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WRKY40 and WRKY6 act downstream of the green leaf volatile E-2-hexenal in Arabidopsis

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SUMMARY

Plants are known to be responsive to volatiles, but knowledge about the molecular players involved in transducing their perception remains scarce. We study the response of Arabidopsis thaliana to E-2-hexenal, one of the green leaf volatiles (GLV) that is produced upon wounding, herbivory or infection with pathogens. We have taken a transcriptomics approach to identify genes that are induced by E-2-hexenal, but not by defence hormones or other GLVs. Furthermore, by studying the promoters of early E-2-hexenal-induced genes we determined that the only statistically enriched cis-element was the W-box motif. Since members of the plant-specific family of WRKY transcription factors act in trans on this cis-element, we focused on WRKY6, 40 and 53 that were most strongly induced by E-2-hexenal. Root elongation of Arabidopsis seedlings of the wrky40 wrky6 double mutant was much less inhibited than in wt plants, similar to the E-2-hexenal-responsive mutant her1, which is perturbed in γ-amino butyric acid (GABA) metabolism. The induction of several of the E-2-hexenal-specific genes was much higher in the wrky40, wrky6 or wrky40 wrky6 mutants, including GAD4, a glutamate decarboxylase that catalyzes the formation of GABA from glutamate. In conclusion, WRKY6 and 40 seem to act as important players transducing E-2-hexenal perception.

Keywords: Arabidopsis thaliana, green leaf volatiles, E-2-hexenal, transcription factors, markers.

INTRODUCTION

Plants can emit a bouquet of volatile organic molecules (VOCs) in the air from their photosynthetic aerial tissues. These VOCs mostly comprise terpenoids, aromatic compounds and C6-volatiles, the latter most commonly referred to as green leaf volatiles (GLVs). GLVs are produced in relatively low amounts by undamaged plants, but their emission increases in response to wounding, pathogen infection or herbivory (Hatanaka et al., 1992; Turlings et al., 1995; Matsui, 2006). Their emission is transient but can be sustained by repetitive wounding as often occurs during insect herbivory (Loughrin et al., 1994; Turlings et al., 1995).

Deacylation of galactolipids from chloroplast membranes provides free fatty acids necessary for the formation of GLVs (Matsui et al., 2000). GLVs are synthesized from ω-linolenic or linoleic acid through dioxygenation by C13-lipoxygenase activity, followed by subsequent cleavage by hydroperoxide lyase (HPL), a member of the cytochrome P450 family. The formation of Z3-hexenal from linolenic acid is followed by isomerization to E-2-hexenal and both aldehydes can be metabolized further by alcohol dehydrogenase activity (ADH) or alkenal reductases (Hatanaka et al., 1992; Mano et al., 2002) to form the corresponding alcohols. The formation of n-hexanal from linoleic acid is followed by conversion to hexanol. These alcohols can then be modified by acyltransferases (D’Auria et al., 2007). The conversion of the aldehydes to alcohols and subsequently to acetates might be an inactivation mechanism (Matsui et al., 2012).

GLVs have been assigned various functions. First, they can have a direct inhibiting effect on phytopathogens (Prost et al., 2005; Matsui, 2006; Kishimoto et al., 2008), although recently it has been shown that E-2-hexenal can actually make Arabidopsis thaliana more susceptible to the pathogen Pseudomonas syringae pv. tomato DC3000.
GLVs, emitted from wounded leaves, can also prime neighbouring plants to react, under the right conditions, with cellular nucleophilic groups. Reactive electrophile species (RES), such as -2-hexenal, can reprogram gene expression and can benefit cells (for a review see Farmer and Mueller, 2013)).

GLVs, emitted from wounded leaves, can also prime defence responses in systemic leaves, by which undamaged distal plant parts are prepared to respond more rapidly and intensively to subsequent biotic attack, without directly activating costly defence mechanisms (Frost et al., 2008b; Sugimoto et al., 2014; Ameye et al., 2015). For instance, E-2-hexenal, released by rice upon planthopper infestation, induces expression of defence-related genes, increasing resistance to bacterial blight (Gomi et al., 2010). E-2-hexenal contains an ω,β-un-saturated carbonyl group and this electrophilic β-carbon will react, under the right conditions, with cellular nucleophilic groups. Reactive electrophile species (RES), such as E-2-hexenal, can reprogram gene expression and can benefit cells (for a review see Farmer and Mueller, 2013)).

To gain insight into the biological processes affected by E-2-hexenal exposure to E-2-hexenal or to the carrier MeOH for the mock treatment and rosette leaves were harvested after 1, 3 and 24 h. E-2-hexenal exposure triggered an extensive change in gene expression, as summarized in Figure S1a. Overall 3494 genes showed a significant change in transcript abundance (P ≤ 0.05, false discovery rate (FDR) 0.05% and ≥ two-fold change), on at least one time point (Data S1 and S2). Specifically, transcript levels of 1939 genes (55%) increased and of 1555 genes (45%) decreased more than two-fold (Figure S1a). The transcriptome responses to E-2-hexenal were transient. The majority (99.5%) of the genes that responded to E-2-hexenal did so within the first 3 h of exposure, whereas an almost complete reset to original levels could be observed after 24 h exposure, when only 16 genes (0.5%) had a significant change in transcript abundance (FDR). Additionally, the transcriptional responses to E-2-hexenal displayed a dynamic temporal pattern with only partial overlap of the differentially regulated genes at the tested time points. The 1-h and 3-h responses had 948 genes (27%) regulated in common, whereas only 2 and 7 genes were common to the 1-h and 24-h response and the 3-h and 24-h response, respectively. Finally, only two genes were co-regulated at all time points (Figure S1a).

Given the limited transcriptional responses to 24 h E-2-hexenal exposure, only the 1 h and 3 h data sets were taken into account for further analysis. To gain insight into the biological processes affected by E-2-hexenal treatment, we used the Gene Ontology (GO) tool at FatiGO (http://babelomics.bioinfo.cipf.es) and identified functional categories overrepresented in the E-2-hexenal-responsive genes compared with the Arabidopsis genome. In the early-point induced genes (1 h) the most represented GO terms in the functional enrichment were related to defence and immune responses, among which
responses to pathogen (bacteria and fungi) dominate (Figure S1b). GO categories associated to programmed cell death, response to ethylene and wounding were also significantly enriched. Similar GO categories were also enriched, although at lower extent, in the mid-point induced genes (1-3 h). Additionally, the latter genes also contained many transcripts responsive to chemical and abiotic stimuli e.g. hormone, abscisic acid (ABA), temperature, heat, light, oxidative, osmotic and salt stress; Figure S1b. Late induced genes (3 h) showed a very different picture being significantly enriched mainly in transcripts associated with protein transport, localization and catabolism. Downregulated genes were significantly enriched in GO categories associated to hormone responses (1 h and 1-3 h), predominantly gibberellin and auxin, and metabolic processes of carboxylic acids, carbohydrates and lipids (1-3 h and 3 h; Figure S1c). In contrast, no defence-related categories were enriched in the downregulated genes.

**Specificity of E-2-hexenal transcriptional responses**

Previous studies identified a few genes, mainly stress- and defence-related (e.g. phenylpropanoid-related genes and lipoxygenase pathway genes), that are induced in response to E-2-hexenal treatment in Arabidopsis (Bate and Rothstein, 1998; Kishimoto et al., 2005). However, induction of these genes was not specific to E-2-hexenal but occurred also in response to other defence hormones (e.g. methyl jasmonate, MeJA). Moreover, comparative studies showed that effects of E-2-hexenal at the transcriptional level were moderate relative to other phytohormones, suggesting that a putative role of E-2-hexenal might be to activate a low level of general defence responses (Bate and Rothstein, 1998). To investigate this hypothesis, we sought to determine to which extent E-2-hexenal transcriptional responses were specific or how they overlapped with transcriptional responses induced by other defence-related hormones. To this end, we analyzed the regulation of the E-2-hexenal upregulated transcripts by other defence hormones. According to the GO analysis, E-2-hexenal-induced genes are especially enriched in functional categories related to defence responses to pathogens (Figure S1b) that are generally regulated by salicylic acid (SA), JA, ABA or ethylene (ET). Therefore, we analyzed the regulation of the E-2-hexenal-induced transcriptome by these phytohormones, making use of publicly available transcript profiles (http://www.ebi.ac.uk/arrayexpress/). We selected, where possible, data sets carried out under experimental conditions similar to the ones used in this study (e.g. leaf tissue, Col-0 ecotype, similar developmental stage, exposure time and microarray platform used (Alonso et al., 2003; Goda et al., 2008; Kuhn et al., 2008; van Leeuwen et al., 2007). This analysis suggests that of the four hormonal treatment experiments analyzed, SA is the signal most closely related to the pattern of gene activation by E-2-hexenal, as about half of the genes upregulated by E-2-hexenal, were also upregulated by this hormone, followed by ABA and JA, with 29 and 13% regulated genes in common, respectively (Table S1). In contrast, ET treatment showed almost no overlap with E-2-hexenal treatment, with only 1% of the genes co-regulated (Table S1). More interestingly, approximately one-third of the genes upregulated by E-2-hexenal seemed to be not affected by the other phytohormones, putatively representing specific targets of E-2-hexenal signalling (Table S1 and Data S3). To confirm the results of this *in silico* analysis, the expression of 12 putatively E-2-hexenal-specific target genes was studied by Q-RT-PCR in response to E-2-hexenal, SA, MeJA, ABA and the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC).

In order to analyze primary targets of E-2-hexenal signalling, representative E-2-hexenal-specific targets were chosen from the 1 h E-2-hexenal-specific responsive genes, with functions related to either defence response (e.g. NDR1-like3, PEN2, TIR-NBS-LRR disease-resistance proteins, ATL2; Table 1) or signal transduction (e.g. ERF98, ERF104, a jumonji (JMJ) transcription factor, AtRAB2b, ATL2, ATL3, AtCMGP2/PUB27; Table 1). Plants were treated with E-2-hexenal, SA, MeJA, ABA, or ACC, or mock treated for 1 h and 3 h. Expression of SUMO3, JAZ10, RD29B and ERF1, genes induced rapidly upon SA, MeJA, ABA and ET treatment, respectively (Yamaguchi-Shinozaki and Shinozaki, 1994; Solano et al., 1998; Yan et al., 2007; van den Burg et al., 2010), was used to confirm activation of downstream responses following the four hormonal treatments. As shown in Figure S2, expression of SUMO3, RD29B and ERF1 was induced both after 1 h and 3 h exposure with the corresponding phytohormone, whereas JAZ10 expression was transiently induced in agreement with previous studies (Yan et al., 2007). Expression of all 12 putative E-2-hexenal-specific targets was strongly induced after 1 h exposure to this C6-aldehyde, confirming the microarray data (Figure 1 and Table 1). Moreover, induction of ERF104, the TIR-NBS-LRR disease-resistance protein At1 g63750, NDR1-like3, ATL2, ATL31 and the ankyrin-family protein was only detected after 1 h exposure to E-2-hexenal, whereas the seven remaining target genes were expressed, although at lower levels, also after 3 h E-2-hexenal treatment (Figure 1). More interestingly, five of the 12 tested genes, *i.e.* ERF98, AtRAB2b, the JMJ transcription factor, AtCMGP2/PUB27 and the TIR-NBS-LRR disease-resistance protein At5g22690, were indeed specifically induced by E-2-hexenal as none of the tested phytohormones altered their expression at any of the tested time points (Figure 1). Expression of the remaining genes was affected also by other hormonal treatments, often being upregulated by SA (e.g. ERF104, PEN2, NDR1-like3, ATL2, ATL31, the ankyrin protein), confirming that E-2-hexenal transcriptional responses are especially related to those induced by SA. However, induction by other phyto-
hormones always occurred at much lower levels compared to E-2-hexenal and generally at a later time point (3 h). We also tested whether these 12 genes were induced by other GLVs after 1 h. Clearly JMJ-TF, AtCMGP2/PUB27, ERF104, PEN2, NDR1-like, At1g63750, and AT4g11100 were specifically induced by E-2-hexenal (Figure 2). Induction of the other five genes by one or two other GLVs was statistically significant but always occurred at very much lower levels compared to E-2-hexenal. All together, these data indicate that E-2-hexenal activates unique defence and signalling targets not shared by the other GLVs or defence hormones tested here.

Identification of cis-regulatory elements and trans-acting factors

In order to identify transcription factors regulating early responses to E-2-hexenal, the promoters of the early upregulated genes were analyzed for the presence of putative cis-regulatory elements by searching for statistically overrepresented sequences compared to all Arabidopsis promoters. Using motif analysis (The Arabidopsis Information Resource; TAIR), Regulatory Sequence Analysis Tool (RSAT; http://rsat.ulb.ac.be; van Helden et al., 2003), ATHENA (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl; O’Connor et al., 2005) and AlignACE (http://atlas.med.harvard.edu; Roth et al., 1998), the W-box motif (TTGACY) was the only known cis-element statistically enriched in this set of promoters (Figure S3a). This W-box motif represents putative binding sites for WRKY transcription factors, a plant-specific family, with 74 members in Arabidopsis, regulating several physiological processes including plant-defence responses (Eulgem and Somssich, 2007; Pandey and Somssich, 2008; Rushton et al., 2010). The same cis-regulatory motif was also statistically enriched in the promoters of the mid-induced genes (1-3 h), but it was not overrepresented in the late induced genes (3 h; Figure S3a) or in the repressed genes, at any time point. Analysis of occurrence of the W-box motif with POBO (http://ekhidna.biocenter.helsinki.fi/pobo; Kankainen and Holm, 2004) further confirmed enrichment of the TTGACY motif in the promoters of the mid-induced genes (1h–3h), but it was not overrepresented in the late induced genes (3 h; Figure S3a) or in the repressed genes, at any time point. Analysis of occurrence of the W-box motif with POBO (http://ekhidna.biocenter.helsinki.fi/pobo; Kankainen and Holm, 2004) further confirmed enrichment of the TTGACY motif in the promoters of the early E-2-hexenal-induced genes compared to a set of random Arabidopsis promoters (Figure S3b). The enrichment of the W-box motif in the early and mid E-2-hexenal-induced promoters, suggests that WRKY transcription factors might play a role in the regulation of E-2-hexenal responses. In agreement with this, 13 WRKY transcription factors were upregulated by E-2-hexenal, with different temporal patterns (Table 2). Among these, we focused on the three that, according to the microarray data, showed the

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Description</th>
<th>1 h</th>
<th>3 h</th>
<th>W-box</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g07410</td>
<td>Rab GTPase (atrab2b)</td>
<td>5.10</td>
<td>0.00</td>
<td>2.33</td>
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<tr>
<td>At1g63750</td>
<td>TIR-NBS-LRR disease-resistance protein</td>
<td>3.73</td>
<td>0.00</td>
<td>0.77</td>
</tr>
<tr>
<td>At2g44490</td>
<td>β-Glucosidase (PEN2)</td>
<td>3.29</td>
<td>0.00</td>
<td>1.13</td>
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<tr>
<td>At3g16720</td>
<td>Zinc finger, RING-H2 ubiquitin ligase, putative (ATL2)</td>
<td>10.13</td>
<td>0.00</td>
<td>1.09</td>
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<tr>
<td>At3g23230</td>
<td>Ethylene-response factor (ERF98)</td>
<td>11.23</td>
<td>0.00</td>
<td>1.63</td>
</tr>
<tr>
<td>At4g11100</td>
<td>Ankyrin repeat family protein</td>
<td>7.62</td>
<td>0.00</td>
<td>1.16</td>
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<tr>
<td>At5g06320</td>
<td>Non-race specific disease-resistance gene (NDR1-like3)</td>
<td>4.44</td>
<td>0.00</td>
<td>1.66</td>
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<tr>
<td>At5g22690</td>
<td>TIR-NBS-LRR disease-resistance protein</td>
<td>4.41</td>
<td>0.00</td>
<td>1.26</td>
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<td>At5g27420</td>
<td>Zinc finger, RING-H2 ubiquitin ligase, putative (ATL31)</td>
<td>25.11</td>
<td>0.00</td>
<td>1.73</td>
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<td>At5g46910</td>
<td>Jumonji transcription factor (JMJ-TF)</td>
<td>3.94</td>
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<td>At5g61600</td>
<td>Ethylene-response factor (ERF104)</td>
<td>11.31</td>
<td>0.00</td>
<td>1.50</td>
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<td>At5g64660</td>
<td>Cys, met, pro, and gly protein 2 (atgad2)</td>
<td>6.82</td>
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<td>At4g23190</td>
<td>Cystein-rich receptor-like protein kinase (CRK11)</td>
<td>5.10</td>
<td>0.00</td>
<td>2.33</td>
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<tr>
<td>At4g02380</td>
<td>Senescence-associated gene 21 (SAG21)</td>
<td>27.28</td>
<td>0.00</td>
<td>32.90</td>
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<tr>
<td>At4g23190</td>
<td>Cystein-rich receptor-like protein kinase (CRK11)</td>
<td>29.24</td>
<td>0.00</td>
<td>5.39</td>
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<tr>
<td>At4g37370</td>
<td>Cytochrome P450 family protein (CYP81D8)</td>
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<td>0.00</td>
<td>66.72</td>
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<tr>
<td>GABA metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At3g22200</td>
<td>γ-Aminobutyric acid transaminase (GABA-TP)</td>
<td>1.44</td>
<td>0.06</td>
<td>2.23</td>
</tr>
<tr>
<td>At1g65960</td>
<td>Glutamate decarboxylase 2 (atgad2)</td>
<td>0.80</td>
<td>0.26</td>
<td>0.72</td>
</tr>
<tr>
<td>At2g02010</td>
<td>Glutamate decarboxylase 4 (atgad4)</td>
<td>9.19</td>
<td>0.05</td>
<td>7.48</td>
</tr>
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</table>
strongest induction: AtWRKY40, AtWRKY53 and AtWRKY6, with about 185-fold, 23-fold and 22-fold increase in expression upon 1 h E-2-hexenal exposure (Table 2). Q-RT-PCR analysis confirmed the rapid, strong and transient induction of these three WRKY transcription factors by E-2-hexenal (Figure 3). In detail, expression of AtWRKY53 was only induced after 1 h exposure and returned to control levels at the later time point, whereas induction of AtWRKY40 and AtWRKY6 could be detected both at 1 h and 3 h, although at a lower level at 3 h (Figure 3).

**AtWRKY40 and AtWRKY6 mediate inhibition of root elongation by E-2-hexenal**

The putative role of AtWRKY40, AtWRKY53 and AtWRKY6 transcription factors in mediating E-2-hexenal signalling was analyzed by various E-2-hexenal responses in the

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knock-out wrky40 and wrky53 mutants (Wang et al., 2006; Murray et al., 2007; Shen et al., 2007) and in the wrky6-2 mutant, producing a truncated version of the WRKY6 protein, lacking the DNA-binding domain (Robatzek and Somssich, 2002).

We have previously shown that E-2-hexenal exposure inhibits root elongation in Arabidopsis seedlings (Mirabella et al., 2008). This response is independent of SA, JA, ABA and ET and, among GLVs, specific for E-2-hexenal. To determine the role of these WRKYs in this process, 3-day-old wild-type Col-0, wrky40, wrky53 and wrky6-2 seedlings were exposed for 24 h to aerial E-2-hexenal, in concentrations ranging from 0.1–0.3 μM, or to MeOH, for the mock treatment, and root growth was measured 3 days after the exposure. Moreover, since redundancy in activity among WRKY transcription factors has been often reported (Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Liu et al., 2012), also wrky40 wrky53, wrky40 wrky6 and wrky53

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wrky6 double mutants were generated and analyzed. Growth of wild-type roots was inhibited by E-2-hexenal in a dose-dependent manner whereas roots of the her1-1 mutant, known to be resistant to E-2-hexenal inhibition (Mirabella et al., 2008), showed sustained growth also at the highest E-2-hexenal concentration, as shown previously (Figure 4; Mirabella et al., 2008). Similarly to wild-type seedlings, E-2-hexenal inhibited root elongation in all mutants, with the exception of the wrky40 wrky6 double mutant, whose root elongation was not inhibited by E-2-hexenal (Figures 4 and S4). These data clearly indicate that AtWRKY40 and AtWRKY6 are required for the E-2-hexenal-induced inhibition of root elongation and have a redundant function in mediating this E-2-hexenal response.

AtWRKY40, AtWRKY6 and AtWRKY53 regulate induction of gene expression by E-2-hexenal

We next determined whether these WRKYs also mediated transcriptional responses downstream of E-2-hexenal, by analyzing the induction of E-2-hexenal-specific target genes, in the double wrky mutants. We focused on the JMJ transcription factor, the two TIR-NBS-LRR resistance proteins, ERF98, PEN2, NDR1-like3, AtCMPG2/PUB27, ATL22 and the ankyrin protein, all containing W-boxes in their promoters (Table 1). ERF104, containing no W-boxes in its promoter, was also included in this analysis. Transcripts of the JMJ transcription factor and NDR1-like3 accumulated at similar levels in all plant genotypes upon E-2-hexenal treatment (Figure 5a). In contrast, transcripts of ERF98, the TIR-NBS-LRR disease-resistance protein At5g22690, AtCMPG2/PUB2, ATL2 and PEN2 accumulated to higher levels in the wrky40 wrky6 compared to wild-type leaves (Figure 5b). This higher induction was especially pronounced for ERF98, whose induction was four-fold higher in wrky40 wrky6 than wild-type leaves (Figure 5b). In addition, expression levels of the TIR-NBS-LRR disease-resistance protein At1 g63750 and the ankyrin protein were also higher in the wrky40 wrky6 mutant (Figure 5c). However, this occurred also in the mock (MeOH) treatment, indicating that WRKY40 and WRKY6 are also important for basal expression levels of these two genes (Figure 5c). Furthermore, induction of the TIR-NBS-LRR resistance protein At5g22690 was higher in the wrky40 wrky53 mutant

Table 2 WRKY transcription factors upregulated by E-2-hexenal

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Description</th>
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<th>3 h</th>
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<td>At4g23810</td>
<td>AtWRKY53</td>
<td>22.47</td>
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<td>At4g31550</td>
<td>AtWRKY11</td>
<td>12.55</td>
<td>0.22</td>
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<td>At5g24110</td>
<td>AtWRKY30</td>
<td>6.68</td>
<td>1.45</td>
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<td>At2g44040</td>
<td>AtWRKY46</td>
<td>5.03</td>
<td>1.43</td>
</tr>
<tr>
<td>At4g01250</td>
<td>AtWRKY22</td>
<td>3.84</td>
<td>1.59</td>
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<td>AtWRKY70</td>
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<td>1.02</td>
</tr>
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<td>At1g80840</td>
<td>AtWRKY40</td>
<td>184.82</td>
<td>16.11</td>
</tr>
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<td>At1g62300</td>
<td>AtWRKY6</td>
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<td>4.05</td>
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<td>At5g49520</td>
<td>AtWRKY48</td>
<td>14.22</td>
<td>2.87</td>
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<td>At2g23320</td>
<td>AtWRKY15</td>
<td>9.71</td>
<td>4.26</td>
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<td>At2g30250</td>
<td>AtWRKY25</td>
<td>3.70</td>
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<td>AtWRKY29</td>
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<td>At3g01970</td>
<td>AtWRKY45</td>
<td>1.15</td>
<td>2.16</td>
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Fl, fold induction; P, P-value.
P-values of less than 0.001 are given as 0.00. The WRKY transcription factors analyzed in this study are in bold.
compared to wild-type plants. Further analysis of the wrky6 and wrky40 single mutants showed that transcript levels of At5g22690 were higher in the wrky40 mutant too (Figure S5). No differences in the expression levels of any of the tested genes was detected in the wrky53 wrky6 mutant (Figure 5b), although AtCMPG2/PUB27 was stronger induced in the single wrky6 mutant (Figure S5). Finally, as expected, ERF104, with no W-boxes in its promoter, responded to E-2-hexenal in a similar manner in all tested genotypes (Figure 5a).

W-boxes were also enriched in the promoters of the mid-point (1 h and 3 h) genes induced by E-2-hexenal (Figure S3a). Three genes were selected as representative of the mid-point E-2-hexenal-induced transcripts, all containing W-boxes in their promoters (Table 1) and known to respond to several abiotic and biotic stresses (Weaver et al., 1998; Taki et al., 2005; Kempema et al., 2007; Little et al., 2007; Tran et al., 2007; Wrzaczek et al., 2010). SAG21, CYP81D8 and CRK11 expression increased from 2-fold to 7-fold in leaves of the wrky40 wrky6 compared to wild-type leaves, both after 1 h and 3 h E-2-hexenal exposure (Figure 6). Induction of these three genes was similarly higher in the wrky40 wrky53 mutant, although only upon 3 h exposure to E-2-hexenal (Figure 6). In contrast, no difference was detected in the expression levels in wrky53 wrky6 leaves compared to wild-type leaves.
et al., 2009; Clark et al., 2009) were induced by E-2-hexenal (Table 1), while AtGAD2 was inhibited. To confirm these data, transcript levels of AtGAD2, AtGAD4 and GABA-TP in Col leaves in response to E-2-hexenal exposure was determined. Expression of AtGAD4 was strongly induced by E-2-hexenal, up to 10- and 60-fold at 1 h and 3 h, respectively (Figure 7a), confirming the microarray data (Table 1). In contrast, transcript levels of AtGAD2 were downregulated about two-fold by 3 h E-2-hexenal exposure (Figure 7b), whereas no significant effect was detected on GABA-TP expression (Figure 7c). Although, both AtGAD4 and AtGAD2 contain a W-box in their promoters (Table 1), only AtGAD4 expression was altered in the wrky40 wrky6 mutant, displaying 12- and 9-fold increases in transcript accumulation at 1 h and 3 h, respectively, compared to wild-type leaves. Similarly, expression of GABA-TP, containing no W-box in its promoter, was unaffected in the wrky double mutants (Figure 7a and b). GAD4 expression was stronger induced in the wrky40 mutant, but not in the wrky6 mutant, indicating a prominent role for WRKY40 in this process (Figure S5).

To determine whether the higher AtGAD4 induction in the wrky40 wrky6 mutant correlated with higher GABA accumulation, steady state GABA levels were measured in Col and wrky40 wrky6 leaves after 1, 3, 6 and 24 h exposure to E-2-hexenal. In agreement with our previous work (Mirabella et al., 2008), GABA levels strongly increased upon E-2-hexenal treatment, however, no difference could be detected between wild-type and mutant leaves (Figure 7d). Still when we exposed gad4 mutant seedlings to E-2-hexenal, this mutant failed to accumulate GABA (Figure S5), indicating a prominent role for GAD4 in this process.

DISCUSSION

In this study we describe that two WRKY transcription factors, WRKYs 6 and 40, act downstream of E-2-hexenal perception by Arabidopsis. We demonstrate that the double wrky6 wrky40 mutant shows a particular response to the C6-aldehyde similar as the hexenal resistance mutant 1, her1. These two WRKY genes are rapidly induced by E-2-hexenal and are involved in repressing the expression of several genes induced by this green leaf volatile. We also established a link between WRKY40 and the transcriptional regulation of a member of the GABA shunt which was previously shown to act downstream of E-2-hexenal. Based on all these observation we propose that these two WRKYs act downstream of E-2-hexenal. Moreover, we have isolated several E-2-hexenal-specific target genes that can be used as markers of E-2-hexenal signalling.

Transcriptomics and E-2-hexenal markers

Although next generation sequencing has recently taken a flight for the analyses of transcriptomes, is has been proven that the robust Affymetrix microarray platform can be successfully used for gene discovery. We thus set out to use this system to discover genes acting downstream of the green leaf volatile E-2-hexenal. Based on transcriptomics...
levels of genes that have been demonstrated to be induced by E-2-hexenal, we tested different concentrations of this volatile and the lowest concentration at which these genes were induced after 24 h was 3 \( \mu \text{M} \). Retrospectively, since the transcriptome of the Arabidopsis plants is almost completely reset after a 24 h treatment (Figure S1), we could have used most likely lower concentrations. However, our paper shows that this relatively high concentration has served its purpose well for gene discovery and that eventually lower concentrations (0.1–0.3 \( \mu \text{M} \)) were used for a biological read-out (Figure 4). Approximately one-third of the genes regulated by E-2-hexenal was not affected by the phytohormones SA, JA, ABA and ET (for 1–3 h) (Data S3).

Subsequently we tested 12 of those by Q-RT-PCR and five of them were only induced by E-2-hexenal and not by the phytohormones (Figure 1). These were ERF98, previously shown to be involved in regulating ascorbic acid biosynthesis (Wang et al., 2013); AtRAB2, a putative GTPase (Vernoud et al., 2003); a JMJ transcription factor, involved in the response to chitin (Libault et al., 2007); AtCMPG/PUB27, a putative ubiquitin ligase (Cho et al., 2008) and a TIR-NBS-LRR disease-resistance protein (Tan et al., 2007).

Two of them, JMJ transcription factor and AtCMPG/PUB27 were not induced by other GLVs (Figure 2) and can thus be used as markers for E-2-hexenal signalling at early time points.

**WRKYs acting downstream of E-2-hexenal**

The large number of genes regulated by E-2-hexenal allowed us to search for cis-regulatory elements that (subsets of) these genes have in common in their promoters. With the algorithms used, it appeared that only the W-box motif (TTGACY) was significantly over represented in the early- and mid-induced genes (Figure S3) and not in the repressed genes. This enrichment for one particular cis-regulatory element was also found for the response to phytoprostanes where the TGA motif and TGA transcription factors play a dominant role (Mueller et al., 2008). This W-box is a putative binding site for WRKY transcription factors of which, coherently, 13 were induced by E-2-hexenal (Table 2), all but one of them already after 1 h. Without regard for their putative function we focused on the three WRKYs that were strongest induced by E-2-hexenal: WRKY6, 40 and 53 (Figure 3). For Arabidopsis, WRKY53 has been reported to be involved in pathogen defence and leaf senescence (Hinderhofer and Zentgraf, 2001; Miao et al., 2004, 2008, 2013; Miao and Zentgraf, 2010; Zentgraf et al., 2010; Hu et al., 2012), WRKY6 also in pathogen
defence and leaf senescence (Robatzek and Somssich, 2002), but more recently in regulating phosphate uptake (Chen et al., 2009) and priming (Jaskiewicz et al., 2011), and WRKY40 in several processes in combination with other WRKYs (Xu et al., 2006; Chen et al., 2010; Jiang et al., 2011; Liu et al., 2012; Schon et al., 2013; Van Aken et al., 2013). Additional functions for WRKY6 are still being reported e.g. in arsenate uptake (Castrillo et al., 2013), suggesting that more functions of the various WRKYs in planta are still to be discovered. AtWRKY18, which has recently been shown to be involved in the response to bacterial, yet to be identified, volatiles (Wenke et al., 2012), was not induced by E-2-hexenal.

Next we set out to address two questions regarding the role of these three WRKYs, either alone or in a combined fashion. Question 1: Which of them is involved in the response of seedlings to E-2-hexenal, i.e. inhibition of root elongation as we previously demonstrated (Mirabella et al., 2008)? The biological significance of this trait is not yet known, but it has been reported that induction of defences in aerial parts leads to reduction in root growth (Hummel et al., 2009), or to bunkering of recently fixed carbon in roots (Schwachtje et al., 2006). A similar bioassay was used for growth promoting volatiles (Zhang et al., 2007). Question 2: Which of them is involved in regulating induction of gene expression by E-2-hexenal?

The roots of the single wrky6, 40 or 53 mutants responded similarly as the wt seedlings (Figure 4), but of all combinations of the double mutants, the wrky40 wrky6 had a similar phenotype as the her1 mutant (Figure 4, Figure S4). Thus WRKY40 and 6 seem to act redundantly in mediating this E-2-hexenal response, with no apparent function for WRKY53 in this process.

Since the single wrky mutants had a similar phenotype as the wt plant in our bioassay, we first tested the double mutants for their role in regulating induction of gene expression by E-2-hexenal. From these analyses, using genes with W-boxes in their promoters, it became clear that in the double wrky40 wrky6 mutant several genes were much higher expressed than in wt plants (Figures 5 and 6). However, several of these genes were also induced in the single wrky6 or wrky40 mutant (Figure S5) indicating that these WRKYs do not act redundantly in regulating the expression of the genes tested. Clearly, these WRKYs act as repressors in this particular situation. This function as repressor is not uncommon for WRKYs (Rushton et al., 2010) and has been shown for WRKY6 (Castrillo et al., 2013) and WRKY40 (Shang et al., 2010; Liu et al., 2012; Van Aken et al., 2013; Yan et al., 2013). The expression of several other genes was not affected in the wrky40 wrky6 mutant as well as that of ERF104, which has no W-boxes in its promoter. This included the very specific E-2-hexenal marker JMJ-TF suggesting that other WRKYs might be involved in regulating this gene.

The role of GABA

Since GABA plays a role in modulating the responsiveness of Arabidopsis seedlings to E-2-hexenal (Mirabella et al., 2008), we especially paid attention to the GABA shunt. We thus discovered that GAD4 is induced by E-2-hexenal, both from our microarray (Table 1) and Q-RT-PCR (Figure 7) analyses. Since GABA levels increase upon E-2-hexenal treatment (Figure 7), it is not unlikely that GAD4 plays an important role in this since the gad4 mutant fails to accumulate GABA upon E-2-hexenal treatment (Figure S5). Interestingly, GAD4 transcript levels are higher not only in the wrky40 wrky6 double mutant but also in the wrky40 mutant indicating that WRKY40 acts upstream of GAD4. Still the wrky40 mutant does not show the root phenotype that the wrky40 wrky6 mutant shows indicating that there are more processes downstream causing this phenotype than just GABA. GABA levels in the leaves of the wrky40 wrky6 double mutant are similar as in wt leaves after E-2-hexenal treatment but it might be that the flux through the GABA shunt is much higher than in wt with steady state GABA levels being similar. This higher flux through the GABA shunt can be beneficial for carbon metabolism (Michaeli et al., 2011) and thus perhaps, similar to higher GABA levels in the her1 mutant (Mirabella et al., 2008), lead to root elongation in the presence of E-2-hexenal, which does not occur in wt seedlings.

In conclusion, our data position WRKY40 upstream of GAD4 and thus the GABA shunt. This WRKY acts as a repressor for GAD4 that has a W-box in its promoter. We have now extended the E-2-hexenal signalling cascade not only with several transcription factors but also with several E-2-hexenal-specific markers downstream of these WRKYs. Further studies are needed to reveal what role these WRKYs play in the various functions that have been assigned to E-2-hexenal.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and chemical treatment
All experiments were performed with A. thaliana plants, ecotype Columbia (Col-0). The following Arabidopsis lines have been described elsewhere: her1-1 (Mirabella et al., 2008), wrky40 (Shen et al., 2007), wrky53 (Murray et al., 2007) and wrky6-2 (Robatzek and Somssich, 2002). Seeds of the wrky53 mutant were obtained from the Nottingham Arabidopsis Stock Centre. Seeds of the wrky40 and wrky6-2 mutants were kindly provided by Dr. I.E. Somssich (Max Planck Institute for Plant Breeding Research, Cologne, Germany). Double homozygous wrky mutants were generated from genetic crosses of single mutants and identified through PCR genotyping, by using primer pairs flanking the T-DNA insertion site for the wrky53 mutant (WRKY53-RRP: 5′-GGGAA GGTCGCTCAATCTCG-3′; WRKY53-RRP: 5′-TCAGGCA CAGCTGCAAGACGC-3′). The Spm transposon tagging site for the wrky40 mutant (WRKY40-RRP: 5′-AGCTTTGAGGAAGGACACAC-3′; WRKY40-RRP: 5′-GGAGGACAAAGCCACATTGT-3′) and the En-1 transposon footprint for the wrky6-2 mutant (WRKY6-RRP:
GACCAGTGTTTTC-3

GLVs, plants were exposed to 3 μM only methanol was applied. For treatment with different GLVs, these three genes following 24 h treatment with a range of 2 μM concentrations and identified 3 μM E-2-hexenal, Z-3-hexenal, E-2-hexenyl acetate or Z-3-hexenyl acetate as described above. The concentration of the different GLVs in the gaseous phase in the desiccators was determined under the exact same conditions as described above, thus including pots with soil but without plants, or plates but without seedlings. The air in the desiccators was collected after 3 h on Porapak Q80/100 mesh (Supelco; sigmaaldrich.com, St. Louis, MO, USA) as described in (Ament et al., 2010). The Porapak was subsequently eluted with 1 mL dichloromethane and the amount of GLVs determined on a GC-Q-TOF-MS as described in (Falara et al., 2014) except that the GC was set to 40°C for 3 min after which the temperature was raised by 15°C min⁻¹ to 250°C. The determined amount of GLV after 3 h exposure in desiccators was compared to the amount of GLV that was normally applied in the desiccators but that was now directly applied to Porapak. The calculated recovery for each GLV is shown in Figure S6. For treatment with phytohormones, plants were sprayed with 300 μM SA, 100 μM MeJA, 50 μM ABA and 100 μM ACC, all in 0.02% Silwett L77. For the control treatment only 0.02% Silwett L77 was applied. Root growth assays were performed as previously described (Mirabella et al., 2008). With a concentration of 0.3 μM E-2-hexenal applied, the calculated recovery was 7.3% (Figure S6), which translates in a concentration of 22.5 nM E-2-hexenal in the gaseous phase.

Microarray experiments and data analysis

A preliminary experiment was conducted to identify the concentration of exogenous E-2-hexenal to use for the microarray experiments. Previous studies showed that transcripts of lipoxigenase (LOX), allene oxide synthase (AOS) and phenylalanine ammonia-lyase (PAL) accumulate upon exposure to 10 μM E-2-hexenal for 24 h (Bate and Rothstein, 1998). We analyzed the expression of these three genes following 24 h treatment with a range of E-2-hexenal concentrations and identified 3 μM as the minimum E-2-hexenal concentration at which a slight but clear induction of LOX, AOS and PAL could be detected. Therefore this E-2-hexenal concentration was chosen to conduct all further experiments. However, it must be noted that, in our microarray data, none of these three genes showed a statistically relevant change in gene expression.

Three independent biological replicates were set up for each treatment (3 μM aerial E-2-hexenal and MeOH) and time point (1, 3 and 24 h). In order to minimize plant-to-plant variation, each biological replicate consisted of the pooled material of six individual plants. RNA was extracted using the TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) extraction procedure followed by purification on RNeasy columns (Qiagen, Germantown, MD, USA). Labelled cRNA was prepared and hybridized to whole-genome Affymetrix ATH1 GeneChip, according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA, USA). All analyses were performed with R (version 2.11.1) and Bioconductor (Gentleman et al., 2004). We performed a set of quality control checks, i.e. visual inspection of the scans, RNA degradation analysis, examination of the consistency among the replicated samples by principal component analysis, checking the arrays for surface anomalies and inspection of MA plots using a pseudo-median reference and examination of the Affymetrix provided QC values. All 18 arrays passed these tests. The probe level data of the arrays was summarized using the robust multi-array average (RMA) algorithm (Irizarry et al., 2003).

Significant differences in expression were determined using analysis of variance (ANOVA) with the MAanova package from Bioconductor (Gentleman et al., 2004). In a mixed effect model, time and treatment were considered fixed effects and batch was considered a random effect. A permutation-based Empirical Bayes test (2000 random permutations) was used for hypothesis testing. To account for multiple testing, P-values were adjusted to represent a FDR of 5% (Benjamini and Hochberg, 1995). Contrast analyses were performed to detect differential gene expression at each time point between mock treated and E-2-hexenal treated plants. Genes were considered to be differentially expressed if the FDR corrected P-values were <0.05 and the fold changes >2.

Transcriptome comparison using publicly available transcript profiles

From the data sets of (Goda et al., 2008; Kuhn et al., 2008), and (van Leeuwen et al., 2007) the appropriate Affymetrix Cel files (Col, 0.5, 1 or 3 h after hormone application) were taken. Probe level data of the arrays was summarized using the RMA algorithm (Irizarry et al., 2003). All data were fitted using a linear model with the hormone treatment as a fixed effect. For all three data sets model coefficients were used to determine fold changes between mock and hormone treatment. For the ethylene data set (Alonso et al., 2003) we used the 244 upregulated probe sets as provided by the paper.

Next we qualitatively compared the upregulated genes in these studies with the upregulated genes from our E-2-hexenal treatment microarray experiment. Genes upregulated by E-2-hexenal were chosen by an FDR corrected P-value <0.05 and by a fold change >2 whereas a lower limit of fold change (>1.5) was chosen to define the upregulated genes in the other studies. Different fold changes were used in order to balance for effects due to different experimental designs: the experiments taken from the public domain are smaller than our E-2-hexenal experiment. For each experiment the number of co-regulated genes was calculated and the E-2-hexenal-specific upregulated genes were determined as those upregulated by E-2-hexenal that did not occur as upregulated in any of the other studies used here (Data S3).

Q-RT-PCR and GABA quantification

Q-RT-PCR experiments and GABA extraction and quantification were performed as described elsewhere (Park et al., 2010). Briefly, all rosette leaves of 19- to 21-day old plants, grown and treated as described above, were collected and directly frozen in liquid nitrogen prior to analysis. Experiments were performed in three independent biological replicates and each replicate consisted of the pooled material of three individual plants. For each newly designed primer pair (Table S2), the PCR product was analyzed on 2% agarose gels to confirm amplification of a single product and sequenced to confirm amplification of specific target genes.

Microarray data deposition

The data presented in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE53957:http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=mnjqfmgsklpmzfatl&acc=GSE53957.
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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Data S1. Differentially expressed genes up-regulated by E-2-hexenal treatment on at least one time point in Arabidopsis.

Data S2. Differentially expressed genes down-regulated by E-2-hexenal treatment on at least one time point in Arabidopsis.

Data S3. Genes up-regulated by E-2-hexenal and not by SA, JA, ABA and ET in Arabidopsis.

Figure S1. Transcriptome profiling of Arabidopsis after treatment with E-2-hexenal.

Figure S2. Q-RT-PCR of Arabidopsis plants treated with SA, MeJA, ABA or ACC for control marker genes.

Figure S3. Enrichment of the WRKY-box motif in the promoters of the early-point (1 h) time and mid-point time (1 h and 3 h) genes up-regulated by E-2-hexenal.

Figure S4. Root growth of wild-type, wrky53, wrky40 wrky53 and wrky6 mutant seedlings after aerial treatment with E-2-hexenal.

Figure S5. WRKY6 and WRKY40 are non-redundant regulators of gene expression and GABA shunt induction by E-2-hexenal in Arabidopsis.

Figure S6. Percentage recovery of GLVs in the gaseous phase in the desicators.

Table S1. Regulation of E-2-hexenal-induced genes by SA, JA, ABA and ET.

Table S2. Sequences of the primer pairs used for the Q-RT-PCR experiments.

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