Glucosinolate accumulation and hydrolysis in leafy *Brassica* vegetables are depending on leaf age

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ABSTRACT

The health-beneficial effects of *Brassica* vegetables are mainly attributed to their high contents of glucosinolates and the products of their enzymatic hydrolysis, especially isothiocyanates. Distribution of glucosinolates across plant organs can strongly vary. Consequently, the effect of leaf age on glucosinolate accumulation as well as hydrolysis was investigated in two leafy *Brassica* vegetables, pak choi and giant red mustard. Furthermore, activity of the hydrolyzing enzyme, myrosinase, was evaluated across the leaves. Additionally, a possible glucosinolate transport from older to younger leaves was monitored. Young leaves of both species contained of more glucosinolates than old ones. Accordingly, more isothiocyanates were released upon glucosinolate hydrolysis in young leaves. Myrosinases fully hydrolyzed the whole glucosinolates regardless of the leaf age. It was confirmed that older leaves can supply younger leaves with glucosinolates. The results can help to improve the health-beneficial value of *Brassica*s in the diet by an increased formation of isothiocyanates.

KEYWORDS: glucosinolate transport; myrosinase; epithiospecifier protein; isothiocyanate; *Brassica rapa*; *Brassica juncea*

INTRODUCTION

Brassica vegetables are of high interest for human nutrition, because they are rich in vitamins, minerals, dietary fiber, and many bioactive compounds.[1] These plants are particularly valued for their high glucosinolate (GLS) content, that are part of the plants defense against insects and pathogenes. GLSs are a group of sulfur-containing secondary plant metabolites, biosynthesized from amino acids, consisting of a β-D-thioglucose connected to an *O*-sulfated (*Z*)-thiohydroximate function and a variable side chain.[2] Biological variety of GLSs is predominantly due to variation of this side chain, which is decisive for the classification into aliphatic, benzenic, or indolic GLSs. Upon plant tissue damage, GLSs are hydrolysed by plant enzymes, commonly known as myrosinases, which hydrolyze the thioglucosidic bond and an unstable aglucone is released (**Figure 1**). This can spontaneously rearrange to an isothiocyanate (ITC) or at low pH to a nitrile (CN). Depending on the chemical structure of the side chain and in the presence of specifier proteins, CNs and epithionitriles (ETNs) can be preferentially formed.[3] ETN formation occurs only from GLS precursors with a terminal unsaturated double bond.[4] Nitrile specifier and epithiospecifier proteins (ESPs) are Fe^{2+}-dependent proteins and have no hydrolytic activity on GLSs themselves[5], but use the GLS aglucon as target.[6]

GLSs have been well studied in an ecological context for their role in plant defense against herbivores and pathogens.[7] Not GLSs themselves, but their hydrolysis products, particularly ITCs, show biological effects. ITCs are antimicrobial compounds and can have health-promoting effects on humans such as anti-inflammatory and chemopreventive actions.[8] Furthermore, epidemiological studies indicated that consumption of *Brassica* vegetables reduces the risk for cancer development and ITCs have been defined as major active constituents.[9] For these reasons, it seems to be desirable that *Brassica* vegetables release predominantly ITCs instead of CNs and ETNs.

In agreement with the optimal defense theory, different organs and leaves within a plant can contain different concentrations of defense compounds.[10] Defenses incur costs because they redirect resources from growth and other plant processes. Plants maximize growth and defense by spatial and temporal variation in specific metabolites in tissues that have the highest value but are most vulnerable to attacks.[11] This leads to an increased defense effectiveness. Previous studies demonstrated that due to the lower GLS accumulation in older leaves, young leaves show higher GLS levels, e.g., in *Arabidopsis thaliana* or various leafy crucifers.[12-14] In different *Barbarea* species, a genus of the Brassicaceae family, GLS contents negatively correlated with leaf size due to the fact that pests that were feeding on the leaves of cruciferous crops laid more eggs per leaf area on smaller leaves than on larger ones.[12] In *A. thaliana* (Col-0 ecotype), both GLS content and composition differed significantly among organs.[13] Moreover, an increasing accumulation of both, aliphatic and indolic GLSs, from the outer leaf layers to the inner leaf layers of headed cabbage was also demonstrated.[14] Recently, Hunziker, et al. [15] were not only able to show leaf age-dependent GLS distribution within *A. thaliana* plants, but also that mature leaves provide young leaves with GLSs to optimize their defense against herbivores by intensified GLS biosynthesis. GLS transporters were identified as essential for translocating GLSs from old to young leaves by loading into phloem.[16] In transporter mutants, GLSs were uniformly distributed in leaves of all ages.[15]

Although the effect of leaf age on GLS distribution within the plant is well understood, it is not clear if GLS hydrolysis is affected as well. As ITCs have strong effects concerning pest control [17-18], plants could benefit not only from accumulating of GLSs in young leaves, but rather from shifting GLS hydrolysis towards enhanced ITC formation, providing also a healthier option regarding human consumption. In this study, the effect of leaf age on GLS accumulation and hydrolysis outcome was investigated in two *Brassica* vegetables; pak choi (*Brassica rapa* L. subsp. *chinensis*) as a leafy vegetable consumed worldwide and giant red mustard (*Brassica*

juncea subsp. *rugosa* cv. Red Giant), a large, leafy vegetable offered mainly in ready-to-eat salad mixes. Moreover, activity of ESPs was assessed. Additionally, distribution and accumulation of allyl GLS, which does not occur naturally in pak choi and was artificially infused into an old leaf, was monitored. Understanding of GLS distribution and the shifts in GLS hydrolysis outcome depending on the leaf age can contribute to the overall aim to increase the health-beneficial value of our daily diet.

MATERIALS AND METHODS

Chemicals. Acetic acid (≥99,5%), allyl ITC (≥99%), aryl sulfatase, benzonitrile (≥99.9%), 3-butenenitrile (≥98%), DEAE-Sephadex A-25, DL-dithiothreitol (≥99%), indol-3-ylacetonitrile (≥98%), iron(II) sulfate heptahydrate (≥99%), 4-pentenenitrile (≥97%), 3-phenylpropanenitrile (≥99%), (-)-sinigrin hydrate (allyl GLS) (≥99%) and thioglucosidase from *Sinapis alba* seeds (≥100 U/g) were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). L(+)-ascorbic acid (≥99%), 4-hydroxybenzyl-GLS (≥99%), imidazole (≥99%) and methylene chloride (GC Ultra Grade) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). DL-goitrin (≥98%) was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). 3-Butenyl-GLS (≥99%), 2-hydroxy-3-butenyl-GLS (≥80%), 4-pentenyl-GLS (≥99%), 4-(methylthio)butyl-GLS (≥99%), and 3-indolylmethyl-GLS (≥99%) were obtained from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). 3-Butenyl-ITC (≥95%) and 4-penten-1-yl-ITC (≥95%) were purchased from TCI Deutschland GmbH (Eschborn, Germany). 1-Cyano-2,3-epithiopropane (≥95%) was synthetized by Sirius Fine Chemicals SiChem GmbH (Bremen, Germany) and 1-cyano-3,4-epithiobutane was synthetized by ASCA GmbH (Berlin, Germany). 3-Hydroxypent-4-enenitrile (95%) was purchased from abcr GmbH (Karlsruhe, Germany). $NaSO_4$ anhydrous (≥99%) was obtained from VWR International GmbH (Darmstadt, Germany). 5-(Methylsulfinyl)pentanenitrile, and 4-(methylsulfinyl)butanenitrile were purchased from Enamine (Riga, Latvia). Acetonitrile (LC-MS grade) and methanol (≥99.95%) were

purchased from Th. Geyer GmbH & Co. KG (Renningen, Germany). Water used in this study was of ultrapure grade.

Effect of leaf age on GLSs and hydrolysis products. Effect of leaf age on GLS contents and GLS hydrolysis outcome was studied in two leafy *Brassica* species. Seeds of pak choi (*Brassica rapa* ssp. *chinensis* cv. Arax) and giant red mustard (*Brassica juncea* ssp. *rugosa* cv. Red Giant) were obtained from HILD Samen GmbH (Marbach am Neckar, Germany) and sown in polypropylene plant pots (15 cm diameter) filled with substrate (Einheitserde Classic T; Einheitserde Werkverband e.V., Germany). Plants were grown under controlled environmental conditions in a phytochamber (ENGIE Deutschland GmbH, Köln, Germany) equipped with mercury to metal halide conversion lamp (Clean Ace™ (Daylight) Mogul Base – 6500K CCT/90CRI; EYE Lighting International, Mentor, OH USA) at the Leibniz Institute of Vegetable and Ornamental Crops in Großbeeren, Germany. The following growth conditions were set up in the phytochamber: temperature 22 – 18 °C (day – night), 12 h photoperiod, light intensity 500 µmol m^{-2} s^{-1} and 70% air humidity. Plants were watered as required and no fertilizers were used during the whole growing phase.

Pak choi plants were harvested at nine-leaf stage and giant red mustard at eight-leaf stage (five weeks after sowing). During the harvest, the aboveground part of the plants was cut off and the leaves were ordered according to their age beginning with L1 as the youngest leaf and ending with L8 (giant red mustard, **Figure 2B**) and L9 (pak choi, **Figure 2A**) as the oldest leaf. Leaves of the same age from two plants were pooled to one sample. Leaves were weighed and halved along the midrib. One half of each leaf was put into a 2 mL tube or 20 mL vial (polyvial V; Zinsser Analytics GmbH, Eschborn, Germany), depending on the leaf size, immediately shock-frozen in liquid nitrogen and stored at -20 °C until subsequent freeze-drying. The other half of the leaf was put into another 2 mL tube, or 20 mL vial. Five metal balls (8 mm ⌀) were added into the vial and the samples were homogenized for two min using an oscillating mill (MM400, Retsch GmbH,

Haan, Germany). After the homogenization, samples were incubated at room temperature for 30 min to achieve enzymatic GLS hydrolysis. Afterwards 250 mg of the homogenates were used for analysis of GLS hydrolysis products. For the assessment of the activity of epithiospecifier proteins (ESP), ESP extracts were prepared from another 250 mg of homogenate which were transferred into a 2 mL tube and 250 µL of water were added. Samples were put into ultrasonic ice bath (SONOREX SUPER RK 102 H, BANDELIN electronic GmbH & Co. KG, Berlin, Germany) for 10 min and afterwards centrifuged at 25 000 g and 4 °C (MegafugeTM 16, Thermo Fisher Scientific, Waltham MA, USA). 200 µL of the supernatant (ESP extract) were transferred into fresh eppendorf tubes, shock-frozen in liquid nitrogen and stored at -80 °C until further analysis. The experiment was carried out three times independently.

Effect of hydrolysis duration on the enzymatic hydrolysis of GLS. To test whether myrosinase activity was sufficient to hydrolyze the high GLS contents of young leaves of giant red mustard within the incubation time, a time course of allyl GLS hydrolysis was carried out. Plants of giant red mustard were grown in a phytochamber as described above. At eight-leaf stage, plants were harvested. First, young leaves (L1 – L3) and the oldest leaf (L8) of three plants were sampled. Plant material for GLS analysis in young and old leaves was immediately shock-frozen in liquid nitrogen and stored at -20 °C until subsequent freeze-drying. The residual young leaves were homogenized and samples for GLS analysis were taken after 1, 5, 15, 30, and 60 min and hydrolysis was stopped by immediately shock freezing aliquots in liquid nitrogen. To study if the amounts of myrosinase in old leaves is sufficient for hydrolysis, old leaves homogenates were enriched with 5 µmol g^{-1} FM of allyl GLS and samples for allyl GLS analysis were taken after 1, 5, 15, 30, and 60 min. Next, young leaves of three further plants were homogenized and 5 µmol g^{-1} FM of allyl GLS were added to the homogenate and mixed well and samples for GLS analysis were taken after 1, 5, 15, 30, and 60 min. ITCs are electrophilic and can react with nucleophiles such as the SH-group or NH_2-group from proteins, thereby also affecting their

function and properties.[19] So to test, whether high amounts of allyl ITC, which is mainly released from allyl GLS in giant red mustard, could affect myrosinase activity in young leaves, 20 µL of allyl ITC were added to one gram of young leaves and homogenated. Samples for GLS analysis as well as for analysis of GLS hydrolysis products were taken 1, 5, 15, 30, and 60 min after the homogenization. Additionally, a homogenate of young leaves was spiked with 5 µmol g^{-1} FM of allyl GLS 120 min after the homogenization, to test, whether the hydrolyzing activity of myrosinase is stable over a longer time. Here, samples for GLS analysis and analysis of GLS hydrolysis products were taken 30 and 60 min after spiking. Quantification of allyl GLS and its hydrolysis products was assessed for every sampling time point of each treatment. GLS hydrolysis products were extracted from fresh plant material immediately after the sampling. Frozen samples for GLS analysis stored at -20 °C until subsequent freeze-drying. The whole experiment was carried out in duplicate.

Transport and accumulation of allyl GLS in pak choi. To investigate transport of GLSs out of old leaves (as the site of biosynthesis) into young leaves in more detail, naturally absent allyl GLS was infused into an old leaf of pak choi. Pak choi plants were grown in polypropylene plant pots (13 cm diameter) filled with quartz sand (0.5 – 1 mm, Eroquarz GmbH, Laußnitz, Germany). Plants were grown under controlled environmental conditions in phytochamber as described above. Plants were watered with a nutrient solution (EC = 2.0, pH = 6) during the whole growing phase. At day zero, allyl GLS solution or water (control group) was infused into leaf number 6 (L6) of five week-old pak choi plants by piercing the midrib of the leaf with a needle and a piece of yarn was pulled through the midrib. Both ends of the yarn were placed into an 2 mL tube filled with 1.5 mL of the 13.3 mM allyl GLS solution (i.e. 20 µmol of allyl GLS were infused) or water (**Supporting information (SI) Figure S4**). Furthermore, to study whether there is a difference in GLS transport within the plant when the site of GLS biosynthesis is shaded or not, L6 was covered in aluminium foil 24 h before the infusion and for the following 72 h in half of the

plants. In total, there were 4 treatment groups: control with non-shaded (NS_control) or shaded (S_control) L6 receiving water, or treatments with infusion of allyl GLS into non-shaded (NS) or shaded (S) L6. GLS analysis of single leaves of 3 plants (treated on three different days) of each group was performed 24, 48, and 72 h after the infusion (n = 3). Control plants were monitored only for 48 h. During the harvest, single leaves were weighted, immediately shock-frozen in liquid nitrogen and stored at -20 °C until subsequent freeze-drying.

Analysis of desulfo-GLS by UHPLC-DAD-ToF-MS. GLS analysis was carried out according to the method described by Renz, et al. [20]. Briefly, 10 mg of freeze-dried and ground plant material were extracted three times using hot 70% methanol in the presence of 0.4 µmol 4-hydroxybenzyl GLS as an internal standard. The extracts were loaded onto DEAE-Sephadex A-25 ion-exchanger columns and desulphated with aryl sulfatase overnight. For elution of desulpho-GLSs, 1.5 mL of water were used. Afterwards, samples were filtrated through 0.22 µm cellulose acetate filters (Sorenson Bioscience Inc., Salt Lake City, UT, USA) and analyzed via HPLC-DAD-ToF-MS (1290 Infitiny II LC System with 6230 TOF LC/MS, Agilent Technologies Deutschland GmbH, Waldbronn, Germany) as reported previously.[20] Desulfo-GLSs were identified by comparing retention times and UV absorption spectra with those of known standards. Quantification was done by using calibration factors of reference compounds relative to the internal standard. For those compounds where no standard was available, the calibration factor reported in EN ISO 9167-1 was used.[21]

Analysis of GLS hydrolysis products using GC-MS. Extraction and quantification of GLS hydrolysis products was carried out from fresh plant material according to Hanschen, et al. [22]. Briefly, 250 mg of homogenized plant material were used for triple extraction of GLS hydrolysis products by methylene chloride in the presence of 0.2 µmol of the internal standard benzonitrile. Afterwards, the extracts were dried using anhydrous sodium sulphate and concentrated under nitrogen gas flow to 300 µL and analyzed by GC-MS (7890 A GC with 5975C Inert XL MSD,

Agilent Technologies Deutschland GmbH, Waldbronn, Germany) according to Hanschen [23] using a SGE^{TM} BPX5 column (30 m × 0.25 mm × 0.25 µm) (Trajan Scientific Europe Ltd, Victoria, Australia). Identification of GLS hydrolysis products was performed by comparing them to standards and using their retention times and mass spectra. Response factors of the standards to the internal standard were used for their quantification of GLS hydrolysis products. For compounds, where no standard was commercially available, the response factor of the chemically most similar compound was used.

Assessment of epithiospecifier protein activity. To evaluate the effect of leaf age on the activity of ESP, the protocol described by Matusheski, et al. [24] was modified and adapted. Briefly, 50 µL of ESP extract, 350 µL of a 50 mM sodiumacetate buffer (pH = 5.5) containing 1 mM dithiothreitol and 0.2 mM of $FeSO_4$, 50 µL of 0.5 U/mL myrosinase, 10 µL of 25.5 mM L(+)-ascorbic acid and 50 µL of allyl GLS (5 mg/mL) were mixed and incubated for 1 h at room temperature. Then, hydrolysis products of allyl GLS (1-cyano-2,3-epithiopropane (CETP), allyl ITC and allyl CN) were extracted and quantified according to the protocol described above. As samples of giant red mustard already contained allyl GLS, blanks with addition of 50 µL of water instead of allyl GLS were also analyzed. The concentrations quantified in these samples were used to correct the values of the positive controls. ESP activity was expressed as a potential to form % ETN = [CETP]/([CETP] + [allyl ITC] + [allyl CN]) * 100%.

Data analysis. All experiments were carried out in three independent repetitions unless stated otherwise. Data is shown as means ± standard deviation. Analysis of GLSs and their hydrolysis products was performed using MassHunter version 10.2 (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). To investigate differences between leaves of different age, means were compared using one-way ANOVA followed by Tukey's *post-hoc* test using TIBCO Statistica 13 (StatSoft Europe GmbH, Hamburg, Germany). A significance level of $p \leq 0.05$ was considered statistically significant.

RESULTS

Leaf age affects GLS levels and hydrolysis. To study the effect of leaf age on GLS levels and on the outcome of GLS hydrolysis, GLSs were analyzed using UHPLC-DAD-ToF-MS, GLS hydrolysis products were measured by GC-MS, and the activity of ESPs was investigated in single leaves of pak choi (L1 – L9) and giant red mustard (L1 – L8) (**Figure 2A and 2B**).

In total, eight different GLSs could be detected in pak choi. Four of them were aliphatic GLSs: 2-hydroxy-3-butenyl-GLS (2OH3But-GLS), 3-butenyl-GLS (3But-GLS), 4-pentenyl-GLS (4Pent-GLS) and 4-(methylthio)butyl-GLS (4MTB-GLS); three were indolic GLSs: indol-3-ylmethyl-GLS (I3M-GLS), 1-methoxy-3-indolylmethyl-GLS (1MOI3M-GLS), and 4-methoxy-3-indolylmethyl-GLS (4MOI3M-GLS). The benzenic GLS 2-phenylethyl-GLS (2PE-GLS) was detected in the samples, as well (**SI Table S1 & S2 and Figure S1**). The most prominent GLS was 3But-GLS, regardless of the leaf age, and its amounts ranged between 3.0 ± 1.4 µmol g^{-1} FM in L2 and 0.2 ± 0.1 µmol g^{-1} FM in L9. It was followed by the amounts of 2OH3But-GLS, 4Pent-GLS, and 4MTB-GLS. In pak choi, the highest total amounts of GLSs were found in L2 (9.1 ± 3.1 µmol g^{-1} FM). GLS levels continuously declined with increasing leaf age down to 0.5 ± 0.3 µmol g^{-1} FM in L9. Compared to L2, significantly lower GLS levels were found in L7, L8 as well as L9. (**Figure 3A**).

Regarding the formation of GLS hydrolysis products, the highest levels of CNs, ETNs, and ITCs were found in L1 of pak choi (3.9 ± 0.9 µmol g^{-1} FM). The levels of GLS hydrolysis products also showed a decrease with increasing leaf age (**Figure 3A**). The amounts of single GLS hydrolysis products are shown in **SI Table S3**.

The ratios of CN, ETN and ITC formation relative to the total amounts of GLS hydrolysis products in pak choi were calculated and are shown in **Figure 3B**. The proportion of ITCs did not change significantly depending on the leaf age. The levels of CNs increased proportionally with increasing

leaf age: from 16.9% in L1 to 41.6% of CNs in L9. The oldest leaf (L9) showed also a reduced relative ETN formation (**Figure 3B**).

To test if the changes in relative ETN formation in older leaves is linked to ESP activity, which is responsible for ETN formation but also enhances release of CNs, ESP activity was assessed as the potential to form 1-cyano-2,3-epithiopropane (CETP) from a defined amount of allyl GLS (**Figure 3C**). The highest formation of CETP in pak choi was detected in L1 (21.0 ± 7.4%). It decreased gradually with increasing leaf age down to 2.2 ± 0.5% in L9.

In giant red mustard, three GLSs were detected and quantified. Allyl GLS and 3But-GLS as aliphatic GLSs and the benzenic, 2PE-GLS, were measured in the samples (**SI Table S4, Figure S3**). Allyl GLS was the most prominent GLS with up to 10.5 ± 0.9 µmol g^{-1} FM in L2. The highest amounts of GLSs within the plants of giant red mustard were found in L2 (11.7 ± 1.0 µmol g^{-1} FM). The amounts of GLSs decreased significantly with increasing leaf age until L5. L5 to L8 contained the lowest GLS levels ranging from 2.5 - 0.4 µmol g^{-1} FM (**Figure 4A**).

With regard to GLS hydrolysis products, giant red mustard released predominantly ITCs, among them mainly allyl ITC (**Figure 4**). L1 - L3 showed the highest ITC levels ranging from 6.8 to 5.0 µmol g^{-1} FM which decreased with increasing leaf age till only 0.3 ± 0.1 µmol g^{-1} FM in L8 (**Figure 4A**). Similarly, the highest amounts of ETNs were found in L1 (2.8 ± 1.2 µmol g^{-1} FM) and much less in older leaves. Only very little (0.07 ± 0.02 µmol g^{-1} FM in L1) to no CNs (L6 - L8) were detected in giant red mustard and the amounts did not differ significantly between the leaves of different age. The relative formation of CNs, ETNs and ITCs in single leaves of giant red mustard is displayed in **Figure 4B**. The formation of ITCs was lowest in L1 (69.7 ± 15.5%) and significantly higher in L3 - L8, ranging from 94.2 (L3) to 98.5% (L8). The relative formation of ETNs behaved the other way round: the youngest leaves, L1 and L2, formed the highest ETN ratios (29.5 ± 15.2 and 15.4 ± 10.5% respectively), while in leaves L3 – L8 the relative ETN formation was

significantly lower and ranged from 5.5 to 1.5%. The ESP assay showed overall very low activity in giant red mustard differing from 1.6% in L1 to 1.0% in L7 and the data did not differ significantly (**SI Table S5**).

Taken together, we found leaf age-dependent differences in GLS amounts as well as in outcome of GLS hydrolysis in both species. Young leaves contained more GLSs and higher absolute amounts of health-promoting ITCs were formed upon GLS hydrolysis in young leaves. However, during GLS hydrolysis in giant red mustard, proportionally more ITCs are formed in old leaves.

Effect of hydrolysis duration on myrosinase activity and enzymatic hydrolysis of allyl GLS in giant red mustard. In some cases, a discrepancy between the amounts of GLSs and their hydrolysis products in the young leaves was found. Especially in L2, GLS levels were higher than the amounts of the analyzed GLS hydrolysis products. To investigate, whether the levels of myrosinases in young leaves are sufficient to hydrolyze the high amounts of the present GLSs, a time course experiment of hydrolysis of allyl GLS was conducted (**Figure 5**) in giant red mustard. First, the content of allyl GLS was analyzed by UHPLC-DAD-ToF-MS in young leaves (YL = L1 - L3), before homogenization and in YL homogenate 1, 5, 15, 30, and 60 min after homogenization.

Around 63% of the large amount of allyl GLS in YL (8.2 ± 0.5 µmol g^{-1} FM) was hydrolyzed within the first minute after the homogenization. After 5 min, only 8% of the initial allyl GLS amount was not yet hydrolyzed (**Figure 5**, green bars). Furthermore, in an YL homogenate enriched with 5 µmol g^{-1} FM of allyl GLS (**Figure 5**, light blue bars), allyl GLS was also sufficiently hydrolyzed after 5 min. Myrosinase has retained its hydrolyzing activity even in an experiment, where allyl GLS was added to an YL homogenate 120 min after the homogenization (**Figure 5**, dark blue bars). 30 min after spiking of the homogenate, allyl GLS was almost completely hydrolyzed.

During the enzymatic hydrolysis of allyl GLS in giant red mustard, predominantly allyl ITC is formed. The increased levels of allyl ITC in the samples seem not to affect the hydrolyzing activity of the present myrosinases. Furthermore, the enrichment of YL homogenate with allyl ITC right after homogenization did not affect the hydrolysis of allyl GLS (**Figure 5**, orange bars).

Additionally, the effect of allyl GLS on myrosinase activity was investigated in a homogenate of the old leaves (OL) of giant red mustard (**Figure 5**, yellow). In OL, only 1.5 ± 0.6 µmol g^{-1} FM of allyl GLS was found. We assumed also lower abundance of myrosinases in OL and therefore a possible inhibitory effect of elevated levels of allyl ITC on the hydrolyzing activity of myrosinases. Nevertheless, allyl GLS was almost completely hydrolyzed within the first minute after the homogenization and enrichment of the OL homogenate with allyl GLS.

More than 90% of the high amounts of allyl GLS degrade within a few minutes of enzymatic hydrolysis after the cell damage in giant red mustard. The activity of myrosinases remained high during the time course and it was not affected by elevated concentrations of allyl ITC in the homogenate.

Glucosinolate transport and accumulation within plant. As the distribution of GLSs varies strongly within the plants of both species, a possible transport of GLSs from old to younger leaves was investigated in pak choi. Transport and accumulation of artificially infused allyl GLS (into L6), which is naturally not present in pak choi, were studied (**Figure 6**). Distribution of allyl GLS within the plant was monitored for up to 72 h after the infusion into a shaded or a non-shaded leaf (L6). Control plants were infused with water. Naturally occurring GLSs (**Figure 6A**) and allyl GLS (**Figure 6B**) were quantified in single leaves 24, 48, and 72 h after the infusion. As the distribution of allyl GLS of the individual plants differed slightly depending on total number of leaves, leaves L1 – L3 were grouped together into young leaves (YL) (**Figure 6**, green bars) and L4 - L8 into old leaves (OL) (**Figure 6**, yellow bars).

Regardless of whether the infused L6 was shaded or not, 24 h after the infusion of allyl GLS or water, natural GLSs were equally distributed between YL and OL of pak choi (**Figure 6A**). However, after 48 h significantly higher amounts of GLSs were found in OL, when allyl GLS was infused or when water was infused in a non-shaded L6. GLSs were again evenly distributed between YL and OL 72 h after the infusion of allyl GLS. When the infusion was inserted into a non-shaded L6, GLS amounts of YL were increased in plants which received allyl GLS 72 h before the harvest compared to controls 48 h after the infusion of water (**Figure 6A**). GLS levels of OL were significantly increased 48 h after infusing allyl GLS into a non-shaded L6 compared to 24 h after the infusion. Infusion into a shaded L6 did not affect the GLS amounts in YL as well as OL between the different sampling time points. No significant differences were found in the amounts of naturally occurring GLSs between YL of the not shaded and shaded plants at each sampling time point. With regard to the shading of L6, amounts of natural GLSs also did not differ in OL at each time point.

Twenty-four hours after infusion, allyl GLS was found exclusively in YL, regardless of whether L6 was shaded or not (**Figure 6B**). After 48 and 72 h, allyl GLS was found in both, YL and OL of pak choi. When allyl GLS was applied into a non-shaded L6, it was distributed equally between YL and OL 48 h after the application. After 72 h, we found significantly more allyl GLS in OL. In contrast, when allyl GLS was infused into a shaded L6, 48 h after the infusion the amount of allyl GLS was significantly higher in OL compared to YL and after 72 h allyl GLS was equally distributed between YL and OL (**Figure 6B**).

In this experiment, GLS transport out of the site of biosynthesis (OL) to YL was confirmed. Naturally not occurring GLS in pak choi (allyl GLS) was exclusively found in YL 24 h after it was infused into an old leaf (L6). However, later (48 and 72 h after the infusion), allyl GLS was distributed between YL and OL. (**Figure 6C**)

DISCUSSION

In order to study the effect of leaf age on GLS accumulation and hydrolysis in leafy *Brassica* vegetables, contents of GLSs and their hydrolysis products were analyzed in single leaves of different age in pak choi and giant red mustard. The GLS profiles of both species reflected the profiles found in previous studies. Similarly to Heinze, et al. [25], pak choi produced predominantely aliphatic GLSs, namely 3But-GLS, 2OH3But-GLS, 4MTB-GLS, and 4Pent-GLS, while allyl GLS represented more than 90% of total GLS content in giant red mustard, as described earlier.[26] In both species, the highest total GLS content was found in L2 and it continuously decreased with increasing leaf age (**Figure 3A & 4A**). This finding is in agreement with studies on *A. thaliana* or headed cabbage showing tissue specificity of GLS distribution.[13-15] For example, accumulation of aliphatic as well as indolic GLSs increased toward the inner core of cabbage.[14] Moreover, three times higher GLS levels were found in young leaves of *A. thaliana* compared to mature or old leaves.[15] The fact, that older leaves have lower GLS contents than younger leaves is in angreement with the optimal defense theory. As GLSs are crucial for plant defense in Brassicaceae, their concentrations vary across the plant and the most valuable parts are protected the most.[11] Brown, et al. [13] showed that this is not due to decreasing GLS concentrations in individual leaves with age, but rather, leaves initiated earlier had lower rates of GLS accumulation throughout their lifetimes.

A possible strategy to enhance the protection of the most valuable parts of Brassicaceae against pathogens and pests could be a shift in GLS hydrolysis towards increased formation of ITCs, which can act antimicrobial and antiinsecticide. This could also improve the nutritional value of our daily diet, as the health-beneficial effect of *Brassica* vegetables is also attributed to ITCs. Here, upon enzymatic degradation of GLSs, total amounts of GLS hydrolysis products found in both species strongly decreased with increasing leaf age (**Figure 3A & 4A**). Pak choi mainly released ETNs (**Figure 3B**), while giant red mustard released particularly ITCs and low levels of

ETNs (**Figure 4B**). This was also reflected in the ESP activity, which was much higher in pak choi compared to giant red mustard.

In both vegetables, the relative ETN formation decreased with increasing leaf age and in pak choi ESP activity also declined significantly. Likely, in young leaves, the ESP activity must be higher due to the higher GLS amounts in young leaves to reach more than 90% of ETN formation in pak choi. Williams, et al. [27] demonstrated that ESP activity reflects GLS levels in broccoli seedlings of different age. In two day-old seedlings GLS amounts as well as ESP activity showed a maximum and both rapidly decreased in further development. Here, amounts of alkenyl GLSs with terminal unsaturated bond correlated with ESP activity in pak choi (**SI Figure S2**). In giant red mustard, a leaf age-dependent ITC formation could be observed: while the absolute amounts of ITCs were higher in young leaves, old leaves formed proportionally more ITCs than young leaves (**Figure 4A & 4B**). However, no change in ESP activity was detectable, likely due to the overall little ESP activity in this vegetable (**SI Table S5**). Even with no change in ESP activity, the outcome of enzymatic GLS hydrolysis could be affected in older leaves, as with lower GLS concentration the possibility of the ESP protein to degrade the aglucon declines so the possibility for spontaneous breakdown to ITCs will increase. Similarly, it was shown that dilution can increase ITC formation.[22] However, in pak choi the decrease in ESP activity with increased leaf age did not significantly increase the ITC ratio but relative CN formation increased correspondingly. Recently it has been reported that alkenyl ITCs such as allyl ITC or 3But-ITC can be quickly converted to amines in *Brassica* plant tissues.[28] So it is likely that here part of the ITC levels degraded to amines as well. With lower ITC levels released in older leaves such enzyme-like conversion to amines will increase [28], so relative analyzed ITC formation could be unaffected although more ITC are formed.

Further, especially in L2 GLS levels were higher than the amounts of the analyzed GLS hydrolysis products and the recovery rate was only about 40%. As GLS contents were highest in these leaves, it was assumed that the present myrosinase activity might not have been sufficient to fully

hydrolyze the given GLS amounts within 30 min. Previous studies showed that myrosinase activity can vary with plant species[22], organ[29], or stage of development[27]. Furthermore, L-ascorbate as a cofactor, affects myrosinase activity and therefore might be affecting hydrolysis outcome in leaves of different age as well.[30] Hence, GLS hydrolysis kinetic of allyl GLS in young and old leaves of giant red mustard was investigated. As more than 90% of the high amounts of allyl GLS was degraded within few minutes in both leaf groups and even after two hours myrosinase activity was maintained, a low enzyme activity can be excluded as a reason for low recovery. The low recovery rates in young leaves point to an elevated level of the ITC hydrolase-like factor that yields amines in *Brassica* tissues as additional products of enzymatic GLS hydrolysis.[28] Contrarily, for L1 relatively low GLS levels but the hightest levels of GLS hydrolysis products were found. As Verkerk and Dekker [31] reported an increase in GLS levels upon heating and myrosinase activity inhibition by up to 240%, there is the possibility that during shock-freezing of plant samples in liquid nitrogen, some of the GLSs still could be degraded by myrosinase and this effect might have been stronger in L1 which is suspected to have the highest myrosinase activity of all leaves.

In order to study the distribution of GLSs within a plant in more detail, the possibility of GLS transport from the old into the young leaves was investigated as already reported in *A. thaliana*.[15] Therefore, distribution of artificially infused allyl GLS into a shaded or non-shaded old leaf, was monitored for up to 72 h after the infusion in pak choi. 24 h after the infusion of allyl GLS into an old leaf, allyl GLS was detected only in young leaves, regardeless of whether the leaf was shaded or not, thereby clearly confirming active GLS transport within the plant (**Figure 6**). Only after 48 h and 72 h allyl GLS was distributed across both, young and old leaves. Similarly, the distribution of native GLSs varied between the different sampling time points. 24 h after the infusion, native GLSs were distributed equaly between young and old leaves, while 48 h – 72 h after the infusion, more GLSs were found in old leaves. Possibly, plants recognized a pierced leaf as a site of

potential attack and therefore native GLS as well as allyl GLS were transported rather to the old leaves. In this context, Hunziker, et al. [15] tested whether risk to plant tissue would be highest when other tissues of the same plant are also under attack. After herbivory, accumulation of several GLSs was demonstrated to increase in the unattacked leaves, which correlated with increased local expression of biosynthesis as well as transporter genes.[15]

In this work, GLS accumulation in young leaves of two leafy *Brassica* vegetables, namly pak choi and giant red mustard, was demonstrated, which also released the highest levels of ITC and other hydrolysis products. A shift of GLS hydrolysis towards enhanced relative ITC formation was detected in old leaves of giant red mustard while in pak choi a decline in ESP activity with older leaves was shown that did not increase relative ITC formation. Myrosinase was shown to sufficiently hydrolyze the given amounts of GLSs within few minutes in each leaf regardeless of the leaf age. Further, GLS transport within the plant as well as from old leaves, as a site of GLS biosynthesis, to young leaves, as the most valuable part of the plant, was confirmed. Our findings deepen the understanding of GLS distribution and hydrolysis within *Brassica* vegetables and the results can contribute to the aim to increase the nutritional value of our food. As in both species, young leaves contained the highest GLS amounts, more health-promoting ITCs might be released during the consumption of leafy *Brassica* vegetables.

ABBREVIATIONS

GLS(s), glucosinolate(s); 2OH3But-GLS, 2-hydroxy-3-butenyl-GLS; 3But-GLS, 3-butenyl-GLS; 4Pent-GLS, 4-pentenyl-GLS; 4MTB-GLS, 4-(methylthio)butyl-GLS; I3M-GLS, 3-indolylmethyl-GLS; 1MOI3M-GLS, 1-methoxy-3-indolylmethyl-GLS; 4MOI3M-GLS, 4-methoxy-3-indolylmethyl-GLS; 2PE-GLS, 2-phenylethyl-GLS; ITC(s), isothiocyanate(s); CN(s), nitrile(s); ETN(s), epithionitrile(s); CETP, 1-cyano-2,3-epithiopropane; ESP(s); epithiospecifier protein(s); L, leaf; YL, young leaves; OL, old leaves; NS, non-shaded; S, shaded.

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REFERENCES

1. Kapusta-Duch, J.; Kopec, A.; Piatkowska, E.; Borczak, B.; Leszczynska, T., The beneficial effects of *Brassica* vegetables on human health. *Rocz. Panstw. Zakl. Hig.* **2012,** *63* (4), 389-95.

2. Blazevic, I.; Montaut, S.; Burcul, F.; Olsen, C. E.; Burow, M.; Rollin, P.; Agerbirk, N., Glucosinolate structural diversity, identification, chemical synthesis and metabolism in plants. *Phytochemistry* **2020,** *169*, 112100.

3. Wittstock, U.; Burow, M., Glucosinolate breakdown in *Arabidopsis*: mechanism, regulation and biological significance. *Arabidopsis Book* **2010,** *8*, e0134.

4. Witzel, K.; Abu Risha, M.; Albers, P.; Bornke, F.; Hanschen, F. S., Identification and characterization of three epithiospecifier protein isoforms in *Brassica oleracea*. *Front. Plant. Sci.* **2019,** *10*, 1552.

5. Tookey, H. L., Crambe thioglucoside glucohydrolase (EC 3.2.3.1): separation of a protein required for epithiobutane formation. *Can. J. Biochem.* **1973,** *51* (12), 1654-60.

6. Mocniak, L. E.; Elkin, K.; Bollinger, J. M., Lifetimes of the aglycone substrates of specifier proteins, the autonomous iron enzymes that dictate the products of the glucosinolate-myrosinase defense system in *Brassica* plants. *Biochemistry-Us* **2020,** *59* (26), 2432-2441.

7. Jeschke, V.; Gershenzon, J.; Vassao, D. G., Insect detoxification of glucosinolates and their hydrolysis products. *Adv. Bot. Res.* **2016,** *80*, 199-245.

8. Sturm, C.; Wagner, A. E., *Brassica*-derived plant bioactives as modulators of chemopreventive and inflammatory signaling pathways. *Int. J. Mol. Sci.* **2017,** *18* (9).

9. Palliyaguru, D. L.; Yuan, J. M.; Kensler, T. W.; Fahey, J. W., Isothiocyanates: translating the power of plants to people. *Mol. Nutr. Food Res.* **2018,** *62* (18), e1700965.

10. Biere, A.; Marak, H. B.; van Damme, J. M., Plant chemical defense against herbivores and pathogens: generalized defense or trade-offs? *Oecologia* **2004,** *140* (3), 430-41.

11. Touw, A. J.; Verdecia Mogena, A.; Maedicke, A.; Sontowski, R.; van Dam, N. M.; Tsunoda, T., Both biosynthesis and transport are involved in glucosinolate accumulation during root-herbivory in *Brassica rapa*. *Front. Plant. Sci.* **2019,** *10*, 1653.

12. Badenes-Perez, F. R.; Gershenzon, J.; Heckel, D. G., Insect attraction versus plant defense: young leaves high in glucosinolates stimulate oviposition by a specialist herbivore despite poor larval survival due to high saponin content. *PLoS One* **2014,** *9* (4), e95766.

13. Brown, P. D.; Tokuhisa, J. G.; Reichelt, M.; Gershenzon, J., Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry* **2003,** *62* (3), 471-81.

14. Zhao, Y.; Yue, Z.; Zhong, X.; Lei, J.; Tao, P.; Li, B., Distribution of primary and secondary metabolites among the leaf layers of headed cabbage (*Brassica oleracea* var. *capitata*). *Food Chem.* **2020,** *312*, 126028.

15. Hunziker, P.; Lambertz, S. K.; Weber, K.; Crocoll, C.; Halkier, B. A.; Schulz, A., Herbivore feeding preference corroborates optimal defense theory for specialized metabolites within plants. *Proc. Natl. Acad. Sci. U S A* **2021,** *118* (47).

16. Nour-Eldin, H. H.; Andersen, T. G.; Burow, M.; Madsen, S. R.; Jorgensen, M. E.; Olsen, C. E.; Dreyer, I.; Hedrich, R.; Geiger, D.; Halkier, B. A., NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. *Nature* **2012,** *488* (7412), 531-4.

17. Jeschke, V.; Kearney, E. E.; Schramm, K.; Kunert, G.; Shekhov, A.; Gershenzon, J.; Vassao, D. G., How glucosinolates affect generalist Lepidopteran larvae: growth, development and glucosinolate metabolism. *Front. Plant. Sci.* **2017,** *8*, 1995.

18. Singh, S.; Diksha; Mahajan, E.; Sohal, S. K., Appraisal of growth inhibitory, biochemical and genotoxic effects of allyl Isothiocyanate on different developmental stages of *Zeugodacus cucurbitae* (Coquillett) (Diptera: Tephritidae). *Sci. Rep.* **2022,** *12* (1), 10363.

19. Rade-Kukic, K.; Schmitt, C.; Rawel, H. M., Formation of conjugates between beta-lactoglobulin and allyl isothiocyanate: effect on protein heat aggregation, foaming and emulsifying properties. *Food Hydrocoll.* **2011,** *25* (4), 694-706.

20. Renz, M.; Dekker, M.; Rohn, S.; Hanschen, F. S., Plant matrix concentration and redox status influence thermal glucosinolate stability and formation of nitriles in selected Brassica vegetable broths. *Food Chem.* **2023,** *404* (Pt A), 134594.

21. DIN Deutsches Institut für Normung e.V., EN ISO 9167-1: Rapssamen - Bestimmung des Glucosinolatgehaltes - Teil 1: HPLC-Verfahren (ISO 9167-1: 1992). **1995**.

22. Hanschen, F. S.; Klopsch, R.; Oliviero, T.; Schreiner, M.; Verkerk, R.; Dekker, M., Optimizing isothiocyanate formation during enzymatic glucosinolate breakdown by adjusting pH value, temperature and dilution in Brassica vegetables and Arabidopsis thaliana. *Sci. Rep.* **2017,** *7*, 40807.

23. Hanschen, F. S., Domestic boiling and salad preparation habits affect glucosinolate degradation in red cabbage (Brassica oleracea var. capitata f. rubra). *Food Chem.* **2020,** *321*, 126694.

24. Matusheski, N. V.; Juvik, J. A.; Jeffery, E. H., Heating decreases epithiospecifier protein activity and increases sulforaphane formation in broccoli. *Phytochemistry* **2004,** *65* (9), 1273-81.

25. Heinze, M.; Hanschen, F. S.; Wiesner-Reinhold, M.; Baldermann, S.; Grafe, J.; Schreiner, M.; Neugart, S., Effects of developmental stages and reduced UVB and low UV conditions on

plant secondary metabolite profiles in pak choi (*Brassica rapa* subsp. *chinensis*). *J. Agric. Food Chem.* **2018,** *66* (7), 1678-1692.

26. Tian, Y.; Deng, F. M., Phytochemistry and biological activity of mustard (*Brassica juncea*): a review. *Cyta-J. Food* **2020,** *18* (1), 704-718.

27. Williams, D. J.; Critchley, C.; Pun, S.; Nottingham, S.; O'Hare, T. J., Epithiospecifier protein activity in broccoli: the link between terminal alkenyl glucosinolates and sulphoraphane nitrile. *Phytochemistry* **2008,** *69* (16), 2765-73.

28. Andernach, L.; Witzel, K.; Hanschen, F. S., Glucosinolate-derived amine formation in *Brassica oleracea* vegetables. *Food Chem.* **2023,** *405* (Pt B), 134907.

29. Phelan, J. R.; Allen, A.; Vaughan, J. G., Myrosinase in *Raphanus sativus* L. *J. Exp. Bot.* **1984,** *35* (159), 1558-1564.

30. Burow, M.; Markert, J.; Gershenzon, J.; Wittstock, U., Comparative biochemical characterization of nitrile-forming proteins from plants and insects that alter myrosinase-catalysed hydrolysis of glucosinolates. *Febs. J.* **2006,** *273* (11), 2432-2446.

31. Verkerk, R.; Dekker, M., Glucosinolates and myrosinase activity in red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra* DC.) after various microwave treatments. *J. Agric. Food. Chem.* **2004,** *52* (24), 7318-23.

FIGURES

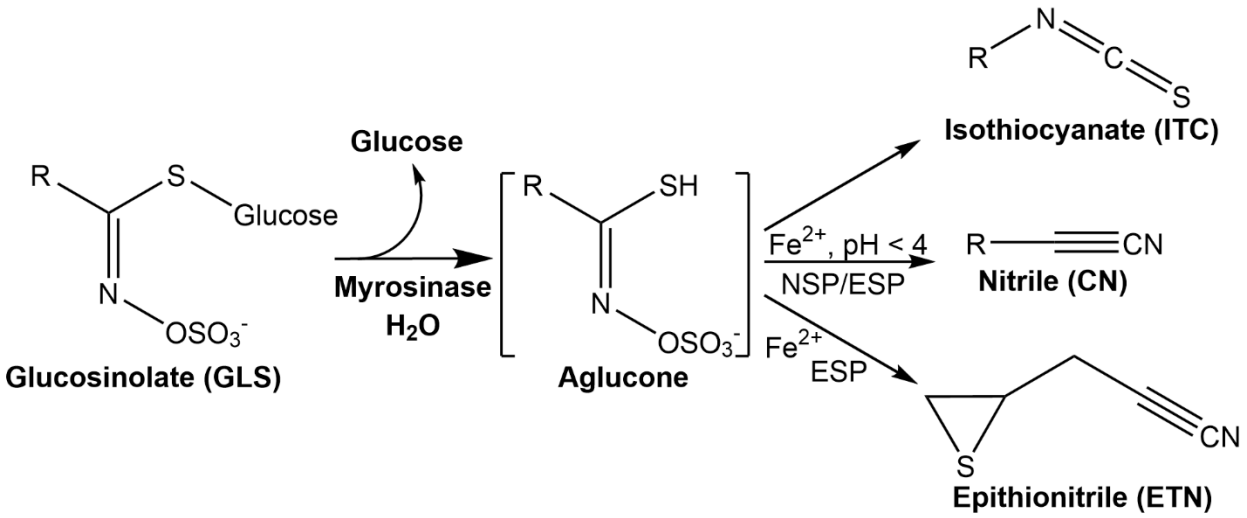
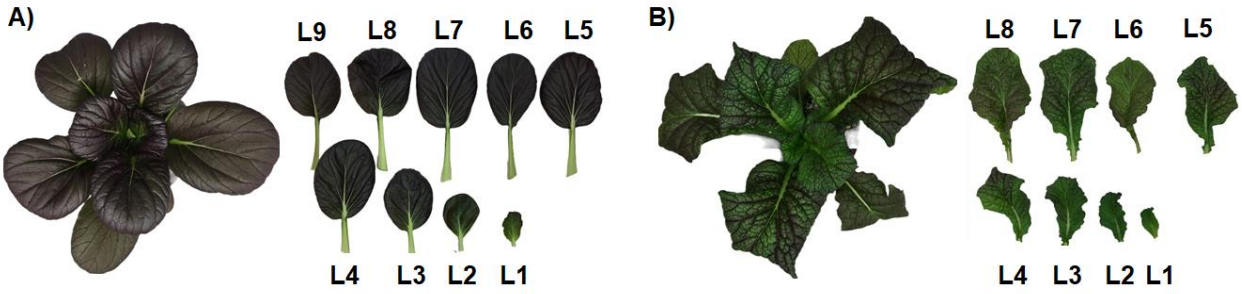


Figure 1 Core structure of glucosinolates (GLSs) and enzymatic degradation of GLSs. Once GLSs and myrosinases come in contact, an unstable aglucone is formed, quickly reacting to isotiocynaates (ITCs), nitriles (CNs) and epithionitriles (ETNs) depending on pH, presence of ions and specifier proteins- nitrilespecifier protein (NSP) or epithiospecifier protein (ESP). Only GLSs with terminal double bond in the side chain can form ETNs in presence of ESPs.



546 **Figure 2 A)** Pak choi (*Brassica rapa* ssp. *chinensis* cv. Arax). During the harvest single leaves
547 were ordered and numbered according to their age beginning with L1 (the youngest leaf) and
548 ending with L9 (the oldest leaf). **B)** Giant red mustard (*Brassica juncea* supbsp. *rugosa* cv. Red
549 giant). During the harvest single leaves were ordered according to their age from L1 (the youngest
550 leaf) to L8 (the oldest leaf).

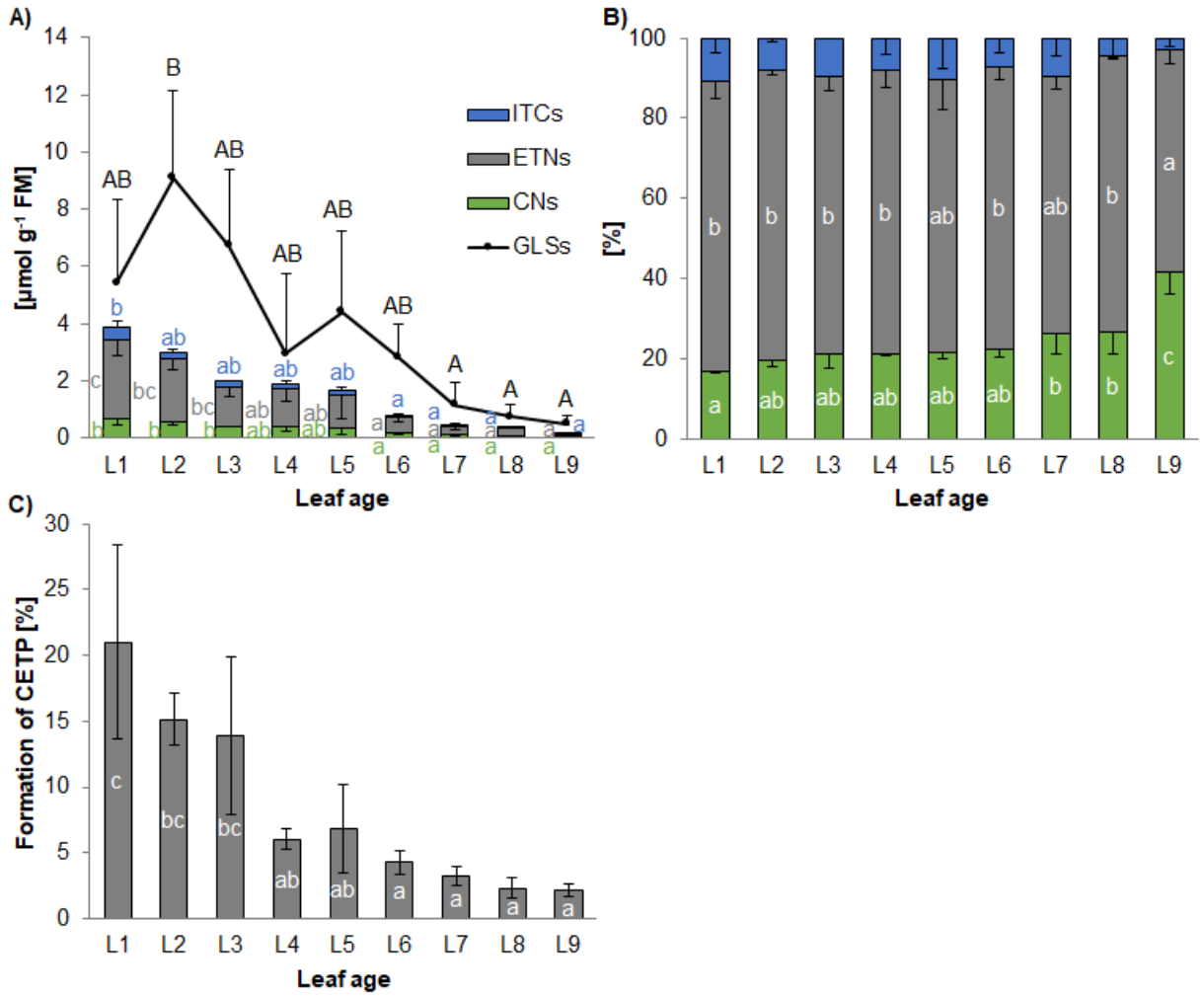


Figure 3 Effect of leaf age on total glucosinolate (GLS) level and GLS hydrolysis in pak choi. **A)** Total amounts of GLSs and their hydrolysis products- nitriles (CNs), epithionitriles (ETNs), and isothiocyanates (ITCs)- in leaves of different age (L1- the youngest leaf, L9- the oldest leaf) [µmol g^{-1} fresh matter]. Different capital letters indicate significant differences in total GLS amounts. Different small letters indicate significant differences in the amounts of ITCs, ETNs, and CNs between leaves of different age (ANOVA, Tukey HSD test, $p \leq 0.05$). **B)** Proportion of CNs, ETNs, and ITCs among total GLS hydrolysis products [%] depending on the leaf age. Different letters indicate significant differences in amounts of ETNs and CNs in leaves of different age. No siginificant differences found in the amounts of ITCs (ANOVA, Tukey HSD test, $p \leq 0.05$). **C)** Activity of the epithiospecifier proteins (ESPs) as a potential to form 1-cyano-2,3-epithiopropane

(CETP) [%] depending on the leaf age in an assay with ESP plant extract and a defined amount of allyl GLS. Different letters indicate significant differences in ESP activity between leaves of different age (ANOVA, Tukey HSD test, $p \leq 0.05$). Values show means and standard deviations of three independent experiments ($n = 3$).

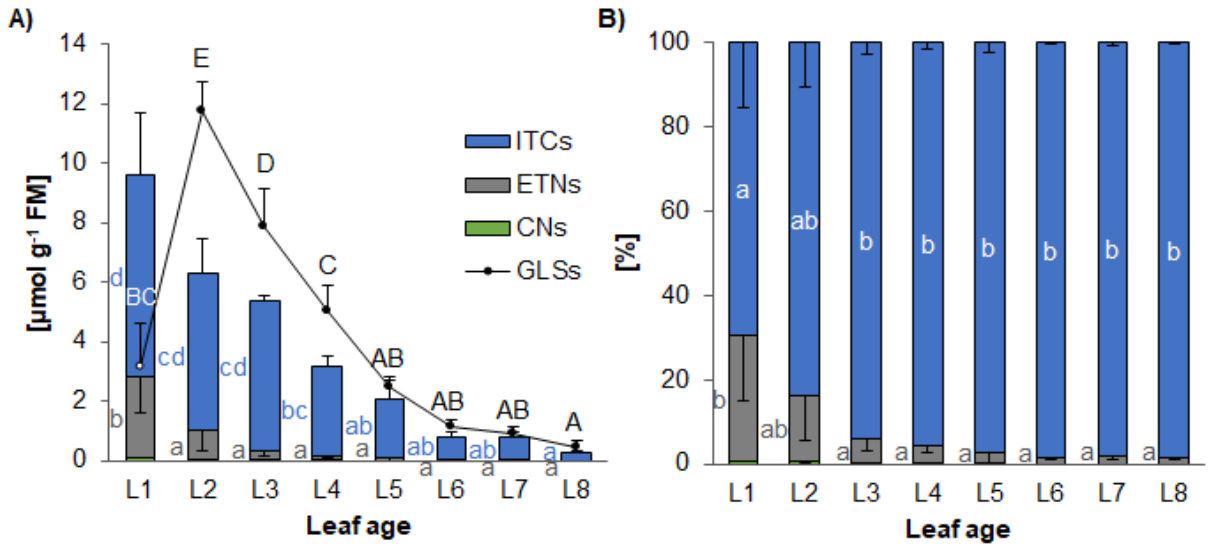
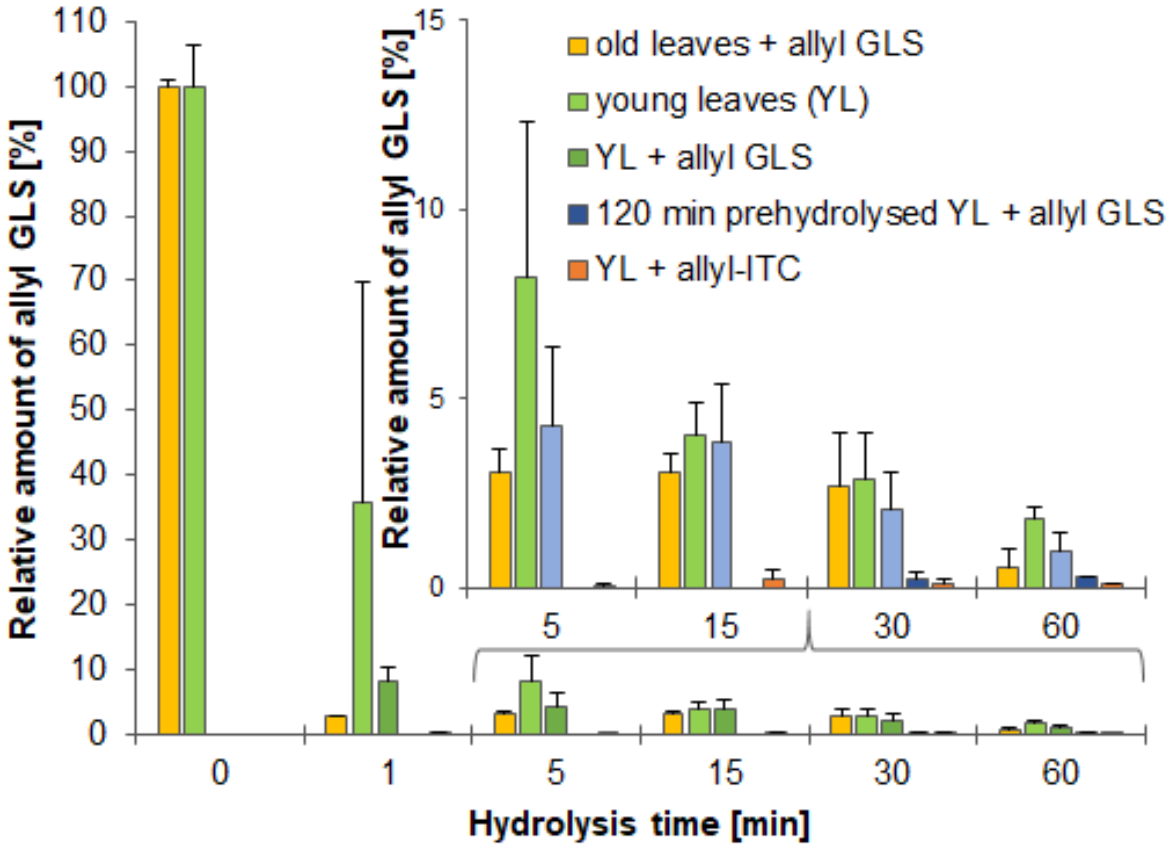
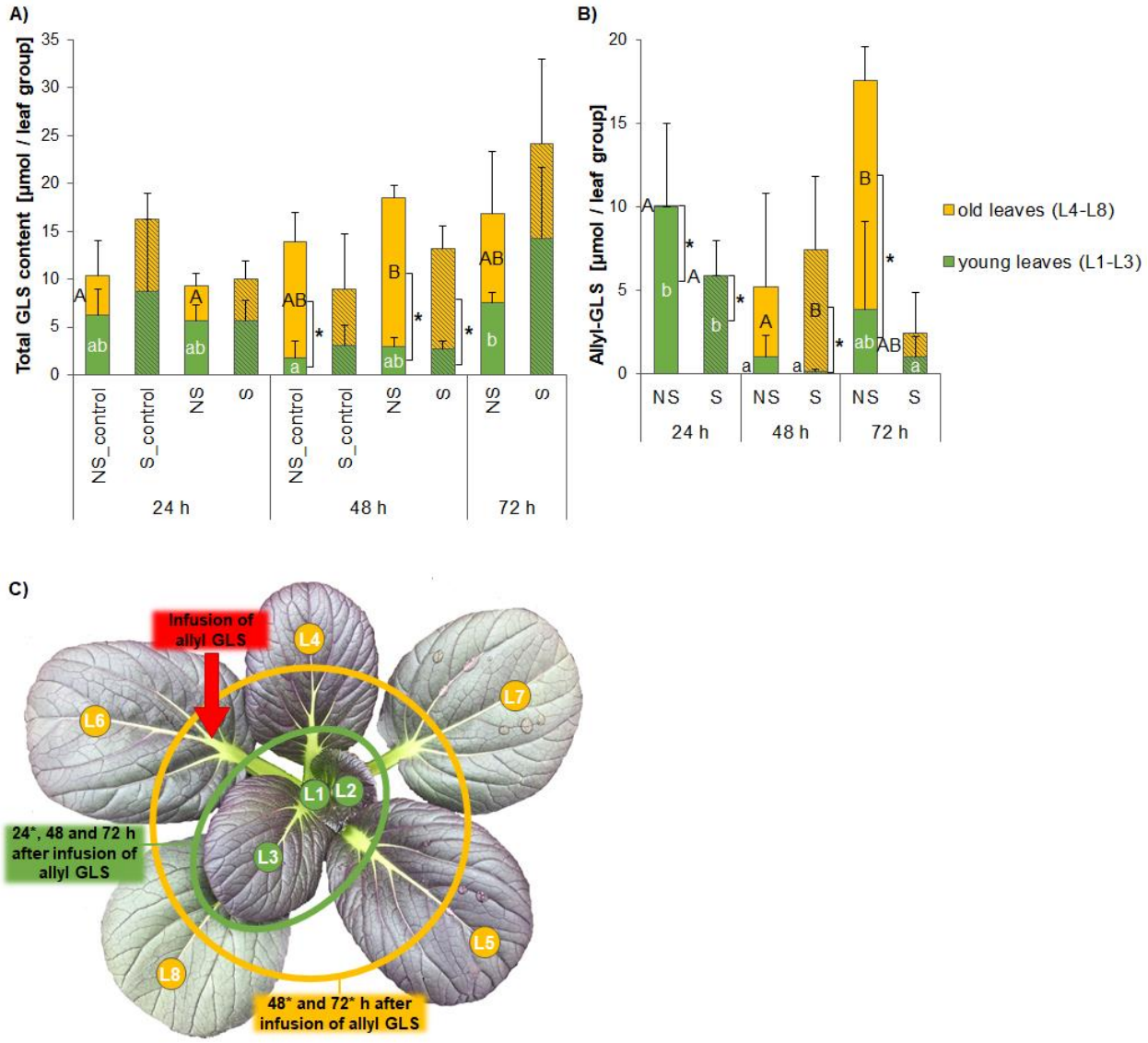


Figure 4 Effect of leaf age on total glucosinolate (GLS) level and GLS hydrolysis in giant red mustard. **A)** Total amounts of GLSs and their hydrolysis products- nitriles (CNs), epithionitriles, (ETNs), and isothiocyanates (ITCs)- in leaves of different age (L1- the youngest leaf, L8- the oldest leaf) [µmol g^{-1} fresh matter]. Different capital letters indicate significant differences in total GLS amounts. Different small letters indicate significant differences in the amounts of ITCs and ETNs between leaves of different age. No significant differences found in the amounts of CNs (ANOVA, Tukey HSD test, $p \leq 0.05$). **B)** Proportion of CNs, ETNs, and ITCs among total GLS hydrolysis products [%] depending on the leaf age in leafy mustard. Different capital letters indicate significant differences in amounts of ITCs and ETNs between leaves of different age. No significant differences found in the proportion of CNs (ANOVA, Tukey HSD test, $p \leq 0.05$). Values show means and standard deviations of three independent experiments (n = 3).



576 **Figure 5** Effect of hydrolysis duration on the enzymatic hydrolysis of allyl glucosinolate (allyl GLS)
577 in giant red mustard. Amounts of allyl GLS [%] relative to amounts of allyl GLS present at time
578 point 0 min. If the samples were enriched with allyl GLS, 100% means sum of the native allyl GLS
579 and the spiked allyl GLS. Yellow = old leaves (L8) homogenate enriched with 5 µmol g^{-1} FM of
580 allyl GLS right after homogenization. Green = young leaves (YL; L1 – L3) homogenate.
581 Light blue = YL homogenate enriched with 5 µmol g^{-1} FM of allyl GLS right after homogenization.
582 Dark blue = YL homogenate enriched with 5 µmol g^{-1} FM of allyl GLS after 120 min of enzymatic
583 hydrolysis. Orange = YL homogenate enriched with allyl ITC right after homogenization. Values
584 show means and standard deviations of two independent experiments (n = 2).



585 **Figure 6** Distribution of glucosinolates (GLSs) and artificially infused allyl GLS between young
586 (L1 - L3) and old leaves (L4- L8) of pak choi after infusing into a shaded or non-shaded leaf. **A)**
587 Total natural GLS content in young (green) and old (yellow) leaves [µmol / leaf group] after
588 infusion with or without allyl GLS. **B)** Distribution of allyl GLS [µmol / leaf group] in young and old
589 leaves 24, 48, and 72 h after infusion with 20 µmol of artificial allyl GLS into a non-shaded (NS)
590 or shaded (S) old leaf (leaf number 6; L6). Asterisks in Figure 5A and B indicate significant
591 differences between total GLSs or allyl GLS of the young and old leaf groups. Different letters
592 indicate significant differences in total GLS or allyl GLS amounts found in young (small letters) or
593 old leaves (capital letters) between the different sampling time points as tested for shaded and

not shaded group separately. Values show means and standard deviations of three independent experiments (n = 3). **C)** Illustration of distribution of allyl GLS between young (green) and old (yellow) leaves 24, 48, and 72 h after infusion with allyl GLS. Asterisks indicate significantly higher amount of allyl GLS in one of the leaf groups at one of the sampling time-points.

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