A metabolomic assessment of NAC154 transcription factor overexpression in field grown poplar stem wood

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Field grown transgenic and wild type poplar wood extracts were analyzed by LC-MS. NAC154 over-expression resulted in chemotypic and phenotypic changes, where arginine levels correlate with phenotype, suggesting NAC154 may be involved in dormancy/senescence processes.

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Abstract

Several xylem-associated regulatory genes have been identified that control processes associated with wood formation in poplar. Prominent among these are the NAC domain transcription factors (NACs). Here, the putative involvement of *Populus NAC154*, a co-ortholog of the Arabidopsis gene *SND2*, was evaluated as a regulator of "secondary" biosynthetic processes in stem internode tissues by interrogating aqueous methanolic extracts from control and transgenic trees. Comprehensive untargeted metabolite profiling was accomplished with a liquid chromatography-mass spectrometry platform that utilized two different chromatographic supports (HILIC and reversed phase) and both positive and negative ionization modes. Evaluation of current and previous year tissues provided datasets for assessing the effects of *NAC154* overexpression in wood maturation processes. Phenolic glycoside levels as well as those of oligolignols, sucrose and arginine were modulated with phenotypic and chemotypic traits exhibiting similar trends. Specifically, increased levels of arginine in the *NAC154* overexpressing tissues supports a role for the transcription factor in senescence/dormancy-associated processes.

Keywords: poplar; *Populus tremula* x *P. alba*; Salicaceae; wood; arginine; LC-MS; metabolomics; NAC transcription factor

1. Introduction

The manipulation of both growth and maturation processes in woody tissues has the potential to increase biomass productivity as well as provide targeted wood and biomass end products (Costa et al., 2013; Van Acker et al., 2014; Wilkerson et al., 2014). Such research endeavors require tools that evaluate wood biochemical processes, which oftentimes necessitates the use of analytical methodologies that allow for assessment of metabolite pools. One technique that can provide such insights into woody tissue growth and maturation is liquid chromatography-mass spectrometry (Morreel et al., 2006; Robinson and Mansfield, 2011). Use of such technologies can identify features (or ions) that are significantly correlated with sample type. That said, feature and metabolite identification processes oftentimes have two problems, each with specific challenges. While it is relatively straightforward to identify ions that are different between treatments or genotypes, the identification process is complicated in poplar metabolomics by the lack of authentic standards and a species specific mass spectrometry database, as are available for plants such as rice, soybean, wheat and Arabidopsis (Matsuda et al., 2009).

NAC transcription factors (TFs) are plant-specific modulators of developmental processes. Their name is derived from the concatenation of three separate genes; *NO APICAL MERISTEM* (from petunia), *ATAF1/ATAF2*, and *CUP-SHAPED COTYLEDON2 (CUC2)*; the latter two in Arabidopsis. As a class, NAC proteins possess a conserved N-terminal DNA binding domain with a C-terminus containing an activation domain (Aida et al., 1997; Duval et al., 2002; Ernst et al., 2004). A recent genomic analysis of *Populus* identified 163 NAC genes that clustered into

18 families (Hu et al., 2010). Grant and coworkers recently evaluated two types of NAC domain TFs, those that positively and negatively regulate secondary cell wall synthesis (Grant et al., 2010). *NAC154* (one of two *Populus* co-orthologs of *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN2*, *SND2*; Potri.017G016700; UniProt ID: B9MYC7) expression was observed in both developing secondary xylem and phloem fibers, and overexpression of *NAC154* resulted in greenhouse-grown trees exhibiting reduced height, more phloem-cambial tissue and less xylem when compared to control plants. The gene has been implicated in tension wood related processes (Andersson-Gunneras et al., 2006; Grant et al., 2010), and interestingly, conversion of *NAC154* to a transcriptional repressor was recently reported to also provide a reduced growth phenotype (Wang et al., 2013). Research efforts with poplar NACs as well as the Arabidopsis co-ortholog *SND2/ANAC073* suggest a hierarchical transcriptional framework where *SND2* expression is controlled to some degree by *SND1* (Zhong et al., 2010; Zhong et al., 2008; Zhong et al., 2011).

The absence of poplar metabolomic databases along with the lack of available information on the effect of NAC TF over-expression on field-grown trees (*P. tremula* x *P. alba* clone 717-1B4) led us to evaluate the extractable metabolites present in stem internode tissues of field-grown trees produced by control and *NAC154* overexpressing trees (N154OE) in the year of harvest (2012) as well as in the previous year (2011). The metabolomic analyses were combined with field evaluations to directly compare the chemotype with the observed phenotype. The datasets obtained are available at the MetaboLights (Haug et al., 2013) data repository (Accession: MTBLS119).

2. Results and Discussion

2.1. Protocol Development

A metabolic profiling experiment by LC-MS is essentially an in-depth analysis of pre-selected time windows. As such, there are several technical issues that must be considered prior to sample collection. In order to gain some insight into the processes associated with perennial tissues, stem internodes were analyzed from current year biosynthesis as well as tissues initially produced the previous year. To control for temporal changes inherent in woody tissues transferring metabolites in a time-dependent fashion, all samples were collected at the same time of day (mid-morning) in late August, presumably when most growth and cell elongation processes have ceased in wood forming tissue. To ensure consistency in developmental stages among the trees sampled, stem wood selection (internodes) was based upon the leaf plastochron index (LPI) (Larson and Isebrands, 1971), an assessment that relies upon morphological development rather than age (**Fig. 1**). Field-collected internodes were placed in dry ice and returned to the laboratory at which point they were freeze-dried to a constant weight. The samples were then ground to a fine powder and extracted with a mixture of methanol:water (1:1, v/v). We chose

not to separate the phloem and xylem as quantitatively separating these tissues with stems harvested in late summer is quite difficult.

A rapid yet informative assessment of the aqueous methanol extracts was next carried out. Short chromatographic separations (less than 10 minutes), utilizing UPLC separation technologies were coupled to a mass spectrometer with a rapid duty cycle and high mass accuracy (Waters SynaptG2-S HDMS). Separation methodologies relied upon gradient-based reversed-phase (C₁₈) and hydrophilic interaction (HILIC) chromatographies, with each biological sample analyzed in triplicate. Analyses were initiated by several injections of a "master mix" comprised of equal volumes of each sample type to equilibrate the system, followed by randomized injections to complete one LC-MS mode analysis. All individual runs in each analysis mode were comprised of two separate MS experiments, the main experiment being a parent ion scan (0.185 sec scan, 0.015 sec delay) in the mass range of 50-1800. The second experiment was the lock mass scan of leucine-enkephalin (0.10 sec scan, 0.10 sec delay), which occurred once every 20 sec. These analyses were followed by a set of MS^E analyses (data-independent high energy fragmentation) at several levels of energy to assess fragmentation patterns, which aid in compound identification. Once an ionization mode was completed, the mass spectrometer was switched from one ionization mode to the other, employing several blanks injections prior to the samples. A re-injection of a sample after the runs were completed indicated that there was no loss of sample integrity from the start to the completion of the LC-MS work. Once the analysis of all samples in one chromatography mode was completed, the UPLC system was switched to the other chromatography mode and the analyses in both ionization modes was repeated along with the MS^E experiments.

2.2. LC-MS Overview and Structural Assignments

Representative chromatograms in base peak mode are shown in **Fig. 2** with selected structures shown in **Fig. 3**. The methods of separation are orthogonal in nature as is indicated by the elution of tremulacin (**4**) near the end of the reversed-phase separation, and near the beginning of the HILIC separation. Sucrose (**10**) and arginine (**11**) eluted near the end of separation in HILIC mode whereas the compounds eluted at the solvent front in reversed-phase mode. It can also be seen that samples analyzed by HILIC fractionations and positive ion modes tend to have increased peak widths relative to reversed-phase and negative ion modes. Positive electrospray ionization (ESI) tended to favor [M+H]⁺, [M+Na]⁺ and associated cluster ions (for example [2M+Na]⁺) whereas the negative mode ESI favored [M-H]⁻ and [M+formate]⁻ and associated clusters. In-source decay was observed with many metabolites, especially in positive ion mode, and in general [M+Na]⁺ and [M-formate]⁻ were resistant to fragmentation in the MS^E (data independent acquisition/fragmentation) and targeted MS² experiments. Efforts to minimize [M+Na]⁺ adduct formation by ion exchange were not successful. Salicortin (**5**) and tremulacin (**4**) have been reported as the major acetone-soluble phenolics in *Populus tremuloides* internodes

(Clausen et al., 1989), and based upon the ion intensities found in the extracts, they are abundant molecules in the methanolic extracts of control and overexpressing internodes as well. All ions identified in this study and their corresponding peak areas can be found in the Supplemental Materials.

Most of the compounds identified in this work are based upon exact mass determinations and fragmentation patterns, with efforts focused on ions that differed in abundance between the control and N154OE lines. Use of both positive and negative ion modes provided a means to assign $[M+H]^+$ and/or $[M-H]^-$ in an unambiguous way, avoiding the assignment of the predominant ion as either $[M+H]^+$ and/or $[M-H]^-$ when it was an adduct ion. Fragmentation studies beyond the initial MS^E studies were approached in a stepwise manner. When $[M+H]^+$ and/or $[M-H]^-$ ions were readily apparent, they were submitted to fragmentation studies. When resistant adduct ions were encountered, manual searches for the weaker corresponding $[M+H]^+$ or $[M-H]^-$ ion were carried out to determine if they were present in high enough levels to obtain fragmentation data. If not, the orthogonal chromatography mode was evaluated, relying upon the reproducible nature of UPLC-MS for locating the ion(s) of interest.

The assignments of some features required a shallower gradient in order to separate isobaric or "near-isobaric" metabolites. One interesting case was that of salicortin (**5**) and the grandidentatin/purpurein diastereomers (**2**) (**Fig. 4**). Salicortin can be envisioned as being formed via acylation of salicin (**1**) with the 1-hydroxy-6-oxocyclohex-2-enecarboxylic acid (HCH group), whereas the grandidentatin/purpurein diastereomers can be derived from the *p*-coumaroylation of the β -glucopyranoside of *cis*-1,2-cyclohexandiol. The pathways for both HCH and 1,2-dicyclohexanol biosynthesis have not been established. These two classes of phenolic glycosides differ in monoisotopic mass by 0.0364 Da, a result of being derived from different phenolic glycoside precursors (Babst et al., 2010; Boeckler et al., 2011). Selected mass scans for each of these compounds in the negative ion mode along with an extended solvent gradient (to better separate the metabolites) indicated that multiple isobaric peaks were present at each mass (**Fig. 4**). The multiple peaks could be derived either from the intact molecules as $[M-H]^-$, diastereoisomers, regioisomers differing sites of acylation, or from in-source decay of larger metabolites such as tremulacin (**4**). The HCH moiety of salicortin-type molecules could be detected in fragmentation studies (targeted MS^2 or MS^E) by the presence of the 1-hydroxy-6-oxocyclohex-2-enecarboxylate anion (calc. 155.0350, obsd. 155.0348) whereas the characteristic feature of the *p*-coumarate derivatives (*cis* or *trans*) was the presence of the dehydrated anion of *p*-coumaric acid (calc. 145.0295, obsd. 145.0295).

A review of the literature indicates that the stereochemistry about the 1-position of the salicortin-type HCH group has not been established for poplar metabolites, although a related dimer from the fruit of *Idesia polycarpa* was reported to have an *S*-configuration (Yamakoshi et al., 2010). In the case of grandidentatin/purpurein (**2**), the structures were originally reported as

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4 diastereomers of 1,2-*cis*-cyclohexandiol (Pearl and Darling, 1962, 1970); thus the exact
5 stereochemical assignments are not established. More recently, related metabolites have been
6 isolated and characterized that place the *p*-coumaroyl ester on both the 4- (Si et al., 2009) and 6-
7 (Bae and Kim, 2009) positions of the carbohydrate, however there is no information available
8 regarding the cyclohexanediol stereochemistry.
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10 11 12 13 *2.3. Significant Features in the NAC154 Overexpressing and Control Internodes and* 14 *Comparison to Field Phenotypes* 15

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17 The raw data collected from the four sets of analyses (reversed-phase and HILIC in both positive
18 and negative ion modes) were processed by MarkerLynx (Waters) software to identify ions that
19 met selection criteria related to ion peak width and minimum thresholds over the mass range of
20 50-1800 amu (see Supplemental Methods). The application of the selection criteria resulted in
21 deisotoped datasets ranging from 384 ions in the reversed-phase negative mode data to 1277 ions
22 in the HILIC positive mode data (**Fig. 1** and Supplemental Materials). Many of these ions
23 corresponded to the same metabolite due to both adduct formation and in-source decay. In order
24 to assess the overall data quality and experimental variability, Principal Components Analysis
25 (PCA) of the HILIC data from the N154OE internodes for variation associated with field
26 location and between internodes was performed (**Fig. 4**). While there was little correlation
27 between field location and the N154OE transgenics, there was a clear separation of the
28 transgenics based upon year of growth. These results suggest that the local conditions within the
29 plot were the same for all trees, indicating this is not a key variable for further analysis.
30 However, the internode growth year resulted in a clean PCA separation, indicating that
31 comparing across the year of deposition (current vs. previous year wood) may provide insight
32 into wood maturation processes.
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41 The main ions/metabolites that were found to be different and detected as parent ions or adducts
42 are shown in **Table 1**, arranged according to metabolite and LC-MS method. Three comparisons
43 are shown, two compare the N154OE and wild type (WT) lines based upon initial year of
44 growth, and the third compares current versus previous year wood regardless of genotype. In
45 general, if an identified metabolite was found to be significantly different in both HILIC and
46 reversed-phase analyses, they trended in the same direction, supporting the claim that the
47 developed protocol is robust. A case in point is *p*-coumaroyl β -D-glucopyranoside (**8**), which
48 eluted coincidentally at 2.81 minutes in both the HILIC and reversed-phase (RP) separations.
49 This metabolite was present in higher levels in previous year growth tissue relative to current
50 year wood when compared regardless of genotype.
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56 When comparing the N154OE lines with the controls for current year growth, the metabolites
57 identified were generally lower in abundance in the transgenic lines. The main exception to this
58 trend was arginine (**11**), which was detected in 20-fold higher amounts in the HILIC-MS positive
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ion mode analyses (**Table 1**) (discussed below). Field phenotype and laboratory chemotype data are further compared in **Fig. 5**. Overexpression of *NAC154* generated a set of transgenic trees with varying degrees of height and stem diameter reductions relative to the WT lines (**Fig. 5A**). The trees with *NAC154* overexpressed also exhibited smaller composite leaf angles, meaning that adaxial leaf surfaces (top face of the leaf) were closer or more upright to the main stem relative to the controls. Chemotypic assessment of the same set of trees by PCA of the reversed-phase separations in positive ion mode for the same transgenic trees are shown in **Fig. 5B** (for all ion modes and chromatographic separations see Supplemental Fig. S1). The older tissues (previous year wood, left panel), in particular, cluster in a way that mirrors the phenotypic data. The samples that have a greater height and stem diameter reduction are further from the control samples on the plot. The independent grouping of the samples in the older tissue PCA suggests the chemotypes and phenotypes correlate with one another.

The reduction in tree biomass due to *NAC154* overexpression could have resulted from a decreased rate of growth throughout the growing season, early growth cessation, or a combination of these two. Seasonal leaf senescence and dormancy in poplar are correlated with increasing levels of arginine (**11**) in stem wood, probably serving as an N storage compound (Couturier et al., 2010). Thus the observed increase in arginine levels suggests that the reduced size of transgenic poplar is associated with some characteristics of dormancy in stems. As can be seen in **Fig. 5A** (right panel), the arginine levels correlate with phenotypic severity. In particular, composite leaf angle (CLA) showed the strongest inverse correlation with arginine peak area ($R = -0.7937$; $p = 0.0012$), and height and diameter were moderately inversely correlated with arginine level ($R = -0.6$; $p = 0.04$; Supplemental Fig. S2). That *NAC154* overexpression is linked with increased arginine (**11**) levels is a novel discovery made possible by the untargeted metabolite profiling. This finding provides insight regarding xylem-associated TFs as mediators of crosstalk between wood formation and overall plant growth and development, and highlights the importance of investigating dormancy control as a key to domestication of trees for enhanced woody biomass production (Ragauskas et al., 2006).

Populus species accumulate high levels of phenolic glycosides as a constitutive defense against herbivores and the levels of these compounds accumulating in bark show seasonal and genotypic variation (Boeckler et al., 2011; Keefover-Ring et al., 2014). The reduced levels observed in the N154OE lines might also reflect an earlier cessation of growth relative to WT trees. While the aforementioned increase in arginine (**11**) levels and the reduced accumulation of phenolic glycosides point to the possibility of early growth cessation, we have not yet determined the basis of the connection between such chemotypic features, *NAC154* overexpression, and reduced plant growth. In addition to direct effects of *NAC154* overexpression, various abiotic stresses interacting with *NAC154* overexpression may have induced earlier growth cessation and changes in metabolites. For example, reduced photosynthetic carbon capture in the N154OE lines

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4 resulting from altered light interception by more vertically oriented leaves (**Fig. 5A**), could
5 reduce growth and affect metabolites.
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9 If a metabolite was found to be higher in abundance in WT wood in the current year samples, in
10 general the trend remained in the older tissues. However, the total number of metabolites that
11 were significantly different between the WT and N154OE transgenics was greater in the
12 previous year wood, (13 vs. 9; **Table 1**) with most of the additional identified metabolites (e.g.,
13 pinoresinol hexoside, **12**) found to be present in higher amounts in the N154OE tissues. Notably,
14 in contrast to current year wood, arginine (**11**) levels did not differ for WT vs. N154OE
15 transgenics in previous year wood (**Table 1**; compare WT:OE, Prev Yr to WT:OE, Curr Yr).
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20 Differences in metabolite levels occurring between WT and N154OE lines were highlighted
21 above. Overall, however, when the transgenic lines were compared to WT in order to determine
22 the metabolites that changed in levels based upon growth years, relatively few metabolites were
23 significantly different (data not shown). Considering this observation, it can be hypothesized
24 that the features that differed in the samples were related more to growth year than *NAC154*
25 overexpression and consequently all genotypes were analyzed as a group against growth year.
26 This analysis (**Table 1**, last column) provided multiple differences including higher levels of
27 oligolignols in the older wood (Morreel et al., 2010; Morreel et al., 2004). The overall
28 oligolignol profile obtained was similar to that reported previously for greenhouse-grown trees
29 (Morreel et al., 2004) although the relative peak abundances were different. This could be due to
30 actual ratio differences, the different methods of extraction and analysis, or a combination of the
31 above. That oligolignols are more prevalent in previous year wood relative to current year wood
32 suggests that lignification processes differ across growth years. It was also determined that
33 sucrose (**10**) levels were higher in current year relative to previous year wood samples (**Table 1**),
34 but were not significantly correlated with *NAC154* over-expression. This may be a mass balance
35 issue as there is more non-living tissues in previous year wood, which would presumably lack
36 sucrose, making the levels lower in the older tissues. However, seasonal cessation of wood
37 formation in poplar is associated with reduced sucrose levels (Deslauriers et al., 2009) and older
38 wood may reach the cessation stage prior to new growth.
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48 The metabolomics data permits the generation of several hypotheses concerning the connection
49 between *NAC154* function and the observed decreases in leaf angle and tree size. Leaf
50 development in poplar and many eudicots is characterized by a gradual shift in orientation from
51 upright leaf primordia toward horizontal or even pendulous orientation at maturity. Considering
52 the observed increase in arginine content of the N154OE trees as a marker for earlier growth
53 cessation, several aspects of growth and development, including attainment of normal orientation
54 of mature leaves, may have been inhibited by early dormancy in the transgenic trees.
55 Overexpression of transcriptional repressor chimeras *SND2-SDRX* in *Arabidopsis* (Zhong et al.,
56 2008) and *NAC154-SDRX* in poplar (Wang et al., 2013) reportedly reduced xylem cell wall
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thickness. However, a complementary increase in xylem cell wall thickness in N154OE plants (Grant et al., 2010) was not observed, as would be predicted based on results from the dominant repression experiments. Thus it appears that *NAC154* is not simply a positive regulator of xylem cell wall thickness. Moreover, the observations that both dominant repression and overexpression of *NAC154* resulted in reduced plant size (Grant et al., 2010; Wang et al., 2013), and that downregulation of *NAC154* is associated with tension wood production (Andersson-Gunneras et al., 2006; Grant et al., 2010) raise additional questions about the role(s) of *NAC154* and the possibility that the absolute level of expression and species-specific effects are important determinants of the phenotypes of *NAC154* transgenic lines (Hussey et al., 2011). Additional loss- and gain-of-function experiments with *NAC154* are necessary to more precisely determine the role of this xylem-associated NAC domain TF and the target pathways responsible for the metabolite profiles and growth-suppressive effects reported here.

3. Conclusions

The availability of the complete poplar genome sequence coupled with facile transgenesis has made poplar a useful model for understanding wood biochemical processes. As research moves from the laboratory to the field, baseline chemotypic information is required to assess the metabolic outputs of targeted gene manipulation as well as the effects of environmental variables on poplar growth and development (Keefover-Ring et al., 2014). As little information was available for internode wood metabolites via LC-MS approaches at the start of this investigation, it was instructive to obtain and make accessible a poplar metabolome database, and in doing so also evaluate the effects of the over-expression of a TF involved in wood maturation. The analysis of methanol:water extracts from whole internodes using a non-targeted approach was used to compare and contrast the effects of *NAC154* overexpression in stems over a two year growth period. The use of UPLC technology coupled to a rapid-scanning high mass accuracy mass spectrometer allowed for the development of an exact mass-retention time map for poplar internode wood extracts. Statistical analysis of the predominant ions in the datasets indicated clear differences between growth years as well as with N154OE, with the data suggesting possible roles for *NAC154* in growth cessation processes. There was a direct correlation between arginine (**11**) levels and field phenotype (height and composite leaf angle) clearly demonstrating that chemotypic evaluation of field grown poplar can be used in combination with phenotypic data to drive further understanding of wood biochemical processes. The dataset obtained (MetaboLights: MTBLS119) provides an initial resource for further annotation of the poplar metabolome as well as aiding in further studies on the effects of environmental conditions and/or genetic manipulation on poplar growth and development.

4. Experimental

4.1. Phenotypic assessment of poplar transgenics

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5 *P. tremula* x *P. alba* clone 717-1B4 was the genotype used for all studies and the production of
6 transgenics overexpressing *Populus NAC154* (N154OE transgenics) as previously described
7 (Grant et al., 2010). The transgenic events generated (i.e., independent insertion events) and
8 non-transgenic WT control plants were propagated in tissue culture, acclimated to soil and grown
9 in a glasshouse for two months before being planted at a field site in Critz, Virginia, USA, in
10 May 2010. Ten ramets of WT trees and four ramets of each N154OE transgenic event were
11 transplanted in two pairs, with each pair being placed randomly on the site at a 2.4 x 2.4 m
12 spacing. Additional types of transgenic poplars (i.e., with different transgenes) were included
13 (data not shown) in this study for a total of 260 test trees surrounded by two border rows.
14 Herbicide application and manual cultivation were used to control weeds. Tree heights were
15 measured using a height pole, and stem diameter (5 cm above ground level) using a caliper in
16 October 2012. ImageJ was used to measure angles of petioles and blades of leaves using
17 photographs taken in October 2011. For each leaf, two angles were measured: 1) between the
18 adaxial side of the leaf petiole and the main stem, and 2) between the adaxial side of the leaf
19 blade and a line extended from the petiole. The resulting values were summed for each leaf to
20 yield a composite leaf angle (CLA). Thus the smaller the CLA value, the closer the adaxial leaf
21 surface was to the main stem. Statistical analyses were done using JMP Pro 10 ((SAS Institute,
22 Inc.). One-way analysis of variance (ANOVA) and unpaired t-test was used to determine the
23 effect of the transgene (*35Spro:NAC154*). The response (Y-data) was the average of the two
24 ramets in a pair, resulting in two independent data points for each transgenic event, and five data
25 points for the control trees. To estimate and test differences between transgenic event and WT
26 means, we used the LSMEANS protocol and Dunnett's adjustment.
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38 4.2. *Poplar internode sample preparation*

39 Samples were collected from the field according to the leaf plastochron index (LPI), tagged and
40 frozen in dry ice. For current year shoot segments, LPI 1 was defined as the first leaf below the
41 shoot tip that was at least 1 cm in length (internode 1 would be directly above this), and counted
42 down to collect internode 12. For the previous year growth, internode 1 was assigned as below
43 the terminal bud scar, counting down the stem to internode 7. Since trees showed different
44 amounts of growth (number of internodes) in a given year, a continuous internode counting
45 down from the shoot tip to the previous year's growth was not applicable. Samples in the
46 laboratory were stored at -80 °C prior to processing, which was initiated by freeze-drying to a
47 constant weight. Twigs were flash frozen in N₂ and subsequently ground in a P14 mill
48 (Pulveristette 14; Fritsch) with a 0.5 mm sieve at maximum speed (20,000 rpm) to generate a
49 fine powder. The samples were collected in labeled, pre-weighed 1.5 mL centrifuge tubes, flash
50 frozen in N₂ and dried, after which the mass was recorded. Sample extraction was performed on
51 10 mg fractions using (50:50 LCMS grade MeOH:H₂O, 1 mL). The tube contents were mixed
52 by vortexing, placed in a shaker (Boekel Sci; Feasterville, PA) and incubated 200 minutes at 30
53 °C. The tubes were then removed, vortexed briefly and allowed to sit overnight at room
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temperature. The samples were then vortexed and centrifuged (13,000 x g, 5 min, RT) with the extracts (450 μ L) transferred to prelabeled, ethanol-washed tubes. The samples were then concentrated via centrifugal concentration until approximately 200 μ L remained and were then flash frozen in N₂ and dried overnight on high vacuum line with a N₂ trap. The resulting dried extract was then weighed to determined yield.

4.3. *Reversed-phase Separations*

The extract was reconstituted with 50 nM acetaminophen (internal standard) in 0.1% aq. HCO₂H:CH₃CN (9:1, v/v) with the volume based upon the weight of the dried extract (100 μ L/mg dry extract). Dissolution was aided by vortexing followed by sonication (10 minutes). The samples were then centrifuged (13,000 x g; 10 min; RT) and an aliquot was removed (100 μ L) making sure not to disturb any precipitate. The sample was further diluted (10x) with 50 nM acetaminophen in 0.1% HCO₂H:CH₃CN (9:1, v/v) and transferred to a LC-MS grade autosampler vial. A "MasterMix" was constructed from a single representative sample from each class. This sample is used to equilibrate the LC-MS system and is also used for statistical validation.

Metabolite separations were performed with an UPLC (Waters I-Class) equipped with a BEH C18 column (1.7 μ m; 2.1 mm $\times$ 50 mm; Waters). The mobile phases were H₂O with 0.1% HCO₂H (Solvent A) and CH₃CN (Solvent B) at 200 μ L/min. The separation initially started with 95% A (0-1 min) with a linear ramp to 85% A (1.0-1.5 min), followed by a second ramp to 40% A (1.5-6.9 min). The gradient was rapidly ramped to 10% A (6.9-7.0 min) where it was held for 0.5 min (7.0-7.5 min). Re-equilibration involved ramping back to 95% A (7.5-8.0 min) and holding at 95% A for 2 min (8.0-10.0 min). Injection volumes were 1 μ L, the column temperature was 40 °C, with the autosampler maintained at 8°C. Under these conditions acetaminophen elutes at 2.07 min (m/z = 152.0708 in positive mode and m/z = 150.0556 in negative mode). Leucine-enkephalin was used as lock mass, scanning for 0.1 sec every 20 sec. Extended run conditions (Fig. 3) were as follows: 95% A (0-1 min) with a linear ramp to 50% A (1.0-20 min), followed by a second ramp to 1% A (20-25 min) and a one minute hold. The gradient was then ramped to 95 % A in one minute where it was held for 3 min (for re-equilibration).

4.4. *HILIC Separations*

The extract was reconstituted with 0.1% HCO₂H:CH₃CN (1:1, v/v) with the volume based upon the weight of the dried extract (100 μ L/mg dry extract). Dissolution was aided by vortexing followed by sonication (10 minutes). The samples were then centrifuged (13,000 x g; 10 min; RT) and an aliquot (100 μ L) was removed making sure not to disturb any precipitate. The sample was further diluted (10x) with 0.1% HCO₂H:CH₃CN (1:1, v/v) and transferred to a LC-MS grade autosampler vial with an insert. A "MasterMix" was constructed from a single representative sample from each class. This sample is used to equilibrate the LC-MS system and is also used for statistical validation.

Metabolite separations were performed with an UPLC (Waters I-Class) equipped with a BEH Amide (1.7 μ m, 2.1 mm $\times$ 50 mm; Waters). The mobile phases were water with 0.1% HCO₂H (Solvent A) and CH₃CN with 0.1% HCO₂H (Solvent B) at 400 μ L/min. The separation initially started with 99% B (0-0.5 min) with a linear ramp to 50% B (0.5-7.0 min). The gradient was rapidly ramped to 99% B (7.0-7.1 min) where it was held for 3.9 min (7.1-10 min). Injection volumes were 1 μ L, the column temperature was 45 °C, and the autosampler maintained at 8°C. Leucine-enkephalin was used as lock mass, scanning for 0.1 sec every 20 sec.

4.5. Mass Spectrometry

A Waters SynaptG2-S HDMS instrument was used in high-resolution mode employing the extended dynamic range feature and electrospray ionization. Capillary voltages were of 2.4 kV and 3.0 kV for negative- and positive-ion modes, respectively, with a cone voltage of 40 V. Cone and desolvation gas flows were 50 and 500 L/hr, respectively with source and desolvation gas temperatures at 125°C and 350°C, respectively. The instrument was calibrated with sodium formate from 50 to 1800 m/z . Infusion flow rate for lock mass was 5 μ L/min. Leucine enkephalin (2 ng/mL) is used as lock mass for positive (m/z 556.2771) and negative (m/z 554.2615) mode experiments with parameters set to 0.2 sec/20 sec intervals. MS spectra are acquired in centroid mode from 50 to 1800 m/z using a scan time of 0.2 s.

4.6. Ion Feature Selection, Data Processing

The commercial MarkerLynx, MetaboLynx and QuanLynx software (Waters) were used to analyze the datasets obtained. If an authentic standard was available it was analyzed under the same conditions as described above. Ions that were identified as significantly different by the processing software were first confirmed manually and subsequently submitted to fragmentation analyses. See the Supplemental for details.

4.7. Compound Identification

Standards were used when available for exact mass-retention time confirmation. These included arginine (**11**), salicin (**1**), sucrose (**10**), tremulacin, *p*-coumaryl β -D-glucopyranoside (**8**; courtesy of J. Ralph, Wisconsin). Master mix samples were also submitted the fragmentation studies using either targeted MS² or data independent fragmentation (MS^E) studies.

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Figure Captions

Fig. 1. *Overview of the Sampling and LC-MS Methodologies.* Internodes were collected according to the leaf plastochron index (LPI). For the current year shoot segment, LPI 1 was the first leaf below the shoot tip that was at least 1 cm in length. Internode 1 was directly above this leaf, and counting down provided internode 12. For the previous year growth, internode 1 was directly below the terminal bud scar, and counting down the stem provided internode 7. Data processing provided between 384-1277 individual features (ions), depending on the analysis mode.

Fig. 2. *LC-MS Separations in Reversed-Phase and HILIC Modes.* The example chromatograms are presented in base peak ion mode. The two separation methods are orthogonal in nature as seen by the elution times of tremulacin (**4**). In the reversed-phase separations, sucrose and arginine eluted at the solvent front.

Fig. 3. *Structures of Compounds.*

Fig. 4. *Salicortin and Grandidentatin/Purpurein Metabolites.* The structures of grandidentatin/purpurein (**2**) and salicortin (**5**) are shown in Panel A with key diagnostic fragmentation ions. Panel B shows the full scan base peak ion chromatogram (i) along with selected ion traces for the intact metabolites (ii, iv) and the key fragment ions (iii, v), all utilizing a reversed-phase separation and negative ion mode. The extended gradient conditions used are described in the Experimental.

Fig. 5. *Principal Components Analyses of the NAC154 Overexpressing Transgenic Lines Based Upon HILIC Separations.* **A**, Negative ion mode analyses based upon field location (top) and year of growth (bottom). **B**, Positive ion mode analyses based upon field location (top) and year of growth (bottom). Samples segregate based upon growth year but not field location.

Fig. 6. *Phenotypic and Chemotypic Assessments of the NAC154 Overexpressing Transgenic Trees.* **A**, Characterization based upon tree height, stem diameter, composite leaf angle (CLA) and arginine peak areas. **B**, Principal components analyses of the LC-MS data (reversed phase, positive ion mode) for previous and current year samples.

Table 1. Metabolites Identified as Changing in Relative Levels Due to *NAC154* Over-Expression and Growth Year.

Compound	RT	Mass	Mode	Ion	ID ¹ Level	WT:OE Current ²	WT:OE Previous ²	Curr:Prev All genotypes ²
arginine (11)	5.84	175.1194	HILIC-(+)	[M+H]	1	0.05	---	---
cinnamoyl hexoside (9)	3.82	333.0945	RP-(+)	[M+Na]	2	---	---	0.29
<i>p</i> -coumaroyl β-D-Glcp (8)	2.81	325.0920	HILIC-(-)	[M-H]	1	2.8	1.9	0.32
	2.81	325.0921	RP-(-)	[M-H]	1	---	---	0.49
	2.82	349.0896	HILIC-(+)	[M+Na]	1	---	---	0.37
grandidentatin/purpurein (2)	2.43	423.1650	HILIC-(-)	[M-H]	2	2.2	1.4	---
	2.62	447.1607	HILIC-(+)	[M+Na]	2	---	0.8	---
populoside (15)	2.85	447.1287	HILIC-(-)	[M-H]	2	---	---	0.53
populoside B/trichocarposide (17,18)	4.60	455.1311	RP-(+)	[M+Na]	2	---	---	0.50
	4.64	431.1339	RP-(-)	[M-H]	2	---	---	0.48
pinoresinol hexoside (12)	3.75	519.1862	RP-(-)	[M-H]	2	---	0.83	---
sakuranin/neosakuranin (13,14)	2.58	447.1287	HILIC-(-)	[M-H]	2	2.1	---	---
salicin (1)	2.50	309.0947	RP-(+)	[M+Na]	1	---	---	2.0
	2.90	309.0949	HILIC-(+)	[M+Na]	1	---	---	2.4
salicortin (5)	3.57	447.1260	RP-(+)	[M+Na]	2	1.2	---	---
sucrose (10)	5.04	341.1080	HILIC-(-)	[M-H]	1	---	---	1.5
	5.05	365.1058	HILIC-(+)	[M+Na]	1	---	---	1.3
tremulacin (4)	1.85	573.1605	HILIC-(-)	[M+formate]	1	---	---	2.3
	5.34	551.1525	RP-(+)	[M+Na]	1	---	1.2	---
	5.38	527.1549	RP-(-)	[M-H]	1	1.3	1.5	1.9
lignan/neolignan	2.87	431.1309	RP-(+)	[M+Na]	2	---	---	0.37
tetrameric oligolignol	5.19	833.2983	RP-(+)	[M+Na]	2	---	---	0.48
trimeric oligolignol	4.96	605.1995	RP-(+)	[M+Na]	2	---	---	0.40
sakuranin/neosakuranin (fragment)	2.60	287.0918	HILIC-(+)	[M+H]	2	3.0	2.1	---
syringic acid hexoside	2.43	359.0974	RP-(-)	[M-H]	2	---	0.71	---
Unknown (feruloylated salicin)	4.70	485.1415	RP-(+)	[M+Na]	3	---	---	0.36
Unknown (flavonone disaccharide)	2.46	617.1841	HILIC-(+)	[M+Na]	3	---	2.4	0.36
Unknown (flavonone)	3.97	287.0917	RP-(+)	?	4	3.4	0.40	---
Unknown (p-coumaroyl ester)	3.93	421.1493	RP-(-)	[M-H]	4	0.60	---	---
Unknown (benzoyl ester)	4.01	429.1155	RP-(+)	[M+Na]	4	---	1.2	---
Unknown (apocarotenoid?)	3.05	431.1912	RP-(-)	[M+formate]	4	---	0.67	---
Unknown (lusitanicoside-like)	3.74	465.1728	RP-(+)	[M+Na]	4	---	---	0.45
Unknown (lusitanicoside-like)	3.78	487.1800	RP-(-)	[M+formate]	4	---	---	0.48
Unknown (apocarotenoid?)	3.75	575.2336	RP-(-)	[M-H]	4	---	0.71	---
Unknown	5.21	591.2077	RP-(-)	[M+formate]	4	---	---	0.59
Total ions						9	13	21

¹Based on Sumner et al. (2007): 1. Identified; 2, Putatively annotated; 3, Putatively characterized; 4. Unknown compounds. ²Factors of change with p-values < 0.01 as calculated by Student's t-test.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A