Phytochemistry Letters 22 (2017) 13–20

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

NMR study of age dependent metabolic adjustments in wild type and *pp2a-b'γ* mutant *Arabidopsis thaliana*

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ARTICLE INFO

Keywords:
Arabidopsis thaliana
Brassicaceae
^{1}H NMR metabolites
Multivariate analysis

ABSTRACT

The model plant *Arabidopsis thaliana* of the cruciferous Brassicaceae family has proven as an excellent tool for genetic modification and understanding of plant metabolic pathways. In this study we set up the methodology of ^{1}H NMR and principal component analysis (PCA) to address metabolic adjustments in *Arabidopsis* leaves. Wild type, together with a prematurely yellowing mutant deficient in a specific protein phosphatase 2A regulatory subunit PP2A-B'γ and a *pp2a-b'γ 35S:PP2A-B'γ* complementation line were analyzed at 4 and 6 weeks of age to reveal metabolic differences between vegetative and maturation phases of rosette growth. A PCA model revealed similar age-dependent metabolic adjustments in all genotypes. The contents of choline, sinapoyl malate, alanine and glutamine were high in four-week-old *Arabidopsis* plants and less abundant in six-week-old plants. Moreover, γ-aminobutyric acid (GABA) was observed only in four-week-old plants, whereas six-week-old plants were devoid of this metabolic compound. In contrast, the contents of fumaric acid, glucose and fructose became elevated at six weeks of age. β-Sitosterol was observed to be more abundant in 4 weeks old *Arabidopsis* rosettes, while α-linolenic and linoleic acids indicated higher content in 6 weeks old *Arabidopsis* rosettes. Total chlorophyll (Chl) content was higher in 4 weeks old *Arabidopsis*, whereas high content of Chl b was observed in 6 weeks old plants. We conclude that NMR based metabolomics analysis is a potential method for identification of age-dependent metabolites in *Arabidopsis* leaves.

1. Introduction

Plant development is tightly intertwined with metabolic interactions, where photosynthetic end products are allocated to support various physiological processes in different plant tissues. Reaching a mature growth state, the onset of flowering and seed production are important developmental phases that are associated with distinct metabolic alterations essential in channeling carbon- and nitrogen-rich compounds to the developing seeds. Studies on various plant species under different growth stages have uncovered the impact of ageing on plant metabolomes (El Senousy et al., 2014; Palama et al., 2010; Yusof et al., 2015).

Within the regulatory networks underlying plant development, protein phosphatase 2A (PP2A), composed of a catalytic subunit C, scaffold subunit A and regulatory subunit B, regulates leaf growth and flowering time in the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*). A combination of genetic approaches and metabolite profiling by GC–MS/MS revealed that the specific cytoplasmic PP2A regulatory subunit B'γ (PP2A-B'γ) is required to control rosette growth and metabolite signatures under oxidative stress (Li et al., 2014; Rasool et al., 2014). Further studies by HPLC showed that PP2A-B'γ modulates cellular *trans*-methylation capacity and the formation of 4-methoxy-indol-3-yl-methyl glucosinolate (4MO-I3 M), a specifically methylated indole glucosinolate, which is known to have deterring activities against microbial pathogens and aphids (Rahikainen et al., 2016). Hence, it is possible that the increased amount of 4MO-I3 M in *pp2a-b'γ* leaves at least partially contributes to its resistance against necrotrophic pathogens (Trotta et al., 2011) and aphids (Rasool et al., 2014). Knock-down *pp2a-b'γ* mutants also display premature yellowing and show senescence-like properties (Trotta et al., 2011) but delayed flowering (Heidari et al., 2013). Whether and how alterations in metabolic pathways integrate with the regulatory networks governing basic production and growth however remains poorly understood.

This study set out to explore the extent of metabolic perturbations in

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http://dx.doi.org/10.1016/j.phytol.2017.07.015
Received 9 January 2017; Received in revised form 8 May 2017; Accepted 24 July 2017

a *pp2a-b'γ* mutants, and to identify the key metabolic differences that can be detected by nuclear magnetic resonance spectroscopy (NMR) in four and six week old *Arabidopsis* plants. 4 and 6 weeks old plants were chosen for analysis to reveal metabolic differences between vegetative and maturation phases of *Arabidopsis* rosette growth. To this aim, leaf extracts of 4- and 6-weeks old *Arabidopsis* wild type, *pp2a-b'γ* mutant and a *pp2a-b'γ 35S:PP2A-B'γ* complementation line were investigated by NMR. NMR is an invaluable and well established experimental approach that has been extensively utilized in metabolomics studies because of minimal sample preparation (Kim et al., 2011).

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) combined with statistical multivariate analysis can discover at the same time different types of compounds in plant extracts, for instance carbohydrates, amino acids and lipids, depending on the selected extraction solvent. NMR spectra of plant extracts display fingerprint signals of the majority of the metabolites (Sekiyama et al., 2011; Ward et al., 2003). Moreover, a large number of research (Mannina et al., 2012) in the fields of food chemistry and food science have applied NMR to distinguish the quality of fruits and vegetables by analyzing metabolite quantities (Kortesniemi et al., 2014, 2015).This type of ^{1}H NMR metabolomics approach provides a comprehensive profile of the studied extract due to clarification of the metabolite structure, simple sample preparation and excellent selectivity (Aretz and Meierhofer, 2016; Lehtonen et al., 2013). However, the limitation of NMR is a low sensitivity in the detection of less abundant compounds in the sample extracts compared to techniques like mass spectrometry. Moreover, due to the limited range of chemical shift, overlapping signals can be obtained in proton spectra and this may create difficulties in the spectral assignment (Emwas, 2015; Kim et al., 2011; Ren et al., 2009). This leads to a decrease in the number of compounds that can be identified based on the corresponding signals in the NMR spectra, especially in the case of natural compounds.

A few studies have utilized NMR techniques combined with multivariate methods to investigate metabolic pathways in *Arabidopsis* under different conditions (Kaiser et al., 2009; Ren et al., 2009; Ward et al., 2003). However, there has not been detailed investigation including (1D) and (2D) experiments to identify age dependent metabolite occurrence in *Arabidopsis*.

In the present study we set up and utilized a NMR methodology to assess 1) how four and six weeks old *Arabidopsis* plants differ with respect to their metabolomes and 2) whether the PP2A-B'γ-mediated developmental effects associate with changes in the metabolome.

NMR combined with statistical analysis revealed similar age-dependent metabolic adjustments in *Arabidopsis* wild type, *pp2a-b'γ* mutant and *pp2a-b'γ 35S:PP2A-B'γ* complementation line. We conclude that NMR is sufficiently reliable in recognition of *Arabidopsis* metabolites in different developmental stages, but the study did not reveal any significant differences between the genotypes. Hence, PP2A-B'γ dependent adjustments in stress-related metabolism (Li et al., 2014; Rahikainen et al., 2016) are not associated with drastic metabolic alterations detectable by comparative NMR analysis of healthy *Arabidopsis* wild type and *pp2a-b'γ* mutant plants.

2. Results and discussion

2.1. Optimization of Arabidopsis sample preparation for NMR

At the beginning several different extraction solvents were tested, such as deuterated water D_2O, chloroform $CDCl_3$, dimethyl sulfoxide DMSO-d_6, methanol MeOD-d_4, methanol: water (MeOD:D_2O 80:20 v/v), acetone-d_6 and acetone-d_6:water (acetone-d_6:D_2O, 70:30 v/v).

In the first step 50 mg of freeze dried *Arabidopsis* rosette powder was mixed with 1.0 ml of solvent, vortexed for 15 min and centrifuged (15000 rpm, 10 min). 600 µl of the solution was pipetted to the 5 mm NMR tube. Measured ^{1}H NMR spectra included 128 scans and were compared to choose the solvent that contained the most versatile distribution of signals. After comparison, D_2O, $CDCl_3$ and MeOD-d_4 were chosen over the other solvents for follow-up tests A-C to examine the extraction efficiency.

Table 1
^{1}H NMR signals of the metabolites in *Arabidopsis* wild type and *pp2a-b'γ* mutant (25 °C, MeOD-d_4) at 4 and 6 weeks of age.

Metabolite	Position	δ_H (ppm)	Multiplicity	*J* (Hz)
Alanine	β-CH_3	1.46	d	6.7
	H2	3.58	m	–
L-Glutamine	H4	2.48	m	–
	H3	2.09	m	–
β-D-Fructopyranose	H1′	3.47	d	11.1
	H1	3.66	d	11.1
	H6′	3.62	dd	10.5; 2.1
	H6	4.02	dd	10.8;1.2
γ-Aminobutyric acid	H2	2.34	t	6.3
	H3	1.88	m	–
	H4	2.97	t	7.1
Fumaric acid	H2a, H3a	6.69	s	–
α-Glucose	H1	5.10	d	3.7
	H2	3.35	dd	9.8, 3.8
	H3	3.66	t	9.1
	H4	3.29	t	9.0
	H5	3.77	m	–
	H6a	3.68	dd	6.2, 12.5
	H6b	3.77	m	–
β-Glucose	H1	4.47	d	7.8
	H2	3.12	dd	9.1, 7.9
	H3	3.34	m	–
	H4	3.27	m	–
	H5	3.27	m	–
	H6a	3.64	m	–
	H6b	3.85	d	13.1
Choline	N-$(CH_3)_3$	3.20	s	–
Sinapoyl malate	CH-α	7.63	d	16.0
	CH-β	6.46	d	16.0
	H2,H6	6.92	s	–
	OCH_3-3/5	3.90	s	–
α-Linolenic acid	CH_3	0.97	t	7.54
	H3, H4, H6, H7, H9, H10	5.28 – 5.43	m	–
Linoleic acid	CH_3	0.92	t	6.96
	H6, H7, H9, H10	5.36	m	–
	H3	1.35	m	–
β-Sitosterol	C18-CH_3	0.71	s	–
	H6	5.40 – 5.43	m	–
	H3	3.42 – 3.44	m	–

Multiplicity: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet.

A: the first test was a repetition of the extraction described above except after vortexing the sample, it was kept in the fridge overnight and on the next day vortexed again for 15 min before a second centrifugation. This longer extraction protocol did not have visible effect when the spectra were compared with the previous ones.

B: In this test the extraction procedure was repeated twice with half amounts of solvent: The sample was mixed with 0.5 ml of solvent, then the supernatant was taken to a new Eppendorf tube and an additional 0.5 ml of solvent was added to the pellet, vortexed for 15 min and centrifuged for 10 min. The supernatant was combined to the previous one. 600 µl of the solution was pipetted to the NMR tube. Again, no clear difference was observed with the previous spectra.

Test C was similar to the test B except 1.0 ml of solvent was used two times rather than 0.5 ml. This time the excess of solvent needed for NMR sample did not improve the extraction efficiency for 600 µl sample volume. Based on the tests MeOD-d_4 was chosen as the most potent extraction solvent and the simple extraction protocol A was found justified. However, after these tests for the experiments containing all three plant lines, the low quantity of *pp2a-b'γ* mutant rosette, because of its small size, prevented us from using 50 mg plant powder amounts. Instead of 50 mg, 25 mg was used and the number of scans in NMR measurements was increased to 1 k. Also slightly smaller solvent