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The roots of plant defenses: Integrative multivariate analyses uncover dynamic behaviors of roots' gene and metabolic networks elicited by leaf herbivory

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Summary

High-throughput analyses have frequently been used to characterize herbivory-induced reconfigurations in plant primary and secondary metabolism in above and below-ground tissues but the conclusions drawn from these analyses are often limited by the univariate methods used to analyze the data. Here we use our previously described multivariate time series data analysis to evaluate leaf herbivory-elicited transcriptional and metabolic dynamics in the roots of *Nicotiana attenuata*. We observed large, but transient, systemic responses in the roots that contrasted with the pattern of co-linearity observed in the up- and down-regulation of genes and metabolites across the entire time series in treated and systemic leaves. Using this newly developed approach for the analysis of whole-plant molecular responses in a time course multivariate data-set, we simultaneously analyzed stress responses in leaves and roots in response to the elicitation of a leaf. We found that transient systemic responses in roots resolved into two principal trends characterized by: (a) an inversion of root-specific semidiurnal (12h) transcript oscillations and (b)

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Supporting information

Figure S1. Principal component analysis of constitutive and OS-elicited modulations of leaf and root transcriptomes in *Nicotiana attenuata*.

Figure S2. Overlap in transcriptional and metabolic responses to OS-elicitation in treated leaves, and systemic leaves and roots.

Figure S3. Overview of SplineCluster clusters.

Figure S4. Temporal profiles of induced *m/z* signals in roots corresponding to free amino acids (tryptophan and phenylalanine), phenolic conjugates of putrescine, glucoside conjugates of 12-hydroxy-jasmonic acid and salicylic acid.

Figure S5. Temporal profiles of unknown *m/z* features showing an increasingly induced accumulation across the time course in roots in response to OS-elicitation.

Figure S6. Temporal profiles of oxo-phydienic acid (OPDA), the jasmonic acid metabolic precursor, and jasmonic acid, showing induction in roots in response to OS-elicitation.

Figure S7. Combined analysis of major transcriptomic responses at 5h and metabolomic responses at 9h in roots.

Figure S8. Temporal profiles of genes known to be involved in sugar transport.

Figure S9. Models used to evaluate oscillating root transcriptome.

Method S1. Metabolite annotation procedure and nomenclature.

Method S2. Metabolomic analysis of root tissues.

Data S1. Processed positive and negative root metabolomic data

transcriptional changes with major amplitude effects that translated into a distinct suite of root-specific secondary metabolites (e.g. alkaloids synthesized in the roots of *N. attenuata*). These findings underscore the importance of understanding tissue-specific stress responses in the correct day-night phase context and provide a holistic framework for the important role played by roots in aboveground stress responses.

Keywords

Systems biology; Transcriptomics; Metabolomics; Plant stress responses; Multivariate analysis; Time course experiments; *Nicotiana attenuata*

Introduction

Advances in high-throughput “-omics” technologies have enabled several efforts to characterize plant physiological responses by analyzing complex networks of interactions at different levels and scales. Most of these analyses aim at deciphering how environmental perturbations sensed by one part of the network impact the entire network, a dynamic process which is often captured by static snapshots framed in a time course experiment. To identify biochemical pathways underlying these complex networks, simplified representations are generated using top-down approaches based on the principle of “guilt-by-association” (Usadel *et al.* 2009). The observation that genes involved in a common biological process tend to be co-regulated has enabled the inference of functional associations among genes and different biological pathways in many studies of plant responses (Bassel *et al.* 2011, Persson *et al.* 2005, Usadel *et al.* 2012). This conceptual approach is also the basis of the widely known databases offering genome-wide representations of networks of co-function in Arabidopsis (AraNet; (Lee *et al.* 2010)) and Rice (RiceNet; (Lee *et al.* 2011)). Although these representations are useful, a major drawback of integrating multiple data-sets to predict co-functional relationships is the lack of context specificity in which functional information depend only on a subset of key interactions rather than on the entire data set (Gillis and Pavlidis 2012). To overcome this limitation, condition-dependent approaches which limit co-functional studies of genes or metabolites to a single tissue are applied to enhance the statistical confidence for inferring tissue-specific regulatory networks. Such approaches, however, are not appropriate for studies that seek to understand the networks at the scale of the entire organism.

Plants have evolved sophisticated mechanisms to withstand herbivore attack which can be classified as defense responses that limit the extent of damage and a suite of more poorly understood tolerance mechanisms that mitigate the negative fitness effects of herbivore attack (Erb *et al.* 2009, Schwachtje and Baldwin 2008). Dramatic reconfigurations of these metabolic pathways spread rapidly throughout a plant during herbivore attack which suggests the existence of complex inter-dependencies in the way that each plant tissue adjusts its intrinsic physiology as part of the whole-plant response to the attack. In the past, to unravel these defense-tolerance trade-off strategies, emphasis was placed only on the response that leaves deploy, but more recently roots have been recognized as playing an

integral role in a plant's aboveground defense mechanisms as well as serving as a dynamic storage organ following herbivory-induced resource (re)-allocation (Schwachtje *et al.* 2006).

Several studies have highlighted the importance of roots in synthesizing nitrogen-rich secondary metabolites involved in leaf defenses, such as nicotine in the roots of tobacco plants (Dawson and Solt 1959), tropane alkaloids in various *Solanaceae* species (Ziegler and Facchini 2008) and pyrrolizidine alkaloids in *Astraceae* species (Ober and Kaltenegger 2009). These systemically-activated changes in root metabolism can have a profound impact on leaf attackers (Steppuhn *et al.* 2004). The role of roots as a sink tissue that sequesters partitioned assimilates to facilitate re-growth after herbivore attack has also been uncovered as a key tolerance strategy to herbivory in many plants and involves specific herbivory signaling networks (Schwachtje *et al.* 2006). Compared to leaf-mediated aboveground defenses, the regulatory mechanisms by which roots contribute to these defenses have however remained underexplored and “-omic” approaches have recently been applied in a few cases (Kaplan *et al.* 2008, Marti *et al.* 2013).

Another important area of research for plant's responses to environmental stress involves the analysis of the physiological importance of oscillations in genes and metabolites controlled by the circadian clock. The diurnal regulation of primary metabolic pathways, such as those of starch and sugar metabolism in leaves and of nitrogen metabolism in roots, involves multiple clock components as demonstrated by previous studies using plants with disrupted clock functions (Espinoza *et al.* 2010, Gibon *et al.* 2006, Gutierrez *et al.* 2008, Lu *et al.* 2005). Interestingly, genes and metabolites involved in secondary metabolic pathways have also been shown to oscillate (Doherty and Kay 2010, Kim *et al.* 2011) but the implication of these rhythms in the roots for rapid defense induction is unknown. A recent systems biology based study on root transcriptional activity identified root-specific short-rhythms of gene expression that determine the periodicity in lateral root development (Moreno-Risueno *et al.* 2010). Bioinformatics analysis of the underlying spatial-temporal transcriptional patterns led to the identification of higher-order behavioral properties commonly termed as “emergent properties” that are specific to root developmental gene expression. This study clearly highlights the power of holistic approaches in identifying unknown key elements of the roots' physiology in a time course experiment.

In order to gain a comprehensive picture of the role of roots in defense-tolerance tradeoffs during shoot herbivory, we studied the dynamic behavior of genes and metabolites and their interactions in the roots of *Nicotiana attenuata* plants to which herbivore attack had been simulated to a leaf. Roots of this plant synthesize alkaloids for aboveground defense (Steppuhn *et al.* 2004) and control tolerance mechanisms essential for survival, such as the bunkering of C and N in roots (Schwachtje *et al.* 2006). To overcome problems associated with gene module detection using integrated data sets or the loss of information that typically accompanies the analysis of context-specific inferences, we investigated responses elicited by leaf herbivory in both treated leaves and roots in parallel using a coordinated multivariate time series analysis, as reported in (Gulati *et al.* 2013). From this work, we established the importance of deriving temporal information from multiple factors for the analysis of systemic responses in roots that are elicited by simulated herbivory to leaves. Distinct functional modules identified by this analysis are used to illustrate the changes in

root-specific entrained gene circadian rhythms elicited by simulated leaf-herbivory and the switching of amplitude effects between leaves and roots. These findings highlight the power of spatio-temporal maps in identifying “emergent network properties” in roots and in depicting the multiple roles of roots in aboveground defense responses of *N. attenuata*.

Results and discussion

A mosaic of co-linear up- and down-regulations in transcripts and metabolites spreads throughout the plant after OS-elicitation

To study tissue-based responses to leaf herbivory, we used a data set consisting of 134 published (Kim et al., 2011) microarray profiles of source/sink leaves and roots collected every 4 h from 3 biological replicates of treated and control *Nicotiana attenuata* plants. In treated plants, diluted oral secretion (W+OS) from larvae of the specialist herbivore *Manduca sexta* was applied into mechanically produced puncture wounds in leaves to mimic herbivory by this insect (Kim et al. 2011). This procedure used to simulate leaf herbivory is hereafter referred to as “OS-elicitation”. Principal component analysis (PCA) clearly separated control and treated leaves at 1 and 5h and roots of control and treated plants at 9 and 13h after OS-elicitation (Figure S1). We investigated OS-elicited transcriptomic and metabolic changes in elicited (treated leaves) and unelicited tissues (untreated systemic leaves and roots) of the same plant in a time course data-set which has been previously reported in (Gulati et al. 2013), using differentially expressed genes (DEG) analysis (FDR=0.05, $-1 \leq \text{fold change} \leq 1$) at each time point for both transcripts and metabolite-derived m/z signals. After plotting the results, we observed a strong coordination between the number of up and down-regulated transcripts and metabolites in leaf tissues (treated and untreated) across the entire time series (Figure 1). While the number of up and down-regulated transcripts decreased after 1h of OS elicitation in treated leaves, there was a clear increase in the number of both up and down-regulated transcripts in systemic (untreated) leaves which further decreased in both the leaf tissues 9h post elicitation, the harvest time-point which marks the beginning of the dark phase. The maximum number of up and down-regulated m/z features derived from the metabolite mass spectrometry (MS)-based root data-set published in Kim et al. (2011) peaked in the dark phase in treated and untreated leaves. However, the number of significantly regulated metabolic features was similar in both treated and systemic leaves, although larger differences were observed in the number of regulated genes in these two tissues. We observed a common pattern of delayed responses that materialized in a sub-set of transcripts and m/z features, peaking 13h after OS-elicitation in both elicited and un-elicited leaves. In clear contrast with leaf tissues, these patterns of co-linearity were not observed for the time series of both the metabolomes and transcriptomes of root tissues. Interestingly, systemic signaling elicited larger transcriptomic changes in roots compared to the systemic (untreated) leaves with direct vascular connection with treated leaves. Despite these larger transcriptomic responses, the metabolic responses of roots reached almost the same magnitude as those of systemic leaves, but the differentially regulated metabolites were more frequently found in the positive than in the negative ionization mode of the MS analysis (Figure 1, pattern b).

Surprisingly, we observed a pattern unique to root tissues that manifest itself in a similar number of induced and suppressed genes separated by a short time lag (Figure 1, pattern a). This temporal uncoupling between the up- and down-regulation of a root's gene expression machinery clearly contrasted with the highly collinear responses described above for leaves. To identify the degree of overlap between the transcriptional and metabolic responses in treated and systemic tissues, we calculated the percentage of genes/metabolites showing significant differential expression in the two tissues and found higher overlap between elicited and systemic leaf tissues (reaching a maximum of 13/15% commonly induced/repressed genes and 47/32% commonly induced/repressed metabolites) than between elicited leaves and roots (4/4% commonly induced/repressed genes and 5/3% induced/repressed metabolites), suggesting a role for distinct molecular players and pathways in roots that respond to simulated leaf herbivory (Figure S2). We computed the enrichment of gene ontology (GO) terms for gene sets obtained by DEG analysis at each time point using best BlastX hit (e value threshold $1e-15$) with the MapMan classification of biological processes for Arabidopsis (TAIRv6, see Material and Methods section) (Thimm *et al.* 2004). Enriched GOs for up-regulated genes in elicited and systemic leaves were related to “stress”, “oxidative pentose pathway” (OPP), “lipid metabolism”, and “cell wall” (Figure 2). The GO class that showed the largest difference between treated and untreated leaves corresponded to “secondary metabolism” and contributed to a significant number of the up-regulated transcripts at all the time points in the series. It is conceivable from the above trends that the underlying regulatory networks might have evolved to provide tight temporal control over resource partitioning in leaves. The enriched GOs for up-regulated root transcriptome signatures occurring 5h and 13h after elicitation that showed a significant match to Arabidopsis proteome included “OPP”, “stress”, “lipid metabolism”, “developmental programs” and “cellular functions” (cell wall, cell cycle/organization, DNA) as main processes. Comparatively, less information was inferred based on homology searches with Arabidopsis regarding enriched gene processes for down-regulated transcripts in the roots. To robustly disentangle the kinetics of these mechanisms, we employed informatics strategies involving coordinated multivariate analysis without merging the time variable as a single functional entity.

Simulated leaf herbivory triggers largely unexplored metabolic changes in roots

To interpret the downstream effects of the large transcriptional changes observed in roots upon leaf herbivory, we analyzed the time series root metabolomics data set. Figure 3A presents the overlaid chromatograms for roots of treated and untreated plants, obtained 21h after OS-elicitation in leaves. For ease of interpretation, we visualized metabolites exhibiting pronounced changes in relative levels (Figure 3A), including many unknown ion peaks, on the chromatogram. The calculation of predicted molecular formulae for many of these unknown ions (Methods S1) revealed that the simulated leaf herbivory-regulated root metabolome was replete with many nitrogen containing compounds which are efficiently ionized in the positive ionization mode (Gaquerel *et al.* 2010), which may provide an explanation for the much larger number of induced ions detected in the positive mode which were not detected in the negative mode.

To facilitate the annotation of m/z signals from the metabolomics data to particular metabolic pathways, we classified patterns for the set of 1728 differentially regulated m/z features (FDR=0.05, 0.5=>FC>=2) using the time series clustering software SplineCluster (Heard *et al.* 2006) (Figure S3). For simplicity, we presented 10 of the 15 clusters retrieved from this analysis which showed major induced changes in the root metabolome (Figure 3B). An overview of the different clusters is presented as supplement material (Figure S3). We labeled the resulting clusters based on the time point at which highest fold-change effects were detected. The set of clusters labeled (a,b,c), (e,f,g) and h and j contained ions which accumulated respectively at 1, 5, 9 and 13h after OS-elicitation. A few of these clusters showed induced accumulations at more than one time point, especially cluster “i” that contained a large number of m/z features with statistically significant differential accumulation at 9, 17 and 21h after OS elicitation. Pronounced accumulation patterns were detected for free tyramine (Figure 3C, for metabolite annotation and elemental formula prediction see Method S1), phenolic conjugates of putrescine with known defensive functions in leaves (Kaur *et al.* 2010) and free amino acids (tryptophan and phenylalanine) (Figure S4). We also detected clear elevations in the levels of glucoside conjugates of 12-hydroxy-jasmonic acid and salicylic acid. Figures S4, S5 and S6 depict temporal profiles for known compounds and a few unknown m/z features from cluster “i” with their predicted molecular formulae.

Multivariate time series analysis captures sequential transcriptomic changes in roots

We next analyzed the nature of the temporal uncoupling in the up- and down-regulation of transcript accumulation in roots following leaf herbivory. To this end, we compared the transcriptional responses at 5h (5318 transcripts up-regulated) and 9h (5955 transcripts down-regulated) after elicitation and found a large overlap (68%) in the gene identities of those 2 groups, but these genes were largely of unknown function with only 40% having close homologs in Arabidopsis (Figure 4A, grey section of the pie chart). Those that did have close homologs (black section of the pie chart) were enriched in lipid metabolism, OPP, cell wall and cell cycle associated pathways. Considering this transient up-regulation of a set of genes in the root transcriptome, we postulated that the simultaneous analysis of all 6 time points would help to deduce the significance of this pattern and to explore new ones from the time series. Recent studies have shown that responses to shoot herbivory in roots are controlled by signaling pathways from aboveground tissues (Machado *et al.* 2013); therefore understanding shoot-root systemic defense signaling necessitated the coordinated analysis of profiled transcriptomic responses in both treated leaves and roots. In a previous study of systemic signaling in aboveground tissues (Gulati *et al.* 2013), we developed a method to mine dynamic changes in gene expression by simultaneously analyzing time series with 2 binary factors (tissue type and treatment) and used it to establish gene-metabolite interactions in systemically induced metabolic pathways in systemic leaves. In the present study, we used this method to characterize co-expression motifs with differential expression between elicited leaves and roots by re-analyzing previously published data sets in Gulati *et al.* (2013) and Kim *et al.* (2011) in order to analyze the temporal uncoupling between the up- and down-regulation of a root's gene expression machinery. By applying the first step of the multifactorial method (Figure 4B, step 1), we obtained 4 clusters of genes derived from bootstrap-based non-parametric ANOVA models (Zhou and Wong

2011) representing statistical structures referred to as “interactive” (treated leaves and roots behaving differently in response to OS-elicitation), “additive” (treatment responses independent of tissue type), “tissue effects” (significant difference in leaves and roots with no response to OS-elicitation), or “treatment effects” (equivalent response to OS-elicitation in both treated leaves and untreated roots).

To understand the differences in the transcriptional responses of elicited leaves and roots, we focused on the set of genes displaying an “interactive” response pattern. Figure 4B (step 2) provides a heatmap representation of the amplitudes of the time response metrics obtained from the above analyses. The time response metric represents the projection of strong effects (interactive) along the time series and was estimated by fitting different ANOVA models. Consistent with results from the above single time point analysis, we observed large differences in the responses of treated leaves and roots at 5 and 13h after elicitation. The end result of the application of this statistical method is the generation of spatio-temporally-resolved OS-elicited gene clusters. These clusters were obtained by superimposing Self-Organizing Maps (BL-SOM) on the scaled data which include information about differences in fold changes of OS responses in both treated leaves and roots and about the amplitude of the differences of responses between the two tissues at each time point in the series (see Material and Methods section). We designated each cluster on the obtained maps (40×18 cells) as “interactive” motifs. Interestingly, genes showing the specific pattern described above of significant induction at 5h and immediate suppression at next time point (9h) (Figure 1) were mapped onto the spatio-temporal maps and found to localize entirely in the “interactive” motif labeled “R5a”: 2385 transcripts exhibiting this pattern of regulation mapped on motif R5a which contains 4501 transcripts. We therefore used SOM “interactive” motifs to refine our interpretation of the transient transcriptional changes that were unique to roots and also to identify new patterns that were not captured by the univariate approach of single time point analyses but appeared in “interactive” motifs labeled “R5b” (3340 transcripts), “R9” (1493 transcripts) and “R13” (4031 transcripts).

The application of this multifactorial approach to the analysis of metabolomics profiles requires samples with somewhat similar metabolomes, such as those analyzed for different leaf positions (Gulati *et al.* 2013). In contrast, the root metabolic profile largely differs from that of elicited leaf tissues. This renders very difficult the application of the leaf-to-root multifactorial analysis for reducing the complete feature set (genes and metabolites) to a small subset showing similar behavior (such as interactive effect) prior integration of gene and metabolite profiles of roots. For this reason, according to major gene expression changes at 5h (Figure 1) and metabolic changes at 9h in roots (Figure 3), we only constructed SOM maps integrating genes and metabolites at these two individual time points. Figure S7 presents a cluster that was extracted from this analysis and which connects induced tyramine accumulation with genes annotated for amino acid metabolism, a MYB transcription factor as well as other genes with no functional assignments.

Simulated leaf herbivory elicits an inversion in root-specific transcriptome rhythms

The endogenous circadian clock regulating biological rhythms allows plants to anticipate fluctuations in environmental conditions as well as certain biotic stresses (Izawa 2012,

Wang *et al.* 2011) and to regulate its physiology accordingly. Importantly, above and belowground tissues of a plant possess autonomous circadian clocks that adapt the rhythmic expression of genes and metabolites (James *et al.* 2008, Moreno-Risueno *et al.* 2010). Here we report and discuss the identification of circadian transcripts that displayed a treatment and root-specific inversion of their rhythm in response to leaf OS-elicitation.

From the spatio-temporally resolved SOM, we detected two dominating motifs at 5h that reflected two different modes of gene regulation (Figure 5A). The motif labeled “R5a” represents the set of genes with large differences in fold changes (OS/C) between elicited leaves and roots but weak time response metrics for the “interactive” effect. As expected, this motif contained all the genes that showed significant differential expression in the single time point analysis (FDR=0.05, $F>2$). Additional genes (2116) that did not pass these univariate analysis statistical thresholds when only roots were considered were also captured by the multifactorial analysis on the basis of their greater response to elicitation in roots than in shoots at this time point. To assess the functional significance of this first group of genes, we computed the enrichment of GO terms using MapMan classification of biological processes in Arabidopsis (hyper-geometric test, $F<0.05$). With only 48% of genes showing a significant match to Arabidopsis genes, this motif appeared enriched with genes implicated in only two main processes that corresponded to transport and cell wall metabolism.

Leaf OS-elicitation triggers the partitioning of recently fixed photoassimilates from the damaged sites to sink tissues, including roots. Passive unloading of sucrose (symplasmically or apoplasmically) diverted actively from attacked leaves has been proposed earlier, but the fact that roots of herbivory-elicited plants recruit sugar much more efficiently than do roots of control plants suggests the importance of transporter activity (Schwachtje *et al.* 2006). We screened sugar-related genes based on literature search and found few genes known to regulate sugar translocation exhibiting rapid modulations characteristic of the motif “R5a” (Figure S8). Reconfigurations of transporter-mediated root functions also contribute to the defense of shoots, especially in species which have evolved the capacity to defend leaves with root-produced secondary metabolites. This is especially well described in *N. attenuata*, in which nicotine synthesized in roots are rapidly transported to aboveground tissues during herbivore attack (Baldwin 1999, Morita *et al.* 2009). The overrepresentation of processes associated with cell wall metabolism in motif “R5a” suggests that leaf OS-elicitation systemically activates physical changes in roots’ cell wall. The plant cell wall is a dynamic structure that plays important roles in growth and in the interactions of plants with their environment. Simulated leaf herbivory has been shown to negatively impact short-term dynamics of root growth (Hummel *et al.* 2007, Schmidt *et al.* 2010), possibly by high amplitude effects in cell wall related gene expression such as those captured by motif “R5a”. Large changes in the expression of genes encoding structural components of the cell wall have been reported as part of the developmental response elicited in roots during abiotic stress (Dinneny *et al.* 2008). The consecutive changes in the cell wall structure can impact root exudation (Badri and Vivanco 2009), have signaling functions and may also reflect changes in root “foraging behavior” that are elicited by leaf herbivory.

To better visualize process-specific gene dynamics, we further classified motif “R5a” into 3 clusters using k-means clustering with averaged gene expression in roots and treated leaves

for all time points. Interestingly, cluster labeled “R5a-1” which covers almost 85% of the motif “R5a” was dominated by genes showing rhythmic expression. To objectively determine which genes exhibited robust circadian expression, we tested for statistically significant ($P < 0.05$) correlation ($PC > 0.75$) between the temporal expression profiles of each gene and defined model types using HAYSTACK (Michael *et al.* 2008). 3204 transcripts (89% of cluster “R5a-1”) were classified as oscillating with a best fit to the “box” model type (Figure S9) in roots of control plants. In a previous study on the same experimental data set, we reported perturbations in the oscillations of genes involved in secondary metabolic pathways in response to simulated leaf herbivory in *N. attenuata* (Kim *et al.* 2011). In contrast to most secondary metabolic transcripts expressed in leaves that had conventional circadian rhythms of 24h period lengths, these root transcripts oscillated with a 12h period length and all clustered into a single group with identical phases. Similarly, 2214 (60%) of these genes also showed a circadian rhythm with a 12h period length in leaves of control plants. Root-specific, short-term fluctuations of gene expression with a 6h period have previously been reported to coordinate lateral root formation in *Arabidopsis* (Moreno-Risueno *et al.* 2010) and semidiurnal (12h) oscillations controlling starch-related gene expression have been described in Cassava storage roots (Baguma *et al.* 2008). One very striking observation from our analysis was that 41% of these rhythmic transcripts following the “box” model type showed a phase inversion of their expression to the “spike” model (Figure S9, Figure 5) in the roots of OS elicited plants. In other words, while still maintaining a 12h period rhythm, these transcripts peaked now both at dusk and at dawn. These oscillating transcripts showed a fold change (OS/C) greater than 2 at 5h which decreased along the time series but the rhythm inversion was observed for all time points. This inversion of the 12h rhythms was not found in treated leaves suggesting that this rhythm change in response to leaf OS-elicitation might be driven by root-specific circadian clock components and/or effects.

To identify whether this phenomenon involved the specific recognition of insect’s OS, we compared the root transcriptional responses to OS-elicitation with those obtained after mechanical wounding alone (W+W treatment) and found no overlap, which reinforced the conclusion that these responses are highly selective to OS-elicitation. Next, we checked for the presence of a similar pattern of inversion in the transcriptional responses for the same set of transcripts in systemic leaves to OS-elicitation but none of these transcripts showed statistically significant rhythmic patterns in systemic leaves after OS elicitation. This demonstrates that the responsiveness of these genes to OS-elicitation is selective to systemic cues directed towards roots.

An interactive motif with opposite amplitude changes in treated leaves and roots

The second component of the large transcriptional responses in roots visualized by the spatial-temporal SOM grids 5h after elicitation corresponded to motif – “R5b” (Figure 5B). This motif is characterized by a strong “interactive effects” as a result of their up-regulation in roots and down-regulation at the same time point in treated leaves. We further partitioned motif “R5b” using Euclidean distance-based k-means clustering and analyzed the two main patterns. The cluster named – “R5b-2” represents the set of genes with a similar inversion of a 12h periodic rhythm in roots in response to OS-elicitation but which exhibits significant

down-regulation in gene expression in treated leaves 5h after elicitation (heatmap of Figure 5B). This set does not show overlap with those found with single time point analysis since the fold change differences are below the threshold taken for DEG analysis. Compared to other clusters, the cluster named – “R5b-1” consists of few genes each of which showed higher constitutive levels in roots compared to leaves and is well represented by genes implicated in the nicotine biosynthetic pathway. Expression of nicotine biosynthetic genes -- *NaPMT1* (Putrescine N-methyltransferase), *NaPMT2*, *NaA622*, *NaQPT* (Quinolate phosphoribosyltransferase), *NaDAO* (D-amino-acid oxidase) -- peaked at 9h and 17h in root tissues. As previously reported, OS-elicitation in leaves induced an increase in the expression of these genes in roots which lasted for all time points but we also noticed that peaking time of expression shifted from 9 to 5h compared to control plants. In treated leaf tissues, expression of these genes showed a significant and consistent down-regulation. Nicotine synthesis takes place in the roots and a role for the residual expression of nicotine structural genes in other plant parts has not been assigned yet. Consistent with our observations, previous work has reported the down-regulation of *PMT* transcript levels in treated leaves in *Nicotiana tabacum* (Sachan and Falcone 2002).

A chronological perspective on the arrangement of root gene expression motifs using SOM

The main benefit of retaining the temporal information of a time series experiment rather than merging it as a single variable within the multivariate analysis is the ability to directly visualize the chronology of activation of previously characterized nodes in metabolic pathways central to signaling, tolerance and defense in *N. attenuata*. Figure 6A reveals the location of a few previously functionally characterized elements in these pathways onto “interactive” motifs of the SOM grid. Although not informative about interactions between these different motifs and regulation mechanisms controlling their activation, this data visualization approach provides precious information on the onset of activation of these different processes needed for conducting additional experimental work.

Consistent with its early activation following leaf herbivory, *NaGAL83*, the β -subunit of the SNF1-related kinase which mediates herbivory-induced allocation of sugars to roots of *N. attenuata* (Schwachtje *et al.* 2006), is detected in the motif named “R1” and mining this motif enriched in signaling processes may help identifying additional components in the network of kinase protein controlling this process. Future experimental work will test whether the modulations discovered for sugar metabolic and transporter genes located in motifs “R5b” and “R5a” are controlled by the upstream action of *NaGAL83*. In this study, we illustrated the use of SOMs for inferring insights into the patterns of gene expression underlying induced nicotine biosynthesis. A similar approach could be employed for discovering genes required for the synthesis of additional root-based defensive small molecules such as oxylipins. Known and putative metabolism-based defense mechanisms are more represented in later established gene interactive motifs: *NaPMT1* from the nicotine biosynthetic pathway is located in motifs “R5b” and “R9” and *NaLOX1* which initiates the production of fatty acid 9-hydroperoxides and hydroxides that serve as precursors for a wide array of oxylipins with signaling and defensive functions (Vellosillo *et al.* 2007) is found in

motif “R13”, a motif that could be mined for elucidating downstream steps in the synthesis of more complex root oxylipins.

Conclusions and perspectives

Approaches used to prioritize genes for functional characterization aim at identifying the most promising genes among a larger pool of candidates through integrative computational analyses. The rationale behind these methods is first to partition genes into modules or clusters, so that these clusters can be readily queried for criteria such as their involvement into a given cellular process. But often, the vector time corresponding to the genes’ temporal dynamics is considered as another variable. In this article, we implemented an approach to mine biologically important transcriptomic patterns in roots by implementing a two step partition. In the first step, we simultaneously handled the time course and the two binary factors (tissue type: treated leaves and roots, treatment: Control and W+OS) and obtained four exclusive clusters. This approach facilitated the retention of information about the timings of gene activation. A second step of classification was applied to the cluster of genes showing an “interactive” effect and we visualized the dynamic behavior of genes separated across the time series and the tissue type (treated leaves and untreated roots) by employing SOM. From this analysis, distinct motifs reflecting various broad functions were identified. As a proof of principle, we studied motifs – “R5a” and “R5b” and discovered two major trends – OS-specific inversions in 12h root-specific rhythms and amplitude effects in nicotine biosynthetic and other metabolic genes. Hence, this two-step reduction approach helped in the identification of “emergent properties” of roots’ transcriptional changes in a lower dimensional space.

We also recovered 2 additional clusters which should be studied intensively in the future to understand the role of roots in orchestrating leaf responses to attack from herbivores (Figure 6B): a cluster with an “additive” effect (genes’ responses to OS elicitation that are independent of the tissue type) and one with a “treatment” effect (genes showing responsiveness to OS elicitation in both treated leaves and untreated roots). *NaLOX6* shows a significant up-regulation in treated leaves at 1h after OS elicitation and in roots at later time-points. Consistent with a major function of this gene in roots, a close homolog in *Arabidopsis* has recently been characterized for stress-induced jasmonate accumulation in roots (Grebner *et al.* 2013). Combining together these sets of interactive, additive and treatment effects allowed us to decipher the complex dynamics that occur in the transcriptomes and metabolomes of roots in response to leaf OS-elicitation with an unprecedented level of resolution.

Regulatory machineries behind OS-elicited remodeling of root-specific transporter gene oscillation are unknown. Future experiments involving longer time series experiments with wild-type and transgenic lines impaired either in upstream nodes of herbivory-induced signaling and tolerance pathways (Machado *et al.* 2013) or core circadian clock components (Yon *et al.* 2012) will be required to examine the time required for these rhythms to revert back to their original “box” model type. Additionally, grafting experiments with these transgenic lines will be used to determine the relative contribution of shoot vs root signals

for this phenomenon and which components were co-opted by OS-elicited systemic signals to regulate these root-specific 12h gene rhythms.

Materials and methods

Microarray Data-set and Processing

We analyzed 134 published microarray expression data of *Nicotiana attenuata* from the Gene Expression Omnibus database (accession no. GSE30287), reflecting responses in treated leaves and untreated leaves and roots to simulated *Manduca sexta* feeding (by applying diluted insect's oral secretions into freshly created puncture wounds to a specific leaf, W+OS) and to a mechanical wounding treatment with the identical leaf damage (in which water was applied to the puncture wounds) for 6 whole-plant harvest time points (1, 5, 9, 13, 17, 21h after treatment). This microarray data-set has been originally published as part of the publication by Kim *et al.* (2011) and re-analyzed for the development of the multifactorial approach by Gulati *et al.* (2013). Raw intensities were log₂ and baseline transformed and normalized to their 75th percentile using the R software package, prior to statistical analysis.

Metabolomics Data-set and Processing

In a previous study, metabolomic analysis was conducted on leaf (treated and untreated systemic leaves) and root methanolic extracts from samples harvested for generating the microarray data platform. The resulting metabolomics data-set has been originally published as part of the publication by Kim *et al.* (2011) and only metabolomic profiles from leaf samples (treated and untreated systemic leaves) were mined to establish the multifactorial statistical approach described in Gulati *et al.* (2013). In the present study, metabolomic data collected from roots for control plants and simulated herbivory treated plants were extracted from the data-set published by Kim *et al.* (2011) and re-analyzed using additional statistical approaches. Analytical conditions for metabolomic measurements and first steps in data processing are described in Kim *et al.* (2011) and Gaquerel *et al.* (2010) and described in Method S2. The metabolite annotation procedure and nomenclature for root metabolites is described in Method S1. Raw intensity values were 75th percentile normalized before statistical analysis (Data S1).

Statistical Analysis and Data Visualization

All statistical tests for DEG analyses were carried out using R software (core module, t.test, p.adjust with method="fdr"). To mine the major biological processes perturbed in response to OS elicitation, we functionally annotated *N. attenuata* genes recognized by these probe sets (NCBI GEO database, accession number GSE30287) using best BlastX hit of Arabidopsis TAIR6 Proteome with an e value cut off of 1e-15. Next, using MapMan classification of biological processes for Arabidopsis, we assigned classes to each probe id of our microarray data set. Enrichment analysis of gene ontology biological processes based on hyper-geometric test was performed using R. Significant enrichments were those with $F < 10e-10$. Multifactorial data analysis was carried out using the methods implemented in the R package TANOV (Zhou and Wong 2011). The cluster showing interactive effect in gene expression between treated leaves and roots was scaled as

$$E_i = [F_1 \dots F_6]_i * [R_1 \dots R_6]_i^2$$

Where E_i : scaled expression, F_i : difference in fold changes (OS-elicitation/control) between treated leaves and untreated roots, R_i : response timing. Results of the multifactorial analysis, including gene annotation and scaled data used for the Self Organizing Maps are available as a spreadsheet (217588Supplemental_File1.xls) in the TvR sheet at: <http://www.plantphysiol.org/content/early/2013/05/09/pp.113.217588/suppl/DC1>. The Self Organizing Maps were constructed using BL-SOM software (http://prime.psc.riken.jp/?action=blsom_index). Hierarchical Clustering Analyses for all heatmaps are based on Euclidean distance measures and average linkage aggregation methods. All heatmaps and box-plots were created using R. The model based HAYSTACK algorithm (Michael *et al.* 2008) was used to identify periodicity in root and leaf transcriptomes. Euclidean distance-based k-means clustering was obtained using R.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Badri DV, Vivanco JM. Regulation and function of root exudates. *Plant Cell Environ.* 2009; 32:666–681. [PubMed: 19143988]
- Baguma Y, Sun C, Boren M, Olsson H, Rosenqvist S, Mutisya J, Rubaihayo PR, Jansson C. Sugar-mediated semidiurnal oscillation of gene expression in the cassava storage root regulates starch synthesis. *Plant Signal Behav.* 2008; 3:439–445. [PubMed: 19513234]
- Baldwin IT. Inducible nicotine production in native *Nicotiana* as an example of adaptive phenotypic plasticity. *J Chem Ecol.* 1999; 25:3–30.
- Dawson RF, Solt ML. Estimated Contributions of Root and Shoot to the Nicotine Content of the Tobacco Plant. *Plant Physiol.* 1959; 34:656–661. [PubMed: 16655293]
- Dinneny JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN. Cell identity mediates the response of *Arabidopsis* roots to abiotic stress. *Science.* 2008; 320:942–945. [PubMed: 18436742]
- Doherty CJ, Kay SA. Circadian control of global gene expression patterns. *Annu Rev Genet.* 2010; 44:419–444. [PubMed: 20809800]
- Erb M, Lenk C, Degenhardt J, Turlings TC. The underestimated role of roots in defense against leaf attackers. *Trends Plant Sci.* 2009; 14:653–659. [PubMed: 19736036]
- Espinoza C, Degenkolbe T, Caldana C, Zuther E, Leisse A, Willmitzer L, Hinch DK, Hannah MA. Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in *Arabidopsis*. *PLoS One.* 2010; 5:e14101. [PubMed: 21124901]
- Gaquerel E, Heiling S, Schoettner M, Zurek G, Baldwin IT. Development and validation of a liquid chromatography-electrospray ionization-time-of-flight mass spectrometry method for induced changes in *Nicotiana attenuata* leaves during simulated herbivory. *J Agric Food Chem.* 2010; 58:9418–9427. [PubMed: 20701244]

- Gibon Y, Usadel B, Blaesing OE, Kamlage B, Hoehne M, Trethewey R, Stitt M. Integration of metabolite with transcript and enzyme activity profiling during diurnal cycles in Arabidopsis rosettes. *Genome Biol.* 2006; 7:R76. [PubMed: 16916443]
- Gillis J, Pavlidis P. “Guilt by association” is the exception rather than the rule in gene networks. *PLoS Comput Biol.* 2012; 8:e1002444. [PubMed: 22479173]
- Grebner W, Stingl NE, Oenel A, Mueller MJ, Berger S. Lipoxigenase6-dependent oxylipin synthesis in roots is required for abiotic and biotic stress resistance of Arabidopsis. *Plant Physiol.* 2013; 161:2159–2170. [PubMed: 23444343]
- Gulati J, Kim SG, Baldwin IT, Gaquerel E. Deciphering Herbivory-Induced Gene-to-Metabolite Dynamics in *Nicotiana attenuata* Tissues Using a Multifactorial Approach. *Plant physiology.* 2013; 162:1042–1059. [PubMed: 23656894]
- Gutierrez RA, Stokes TL, Thum K, Xu X, Obertello M, Katari MS, Tanurdzic M, Dean A, Nero DC, McClung CR, oruzzi GM. Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene CCA1. *Proc Natl Acad Sci U S A.* 2008; 105:4939–4944. [PubMed: 18344319]
- Heard NA, Holmes CC, Stephens DA. A quantitative study of gene regulation involved in the immune response of anopheline mosquitoes: An application of Bayesian hierarchical clustering of curves. *J Am Stat Assoc.* 2006; 101:18–29.
- Hummel GM, Naumann M, Schurr U, Walter A. Root growth dynamics of *Nicotiana attenuata* seedlings are affected by simulated herbivore attack. *Plant Cell Environ.* 2007; 30:1326–1336. [PubMed: 17727422]
- Izawa T. Physiological significance of the plant circadian clock in natural field conditions. *Plant Cell Environ.* 2012; 35:1729–1741. [PubMed: 22681566]
- Kaplan I, Halitschke R, Kessler A, Sardanelli S, Denno RF. Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology.* 2008; 89:392–406. [PubMed: 18409429]
- Kaur H, Heinzel N, Schottner M, Baldwin IT, Galis I. R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. *Plant Physiol.* 2010; 152:1731–1747. [PubMed: 20089770]
- Kim SG, Yon F, Gaquerel E, Gulati J, Baldwin IT. Tissue specific diurnal rhythms of metabolites and their regulation during herbivore attack in a native tobacco, *Nicotiana attenuata*. *PLoS One.* 2011; 6:e26214. [PubMed: 22028833]
- Kuhl C, Tautenhahn R, Bottcher C, Larson TR, Neumann S. CAMERA: An Integrated Strategy for Compound Spectra Extraction and Annotation of Liquid Chromatography/Mass Spectrometry Data Sets. *Anal Chem.* 2012; 84:283–289. [PubMed: 22111785]
- Lee I, Ambaru B, Thakkar P, Marcotte EM, Rhee SY. Rational association of genes with traits using a genome-scale gene network for *Arabidopsis thaliana*. *Nat Biotechnol.* 2010; 28:149–156. [PubMed: 20118918]
- Lee I, Seo YS, Coltrane D, Hwang S, Oh T, Marcotte EM, Ronald PC. Genetic dissection of the biotic stress response using a genome-scale gene network for rice. *Proc Natl Acad Sci U S A.* 2011; 108:18548–18553. [PubMed: 22042862]
- Lu Y, Gehan JP, Sharkey TD. Daylength and circadian effects on starch degradation and maltose metabolism. *Plant Physiol.* 2005; 138:2280–2291. [PubMed: 16055686]
- Machado RA, Ferrieri AP, Robert CA, Glauser G, Kallenbach M, Baldwin IT, Erb M. Leaf-herbivore attack reduces carbon reserves and regrowth from the roots via jasmonate and auxin signaling. *The New phytologist.* 2013
- Marti G, Erb M, Boccard J, Glauser G, Doyen GR, Villard N, Robert CA, Turlings TC, Rudaz S, Wolfender JL. Metabolomics reveals herbivore-induced metabolites of resistance and susceptibility in maize leaves and roots. *Plant Cell Environ.* 2013; 36:621–639. [PubMed: 22913585]
- Michael TP, Mockler TC, Breton G, McEntee C, Byer A, Trout JD, Hazen SP, Shen R, Priest HD, Sullivan CM, Givan SA, Yanovsky M, Hong F, Kay SA, Chory J. Network discovery pipeline

- elucidates conserved time-of-day-specific cis-regulatory modules. *PLoS Genet.* 2008; 4:e14. [PubMed: 18248097]
- Moreno-Risueno MA, Van Norman JM, Moreno A, Zhang J, Ahnert SE, Benfey PN. Oscillating gene expression determines competence for periodic Arabidopsis root branching. *Science.* 2010; 329:1306–1311. [PubMed: 20829477]
- Morita M, Shitan N, Sawada K, Van Montagu MCE, Inze D, Rischer H, Goossens A, Oksman-Caldentey KM, Moriyama Y, Yazaki K. Vacuolar transport of nicotine is mediated by a multidrug and toxic compound extrusion (MATE) transporter in *Nicotiana tabacum*. *P Natl Acad Sci USA.* 2009; 106:2447–2452.
- Ober D, Kaltenegger E. Pyrrolizidine alkaloid biosynthesis, evolution of a pathway in plant secondary metabolism. *Phytochemistry.* 2009; 70:1687–1695. [PubMed: 19545881]
- Persson S, Wei H, Milne J, Page GP, Somerville CR. Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. *Proc Natl Acad Sci U S A.* 2005; 102:8633–8638. [PubMed: 15932943]
- Sachan N, Falcone DL. Wound-induced gene expression of putrescine N-methyltransferase in leaves of *Nicotiana tabacum*. *Phytochemistry.* 2002; 61:797–805. [PubMed: 12453572]
- Schmidt L, Hummel GM, Schottner M, Schurr U, Walter A. Jasmonic acid does not mediate root growth responses to wounding in *Arabidopsis thaliana*. *Plant Cell and Environment.* 2010; 33:104–116.
- Schwachtje J, Baldwin IT. Why does herbivore attack reconfigure primary metabolism? *Plant Physiol.* 2008; 146:845–851. [PubMed: 18316639]
- Schwachtje J, Minchin PE, Jahnke S, van Dongen JT, Schittko U, Baldwin IT. SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proc Natl Acad Sci U S A.* 2006; 103:12935–12940. [PubMed: 16912118]
- Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: Processing mass spectrometry data for metabolite profiling using Nonlinear peak alignment, matching, and identification. *Anal Chem.* 2006; 78:779–787. [PubMed: 16448051]
- Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT. Nicotine's defensive function in nature. *PLoS Biol.* 2004; 2:E217. [PubMed: 15314646]
- Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt M. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J.* 2004; 37:914–939. [PubMed: 14996223]
- Usadel B, Obayashi T, Mutwil M, Giorgi FM, Bassel GW, Tanimoto M, Chow A, Steinhauser D, Persson S, Provart NJ. Co-expression tools for plant biology: opportunities for hypothesis generation and caveats. *Plant Cell Environ.* 2009; 32:1633–1651. [PubMed: 19712066]
- Vanholme R, Storme V, Vanholme B, Sundin L, Christensen JH, Goeminne G, Halpin C, Rohde A, Morreel K, Boerjan W. A systems biology view of responses to lignin biosynthesis perturbations in *Arabidopsis*. *Plant Cell.* 2012; 24:3506–3529. [PubMed: 23012438]
- Vellosillo T, Martinez M, Lopez MA, Vicente J, Cascon T, Dolan L, Hamberg M, Castresana C. Oxylipins produced by the 9-lipoxygenase pathway in *Arabidopsis* regulate lateral root development and defense responses through a specific signaling cascade. *Plant Cell.* 2007; 19:831–846. [PubMed: 17369372]
- Wang W, Barnaby JY, Tada Y, Li H, Tor M, Caldelari D, Lee DU, Fu XD, Dong X. Timing of plant immune responses by a central circadian regulator. *Nature.* 2011; 470:110–114. [PubMed: 21293378]
- Yon F, Seo PJ, Ryu JY, Park CM, Baldwin IT, Kim SG. Identification and characterization of circadian clock genes in a native tobacco, *Nicotiana attenuata*. *Bmc Plant Biol.* 2012; 12:172. [PubMed: 23006446]
- Zhou BY, Wong WH. A Bootstrap-Based Non-Parametric Anova Method with Applications to Factorial Microarray Data. *Stat Sinica.* 2011; 21:495–514.
- Ziegler J, Facchini PJ. Alkaloid biosynthesis: metabolism and trafficking. *Annu Rev Plant Biol.* 2008; 59:735–769. [PubMed: 18251710]

Figure 1. Tissue-specific temporal patterns of up- and down-regulated transcriptional and metabolic changes after simulated leaf herbivory in *Nicotiana attenuata*

Leaf herbivory-elicited differentially regulated transcripts and m/z features derived from metabolite MS analysis (Wound + OS/Control, $P \leq 0.05$ and $-1 \geq \text{fold change} \geq 1$) were identified for treated (leaves) and untreated systemic (leaves and roots) tissues using normalized and log2 transformed expression values at each harvest time. Two properties were particularly striking: (a) a pronounced temporal shift in the peaks of up- and down-regulation in gene expression that were unique to roots and (b) a predominance in the regulation of the positively-charged metabolome.

Figure 2. Functional categorization of OS-elicited whole organism transcriptional responses Gene ontology (GO) enrichment (Hyper-geometric test, $F < 10e-10$) was performed to identify significantly up- (a) and down-regulated (b) transcripts in treated leaves and untreated systemic tissues (leaves and roots) using MapMan classification of biological processes for Arabidopsis (TAIRv6).

Figure 3. Leaf OS-elicitation triggered metabolic reconfigurations in roots

(a) Representative ultrahigh performance liquid chromatography quadrupole-time-of-flight mass spectrometry (UHPLC-qTOFMS, positive ionization mode) metabolic profiles of root samples collected after 21 h from elicited and control plants. Colored peaks correspond to compound spectra detected by FindDISSECT that were differentially regulated (W+OS/C, Figure 1). (b) We used SplineCluster to classify the dynamics shared by different groups of metabolites. Ten of the 15 clusters are presented. Clusters are colored according to the time of maximum differential regulation and red lines denote the threshold used to identify up-

regulated metabolites. (c) Temporal profiles of three representative m/z features from clusters labeled – “l” and “k” whose accumulation was differentially amplified in roots by the OS-elicitation in leaves and mechanical wounding treatments compared to controls.

Figure 4. Multivariate analysis deciphers the complex root-specific transcriptional responses activated 5, 9 and 13h after OS-elicitation

(a) Temporally-shifted peaks of up- and down-regulation in gene expression unique to roots (Figure 1, pattern a) were observed via a single time point analysis. A large overlap (68%) is observed between induced transcripts at 5h and those suppressed at 9h. Remarkably, only 40% of these common transcripts have close homologs in *Arabidopsis*. (b) Using multivariate time series analysis, a coordinated comparison of transcriptional responses in treated leaves and untreated roots in the time series was implemented. Step 1 summarizes the experimental design of the microarray analysis. The heatmap depicts the extracted time response metrics which represent the significant interactive effect -- genes with statistically different transcriptional responses in treated leaves and untreated roots -- along the time series. In step 2, the genes showing interactive effect were spatio-temporally-resolved after appropriate scaling and classified by Self Organizing Maps (SOM). In step 3, transcripts common between roots' transcriptional responses at 5 and 9h that were extracted using single time point analysis were found localized in the motif labeled as "R5a" in the SOM maps.

Figure 5. Induced root responses involve changes of transcriptional rhythms and amplitude effects inferred from a coordinated multivariate time series analysis

(a) Simulated leaf-herbivory elicits an inversion of the rhythm in the roots transcriptome. Interactive motif “R5a” from the SOM is represented by only 48% genes showing homologs in Arabidopsis and is enriched with genes implicated in “transport” and “cell wall” processes. A major section of this motif, represented by sub-cluster “R5a-1” showed strong induction in roots 5h after elicitation. 89% of the genes in sub-cluster “R5a-1” oscillated in control plants and their pattern of expression best fit the “box” model type (Haystack, $PC > 0.75$, $pvalue < 0.05$). Strikingly, the expression of 41% of these genes showed an inverted pattern of peak expression timing to dusk and dawn in response to simulated leaf

herbivory. **(b)** A transcriptomic response with major amplitude changes in both treated leaves and roots. Interactive motif “R5b” includes genes with strong significant interactive behavior between treated leaves and roots (gene expression being induced in roots while suppressed in treated leaves) compared to fold change (W+OS/C) and hence were not identified by the single time point analysis. Enriched GO terms are represented by 63% of the genes having close homologs in *Arabidopsis*. Sub-cluster “R5b-1” contains, among others, genes of the nicotine biosynthetic pathway. These genes show a root-specific shift of their peaking time only at 5h after elicitation but a major amplitude effect for all the time points in the series.

Figure 6. Sequential arrangement of major transcriptional responses identified using a multivariate time series analysis

(a) Three components of plant responses to herbivory were represented by distinct interactive motifs in spatio-temporally resolved SOM. Schematic pathway summarizing signaling, defense and tolerance processes in roots is based on previously published experimental knowledge (Schwachtje *et al.* 2006, Schwachtje and Baldwin 2008, Steppuhn *et al.* 2004) and the localization of known nodes in this pathway on the SOM is presented.

(b) Temporal profiles of NaLOX6, Na_F-Box-like and Na_42948 represent three additional clusters with genes showing – Additive, Tissue and Treatment effects obtained from multivariate time series analysis.