Light exerts multiple levels of influence on the Arabidopsis wound response.

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Abstract

Light plays important roles in modulating plant responses to attack by pests and pathogens. Here, we test the hypothesis that darkness modifies the response to wounding, and examine possible mechanisms for such an effect. We investigated changes in the Arabidopsis transcriptome following a light-dark transition, and the response to wounding either in the light or in the dark. The transcriptional response to the light-dark transition strongly resembles responses associated with carbon depletion. The dark shift and wound responses acted largely independently, but more complex interactions were identified at a number of levels. Darkness attenuates the overall transcriptional response to wounding, and we identified a number of genes and physiological processes, such as anthocyanin accumulation, that exhibit light-dependent wound responses. Transcriptional activation of light-dependent wound-induced genes requires a chloroplast-derived signal originating from photosynthetic electron transport. We also present evidence of a role for the circadian clock in modifying wound responses. Our results show that darkness impacts on the wound response at a number of levels, which may have implications for the effectiveness of herbivore defence over a diurnal cycle.

Key Words: wounding; defence; darkness; microarray; photosynthesis; resource allocation

Introduction

Mechanical wounding results in major transcriptional, biochemical and physiological changes in the leaves of higher plants (de Bruxelles and Roberts, 2001) and has been studied both because of it's relevance as a natural abiotic stress and as a model for the response to herbivore-induced damage. A number of signalling pathways have been identified with roles in regulating the wound response (de Bruxelles & Roberts, 2001; León, Rojo & Sanchez-Serrano, 2001). Central amongst these is the jasmonic acid (JA) pathway, which controls the expression of many wound-induced genes and is essential for resistance against arthropod herbivores (Howe & Jander, 2008). Other hormones which play important roles in the wound response are ethylene, which acts in concert with JA (O'Donnell et al., 1996), and ABA, which probably mediates responses local to the wound site where tissue desiccation occurs (Birkenmeier and Ryan, 1998; Delessert et al., 2004). There is also evidence that oligogalacturonic acids and extracellular ATP, released from the walls and cytoplasm of damaged cells, are important signals activating wound-induced changes in gene expression (Song et al., 2006; Heil, 2009). Together, these signals orchestrate responses to wounding which include localised tissue repair, desiccation tolerance and the up-regulation of the production of defensive anti-herbivore and antimicrobial secondary metabolites and proteins. Inducible responses to stress are generally assumed to have evolved because defence imposes a cost on the plant (Cipollini, Purrington & Bergelson, 2003; Zangerl, 2003; Walters & Heil, 2007), and these complex signalling networks most likely exist, therefore, to optimise the costs and benefits of defence. As a consequence, it is probable that inducible defences are further modified by interactions with other environmental signals which impact on the availability of resources that can be allocated to defence.

Light is of fundamental importance for plant growth because of its role in driving photosynthetic carbon fixation. Because of this, plants have evolved many mechanisms to sense and respond to light so that they might optimise growth and development according to the prevailing conditions. It has recently become apparent that this influence also extends to plant defence. Light has increasingly been recognised as having important effects on plant responses to biotic stress, particularly in relation to pathogen resistance (Karpinski et al., 2003; Bechtold, Karpinski & Mullineaux, 2005; Roberts and Paul, 2006; Roden and Ingle, 2009). For example, activation of defence against pathogens is affected by the duration of exposure to light following infection, whilst systemic acquired resistance (SAR) is regulated by phytochromes (Genoud et al., 2002; Griebel and Zeier, 2008). Herbivore resistance responses are also impacted by light. Induced emissions of volatile compounds which attract natural enemies of herbivores have been found to be light-dependent in a number of systems (Loughrin et al., 1997; Halitschke et al., 2000; Maeda et al., 2000; Gouinguené & Turlings, 2002). On a broader level, the phytochrome-mediated shade avoidance response has a repressive effect on jasmonic acid (JA)-dependent responses to herbivory, indicating that light signalling modulates herbivore defence as a mechanism to integrate different environmental signals within the plant, regulating potential competition between these two different stress responses (Izaguirre et al., 2006; Moreno et al., 2009).

At the molecular level, many studies have contributed to our understanding of induced defence responses to wounding and herbivory. Amongst these, a number of transcriptomic approaches have shown that not only does wounding up-regulate the expression of genes with direct roles in defence, but that expression of many genes associated with photosynthesis and other aspects of primary metabolism is repressed (Reymond et al., 2000; Hermsmeier, Schittko & Baldwin, 2001; Delessert et al., 2004). Alongside this, a wide range of

physiological and biochemical data also show that wounding and herbivory commonly cause widespread changes in primary metabolism, including reductions in photosynthesis (Schwachtje and Baldwin, 2008). The functional significance of these changes is still not fully understood, but it is likely that both wound and light signalling pathways will contribute to the regulation of such responses. Whilst the transition from darkness to light has been extensively studied in plants, particularly with regard to photomorphogenesis (Jiao, Lau & Deng, 2007), the transition from light to dark has received relatively little attention. Kim and von Arnim (2006) used transcriptome microarrays to investigate the effects of darkness on Arabidopsis seedlings grown under continuous light, and found that responses partially overlapped with those seen under sugar starvation. A related, more comprehensive study on the effects of an elongated night period in Arabidopsis, identified interactions between C signalling and the circadian clock in the regulation of metabolism and gene expression, again driven partially by sugar/C depletion (Udsadel et al., 2008). In the work described here, we aimed to extend our understanding of the interactions between the light environment and defence responses by using a dark period imposed during the morning to investigate early responses to darkness and their effects on the wound response in Arabidopsis. Our results indicate that the response to darkness is distinct from, but overlapping with the wound response, and that darkness modifies the wound response at a range of organisational scales.

Materials and Methods

Plant material

Seeds of *Arabidopsis thaliana*, ectotpye Col-0, were surface-sterilised and germinated in Petri dishes containing half-strength MS medium (Murashige and Skoog, 1962), with 1% sucrose. Plates were kept in controlled environment rooms at 20 ± 2ºC following a 10/14

hour light/dark regime with PAR of 250 μmol m^{-2} s^{-1}. At 10-days-old, seedlings were transplanted to a sieved, pre-autoclaved compost/horticultural silver sand mixture (3:1) and grown under the same conditions. Leaves were wounded twice across the lamina, perpendicular to the main vein, using a haemostat. Where appropriate, 10 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) or 10 μM DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) were sprayed onto rosettes 1 h before wounding.

Transcriptomics experiments

Four-week-old plants were left to acclimatise in controlled environment cabinets (Percival) at 22ºC and relative humidity 55-60%, either in the light (PAR = 250 μmol m^{-2} s^{-1}) or in the dark for 30 min. Subsequently, leaves of half of the plants in each group were wounded either in the light or in the dark under a 15 W Kodak safe light with a yellow 0B filter. All plants were returned to their respective growth cabinets for a further hour, after which rosettes were harvested into liquid nitrogen. Typically 15 – 17 plants were used per treatment, and the experiment was conducted on three separate occasions. RNA was extracted and purified as described below. Labelling and hybridisation to the Affymetrix ATH1 gene chip were performed at the VIB Microarray Facility, (K. U. Leuven, Belgium; www.microarrays.be). Data are deposited in the GEO database, accession number GSE13803.

Microarray data analysis and bioinformatics

Raw data were normalised using GCRMA (Wu et al., 2004) and the resulting expression values were then filtered to eliminate probe sets for which the mean signal was less than 10 or for which expression was not scored as present in all three replicate arrays for at least one treatment. This resulted in the inclusion of 13,821 probe sets for further analysis. Differentially-expressed genes were identified using Rank Products (Breitling et al., 2004)

and hiercarchical clustering (Eisen et al., 1998) was performed in the D-Chip package (Li and Wong, 2003). For analysis using MapMan (Thimm et al., 2004) and PageMan (Usadel et al., 2006), mean log fold-change values for all 13,821 probe sets included in our analysis were derived from the Rank Products output and used for display and statistical testing. Gene ontology term enrichment analysis was performed using the online AmiGO toolkit (Carbon et al., 2009).

Measurement of gene expression by RT-PCR

RNA was extracted using a scaled-up version of the method described by Verwoerd, Dekker & Hoekma (1989) and purified using the Qiagen RNeasy kit, as per manufacturers instructions (Qiagen; www.qiagen.com). Prior to cDNA synthesis, 10 µg RNA was treated with DNaseI (Invitrogen; www.invitrogen.com). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) using the primer GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTVN. PCR was carried out using Taq DNA polymerase (REDTaq; Sigma-Aldrich). The number of cycles used (25-35) was adjusted for each primer set so that amplification was in the linear range. The following oligonucleotides were used: ACT2-F; TGGTGATGGTGTGTCT, ACT2-R; ACTGAGCACAATGTTAC, CYP82G1-F; GGCGGTATCGCTGCTACTC, CYP82G1-R; GGCTAAACCAGGCCCTTCAG, DDF1-F; ACGTCACCCAGTTTACAGAG, DDF1-R; TCCAAATCCATACGAAGAAG, MAPKKK18-F; CGAGAGAGCCCTTCCACAAC, MAPKKK18-R; GACTCGCTGTCCATCTCTCC, NCED3-F; GAGCTGCAGCCGGTATAGTC, NCED3-R; CAGGACCCTATCACGACGAC, OXI1-F; TACGCGGCGGAGCTTGTATTAGCAC, OXI1-R; CAACCCTTAACCCATTCCCCACTAGT, RBOHD-F; GGAGTGGAAGGATGGACTGG,

RBOHD-R; GCCGAGACCTACGAGGAGTA, WD-40-F;
GATCGGTACGGTCGTGAGAC, WD-40-R; CCCAAGAACCGGAGTAGAGC.

Phenylpropanoids assay

Plants were grown as previously described until 4-5 weeks old. One hour into their normal 10 h light period, plants were placed in controlled environment cabinets (Percival) at 22ºC with 60% relative humidity either in the light (PAR of 250 μmol m^{-2} s^{-1}) or in the dark. After 30 min, half of the plants in each cabinet were wounded. For the dark treatment, plants were wounded under a 15 Watt Kodak safe light with a yellow 0B filter. Plants were returned to their respective controlled environments for 10 h, after which, rosettes were weighed and harvested into liquid nitrogen.

Metabolites were extracted by grinding each rosette individually to a fine powder with liquid nitrogen in a pestle and mortar, and homogenised following addition of 2 ml of acidified methanol (methanol:water:HCl 70:29:1). 1.5 ml of homogenate was separated by centrifugation at full speed in a benchtop microcentrifuge for 15 min. Supernatants were scanned from 240 to 750 nm using an Ultrospec 2100 Pro UV/visible spectrophotometer. Absorbance values were normalised against the sample fresh weight.

Results

To explore the early response to wounding, and in particular the impact of light on this response, we investigated changes in gene expression in Arabidopsis leaves using Affymetrix ATH1 microarrays. We used a 2 x 2 factorial design, with 1 hour incubations of unwounded and wounded leaves left either in the light or in the dark. Following initial filtering of data, we used Rank Products analysis (Breitling et al., 2004) to identify differentially-regulated

genes from relevant pair-wise comparisons to examine the responses to wounding and the transfer to darkness. A false discovery rate (FDR) of 0.05 was set as a threshold for the identification of differentially-expressed genes.

Transfer to darkness imposes rapid transcriptional responses similar to those occurring under an extended night period

We first examined the response of plants following transfer from the light to the dark (hereafter referred to as a 'dark shift'), by comparing gene expression in unwounded leaves left in the light with that from leaves transferred to the dark for one hour. The dark shift resulted in the differential regulation of 536 probe sets at FDR < 0.05 (275 up-regulated and 261 down-regulated). Differentially-regulated genes include those involved in transcriptional regulation, metabolism and stress responses. The full lists of genes are available as Supporting Information Table S1. To gain a more holistic understanding of the biological processes affected by the dark shift response, we used MapMan software (Thimm et al., 2004) to display the microarray data on biological pathway maps. In addition, MapMan uses a Wilcoxon rank sum test to identify functionally-related groups of genes which show different patterns of responses compared with the complete collection of genes under analysis. A graphical summary illustrating differentially-regulated functional groups is shown in Fig. 1. (The full statistical analysis is presented in Supporting Information Table S2). The most noteworthy responses following the dark shift are the highly significant down-regulation of protein synthesis and concurrent up-regulation of the ubiquitin-dependent proteolytic pathway, along with the down-regulation of various aspects of primary and secondary metabolism, including glycolysis, carbohydrate, amino acid and nucleotide biosynthesis and phenylpropanoid metabolism, and the up-regulation of ethylene biosynthetic and signalling genes. This profile is very similar to that observed by Usadel et al. (2008) for

plants responding to carbon depletion under an extended night. In addition to the similarity with the response to an extended night, a meta-analysis of public microarray gene expression data using Genevestigator (Zimmermann et al., 2004), indicated that genes induced by the dark shift in our experiments also show increased expression in response to low CO_2 (*i.e.* under carbon deficit), but reduced expression in response to supplemental glucose, and to a lesser extent, sucrose (Supporting Information Fig. S1).

Darkness imposes a wide-scale attenuation of the wound response

Upon examination of the responses to wounding, we found a total of 663 probe sets responsive to wounding in the light (445 up-regulated and 218 down-regulated), whilst in the dark, only 444 probe sets showed differential regulation (335 up-regulated and 109 down-regulated) (Supporting Information Table S1). As expected, inspection of these lists revealed large numbers of genes involved in signalling, such as transcription factors, protein kinases and phosphatases, and genes related to hormone biosynthesis and signalling. MapMan analysis (Fig. 1) showed that the responses to wounding and the dark shift are mainly non-overlapping and involve distinct biological processes. In wounded leaves, notable responses include the up-regulation of a number of classes of genes involved in signalling, including calcium signalling, ethylene and jasmonate biosynthesis and signalling, receptor kinases, and members of the AP2/EREBP, WRKY and PHOR1 transcription factor families. Meta-analysis using Genevestigator suggested that genes responsive to wounding in our experiments were also responsive to infection by several microbial pathogens and to abiotic stresses, including salt and drought stress (Supporting Information Fig. S1). Interestingly, the meta-analysis also indicated that basal expression of wound-induced genes is repressed under elevated CO_2.

We next made comparisons of the wound responses in light- and dark-treated leaves. The Venn diagrams in Fig. 2a illustrate that although fewer genes responded to wounding in the dark than in the light, there was high degree of overlap between the responses under each condition. Only 83 (19%) of the total of 444 differentially-regulated probe sets were unique to the dark. Hence, the group of genes regulated by wounding in the dark is a subset of those regulated in the light. Comparisons of the distributions of mean fold-change values for probe sets responsive to wounding under both light regimes suggested that the magnitude of up- or down-regulation of expression was generally lower in the dark (Fig. 2b). Closer analysis revealed that 81% of probe sets that were initially identified as differentially-regulated by wounding under both light treatments, showed a greater magnitude of change in expression in the light. This difference was statistically significant ($P<0.0001$) as determined by a Wilcoxon matched pairs test.

Interactions between the wound response and the light environment

To provide a more detailed overview of the different patterns of expression exhibited by the wound- and dark-regulated genes identified above, we performed a hierarchical clustering analysis of all differentially-regulated genes. By inspection of the resulting tree diagram, we identified 12 major groups of genes with distinct expression profiles (Fig. 3). For the majority of genes, the dark shift and wound responses acted independently and additively. One outcome of this superimposition of responses is that for genes affected by both treatments, the dark shift can either exaggerate or attenuate the wound response, depending whether the two responses operate in the same or opposite directions (*e.g*. clusters 1 & 12). However, there are also significant numbers of genes for which there are more complex interactions between the two treatments. For example, cluster 4 contains genes that are induced by wounding only in the dark. Gene ontology analysis revealed that this cluster is significantly

($P = 1.9x10^{-7}$) enriched in genes associated with the biosynthesis of glucosinolates, compounds with important functions in defence against herbivores and pathogens. Conversely, the down-regulation of genes in cluster 7 by wounding appears to be dominant over the transcriptional activation conferred by the dark shift. This cluster is enriched in genes associated with auxin responses ($P = 3.2x10^{-5}$).

We were particularly interested in cluster 8, which contains wound-induced genes for which transcriptional activation is reduced in the dark. We selected sub-clusters from within cluster 8 (Supporting Information Fig. S2), that include light-dependent wound-induced genes that exhibit only minor responses to the dark shift alone. The genes comprising these sub-clusters and their responses to wounding are listed in Table 1. To examine the regulation of these genes further, and to validate the results from the microarray experiments, the steady-state mRNA levels of representative genes from Table 1, along with wound-induced genes whose expression was light-independent, were measured using semi-quantitative RT-PCR. The results (Fig. 4) confirmed the results from the microarrays. The genes *OXI1* and *DDF1* showed light-independent wound-induced expression, whereas genes from cluster 8 identified as light-dependent, *CYP82G1*, *MAPKKK18*, *NCED3* and a gene encoding a WD40 repeat-containing protein, all showed attenuated wound-induced expression in the dark.

The effect of wounding on specific metabolic pathways is modified by light

We also used MapMan to search for interactions between the wounding and dark treatments at the level of functional groups. As for the analysis at the individual gene level, we found that most categories showed similar responses to wounding irrespective of the light conditions. However, we observed a number of instances where individual MapMan categories were significantly affected by wounding only in the light or in the dark, but not

both, (Fig. 1 and Supporting Information Table S2). These include photosynthesis, isoprenoid metabolism, glucosinolate biosynthesis, thiroedoxins, glutathione S-transferases, light signalling, and auxin and ABA responsive genes. Genes associated with the light reactions of photosynthesis and genes involved in light signalling are down-regulated by wounding only under dark conditions. At least in the case of the photosynthetic genes, this may represent a synergistic effect, since although both darkness and stress generally down-regulate such genes, neither wounding nor the dark shift alone produce a significant effect on these groups within the time frame of our experiment. We also made the observation that a number of functional classes that are wound-regulated only in the dark are also regulated by the dark shift. Generally, these represent groups that respond to wounding from an altered baseline imposed by the dark shift, to achieve a final absolute level of expression that is closer to the value in light wounded plants (*c.f.* Fig. 3, Cluster 4). For example, many genes encoding proteins involved in glucosinolate metabolism and protein synthesis are repressed by the dark treatment, but show a significant increase in expression from this depressed baseline following wounding, despite the fact that they are not significantly affected by wounding in the light.

One family of MapMan gene classes showing differential regulation by wounding in the light compared with the dark was that of secondary metabolism, particularly genes involved in phenylpropanoid and flavonoid metabolism (Fig. 1). It is already well known that wounding, herbivory and pathogen attack tend to increase the biosynthesis of phenylpropanoid compounds, and that phenylpropanoid metabolism is increased under high light stress and in response to UV radiation (Dixon & Paiva, 1995; Davies & Schwinn, 2003). However, much less is known about possible interactions between wounding or biotic stress and light. Based on the differential responses of phenylpropanoid and flavonoid biosynthetic gene classes, we

tested whether there were corresponding differences in metabolite accumulation in wounded plants. We found that the levels of total UV-absorbing compounds (which include various flavonoids and other phenolics) showed no consistent responses in our experiments (data not shown). By contrast, wounding led to a significant accumulation of metabolites with a peak absorption around 524 nm, typical of anthocyanins, but only in plants left in the light (Fig. 5).

Light-dependent wound-induced changes in gene expression are regulated by a chloroplast signal, but independent of ABA signalling.

Having identified a number of levels of influence of light on the wound response, we wanted to investigate possible regulatory mechanisms. Several studies have linked chloroplast function with light-mediated effects on defence responses (Chen et al., 1998; Genoud et al. 2002; Chang et al., 2004), and the interruption of photosynthesis is one obvious impact of the transfer to darkness. We therefore used inhibitors of photosynthetic electron transport (PET) to test whether loss of photosynthetic activity might impact the wound response. Wounded and control leaves pre-treated with DCMU or DBMIB were analysed by RT-PCR to examine expression of wound-induced marker genes. Fig. 6a shows that the wound-induced expression of all four light-dependent genes tested, but not that of the light-independent marker *DDF1*, was substantially reduced by these inhibitors, indicating that active PET is required for the wound-induced expression of these genes.

One of the genes we identified whose response to wounding is strongly modulated by light, *NCED3*, encodes an isoform of 9-*cis*-epoxycarotenoid dioxygenase, which is required for stress-induced abscisic acid biosynthesis (Ruggiero et al., 2004). ABA is well known as a regulator of wound-induced gene expression, and it has recently been shown that it is also an important signal in the high light stress response in Arabidopsis (Galvez-Valdivieso et al.,

2009). We therefore tested whether ABA signalling might be important for wound-induced expression of light-dependent genes. Plants with mutations in the ABA signalling genes *ABI1*, *ABI2* and *ABI3* were wounded and marker gene expression assayed by RT-PCR (Fig. 6b). The results show that expression of those genes identified as light-dependent was not affected by deficiencies in ABA signalling. The light-independent wound-induced marker genes *DDF1* and *OXI1*, showed differing requirements for ABA signalling. *DDF1* was expressed normally in the ABA mutants, whereas in contrast, wound-induced expression of *OXI1* was undetectable.

Regulation of wound response genes by the circadian clock

Because darkness also has a broader quantitative effect on the wound response, we considered other potential regulatory mechanisms underlying this effect. One mechanism which governs plant interactions with natural light/dark transitions is the circadian clock (McClung, 2006). As well as regulating many aspects of growth and development, the clock also influences plant responses to the environment, including defence (Hotta et al., 2007; Roden & Ingle, 2009). We therefore sought to identify relationships between wound responsive genes and those under the control of the circadian clock. From the total of 13,822 probe sets under consideration in our microarray analysis, 1522 were identified as circadian-regulated by Covington & Harmer (2007). Table 2 compares the frequencies of occurrence of these circadian genes amongst those genes defined as differentially regulated by the dark shift or by wounding in our experiments. As might be expected, we found that circadian-regulated genes were significantly over-represented amongst genes responding to the dark shift, especially those which are down-regulated in the dark. When we looked at the wound response, we found that circadian-regulated genes were slightly (but significantly) under-represented amongst wound-induced genes, but heavily over-represented amongst genes

down-regulated by wounding. Genes in this category may highlight a convergence of wound- and circadian-regulated growth inhibition, since they include a high proportion of genes involved in auxin responses ($P = 1.43x10^{-5}$ by GO term analysis), as well as several expansins, proteins which mediate cell wall extensibility (Supporting Information Table S3). The majority of these circadian-regulated, wound-repressed genes, show peak expression during the second half of the subjective day in the experiments performed by Covington & Harmer (2007), (Supporting Information Fig. S3). We found no significant over- or under-representation of circadian-regulated genes in any of the clusters from Fig. 3 that show light-dependent wound responses.

Discussion

Using a transcriptomic approach as a starting point, the work presented here identifies a number of levels of influence of light upon the response to wounding, and suggests some mechanisms by which these influences may be exerted. In addition, our data also identify biological processes which show rapid changes in response to a shift from light into darkness. The profile produced by MapMan analysis for the dark shift response in our experiments was remarkably similar to the response of plants to an extended night, which suffer carbon depletion when photosynthesis does not begin at the time predicted by the circadian clock and starch stores become exhausted (Usadel et al., 2008). Meta-analysis in Genevestigator also revealed that dark shift-responsive genes were regulated in other experiments by CO_2 concentration and exogenous glucose. Together, these observations suggest that the dark shift used in our experiments rapidly activates transcriptional responses associated with carbon depletion and affects processes associated with resource allocation.

A substantial proportion of dark shift-responsive genes are also regulated by wounding (147/746 probe sets; 19.7%), but the two responses are largely independent of one another, as shown by the clustering and MapMan analyses. Consistent with previous studies (Reymond et al., 2000; Cheong et al., 2002; Delessert et al., 2004), we identified substantial overlaps between the wound response and responses to several microbial pathogens and elicitors, as well as to abiotic stresses, notably salt and drought stress. Interestingly, we found evidence via Genevestigator, that CO_2 concentration may also influence the wound response, since the basal expression levels of wound-inducible genes are generally lower at elevated CO_2. This observation is consistent with previous studies which show increased susceptibility of plants to herbivory at elevated CO_2 via repression of JA-dependent defences (Zavala et al., 2008), but is contrary to the idea that provision of resources from photosynthesis may limit defence.

In terms of the interaction between light and the wound response, we found that at the whole genome level, the magnitude of the wound response is lower in the dark than in the light (Fig. 2). This is true both in terms of the numbers of genes showing differential expression in response to wounding, and the relative changes in expression levels of individual genes. In fact, the former is largely a consequence of the latter, in that many genes classified as wound responsive in the light fail to pass the threshold criteria for differential expression in the dark because of the reduced magnitude of their response. Hence, the major effect of darkness on the wound response is quantitative rather than qualitative. A similar effect of darkness on the cold response in Arabidopsis was recently reported (Soitamo et al., 2008). Fewer genes were regulated by exposure to low temperature in the dark than in the light, and phenylpropanoid and photosynthetic carotenoid-related genes in particular were identified as showing light-dependent cold responses. There are a number of possible explanations for the reduced transcriptional response under darkness. The first relates directly to resource allocation,

which is a major driver of transcriptional re-programming during the shift from light to dark (Kim & von Arnim, 2006). Loss of photosynthesis in the dark causes a reduction in available energy and carbon skeletons for metabolism, which will directly impact on transcriptional activity. Superimposed on potential metabolic constraints will be changes in the activity of signalling pathways, such as those responding to light and C-depletion, which are likely to have a general suppressive effect on cellular activity. For example, the significant reduction in protein synthesis in the dark inferred from the MapMan analysis, is likely to have knock-on effects on transcriptional activity in response to wounding. Interestingly, we did observe a compensatory affect of wounding in the dark on the expression of genes involved in translation, which may be an adaptive response to minimise the attenuation of defence in the dark.

As well as the broad-scale quantitative differences discussed above, our analyses also identified a number of processes and individual genes that are more strongly light-dependent. MapMan identified phenylpropanoid and flavonoid metabolism as showing different responses to wounding under different light conditions, and an analysis of products of these pathways confirmed that wound-induced anthocyanin production is light-dependent. *AtMYBL2* is a negative regulator of anthocyanin biosynthesis (Matsui, Umemura & Ohme-Takagi, 2008), and was identified in Cluster 7 (Fig. 2) as exhibiting light-dependent repression by wounding. This is consistent with our finding that wound-induced anthocyanin accumulation only occurs in the light, and suggests that *AtMYBL2* may be an important regulator of this process. The MYB factor *PAP1*, which is a key positive regulator of the anthocyanin biosynthetic pathway in leaves (Borevitz et al., 2000), and the related *PAP2* gene, were strongly down-regulated by darkness, but showed only small responses to wounding in our microarray experiments (data not shown). Isoprenoid metabolism was

another process identified in MapMan as showing different responses to wounding in the light and in the dark, which is consistent with the fact that the emission of many herbivore-induced volatiles requires light (Loughrin et al., 1997; Halitschke et al., 2000; Maeda et al., 2000; Gouinguené & Turlings, 2002).

We also identified a number of individual genes whose wound-induced expression was strongly influenced by light. An earlier study by Chang et al., (2004), presented evidence that wound-induced expression of Arabidopsis *APX2* requires PET, suggesting one possible mechanism for the light-dependent effects on the wound response seen here. Our experiments using PET inhibitors extend this idea to suggest that this may be a more general mechanism for the light-dependent regulation of wound-induced genes. We found that transcriptional activation of all tested light-dependent, but not light-independent wound-induced genes, is blocked by both DCMU and DBMIB. These inhibitors prevent the reduction and oxidation of plastoquinone (PQ) respectively, indicating a site downstream of PQ in PET as the source of a chloroplast signal promoting wound-induced gene expression. The existence of such a signal suggests that the attenuation of the wound response in the dark is controlled in part by direct signalling of the loss of photosynthesis, and therefore resource provision, rather than simply by direct metabolic constraints. The light-dependent, wound-induced expression of *NCED3* suggested another possible mechanism for modulation of the wound response by light. *NCED3* is required for stress-induced ABA biosynthesis (Ruggiero et al., 2004), and since ABA has been implicated previously in regulating transcriptional responses to wounding, we were interested to test whether there might be any link between ABA signalling and light-dependent gene expression. However, analysis of wound-induced gene expression in ABA-insensitive mutants failed to identify any requirement for ABA signalling in the regulation of the light-dependent marker genes tested. This is consistent with the

response of *APX2* to wounding, which requires active PET, but not ABA signalling (Chang et al., 2004). For light-independent wound-induced genes, both ABA-dependent and ABA-independent pathways exist, since transcriptional activation of *OXI1*, but not *DDF1*, is blocked in the ABA mutants.

Because of the similarity between the dark shift response in our experiments and the circadian clock-driven response to an extended night (Usadel et al., 2008), we also considered possible influences of the clock on the wound response. Although we found no evidence for clock regulation of light-dependent effects, we did identify a more general interaction with the wound response. In particular, we found that circadian-regulated genes are significantly over-represented amongst genes down-regulated by wounding. This observation is consistent with other studies which also identify over-representation of stress-responsive genes amongst circadian-regulated genes, especially those responding to JA, a major regulator of the wound response (Covington et al., 2008; Mizuno & Yamashino, 2008). Genes associated with cell expansion and growth were particularly prevalent amongst these genes in our experiments, and this may reveal a point of convergence for light-, circadian- and stress-mediated effects on growth. Beyond this, the circadian clock may present a useful mechanism for the plant to modulate defence responses based on resource availability. It will be of interest in the future to test whether other responses to wounding and herbivory are gated by the circadian clock.

Whilst much of this analysis has focussed on the effect of wounding on plants either in the light or in the dark (that is, by consideration of pair-wise comparisons made using the relevant light/dark treated controls), it is also of interest to understand the differences that might be expected if plants in normal light conditions were wounded and then either left in the light, or moved into darkness. Biologically, such a scenario might reflect the difference

between mechanical wounding or herbivore attack either early in the day, or in the evening, just prior to nightfall. The gene expression profiles presented in Fig. 3 suggest that in the latter case, the outcome would be similar to the additive effects of the wound and dark shift responses. Hence, whether a plant is exposed to light or dark conditions after wounding has a profound impact on the outcome, whereby the response to darkness is superimposed on the wound response. In our experiments, this leads in some instances to the loss or attenuation of transcriptional responses of many genes that normally show opposing patterns of regulation in the two responses, such as phenylpropanoid metabolism, protein synthesis and the ubiquitin-mediated protein degradation pathways, whereas the expression of other genes is increased additively. The ecological implications of the interactions between light and defence at a range of scales are discussed in more detail by Roberts & Paul (2006), and it will be of interest in the future to determine how the relationships identified here at the molecular and physiological levels might impact on plant-herbivore interactions under different light conditions.

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Table 1. Identities of light-dependent, wound-induced genes in selected sub-clusters from within cluster 8 identified by hierarchical clustering of probe sets. Changes in gene expression are expressed as $\log_2$ ratios.

Affy ID	**AGI ID**	**Annotation** (TAIR9)	LW Ratio	DW Ratio
Sub-cluster 1				
253060_at	At4g37710	VQ motif-containing protein	3.39	0.74
248253_at	At5g53290	CRF3 (CYTOKININ RESPONSE FACTOR 3)	2.51	0.27
253679_at	At4g29610	cytidine deaminase, putative	2.24	0.17
256991_at	At3g28600	ATP binding / ATPase/ nucleoside-triphosphatase	2.31	0.43
266232_at	At2g02310	AtPP2-B6 (Phloem protein 2-B6)	2.26	0.41
266878_at	No gene		2.25	0.43
249540_at	At5g38120	4-coumarate--CoA ligase family protein / 4-coumaroyl-CoA synthase family protein	2.25	0.97
250161_at	At5g15240	amino acid transporter family protein	2.69	1.51
260152_at	At1g52830	SHY1/IAA6 (INDOLE-3-ACETIC ACID 6)	2.05	1.02
261431_at	At1g18710	AtMYB47	2.14	1.95
Sub-cluster 2				
257280_at	At3g14440	NCED3/SIS7/STO1 (9-cis-epoxycarotenoid dioxygenase)	3.86	1.76
248358_at	At5g52400	CYP715A1	2.99	1.05
265216_at	At1g05100	MAPKKK18	3.15	1.50
248226_at	No gene		3.75	2.16
254809_at	At4g12410	auxin-responsive family protein	2.45	0.88
247393_at	At5g63130	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein	3.20	1.65
266590_at	At2g46240	BAG6 (BCL-2-ASSOCIATED ATHANOGENE 6)	2.59	1.37
266456_at	At2g22770	NAI1	2.00	0.96
260210_at	At1g74420	FUT3 (FUCOSYLTRANSFERASE 3)	2.11	1.10
263465_at	At2g31940	unknown protein	1.92	1.03
Sub-cluster 3				
262448_at	At1g49450	transducin family protein / WD-40 repeat family protein	3.66	1.84
259866_at	At1g76640	calmodulin-related protein, putative	3.80	2.12
257835_at	At3g25180	CYP82G1	3.27	1.71

267058_at	At2g32510	MAPKKK17	3.12	1.64
265479_at	At2g15760	calmodulin-binding protein	2.15	1.07
250956_at	At5g03210	unknown protein	2.03	1.05
263972_at	At2g42760	unknown protein	2.51	1.66
261922_at	At1g65890	AAE12 (ACYL ACTIVATING ENZYME 12);	2.50	1.67
247723_at	At5g59220	protein phosphatase 2C, putative	2.05	1.36
254996_at	At4g10390	protein kinase family protein	3.40	2.85
264886_at	At1g61120	GES/TPS04 (TERPENE SYNTHASE 04; (E,E)-geranyllinalool synthase)	2.07	1.55
258551_at	At3g06890	unknown protein	2.55	2.09
247175_at	At5g65280	GCL1 (GCR2-LIKE 1)	2.59	2.27

Table 2. Occurrence of circadian-regulated genes amongst dark shift and wound-responsive gene classes, tested using Fisher's exact test.

	Total number of differentially-regulated genes	Expected number of circadian genes	Observed number of circadian genes	p-value
Dark shift; up-regulated	275	30	67	8.34×10^{-10}
Dark shift; down-regulated	261	29	79	7.22×10^{-17}
Wounding - Light; up-regulated	445	49	32	0.0105
Wounding - Light; down-regulated	218	24	79	3.90×10^{-22}
Wounding - Dark; up-regulated	335	37	17	0.0002
Wounding - Dark; down-regulated	109	12	52	1.79×10^{-21}

Figure Legends

Figure 1. PageMan display of MapMan gene categories affected by the dark shift and wound responses. The Wilcoxon rank sum test was used to identify functional groups where the distribution of responses within a group differed from the response of the entire gene set under test. Coloured boxes indicate statistically-significant groups (Benjamini & Hochberg-corrected P-value below 0.05). Colour scale represents z-transformed P-values, with red indicating a trend within the group for up-regulation of expression relative to the control, and blue, down-regulation.

Figure 2. Summary of the effect of darkness on the response to wounding. (a) Venn diagrams showing the overlap between wound-responsive probe sets (as identified by Rank Products analysis at FDR < 0.05) in leaves wounded either in the light or in the dark. (b) Box-and-whiskers plots showing the distribution of mean fold change values for probe sets that are differentially-regulated by wounding under both light and dark conditions. For down-regulated probe sets, the negative sign was removed, so that the plot shows the magnitude of all changes in gene expression irrespective of the direction.

Figure 3. Hierarchical clustering of differentially-expressed genes. Columns indicate mean $\log_2$ Affymetrix signals for the three replicate arrays for each treatment; LU, light unwounded; LW, light wounded; DU, dark unwounded; DW, dark wounded. Expression values for each probe set are coloured relative to the mean expression value such that red indicates high and green low expression signals. Clusters of probe sets (genes) with different

patterns of regulation are shown to the right, including standardised mean expression profiles for the probe sets in each cluster.

Figure 4. Validation of microarray expression data by RT-PCR. RT-PCR products amplified from cDNA from control, unwounded (C) and wounded (W) plants left either in the light or the dark for 1 h after treatment. cDNA was amplified using gene-specific primers corresponding to the genes *ACT2* (At3g18780), *OXI1 (*At3g25250*)*, *DDF1* (At1g12610), *CYP82G1 (*At3g25180*)*, a gene encoding a WD40 repeat-containing protein (At1g49450), *MAPKKK18 (*At1g05100*)* and *NCED3* (At3g14440).

Figure 5. Wound-induced anthocyanin accumulation is light-dependent. Anthocyanin content in leaves of unwounded (open bars) and wounded (hatched bars) plants left either in the light or in the dark. ** Denotes mean value statistically significant at $p \leq 0.001$ in Tukey post-test following two-way ANOVA. Data shown are from one representative experiment.

Figure 6. Wound-induced expression of light-dependent genes is blocked by PET inhibitors, but is not altered in ABA signalling mutants. (a) RT-PCR products amplified from cDNA from control, unwounded (C) and wounded (W) plants from leaves of plants sprayed prior to wounding with either water (Control), 10 µM DCMU, or 10 µM DBMIB. (b) RT-PCR products amplified from cDNA from control, unwounded (C) and wounded (W) wild-type Col-0 plants and the *abi1-1*, *abi2-1* and *abi3-1* ABA signalling mutants. cDNA was amplified using gene-specific primers corresponding to the genes *ACT2* (At3g18780), *OXI1*

*(*At3g25250*)*, *DDF1* (At1g12610), *CYP82G1 (*At3g25180*)*, a gene encoding a WD40 repeat-containing protein (At1g49450), *MAPKKK18 (*At1g05100*)* and *NCED3* (At3g14440).

Supporting Information

Supporting Information Table S1. Genes differentially-regulated by wounding and the dark shift.

Supporting Information Table S2. Statistical analysis of the dark shift and wound responses using MapMan functional categories.

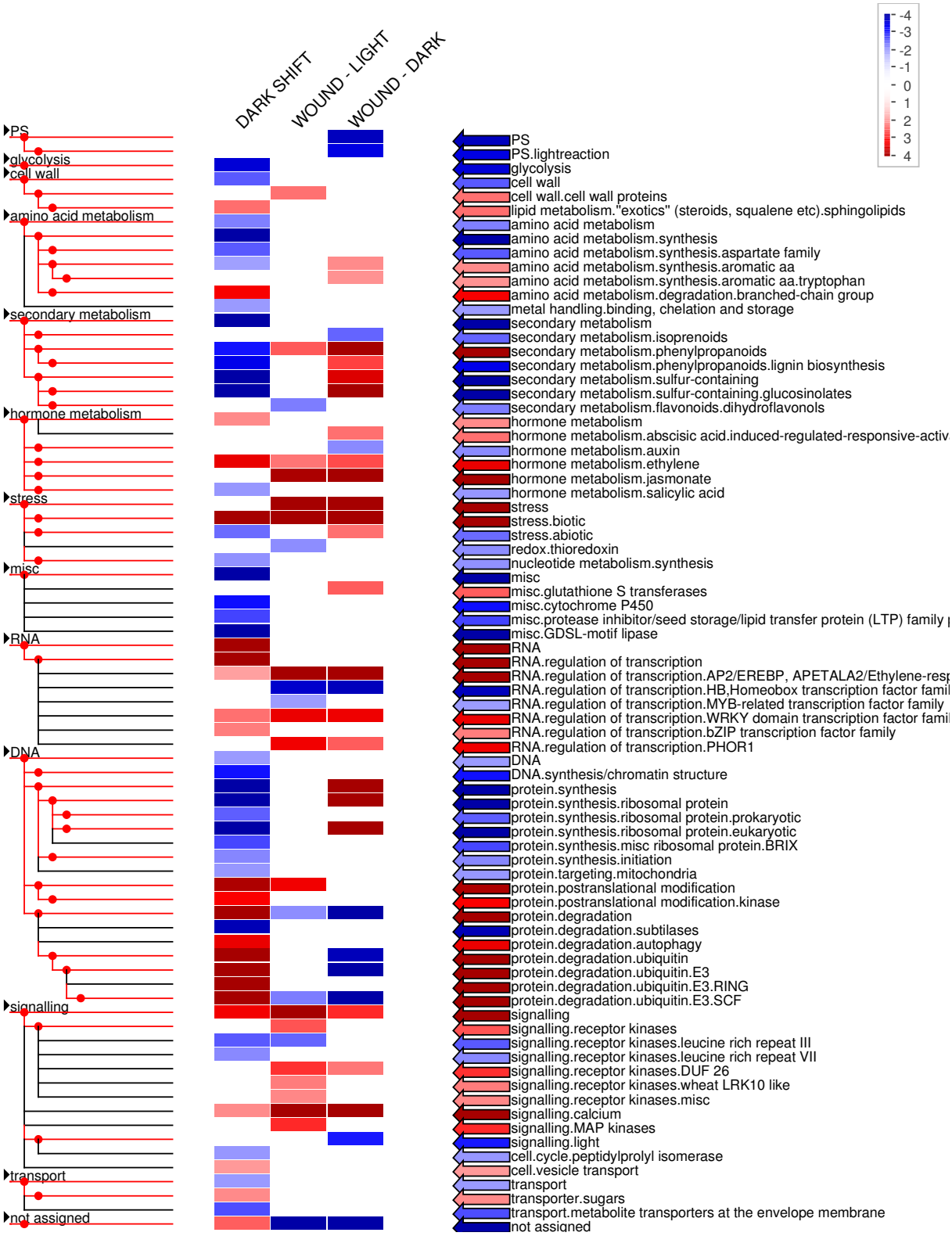
Supporting Information Table S3. Identities of wound-repressed genes that are also under the control of the circadian clock.

Supporting Information Figure S1. Meta-analysis of wound and dark-regulated genes using the Genevestigator tool.

Supporting Information Figure S2. Sub-clusters within cluster 8 (Fig. 3) including light-dependent wound-induced genes.

Supporting Information Figure S3. Circadian phasing of wound-repressed genes.

Figure 1.


DARK SHIFT
WOUND - LIGHT
WOUND - DARK
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-3
-2
-1
0
1
2
3
4
PS
glycolysis
cell wall
amino acid metabolism
secondary metabolism
hormone metabolism
stress
misc
RNA
DNA
signalling
transport
not assigned
PS
PS.lightreaction
glycolysis
cell wall
cell wall.cell wall proteins
lipid metabolism."exotics" (steroids, squalene etc).sphingolipids
amino acid metabolism
amino acid metabolism.synthesis
amino acid metabolism.synthesis.aspartate family
amino acid metabolism.synthesis.aromatic aa
amino acid metabolism.synthesis.aromatic aa.tryptophan
amino acid metabolism.degradation.branched-chain group
metal handling.binding, chelation and storage
secondary metabolism
secondary metabolism.isoprenoids
secondary metabolism.phenylpropanoids
secondary metabolism.phenylpropanoids.lignin biosynthesis
secondary metabolism.sulfur-containing
secondary metabolism.sulfur-containing.glucosinolates
secondary metabolism.flavonoids.dihydroflavonols
hormone metabolism
hormone metabolism.abscisic acid.induced-regulated-responsive-activ
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hormone metabolism.jasmonate
hormone metabolism.salicylic acid
stress
stress.biotic
stress.abiotic
redox.thioredoxin
nucleotide metabolism.synthesis
misc
misc.glutathione S transferases
misc.cytochrome P450
misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family
misc.GDSL-motif lipase
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RNA.regulation of transcription.WRKY domain transcription factor famil
RNA.regulation of transcription.bZIP transcription factor family
RNA.regulation of transcription.PHOR1
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protein.degradation.autophagy
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protein.degradation.ubiquitin.E3
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protein.degradation.ubiquitin.E3.SCF
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signalling.receptor kinases
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signalling.calcium
signalling.MAP kinases
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transport.metabolite transporters at the envelope membrane
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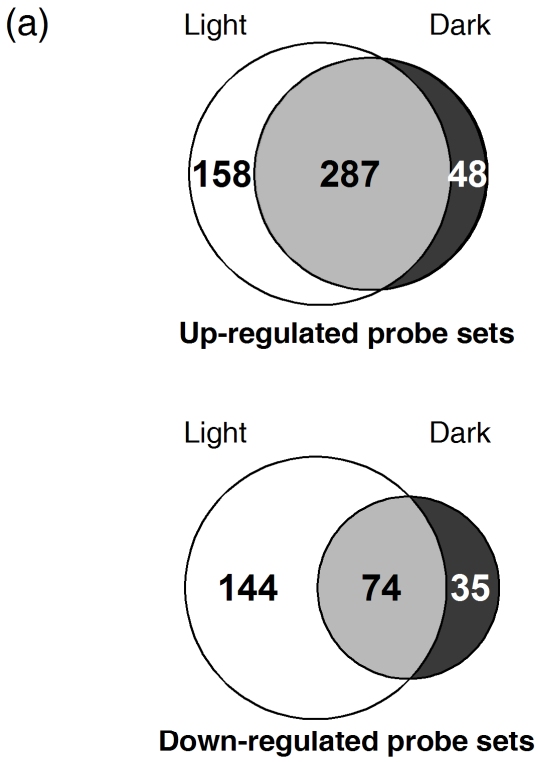
Figure 2.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Light
Dark
158
287
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Up-regulated probe sets
Light
Dark
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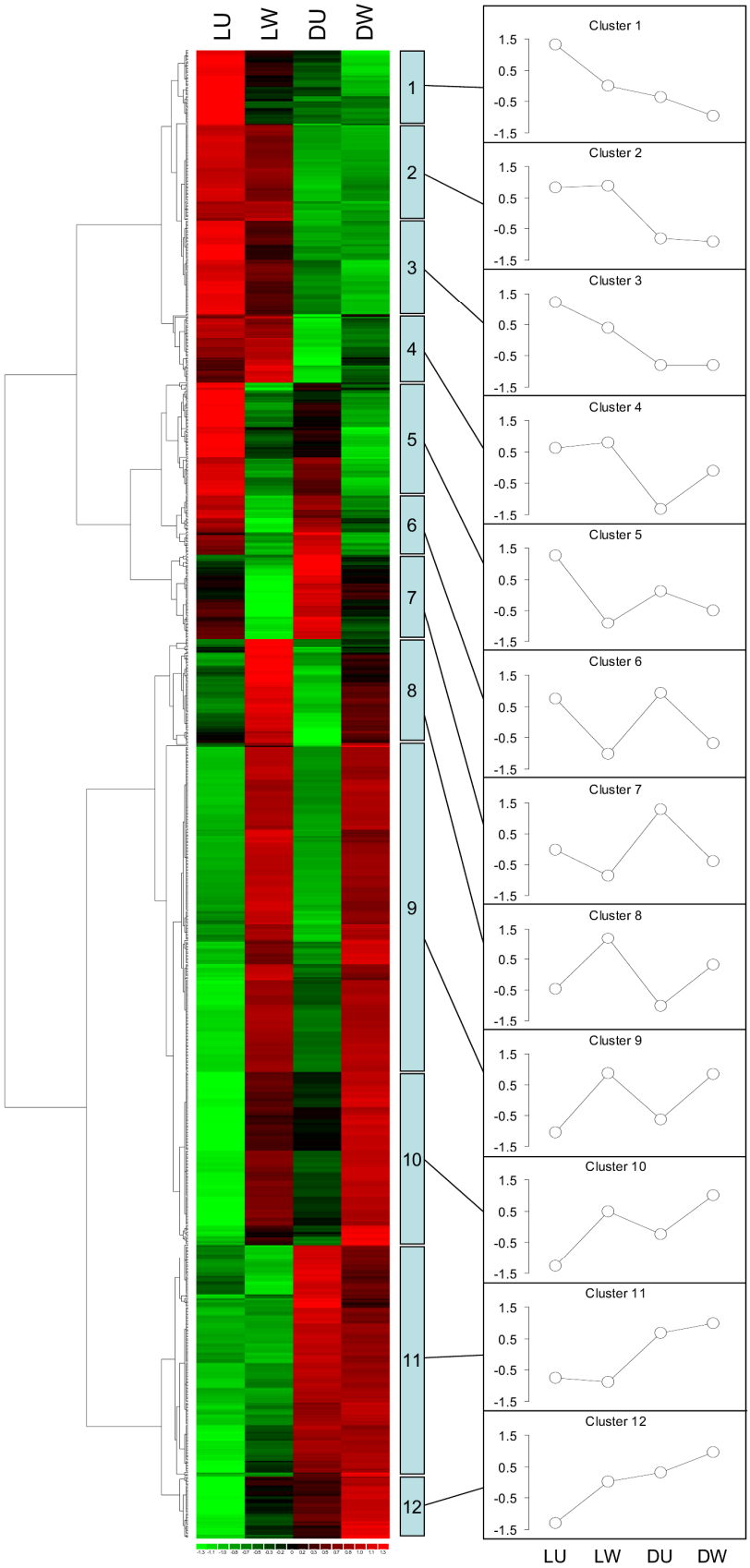
2
3
4
5
6
7
Light
Dark

Figure 3.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Cluster 6
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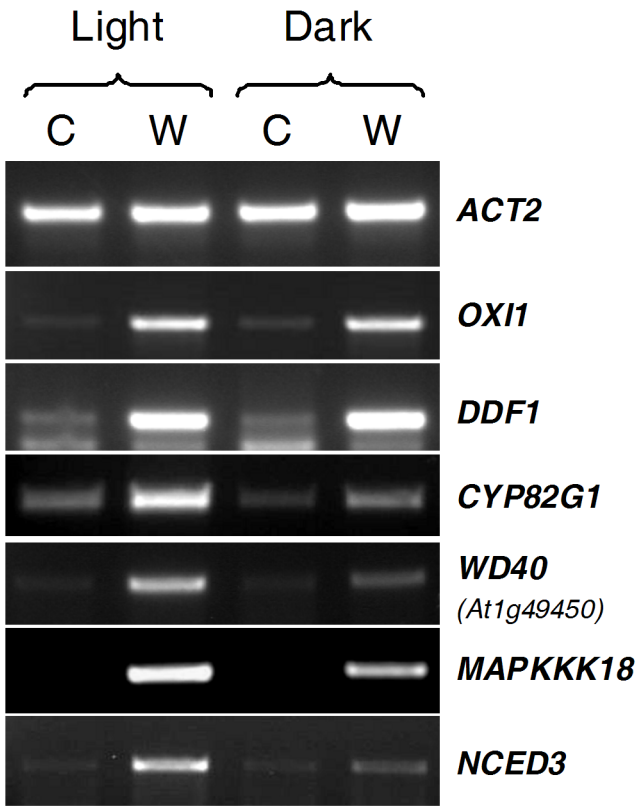
-0.5
-1.5
LU
LW
DU
DW

Figure 4.



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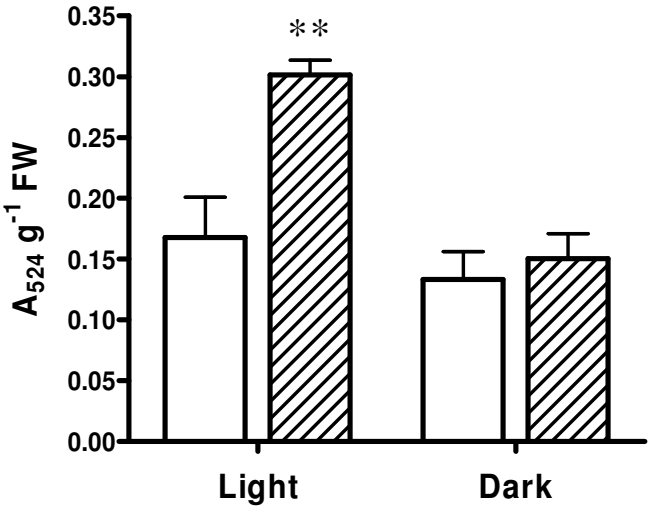
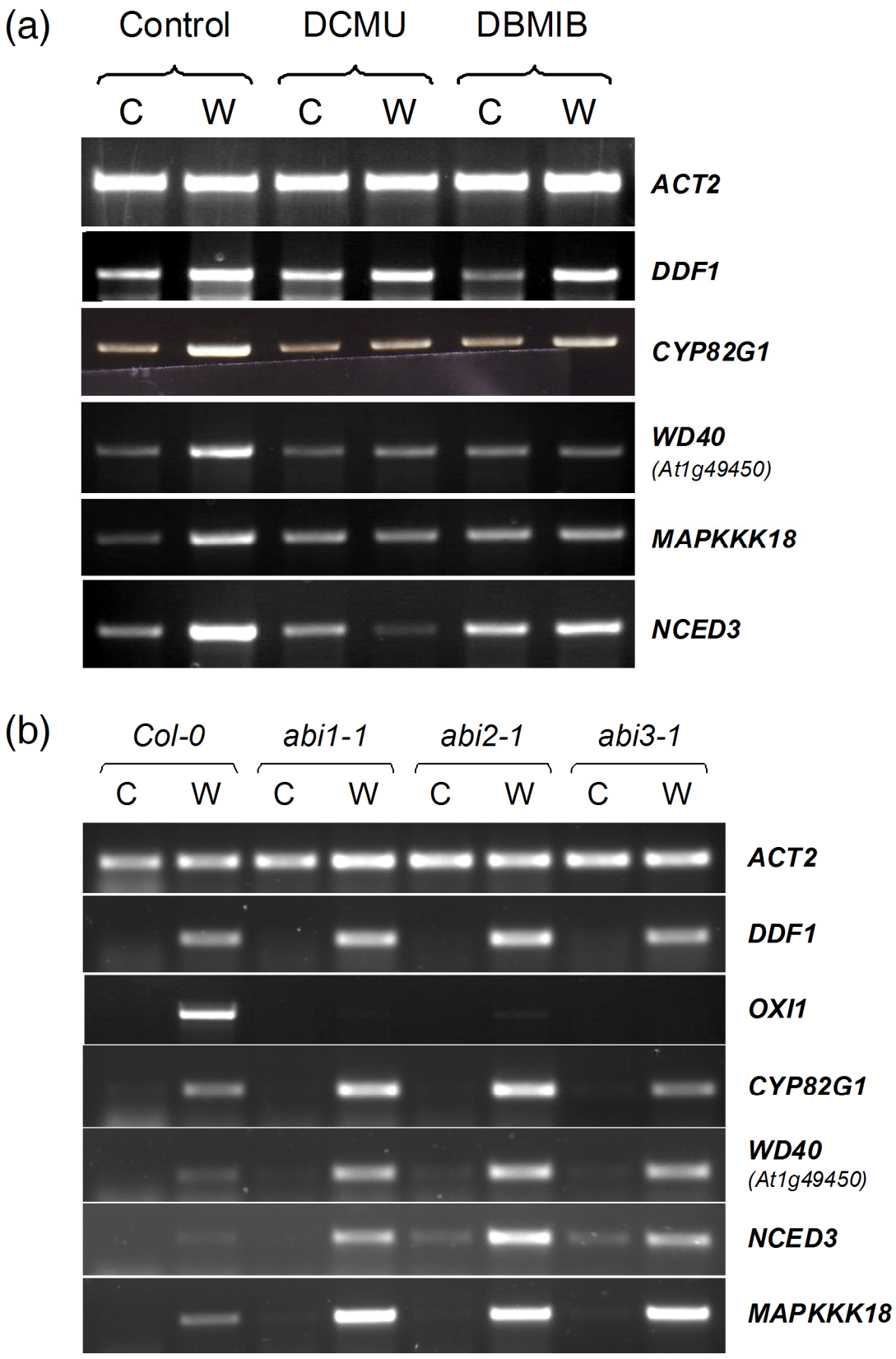


Figure 6.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DBMIB
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W
C
W
C
W
ACT2
DDF1
CYP82G1
WD40
(At1g49450)
MAPKKK18
NCED3
(b)
Col-0
abi1-1
abi2-1
abi3-1
C
W
C
W
C
W
C
W
ACT2
DDF1
OXI1
CYP82G1
WD40
(At1g49450)
NCED3
MAPKKK18