How potato fights its enemies – Unsupervised MS/MS similarity metabolite profiling reveals novel defense-related metabolites in potato

Solanum tuberosum (potato) wildtype and overexpression lines with an enhanced *Phytophtora infestans* resistance [1] were analysed for their metabolic response to simulated herbivory.

Using the data acquired with Bruker LC-ESI-Q-TOF MS and MS/MS instrumentation and methods, the MetaboScape software provided an efficient platform for the straightforward linkage of structural similarities and metabolite abundances. The described data mining approach we refer to as "unsupervised MS/MS similarity metabolite profiling" enabled a rapid annotation of 17 metabolites which were previously unknown in our analyses.

Introduction

Solanum tuberosum (potato) is an important crop for human nutrition with high agronomical and cultural importance. Resistance against biotic attacks is desirable to reduce pesticide treatments

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Table 1. UPLC-MS equipment and setup for metabolite profiling

Mass Spectrometry	
Instrument	MicrOTOF-Q II
Source	Apollo II ESI source
Ionization	ESI(+)
Scan Range	m/z 50–1000
Aquisition Rate	3 Hz, autoMS/MS: 3Hz
Calibration	Internal calibration through automation at 12min, Lithium Formate, HPC, Polarity: Positive
UPLC	Waters Acquity
Column	HSS T3 (100 x 1.0 mm)
Flow Rate	0.15 ml/min
Mobile Phase	A = water + 0.1% formic acid, B = acetonitrile + 0.1% formic
Gradient	0 – 1 min 5% B 1 – 5 min to 75% B 5 – 10 min to 95% B 10 – 12 min 95% B 12 – 15 min 5% B
Data Processing	MetaboScape 3.0 and DataAnalysis 4.4

and to sustain potato quality. For a better understanding of the herbivory defense, we simulated insect biting on the plants. In addition to the wildtype cultivar Désirée we analysed the overexpression lines "*AtACT-AtDTX18*". These express a *p*-coumaroyl-CoA:agmatine N4-*p*-coumaroyltransferase from *Arabidopsis thaliana* as well as an *Arabidopsis thaliana* MATE transporter for the export of several hydroxycinnamic acid amides. The *AtACT-AtDTX18* overexpression plants are more resistant against *Phytophthora infestans*, causal agent of late blight in potato, which was the cause for Irish potato famine (1845 – 1852) and still leads to severe crop losses today. One of these hydroxycinnamic acid amides, *p*-coumaroylagmatine, inhibits spore germination of this agriconomically important oomycete [1].

Metabolic changes of the *AtACT-AtDTX18* overexpression lines after *Phytophthora infestans* infection were previously described in a targeted analysis for hydroxycinnamic acid amides [1]. Here, untargeted metabolite profiling was applied with focus on the early metabolic responses to herbivory, which was simulated by wounding the plant leaves with tweezers. We further addressed the question, whether the wounding responses in the overexpression lines with higher *Phytophthora infestans* resistance differ from those of the wildtype plants.

Metabolite profiling always reveals many peaks of unknown metabolite structures. Thus, we used this data set for an approach we refer to as "MS/MS similarity metabolite profiling". With the help of MetaboScape we annotated numerous compounds based on their MS/MS and thereby extended our knowledge on potential potato defense metabolites.

Experimental

Potato plants (*Solanum tuberosum* L. cv. Désirée and *AtACT-AtDTX18* OE) were generated and grown for 4 weeks in a phytochamber as described in Dobritzsch et al. [1]. For simulation of herbivore biting, leaves were wounded with tweezers and approximately 500 mg of leaf material was collected after 8 hours. Leaf metabolites were extracted twice with 200 µl 80% methanol. The consecutive extracts were pooled, evaporated and redissolved in 30% methanol with the final volume adjusted to the sampled material (final concentration of 50 mg fresh weight per 100 µl extract). Extracts were subjected to UPLC-QTOF-MS/MS analysis with devices and settings provided in Table 1. AutoMS/MS was performed on three samples of each sample group. Recalibration of the MS data was conducted on lithium formate clusters at the end of each run using DataAnalysis 4.4 (Bruker) for raw data or fully automatically by T-ReX 3D in MetaboScape 3.0 for metabolite profiling data.

Untargeted metabolite profiling was performed in MetaboScape with the settings provided in Table 2. Afterwards, autoMS/MS were matched on the created bucket table. The four replicate groups (*AtACT-AtDTX18* wounding, *AtACT-AtDTX18* no wounding, *S.t.* cv. Désirée wounding, *S.t.* cv. Désirée no wounding) were analysed with 15 to 25 biological replicates each. "Analyte Lists" with more than 2000 entries for targeted annotation were created in MetaboScape based on analytical standards and annotations in previous experiments. The confidence criteria for analyte list matches were

(annotation quality: very good/acceptable): m/z: 2 ppm/20 ppm, retention time: 0.1 min/0.2 min, mSigma: 20/60, MS/MS score: 900/800.

Pathway maps were drawn in PathVisio 3.3.0 [2] based on information from KEGG pathways [3] (arginine and proline metabolism/phenylpropanoid biosynthesis) with *Solanum tuberosum* and *Arabidopsis thaliana* as reference organisms.

Results

MetaboScape - Metabolite profiling of wounded and unwounded *Solanum tuberosum* plants

With the selected settings for peak picking (Table 2) we focused on the higher abundant leaf metabolites. The MetaboScape T-ReX 3D peak picking algorithm generated a bucket table of 440 region complete extracted features. For most of these, the MS/MS were assigned from autoMS/MS. The analyte lists provided annotations for 96 of the 440 metabolites that matched the criteria of correct retention time, m/z and isotopic pattern. The intuitive graphical annotation quality symbols enabled a fast and reliable

Table 2. MetaboScape 3.0 processing parameters

Intensity threshold [counts]	1500
Minimum peak length [spectra]	10
Minimum peak length (recursive) [spectra]	8
Minimum # Features for Extraction	31
Presence of features in minimum # of analyses	31
Lock mass calibration	false
Mass calibration	true
Calib. RT start	723
Calib. RT end	732
Filter by sample group	*S.t. AtACT-AtDTX18* overexpression vs *S.t. cv. Désirée*
Minimal value of features in sample group (type)	PERCENTAGE
Minimal value of features in sample group	75
Area	true
Primary Ion	$[M+H]^+$
Seed Ions	$[M+Na]^+$
EIC correlation	0.9
Mass range: Start [m/z]	50
Mass range: End [m/z]	1000
Retention time range: Start [min]	0.6
Retention time range: End [min]	10
Perform MS/MS import	false
Group by collision energy	false
MS/MS import method	maxsum

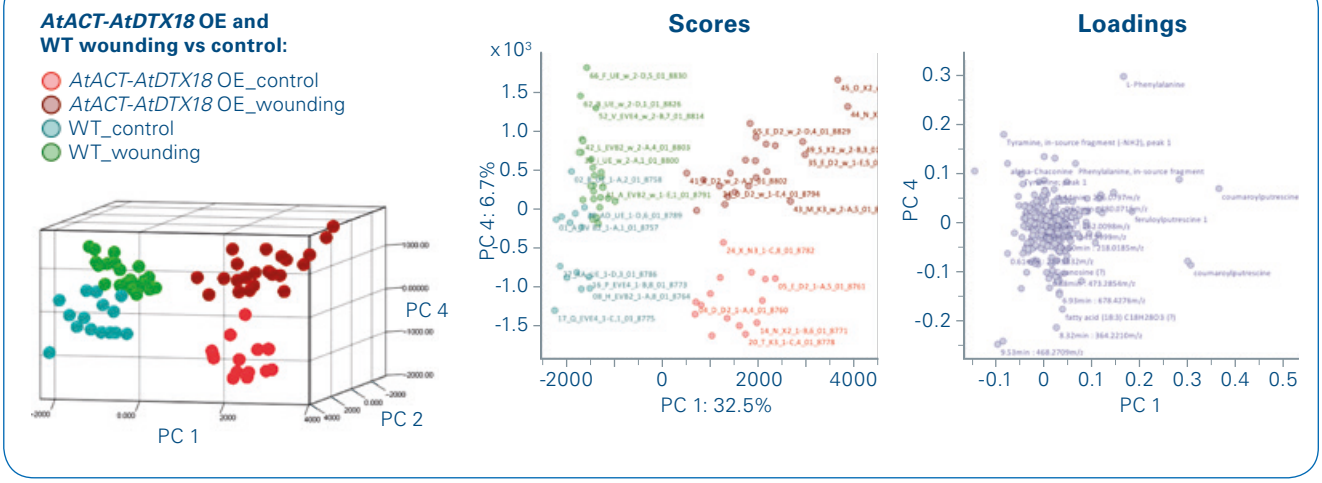

Figure 1: Principal Component Analysis of wounded and unwounded potato wildtype (WT) and AtACT-AtDTX18 *overexpression (OE) lines*

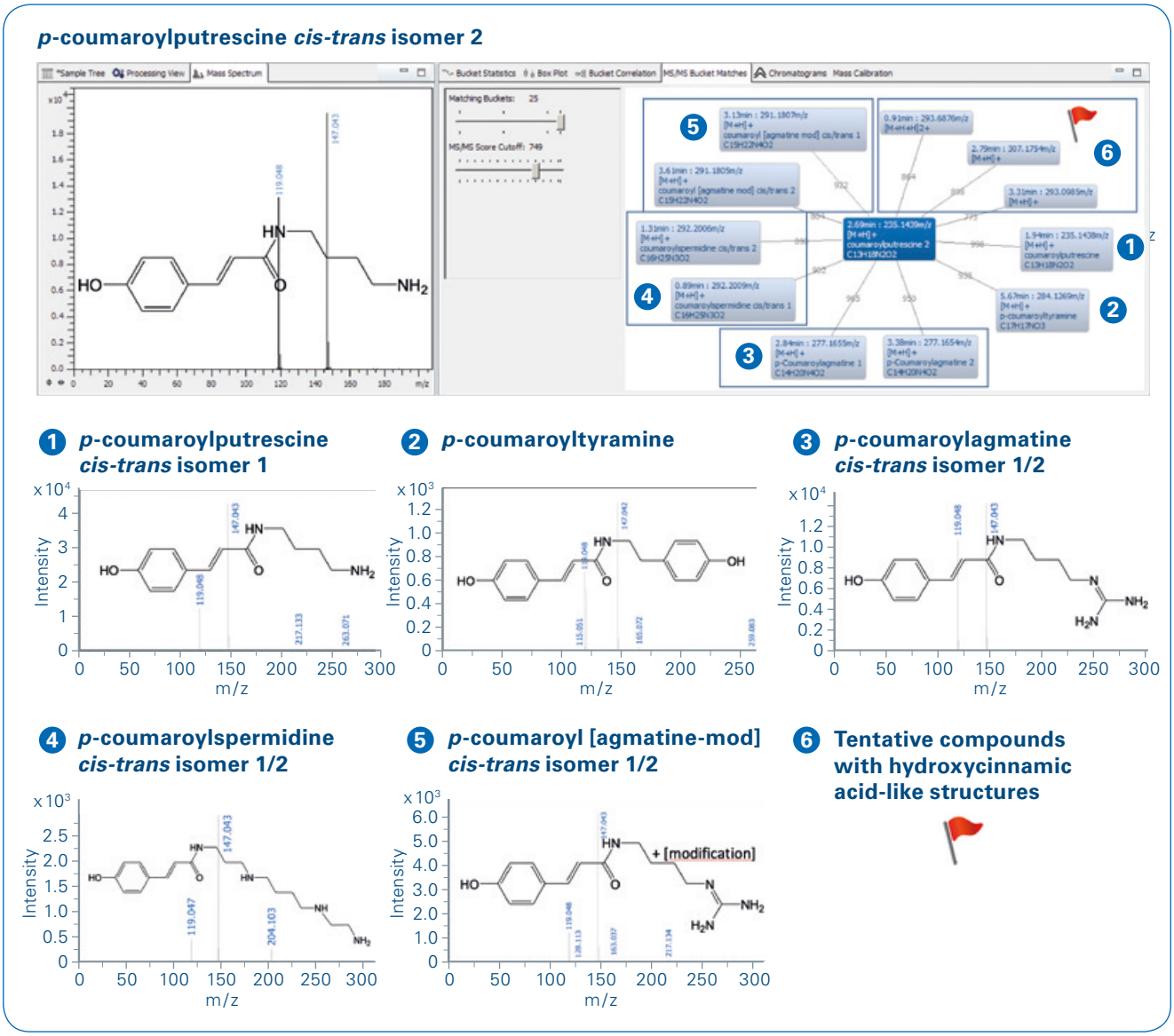

Figure 2: MS/MS similarity metabolite profiling. The MS/MS of p-coumaroylputrescine *(cis/trans-isomer 2) was selected for «MS/MS bucket matches» (bucket highlighted in blue in the center). Hydroxycinnamic acid amides (HCAAs) are stereoisomers and occur as two LC-separated peaks, which are termed as «cis/trans 1 or 2». Of the 11 matching MS/MS, 6 compounds were identified as HCAAs [compounds* 1, 2, 3 *[2 isomers] and* 4 *[2 isomers]]. Compound* 5 *[2 isomers] could be described by in-depth MS/MS analysis as coumaroylagmatine with a modified agmatine residue; the compounds in box* 6 *were flagged as tentative HCAA-like compounds.*

review of the identification confidence. Thus, the straightforward data processing in Metaboscape provided identification for approximately 25% of the features. This emphasized that potato contains many specific metabolites which are not yet covered by the utilized Analyte List.

A principal component analysis (PCA) provided a first impression of sample similarity and treatment-dependent metabolic differences. A clear distinction between the wildtype and the overexpression lines (OE) was present in PC 1, whereas in PC 4 the groups of wounded vs. unwounded plants separated (Figure 1). The loading values of the PCA depicted coumaroylagmatine, coumaroylputrescine, feruloylputrescine and caffeoylputrescine as the most relevant PC 1 metabolites. The enhancement of these hydroxycinnamic acid amides (HCAAs) in the OE lines was described in a targeted analysis by our group before [1]. From this work we knew about the high MS/MS similarity of HCAAs with indicative main fragments and systematic neutral losses. This was the basis for a novel investigation of the data, which we refer to as "unsupervised MS/MS similarity metabolite profiling".

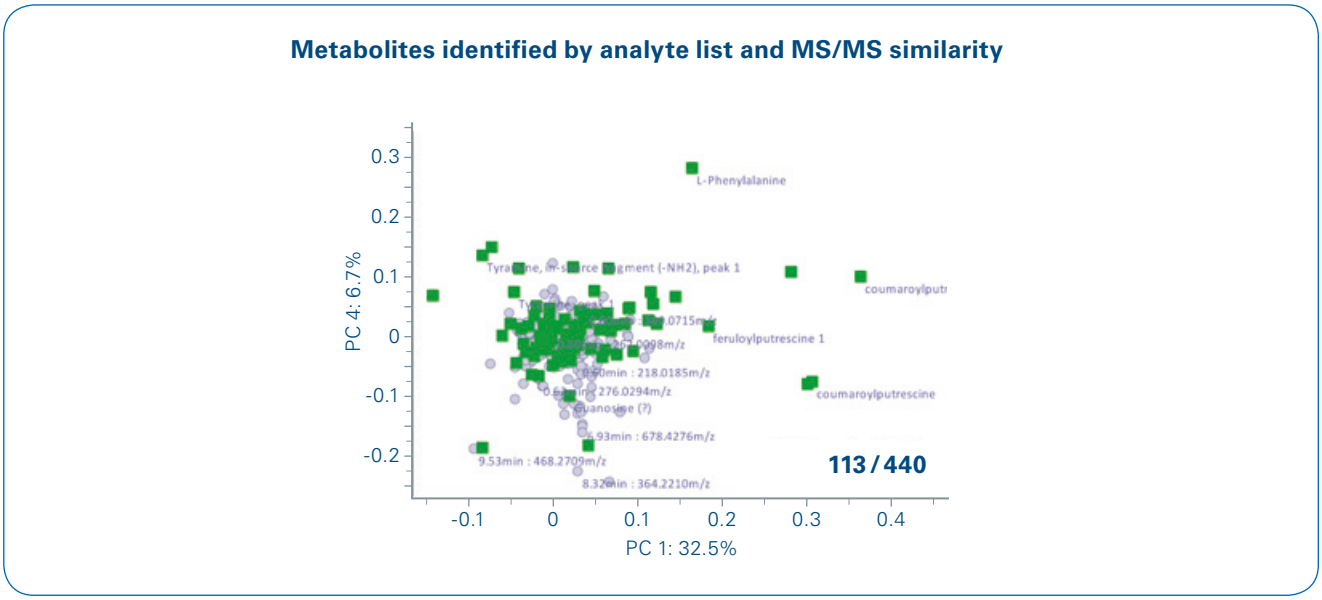

Figure 3: Metabolite identification and annotation in MetaboScape. 13 out of 440 buckets were annotated in this work. 96 buckets were assigned based on Analyte Lists and 17 could be assigned as HCCAAs by MS/MS similarity analyses. To visualize that these 113 metabolites correspond to the main PCA loadings relevant in this study (see Figure 1) all annotated loadings are highlighted in green in the PC1 vs. PC 4 loadings plot.

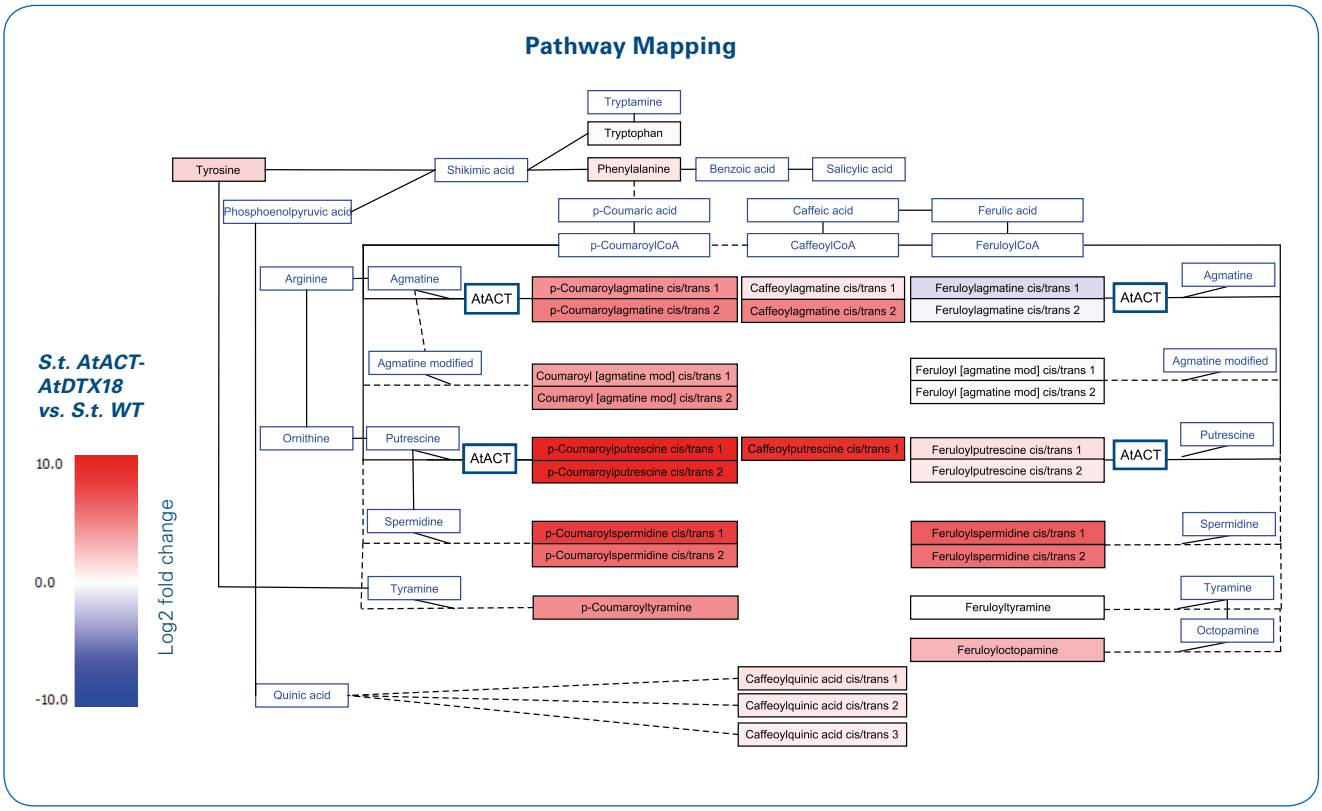

*Figure 4: Extended biosynthetic pathway of hydroxycinnamic acid amide biosynthesis in potato (*Solanum tuberosum *(S.t.)), that was genetically modified by the introduction of the* Arabidopsis *genes* AtACT *and* AtDTX18. AtACT *catalyses the biosynthesis of* p*-coumaroylagmatine and* p*-coumaroylputrescine as well as feruloylagmatine and feruloylputrescine in* Arabidopsis *(KEGG pathways). The HCAA pathway was adapted from KEGG to include all detected HCAA structures. Colors indicate the fold change of the overexpression lines versus the wildtype (without wounding).*

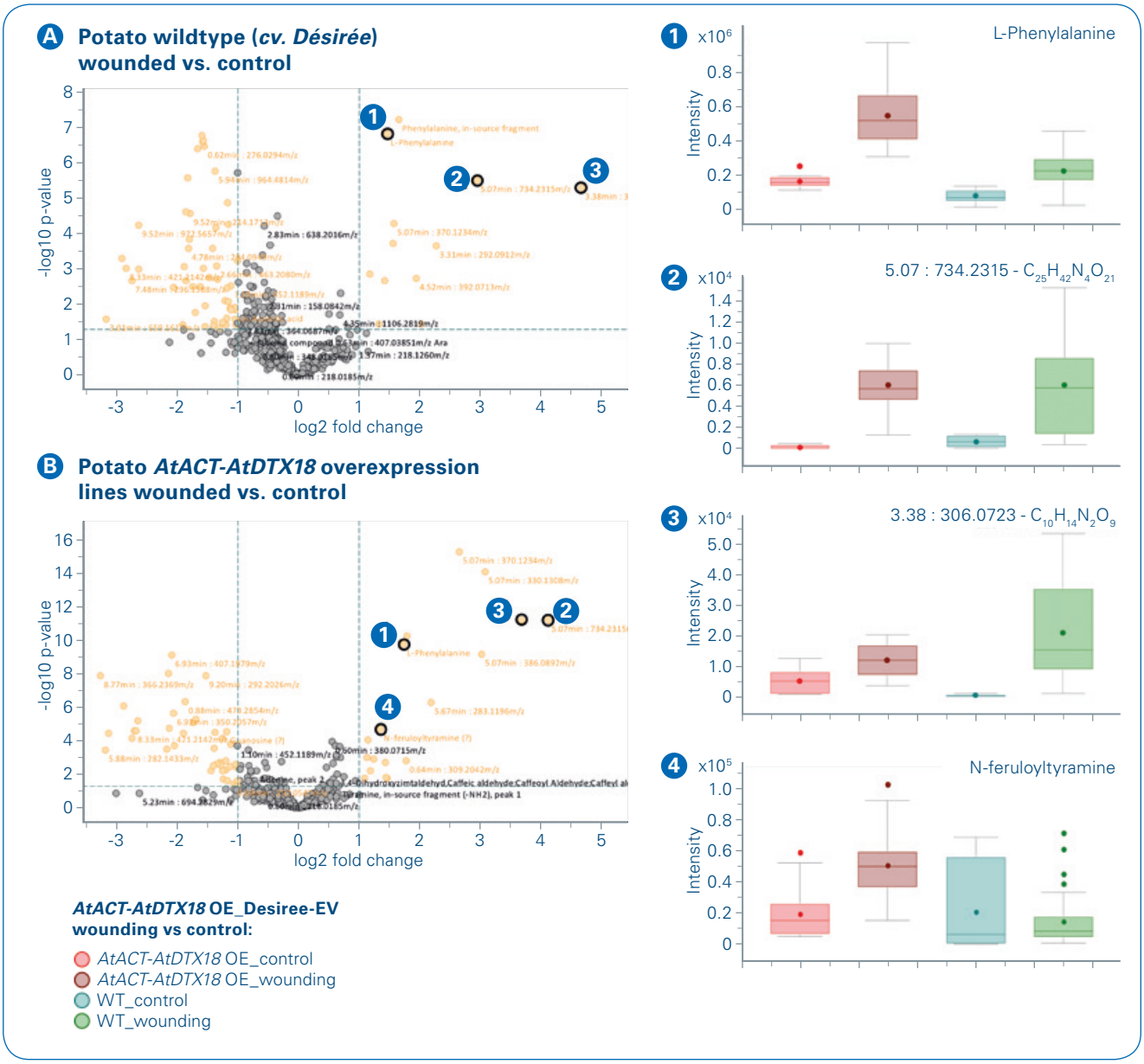

*Figure 5: Wound-responsive buckets in potato (*Solanum tuberosum *cv. Désirée) wildtype* A *and* AtACT-AtDTX18 *overexpression lines* B *: MetaboScape generated volcano plots based on* t-test results (A *and* B*) and boxplots (*1 - 4*) are shown.*

Annotation of metabolites by unsupervised MS/MS similarity metabolite profiling

For many of the non-identified compounds, MS/MS information was available and therefore a high degree of structural information in general. Structurally similar metabolites often exhibit similar MS/MS. Therefore the comparison of MS/MS from unknowns to annotated features holds the potential of unknown identification. However, one by one comparison of hundreds of MS/MS is hard to manage manually.

MetaboScape offers the required tools to investigate MS/MS information with focus on structurally similar compounds. We applied the following strategy: The main PC1 features were selected for "MS/MS bucket matches" (Figure 2). This tool compares the MS/MS of the selected compound to all MS/MS in the bucket table. This revealed instantly numerous features that had potential structural similarity to the given hydroxycinnamic acid target. Among the MS/MS matching buckets were compounds of already annotated HCAAs (e.g. *p*-coumaroylspermidine, *p*-coumaroylagmatine), which validated the approach. In addition, this also pointed to several unknown, previously unidentified

compounds with MS/MS similarity. MetaboScape enables to flag compounds of interest for example for detailed investigation at a later stage. As shown in Figure 2 this flagging was used for highlighting several HCAA-like compounds. After additional detailed investigation of the MS/MS, we could annotate numerous so far undescribed structures of the HCAA compound family. We applied this approach on other features (feruloyl- and caffeoylamides) and found in total 24 HCAAs. Consequently, the MetaboScape software allowed extracting many compounds with one click that provided structural similarity. As shown in Figure 3 in total 113 metabolites could be annotated.

It should be emphasized that nearly all HCAAs were of higher abundance in the overexpression lines. We used the tool "Pathway Mapping" in MetaboScape to visualize this common enhancement and to emphasize common biosynthetic origins. A pathway map was extended by the many additional novel metabolites that were annotated in course of the MS/MS similarity approach described above. Furthermore, we could integrate the reactions that were catalyzed by the overexpressed *At*ACT gene product according to information derived from KEGG [3]. The fold change mapping in MetaboScape (Figure 4) pointed to two main findings: A) The *AtACT* overexpression did not only influence the abundance of its reported direct metabolic products; nearly all HCAAs were changed and increased in the OE lines. B) Feruloylagmatine, although described to be a catalytic product of the *At*ACT enzyme, was decreased after overexpression of the enzyme. Thus, immediate downstream degradation of feruloylagmatine might be present, precursors might be limited or the *Arabidopsis* enzyme might exhibit distinct substrate specificity in the metabolic context of potato. This underlines, that the MetaboScape workflow provided numerous findings for exciting follow up experiments.

Wound-induced metabolites in potato

The metabolic changes in response to wounding became obvious by the comparison of control and treatment in both genotypes (wildtype and overexpression lines) separately. The *t*-test results revealed common wound-induced features (Figure 5). Phenylalanine was significantly increased after wounding in both WT and OE plants (Figure 5, compound 1), which was also implied by the PCA with phenylalanine as PC 4 loading value (Figure 1). The molecular formulae of other common wound-induced features were putatively assigned with Smart Formula (Figure 5, compounds 2 and 3). A few metabolites were wound-induced in the *AtACT-AtDTX18* overexpression lines, but not significantly in the wildtype plants 8 hours after wounding. The most interesting feature was feruloyltyramine (Figure 5, compound 4). Feruloyltyramine is a compound of the HCAA metabolite clade and was already described as a *Phytophthora infestans*-responsive metabolite in potato by Keller et al., 1996 [4] and as wound-responsive compound in potato tubers by Negrel et al., 1993 [5]. In contrast to both groups, we did not observe significant increases of feruloyltyramine in the wounded WT plants, which might be explained by an earlier sampling time point (8 hours instead of 24 and 48 hours (Keller et al.), 6 days (Negrel et al.)) or differential reactions towards the oomycete elicitor and in response to wounding.

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Conclusion

- Using the data acquired with Bruker LC-ESI-Q-TOF MS and MS/MS instrumentation and methods, the MetaboScape software provided an efficient platform for the straightforward linkage of structural similarities and metabolite abundances. We used various statistical tools (PCA, *t*-test) in MetaboScape to focus on relevant features. The unsupervised MS/MS similarity metabolite profiling approach enabled a rapid annotation of 17 metabolites which were previously unknown in our analyses.
- The common abundance patterns together with common MS/MS patterns pointed to an overflow of the hydroxycinnamic acid amide biosynthesis in the *AtACT-AtDTX18* overexpression lines. This is in perfect accordance with the function of the overexpressed enzyme *At*ACT that is responsible for the biosynthesis of four HCAAs. The increase of the many other HCAAs might be a metabolic side effect of the plant to compensate for this overflow and to bypass toxic effects for the cell. MetaboScape was very valuable for easy data processing, the annotation of many formerly unknown compounds and an intuitive visualization of metabolic changes.

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