Herbivore-simulated induction of defenses in clonal networks of trembling aspen (Populus tremuloides)

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Trembling aspen (Populus tremuloides Michx.) as a clonal tree species possesses a complex root system through which trees of the same or different clones are connected. Root connections have been studied with respect to resource sharing, but the nature, quantities or extent of what is shared between trees is relatively unknown. In this study, we posed the hypothesis that systemic defense induction signals could also spread through these root networks and trigger defenses in neighboring ramets before arrival of pests. Temporal expression pattern of Kunitz trypsin inhibitor (KTI) and dihydroflavonol reductase (DFR) genes, two markers of poplar defense, was followed by quantitative real-time polymerase chain reaction. The expression was quantified in systemic leaves of wounded and healthy plants that shared the same parental root and in untreated controls grown in separate pots. Untreated interconnected plants did not show induced resistance upon herbivore-simulated attack. Although wound-treated ramets induced defense genes, untreated interconnected plants produced an expression pattern similar to non-connected controls. Root connections do not automatically lead to induction of defensive traits that are expressed in plants directly under damage thought to simulate herbivory. Rather, it seems that other communication means such as airborne volatiles can serve as signal transmission pathways among neighboring plants.

Keywords: gene expression, herbivory, interplant communication, roots.

Introduction

Physiological integration through physical root connections in plants has been thoroughly studied in various plant species for the last three decades. Most studies dealt with the mechanisms of carbohydrate, water and mineral nutrients sharing (Stone 1974, Kozlowski 1992, Stuefer et al. 1994, Alpert 1996, Stuefer et al. 1998, Magori et al. 2003, Nilsson and D'Hertefeldt 2008, Liu et al. 2009, Du et al. 2010, Mony et al. 2011). Other authors investigated the physiological integration in terms of exploitation of patchy resources (Evans and Cain 1995, Shumway 1995, Hutchings and Wijesinghe 1997, Ikegami et al. 2008), resistance to environmental stresses (Pennings and Callaway 2000, Yu et al. 2008) or improvement of competitive ability (Oborny et al. 2000, Peltzer 2002, Yu et al. 2009). Much less attention has been paid to circulation of non-resource agents such as pathogens (Bruhn et al. 1991, He et al. 2000, Koubek and Herben 2008) and hormones (Alpert et al. 2002). In 2004, Stuefer and Gomez proposed that clonal plant networks could represent highways for long-distance transport of systemic defense induction signals. Sharing of these signals among network members could allow an early response to impending herbivore attacks by upregulation of defense traits. To date, however, such a network-specific early-warning system has only been studied in a single species of stoloniferous herb Trifolium repens L. (Gomez and Stuefer 2006, Gomez et al. 2007, 2008, 2010).

Clonal trees are extremely long-lived and successful life-forms that possess efficient defense mechanisms (Maleck and...
Dietrich 1999, Bruxelles and Roberts 2001, Philippe and Bohlmann 2007, Chen et al. 2009, Duplessis et al. 2009, Ralph 2009). Physical barriers and constitutively produced phytochemicals make up the first line of defense. However, when these barriers are breached, inducible defenses take over reducing herbivory (Philippe and Bohlmann 2007). Some inducible biochemicals directly inhibit insect growth and development, while others serve as airborne signals that reduce herbivory indirectly by deterring herbivores or attracting their predators or parasites (Havill and Raffa 2000). In the genus *Populus*, defense mechanisms against herbivory are well understood and many genes encoding enzymes involved in induced direct or indirect defenses and their corresponding spatial and temporal expression profiles are known (Haruta et al. 2001a, 2001b, Peters and Constabel 2002, Arimura et al. 2004, Christopher et al. 2004, Wang and Constabel 2004, Lawrence et al. 2006, Ralph et al. 2006, Tsai et al. 2006, Major and Constabel 2008, Philippe et al. 2009, 2010).

Among the 1100 genes upregulated upon herbivory feeding, the Kunitz protease inhibitors (KPIs) feature as a prominent marker of poplar defense response. KPIs belong to a large and rapidly evolving gene family whose members are differentially expressed throughout the plant (Haruta et al. 2001a, Christopher et al. 2004, Lawrence et al. 2006, Major and Constabel 2006, Ralph et al. 2006, Miranda et al. 2007, Philippe et al. 2009). They show diverse biochemical properties and functional specialization against various types of proteases in insect gut where they intervene with normal digestion leading to loss of essential amino acids (Major and Constabel 2008). Among them, mRNA encoding Kunitz trypsin inhibitors (KTI) were found to be one of the 10 most abundant expressed sequence tags in the leaf transcriptome following wounding to simulate herbivory, suggesting their important role in poplar defense (Christopher et al. 2004).

Besides protein-based anti-herbivore defense, poplars produce substantial amounts of phytochemicals functioning as anti-nutrients or feeding deterrents (Philippe and Bohlmann 2007). Salicin-based phenolic glycosides, hydroxyxccinnamate derivatives and flavonoid-derived condensed tannins represent an array of complex compounds that can constitute up to one-third of *Populus* leaf dry mass (Tsai et al. 2006). The last-mentioned condensed tannins are produced by the flavonoid biosynthetic pathway. Dihydroflavonol reductase (DFR), a key enzyme of this pathway, is encoded by a single-copy gene in the genome of trembling aspen (Peters and Constabel 2002). Both its expression and enzyme activity was shown to be dramatically induced 24 h after herbivory in local and as well as systemic leaves (Peters and Constabel 2002). Levels of condensed tannins correlated with negative impacts on the performance of gypsy moth larvae and forest tent caterpillars (Hemming and Lindroth 1995, Hwang and Lindroth 1997, Osier et al. 2000, Osier and Lindroth 2001).

Herbivore feeding initiates production of defense induction signals at the site of the damage. These systemic signals travel together with assimilate movement through the vascular architecture of phloem (Davis et al. 1991, Jones et al. 1993, Metraux et al. 2002). Even though the nature of these signals is not yet well known, their movement seems to be affected by the source–sink gradient and constrained by vascular connectivity (Arnold and Schultz 2002, Arnold et al. 2004, Babst et al. 2005). Hence, maximum systemic upregulation was observed in leaves directly connected to the damaged leaves. However, Major and Constabel (2007) clearly demonstrated that some insect herbivory defense genes are upregulated even in roots of plants with methyl jasmonate treated or wounded leaves. Their findings imply that systemic signals are transmitted not only upward from damaged to the top systemic leaves but also downward, basipetally, from shoots to roots.

In this work, we wanted to verify if systemic defense induction signals can spread through the clonal root system of trembling aspen (*Populus tremuloides* Michx.) and trigger expression of defense traits in undamaged neighboring ramets. The aspen clonal root system is made up not only by new roots grown from suckers but also original parental roots that gave rise to the new sucker generation. These original parental roots can be found even in mature aspen stands where they keep connecting stems (ramets) that have regenerated from the same parental roots (DeByle 1964, Strong and LaRoi 1983, DesRochers and Liefers 2001, Jelinkova et al. 2009). Moreover, besides connecting genetically identical ramets, aspen root systems also integrate stems of different clones (Jelinkova et al. 2009). These inter-clonal connections are enabled by the existence of natural root grafts, which result in morphological union of cambium, phloem and xylem of previously distinct roots (Graham and Bormann 1966). Trembling aspen creates large physiological networks rather than stands of discrete individuals or clones.

We followed the temporal expression pattern of *KTI* and dihydroflavonol reductase (DFR) genes using quantitative real-time polymerase chain reaction (PCR). The expression was quantified in systemic leaves of wounded and healthy plants sharing the same roots and in untreated controls. To our knowledge, this is the first study attempting to investigate clonal networks in respect to information sharing among network members in long-living tree species.

**Materials and methods**

**Plant material**

Different genotypes of *Populus tremuloides* Michx. originating from the mixed boreal forest of northwestern Quebec, Canada, were collected as root cuttings in autumn 2007 and stored at 4 °C in moist peat moss. In spring 2008, 60-cm-long root segments were transferred into large pots filled with PRO-MIX®.
BX potting substrate (75–85% sphagnum peat moss, perlite, vermiculite, limestone and micronutrients). Planted root cuttings were kept in a greenhouse under the conditions promoting root suckering (16/8-h photoperiod, 28 °C and 60% humidity). Suckers used for the experiment were from 12 to 15 weeks old and ~1.5 m tall and had only one main stem bearing around 30 leaves.

**Stress treatment simulating insect herbivory**

Aspen suckers growing on the same root (stem bases not farther than 30 cm from each other) were selected for the experiment. The sucker growing at the root’s proximal end underwent a stress treatment and the one at the distal part was left untreated. A third plant in a separate pot was designated as a control. The stressed, untreated and control plants were placed so they would be at the same distance from each other. The experiment was done in biological replicates I–III (three separate experiments comprised a stressed, untreated and control plant) using plant material of the same genotype. This genotype had been selected in a preliminary experiment as the one providing the strongest systemic response to the stress type had been selected in a preliminary experiment as the one providing the strongest systemic response to the stress treatment. The sucker growing at the root’s proximal end underwent a stress treatment and the one at the distal part was left untreated. A third plant in a separate pot was designated as a control. The stressed, untreated and control plants were placed so they would be at the same distance from each other. The experiment was done in biological replicates I–III (three separate experiments comprised a stressed, untreated and control plant) using plant material of the same genotype. This genotype had been selected in a preliminary experiment as the one exhibiting the strongest systemic response to the stress treatment.

Leaves of all plants were numbered basipetally according to the leaf plastochron index (LPI) with the first unfolded leaf being designated as leaf 1 (Constabel et al. 2000). Leaves at LPI 7–25 of the stressed plants were wounded by crunching the blade margins with pliers 3 times at 1 h intervals (Major and Constabel 8–25 of the stressed plants were wounded by crunching the blade margins with pliers 3 times at 1 h intervals (Major and Constabel 2006). Leaves at LPI 7–3 of stressed, untreated and control plants were collected across five time points. Collection started with the leaves at LPI 7 before the wound treatment (time 0) and continued after 24, 36, 72 and 94 h from the beginning of the treatment. In this way, at every time point, LPI of the controls corresponded to LPI of stressed and untreated plants. Sampled leaf tissue was immediately frozen in liquid nitrogen and then stored at −70 °C until RNA extraction.

**Selection of target genes and normalization standards**

Gene expression was followed in two target genes: dihydroflavonol reductase (DFR; GenBank AY147903) and Kunitz trypsin inhibitor III (KTI; GenBank AF349441-AF349443). These genes were selected based on the following criteria: (i) a maximum increase in the systemic (in untreated leaves of stressed plants) expression after wounding, (ii) known direct roles in anti-herbivore defense, (iii) a selection representing different types of anti-herbivore defense (phytochemical and proteolytic) and (iv) sequences of *P. tremuloides* available in public databases. Two house-keeping genes, actin (*ACT*) and translation initiation factor 5A (*TIF*), were chosen as endogenous standards for transcript abundance normalization (Brunner et al. 2004). These genes were shown not to be significantly up or downregulated by herbivory feeding (Ralph et al. 2006) and hence represented suitable candidates for robust normalization.

**Primer and TaqMan probe design**

Gene-specific primers (Table 1) were designed in FastPCR v. 6.0 software (Kalendar 2008) using a stringent set of criteria. Primer specificity was verified in silico by megablast against the nucleotide database at NCBI, by gel electrophoresis in Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany), and by sequencing of PCR amplicons.

Dual-labeled TaqMan probes with Black Hole Quencher™ dyes (Biosearch Technologies, Novato, CA, USA; Table 1) were designed to meet the following parameters: melting temperature range 68–70, guanine–cytosine content range 30–80%, melting temperature range at the 3′ end 34–48 and a maximum length of 30 bp. Runs of more than three Gs and Cs in a row within the last five nucleotides at the 3′ end were avoided whenever possible. All probes were designed in forward orientation and so that the annealing would take place close to the forward primer. Specificity verification was done by nucleotide BLAST against the NCBI database.

**Quantitative real-time PCR assay**

Frozen leaf tissue was ground in liquid nitrogen and total RNA was isolated using the Qiagen RNeasy Plant MiniKit according to the manufacturer’s instructions. Real-time PCR was done in

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**Table 1. Gene-specific primers and probes used for the real-time amplification of Kunitz trypsin inhibitors (KTIs) and dihydroflavonol reductase (DFR) genes, two markers of poplar defense, and actin (ACT) and translation initiation factor 5A (TIF) used as endogenous standards for transcript abundance normalization.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers 5′–3′</th>
<th>Probe 5′–3′</th>
<th>Amplicon size (bp)</th>
<th>Amplicon Tm (°C)</th>
<th>PCR efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>tgacaaccttgcaagatg</td>
<td>cgcagcttgtgacctccagcaga 115 75.0 108.1</td>
<td>102</td>
<td>77.0</td>
<td>101.1</td>
</tr>
<tr>
<td>TIF</td>
<td>acggtaaaccaaggatgctgcagcagg</td>
<td>agcgacagctgtgacccag 123 74.5 97.8</td>
<td>134</td>
<td>73.5</td>
<td>99.7</td>
</tr>
<tr>
<td>KTI</td>
<td>accatgtaagatgatgcagctgg</td>
<td>agcgacagctgtgacccag 123 74.5 97.8</td>
<td>123</td>
<td>74.5</td>
<td>97.8</td>
</tr>
<tr>
<td>DFR</td>
<td>cggatcatttgctgaatctgctgactacttgatgcttttgcgagcagcttgccag</td>
<td>agtgtagctgtgccagcaccgtccag 115 75.0 108.1</td>
<td>115</td>
<td>75.0</td>
<td>108.1</td>
</tr>
</tbody>
</table>
two steps. The total RNA was first reverse transcribed to cDNA with an AffinityScript™ QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA) and 1 µg of the total RNA. Quantitative PCR was done in a total reaction volume of 25 µl containing 10× PCR buffer, 3.5 mM of magnesium chloride, 300 nM of each primer, 300 nM of probe, 0.8 mM dNTP, 30 mM of reference ROX dye, 1.25 U of SureStart Taq DNA polymerase and 1 µl of cDNA template. A two-step amplification protocol started with 10 min of denaturation at 95 °C and was followed by 40 cycles of 15-s denaturation at 95 °C and 1-min annealing and synthesis at 60 °C. Amplification and fluorescence data collection was done in optical 96-well plates on an Mx3000P® System (Stratagene). All PCR reactions were run in three technical replicates.

Data analysis and statistics

Raw fluorescence data (R) were normalized to ROX reference dye (R_R) and the amplification plots were corrected employing the adaptive baseline algorithm (ΔR_R) in MxPro™ QPCR software (Stratagene). Amplification-based threshold was used to obtain threshold cycle values (C_t) in the same program. Standard curves were created using five dilutions of PCR products. The real-time PCR efficiency values were calculated from the slope according to the equation \( E = 10^{-\frac{1}{\text{slope}}} \) in REST-384© v.2 software ((http://www.gene-quantification.info/) (Pfaffl et al. 2002). The same software was adopted to calculate relative concentrations of target mRNA (expression ratios) using the ΔΔC_t method. The calculated expression ratios were tested for significance by a pair wise fixed reallocation randomization test. Each of the five time points was analyzed separately; gene expression was related within the same plant to time 0.

Results

Expression of KTI

All stressed plants responded to the simulated herbivory by a statistically significant upregulation of KTI gene expression (Table 2, Figure 1a). In Replicates II and III, the levels of KTI transcript abundance peaked 24 h after treatment with an ~34-fold increase and then gradually decreased. In Replicate I, KTI gene was upregulated five times at the 36-h time point and significantly downregulated at the 96-h time point. No control plant showed upregulation of the KTI gene 24 h after treatment (Table 2, Figure 1a). There was on average a 2.4-fold increase of transcript abundance 36 h after treatment. This transient change was statistically significant in Replicate II, followed by a significant decrease in all replicates.

In untreated plants, expression pattern of the KTI gene greatly resembled the one of healthy controls (Table 2, Figure 1a). Untreated suckers did not respond to the simulated herbivory immediately after treatment, nevertheless they showed a similar transient increase of KTI transcript abundance 36 h after treatment as well as a similar drop at the 72-h time point.

Expression of DFR

Expression of the DFR gene was significantly upregulated in all stressed plants (Table 3, Figure 1b). Number of DFR transcripts started increasing at first sampling 24 h after treatment and reached the maximum levels at the 36-h time point. No significant increase of DFR mRNA abundance was observed in either control or untreated plants 24 h after treatment. Similar to the KTI expression pattern, there was a transient but

Table 2. Temporal pattern of KTI relative expression in control (C), stressed (S) and untreated (UT) plants of P. tremuloides after simulated herbivory.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Replicate I</th>
<th>Replicate II</th>
<th>Replicate III</th>
<th>Average</th>
<th>SE</th>
<th>Replicate I</th>
<th>Replicate II</th>
<th>Replicate III</th>
<th>Average</th>
<th>SE</th>
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<tbody>
<tr>
<td>Sampling time (hours after treatment)</td>
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</tr>
<tr>
<td>24</td>
<td>–2.7</td>
<td>–1.5</td>
<td>–0.8</td>
<td>–2.1</td>
<td>0.8</td>
<td>–1.5</td>
<td>–0.6</td>
<td>–0.6</td>
<td>–1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>S</td>
<td>3.31</td>
<td>3.48</td>
<td>33.82</td>
<td>24.0</td>
<td>18.0</td>
<td>1.7</td>
<td>5.1</td>
<td>4.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>UT</td>
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<td>–1.0</td>
<td>–0.3</td>
<td>–1.21</td>
<td>1.0</td>
<td>–1.1</td>
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<td>0.8</td>
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<tr>
<td>36</td>
<td>2.1</td>
<td>3.71</td>
<td>1.3</td>
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<td>1.9</td>
<td>4.4</td>
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<tr>
<td>S</td>
<td>5.1</td>
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<td>12.9</td>
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<tr>
<td>72</td>
<td>–12.82</td>
<td>–4.21</td>
<td>–7.22</td>
<td>–8.1</td>
<td>4.4</td>
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<tr>
<td>S</td>
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<tr>
<td>UT</td>
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<td>–1.6</td>
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<tr>
<td>S</td>
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<td>UT</td>
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<td>–1.3</td>
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<td>1.2</td>
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</tr>
</tbody>
</table>

1Expression of KTI gene significantly different (P < 0.05) from time 0.
2Expression of KTI gene significantly different (P < 0.001) from time 0.
statistically significant increase of DFR transcript abundance in both untreated and control plants 36 h after treatment.

**Discussion**

In this work, we found no evidence that interconnected ramets of trembling aspen are able to systemically induce resistance upon herbivore simulated attack. While wound-treated ramets showed systemic induction of defense genes, untreated interconnected suckers produced an expression pattern similar to controls. This was the first study attempting to investigate clonal networks with respect to information sharing among root systems in long-living tree species. Even though trembling aspen comprises an important component of the boreal forest (Bradshaw et al. 2000) and as many aspects of its biology have been thoroughly studied, root connections in this clonal species and their ecological significance have received considerably less attention.

It is unlikely that the lack of systemic defense induction in sibling ramets was caused by factors such as marker gene selection. Kunitz-type trypsin inhibitor is among the genes most strongly induced in both mature and young leaves and in almost all plant tissues including roots (Major and Constabel 2007). Dihydroflavonol reductase is a marker for the biosynthesis of condensed tannins (Robbins et al. 1998). Their function as feeding deterrents and their formation was repeatedly shown to be induced by wounding and insect attacks (Osier et al. 2000, Osier and Lindroth 2001, Kao et al. 2002, Peters and Constabel 2002, Tsai et al. 2006). Thus, both genes represent good markers of the systemic defense response.

We used a highly sensitive and robust detection method. Quantitative real-time PCR assays using gene-specific primers showed a significant upregulation of the selected genes in systemic leaves of wound-treated aspen ramets. These ramets showed upregulation by more than 30 and 80 times in comparison with the same plant before treatment. Even though the systemic defense induction tends to be much weaker than the local response (Haruta et al. 2001a, Christopher et al. 2004), the plier-wounding we applied was sufficient to provoke a profound systemic effect. We observed a rather substantial variation of upregulation intensity among biological replicates. As the plants used were genetically identical, we assume that this variation can be attributed mainly to differences in damage applied to leaves. It was shown that damaging different proportion of leaves produced varying intensity of defense response (Haruta et al. 2001a). Hence, among-replicate variation could be diminished by better standardization of wound treatment.

In addition, we assume that the lack of defense response in interconnected sibling ramets was not caused by time point selection in this experiment. Our sampling times stretched from 24 to 96 h after mechanical wounding. With respect to DFR gene expression, Peters and Constabel (2002) did not observe any delay between local and systemic upregulation. DFR transcripts started accumulating in both local and systemic leaves 12 h after treatment and reached the maximum levels after 24 h. Similarly, Haruta et al. (2001a, 2001b) observed only a small time lapse of KTI gene expression in systemic and local leaves. While a local increase in mRNA levels was detectable already 6 h after treatment, systemic leaves started upregulating KTI gene expression only at the 24-h time point. Moreover, 24 h after treatment, KTI mRNA was also detectable in roots of damaged plants (Major and Constabel 2007).
Table 3. Temporal pattern of DFR relative expression in control (C), stressed (S) and untreated (UT) plants of P. tremuloides after simulated herbivory.

<table>
<thead>
<tr>
<th>Sampling time (hours after treatment)</th>
<th>Factor</th>
<th>Replicate I</th>
<th>Replicate II</th>
<th>Replicate III</th>
<th>Average</th>
<th>SE</th>
<th>Replicate I</th>
<th>Replicate II</th>
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<th>Average</th>
<th>SE</th>
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<tbody>
<tr>
<td>24</td>
<td>C</td>
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<tr>
<td></td>
<td>S</td>
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<td>50.4(^1)</td>
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<tr>
<td></td>
<td>UT</td>
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<td>-2.6</td>
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<td>0.7</td>
<td>-0.6</td>
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<td>96</td>
<td>C</td>
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</table>

\(^1\)Expression of KTI gene significantly different \((P < 0.05)\) from time 0.
\(^2\)Expression of KTI gene significantly different \((P < 0.001)\) from time 0.
As far as we know, there is no available information on volatile-induced K713 gene expression. As even the control plants responded with defense induction, other factors cannot be excluded.

This study has focused specifically on the expression of two representative genes of poplar defense after herbivory simulated by mechanical wounding. It has been shown that responses to mechanical wounding and insect feeding are qualitatively similar; nonetheless, they differ quantitatively (Major and Constabel 2006). To clarify the role of aspen root networks in anti-herbivore defense, further studies employing real insect feeding are needed.

Conclusion

Even though root connections can trigger physiological responses to defoliation in non-defoliated aspen suckers (Baret and DesRochers 2011), our results suggest that these root pathways do not automatically lead to induction of defensive traits that are expressed in plants directly under herbivore attack. Rather, it seems that other communication means such as airborne volatiles can serve as signal transmission pathways among neighboring plants as indicated by a transient mRNA accumulation of defense marker genes.

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Conflict of interest

None declared.

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References


