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Identification of complex, naturally occurring flavonoid glycosides in *Vicia faba* and *Pisum sativum* leaves by HPLC-DAD-ESI-MS^n and the genotypic effect on their flavonoid profile

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ABSTRACT

Vicia faba and *Pisum sativum* are legumes belonging to the plant family Fabaceae. As their flavonoid profile is highly diverse with quite complex structures, the aim of this study was to identify the flavonol glycosides in the leaves of different cultivars of *V. faba* and *P. sativum*. In *V. faba* the main flavonol aglycon is kaempferol, whereas in *P. sativum* the main flavonol aglycon is quercetin. Furthermore, *V. faba* leaves contain mono-, di-, tri- and tetraglycosides of quercetin and kaempferol with primarily glucose, rhamnose, and galactose as sugar moieties. Two of these structures have been identified to be acylated with acetic acid. In contrast, in *P. sativum* leaves only triglycosides were identified being sophotriosides. Nine of the 11 quercetin and kaempferol glycosides were acylated with selected hydroxycinnamic acids. Depending on cultivar, *V. faba* and *P. sativum* provided different flavonol profiles. Here, shown for the first time in legume leaves. For example, the summer cultivars of *V. faba* showed a high concentration of kaempferol-3-*O*-rhamnoarabinoside-7-*O*-rhamnoside. However, *P. sativum* with especially the cv Salamanca, had the most desired flavonoid glycoside profile referring to high concentrations of quercetin glycosides and exemplarily the acylation with *p*-coumaric acid.

1. Introduction

Flavonoids consist of two aromatic rings and one heterocyclic ring containing an oxygen atom. In plants, flavonoids mainly occur as glycosides and are usually conjugated with glucose. So far, a large number of flavonoid glycosides have been identified within the family of Fabaceae that includes also the legumes. Noteworthy is the high diversity of flavonoid glycosides in legumes. So, flavone glycosides are mainly present in the leaves of *Lupinus angustifolius* (Duenas, Hernandez, Estrella, & Fernandez, 2009) and *Medicago truncatula*. Isoflavones are present in the seeds of *Lens culinaris*, *Phaseolus vulgaris*, and *Cicer arietinum* (Konar, Poyrazoglu, Demir, & Artik, 2012) and *M. truncatula* (Farag, Huhman, Lei, & Sumner, 2007). In contrast, *V. faba* and *P. sativum* leaves are mainly characterized by flavonols (Ferreres, Esteban, Carpenaruiz, Jimenez, & Tomas-Barberan, 1995; Tomas-Lorente, Garciagrau, Tomas-Barberan, & Nieto, 1989). These studies highlighted that the family of Fabaceae is more diverse in their flavonoid profile compared to *e.g.* the family of Brassicaceae with the genus *Brassica* (Cartea, Francisco, Soengas, & Velasco, 2011) or *Arabidopsis thaliana* (Hectors et al., 2014).

So far, *V. faba* is mainly grown for its seeds that are used in human and animal nutrition. Its leaf flavonoids are to a large extent non-acylated and acetylated kaempferol glycosides (Spanou, Aligiannis, Skaltsounis, & Kouretas, 2008; Spanou et al., 2012; Tomas-Lorente et al., 1989). They might be of certain interest for their antioxidant potential and chemopreventive properties (Spanou et al., 2008). *P. sativum* is one of the most consumed vegetable worldwide and therefore of great economic importance (Adsule, Lawande, & Kadam, 1989). The grains of *P. sativum* are a well-known source of plant proteins. However, sprouts of *P. sativum* nowadays gain attention as ready-to-eat vegetables (Santos, Herrero, et al., 2014; Santos, Oliveira, Ibanez, & Herrero, 2014). They contain non-acylated and monoacylated triglycosides of quercetin and kaempferol (Ferreres et al., 1995; Santos, Herrero, et al., 2014; Santos, Oliveira, et al., 2014; Weissenböck, Hedrich, & Sachs, 1986). There are future ideas pending to consume also the leaves of *P. sativum* and further legumes, as it is already done in many developing countries. Leaves especially contain high concentrations of these flavonoids that are interesting as bioactive compounds *e.g.* quercetin was shown to act as anti-inflammatory compound *in vivo* (Boesch-Saadatmandi, Wagner, Wolffram, & Rimbach, 2012).

For the plant's secondary metabolism and the corresponding flavonoid profile, cultivar is a predominant influencing factor. In a recent survey with eight kale (*Brassica oleracea* var. *sabellica*) cultivars, the quercetin and kaempferol glycoside concentration differed and

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were highest in old, traditional cultivars (Schmidt et al., 2010a). This genotypic effect had also been shown for other *B. oleracea* (Kim, Padilla-Zakour, & Griffiths, 2004; Krumbein, Saeger-Fink, & Schonhof, 2007) and *Brassica rapa* species (Harbaum et al., 2007; Rochfort, Imsic, Jones, Trenerry, & Tomkins, 2006). Flavonoid glycoside profiles of different cultivars of *V. faba* and *P. sativum* have not been quantified comprehensively, so far, but the chemical structure of the flavonoid glycosides including glycosylation pattern and acylation pattern is important to reveal information on possible bioactive effects of the compounds. With regard to even improve the nutritional value legumes (and *Brassica* species), it is important to fully characterize their flavonoid profile, as this will give a background for further techniques (*e.g.* SMART breeding) to enhance the flavonoid concentrations. Aside from the genotype a further strong influencing factor is UV-B radiation. UV-B radiation is known to induce a shift of mono-hydroxylated flavonoids to poly-hydroxylated flavonoids (Agati, Cerovic, Pinelli, & Tattini, 2011; Majer, Neugart, Krumbein, Schreiner, & Hideg, 2014). Additionally, besides quercetin glycosides in kale also monoacylated kaempferol di-, tri- and tetraglycosides containing a catechol structure in the hydroxycinnamic acid residue were increased with subsequent doses of moderate UV-B (Neugart et al., 2014). The same dependency of the chemical structure can be expected for other species. In this context a comprehensive overview of major and minor flavonoid glycosides is relevant to explain the changes in the flavonoid profile as response to influencing factors.

However, various *V. faba* species have been characterized only with regard to the aglycons quercetin and kaempferol, even without quantification (Webb & Harborne, 1991). *Vicia* species including *Vicia kalakhensis*, *Vicia narbonensis*, *Vicia galilaea* and *Vicia eristalioides* were shown to have diverse flavonol glycoside profiles (Campeol, Catalano, Cremonini, & Morelli, 2000; Campeol, Cioni, Flamini, Rossi, & Cremonini, 2003)

The aim of the present study were to characterize the flavonoid profile in *V. faba* and *P. sativum* leaves in order to define the future potential of these legume leaves. The chemical structure of the flavonoid glycoside including glycosylation pattern and acylation pattern is important to reveal information on possible bioactive effects of the compounds. Based on testing genetic variation, it was aimed at determining the genotype-depending profile of flavonoid glycosides in various cultivars including winter and summer cultivars that have different optimum growing conditions. For the first time a wide selection of *V. faba* and *P. sativum* cultivars has been screened for their flavonoid glycoside profile.

2. Materials and methods

2.1. Plant material

Different *V. faba* and *P. sativum* cultivars were grown for eight weeks (17th May 2011–12th July 2011) in a greenhouse under typical Northern European environmental conditions with a temperature range between 7 and 16 °C mean daily temperature and radiation between 356 and 1045 μmol m^{-2} s^{-1} mean daily photosynthetic active radiation. For *V. faba* the winter cultivars Hiverna and Nordica and the summer cultivars Fuego and Espresso (all provided kindly by Norddeutsche Pflanzenzucht (NPZ) Hans-Georg Lembke KG, Holtsee, Germany and Gemeinschaft zur Förderung der privaten deutschen Pflanzenzüchtung e.V. (GFP), Bonn, Germany) and for *P. sativum* the winter cultivar James and the summer cultivars Gregor, Navarro, Salamanca, and Starter (NPZ and GFP) were grown with three biological replicates of three adult plants each.

2.2. Sample preparation

For each replication, the shoots taken from three plants were frozen in liquid nitrogen, stored until lyophilization at −40 °C, lyophilized, and then ground to a powder. The samples were stored in darkness at room temperature until being required for analysis.

2.2.1. Flavonol aglycons

A lyophilized sample (0.5 g) was hydrolyzed with 50% aqueous methanol and 1.6 M HCl in double determination (Schmidt et al., 2010a). After refluxing at 90 °C for 2 h, the extract was cooled down to room temperature, adjusted to 100 mL and sonicated for 5 min. The extract was then filtered through a 0.45 μm PTFE filter for HPLC analysis.

2.2.2. Flavonol glycosides and hydroxycinnamic acid derivatives

To analyze the (intact) flavonol glycosides and hydroxycinnamic acid derivatives, samples were extracted as previously described (Neugart et al., 2012) with the following modifications: In analytical duplicates, 20 mg lyophilized and ground powder were extracted with 600 μL 60% aqueous methanol for 1 h under continuous stirring at room temperature. Samples were centrifuged at 19,000 ×*g* for 10 min and the supernatants were collected in a new tube. The extraction procedure was repeated with 400 μL and 200 μL for 20 min and 10 min, respectively. The supernatants were combined and were evaporated to dryness with a rotary evaporator. The residue was dissolved in 200 μL distilled water. The extract was then filtered through centrifuge tube filters with a cellulose acetate membrane 0.22 μm (Corning® Costar® Spin-X®, Sigma Aldrich Chemical Co., St. Louis, MO) for HPLC analysis.

2.3. HPLC-DAD-ESI-MS^n

The HPLC-DAD-ESI-MS^n method for the identification (Schmidt et al., 2010b) and quantification (Neugart et al., 2012) of the flavonol glycosides and hydroxycinnamic acid derivatives in *V. faba* and *P. sativum* was done with an HPLC series 1100 from Agilent Technologies Sales & Services GmbH & Co.KG (Waldbronn, Germany) consisting of a degasser, binary pump, autosampler, column oven and a photodiode array detector was used. An ion trap (6300 series) with an ESI ion source in negative ionization mode was used as the mass spectrometer.

2.3.1. Flavonol aglycons

The flavonol aglycons were quantified after acid hydrolysis using a modified HPLC-DAD-ESI-MS^n method according to Schmidt et al. (2010a). The extracts were separated on a Prodigy column (ODS 3, 150 × 3.0 mm, 5 μm, 100 Å; Phenomenex Inc., Aschaffenburg, Germany) with a security guard C18 (ODS 3, 4 × 3.0 mm, 5 μm, 100 Å) at a temperature of 25 °C using a water/acetonitrile gradient. Solvent A consisted of 99.5% water and 0.5% acetic acid; and solvent B contained 100% acetonitrile. The following gradient was used for eluent B: 30–35% (0–5 min), 35–39% (5–17 min), 39–90% (17–21 min), 90% isocratic (21–26 min), 90–30% (26–29 min), and 30% isocratic (29–34 min). Flow was performed using 0.3 mL min^{-1}, and the measured detector wavelength was 370 nm. The standards dihydroquercetin and kaempferol (Carl Roth GmbH, Karlsruhe, Germany) were used to obtain an external calibration curve in the range of 0.1 to 10 mg 100 mL^{-1}. Quercetin and kaempferol were identified as deprotonated molecular ions and characteristic mass fragment ions by HPLC-DAD-ESI-MS^2. The mass optimization was performed for quercetin $[M-H]^-$ *m/z* 301.

2.3.2. Flavonol glycosides

The flavonol glycosides were analyzed using the same column and eluent composition as described above. In contrast, the following gradient was used: eluent B (100% acetonitrile) at a temperature of 30 °C: 5–7% (0–12 min), 7–9% (12–25 min), 9–12% (25–45 min), 12–15% (45–100 min), 15% isocratic (100–150 min), 15–50% (150–155 min), 50% isocratic (155–165 min), 50–5% (165–170 min), and 5% isocratic (170–175 min) (Schmidt et al., 2010b). The flow was 0.4 mL min^{-1} and the measured detector wavelength for the