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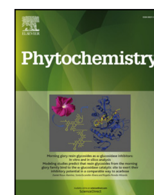
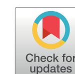


# A promiscuous beta-glucosidase is involved in benzoxazinoid deglycosylation in *Lamium galeobdolon*



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ABSTRACT

In the plant kingdom beta-glucosidases (BGLUs) of the glycosidase hydrolase family 1 have essential function in primary metabolism and are particularly employed in secondary metabolism. They are essential for activation in two-component defence systems based on stabilisation of reactive compounds by glycosylation. Based on *de novo* assembly we isolated and functionally characterised BGLUs expressed in leaves of *Lamium galeobdolon* (*Lg*GLUs). *Lg*GLU1 could be assigned to hydrolysis of the benzoxazinoid GDIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one glucoside).

Within the Lamiaceae *L. galeobdolon* is distinguished by the presence GDIBOA in addition to the more common iridoid harpagide. Although *Lg*GLU1 proved to be promiscuous with respect to accepted substrates, harpagide hydrolysis was not detected. Benzoxazinoids are characteristic defence compounds of the Poales but are also found in some unrelated dicots. The benzoxazinoid specific BGLUs have recently been identified for the grasses maize, wheat, rye and the Ranunculaceae *Consolida orientalis.* All enzymes share a general substrate ambiguity but differ in detailed substrate pattern. The isolation of the second dicot GDIBOA glucosidase LgGLU1 allowed it to analyse the phylogenetic relation of the distinct BGLUs also within dicots. The data revealed long periods of independent sequence evolution before speciation.

## 1. Introduction

Chemical defence by secondary metabolites (also named specialised metabolites) is a potent weapon of plants against microbial pathogens and herbivores. Strategically important are toxicity of the metabolites and diversity within the plant kingdom, to guarantee efficient control and to avoid the appearance of general resistance. Classes of secondary metabolites can be characteristic for phylogenetic groups (Wink and Waterman, 1999). Other secondary metabolites have a scattered distribution in the plant kingdom (Ober and Hartmann, 2000). The mode of action of the metabolites is reactivity towards essential structures (amino acids, nucleic acids, fatty acids). This implies the risk of autotoxicity. Glycosylation, in most cases by UDP-glucosyltransferases, stabilises the compound, increases the water solubility and facilitates the transport into the vacuole, often acting as storage organelle (Jones and Vogt, 2001), whereby autotoxicity is avoided. However, the subsequent generation of the toxic aglycon is required for defence. A solution to the problem is the invention of so-called two-component plant defence that consists of the stabilised form of the metabolite and an enzyme for activation. Beta-glycosidases (BGLUs) stored in different cellular compartments (Morant et al., 2008) can be employed for generation of the reactive aglycon upon disintegration of the cell. The principle is established for a wide spectrum of structural diverse defence compounds including alkaloid glucosides, benzoxazinoid glucosides, cyanogenic glucosides, glucosinolates, iridoid glucosides and salicinoids (see Pentzold et al. (2014), for review).

BGLUs (EC 3.2.1.21) belong to the glycoside hydrolase family 1 and are present in bacteria, fungi, animals and plants. In plants *BGlus* constitute a small gene family comprising about 40 members each in *A. thaliana* and *O. sativa*. Most plant BGLUs are beta-O-glucosidases,

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exceptions are the beta-S-glucoside hydrolysing myrosinases (Morant et al., 2008). Plant BGLUs are relevant for developmental processes, e.g. mobilisation of storage compounds during germination (Leah et al., 1995), reconstruction of cell walls (Dharmawardhana et al., 1995), and phytohormone homeostasis (Falk and Rask, 1995). Due to the essential functions in primary metabolism, these BGLUs can be considered as basic set. A prerequisite for the establishment of the different two-component defence pathways is the availability of *BGlus* that are competent to hydrolyse distinct glycosides. The basic gene set constitutes the resource for evolution of new specificities by means of gene duplication and functionalisation. Isolation and characterisation of BGLUs integrated in the same defence pathway but in unrelated plant species can shed light on prerequisites for a *BGlu* to be successfully recruited for activation of a specific defence compound. An example of such a defence pathway shared by unrelated plant species is biosynthesis of benzoxazinoids (BX).

Benzoxazinoid aglucons interfere with $NH_2$ and SH nucleophilic groups of biomolecules and can influence the function of proteins, e.g. papain (Pérez and Niemeyer, 1989) and H+-ATPase (Friebe et al., 1997), but also the herbicide atrazine (Hamilton, 1964). Highly reactive metabolites that are toxic to a wide range of chewing and phloem-feeding insect herbivores and plant pathogens are generated by non-enzymatic breakdown of the unstable aglucons (Handrick et al., 2016). BXs are characteristically found in the Poales. The complete pathway has been elucidated in maize (*Bx1-Bx9*; Frey et al., 1997; von Rad et al., 2001; Jonczyk et al., 2008). In addition to the grasses, BX have been detected in distant orders of the eudicots, the Ranunculaceae and the Lamiaceae, where just one single species namely *Consolida orientalis* and *Lamium galeobdolon*, respectively, have been reported to possess benzoxazinoids. The more common class of secondary metabolites in the Lamiaceae are the iridoids. Harpagide and derivatives thereof were detected together with the benzoxazinoid DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) and its glucoside (GDIBOA) in *L. galeobdolon* leaves (Alipieva et al., 2003). Like in the other dicots, the main benzoxazinoid in several grasses, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its glucoside (GDIMBOA), was not traceable in *L. galeobdolon*. The maize BGLUs *Zm*GLU1 and *Zm*GLU2 that hydrolyse GDIMBOA and GDIBOA (BXBGLUs) were among the first plant BGLUs characterised (Cicek and Esen, 1999). *Zm*GLUs were in parallel described as cytokinine glucosidases (Brzobohatý et al., 1993). The BXBGLUs from wheat (*Ta*GLUs) and rye (ScGLU) have been isolated and characterised subsequently (Sue et al., 2006). The only BXBGLU outside of the grasses, *Co*GLU, was isolated from *C. orientalis* (Dick et al., 2012). Phylogenetic analysis of *Co*GLU revealed independent evolution in the grasses and the Ranunculaceae.

We isolated and characterised four *L. galeobdolon BGlus* that are expressed in leaves. *Lg*GLU3 and *Lg*GLU4 might be considered as enzymes with general function. *Lg*GLU1 and *Lg*GLU2 hydrolyse substrates of secondary metabolism. While the natural substrate of *Lg*GLU2 is unknown, *Lg*GLU1 is the *L. galeobdolon* GDIBOA glucosidase. The catalytic data for benzoxazinoids are similar to *Co*GLU and similarly the enzyme accepts several additional substrates including dhurrin. Contrary to *Co*GLU hydrolysis of the iridoid oleuropein by *Lg*GLU1 is highly efficient. Hydrolysis of the intrinsic iridoid harpagide however was not detected for *Lg*GLU1. Phylogenetic analysis indicates that all monocot and dicot BX*Bglu* genes evolved independently.

## 2. Results

### 2.1. DIBOA glucoside (GDIBOA) is the major benzoxazinoid in Lamium galeobdolon leaves

Two classes of defence related secondary metabolites have been described for *Lamium galeobdolon*, benzoxazinoids (DIBOA), and iridoids (harpagide) (Alipieva et al., 2003; Schullehner et al., 2008). Using HPLC and LC-MS analysis we verified these data (Supplementary data Table S1). The concentration of harpagide in leaves was around 20–times lower than GDIBOA (9 mmol/kg fresh weight). The aglucon DIBOA was not detectable in extracts of intact leaves if enzymatic activities are prevented (see Experimental), but within 30 min of incubation of macerated leaves at room temperature more than 99 percent of the benzoxazinoids consisted of the aglucon. (Supplementary data Table S1). The data showed that GDIBOA is the major defence metabolite of *L. galeobdolon* in leaves and that protection from BGLU activity is lost in damaged cells resulting in complete liberation of the reactive aglucon. Hence, GDIBOA glucosidases activating the toxic DIBOA are efficient. To identify the involved enzymes we aimed to identify the *L. galeobdolon BGlus* expressed in leaves employing *de novo* assembly based on cDNA sequence reads.

### 2.2. De novo assembly identifies four of L. galeobdolon BGlu genes

Leaf, flower and adventitious root tissue were used for isolation of total RNA. Equal amounts of the RNA were combined for generation of a normalised random primed cDNA library. Contigs were assembled from 18 to 20 million paired-end reads. To estimate the quality of the data we screened for the presence of the previously isolated *L. galeobdolon* genes *LgIgl1* and *LgIgl2* (Schullehner et al., 2008) and found the sequences fully and without mistakes represented in the sequence library. Based on published BGLU sequences from monocots and dicots (Dick et al., 2012; Rouyi et al., 2014) the data were screened for putative *BGlu* genes. Four full size-genes termed *LgGlu1* to *LgGlu4* (Supplementary data Table S2) were identified that have in BLAST analysis *E*-values below 1e-100 for query BGLU amino acid sequences (Supplementary data Table S3). *Lg*GLU3 differs from the other enzymes by the mannosidase signature around the catalytic glutamate residue (Czjzek et al., 2000; Xu et al., 2004). In addition, *Lg*GLU3 has two in frame potential start codons and can possibly encode two proteins that differ by 18 amino acids at the amino-terminus. For the longer version localisation in mitochondria or plastids is predicted by iPSORT and WoLF PSORT (Bannai et al., 2002; Horton et al., 2007). The shorter protein displays a putative signal peptide for channelling into the secretion pathway or the vacuole. The secretion pathway is clearly predicted by the programs SignalP-4.1 and iPSORT for the other three BGLU enzymes. WoLF PSORT indicates location at the endoplasmatic reticulum for *Lg*GLU4.

Expression of *L. galeobdolon BGlu* genes was analysed by quantitative RT-PCR (Fig. 1, see Experimental). Transcripts of all four genes were detectable in leaf, flower and root (Fig. 1A). Compared to the housekeeping gene *GAP C* the steady state expression levels in leaves were moderate comprising 2–10% thereof. An exception is *LgGlu2,* for which extreme variation of transcript levels was displayed, reaching amounts equal to *GAP C* in single biological replicates. What is causing this variability in leaves is unknown. For other organs, the *LgGlu2* values are relatively constant and the lowest expression level was determined for the root. *LgGlu3* steady state transcript levels are higher for flower and root than for leaves. *LgGlu1* and *LgGlu4* had similar low mRNA levels in all organs. To investigate a potential impact of physical damage on gene expression we crushed leaves (see Experimental) and isolated RNA at different time points (Fig. 1B). Only for *LgGlu4* an impact of wounding on mRNA levels was detected. The RNA amount increased transiently, peaking at 6 h after damage reaching about 20-fold elevated amounts.

### 2.3. Heterologously expressed LgGLUs exhibit different substrate spectra

Dicot BGLUs that contain amino-terminal signal peptides for direction into the secretory pathway are frequently co-translationally glycosylated. This modification is often essential for enzyme function (Morant et al., 2008). In *E. coli* the correct modification is not guaranteed and bacteria are no reliable expression systems. The problem is circumvented by transient expression in plants. A viral replicon system

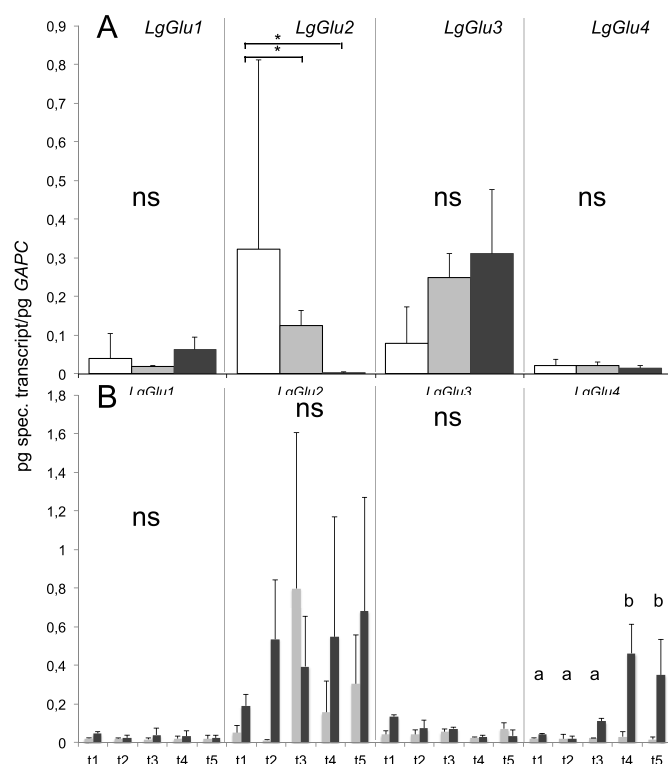


**Fig. 1.** Steady state RNA levels of *LgGlus.* (A) Expression in leaves (white column), flower (light grey column) and root (dark grey column). (B) Expression after wounding. Light grey columns, controls; dark grey columns, wounded leaves. All values are normalised to *GAP C* expression. The mean values of three biological replicates for flower and root, and nine replicates for leaves are displayed. The standard deviation is indicated. t1: 2 min, t2: 30 min, t3: 60 min, t4: 240 min, t5: 480 min. Significant differences with respect to tissue steady state levels are demonstrated for *LgGlu2* ($p < 0.05$). Expression in leaves is higher for LgGlu2 compared to the other genes ($p < 0.001$ *LgGlu1*, *LgGlu4*, $0 < 0.01$ for *LgGlu3*). *LgGlu3* has the highest mRNA concentration in the flower, the difference is significant ($p < 0.05$) in comparison with *LgGlu1* and *LgGlu4*. Root transcription levels are highest for *LgGlu3*, the difference is significant at $p < 0.05$ with respect to the other genes. Wounding does only cause significant differences in the case of *LgGlu4*. The increase of transcript level after wounding at the time point t4 and t5 is significant ($p < 0.001$) compared to all other induced and control plants. Statistical analysis was by ANOVA tests with RSTudio. Pairwise t-tests were performed for statistical significant results.

(Marillonnet et al., 2005) was used successfully in *Nicotiana benthamiana* for characterisation of *Co*GLU (Dick et al., 2012). Analogous expression constructs for *Lg*GLU1, *Lg*GLU2 and *Lg*GLU4 delivered the respective enzymes as major proteins in infected leaves (Supplementary data Fig. S1). Expression of *Lg*GLU3 could not be achieved. *Co*GLU was included in the following analyses as a positive control.

To prove functionality of the heterologously expressed BGLUs, hydrolysis of the substrate *para*-nitrophenyl-beta-D-glucopyranoside (*p*NPG) was analysed (Table 1). All expressed *L. galeobdolon* enzymes proved to be functional glucosidases. Next, we tested the enzymes for the specificity towards other glycosides using *para*-nitrophenyl-beta-D-mannopyranoside (*p*NPM), *para*-nitrophenyl-beta-D-fucopyranoside (*p*NPF) and *para*-nitrophenyl-beta-D-cellobioside (*p*NPC). None of the BGLUs hydrolysed *p*NPM. Fucosidase activity was revealed for *Lg*GLU2 and *Lg*GLU4. While for *Lg*GLU4 this activity seems to be negligible, fucosidase activity by *Lg*GLU2 is substantial and reaches about 25% of the glucosidase activity. All enzymes tested had cellobiose activity at about 10% of the level determined for *p*NPG. All *Lg*GLU enzymes preferred the glucoside as substrate.

To get a hint of the *in vivo* function of the *Lg*GLUs we employed representative glucosides of different families of plant secondary metabolites, benzoxazinoids (GDIBOA, GDIMBOA), iridoids (oleuropein, harpagide, geniposide), cyanogenic glucosides (dhurrin), isoflavonoids (daidzin, phloridzin, genistin), the hydroquinone arbutin, and the cytokinin glucoside *trans*-zeatin-O-glucoside (*t*ZOG) (Table 1, Supplementary data Fig. S2) as substrates. Hydrolysis of the hydroquinone and cytokinin glucosides was not detectable or minor for all *Lg*GLUs. The glucosidase activity of *Lg*GLU4 for any of the tested substrates was no more than 6% of the activity with *p*NPG implying that no glucoside with significant similarity to the natural substrate was represented. Generally, a low enzyme activity was determined for *Lg*GLU2 but hydrolysis increased for dhurrin (200%) and the iridoid oleuropein (120%) relative to *p*NPG hydrolysis (100%, Table 1). *Lg*GLU1 was identified as the *L. galeobdolon* benzoxazinoid glucosidase since the enzyme displayed significant enzymatic activity in GDIBOA hydrolysis reaching threefold level compared to glucosidase activity with *p*NPG (Table 1). Furthermore, crude extract from *L. galeobdolon* leaves and heterologously expressed *Lg*GLU1 had the same pH and temperature optimum (pH 5, 30–40 °C) for GDIBOA glucosidase activity (Supplementary data Fig. S3).

### 2.4. GDIBOA BGLUs of the dicots are promiscuous enzymes

The enzymatic activity of *Lg*GLU1 as GDIBOA hydroxylases (34 micromol $mg^{-1}min^{-1}$) equalled the *Co*GLU enzyme activity (41 micromol $mg^{-1}min^{-1}$). Both dicot benzoxazinoid hydroxylases discriminate between GDIBOA and the 7-methoxy derivate GDIMBOA, and for the latter specific activities were 0.5 micromol mg-1min-1 and 1.0 micromol $mg^{-1}min^{-1}$, respectively (Table 1). Both dicot GDIBOA BGLUs had substantial activity with non-benzoxazinoid substrates (Fig. 2A and B). Surprisingly specific activities of both enzymes were significantly higher with cyanogenic glucosides, isoflavonoid glucosides, and in the case of *Lg*GLU1 for the iridoid oleuropein than with GDIMBOA. Dhurrin hydrolysis was shown before for *Co*GLU (Dick et al., 2012). Likewise *Lg*GLU1 hydrolysis of the cyanogenic glucoside (7 and 9 micromol mg-1min-1) equalled the efficiency with *p*NPG. *Lg*GLU1 and *Co*GLU possessed isoflavonoid glucosidase activity. *Co*GLU however had a preference for daidzin that was not displayed by *Lg*GLU1 (Table 1, Fig. 2 A, B). The most significant difference between *Lg*GLU1 and *Co*GLU was glucosidase activity with the iridoid oleuropein (Table 1, Fig. 2A), which was close to the *p*NPG values (8 micromol $mg^{-1}min^{-1}$) for *Lg*GLU1 and only 15% thereof for *Co*GLU (2 micromol $mg^{-1}min^{-1}$). Inhibition of GDIBOA hydrolysis by the two respective second best substrates oleuropein and dhurrin was analysed for *Lg*GLU1 and *Co*GLU (Fig. 2C). Similar to the differences in hydrolysis, the level of inhibition by the iridoid oleuropein was significantly higher (about 80%) for *Lg*GLU1 compared with *Co*GLU (about 50%). Inhibition by dhurrin was substantial for both glucosidases and reduced GDIBOA hydrolysis to about 50%. Inhibition of CoGLU by dhurrin has been shown before (Dick et al., 2012).

To further characterise *Lg*GLU1, the steady state constants of the heterologously expressed enzyme were determined for the substrates GDIBOA, dhurrin, and the iridoid oleuropein. *Co*GLU was included for comparison. For all substrates, the $K_m$ values were in the millimolar range (Table 2). The $K_m^{GDIBOA}$ value of *Lg*GLU1 (2.5 ± 0.3 mM) fits well with the constant determined in the analysis of the crude extract (2.1 ± 0.45 mM) giving support that *LgGlu1* represents the *L. galeobdolon* GDIBOA glucosidase. The kinetic constant $k_{cat}/K_m^{GDIBOA}$ for *Lg*GLU1 and *Co*GLU prove the competence of both enzymes to efficiently hydrolyse GDIBOA (Table 2). Both constants differ by a factor of two. However, the differences in hydrolysis of the non-benzoxazinoid substrates are more pronounced. Dhurrin hydrolysis is significantly more efficient by *Co*GLU due to higher $k_{cat}$; the specific constant is even higher for dhurrin than for GDIBOA. Both determined values, $K_m$ and $k_{cat}$, demonstrate that *Lg*GLU1 is compared to *Co*GLU a competent oleuropein glucosidase.

**Table 1**
Specific activity of *L. galeobdolon* BGLUs with different substrates. *Co*GLU is included for comparison. Mean value of three replications and the standard deviation are given. nd = not detected. Enzyme extracts of heterologously expressed green fluorescent protein generated in parallel to *Lg*GLU proteins were used as negative controls. Hydrolysis of the glucosides was not detectable in the controls.

| Substrate class | Substrate | Specific activity [nmol min$^{-1}$ mg$^{-1}$] | | | |
|---|---|---|---|---|---|
| | | *Lg*GLU1 | *Lg*GLU2 | *Lg*GLU4 | *Co*GLU |
| Artificial substrates | pNPG | 8660 ± 867 | 472 ± 33 | 5814 ± 395 | 13549 ± 1027 |
| | pNPM | nd | nd | nd | nd |
| | pNPF | nd | 114 ± 22 | 104 ± 34 | nd |
| | pNPC | 283 ± 11 | 22 ± 3 | 291 ± 116 | 523 ± 282 |
| Benzoxazinoids | GDIBOA | 34083 ± 892 | 86 ± 23 | 5 ± 2 | 40919 ± 1855 |
| | GDIMBOA | 492 ± 27 | nd | nd | 1002 ± 24 |
| Iridoids | Oleuropein | 7967 ± 598 | 580 ± 37 | 77 ± 1 | 2112 ± 553 |
| | Hapagide | nd | nd | nd | nd |
| | Geniposide | 71 ± 8 | nd | nd | 84 ± 28 |
| Cyanogenic glycoside | Dhurrin | 7139 ± 572 | 949 ± 69 | 340 ± 61 | 14122 ± 1085 |
| Isoflavonoid glucosides | Daidzin | 2436 ± 104 | 53 ± 3 | 149 ± 16 | 4996 ± 446 |
| | Phloridzin | 65 ± 5 | 2 ± 1 | nd | nd |
| | Genistin | 1933 ± 146 | nd | 78 ± 26 | 1924 ± 1029 |
| Hydroquinone glucoside | Arbutin | nd | nd | nd | nd |
| Cytokinin glucoside | tZOG | 13 ± 1 | nd | nd | 61 ± 2 |

Taken together, *Lg*GLU1 and *Co*GLU are beta-glucosidases that hydrolyse specifically one benzoxazinoid, GDIBOA, the main secondary metabolite present in the *L. galeobdolon* and *C. orientalis*. In addition, alien natural substrates are hydrolysed and the substrate spectrum is overlapping but unique for each of the two dicot GDIBOA glucosidases.

### *2.5. The GDIBOA BGLUs LgGLU1 and CoGLU do not share a recent phylogenetic root*

Phylogenetic analyses of plant BGLUs have been published recently (Cao et al., 2017; Rouyi et al., 2014). The choice of sequences was mainly based on full genome sequences (e.g. *A. thaliana*, *O. sativa*, *S. bicolor*, *G. max*, *V. vinifera*, *Z. mays* etc.) and *BGlu* annotations. Using phylogenetic analysis, we aimed to get insight into the evolution of GDIBOA hydrolysis. For construction of the phylogenetic tree (see Experimentals) we employed the BGLU sequences of angiosperms (870 sequences) annotated in UniRef, including the Arabidopsis/Oryza clades (At/Os) defined by Rouyi et al. (2014), and the functional characterised enzymes (Supplementary data Table S4) to give a hint on putative enzymatic functions within clades. The defined grasses, dicot GDIBOA glucosidases, and *L. galeobdolon* BGLUs were included. For the sake of clarity, the cladogram is displayed in Fig. 3. The phylogenetic tree with real branch lengths and bootstrap values is given in Supplementary data Fig. S5.

The analysis (Fig. 3, Supplementary data Fig. S5) gave support for most of the clades defined by Rouyi et al. (2014). Classified At/Os clades were expanded by further monocot and dicot sequences (clades At/Os1, 2, 4, 5, 6) and the clades can be considered as the result of early gene duplications, which happened before the split of monocots/dicots. These clades are characterised by members with defined basic functions like mannosidases (clade At/Os4) and lignolases (clade At/Os5). Enzymes in these clades might represent the postulated basic portfolio of plant BGLUs. For At/Os3 and 7 a shared recent root of monocot and dicot genes got low bootstrap support (30%, 2%, Supplementary data Fig. S5). Former pure *Arabidopsis* clades were extended mainly by BGLUs from the Brassicaceae, e.g. AtII/myrosinases. Additional new clades were established. Based on characterised function of BGLUs therein, these can be assigned to *trans*-glucosidase activity and be defined as independent clades involved in secondary metabolism. Interestingly, cyanogenic glycosides (CG) are hydrolysed by enzymes located in a pure dicot clade, and in the benzoxazinoid-dhurrin-avenacoside clade. The latter traces back to a progenitor existing before the split of monocots and dicots, which however is not shared by the dicot CG glucosidase clade.

*L. galeobdolon* BGLUs were integrated in different clades. *Lg*GLU3 is found in the At/Os4/mannosidase clade, embedded in a sub-clade of BGLUs from other Lamiales. *Lg*GLU4 is a member of a distinct Lamiales clade but none of the enzymes was characterised for its enzymatic function. A displayed connection of the clade with any other clade has low bootstrap support (4%, Supplementary data Fig. S5). *Lg*GLU1 and *Lg*GLU2 locate in clades functionally characterised by the presence enzymes of secondary metabolism, and are each connected to further Lamiales BGLUs. The *Lg*GLU2 clade comprises also the furcatin hydrolase of *Viburnum furcatum*. The *Lg*GLU1 clade is connected with a clade embracing enzymes of raucaffricine and strictosine metabolism (Barleben et al., 2007; Xia et al., 2012) and additional BGLUs from Lamiales species. The biochemically characterised oleuropein glucosidase *Oe*GLU from *Olea europaea* is a member thereof. Although the bootstrap support is low (12%, Supplementary data Fig. S5) it can be speculated that the *Lg*GLU1 clade and the *Oe*GLU/secondary metabolite BGLU clade share a recent evolutionary progenitor. *Co*GLU and a *Delphinium grandiflorum* BGLU characterised as acyl-glucose-dependent anthocyanin glucosyltransferase, establish a distinct *Ranunculaceae*-specific clade that has no supported connection with any other clade. Hence, for the two clades that include the dicot GDIBOA glucosidases no recent shared root was detected in the phylogenetic analysis. On the other hand, the monocot benzoxazinoid glucosidases (*Zm*GLU1, *Zm*GLU2, *Ta*GLU1, *Ta*BGLU2, *Ta*GLU3, *Sc*GLU) clearly display monophyletic evolution. Hence monocot and each of the dicot GDI(M)BOA glucosidases had a long time of independent evolution, so they are no orthologues.

## 3. Discussion

### *3.1. BGLUs involved in primary function and secondary metabolism are expressed in L. galeobdolon leaves*

Identification of putative beta-glycosidase genes by *de novo* gene assembly and heterologous expression allowed the characterisation of four *L. galeobdolon* BGLUs. *Lg*GLU3 displayed the characteristic sequence feature of beta-mannosidases, namely the amino acid motif L(S/A)ENG instead of I/VTENG. This motif positions the sugar moiety in proximity to the two glutamate residues that are the nucleophile and general acid/base catalyst in the reaction (Czjzek et al., 2000; Xu et al., 2004). Mannosidases might be required for cell wall modification. This suggestion is substantiated by the fact that *LgGlu3* displayed high mRNA levels in roots, flowers and leaves. Similarly the mannosidase *OsBglu7* is the only rice *Bglu* that is ubiquitously highly expressed

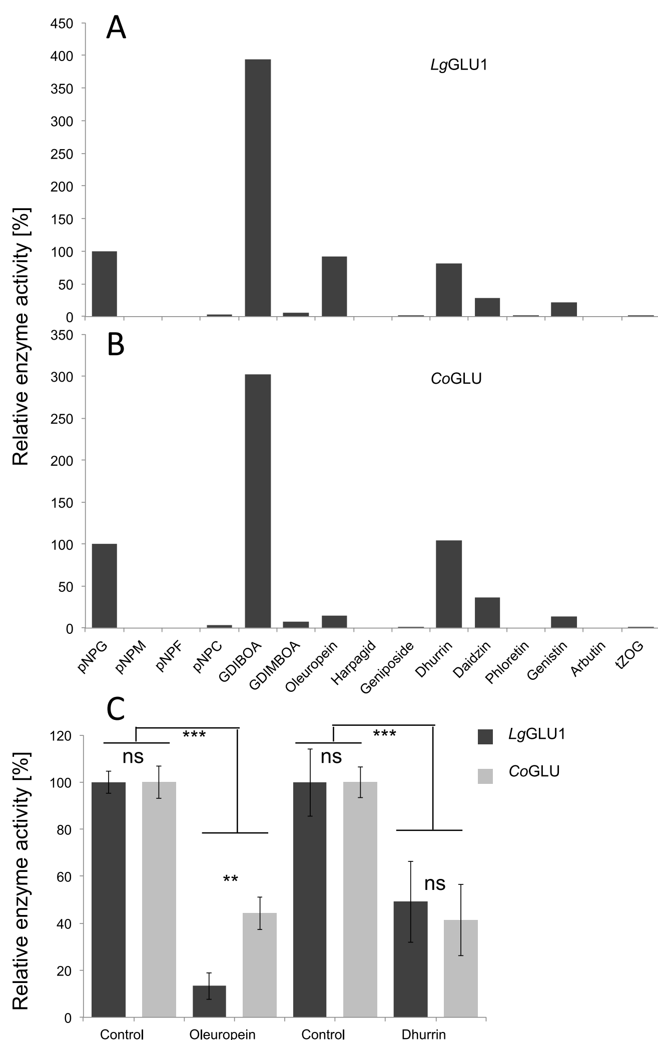


**Fig. 2.** Substrate preference of the GDIBOA glucosidases *Lg*GLU1 and *Co*GLU. Enzyme activity of *Lg*GLU1 (A) and *Co*GLU (B) with different artificial and natural glucosides. The values are normalised to *p*NPG activity. Activities with the non-benzoxazinoid substrates are significantly different for *Lg*GLU1 and *Co*GLU (oleuropein $p < 0{,}001$, dhurrin $p < 0.05$, daidzin $p < 0.05$) While *Co*GLU prefers the isoflavonoid daidzin compared to to genistin ($p < 0.01$) no significantdifference is detected for *Lg*GLU1. (C) Inhibition of GDIBOA hydroxylation by incubation with oleuropein and dhurrin. Dark grey columns *Lg*GLU1, light grey columns *Co*GLU. The means of three replicates are given and the standard deviations are indicated. GDIBOA hydrolysis is significantly inhibited by oleuropein and dhurrin, inhibition of *Lg*GLU1 by oleuropein is stronger compared to inhibition of *Co*GLU. The concentrations of glucosides as substrates and inhibitors were 0.5 mM in all experiments.The values are given as micromole $mg^{-1}min^{-1}$. *** $p < 0.001$, **$p < 0.01$. Statistical analysis was by ANOVA tests with RSTudio. Pairwise t-tests were performed for statistical significant results.

(Ketudat Cairns et al., 2015) and the pattern is shared (Arabidopsis eFP browser; Winter et al., 2007) by the Arabidopsis mannosidase *AtBglu44* (Xu et al., 2004). However, since enzymatic data of *Lg*GLU3 could not yet be gained, mannosidase activity still has to be verified.

The enzymatic function of *Lg*GLU1 as GDIBOA glucosidase was definitely established. It was shown that the enzymatic properties determined for the GDIBOA hydrolysis by crude protein extract of *L. galeobdolon* leaves and heterologously expressed *Lg*GLU1 fit well (Supplementary data Fig. S3) and identical $K_m^{GDIBOA}$ values were determined. Beside GDIBOA, *Lg*GLU1 accepts several metabolites of different families. The same glucosidase activities were found in the crude extract from *L. galeobdolon* leaves and *Lg*GLU1 can account for all glucosidase reactions found therein with the exception of mannosidase activity (Supplementary data Fig. S4). *LgGlu1* transcript levels are moderate in all tissues analysed. Given the enzymatic properties of *Lg*GLU1 and crude extract in GDIBOA hydrolysis activity, *Lg*GLU1 is calculated to account for about 1% of the total protein in leaves.

The *in planta* functions of *Lg*GLU2 and *Lg*GLU4 could not be assessed, although heterologous expression was successful and glucosidase activity could be verified. *Lg*GLU4 accepts none of the natural substrates tested by a rate similar to the hydrolysis of the artificial glucoside *p*NPG. In phylogenetic analysis, the *Lg*GLU4 is a member of an independent clade with no assigned function. The induced transcript level increase by wounding suggests an involvement in repair or defence reactions. Cellobiose hydrolysis, that was determined for the wound inducible *Os*BGLU4 (Rouyi et al., 2014), however can be excluded (Table 1). The cyanogenic glucoside dhurrin and the iridoid oleuropein were hydrolysed by *Lg*GLU2 with similar rates as *p*NPG (Table 1). This activity spectrum can be an indication that secondary metabolites are natural substrates of *Lg*GLU2. The intrinsic iridoid harpagide however proved to be not hydrolysed by the enzyme.

Recent comprehensive expression analyses of different members of the rice *OsBGlu* family by Ketudat Cairns et al. (2015) and Cao et al. (2017) revealed a tissue and condition specific expression pattern for individual *Bglus*. These findings are in accordance with the expression atlas of the *AtBGlus* at the Arabidopsis eFP browser (Winter et al., 2007). The largest part of the Arabidopsis and rice *Bglus* is expressed in the seed. At most 10 of the about 40 members of the gene families are significantly expressed in leaves and other vegetative tissues. Hence, the four *L. galeobdolon* genes *LgGlu1* to *LgGlu4* might represent an essential part of the *LgBglu* genes expressed in leaves. The repertoire of BGLUs includes an enzyme of cell wall metabolism, *Lg*GLU3, and the BGLU dedicated to chemical defence, *Lg*GLU1.

### 3.2. BXBGLUs of dicots and monocots have distinct substrate preferences

In the grasses, benzoxazinoids are constitutively present at high levels in the seedling. In dicots, the constitutive expression is not restricted to juvenile stages. While in wheat and maize seedlings almost exclusively the 7-methoxy derivative (G)DIMBOA is detected, in rye shoots (G)DIBOA is predominant and in the root the major BX is (G) DIMBOA. Beside *Aphelandra squarrosa* and *Aphelandra auriantica* that exhibit traces of the 7-methoxy benzoxazinoids, (G)DIBOA is found in dicots as unique terminal product of benzoxazinoid biosynthesis (Sicker et al., 2000). These substrate differences are reflected by the

**Table 2**
Steady-state constants of *Lg*GLU1 and *Co*GLU for different substrates. The best fit value +/−SE determined from data of three replicate measurements at each of seven different substrate concentrations are given.

| Substrate | *Lg*GLU1 | | | *Co*GLU | | |
|---|---|---|---|---|---|---|
| | $K_m$ [mM] | $k_{cat}$ [$s^{-1}$] | $k_{cat}/K_m$ [$mM^{-1}s^{-1}$] | $K_m$ [mM] | $k_{cat}$ [$s^{-1}$] | $k_{cat}/K_m$ [$mM^{-1}s^{-1}$] |
| GDIBOA | 2.5 ± 0.3 | 211.1 ± 11.3 | 81.6 | 6.8 ± 1.3 | 307.9 ± 36.1 | 45.3 |
| Dhurrin | 1.9 ± 0.7 | 112.5 ± 22.6 | 59.0 | 1.9 ± 0.2 | 236.5 ± 6.9 | 125.8 |
| Oleuropein | 4.1 ± 0.8 | 104.4 ± 11.4 | 25.5 | 8.3 ± 3.5 | 69.6 ± 3.4 | 8.4 |

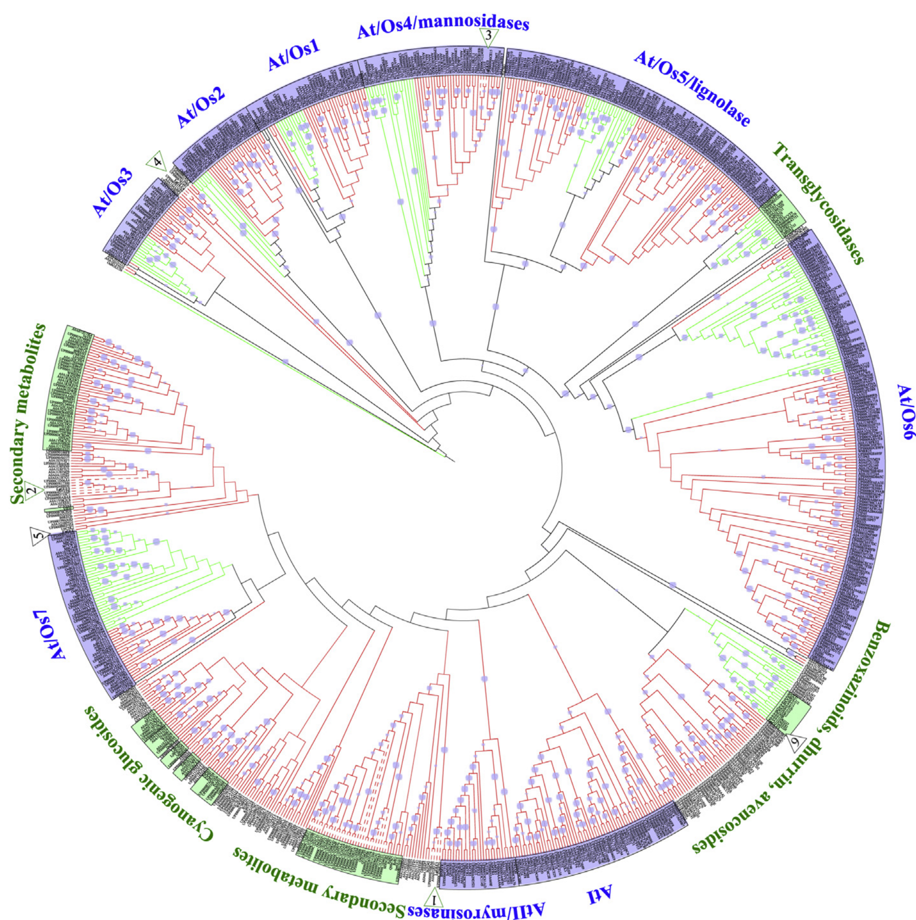


**Fig. 3.** Phylogenetic analysis of plant beta-glucosidases. 870 sequences extracted from UniRef100 and complemented with defined BGLU sequences (see Experimental) were used to build a phylogenetic tree. The multiple sequence alignment was done with MUSCLE 3.8.31 (Edgar, 2004) and the tree was calculated with RAxML 8.2.10 (Stamatakis, 2014) in 100 bootstrap samples (Experimental). Branches with bootstrap values higher than 25% are marked with blue dots; the size is proportional to the value. The tree was rooted with *Os*BGLU36 (SFR2). The tree is coloured by organism, monocots in green and dicots in red. The identifiers of the BGLUs refer to UniProtKB and UniRef100. Arabidopsis, rice enzymes and enzymes with defined function are supplied with systematic names. The respective identifiers are compiled in Table S4 in Supplementary Data. Identifiers are shaded in blue for At/Os and At clades as defined by Rouyi et al. (2014). Clades that include enzymes with experimentally defined functions have green-shaded identifiers. The green triangles numbered 1 to 4 indicate the positions of the *L. galeobdolon* genes *Lg*GLU1 to *Lg*GLU4 respectively. The black triangles point to *Co*GLU (5) and *Zm*GLU (6). The respective phylogenetic tree with real branch length and bootstrap values is displayed in Fig. S5 in Supplementary Data. Dashed lines indicate Lamiales species. U6C5K2 (DgAA7BGGT1) represents the Acyl-glucose-dependent anthocyanin glucosyltransferase from *Delphinium grandiflorum* (Nishizaki et al., 2013). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

preferences of the glucosidases. The *Zm*GLUs and *Ta*GLUs are most effective with GDIMBOA (Oikawa et al., 1999; Sue et al., 2000), *Sc*GLU does not greatly differentiate between the two glucosides (Sue et al., 2006), and both dicot BXGLUs, *Lg*GLU1 and *Co*GLU have only minor GDIMBOA glucosidase activity (Table 1; Fig. 2; Dick et al., 2012). A second analogy displayed by the dicot enzymes is a relatively high $K_m$ value for the preferred substrate (about 2 mM) compared to the grasses (0.1–0.4 mM). However, the substrate concentration in the plants given (Supplementary data Table S1; Schullehner et al., 2008), the lower affinity is not limiting for the reaction in the dicots as the efficiencies of the maize, rye, *L. galeobdolon*, and *C. orientalis* enzymes are in the same range ($k_{cat}/K_m$ about 100 [$mM^{-1}s^{-1}$]).

BGLUs in general often exhibit hydrolysis of several substrates. These off-activities have been subdivided into "multitasking" and "moonlighting" by Ketudat Cairns et al. (2015). For *Zm*GLU multitasking can be proposed since hydrolyses of the cytokinin tZOG and GDIMBOA occur with similar efficiency and it can be expected that both substrates are present in some tissues at the same time. None of the dicot BXBGLUs functions as efficient cytokinin glucosidase (Table 1; Dick et al., 2012). Glucosidase activity with substrates that are not detected in the respective plants can be considered as a type of moonlighting. Although isoflavonoids are not innate secondary metabolites, the dicot enzymes and *Sc*GLU share some isoflavonoid glucosidase activity (Table 2; Sue et al., 2006). Furthermore, the dicot and maize BXGLUs all interact with the cyanogenic glucoside (CG) dhurrin, resulting in hydrolysis or in case of the maize enzymes in inhibition of enzyme activity. CGs consist of alpha-hydroxynitrile type aglycons and of a sugar moiety, and are wide-spread two-component defence compounds in the plant kingdom (Bak et al., 2008). CG biosynthesis most probably evolved several times independently (Takos et al., 2010). Speculatively one can assume that a structure that allowed acceptance of CGs was a required attribute for precursors of nowadays BXBGLUs present in monocots and dicots.

Remarkably, the most distinct difference between the GDIBOA BGLUs *Lg*GLU1 and *Co*GLU is the efficiency in hydrolysis of the iridoid oleuropein and its function as inhibitor of the GDIBOA glucosidase activity of *Lg*GLU1 (Table 1, Table 2, Fig. 2). The phylogenetic analysis revealed both dicot BXBGLUs embedded in unique family or order specific clades indicating a diversification of the BGLUs in parallel to diversification of the Ranunculaceae and Lamiales respectively. Interestingly the oleuropein glucosidase *Oe*GLU (Velázquez-Palmero et al., 2017) of the oil tree (*Olea europaea*) is a member of a large Lamiales clade that is associated, although with moderate bootstrap support (Supplementary data Fig. S5) with the *Lg*GLU1 clade. This might indicate that acceptance and hydrolysis of iridoids was tentatively featured in the evolving Lamiales clade. Iridoids are common secondary metabolites in the Lamiales but not described for Ranunculaceae (Watson and Dallwitz, 1992), hence *Co*GLU iridoid hydrolysis is not connected to biological function but might rather be accidental. The differences in the choice of individual BGLU from the pool of co-existing precursors and distinct enzyme-substrate interactions might have been selected during the long time of independent sequence evolution demonstrated by the phylogenetic analysis. These features limit structure-function analysis across the BXBGLUs.

### 3.3. Evolution of two component secondary metabolite pathways

Evolution of a biosynthetic pathway is dependent on the contribution of the synthesised compound to the vitality of the organism. In the case of two-component defence metabolites, two features conflict the fixation. Obviously, autotoxicity of the intermediate is deleterious, attenuation of reactivity reduces efficiency and at the same time,

metabolic costs are maintained. Hence, BGLU function that activates the stabilised metabolite in case of challenge by herbivores and pathogens is the necessary component to economise the defence pathway. Direct proof for the importance of BGLUs for efficiency of defence is given by transgenic expression of *Sb*DHR from sorghum in barley, which leads to hydrolysis of the leucine-derived cyanogenic glucoside epiheterodendrin (Nielsen et al., 2006). Barley is missing an appropriate glucosidase (Nielsen et al., 2002). *Trans*-genetically established cyanogenesis led to increased resistance against the pathogen *Blumeria graminis* (Nielsen et al., 2006). Horowitz (1945) developed the concept of retrograde biosynthetic pathway evolution putting emphasis on the terminal step for the establishment of the complete pathway. In this light, a fundamental role can be assigned to glucosidases that catalyse the indispensable final reaction in two-component defence strategy.

Experimental determination of substrate specificity of BGLUs has generally revealed substantial promiscuity (Ketudat Cairns et al., 2015; Weng et al., 2012). In limited cases the *in planta* function has been determined by mutation analysis (Barth and Jander, 2006; Chapelle et al., 2012; Czjzek et al., 2000; Lai et al., 2014, 2015; Lee et al., 2006; Miyahara et al., 2012; Takos et al., 2010; Wang et al., 2011; Xu et al., 2012; Zamioudis et al., 2014). In most cases however *in vitro* activity led to the classification. Substrate ambiguity of enzymes, as demonstrated for BGLUs, was proposed as basis for proliferation of pathways (Jensen, 1976). Latently available substrate conversion has no selection pressure and can evolve possibly without affecting the original function (e.g. Aharoni et al., 2005). Further mechanisms for recruitment are gene duplications and neo-functionalisation (Moghe et al., 2017; Ober and Hartmann, 2000). Plasticity with respect to the accomplished ultimate function is demonstrated in the lineage of grasses BXBGLUs (Fig. 3, Supplementary data Fig. S5). *Zm*GLUs, *Ta*GLUs, *Sc*GLUS, *As*GLU, and *Sb*DHR are of monophyletic origin and the enzyme phylogeny follows the phylogenetic relation of the species. However, different substrates (BX, CG, and saponines) are hydrolysed. A similarly case of streamlining of homologous genes is found in *Lotus japonicus* that produces both cyanogenic and non-cyanogenic hydroxynitrile glucoside. In this case, the BGLU repertoire, the enzymes *Lj*BDG2, 3, and 4, shows differential expression in leaves and flowers, and the enzymes have different substrate spectra. *Lj*BDG4 is flower-specific and hydrolyses exclusively non-cyanogenic γ- and β-HNGs (Lai et al., 2015).

Although the second best substrate of *Lg*GLU1 is oleuropein, hydrolysis of the innate iridoid harpagide is not detectable for the heterologous enzyme and leaf extracts (Table 1, Supplementary data Fig. S4). The examples of productive/non-productive substrate pairs, *Lg*GLU1 and GDIBOA/harpagide, *Zm*GLU and GDIMBOA/dhurrin, and LjBDG4 and γ- and β-HNG/α-HNGs, might point to a further line in the evolution of secondary metabolite pathways, the exclusion of innate substrate hydrolysis and thereby the selection of unique defence compounds. Mutual exclusion of secondary metabolite families in plants is wide-spread (e.g. Wink, 2003) and was often attributed to limitations by the share of intermediates generated in primary metabolism. For example, in *Hordeum* ssp., presence of gramin and benzoxazinoids are exclusionary (Grün et al., 2005) and both are connected to tryptophan biosynthesis. However, confinement to one toxic compound in general might be evolutionary advantageous and triggering substrate exclusion by BGLUs.

## 4. Conclusions

The specific BGLUs involved in activation of benzoxazinoids in defence reactions are monophyletic in the monocots but the result of independent convergent evolution in the dicots. Similarly, the branch-point or signature enzyme of the pathway termed BX1 demonstrates independent evolution. Although the modifying enzymes of the pathway have not yet been elucidated for the dicots, it can be speculated that the complete pathway is the result of repeated evolution. For the monophyletic grasses BGLU clade that includes enzymes with high homology but different substrate specificities, crystal structure analysis in connection with mutagenesis elucidated enzyme function down to single amino acids and motives. Due to the long time of independent evolution recognised for the dicot BGLUs and preserved substrate ambiguity no structure function relations for benzoxazinoid hydrolysis can be determined. Inherent promiscuity might be the key for evolution of two-component defence strategies.

## 5. Experimental

### 5.1. Standards and reference chemicals

Benzoxazinoids were gifts from Prof. D. Sicker, University of Leipzig, Germany, or prepared as described by von Rad et al. (2001). Harpagide, daidzin, daidzein, phloretin, phloridzin, and arbutin were purchased from Extrasynthese, Genay, France, *trans*-zeatin-O-glucoside and *trans*-zeatin from OlChemlm Ltd. Olomouc, Czech Republic and oleuropein, geniposide, genipin, genistin, genistein, *para*-Nitrophenyl beta-D-glucopyranoside, *para*-Nitrophenyl-beta-D-mannopyranosid, *para*-Nitrophenyl beta-D-fuco⌐pyran⌐oside, *para*-Nitrophenyl beta-D-cellobioside from SIGMA-Aldrich, Germany. The structures of the substrates for glucosidase analysis are given in Supplementary data Fig. S2.

### 5.2. Plant materials and growth conditions

*Lamium galeodolon* shoots and adventitious roots, flowers and buds were harvested at the Staudengarten Weihenstephan, FH Freising. Rooted shoots were grown as described by Schullehner et al. (2008). *Nicotiana benthamiana* was grown on soil and used for infiltration 5–6 weeks after germination.

### 5.3. Generation of transgenic plants

Transient transgenic expression of BGLU in *N. benthamiana* was as described by Marillonnet et al. (2005) with vectors described by Engler et al. (2008).

### 5.4. Molecular biology methods

DNA and RNA isolation, cDNA synthesis, cloning, and PCR amplification was as described by Schullehner et al. (2008). A list of primers used for cloning and expression analysis is given in Supplementary data Table S5.

### 5.5. Transcriptome

To generate the data for the *de novo* assembly, RNA from leaves, flowers and adventitious roots was isolated and combined to establish a random primed normalised library. Sequencing was by MiSeq generating paired-end reads (Eurofins Genomics GmbH, Ebersberg, Germany). About 20 million library fragments were generated. The data are available at ftp://ftpmips.helmholtz-muenchen.de/plants/data_dump/Lamium/

The reads obtained from high-throughput sequencing were assembled using the Trinity *de novo* tool (version trinityrnaseq_r2013_08_14; Haas et al., 2013) under default parameter setting. Subsequently, the transcript annotation was performed by comparing nucleotide sequences from *A. thaliana* database TAIR9 (Arabidopsis Genome Initiative, 2000) using BLASTn algorithms with a maximal *E*-value of 10-e5. In case of multiple hits, the best fitting one was considered.

### 5.6. Quantification of metabolites from L. galeobdolon

Benzoxazinoids were extracted from *L. galeobdolon* as described by von Rad et al. (2001) with one volume Folch solution

(Chloroform:MeOH 2:1 (v:v), 1% HCl; Folch et al., 1957). To determine the amount of glucosides in native conditions, samples were snap frozen and immediately boiled for 10 min prior to extraction to ensure immediate inactivation of BGLU activities. For analysis of the metabolisation in damaged cells, leaves were macerated and incubated at room temperature for 30 min. The metabolites were quantified by HPLC analysis using authentic standards for each compound for verification and quantification. To determine the amount of harpagide, fresh plant material was snap frozen, macerated and extracted with three volumes EtOH. The concentrated material was suspended in MeOH and submitted for analysis with HPLC and LC-MS. Details of chromatographic conditions are given in Supplementary data Table S6.

### 5.7. Enzymatic analysis

Plant protein extracts from *N. benthamiana* with heterologously expressed protein were prepared as described by Marillonnet et al. (2005). Protein crude extracts from *L. galeobdolon* were passed through gel filtration columns (Illustra NAP-10 columns, GE Healthcare) to remove endogenous substrates and products. Boiled protein extracts were used as negative controls.

BGLU activity with the *p*NP-glycosides was determined in citrate-phosphate buffer pH 5.5 at 30 °C for 15 min and 5 mM substrate concentration. Reactions were stopped by addition of five volumes 2% sodium carbonate and the liberated *p*-nitrophenol was quantified at 405 nm.

The other assays were carried out with 0.5 mM substrate concentration in citrate-phosphate buffer pH 5.5 at 37 °C for 5 min (GDIBOA, oleuropein, harpagide), 10 min (daidzin, genistin), or 30 min (GDIMBOA, dhurrin, geniposide, phloretin, arbutin, tZOG). When authentic glycosides and aglucons were available analysis was by HPLC using the glycosides and aglucons as standards for quantification and verification. Reaction assays were either stopped by addition of one volume MeOH, spun down, and submitted directly for analysis or in case of the benzoxazinoids, substrates and products were extracted with four volumes Folch solution.

For substrates missing aglucon standards (dhurrin, arbutin, and iridoids oleuropein and harpagide), assays were inactivated at 85 °C for 5 min and glucose released by beta-glucosidase hydrolysis was measured as described by Cicek et al. (2000), with the Glucose (GO) Assay Kit (Sigma, Germany) according to manufacturer's instructions.

### 5.8. pH and temperature optimum

pH and temperature of the GDIBOA hydrolysis was determined for heterologous *Lg*GLU1 and crude extract. To determine the pH optimum the reactions were carried out in citrate-phosphate buffers with pH adjusted to a range from 3 to 8 at a temperature of 37 °C. For determination of the temperature optimum, the assays were performed in citrate-phosphate buffer of pH 5.5 at temperatures ranging from 30 °C to 70 °C in 10 °C increments. After preincubation at the respective temperature, the reactions were started by addition of enzyme extract and incubated for 5 min. Heterologously expressed *Lg*GLU1 and *L. galeobdolon* crude extract was used for comparison.

### 5.9. Determination of catalytic parameters

For the determination of catalytic parameters the substrates GDIBOA and oleuropein were used at concentrations between 0.175 and 5.6 mM, and dhurrin was used in concentrations between 0.313 and 10 mM. All reactions were carried out in citrate-phosphate buffer pH 5.5 at 37 °C, and within the initial rate period for all substrates (5 min). Determination was in three replicates. Enzyme parameters were determined using GraphPad Prism Version 4.03 with non-linear regression.

### 5.10. Inhibition of GDIBOA hydrolysis

To analyse the inhibition of GDIBOA hydrolysis by dhurrin and oleuropein a preincubation with either at 0.5 mM concentration at 30 °C for 5 min was executed. Subsequently, 0.5 mM GDIBOA was added and the reaction was stopped after incubation at 37 °C for 5 min by mixing with four volumes Folch solution. GDIBOA and DIBOA concentrations in the upper aqueous phase were measured by HPLC analyzation.

### 5.11. Transcriptional analysis after wounding stress

Wounding of *L. galeobdolon* was by gently crushing mature leaves with metal forceps at 5 mm distances. The plant material was snap frozen in liquid nitrogen after 2, 30, 60, 240 and 480 min and subjected to RNA isolation. cDNA synthesis and qRT-PCR reaction were as described by Schullehner et al. (2008). qRT-PCR primers used for transcription analysis are listed in Supplementary data Table S5.

### 5.12. Phylogenetic analysis

The dataset of 870 sequences contained BGLU protein sequences identified with PSI-BLAST (BLOSUM62; max E-value 0.005; Altschul et al., 1997) in the UniRef100 database as homologs of LgGLU1. Sequences without annotation as beta-glucosidases in protein descriptions were manually removed. 121 A. thaliana, O. sativa BGLUs and functional defined enzyme sequences (Table S4 in Supplementary Data) are included. These sequences were aligned using the multiple alignment program MUSCLE 3.8.31 (Edgar, 2004). Alignment columns with more than 20% gap chars were removed with trimAl v1.2 (Capella-Gutierrez et al., 2009). Phylogenetic trees were calculated with the program RAxML 8.2.10 (Stamatakis, 2014) using the PROTCATJTTF model, fast bootstrap mode (-f a option) and 100 bootstrap samples. As the comparison of the derived topologies with trees generated by an alternative method (Bayesian approach) did not result in major differences, we used the RAxML trees for further analysis. The trees were manually rooted to sequence OsBGLU36, visualized, and coloured by organism (monocots in green and dicots in red) using the iTOL software (Letunic and Bork, 2016). Internal nodes found in more than 25 of the bootstrap samples were visualized by circles, which were scaled according to their bootstrap values.

## 6. Accession numbers

Sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: MH271221 (*LgBglu1*); MH271222 (*LgBglu2*); MH271223 (*LgBglu3*); MH271224 (*LgBglu4*).

Data deposit for RNAseq data is ftp://ftpmips.helmholtz-muenchen.de/plants/data_dump/Lamium/.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2018.10.012.

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