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SEED α-D-GALACTOSIDES OF SELECTED *VICIA* SPECIES AND ENZYMES INVOLVED IN THEIR BIOSYNTHESIS

LESŁAW B. LAHUTA[1*], JOANNA GOSZCZYŃSKA[1], AND MARCIN HORBOWICZ[2]

[1]*Department of Plant Physiology and Biotechnology, University of Warmia and Mazury, ul. Oczapowskiego 1A, 10-718 Olsztyn, Poland*
[2]*Marcin Horbowicz, Department of Plant Physiology and Genetics, University of Podlasie, ul. Prusa 12, 08-110 Siedlce, Poland*

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We compared the soluble carbohydrate composition of seeds of ten wild and cultivated species of the genus *Vicia*. In some *Vicia* species (*V. angustifolia*, *V. grandiflora*, *V. sativa*, *V. sepium*) they contained only raffinose family oligosaccharides (RFOs) and in others also D-pinitol and its α-D-galactosides. In terms of galactosyl pinitol composition they were divided into three groups: those accumulating small amounts of mono-, di-, tri-galactosyl pinitol A (GPA, ciceritol and TGPA, respectively) and unknown compound (*V. sylvatica* and *V. hirsuta*); those accumulating more ciceritol than TGPA (*V. tetrasperma* and *V. villosa*); and those accumulating more TGPA than ciceritol (*V. cracca* and *V. tenuifolia*). The differences in the activity of galactosyltransferases engaged in RFOs and galactosyl pinitol synthesis confirmed this classification. Seeds of *V. angustifolia*, naturally accumulating only RFOs, showed an ability to accumulate exogenously applied D-pinitol or D-*chiro*-inositol and to form the respective α-D-galactosyl cyclitols. Levels of synthesized galactosides depended on the type and concentration of cyclitol in the feeding solution, and seed maturation stage. However, even a high level of D-pinitol or D-*chiro*-inositol in the feeding solution caused accumulation of only small amounts of mono- and di-galactosyl pinitols, or tri-galactosyl D-*chiro*-inositol in seeds of *V. angustifolia*. Enhanced synthesis of galactosyl cyclitols, mainly mono- and di-galactosides of D-*chiro*-inositol (fagopyritols), clearly reduced production of verbascose. We suggest that exogenously applied free cyclitols inhibit biosynthesis of tri- and di-galactosides and/or cause substrate competition in enzymes of *Vicia* species.

Key Words: D-*chiro*-inositol, D-pinitol, raffinose family of oligosaccharides, galactosyl pinitols, enzymes, *Vicia*, seed.

INTRODUCTION

Galactosides of sucrose, called raffinose family oligosaccharides (RFOs), are common carbohydrates of legume seeds (Horbowicz and Obendorf, 1994). RFOs are considered anti-nutritional compounds because they are poorly digested by monogastric animals and humans, causing flatulence and decreasing the metabolizable energy of the diet as much as 20% (Coon et al., 1990). The level of RFOs in seeds can be altered by breeding mutants accumulating low raffinose or low stachyose (Kerr and Sebastian, 2000; Hitz et al., 2002; Dierking and Bilyeu, 2008) or genetically manipulating the level of RFOs by inhibiting galactinol synthase activity (Bock et al., 2009) and by over-expression of α-D-galactosidase in seeds (Polowick et al., 2009). Another way is to reduce the level of RFOs while promoting the synthesis of related compounds such as galactosyl cyclitols (Frias et al., 1999), which show lower flatus potential (Fleming, 1981). Although genetic transformation of soybean with the *inositol methyl transferase* gene from *Mesembryanthemum crystallinum* increased accumulation of D-ononitol and D-pinitol in vegetative tissues (Chiera et al., 2006) there are no data on the effect of this transformation on the accumulation of galactosyl cyclitols in seeds.

The relationships in biosynthesis of RFOs and galactosyl cyclitols need to be explained in regard to the physiological role of RFOs. RFOs are typical seed storage compounds which are quickly hydrolyzed during initial germination steps. Inhibition of RFO degradation delayed germination of seeds of pea (Blöchl et al., 2007), winter vetch (Lahuta and

*e-mail: lahuta@uwm.edu.pl

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Goszczyńska, 2009) and wild type soybean but not soybean lines with decreased levels of RFOs (Dierking and Bilyeu, 2009). Overexpression of α-galactosidase in pea seeds leads to 40% reduction of RFOs without any effect on seed viability and germination (Polowick et al., 2009; Obendorf et al., 2009).

RFOs are thought to play important roles in seed viability and desiccation tolerance (for review see Obendorf, 1997). In seeds of some legumes, besides galactosides of sucrose, galactosyl derivatives of such cyclitols as D-pinitol, D-ononitol, D-*chiro*-inositol and L-bornesitol are accumulated, although at levels much lower than RFOs (Horbowicz and Obendorf, 1994; Peterbauer and Richter, 2001; Lahuta et al., 2005; Obendorf et al., 2005). Accumulation of RFOs and galactosyl cyclitols in seeds starts at the stage of pod filling and increases during seed maturation and desiccation steps (Obendorf, 1997). The RFO biosynthesis pathway is initiated by galactinol synthase (GolS, EC 2.4.1.123), which produces galactinol from UDP-galactose and *myo*-inositol (Liu et al., 1995). Galactinol (α-D-galactopyranosyl-(1→1)-1L-*myo*-inositol) serves as the main galactosyl donor for galactosylation of sucrose by raffinose synthase (RS, EC 2.4.1.82) (Peterbauer et al., 2002b) and/or for galactosylation of raffinose by stachyose synthase (STS, EC 2.4.1.67) (Hoch et al., 1999; Peterbauer et al., 2002a, 2003). The enzyme responsible exclusively for galactosylation of stachyose, which forms verbascose, has not been identified yet. According to some suggestions, STS probably is responsible for biosynthesis of verbascose too, due to its multifunctional character (Peterbauer et al., 2003). Besides synthesis of galactinol, GolS can produce the monogalactoside of D-*chiro*-inositol (fagopyritol B1) (Obendorf et al., 2004). RS and STS can also transfer the galactose moiety from galactinol to cyclitols (Hoch et al., 1999; Peterbauer et al., 2002a; 2002b). The multifunctional properties of enzymes of RFOs synthesis suggest that the same set of enzymes synthesizes galactosyl cyclitols (for review see Peterbauer and Richter, 2001). In this situation, simultaneous biosynthesis of RFOs and galactosyl cyclitols in seed tissues could be dependent on the concentration of galactosyl acceptors such as sucrose and the cyclitols *myo*-inositol, D-*chiro*-inositol and D-pinitol. In developing seeds of *Vicia hirsuta* (tiny vetch), *Vicia villosa* (winter vetch) and *Vicia cracca* (bird vetch), higher concentrations of D-pinitol than *myo*-inositol throughout seed development and maturation coincided with increasing accumulation of galactosyl pinitols and a decrease in RFOs (Lahuta et al., 2005a).

Indirect evidence for this hypothesis was found in developing seeds of two *Vicia* species – *V. hirsuta* (Lahuta et al., 2005b) and *V. tetrasperma* (Lahuta et al., 2005c) – and in soybean seeds fed with free cyclitols (Obendorf et al., 2004; Gomes et al., 2005). In vetch and soybean seeds, increased levels of D-pinitol and D-*chiro*-inositol enhanced accumulation of their galactosides and inhibited biosynthesis of RFOs. However, D-pinitol occurs naturally in seeds of the investigated *Vicia* species and both cyclitols are present in soybean seeds. It is possible that in these species another enzyme system besides the RFO pathway operates. The pattern of change in the activities of enzymes synthesizing RFOs and galactosyl pinitols in developing *Vicia villosa* seeds indicated that synthesis of stachyose and mono- and di-galactosides of D-pinitol can be catalyzed by one enzyme which is distinct from the enzyme catalyzing synthesis of verbascose and tri-galactosyl pinitol A (Lahuta, 2006).

Here we used seeds of different *Vicia* species as a model to study the relationships between accumulation of RFOs and accumulation of galactosyl cyclitols. First we analyzed the composition of soluble carbohydrates in mature seeds of different species and then analyzed the activities of galactosyltransferases involved in the RFO and galactosyl pinitol biosynthetic pathways. The results of these analyses could prove important for *Vicia taxonomy*. Then we tested whether developing seeds of *Vicia angustifolia*, naturally accumulating only RFOs, are able to transform exogenously applied free cyclitols to the corresponding α-galactosides, and whether the process would affect accumulation of RFOs. If the same set of enzymes is engaged in biosynthesis of both RFOs and galactosyl cyclitols, high concentrations of cyclitols should promote the accumulation of mono-, di- and tri-galactosyl cyclitols instead of RFOs in seeds.

MATERIAL AND METHODS

SEED MATERIAL

Mature seeds of wild-growing *Vicia* species were collected from plants growing in natural ecosystems in Poland: *V. angustifolia* L. (common vetch), *V. cracca* L. (bird vetch), *V. grandiflora* Scop. (yellow-flowered vetch), *V. hirsuta* (L.) S.F. Gray (hairy tare), *V. sepium* L. (bush vetch), *V. sylvatica* L. (wood vetch), *V. tenuifolia* Roth. (fine-leaved vetch) and *V. tetrasperma* (L.) Schreb. (smooth tare). Seeds of cultivated *Vicia* species were obtained from Rolnas (Poland): *Vicia sativa* L. (garden vetch cv Kamiko) and *Vicia villosa* Roth. (winter vetch cv Minikowska). Mature seeds were used for estimation of soluble carbohydrate content and composition and for galactosyltransferase activity assays.

EXPLANT FEEDING EXPERIMENT

Plants of *Vicia angustifolia* L. (common vetch) were grown in a greenhouse in 1 dm^3 pots (two plants per pot) containing a mixture of soil and sand (4:1) under

a 14 h photoperiod at 20°C/16°C (day/night). Plants were illuminated with fluorescent lamps (light intensity 190 μmol m^{-2} s^{-1}), watered daily and fertilized weekly with NPK fertilizer (3:1:1, 0.5 g pot^{-1}).

Stem-leaf-pod explants of common vetch were used in feeding experiments with D-pinitol and D-*chiro*-inositol. Explants were excised 2 cm below and 0.5 cm above the node with the first pod (at 22–30 days after pollination, DAP). Each explant included part of the stem with one node, one leaf and one pod. Immediately after excision the bottom part of the stem was placed in a 1.5 mL micro-tube containing 1 mL mixture of sucrose (10 mM), D-pinitol or D-*chiro*-inositol (at 1, 5 or 10 mM). The control feeding medium contained only sucrose (10 mM). Temperature and light conditions were the same as for the plants grown in the greenhouse. After two days of feeding, all the solution was absorbed by the explants. Then the explants with pods were dried at 24°C and 25% RH for 14 days.

ANALYSIS OF SOLUBLE CARBOHYDRATES

Carbohydrates were extracted from seeds with a mixture of ethanol:water (1:1, v/v) and analyzed by high resolution gas chromatography, as described previously (Lahuta, 2006). Carbohydrates were quantified with the following standards: *myo*-inositol, sucrose, raffinose, stachyose (Sigma-Aldrich), verbascose (Megazyme, Australia), D-pinitol, D-*chiro*-inositol and galactinol (Research Industries, New Zealand). Standards of galactosyl cyclitols not available commercially were isolated and purified from natural sources: fagopyritols (mono-, di- and tri-galactosyl D-*chiro*-inositol, called fagopyritol B1, B2 and B3, respectively) from buckwheat seeds (Horbowicz et al., 1998), galactosyl pinitol A (GPA), galactosyl pinitol B (GPB), di-galactosyl *myo*-inositol (DGMI), ciceritol (di-galactosyl pinitol A) and tri-galactosyl pinitol A (TGPA) from winter vetch seeds (Szczeciński et al., 2000). Carbohydrate content was calculated from standard curves of the corresponding pure compound. Xylitol (Fluka) was used as internal standard. Results of analyses are means ±SE of three independent replicates.

ENZYME ACTIVITY ASSAY

Samples of mature seeds (200 mg) were homogenized with a mortar and pestle in 1 mL ice cold extraction buffer (pH 7.0, 50 mM HEPES-NaOH, 1 mM dithiothreitol [DTT], 1% (w/v) polyvinyl polypyrrolidone, 1% protease inhibitor cocktail (Sigma, no. P2714-1EA) and centrifuged (21,000 g for 30 min at 4°C). Aliquots of the supernatant were desalted by centrifugal gel filtration (Sephadex G25 superfine) and concentrated by centrifugal ultrafiltration (Centricon-10, Millipore). The activity of galactinol synthase (GolS), raffinose synthase (RS), stachyose synthase (STS) and verbascose synthase (VS) was determined according to the method described by Peterbauer et al. (2001). The enzymes responsible exclusively for synthesis of α-galactosides of D-pinitol have not been identified or named yet, so enzymes catalyzing the reactions of di- and tri-galactosyl pinitol synthesis were arbitrarily named ciceritol synthase (CICS) and TGPA synthase (TGPAS), respectively. As stachyose synthase may be responsible also for synthesis of GPA, ciceritol and/or TGPA (Peterbauer et al., 2002b; 2003), CICS and TGPAS activity was analyzed similarly to stachyose synthase activity, but GPA or ciceritol were used instead of raffinose as galactose moiety acceptor (Lahuta, 2006). Synthesis of galactosyl pinitols was estimated in a total volume of 30 μL containing 50 mM HEPES-NaOH (pH 7.0), 1 mM DTT, 10 mM galactinol and 20 mM GPA (for ciceritol synthesis) or 20 mM ciceritol (for TGPA synthesis). Reactions were stopped after 2 h (ciceritol synthesis) or 4 h (TGPA synthesis) of incubation by adding 70 μL 70% ethanol and boiling the mixture for 5 min. The reaction products were determined by gas chromatography. Buffer solution was used instead of galactosyl acceptors in the control. Soluble protein was determined using Bradford's dye-binding procedure (Bio-Rad) with bovine serum albumin as standard. All reactions were run with three independent seed samples.

DATA ANALYSIS

The significance of results was tested by ANOVA and Tukey's post test (if overall $P<0.05$) for multiple comparisons.

RESULTS

SOLUBLE CARBOHYDRATES IN SEEDS

The seeds of all studied *Vicia* species contained sucrose, *myo*-inositol, galactinol and sucrose galactosides (RFOs) as common soluble carbohydrates (Tab. 1). Besides the carbohydrates, some *Vicia species* (*V. sylvatica*, *V. hirsuta*, *V. tetrasperma*, *V. villosa*, *V. cracca*, *V. tenuifolia*) contained D-pinitol and its α-D-galactosides. All the studied *Vicia* species can be divided into four types in respect to galactosyl pinitol and RFO composition found in their seeds. In the first type (I) of seeds (*V. augustifolia*, *V. grandiflora*, *V. sativa*, *V. sepium*) only *myo*-inositol and its common galactoside, galactinol, were found, although in relatively low amounts. In these seeds the RFO level reached almost 90% of total soluble carbohydrates. The second type (II) of *Vicia* seeds (*V. sylvatica*, *V. hirsuta*) contained, besides *myo*-inositol, D-pinitol as well as its α-D-galactosides (galactosyl pinitols), mainly GPA and ciceritol. The seeds of both species also con-

TABLE 1. Carbohydrate composition (mg g^{-1} DW) in seeds of vetch (*Vicia*) species. Means ± SE of three replicates. Values within rows followed by the same letter do not differ significantly at $p<0.05$

Carbohydrate	*V. angustifolia*	*V. grandiflora*	*V. sativa* (cv Kamiko)	*V. sepium*	*V. sylvatica*	*V. hirsuta*	*V. tetrasperma*	*V. villosa*	*V. cracca*	*V. tenuifolia*
Sucrose	7.8^{a} ±1.5	5.9^{a} ± 0.1	8.9^{a} ± 0.5	14.9^{b}±1.2	12.6^{ab}±0.7	10.6^{ab}±1.8	4.6^{ab}±0.4	16.1^{b}±0.4	24.9^{c}±1.8	7.3^{a} ± 0.7
Raffinose	0.7^{a} ±0.1	0.9^{a} ± 0.0	0.9^{a} ± 0.1	1.9^{b} ± 0.1	0.8^{a} ± 0.0	2.3^{b} ± 0.4	1.4^{ab}±0.1	1.2^{a} ± 0.1	2.9^{c} ± 0.2	0.5^{a} ± 0.0
Stachyose	8.0^{a} ±1.1	4.7^{b} ± 0.0	8.6^{a} ± 0.5	8.3^{a} ± 0.6	1.6^{c} ± 0.1	10.1^{a}±1.8	2.7^{bc} ± 0.2	5.69^{a}±0.3	10.4^{a}±0.4	1.0^{c} ± 0.0
Verbascose	59.6^{a} ±1.2	24.9^{b}±0.5	27.2^{b}±2.9	33.1^{bc}±2.6	19.7^{b}±0.8	36.9^{c}±3.2	20.1^{b}±2.1	17.1^{b}±1.1	17.2^{b}±1.0	1.9^{d} ± 0.1
myo-Inositol	0.3^{a}±0.1	0.3^{a}±0.0	0.3^{a}±0.0	0.2^{a}±0.0	0.6^{b}±0.1	0.3^{a} ± 0.0	0.4^{ab}±0.0	0.5^{ab}±0.0	0.4^{ab}±0.0	0.4^{ab}±0.0
Galactinol	0.9^{a}±0.1	0.7^{a}±0.0	0.6^{b}±0.1	0.3^{b}±0.0	0.5^{b}±0.0	0.7^{ab}±0.1	1.5^{c} ± 0.1	0.6^{ab}±0.0	1.0^{ac}±0.1	1.6^{c} ± 0.0
di-galacto-*myo*-Inositol	–	–	–	–	2.2^{a}±0.2	1.5^{ab}±0.2	0.6^{b} ± 0.0	1.7^{ab}±0.0	1.4^{ab}±0.1	1.9^{a} ± 0.6
D-Pinitol	–	–	–	–	1.4^{a}±0.1	4.8^{b}±0.7	3.5^{c} ± 0.3	1.5^{ac}±0.5	6.4^{b}±0.5	8.4^{d} ± 0.6
Galactosyl pinitol A	–	–	–	–	1.2^{a}±0.1	5.4^{b}±0.9	1.6^{ac} ± 0.2	1.7^{ac}±0.0	3.1^{c}±0.2	3.0^{c} ± 0.3
Galactosyl pinitol B	–	–	–	–	0.3^{a}±0.1	1.1^{b}±0.2	0.6^{a} ± 0.0	0.6^{ab}±0.0	2.0^{c}±0.2	2.8^{c} ± 0.2
di-Galactosyl pinitol A	–	–	–	–	2.4^{a}±0.1	4.4^{a}±0.8	9.9^{b} ± 1.0	18.9^{c}±0.9	12.2^{b}±0.8	13.9^{b}±1.4
tri-Galactosyl pinitol A	–	–	–	–	1.1^{a}±0.1	1.5^{a}±0.2	5.8^{ab}±0.6	12.8^{bc}±0.7	25.9^{c}±2.1	46.9^{c}±3.7
tetra-Galactosyl pinitol A	–	–	–	–	–	–	0.6^{a} ± 0.0	0.9^{ab}±0.0	1.5^{b}±0.1	3.5^{c}±0.2

tained an unknown compound (its retention time was between that of *myo*-inositol and sucrose during GC separation) whose concentration was 3 times higher than that of sucrose (data not shown). This compound was also previously detected but not identified by Yasui et al. (1987) only in seeds of the same two *Vicia* species among 29 species they investigated. The third type (III) of seeds (*V. tetrasperma*, *V. villosa*) contained a high concentration of galactosyl pinitols (GPs), and the major galactosides were ciceritol (di-galactosyl pinitol) and tri-galactosyl pinitol A (TGPA). In the last type (IV) of seeds (*V. cracca* and *V. tenuifolia*) the concentration of D-pinitol galactosides (mainly TGPA) was much higher than that of RFOs. In these seeds the GP level reached 60% (for *V. cracca*) and 96% (*V. tenuifolia*) of total α-D-galactosides. Among the RFOs present in seeds of all studied *Vicia* species, verbascose dominated. In seeds that contained elevated amounts of ciceritol and TGPA (groups III and IV) a higher homologue of TGPA was found as well, tentatively identified as tetra-galactoside of D-pinitol A (Tab. 1). Its concentration increased with the increase of TGPA.

GALACTOSYLTRASNFERASE ACTIVITY IN MATURE SEEDS

In crude desalted extracts from dry vetch seeds the activity of enzymes synthesizing both RFOs and galactosyl pinitols was measured (Fig. 1). Galactinol synthase (GolS) activity was highest (Fig. 1a) of all investigated galactosyltransferases. There were no significant differences in GolS activity between the seeds of the four *Vicia* groups ($p<0.05$). RS activity was lowest (Fig. 1b). Increases of the galactosyl pinitol concentration in seeds (Tab. 1) were associated with decreased stachyose synthase (STS) and verbascose synthase (VS) activity (Fig. 1c,d). In seeds from group IV (with the highest concentration of galactosyl pinitols), STS and VS activity was lowest (Fig. 1c,d), and ciceritol synthase (CICS) and TGPA synthase (TGPAS) activity was significantly higher than in the seeds of groups I and II (Fig. 1e,f). STS and CICS activity were negatively correlated (r = -0.28), as were VS and TGPAS activity (r = -0.12).

EXPLANT FEEDING EXPERIMENT

Feeding of common vetch explants containing pods with maturing seeds (22–30 DAP) and followed explants drying resulted in uptake of free cyclitols and the formation of their galactosides (Figs. 2, 3). Before the feeding experiment, seeds at 22 DAP contained only galactinol (0.11 mg g^{-1} dry weight, DW); at 26 DAP the concentration of galactinol doubled and the seeds also contained raffinose (0.28 mg g^{-1} DW). During the next 4 days of maturation, biosynthesis of RFOs increased (total RFO content reached

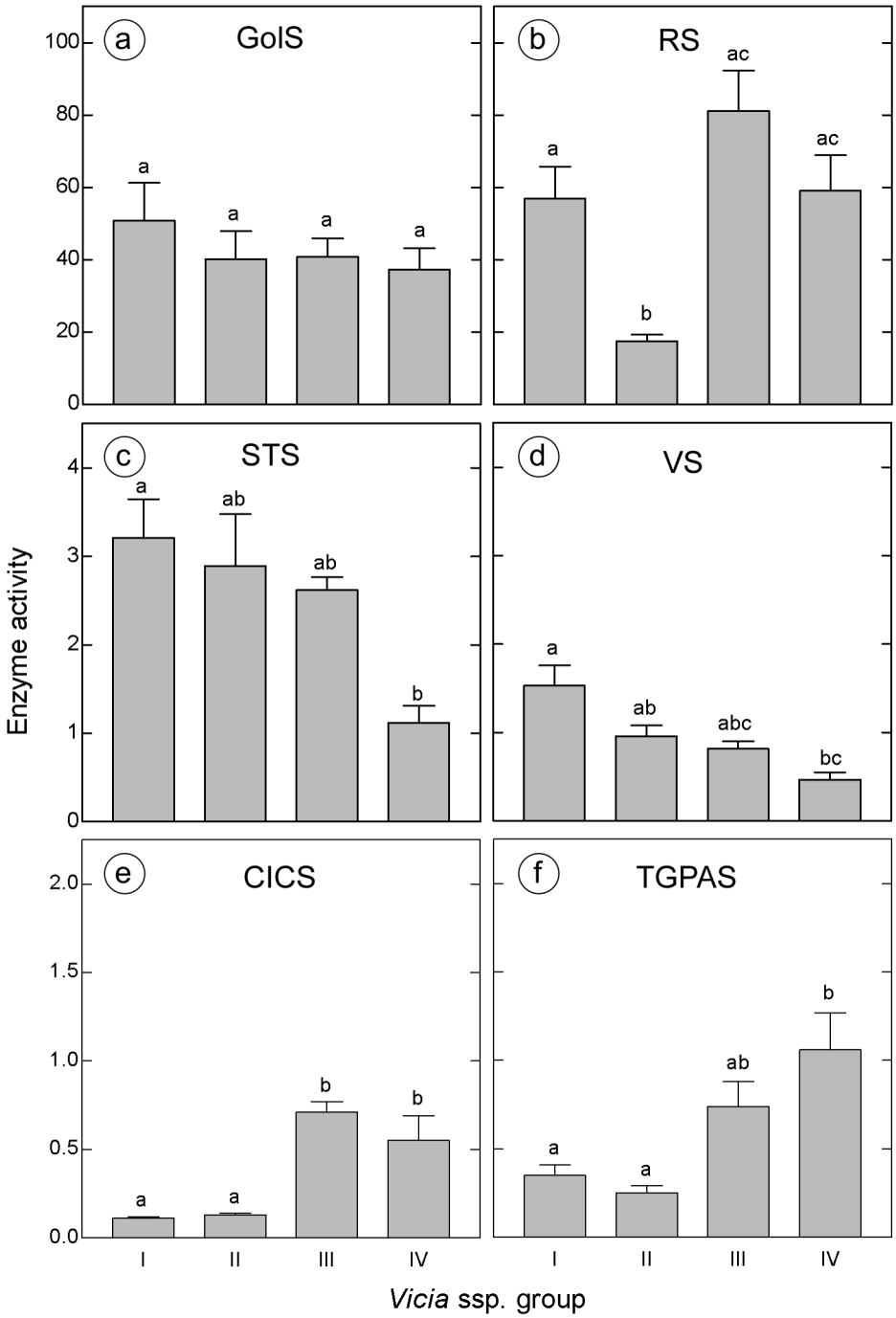

Fig. 1. Activity of enzymes synthesizing galactinol **(a)**, raffinose **(b)**, stachyose **(c)**, verbascose **(d)**, ciceritol **(e)**, and TGPA **(f)** in mature seeds of *Vicia* spp., grouped according to the composition of α-D-galactosides (Tab. 1) into four groups: I (*V. angustifolia*, *V. grandiflora*, *V. sativa*, *V. sepium*), II (*V. sylvatica*, *V. hirsuta*), III (*V. tetrasperma*, *V. villosa*), and IV (*V. cracca*, *V. tenuifolia*). Activity of enzymes (nmol min^{-1} mg^{-1} protein – a, c-f; pmol min^{-1} mg^{-1} protein – b) was estimated by analysis of amounts of products in reactions: galactinol synthase, GolS (EC 2.4.1.123): UDP-galactose + *myo*-inositol → galactinol + UDP; raffinose synthase, RS (EC 2.4.1.82): sucrose + galactinol → raffinose + *myo*-inositol; stachyose synthase, STS (EC 2.4.1.67): raffinose + galactinol → stachyose + *myo*-inositol; verbascose synthase, VS (EC 2.4.1.x): stachyose + galactinol → verbascose + *myo*-inositol; ciceritol synthase, CICS: galactosyl pinitol A + galactinol → ciceritol (di-galactosyl pinitol A) + *myo*-inositol; *tri*-galactosyl pinitol A synthase, TGPAS: ciceritol + galactinol → TGPA + *myo*-inositol. Means ± SE. Bars with the same letters do not differ significantly ($p<0.05$) after Tukey's correction for multiple comparisons.

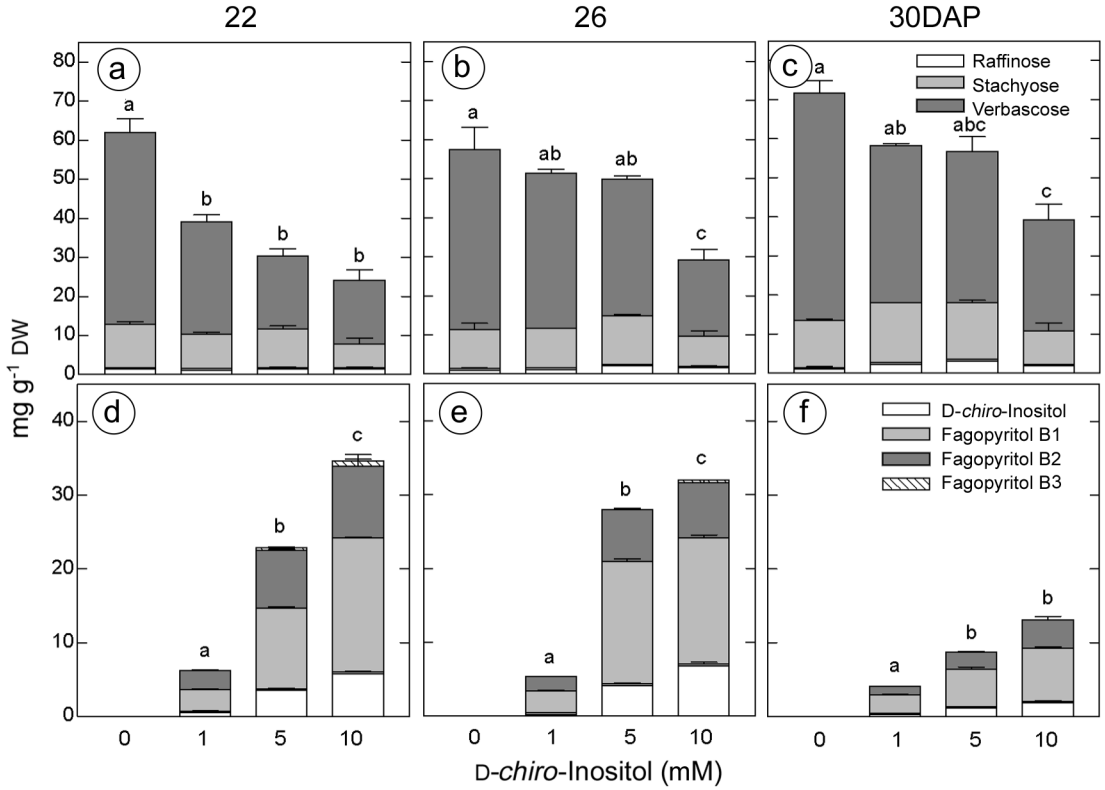

Fig. 2. Effect of feeding explants D-*chiro*-inositol on accumulation of RFOs **(a–c)**, free D-*chiro*-inositol (*chiro*-In) and its respective mono-, di- and tri-galactosides: fagopyritol B1 (Fag B1), fagopyritol B2 (Fag B2) and fagopyritol B3 (Fag B3), (**d–f**) in maturing (22, 26 and 30DAP) *Vicia angustifolia* seeds. Means ±SE (mg g^{-1} DW) of three replicates. Bars with the same letters do not differ significantly ($p<0.05$) after Tukey's correction for multiple comparisons.

15.11 mg g^{-1} DW. Regardless of maturation stage, seeds from explants fed D-*chiro*-inositol accumulated free cyclitol and formed its mono- and digalactosides: fagopyritol B1 and B2 (Fig. 2d–f). In seeds that accumulated D-pinitol, its galactosides GPA, GPB and ciceritol were formed (Fig. 3d–f). Seeds at 22 DAP were also able to synthesize small amounts of fagopyritol B3 (Fig. 2d), but no TGPA (Fig. 3d). The concentration of free cyclitols and their galactosides was increased with the increase of exogenous cyclitols. Enhanced biosynthesis of galactosyl cyclitols was associated with a decline of RFO levels (mainly verbascose) (Fig. 2a–c, 3a–c), but the effect gradually disappeared during seed maturation. Regardless of the stage of maturation, seeds accumulated more fagopyritols (Fig. 2d–f) than galactosyl pinitols (Fig. 3d–f).

DISCUSSION

We found that sucrose, *myo*-inositol, galactinol and RFOs were ubiquitous soluble carbohydrates in the seeds of all investigated *Vicia* species. Among the RFOs, verbascose was the major oligosaccharide. Seeds of six *Vicia* species (*V. sylvatica*, *V. hirsuta*, *V. tetrasperma*, *V. villosa*, *V. cracca*, *V. tenuifolia*) contained D-pinitol and its α-D-galactosides. In terms of variation of galactoside levels, seeds of *Vicia* species can be classified in four types, as Yasui (1987) proposed. This classification can be amended to include the occurrence of tetra-galactosyl pinitol A (tentatively identified), whose concentration in seeds correlated positively with the level of tri-galactopinitol A (TGPA) (Tab. 1). Additionally, a unique composition of α-D-galactosides, with galactosyl pinitols accumulated instead the RFOs, was found in seeds of *V. tenuifolia*. The presence of galactosyl cyclitols without RFOs was found earlier only in buckwheat seeds (Horbowicz et al., 1998). However, buckwheat seeds contain galactosides of D-*chiro*-inositol (called fagopyritols), and *V. tenuifolia* seeds contain mainly tri- and di-galactosyl pinitol A. Biosynthesis of fagopyritols in buckwheat (Ueda et al., 2005) and soybeans (Obendorf et al., 2004) depends on the catalytic properties of galactinol synthase (GolS), synthesizing galactinol as well as two isomers of D-*chiro*-inositol galactosides (fagopyritol B1 and A1). Accumulation of these galactosides

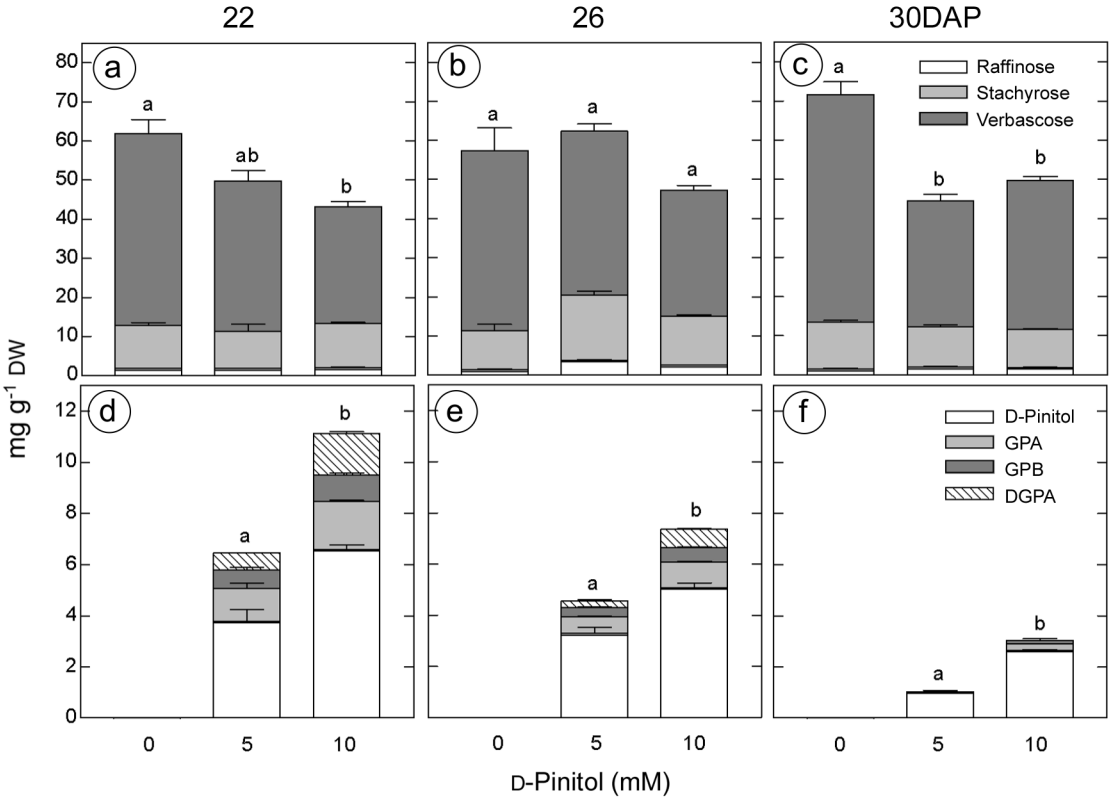

Fig. 3. Effect of feeding explants D-pinitol on accumulation of RFOs **(a–c)**, free D-pinitol and its respective mono- and di-galactosides: galactosyl pinitol A, B (GPA, GPB), and di-galactosyl pinitol A (DGPA, common name ciceritol) (**d–f**) in maturing (22, 26 and 30DAP) *Vicia angustifolia* seeds. Means ±SE of three replicates. Bars with the same letters do not differ significantly ($p<0.05$) after Tukey's correction for multiple comparisons.

depends on the concentration of D-*chiro*-inositol transported from the maternal tissue to the seed (Gomes et al., 2005). However, GolS does not participate in galactosyl pinitol biosynthesis (Obendorf et al., 2004).

We suggest that preferential accumulation of galactosyl pinitols in seeds of *V. tenuifolia* and *V. cracca* (Tab. 1) can be explained by study of RFO pathway enzymes. In this work the seeds of all investigated *Vicia* species were found able to synthesize both RFOs and di- and tri-galactosyl pinitol A (Fig. 1). Synthesis of galactosyl pinitol A (GPA) was possible with galactinol as sole galactosyl donor, indicating that GolS does not participate in GPA formation (data not shown). The differences between the *Vicia* species in the levels of galactosyltransferase activity in di- and tri-galactoside synthesis (stachyose, verbascose, RFOs, ciceritol and TGPA) can be used for taxonomic classification of the species. Although it is still not clear whether digalactosides (stachyose and ciceritol) or trigalactosides (verbascose and TGPA) are synthesized by the same enzymes, we found large differences in the activity of enzymes synthesizing both of these types of carbohydrates (Fig. 1c–f). Probably the variation is due to differences in enzyme structure, leading to different substrate specificity. Unfortunately, unlike isozyme analysis via electrophoretic separation, which is a useful method for evaluation of phylogenetic relations between species and sections (Jaaska, 2005), there are no methods for isozyme analysis of the RFO pathway. Our assays of enzyme activity with various substrates at the same concentrations may serve as an indirect method for screening for the possible presence of different forms of the same enzymes.

The main goal of our study was to determine the regulatory role of free cyclitols in accumulation of α-D-galactosides in *Vicia* seeds. In previous studies we introduced feeding of stem-leaf-pod explants with D-pinitol and D-*chiro*-inositol as an effective method for evaluating the regulatory role of cyclitols in galactoside biosynthesis in seeds of *V. hirsuta* (Lahuta et al., 2005b) and *V. tetrasperma* (Lahuta et al., 2005c). However, the seeds of those two species naturally accumulate D-pinitol and pinitol galactosides. Thus, increasing the concentration of free D-pinitol could only enhance the activity of the enzymatic

pathway, leading to accumulation of higher amounts of galactosyl pinitols, as suggested previously (Lahuta et al., 2005a). Therefore we decided to feed free cyclitols to explants of *Vicia angustifolia*, whose mature seed contains the highest level of RFOs among the investigated *Vicia* species (Tab. 1), and not D-pinitol or D-*chiro*-inositol and their galactosides. As expected, feeding explants (at 22-30 DAP) with those cyclitols resulted in accumulation of free cyclitols in seed. However, D-pinitol applied even at high concentration caused accumulation of mono- and di-galactosyl cyclitols but not tri-galactosyl pinitol A, regardless of seed maturation stage (Fig. 2a–d). Similarly to earlier-investigated *V. hirsuta*, *V. tetrasperma*, soybean (Lahuta et al., 2005b, 2005c; Obendorf et al., 2004; Gomes et al., 2005) and buckwheat (Ma et al., 2005), common vetch seed converted the D-*chiro*-inositol to galactosides (fagopyritols) much more effectively (Fig. 2d–f) than it converted D-pinitol to the corresponding galactosides (Fig. 3d–f). This was probably due to utilization of D-*chiro*-inositol by GolS, indicating the highest activity among the analyzed enzymes (Fig. 1). During estimation of GolS activity in the presence of D-*chiro*-inositol as galactosyl acceptor, we observed the formation of fagopyritol B1 only (data not shown), as in the case of *V. hirsuta* (Lahuta et al., 2005b). The higher level of fagopyritols accumulated in seeds at 22 and 26 DAP as compared to seed at 30 DAP may be attributable to higher activity of GolS and STS. At the stage of cotyledon growth and dry mass accumulation, the activity of GolS, RS and ST reached maximum in maturing pea (Peterbauer et al., 2001) and winter vetch seeds (Lahuta, 2006), and then dramatically decreased up to full seed maturity. In our experiment the explants were fed limited amounts of solution and then dried. Our results suggest that biosynthesis of α-D-galactosides can be inhibited by seed desiccation.

The uptake of cyclitols by seeds and the formation its galactosides decreased the synthesis of verbascose. Although increasing concentrations of free cyclitols gradually reduced verbascose accumulation, clear inhibition of verbascose synthesis by D-*chiro*-inositol and D-pinitol was observed. This seems to mean that reduction of verbascose accumulation was not a result of the competition between the levels of galactose moiety acceptors and donors, but rather due to inhibition of STS and/or VS activity by the free cyclitols.

On the other hand, the high concentrations of galactosyl pinitols in seeds of *V. cracca* and *V. tenuifolia* may be a result of both high concentration of D-pinitol transported from vegetative tissues into seeds and modification of STS and/or VS. Of all the studied species, only *V. cracca* and *V. tenuifolia* are perennials with over-wintering underground stolons. In stolons of white clover the concentration of D-pinitol increased during winter (Turner and Pollock, 1998); its presence may be associated with plant hardiness. In vegetative tissues of *V. tenuifolia* the concentration of D-pinitol was higher than the sucrose concentration (our unpublished data). Intensive synthesis of D-pinitol in vegetative tissues may be the reason its concentration is higher than sucrose in phloem. In effect, developing seeds contained elevated amounts of D-pinitol (Lahuta et al., 2005a) and probably adapted the RFO pathway enzymes for efficient utilization of D-pinitol as the main galactosyl acceptor.

According to Yasui and Ohashi (1990), the primitive ancestors of *Papilionoideae* (including *Vicia* spp. from groups II-IV in our study) had the ability to synthesize and accumulate both D-pinitol galactosides and raffinose family oligosaccharides. Some of the descendants lost the ability to synthesize D-pinitol. Other descendants lost the ability to accumulate raffinose family oligosaccharides or only verbascose (*Vigna, Phaseolus*) (Yasui and Ohashi, 1990). We suggest that *Vicia* spp. seeds may provide the best source for isolation, purification and analysis of the catalytic properties of enzymes synthesizing digalactosides and trigalactosides of D-pinitol to show the genetic differences and explain the reason various legumes replaced raffinose family oligosaccharides with the corresponding galactosyl cyclitols.

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