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Intraspecific comparative analyses of metabolites between diploid and tetraploid *Arabidopsis thaliana* and *Pyrus communis*

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ABSTRACT

Background: It has been said that naturally occurring autopolyploid strains are more tolerant of biotic and/or abiotic stresses, due at least in part to the higher accumulation of secondary metabolites. Data supporting this hypothesis come from comparisons between naturally established autopolyploids and diploids; thus the high accumulation of metabolites in polyploid strains may be a secondarily acquired feature and not a direct effect of the autopolyploidy. But no detailed studies on this issue have been carried out.
Results: Here we carried out metabolome analyses between newly created tetraploids and the parent diploid in a model plant, *Arabidopsis thaliana*, and the agriculturally important pear fruit tree (*Pyrus communis* var. *sativa*). Our data showed that small numbers of metabolite species differ in amount between diploids and tetraploids in both species, but the differences were not reproducible among growth conditions and species.
Conclusions: These results strongly indicate that metabolite content is not universal nor the direct target of polyploidy-dependent changes. Instead, naturally occurring hyperaccumulation of metabolites in autopolyploids may be the result of secondary natural selection.

1. Introduction

Autopolyploidy has been utilized to improve crops, fruits, vegetables and medicinal plants [1–3]. This is because polyploidization is believed to be associated with larger stature, prolonged growth and a higher concentration of metabolites, which has frequently been discussed as possibly linked to stronger tolerance to biotic and abiotic stresses [3–6]. Among these characters, larger stature has been investigated extensively and was confirmed to be generally associated with autopolyploidy in all examined species and is also known to be attributable to larger cell size, not only in plants but also in animals and fungi [7–10]. To determine the molecular background of this ploidy-dependent cell size enlargement, transcriptome analyses have been performed for several model cases, but most failed to detect meaningful differences in the mRNA profiles between diploids and polyploids, with the exception of a few reports [11]. On the other hand, how much higher are the concentrations of primary/secondary metabolites in polyploids compared to diploids is still unclear, and some reports showed higher concentrations of some metabolites in high ploidy strains [5,12,13]. This is partly because studies on the effects of autopolyploidy on metabolites have been carried out on naturally occurring polyploids that are thought have been under a long period of natural selection after polyploidization or on horticulturally selected cultivars that have been selected artificially for better traits.

Studies of the influence of autopolyploidy have been performed in the model plant *Arabidopsis thaliana* (L.) Heynh. (*A. thaliana*, hereafter). Many studies have already confirmed that tetraploids have larger cells and larger organs than diploids (e.g., [10]), but

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polyploidy at a higher level than hexaploidy results in smaller stature in organs with larger cells due to "high-ploidy syndrome" [14]. Microarray analysis and RNAseq analysis have also been performed in *A. thaliana* to determine the molecular mechanisms of the ploidy-dependency of cell size, but were largely unsuccessful. A report by Yu et al. [15] was most important in revealing that the difference in the transcriptional profile between tetraploids and diploids varied significantly between two accessions: Columbia-0 (Col-0) and Landsberg *erecta*, with no meaningful overlap.

In contrast to the above analyses of changes in cell size, no comparative analysis of the amounts of secondary metabolites among *A. thaliana* autopolyploids has been performed. As mentioned above, to determine whether polyploidy is indeed linked to high accumulation of metabolites, experiments must be performed in newly induced polyploids rather than naturally established and/or artificially selected strains. In this study, we used *A. thaliana* tetraploids as the ideal model system to examine whether polyploidy is associated with changes in metabolites because it is the best-studied plant species and easily forms tetraploids [10]. We compared metabolites in rosette leaves between diploids and tetraploids in the Col-0 accession, grown under various conditions, to evaluate whether any differences detected are stable or affected by environmental/physiological conditions. Furthermore, we compared the *A. thaliana* data with data on naturally occurring tetraploids and the diploid parent of pear to evaluate whether the ploidy-dependent metabolite changes are common to two species. We dared to compare these two different species and two different organs, to examine if changes in metabolites are shared between them. If metabolic changes are direct results from autopolyploidy, it must be shared between differed organs, as the increased cell volume shared in every polyploids. We found only a slight change in the metabolite content between diploids and tetraploids, indicating that differences in metabolite concentration in naturally occurring/horticulturally selected polyploids are not a direct result of genome polyploidization, but may be due to selection. Polyploidization could increase the ability to acquire new traits via genome rearrangement.

2. Materials and methods

2.1. Tetraploid materials

Tetraploidization of *A. thaliana* accessions was performed as described previously [16]. We chose Columbia-0 (Col-0) since this is the most studied accession. Diploid and tetraploid plants were maintained at 23 °C or 16 °C under continuous illumination (ca. 60 μmol/s/m^2) on rock wool supplied with 0.2 g/L Hyponex solution as described elsewhere [17]. For metabolome analyses, diploids (2C) and autotetraploids (4C) of *A. thaliana* were grown on soil or agar-solidified medium. Surface-sterilized seeds were sown on half-strength Murashige and Skoog medium (Murashige and Skoog Plant Salt Mixture, Wako Pure Chemical Industries) containing Murashige and Skoog vitamins (Sigma), 1% sucrose and 0.5 g/L MES, solidified with 0.8% agar (pH 5.8) (1/2 MS plate). Plants were grown under continuous light (CL) or long-day conditions (16-h light/8-h dark; 16L8D) for 2 or 3 weeks. For soil culture, 1-week-old plants grown on 1/2 MS plates were transferred to soil (PRO-MIX (Premier Horticulture, Canada): vermiculite = 2:1) and grown under 16L8D conditions for 1 or 2 weeks with the nutrient solution described previously as the control medium [18]. Rosette leaves were harvested, immediately weighed, frozen in liquid nitrogen and stored at −80 °C.

We also examined naturally occurring tetraploids of pear (*Pyrus communis* L. var. *sativa* (DC.) DC. 'La France') reported previously [19,20]. Pear flower buds and fruits were harvested from an orchard in Yamagata Prefecture, Japan in 2010. The samples were rapidly transferred from the orchard to the laboratory and receptacles were cut from flower buds at blooming or from small fruits 2 weeks after blooming, or fruit flesh was cut from fruits at 1, 2, 3 and 4 months after blooming, at harvesting (5 months after blooming) and 1 month after harvesting (ripened fruits). The cut receptacle or fruit flesh was immediately frozen in liquid nitrogen and stored at −80 °C. The frozen samples were crushed using a homogenizer and used for metabolomic analysis. We prepared at least three biological replicates of each sample. Sample extraction and metabolomic analysis using capillary electrophoresis mass spectrometry were conducted using a previously described method [21,22]. Plant hormones in pear fruits were also quantified following a previous report [21].

2.2. Widely targeted metabolomics data collection for A. thaliana

Metabolites were extracted from rosette leaves as described previously [23], Briefly, frozen rosette leaves from *A. thaliana* (approx. 5–200-mg fresh weight) were crushed in 80% (v/v) methanol with 0.1% (v/v) formic acid in a 2-mL microtube with 5-mm zirconia beads using Shake Master Neo (Biomedical Science) for 5 min at 1000 rpm. After centrifugation, the supernatant was subjected to widely targeted metabolome analysis using liquid chromatography-tandem quadrupole mass spectrometry as described previously [24]. Some compounds were co-eluted in the liquid chromatography and indistinguishably detected. Such groups of compounds were termed as follows: 4i_kaempferol-Rha-Rha for kaempferol-3-rhamnoside-7-rhamnoside, vitexin-2″-*O*-rhamnoside, kaempferol-3,7-*O*-bis-alpha-L-rhamnoside, and kaempferol-3,7-*O*-di-rhamnopyranoside; 2i_kaempferol-Glc-Rha-Rha for kaempferol-3-glucoside-2″-rhamnoside,-7-rhamnoside and kaempferol-3-*O*-alpha-L-rhamnopyranosyl(1 → 2)-beta-D-glucopyranoside-7-*O*-alpha-L-rhamnopyranoside; 7i_kaempferol-Gal-Rha for kaempferol-3-*O*-beta-D-galactoside-7-*O*-alpha-L-rhamnoside, kaempferol-3-*O*-beta-D-glucoside-7-*O*-alpha-L-rhamnoside, and 5 other kaempferol diglycosides; 3i_D-erythrose for D-erythrose, glycolaldehyde dimer, and D(−)-threose.

3. Results

3.1. Comparative widely targeted analysis of metabolites in rosette leaves from the Columbia accession

First we compared the metabolite profile between diploids and autotetraploids of *A. thaliana*. We chose rosette leaves from plantlets grown under various conditions to determine whether common metabolites showed differences in accumulation between diploids and autotetraploids. Our widely targeted analysis [24,25] detected several differences between tetraploids and diploids (Table 1).

In the leaves of plants grown on 1/2 MS plates, tyramine, 3-methylsufinylpropyl-glucosinolate (GSL), 4-methylthiobutyl-GSL and indol-3-ylmethyl-GSL showed a tendency for greater accumulation in tetraploids than in diploids, but their higher accumulation was not stable in the various day lengths (16L8D vs. CL) or developmental stages (2 or 3 weeks old). Only tyramine had stable high accumulations under the 16L8D condition between the 2- and 3-week-old stages (Table 1). When grown on soil, AMP, fumarate, succinate, tyramine, cyanidin 3-*O*-rutinoside, 3-benzoyloxypropyl-GSL, indol-3-ylmethyl-GSL, 4i_kaempferol-Rha-Rha (see Section 2), GMP and nicotinate showed higher accumulation between the 2- and 3-week-old stages (Table 1). Notably, tyramine and indol-3-ylmethyl-GSL also accumulated at higher levels in tetraploid plants grown on 1/2 MS medium.