Dynamics of the Tissue-Specific Metabolism of Luteolin Glucuronides in the Mesophyll of Rye Primary Leaves *(Secale cereale)*

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Secale cereale L., UDP-glucuronate: flavone-glucuronosyltransferases, β-Glucuronidase, Luteolin 7-O-Diglucuronosyl-4′-O-Glucuronide

Developing primary leaves of *Secale cereale* L. exhibit a dynamic metabolism of the major flavonoid luteolin 7-O-[β-D-glucuronosyl(1→2)β-D-glucuronide]-4′-O-β-D-glucuronide (R_1). Final steps of R_1 biosynthesis are sequential glucuronidations of luteolin, which are catalyzed by three specific UDP-glucuronate: flavone glucuronosyltransferases. These enzymes reach highest activities at the fourth and fifth day of leaf development, coinciding with maximal R_1 accumulation. The activities decrease with advancing age of the leaves. In contrast, a R_1-specific β-glucuronidase, responsible for the hydrolysis of glucuronic acid in position 4′, shows increasing activity up to the 5th or 6th day; but this activity, leading to luteolin 7-O-diglucuronide (R_2), is not reduced in later developmental stages. In this phase of leaf development, the amount of R_1 drastically drops, whereas R_2 accumulates only slightly. From *in vitro* results and from feeding experiments using [^{14}C]cinnamic acid, a precursor of R_1 biosynthesis, we conclude that the anabolic sequential glucuronidation takes place in young and expanding leaf tissue, whereas deglucuronidation occurs in nearly mature and mature tissue. The three glucuronosyltransferases as well as the β-glucuronidase, and the flavonoids R_1 and R_2 are localized in the mesophyll.

Introduction

During the last three decades, substantial progress has been made in the elucidation of biochemical and physiological processes of secondary plant products including phenylpropanoids and flavonoids [1, 2]. Their biosynthesis, accumulation, turnover and degradation are often integrated into the ontogenesis of higher plants. Secondary metabolism can reflect the physiological state of a whole plant, its organs, certain tissues or even particular cells. In particular, the relation between differentiation processes of seedlings and the biosynthesis of phenylpropanoids and flavonoids has been studied extensively [1–6, 11].

In a recent study [6], we described the accumulation pattern of six different flavonoid compounds in developing primary leaves of rye. A close correlation was found between organogenesis, and complex processes in flavonoid metabolism. In particular, the major flavone luteolin 7-O-[β-D-glucuronosyl(1→2)β-D-glucuronide]-4′-O-β-D-glucuronide, R_1 [7], exhibits marked quantitative changes during leaf growth. Maximal accumulation of R_1, ca. 50 nmoles per leaf, is reached on the 5th day of seedling development. In the following phase, a rapid decrease occurs, and after 10 days only 20% of the maximum amount is left. In contrast, the minor compound luteolin 7-O-[β-D-glucuronosyl(1→2)β-D-glucuronide], R_2, slightly increases continuously over the growth period studied. Both compounds R_1 and R_2 are exclusively localized in the mesophyll of the primary leaf [8].

Recently we reported on a R_1-specific β-glucuronidase from rye primary leaves, which is responsible for the hydrolysis of the glucuronic acid moiety in position 4′, with R_2 as reaction product [9]. This deglucuronidation is supposed to be the first step of the turnover or degradation of R_1 *in vivo*. In addition to the β-glucuronidase, three specific UDP-glucuronate: flavone glucuronosyltransferases involved in R_1 biosynthesis were characterized [10]. They catalyze the sequential glucuronidation of

Abbreviations: 2-ME, 2-mercaptoethanol; -glur, glucuronide; LGT, UDP-glucuronate: luteolin 7-O-glucuronosyltransferase; LMT, UDP-glucuronate: luteolin 7-O-glucuronide glucuronosyltransferase; LDT, UDP-glucuronate: luteolin 7-O-diglucuronide-4′-O-glucuronosyltransferase; βGL, β-glucuronidase; L, luteolin; N, luteolin 7-O-glucuronide; R_2, luteolin 7-O-[β-D-glucuronosyl(1→2)β-D-glucuronide]; R_1, luteolin 7-O-[β-D-glucuronosyl(1→2)β-D-glucuronide]-4′-O-β-D-glucuronide.

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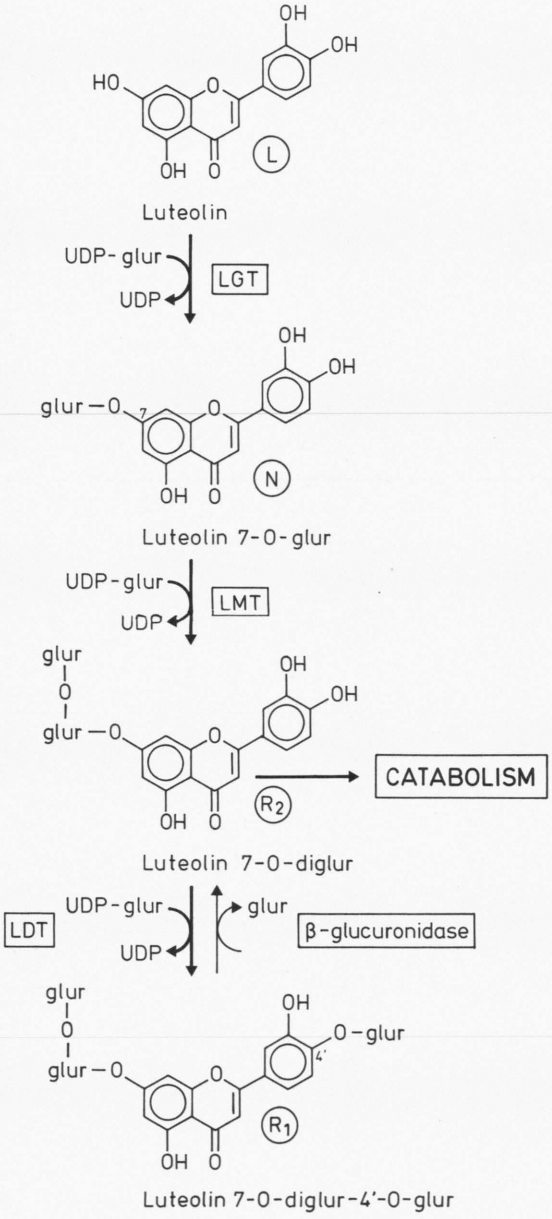
luteolin *via* luteolin 7-O-glucuronide (N) and luteolin 7-O-diglucuronide (R_2) to luteolin 7-O-diglucuronide 4-O-glucuronide (R_1) as the final product. The metabolic sequence is summarized in Fig. 1.

The rye primary leaf is a suitable model for investigations on the relation between a distinct part of the biosynthesis and degradation of the major flavonoid, R_1, and organogenesis, tissue specificity and cell differentiation. In this paper we present activity profiles of the four enzymes mentioned [9, 10] with regard to leaf development, gradient of cell differentiation within the leaf, and distribution in tissues. The results are compared with the accumulation patterns of R_1 and R_2.



Materials and Methods

Plant material

Caryopses of *Secale cereale* L. var. Kustro were purchased from F. von Lochow-Petkus (Bergen). Seedlings were grown in a phytotron under conditions described elsewhere [6]. Primary leaves of 3 to 10 day old seedlings were harvested at the beginning of the light phases of the photoperiod (13 h light, 11 h darkness).

Separation of the leaf tissues and preparation of mesophyll protoplasts

Leaf tissues were prepared as described previously [11]. Mesophyll protoplasts were prepared from 40 to 60, 4 and 5 day old leaves as described in [12].

Chemicals

Common chemicals were purchased from Merck (Darmstadt, FRG), luteolin 7-O-glucuronide was isolated from petals of *Antirrhinum majus*, R_1 and R_2 from rye primary leaves, and purified to 95% as determined by HPLC, according to [7]. Biochemicals were purchased from Boehringer (Mannheim, FRG), Serva (Heidelberg, FRG) or Sigma (München, FRG). Cellulysin (*Trichoderma viride*, 10,000 units $\times$ g^{-1}), and Miracloth were obtained from Calbiochem (La Jolla, USA). [^{14}C]cinnamic acid (1.6–2.0 GBq/mmol) was purchased from Amersham (Braunschweig, FRG). Thin layer chromatography was performed on microcrystalline cellulose (Macherey & Nagel, Düren, FRG).

Fig. 1. Part of luteolin glucuronide metabolism in rye primary leaves. Sequential glucuronidation of luteolin catalyzed by three specific UDP-glucuronate: flavone glucuronosyltransferases (LGT, LMT, LDT); intermediates are luteolin 7-O-glucuronide (N), and luteolin 7-O-diglucuronide (R_2). The end product luteolin 7-O-diglucuronosyl-4'-O-glucuronide (R_1) is substrate of a specific β-glucuronidase (βGL), catalyzing the hydrolysis of glucuronic acid in position 4'. The product R_2 might succumb further catabolic reactions.

Preparation of enzyme extracts

Unless stated otherwise, all steps were carried out at 4 °C. Primary leaves (3 to 10 days old) or their 1.5 cm sections were frozen in liquid nitrogen and ground to a powder. Separated leaf tissues and mesophyll protoplasts were homogenized with quartz sand in 1–2 ml of extraction buffer (0.1 M K-Pi, pH 7.0, 1 mM 2-ME). Powders were extracted with 10 ml buffer/g leaves. Extraction mixtures contained 15% Polyclar AT and 50% Dowex AG 1×2 (200–400 mesh) weight to fresh weight of plant material, for binding phenolic constituents. After stirring (30 min), the mixtures were squeezed through Miracloth and centrifuged for 10 min at 40,000×*g*. Protein solutions used were without significant contaminations of phenolics.

Enzyme assays

The activity of the β-glucuronidase was measured as described previously [9], but reactions were stopped within linearity after three hours. UDP-glucuronate: flavone glucuronosyltransferases were assayed as described elsewhere [10] with the following modifications: UDP-glucuronate: luteolin 7-O-glucuronosyltransferase (LGT) was incubated in 10 mM citrate buffer pH 5.5, 10 mM 2-ME, and UDP-glucuronate: luteolin 7-O-diglucuronide 4′-O-glucuronosyltransferase (LDT) in 10 mM citrate buffer pH 6.5, 10 mM 2-ME. Both activities were stopped after 30 min. UDP-glucuronate: luteolin 7-O-glucuronide glucuronosyltransferase (LMT) was assayed with 10 mM citrate buffer pH 6.0, 10 mM 2-ME, for 15 min. Incubations were performed with 5–15 µl protein solutions to determine the transferase activities, and with 10–30 µl for measurements of β-glucuronidase activity. Assays were analyzed and quantified according to [9, 10] by HPLC, using Zorbax C-8 (Dupont Instruments, Bad Nauheim, FRG) and Lichrosorb RP-8, standard 250-4, cat. 15540 (Merck) columns. In all cases, product formation was linear with time and protein concentration.

Protein was determined by Bradford's method [14], using BSA as standard. Chlorophyll content of extracts prepared from epidermal layers and from mesophyll protoplasts were estimated as described in [15]. Chlorophyll recovery in isolated protoplasts was used to calculate protoplast yield.

Feeding experiments with [^{14}C]cinnamic acid and determination of radioactivity

3, 4 and 5 day old primary leaves (10 of each age) were prepared and floated on a 50 µM [^{14}C]cinnamic acid solution for three hours (13). After the treatment, the leaves were washed three times with water and cut into sections (see legend of Fig. 4). The leaf sections were extracted for flavonoids [7, 13]. Separations of the different phenolic compounds were performed by chromatography on SC 6 polyamide columns using sequentially H_2O, CH_3OH, and CH_3OH with 0.01% NH_4OH as eluents. CH_3OH/NH_4OH fractions, containing R_1 and R_2, were evaporated and dissolved in 0.4 ml H_2O. Aliquots (100 µl) were separated by TLC, using two solvent systems: 1. $CHCl_3$/HOAc (3:2, $H_2O_{sat.}$) and 2. 15% HOAc. R_1 and R_2 were identified by cochromatography with authentic compounds. Radioactively labelled compounds on TLC plates were located using a TLC scanner with a TLC linear analyzer LB 2820/1 and a display unit model 7940 from Berthold (Wildbad, FRG). Radioactivity was further determined with a Packard Instruments liquid scintillation spectrometer Tricarb model 3380, using Unisolve type I cocktail from Zinsser (Frankfurt, FRG).

Result and Discussion

1. Profiles of enzyme activities

Previous work demonstrated that each UDP-glucuronate: flavone glucuronosyltransferase has a high affinity for its natural substrate luteolin, luteolin 7-O-glucuronide (N), and luteolin-7-O-diglucuronide (R_2), respectively [10]. No other transferase capable of catalyzing one of these reactions was detected. High affinity and specifity to the natural substrate R_1 was also found for the β-glucuronidase, that produces R_2 [9]. The latter compound is an intermediate of the anabolic as well as of the catabolic sequence (*cf.* Fig. 1). Using the standard assays, there was no evidence of reverse reactions, catalyzed by the corresponding enzymes.

These results were prerequisits to the study of enzyme activities in developing primary leaves and their specific distribution in the leaf. The activities of the four enzymes were measured over a period of 7 days, starting with 3 day old seedlings. In crude

protein extracts of the leaves, the product of LGT, luteolin 7-O-glucuronide (N), did not substantially accumulate, since it was further glucuronidated by LMT and LDT. Therefore the over all activity was measured under optimized conditions for LGT in crude extracts. Fig. 2a, b, c exhibits the activity profiles of the glucuronosyltransferases from the primary leaves as a function of plant age. The over all activity (Fig. 2a) reaches its maximum (38 pkat) on the fourth day, declining to 45% of the maximal amount by the tenth day. A similar profile of activity, but with a less rapid decrease was found for the LDT (Fig. 2c), (4 d: 43 pkat, 10 d: 30%). Individual determinations of the LMT activity with R_2 as the major reaction product could be made using short incubation times. The LMT enzyme exhibits highest activity at the fifth day (Fig. 2b), (107 pkat, 10 d: 27%).

Maximal activity of the sequential glucuronidation is revealed in leaf stages with nearly linear R_1 accumulation [6]. In older leaves, after day 5, R_1 biosynthesis is considerably reduced, and the amount of R_1 decreases rapidly. Nevertheless the UDP-glucuronate: flavone glucuronosyltransferases are probably not the rate limiting enzymes of R_1 biosynthesis. Chalcone synthase (CHS), the key enzyme of flavonoid biosynthesis may be rate limiting as was shown with oat primary leaves [11].

The reduction of glucuronosyltransferase activities alone cannot explain the rapid decrease of R_1 after the 5th day of development, but the R_1-reduction would be explained by a prevailence of β-glucuronidase activity at later stages in development. In fact, this activity increases during early developmental phase reaching its maximum between day 5 and 6 (Fig. 2d). No reduction was observed up to day 10.

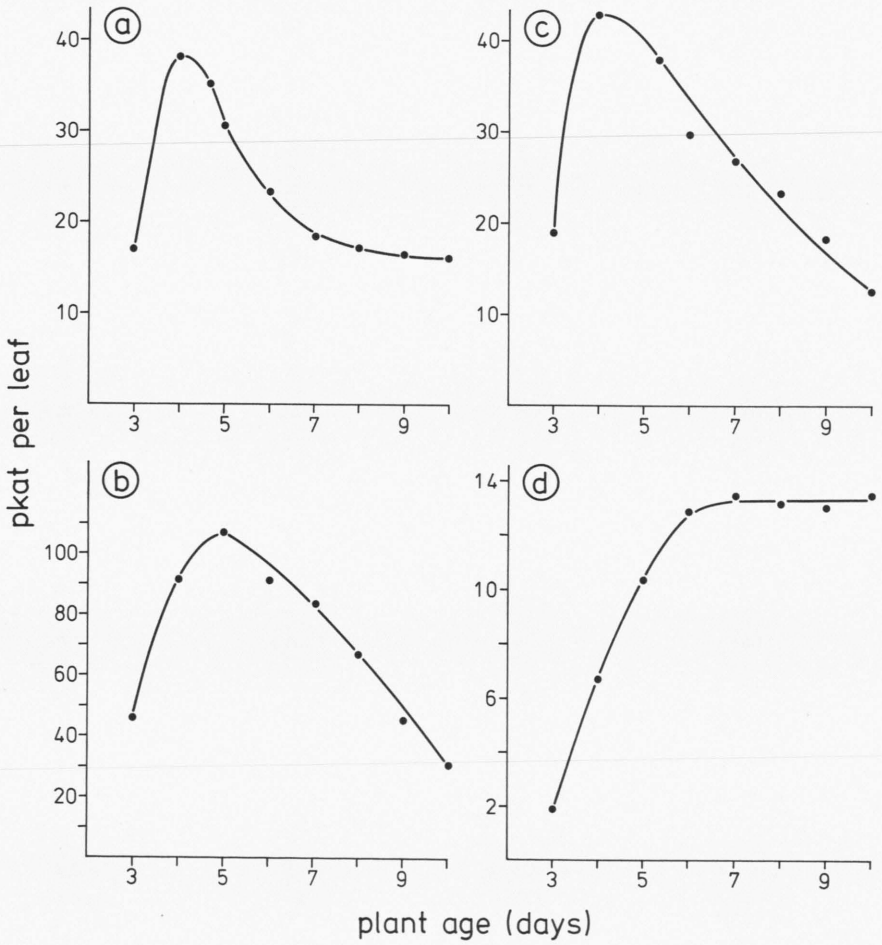


Fig. 2. Leaf age dependent profiles of the enzyme activities: a) LGT (over all activity), b) LMT, c) LDT, and d) β-GL. Values are the averages of three different series of experiments.

The major part of the R_2 quantity accumulating in the primary leaf may be due to this hydrolytic activity. Deglycosilation of flavonoid glycosides is a known mechanism to initiate further catabolic reactions of the aglyca [16, 17]. Deglucuronidation of R_1 to R_2 might be the first step of a catabolic sequence. Since R_2 remains a minor compound during all stages of leaf development (*cf.* [6]), and further catabolites of R_1 or R_2 were not detected, R_2 may turnover or degradate rapidly by still unknown reactions. From results of *in vitro* measurements, polymerization of R_2, catalyzed by peroxidases, cannot be excluded (data not shown).

2. *Distribution of enzyme activities*

Rye primary leaves exhibit a continuously proceeding cell differentiation and expansion from the meristematic basal region towards the tip as a result of basiplastic growth. The major amount of R_1 was found in the middle and upper part of the leaf (4 to 6 day old), and it was concluded, that R_1 is synthesized and accumulated primarily in the expansion zone of the leaf [6]. In 4 and 4.5 day old leaves, LGT (over all activity) is present in similar amounts throughout the leaf. At day 6, the activity is retained in the middle to the lower leaf parts, but is reduced in the upper ones (Fig. 3a). A comparable distribution was found for individual measurements of LMT and LDT activities (data not shown). The activity of β-glucuronidase increases from the base to the tip, and it is not reduced during further development (Fig. 3b). These *in vitro* data may indicate that the hydrolysis of R_1 to R_2 occurs in nearly mature or mature tissue. The results obtained from feeding experiments with [^{14}C]cinnamic acid, a precursor of flavonoid biosynthesis, support this interpretation. Highest amounts of labelled R_1 plus R_2 were found in the middle sections of the leaf. In the upper part, the yield of labelled luteolin glucuronides was drastically diminished (Fig. 4).

Luteolinglucuronides (R_1 and R_2) are only present in the mesophyll of rye primary leaves [6, 8]. The intermediate, N, is not detectable at all. To study the tissue distribution of the transferases and of the β-glucuronidase, peeled lower and upper epidermal layers and the corresponding leaf complements including mesophyll cells were measured for enzyme activities. Fig. 5a presents the tissue-specific distribution of LGT (over all) activity for 4 day old leaves. This result is representative also for the separately measured LMT and LDT activities as well as for other stages of the leaf development. The enzymes showed a clear localization in the mesophyll; extracts of epidermal layers contained only ca. 5% of

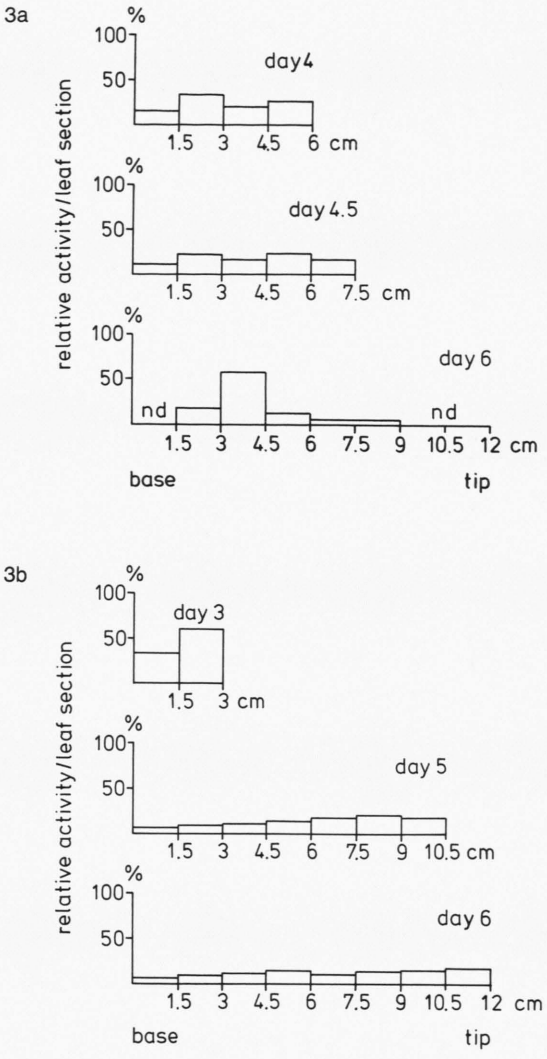


Fig. 3. Rye primary leaves of different ages were cut into 1.5 cm sections and extracted to determine enzyme activities. Fig. 3a represents the distribution of the relative glucuronosyltransferase activity (LGT, over all reaction). Comparable data were obtained for individual measurements of LMT and LDT activities (not shown). Fig. 3b shows the distribution of relative β-glucuronidase activity throughout the leaf. For 100% values of the different ages, compare profiles of activities in Fig. 2. Values represent the means of five (3a) and three (3b) different series of experiments with maximal variation of ±5%.

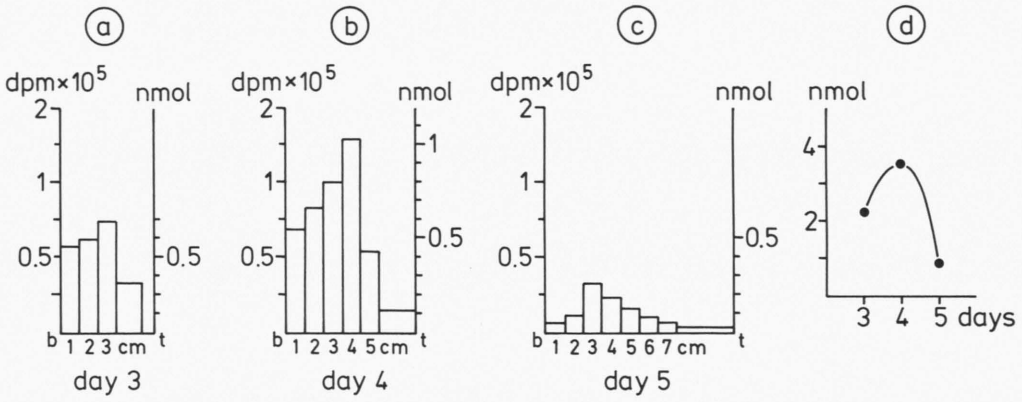


Fig. 4. Feeding experiments with [^{14}C]cinnamic acid using 3, 4, and 5 day old leaves. Ten treated leaves of each stage were cut into 1 cm section and the remaining tip. Identical sections were extracted and labelled flavonoids R_1 and R_2 were determined (dpm, nmol). The distribution of radioactively labelled R_1 plus R_2 is shown in 4a, b, and c; 4d exhibits the total amount of labelled R_1 plus R_2 (nmol) per 10 leaves *vs.* leaf age.

the total activity. A similar distribution was found for the β-glucuronidase; about 90% of the activity was located in the autotrophic mesophyll and ca. 10% in each epidermal layer (Fig. 5b). The activities present in epidermal extracts are probably due to contaminations with some remaining mesophyll, since mesophyll cells were observed microscopically sticking to the peels. The remaining activities found in epidermal preparations coincided with chlorophyll contaminations.

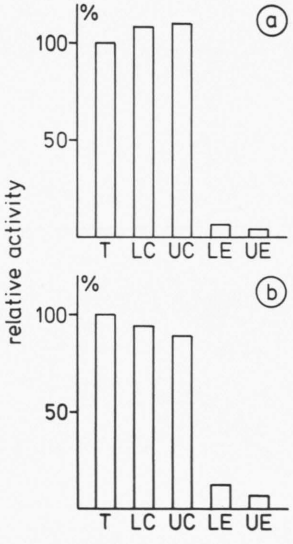


Fig. 5. Tissue-specific distribution of a) LGT (over all activity) for 4 day old leaves and b) β-glucuronidase for 5 day old leaves (%). T: total leaf, LC: leaf complement (mesophyll plus upper epidermis, lower epidermis peeled off), UC: leaf complement (mesophyll plus lower epidermis, upper epidermis peeled off), LE: lower epidermis, UE: upper epidermis.

To obtain further evidence for tissue-specificity, mesophyll protoplasts were isolated and enzyme activities were determined. Nearly 80% of the glucuronosyltransferase activities, but only 20–25% of the β-glucuronidase activity could be recovered, compared to whole leaves (protoplast yield was 90%). Mixing experiments did not point to the presence of endogenous inhibitors for either glucuronosyltransferases or β-glucuronidase. The reason for β-glucuronidase loss is still unclear. Vascular bundles did not contain any of the activities.

The age dependent profiles of enzyme activities and their specific distribution reflect the dynamic accumulation pattern of the luteolin glucuronides in rye primary leaves. A strong relationship between a major part of R_1 metabolism and the ontogenesis of the leaf was demonstrated. The properties of the glucuronosyltransferases point to a channeling of the luteolin glucuronidation [10]. Neither luteolin nor luteolin 7-O-glucuronide (N) are detectable in the primary leaves of any stage. An ordered interaction between anabolic and catabolic sequences of the R_1 metabolism is only possible by a strict subcellular separation of the different reactions. Therefore, our future studies will concentrate on the subcellular localization of R_1 metabolism in leaf mesophyll cells.

The further catabolism of R_2 will be another centre of interest. Functions of the luteolin glucuronides in rye primary leaves are still unclear. Known functions of flavonoids in general are for example, protection against harmful UV radiation or microbial attack [18]. Recently, a novel function of luteolin was found. This compound may serve to control the expression of *Rhizobium meliloti* nodulation genes [19]. Further studies of exogenous influences on R_1 metabolism will help to elucidate the role of the luteolin glucuronides in rye primary leaves.

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