Gene Expression Profiling of the Tetrapyrrole Metabolic Pathway in Arabidopsis with a Mini-Array System[1[w]]

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Tetrapyrrole compounds, such as chlorophylls, hemes, and phycobilins, are synthesized in many enzymatic steps. For regulation of the tetrapyrrole metabolic pathway, it is generally considered that several specific isoforms catalyzing particular enzymatic steps control the flow of tetrapyrrole intermediates by differential regulation of gene expression depending on environmental and developmental factors. However, the coordination of such regulatory steps and orchestration of the overall tetrapyrrole metabolic pathway are still poorly understood. In this study, we developed an original mini-array system, which enables the expression profiling of each gene involved in tetrapyrrole biosynthesis simultaneously with high sensitivity. With this system, we performed a transcriptome analysis of Arabidopsis seedlings in terms of the onset of greening, endogenous rhythm, and developmental control. Data presented here clearly showed that based on their expression profiles at the onset of greening, genes involved in tetrapyrrole biosynthesis can be classified into four categories, in which genes are coordinately regulated to control the biosynthesis. Moreover, genes in the same group were similarly controlled in an endogenous rhythmic manner but also by a developmental program. The physiological significance of these gene clusters is discussed.

Tetrapyrrole compounds play an essential role in all living organisms. They are involved in various metabolic processes such as energy transfer, signal transduction, and catalysis (Dailey, 1990). In plants, at least three structurally and functionally distinct classes of tetrapyrroles can be distinguished, Mg-porphyrins, Fe-porphyrins, and phycobilins. Mg-porphyrins, such as chlorophylls (Chls), are the most abundant tetrapyrrole compounds on earth. As part of the photosynthetic apparatus, the Mg-porphyrin rings of Chl *a* and Chl *b* are involved in light trapping and charge separation for photosynthesis. In addition, Mg-porphyrin intermediates have been shown to regulate the transcription of nuclear-encoded photosynthetic genes in plant systems (Susek and Chory, 1992; Rodermel, 2001). Fe-porphyrins, hemes, constitute the second class of tetrapyrroles. In particular, protoheme functions as a redox-active cofactor or prosthetic group in many proteins. For instance, protoheme is bound to the various cytochromes of the plastidic and mitochondrial electron transport chains and to soluble enzymes, such as catalase and peroxidase. Apart from these functions, heme and its derivatives have also been shown to regulate a variety of cellular processes in animal and bacterial systems, such as transcription (Guarente and Mason, 1983), translation (Chen et al., 1989; Joshi et al., 1995), and posttranslational protein translocation (Lathrop and Timko, 1993). Phytochromobilin belongs to the third class of plant tetrapyrroles, the phycobilins. In contrast to Chls and hemes, phycobilins are linear pigments. Phytochromobilin is covalently bound to the phytochrome apoprotein (Lagarias and Rapoport, 1980), and this holocomplex is known to act as a photoreceptor that controls various developmental processes (Furuya, 1993).

In plants and many bacteria, all of the tetrapyrroles originate from a common biosynthetic pathway (Beale, 1999). This pathway, the so-called C_5 pathway, starts at glutamyl-$tRNA^{Glu}$, and the subsequently formed 5-aminolevulinic acid (ALA) is metabolized to form tetrapyrroles through a variety of reactions. All of the steps in the biosynthesis of Mg-porphyrins and Fe-porphyrins are shared up to protoporphyrin IX. As two or more products are often formed simultaneously or at different stages of development within a single organism, the process obviously requires a high degree of metabolic regulation. During the last decade, considerable progress has been made in elucidating the biosynthetic enzymes, cloning their genes, and studying their regulated expression in response to light and other environmental and developmental factors. A regulatory model has emerged as to how their expression can affect the flow of metabolites

[1] This work was supported by a grant from the 21st Century COE Program, Ministry of Education, Culture, Sports, Science and Technology, and by a project titled Development of Fundamental Technologies for Controlling the Process of Material Production of Plants based on funds provided by the Ministry of Ecology, Trade and Industry in Japan.

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[w] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.042408.

through the C_5 pathway, reflecting the varying needs for tetrapyrroles in response to different environmental and developmental conditions. It is generally considered that several specific isoforms catalyzing particular enzymatic steps control the flow of tetrapyrrole intermediates via the differential regulation of gene expression depending on environmental and developmental factors, as well as the potent negative feedback regulation of enzyme activity (Reinbothe and Reinbothe, 1996; Grimm, 1998). The light-, tissue-, and developmental-dependent expression as well as diurnal and circadian expression of genes in the tetrapyrrole pathway have been described earlier (He et al., 1994; Papenbrock et al., 1999). All these conclusions, however, are based on experiments with a limited number of genes, and the coordination of such regulatory steps and orchestration of the overall tetrapyrrole metabolic pathway are still poorly understood. It is therefore necessary to test these ideas with as many genes as possible.

With the sequencing of the entire genome of Arabidopsis (Arabidopsis-Genome-Initiative, 2000), it has become feasible to study gene expression in a genome-wide fashion using high-density oligonucleotide microarrays or DNA microarrays. Unfortunately, the high cost of the necessary instruments and supplies has prevented widespread use of this technique. In addition, although an enormous amount of data obtained with microarrays is needed to elucidate physiological events in a genome-wide fashion, the amount is often too great to handle for the analysis of a particular metabolic pathway. Alternatively, cDNA macroarrays using PCR-amplified DNA fragments, such as expressed sequence tags, spotted on nylon membranes are much less expensive and use relatively standard laboratory equipment. However, the cross-hybridization of multiple labeled cDNA species cannot be identified with this method. An alternative solution to such limitations may be a mini-array membrane hybridization method using gene-specific probes from a small biosynthetic pathway. Recently, laboratory-made mini-array systems have been shown to reliably assess the expression of genes simultaneously from yeast (Hauser et al., 1998; Cox et al., 1999; Fukuzawa et al., 2001), rats and mice (Dai et al., 2002), and Arabidopsis (Matsuyama et al., 2002).

In this study, we developed a novel mini-array system to monitor the expression profile of the tetrapyrrole biosynthetic pathway in Arabidopsis. Using this system, we illustrate the profiles of changes in gene expression in terms of the onset of greening, endogenous rhythm, and developmental control. Our findings demonstrated the potential of our mini-array system for detection and analysis of the expression of genes involved in tetrapyrrole biosynthesis in a genome-wide fashion. With this system, we clearly showed that the genes corresponding to regulatory enzymes of tetrapyrrole biosynthesis can be classified into four categories, in which genes are coordinately regulated by endogenous factors to control the biosynthesis.

RESULTS

Development of the Mini-Array System

Our database search of the Arabidopsis genome identified 35 genes involved in the biosynthesis of tetrapyrrole (Table I) from numerous previous studies and a recent database search by Lange and Ghassemian (2003). These genes encode enzymes of the common pathway leading from glutamyl-$tRNA^{Glu}$ to protoporphyrin IX via the C_5 pathway (17 genes), Mg-branch to synthesize Chl *a* and *b* (11 genes), and Fe-branch to synthesize protoheme and phytochromobilin (7 genes). Among 18 enzymatic steps tested, isoforms were found in 9 steps, although some isoforms were presumed to be pseudogenes, such as *CPO2*, or their functions were not identified experimentally. To monitor the quantitative expression of all 35 genes simultaneously, and, specifically, we developed an original mini-array system, which is based on the membrane-supported macroarray technique (Pietu et al., 1996).

To enable quantitative analysis of the expression of each gene with high sensitivity, precautions were taken to produce long (150–300 bp) DNA probes immobilized on the membrane with high gene specificity. When coding sequences with high homology were found between isoforms, we chose gene-specific regions, such as the 3′-untranslated region, and amplified them by reverse transcription (RT)-PCR (Supplemental Fig. 1, available at www.plantphysiol.org). Sequences of primers used for amplification are listed in Supplemental Table I. The specific hybridization of each RT-PCR product was confirmed by genomic southern analysis to give a single band (Supplemental Fig. 2). Resultant DNA fragments were purified and used as DNA probes for the mini-array system. Probes for ubiquitin (*UBQ10*; Sun and Callis, 1997) and actin (*ACT8*; An et al., 1996) as housekeeping genes, the light-harvesting Chl *a*/*b* complex of photosystem II (*Lhcb*) as a marker gene of chloroplast development, and λDNA as negative control were also spotted. We also spotted various chloroplast-related and phytohormone-responsive genes (Table I). Consequently, a total of 48 probes were spotted in duplicate in a nylon membrane, in which one set of all probes was designed to spot in one grid area (Fig. 1A). Since the amount of probe material has a strong impact on the sensitivity and reproducibility (Bertucci et al., 1999), exactly 4 ng (equivalent to 1×10^{10} molecules) of each probe was immobilized to each spot to facilitate quantitative analysis.

To evaluate the quality of this mini-array, we carried out a pilot hybridization experiment with two independent samples: total RNA isolated from 3-d-grown etiolated seedlings and subsequently 9-h-illuminated seedlings. In both samples, two mini-array membranes

Table I. *List of genes spotted on the mini-array membrane*

A total of 35 genes involved in tetrapyrrole biosynthesis found in the database of the complete genome sequence of Arabidopsis were spotted together with control genes. It should be noted that the annotation of isoforms may vary from those in previous publications.

Col	Row	Gene Product	No.	Annotation	AGI Code	Comment
		Tetrapyrrole Biosynthetic Genes				
1	1	Glutamyl-tRNA reductase	3	*HEMA1*	At1g58290	
1	2			*HEMA2*	At1g09940	
1	3			*HEMA3*	At2g31250	By homology
1	4	Glu 1-semialdehyde aminotransferase	2	*GSA1*	At5g63570	By homology
2	1			*GSA2*	At3g48730	By homology
2	2	5-aminolevulinic acid dehydratase	2	*ALAD1*	At1g69740	By homology
2	3			*ALAD2*	At1g44318	By homology
2	4	Porphobilinogen diaminase	1	*PBGD*	At5g08280	
3	1	Uroporphyrinogen III synthase	1	*UROS*		By homology, GenBank; AC002504
3	2	Uroporphyrinogen III decarboxylase	2	*URO1*	At2g40490	By homology
3	3			*URO2*	At3g14930	By homology
3	4	SAM uroporphyrinogen III methyltransferase	1	*UPM1*	At5g40850	Complemented yeast *UPM* mutant
4	1	Coproporphyrinogen III oxidase	2	*CPO1*	At1g03480	Mutant isolated as *lin2*
4	2			*CPO2*		GenBank; AC005275, Pseudogene?
4	3			*CPO3*	At5g63290	Bacterial O_2-independent type
4	4	Protoporphyrinogen IX oxidase	2	*PPO1*	At5g14220	By homology
5	1			*PPO2*	At4g01690	
5	2	Ferrochelatase	2	*FC1*	At5g26030	
5	3			*FC2*	At2g30390	
5	4	Heme oxygenase	4	*HO1*	At2g26670	Mutant isolated as *hy1*
6	1			*HO2*	At2g26550	
6	2			*HO3*	At1g69720	By homology
6	3			*HO4*	At1g58300	By homology
6	4	Phytochromobilin synthase	1	*HY2*	At3g09150	Mutant isolated as *hy2*
7	1	Mg-chelatase ChlI	2	*CHLI1*	At4g18490	
7	2			*CHLI2*	At5g45930	
7	3	ChlH	1	*CHLH*	At5g13630	Mutant isolated as *gun5*
7	4	ChlD	1	*CHLD*	At1g08520	
8	1	SAM Mg-protoporphyrin IX methyltransferase	1	*CHLM*	At4g25080	
8	2	Mg-protoporphyrin IX monomethylester cyclase	1	*CRD1*	At3g56940	
8	3	Protochlorophyllide oxidoreductase	3	*PORA*	At5g54190	
8	4			*PORB*	At4g27440	
9	1			*PORC*	At1g03630	
9	2	Chlorophyll synthetase	1	*CHLG*	At3g51820	
9	3	Chlorophyll *a* oxygenase	1	*CAO*	At1g44446	
		Control Genes				
9	4	Monogalactosyl diacylglycerol synthetase	3	*MGD1*	At4g31780	Chloroplast marker
10	1			*MGD2*	At5g20410	Chloroplast marker
10	2			*MGD3*	At2g11810	Chloroplast marker
10	3	Response regulator	1	*ARR5*	At3g48100	Cytokinin-responsive
10	4	Low temperature and salt responsive protein	1	*RCI2a*	At3g05880	Abscisic acid responsive
11	1	12-Oxophytodienoate reductase	2	*OPR1*	At1g76680	Jasmonate-responsive
11	2			*OPR2*	At1g76690	Jasmonate-responsive
11	3	Aconitate hydratase	1	*ACO*	At4g35830	Ethylene-responsive
11	4	Pathogenesis related protein 1 (PR1)	1	*PR1*	At2g14610	Salicylate-responsive
12	1	Actin (housekeeping)	1	*ACT8*	At1g49240	House keeping
12	2	Polyubiquitin (UBQ10)	1	*UBQ10*	At4g05320	House keeping
12	3	Light-harvesting Chl *a/b* binding protein	1	*Lhcb6*	At1g15820	Chloroplast marker
12	4	Negative control (λDNA)	1			Negative control

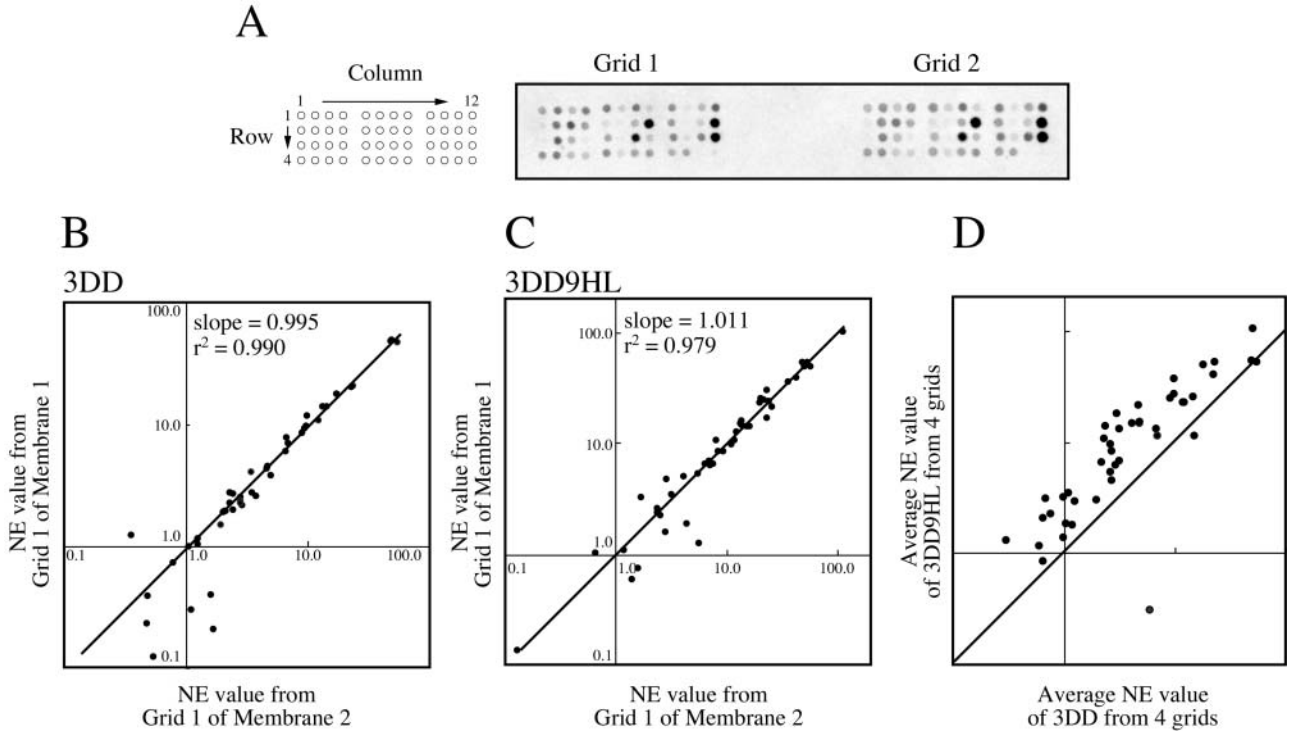

Figure 1. Evaluation and reproducibility of the developed mini-array system. A, Design and scanned image of the mini-array. On one membrane, two grids consisting of 48 genes as described in Table I were spotted. Two scanned images from one membrane after hybridization (right). Results from two different array membranes hybridized with target DNA prepared from 3-d-old etiolated seedlings (3DD; B) and subsequently illuminated for 9 h (3DD9HL; C). NE values obtained from grid 1 of membrane 1 and 2 were plotted. A fitted line was drawn, and the obtained slope and correlation factors are described in the inset. D, Comparison between 3DD and 3DD9HL. In this plot, averaged NE values from four images of each sample are plotted. A line with slope 1 was drawn in the plot. The data showed that most genes are induced in 3DD9HL, while two spots below the line represent suppressed genes.

were hybridized simultaneously with ^{33}P-labeled cDNA probe synthesized from each sample. Consequently, four images were obtained from each sample, which were almost indistinguishable either in the same or in another mini-array membrane (Fig. 1A). To make quantitative evaluations, the filter background, which is 0.8 times the lowest signal, was subtracted from the hybridization value, and then normalized by division with the signal intensity of the housekeeping *ACT*. This lower limit was selected since at levels closer to the background, a slight variation in background levels across the filter artificially affects the profile (see ''Materials and Methods''). Figure 1, B and C, shows plots of two representative sets of data on row signal intensity from two samples. A good correlation was obtained in all data sets. We observed a linear correlation with a mean correlation factor of more than 0.98 and mean slope of 1.03 to 1.04 with a SD of less than 0.09. Thus, the developed mini-array system has a high degree of reproducibility and is valid for linear quantitative determination of the expression of genes of tetrapyrrole biosynthesis in a genome-wide fashion. When averaged data from two samples were compared, most of the spots showed significant changes in their expression level (Fig. 1D).

Gene Expression Profiling of Genes of Tetrapyrrole Biosynthesis at the Onset of Greening

During chloroplast development, higher plants need to synthesize Chl synchronously with Chl-binding proteins. In fact, it has been demonstrated that the expression of Chl-binding proteins and key enzymes of tetrapyrrole biosynthesis, such as glutamyl-tRNA reductase (Tanaka et al., 1996), Glu 1-semialdehyde aminotransferase (Ilag et al., 1994), and subunits of Mg-chelatase (Jensen et al., 1996), is induced by illumination. To clarify the expression profiles of the genes involved in the biosynthesis of tetrapyrrole during chloroplast development, total RNA was extracted from 3-d-old etiolated seedlings illuminated for various periods up to 24 h and analyzed with the proposed mini-array system. Hybridization experiments were carried out with triplicate independent sampling of seedlings (Supplemental Table II).

After subsequent data processing, normalized expression (NE) values (see ''Materials and Methods'') were obtained for each gene. The NE values were further transformed into values relative to the NE values of a dark control (0 h), since these relative values are generally favorable for categorizing expression

profiles, while the NE is valuable for evaluating absolute expression levels. Accuracy of resultant expression profiles was confirmed by northern-blot analysis (Supplemental Fig. 3). Subsequently, using the profile of the relative value from each gene, we performed clustering for all the genes using self-organizing mapping (SOM; Tamayo et al., 1999) with the GeneCluster2 software. Subsequently, genes were grouped into 4 categories from cluster 1 (c1) to cluster 4 (c4). The resulting four categories showed a good correlation with the fold changes of the maximum or minimum NE value during greening to that of the dark control, which we termed the maximum expression ratio (Fig. 2). This value indicates the level of induction or repression of gene expression during the onset of greening. The maximum expression ratio was 3.5 to 22.7 for c1, 2.0 to 4.5 for c2, 1.8 to 2.3 for c3, and less than 0.3 for c4. As shown in Figure 2A, c1 represents a group for genes the expression of which was rapidly induced by light within 1 h and reached a plateau after 3 h of illumination. The expression of *Lhcb* was rapidly induced within 3 h, and then a second peak appeared after 12 h of illumination. In this cluster, four genes were involved, *HEMA1*, an isoform of glutamyl-tRNA reductase, *CHLH*, a subunit of Mg-chelatase, *CRD1*, a putative Mg-protoporphyrin IX monomethylester cyclase, and *CAO*, chlorophyll(ide) *a* oxygenase. c2 is the biggest cluster with 16 genes, showing a gradual increase in expression and a maximum after 9 to 12 h of illumination (Fig. 2B). Interestingly, except for *PORC*, a light-inducible isoform of NADPH: protochlorophyllide oxidoreductase (POR), *CHLG* encoding Chl synthetase, and *HO2* encoding heme oxygenase, all other genes encode enzymes involved in the earlier steps of tetrapyrrole biosynthesis up to the insertion of metal into protoporphyrin IX. Thirteen genes belonging to c3 show an almost constitutive expression, although the expression of some genes fluctuated slightly after illumination (Fig. 2C). These fluctuations are probably caused by a slight variation in the expression levels of genes with very low NE values. Two genes, *PORA* and *PORB*, both isoforms of POR, belonged to c4 (Fig. 2D). These were the only two genes that were negatively regulated by light. They are characteristic as they showed a high level of NE in the dark control, but this value decreased dramatically within 3 to 6 h, which is consistent with a previous study by Armstrong et al. (1995).

Rhythmic Regulation of the Genes of Tetrapyrrole Biosynthesis in Mature Plants

In mature leaves that possess developed chloroplasts, the synthesis of Chl and Chl-binding proteins is closely coordinated and regulated by light and the endogenous clock for the assembly of a functional photosynthetic apparatus (Beator and Kloppstech, 1993). To determine the gene expression profiles for tetrapyrrole biosynthesis under diurnal and circadian rhythms, samplings were carried out from 3-week-old seedlings of Arabidopsis harvested after growth under a cyclic 12-h light/12-h dark regime or subsequently after a second round of continuous illumination. Total RNA isolated from the samples was analyzed using the mini-array system in duplicated hybridization experiments (Supplemental Table III).

After data processing, the SOM analysis clustered genes into three categories: the first group is regulated by both diurnal and circadian rhythms, the second is only regulated by diurnal rhythm, and the third is nonrhythmic (Fig. 3). In these plots, to compare the relative changes in gene expression throughout the tested periods, the NE value from each gene was normalized by dividing by the average NE value of each gene. In this case, the resulting relative ratio gives the relative fluctuation of gene expression and the average of NE is the average expression level relative to that of *ACT*.

The first group contained six genes. Interestingly, this group was composed of the four genes in cluster c1 and the two genes in cluster c4 (Fig. 3, A and B). The phase and amplitude of the four genes in c1 were synchronized and coincided with that of *Lhcb*, whereas those of the two genes in c4 (*PORA* and *PORB*) were synchronized to each other, but the peak of oscillation was somewhat delayed compared to that of c1. These results suggest that four genes in c1, which are dramatically induced by illumination at the onset of greening, are also important for coordinating the assembly of a functional photosynthetic apparatus with the endogenous clock, and two genes in c4 may be differentially regulated by the endogenous clock to maintain Chl biosynthesis. As shown in Figure 3, C and D, 19 genes were clustered into the second group, which was regulated only by diurnal rhythm. This group was composed of the c2 and c3 clusters and 16 genes corresponding to the earlier steps of tetrapyrrole biosynthesis up to the metal insertion stage. Moreover, the phase and amplitude of all genes coincided well under diurnal light/dark cycles (Fig. 3C). When plants were transferred to continuous light, the rhythmic expression of these genes disappeared, although some fluctuations remained (Fig. 3D). The remaining genes involved in the c2 and c3 cluster did not show a pronounced change in expression under diurnal or circadian growth conditions (Fig. 3, E and F).

Development-Dependent Expression of the Genes of Tetrapyrrole Biosynthesis

Finally, we determined the development-dependent control of the expression of these genes. Whole plants were sampled every week after sowing and analyzed using the mini-array system (Supplemental Table IV). The relative expression level in each developmental stage was calculated by dividing the NE value of each developmental stage by the average NE value for all the stages. In this experiment, regardless of the clusters of light responsiveness, all genes basically showed a similar profile of expression (Fig. 4). The highest

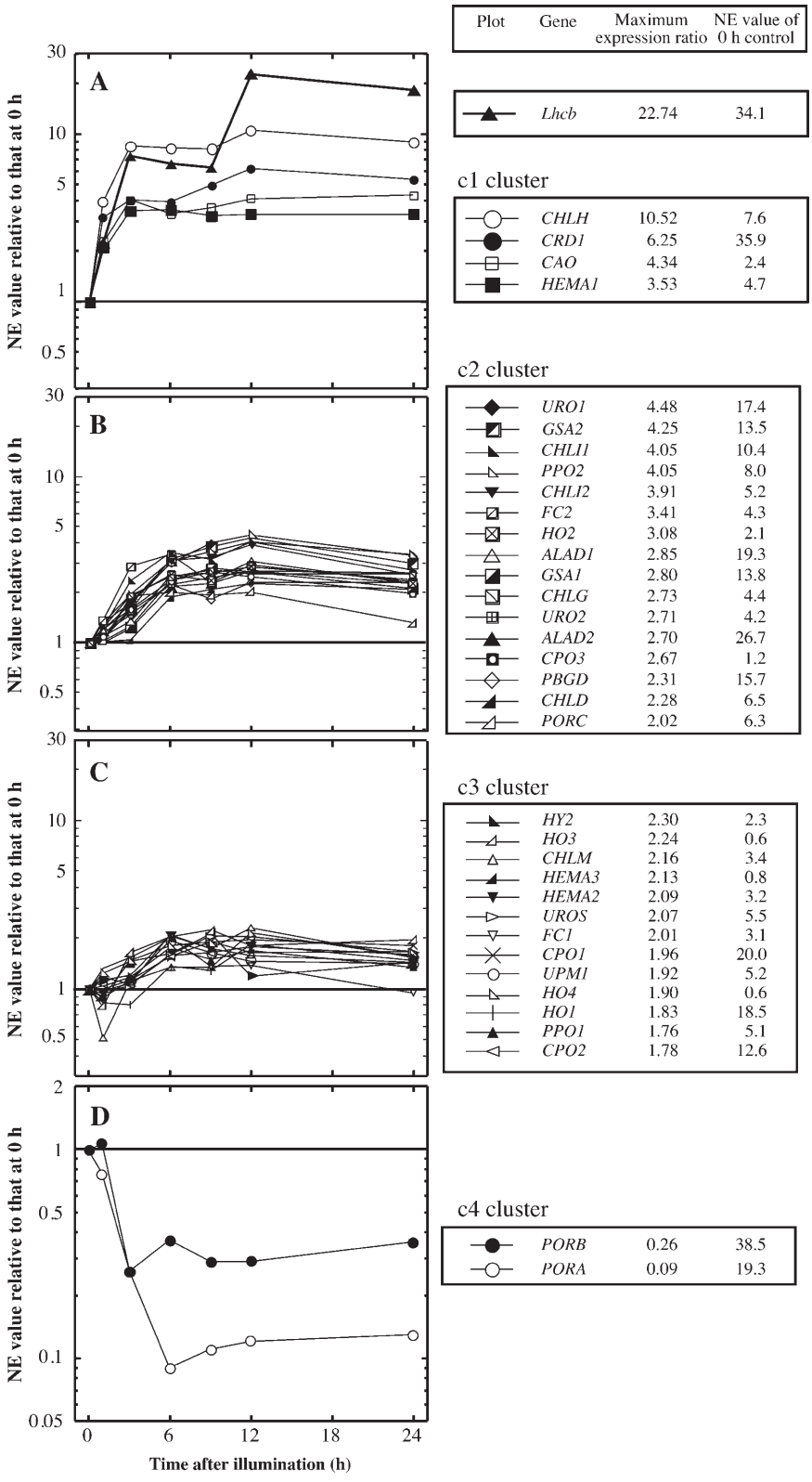

Figure 2. Clustering of genes involved in tetrapyrrole biosynthesis at the onset of greening. Expression profiles of each gene at the onset of greening (0, 1, 3, 6, 9, 12, and 24 h after illumination) were clustered into four groups based on SOM analysis. In each plot, relative NE values to that of 0 h control were plotted on a semilogarithmic scale. Genes clustered in c1 and *Lhcb* (A), c2 (B), c3 (C), and c4 (D) are plotted. The maximum expression ratio and NE value of the 0 h control of each gene are shown in the right section.

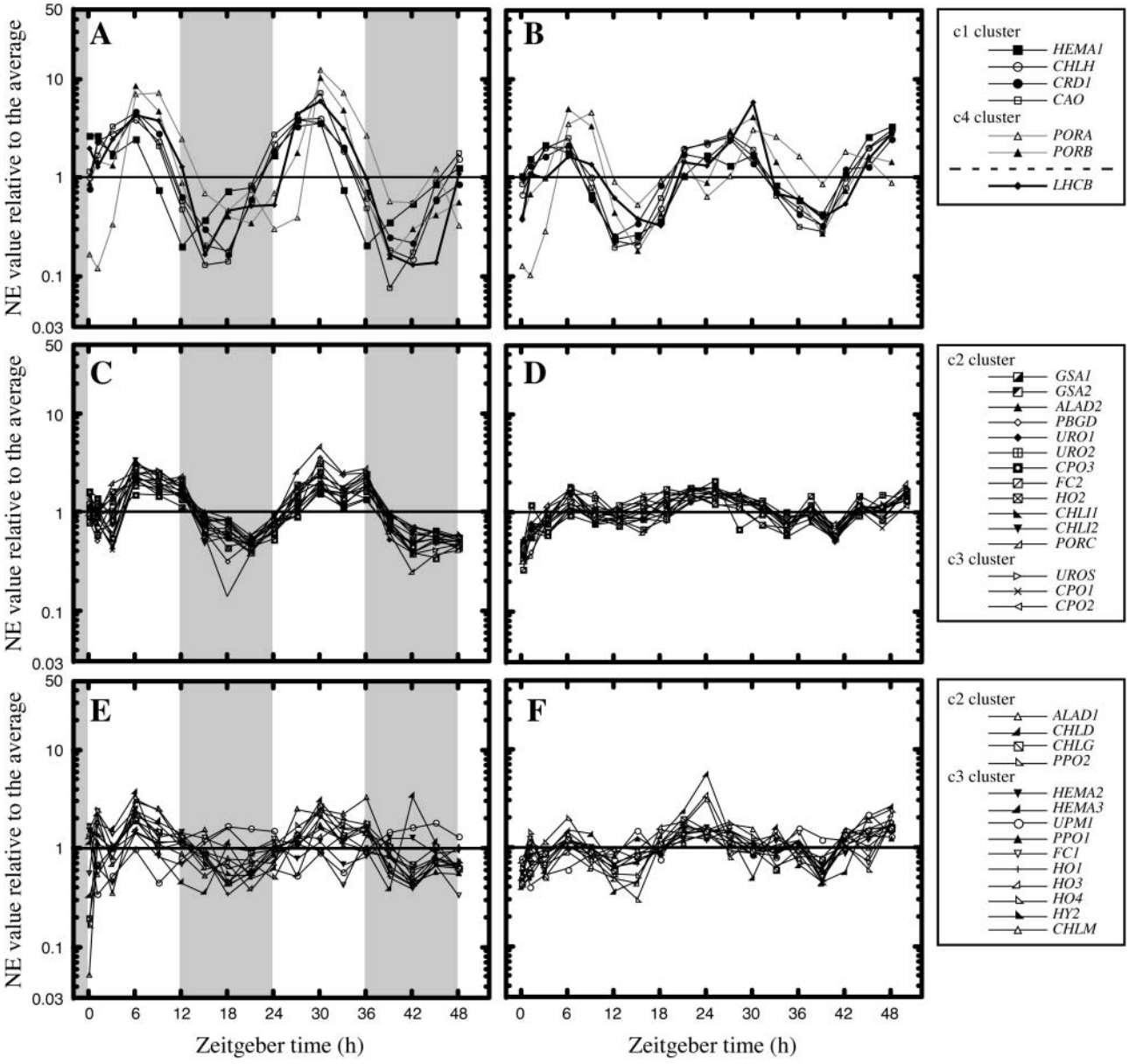

Figure 3. Rhythmic regulation of the genes involved in tetrapyrrole biosynthesis in mature plants. Expression profiles of each gene under diurnal (A, C, and E) and circadian (B, D, and F) growth conditions were clustered into three groups based on SOM analysis. In the diurnal cycle, 3-week-old seedlings were maintained on a 12-h light/12-h dark (gray) cycle, while they were transferred under continuous light for the circadian experiment. Zeitgeber time (hours after onset of experiments) is indicated at the bottom. In each plot, the NE value relative to the average NE was plotted on a semilogarithmic scale. Genes regulated by both diurnal and circadian rhythms (A and B), genes only regulated by diurnal rhythm (C and D), and nonrhythmic genes (E and F) are plotted. Genes in each group are depicted in the right section.

level of gene expression was observed after 1 week of germination, after which there was a decline to the average level, which was retained until week 4. Although several genes showed a peak of expression after 3 weeks of germination, we could not find definite correlation of these genes with the gene clusters. The level then declined after 5 weeks of germination. Thus, it is likely that the genes of tetrapyrrole biosynthesis tested with this mini-array system are similarly controlled by development, although they markedly differed in the light-responsive and rhythmic control of their expression.

DISCUSSION

In this study, we developed a mini-array system to monitor the genes of tetrapyrrole biosynthesis in

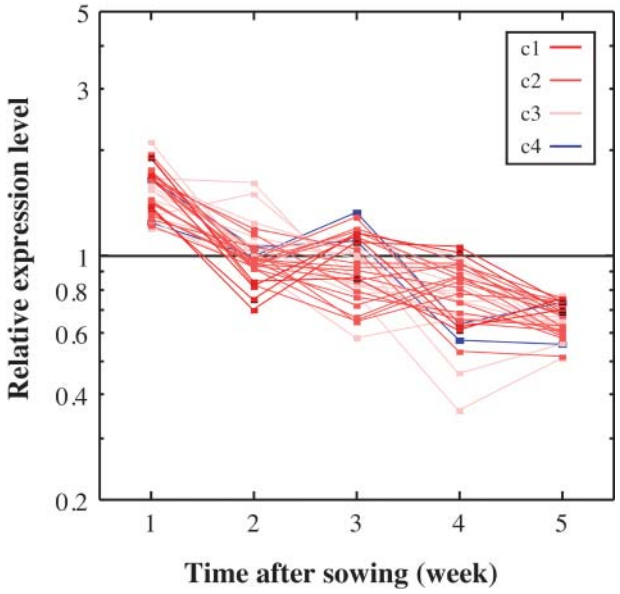

Figure 4. Development-dependent expression of the genes of tetrapyrrole biosynthesis. Development-dependent gene expression profiles are plotted. Samples were obtained every week after sowing. In each plot, the NE value relative to the average NE was plotted on a semi-logarithmic scale. Since all genes basically showed a similar profile of expression, they are plotted in one chart.

Arabidopsis. While the technical principle is not new, owing to precautions taken to prepare DNA probes of 150–350 bp with high gene specificity and to immobilize an exact quantity (4 ng) of highly purified probe to nylon membranes, the data presented here show that this mini-array method has good reproducibility and sensitivity (Fig. 1). It has been reported that the sensitivity of mini-arrays was dependent on the concentration of probe on the filter (Bertucci et al., 1999), and 4×10^9 molecules/spot are necessary for a quantitative analysis using the mini-array method with colorimetrical detection (Quere et al., 2002). In this study, 4 ng of probe DNA is equivalent to 1×10^{10} target molecules/spot, if one assumes that the average probe size is 300 bp and all the materials spotted bind to the membrane and are available for hybridization. Thus, this mini-array system is considered to have a sensitivity comparable to that of other macro- and microarray systems. Furthermore, a significant advantage in cost enabled gene expression analysis of various physiological events, such as greening, rhythmic control, and developmental control. We have confirmed that it is possible to reuse membranes several times after stripping the target DNA (data not shown). Thus, this method is effective and inexpensive for quantitative analysis of the expression of each gene involved in tetrapyrrole biosynthesis in Arabidopsis.

Using this system, we obtained information on the absolute value (NE) and the value relative to appropriate control levels of gene expression, which is important to evaluate the exact contribution of each gene and the profiles of each expression, respectively. The calculation of NE value comprises two steps, subtraction from an appropriate background value and normalization to that of *ACT*. The choice of background was critical as the profiles of genes expressed at very low levels were greatly affected by slight variations in the background level. We found that a background 0.8 times the level of the lowest signal usually gave a linear correlation and high reproducibility, as in the case of cDNA macroarray experiments (Obayashi et al., 2004).

Clustering of Expression Profiles by SOM

So far, several algorithms are available for clustering array data, such as one-dimensional and two-dimensional SOM. In this study, we use one-dimensional SOM for clustering, since this procedure is simple to understand and appropriate for categorizing small groups of genes. In clustering, a smaller number of categories is better for understanding the physiological characteristics in each cluster. In this study, the genes of tetrapyrrole biosynthesis were clustered into four major groups based on their expression profiles during greening. Genes in c1 and c4 were clearly distinguishable as having pronounced changes in expression, although the classification of genes in c2 and c3 was somewhat obscure. We carried out repetitive SOM analyses by modifying the number of categories, and finally determined four gene clusters. These four categories showed a good correlation with the maximum expression ratio. Moreover, genes in the same group were similarly controlled in an endogenous rhythm. A summary of gene clustering and the NE value of each gene in 3-d-old etiolated and 3-week-old mature seedlings are shown in Figure 5.

The c1 Cluster Comprises the Most Important Regulatory Genes in Tetrapyrrole Biosynthesis

Four genes in the c1 cluster were rapidly induced by light at the onset of greening and synchronously fluctuated under diurnal and circadian rhythms. It should be noted that the oscillation and amplitude of the rhythmic regulation of these genes are synchronized to those of *Lhcb*. The pronounced regulation of these genes suggests that they are the most important regulatory gene group for the biosynthesis and flow into the Chl branch pathway of tetrapyrroles.

The first gene in c1 was *HEMA1* encoding glutamyl-tRNA reductase, which is the first committed enzyme of ALA biosynthesis. The formation of ALA is the rate-limiting step for tetrapyrrole biosynthesis and is the primary determinant of the rate of Chl synthesis in light-grown plants (Beale and Weinstein, 1991). Among three isoforms of *HEMA*, *HEMA1* showed the highest NE values in young and mature tissues. Consistent with our results, the expression of *HEMA1* of Arabidopsis is reported to be positively regulated

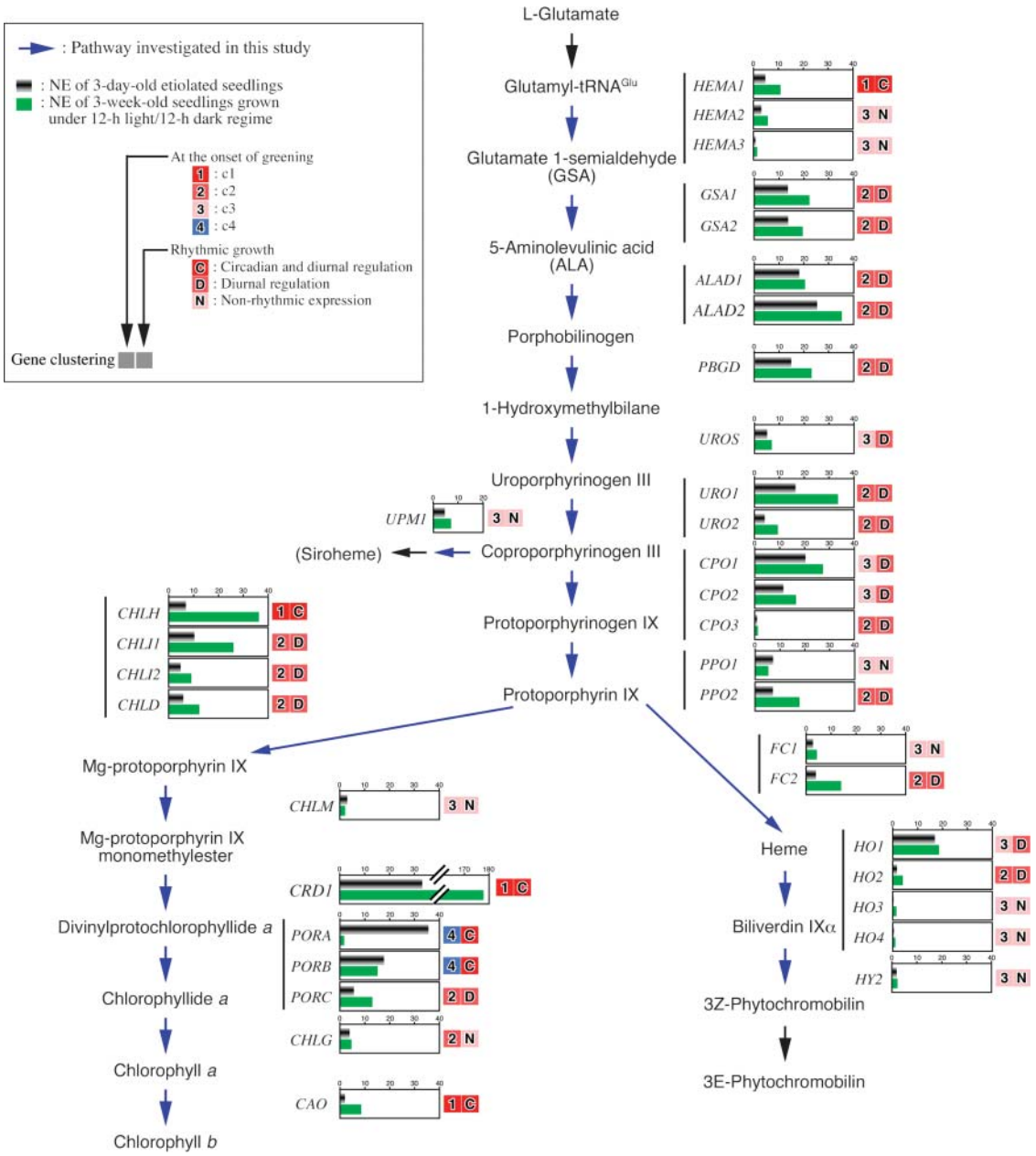

Figure 5. Summary of gene expression analysis with the mini-array system. The tetrapyrrole biosynthetic pathway is depicted. Genes investigated in this study are shown by blue arrows in the pathway. For each gene, the NE values of 3-d-old etiolated seedlings (black bar) and 3-week-old seedlings grown under 12-h light/12-h dark regime (green bar) are shown. Results of gene clustering at the onset of greening, and rhythmic growth are shown on the right.

by light (Ilag et al., 1994) through phytochrome (McCormac et al., 2001), while that of *HEMA2* is independent of light (Kumar et al., 1996; Ujwal et al., 2002). Thus, it is apparent that the level of *HEMA1* expression primarily determines the flow of total tetrapyrroles in photosynthetic tissues.

The second gene in c1 was *CHLH* encoding a subunit of Mg-chelatase, which is the branch point of Chl and heme biosynthesis. Mg-chelatase is composed of three subunits, which are commonly referred to as ChlI, ChlD, and ChlH. CHLH is demonstrated to be the protoporphyrin IX-binding subunit (Gibson et al., 1995; Karger et al., 2001). The expression of the *CHLH* gene is known to be induced by light in Arabidopsis (Gibson et al., 1996), soybean (Nakayama et al., 1998), and barley (Jensen et al., 1996). Thus, CHLH is the determinant of the flux of protoporphyrin IX into Chl biosynthesis at the branch point of the tetrapyrrole biosynthetic pathway. Furthermore, experiments with the Arabidopsis mutant GUN5 revealed the involvement of the CHLH subunit in the plastid-to-nucleus signal transduction. Currently, it is thought that Mg-

protoporphyrin IX accumulation triggers an intracellular signaling pathway that regulates nuclear gene expression (Rodermel and Park, 2003; Strand et al., 2003). Therefore, it is likely that CHLH is important for the coordinated assembly of nuclear-encoded proteins with Chls and plastid-encoded proteins (Mochizuki et al., 2001; Rodermel, 2001).

The third gene in c1 was *CRD1*, which had the highest NE value among all the genes tested. This gene was first isolated from pea as a mesophyll-specific cDNA that is regulated by a phytochrome and circadian rhythm (Zheng et al., 1998). Then, this gene was isolated from *Chlamydomonas reinhardtii* by screening for mutants that failed to accumulate photosystem I and light-harvesting complex I under copper-deficient conditions (Moseley et al., 2000). It is reported that the expression of *CRD1* in Chlamydomonas is increased in copper-deficient or anaerobic conditions (Moseley et al., 2002). Subsequently, the disruption of this gene in *Rubrivivax gelatinosus* (*acsF*) resulted in the accumulation of Mg-protoporphyrin IX monomethylester under aerobic conditions (Pinta et al., 2002), indicating that the *CRD1* gene encodes an oxidative Mg-protoporphyrin IX monomethylester cyclase. Recently, the physiological function of CRD1 has been confirmed in Arabidopsis (Tottey et al., 2003). As the *CRD1* gene showed a similar expression profile to other genes in c1, it is likely that the Mg-protoporphyrin IX monomethylester cyclase is also responsible for the regulation of tetrapyrrole biosynthesis. One possibility is that CRD1 is responsible for the plastid-to-nucleus signal transduction like the CHLH protein, since Mg-protoporphyrin IX monomethylester is also known to function as a plastid-derived signal (Kropat et al., 1997). Further analysis is definitely necessary to clarify the regulatory role of CRD1.

The fourth gene in c1 is *CAO* encoding chlorophyll(ide) *a* oxygenase, which is responsible for Chl *b* biosynthesis (Tanaka et al., 1998). It was presumed that the biosynthesis of Chl *b*, which binds to the light-harvesting Chl antenna complex, is regulated by environmental conditions, such as light intensity, rather than coordinated control with other regulatory genes. In fact, it has been reported that the expression of Arabidopsis *CAO* decreased under dim-light conditions (Espineda et al., 1999). Thus, it is possible that the expression of *CAO* is synchronized to that of other genes in c1 to regulate the assembly of the light-harvesting Chl antenna during greening and in matured leaves, and in addition is regulated by light conditions to fine-tune the size of the antenna.

Characteristics of the c2 and c3 Clusters

Most genes in the biggest cluster c2 were involved in the earlier steps of tetrapyrrole biosynthesis up to the insertion of metal. All genes in c2 were induced by light at the onset of greening and showed a diurnal fluctuation in mature leaves. Thus, it is likely that the genes in c2 encoding the enzymes of tetrapyrrole biosynthesis are similarly controlled to provide tetrapyrrole intermediates for the production of Chl and heme in photosynthetic tissues. As these genes were not regulated by circadian rhythm, light may be the primary determinant for the gene expression. A POR isoform, *PORC*, and *CHLG* encoding Chl synthetase were in this group. The light-induced induction of *PORC* was previously reported (Oosawa et al., 2000), but currently we do not know whether such light-regulation is coordinated with the early steps of tetrapyrrole biosynthesis.

The cluster c3 comprised genes that were constitutively expressed with high NE values, such as *HO1*, *CPO1*, and *CPO2*, and with very low NE values, such as *HEMA3*, *HO3*, and *HO4*. It is probable that some genes in this group are pseudogenes or their functional contribution is not significant. Alternatively, it is possible that they are induced under particular environmental conditions, such as stress. In fact, the expression of *HEMA2* and *FC1* in Arabidopsis is known to be induced by a cytoplasmic protein synthesis inhibitor, cycloheximide (data not shown), as well as cucumber (Suzuki et al., 2002). A characteristic of this cluster is that all genes in the heme branch, except for *FC2* and *HO2*, both of which are in c2, were in the c3 cluster. These results indicate the ubiquitous biosynthesis of heme in higher plants, which is independent of environmental changes. Such a constant heme supply may facilitate the assembly of heme- and phycobiliprotein, such as phytochrome, to sense the environmental conditions.

The c4 Cluster Comprises Negatively Light-Regulated POR Isoforms, PORA and PORB

Two genes in the cluster c4 were well-known isoforms of *PORA* and *PORB*, which were negatively regulated by light. The proteins encoded by these two genes are known to be the major components of prolamellar bodies in etioplasts of dark-grown seedlings (Masuda et al., 2003). It has been reported that both mRNAs are accumulated in etiolated seedlings but only *PORB* mRNA continues to accumulate in light-grown plants, while *PORA* mRNA rapidly disappeared after illumination. This study, however, showed that the expression of *PORA* persisted in mature leaves under circadian rhythmic control, as well as that of *PORB*, although the NE value of *PORA* was 7 to 15 times lower than that of *PORB*. Thus, it is likely that *PORA*, as well as *PORB*, persists in its expression in mature leaves with rhythmic fluctuation to provide a way to adjust the synthesis of POR to meet the varying needs of fully green plants for Chl. It should be noted that oscillations of PORA and PORB were somewhat delayed to those of genes in c1. Database searches of plant cis-acting regulatory DNA elements (PLACE; Higo et al., 1999) detected a conserved motif, the nine nucleotide AAAATATCT "evening element" in the 5′-upstream regions of *PORA* and *PORB* but not in c1 (Harmer et al., 2000).

Thus, it is likely that these two genes are differentially controlled to maintain Chl biosynthesis.

CONCLUSION

In summary, we have developed a mini-array of the genes involved in the biosynthesis of tetrapyrrole in Arabidopsis. The system is reasonably cheap, is highly sensitive, and can be used to monitor the expression profiles of all genes specifically and simultaneously. All these parameters will contribute to the study of other metabolic or regulatory genes of interest. With this system, we could group genes into four categories based on their expression profile during greening, although c2 and c3 have insufficient features to classify further. Clustering of c1 and c2 was also applicable to the rhythmic expression of the genes of tetrapyrrole biosynthesis. Interestingly, a good coordination of gene expression was observed in each group, suggesting that each group of genes is regulated by common regulatory machinery. Among them, the c1 cluster contained the most important genes that control the biosynthesis and efflux of tetrapyrroles in Arabidopsis. Further analysis with the aid of this system will contribute to the elucidation of the complete regulatory circuit of tetrapyrrole biosynthesis in Arabidopsis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seed stocks of Arabidopsis L. Heynh from the Columbia (Col) ecotype were used for all experiments. Surface-sterilized seeds were plated on a 0.8% (w/v) agar medium containing Murashige and Skoog (1962) salts. Plates were placed at 4°C in darkness for 3 d prior to receiving 1 h of white light irradiation to synchronize germination. Then, plates were exposed to white fluorescent light (50 μmol m^{-2} s^{-1}). For rhythm experiments, seedlings were grown on the same medium supplemented with 1% (w/v) Suc at 23°C in an environmental growth chamber under a 12-h light/12-h dark cycle. For development experiments, seedlings were grown under continuous white light illumination.

Preparation of Probes and Production of Mini-Array Membranes

Total RNA was prepared from 3-d-old etiolated or 2- to 4-week-old mature plants with an RNeasy Mini Kit (Qiagen, Chartsworth, CA) as recommended by the manufacturer. RT-PCR was carried out with RNA PCR kit (Avian Myeloblastosis Virus) version 2.1 (Takara, Otsu, Japan) with 1 μg of total RNA according to the manufacturer's instruction. RT-PCR amplification was carried out with cognate gene-specific primers (Supplemental Table I) as follows: 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The DNA fragments were checked by agarose electrophoresis (Supplemental Fig. 1), cloned into a TA-cloning vector, and subsequently sequenced using a Thermo Sequenase Sequencing kit (Amersham Bioscience, Piscataway, NJ). The inserted DNA fragment of each clone was amplified by PCR with universal M13 forward and reverse primers. PCR products were purified with a Qiaquick PCR Purification kit (Qiagen, Valencia, CA), and their DNA concentration was determined by spectrometry and agarose electrophoresis. Using the spotting machines, the Hydra Microdispenser and Tango Liquid Handling system (Shieh et al., 2002), 4 ng of each probe was spotted onto a nylon membrane (1.5 cm × 8 cm) in duplicate and fixed with UV-crosslinking (Labo, Tokyo).

Genomic Southern Hybridization Analysis

The gene specificity of the DNA probes was confirmed by genomic southern hybridization analysis (Supplemental Fig. 2). Genomic DNA was isolated from Arabidopsis seedlings according to Murray and Thompson (1980). The DNA (3 μg) was digested with restriction enzymes, separated on agarose gel, transferred onto nylon membrane, and hybridized with ^{32}P-labeled probes. The labeling of probes was carried out with Random Primer DNA Labeling kit Ver.2 (Takara). After overnight hybridization at 65°C, blots were washed twice with 0.2 × SSC, 0.1% SDS at 65°C, and then exposed to an imaging plate (Fuji Film, Tokyo) for detection.

Mini-Array Hybridization and Image Analysis

Total RNA was prepared from samples as described above. With the resultant RNA sample (10 μg) as a template, the labeling of the target DNA was carried out by RT in the presence of [α-^{33}P] dCTP and 1 μg of oligo(dT)$_{16-18}$ primer with a SuperScript First-Strand Synthesis system (Invitrogen) according to the manufacturer's directions. The labeled cDNA was denatured and used as target DNA for hybridization. Hybridization with the labeled target was performed in the presence of 0.5 M Na_2HPO_4, 1 mM EDTA, and 7% SDS (Church and Gilbert, 1984) at 65°C for 16 h. After incubation, the membranes were washed twice with 0.2 × SSC, and 0.1% SDS at 65°C, and then exposed to an imaging plate (Fuji Film) for detection.

Radioactive images were obtained with a high-resolution scanner (Storm, Amersham Bioscience), and the signal intensity was quantified with Array Vision software (Amersham Bioscience). Quantitative analysis of hybridization with the same probe resulted in high degree of reproducibility (Fig. 1). To normalize the hybridization signal intensities of each membrane, it was first necessary to subtract the appropriate background value. The subtraction of a real value, such as the signal intensity of nonspotted area or λDNA spotted area, as a background from the intensity of each spot sometimes caused large fluctuations of particularly low intensity signals. According to our experiments using cDNA macroarray data (Obayashi et al., 2004), we adopted 0.8 times the lowest signal intensity of each membrane as the appropriate background value. Then, the value of each spot was normalized by that of *ACT*, a housekeeping control, as percentage of *ACT*. The average of this value from replicated experiments was calculated, and resultant value was defined as the NE of each gene.

Clustering of Gene Expression Profiles

For the clustering of gene expression profiles, one-dimensional SOM was carried out using GeneCluster2 released by the Whitehead Institute (http://www-genome.wi.mit.edu/cancer/software/software.html). As a result of a trial altering the number of categories, four major clusters were obtained by the SOM of gene profiles during greening. Similarly, analysis was carried out in terms of profiles of rhythmic and development-dependent expression.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

ACKNOWLEDGMENTS

We are grateful to Hiroshi Shimada for valuable discussions. We would like to thank Nozomi Taki for technical assistance in preparing the radiolabeled cDNA targets for hybridization.

Received March 10, 2004; returned for revision April 30, 2004; accepted May 3, 2004.

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