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Polyphenol metabolism provides a screening tool for beneficial effects of *Onobrychis viciifolia* (sainfoin)

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ABSTRACT

Onobrychis viciifolia (sainfoin) is a traditional fodder legume showing multiple benefits for the environment, animal health and productivity but weaker agronomic performance in comparison to other legumes. Benefits can be mainly ascribed to the presence of polyphenols. The polyphenol metabolism in *O. viciifolia* was studied at the level of gene expression, enzyme activity, polyphenol accumulation and antioxidant activity. A screening of 37 accessions regarding each of these characters showed a huge variability between individual samples. Principal component analysis revealed that flavonols and flavan 3-ols are the most relevant variables for discrimination of the accessions. The determination of the activities of dihydroflavonol 4-reductase and flavonol synthase provides a suitable screening tool for the estimation of the ratio of flavonols to flavan 3-ols and can be used for the selection of samples from those varieties that have a specific optimal ratio of these compounds for further breeding.

1. Introduction

Legumes provide a rich source of proteins and polyphenols (Aoki et al., 2000) and can therefore play a major role in the nutrition of humans and animals. Most of the commonly used fodder legumes, such as alfalfa, lupin and a number of clover species cause bloat, which is a cause of considerable economic risk in ruminant farming (Majak et al., 1995). There are, however, a few non-bloating legumes, including sainfoin (*Onobrychis viciifolia*), cicer milkvetch (*Astragalus cicer*) and bird's-foot trefoil (*Lotus corniculatus*). These species are currently not competitive with alfalfa in terms of yield, re-growth and persistence in the stand. Sainfoin is a traditional fodder legume, which was replaced by other legume forages in the middle of the 20th century, due to low productivity, and problems with establishment (Borreani et al., 2003) despite showing multiple benefits on environment and animal health (Hayot Carbonero et al., 2011; Marais et al., 2000). Consumption of sainfoin by many ruminant species leads to increased absorption of amino acids and reduced urinal N-excretion and therefore, to a reduced nitrogen emission (Scharenberg et al., 2007). In addition, methane emission is reduced, one of the major gases that are associated with global warming. Furthermore sainfoin is an excellent food source for bees and other pollinators (Hayot Carbonero et al., 2011).

Abbreviations: 5-DLCy, 5-deoxyleucocyanidin; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; BSA, bovine serum albumin; BUT, butin; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; DHF, dihydrofisetin; DHK, dihydrokaempferol; DHM, dihydromyricetin; DHQ, dihydroquercetin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTE, dithioerythritol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ERI, eriodictyol; F3′H, flavonoid 3′-hydroxylase; FGT, flavonoid glucosyl-transferase; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; GAPDH, glycerinaldehyde 3-phosphate dehydrogenase; GAR, garbanzol; HAC, hierarchical cluster analysis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IFS, isoflavone synthase; LAR, leucoanthocyanidin reductase; LIQ, liquiritigenin; MeOH, methanol; NADPH, nicotinamide adenine dinucleotide phosphate; NAR, naringenin; OMT, *O*-methyl transferase; ORF, open reading frame; PAL, phenylalanine ammonia-lyase; PCA, principal component analysis; PEG, polyethylene glycol; PHF, pentahydroxyflavanone; PKR, polyketide reductase; POX, peroxidase; RACE, rapid amplification of cDNA ends; SAM, *S*-adenosylmethionine; TLC, thin layer chromatography.

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Sainfoin's benefits can mainly be ascribed to its polyphenols (Ingham, 1978; Koupai-Abyazani et al., 1993; Lu et al., 2000; Marais et al., 2000; Mueller-Harvey, 2006; Regos et al., 2009; Theodoridou et al., 2010; Yeap Foo et al., 1982). The effects are dependent on both the concentration and the composition. Polyphenols show antiparasitic properties; in particular anthelminthic effects against nematodes are well established (Barrau et al., 2005; Heckendorn et al., 2006; Hoste et al., 2005). Flavonols are the predominant polyphenols in sainfoin (Regos et al., 2009) and have been shown to inhibit the larval migration of nematodes (Heckendorn et al., 2006; Hoste et al., 2005; Manolaraki et al., 2010). In addition, proanthocyanidins (synonyms: condensed tannins, polymeric flavan 3-ols) are found in sainfoin, which are absent in many other common fodder legumes (Li et al., 1996). The beneficial effect of proanthocyanidins with respect to bloat prevention is ascribed to their ability to form complexes with proteins in the rumen of the animals (Jones and Lyttleton, 1971; Jones and Mangan, 1977). Apart from the very commonly occurring polyphenol classes, a number of isoflavonoids and 5-deoxy(iso)flavonoids could be found, which are typical for legumes (Ingham, 1978) and are known to act as phytoalexins.

Whilst the agronomic performance of other legumes like lucerne and clover has benefitted from extensive breeding programmes, very few breeding programmes have been undertaken for sainfoin (Hayot Carbonero et al., 2011). The existing sainfoin germplasm is highly diverse in its agronomic behaviour (Hayot Carbonero et al., 2011, 2012), morphology and in the polyphenol spectrum (Regos et al., 2009). Therefore, identification of suitable germplasm resources with a known polyphenol spectrum is a promising approach for future breeding of sainfoin varieties with improved agronomic performance whilst maintaining the beneficial effects. A collection of 360 sainfoin accessions at the National Institute of Agricultural Botany (NIAB, Cambridge, UK) was recently evaluated (Hayot Carbonero et al., 2011). A subset of 37 accessions was preselected based on their agronomic performance (Hayot Carbonero, 2011). The accessions were sourced from Europe, Eastern block countries, North America and Asia, and accessions were chosen from this large group to ensure a good cover of countries, with particular emphasis in including environmental conditions. In addition, the three main types were included: the so called single cut or 'common' types, the so called double cut or giant' types and those that were a mixture of the two. Finally attention was paid to good condition in terms of homogeneity or uniformity, within the context of an outbreeding species. Proanthocyanidin composition and anthelmintic data are also available (Manolaraki, 2011; Stringano et al., 2012). This offered the opportunity to study the polyphenol metabolism in terms of gene expression, enzyme activity, antioxidative activity and polyphenol composition, and to identify possible germplasm screening tools. The variety 'Cotswold Common' was chosen as a standard, since already a lot of information about this variety from previous growing trials was available, such as assurance in terms of distinctness, uniformity and stability. Furthermore seeds of this variety are commercially available.

2. Results/discussion

2.1. Determination of polyphenol enzymes in O. viciifolia

A large variety of polyphenols, namely hydroxycinnamic acids, chalcones, flavanones, dihydroflavonols, flavonols, flavan 3-ols, anthocyanins, and a number of isoflavonoids and 5-deoxyflavonoids were identified in *O. viciifolia* (Ingham, 1978; Koupai-Abyazani et al., 1993; Lu et al., 2000; Marais et al., 2000; Regos et al., 2009; Yeap Foo et al., 1982), which are present in various glycosylated and methylated forms (Lu et al., 2000; Regos et al., 2009). Therefore the presence of the main enzymes of the polyphenol pathway as well as modifying enzymes was expected in tissues of *O. viciifolia*. Preparations from *O. viciifolia*, which were obtained by standard methods (Claudot and Drouet, 1992; Stich et al., 1992) showed no or only low enzyme activities. An optimised extraction method for polyphenol rich material (Dellus et al., 1997) could be successfully adapted for tissues of *O. viciifolia* (refer to Section 3.5) and provided preparations showing high activities for all tested enzymes. The activity of phenylalanine ammonia-lyase (PAL), chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR), flavonol synthase (FLS) and flavonoid glucosyl-transferase (FGT) was determined in 11 different tissues of *O. viciifolia* line Cotswold Common: young leaves (still folded), fully developed leaves, young petiols, middle-aged petiols, stems, young flowerstalks, flower buds, open flowers, senescent flowers, unripe seeds, and seedstalks (Table 1). Furthermore the activity of *O*-methyltransferase (OMT) was determined in young leaves. In general, young leaves (still folded) showed the highest enzyme activities and were therefore used as standard material for further investigations. The enzymatic reactions of PAL, CHS/CHI, FHT, DFR, FLS, FGT and OMT were optimised regarding pH optimum, temperature stability and optimum, linearity with time and protein concentration, and resulted in the standard enzyme assays for *O. viciifolia*, described in Section 3.6. IFS activity could not be determined in preparations from leaves, but was demonstrated successfully with microsomes prepared from seedlings of *O. viciifolia*, which had been treated 6 h before harvest with glutathione, an elicitor for the formation of flavonoids (Jung et al., 2000). Flavonoid compounds of three different B-ring hydroxylation patterns were reported to be present in *O. viciifolia* (Regos et al., 2009; Stringano et al., 2012). However, the activity of the microsomal enzymes flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) activity could not be demonstrated in this study despite many attempts. It is likely that this is due to the high polyphenol content which negatively affects the stability of membrane associated enzymes during preparation.

The enzymes from plant crude extracts were tested for their substrate acceptance (Suppl. Table 1). This included also intermediates for 5-deoxyflavonoid formation, because 5-deoxy(iso)flavonoids such as liquiritigenin, garbanzol, vestiton, vestitol, afrormosin, and formononetin were identified in *O. viciifolia* (Ingham, 1978). Structures of the substrates that were used are shown in Fig. 1.

CHS accepted *p*-coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA and feruloyl-CoA as substrates. Highest conversion rates were observed with *p*-coumaroyl-CoA, which was also preferred, when offered together with a second substrate in equimolar amounts. FHT accepted flavanones of all tested hydroxylation patterns, but converted 5-hydroxyflavanones to a greater extent than 5-deoxyflavanones. DFR was not able to convert DHK to leucopelargonidin by enzyme preparations from *O. viciifolia* indicating the presence of a DFR with narrow substrate specificity as reported for other plants such as tobacco and petunia (Forkmann, 1993; Forkmann and Ruhnau, 1987). This is in agreement with the fact that sainfoin only accumulates anthocyanidins and proanthocyanidins with a 3′,4′- or 3′,4′,5′-hydroxylation pattern, whereas the flavonols carry 1–3 hydroxyl groups in the B-ring. The 5-deoxydihydroflavonols garbanzol and dihydrofisetin were accepted even to a higher extent than DHQ. Since 5-deoxyflavonoids such as garbanzol and liquiritigenin have been identified in *O. viciifolia* (Ingham, 1978), these compounds are natural substrates for the *O. viciifolia* DFR. However, in contrast to DFR, FLS showed a low acceptance of 5-deoxydihydroflavonols reflecting the lack of 5-deoxyflavonols in *O. viciifolia*. DHK was the preferred substrate of FLS. It is obvious that this favours the formation of flavonols at the expense of flavan 3-ol