Top-down Phenomics of Arabidopsis thaliana

METABOLIC PROFILING BY ONE- AND TWO-DIMENSIONAL NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND TRANSCRIPTOME ANALYSIS OF ALBINO MUTANTS

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Elucidating the function of each gene in a genome is important for understanding the whole organism. We previously constructed 4000 disruptant mutants of Arabidopsis by insertion of Ds transposons. Here, we describe a top-down phenomics approach based on metabolic profiling that uses one-dimensional 1H and two-dimensional 1H,13C NMR analyses and transcriptome analysis of albino mutant lines of Arabidopsis. One-dimensional 1H NMR metabolic fingerprinting revealed global metabolic changes in the albino mutants, notably a decrease in aromatic metabolites and changes in aliphatic metabolites. NMR measurements of plants fed with 13C-glucose showed that the albino lines had dramatically different 13C-labeling patterns and increased levels of several amino acids, especially Asn and Gln. Microarray analysis of one of the albino lines revealed a unique expression profile and showed that changes in the expression of genes encoding metabolic enzymes did not correspond with changes in the levels of metabolites. Collectively, these results suggest that albino mutants lose the normal carbon/nitrogen balance, presumably mainly through lack of photosynthesis. Our study offers an idea of how much the metabolite network is affected by chloroplast function in plants and shows the effectiveness of NMR-based metabolic analysis for metabolic profiling. On the basis of these findings, we propose that future investigations of plant systems biology combine transcriptomic, metabolomic, and phenomic analyses of gene disruptant lines.

Plants produce energy sources, foods, and industrial or medical materials that are necessary and useful for us. It has been expected to handle the metabolic flows so as to increase the amount of interested chemical resources in plants. For that purpose, comprehensive understanding of the metabolite network is required. Presumably, the metabolite network is regulated by both genetic information and cellular conditions. In recent years, to address the key factors regulating the metabolite network, metabolomics, the nontargeting comprehensive metabolite analysis of plants, has been introduced in plant research as well as other organisms. To elucidate the linkage between genetic information and metabolites, attempts to integrate transcriptome and metabolomics data have been made. Hirai et al. (1) described the linkage between the levels of some secondary metabolites, such as glucosinolates and the transcripts for genes encoding corresponding metabolic enzymes. However, such approaches for the primary metabolic pathway did not give us clear information on the regulatory systems, presumably due to higher complexity (1–3).

To understand such complex biological phenomena, a systems biological approach should be one of the promising ways. To establish systems biology, a vast amount of information on cellular entities, such as transcript, proteins, and metabolites is required and combined functionally. Computational models have been developed for Escherichia coli and Saccharomyces cerevisiae based on data from gene disruption mutants (4–8) and have proven to be useful for predicting the phenotypes of mutants, cellular behavior, and evolution (9–13).

Completion of the Arabidopsis and rice genome sequences (14–17) has allowed the application of systems biology to plants (18, 19). Furthermore, a large number of gene disruption mutants have been constructed in various plant species, especially Arabidopsis and rice. By taking advantage of these resources, functional genomic approaches can be used to elucidate the function of plant genes (20). In a previous report, we described a phenomic approach analyzing the effects of 4000 gene disruptions in Arabidopsis (21). We found that fewer than 5% of the gene disruptions caused a visible phenotype in the aerial parts, suggesting that a more detailed and practical methodology is needed to determine the effects of losses in gene function.

For the purpose of establishing systems biology in plants, we propose the comprehensive analysis of gene disruptant lines by...
Metabolic Profiling of Albino Mutants

In this study, we analyzed three Ds transposon insertion mutants of Arabidopsis that display albino phenotype. The albino phenotype is often due to retarded or disrupted chloroplast development. The effects of the loss of chloroplast function on the metabolic pathways, however, have not been previously investigated. Analysis of extracts from albino plants by one-dimensional $^1$H NMR spectroscopy with stable isotope labeling, along with transcriptional and phenomic analyses. In the current study, we analyzed the loss of chloroplast function on the metabolic pathways, including amino acids. Our results indicate that the albino mutations substantially altered the metabolite profile. Microarray analysis of one of the albino lines revealed a unique pattern of gene expression. We discuss the mechanism of changes in metabolites on the basis of this microarray data and the usefulness of metabolic analysis of disruptive mutants in determining gene functions in plants.

EXPERIMENTAL PROCEDURES

Plant Materials and Sample Preparation—Three albino mutants of Arabidopsis were obtained from the RIKEN Biological Resource Center (available on the World Wide Web at rarge.gsc.riken.jp/) (21, 22). One of the parents of the Ds insertion lines, Ds13, was used as the control line (22). The seed were sterilized in 2.5% NaClO containing 0.1% Triton X-100 for 5 min and then washed five times with water. The sterilized seeds were kept for 4 days at 4 °C and then germinated and grown on agar plates containing half-strength Murashige and Skoog medium containing 0.5% glucose or $^{13}$C$_6$-glucose (Cambridge Isotope Laboratories, Inc., Andover, MA). The plates were kept at 23 °C under a 16-h light/8-h dark cycle. After 3 weeks, ~8 h into the photoperiod, the aerial parts of 20 plants were harvested randomly and immediately plunged into liquid nitrogen before freeze-drying. NMR samples were prepared essentially as described previously (23, 24). Briefly, 5 mg of the freeze-dried material was extracted with 600 μl of hexafluoroacetone triioderate at 50 °C for 5 min with gentle vortexing. After centrifugation, the extracted supernatant was transferred into a 5-mm Ø NMR tube for NMR measurements.

One- and Two-dimensional NMR Measurements—One-dimensional $^1$H NMR spectra of unlabeled samples were acquired at 298 K on a Bruker DRX-500 NMR spectrometer equipped with a $^1$H inverse probe and a triple-axis gradient. $^{13}$C-Labeled plants were compared by $^{13}$C-decoupled and coupled Watergate $^1$H NMR pulse sequences to assess their incorporation of $^{13}$C. Two-dimensional HSQC experiments were performed at 298 K with a Bruker DRU-700 NMR spectrometer equipped with a $^1$H inverse cryogenically cooled probe with a z axis gradient. A total of 200 complex f1 ($^{13}$C) and 2048 complex f2 ($^1$H) points were recorded with 36 scans per f1 increment. The spectral widths were 13,381 Hz for f1 and 11,161 Hz for f2. To quantify the signal intensities, a Lorentzian-to-Gaussian window with a Lorentzian line width of 5 Hz and a Gaussian line width of 10 Hz were applied in both dimensions before Fourier transformation. A fifth order polynomial base-line correction was subsequently applied in the f1 dimension. The indirect dimension was zero-filled to 1024 points in the final data matrix. For the $^{13}$C-$^{13}$C coupling analysis, a total of 400 complex f1 points over a spectral width of 7000 Hz was acquired by ultrahigh resolution (UHR) HSQC. NMR spectra were processed using NMRPipe software (25, 26). The $^1$H and $^{13}$C chemical shifts were determined using sodium 2,2’-dimethyl 2-silapentane 5-sulfonate as a reference. The NMR measurements were repeated three times for each sample.

Quantitative Multivariate Analysis of One- and Two-dimensional NMR Spectra—The one-dimensional NMR spectra were integrated between 0.5 and 10.5 ppm over a series of 0.04-ppm integral regions using our custom integration software. After exclusion of the water resonance, each integral region was normalized to the total integral region. Two-dimensional spectral signal assignments were made using our custom software. 4 These well separated two-dimensional spectral signals, aided by HSQC and our data base of standards, allowed the accurate identification of major metabolites. The data were analyzed by partial least-squares projection based on the spectral bins obtained from one- and two-dimensional spectral analyses using the pls package (version 2.0) with the “simples” method running on R software.

Microarray Analysis—Total RNA was isolated from 3-week-old plants of the control line and albino line 2198 at 6 h after the start of the light period. Total RNA was isolated using TRIzol extraction reagent (Invitrogen) and purified with an RNase MinElute Cleanup Kit (Qiagen). First strand cDNA was synthesized from 16 μg of total RNA using a SuperScript double-stranded cDNA synthesis kit (Invitrogen). The labeled cRNA was synthesized using a BioArray RNA transcript labeling kit (T7) (ENZO Life Sciences, Inc., Farmingdale, NY). After labeling, the cRNA was partially digested by incubating it for 35 min at 94 °C in 40 mM Tris acetate (pH 8.0), 100 mM potassium acetate, and 30 mM magnesium acetate. Microarray hybridization was carried out using 30 μg of cRNA. The hybridization and data detection were performed according to the manufacturer’s recommendations (Affymetrix). The data were normalized by robust multiaarray normalization and analyzed using affyInGUI running on R software (27). The normalized data were also analyzed by MapMan (28) and Mev version 4.0 (29) software. Primary array data sets were published in the gene expression and hybridization array data repository of the National Center for Biotechnology Information (Gene Expression Omnibus) (available on the World Wide Web at www.ncbi.nlm.nih.gov/geo/) (accession number GSE6788).

RESULTS

Metabolite Fingerprinting Based on $^1$H One-dimensional NMR Spectral Analysis—In addition to T-DNA-tagged and multielement transposon-tagged lines, gene disruption lines generated using transposon-tagging systems based on the

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The abbreviations used are: HSQC, heteronuclear single quantum coherence; PLS-DA, partial least-squares discriminant analysis; pTAC, plastid active chromosomal protein; UHR, ultrahigh resolution.

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4 E. Chikayama and J. Kikuchi, unpublished data.
Metabolic Profiling of Albino Mutants

FIGURE 1. Establishment of systems biology for Arabidopsis using a combination of phenomic, metabolomic, and transcriptomic analyses. A, to establish systems biology for plants, we propose a comprehensive analysis of Ds gene disruption mutant lines by integrating phenomic, metabolomic, and transcriptomic data. The yellow arrows indicate the flow of the genetic information from the genome to the phenotype, in this case the effects of albino mutations. Metabolites comprise a network(s), and the metabolite status of the plant results in the phenotypes. Information on metabolic fingerprints and profiles of disrupted lines together with gene expression profiles are required for the establishment of systems biology in plants. B, phenotypes of three albino lines and the control line (plus albinos in close-up). Plants were grown on half-strength Murashige and Skoog plates containing 0.5% glucose for 3 weeks. Scale bars, 5 mm.

maize Activator (Ac)/Dissociation (Ds) system are very useful for genetic studies (30, 31). To help establish systems biology in plants, we propose the systematic analysis of gene-disrupted lines by metabolic fingerprinting using one-dimensional $^1$H NMR spectroscopy and metabolic profiling by multidimensional NMR spectroscopy with stable isotope labeling, combined with transcriptomic and phenomic analyses (Fig. 1A). We selected three albino mutants (12-2658-1, 13-2198-1, and 15-1824-1) that have single gene disruptions from RIKEN Ds transposon stocks (21, 22). We refer to these lines as 2658, 2198, and 1824, respectively. All three mutants had an obvious albino phenotype. Line 2658 was slightly bluish, but the three lines were otherwise nearly indistinguishable (Fig. 1B).

The disrupted gene in line 2658 is At1g63970, which encodes 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, an enzyme that catalyzes the fifth step of the nonmevalonate pathway of isoprenoid biosynthesis (32). The biosynthesis of plastid isoprenoids is essential for chloroplast development and plant growth (32–34). The gene disrupted in line 2198 was At5g04260, which encodes one of the plastid active chromosomal proteins, pTAC3 (35). pTACs are required for transcriptional or posttranscriptional processing of mRNA in plastids. T-DNA insertion mutants of other pTACs, namely pTAC2, pTAC6, and pTAC12, have albino or pale green phenotype (35), which is consistent with the albino phenotype of line 2198. In line 1824, At5g08400 was disrupted. Although the function of this gene is still unknown, its gene product is predicted to localize in chloroplasts (36). The obvious albino phenotype of line 1824 suggests that this gene is important for chloroplast function.

We next examined the effect of the albino mutations on the cellular metabolite profile by one-dimensional $^1$H NMR analysis of the three albino lines and the control line. After plants were grown on plates for 3 weeks, extracts were prepared and analyzed by NMR. We did three biologically replication for each line in one experiment set. There were some clear differences in the one-dimensional $^1$H NMR spectra of the albino lines and the control line (Fig. 2). For example, several signals around 1.0 and 7.0 ppm, which correspond to lipids and aromatic compounds, respectively, were stronger in the spectrum of the control line. On the other hand, many signals between 2.5 and 4.0 ppm, corresponding to organic acids and sugars, were stronger in the spectra of the albino lines.

To gain further insight into the metabolic changes caused by the albino mutations, we performed partial least-squares discriminant analysis (PLS-DA). In the PLS-DA, each one-dimensional $^1$H spectrum between 0.5 and 10.5 ppm was subdivided into 250 segments of sequential 0.04-ppm regions (segments for the signal from water were removed). Each segment was normalized by the total intensity of the one-dimensional $^1$H spectrum. PLS-DA allowed differentiation of the different lines (Fig. 2). Components 1 and 2 were clearly different between the albino and control lines. A loading plot showed that chemical shifts around 2.96 and 3.72 ppm and the regions around them made strong positive contributions, and those around 0.96 and 1.32 ppm made negative contributions to component 1 (Fig. 2). These two regions correspond mainly to organic acids and lipids, respectively. For component 2, the regions around 2.96 and 3.72 ppm made positive contributions and that around 2.52, 2.72, and 4.16 ppm made a negative contribution. These results indicate that the albino mutants had a common metabolic phenotype and that the gene malfunctions had different metabolic effects in each mutant although their visible phenotypes were very similar.

Metabolite Profiling and Comparison based on $^1$H/$^13$C HSQC—To obtain more detailed metabolite profiles, we conducted $^1$H/$^13$C HSQC measurements. For these experiments, plants were labeled with $^{13}$C$_6$-glucose according to our previously described methods (23, 24). Briefly, each line was grown on medium containing $^{13}$C$_6$-glucose. After labeling with $^{13}$C$_6$-glucose for 3 weeks, the greening portions of each line were collected and analyzed by NMR. First, we performed $^1$C decoupling $^1$H NMR to estimate the labeling efficiency. Photosynthesis dilutes $^{13}$C incorporated from the roots with $^{12}$C incorporated from CO$_2$. Therefore, we expected to detect the difference of labeling efficiencies between the control and albino lines. The differences between decoupled and coupled spectra were greater in albino lines than in the control line (Fig. S1A). PLS-DA showed a clear separation between the decoupled and coupled data of the albino lines (Fig. S1B). This suggests that the albino lines were labeled more efficiently, as expected. Loading plots identified the peaks that contributed to
the discrimination of these samples. Peaks at 2.72, 2.56, and 4.16 ppm and at 2.64, 3.08, 4.08, and 2.24 ppm were prominent in the coupled and decoupled spectra, respectively (Fig. S1C). These peaks correspond to organic acids, such as amino acids. Although it is difficult to assign these peaks, those at 2.64 and 3.08 ppm may originate from Gln and Asn, respectively. These results support the idea that the albino mutations cause drastic changes in the metabolite profiles.

We detected many 1H,13C HSQC signals in the samples (Fig. 3). These signals originated from 13C atoms bound to 1H in the metabolites. Because there were many peaks with the same 1H chemical shifts, two-dimensional NMR provided better discrimination of the different metabolites. To determine the molecular basis for the effects of mutations or physiological stimuli, it is necessary to know which metabolites change and by how much. Using these clearly separated signals (Fig. 3), our custom program, and data base of chemical shifts for standard metabolites, we were able to assign 80 peaks from 22 metabolites, including amino acids and sugars (Fig. 4A and Table S1). The assignments were supported by the presence of peaks corresponding to multiple carbon atoms in each metabolite (Fig. 4B).

The assignment of metabolites from PLS-DA based on the 1H,13C HSQC spectra is shown in Fig. 4B. The control and albino samples could again be separated according to component 1, suggesting a unique profile of metabolites in the albino mutants. Asn and Gln were the main contributors to the differences between the albino and control lines. Component 2 separated the albino samples. These results agreed well with those obtained by one-dimensional NMR, and confirmed that the metabolite profiles of the albino and control plants were different. The results also indicated some differences in metabolite profiles among the different albino mutants.

We examined the 13C–13C coupling status using UHR HSQC. A typical example of the Asp β and Asnβ peaks is shown in Fig. 5. The 13C–13C coupled signals due to Cα and Cγ in both Asp and Asn were stronger in the albino lines than in the control line. All three albino mutants exhibited clear dd couplings, whereas control signals showed a mixture of s, d1, and d2 couplings. The Asp β signals, however, appeared to be weaker in the albino lines than in the control line, suggesting that the weak peak strengths for Asp β were caused not by a low labeling efficiency, but rather by a low amount of this compound. Although it is difficult to estimate the precise labeling efficiency from these data, the labeling efficiency in the control line was at least half of those in the albino mutants. Similar results were found for other amino and organic acids. Therefore, the relative amounts of metabolites shown in Fig. 4A are a reasonable reflection of the actual differences in their absolute quantities.

It should be noted that the absolute signal strengths were different among experiments. Presumably, the subtle differences in growth conditions, such as humidity, caused such differences (Fig. S2). However, albino samples always showed distinct metabolite profiles. We also prepared plants growing modified media that had only KNO3 for a nitrogen source. The metabolite profiles of albino mutants were different from those described above (Fig. S2). Using these data obtained from different experiments.
that showed the largest differences in expression between the two lines are listed in Table S2.

Using a public microarray data base, Genevestigator (available on the World Wide Web at www.genevestigator.ethz.ch/at/), we compared the expression profiles of the top 100 up- or down-regulated genes in the albino mutants with those affected by other treatments (light, dark, UV exposure, plant hormones, etc.). None of the treatments or stimuli appeared to cause expression patterns similar to that in the albino mutant. This suggests that the expression profile of the albino mutant is unique (Fig. S3A). We also analyzed the expression pattern in the context of the metabolic pathway using the MapMan program (28). Classification of these changes revealed several interesting features of the albino mutant (Fig. 7A). Specifically, the expression of genes involved in photosynthesis was up-regulated in the albino mutant, whereas there was a decrease in the expression of genes related to glycolysis, the TCA cycle, amino acid synthesis and metabolism, phenylpropanoid synthesis, and lipid degradation.

Because the UHR HSQC experiments indicated that the albino lines have different amino acid contents than the control line, we also examined the expression of genes involved in amino acid biosynthesis (Table S3). The mRNA levels of two Arabidopsis nitrate reductase genes, NIA1 and NIA2, were decreased 0.8 and 0.2 times, respectively, and those for all of the glutamine synthase genes were unchanged or slightly decreased. The expression of ASN1 and ASN2 was 1.3 times up-regulated and 2 times down-regulated, respectively. The expression patterns of these genes implied that albino line 2198 has a high nitrogen status. The expression of genes encoding transcription factors was also strongly affected by the albino mutation. Specifically, the expression of genes encoding AUX/IAA transcription factors, response regulators, and C2C2-type transcription factors, such as GATA (37, 38), were considerably up-regulated, whereas the expression of WRKY genes was down-regulated (Fig. S3B). Interestingly, an analysis using Genevestigator of 28 genes encoding WRKY, AUX/IAA transcription factors, and response regulators revealed that the expression pattern in the albino line was similar to that of plants treated with 6-benzyl adenine (Fig. 7B). In addition, several genes whose products were involved in the regulation of circadian rhythm showed different expression levels in albino line 2198. For example, phyA, GIGANTEA, APRRS, APRRT7, CRY1, and LHY1 were up-regulated, whereas CCA1 was down-regu-
lated in the albino line (Table S4). The reported expression phases of these genes do explain these differences (39), suggesting that circadian rhythm is somehow affected in the albino mutant. Blasing et al. (40) demonstrated that sugar levels affect the circadian rhythm. They extensively analyzed the effects of sugars, nitrogen sources, and lights on the diurnal expressions of genes. We compared the expression profiles of these genes in the previous report and this study and failed to find consistent explanation for the effect of the albino mutation.

**DISCUSSION**

By analyzing one-dimensional NMR, we demonstrated that all three albino mutants had metabolite fingerprints distinct from that of the control line. In addition, we analyzed the metabolite profiles by $^{13}$C two-dimensional NMR using the plants grown in the presence of $^{13}$C-glucose and obtained not only the supporting data for one-dimensional NMR results but also the data that show how the carbon source is distributed to metabolites in the absence of chloroplast function in Arabidopsis. The metabolite profiles, especially increased amide amino acid levels, were consistent among all three albino lines even under different growth conditions (Fig. 6), suggesting the strong linkage between phenotype and metabolite profiles. It is not surprising that albino mutants have higher $^{13}$C signals because of less dilution by photosynthesis (Fig. S1). However, the $^{13}$C distributions of albino mutants were quite different from the control line, although the control line also was fed excess $^{13}$C-glucose (Fig. 4). The changes in amino acids do not seem to reflect the reduced function of plastidic amino acid synthesis, indicating rather the indirect effect of the defect in chloroplast development. These observations suggest that chloroplast function or photosynthetic activity is deeply implicated in the regulation of metabolic flow in plants.
Metabolic Profiling of Albino Mutants

Because all albino mutants have higher amide amino acid levels than the control line (Figs. 4A and 6). The metabolic pathways controlling carbon and nitrogen are tightly linked and regulate each other, and their balance is usually referred to as the “C/N balance” (2, 41, 42). The expression patterns of the genes encoding enzymes involved in nitrogen assimilation, such as nitrate reductase and glutamine synthase, are regulated not only by the defect of photosynthesis but also via other physiological functions. Therefore, albino mutations affect the metabolite profiles not only by the defect of photosynthesis but also via other physiological functions.

In addition, because all three albino lines had 6 times higher sucrose levels (Fig. 4A), the disturbed C/N balance cannot be explained by a lower sugar content. Although it has been shown that the addition of sugar activates genes related to nitrogen assimilation, metabolic changes other than those related to photosynthesis may be involved in the regulation of these genes. The change in the cytosolic redox and pH status by photosynthesis could override the effect of sugar on the expression of sugar-repressive nuclear genes (49). It is possible that enzymes in addition to nitrate reductase, which is regulated by post-translational modifications (3, 50, 51), are regulated not only by the metabolite levels but also by the conditions of the cell. Fritz et al. recently showed that amino acids did not affect each other’s level (48). Therefore, there should be other mechanisms for maintaining the cellular amino acid levels. Detailed analysis of metabolic flow should offer some clues in this regard. In the 2198 line, the expression of several circadian rhythm-related genes seemed disturbed, consistent with the report that sugar levels affect the expression of circadian rhythm-related genes (40). However, the reported expression profiles of some of these genes are not consistent with the metabolic conditions in 2198 line, indicating the different effects other than sugar or nitrogen sources on circadian rhythm in the albino line. The relationship between gene functions, albino phenotype, and metabolic profiles will be clearer when data of pale green mutants and other mutants with similar metabolic phenotypes are accumulated in this proposed approach.

The metabolite profile of line 2658 was different from those of the other two albino lines. Although the reason for these differences is not currently clear, it might not be surprising that the lack of the nonmevalonic acid pathway affects more than chloroplast development and therefore produces a distinct metabolic profile. However, this result strongly indicates the usefulness of metabolite profiling in characterization of mutant lines. It is also possible that a slight difference in the strength of the albino phenotype can result in different metabolite profiles. Although the phenotypes of lines 2198 and 1824 were indistinguishable, one-dimensional NMR and two-dimensional NMR indicated that they have distinct metabolite compositions. This supports the latter possibility and emphasizes the effectiveness of metabolite fingerprinting and profiling in the characterization of mutants and transgenic lines. We noticed the differences in metabolite profile among experiments. However, the distinct metabolite profiles in albino mutants were always observed as shown in PLS-DA analysis and correlation analysis. Such difference among experiments should be caused by slight difference in growth conditions. Therefore, in order to isolate mutants or characterize mutants by means of metabolite profiling, comparing relative metabolites levels among several lines, including the control line, would be safer than comparing absolute metabolite levels.

NMR has become an important tool in investigating the physiology and metabolism of living systems (52–55). The

![FIGURE 6. Metabolite-metabolite correlations among multiple two-dimensional HSQC experiments. Correlations among metabolites were calculated using signal intensity of a carbon of each metabolite detected in multiple samples and shown in a heat map. Since analytical errors of NMR experiments are negligible relative to biological error (due to variation of slightly changed growth conditions), the metabolite-metabolite correlation indicates the maintained homeostasis of cellular metabolism (69). Red and blue, higher or lower correlations. Lower left, correlations among albino samples; upper right, correlations among control samples.](Image 61x477 to 289x734)
application of multitarget profiling by NMR to microbes (e.g. in the discrimination of mutant and wild-type yeast based on metabolic fingerprints) has greatly advanced the understanding of how metabolic pathways are regulated (56, 57). NMR spectrometry has also been applied to the screening of Arabidopsis ecotypes for metabolic differences and to analyze plant hormone responses (58, 59). In addition, one-dimensional $^1$H NMR spectral analysis can be used for rapid and inexpensive metabolic fingerprinting. Thus, one-dimensional $^1$H NMR is very useful at the initial screening stage in top-down phenomics studies; however, this method is limited by the fact that most of the chemical compounds contributing to the various chemical shifts remain unknown, and many of the chemical shifts overlap. An attractive alternative is to introduce $^{13}$C chemical shifts as a second dimension. The problem of the low signal strength of $^{13}$C signals due to its low natural abundance can be overcome by labeling materials with $^{13}$C (23, 24, 26, 60, 61). Plant materials can be labeled by feeding with $^{13}$C-labeled sugars or CO$_2$ gas (23, 24, 26). This would also provide an excellent method for tracing the carbon movements among metabolites and could allow determination of the metabolic flow at the atomic level, since we showed that the labeling efficiency was higher in albino mutants, presumably caused by the loss of photosynthesis that dilutes the $^{13}$C level with $^{12}$C from CO$_2$. This fact should be characterized by metabolic fluxomic approach that was recently proposed by us (62). We did not examine in detail, but it is interesting to know how the root uptake of nutrition is enhanced in the albino mutants.

The correlation between two-dimensional NMR-based profiling of primary metabolites and transcriptome analysis was weak as previously reported (1). For example, the higher levels of Gln, Glu, and Asn could not be explained by the expression of the corresponding

FIGURE 7. Comparison of expression patterns for genes affected in the albino mutant with those affected by abiotic stress or hormone treatment. A, MapMan projections of the expression of genes involved in metabolic pathways. Each square corresponds to a gene. Red and blue indicate higher and lower expression than the control, respectively. The scale bar is shown in log$_2$. Regions boxed in red indicate the set of genes showing significant changes in expression (Benjamini-Hochberg-corrected probability < 1.0 $\times$ 10$^{-10}$). B, the expression patterns of the genes encoding WRKY, AUX/IAA, and response regulators whose expression was affected in albino line 2192 were compared with public microarray data in Genevestigator. The scale bar is shown in log$_2$. TCA, trichloroacetic acid.
genes. Similarly, the lower level of Pro in the albino line did not correspond with the changes in the expressions of \( P5CS \) encoding a key enzyme for Pro biosynthesis and genes encoding proline dehydrogenase and pyrroline 5-carboxylate dehydrogenase, which participate in Pro catabolism (63, 64). Therefore, a combination of metabolomic, transcriptomic, and other “omic” approaches is needed for understanding the regulation of plant metabolism, because these data elucidate “static” features of biological systems, but their dynamic nature could be investigated by network analysis of each biomolecule.

Combining top-down metabolic analysis of gene disruptants with transcriptomic and phenomic studies is a powerful method for establishing systems biology in plants. A recent study based on network theory showed that metabolic pathways comprise a scale-free network (65). This structural property of metabolic networks provides a high degree of robustness and tolerance to local error but vulnerability to the removal of important pathways or metabolites (66). These network theory studies suggest that there should be hub metabolites, which play key roles in metabolite networks (65, 67, 68). Collecting data on mutations that affect metabolite compositions in plants should allow identification of hub metabolites and key genes in the metabolic network and should offer clues to help dissect the complex metabolic systems of plants.

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