Transcription Profiling of the Early Gravitropic Response in Arabidopsis Using High-Density Oligonucleotide Probe Microarrays[1][w]

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Studies of plant tropisms, the directed growth toward or away from external stimuli such as light and gravity, began more than a century ago. Yet biochemical, physiological, and especially molecular mechanisms of plant tropic responses remain for the most part unclear. We examined expression of 8,300 genes during early stages of the gravitropic response using high-density oligonucleotide probe microarrays. Approximately 1.7% of the genes represented on the array exhibited significant expression changes within the first 30 min of gravity stimulation. Among gravity-induced genes were a number of genes previously implicated to be involved in gravitropism. However, a much larger number of the identified genes have not been previously associated with gravitropism. Because reorientation of plants may also expose plants to mechanical perturbations, we also compared the effects of a gentle mechanical perturbation on mRNA levels during the gravity response. It was found that approximately 39% of apparently gravity-regulated genes were also regulated by the mechanical perturbation caused by plant reorientation. Our study revealed the induction of complex gene expression patterns as a consequence of gravitropic reorientation and points to an interplay between the gravitropic and mechanical responses and to the extreme sensitivity of plants to even very gentle mechanical perturbations.

Though studies of plant tropisms began more than a century ago (Knight, 1806; Ciesielski, 1872; Darwin, 1880), the mechanisms of plant tropic responses, including gravitropism, are for the most part still unknown. It is believed that the gravitropic response is a well-coordinated process regulated through gravity signal perception and transduction, gene transcription, and translation. Previous research findings, based largely on physiological, biochemical, and genetic experimental evidence, have implicated a role for starch-filled plastids, amyloplasts, as statoliths in gravity perception (Volkmann and Sievers, 1979; Sack, 1991; Blancaflor et al., 1998; Moctezuma and Feldman, 1999a), and Ca^{2+} (Belyavskaya, 1996; Lu and Feldman, 1997; Sinclair and Trewavas, 1997), H^+ (Mulkey and Evans, 1981; Zieschang et al., 1993; Scott and Allen, 1999), K^+ (Philippar et al., 1999), auxin (Cholodny, 1928; Went, 1928; Feldman, 1985; Parker and Briggs, 1990; Konings, 1995; Chen et al., 1999; Moctezuma and Feldman, 1999b), the cytoskeleton (Baluska and Hasenstein, 1997), and the cell wall (Cosgrove, 1997; Edelmann, 1997; Hejnowicz, 1997) in gravity signal transduction. Earlier work has also implicated a need for both transcription and translation regulation in the root gravity response (Feldman, 1981). Yet in only a few studies have attempts been made to analyze gravity-induced changes at the transcriptional level (Guilfoyle et al., 1993; Li et al., 1999; Philippar et al., 1999). Recently developed cDNA and oligonucleotide probe microarray technologies now allow for accurate measurement of mRNA transcript abundance for hundreds or thousands of genes in parallel (Schena et al., 1995, 1996; Chee et al., 1996; Lipshutz et al., 1999). In some organisms with completed genome sequences, such as in yeast and *Caenorhabdtis elegans*, global gene expression profiling at the transcription level becomes possible (De Risi et al., 1997; Hill et al., 2000). Though relatively new, microarray technology has already been successfully employed in a number of studies of gene expression in plants (Desprez et al., 1998; Giegé et al., 1998; Ruan et al., 1998). For example, it has been used to examine gene expression profiles during organ development (Aharoni et al., 2000; Girke et al., 2000; Zhu et al., 2001), during the defense response (Maleck et al., 2000; Reymond et al., 2000; Schenk et al., 2000), and during nutrient uptake (Wang et al., 2000).

In our work, we attempted to identify genes involved in early stages of the plant gravitropic response using high-density oligonucleotide probe microarrays representing 8,300 unique genes, or approximately one-third of the genome of the model

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plant Arabidopsis. The Arabidopsis oligonucleotide probe array was designed based on the Unigene set selected mainly from the Arabidopsis genomic sequences. It includes probes for 8,300 Arabidopsis genes and forty probes for spiking and negative controls. Gene probes on the array are represented by known genes, predicted genes and approximately 100 expressed sequence tag clusters (Zhu and Wang, 2000). In this paper, we describe our attempt at obtaining the first comprehensive view of global gene expression changes during early stages of the gravitropic response.

RESULTS

Experimental Design and Application of High-Density Oligonucleotide Probe Microarrays to Identification of Gravity-Regulated Genes on a Large-Scale Basis

We applied microarray technology to characterize and compare early changes in gene expression profiles in gravitropically stimulated plants. For our experiments, we used 3-week-old seedlings of Arabidopsis (Columbia ecotype) growing in vitro in vertically oriented square petri plates (see "Materials and Methods" for description of the growth conditions). To minimize possible "side effects" of phototropic and blue light-activated signal transduction in plant gravitropism, experiments were conducted in a dark room under dim green light (bandpass, 525 ± 15 nm; fluence, 0.01 μmol s^{-1} m^{-2}). Before the beginning of experiments, plates with 3-week-old Arabidopsis seedlings were transferred from the growth chamber into the dark room with dim green light for overnight exposure and adaptation of plants to the experimental conditions. Sixteen hours later, four experiments were conducted: (a) plants growing vertically and with no mechanical disturbances, and total RNA extracted (control, Fig. 1A); (b) plants reoriented from the vertical to the horizontal position for 15 min, and total RNA extracted (Fig. 1B); (c) plants reoriented from the vertical to the horizontal position for 30 min, and total RNA extracted (Fig. 1C); and (d) plants gently rotated 360° (10-s rotation) ending in the original vertical position, and total RNA extracted 30 min later (Fig. 1D). The rationale for these experiments was to detect the earliest gravity-induced changes in gene expression profiles and to detect and evaluate effects on the transcription machinery of mechanical perturbations associated with plant reorientation. The gravitropic response in Arabidopsis requires at least 1 min of presentation time; when stimulation times are shorter than 1 min, no gravitropic curvature can be detected (Blancaflor et al., 1998). Therefore, a 360° change in orientation of the gravity vector in a period of 10 s should not trigger the gravitropism-specific signal transduction pathway leading to gravitropic curvature.

To minimize biological and technological variance, for each experiment, RNA samples were extracted and pooled from a total of 300 to 600 whole seedlings from three different plates with the same condition, and each microarray experiment was repeated three times. Thus, 12 microarray experiments were conducted, and more than 99,600 expression measurements (8,300 × 3 × 4 = 99,600) were made (Supplemental Table I can be viewed at www.plantphysiol.org). After cDNA and cRNA synthesis (antisense RNA synthesized in vitro using cDNA as a template in the presence of biotinylated ribonucleotides), array hybridization, and data acquisition, expression measurements for the 8,300 genes were examined. For each experiment, the mean and the SE were computed based on three replicates, and only genes that showed a signal threshold above the background and expression changes of ≥2-fold were further analyzed. The reproducibility of the Arabidopsis high-density oligonucleotide probe array was characterized in previous work (Lipshutz et al., 1999; Zhu et al., 2000, 2001) and in this work by calculating the rate of false changes. Genes that showed changes of ≥2-fold, and a signal threshold above the background were counted as false changes. In this study, data from 12 pairs of array experiments indicated that the average rate of false changes between two array experiments was 0.61% and did not exceed 1.13% in any pair of compared experiments.

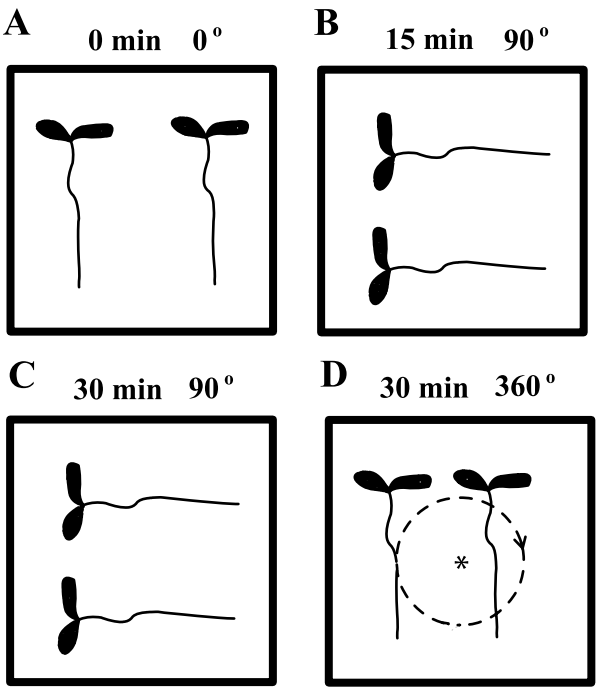

Figure 1. Schematic diagram showing experimental design. A, Total RNA was extracted from vertically oriented control plants after their adaptation to the experimental conditions. B, Total RNA was extracted 15 min after plants were reoriented from the vertical to horizontal position. C, Total RNA was extracted 30 min after plants were reoriented from the vertical to horizontal position. D, Total RNA was extracted 30 min after plants were gently rotated 360° in the gravity vector plane (around an axis parallel to the earth surface, and which is displayed as * in the middle of the petri plate).

Thus, the array experiments were highly reproducible (Fig. 2). Analysis of the genes that displayed false changes indicated that, in most cases, their expression levels were low and close to the background, and their -fold changes were close to the 2-fold signal threshold. Therefore, a significant gene expression change was defined as a 2-fold or above for a given gene between any two experiments.

Sample quality, specifically labeled cRNA quality, was monitored by comparing the ratio of the hybridization signal of 3′- and 5′-probe sets for glyceraldehyde-3-phosphate dehydrogenase and ubiquitin 11. Only data with consistent 3′ to 5′ ratio were used in this study. Because the major concern in cRNA synthesis is achieving full-length products, the 3′ to 5′ ratio permits an assessment of the sample quality before placing cRNA on the expensive array and hybridizing. If the 5′-prime region signal exceeded the 3′-prime signal by more than 3-fold, the sample failed quality control, and was prepared again.

Selected housekeeping genes were used to ensure the quality of the array experiments. Comparison of average differences (which indicate the level of expression of a transcript) for the housekeeping genes in all four experiments showed that they did not exceed the defined 2-fold change threshold for a significant gene expression change (Table I). The fact that the constitutively expressing genes with average differences in the range between 17 and 1,138 did not show significant expression changes, neither in the gravitropic stimulation nor in the mechanical perturbation experiments, adds support to the validity of the data obtained in this study.

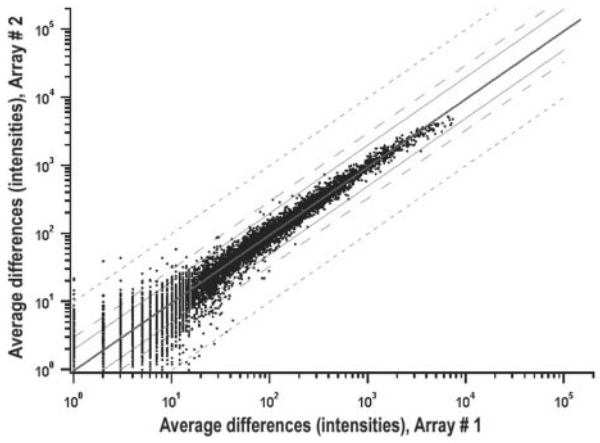

Figure 2. Assessment of microarray reproducibility. Biotin-labeled cRNA prepared from control plants was hybridized sequentially to two Affymetrix Arabidopsis GeneChip arrays manufactured on the same silicon wafer. The solid line indicates a difference of a factor of 2, the long dashed lines a factor of 3, and the short dashed lines a factor of 10, between the two hybridizations. The rate of false changes between the shown two-array experiments was 0.82% after applying a 2-fold signal threshold.

Analysis of Gene Expression Data

Because of several practical obstacles, the rate-limiting step in Arabidopsis large-scale gene expression studies is often not the data generation step but rather the data analysis step (Ghassemian et al., 2001). To facilitate analysis of data obtained with high-density oligonucleotide probe microarrays, we have developed a computer program named VIZARD. The program includes several integrated tools for filtering, sorting, clustering, and visualization of gene expression data as well as tools for discovery of regulatory motifs in upstream sequences. VIZARD also includes annotation and upstream sequence databases for the majority of genes represented on the Affymetrix Arabidopsis GeneChip array (Affymetrix, Santa Clara, CA). The program was written in the Java language and runs on all computer platforms supporting Java 2 (JRE 1.2.2 and later). VIZARD is available free of charge for educational, research, and not-for-profit purposes and can be downloaded at http://www.anm.f2s.com/research/vizard/.

The Transcription Machinery of Arabidopsis Is Sensitive to Mechanical Perturbations Caused by Plant Reorientation

Because reorientation of plants during gravistimulation may also expose plants to mechanical perturbations, we analyzed the effects of a gentle mechanical perturbation on mRNA levels. Comparison of the transcripts present in the control (Fig. 1A) and in the mechanical perturbation (Fig. 1D) experiments revealed that 183 of 8,300 genes represented on the array exhibited significant expression changes 30 min after plants were gently rotated in a 10-s period 360° in the gravity vector plane. Notably, the majority of genes showing differential expression in response to the mechanical perturbation were down-regulated (169 of 183 genes, or 92%). Genes regulated by the mechanical perturbation (Supplemental Table II can be viewed at www.plantphysiol.org) belonged to the following functional categories: transcription/transcription factors, splicing, oxidative stress/disease resistance, cell wall/plasma membrane, protein kinases/phosphatases, calcium-binding proteins, heat shock proteins, cell division/growth, and cytoskeleton. At present, approximately 26% of the identified genes have no functional assignment in public databases.

The "Description" columns of all tables in this paper have functional annotations retrieved from public databases using the BLAST search engine (Altschul et al., 1990) and Munich Information Center for Protein Sequences (MIPS) Arabidopsis Data Base (MATDB) at the Munich Information Center for Protein Sequences (http://mips.gsf.de). Because publicly available data changes at a very fast pace, these annotations will be outdated as soon as new information is available or changes to the current annota-

Table I. *The mean SE computed based on three replicates (for each experiment, Exp A, Exp B, Exp C, and Exp D) for the selected housekeeping genes*

Exp A corresponds to an experiment displayed schematically in Fig. 1A, Exp B to Fig. 1B, Exp C to Fig. 1C, and Exp D to Fig. 1D.

Probe Set ID	Description	Exp A		Exp B		Exp C		Exp D	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
12827_S_AT	gb|AAA32794.1| (M64116) cystolic glyceraldehyde-3-phosphate dehydrogenase	986.1	108.4	989.9	82	1,022.8	141.6	1,058.7	41.2
16158_F_AT	gb|AAB52506.1| (U27811) actin7	947.9	13.5	896.9	47	1,019.6	53.4	1,137.8	73.7
15213_S_AT	emb|CAA04115.1| (AJ000476) Omithine carbamoyltransferase	831.1	53.1	771.5	54.3	685.7	51.6	946.6	83.3
16612_S_AT	gb|AAD55279.1|AC008263_10 ribosomal protein S9	599.7	16.9	531.8	13	524.8	22.9	635.0	32.5
16975_AT	emb|CAA73156.1| (Y12576) histone H2B	564	14	498.9	28.7	525.1	28.6	574.4	22.7
15534_F_AT	gb|AAA33476.1| (M13377) histone H4	216.3	5	254.4	5.8	180.1	12.3	282.2	27
17454_AT	gb|AAC69132.1| (U78721) 30S ribosomal protein S5	183.6	6.8	203.4	3	168.9	5.9	221.5	11.9
17409_S_AT	gb|AAB81995.1| (AF026803) histone H3	72	2.1	74.4	1.6	84.7	7.4	98.8	8
13081_S_AT	gb|AAD20138.1| (AC006282) 60S ribosomal protein L24	63.6	2.6	74.1	3	55.1	4.4	60.9	3.8
14518_AT	gb|AAC31838.1| (AC002388) 60S ribosomal protein L30	50.6	5	52.7	8.2	40.4	4.1	40.5	2.7
14895_S_AT	emb|CAB10562.1| (Z97344) acetylomithine deacetylase	19.5	4.1	24.2	2.5	24.4	2.4	17.3	1.4

tions are made. The above described VIZARD program has tools for retrieving up-to-date functional annotations from the MATDB. In addition, one can obtain up-to-date gene descriptions (and perform a homology search) via a "Probe Set ID," an ID for sequences of the oligonucleotides probes on the array. Sequences for any particular Probe Set ID can be found at ftp://tairpub:tairpub@ftp.Arabidopsis.org/home/tair/Microarrays/Affymetrix/.

The fact that Arabidopsis seedlings gently rotated 360° and then, remaining undisturbed for 30 min, exhibited large changes at the transcription level suggests high sensitivity of the transcription machinery to even very gentle mechanical perturbations and emphasizes the importance of taking into consideration the mechanical perturbations in gravitropism-related research.

Identification of Gravity-Regulated Genes

Comparison of the transcripts present or absent in the control (Fig. 1A), the gravitropic stimulation (Fig. 1, B and C), and the mechanical perturbation (Fig. 1D) experiments revealed that 141 genes of 8,300 genes (approximately 1.7%) represented on the array exhibited significant expression changes within 30 min of the gravitropic stimulation. However, 55 of the identified 141 genes also exhibited significant expression in response to the mechanical perturbation. Thus, only 86 genes displayed differential expression in the gravistimulation experiments but not in the mechanical perturbation experiment. The identified gravity-regulated genes are listed in Supplemental Table III (which can be viewed at www.plantphysiol.org). The number of genes showing differential expression at the 15-min time point (Fig. 1B) was 39, and this number increased to 132 within the next 15 min of gravity stimulation (Fig. 1C). Because 30 genes were differentially expressed both at the 15 and at the 30-min time points, total number of identified gravity-regulated genes was 141.

The majority of the gravity-regulated genes belonged to the following functional categories: oxidative stress/plant defense, metabolism, transcription, cell wall/plasma membrane, signal transduction, heat shock proteins, ethylene-responsive element-binding factors, and calcium-binding proteins (Table II). At present, approximately 28% of the identified genes have no functional assignment in public databases. It should be noted that the oxidative burst/plant defense group (peroxidase ATP N, cytochrome P450, glutathione *S*-transferase, β-glucosidase, li-

Table II. *Functional distribution of the 141 gravity-regulated genes*

The no. of gravity-regulated genes in each functional category is based on the no. of genes showing expression changes above the defined 2-fold change threshold in experiments displayed schematically in Fig. 1, B and C.

Functional Category	No. of Gravity-Regulated Genes	Percentage of Category
Oxidative stress/plant defense	32	22.7
Metabolism	21	14.9
Transcription	12	8.5
Cell wall/plasma membrane	10	7.1
Signal transduction	9	6.4
Heat shock proteins	6	4.3
Ethylene-responsive element binding factors	6	4.3
Calcium binding proteins	3	2.1
Energy	2	1.4
Cytoskeleton	1	0.7
Unknown/unclassified proteins	39	27.7

poxygenase 1, anthranilate synthase, AIG2, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, etc.) was the largest functional category of the gravity-regulated genes. Involvement of oxidative burst/plant defense genes in the gravitropic response was somewhat unexpected, although rapid non-pathogen-related induction of the oxidative burst is known to occur in response to wounding, extreme temperatures, UV irradiation, salt, and osmotic and mechanical stimulation (Bradley et al., 1992; Yahraus et al., 1995; Foyer et al., 1997; Cazale et al., 1998; Depège et al., 2000). The oxidative burst/plant defense genes have not yet been considered to play a role in gravitropism, except for the recent study on role of auxin-induced reactive oxygen species (ROS) in root gravitropism (Joo et al., 2001). In the work of Joo et al. (2001), it was demonstrated that both gravity- and auxin-induced asymmetric ROS generation in roots of maize (*Zea mays*), unilateral application of ROS to vertical roots stimulated root bending, and scavenging of ROS by antioxidants inhibited root gravitropism. Results of the above mentioned work support our findings of involvement of oxidative burst/plant defense genes in the gravitropic response.

Transcription Profiling Indicates Complex Gene Expression Changes during the Gravitropic Response

Analysis of transcript abundance profiles in all experiments indicates complex changes in gene expression patterns during the early stages of the gravitropic response. In most instances, genes regulated by gravity were not simply "switched on" or "switched off": It was the level of gene transcript abundance that changed, and the amplitude of these changes was up to 11.5-fold.

Concerning dynamics of the plant response to gravitropic stimulation, the majority of the gravity-regulated genes were detected at the 30-min time point (132 of 141 genes). Except for the putative expansin gene and putative thaumatin gene, all other genes exhibiting significant expression changes at the 15-min time point were notably down-regulated (37 of 39 genes). On the other hand, 100 of 132 genes (approximately 76%) exhibiting significant expression changes at the 30-min time point were up-regulated. Temporal expression patterns for 26 of 39 genes indicated down-regulation at the 15-min time point and up-regulation at the 30-min time point. This also did not fit into a simple "switch on/switch off" model. Because we had only two time points, 15 and 30 min, a thorough gene clustering analysis was not feasible, however, comparison of gene expression profiles in all experiments indicated existence of several clusters of coregulated genes (Supplemental Fig. 1 can be viewed at www.plantphysiol.org). As mentioned earlier, 55 of the identified 141 gravity-regulated genes (i.e. approximately 39%) also exhibited significant expression changes in response to the mechanical perturbation. This implies that the gravitropic and mechanical responses may be partially overlapping and share some common mechanisms of gene expression regulation and/or compete for some key regulatory elements.

Identification of cis-Regulatory Elements Associated with Gravity-Regulated Genes

To elucidate whether there is any commonality in the regulatory elements of the gravity-regulated genes, promoter sequences of the identified genes were analyzed using AlignACE software (Hughes et al., 2000). Because most Arabidopsis cis-elements are found within 1-kb distance from the translation initiation codon, we analyzed 1-kb upstream sequences of 40 genes that belonged to a cluster of genes up-regulated at the 30-min time point after gravistimulation (Fig. 3A). It was found that these genes share six potential cis-acting elements in the 6- to 10-bp size range (Fig. 3, B–G). Promoters of the majority of genes belonging to this group contained two or three repeats of the identified sequence motifs, suggesting that these motifs represent cis-elements shared by coregulated, gravity-induced genes. All of the observed motifs notably contained either GAGAGA or GAAAAAG sequence as a consensus sequence. The identified motifs did not have significant matches with known transcription factor-binding sites contained in the TRANSFAC database (Transcription Factor Database, http://transfac.gbf.de/TRANSFAC) and should be considered novel cis-regulatory elements, if their biological role in the gravitropic response is proved.

DISCUSSION

In this study, we present the first map of global gene expression patterns, composed of transcription profiles of 8,300 genes and 33,200 gene expression measurements, in a model plant Arabidopsis, during the early gravitropic response, as well as after the mechanical perturbation. To quantitatively characterize gravity-induced changes at the transcription level, we used highly accurate and reproducible Affymetrix high-density oligonucleotide probe arrays (Lipshutz et al., 1999; Zhu et al., 2000). Analysis of the obtained microarray data allowed large-scale identification of genes regulated by gravity and by mechanical perturbation. Several lines of evidence support the experimental reliability of this study: (a) each microarray experiment was repeated three times; the mean and the SE of the mean were computed and used for further analysis; (b) data from 12 pairs of array experiments indicated that the average rate of false changes between two array experiments was 0.61%, and did not exceed 1.13% in any pair of compared experiments; (c) a rather conservative threshold for minimal average differences between

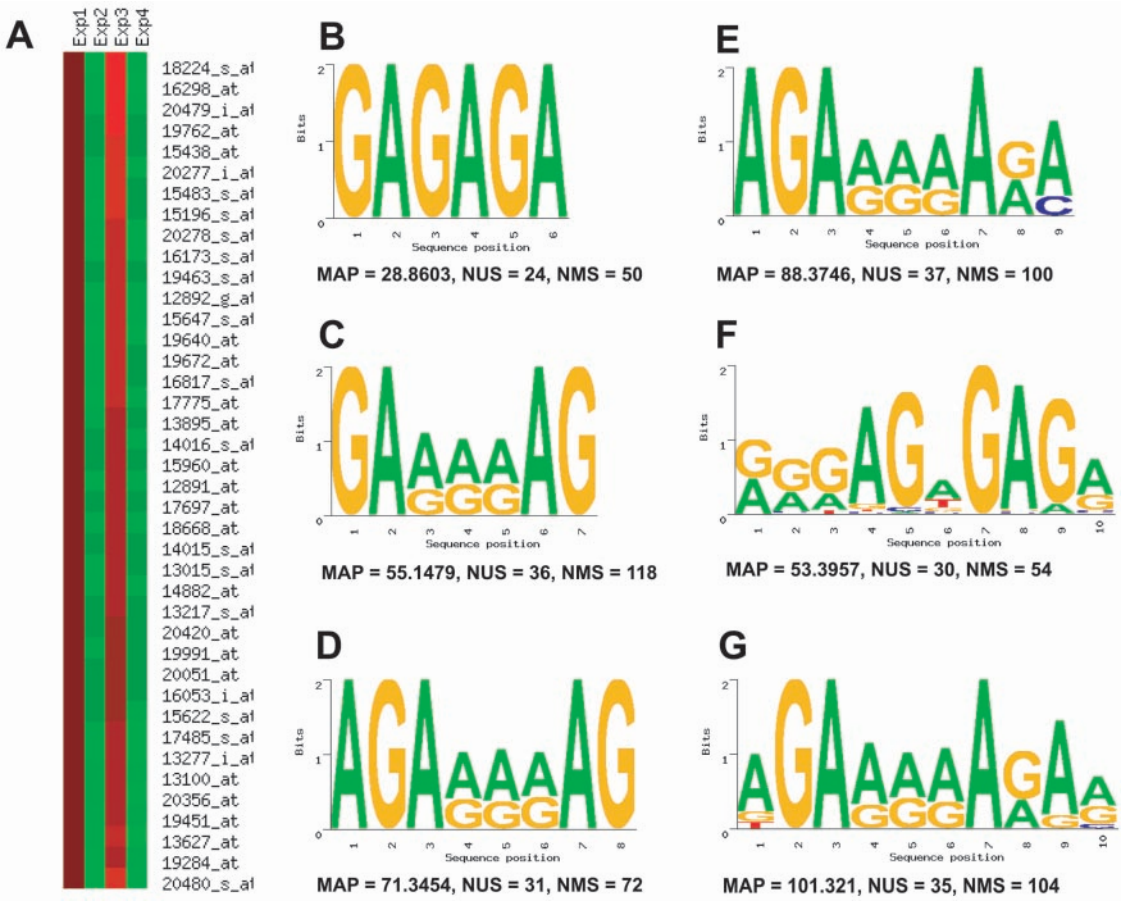

Figure 3. Transcription profiles of 40 genes that belonged to a cluster of genes up-regulated at the 30-min time point after gravistimulation (A), and sequence logos of potential cis-regulatory motifs identified in upstream sequences of these genes (B–G). Increase in transcript abundance is shown in red, decrease in green. Sequence logos (Schneider and Stephens, 1990) are graphic representations of sets of binding sites. The logos display the frequencies of bases at each position along with the degree of sequence conservation, measured in bits of information. The vertical scale is in bits, with a maximum of two bits possible at each position. The MAP, NUS, and NMS values represent the MAP score, number of upstream sequences sharing the motif, and number of motif sites in these sequences, respectively. The MAP score measures the degree to which a motif is over-represented relative to the expectation for the random occurrence of such a motif in the sequence under consideration (Hughes et al., 2000).

experiments was used (27), thus sacrificing some of the genes in the "gray area," close to the background (such as those encoding transcription factors, which normally have low transcript abundance levels), but thereby increasing reliability of the data; (d) selected, constitutively expressed housekeeping genes with average differences in the 17 to 1,138 range did not significantly change their expression profiles in any gravitropic or mechanical stimulation experiment; (e) previous reports on quantitative assessment and comparison of microarrays with other technologies demonstrated that data obtained with different methods were consistent, and, if different, microarrays produced -fold changes that were lower than other conventional methods such as northern blot, reverse transcriptase-PCR, and RNase protection assay (for example, see Taniguchi et al., 2001), therefore, we are not systematically overestimating the data and we are reporting on the conservative side; and (f) the microarray data are in agreement with previous research findings suggesting the involvement of several identified genes in gravitropism.

Approximately 1.7% of all genes represented on the array (141 of 8,300 genes) exhibited significant expression changes within 30 min of the gravitropic stimulation. The relatively low number of genes regulated by gravity may be attributable to a masking effect, because the total RNA samples were extracted from heterogeneous tissues from whole seedlings. Among the identified genes were those whose gene products were previously implicated to be involved in gravitropism, such as calcium-binding/calmodulin/calmodulin-like proteins (Feldman and Gildow, 1984; Björkman and Leopold, 1987; Hasenstein and Evans, 1988; Lu and Feldman, 1997), Na^+/H^+-exchanging protein (for review, see Wiesenseel and Meyer, 1997; Scott and Allen, 1999) expansin (Caderas et al., 2000) and putative auxin-induced protein IAA12 (there

have been numerous reports of involvement of auxin in gravitropism). On the other hand, genes encoding polar auxin carriers AUX1 and EIR1 (also known as AtPIN2 and AGR1), which are considered to be necessary for the gravitropic response (Bennett et al., 1996; Luschnig et al., 1998) and which were present on the array, did not exhibit significant gravity-induced expression changes. This observation is in agreement with the recent study that demonstrated that EIR1 is controlled at the posttranscriptional level (Sieberer et al., 2000). Regarding a role of ethylene in the gravitropic response, previously published experimental evidence is contradictory. The microarray data obtained in this study provide support for the idea that ethylene is involved in the early gravitropic response: several ethylene-responsive element-binding factors significantly changed their expression levels after gravistimulation. This is also in agreement with recent research findings supporting an ethylene role in gravitropism (Philosoph-Hadas et al., 1996; Madlung et al., 1999; Ferrari et al., 2000).

Many of the genes we have identified were not known previously to be involved in the gravitropic response or gravity signal transduction pathway. A role for many of these, such as for those encoding oxidative burst proteins, transcription factors, heat shock proteins, and ethylene-responsive element-binding factors remains to be elucidated and points to new directions for studying the gravitropism mechanism. Somewhat surprising was the apparent involvement of the oxidative burst/plant defense genes that formed the largest functional category of the gravity-regulated genes. Though the oxidative burst/plant defense genes have not yet been considered to play a role in gravitropism, our findings as well as results of the recent study on role of auxin-induced ROS in root gravitropism (Joo et al., 2001) imply involvement of oxidative burst/plant defense genes in the gravitropic response.

We have also identified several potential cis-regulatory elements of the gravity-induced genes using a computational approach. It was found that 40 genes, which were up-regulated at the 30-min time point after gravistimulation and which belonged to the same expression pattern cluster, have common promoter motifs (Fig. 3). This observation suggests the existence of a tightly regulated genetic network of the gravitropic response in plants.

It was found that Arabidopsis seedlings have highly sensitive transcription machinery responding to even very gentle mechanical perturbations. Significant changes in the mRNA levels of 183 genes were detected 30 min after a single, gentle 360° reorientation (10-s rotation). It is surprising that the vast majority (92%) of the "mechano"-sensitive genes, including gene-encoding putative calmodulin (AAC34487.1), were down-regulated. In previous studies of plant responses to mechanical stresses such as touch, wind, rain, and wounding, calmodulin and calmodulin-related genes were up-regulated (Braam and Davis, 1990). The differences between our observations and previous research findings with regard to the putative calmodulin gene may be explained by the different application of mechanical stimuli: In our work, a very gentle mechanical perturbation was applied, compared with a relatively strong mechanical stress caused by wounding, wind, or rain. Also, the putative calmodulin gene, identified in our work, was assigned a putative function based on homology search results; however, this gene may be under a completely different transcriptional regulation mechanism compared with the calmodulin gene induced in the previous work.

The high sensitivity of the transcription machinery to mechanical perturbations emphasizes the importance of considering this stimulus in studies of gravitropism. Using a smooth rotating mechanical stage to reorient the root and a feedback system to connect the stage to a video digitizer system, Mullen et al. (2000) recently reported a mean time lag of approximately 10 min for onset of root gravicurvature in Arabidopsis, although previous reports of Arabidopsis root gravitropism suggested latent periods of approximately 30 min. The authors suggested that this more rapid onset of gravicurvature can, in part, be explained by reduced mechanical perturbation during the process of gravistimulation. This observation adds support to the idea of the importance in the early gravitropic response of mechanical perturbations associated with plant reorientation. In our study, approximately 39% of the identified gravity-regulated genes also exhibited significant expression changes in response to the mechanical perturbation. The cluster analysis of the microarray data indicated complex changes in gene expression patterns during the gravitropic response and a possible interplay between the gravitropic and mechanical responses (Supplemental Fig. 1). Therefore, it seems plausible that the gravitropic and mechanical responses may share, in part, some common mechanisms of gene expression regulation, or they may compete for some key regulatory elements.

Thus, microarray technology may offer significant advantages for the discovery of gravitropism-related genes and functional characterization of genes on a genomic-scale basis. Combined with more traditional biochemical and molecular methods, microarray technology promises to become a very significant tool in the hands of researchers. In this paper, we demonstrated the utility and potential of the parallel gene expression analysis approach in the study of gravitropism.

MATERIALS AND METHODS

Plant Material and Experimental Conditions

Wild-type Arabidopsis seeds of the Col-1 ecotype were sterilized in 20% (w/v) bleach and sown on nylon membranes placed on top of the agar (to

prevent plants from growing into the growth media) in square petri plates (100 × 100 × 15 mm) containing Murashige and Skoog salts, B5 vitamins, and 1.2% (w/v) agar, pH 5.7. The number of seeds placed per petri plate was between 100 and 200. Seeds were stratified at 4°C for 3 d and then placed vertically in growth chambers held at 22°C, a 12-h light/dark cycle, and 80% humidity. After 3 weeks, the petri plates were transferred to a "dark" room illuminated by dim green light (bandpass, 525 ± 15 nm; fluence, 0.01 μmol s^{-1} m^{-2}). The plates were maintained vertically under these conditions for 16 h to adapt the plants to the experimental conditions and to minimize phototropic effects and mechanical perturbations associated with transfer of the seedlings from the growth chamber to the dark room. Sixteen hours later, the experiments were conducted in the dark room under the dim green light. The gravitropic response was induced using a one-time gentle reorientation of the plates in the gravity vector plane (Fig. 1, B and C). For analysis of mechanical perturbation, a vertically held plate was gently rotated (10-s rotation) 360° in the gravity vector plane (around an axis parallel to the earth surface) and left undisturbed for 30 min (Fig. 1D).

RNA Extraction

For each experiment, RNA samples were extracted and pooled from 300 to 600 whole seedlings growing on three different plates with the same condition (100–200 seedlings per petri plate). At the termination of the various time intervals, in dim green light, tissue for each time point was rapidly frozen in liquid nitrogen, and the total RNA was extracted. Samples were homogenized in liquid nitrogen, and the total RNA was obtained using the RNAwiz (Ambion, Austin, TX) kit according to the manufacturer's protocol.

cDNA Synthesis

Total RNA (5 μg) from each sample was reverse transcribed at 42°C for 1 h using 100 pmol of oligo$(dT)_{(24)}$ primer containing a 5′-T7 RNA polymerase promoter sequence (5′-GGCCAGTGAATTGTAATA-CGACTCACTATAG-GGAGGCGG-$(dT)_{24}$-3′), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol (DTT), 0.5 mM dNTPs, and 200 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The second strand of cDNA was synthesized using 40 units of *E. coli* DNA polymerase I, 10 units of *E. coli* DNA ligase, and 2 units of RNase H in a reaction containing 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 10 mM (NH_4) SO_4, 0.15 mM b-NAD $^+$, 1 mM dNTPs, and 1.2 mM DTT. The reaction proceeded at 16°C for 2 h and was terminated using EDTA. Double-stranded cDNA products were purified by phenol/chloroform extraction and ethanol precipitation.

cRNA Synthesis

Biotinylated cRNAs were in vitro transcribed from synthesized cDNA by T7 RNA polymerase (BioArray high yield RNA transcript labeling kit, Enzo Diagnostics, New York). cRNAs were purified using affinity resin (RNeasy spin columns, Qiagen USA, Valencia, CA) and randomly fragmented by incubating at 94°C for 35 min in a buffer containing 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate to produce molecules of approximately 35 to 200 bases long.

Array Hybridization

The labeled samples were mixed with 0.1 mg mL^{-1} sonicated herring sperm DNA in a hybridization buffer containing 100 mM MES, 1 M NaCl, 20 mM EDTA, and 0.01% (w/v) Tween 20, denatured at 99°C for 5 min, and equilibrated at 45°C for 5 min before hybridization. The hybridization mix was then transferred to the Arabidopsis GeneChip genome array (Affymetrix) cartridge and hybridized at 45°C for 16 h on a rotisserie at 60 rpm. The hybridized arrays were then rinsed and stained in a fluidics station (Affymetrix). They were first rinsed with wash buffer A (6× SSPE [0.9 M NaCl, 0.06 M NaH_2PO_4, 0.006 M EDTA], 0.01% [w/v] Tween 20, and 0.005% [w/v] Antifoam) at 25°C for 10 min and incubated with wash buffer B (100 mM MES, 0.1 M NaCl, and 0.01% [w/v] Tween 20) at 50°C for 20 min, and then stained with streptavidin phycoerythrin (SAPE; 100 mM MES, 1 M NaCl, 0.05% [w/v] Tween 20, 0.005% [w/v] Antifoam, 10 mg mL^{-1} SAPE, and 2 mg mL^{-1} bovine serum albumin) at 25°C for 10 min, washed with wash buffer A at 25°C for 20 min, and stained with biotinylated antistreptavidin antibody at 25°C for 10 min. After staining, arrays were stained with SAPE at 25°C for 10 min and washed with wash buffer A at 30°C for 30 min. The probe arrays were scanned twice, and the intensities were averaged with a Hewlett-Packard GeneArray Scanner.

Data Analysis

GeneChip Suite 3.2 (Affymetrix) was used for background subtraction and data normalization. The average intensity of each array was scaled to 100, so that average hybridization intensities of all arrays are equivalent. False positives were defined based on experiments in which samples were split and hybridized to GeneChip expression arrays, and the results were compared. A false positive was indicated if a probe set was scored qualitatively as an "increase" or "decrease" and quantitatively as changing by at least 2-fold and with an average difference greater than 27. A significant change was defined as 2-fold change or above with an expression baseline of 27, which was determined as the threshold level according to the scaling. Further analysis and visualization of microarray data was performed using an in-house built program named VIZARD (http://www.anm.f2s.com/research/vizard/). Promoter sequences were extracted from the MIPS Arabidopsis database at http://mips.gsf.de/proj/thal/db/, and were analyzed using AlignACE software (Hughes et al., 2000). Sequence logos (Schneider and Stephens, 1990) were generated using the European Bioinformatics Institute (EBI) SEQUENCE LOGO interface at http://ep.ebi.ac.uk/EP/SEQLOGO/.

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