Changes in isovitexin-O-glycosylation during the development of young barley plants

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Isovitexin-2"-O-β-D-glucoside
Scarlett
Morex

A B S T R A C T

Phenylpropanoids are a class of plant natural products that have many biological functions, including stress defence. In barley, phenylpropanoids have been described as having protective properties against excess UV-B radiation and have been linked to resistance to pathogens. Although the phenylpropanoid composition of barley has recently been addressed in more detail, the biosynthesis and regulation of this pathway have not been fully established. Barley introgression lines, such as the S42IL-population offer a set of genetically diverse plants that enable the correlation of metabolic data to distinct genetic regions on the barley genome and, subsequently, identification of relevant genes.

The phenylpropanoid profiles of the first and third leaf of barley seedlings in Scarlett and four members of the S42IL-population were obtained by LC-MS. Comparison of the leaf profiles revealed a change in the glycosylation pattern of the flavone-6-C-glucoside isovitexin in the elite cultivar Scarlett. The change was characterized by the stepwise decrease in isovitexin-7-O-glucoside (saponarin) and an increase in isovitexin-2"-O-β-D-glucoside content.

The lines S42IL-101- and -177 and -178 were completely devoid of isovitexin-2"-O-β-D-glucoside. Parallel glucosyltransferase assays revealed parallel metabolic patterns. The genetic region responsible for this metabolic effect was located on chromosome 1H between 0.21 and 15.08 cM, encompassing 505 gene candidates in the genome of the sequenced cultivar Morex. Only one of these genes displayed sequence similarity with glucosyltransferases of plant secondary metabolism that possessed the characteristic PSPG motif.

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1. Introduction

Phenylpropanoids are a class of plant natural products derived from the aromatic amino acid L-phenylalanine and include coumarins, monolignols, hydroxycinnamic acids and flavonoids. Methyl-, glucosyl-, prenyl-, sulfo- or acyltransferases contribute to the modification of the phenypropanoid backbone, resulting in a large diversity of compounds (Geiger, 1986; Ibrahim, 2005; Vogt, 2010). For example, more than 900 different flavonoids have been described in the plant kingdom (Buer et al., 2010; Vogt, 2010).

The investigation of phenylpropanoids in barley (Hordeum vulgare spp. vulgare) was initiated in the 1950s. The first studies of the flavonoid composition in leaves of barley seedlings revealed large amounts of the C-glycosylated flavones, isovitexin-7-O-glucoside (saponarin) and isoorientin-7-O-glucoside (lutonarin), as shown in Fig. 1 (Seikel and Bushnell, 1959; Seikel et al., 1962; Seikel and Geissman, 1957). Additional studies revealed that both compounds were the most abundant flavonoids in barley seedlings and were determined to be protectants against excess UV-B radiation (Markham and Mitchell, 2003; Reuber et al., 1996; Schmitz-Hoerner and Weissenböck, 2003). Thin layer chromatography enabled the identification of 27 different flavonoid species in barley leaves, revealing different glycosylation patterns of (iso)-vitexin and (iso)-orientin, showing the glycosylation of these C-glycosides...
resistance against the pathogen Fusarium graminearum, were also identi-
cified in barley leaves. The isolation of phenolic compounds and NMR
et al., 2010, 2011).

C

Accelerated acylation of flavones could be identified in young
barley leaves. The isolation of phenolic compounds and NMR
analysis revealed the presence of 6”-sinapoyl-saponarin, 6”-fer-
uloylsaponarin and 4”-glucosyl-6”-sinapoylsaponarin in barley
(Ohkawa et al., 1998). Furthermore isoorientin-7-O-glucoside and iso-
hermetin-3-O-rutinoside-7-O-glucoside, were also identified in barley and were correlated to
resistance against the pathogen Fusarium graminearum (Bollina
et al., 2010, 2011).

Additional acylated flavones could be identified in young
barley leaves. The isolation of phenolic compounds and NMR
analysis revealed the presence of 6”-sinapoyl-saponarin, 6”-fer-
uloylsaponarin and 4”-glucosyl-6”-sinapoylsaponarin in barley
(Ohkawa et al., 1998). Furthermore isoorientin-7-O-[6-feruloyl]-
glucoside-2”-glucoside, isoorientin-7-O-[6-sinapoyl]-glucoside and iso-
scoparin-7-O-[6-sinapoyl]-glucoside-4”-O-glucoside were described
in barley (Ferreres et al., 2008; Norbaek et al., 2003).

Several other studies led to the identification of p-coumaroyl
and p-feruloyl agmatine. Hordatine A and B, which are condensed
dervatives of acylated polyamines, were isolated and were refer-
enced for their anti-fungal and anti-pathogenic activities (Nomura
et al., 1999; Smith and Best, 1978; Stoessl, 1966). For example, the
antifungal activity of the acylated polyamine conjugate p-cou-
maroyl-hydroxyagmatine against the mildew Erysiphe graminis
specifically the S42IL-population, which was derived from an initial cross of
the elite cultivar Scarlett with the wild barley accession ISR42-8
(Hordeum vulgare ssp. spontaneum). After further backcrossing
with the elite parent cultivar Scarlett, selfing and marker assisted
selection, the S42-introgen lines were developed, which con-
sisted of single wild barley chromosome segments in the genetic
background of the elite cultivar Scarlett. Currently, the whole
library includes 73 different lines, covering 87.3% of the wild barley
donor genome (Schmalenbach et al., 2008, 2011; von Korff et al.,
2004). Additionally, the S42IL population was fully genotyped using
the 15k ILLUMINA SNP array and genotyping by sequencing
(Honsdorf et al., 2014b; Schmalenbach et al., 2011).

The main objective of our study was to identify candidate genes
that could be responsible for the biosynthesis and modification of
phenylpropanoids, specifically flavone-C-glycosides in barley. To
achieve this goal, we investigated the phenolic profiles of different
leaves from barley seedlings of the elite cultivar Scarlett. Using LC-
MS, LC-MS/MS and NMR we were able to demonstrate changes in
the phenolic profile from the oldest (first leaf) to the youngest
(third) leaves of two-week-old barley seedlings. A survey of
candidate genes identified by metabolic and genetic characterization
of the S42IL-population compared with the elite cultivar
Scarlett resulted in the annotation of a single glucosyltransferase
gene in the sequenced Morex genome that could be involved in the
shift from isovitexin-7-O-glucoside to other isovitexin glycosides.

2. Results

2.1. Phenolic profiling of leaf samples of Hordeum vulgare ssp.
vulgare cv. Scarlett

In the first part of our study, the elite cultivar Scarlett was ana-
yzed for derivatives of the phenylpropanoid metabolism. Annotation
of phenylpropanoids was performed by LC-MS-analysis, database search, and comparison of the MS and MS/MS data with
literature data. Table 1 provides a summary of the detected phenyl-
propanoids, with a mass accuracy below 5 ppm. LC-MS and LC-
MS/MS analysis resulted in the annotation of 16 different phenolic
markers for them were developed (Peukert et al., 2013). Certainly
other genes of enzymes that are important for the biosynthesis of
flavonoids, such as UDP-glucose- dependent glycosyltransferases
are poorly studied in barley. Although the activity of an isovitexin-
7-O-glycosyltransferase was recently addressed, candidate genes
for natural product C-glycosyl- and O-glycosyltransferases are still
missing in barley. Additionally the biosynthetic pathways of
C-glycosides formation are poorly described in barley, e.g. the com-
plex conversion of naringenin to isovitexin has only been adapted
from buckwheat and not demonstrated in barley (Marinova et al.,
2007). Recombinant enzymes for the biosynthesis of C-glycosy-
lated flavonoids have been described in rice (Brazier-Hicks et al.,
2009; Du et al., 2010). The lack of suitable mutants makes the
identification of new genes difficult and existing mutant lines, such
as the members of the proanthocyanidin free seed collection of the
Carlsberg laboratory have been poorly characterized both geneti-
cally and biochemically (Jende-Strid, 1993; Reuber et al., 1997).

Barley is one of the most important crop plants worldwide,
because of its multipurpose use as animal feed stock, human food
and malting substrate (Honsdorf et al., 2014a). However, although
domestication of barley leads to elite cultivars with high yields, the
allelic diversity was lost over time, leading to a limited variation in the
genepool which is currently affecting the success of breeding
for enhanced abiotic and biotic stress resistance (Tanksley and
McCouch, 1997; Zhao et al., 2010). Several approaches in breeding
have been conducted to increase the genetic diversity in these elite
cultivars, e.g., the generation of barley introgression lines (IL), such
as the S42IL-population, which was derived from an initial cross of
the elite cultivar Scarlett with the wild barley accession ISR42-8
(Hordeum vulgare ssp. spontaneum). After further backcrossing
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Scarlett resulted in the annotation of a single glucosyltransferase
gene in the sequenced Morex genome that could be involved in the
shift from isovitexin-7-O-glucoside to other isovitexin glycosides.

![Fig. 1. Structural formula of common flavones from barley, isovitexin (A), isoorientin (B), isoorientin-7-O-glucoside (C) and isoorientin-7-O-glucoside (D).](image-url)
<table>
<thead>
<tr>
<th>No</th>
<th>Negative ion mode</th>
<th>UV</th>
<th>Positive ion mode</th>
<th>Sum formula</th>
<th>Annotation</th>
<th>Scarlett S42IL-101</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>m/z MS1</td>
<td>m/z MS2</td>
<td>m/z MS1</td>
<td>m/z MS2</td>
<td>a</td>
<td>Leaf 1</td>
<td>Leaf 2</td>
</tr>
<tr>
<td>1</td>
<td>367.1035</td>
<td>134, 193</td>
<td>369.1178</td>
<td>C_{17}H_{20}O_{9}</td>
<td>4-Feruloylquinic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>355.1032</td>
<td>300(s), 330</td>
<td>-</td>
<td>C_{16}H_{20}O_{9}</td>
<td>Ferulic acid glucoside</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>385.1145</td>
<td>322</td>
<td>-</td>
<td>C_{17}H_{22}O_{10}</td>
<td>Sinapic acid glucoside</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>609.1458</td>
<td>489, 447, 327</td>
<td>611.1618</td>
<td>C_{27}H_{30}O_{16}</td>
<td>Isoorientin-7-O-hex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>609.1463</td>
<td>489, 429, 309</td>
<td>611.1635</td>
<td>C_{27}H_{30}O_{16}</td>
<td>Isoorientin-2-O-hex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>549.2943</td>
<td>280</td>
<td>276.1585</td>
<td>C_{28}H_{38}N_{8}O_{4}</td>
<td>Hordatine A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>579.3043</td>
<td>267, 423</td>
<td>291.1639</td>
<td>C_{29}H_{40}N_{8}O_{5}</td>
<td>Hordatine B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>593.1521</td>
<td>473, 431, 311</td>
<td>595.1664</td>
<td>C_{27}H_{30}O_{15}</td>
<td>Isovitexin-7-O-glc</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>623.1618</td>
<td>503, 461, 323, 309</td>
<td>625.1799</td>
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<td>+</td>
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<td>593.1522</td>
<td>473, 413, 293</td>
<td>595.1667</td>
<td>C_{27}H_{30}O_{15}</td>
<td>Isovitexin-2-O-D-glc</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>623.1612</td>
<td>503, 443, 323, 309</td>
<td>625.1774</td>
<td>C_{28}H_{32}O_{15}</td>
<td>Isoorientin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>815.2043</td>
<td>609, 489, 447, 327</td>
<td>817.2213</td>
<td>C_{38}H_{40}O_{19}</td>
<td>Isoorientin</td>
<td>+</td>
<td>+</td>
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<tr>
<td>13</td>
<td>785.1940</td>
<td>609, 489, 447, 327</td>
<td>787.2080</td>
<td>C_{37}H_{38}O_{19}</td>
<td>Isoorientin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>799.2087</td>
<td>593, 473, 431, 311</td>
<td>801.2237</td>
<td>C_{37}H_{38}O_{19}</td>
<td>Isoorientin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>799.2089</td>
<td>593, 431, 413, 293</td>
<td>801.2237</td>
<td>C_{37}H_{38}O_{19}</td>
<td>Isoorientin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>769.1988</td>
<td>593, 473, 431, 311</td>
<td>771.2131</td>
<td>C_{37}H_{38}O_{19}</td>
<td>Isoorientin</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Annotations were conducted for m/z values with a mass accuracy lower than 5 ppm. Chemical formulas were calculated with the smart formula tool of the software package Data Analysis 4.1 (Bruker, Bremen). Database search was performed on the databases, ChemSpider, Analyte DB, Metlin, KEGG and ChEBI. Results of database search and MS/MS data were compared with recently published results. (þ) denotes the occurrence of the compound in the leaf and the genotype, whereas (–) indicates the absence of the compound. References are marked with the following numbers [1] – Piasecka et al., 2015; [2] – Ferreres et al., 2008; [3] – Ferreres et al., 2007; std – identified by standard; NMR – identified with NMR; Hex – hexose, glc – glucose.

Bold signifies the precursor ions.

a Fragment ions, which could be related to published fragmentation behaviour of flavonoids, other ions were also observed but not included.
b No clear MS2 results were obtained because of poor ionization of the [M+H]⁺/[M-H]⁻
c Tentative annotation of compounds.
d Fragment ions referenced in literature with low intensities in our MS2.
replicates was determined by a calibration curve of an authentic isovitexin-7-O-glucoside. The standard deviation of the content of three biological replicates is indicated by the error bars. The content of phenolic in three independent biological replicates differed with respect to its retention time and fragmentation behaviour in both polarities with an authentic standard (see Figs. S1, S2 and S3). The quantification of phenolics in all three leaves revealed a decreasing content of isovitexin-7-O-glucoside from the first to the third leaf (Fig. 3). The content of isovitexin-7-O-glucoside changed from 3.9 μmol/g fr. wt (74% of total phenolic content) in the first leaf to 0.3 μmol/g fr. wt (7.8% of the total phenolic content) in the third leaf.

Instead of isovitexin-7-O-glucoside, compound 10 increased from the first to the third leaf of Scarlett from 0.1 μmol/g fr. wt to 2.7 μmol/g fr. wt. It displayed the same UV-absorption maxima, m/z of the [M+H]\(^+\) and [M-H]\(^-\) as isovitexin-7-O-glucoside, but it differed with respect to its retention time and fragmentation behaviour. CID experiments of m/z = 593.1522 (−35 eV) revealed fragment ions at m/z 473 [M–H–120]\(^-\), 413 [M–H–180]\(^-\) and 293 [M–H–180–120]\(^-\) (see Fig. S1), which is typical for 2"-O-β-D-glycosylated C-glycosyl flavones (for fragmentation scheme, see Fig. 4B and C). High resolution mass spectrometry (HRMS) – experiments in positive ion mode showed in addition to m/z 595.1667 [M+H]\(^+\) the m/z 433.1134 [M+H–162]\(^+\), which enabled annotation of the compound with pseudo MS\(^2\). Pseudo MS\(^2\) in positive ion mode showed a similar fragmentation pattern to an authentic isovitexin standard.

### 2.2. Modification of the O-glycosylation pattern during the Development of young barley plants

A comparison of the phenolic profiles among the first, second and third leaf revealed quantitative changes in several flavone glycosides. Fig. 2A shows the UV-chromatograms recorded at 280 nm for all three leaf samples of the elite cultivar Scarlett. In the first leaf of Scarlett, compound 8 was the most abundant and was identified as isovitexin-7-O-glucoside (saponarin), by comparison of the retention time, UV-spectra and fragmentation behaviour in both polarities with an authentic standard (see Figs. S1, S2 and S3). The quantification of phenolics in all three leaves revealed a decreasing content of isovitexin-7-O-glucoside from the first to the third leaf (Fig. 3). The content of isovitexin-7-O-glucoside changed from 3.9 μmol/g fr. wt (74% of total phenolic content) in the first leaf to 0.3 μmol/g fr. wt (7.8% of the total phenolic content) in the third leaf.

Compounds 6 and 7 were tentatively annotated as the condensed polyamines Hordatine A and B. Both compounds showed singly charged ions in the negative ion mode and doubly charged ions with very high intensities in the positive ion mode. The largest portion of the annotated compounds was derived from flavonoids, revealing hexosides of isoorientin (compounds 4 and 5) and isovitexin (compounds 8 and 10). Compounds 4 and 5, as well as 8 and 10, respectively, showed similar UV-absorption maxima and the same m/z values, indicating an isomeric structure. The fragmentation pattern in negative ion mode enabled the identification of the position of glycosylation at the 7-OH for compounds 4 and 8 and accordingly at 2"-OH for compounds 5 and 10. Compound 9 was annotated as isoscoparin-7-O-hexoside and compound 11 as the corresponding 2"-O-hexoside. Additionally, the masses and corresponding fragment ions of the acylated derivatives of isovitexin (compounds 14–16) and isoorientin (compounds 12 and 13) could also be identified in leaf samples of Scarlett.

### Fig. 2. HPLC-analysis recorded at 280 nm for a methanolic extract of Scarlett (A) and the introgression lines 542IL-101 (B) from the first (black), second (blue), and third (green) leaf in a Z-axis plotted overlay (angle – 15°). Intensity is shown in absorption units (AU), retention time in minutes. Phenolics from different leaves were extracted with methanol and analysed with UPLC/LC-MS. 8, isovitexin-7-O-glucoside, 10, isovitexin-2"-O-β-D-glucoside, see also Table 1. The overlays show a stepwise decrease in isovitexin-7-O-glucoside from the first to the third leaf of the elite cultivar. The abundance of isovitexin-2"-O-β-D-glucoside increases from the first to the third leaf. In comparison to the elite cultivar, the introgression line 101(B) showed no strong decrease in isovitexin-7-O-glucoside and no induction of isovitexin-2"-O-β-D-glucoside.

### Fig. 3. Content of total phenolics (A), content of isovitexin-7-O-glucoside (B) and content for isovitexin-2"-O-β-D-glucoside (C) in different leaves: Scarlett is marked in black and 542IL-101 in blue. The standard deviation of the content of three biological replicates is indicated by the error bars. The content of phenolic in three independent biological replicates was determined by a calibration curve of an authentic isovitexin-7-O-glucoside standard.
**Table 2**

NMR spectroscopic data for compound 10 (600/150 MHz) in acetone-d$_6$ at $-25^\circ$C.

<table>
<thead>
<tr>
<th>No.</th>
<th>$\delta$ 13C</th>
<th>$\delta$ 1H, mult. (J in Hz)</th>
<th>HMBC (H $\rightarrow$ C)</th>
<th>NOE$^a$</th>
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<tr>
<td>2</td>
<td>164.9</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>104.1 (102.6)$^b$</td>
<td>6.65 s</td>
<td>2, 4, 10, 1$^c$</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>183.3</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>163.8$^c$</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>108.4 (108.7)$^d$</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>158.0$^e$</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>95.1 (92.9)$^f$</td>
<td>6.54 s</td>
<td>4 (w), 5, 6, 7, 10</td>
<td>3, 3'/5', 8 (v w)$^g$</td>
</tr>
<tr>
<td>9</td>
<td>n.d.</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>105.0</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1$^h$</td>
<td>123.3</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'/6'</td>
<td>129.3</td>
<td>7.95 d-like (8.9)</td>
<td>2, 4', 6'/2'</td>
<td>3, 3'/5', 8 (v w)$^g$</td>
</tr>
<tr>
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<td>7.03 d-like (8.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4'</td>
<td>161.8</td>
<td>–</td>
<td></td>
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</tr>
<tr>
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<tr>
<td>1$^h$</td>
<td>73.4 (71.0)$^h$</td>
<td>5.02 d (9.9)</td>
<td>5, 6, 2', 3' (w)</td>
<td>3', 5$^i$</td>
</tr>
<tr>
<td>2</td>
<td>82.8 (80.9)$^i$</td>
<td>4.08 very br s-like$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>79.3</td>
<td>3.72 br t-like (8.6)</td>
<td>2', 4'</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>70.8</td>
<td>3.66 br t-like (8.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>81.9</td>
<td>3.50 br d-like (8.9)</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>61.9</td>
<td>3.84' $^j$, 3.82'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1'</td>
<td>106.1 (105.3)$^k$</td>
<td>4.36 d (7.8)</td>
<td>2'</td>
<td>3', 5'</td>
</tr>
<tr>
<td>2'</td>
<td>75.7</td>
<td>3.09' $^l$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'</td>
<td>77.8</td>
<td>3.28 dd (9.1/9.1)</td>
<td>3', 1' $^m$</td>
<td></td>
</tr>
<tr>
<td>4'</td>
<td>71.5</td>
<td>3.10' $^n$</td>
<td>2', 4'</td>
<td></td>
</tr>
<tr>
<td>5'</td>
<td>76.8</td>
<td>2.95 ddd (9.5/5.2/3.4)</td>
<td>6' (w)</td>
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</tr>
<tr>
<td>6'</td>
<td>62.9</td>
<td>3.34 dd (11.3/3.4); 3.23 dd (11.3/5.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Chemical shift of HSQC or HMBC correlation peaks.
$^b$ Solvent: DMSO-d$_6$.
$^c$ Interchangeable.
$^d$ Chemical shift of selective TOCSY1D peak.
$^e$ Chemical shift of HSQC correlation peak.
$^f$ ROESY correlations (mutual correlations are shown only once); mixing time 0.3 s.
$^g$ Selective ROESY1D correlation, irradiation of H-2/H6', mixing time 0.3 s; n.d. not detected; (w) weak correlation signal; (v w) very weak correlation signal.

(Figs. S2 and S4), so the MS results enabled the tentative annotation of compound 10 as isovitexin-2'-O-β-D-hexoside.

For further identification compound 10 was purified and analysed by NMR (see Table 2 for NMR-results). Two 1H NMR singlets at 6.65 and 6.54 ppm as well as an AA'XX' four spin system (7.95, d-like, 2H; 7.03 d-like, 2H) together with an OH singlet at 13.60 ppm revealed a 7,8,4'-tri-OH substituted flavonoid aglycone. The HSQC spectrum clearly showed the presence of one O-linked (3H 4.36 ppm, d, J = 7.8 Hz; 13C 106.1 ppm) and one C-linked (3H 5.02 ppm, d, J = 9.9 Hz; 13C 73.4 ppm) hexose residue. The ROESY correlations of both anomeric protons with the corresponding proton signals at position 3 and 5, as well as the double doublet pattern with coupling constants $\approx 8$ Hz for both protons at position 2 confirmed the presence of two B-D-glucose units. Due to a very broad proton signal for H-2', caused by a restricted rotation of the C-glycosidic bond - no HSQC correlation peak was observed. However, the COSY spectrum as well as a 1D-TOCSY experiment with selective irradiation of H-1' showed a correlation of H-1' (5.02 ppm) with H-2' (4.08 ppm). Furthermore, the HMBC correlation of H-1'' (4.36 ppm) with a carbon signal at 82.8 ppm indicated the presence of a 2''-O-glucoside, as the downfield shift of C-2'' is characteristic of a glucose substituted at the 2'-position with another glucose. The presence of a 6-C-sophoroside was indicated by comparison of the 13C NMR chemical shifts with published data (Geiger, 1986) as well as a weak ROESY correlation between H-2'/6' and the singlet at 6.54 ppm, which therefore was assigned to H-8. A similar weak correlation is shown in the 1D ROESY of an authentic sample of isovitexin with selective irradiation of H-2'/6', whereas for vitexin, weak correlations to glucose signals were found (Figs. S19 & S20).

Because of these data, compound 10 was identified as isovitexin-2'-O-β-D-glucoside (Fig. 4 A), revealing a change in the isovitexin-O-glycosylation pattern during the development of young barley plants. A similar change of the O-glycosylation pattern from the first to the third leaf was also observed for isoorientin derivatives (for EIC...
see Fig S9). Compounds 4 and 5 could be annotated as isoorientin derivatives, where compound 4 showed the typical fragmentation behaviour of a 7-O-glycosylated compound and compound 5 for the 2’-O-glycosylated species respectively. Similar results were obtained for the flavone isoscoparin (for EIC see Fig S10), but these effects were not further quantified.

2.3. The barley introgression lines S42IL-101 and S42IL-102 displayed a modified O-glycosylation pattern

We next analysed the phenolic profile of the second leaf from 49 members of the S42IL-population using HPLC. From these 49 lines, three lines showed differences in the phenolic profile compared to the elite cultivar Scarlett and were selected for further analysis (data not shown).

In the HPLC profiles of all three leaves of the lines, S42IL-101 (Fig. 2B), S42IL-177 and S42IL-178 isovitexin-2”-O-β-D-glucoside was completely absent (See Fig. S8 and Fig. 2C). S42IL-101 was then selected for the quantification of isovitexin-7-O-glucoside and -2”-O-β-D-glucoside and enzyme activity tests.

The content of isovitexin-7-O-glucoside, isovitexin-2”-O-β-D-glucoside and the total amount of phenolics for three biological replicates is summarized in Fig. 3 (UV profiles of all three biological replicates are shown in Figs. S6–S7). Although the total amount of phenolics did not change between the two genotypes (Scarlett, S42IL-101), there were distinct qualitative and quantitative differences between the lines compared to Scarlett.

In all three leaves of S42IL-101 seedlings isovitexin-7-O-glucoside was the most abundant flavonoid with ca. 77% of the total phenolic content. Furthermore other 2”-O-glucosides of isoorientin and isoscoparin could not be detected (see Table 1).

2.4. Isovitexin-2”-O-β-D-glucoside is produced via a specific UDP-glycosyltransferase reaction in planta

To characterize the biosynthesis of isovitexin-2”-O-β-D-glucoside and isovitexin-7-O-glucoside in planta, enzyme activity tests of leaf protein extracts were performed. A scheme of the investigated enzymatic reactions is shown in Fig. 5. The aim was to verify, whether both compounds could be produced from isovitexin in the presence of UDP-glucose and whether the metabolic findings for the three genotypes correlated with the enzyme activities.

The enzymatic tests of crude protein extracts from the first leaf of the elite cultivar Scarlett, as well as the introgression line S42IL-101 revealed the synthesis of isovitexin-7-O-glucoside (Fig. 6). The reaction was strictly dependent on isovitexin and on the presence of UDP-glucose and isovitexin. B: Enzyme test of the crude protein extract of the third leaf. Chromatograms of the enzyme activity tests are marked by a red line; (—) isovitexin-7-O-glucoside standard; (——) purified isovitexin-2”-O-β-D-glucoside, the substrate peak (isovitexin) was marked by (S). Isovitexin-7-O-glucoside (8) production was observed in both enzyme assays, whereas isovitexin-2”-O-β-D-glucoside (10) was observed only in the third leaf. C: Comparison of specific isovitexin-7-O-glycosyltransferase activity in the first leaf (black) and the third leaf (white) of Scarlett and S41IL-101. D: Comparison of specific isovitexin-2”-O-glycosyltransferase activity for the first leaf (black) and the third leaf (white) of Scarlett and S41IL-101. Standard deviation of the specific activity of three biological replicates is indicated by error bars.

Fig. 5. Reaction schemes of the investigated glycosyltransferase activities in the first and the third leaf of Scarlett and the introgression line S42IL-101. Isovitexin is converted in the presence of UDP-glucose to isovitexin-7-O-glucoside via a 7-O-glycosyltransferase reaction (upper reaction). The second UGT-activity that was investigated is the conversation of isovitexin to isovitexin-2”-O-β-D-glucoside.

Fig. 6. HPLC profiles of enzyme tests of the first (A) and third leaf (B) of the barley cultivar, Scarlett, recorded at 280 nm, and comparison of the specific isovitexin-7-O-glycosyltransferase and the isovitexin-2”-O-glycosyltransferase activities (C and D). A: Crude protein extract of the first leaf of the elite cultivar Scarlett, incubated for two hours at 30 °C in the presence of UDP-glucose and isovitexin. B: Enzyme test of the crude protein extract of the third leaf. Chromatograms of the enzyme activity tests are marked by a red line; (—) isovitexin-7-O-glucoside standard; (——) purified isovitexin-2”-O-β-D-glucoside, the substrate peak (isovitexin) was marked by (S). Isovitexin-7-O-glucoside (8) production was observed in both enzyme assays, whereas isovitexin-2”-O-β-D-glucoside (10) was observed only in the third leaf. C: Comparison of specific isovitexin-7-O-glycosyltransferase activity in the first leaf (black) and the third leaf (white) of Scarlett and S41IL-101. D: Comparison of specific isovitexin-2”-O-glycosyltransferase activity for the first leaf (black) and the third leaf (white) of Scarlett and S41IL-101. Standard deviation of the specific activity of three biological replicates is indicated by error bars.
of UDP-glucose. Formation of isovitexin-2'-O-β-D-glucoside could not be detected. Additionally the specific activity of the isovitexin-7-O-glycosyltransferase was not significantly different among the two lines (see Fig. 6 C).

In the third leaf, the synthesis of isovitexin-7-O-glucoside increased 2–3-fold in all genotypes. Additionally, the production of isovitexin-2'-O-β-D-glucoside could only be detected in Scarlett (Fig. 6 B and D).

Although the metabolite profile showed low amounts of isovitexin-7-O-glucoside in the third leaf of Scarlett, our results indicated an increased activity of a 2'-O-glucosyltransferase from the first to the third leaf. This increase indicates that there will be a genetic factor, determining the different accumulation of isovitexin-2'-O-β-D-glucoside in the different genotypes.

2.5. Identification of candidate genes, which alter the isovitexin-O-glycosylation pattern

Detailed genetic analysis using the map from Honsdorf et al. (2014b) showed an overlap of the introgression for the selected introgression lines on the chromosome 1H. The introgression of the wild barley genome was located between 0.21 cM and 15.08 cM in line S42IL-101 (Honsdorf et al., 2014b), whereas lines S42IL-177 and -178 partly cover this region (data not published).

Using the data of the Morex genome 505 candidate genes (212 of them were described as “unknown protein” or as undescribed protein) could be identified. Among these genes, two genes could be annotated as a UDP-glycosyltransferase (HORVU1Hr1G002710.1 and HORVU1Hr1G002660.1). HORVU1Hr1G002710.1 is the most promising candidate gene based on the highly conserved plant secondary product glucosyltransferase consensus motif (PSPG-Box). HORVU1Hr1G002710.1 showed 73% sequence homology for the highly conserved residues of the PSPG-box, revealing its identity as a natural product UGT. Conserved amino acids include the HCGWNS motif, in which the amino acids WN are replaced by PA.

Further BlastP searches showed the highest sequence similarities of HORVU1Hr1G002710.1 with an UGT91D1 like enzyme (accession no. XP_003595503) from Brachyphyllum distachyon (59% sequence identity) and UGT91B1 from Triticum urartu (61% sequence identity).

In contrast the gene product of HORVU1Hr1G002660.1 consists of 139 amino acids and is lacking of the important HCGWNS sequence motif. These results indicate that HORVU1Hr1G002710.1 is the prime candidate gene for a natural product UGT. Genes, which could be related to phenylpropanoid metabolism are shown in Table 3.

Table 3

<table>
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<tr>
<th>Marker</th>
<th>Gene</th>
<th>cM</th>
<th>Description</th>
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<td>BOPAI_1714-365</td>
<td>HORVU1Hr1G000430</td>
<td>0.21</td>
<td>Chalcone isomerase, subgroup</td>
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<tr>
<td>SCRI_RS_207257</td>
<td>HORVU1Hr1G002710.1</td>
<td>4.11</td>
<td>UDP-glucuronosyl/UDP-glucosyltransferase O-methyltransferase family protein</td>
</tr>
<tr>
<td>SCRI_RS_66630</td>
<td>HORVU1Hr1G001570.1</td>
<td>4.96</td>
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</tbody>
</table>

3. Discussion

Our study showed a change of the isovitexin-O-glycosylation pattern from the first to third leaf in barley seedlings of the cultivar Scarlett. In the first leaf isovitexin-7-O-glucoside was the most abundant flavonoid, whereas the phenylpropanoid profile of the third leaf was dominated by isovitexin-2'-O-β-D-glucoside. The screening of the S42IL-population revealed three lines with different phenylpropanoid profiles compared to Scarlett, which missed the 2'-O-glycosylated form of isovitexin in leaf three completely. With these lines we could correlate the metabolic and enzymatic findings to a genetic region on the chromosome 1H between 0.21 cM and 15.08 cM, carrying one candidate UDP-glucosyltransferase gene. Sequence analysis of the amino acid sequence showed high sequence similarities with the PSPG-box motif of plant natural products UGTs.

3.1. The O-glycosylation pattern is changed during the leaf development in barley cv Scarlett

Glycosylation is a key modification of plant natural products, which increases their solubility in water, improves their chemical stability, reduces the chemical reactivity and changes their bioactivity (Jones and Vogt, 2001). This reaction is performed by UDP-dependent glycosyltransferases (UGT), which transfer the sugar moiety of activated sugars to the natural product. However, in vitro studies demonstrated that UGTs had broad substrate specificity; the enzymes were also described to be regioselective for the position of glycosylation. For example the regioselective glycosylation of several phenylpropanoids by the UGT73A4 (preference for 4' and 7-OH of flavonoids) and UGT71F1 (preference for 3- or 7-OH position of flavonoids) from Beta vulgaris has been described (Isayenkova et al., 2006).

Our studies showed a switch in the O-glycosylation pattern in two week old barley seedlings from isovitexin-7-O-glucoside towards the 2'-O-glucoside formation in barley. Changes in the composition of phenolics in different leaves have also been shown in oat, in which the content of vexitexin-rhamnosides decreased from the primary to the sixth leaf and the content of an orientin-arabinoside increased (Popovici and Weissenböck, 1976).

Using crude leaf protein extracts an enzyme activity test for the formation of the isovitexin-2'-O-β-D-glucoside could be established. Within the same enzyme assay, also the formation of isovitexin-7-O-glucoside was recorded. Despite the decreasing levels of isovitexin-7-O-glucoside in leaf 3, the in vitro activity of the enzymatic reaction remained high, which is an indication of further control mechanisms active in in vivo conditions. Because of the additional activity of the 2'-UGT and the described regioselectivity of UGTs in the literature, we can draw the conclusion, that an additional UGT gene, which is also specific for isovitexin, is coexpressed with the 7-O-UGT in the second and third leaf of the elite cultivar Scarlett.

3.2. Barley introgression lines enable the tentative annotation of genetic regions for the biosynthesis of phenolic compounds

C-glycosylated flavonoids are compounds derived from 2-hydroxy-flavonones via the action of specific UDP-glucose-dependent C-glycosyltransferases. For example, the biosynthesis of isovitexin-7-O-glucoside is initiated with naringenin, which is hydroxylated at position 2 via the activity of a P450 dependent enzyme. The 2-hydroxynaringenin is converted to isovitexin, which is then O-glycosylated by an UDP-glucose-dependent O-glycosyltransferase To date, this pathway has only been established from Fagopyrum esculentum and has not been completely shown in barley (Kerscher and Franz, 1987, 1988; Marinova et al., 2007). Although there was the description of a 7-O-glycosyltransferase activity in barley the responsible enzymes and genes have been poorly characterized. The enzymes involved in the biosynthesis of
C-glycosylated flavones have only been described in rice, where a C-glycosyltransferase and a P450-enzyme for the production of 2-OH naringenin was described in vitro (Brazier-Hicks et al., 2009; Du et al., 2010). Initial studies examining the production of an isovitexin-2'-O-β-D-glucoside from isovitexin date back to the 1980s in petals of Silene alba (Heinsbroek et al., 1980) but a candidate gene for a isovitexin-2'-O-glycosyltransferase has never been identified.

Investigation of the S42IL introgression population revealed 3 different lines, S42IL-1, -177 and 178) which completely lacked the 2'-O-glycosylated derivatives of isovitexin and line 101 was devoid of the corresponding enzyme activity in crude protein extracts. S42IL-101 appears to be homozygous for the introgression of the wild barley allele is affecting the glycosylation pattern in the introgression lines studied. Eventually the gene product of the glycosyltransferase of the wild barley allele is altered in its enzyme properties, caused by changes in the nucleotide and the amino acid sequence, respectively. Furthermore the transcription of the UGT gene could be blocked by changes in the nucleotide sequence of the promotor region.

However, in our studies detailed sequence data was only derived from the elite cultivar Morex and, sequence data from the elite cultivar Scarlett is not available. Recently published data from Mascher et al. (2017), where they analysed the genetic variation among 48 spring barley cultivars, indicated genetic variation between different cultivars. Because of this the presence of the UGT-gene in the elite cultivar Scarlett has to be proven. The DNA-sequence of HORVU1Hr1G002710.1 has to be obtained and the activity of the gene product as a secondary UGT has to be demonstrated. Furthermore sequencing of homologous genes from the other introgression lines may also contribute to an explanation of the different metabolic profiles between the selected lines.

However, genetic markers allowed us, to correlate our metabolic data to a distinct genetic region on the barley chromosome 1H, leading to the identification of a candidate UGT with the required PSPG-box motif, which is essential for an enzyme to function as a natural product UGT. The two peptide sequences, WAPQ and HCGWNS, are conserved in 95% of all secondary compound UGTs, where the underlined amino acids are completely conserved. These conserved amino acids (together with other amino acids of the PSPG-Box motif) were also located in the amino acid sequence of our UGT candidate and revealed a function as a secondary compound UGT (Vogt and Jones, 2000). Furthermore the enzyme showed a high similarity to other natural products UGTs from Brachypodium or rice. Subsequent cloning, functional expression and biochemical characterization of the candidate gene will verify the activity of the gene product as a natural product UGT, involved in the synthesis of isovitexin-2'-O-β-D-glucoside.

4. Conclusion

Glycosylation of isovitexin is a regiospecific process, which leads to the favoured production of isovitexin-7-O-glucoside in the first leaf of young barley plants (Scarlett). During the early plant development this regiospecificity is changed from 7-O-glycosylation to 2'-O-glycosylation. Using barley introgression lines among 50% different genes on chromosome 1H, one UGT-candidate gene was tentatively linked to the observed shift in glucosylation and was subsequently annotated based on its conserved and characteristic PSPG-consensus motif as a secondary compound UGT; however additional characterization is required. The discrepancy between the initial enzymatic tests and the metabolite profiles reveal further control mechanisms for the position specific glycosylation of isovitexin in planta, which have to be characterized in further studies.

5. Experimental

5.1. Reagents

Chemicals were purchased from Sigma-Aldrich (Darmstadt, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and Th. Geyer GmbH & Co. KG (Renningen, Germany). Solvents for HPLC- and UPLC analysis were LC-MS grade. Isovitexin-7-O-glucoside and isovitexin standards were purchased from Extrasynthese (Lyon, France).

5.2. Plant material and growth conditions

Seeds of barley (Hordeum vulgare spp. vulgare cv. Scarlett, S42IL-101) were obtained from the laboratory of Prof. Dr. Klaus Pillen (Martin Luther University Halle-Wittenberg) and were cultivated in the fields of the IPK Gatersleben during spring and summer in 2015. Seeds from the cultivation period 2015 were sown in plastic pots with soil and cultivated for two weeks in a growth chamber (Peri-cival, Model: E-411X-LT, Percival Sciientific Inc., Perry, USA) at 20 °C/18 °C day/night with a 12 h photoperiod and 380 μmol photons/m² s with 100% light intensity. The photon flux was checked every three days. At the end of cultivation the first, second and third leaf were harvested separately, pooled (16 leaves/pool) and frozen in liquid nitrogen. The samples were frozen at −80 °C for long-term storage.

5.3. Extraction, sample preparation and analysis of phenolic compounds with rp-UPLC-PDA-ESI-QTOF-MS and rp-UPLC-PDA-ESI-QTOF-MS/MS

Extraction of phenolic compounds and sample preparation for the analysis were performed according to established methods (Petridis et al., 2016).

Analysis of phenolic compounds was carried out with rp-UPLC-PDA-ESI-QTOF-MS and - MS/MS with an Acquity H-class UPLC-System (Waters, Eschborn), equipped with an Acquity photo diode array detector (Waters, Eschborn) coupled to an ESI-QTOF-MS (maXis impact, Bruker, Bremen). The chromatographic separation was performed according to described methods (Petridis et al., 2016). PDA-detection (photo diode array) of phenolics was done in a range between 210 and 800 nm with a sampling rate of 20 points/s and a resolution of 1.2 nm.

MS experiments were conducted in positive and negative ion mode for molecules with an m/z between 50 and 1000 using the following parameters for positive ion mode: capillary voltage: 4 kV; nebulizer pressure: 3 bar; dry gas 8 l/min; dry temperature 200 °C; hexapole radiofrequency (RF) voltage: 60 Vpp; funnel RF 1: 300 Vpp; funnel RF 2: 300 Vpp; prepulse storage time: 5 μs; transfer time: 50 μs; ion energy: 5 eV, low mass: 40 m/z; collision energy 10 eV; collision cell RF: 500 Vpp.

In negative ion mode, the following parameters were used: capillary voltage: 3.5 kV; nebulizer pressure: 3 bar; dry gas 8 l/min; dry temperature 200 °C; hexapole radio frequency voltage: 60 Vpp; funnel RF 1: 1300 Vpp; funnel RF 2: 300 Vpp; prepulse storage time: 8 μs; transfer time: 50 μs; ion energy: −5 eV, low mass: 40 m/z; collision energy −10 eV; collision cell RF: 800 Vpp. At the beginning of each run, the system was internally calibrated with 10 mM sodium formiate in the calibration mode “quadratic HPC”.

MS/MS-experiments were performed in auto MS/MS mode using CID with following parameters: absolute area threshold 5000 cts, activation voltage 15 spectra, exclusion release 60 s, collision energy values (z = 1, z = 2, z = 3 (isolation mass = 500)); 25 eV, 20 eV, 10 eV; collision energy values (z = 1, z = 2, z = 3 (isolation mass = 1000)): 50 eV, 40 eV, 35 eV, and collision energy...
values for pseudo MS$^3$ ($z = 1, z = 2, z = 3$ (isolation mass = 500)): 20 eV, 15 eV, 10 eV respectively. Data interpretation was performed using Compass Data Analysis V 4.1 and Quant Analysis V 2.1 (Bruker, Bremen). Quantification of isovitexin-7-O-glucoside and isovitexin-2''-O-β-D-glucoside was conducted with an authentic standard of isovitexin-7-O-glucoside on an Acquity UPLC system (Waters, Eschborn) under the conditions described above.

5.4. Purification of isovitexin-2''-O-β-D-glucoside

To identify the unknown isovitexin derivative, the compound was purified for NMR. For purification of isovitexin-2''-O-β-D-glucoside, 10 g of ground fresh leaf material of the second leaf was suspended in 40 ml of 100% methanol and incubated over night at 4°C. The extract was clarified by filtration through a nylon mesh with 20 µm pore size (GE Healthcare, Chalfont St Giles, Great Britain) and the remaining residue was extracted for 1 h with 40 ml of 100% methanol. After a second filtration through 20 µm nylon mesh, both filtrates were combined.

The combined extract was concentrated to approx. 30 ml under vacuum with a rotavapor evaporator and loaded on a Redisep RP C18 4.3 g flash chromatography column, 60 A, with 40-63 µm particle size (Tyledone ISCO, Lincoln). Flash chromatography to prefractioante the sample was carried out using a Combiflash RF System (Axel Semrau GmbH & CO KG, Sprockhövel, Berlin) under following conditions: flow rate: 18 ml/min, conditioning of column: 28.7 ml of 100% A (0.1% formic acid in water), 0-2 min with 100% A, 2-17 min in a linear gradient from 100% A to 0% A and 100% B (0.1% formic acid in methanol). Subsequently, the column was washed with 100% B for 5 min. The fractionation was followed at 280 nm and 336 nm. The purity of the fractions was controlled on an Acquity UPLC system with PDA detection under the conditions described above (see section 4.3). Fractions containing isovitexin-2''-O-β-D-glucoside were concentrated under vacuum and purified with semi preparative HPLC, using a Waters 600 HPLC system, coupled to a Waters 996 Photo Diode Array Detector (Waters, Eschborn). Chromatographic separation was performed on an XBridge™ Prep C18 ODB™ column (150 × 19 mm, 5 µm) (Waters, Eschborn), which included an isocratic step for the first two minutes with 97% A (0.1% formic acid in Water) and 3% B (0.1% formic acid in acetonitrile) and an additional linear gradient of 97% A and 3% B to 40% A and 60% B in 128 min. The flow rate was set to 3 ml/min. Afterwards the column was washed with 97% B. Further impurities were removed on an analytical Alliance HPLC System (Waters, Eschborn), equipped with a photo diode array detector according to established methods with some modifications (Mock et al., 1999). Chromatographic separation was performed with a Phenomenex Gemini-NX, C-18 column (250 × 4.6 mm, 5 µm, 110 Å) (Phenomenex, Torrance, USA) using the following three stepped linear gradient: 0–20 min: 97% A (A = 0.1% formic acid in water) and 3% B (B = 0.1% formic acid in acetonitrile) to 85% A and 15% B; 20 min - 50 min: 85% A and 15% B to 60% A and 40% B; 50 min - 60 min: 60% A and 40% B to 0% A and 100% B. Washing and equilibration were conducted using the following conditions: 60 min - 65 min: 0% A and 100% B; 65 min - 67 min: 0% A and 100% B to 97% A and 3% B; 67 min - 77 min 97% A and 3% B. The flow rate was set to 1 ml/min.

5.5. NMR analysis

NMR spectra were obtained with an Agilent VNMRS 600 system at ~25 °C. 1D (${^{1}H, ^{13}C, ^{1}H}$H zTOCSY, 1D, $^{1}H, ^{1}H$ ROESY) and 2D ($^{1}H, ^{13}C$ gHSQCAD, $^{1}H, ^{13}C$ gHMBCAD, $^{1}H, ^{1}H$ DQF COSY, $^{1}H, ^{1}H$ zTOCSY, $^{1}H, ^{1}H$ ROESY) NMR spectra were measured using standard CHEMPACK 7.1 pulse sequences implemented in the VNMRJ 4.2A spectrometer software. Chemical shifts were referenced to internal TMS ($\delta$H 0.0, $\delta^{1}$H 29.8, $\delta^{13}$C) or DMSO-d$_6$ ($\delta^{1}$C 39.5, $\delta^{13}$C). The mixing time for the $^{1}$H,$^{1}$H-ROESY experiments was set to 0.3 s.

5.6. Extraction of crude plant protein

Proteins were extracted from ground fresh leaf material of the first and third leaf using an established protocol, but with some modifications (Marinova et al., 2007). One gram of fresh leaf material was suspended in 6 25 ml 100 mM potassium phosphate buffer, pH 7.6 and 2 mM DDT (extraction buffer). Next, 0.200 g fr. wt polyclar AT was added and incubated on ice for 20 min. The supernatant was clarified by centrifugation at 20000 g at 4°C for 10 min. After precipitation with 70% ammonium sulphate, the pellet was dissolved in 0.5 ml extraction buffer and desalted with NAP-5 and NAP-10 columns (GE Healthcare, Chalfont St Giles, Great Britain) according to the manufacturer’s instructions. The protein content was determined using the Bradford test (Bradford, 1976).

5.7. Enzyme activity tests

The activity of the UDP-glucosyl transferase reaction was determined using a fresh enzyme preparation in presence of UDP-glucose and isovitexin as a substrate. Ten micrograms of a crude protein preparation and 5 μl 2.3 mM isovitexin were adjusted with extraction buffer to a total volume of 97 μl. The reaction was initiated by adding 3 μl 100 mM UDP-glucose solution. The mixture was incubated at 30°C under continuous shaking (900 rpm/min) for 0, 5, 10, 20, 60, 120 min. The reaction was stopped by adding 20 μl 50% TCA. After precipitation of the proteins on ice for ten minutes, the protein was removed by centrifugation (20000 g, 10 min, 4°C). The supernatant was analysed by UPLC (see section 4.3). As a control, an aliquot of the crude protein was deactivated at 95°C for 20 min and then used for the activity test.

5.8. Identification of candidate genes

Identification of candidate genes was done with the online tool Barleymap (http://floresta.eead.cisic.es/barleymap/find/) between the genetic markers BOPA1_7174-365 and BOPA2_12_30588 (Cantalapiedra et al., 2017). The search was conducted against the genome data of Morex (Mascher et al., 2017). The genes/marker search of the Morex genome was extended to 0.5 cm. A detailed list of the used markers is found in the supplementary files (see Supplemental Genetic Markers 0.21–15.08 cm).

Funding

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.phytochem.2018.01.001.


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