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Spatial and temporal analysis of the local response to wounding in *Arabidopsis* leaves

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Abstract

We studied the local response to wounding in *Arabidopsis thaliana* leaves using a two-step microarray analysis. A microarray containing 3500 cDNA clones was first screened to enrich for genes affected by wounding in the immediate vicinity of the wound (4 h post wounding). 359 non-redundant putative wound responsive genes were then spotted on a smaller wound-response array for detailed analysis of spatial expression (local, adjacent and systemic), timing of expression (0.5, 4, 8, 17 h), and effect of hormone treatments (methyl jasmonate, ethylene and abscisic acid). Our results show that genes that respond early at the site of the wound also respond throughout the plant, with similar kinetics. Early-induced genes which respond systemically encode predominantly signal transduction and regulatory factors (36%), and the expression of many of them is also controlled by methyl jasmonate (about 35% of the 36%). Genes specific to the wound site and the wounded leaf have a slower response to wounding and are mainly metabolic genes. At the wound, many genes of the lignin biosynthesis pathway were induced. *In silico* analysis of the 5′ promoter regions of genes affected by wounding revealed G-box-related motifs in a significant proportion of the promoters. These results show that the establishment of a systemic response to wounding is a priority for the plant, and that the local response at the wound site is established later. Ethylene and abscisic acid are involved in the local response, regulating repression of photosynthetic genes and expression of drought responsive genes respectively.

Introduction

As sessile organisms, plants are forced to react to a number of biotic and abiotic stresses. Agents that inflict wounding and immediate tissue damage to the plant, such as herbivore feeding and adverse weather conditions, endanger plant survival by exposing the plant to water loss and further invasion by pathogens. The response of plants to mechanical wounding has been the subject of extensive investigations (for reviews, see: Bowles, 1990; Ryan, 2000; de Bruxelles and Roberts, 2001; Leon *et al.*, 2001). Some plant species have developed complex natural defense mechanisms to protect against wounding, ranging from physical barriers, including cuticle formation, lignification, thorns and trichomes (Levin, 1973), to the production of toxic components such as alkaloids (Baldwin, 1989) and tannins (Scalbert, 1991).

The wounding response (WR) involves repair of damaged wound tissue, production of compounds that limit herbivore feeding (e.g., proteinase inhibitors that limit digestibility; Peña-Cortés *et al.*, 1995), protection against subsequent infec-

tion by opportunistic pathogens, and the adjustment of plant metabolism to cope with all these changes. The WR can be subdivided into a local response, occurring in the immediate vicinity of the wound, and a systemic response, occurring throughout the plant (Bowles, 1993). Genes involved in the local response are predicted to play a role in wound healing and repair, as well as protection against water loss and invasion of pathogens. The systemic genes produce a defense mechanism against further attack by herbivores or pathogens.

Recently, microarrays have enabled the large-scale identification of WR genes and the functional dissection of the response. A substantial overlap was demonstrated between wounding and the response to water stress (Reymond *et al*., 2000), and between wounding, pathogen response and other signaling pathways (Cheong *et al*., 2002). The finding that the *ADC2* (arginine decarboxylase) gene is induced by wounding, suggested an involvement of polyamines in the response (Perez-Amador, 2002).

The systemically induced genes and the long-distance signaling aspects of the WR have been the subject of extensive investigations. Several signaling mechanisms have been identified (for review, see: Ryan, 2000; de Bruxelles and Roberts, 2001; Leon *et al*., 2001). Oligogalacturonides derived from damaged plant cell walls or from fungal pathogens induce proteinase inhibitor (*pin*) genes in tomato (Doares *et al*., 1995; Bergey *et al*., 1999). The mobility of oligogalacturonides is limited and they are involved in the local response to wounding. In solanaceous plants, the 18 amino acid peptide systemin was shown to function as a long distance signal (Ryan, 2000). Systemin activates a lipid-derived signalling pathway, leading to the local and systemic accumulation of the hormone jasmonic acid (JA) and its ester methyl jasmonate (MeJA; Farmer and Ryan, 1990). More recently, reciprocal grafting experiments demonstrated that activation of the JA biosynthetic pathway by wounding or systemin is required for the production of a long-distance signal whose perception in distal leaves depends on JA signaling, suggesting that JA or a related compound of the octadecanoid pathway may act as a transmissible wound-signal (Li *et al*., 2002; Lee and Howe, 2003). However, in systemic leaves, accumulation of JA or its bioactive precursor OPDA (12-oxophytodienoic acid) could not be detected even though activation of defense gene expression occurred (Strassner *et al*., 2002). Wounded plants also accumulate abscisic acid (ABA) in the region surrounding the wound site (Peña-Cortés *et al*., 1995; Birkenmeier and Ryan, 1998). ABA may function in a dehydration response pathway at the wound site (Reymond *et al*., 2000). Wounding also induces ethylene biosynthesis and ethylene acts in conjunction with JA to regulate *pin* expression (O'Donnell *et al*., 1996). In addition to these signals, the WR was shown to involve reactive oxygen species (ROS; Orozco-Cardenas and Ryan, 1999), secondary messengers (e.g., calcium), and phosphorylation events (for review, see Leon *et al*., 2001).

In order to study which factors are involved in the establishment and regulation of the WR we focused our microarray approach on the analysis of gene expression at the wound site. To achieve this, we used a two-step microarray approach. We enriched for genes expressed at the wound site in the first step, and then printed these genes on a smaller wound responsive (WR) array. On this specialized array we carried out a detailed study of spatial and temporal expression, and response to phytohormones of WR genes. Our results show that the systemic response is established earlier than the local response, involves mainly signaling and regulatory factors, and is partly regulated by MeJA. Genes that are specific to the local response encode mainly proteins with metabolic functions.

Materials and methods

Plant material

Arabidopsis thaliana ecotype C24 plants were grown in 80mm petri dishes on Murashige–Skoog (MS) medium containing 3% sucrose and 0.8% agar, under 16 h day-light (75–100 μmole/m^2/s) at 22 °C. After a week, the plantlets were transferred to new petri dishes containing MS-agar medium (3% sucrose), at a density of 5 plantlets/dish to improve growth and expansion of the leaves. In the case of plants used in phytohormone treatments, the plants were grown under the same conditions and were transferred to petri dishes containing 15 ml of liquid MS medium (3% sucrose) 3 days prior to the start of the treatment

with gentle shaking at 60–80 rpm, under identical lighting and temperature conditions.

Wounding and phytohormone treatments

Five weeks old plants were used for all treatments. Wounding treatment involved mechanically wounding about 50% of the area of the leaves with a pair of sterilized serrated forceps; about half the leaves of the rosette of a plant were wounded. Each pinch was made transversally across the whole leaf, leaving non-wounded tissue on both sides of the wounded area (Figure 1). The plants were then returned to the growth room for the duration of the treatment. For MeJA and ABA, 0.1 M stock solutions were prepared in DMSO. Plants that were growing in liquid medium for 3 days were transferred to fresh liquid MS medium supplemented with 0.1 mM (final concentration) of the hormone. The plates were returned to the growth room with gentle shaking for 5 h. For the ethylene treatment, plants that were transferred in liquid medium for 3 days were then transferred to fresh liquid MS medium and put in an airtight jar. Pure ethylene gas was then injected in the air space to a final concentration of 5 or 200 ppm and the jar was returned to the growth room for 5 h with gentle shaking. Control plants were subjected to the same treatment but were transferred to fresh liquid MS instead.

Description and screening of the 3.5 K array

The 3.5 K array used in this study was previously described by Klok *et al*. (2002). The array contains 2500 sequenced EST clones that were representative of a wide range of developmental and metabolic processes from different organs and at different developmental stages (Schenk *et al*., 2000). In addition, a total of 1000 clones were added randomly from a non-normalized cDNA library prepared from low-oxygen stressed hairy root cultures (Klok *et al*., 2002).

Results were obtained from four hybridizations: two biological replicates, and two repeats in which the probe was made using the same first strand cDNA, but labelled with the opposite dye (technical repeat). Microarray data were normalized using the 'SlideNormalise' function in the tRMA package (Wilson *et al*., 2003) and data from replicates were averaged (median value). Genes with significant differential expression were defined as those genes with an induction or repression ratio greater or equal to two.

Preparation and screening of the specialized WR array

All clones (133 housekeeping and control genes, as well as 413 independent clones representing 359 non-redundant putative WR genes) were amplified

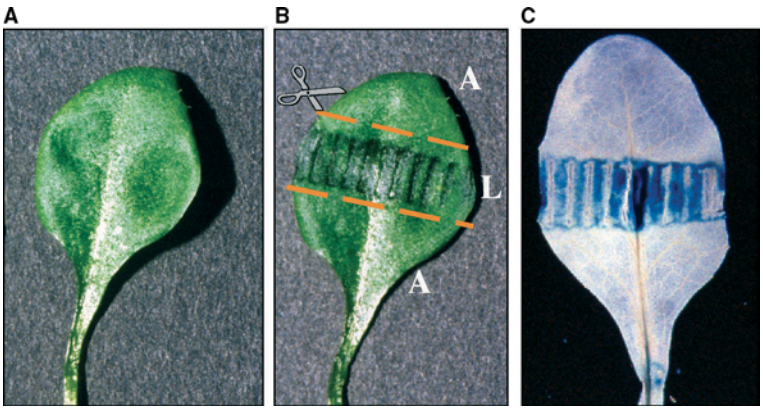

Figure 1. The leaf of an *ADH1*-GUS (*Arabidopsis* alcohol dehydrogenase promoter fused to the GUS reporter gene) *Arabidopsis* transformant before wounding (A), after wounding (B) and stained for GUS activity 8 h post-wounding (C). The localized tissue corresponds to the area where GUS expression is observed and the orange lines in B show the part of the wounded leaf that was harvested for microarray screenings. The adjacent tissue corresponds to the rest of the wounded leaf (contained outside the orange cuts in B). L: localized, A: adjacent.

as individual clones from a λZipLox library (Life Technologies, Rockville, MD) using M13 forward and reverse primers, or from total cDNA using specific primers to amplify 500 bp of the 3′ end region of the gene (MIAME (Brazma *et al*., 2001) compliant details of the scans and arrays can be accessed at www.pi.csiro.au/gena). The PCR products were ethanol precipitated, dissolved in 6 μl of 50% DMSO and transferred to 384-well microtiter plates. PCR products were printed onto CMT-GAPS™ slides (Corning, NY, USA) using a Bio-Rad VersArray ChipWriter Pro microarrayer with 16 pins (SMP3 stealth microspotting pins, TeleChem International, Inc., CA, USA). The DNA was then immobilized by baking the slides for 2–4 h at 80 °C. Each WR clone was printed three times on each array by at least two different pins, allowing us to obtain statistically robust expression data. Two arrays were printed on each slide and hybridized as duplicates at the same time, thereby limiting technical variation.

Selection of 'housekeeping' and control genes

Because the genes on the WR array were affected by wounding, a set of genes that were not affected by wounding needed to be added to the array for normalization purposes. We therefore added 133 'housekeeping' genes that were selected as follows. Firstly, from our analysis of the WR on the 3.5 K array and experiments carried out previously on the same array (Klok *et al*., 2002) we identified a set of stable genes using the 'SelectHousekeepingGenes' function implemented in tRMA (Wilson *et al*., 2003). This function selects those genes that have the most stable expression across all experiments and showing statistically insignificant variability. To ensure a selection that is representative of the whole fluorescence range (critical for a reliable normalization procedure), the dataset is divided into 20 segments ranging from the lowest fluorescing spots to the highest ones. A defined number of housekeeping genes (usually 3) are then selected from each segment to span the range. The second approach was to look at the scientific literature for reports of stably expressed genes. Some genes that have been previously shown to be stable in numerous conditions (Wu *et al*., 2001; Todd Richmond, personal communication) were also added after verification of their stable expression behavior on the wounding experiment on the 3.5 K slide. A set of negative controls was also printed, including genes that are not present in the *Arabidopsis* genome (i.e., luciferase, GUS), as well as *Arabidopsis* genomic segments that are not transcribed, like introns and genomic repeats. These negative controls were used for quality control as well as for the normalization procedure. We also used controls specific for the 3′ and 5′ end of a gene to ensure that no degradation of the labeled RNA occurred.

Sample preparation and slide hybridization

Tissue samples were collected as shown in Figure 1. We harvested three distinct regions of the wounded plants at four different time-points (0.5, 4, 8 and 17 h). Firstly, the local area at the wound site corresponding to the region where wound-induction of the *ADH1* (alcohol dehydrogenase, E.C. 1.1.1.1) gene was observed (de Bruxelles *et al*., in preparation). Secondly, the non-wounded tissue of the wounded leaves. Thirdly, non-wounded leaves from the wounded plant (systemic leaves). Wounding experiments were carried out using young plantlets (5 weeks old) at the rosette stage, and unhealthy plants were avoided. Only well-developed leaves were used in all experiments, and leaves showing signs of senescence were not used. Control samples were from non-wounded leaves of non-wounded plants harvested at the same time as the wounded samples. For the phytohormone treatments, whole leaves were collected at the end of each treatment. Tissues were immediately frozen in liquid nitrogen after collection and total RNA was extracted using a Qiagen RNeasy Plant kit according to the manufacturers protocol. RNA was eluted twice in 50 μl of RNase-free H_2O and was then treated with DNase to remove all traces of DNA: RQ1 RNase-free DNase (3 units) and 10 μl of 10× RQ1 buffer were added to 100 μl of RNA and incubated for 45 min at 37 °C. To further remove any risk of contamination by DNA, the RNA was precipitated O/N at 4 °C after addition of 28 μl of 10 M LiCl. The RNA was then resuspended in RNase-free water, quantified by spectrometry, and re-dissolved to 5 μg/μl after EtOH precipitation. Labeling of the samples with Cy3 and Cy5, pre-hybridization, hybridization and subsequent washes were performed according to Schenk *et al*. (2000) for the 3.5 K array, and according to the manufacturer's

protocol for DMSO-printed slides (Corning, NY), except that the last washing step was in 0.1× SSC twice for 20 min instead of 4 × 1′. Slides were scanned with a GenePix 4000A microarray scanner (Axon Instruments, Union CA, USA) and spots analyzed using the GenePix Pro software. Spots that were poorly recognized by the GenePix Pro software were manually corrected.

Data analysis of the specialized WR array

Because of the special composition of the WR array, we developed a new normalization method that was implemented in the tRMA package (Wilson *et al*., 2003). This method uses a set of dedicated housekeeping genes on the array to carry out the normalization process. This set of genes spans a wide range of expression values and is stable across all experiments performed (see above). A Lowess curve was determined and used for the subsequent normalization of all the genes after 'slide normalization'. Normalized data from replicates were averaged (median value). Due to the nature of WR array possessing mostly genes selected as being differentially expressed, current statistical methods for selecting differentially expressed genes could not be performed. Therefore, differentially expressed genes were defined as the genes having a ratio greater than 2 or smaller than 0.5.

k-means clustering according to the time-course experiment was done using the Genesis software (Sturn *et al*., 2002). Clusters were moved manually in Microsoft Excel according to their spatial distribution.

Functional classification

A flat file containing the automatically derived classification of *Arabidopsis* genes from PEDANT was used (version 1.0.2, September 2002, http://pedant.gsf.de). To avoid having a gene classified in more than one functional group, the following changes were introduced: a gene found in either the 'transcription' (04) or 'cellular communication/signal transduction mechanism' (10) group was classified in the 'transcription/signal transduction' group. Genes that were in the 'protein synthesis' (05) or the 'protein fate' (06) group were classified in a combined 'protein synthesis and fate' group. Finally, the remaining unclassified genes that were found in the 'metabolism' (01) or the 'energy' (02) group were classified in the 'metabolism and energy' group. All the remaining genes were put into the 'others/unknown/unclassified' group.

Promoter motif searches

We created a genome file for the GeneSpring program (Silicon Genetics, CA, USA), based on the complete *Arabidopsis* genome sequence (ftp.tigr.org, version of June 13, 2001). The genome annotation from TAIR was used to determine the position of each gene (file sv_gene.data, version from April 26, 2002). GeneSpring was used to identify shared motifs in the promoter regions (−10 to −500 bp upstream of the start codon) of each gene cluster. We searched for motifs 5–8 bp in length (allowing one single mismatch) that were significantly over represented in these promoter regions when compared to promoter regions of all the other genes (P-value ≤ 0.05).

Semi-quantitative real-time reverse transcriptase-mediated PCR

Reverse transcriptase semi-quantitative real-time PCR (qRT-PCR) reactions were as described in Klok *et al*. (2002). As starting material, we used the same RNA or first strand cDNA used to make the probes used in the screening of the microarray, and each quantification was done in triplicate. As a control, we used the formaldehyde dehydrogenase gene (*FDH*, At5g43940; Dolferus *et al*., 1997) which is not affected by wounding (data not shown). Each sample was then normalized to *FDH* levels to compensate for variation in the amounts of template cDNA. The expression ratio for each clone was calculated compared to its expression level in non-wounded plants. As a second stable control gene, we used the *Cylophilin* gene (At2g29960) and its expression was always found to be close to 1 when normalized to *FDH* levels (data not shown).

Gene list comparisons

Genes in each cluster of interest (spatial, temporal, phytohormone responsive) were entered in separate genelists. We then used the hypergeometric equation to calculate the probability of overlap

corresponding to k or more genes between a gene list of n genes compared against a gene list of m genes when randomly sampled from a universe of u genes. This equation is implemented in GeneSpring 5.0 (Silicon Genetics, CA, USA):

$$P = \frac{1}{\binom{u}{m}} \sum_{i=k}^{n} \binom{m}{i} \binom{u-m}{n-i}.$$

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Results and discussion

Identification and analysis of genes expressed locally at the wound site

A 3500 cDNA clone-containing (3.5 K) microarray (see Materials and methods; Klok *et al*., 2002) was screened for genes that are differentially expressed between the wounded area of a leaf and a non-wounded leaf from a non-wounded plant, 4 h after wounding. As the wounded area, we harvested approximately 10 cell layers flanking the wound site (including the wounded tissue), based on the expression pattern of the alcohol dehydrogenase gene (*ADH1*) following wounding (de Bruxelles *et al*., in preparation; Figure 1). The data from the screening of this array can be accessed at www.pi.csiro.au/gena.

Eleven percent of the clones (400 clones) on the 3.5 K array were differentially expressed at the wound site. These WR clones represented 228 non-redundant genes, of which 110 were induced and 118 were repressed. We verified the expression changes of 25 genes by northern blot hybridization using RNA extracted from the entire aerial tissues of non-wounded control plants and wounded plants (local, adjacent and systemic tissues harvested in bulk); a representative subset of 11 genes out of the 25 tested genes is shown in Figure 2. We found that the northern results confirmed the microarray expression data in all cases (Figure 2). Fifty-three percent (53%) of the induced genes were derived from the stress-related library, which represented only 30% of the total spotted clones. Conversely, a negative bias towards this library was observed for the repressed genes (10%), indicating a significant overlap between the wound-response and the anaerobic response. Comparison of our data with those from a study of the response to hypoxia in *Arabidopsis* roots using the same array (Klok *et al*., 2002) revealed an overlap greater than 23%, even though the hypoxia response was studied in the roots.

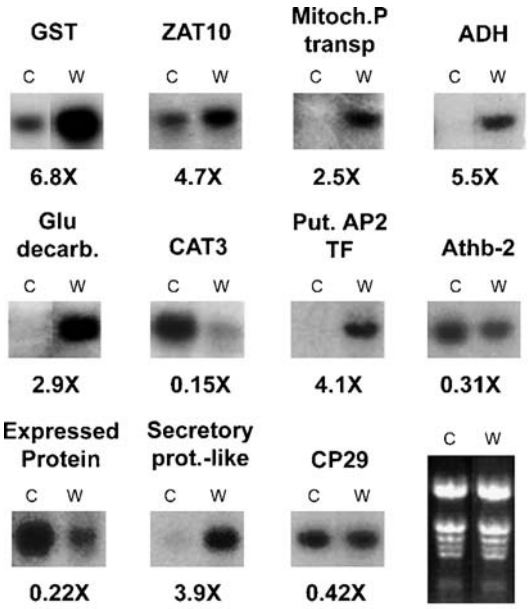

Figure 2. Verification by northern blot analysis of a subset of WR genes detected on the 3.5 K array. Northern blot hybridizations were carried out with RNA extracted from the whole aerial tissue of wounded plants 5 h after wounding (W) and from the whole aerial tissue of non-wounded plants at the same time (C). Ethidium bromide staining of one of the gels before transfer is shown as representative control for the amount of RNA loaded on the gel. Expression ratio from the wound-site compared to non-wounded leaves as measured on the 3.5 K array is shown under each northern blot. Due to the different samples used for northern blots or microarray analysis, only relative comparisons can be made between them (induced or repressed). The analysed genes are: GST: putative glutathione S-transferase (At1g02930); ZAT10: salt-tolerance zinc finger protein (At1g27730); Mitoch.P Transp.: mitochondrial phosphate transporter (At3g48850); ADH1: alcohol dehydrogenase (At1g77120); Glu decarb.: putative glutamate decarboxylase (At2g02010); CAT3: catalase 3 (At1g20620); Put.AP2 TF: putative protein contains similarity to transcription factor (At5g64750); Athb-2: DNA-binding homeotic protein Athb-2 (At4g16780); Expressed protein: expressed protein (At2g46220); Secretory protein-like: 33 kDa secretory protein-like (At5g48540); CP29: RNA-binding protein cp29 protein (At3g53460).

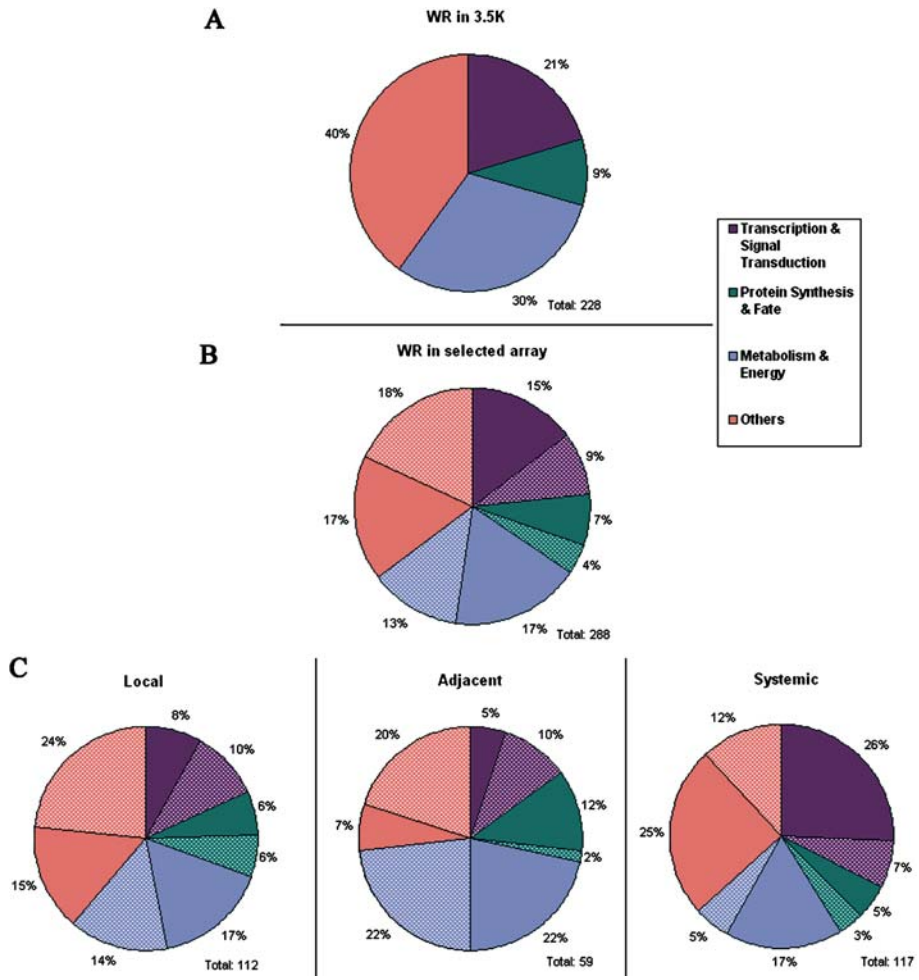

Figure 3. Repartition of the wound-responsive genes into functional groups. Results from the original wounding experiment on the 3.5 K array are shown (A) as well as for the localized-wound array (B). Functional classification according to the spatial specificity of gene regulation is also shown (C). Functional classification in the group named 'others' include genes not classified in any of the three other groups as well as unknown and unclassified genes. In C, the solid colors represent induced genes while hashed colors represent repressed genes.

The WR genes were placed in functional categories according to the PEDANT classification (http://pedant.gsf.de; Figure 3A, see Materials and methods). Twenty-one percent (21%) of the WR genes were implicated in transcription regulation and signal transduction, including several AP2-like, WRKY and zinc-finger transcription factors, consistent with the figures from Cheong *et al.* (2002). Nine percent were involved in protein synthesis and fate (e.g., cysteine proteinase RD19A, At4g39090; ubiquitin-conjugating enzyme 5, At1g63800; serine acetyltransferase, At1g55920; UDP-glucose:indole-3-acetate β-D-glucosyl-transferase, At1g05560), and 30% were involved in metabolism and energy supply, with many genes specifically involved in sugar metabolism and transport (e.g., β-glucosidase, At1g02850; glucose transporter, At1g11260) or photosynthesis (e.g., photosystem II type I chlorophyll *a*/*b*-binding protein, At2g34430; Lhca2 protein, At3g61470). A significant proportion (50%) of the metabolic genes was associated with lipid/fatty acid or carbon metabolism (e.g., 4-coumarate:CoA ligase 1, At1g51680; omega-6 fatty acid desaturase FAD2, At3g12120; hydroxypyruvate reductase (HPR), At1g68010). Finally, 40% of the WR genes were unclassified or had an unknown function. More than 25% of the WR genes were associated with

stress response, detoxification or defense response (not shown). In addition to known and putative genes, we also observed the differential expression of 3 cDNA clones that have no annotated gene model associated but do match genomic DNA (submitted to GenBank as CF074504, CF074506 and CF074505).

A non-redundant set of putative WR genes, enriched for genes that are expressed locally at the wound site, was spotted on a smaller WR array for detailed analysis of spatial and temporal expression pattern, and response to hormones. Half (185) of these genes were derived from the previous selection of WR genes on the 3.5 K array. Another 130 candidate genes were added from the 3.5 K slide as these genes just failed to meet our two fold cutoff ratio and/or were differentially expressed in only three out of the four replicates. Finally, 44 genes previously shown to be wound-responsive but not present on the 3.5 K array were also added. Details of the 3.5 K and the WR arrays can be accessed at www.pi.csiro.au/gena. The WR array made it possible to do subsequent characterizations on this gene set enriched for genes that are expressed locally at the wound site with higher confidence and accuracy, because (1) duplicates can be assayed under the same conditions as two arrays were printed on the same slide, (2) multiple spots of the same clone were printed on the array, and (3) all clones were re-amplified from their original library or from cDNA and PCR-verified before printing. Overall, 288 of the 359 putative WR genes printed on the WR array were modulated upon wounding at at least one time-point investigated.

Clustering of WR genes in three groups according to spatial expression profile

The genes on the WR array were clustered in three groups according to their spatial expression profile: genes that were systemically expressed throughout the wounded plant (distant unwounded leaves vs. leaves of a non-wounded control plant), and genes expressed in two zones from the wounded leaf: the 'local' group containing genes that were differentially expressed at the wound site only, and the 'adjacent' group containing genes that were differentially expressed both at the wounded site and in the non-wounded tissue next to it. Fifty two percent of the induced genes on the WR array appeared to be expressed systemically (86 genes), while 31% (51 genes) and 17% (27 genes) were induced locally and in the adjacent region respectively. In contrast, the majority of repressed genes were of the locally expressed cluster (50%, or 61 genes), while 31 (25%) and 32 (25%) genes were repressed systemically or in the adjacent region respectively. The proportion of induced and repressed genes in the local and adjacent cluster was about the same, but in the systemic group most of the genes were up-regulated by wounding (72%).

The distribution in functional categories was similar for the WR array and for the 3.5 K array (Figure 3B). However, when the WR genes were subdivided into the three spatial expression groups, differences appeared (Figure 3C). This signifies that the response to wounding in the three groups is functionally different. Genes involved in the regulation of transcription and signal transduction were more highly represented in the systemic cluster and were, in contrast to the local and adjacent groups, mostly up-regulated (~80%). Genes encoding proteins of the metabolism/energy class were relatively more represented in the local and adjacent clusters. This was also the case for the protein synthesis/fate class (Figure 3C). Most of the photosynthetic genes present on the WR array were present in the adjacent cluster, and most were repressed.

These results indicate that the systemic-responsive genes are a predominant class on the WR array, even though genes from the 3.5 K array were selected on the basis of local response to wounding. The systemic response originates at the wound site and involves relatively more signal transduction components and transcription factors, presumably involved in establishing a defense response in the rest of the plant. In the wounded leaf, as compared to more distant leaves on the same plant, changes in response to wounding are more metabolic in nature and predominantly involve the repression of these functions. Our results show that there are also significant differences between the wound site (local) and the rest of the wounded leaf (adjacent), in that metabolic changes are more pronounced in the adjacent group. The lowering of the photosynthetic rate in response to a wide variety of stresses is well documented (Krause and Weis, 1991; Pfundel and Bilger, 1994), as is the down-regulation of photosynthetic

genes by a variety of stress situations, including cold and water loss (Stitt and Hurry, 2002).

Clustering of WR genes according to their temporal expression profile

Differentially expressed genes of the three areas were clustered according to their temporal expression profiles (0.5, 4, 8, 17 h) using *k*-means clustering. A total of seven distinct temporal clusters were identified using hierarchical clustering and used as a parameter in the *k*-means clustering algorithm (see Supplementary data). Biplot analysis (Chapman *et al*., 2002) revealed that the 30 min time point was significantly different from the later time points ($0.33 < r < 0.53$), while the three later time points were more similar to each other ($0.7 < r < 0.93$). We therefore classified the genes in two temporal groups: early responsive genes, differentially expressed at 30 min; and late responsive genes, with expression peaking at 4–8 h post wounding. These two groups were then subdivided into induced and repressed genes.

Most genes that were systemically expressed showed the same kinetics of induction or repression in all three expression zones. There was no delay in expression of these genes between the local area, the adjacent tissue, and in distant non-wounded leaves as early as 0.5 h after wounding, suggesting that they must be regulated by a signal that spreads quickly in the plant (Figure 4). However, some genes showed a different regulation pattern in the three different zones. A pathogen response gene (*PR1*-like, At2g14610) was locally repressed early on, but was induced after 4 h in the adjacent tissue, and was not affected systemically (Figure 4). Another potential pathogen response gene (putative pectinesterase, At1g76160) was induced locally after 4 h, but was repressed after 8 h in the adjacent tissue and induced early systemically. The induction of pathogen response genes in the wounded leaf is not unexpected as JA, together with ethylene and salicylic acid (SA), is involved in pathogen response pathways. The induction of these genes in the wounded leaf may provide a defense mechanism against opportunistic pathogens. Constitutive JA-signalling mutants (*cev1*) resulted in suppression of SA-dependent defense responses (Ellis and Turner, 2001), but some defense response genes were shown to require intact JA, ethylene and SA signaling pathways (Campbell *et al*., 2003). Overlap between the wound- and pathogen-response pathways has been reported on several occasions, and recently using microarray approaches (Schenk *et al*., 2000; Cheong *et al*., 2002).

A group of six genes (At1g21120, At3g28180, At1g59870, At2g31880, At2g41100, and At3g45640) were induced very early but were then repressed 8 h after wounding, especially at the local site of wounding (Figure 4). Another two sets of genes were more rapidly induced or repressed in the non-wounded tissues (both adjacent and systemic) than at the wound site itself (Figure 4). We verified four of the genes whose expression pattern was different in the three different expression zones by qRT-PCR and found that the qRT-PCR results confirmed the microarray expression data (Figure 5).

Correlation analysis between genes clustered into different spatial and temporal groups and their functional classification (see Materials and methods) indicated that the majority of the genes involved in the regulation of transcription and signal transduction were induced (42/67 or 63%) and were significantly associated with early and systemically induced genes (30/67, $P = 1.1 \times 10^{-39}$). Photosynthetic genes were repressed late in the whole wounded leaf (19/19, $P = 1.67 \times 10^{-20}$) and none of them were systemically regulated. Genes involved in general metabolism and energy supply to the cell, as well as genes involved in the regulation of protein synthesis and destination, were not confined to a specific spatial or temporal group.

Amongst the transcription factors identified in this study are two WRKY transcription factors belonging to group I of the AtWRKY family (for review, see Eulgem *et al*., 2000); these factors were induced early and systemically. Zinc-finger protein-encoding genes (8) were affected early and systemically (At2g41940 was very close to the cutoff value in the adjacent tissue), and were either induced or repressed. However, AP2 domain transcription factors were more diverse: *RAP2.3* is repressed systemically 4 h post wounding; *RAP2.5* (*AtERF4*) is induced early systemically; *RAP2.4* is induced early in the whole wounded leaf; *RAP2.9* is induced locally after 4 h. The AP2 type transcription factor genes were differentially regulated in specific parts of the wounded plant while WRKY genes all appeared to be induced systemically. The expression kinetics of

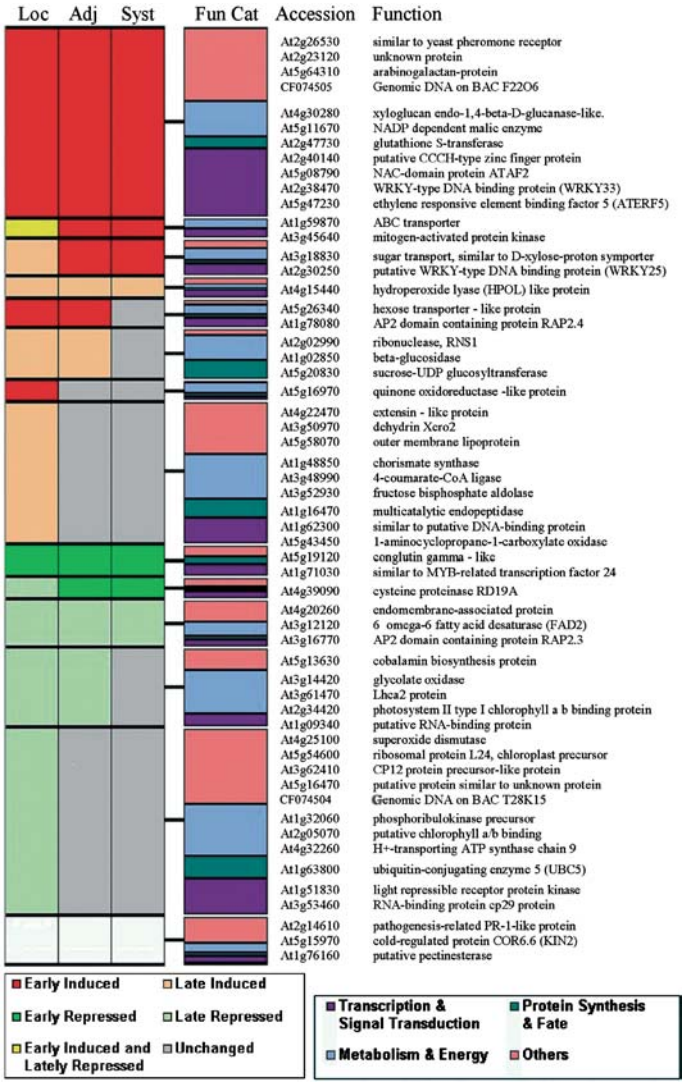

Figure 4. Spatial, temporal and functional classification of the wound responsive genes. The height of each cluster is proportional to the number of genes it contains. Similarly, the height of each bar for the functional categories is proportional to the percentage of genes in each specific category. Only the clusters containing more than Five genes are considered relevant. The white cluster contains 15 genes whose expression profile cannot be placed in any of the other clusters. Only a subset of the genes present in each cluster is represented for each cluster on the right. Loc: local, Adj: adjacent, Syst: systemic, Fun Cat: functional category.

these three classes of regulatory proteins is fully consistent with the observations of Cheong *et al.* (2002).

Taken together, the results of detailed spatial and temporal expression profiling indicate firstly, that genes affected by wounding systemically include a high proportion of regulatory factors and signal transduction components, and that these genes are induced very early in the response, spreading quickly throughout the plant. Secondly, in the wounded leaf (local and adjacent tissue), the response includes a higher proportion of metabolic genes, especially photosynthetic genes, and these genes are repressed later in the WR. The majority of the genes that were induced systemically were early responsive while the majority of the genes whose differential expression was restricted to the wounded leaf were late responsive. This suggests that the immediate reaction of a plant to wounding is to signal the threat to the whole plant and that the healing process in the wounded area is established later.

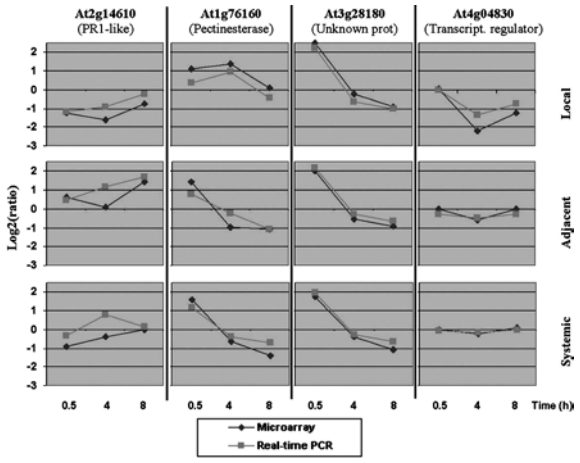

Figure 5. Confirmation of some selected gene expression profiles by qRT-PCR. Ratios represented on the *Y* axis are from either the local, adjacent or systemic tissue compared to a non-wounded plant at the different time points indicated on the *X* axis and log 2 transformed. Ratios obtained from both microarray and quantitative RT-PCR techniques are shown.

Role of phytohormones

We investigated the involvement of hormones that have previously been implicated in the WR: MeJA, ABA and ethylene (Figure 6). MeJA has been shown to be a systemic signal for WR gene regulation (Farmer and Ryan, 1990; Li *et al*., 2002). Amongst the phytohormones studied, MeJA affected the highest number of WR genes. About 20% (55) of the WR genes showed differential expression, consistent with the fact that a high proportion of WR genes are systemically expressed (41%). There was a significant correlation between regulation by MeJA and the regulation of genes systemically induced by wounding (26/55, $P = 4 \times 10^{-42}$), providing further support for the involvement of MeJA in the systemic response to wounding (Li *et al*., 2002; Ryan and Moura, 2002; Strassner *et al*., 2002). Also the correlation between MeJA-regulated genes and genes involved in signal transduction and regulation of transcription was high (14/55, $P = 1.7 \times 10^{-23}$). Overall, a third of the systemically early-induced genes that are involved in regulatory processes are also induced by MeJA.

Many genes that respond to JA also respond to ethylene, especially genes that are important in pathogen response (Rojo *et al*., 2003). Ethylene affected about 10% or 27 WR genes. Analysis of the correlation between the spatial and temporal clusters indicated that ethylene responsive genes are closely associated with the down-regulation in local and adjacent tissues (17/27, $P = 2.1 \times 10^{-24}$; Figure 6). The correlation with the decreased expression of photosynthetic genes is also very high (9/27, $P = 2.4 \times 10^{-20}$). A reduction of photosynthesis rate by ethylene has previously been demonstrated (Kays and Pallas, 1980; Pallas and Kays, 1982), and may be part of the role ethylene plays in the senescing response in plants. In *Arabidopsis* ethylene acts as a cross-talk regulator between JA-dependent and -independent pathways (Rojo *et al*., 1999; Leon *et al*., 2001; Pieterse *et al*., 2001). The ethylene biosynthetic genes ACC synthase and ACC oxidase are both induced by wounding (Li *et al*., 1992; Barry *et al*., 1996). Our data show wounding induction of ACC oxidase in the local and slightly in the adjacent area, but not systemically. These results suggest that ethylene plays a role only in the local and adjacent tissue. There is no direct effect of MeJA on ACC oxidase expression or other ethylene responsive genes. However, several ethylene-responsive factors transcripts are rapidly and highly induced following wounding both in wounded and systemic leaves (Cheong *et al*., 2002; Nishiuchi *et al*., 2002). Accordingly, both ethylene response element binding factors present on the WR array, ERF4 and *ERF5*, were rapidly induced by wounding, but they were not affected by our ethylene treatments (5 h with 5 or 200 ppm). *ERF4* and *ERF5* are maximally induced by ethylene after 12 h (Fujimoto *et al*., 2000), indicating that our treatment was too short to show an effect on these genes and that another factor may trigger their induction in the wound-response. The genes that were affected by our ethylene treatments were predominantly photosynthetic genes.

ABA affected about 7% of the WR genes (21 genes). All ABA-induced genes on the WR array are genes known to be affected by water or salt stress (e.g., *ADH1*: Dolferus *et al*., 1994; *ENOD20*, *Lti78*: Seki *et al*., 2001; *UDP-glucosyltransferase* and *VSP2*: Gong *et al*., 2001). The genes that are affected by ABA are predominantly expressed in the wounded leaf, and only few of them are also induced systemically (Figure 6). This strongly suggests that these genes are induced as a consequence of water-loss occurring at the wound site. This is consistent with the work of Reymond *et al*. (2000), who also found that water stress is

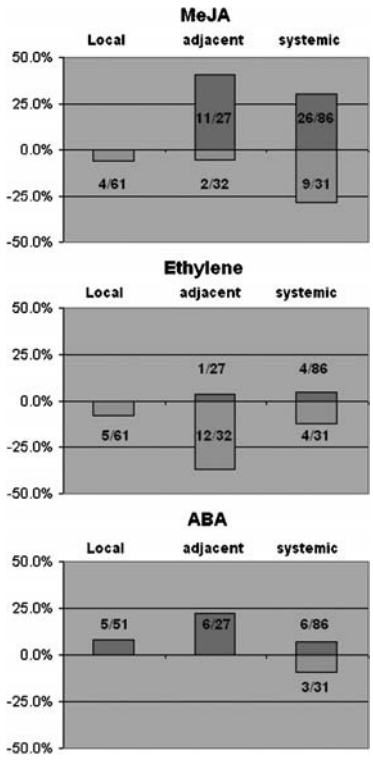

Figure 6. Effect of different phytohormones on wound regulated genes. Red bars represent genes induced by both wounding and the hormone treatment while green bars represent genes repressed by both treatments. Data are shown as the percentage of genes in each group similarly affected by the hormone treatment. Genes having opposite regulation by wounding or the hormone treatment are not shown, as they were insignificant in number.

an important component in the response to mechanical wounding. In addition, using a cDNA microarray containing around 7000 *Arabidopsis* full-length cDNAs, Seki *et al*. (2002) reported that half of the genes induced by dehydration are also induced following ABA treatment. Only five of the ABA-regulated genes were also regulated by JA (GAST1, At1g74670; AcylCoA oxidase, At4g16760; VSP2, At5g24770; COR6.6, At5g15970; unknown gene, At2g10940).

Genes involved in auxin signalling were also affected by wounding. Two genes encoding UDP-glucose:indole-3-acetate β-D-glucosyltransferase were induced locally 4 h after wounding. These genes are involved in the inactivation of indole-3-acetic acid auxin (IAA) by conjugation with a glucosyl group. IAA-Ala hydrolase (*IAR3*) was induced systemically; a multi-catalytic endopeptidase and an auxin response factor were induced locally. *NPK1*, encoding an activator of a MAP-kinase signal transduction cascade that leads to the suppression of early auxin response gene transcription (Kovtun *et al*., 1998) is also induced systemically. This observation confirms a recent finding showing repression of auxin action following wounding (Cheong *et al*., 2002). Also, auxins are known to have a negative effect on the WR and IAA levels decline upon wounding (Thornburg and Li, 1991).

Identification of promoter elements in WR genes

Genes having a similar expression profile are likely to be regulated by similar regulatory factors and therefore may possess common regulatory elements in their promoters. We examined the upstream region (−10 to −500 bp) of the WR genes identified on the 3.5 K array for the presence of significantly over-represented motifs. The G-box-like (5′-CACGTGG) core-motif, which was present in 92 out of the 196 promoters for which we found an annotation in TAIR, was the only motif we could find (Table 1). When a similar analysis was performed for all the differentially expressed genes on the WR microarray, a composite motif could be assembled from five over-represented overlapping motifs (Table 1). This 5′-CCCA-CACGT composite motif also shows strong homology to the G-box sequence. Analysis of the 5′ upstream region of 158 genes induced on the WR microarray showed that 110 of them contained an ACGCGT motif (allowing a single mismatch). In the promoter of the 123 repressed genes analyzed, a GATAAG motif was found in 68 promoters in either sense (34) or antisense orientation (37). In the same subset of genes, a CGTATCC motif was also significantly over-represented. In genes that were repressed adjacent to the wound site we identified a GCCACGT motif (one mismatch allowed) in 22 out of the 32 upstream regions.

We were unable to identify any specific motifs in the other clusters, nor could over-represented promoter elements be identified for the early (induced/repressed) or late (induced/repressed) genes. G-box-like elements were found in all spatial groups. G-box elements (5′-CACGTGG core motif) and ABA response elements (ABRE) are present in the promoter of many ABA and water stress responsive genes (reviewed by Leung and

Giraudat, 1998). These promoter elements can therefore be expected in genes that are differentially expressed locally or adjacent to the wound, where water loss and ABA function play a major role. However, the G-box element is very similar to the ACGT-box (Box II, CACGTG-motif, LRE) found in the promoter of many light-regulated genes (Foster *et al.*, 1994; Terzaghi and Cashmore, 1995). The element is a binding site of a redundant class of bZIP transcription factors that play a role in a variety of stress responses.

The ACGCGT motif found in all spatial locations is similar to CE3 (coupling element). CE3 is found in conjunction with the ABRE and is the functional equivalent of it (Hobo *et al.*, 1999). The GATAAG motif found in the repressed genes is similar to the I-box, another light responsive element (Donald and Cashmore, 1990; Rose *et al.*, 1999). As the majority of the genes repressed by wounding are involved in photosynthesis, it is not unexpected to find this promoter element. The CGTATCC motif found in the same set of genes is not similar to any previously identified promoter element, although it shows some similarity with the complement of the I-box.

The response in the wounded leaf

In this study, we treated the wound site (local) and the rest of the wounded leaf (adjacent) as two different zones, in order to elucidate differences between these two areas of the wounded leaf and to further discriminate the local response from the systemic WR. The overlap between the local and adjacent area is quite high. This could be partially due to contamination of tissue between the two areas, as it is impossible to physically separate the tissues. Many overlapping genes show higher expression ratios in the local area than in the adjacent area, but for some genes the opposite is true. Also, some highly induced genes are restricted to the wounded area (e.g., HSP90, probable quinone oxidoreductase), while some genes that are affected in one direction in one tissue (induction/repression) show the opposite regulation (repression/induction) in the other tissue (e.g., PR1-like and pectinesterase). This indicates that, although contamination may occur, there are sets of genes that are specific to each of the two regions, and some wound responsive processes take place specifically in the region of the wound.

The WR genes expressed locally at the wound site are likely to be involved in repairing and sealing of the wound site. A well-known physiological event occurring in the cells located at the boundary of wounded tissues is cell wall lignification (Rittinger *et al.*, 1987). Using confocal laser microscopy, we observed a clear deposition of phenolics in damaged *Arabidopsis* leaves 24 h post-wounding. This deposition was limited to the damaged area (Figure 7). Lignification of cell walls strengthens the cells around the damaged area and

Table 1. Significant DNA motifs found within 500bp upstream of translation start.

Cluster	Motif	Genes in cluster	Frequency	*P*-value	Similar to
All WR on 3.5 K	CACGTGG[1]	228	92/196	5.8×10^{-6}	G-box
All WR	ACACGT[1]	288	256/281	1.1×10^{-3}	G-box
	CCCACA[1]	288	246/281	2.0×10^{-4}	Pc-CMA2c (S.o.)
	CACACG[1]	288	223/281	7.6×10^{-4}	G-box
	CACACGT[1]	288	142/281	1.8×10^{-3}	G-box
	CCACACG[1]	288	117/281	4.5×10^{-6}	–
Induced	ACGCGT[1]	164	110/158	3.2×10^{-2}	CE3 (O.s.)
Repressed	GATAAG	124	34/123	3.9×10^{-2}	I-box
	CTTATC	124	37/123	3.2×10^{-2}	I-box
	CGTATCC[1]	124	58/123	1.1×10^{-2}	–
Repressed adjacent	GCCACGT[1]	32	22/32	7.9×10^{-3}	G-box

All possible clusters were considered and the data shown are for those clusters where one or more significant motifs were detected. The 'Genes in cluster' column shows the total number of genes present in the cluster considered, while the frequency shows the number of genes possessing a specific motif and the number of upstream sequences used for the analysis. The numbers in square brackets next to the motif sequence indicate the number of mismatches allowed in the motif.

supplies protection against water-loss and invasion by pathogens. Several lignin biosynthetic genes are induced locally, including genes encoding enzymes of the shikimate pathway leading to the production of Phe (putative chorismate synthase; anthranilate *N*-benzoyltransferase), and genes of the lignin pathway (4-coumarate CoA ligase-like, cinnamyl-alcohol dehydrogenase ELI3-2 and a putative cinnamoyl-CoA reductase). An extensin-like gene which may be involved in lignin deposition (Tire *et al.*, 1994) was also induced specifically at the wound site. This localized expression of lignin biosynthetic genes correlates with the observation that phenolics accumulate at the wound-site (Figure 7).

Down-regulation of photosynthetic genes was typical for the wounded leaf (local + adjacent). Many photosynthetic genes were locally repressed, including a plastocyanin, several proteins from the photosystem II complex, several chlorophyll A–B binding proteins, H^+-transporting ATP synthase, ribulose bisphosphate carboxylase, and magnesium-protoporphyrin IX methyltransferase. Genes encoding proteins for the formation of chloroplast ribosomes (e.g. 50S ribosomal protein L12-A, L24, L1, S13 and S1) were also repressed. In the adjacent cluster, the expression of photosynthetic genes encoding several proteins from the PSII complex, the light harvesting complex a2 protein, and an oxygen-evolving enhancer protein precursor-like protein were affected by wounding. Some genes involved in photorespiration (hydroxypyruvate reductase; putative glycolate oxidase) were down-regulated by wounding.

A high proportion of differentially expressed genes involved in metabolism and energy supply are induced in the region adjacent to the wound site compared to the other locations. These include genes that code for proteins involved in sugar transport and metabolism (hexose transporter-like, sucrose synthase, fructokinase-1 and beta-glucosidase). This may reflect a change in the sink-source relationship in response to the repression of photosynthetic activity, and that the wounded leaf is becoming a sink tissue dependent on sugar supply.

WR genes expressed throughout the plant

A majority of these genes are involved in signal transduction and regulation of transcription, supporting the notion that the systemic signal prepares the plant for further response to wounding or pathogen attack. These factors are also induced very early in the response. Amongst the induced metabolic genes are genes that may play a role in a response against pathogens. Anthocyanidin synthase catalyses the penultimate step in the biosynthesis of the anthocyanin class of flavanoids (Wilmouth *et al.*, 2002). HPOL is involved in the production of the aliphatic acids, hexanal or 3-hexenal, which have potent antimicrobial activity *in vitro* (Deng *et al.*, 1993). Antisense-mediated depletion of HPOL in potato led to an increased performance of aphid feeding on these plants (Vancanneyt *et al.*, 2001).

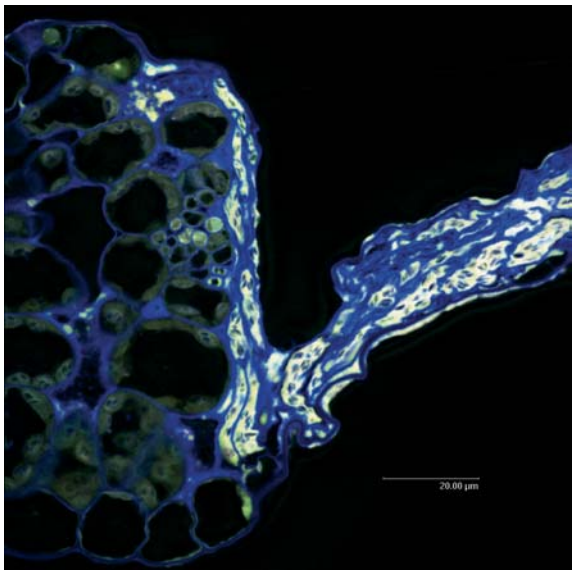

Figure 7. Section of a wounded leaf stained with 0.1% aqueous berberine sulfate for 30 min followed by 30 s in 0.05% aqueous crystal violet, 24 h post-wounding. Tissue was observed using a confocal microscope with UV/blue excitation. Phenolics appears with a white color around and in the wounded area.

Conclusion

Our detailed analysis of the local response to mechanical wounding in *Arabidopsis* shows that many genes which are induced locally are also systemically expressed. The enrichment strategy using the two-step microarray screening has led to the identification of a set of signal transduction components and transcription factors which are induced very early in the response. The relatively

large proportion of regulatory factor genes in the systemically expressed genes and the induction of these genes very early in the response, suggests that the systemic response plays a crucial role in the regulation and establishment of the entire WR. The systemic response may not only be important for the establishment of a systemic defence against pathogen invasion throughout the plant, but it may also play a role in the establishment of the local response in the wounded leaf. MeJA is critical for the regulation of the systemic response. The genes that are specific for the WR in the wounded leaf are mainly metabolic genes, and they are induced later than the systemic response (4–8 h). Some of these genes are regulated by ABA and are likely to be involved in prevention of water loss at the wound site. Ethylene mainly regulates the repression of photosynthetic genes. A regulatory connection between the local and systemic response remains to be established. The fact that the local response is induced later, leaves open the possibility that the local response is activated by regulatory factors that are induced by the systemic response. Alternatively, a wound signal may be responsible for the induction of both the local and systemic response.

Acknowledgments

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References

Baldwin, I.T. 1989. Mechanism of damage-induced alkaloid production in wild tobacco. J. Chem. Ecol. 15: 1661–1680.

Barry, C.S., Blume, B., Bouzayen, M., Cooper, W., Hamilton, A.J. and Grierson, D. 1996. Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J. 9: 525–535.

Bergey, D.R., Orozco-Cardenas, M., de Moura, D.S. and Ryan, C.A. 1999. A wound- and systemin-inducible polygalacturonase in tomato leaves. Proc. Natl. Acad. Sci. USA 96: 1756–1760.

Birkenmeier, G.F. and Ryan, C.A. 1998. Wound signaling in tomato plants. Evidence that aba is not a primary signal for defense gene activation. Plant Physiol. 117: 687–693.

Bowles, D.J. 1990. Defense-related proteins in higher-plants. Ann. Rev. Biochem. 59: 873–907.

Bowles, D.J. 1993. Local and systemic signals in the wound response. Semin. Cell Biol. 4: 103–111.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C.A., Causton, H.C., Gaasterland, T., Glenisson, P., Holstege, F.C., Kim, I.F., Markowitz, V., Matese, J.C., Parkinson, H., Robinson, A., Sarkans, U., Schulze–Kremer, S., Stewart, J., Taylor, R., Vilo, J. and Vingron, M. 2001. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat. Genet. 29: 365–371.

Campbell, E.J., Schenk, P.M., Kazan, K., Penninckx, I.A., Anderson, J.P., Maclean, D.J., Cammue, B.P., Ebert, P.R. and Manners, J.M. 2003. Pathogen-responsive expression of a putative ATP-binding cassette transporter gene conferring resistance to the diterpenoid sclareol is regulated by multiple defense signaling pathways in *Arabidopsis*. Plant Physiol. 133: 1272–1284.

Chapman, S., Schenk, P., Kazan, K. and Manners, J. 2002. Using biplots to interpret gene expression patterns in plants. Bioinformatics 18: 202–204.

Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T. and Luan, S. 2002. Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress and hormonal responses in *Arabidopsis*. Plant Physiol. 129: 661–677.

de Bruxelles, G.L. and Roberts, M.R. 2001. Signals regulating multiple responses to wounding and herbivores. Crit. Rev. Plant Sci. 20: 487–521.

Deng, W.L., Hamiltonkemp, T.R., Nielsen, M.T., Andersen, R.A., Collins, G.B. and Hildebrand, D.F. 1993. Effects of 6-carbon aldehydes and alcohols on bacterial proliferation. J. Agri. Food Chem. 41: 506–510.

Doares, S.H., Syrovets, T., Weiler, E.W. and Ryan, C.A. 1995. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc. Natl. Acad. Sci. USA 92: 4095–4098.

Dolferus, R., Jacobs, M., Peacock, W.J. and Dennis, E.S. 1994. Differential interactions of promoter elements in stress responses of the *Arabidopsis* Adh gene. Plant Physiol. 105: 1075–1087.

Dolferus, R., Osterman, J.C., Peacock, W.J. and Dennis, E.S. 1997. Cloning of the *Arabidopsis* and rice formaldehyde dehydrogenase genes: implications for the origin of plant ADH enzymes. Genetics 146: 1131–1141.

Donald, R.G. and Cashmore, A.R. 1990. Mutation of either G box or I box sequences profoundly affects expression from the *Arabidopsis* rbcS-1A promoter. EMBO J. 9: 1717–1726.

Ellis, C. and Turner, J.G. 2001. The *Arabidopsis* mutant cev1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. Plant Cell 13: 1025–1033.

Eulgem, T., Rushton, P.J., Robatzek, S. and Somssich, I.E. 2000. The WRKY superfamily of plant transcription factors. Trends Plant Sci. 5: 199–206.

Farmer, E.E. and Ryan, C.A. 1990. Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc. Natl. Acad. Sci. USA 87: 7713–7716.

Foster, R., Izawa, T. and Chua, N.H. 1994. Plant bZIP proteins gather at ACGT elements. FASEB J. 8: 192–200.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. 2000. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell 12: 393–404.

Gong, Z.Z., Koiwa, H., Cushman, M.A., Ray, A., Bufford, D., Kore-eda, S., Matsumoto, T.K., Zhu, J.H., Cushman, J.C., Bressan, R.A. and Hasegawa, P.M. 2001. Genes that are uniquely stress regulated in salt overly sensitive (sos) mutants. Plant Physiol. 126: 363–375.

Hobo, T., Asada, M., Kowyama, Y. and Hattori, T. 1999. ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. Plant J. 19: 679–689.

Kays, S.J. and Pallas, J.E. 1980. Inhibition of photosynthesis by ethylene. Nature 285: 51–52.

Klok, E.J., Wilson, I.W., Wilson, D., Chapman, S.C., Ewing, R.M., Somerville, S.C., Peacock, W.J., Dolferus, R. and Dennis, E.S. 2002. Expression profile analysis of the low-oxygen response in *Arabidopsis* root cultures. Plant Cell 14: 2481–2494.

Kovtun, Y., Chiu, W.L., Zeng, W.K. and Sheen, J. 1998. Suppression of auxin signal transduction by a MAPK cascade in higher plants. Nature 395: 716–720.

Krause, G.H. and Weis, E. 1991. Chlorophyll fluorescence and photosynthesis: the basics. Annu. Rev. Plant Phys. Plant Mol. Biol. 42: 313–349.

Lee, G.I. and Howe, G.A. 2003. The tomato mutant spr1 is defective in systemin perception and the production of a systemic wound signal for defense gene expression. Plant J. 33: 567–576.

Leon, J., Rojo, E. and Sanchez-Serrano, J.J. 2001. Wound signalling in plants. J. Exp. Bot. 52: 1–9.

Leung, J. and Giraudat, J. 1998. Abscisic acid signal transduction. Annu. Rev. Plant Phys. Plant Mol. Biol. 49: 199–222.

Levin, D.A. 1973. Role of trichomes in plant defense. Q. Rev. Biol 48: 3–15.

Li, L., Li, C., Lee, G.I. and Howe, G.A. 2002. Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. Proc. Natl. Acad. Sci. USA 99: 6416–6421.

Li, N., Parsons, B.L., Liu, D.R. and Mattoo, A.K. 1992. Accumulation of wound-inducible acc synthase transcript in tomato fruit is inhibited by salicylic-acid and polyamines. Plant Mol. Biol. 18: 477–487.

Nishiuchi, T., Suzuki, K., Kitajima, S., Sato, F. and Shinshi, H. 2002. Wounding activates immediate early transcription of genes for ERFs in tobacco plants. Plant Mol. Biol. 49: 473–482.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O. and Bowles, D.J. 1996. Ethylene as a signal mediating the wound response of tomato plants. Science 274: 1914–1917.

Orozco-Cardenas, M. and Ryan, C.A. 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proc. Natl. Acad. Sci. USA 96: 6553–6557.

Pallas, J.E. and Kays, S.J. 1982. Inhibition of photosynthesis by ethylene – a stomatal effect. Plant Physiol. 70: 598–601.

Peña-Cortés, H., Fisahn, J. and Willmitzer, L. 1995. Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. Proc. Natl. Acad. Sci. USA 92: 4106–4113.

Perez-Amador, M.A., Leon, J., Green, P.J. and Carbonell, J. 2002. Induction of the arginine decarboxylase ADC2 gene provides evidence for the involvement of polyamines in the wound response in *Arabidopsis*. Plant Physiol. 130: 1454–1463.

Pfundel, E. and Bilger, W. 1994. Regulation and possible function of the violaxanthin cycle. Photosynth. Res. 42: 89–109.

Pieterse, C.M.J., Ton, J. and Van Loon, L.C. 2001. Cross-talk between plant defence signalling pathways: boost or burden? AgBiotechNet 3: 1–8.

Reymond, P., Weber, H., Damond, M. and Farmer, E.E. 2000. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell 12: 707–720.

Rittinger, P.A., Biggs, A.R. and Peirson, D.R. 1987. Histochemistry of lignin and suberin deposition in boundary-layers formed after wounding in various plant-species and organs. Can. J. Bot – Rev. Can. Bot. 65: 1886–1892.

Rojo, E., Leon, J. and Sanchez-Serrano, J.J. 1999. Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*. Plant J. 20: 135–142.

Rojo, E., Solano, R. and Sanchez-Serrano, J.J. 2003. Interactions between signaling compounds involved in plant defense. J. Plant Growth Reg. 22: 82–98.

Rose, A., Meier, I. and Wienand, U. 1999. The tomato I-box binding factor LeMYBI is a member of a novel class of Myb-like proteins. Plant J. 20: 641–652.

Ryan, C.A. 2000. The systemin signaling pathway: differential activation of plant defensive genes. Biochim. Biophys. Acta 1477: 112–121.

Ryan, C.A. and Moura, D.S. 2002. Systemic wound signaling in plants: a new perception. Proc. Natl. Acad. Sci. USA 99: 6519–6520.

Scalbert, A. 1991. Antimicrobial properties of tannins. Phytochemistry 12: 3875–3883.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C. and Manners, J.M. 2000. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. Proc. Natl. Acad. Sci. USA 97: 11655–11660.

Seki, M., Ishida, J., Narusaka, M., Fujita, M., Nanjo, T., Umezawa, T., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y. and Shinozaki, K. 2002. Monitoring the expression pattern of around 7,000 *Arabidopsis* genes under ABA treatments using a full-length cDNA array. Funct. Integr. Genomics 2: 282–291.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y. and Shinozaki, K.

2001. Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell 13: 61–72.

Stitt, M. and Hurry, V. 2002. A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. Curr. Opin. Plant Biol. 5: 199–206.

Strassner, J., Schaller, F., Frick, U.B., Howe, G.A., Weiler, E.W., Amrhein, N., Macheroux, P. and Schaller, A. 2002. Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. Plant J. 32: 585–601.

Sturn, A., Quackenbush, J. and Trajanoski, Z. 2002. Genesis: cluster analysis of microarray data. Bioinformatics 18: 207–208.

Terzaghi, W.B. and Cashmore, A.R. 1995. Photomorphogenesis. Seeing the light in plant development. Curr. Biol. 5: 466–468.

Thornburg, R.W. and Li, X. 1991. Wounding *Nicotiana tabaccum* leaves causes a decline in endogenous indole-3-acetic acid levels. Plant Physiol. 96: 802–805.

Tire, C., De Rycke, R., De Loose, M., Inze, D., Van Montagu, M. and Engler, G. 1994. Extensin gene expression is induced by mechanical stimuli leading to local cell wall strengthening in *Nicotiana plumbaginifolia*. Planta 195: 175–181.

Vancanneyt, G., Sanz, C., Farmaki, T., Paneque, M., Ortego, F., Castanera, P. and Sanchez-Serrano, J.J. 2001. Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. Proc. Natl. Acad. Sci. USA 98: 8139–8144.

Wilmouth, R.C., Turnbull, J.J., Welford, R.W.D., Clifton, I.J., Prescott, A.G. and Schofield, C.J. 2002. Structure and mechanism of anthocyanidin synthase from *Arabidopsis thaliana*. Structure 10: 93–103.

Wilson, D.L., Buckley, M.J., Helliwell, C.A. and Wilson, I.W. 2003. New normalization methods for cDNA microarray data. Bioinformatics 19: 1325–1332.

Wu, S.H., Ramonell, K., Gollub, J. and Somerville, S. 2001. Plant gene expression profiling with DNA microarrays. Plant Physiol. Biochem. 39: 917–926.