified the genes into 3 groups: slow, fast, and transient induction (Fig. 4, A, B, and C, respectively). *ELIP2* was classified in to the “slow” group (Fig. 4A) which is consistent with the kinetic characteristics of *ELIP2* induction determined by quantitative RT-PCR analysis in a previous report (Kimura et al. 2001). In Figure 4, each group was further classified according to its response to hydrogen peroxide. In the “transient” group, H_2O_2-activated genes constituted more than half (Fig. 3C, orange), where as most of the genes in the “slow” group were H_2O_2-insensitive (Fig. 4A, gray). In Panels D and E, the HL-inducible genes were re-divided into H_2O_2-activated (D) and insensitive (E) groups, and then sub-classified into the “slow”, “fast”, and “transient” categories. As we observed before, the majority of the H_2O_2-insensitive genes belong to the “slow” group. Therefore, a tight relationship has been found between H_2O_2-insensitivity and the “slow” characteristics. The majority of the H_2O_2-activated genes are classified in the “transient” group, and this is their largest category (Fig. 4D, yellow).

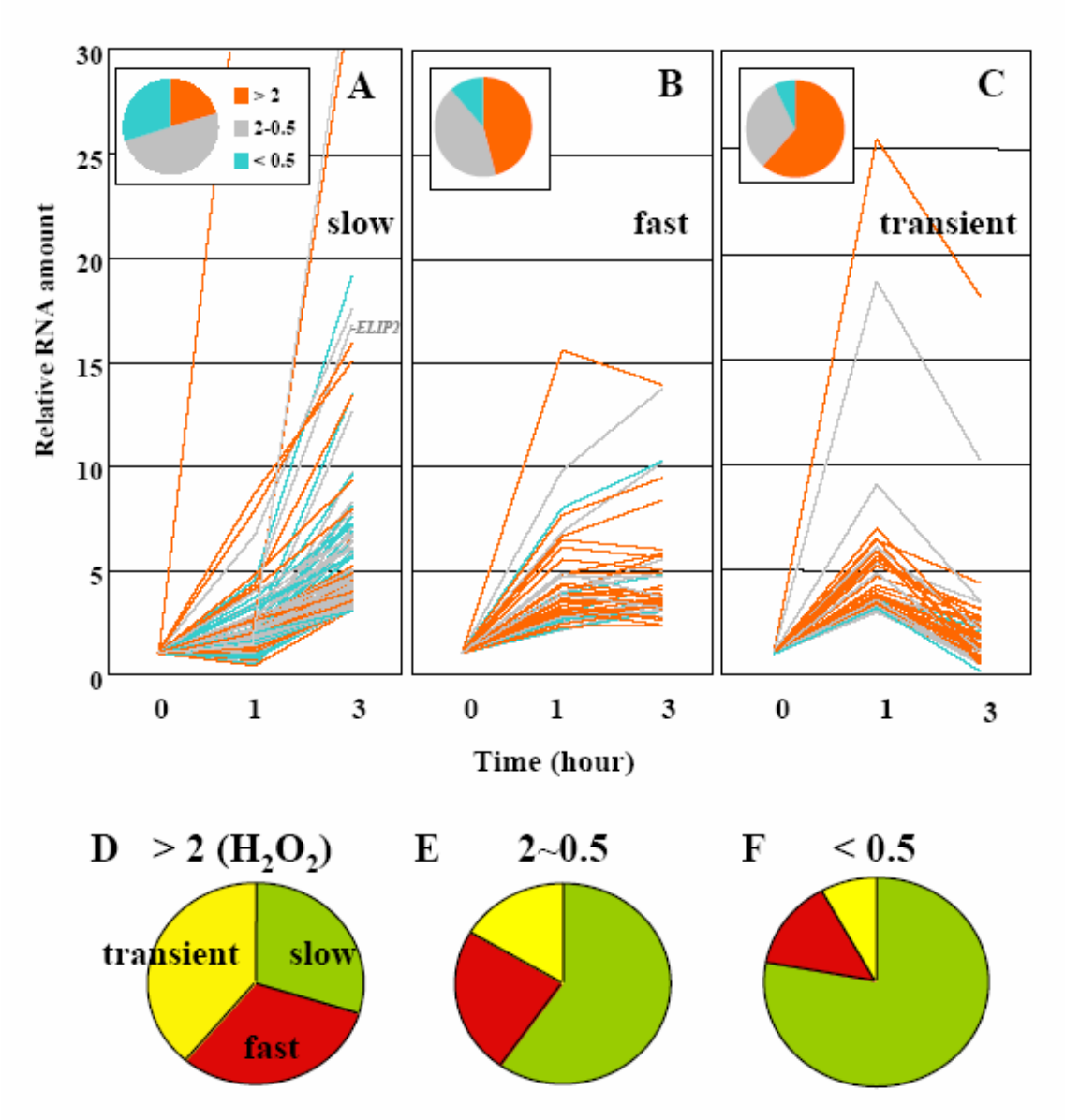

Figure 4: Kinetic classification of HL induction
A, B, and C: HL-inducible genes with more than 3 fold activation were separated into three groups according to the ratio of expressional level at 3 h to that at 1 h.: more than 2 fold (A), between 0.5 to 2 fold (B), and less than 0.5 fold (C). Colors of line and pie graphs indicate response to H_2O_2 as shown in the inserted panel in A. Percentages of the inserted pie graphs are : (A, >2: 21%, 2~0.5: 50 %, <0.5: 30 %); (B, >2: 46%, 2~0.5: 43 %, <0.5: 11 %); (C, >2: 61%, 2~0.5: 32 %, <0.5: 7 %). D, E, and F: The HL-inducible genes were classified into 3 groups according to the response to H_2O_2 as indicated on the pie graphs. Each group was then sub-divided in regard to their kinetic characteristics

To our surprise, genes down-regulated by H_2O_2 were also found among HL-activated genes. Most of them were found in the “slow” group as well (Fig. 4A, blue and F, green). The typical responses of H_2O_2 activated, -insensitive, and –repressed genes to HL are summarized in Figure 6A.

Comparison of responses to light intensities were also investigated. Seedlings were irradiated for 3 h with intensities of 6, 150, and 350 W/m^2, which cor-

correspond to 30, 800, and 1,800 µE/m^2/s, respectively, and the transcriptional responses were analyzed. According to the dose response, genes were divided into 4 groups, “leap”, “rise”, “saturated”, and “stall” (Fig. 5 A, B, C, and D, respectively).

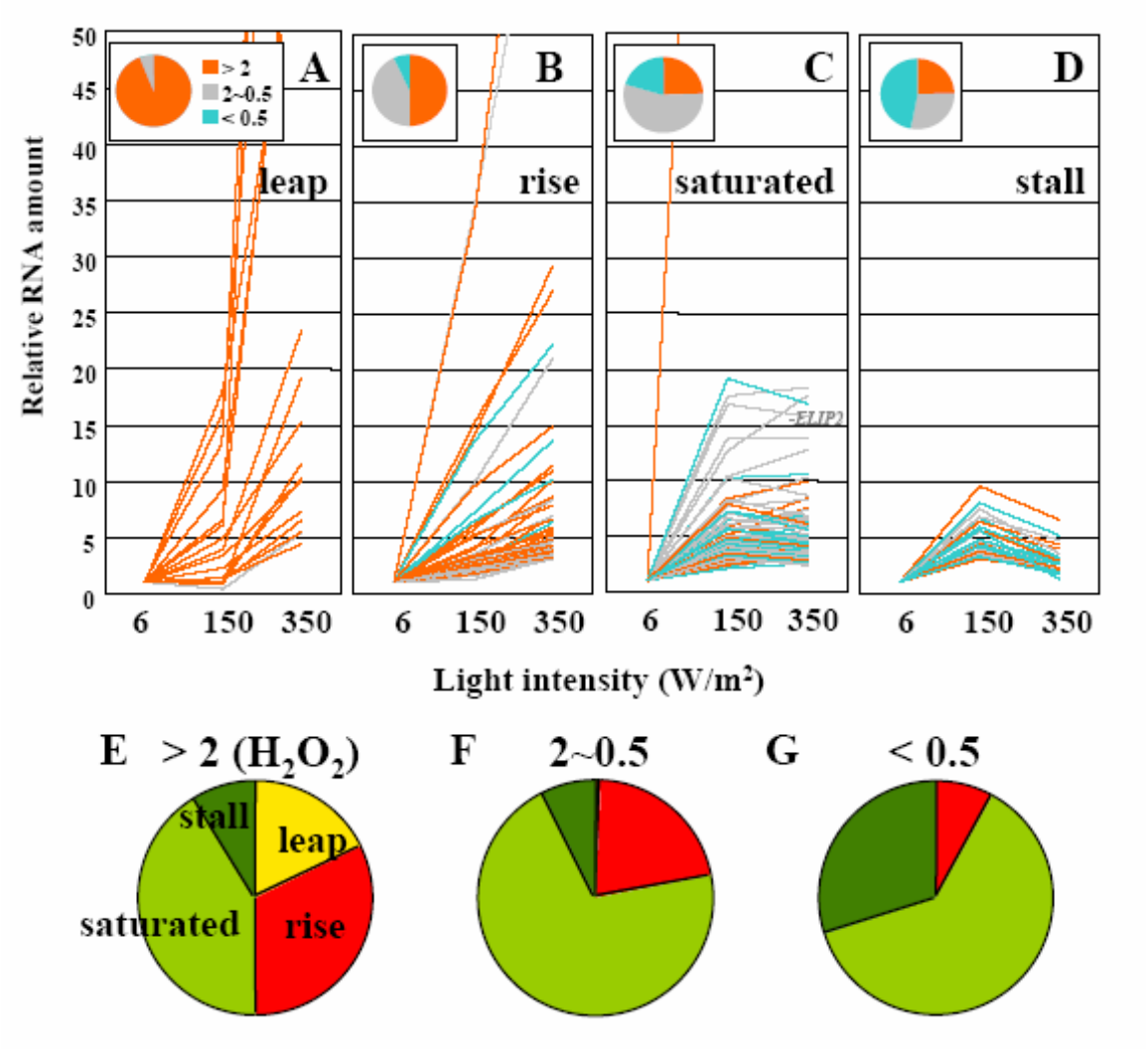

Figure 5: Classification by dose response
HL-inducible genes with more than 3 fold activation were separated into four groups according to the ratio of expression levels at 350 W/m^2 and 150 W/m^2: more than 3 fold (A), between 3.0 and 1.5 fold (B), between 1.5 and 0.75 fold (C), and less than 0.75 fold (D). Colors of line and pie graphs indicated response to H_2O_2 as shown in the inserted panels.
Percentages of the inserted pie graphs are: (A, >2: 94%, 2~0.5: 6 %, <0.5: 0 %); (B, >2: 50%, 2~0.5: 43 %, <0.5: 7 %); (C, >2: 24%, 2~0.5: 55 %, <0.5:20 %); (D, >2: 25%, 2~0.5: 28 %, <0.5: 47 %). E, F, and G: The HL-inducible genes were classified into 3 groups according to the response to H_2O_2 as indicated on the pie graphs. Each group was then sub-divided in regard to their dose response.

ELIP2 was found in the “saturated” group, and again this was consistent with previous results determined by quantitative RT-PCR analysis (Kimura et al. 2001). The figure revealed that almost all of genes of the “leap” group

were up-regulated by H_2O_2 (Panel A, orange). In addition, the most abundant genes in the ”saturated” group were H_2O_2-insensitive. The hydrogen peroxide-suppressed genes were mostly found in the “stall” group (Panel D). the majority of both the H_2O_2-insensitive and –suppressed genes (Panels F and G) fell into the ”saturated” category. The simplified responses are illustrated in Figure 6B.

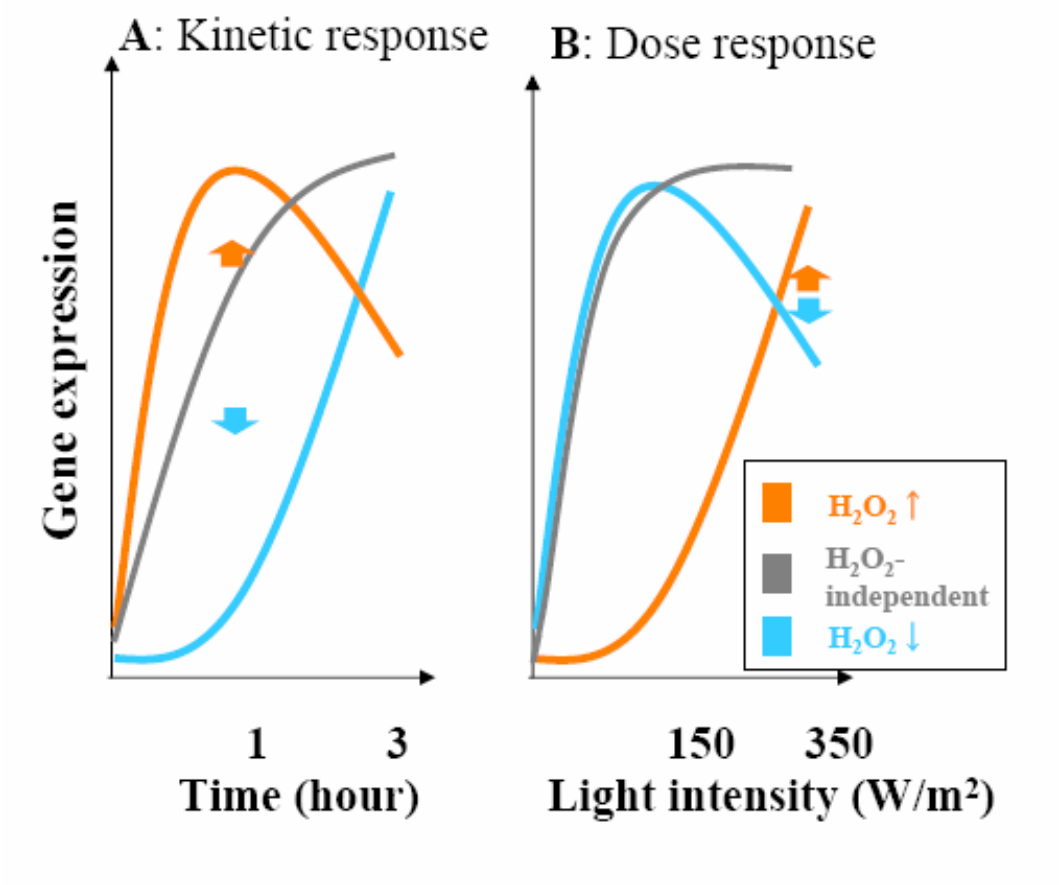

Figure 6: A model of H_2O_2 action in the high light response
A. The effect of H_2O_2 as an activator (orange arrow) or a repressor (blue arrow) is evident at 350 W/m^2 rather than 150 W/m^2. B. The effect of H_2O_2 is dominant at 1 h after the start of high light irradiation. The H_2O_2 repressed genes (blue) are thought to display a combination of HL activation and H_2O_2 suppression, giving expression profiles as shown in blue. On the other hand, a single activation by H_2O_2 of the H_2O_2-inducible genes explains their HL induction.

Screening of Mutants Defective in High Light Responsive Gene Expression

Our expression profiling of the high light response suggests a rather complex signaling network of gene regulation for light stress response. To further our understanding, utilization of genetic mutants is helpful. We decided to isolate mutants that show altered high light responses. Transgenic *Arabidopsis* containing *ELIP2::LUC* responds to high light in a H_2O_2-independent manner, and the response can be monitored in a non-destructive way (Kimura et al. 2001). Taking advantage of this transgenic line,

we screened for mutants with a high luciferase signal under low light conditions, those with a low signal after high light irradiation, and also for those with high stress sensitivity to high light.

A combination of the multiple screening strategies gave us two types of mutants: *no ELIP2 suppression* (*nes*) type, and the *no ELIP2 activation* (*nea*) type. The *nes* type had higher luciferase activity in low light, and the *nea* type had lower luciferase activity after high light-irradiation. Some mutants showed no HL-response at all, showing the same luciferase activity in low light and after HL-treatment (data not shown). They were found in both *nes* and *nea* types. It was noticed that some of the *nes* mutants were high light-sensitive, while others were more tolerant than the wild type.

Table 2: Mutants of light stress response

	Transposon[1]	EMS[1]	Enhancer tag[1]	Heavy ion beam[1]
nes[2]	3	13	1	13
nea[2]	0	8	0	0

Number of mutants is shown. [1]Type of mutagen. [2]Type of high light response.

Discussion

Responses to High Light and Hydrogen Peroxide

Our results have revealed characteristic responses of the hydrogen peroxide-dependent and –independent groups. Figure 6 summarizes their typical responses. In this illustration, H_2O_2-dependent HL activation occurs more quickly than that of H_2O_2-independent activation (Panel A, gray and orange). At 3 h, expression of the genes in the H_2O_2-dependent group was reducing, while expression of those in the H_2O_2-independent group remained high. The expression profile of the H_2O_2-repressible group (Panel A, blue) suggests a combined pattern of HL-activation and suppression by H_2O_2. Because this group is suppressed by it, action of H_2O_2 does not explain their HL-activation. Therefore, they would be activated independently of H_2O_2, as in the case of the gray group. Assuming this HL-activation, suppression by the accumulated H_2O_2 modifies the kinetic response, shifting the rising phase to later. Although the physiological significance of this modulation is not clear, as much as half of this group were found to be activated by drought stress (data not shown). In short, H_2O_2 transiently acts on transcription at 1 h whether it activates or represses gene expression (Panel A, arrows).

The responses to irradiance are summarized in Panel B. The H_2O_2-dependent group is activated at the higher irradiance (350 W/m^2), while the response of the H_2O_2-independent group is saturated at the lower irradiance (150 W/m^2). In the H_2O_2-suppressed group, there is again a combination of HL-response and H_2O_2-suppression, the latter reducing the HL response at the higher irradiance. These models fit the idea that H_2O_2 has a greater effect at

350 W/m^2 than at 150 W/m^2, where it activates or represses transcription (Panel B, arrows).

The discussion above suggests that hydrogen peroxide is not only a mediator of the HL signal transduction, as reported previously (Karpinski et al. 1999), but also a modulator of the high light response for some genes by acting as a repressor of their gene expression. As illustrated in Figure 6, the mode of H_2O_2 action that emerged from our transcriptome analysis is to be confirmed by biochemical or genetic analysis.

Although high light causes accumulation of H_2O_2, only a limited number of the H_2O_2-inducible genes were activated by high light (Fig. 1, 20 + 61 out of 369). This can be explained in part by local accumulation of H_2O_2 under light stress (Fryer et al. 2003). Because H_2O_2 is reported to be a mediator of various types of stress signaling including ozone (Sharma et al. 1996), wounding (Orozco-Cardenas and Ryan, 1999), UV-B (Mackerness et al. 2001), and also pathogen infection (Wojtaszek 1997), the H_2O_2-specific" genes in Figure 1 might be stimulated by such stresses rather than by high light. It is not known how hydrogen peroxide mediates such a variety of signals with relatively little cross talk, as exampled in Figure 1. Because simple application of hydrogen peroxide does activate a massive number of genes (Fig. 1), its accumulation appears to be enough for up-regulation of the H_2O_2-mediated responses to various biotic and abiotic stresses. Therefore, it might be a matter of timing and location of the accumulation, as mentioned above.

Strategy of Mutagenesis

While EMS causes base substitutions form C to U (Lightner and Caspar 1998), irradiation by heavy ion beam mainly causes small deletion of less than 100 bp long (Shikazono et al. 2003; Kagawa et al. 2004). Because such small deletions cause frameshift mutations, heavy ion beam irradiation is expected to give null alleles, which are suitable for clear analysis of gene function. On the other hand, EMS mutagenesis can produce dominant alleles caused by missense mutations. This future is useful when approaching redundant gene sets. A combination of multiple strategies of mutagenesis will help to isolate a wider range of mutants.

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