BIOSYNTHESIS OF RAFFINOSE FAMILY OLIGOSACCHARIDES AND GALACTOSYL PINITOLS IN DEVELOPING AND MATURING SEEDS OF WINTER VETCH (*VICIA VILLOSA* ROTH.)

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ABSTRACT

Changes in the accumulation of two types of α-D-galactosides: raffinose family oligosaccharides and galactosyl pinitols were compared with changes in the activities of galactosyltransferases during winter vetch (*Vicia villosa* Roth.) seed development and maturation. Occurrence of galactinol and raffinose in young seeds and changes in activities of galactinol synthase and raffinose synthase during seed development indicated that formation of raffinose oligosaccharides (RFOs) preceded synthesis of galactopinitols. Although transfer of galactose residues into raffinose oligosaccharides increased as seeds were maturing, at late stages of seed maturation the accumulation of galactopinitols was preferred to that of RFOs. In the present study, activities of enzymes transferring galactose moieties from galactinol to D-pinitol forming galactopinitol A, and further transfer of galactose moieties from galactinol to mono- and di-galactopinitol A were detected throughout seed development and maturation. This is a new observation, indicating biological potential of winter vetch seeds to synthesize mono-, di- and tri-galactosides of D-pinitol in a pathway similar to RFOs. The pattern of changes in activities of stachyose synthase and enzymes synthesizing galactopinitols (named galactopinitol A synthase and ciceritol synthase) suggests that formation of stachyose, mono- and di-galactopinitol A (ciceritol) is catalyzed by one enzyme. High correlation between activities of verbascose synthase and enzyme catalyzing synthesis of tri-galactopinitol A from galactinol and ciceritol (named tri-galactopinitol A synthase) also suggests that biosynthesis of both types of tri-galactosides was catalyzed by one enzyme, but distinct from stachyose synthase. Changes in concentrations of galactosyl acceptors (sucrose and D-pinitol) can be a factor which regulates splitting of galactose moieties between both types of galactosides in winter vetch seeds.

KEY WORDS: cyclitol, galactopinitol, galactosyltransferase, pinitol, raffinose family oligosaccharides, seeds, *Vicia villosa*.

INTRODUCTION

Biosynthesis of raffinose family oligosaccharides (RFOs) starts during seed development and increases in response to seed drying or precocious desiccation (for reviews see Obendorf 1997; Peterbauer and Richter 2001). The initial step involves formation of galactinol (*O*-α-D--galactopyranosyl-(1→1)–l-*myo*-inositol) from UPD-D-galactose and *myo*-inositol by the action of galactinol synthase (GolS, EC 2.4.1.123) (Peterbauer and Richter 2001). Galactinol acts as the main galactosyl donor in biosynthesis of RFOs, which occurs via sequential transfer of galactosyl residues from galactinol to sucrose, raffinose or stachyose, forming raffinose, stachyose and verbascose, respectively (Keller and Pharr 1996). Biosynthesis of raffi-

Abbreviations:
RFOs – raffinose family oligosaccharides (raffinose, stachyose, verbascose); galactinol – α-D-galactopyranosyl-(1→1)-1L-*myo*-inositol; DGMI, di-galactosyl-*myo*-inositol – α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→1)-1L-*myo*-inositol; GPs – galactosyl pinitols; GPA, galactopinitol A – α-D-galactopyranosyl-(1→2)-4-*O*-methyl-1D-*chiro*-inositol; GPB, galactopinitol B – α-D-galactopyranosyl-(1→2)-3-*O*-methyl-1D-*chiro*-inositol; ciceritol, di-galactosyl-D-pinitol A – α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-1D-*chiro*-inositol; TGPA, tri-galactosyl-D-pinitol A – α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-1D-*chiro*-inositol; GolS – galactinol synthase; RS – raffinose synthase; STS – stachyose synthase; VS – verbascose synthase; GGT – galactan: galactan galactosyltransferase; GPAS – galactopinitol A synthase; CicS – ciceritol synthase; TGPAS – tri-galactopinitol A synthase; DAF – day after flowering; FW – fresh weight; DW – dry weight

nose and stachyose is catalyzed by two distinct enzymes: raffinose synthase (RS, EC 2.4.1.82) and stachyose synthase (STS, EC 2.4.1.67). Biosynthesis of verbascose (higher homologues of stachyose), which is the major galactoside in seeds of several agriculturally important legume species (like pea, field bean, faba bean, vetch) is not fully explained. In pea seeds verbascose can be synthesized from stachyose and galactinol or from two stachyose molecules without galactinol (Peterbauer et al. 2001) by multifunctional STS (Peterbauer et al. 2003). Verbascose synthase (VS, EC 2.4.1.x) responsible exclusively for formation of verbascose was not yet purified.

Seeds of some legume species beside RFOs accumulate also galactosides of cyclitols (Horbowicz and Obendorf 1994). The same set of enzymes can be engaged in biosynthesis of galactosyl cyclitols as in the RFOs pathway (Peterbauer and Richter 2001). However, utilization of cyclitols as galactosyl acceptors is strongly correlated to their molecular structure and concentration. GolS activity is inhibited by low concentration of *myo*-inositol and in effect accumulation of RFOs in developing seeds is reduced, too (Hitz et al. 2002; Karner et al. 2004). GolS can utilize other *myo*-inositol isomer – D-*chiro*-inositol as galactosyl acceptor instead of *myo*-inositol, forming fagopyritol B1 (Frydman and Neufeld 1963; Obendorf et al. 2004; Lahuta et al. 2005a). In seeds of buckwheat (*Fagopyrum esculentum*, Polygonaceae) one form of GolS (FeGolS-1) synthesizes two galactosides of D-*chiro*-inositol – fagopyritol A1 (α-D--galactopyranosyl-(1→3)-1D-*chiro*-inositol) and fagopyritol B1 (α-D-galactopyranosyl-(1→2)-1D-*chiro*-inositol), and the second one (FeGolS-2) – only fagopyritol B1 (Ueda et al. 2005). Several-fold higher concentration of D-*chiro*-inositol than *myo*-inositol throughout buckwheat embryo development can be a reason why fagopyritols are major soluble carbohydrates in buckwheat seeds (Horbowicz et al. 1998; Ma et al. 2005). Feeding of immature buckwheat or soybean seeds with D-*chiro*-inositol increased accumulation of fagopyritols and decreased that of RFOs (Gomes et al. 2005; Ma et al. 2005). In tiny vetch seeds fed with D-*chiro*-inositol (which naturally does not occur in this species) at concentrations higher than *myo*-inositol inhibited biosynthesis of galactinol. Besides, accumulation of fagopyritol B1 was preferred to that of galactinol and RFOs (Lahuta et al. 2005a). Those results indicate a competition between *myo*-inositol and D-*chiro*-inositol as galactosyl acceptors for GolS. Although D-*chiro*-inositol is present in soybean (Horbowicz and Obendorf 1994) and lupin seeds (Górecki et al. 1997), several other legumes contain mainly D-pinitol or D-ononitol (methyl ethers of D--*chiro*-inositol) and their galactosides as an important group of soluble galactosides (Yasui 1985; Yasui et al. 1987; Horbowicz and Obendorf 1994; Frias et al. 1999; Peterbauer et al. 1998; Lahuta et al. 2005b). Formation of mono-galactosides of D-pinitol or D-ononitol can be catalyzed rather by RS and STS, than GolS, which is not able to synthesize of galactopinitol or galactoononitol (Frydman and Neufeld 1963; Hoch et al. 1999; Obendorf et al. 2004; Lahuta et al. 2005a; Ueda et al. 2005). Purified (or recombinant) RS from pea seeds indicates broad substrate specificity and catalyzes formation of galactopinitol A from galactinol and D-pinitol (Peterbauer et al. 2002a). Similarly, STS purified from seeds of *Vigna umbellata* (Peterbauer et al. 1998) and *Lentil* (Hoch et al. 1999) effectively utilizes D-ononitol and D-pinitol instead of raffinose as acceptors of galactose. However, in contrast to RS, STS can also utilize some of galactosyl cyclitols other than galactinol, as galactose donors for synthesis of stachyose or di-galactosyl cyclitols. In the previous studies it was found that in seeds of some *Vicia* species a quantitative predominance of D-pinitol over *myo*-inositol during seed development favored accumulation of D-pinitol galactosides (Lahuta et al. 2005b). Feeding of tiny vetch and smooth tare explants with D-pinitol or D-*chiro*-inositol increased the formation of high amounts of mono-, di- or tri-galactosides of cyclitols in seeds and dramatically decreased the level of verbascose, and at lesser extent, stachyose (Lahuta et al. 2005a, c). Similarly, feeding of soybean seeds with free cyclitols increased accumulation of their galactosides and inhibited accumulation of stachyose (Gomes et al. 2005). Negative correlation between concentration of verbascose and ciceritol in seeds was demonstrated in genetic studies performed on lentil (Frias et al. 1999). This inverse correlation between RFOs and galactosyl pinitols (GPs) confirms hypothesis that the same biosynthetic pathway operates in formation of both type of galactosides. In *V. umbellata* and lentil seeds, containing mainly RFOs, biosynthesis of galactosyl cyclitols can facilitate accumulation of RFOs (Peterbauer et al. 1998; Hoch et al. 1999).

Explanation of the correlations in biosynthesis and accumulation of both types of galactosides should be useful for research focusing on the modification of α-D-galactosides composition in legume seeds (Wang et al. 2003). It is considered that GPs characterize lower flatulence potential than RFOs (in digestive tract of human and monogastric animals) (Guillon and Champ 2002). In addition, some of galactosyl cyclitols indicate a potential health benefits (Kawa et al. 2003; Larner et al. 2003). Therefore, the reduction or elimination of RFOs from seeds or their replacement by GPs is an attractive breeding target.

In the present study the accumulation of RFOs and galactopinitols was compared with changes in the activities of galactosyltransferases during maturation of winter vetch (*Vicia villosa* Roth) seeds. Seeds of this vetch species accumulate galactosyl pinitols and RFOs in equal amounts (Lahuta et al. 2005b), which is unique among legumes. Therefore, they are a convenient object for studies of relationships between biosynthesis of both types of galactosides.

MATERIALS AND METHODS

Plant material

Plants of winter vetch (*cv*. Minikowska) were grown on experimental fields from April to July. Fully opened flowers were tagged, and pods were taken to analyses at 4 days intervals beginning from 14 day after flowering (DAF) to full seed maturity (46 DAF). Fresh and dry weights of the seeds were determined in five replicates (20 seeds each).

Analysis of soluble carbohydrates

Extraction of soluble carbohydrates from seeds was performed according to the method described previously (Peterbauer et al. 2001) with some modifications. Seeds were homogenized with mortar and pestle in ethanol: water (1:1,

v/v) containing 100 µg of xylitol as internal standard. The homogenate was heated at 90°C for 30 min and was centrifuged at 20.000 *g* for 30 min at 4°C. Aliquots (0.4 mL) of clear supernatant were transferred to 1.5 mL eppendorf tubes and 200 mg of a 1:1 mixture (w/w) of ion-exchange resins (Dowex 50W × 8, H^+ and Dowex 2W × 8, formate) was added. The samples were shaken at 600 rpm for 50 min and were centrifuged. Aliquots of the supernatants (200 µL) were evaporated to dryness in rotary evaporator centrifuge. To remove traces of water, residues were stored overnight over silica gel in a desiccator. Carbohydrates were derivatized with a mixture of trimethylsilyl imidazole: pyridine (1:1, v/v). Trimethylsilyl-derivatives of soluble carbohydrates were analyzed by capillary gas chromatography. The gas chromatograph (GC 2010, Shimadzu, Japan) was equipped with a Zebron ZB-1 capillary column (15 m length, 0.25 mm diameter, 0.1 µm film, Phenomenex, USA) and flame-ionization detector. Helium was used as a carrier gas with a linear velocity of 40 cm s^{-1}. The column was operated with an initial temperature of 160°C, adjusted to 335°C at 20°C min^{-1} and the final temperature was held for 12 min. The injector port was operated in the split mode (1:10) at 335°C, and the detector was maintained at 350°C.

Carbohydrates were quantified by using standards: glucose, fructose, galactose, sucrose, raffinose, stachyose, *myo*-inositol, D-pinitol (purchased from Sigma), verbascose (Megazyme, Australia), D-*chiro*-inositol and galactinol (Research Industries, New Zeeland). Other standards were extracted and purified from natural sources. DGMI (di-galactosyl *myo*-inositol) was isolated from seeds of *Vicia cracca* and tentatively identified after its hydrolysis to galactose and *myo*-inositol according to method described by Kuo (1992). Standards of galactosyl pinitols (galactopinitol A, di-galactopinitol A – named ciceritol, and tri-galactopinitol A) were isolated and purified from seeds of *Vicia villosa* as was described earlier (Szczeciński et al. 2000). Carbohydrates content was calculated from standard curves of the appropriate component. As an internal standard the xylitol (Sigma) was used. Results of analyses are means of three independent replicates ± SE.

Enzymes activity assay

Enzymatic activity of galactinol synthase was determined according to the method described by Peterbauer et al. (2001) with minor modifications. Sample of developing seeds (approximately 200 mg of fresh weight) was homogenized with a mortar and pestle in 1 mL of ice-cold extraction buffer (pH 7.0, 50 mm HEPES-NaOH, 1 mM dithiothreitol (DTT), 1% (w/v) polyvinyl polypyrrolidone, and 1% protease inhibitor cocktail [SIGMA, No. P2714-1EA]. The homogenate was then centrifuged (23.000 *g* for 30 min at 4°C). Aliquots of the supernatant (0.4 mL) were desalted by centrifugal gel filtration (Sephadex G-25 superfine columns, 2 mL bed volume). The desalted extracts were diluted 2-fold and concentrated at 4°C by centrifugal ultra filtration (Centricon-10, Millipore).

Galactinol synthase activity was estimated by incubation of desalted seed extract with appropriate substrates in a gel filtration buffer (50 mM HEPES-NaOH, pH 7.0, 1 mM DTT) in a final volume of 30 µL. Reaction mixtures for the determination of galactinol synthase activity contained desalted enzyme extract (10 µL), 5 mM $MnCl_2$, 5 mM UDP-galactose, and 20 mM *myo*-inositol. After 30 min of incubation at 30°C the reaction was stopped by adding 70 µL of 70% ethanol (7:3, v/v) and boiling the mixture for 5 min. Product of the reaction – galactinol was determined by gas chromatography method. As a control the buffer solution was used instead of UDP-galactose.

The raffinose synthase (RS; EC 2.4.1.82), stachyose synthase (STS; EC 2.4.1.67) and verbascose synthase (VS; E.C. 2.4.1.x) or galactan: galactan galactosyltransferases (GGT; EC 2.4.1.x) assay was performed according method described by Peterbauer et al. (2001). Activity was assayed at 30°C in a total volume of 30 µL containing enzyme extracts from vetch seeds (20 µL), 50 mM HEPES-NaOH, pH 7.0, 1 mM DTT, 10 mM galactinol and appropriate galactosyl acceptor. For RS estimation it was sucrose (at final concentration 40 mM), for STS – raffinose (20 mM), and for VS – stachyose (20 mM). Activity of GGT was estimated in the same mixture as for VS assay but without galactinol. Reactions were stopped after 3, 2 and 4 hours of incubation (for RS, STS and VS or GGT, respectively) as was described above. 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 D-pinitol (for galactosyl pinitol A – GPA synthesis), 20 mM GPA (for ciceritol synthesis) or 20 mM ciceritol (for tri-galactosyl pinitol A synthesis). Reactions were stopped after 2 (GPA and ciceritol synthesis) or 4 hours (tri-galactosyl pinitol A synthesis). Products of reactions were assayed by gas chromatography. Soluble protein was determined using Bradford dye-binding procedure (Bio-Rad protein assay; Bio-Rad) with bovine serum albumin as a standard. All reactions were performed on three independent seeds samples.

RESULTS

Changes in soluble carbohydrates

The first seeds sample was collected at the beginning of seed filling stage (Lahuta et al. 2005b). Under the conditions used in this study, embryo intensive growth occurred up to 34 DAF, and then accumulation of dry weight stopped (Fig. 1A). Although embryo reached physiological maturity at 30 DAF, seed germinability increased during maturation drying. Qualitative and quantitative changes in soluble carbohydrates were associated with seed development and maturation. Initially, seed contained mainly sucrose (155.20 mg g^{-1} DW at 14 DAF) and low amounts of fructose and glucose (data not shown). The concentration of sucrose decreased two-fold between 14-18 DAF (up to 78.22 mg g^{-1} DW) and later decreased more slowly. At full seed maturity (46 DAF) the content of sucrose (16.43 mg g^{-1} DW) was 10-fold lower than in seeds collected at 14 DAF. Beside sucrose, seeds on 14 DAF contained two free cyclitols – *myo*-inositol and D-pinitol (13.55 and 18.85 mg g^{-1} DW, respectively). The content of both cyclitols gradually decreased toward seed maturity. Similarly, as in the previous study (Lahuta et al. 2005b), the level of D-pinitol was higher than the level of *myo*-inositol throughout seed development and maturation (Fig. 1B).

Accumulation of RFOs preceded accumulation of galactosyl pinitols (GPs) and initially the concentration of RFOs was higher than GPs (14-34 DAF). However, from the be-

ginning of seed maturation drying (34-38 DAF), the rate of accumulation of GPs dramatically increased and in effect mature seeds contained more galactosides of D-pinitol than RFOs (43.9 and 35.4 mg g^{-1} DW, respectively) (Fig. 1C). Preferential utilization of galactose to biosynthesis of GPs coincided with changes in molar ratios of main galactose acceptors – sucrose (for RFOs) and D-pinitol (for GPs). Between 14-18 DAF the sucrose: D-pinitol molar ratio decreased from 4.5 to 3, to remain constant during the next 8 days, and decrease below 2 after 30 DAF (Fig. 1D). At the same time, incorporation of galactose into GPs pool increased above RFOs (Fig. 1C).

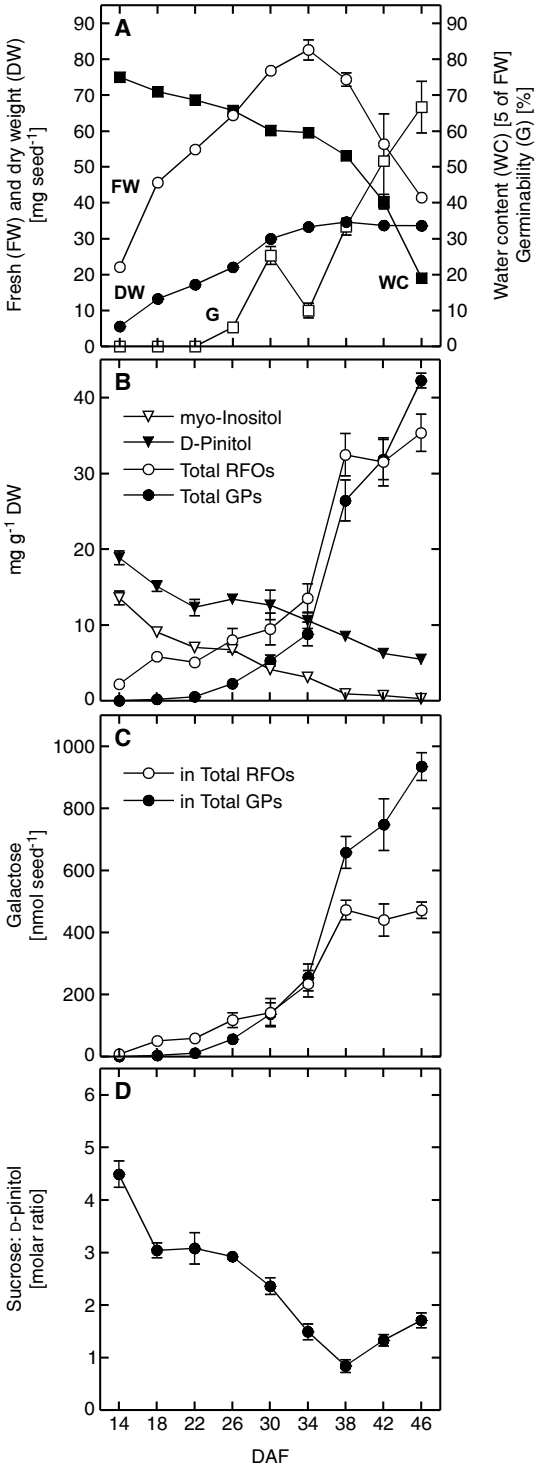

Fig. 1. Changes in fresh weight (FW), dry weight (DW), water content (WC, as percent of fresh weight) and germinability (G) during development and maturation of winter vetch (*Vicia villosa* Roth cv. Minikowska) seed (A). Changes in the concentrations of: *myo*-inositol, D-pinitol, total raffinose family oligosaccharides (RFOs) and total galactosyl pinitols (GPs) (B). Contribution of galactose units in α-D-galactosides accumulated in winter vetch seed (C). The amount of galactose in total RFOs or total galactosyl pinitols (GPs) was calculated as a sum of galactose units in each of the present galactoside according its molecular formula. Changes in the molar ratio of the main galactosyl acceptors: sucrose: D-pinitol (sucrose for RFOs and D-pinitol for GPs) (D). Mean of three to five measurements. Vertical bars correspond to SE. Where no bars are shown, SE is less than the size of the symbols.

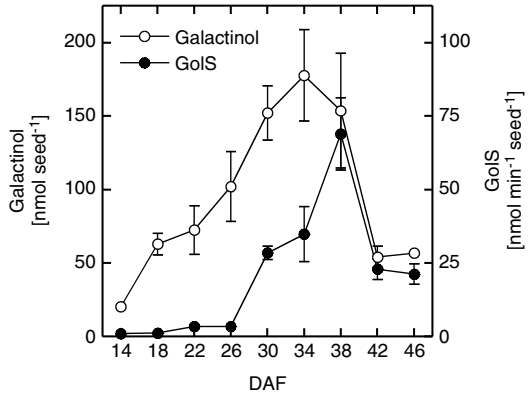

Fig. 2. Changes in the activity of galactinol synthase (GolS) and accumulation of galactinol in developing and maturing winter vetch seeds. Mean of three independent replicates. Vertical bars correspond to SE. Where no bars are shown, SE is less than the size of the symbols.

Biosynthesis of RFOs

Activities of galactinol synthase (GolS) and raffinose synthase (RS) were detected at 14 DAF. GolS activity increased slowly up to 26 DAF, increasing rapidly afterwards to reach the maximum level at 34-38 DAF (69 nmol min^{-1} $seed^{-1}$). During seed maturation drying the content of galactinol and activity of GolS decreased three-fold (Fig. 2). RS activity increased rapidly from 14 DAF, peaked at 26 DAF (0.88 nmol min^{-1} $seed^{-1}$) and subsequently decreased to a plateau (Fig. 3A). Accumulation of raffinose changed parallel to the activity of RS, reaching the level of 300 nmol $seed^{-1}$ at 30 DAF and later decreasing by two-fold. Although activity of stachyose synthase (STS) was detectable already at 18 DAF, the product of enzyme – stachyose appeared several days later (22-26 DAF). Accumulation of stachyose increased up to 38 DAF (350 nmol $seed^{-1}$) and decreased thereafter (to 280 nmol $seed^{-1}$). STS activity increased only from 18 to 26 DAF (reaching maximum level – 7.65 nmol min^{-1} $seed^{-1}$) and later was constant (Fig. 3B). In contrast to STS, activity of verbascose synthase (VS) initiated four days later, increased transitorily (22-26 DAF) and decreased gradually during seed maturation. Formation

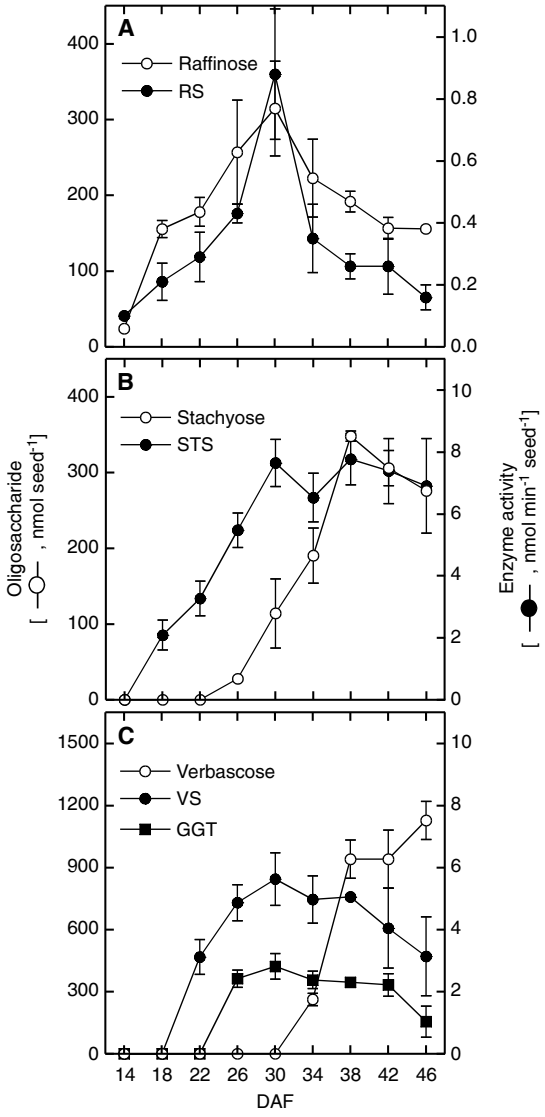

Fig. 3. Accumulation of raffinose (A), stachyose (B) and verbascose (C) and changes in the activity of appropriate galactosyltransferases: raffinose synthase, RS (A); stachyose synthase, STS (B); verbascose synthase, VS and galactan: galactan galactosyltransferase, GGT (C) in developing and maturing winter vetch seeds. Mean of three independent replicates. Vertical bars correspond to SE. Where no bars are shown, SE is less than the size of the symbols.

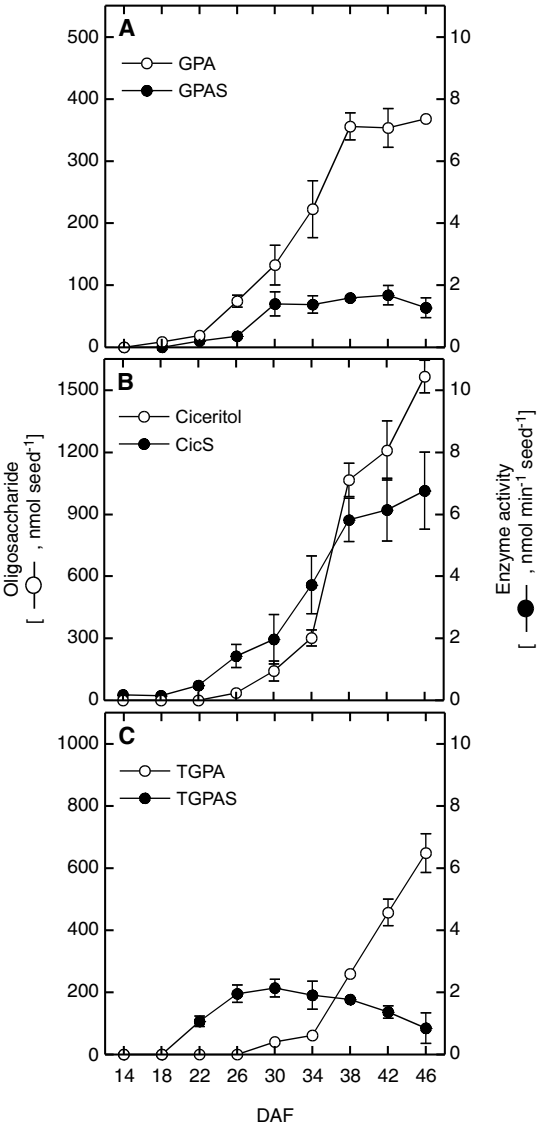

Fig. 4. Accumulation of galactopinitol A, GPA (A), ciceritol (B) and tri-galactopinitol A, TGPA (C) and changes in the activity of appropriate galactosyltransferases: galactopinitol A synthase, GPAS (A); ciceritol synthase, CicS (B) and tri-galactopinitol A synthase, TGPAS (C) in developing and maturing winter vetch seeds. Mean of three independent replicates. Vertical bars correspond to SE. Where no bars are shown, SE is less than the size of the symbols.

of verbascose in reaction without galactinol (GGT) started at 26 DAF and changed in the same manner as the activity of VS (Fig. 3C). However, GGT indicated two-fold lower activity as compared to VS. Accumulation of verbascose started several days later than the activities of VS and GGT had been detected, and continuously increased toward seed maturity (1100 nmol seed-^{1}). At the last stages of seed maturation, formation of verbascose (main oligosaccharide among RFOs) coincided with a decrease in stachyose level (Fig. 5B).

Biosynthesis of galactosyl pinitols

Galactopinitol A (GPA) was present in seeds at 18 DAF; ciceritol (di-galactopinitol A) appeared 8 days later than GPA and tri-galactopinitol A (TGPA) – 4 days later than ciceritol (Fig. 4A-C). Accumulation of GPA increased gradually up to 38 DAF (360 nmol seed-^{1}) and accumulation of its higher homologues – ciceritol and TGPA was continued up to full seed maturity (46 DAF). The content of ciceritol increased to 50% without changes in the level of GPA. Accumulation of TGPA occurred without decrease in the level of ciceritol (Fig. 5A), opposite to accumulation of verbascose, where synthesis of verbascose coincided with an appropriate decrease in stachyose (Fig. 5B). In mature seeds the level of ciceritol was two-fold higher than TGPA and *ca* four-fold higher than GPA (1500, 640 and 350 nmol seed-^{1}, respectively). Accumulation of two other galactosides – galactopinitol B (GPB, isomer of GPA) and

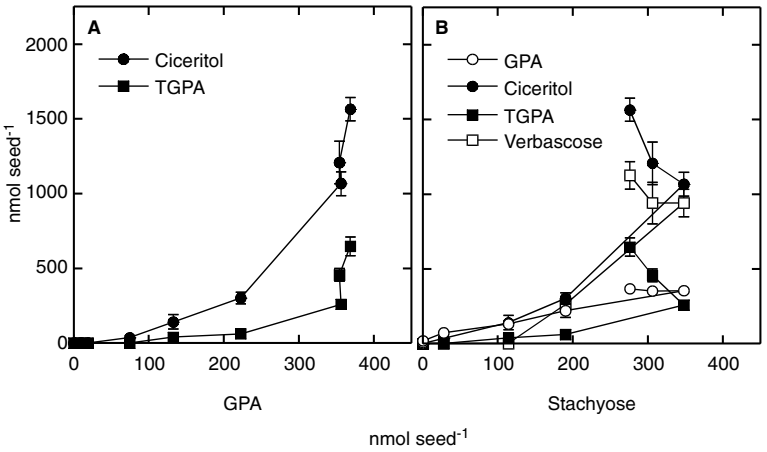

Fig. 5. Relationships in accumulation of GPA and its higher homologues – ciceritol and TGPA (A) and relationships in accumulation of stachyose and galactosyl pinitols (GPA, ciceritol and TGPA) and verbascose (B) during winter vetch seed development and maturation.

di-galactosyl *myo*-inositol (DGMI) started 4-days later than GPA and parallel with ciceritol, respectively. Concentration of both galactosides reached level three- and twenty-fold lower than GPA and ciceritol, respectively (data not shown).

The pattern of activity of enzyme catalyzing formation of GPA from D-pinitol and galactinol – named galactopinitol A synthase (GPAS), was completely different from GolS or RS (Fig. 2, Fig. 3A, Fig. 4A), but similar to STS (Fig. 3B). However, activity of GPAS was four-fold lower than STS. Biosynthesis of ciceritol from GPA and galactinol by enzyme named ciceritol synthase (CicS) increased rapidly from 14 DAF to 38 DAF (Fig. 4B), and thereafter CicS increased only at 10%. Changes in the activity of the enzyme catalyzing formation of TGPA from ciceritol and galactinol, named TGPA synthase (TGPAS) were identical to VS, but at the level similar to GGT (Fig. 3C, Fig. 4C). High positive correlations were found between changes in activities of VS (or GGT) and TGPAS (r^2=0.97 or 0.82, P=0.001, respectively) and between STS and GPAS (r^2=0.84, P=0.01).

DISCUSSION

Comparison of the patterns of sucrose, free cyclitols and α-D-galactosides accumulation clearly demonstrated that synthesis of RFOs and GPs started as the concentration of sucrose and cyclitols decreased, and was closely associated with maturation processes. In winter vetch seeds, like in seeds of other legumes (Blackman et al. 1992; Górecki et al. 1997; Obendorf et al. 1998; Hoch et al. 1999; Górecki et al. 2000; Peterbauer et al. 2001; Lahuta et al. 2005b), maturation drying dramatically increased biosynthesis of α-D-galactosides. However, up to date preferable accumulation of GPs in seeds has been documented only in two other vetch species (*Vicia cracca* and *Vicia tetrasperma*) (Lahuta et al. 2005b, c). Biosynthesis of galactinol in winter vetch seed preceded accumulation of sucrose α-galactosides (RFOs). Activity of galactinol synthase (GolS), detectable at 14 DAF, increased during seed development and reached maximum level at the beginning of seed desiccation. A similar pattern of changes in GolS activity were found in maturing seeds of soybean (Lowell and Kuo 1989), *Vigna umbellata* (Peterbauer et al. 1998) and pea (Peterbauer et al. 2001). In these species, like in winter vetch seeds, GolS indicated the highest activity among RFOs enzymes, confirming its crucial role in flux of galactose moieties into α-galactosides biosynthetic pathway.

Changes in raffinose levels and RS activity in winter vetch seed indicated that accumulation of raffinose was only an intermediary step in biosynthesis of its higher homologues – stachyose and verbascose. Although activities of stachyose synthase (STS) and verbascose synthase (VS) (or galactan: galactan galactosyltransferase, GGT) were detected at 18, 22 and 26 DAF, products of these enzymes appeared several days later: stachyose – between 22-26 DAF and verbascose – 30-34 DAF. It can mean that expression of both enzymes was controlled developmentally, and formation of each galactoside needs accumulation of its precursor at sufficient concentration. The same situation was found earlier in developing pea seeds, in which expression of STS occurred at the early stages of embryo growth and several days preceded accumulation of stachyose (Peterbauer et al. 2001). In *Vigna angularis* high levels of stachyose synthase mRNA were transiently accumulated midway through seed development (Peterbauer et al. 1999). At late stages of pea seed maturation the activity of STS decreased, opposite to the activity of STS, which continued to increase in *Vigna umbellata* seeds (Peterbauer et al. 1998), or to the constant activity of STS in winter vetch seed presented in this study. Winter vetch seeds revealed an ability to form verbascose in two different ways: with galactinol (VS) or without galactinol (GGT) from two molecules of stachyose. The activity of VS was two-fold higher than that of GGT, similarly to activities of VS and GGT found in pea seed (Peterbauer et al. 2001; Peterbauer et al. 2002b). Changes in activities of VS and GGT throughout vetch seeds maturation were significantly correlated (r^2=0.80, P=0.001). In regard to the similarity of activities of both VS and GGT and coincidences in changes of verbascose and stachyose levels, it can be suggested that formation of verbascose at the later stages of seed maturation occurs via transfer of galactose moiety from one molecule of stachyose to another one, without galactinol as galactosyl donor.

In the present study, activities of galactosyltransferases responsible for synthesis of galactopinitols were detected throughout the whole vetch seed development and maturation. This is a new observation, indicating the biological potential of winter vetch seeds to synthesize mono-, di- and

tri-galactosides of D-pinitol. Galactopinitol A (GPA), the first member of GPs, appeared four days later than raffinose, ciceritol – simultaneously with stachyose and TGPA – after ciceritol, but earlier than verbascose. Unlike raffinose, concentration of GPA (the first member in GPs) increased rapidly up to 38 DAF and later was stable. Ciceritol and TGPA were rapidly accumulated during seed maturation drying, like in seeds of *Vicia hirsuta* (Lahuta et al. 2005a) and *Vicia tetrasperma* (Lahuta et al. 2005c).

Because of the high ability of purified STS to synthesize of mono- and/or di-galactosides of D-pinitol or D-ononitol, it is believed that STS is a key regulatory enzyme responsible for synthesis of galactosyl cyclitols (Peterbauer and Richter 2001). However, differences in the ability of purified STS from rice bean (Peterbauer et al. 1998), adzuki bean (Peterbauer and Richter 1998), lentil (Hoch et al. 1999) and pea (Peterbauer et al. 2003) to synthesize di-galactosyl cyclitols and verbascose suggested that different forms of STS could be present in each investigated species. In the present study, similar patterns of activities of VS, GGT and TGPAS suggest that in biosynthesis of tri-galactosides in vetch seeds the same enzyme operates, but it is distinct from STS. Changes in STS activities were similar to GPAS and CicS. Moreover, biosynthesis of GPA and ciceritol was catalyzed by STS, as it was suggested for lentil seeds (Hoch et al. 1999). Although the GPAS activity was *ca* five-fold lower than STS, vetch seed accumulated both galactosides at the same time in equal amounts. A similar pattern of galactosyl ononitol and stachyose accumulation has been reported for rice bean (Peterbauer et al. 1998) and adzuki bean (Peterbauer and Richter 1999). Galactosyl ononitol initially increased faster than that of stachyose, but remained considerably lower during later stages of maturation, although both substances are synthesized by one enzyme. In vetch seeds accumulation of GPA preceded that of stachyose too, but later indicated a similar pattern to stachyose, in contrast to the situation found in *Vigna* species (Peterbauer et al. 1998; Peterbauer and Richter 1999).

The ability of vetch seeds to synthesize ciceritol was detectable as early as 14 DAF and increased gradually during seed maturation process. Contrary to GPAS, ciceritol synthase (CicS) reached the activity as high as STS. However, in comparison to stachyose, accumulation of ciceritol was more efficient (at 38 DAF seeds contained three-fold more of ciceritol than stachyose) and increased rapidly up to seed full maturity. Initial stachyose synthesis might have been limited by raffinose availability during early stages of RFOs accumulation. Synthesis of GPA and ciceritol was less likely to be restricted by a substrate supply, since high concentrations of D-pinitol are already present in young seeds. GPA concentration during late stages of seed development dramatically exceeded that of galactinol, suggesting favourable conditions for stachyose synthesis to proceed via GPA as galactosyl donor, as it was detected in the case of stachyose synthesis from galactosyl ononitol and raffinose in *Vigna umbellata* seeds (Peterbauer et al. 1998). However, in vetch seeds accumulation of stachyose was stopped at the late maturation stages and formation of tri-galactosides – verbascose and TGPA was preferred. Thus, both GPA and ciceritol can be accumulated only as intermediary compounds in TGPA synthesis.

Although accumulation of RFOs started prior GPs, unknown factors changed the splitting of galactose residues from RFOs to GPs. The latest experiments demonstrated that high concentration of free cyclitols during seed development directly inhibited biosynthesis of di- and tri-galactosides of sucrose (RFOs) and increased biosynthesis of galactosyl cyclitols (Obendorf et al. 2004; Gomes et al. 2005; Lahuta et al. 2005a, c). Therefore, high concentration of D-pinitol in winter vetch seeds can disturb RFOs synthesis. However, up to present, there is no evidence supporting the occurrence of biosynthesis of D-pinitol in seeds. Some data suggest that methylated cyclitols are synthesized in vegetative tissues (Wanek and Richter 1995) and transported into developing embryos through the pod and seed coat tissues (Kuo et al. 1997; Gomes et al. 2005; Lahuta et al. 2005a, c). In somatic embryos of alfalfa (*Medicago sativa* L.) pinitol and its galactosides were not synthesized (Horbowicz et al. 1995; Blöchl et al. 2005), while during embryogenesis on mother plants seeds accumulated high amounts of ciceritol (Horbowicz et al. 1995). Seeds of hairy tare, smooth tare (Lahuta et al. 2005a, c) and soybean (Obendorf et al. 2004) fed with D-pinitol synthesized higher amounts of its galactosides. If D-pinitol was synthesized naturally in vetch or soybean embryos, feeding this exogenous source should not be expected to accumulate such a noticeable increase in galactopinitols. Thus, in developing winter vetch seeds, formation of GPs at the stage of seed desiccation should be restricted by the amount of free D-pinitol. Beginning from 34 DAF seeds lost contact with the pod, and potential flux of additional amounts of D-pinitol to the seed was stopped. The comparison of the content of D-pinitol (free or bound into GPs), prior and after seed natural desiccation, indicated that synthesis of GPs could occur without additional amounts of D-pinitol.

On the other hand, biosynthesis of α-galactosides at the late maturation stages needs sufficient flux of galactose moieties from unknown sources. Initially, sucrose transported from the mother plant into seeds (middle stage of embryo growth) can be cleaved by sucrose synthase (Weber et al. 1996) releasing UDP-glucose, which after conversion to UDP-galactose (in Leloir pathway) (Holden et al. 2003) can be further used for galactinol synthesis. However, at the beginning of seed desiccation (34-38 DAF), the amount of sucrose in vetch seeds was not sufficient for galactosides formation. Additionally, from 42 DAF to 46 DAF the content of sucrose increased at 10%. In regard to very low amounts of monosaccharides (data not shown) mobilization of galactose from other compounds (hemicelluloses, membranes) can take place. It is important to note that following water withdrawal from tissues, concentrations of soluble compounds increased dramatically, changing availability of galactose acceptors and donors for enzymes. Thus, understanding the metabolism of galactosides at low water concentration needs further studies.

In summary, results presented in this study do not support the hypothesis of a presence of a single key enzyme controlling RFOs and GPs accumulation. Purification of an enzyme catalyzing formation of verbascose and tri-galactopinitol A can be a further step towards better explanation of biosynthesis of these galactosides. Additionally, studies concerning D-pinitol synthesis in vegetative tissues and mechanisms regulating its transport into developing seeds may be important for the understanding of the role of cyclitols in α-D-galactosides pathway.

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