INHIBITION OF RAFFINOSE FAMILY OLIGOSACCHARIDES AND GALACTOSYL PINITOLS BREAKDOWN DELAYS GERMINATION OF WINTER VETCH (Vicia villosa Roth.) SEEDS

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ABSTRACT
Beside RFOs, which are commonly present in legume seeds, seeds of some species contain galactosyl pinitols (GPs). These carbohydrates, like RFOs, have been hypothesized to constitute an important energy and carbon skeletal source during germination. To test this hypothesis we have applied a specific α-galactosidase inhibitor (1-deoxygalactonojirimycin, DGI) to germinating winter vetch (Vicia villosa Roth.) seeds, containing more galactosyl pinitols than RFOs. The breakdown of RFOs but not that of GPs was completely blocked in both embryonic axes and cotyledons tissues, during the first 18 h of imbibition in DGI. The inhibitor only decreased the rate of GPs degradation. The inhibitory effect of DGI on GP degradation was partially alleviated by addition of sucrose or galactose to DGI solutions. After three days of germination in water, RFOs and GPs disappeared in axial tissues of seeds imbibed in water, galactose or sucrose. Eighteen-hour imbibition of seeds in DGI drastically reduced germination, by ca 50%, during the first three days. The inhibitory effect of DGI decreased during the next seven days of germination. The presence of galactose or sucrose in imbibition solution initially stimulated seed germination, but later this effect was not statistically significant. Our study provides clear evidence that galactosyl pinitols play an important role in early winter vetch seeds germination. Additionally, we suggest that galactosyl pinitols can replace RFOs as reserve material necessary for early germination.

KEY WORDS: germination, galactosyl pinitols, raffinose family oligosaccharides, seed, winter vetch.

INTRODUCTION

The major soluble carbohydrates in legume seeds are sucrose and its α-D-galactosides: raffinose, stachyose and verbascose (raffinose family oligosaccharides, RFOs). RFOs are rapidly disappearing after the initiation and breakdown of seeds, often completed before polymeric carbohydrates are mobilized (Bewley and Black 1994; Vidal-Valverde et al. 1998; Frias et al. 2000). The RFOs in axes are lost during the first two days of imbibition of soybean, pea and lupine seeds, whereas in cotyledons RFOs hydrolisis is prolonged for four-six days (Görecki and Obendorf 1997; Görecki et al. 1997). Verbascose, stachyose and raffinose are degraded progressively, while the level of mo-
pine seeds, loss of RFOs and galactosyl cyclitols in axial tissues preceded visible germination (Górecki et al. 1997). However, all the above species contain only small amounts of galactosyl cyclitols (mainly α-D-galactosides of D-pinitol and less D-chiro-inositol). In seeds of buckwheat (Fagopyrum esculentum Moench. Polygonaceae), containing predominantly galactosides of D-chiro-inositol instead of RFOs, disappearance of these compounds in axes and cotyledons is completed after 18-20 hours of dehulled achenes germination. The loss of fagopyritol B1 (mainly galactoside) in the axes was closely associated with the onset of rapid germination (Horbowicz et al. 1998). In the present study we have tested the effect of α-galactosidases inhibitor (DGJ) on the degradation of RFOs and α-D-galactosides of D-pinitol (galactosyl pinitols) in relation to early germination of winter vetch (Vicia villosa Roth.) seeds. This species has been chosen because of its higher concentration of galactosyl pinitols than RFOs in seeds (Lahuta 2006). Therefore, inhibition of α-galactosidase by DGJ can be an appropriate method for understanding the involvement of both types of α-D-galactosides (RFOs and galactosyl pinitols) in seed germination.

MATERIALS AND METHODS

Winter vetch seeds (Vicia villosa Roth, cv. Minikowska, from Rolnas, Poland) were surface-sterilized and imbibed in sterile water (controls) or water solutions of 250 µM DGJ (Sigma-Aldrich, Vienna, Austria), 50 mM galactose, 25 mM sucrose and combination of these for 18 h. Imbibed seeds were then short washed three times to remove DGJ and sugars, transferred to Petri dishes (wetted filter paper with water) and kept at 24°C in the dark for germination for 10 days. A seed was considered to be germinated when the radicle pierced the seed coat. Soluble carbohydrates were extracted and assayed in axes and cotyledons of dry, imbibed (18 h) and germinated seeds (after three days of germination), as described previously (Lahuta 2006).

Statistical analysis. The results were subjected to analysis of variance (ANOVA) and Tukey post test (if overall P<0.05) for multiple comparisons.

RESULTS AND DISCUSSION

Dry winter vetch embryos contained sucrose, RFOs, D-pinitol and galactosyl pinitols (GPs) as main soluble carbohydrates (Table 1). In the RFOs fraction verbascose dominated, whereas the main GP was ciceritol. The concentration of both types of α-D-galactosides in the axes was ca 2-fold higher than that in cotyledons. Higher concentration of α-D-galactosides in the axis than in cotyledons is characteristic for legumes (Obendorf 1997; Peterbauer and Richter 2001) and can be a result of faster maturation of axial tissues than cotyledons. Imbibition of winter vetch seed initiated fast degradation of GPs and RFOs in the embryo (Fig. 1), which initially involved oligosaccharides of a higher degree of polymerization: verbascose (among RFOs, Fig. 1A), ciceritol and TGPA among GPs (Fig. 1B). In seeds of other legumes (lupine, soybean, pea) degradation of RFOs or GPs and galactosyl cyclitols starts during seed imbibition (Bewley and Black 1994) and, similarly to

### Table 1. The concentration of soluble carbohydrates in embryonic axes and cotyledons of dry winter vetch (Vicia villosa Roth, cv. Minikowska) seeds. Data represent means ± se (n = 3).

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Axis</th>
<th>Cotyledon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>28.6±0.56</td>
<td>12.3±0.44</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>0.86±0.03</td>
<td>0.30±0.02</td>
</tr>
<tr>
<td>Galactinol</td>
<td>1.01±0.03</td>
<td>0.37±0.00</td>
</tr>
<tr>
<td>DGMI</td>
<td>2.13±0.08</td>
<td>0.85±0.03</td>
</tr>
<tr>
<td>Total RFOs</td>
<td>33.08±1.74</td>
<td>14.30±0.42</td>
</tr>
<tr>
<td>Raffinose</td>
<td>2.54±0.04</td>
<td>0.99±0.03</td>
</tr>
<tr>
<td>Stachyose</td>
<td>8.90±0.60</td>
<td>4.08±0.16</td>
</tr>
<tr>
<td>Verbascose</td>
<td>21.64±1.09</td>
<td>9.23±0.24</td>
</tr>
<tr>
<td>D-Pinitol</td>
<td>4.76±0.03</td>
<td>2.09±0.12</td>
</tr>
<tr>
<td>Total GPs</td>
<td>46.88±0.97</td>
<td>28.24±0.75</td>
</tr>
<tr>
<td>GPA</td>
<td>4.22±0.17</td>
<td>1.92±0.06</td>
</tr>
<tr>
<td>GPB</td>
<td>1.53±0.04</td>
<td>0.69±0.02</td>
</tr>
<tr>
<td>Ciceritol</td>
<td>26.31±0.31</td>
<td>15.75±0.38</td>
</tr>
<tr>
<td>TGPA</td>
<td>14.81±0.83</td>
<td>9.87±0.29</td>
</tr>
<tr>
<td>Total RFOs/GPs ratio</td>
<td>117.35±2.13</td>
<td>58.49±1.75</td>
</tr>
<tr>
<td>RFOs/GPs ratio</td>
<td>0.70±0.02</td>
<td>0.51±0.00</td>
</tr>
</tbody>
</table>

Fig. 1. The content of RFOs (A) and GPs (B) in winter vetch cotyledon before (Dry) and after 18 hours of seeds imbibition in water (Control), DGJ, sugars (S – sucrose, G – galactose), mixture of DGJ plus galactose (DGJ+G), and DGJ plus sucrose (DGJ+S). Data represent means ± se (n = 3). For statistical analysis were subjected total RFOs and total GPs. Bars with the same letters are not significantly different (P<0.05) after a Tukey correction for multiple comparisons.

Winter vetch, galactosides of a higher degree of polymerization are degraded earlier. Quemener and Brillouet (1983) reported that the in vitro hydrolysis rate of ciceritol by α-D-galactosidase is much lower than for raffinose, stachyo-
produces D-pinitol, which can by a factor that reduces the sensitivity of α-galactoside to hydrolysis by α-galactosida-
se (Quemener and Brillouet 1983).

During the first 18 hours of imbibition, degradation of RFOs in winter vetch embryos was completely inhibited by DGI at 250 μM concentration (Figs 1A and 2A). However, DGI only partially blocked degradation of GPs (Figs 1B and 2B). In pea seed treatment with 50 μM DGI, RFOs breakdown was blocked completely during 42 hours after imbibition (Blöchl et al. 2007). Addition of galactose to DGI solution slightly decreased the inhibitory effect of DGI on degradation of both types of galactosides in cotyle-
dons, but not in embryonic axes of winter vetch seeds. Ad-
dition of sucrose to DGI did not release the inhibitory effect of DGI on RFOs degradation, but stimulated hydroly-
sis of GPs in cotyledons (Fig. 1), and completely blocked degradation of GPs in embryonic axes (Fig. 2). Galactose
and sucrose used instead of DGI also inhibited degradation of RFOs, but not that of GPs in cotyledons (Fig. 1). Both
sugars did not change degradation of galactosides in em-
byronic axes (Fig. 2). Although the levels of sucrose and D-
pinitol increased according to hydrolysis of galactosides, as expected, no noticeable increase in the content of free
galactose occurred (data not shown).

The inhibition of GPs and RFOs degradation by DGI de-
layed winter vetch seed germination by approximately
50% during the first three days after imbibition (Fig. 3). In
pea imibed in 50 μM DGI the germination rates decreased by approximately 70% (Blöchl et al. 2007). Exogenous
supply of galactose only, but not that of sucrose, was capa-
bile of relieving most of the inhibitory effect of DGI on peas
germination (Blöchl et al. 2007). In our study, winter vetch
seeds imibed in galactose and sucrose solutions initially
germinated faster than control seeds, indicating high em-
byro demand on easy metabolizable carbohydrates even in
the initializing seed germination. Initially, differences in
percentage of germinated seeds were observed up to 3rd
day of germination. Although later the germination of se-
eds imibed in galactose and DGI/sugars solution gradually
increased, the effect of DGI was still evident.

In the axes of seeds germinated for three days (previ-
uously imibed in water, galactose and sucrose), RFOs were
completely degraded (Fig. 4A) and the content of GPs de-
clined to a very low level (Fig. 4C). Following the hydroly-

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**Fig. 2.** The content of RFOs (A) and GPs (B) in winter vetch embryonic axes before (Dry) and after 18 hours of seeds imbibition in water (Control), DGI, sugars (S – sucrose, G – galactose), mixture of DGI plus galactose (DGI+G), and DGI plus sucrose (DGI+S). Data represent means ± SE (n = 3). For statistical analysis were subjected total RFOs and total GPs. Bars with the same letters are not significantly different (P<0.05) after a Tukey correction for multiple comparisons.

**Fig. 3.** Germination of winter vetch seeds imibed for 18 hours in water (Control) or DGI, galactose (Gal), sucrose (Suc) and mixture of them (DGI+Gal, DGI+Suc), and then kept on wet (in water) germination paper for 10 days. Data represent means ± SE (n = 3). Bars with the same letters are not significantly different (P<0.05) after a Tukey correction for multiple comparisons.
sis of oligosaccharides and galactosyl pinitols, the levels of sucrose, fructose (Fig. 4B), D-pinitol and myo-inositol (Fig. 4D) increased. In control cotyledons, the content of RFOs and GPs decreased two- and three-fold, respectively, as compared to dry seeds (Figs 5A and C). It should be noted that in cotyledons of seeds imbibed in galactose and sucrose the content of RFOs and GPs was the lowest among tested seeds. Analogously to the axes, cotyledons were observed to contain higher levels of sucrose and D-pinitol (Figs 5B and D). The concentration of free galactose was very low and comparable to the level of glucose (below 0.15 mg g\(^{-1}\) DW, data not shown). Previous findings indicated that the rate of degradation of ciceritol, presented in lentil, chickpea and white lupine seeds, by \(\alpha\)-galactosidase is lower than in the case of raffinose or stachyose (Quemener and Brillouet 1983; Frias et al. 2000), presumably as a re-

Fig. 4. The content of RFOs (A), sucrose and fructose (B), GPs (C), cyclitols (D) in embryonic axes of winter vetch seed before (Dry) and after three days of germination of seed imbibed for 18 hours in water (Control), DGI, sugars (S = sucrose, G = galactose), and mixture of DGI/sugars (DGI+G, DGI+S). Data represent means ± SE (n = 3). For statistical analysis were subjected total RFOs, total GPs, sucrose + fructose and myo-inositol + D-pinitol. Bars with the same letters are not significantly different (P<0.05) after a Tukey correction for multiple comparisons.

Fig. 5. The content of RFOs (A), sucrose and fructose (B), GPs (C), cyclitols (D) in cotyledon of winter vetch seed before (Dry) and after three days of germination of seed imbibed for 18 hours in water (Control), DGI, sugars (S = sucrose, G = galactose), and mixture of DGI/sugars (DGI+G, DGI+S). Data represent means ± SE (n = 3). For statistical analysis were subjected total RFOs, total GPs, sucrose + fructose and myo-inositol + D-pinitol. Bars with the same letters are not significantly different (P<0.05) after a Tukey correction for multiple comparisons.
result of specific activity of α-D-galactosidase (Petek et al. 1969; Dey et al. 1983).

In winter vetch seeds treated with DGJ and DGJ/sugars, the content of RFOSs and GPs in axial tissues increased in comparison to axes of dry seeds (Figs 4A and C), presumably because of the transport of galactosides from cotyledons to growing roots and epicotyl. However, it is also possible that enzymes for RFOSs synthesis can remain functional at early germination stages, as has been shown in germinating tomato seeds (Downie et al. 2003). In pea seeds, addition of galactose to the inhibitor solution significantly increased the RFOSs content (Bloehl et al. 2007). In our study, galactose and sucrose added to DGJ solution also increased the content of both types of galactosides in the axes, but not in cotyledons.

For comparison of the amounts of galactose released from oligosaccharides and galactosyl pinitol, the amounts of galactose bound in each oligosaccharide or galactosyl pinitol were computed (based on the molecular formula of each galactoside) in a whole embryo before imbibition, after 18 hours of imbibition and after three days of germination. Then, the differences between the total amounts of galactose in RFOSs and GPs before and after imbibition (Fig. 6A) or between 18th hour and third day of germination (Fig. 6B) were calculated. Thus, an average amount of released galactose clearly indicates that during the first 18 hours of germination most of galactose was released from GPs. In seeds imbibed in DGJ, degradation of RFOSs was completely stopped, whereas that of GPs was decreased by ca 45% (Fig. 6A). Additionally, the content of galactose bound in RFOSs slightly increased, presumably because the enzymes for RFOSs biosynthesis remain functional in germinating seeds. A similar effect was found in the case of seeds imbibed in a mixture of DGJ and sucrose. Addition of galactose to DGJ solution partially relieved the inhibitory effect of DGJ on degradation of RFOSs and GPs. Galactose and sucrose stimulated faster release of galactose from GPs and partially inhibited breakdown of RFOSs (Fig. 6A), which coincided with a higher germination rate (Fig. 3). During the germination, control embryos utilized similar amounts of galactose released from RFOSs as during the first 18 hours of imbibition (ca 100 µg embo−1). Comparable amounts of galactose derived from RFOSs were found in embryos initially imbibed in DGJ and DGJ/sucrose (Fig. 6B). However, the amount of galactose released from GPs was still two-fold higher than that from RFOSs.

The same type of chemical linkage [α-(1→6)-O-D-galactoside bound] between galactosyl residues is present in both types of galactosides. Thus, it can be expected that inhibition of degradation of galactosides by DGJ should stop degradation of both types of galactosides. Our results indicate that each treatment leading to inhibition of RFOSs degradation simultaneously stimulated degradation of GPs (Figs 1B and 6A). The reason for this is not clear. In our study seeds were imbibed in DGJ solutions for only 18 hours and later were transferred to Petri dishes containing only water. Therefore, the effect of DGJ was associated with its uptake by imbibed embryos. On the other hand, the stimulatory effect of galactose and, to a lesser extent, sucrose on the GPs degradation can suggest that galactose induces expression of new form of α-galactosidase, preferentially degrading galactosides of D-pinitol. It is also possible that faster degradation of GPs than RFOSs in winter vetch seeds is associated with substrate specificity of α-galactosidase present in winter vetch seeds. Both hydrolytic and galactosyltransferase activity of α-galactosidase was found in seeds of V. sativa (Petek et al. 1969). However, seeds of this species accumulate only RFOSs (Lahuta et al. 2005). The data obtained during our study indicate that investigations on the characteristic of α-galactosidase from winter vetch seeds during germination may be necessary to discover the causes of preferential degradation of GPs rather than RFOSs.

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LITERATURE CITED


