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Differential Gene Expression in Response to Mechanical Wounding and Insect Feeding in Arabidopsis

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Wounding in multicellular eukaryotes results in marked changes in gene expression that contribute to tissue defense and repair. Using a cDNA microarray technique, we analyzed the timing, dynamics, and regulation of the expression of 150 genes in mechanically wounded leaves of Arabidopsis. Temporal accumulation of a group of transcripts was correlated with the appearance of oxylipin signals of the jasmonate family. Analysis of the coronatine-insensitive *coi1-1* Arabidopsis mutant that is also insensitive to jasmonate allowed us to identify a large number of *COI1*-dependent and *COI1*-independent wound-inducible genes. Water stress was found to contribute to the regulation of an unexpectedly large fraction of these genes. Comparing the results of mechanical wounding with damage by feeding larvae of the cabbage butterfly (*Pieris rapae*) resulted in very different transcript profiles. One gene was specifically induced by insect feeding but not by wounding; moreover, there was a relative lack of water stress–induced gene expression during insect feeding. These results help reveal a feeding strategy of *P. rapae* that may minimize the activation of a subset of water stress–inducible, defense-related genes.

INTRODUCTION

Wounding is a continual threat to the survival of all organisms. Responses to wounding have been extensively studied in plants, which in the wild seldom escape some degree of damage from environmental stresses such as wind, sand, hail, and rain. An open wound caused by mechanical wounding is a potential infection site for pathogens; thus, expression of defense genes at the wound site is a barrier against opportunistic microorganisms. Plants respond to mechanical wounding with the induction of numerous genes. The first identified wound-inducible defense proteins in plants include proteinase inhibitors I and II from potato and tomato (Graham et al., 1986; Ryan, 1990).

In Arabidopsis, many genes have been shown to be induced by mechanical wounding (reviewed in Reymond and Farmer, 1998). The expression of many of these genes is induced by treatment with jasmonic acid (JA) or with its precursor oxophytodienoic acid (OPDA); these compounds, which are both members of the jasmonate family (Creelman and Mullet, 1997; Farmer et al., 1998), are essential in vivo regulators of defense gene expression (Reymond and Farmer, 1998). Other signals and stimuli also lead to the expression of genes in wounded plant tissues, although the relative contribution of molecules such as ethylene (O'Donnell et al., 1996; Rojo et al., 1999) and abscisic acid (Pena-Cortés et al., 1989; Birkenmeier and Ryan, 1998) and of electrical signals (Wildon et al., 1992) is still unclear. The importance of water stress/hydraulic pressure changes to gene expression during wounding has received even less attention (Malone and Alarcon, 1995).

A large proportion of multicellular eukaryotes eat plants, and a particularly common source of injury to plants is insect herbivory. Inevitably, insect feeding causes wounding of the plant, but little is known about how plants distinguish and respond to the very different threats posed by mechanical wounding and herbivory. Although reports show that some genes or proteins can be activated by both mechanical wounding and insect challenge (Howe et al., 1996; Stratmann and Ryan, 1997), other observations have revealed responses that are induced specifically or activated more rapidly by damage from insects. Differences have been observed in the expression of several wound-induced genes (Korth and Dixon, 1997) and also in the release of volatiles (Paré and Tumlinson, 1997). A study of insect damage to plants has led to the discovery of volicitin, a factor in insect saliva that elicits the production of plant volatiles, which then attract predatory insects to the herbivore insects (Alborn et al., 1997). As occurs with mechanical wounding, in which jasmonates play important roles in gene expression, the ability of plants to produce or perceive members of the jasmonate family of regulators is essential for their defense against

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tobacco hornworm (Howe et al., 1996) and fungus gnats (McConn et al., 1997).

In this study, our goal was to better understand how plant responses to mechanical wounding differ from those to insect feeding. An answer to this question is important because insects have probably evolved strategies to avoid activating the expression of at least some plant defense processes. We first studied gene expression dynamics in mechanically wounded Arabidopsis leaves by using a cDNA microarray that included 150 defense-related genes. We then dissected the signal requirements for the expression of wound-inducible genes, using Arabidopsis mutants impaired in the jasmonate and ethylene perception pathways. From these results, we determined which signal pathways are selectively activated by a feeding insect and which categories of genes escape activation during feeding. Our results illustrate fundamental differences in responses to damage caused by mechanical wounding and to damage from insect feeding; they also help to link feeding strategy to molecular responses in the plant.

RESULTS

Construction of a cDNA Microarray Containing Arabidopsis Defense-Related Genes

We used a previously described method (Eisen and Brown, 1999) to array a total of 150 polymerase chain reaction (PCR)–amplified double-stranded expressed sequence tags (ESTs) onto glass slides. Data on the ESTs that were used, the layout of the microarray, as well as extensive technical details can be found at http://www.unil.ch/ibpv. The array contained many of the commonly studied genes implicated in Arabidopsis defense and included many genes for which we only recently collated data on inducible expression (Reymond and Farmer, 1998). Genes of potentially related function are displayed in five separate domains: pathogenesis-related (PR) genes; general defense and stress-related genes (e.g., those encoding components of the myrosinase system as well as oxidative stress-related genes); genes involved in fatty acid signaling and metabolism; genes of aromatic amino acid metabolism; and genes involved in signal transduction, regulatory functions, or other (unknown) functions.

We included 16 genes for which the expression was unlikely to vary greatly during experiments (e.g., tubulin, actin, and translation elongation factors); these allowed us to calibrate the signal output and correct for sample-to-sample variability. In some cases, a so-called control gene showed a more than twofold variation in expression after wounding and thus could not be used for calibration. This speaks for the use of as many control genes as possible for data normalization.

Finally, three animal genes having no substantial homology to any sequence in the Arabidopsis database were printed on the microarray to assess for nonspecific hybridization. One of them, the peroxisome proliferator-activated receptor (*PPAR*α) gene, consistently produced a hybridization signal well above background and might have some degree of homology to an as yet unknown Arabidopsis sequence. The other two clones always produced a signal close to the background level (data not shown).

Dynamics of Wound-Inducible Gene Expression

The temporal program of transcription was studied in mechanically wounded Arabidopsis leaves. Leaves of 6- to 7-week-old plants were wounded with a forceps across the apical 40% of the lamina surface. At seven time points up to 24 hr after the wounding, leaves were detached and mRNA was purified. The cDNA made from each sample was labeled with the fluorescent dye Cy5 and mixed with a reference probe consisting of cDNA made from mRNA from unwounded plants and labeled with a second fluorescent dye, Cy3. The two populations of labeled cDNAs were simultaneously hybridized with the cDNA microarray; after scanning each fluor, the signal intensity for each gene was integrated. A pseudocolor image of the results obtained for one time point (60 min after wounding) is shown in Figure 1. Here, marked changes in transcript levels relative to those in the control plants are visible. The expression of each gene was calculated for the complete time course, and a hierarchical clustering program (Eisen et al., 1998) was used to analyze a subset of 91 genes for which expression changed substantially in response to wounding.

Figure 2 illustrates that use of this clustering program allowed grouping of genes with similar expression profiles during the time course. Various patterns of gene expression were observed, including early, mid-, and late gene induction as well as early repression of gene expression. By 15 min after wounding, the expression of 20 genes was already induced, including, for example, *PR-1, PR-2, PR-5*, touch genes (*TCH2, TCH3,* and *TCH4*), and genes encoding mitogen-activated kinases (*MPK3* and *MEKK1*). In several cases (e.g., *PR-1* and *MPK3*), the increase in transcript abundance was short-lived and fell rapidly to the base value. The number of upregulated genes increased to 39 at 90 min but was only 13 at 9 hr after wounding and seven by 24 hr after wounding.

One measure of the reproducibility of the changes we observed in gene expression is exemplified in Figure 2, in which, for most genes, we could see a gradual change over a few time points. This effectively provided independent measurements for all of the observations. To better assess the reproducibility of the microarray technique under our laboratory conditions, we performed nine independent replications of the same experiment. Arabidopsis leaves were wounded, RNA was isolated after 90 min, and labeled

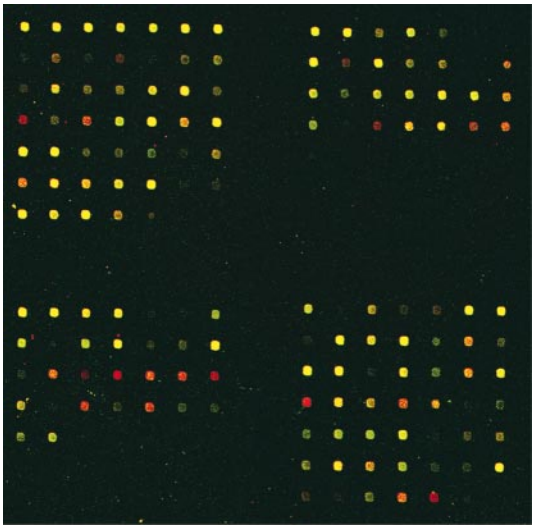

Figure 1. cDNA Microarray Analysis of Gene Expression after Mechanical Wounding.

A fluorescently labeled cDNA probe was prepared from mRNA isolated from control Arabidopsis leaves by reverse transcription in the presence of Cy3-dCTP. A second probe, labeled with Cy5-dCTP, was prepared from leaves that were mechanically wounded (60 min). After the simultaneous hybridization of both probes with a cDNA microarray containing 150 defense-related Arabidopsis ESTs and scanning of the array, a pseudocolor image was generated. Genes induced or repressed after mechanical wounding are represented as red or green signals, respectively. Genes expressed at approximately equal levels between treatments appear as yellow spots. The intensity of each spot corresponds to the absolute amount of expression of each gene. The actual size of the array is 8 × 8 mm. Control genes are in the first row of top left, top right, and bottom left quadrants.

mRNA samples from control and treated plants were hybridized with a microarray. The average expression ratios calculated for the nine independent experiments are shown in Figure 3 for a set of representative genes and illustrate the small variability in the measurements. In some cases in which duplicate genes were included, highly similar values were obtained (Figure 3). In addition, hybridization of different microarrays with the same mRNA samples indicated good correlation (data not shown).

The time-course analysis of gene expression revealed groups of genes with similar behavior (Figure 2). One implication of a common temporal pattern of expression is that genes might share similar or related roles in cellular processes, or they might be regulated by the same signal molecules. Figure 4A shows the mean expression ratios of a group of 17 transcripts that had similar temporal expression profiles. Among these are two genes (*LOX2* and *AOS;* Table 1) implicated in the synthesis of JA as well as a gene known to be induced by jasmonate, *JR3*. Moreover, JA and its

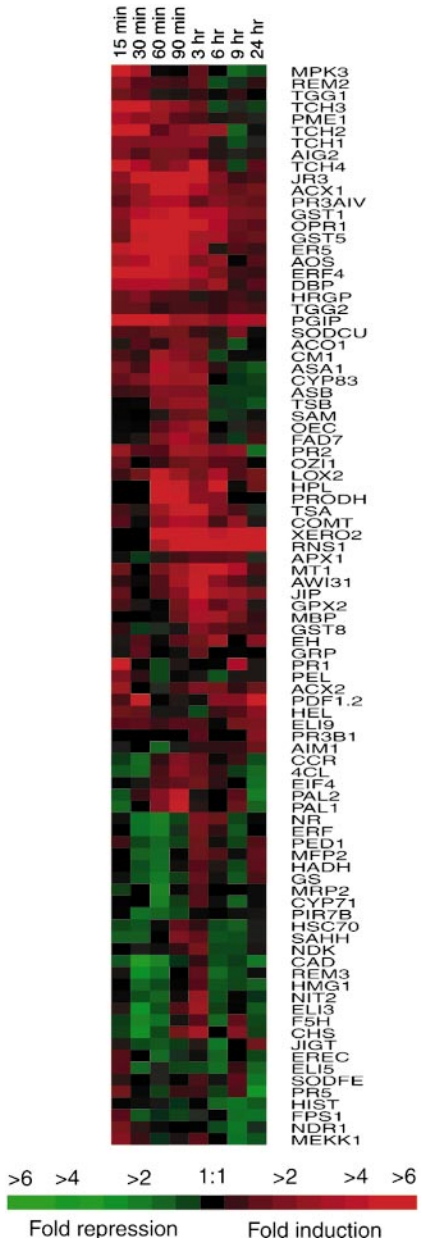

Figure 2. Clustered Display of Data from the Time Course of Mechanical Wounding.

A time course of wound-inducible gene expression in Arabidopsis leaves was constructed using cDNA microarrays. For simplicity, only those genes for which the transcript levels changed substantially as a result of wounding are included. Genes were ordered using a clustering program (see Methods) so that those with similar expression patterns would be grouped together. Each gene is represented by a single row of colored boxes, and each time point is represented by a single column. Induction (or repression) ranges from pale to saturated red (or green). The numbers of independent experiments were as follows: 15 min, 2; 30 min, 1; 60 min, 2; 90 min, 9; 3 hr, 3; 6 hr, 2; 9 hr, 1; and 24 hr, 1.

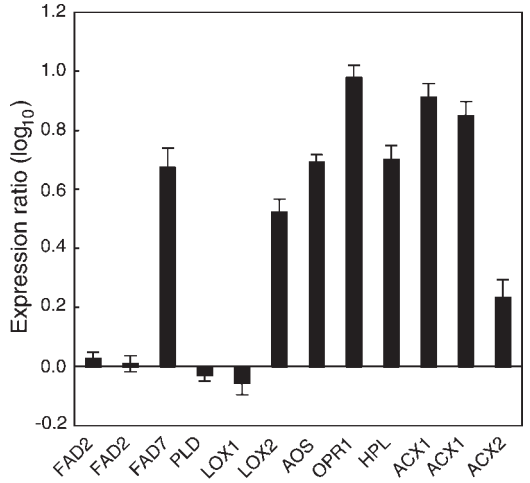

Figure 3. Reproducibility of cDNA Microarray Experiments.

mRNA samples (2 μg) from Arabidopsis leaves harvested 90 min after wounding or from control Arabidopsis leaves were labeled with Cy5 or Cy3, respectively, and hybridized with a cDNA microarray. After scanning each fluor separately, the fluorescent signal intensity was integrated and corrected for local area background. Expression ratios between treated and control samples were calculated. Results are shown for a set of representative wound-inducible genes. Values ±SE represent the average of nine independent experiments. Genes shown in duplicate (*FAD2* and *ACX1*) are represented by two different ESTs on the microarray, which show highly similar expression ratios.

precursor OPDA as well as its C_{16} carbon homolog dinor OPDA are known to accumulate in wounded plant tissues (Albrecht et al., 1993; Parchmann et al., 1997; Weber et al., 1997). By using the oxylipin signature technique (Weber et al., 1997), we were able to measure simultaneously the concentrations of OPDA, dinor OPDA, and JA throughout the time course of the wounded Arabidopsis leaves and to compare these contents with the induction of a subset of genes likely to be controlled by jasmonates. Figure 4B shows that JA reached a peak 2 hr after wounding, in striking agreement with the rise of the transcript levels shown in Figure 4A. In contrast, both OPDA and dinor OPDA levels rose more slowly, peaking ~6 hr after wounding.

Several Signal Pathways Regulate Wound-Inducible Gene Expression

To assess the in vivo role of jasmonates in wound-induced gene expression, we conducted experiments using jasmonate-insensitive mutants. The well-characterized coronatine-insensitive *coi1-1* mutant is insensitive to JA (Feys et al., 1994). Wound-inducible gene expression was analyzed in wild-type and *coi1-1* Arabidopsis plants. As Figure 5A shows, half of the genes that are normally induced after wounding in wild-type plants were no longer induced in the mutant, and two transcripts (*NPR1* and *MPK;* Table 1) were induced only in *coi1-1* plants. This latter finding was confirmed by RNA gel blot analysis for *MPK3* (Figure 5B). Results from microarray experiments allowed us to define two basic classes of wound-inducible genes, as shown in Table 1: a group of *COI1*-dependent genes for which induction, or repression, by wounding depends strictly on the ability of

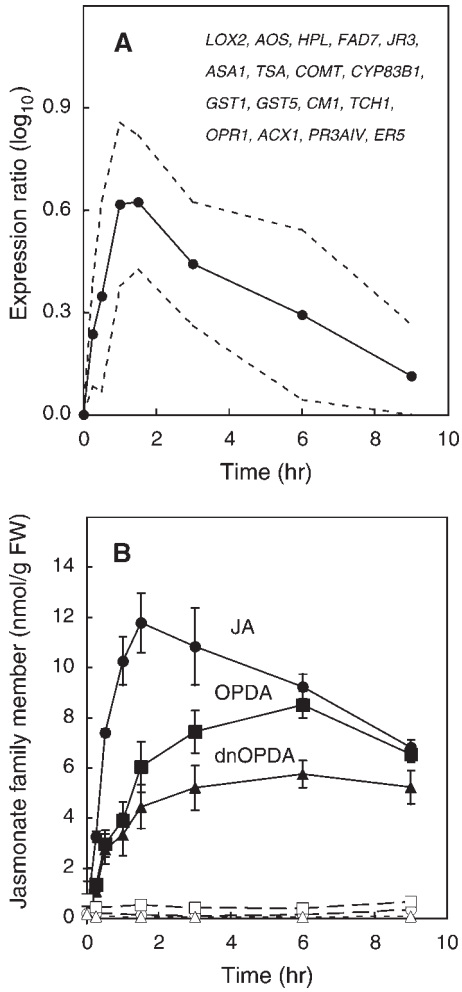

Figure 4. Comparison between the Expression of a Subset of Genes and the Levels of Jasmonate Family Members after Mechanical Wounding.

(A) The average expression profile of a cluster of genes showing a similar temporal expression profile is represented. Dashed lines indicate standard deviation. For experimental details, see Figure 2.

(B) Arabidopsis leaves were extracted at different times, and the tissues were analyzed for JA (circles), OPDA (squares), and dinor OPDA (dnOPDA, triangles) content in both wounded (solid lines) and control (dashed lines) plants. Mean values ±SE were calculated for three plants. FW, fresh weight.

Table 1. Relative Transcript Abundance after Wounding, Dehydration, or Insect (*Pieris rapae*) Feeding[a]

		Wounding		Dehydration	*P. rapae*
Gene	Description[b]	Wild Type	*coi1-1*	Wild Type	Wild Type
COI1-dependent genes[c]					
ACO1	Aminocyclopropane–carboxylic acid oxidase	2.2	1.8	1.2	1.5
ASA1	Anthranilate synthase (α subunit)	3.1	1.0	2.7	1.7
ASB	Anthranilate synthase (β subunit)	2.3	1.2	2.5	1.7
AOS	Allene oxide synthase	4.8	0.6	4.8	2.1
AWI31	Unknown	2.7	1.7	2.2	1.8
CCR	Cinnamoyl–coA reductase	2.1	1.2	2.4	1.3
CHS	Chalcone synthase	2.0	1.0	3.0	2.1
4CL	4-Coumarate:coA ligase	2.8	1.5	2.0	1.3
COMT	*O*-methyltransferase	4.7	1.6	3.6	1.4
CYP83B1	Cytochrome P450	2.9	1.0	4.4	1.8
FAD7	Fatty acid desaturase	4.7	1.2	2.7	1.5
HEL	Hevein-like protein	1.1	1.2	0.7	2.8
HPL	Hydroperoxide lyase	4.8	1.0	7.1	2.2
JIP	Jasmonate-inducible protein	2.9	1.6	2.5	3.1
JR3	Aminohydrolase	9.3	1.9	8.9	3.6
LOX2	Lipoxygenase	3.2	0.7	4.9	2.3
MBP	Myrosinase binding protein	2.6	1.0	2.2	2.1
MPK3	Mitogen-activated protein kinase	0.9	2.2	1.1	1.5
MT1	Metallothionein	2.2	1.5	1.9	2.1
NPR1	Transcription factor inhibitor	1.2	2.4	0.9	1.0
PAL2	Phenylalanine ammonia-lyase	5.6	1.4	1.1	0.3
PR-2	β-1-3-Glucanase	3.0	1.5	3.8	1.6
SAHH	*S*-adenosyl-L-homocysteine hydrolase	2.2	1.4	1.2	1.0
TSA	Tryptophan synthase (α subunit)	6.3	1.0	3.7	2.0
TSB	Tryptophan synthase (β subunit)	2.7	1.3	2.6	1.8
COI1-independent genes[c]					
ACX1	Acyl-coA oxidase	8.3	2.4	12.6	1.7
CM1	Chorismate mutase	2.1	2.1	2.6	1.7
DBP	Oligogalacturonide binding protein homolog	2.7	2.6	2.3	1.4
ER5	Late embryogenesis abundant-like protein	6.6	13.8	11.2	2.4
ERF4	Ethylene-responding factor	4.0	3.1	4.6	2.2
GPX2	Glutathione peroxidase	2.0	2.3	4.9	1.7
GST1	Glutathione *S*-transferase	9.2	12.0	2.2	2.1
GST5	Glutathione *S*-transferase	6.2	2.7	11.5	2.9
OEC	Oxygen-evolving protein	3.0	2.5	2.1	1.2
OPR1	OPDA reductase	9.3	7.7	1.5	1.7
PAL1	Phenylalanine ammonia-lyase	3.8	2.1	3.5	1.6
PGIP	Polygalacturonase-inhibiting protein	2.9	7.3	4.5	1.8
PME1	Pectin methyl esterase	2.0	2.2	0.7	1.1
PR3AIV	Chitinase	2.9	7.6	0.7	1.8
PRODH	Proline dehydrogenase	11.6	10.4	0.03	2.4
TCH1	Calmodulin	2.4	5.2	1.4	1.6
TCH2	Calmodulin-related protein	2.4	4.5	0.8	1.3
TCH3	Calmodulin-related protein	2.4	4.6	0.3	1.3
TCH4	Endotransglycosylase	3.7	3.7	1.0	1.9
RNS1	RNase	64.6	52.4	22.1	2.3
XERO2	Dehydrin-like protein	23.8	83.4	13.9	3.1

[a] Samples from wounded (90 min), dehydrated (120 min), or insect-challenged (180 min) Arabidopsis leaves were fluorescently labeled with Cy5-dCTP, and respective control samples (untreated) were labeled with Cy3-dCTP. After hybridization with a cDNA microarray and scanning, expression ratios were calculated. Ratios correspond to fluorescent values from treated plants relative to untreated plants.

[b] For further details, see http://www.unil.ch/ibpv.

[c] Genes induced (having a ratio >2.0) in wild-type plants after wounding as well as the *P. rapae*–inducible *HEL* and two genes (*MPK3*, *NPR1*) only induced in the wounded *coi1-1* mutant are included. Genes that are induced after wounding in both wild-type and *coi1-1* plants are considered independent of the *COI1* pathway. Our classification of *COI1*-dependent or -independent genes simplifies data analysis; we do not imply that this simplification exists in nature.

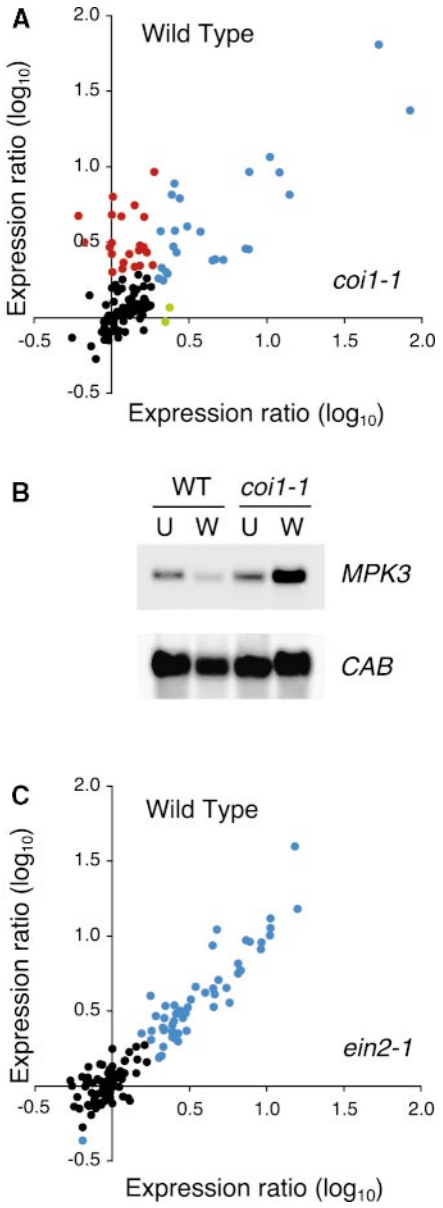

Figure 5. Contribution of Jasmonates and Ethylene to Wound-Inducible Gene Expression.

Relative changes in Arabidopsis gene expression after wounding of leaves for 90 min were studied in mutants. Expression ratios calculated from experiments comparing unwounded with wounded wild-type plants are plotted against expression ratios from experiments comparing unwounded with wounded mutant plants (**[A]** and **[C]**). Black dots represent genes that did not substantially change expression after wounding in both the wild type and mutants (based on the threshold of a twofold change). Blue dots represent genes that were induced (or repressed) in both wild-type and mutant plants. Red dots represent genes that were induced only in wild-type plants. Green dots represent genes (*NPR1* and *MPK3*) that were induced only in mutant plants.

(A) Jasmonate-insensitive mutant *coi1-1*.

(B) RNA gel blot analysis of *MPK3* mRNA accumulation 90 min after wounding of wild-type (WT) or *coi1-1* plants. A chlorophyll *a/b* binding protein probe (*CAB*) was used as a control for equal RNA loading. U, unwounded; W, wounded.

(C) Ethylene-insensitive mutant *ein2-1*.

Each scatter plot represents the mean of two independent experiments.

the plant to respond to JA; and a group of *COI1*-independent genes that are induced in the absence of JA signal transduction. Among the *COI1*-independent genes were some transcripts that had a temporal pattern of expression similar to the changes in JA concentrations (Figures 4A and 4B).

To find a potential signal involved in the induction of the *COI1*-independent group of genes, we investigated the role of ethylene in wounding. Ethylene is essential for the wound induction of proteinase inhibitors in tomato (O'Donnell et al., 1996), and the induction of a transcript encoding an ethylene-responding factor (*ERF4*) during wounding was noted in our experiments. Therefore, we investigated the effects of wounding the leaves of an Arabidopsis *ein2-1* mutant, which is insensitive to ethylene (Guzman and Ecker, 1990). Surprisingly, in plants from two different batches of *ein2-1* seeds, wounding induced the same sets of genes as those induced in wild-type plants, and the degree of induction was very similar in both cases, as depicted in Figure 5C. This implies that none of the wound-inducible genes represented on this microarray requires ethylene to respond to wounding under the experimental conditions described in this study.

Searching for another potential stimulus responsible for the induction of *COI1*-independent genes, we noticed the strong wound activation of genes (*XERO2* and *ER5;* Table 1) that are also induced by water stress (Rouse et al., 1996; Zegzouti et al., 1997). Therefore, we investigated the possible contribution of water stress to the expression of wound-inducible genes. Intact rosettes were gently cut away from their roots and were allowed to dehydrate in the air until they had lost 20% of their water (2 hr). Gene expression profiles were compared with those in control plants. Water stress was found to have a powerful effect on transcript levels: 58 genes on the array more than doubled their expression, and 31 of those were wound inducible (Table 1), including 13 *COI1*-independent genes. An interesting case was the gene encoding proline dehydrogenase (*PRODH*), which is repressed by water stress but induced by wounding.

Transcript Signatures in Wounding, Dehydration, and Insect Feeding

To investigate differences between mechanical damage and insect feeding, we allowed the larvae of the cabbage butterfly *Pieris rapae* to feed on the leaves of wild-type Arabidopsis plants until ~40% of the leaf surface had been removed.

At this point, a range of abandoned and newly started feeding sites was observed on the leaves. Insects were removed, and mRNA was extracted from the leaves for analysis. Figure 6A shows a direct comparison of gene expression in leaves damaged by *P. rapae* and in mechanically wounded leaves. Many transcripts were induced by both treatments, but generally, they were induced to higher levels in the mechanically wounded leaves. Many wound-inducible genes were not induced by the insect feeding (e.g., *PGIP, COMT, TCH1, OPR1,* and *ASA1*; Table 1). Only one gene, encoding a hevein-like protein (*HEL*), was induced by *P. rapae* but not by mechanical wounding. This observation was confirmed by RNA gel blot analysis (Figure 6B). Experiments with larvae of the closely related pierid *P. brassicae* yielded remarkably similar results (data not shown). Again, *HEL* was specifically induced by the insect.

A comparison of data sets from mechanical wounding, dehydration, and insect feeding allowed us to recognize different patterns of gene expression, as illustrated in Figure 7 with a set of representative genes. We observed that some genes were induced in all treatments, some induced transcripts were common to mechanical wounding and dehydration, and others were unique to each treatment. Some of these marker genes (Figures 7A and 7C) will provide a good tool for the further analysis of signaling pathways specifically involved in the responses to mechanical wounding or insect damage. We found no examples of genes that were induced by both dehydration and insect feeding but not by mechanical wounding. The transcript signature of mechanical wounding was more similar to that of dehydration than to that of insect feeding (Figure 7).

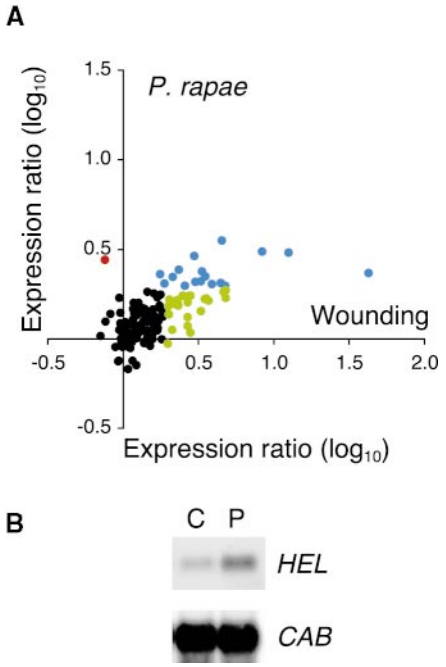

Figure 6. Comparison between Effects of Mechanical Wounding and Insect Feeding.

(A) Relative changes in gene expression were measured 3 hr after wounding Arabidopsis leaves and after challenging leaves with *P. rapae* larvae for 3 hr. Expression ratios calculated from experiments comparing unwounded with wounded plants are plotted against expression ratios from experiments comparing unchallenged with insect-challenged plants. Black dots represent genes that showed no marked change in expression after wounding or insect challenge (based on the threshold of a twofold change). Blue dots represent genes that were induced in both treatments. The red dot represents a gene (*HEL*) that was induced only in insect-challenged plants. Green dots represent genes that were induced only after mechanical wounding.

(B) RNA gel blot analysis of *HEL* mRNA accumulation in leaves challenged for 3 hr with *P. rapae* larvae. A chlorophyll *a/b* binding protein probe (*CAB*) was used as a control to assess equal RNA loading. C, unchallenged; P, *P. rapae*.

DISCUSSION

In this study, we used cDNA microarrays to identify a number of Arabidopis genes for which we were unable to find reports of wound induction in the literature. The data also confirmed previous studies of other genes for which activation by wounding was described (Reymond and Farmer, 1998). Mechanical wounding of Arabidopsis leaves initiated important and dynamic changes in gene expression (Figures 1 and 2). In response to a punctual mechanical wound, the transcript levels for many genes increased to maximum values 90 to 120 min after wounding and then began to subside toward the baseline. Several families of transcripts showed coordinated induction, including genes from the tryptophan pathway (*ASA1, ASB, TSA,* and *TSB*), touch genes (*TCH1, TCH2, TCH3,* and *TCH4*), or genes implicated in aromatic metabolism (*CHS, CCR, 4CL COMT,* and *PAL*). The concerted induction of metabolic cassettes implies tight control of expression among genes with potentially related functions and opens the door for comparative studies using conserved elements in the regulatory regions of these genes.

Another example is provided by genes involved in the synthesis or metabolism of members of the jasmonate family (*FAD7, LOX2,* and *AOS*), which are coordinately induced during wounding (Figure 4A). Concomitantly with changes in gene expression, the amounts of three members of the jasmonate family—OPDA, dinor OPDA, and JA—transiently increased (Figure 4B). The results revealed that temporal changes in JA content correlate tightly with the induction of a group of genes, including genes involved in JA biosynthesis. Moreover, the relative proportion of JA to the cyclopentenones OPDA and dinor OPDA constantly changed during wounding. Shortly after the wound stimulus, molar amounts of JA exceeded the combined quantities of OPDA and dinor

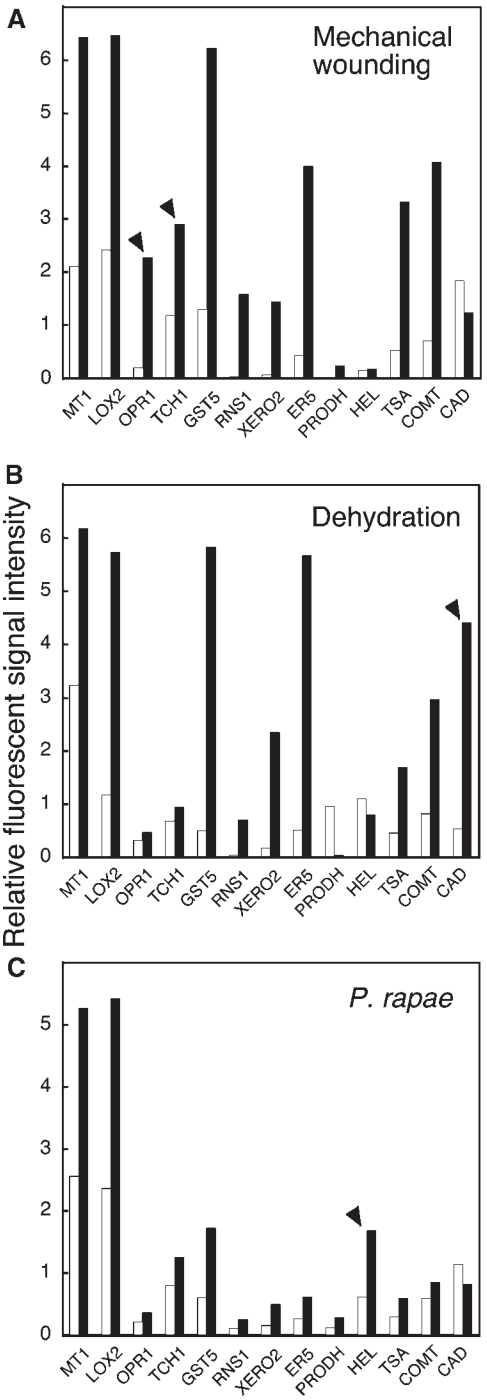

Figure 7. Transcript Signatures for Mechanical Wounding, Dehydration, and Insect Feeding.

Arabidopsis leaves were mechanically wounded (90 min), dehydrated (120 min), or challenged with *P. rapae* larvae (180 min). Cy3- or Cy5-labeled cDNA probes were prepared with mRNA samples from control (open columns) or treated (filled columns) plants, respectively, and were hybridized with a cDNA microarray. After scanning each fluor separately, the fluorescent signal intensity was integrated and corrected for local area background. Results are shown for a set of genes illustrating typical patterns of expression. Genes marked with an arrowhead were induced in only one treatment.

(A) Mechanical wounding.

(B) Dehydration.

(C) *P. rapae*.

OPDA. However, 2 to 3 hr after wounding, the combined amounts of the cyclopentenone oxylipins exceeded those of JA. This crossover point might be relevant to regulation of gene expression by different jasmonates. Future work should address the specific role of each of these molecules in gene expression as determined with cDNA microarrays. The experiment shown here highlights the potential of combining "oxylipin signatures" with "transcript signatures"; it will be interesting to make comparisons in larger databases containing more values for gene expression and more complex pools of oxylipins.

The fact that genes showed similar expression profiles when wounded does not imply that they are regulated by the same signal. Indeed, we found several genes (e.g., *GST1, TCH1,* and *ER5*) for which the temporal expression profile was similar to that of JA-related genes (Figure 4A) but that were still induced by wounding in JA-insensitive *coi1-1* plants (Table 1). Surprisingly, one of these was *OPR1*, which possibly participates in the synthesis of JA (Schaller and Weiler, 1997). The reason that *OPR1* induction is independent of the JA perception, whereas two other genes in the same pathway (*LOX2* and *AOS*) are dependent on an intact JA signaling pathway, is not known. The exact role of *OPR1* induction in wounding requires additional studies.

The use of the *coi1-1* mutant yielded two broad categories of wound-inducible genes: *COI1*-dependent and *COI1*-independent genes (Table 1). It is important to note that a gene defined as *COI1*-dependent or -independent in this study might be controlled differently under other conditions, and we used these two categories only for the present study. We assume that most, if not all, *COI1*-dependent genes are jasmonate dependent and that their expression is altered by a direct or indirect effect of the loss of a functional *COI1* gene. Additionally, the category of *COI1*-independent genes contains genes that were differentially expressed in wounded wild-type and *coi1-1* plants. Some genes were more highly induced in wild-type plants (e.g., *ACX1* and *GST5*), whereas others (e.g., *ER5* and *PGIP*) were more highly induced in *coi1-1* plants. These results merit further attention because they indicate that *COI1* might play subtle roles as a positive or negative regulator of other signal pathways controlling wound-inducible gene expression.

Interestingly, the transcript levels of two genes (*NPR1* and

MPK3; Figure 5A) were induced in wounded *coi1-1* plants after 90 min but not in wounded wild-type plants. *MPK3* shows an early induction after wounding of wild-type plants, but its transcript level returns to the control value 60 min after wounding (Figure 2). These two genes have been implicated in the signaling pathway that leads to resistance to bacterial and fungal pathogens (Cao et al., 1997; Ligterink et al., 1997; Ryals et al., 1997). This observation indicates that sensitivity to endogenous JA might downregulate gene expression. Similarly, exogenous JA has been shown to downregulate the amounts of various transcripts (Wasternack and Parthier, 1997). Our results demonstrate a possible case of signal pathway interaction (cross-talk) in which a wounding pathway might override a pathogen defense pathway.

Our finding that many genes (21) are regulated in a *COI1*-independent manner (genes that are wound-regulated in both the wild-type and the *coi1-1* plants) is in strong agreement with the literature, and several wound-inducible but jasmonate-independent genes are known (Titarenko et al., 1997; Leon et al., 1998; Rojo et al., 1998). We concentrated on the *COI1*-independent genes, because little information is available in the literature on how this large group of genes is regulated during wounding (Rojo et al., 1998), and tested the role of ethlyene as a signal during mechanical wounding. The observation that the lack of ethylene sensitivity did not affect the wound-inducible expression of several genes in the Arabidopsis mutant *ein2-1* is somewhat surprising (Figure 5C). Ethylene has been shown to be necessary for the wound induction of the proteinase inhibitor II gene in tomato (O'Donnell et al., 1996), but touch genes (*TCH2, TCH3,* and *TCH4*) are known to respond to mechanical stimulation even in *ein* mutants (Johnson et al., 1998). A recent study has implicated ethylene in the downregulation of a subset of wound-inducible, JA-dependent genes in Arabidopsis (Rojo et al., 1999). Thus, a more complete picture of apparently complex roles of ethylene in wound-inducible gene expression awaits the analysis of cDNA microarrays containing a larger number of genes.

Water Stress Is an Important Component in the Response to Mechanical Wounding

Because ethylene perception was not required for the expression of the *COI1*-independent genes in our study, we decided to determine whether other factors might contribute to the expression of these genes during wounding. Sensitivity to touch is one factor, and not surprisingly, four of these genes were touch genes (*TCH1, TCH2, TCH3,* and *TCH4*), which are known to be regulated by signal networks involving calcium flux (Braam and Davis, 1990). This left 17 genes for which the inducing stimulus was unclear. Because some of the genes are known to be induced by drought, we looked at gene expression in dehydrating leaves that had lost 20% of their water content. For these experiments, *PRODH*, a gene known to be strongly downregulated during water stress (Kiyosue et al., 1996), served as an excellent control, and in our experiments, transcript levels for *PRODH* were reduced 33-fold (relative to controls) as a consequence of rosette detachment (Table 1). We found that the expression of many wound-inducible genes was induced by dehydration (Table 1), including 13 *COI1*-independent genes. This result implies that water stress might play a pivotal role during the response to the mechanical wounding we used, which generated crushed tissue.

Exactly how water stress leads to changes in gene expression is not clear. In tomato, at least two wound-inducible genes are upregulated by water deficit, abscisic acid, and salinity (Chao et al., 1999). For *COI1*-independent genes described in this study, several factors might contribute to changes in gene expression, for example, the decrease in hydraulic pressure resulting from wounding tissue or from dehydration itself. From our results, we cannot distinguish which of these factors, or others, are associated with gene activation, and we did not assess the contribution of abscisic acid to the regulation of gene expression. Several *COI1*-dependent genes were also induced by dehydration (Table 1), and JA contents are known to increase in tissues undergoing water stress. JA or its precursors may thus mediate at least some drought stress signaling events (Creelman and Mullet, 1997). Because the treatment we used to induce water stress (detachment of rosettes from the root system) may have itself resulted in a wound stimulus, the increase in some transcripts might also be due to this distal wound stress in addition to a water stress.

Finally, for four genes (*PME1, PR3AIV, OPR1,* and *PRODH*), the signal or stimulus that controls their induction during wounding is not known. Possible candidates would be oxylipins (different from jasmonates), cell wall–derived oligosaccharides (Rojo et al., 1999), touch, ion fluxes, or plant hormones. Reactive oxygen intermediates involved in plant defense (Alvarez et al., 1998) could also be envisaged.

In summary, our results lead to a more comprehensive view of gene expression in response to mechanical wounding. Several factors, including tissue damage and water loss, lead to a complex, dynamic pattern of transcript levels in which waves of gene expression involving groups of similarly behaving transcripts were observed. Underlying these patterns is the complex interplay of stimuli that control gene expression, in which one input (e.g., wounding) can override another (e.g., water stress), as demonstrated by the interesting behavior of *PRODH*, which was strongly upregulated by mechanical wounding and downregulated by water stress (Table 1).

Reduced Water Stress–Inducible Gene Expression during *P. rapae* Feeding

Having characterized the expression of an array of genes during mechanical wounding, we then compared the effect of insect damage, asking whether insects preferentially

induce *COI1*-dependent or *COI1*-independent genes. We also estimated the contribution of water stress to the induction of gene expression during insect feeding. The lepidopteran *P. rapae* is a common and economically important insect pest, and the association between members of the Brassicaceae and cabbage butterflies, in particular *P. rapae* and *P. brassicae*, has long been used as a model plant–insect system (Renwick, 1995). On Arabidopsis, we observed that the sharp mouth parts of *P. rapae* larvae successively removed tissue from the edge of the leaf, leaving an oval, semioval, or semicircular hole in the leaf without cutting the midvein. Once the feeding site approached the midvein, the larvae moved to another leaf or to a different site on the same leaf (often on the opposite side of the midvein). This behavior probably not only requires the least amount of movement for the insect but also allows a larva to efficiently and successively remove strips of already wounded tissue at the cut leaf edge while exposing the least amount of damaged surface to the air. When we allowed larvae of *P. rapae* to feed on Arabidopsis leaves until ~40% of the leaf area had been removed, we found that insects induced gene expression in a manner very different from mechanical wounding.

As our results definitively show, many genes that were strongly induced by mechanical damage were less or not at all induced when a plant was attacked by *P. rapae*. The fact that the insect did not induce the expression of many wound-inducible genes is likely to be to the insect's advantage. Several genes that were not induced by insect feeding include *PR* genes (e.g., *PR-2* and *PAL*) or genes involved in the synthesis of aromatic metabolites (e.g., *CCR* and *COMT*), which might reduce insect fitness. On the other hand, the defense gene *HEL* was reproducibly induced by *P. rapae* larvae but not by mechanical wounding. *HEL* is known to be induced by microbial pathogens by way of a JA-dependent pathway (Potter et al., 1993; Thomma et al., 1998), but we found that this gene is also induced by insect feeding. *HEL*, which might be induced by an elicitor released from the insect, thus provides a good marker for further studies of insect interaction with Arabidopsis.

Use of cDNA microarrays containing many more genes might reveal a new class of insect-specific genes that might be useful in developing biotechnological tools for insect control. Although our array contained only 150 genes, it was already large enough to permit the detection of a gene (*HEL*) induced by feeding *P. rapae* larvae but not by mechanical wounding. Our results illustrate the advantages of using small boutique arrays. First, the quality of each clone on the array can be controlled and printing errors can be rapidly rectified. Second, assembly of boutique arrays is possible in small laboratories that currently lack the resources to print genome-scale arrays.

Concerning the signal pathways activated by feeding *P. rapae,* several *COI1*-dependent genes (e.g., *LOX2, MT1, TSA,* and *JIP*) as well as several *COI1*-independent genes (e.g., *GST1, ERF4, RNS1,* and *PRODH*) were induced. Thus, the insect did not appear to preferentially activate either jasmonate-dependent or -independent genes. It is remarkable that the feeding of *P. rapae* had little effect on genes such as *XERO2*, *RNS1*, *ER5*, *TSA,* and *COMT* (Table 1), all of which are water stress inducible. Perhaps *P. rapae* minimizes the effects of water stress on gene expression when feeding by reducing the crushing of tissue and by keeping to a minimum the cut edge of the lamina while removing the maximum tissue mass. In other words, it might not be a coincidence that cabbage butterfly larvae often leave circular or semicircular holes in host plant leaves in contrast to following a feeding strategy that might expose a greater length of ragged or crushed leaf edge to the air. Indeed, some specialist insects use elaborate vein-cutting strategies to cut the flow of defense chemicals to the feeding site (Dussourd and Eisner, 1987). Our results are consistent with the idea that the larvae of cabbage butterflies, such as *P. rapae*, may utilize feeding approaches designed to minimize the activation of a subset of host defense genes.

METHODS

Plant Materials and Growth Conditions

Seeds from plants (*Arabidopsis thaliana* ecotype Columbia) were sown on potting compost and vernalized for 4 days at 4°C. After 17 days of incubation in a growth chamber (10 hr of light at 150 μmol m^{-2} sec^{-1}) at 23°C during the day and 18°C at night, the young plants were transferred to pots (7 cm in diameter, with two plants per pot) containing potting compost and were grown for 3 to 4 weeks in a growth room (20°C at 70% relative humidity and with 10 hr of light at 100 μmol m^{-2} sec^{-1}). The jasmonate response mutant *coi1-1* (Feys et al., 1994) was obtained from J. Turner (University of East Anglia, Norwich, UK). Because this mutation is recessive and causes male sterility, we identified *coi1-1* mutants in F_2 plants grown from self-fertile F_1 plants. Seeds were germinated on Murashige and Skoog medium (Sigma, Buchs, Switzerland) containing 3% sucrose and 30 μM jasmonate and incubated under light (150 μmol m^{-2} sec^{-1}) for 10 days in a growth chamber. Homozygous *coi1-1* mutants showing normal greening of leaves and no inhibition of root growth (Feys et al., 1994) were transferred to pots, as described earlier. The presence of the mutation was confirmed in all plants by using a described cleaved amplified polymorphic sequence marker (Xie et al., 1998). Two separate batches of the ethylene-insensitive *ein2-1* mutants were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK).

Plant Treatments

For wounding treatments, we crushed all rosette leaves of Arabidopsis plants several times across the apical lamina with a forceps, which effectively wounded ~40% of the leaf area. Plants were incubated for various periods, after which the leaves were harvested and immediately immersed in liquid nitrogen. For dehydration experiments, we detached whole Arabidopsis rosettes from the roots by cutting with a clean razor blade; we then weighed them and placed them on chromatography paper (3MM; Whatman, Maidstone, UK) at

20°C and 70% humidity. After 2 hr, plants were weighed again to estimate water loss and were then immediately immersed in liquid nitrogen. For feeding experiments, *Pieris rapae* and *P. brassicae* larvae collected near Lausanne were maintained on cabbage (*Brassica oleracea*) plants in a greenhouse. Fourth and fifth instar larvae were placed on Arabidopsis plants (three larvae per plant) and were allowed to feed under light (100 μmol m^{-2} sec^{-1}) for 3 to 4 hr at 20°C until ~40% of the leaf surface was removed. Larvae were then removed, and all plant leaves were immediately frozen in liquid nitrogen. Mechanical wounding experiments designed to damage leaves in patterns similar to those of feeding insects, including wounding the lamina but not the midribs, were performed. All gene expression patterns were similar to those found in response to normal wounding treatments (data not shown).

Quantitative Analysis of Jasmonate Family Members

Extraction and quantitative analyses of jasmonic acid (JA), oxophytodienoic acid (OPDA), and dinor OPDA were performed with 1 g of leaf material, as previously described (Weber et al., 1997).

cDNA Clones and Microarray Preparation

Arabidopsis cDNA clones (expressed sequence tags [ESTs]) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Inserts of cDNA clones were amplified by polymerase chain reaction (PCR) in 100-μL reaction volumes by using primers that were complementary to vector sequences flanking both sides of the cDNA insert. At the end of each reaction, 5 μL of product was electrophoresed on agarose gels to confirm amplification quality and quantity. PCR products were purified on QIAquick-96 columns (Qiagen, Basel, Switzerland), lyophilized, and resuspended in 8 μL of 3 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) in a 384-well microtiter plate. Final insert concentration was >500 ng/μL.

Microarray fabrication was performed according to published methods (Schena et al., 1995; Shalon et al., 1996). Briefly, PCR products were arrayed onto silylated microscope slides (CEL Associates, Houston, TX) by using a high-precision gridding robot (GeneMachines, San Carlos, CA) equipped with four printing tips (TeleChem International, San Jose, CA). After printing, silylated slides were allowed to dry, and nonbound DNA was removed with 0.2% SDS and double-distilled H_2O; covalently bound DNA was denatured for 2 min in boiling water. Free aldehydes were reduced by soaking slides for 5 min in 68 mM sodium borohydride (dissolved in PBS containing 25% ethanol). Several washing steps were performed with 0.2% SDS and double-distilled H_2O; then slides were dried by centrifugation (model MSE Mistral 2000R; Kleiner, Wohlen, Switzerland) at 500*g* for 5 min and stored at room temperature for further hybridizations. For full details, see http://www.unil.ch/ibpv.

mRNA Isolation and Preparation of Fluorescent Probes

Total leaf RNA was extracted with 2:1 (v/v) extraction buffer (0.5 M Tris-HCl, pH 8.2, 0.25 M EDTA, and 5% SDS):phenol solution, followed by two washes with chloroform and overnight precipitation with LiCl (3 M final concentration). The RNA pellet was resuspended in H_2O and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The pellet was rinsed with 70% ethanol, air dried, and resuspended in H_2O. Poly(A)$^+$ mRNA was prepared by using an Oligotex Midi kit (Qiagen) according to the manufacturer's instructions.

Each mRNA sample (one control and one treated sample) was reverse-transcribed in the presence of Cy3-dCTP or Cy5-dCTP (Amersham Pharmacia Biotech, Dübendorf, Switzerland). Each reaction was performed in a 30-μL volume containing 2 μg of mRNA, 2 μg of oligo(dT) 21-mer, 500 μM each for dATP, dGTP, and dTTP, 200 μM dCTP, 100 μM Cy3-dCTP or Cy5-dCTP, 30 units of RNase inhibitor (Life Technologies, Basel, Switzerland), 10 μM DTT, and 400 units of SuperScriptII reverse transcriptase (Life Technologies) in SuperScript buffer (Life Technologies). After incubation at 42°C for 1 hr, the sample tubes containing Cy3 and Cy5 labeling were pooled and treated with 2.65 μL of 25 mM EDTA and 3.3 μL of 1 M NaOH for 10 min at 65°C to degrade the RNA. After the addition of 3.3 μL of 1 M HCl and 5 μL of 1 M Tris-HCL, pH 6.8, labeled single-stranded DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol, and the pellet was washed with 80% ethanol, dried under vacuum, and resuspended in 10 μL of hybridization solution containing 3 × SSC, 0.2% SDS, and 0.02% yeast tRNA (Life Technologies). Probes were purified by Millipore (Volketswil, Switzerland) Ultrafree-MC filters.

Hybridization Reaction and Microarray Analysis

Before hybridization, the probe solution was boiled for 1 min and then rapidly applied to the microarray under a cover slip. Slides were placed in hybridization chambers (TeleChem International), and 20 μL of 3 × SSC was placed inside each chamber before sealing. Slides were incubated for 14 to 16 hr in a water bath at 64°C and then were sequentially washed in the following solutions: 2 × SSC, 0.1% SDS twice for 5 min, 0.2 × SSC twice for 1 min, and 0.1 × SSC twice for 1 min. Slides were dried by centrifugation at 900*g* for 2 min (MSE Mistral 2000R) before they were scanned.

Microarrays were scanned with a scanning laser microscope (ScanArray3000; GSI Lumonics, Watertown, MA). Separate images were acquired for each fluor at a resolution of 10 μm per pixel. To normalize the two channels with respect to signal intensity, we adjusted photomultiplier and laser power settings such that the signal ratio of the majority of control genes was as close to 1.0 as possible. The average fluorescence intensity for each fluor and for each gene was determined by using the ScanAlyze program (written by M. Eisen, Stanford University; available at http://rana.stanford.edu/software). Background fluorescence was calculated as the median fluorescence signal of nontarget pixels around each gene spot. Genes showing a signal value <1000 (which was typically twice the mean background value) in both Cy3 and Cy5 channels were not considered for the analyses. For all of the experiments, we defined induction or repression of a gene as a minimum twofold change in its transcript level. Gene-clustering analysis was performed as described previously (Eisen et al., 1998).

RNA Gel Blot Analysis

Two micrograms of poly(A)$^+$ mRNA was electrophoresed in formaldehyde-containing agarose gel and transferred to nylon membrane (Hybond N$^+$; Amersham). ESTs for *HEL*, *MPK3*, and chlorophyll *a*/*b* binding protein were labeled with digoxigenin (Roche Molecular Biochemicals, Rotkreuz, Switzerland) by PCR amplification and used as

probes. Hybridization and detection of digoxigenin-labeled probes were performed according to the manufacturer's instructions.

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