Temporal dynamics of pathogenesis related metabolites and their metabolic pathways following inoculation of potato leaves with *Phytophthora infestans*.

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INTRODUCTION

The resistance in potato cultivars against late blight caused by *Phytophthora infestans* is classified into vertical and horizontal. Several metabolites associated with resistance have been detected: phytoalexins such as rishitin, phytuberin, lubimin, solavetivone etc. Metabolite profiling is a novel technology for the discrimination of quantitative resistance in plants against pathogen stress. Metabolite profiles based on GC/MS analysis have been used to discriminate resistance in wheat against *Fusarium graminearum* (http://www.metabolomics.mcgill.ca).

OBJECTIVE

The objective of this study was to use metabolomics approach to study the temporal dynamics of pathogenesis metabolites and their pathways following inoculation of potato leaves with *P. infestans*.

MATERIALS AND METHODS

Plant inoculation and incubation

Single-tuber-plants of cv AC Novachip was produced under greenhouse conditions and the leaflets were inoculated with *P. infestans* (US-8 genotype, A2 mating type) or water as a control. The plants were covered with a plastic bags to maintain saturated atmosphere (24 h) and kept in growth chambers at 20 C.

Metabolite extraction, profiling and analysis

Leaf discs containing the inoculation sites were cut on 1, 2 and 4 days after inoculation (Treatments: W1,P1, P2, P4) and ground in liquid nitrogen. The polar and the non-polar metabolites were extracted following methods developed by Fiehn et al. (2000 a,b) and analyzed using a GC/MS (GC 3400XC with Voyager® ion trap mass analyzer). The GC was equipped with a capillary column (30 m DB-5MS). Peaks were identified based on NIST library match. Factor analysis (SAS), based on principal components, was used to identify metabolites significantly loading to factor scores which in turn clustered the treatments identifying plant-pathogen interaction functions.

RESULTS AND DISCUSSION

Temporal dynamics of PR-metabolites

- A total of 106 consistent peaks were detected, of which 95 metabolites were tentatively identified.
- The metabolites significantly increased or decreased in abundance following the pathogen inoculation were designated as pathogenesis related (PR) metabolites, either up (PRU) or down (PRD) regulated, respectively. 42 (PR)-Metabolites were identified, some examples are listed in (Table 1).
- The PR-metabolites consisted of 9 amino acids (AA), 9 fatty acids (FA), 10 organic acids (OA), 4 sugars (SR), 3 other groups (OG) and 7 unidentified.
- Several AAs of aspartate, serine, glutamate and alanine families were highly up-regulated in P1 treatment, and the regulation was slightly reduced in P2, while highly reduced in P4 treatment, with P1/W > P2/W > P4/W.
- Some AAs detected at P1 were the primary precursors of:
  - structural proteins such as microfilaments or actin filaments that are reported to be involved in the defense response against *P. infestans* attack.
  - microtubules, enzymes and PR-proteins that play important roles in plant-pathogen interactions.

Factor analysis and plant-pathogen interaction functions

The factor vectors (first 3 explained 100% variance) with the highest factor-scores, along with their respective sets of metabolites with significant factor-loadings enabled identification of four hidden plant-pathogen interaction functions:

i) Homeostasis function (W1 = high F1): FAs that loaded highly to F1 (homeostasis) were unsaturated, abundances of which reduced with incubation time following pathogen inoculation. Several sugars loaded to F1, and these are known precursors of several plant structure and functions:

- the building blocks of cell walls, middle lamella.
- participate in the post modification of proteins and fatty acids.
- the production of structural defense materials such as callose and papillae.

ii) Primary defense function (P1 = high F2): The glycoalkaloid metabolite, solanidine (#11) had high factor-loadings to F2 and F3-scores with P1 > P2 > P4. This metabolite was reported to have anti-microbial activity and is produced from the cytoclic acetyl-Co-A through the mevalonate pathway.
The aromatic AAs (tyrosine and phenylalanine) and L-Glutamic acid (glutamate family) that were slightly up-regulated in P1 treatment were highly up-regulated in P2 and the P2 > P1 > P4.

iii) Secondary defense function (P2 = high F3): The aromatic AAs L-phenylalanine (#35) and L-Tyrosine (#31) had the highest loading to F3 and also had the highest P2/W-ratio in 2 DAI and they are the primary precursors of many secondary metabolites such as:

- Small molecular weight defense compounds such as phenols, flavones, coumarines, isoflavones, isoflavanones, lignins, tannins, nitrogen and sulfur containing anti-microbial compounds.
- The secondary messenger metabolite salicylic acid is very important in the defense response against pathogens and it activates several defense pathways leading to more complex defense.

The FA 7,10,13-Hexadecatrienoic acid (C16:3) was significantly down-regulated in P2 and P4 with P4/W < P2/W < P1/W. Following pathogen inoculation, this FA is auto oxidized to give dinor-oxo-phytodienoic acid, a potential wound signaling metabolite.

iv) Collapse of defense function (P4 = low F1, F2, F3): The treatment P4 = at 4 DAI, had the lowest scores for all the three factors and accordingly the metabolites with significantly negative factor-loadings were used to explain the function of collapse of defense responses. Only 6 metabolites including 2 OA, 2 FA and 2 SR had negative loading to F1, F2, F3 and their P/W ratios were: P4/W >P2/W > P1/W.

No.	Metabolite Name	Group[1]	W1[2]	P1/W1[3]	P2/W1	P4/W1	F1[4]	F2	F3
27	L-Aspartic acid, N-(trimethylsilyl)-,	AA	1.24^{B}	2.50^{A}	1.67^{B}	1.03^{B}	0.12	0.88	0.46
34	N,O,O-Tris(trimethylsilyl)-L-threonine	AA	0.36^{C}	3.04^{A}	2.09^{B}	1.27^{BC}	0.04	0.88	0.48
15	1,2,3-Propanetricarboxylic acid, 2- (isocitric acid)	OA	0.77^{B}	1.50^{A}	1.31^{AB}	1.01^{B}	0.13	0.84	0.52
94	L-Serine, N,O-bis(trimethylsilyl)-, tr	AA	0.73	2.88	2.22	2.04	-0.40	0.84	0.36
5	9,12-Octadecadienoic acid (Z,Z) (Linoleic acid)	FA	8.33^{BC}	1.23^{A}	1.06^{B}	0.92^{C}	0.41	0.81	0.42
22	L-Alanine (3TMS) (K)	AA	0.71^{B}	1.77^{A}	0.81^{B}	0.38^{B}	0.61	0.78	0.15
30	L-Proline, 5-oxo-1-(trimethylsilyl)-, tri	AA	1.77^{BC}	1.60^{A}	1.22^{AB}	0.71^{C}	0.48	0.76	0.45
7	9-Octadecenoic acid (Z)-, methyl (Oleic acid)	FA	0.11^{C}	1.50^{A}	1.45^{AB}	1.19^{BC}	-0.26	0.75	0.61
81	Butanedioic acid, bis(trimethylsilyl) (Succinic acid)	OA	1.39	1.11	0.91	0.95	0.64	0.74	-0.20
69	2-Butenedioic acid (E)-, bis(trime (Fumaric acid)	OA	0.35	1.12	0.87	0.99	0.24	0.74	-0.63
32	L-Valine, N-(trimethylsilyl)-, trimeth	AA	0.54^{B}	2.68^{A}	2.21^{A}	1.19^{B}	0.01	0.73	0.68
11	Solanid-5-en-3-ol, (3à)-	O(G)	0.42^{B}	2.17^{A}	2.13^{A}	1.70^{A}	-0.45	0.63	0.64
29	L-isoleucine, N-(trimethylsilyl)-, tri	AA	0.24^{B}	3.43^{A}	3.29^{A}	2.60^{B}	-0.52	0.63	0.57
58	Octadecanoic acid, trimethylsilyl	FA	1.27	1.17	1.20	0.99	0.06	0.57	0.82
93	L-Proline, 1-(trimethylsilyl)-, trimeth	AA	0.11	1.89	1.99	1.74	-0.59	0.52	0.62
31	L-Tyrosine, N,O-bis(trimethylsilyl)-, tri	AA	0.24^{B}	2.36^{A}	2.62^{A}	1.31^{B}	-0.12	0.49	0.86
4	9,12,15-Octadecatrienoic acid, (Linolenic acid)	FA	16.96^{AB}	1.06^{A}	0.87^{B}	0.66^{C}	0.89	0.39	0.23
2	7,10,13-Hexadecatrienoic acid, me (N)	FA	2.04^{A}	1.02^{A}	0.76^{B}	0.47^{C}	0.92	0.38	0.10
26	Glutamine, tris(trimethylsilyl)-	AA	0.32^{B}	2.47^{A}	2.96^{A}	2.33^{A}	-0.63	0.38	0.68
3	7,10-Hexadecadienoic acid, meth (N)	FA	0.25^{A}	1.04^{A}	0.71^{B}	0.45^{C}	0.93	0.36	0.08
35	N,O-Bis(trimethylsilyl)-L-phenylalanine	AA	0.42^{B}	1.51^{AB}	1.82^{A}	1.43^{AB}	-0.56	0.26	0.79
101	TMS 1-TMS-5-TMSoxy-3-(2-TMSamino (Tryptophan)	AA	1.09	0.61	0.63	0.71	0.58	-0.60	-0.55
Percentage of Variance explained							45.56	27.47	26.97

Table 1: Abundances (x105) of selected metabolites (out of 106) from W1 and their P/W-ratios detected in potato leaves inoculated with *P. infestans* or water (control) and incubated for 1, 2, 4 d. The significant factor loadings of metabolites to first three factors (in color) are given.

1. Chemical groups of compounds: AA= Amino Acid; FA= Fatty Acid; OA= Organic Acid; O(G)= Other Glykoalkaloids;
2. Water inoculated plants (control);
3. The metabolite regulation ratio = pathogen inoculated over water inoculated (P/W>1.0 is up-regulated; P/W<1.0 is down regulated) at 1, 2 and 4 DAI; different letters indicate significance at *P=0.05* by comparing the means of the total abundance of the four treatments.
4. Factor-loadings of metabolites to F1, F2 and F3-scores, based on factor analysis of abundances of 106 significant metabolites; the loadings can be positive or negative. Factor loadings > 0.50 are considered significant.

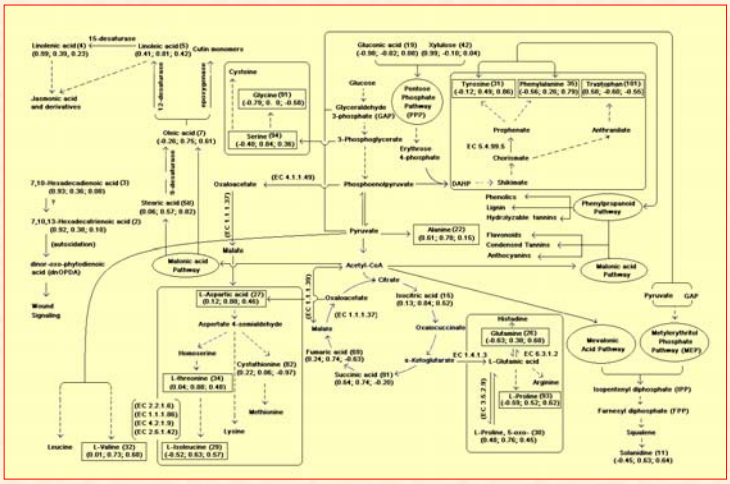

Fig. 2. Factor loadings of metabolites to F1, F2 and F3 in the metabolic pathways of potato leaves inoculated with *P. infestans* .

The Factor loadings of metabolites to F1, F2, F3 vectors are given in the pathway, below the metabolites that are detected in this study. Boxes= detected amino acids; Ellipses= metabolic pathways; F1= Homeostasis; F2= Primary defense response; F3= Secondary defense response; Low for all 3 factor vectors = Collapse of defense. EC= Enzyme commission number (NC-IUBMB). DAHP= 3-deoxy—D-arabino-heptulosonate-7-phosphate.

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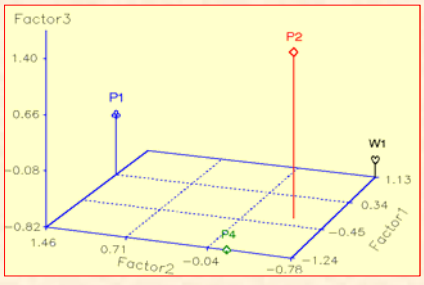

Fig. 1. Scatter plot of treatments using factor scores based on factor analysis of the abundances of 106 metabolites.

The treatments/inoculations are:
W1 = Water 1 DAI,
P1 = Pathogen 1DAI,
P2 = Pathogen 2DAI,
P4 = Pathogen 4DAI.

The metabolites differentially and significantly loaded to different factors are given in Table 1.

TAKE HOME MESSAGE

1. Primary defense metabolites (F2=P1):

- Several AAs that belong to Serine, Aspartate, and Alanine families are up-regulated.
- Different C18 FAs that may be involved in the activation of the jasmonic acid signaling pathways.
- The activation of one antimicrobial alkaloid metabolite.
- Activation of these metabolites occurred in different satellites or neurons of network of pathways.

2. Secondary defense metabolites (F3=P2):

- The pathways activated were: i) glutamate family AAs and the aromatic AAs phenylalanine and tyrosine - the primary blocks of the phenylpropanoid pathway; ii) the C16 FAs that activate the wound signaling response of the plant.

3. Collapse of defense metabolites (low F1,F2,F3=P4):

- At 4 DAI the primary and the secondary plant defense responses were collapsing and the P4/W ratios were the lowest for the AAs, FAs and OAs. At this stage the plant defense responses were failing and the plant was unable to stop the necrotrophic phase of the pathogen that usually take place in 2-3 DAI.

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