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Age-specific allocation of glucosinolates within plant reproductive tissues

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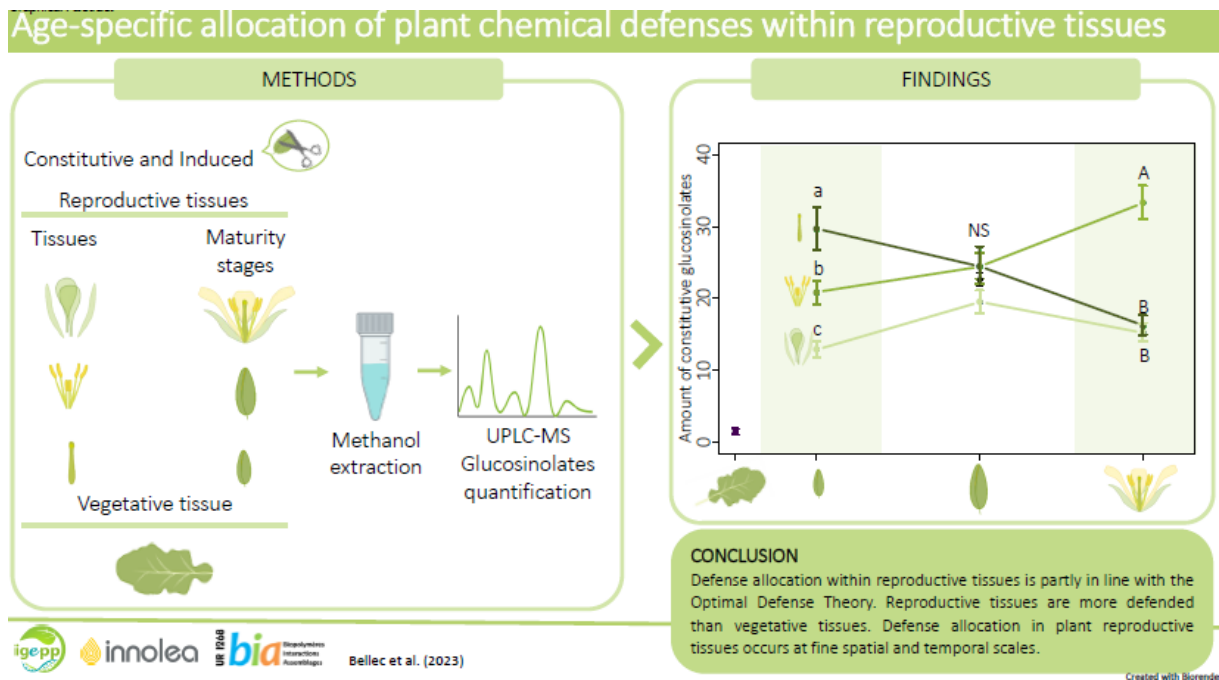
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Abstract

The Optimal Defense Theory (ODT) predicts that the distribution of defenses within a plant should mirror the value and vulnerability of each tissue. Although the ODT has received much experimental support, very few studies have examined defense allocation among reproductive tissues and none assessed simultaneously how these defenses evolve with age. We quantified glucosinolates in perianths, anthers and pistils at different bud maturity stages (i.e., intermediate flower buds, old flower buds and flowers) of undamaged and mechanically damaged plants of an annual brassicaceous species. The youngest leaf was used as a reference for vegetative organs, since it is predicted to be one of the most defended. In line with ODT predictions, reproductive tissues were more defended than vegetative tissues constitutively, and within the former, pistils and anthers more defended than perianths. No change in the overall defense level was found between bud maturity stages, but a significant temporal shift was observed between pistils and anthers. Contrary to ODT predictions, mechanical damage did not induce systemic defenses in leaves but only in pistils. Our results show that defense allocation in plant reproductive tissues occurs at fine spatial and temporal scales, extending the application framework of the ODT. They also demonstrate interactions between space and time in fine-scale defense allocation.

Graphical abstract



Keywords: constitutive defenses, glucosinolates, induced defenses, maturity stages, Optimal Defense Theory, phytochemistry, reproductive tissues

1. Introduction

Plants have evolved sophisticated defense strategies to cope with herbivores, including chemical defenses that can be constitutive, i.e., present in the absence of attack, or induced, i.e. overexpressed after damage or upon risk of damage [1–7]. A substantial body of literature has shown that these chemical defenses are not uniformly distributed across the different plant tissues [8–11]. Over the past six decades several theories have been put forth to explain this heterogeneous spatial and temporal allocation of plant defenses (reviewed in Schuman & Baldwin, 2016 [12]), one of the most influential being the optimal defense theory (ODT). Based on the assumption that the production of chemical defenses is costly and therefore limited, the ODT predicts that the distribution of defenses within the plant should mirror the fitness value of the tissues and their probability of being attacked [13–15]. In short-lived plant species, constitutive defenses should be allocated to the most valuable and vulnerable structures (e.g., primary roots, young leaves, flower buds, flowers and fruits) while induced defenses should be allocated to structures of low value or probability of attack (e.g., secondary roots, fully expanded leaves) [16,17]. These predictions have received considerable

experimental support, with young leaves being generally more defended than older ones [18–20], primary roots being more defended than secondary ones [21,22], and stems being more defended than other vegetative organs [23]. Since reproductive organs are closely tied to the reproductive success of plants [17,24] and constitute high-quality nutritive targets for herbivores [25–27], it is consistent with the ODT that they also are generally more defended than vegetative organs (reviewed in Kessler & Halitschke, 2009 [28]). To date, most studies performed on reproductive organs have been performed on entire, open flowers. However, the reproductive tissues (i.e., perianths, anthers and pistils) that constitute the flower can have specific values and vulnerabilities [29–33], which can evolve with age [25]. The ODT can therefore be applied at such a fine scale, but this area of research is still in its infancy.

Defense allocation in reproductive organs is likely shaped by conflicting selective pressures imposed by pollen foragers. Indeed, plants should both attract pollinators to maximize outcrossing success and defend the pollen from non-pollinating pollen consumers [29–33]. Based on predictions of the ODT, it can be hypothesized that since primary tissues (i.e. anthers and pistils) are directly involved in reproduction they should be more intensely defended than accessory tissues (i.e. perianths), and that among primary tissues the gamete-limited pistils should be more defended than the gamete-unlimited anthers [27]. Although the very few studies describing tissue-specific defense allocation in reproductive organs have indeed shown higher defense concentrations in primary tissues than in accessory tissues [27,34], such hypotheses are still poorly tested.

Besides spatial variations, plant defense levels can also vary over time [35–37]. Indeed, plants undergo major physiological changes during their ontogeny that can lead to significant shifts in the nature and level of defenses [25]. Such so-called ontogenetic trajectories have been well documented in vegetative organs where, in most cases, overall defenses decrease with age [38]. However, they have received much less attention for reproductive organs, with only Li et al. (2021) [39] showing some temporal changes in the defense level of flower buds in *Brassica oleracea* and Chrétien et al. (2022) [25] showing no clear evidence of temporal changes in the defense level of inflorescences in *Brassica nigra*. Thus, as for spatial variations, temporal variations in defense allocation in reproductive organs are still mostly unknown.

In this study, we tested whether predictions of the ODT apply to reproductive organs of an annual brassicaceous plant. We focused on glucosinolates (GSLs), a widely diversified group of sulphur-containing specialized metabolites found mainly in Brassicaceae [40–42]. GSLs and their degradation products (i.e., isothiocyanates, thiocyanates and nitriles) are well

studied plant defenses that mostly deter generalist herbivores and pathogens but attract and stimulate specialists [42,43]. Since the ODT predicts that the youngest leaf should be one of the most-defended vegetative organs, we used it as a reference to compare with reproductive organs. Within the latter, we examined separately accessory and primary tissues (i.e., perianths vs. pistils and anthers, respectively). We took a dynamic approach by assessing GSLs levels at three maturity stages of flowers: intermediate flower buds, old flower buds and open flowers. In addition, we compared levels of constitutive and systemically induced defenses. The following predictions for short-lived plants were tested: *Prediction 1*: vegetative organs should be less defended than reproductive ones; *Prediction 2*: within reproductive organs and all maturity stages confounded, perianths should be less defended than anthers and pistils; *Prediction 3*: among primary tissues and all maturity stages confounded, anthers should be less defended than pistils; *Prediction 4*: defense levels should be constant over maturity stages in primary tissues, while they should decrease after flower opening in perianths as these do not act as barriers anymore; *Prediction 5*: damage should induce systemic defenses only in structures of low values, i.e. leaves.

2. Material and Methods

2.1. Plant cultivation

Spring oilseed rape plants (*Brassica napus* L., ‘Liho’ genotype) were cultivated in seedling trays with 3.5 x 3.5 x 5 cm cells filled with a fertilized substrate (Premier Tech Horticulture), grown in a climatic chamber (20 ± 1 °C, 16L:8D and 70 ± 10 % RH) and watered two times a week with a fertilizer solution (N-P-K: 2.5-5-2.5). Two weeks after sowing, plants were transplanted individually into 7 x 7 x 6.5 cm plastic pots filled with the same substrate. All plants used in experiments were at early flowering stages, i.e. stages 61-64 on the BBCH scale [44].

2.2. Sample collection

On a single plant, reproductive tissues were harvested from five intermediate floral buds (2.5-5 mm long), five old buds (>5 mm long) and five flowers. Since physiological changes are known between these different maturity stages (e.g., pollen development, Scott et al. (1991) [45]), different levels of defense can indeed be expected. The youngest leaf of the plant was also harvested (Figure 1). The whole harvesting procedure was performed on two groups of plants, one undamaged and one mechanically-damaged. Mechanical damage consisted in clipping five flower buds of random maturity stage in half. The systemic response of the plant

was studied by collecting tissues of undamaged flower buds three days later. Eight batches of 10 plants each were constituted per treatment. In order to collect enough material for chemical analyses, batches were pooled by two for pistils of intermediate and old flower buds.

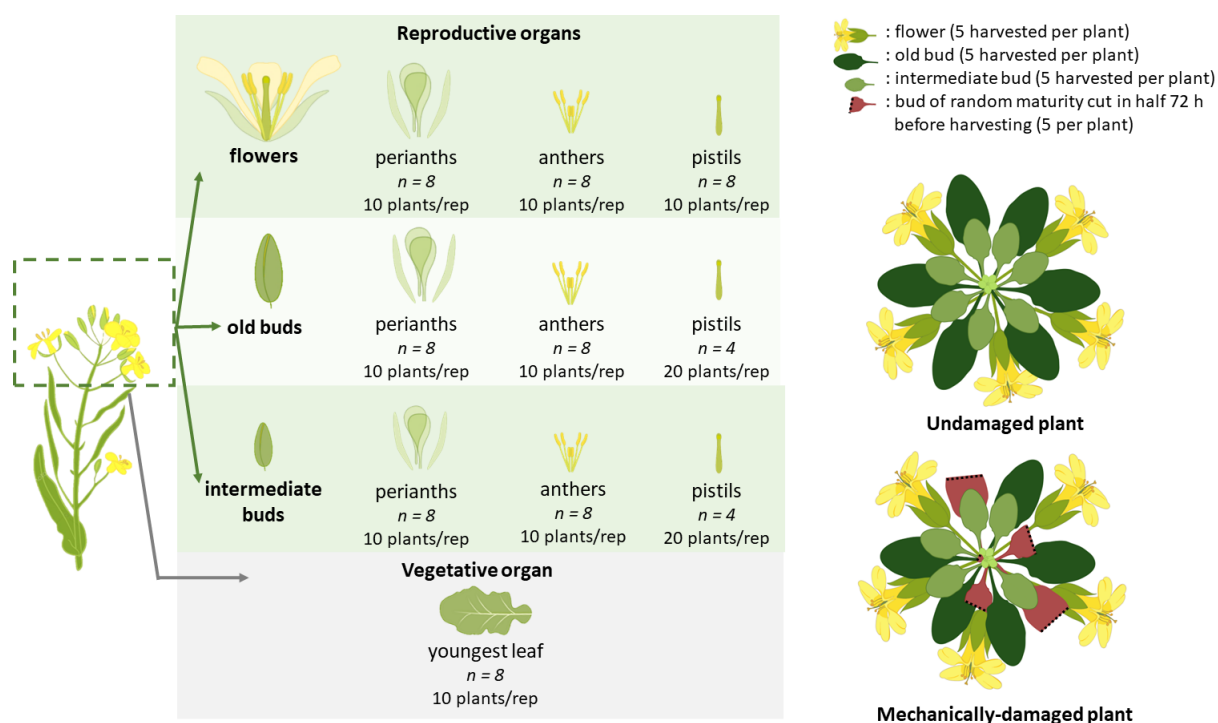


Figure 1: Detailed experimental design.

2.3. Glucosinolate analysis

GSL extraction and analysis was performed as described in Missinou et al. (2022) [46]. In brief, tissues were frozen in liquid nitrogen immediately after harvesting, freeze-dried and ground into powder. For each sample, 1-5 mg DW were extracted in 1 ml of acidified methanol (1 % formic acid). Samples were vortexed, then sonicated in a water bath at 25 °C for 10 min. After centrifugation at 7 000 rpm for 10 min, supernatants were collected and filtered using a 0.22 µm PTFE membrane. GSLs were quantified on a UPLC-DAD-TQD system (Waters Corporation) equipped with a CSH C18 column (1.7 µm × 2.1 mm × 150 mm) and using a water:acetonitrile mobile phase. GSLs were identified based on retention time, mass-to-charge ratio (m/z) and UV spectra, using a mix of pure analytical standards as reference. Each of the GSLs analyzed was quantified using MS data (in Selection Ion Monitoring mode) and a five-point external calibration curve.

2.4. Statistical analyses

All statistical analyses were performed using the R software version 4.2.1 [47]. The constitutive amounts of GSLs were analyzed using ANOVAs that included the tissue, the

organ maturity and the interaction between these two factors as explanatory variables. The effect of mechanical damage on GSLs amounts was analyzed using ANOVAs, which included the treatment (undamaged vs. damaged), the tissue and the interaction between these two factors as explanatory variables. Response variables were systematically square-root transformed to ensure a better model fitting. Pairwise comparisons of estimated marginal means (EMMeans) were performed using the ‘emmeans’ R package [48] and p-values adjusted using the false discovery rate correction [49].

3. Results

Twelve constitutive GSLs, all common in *B. napus* [46], were detected in our samples: 10 aliphatic, one indolic and one aromatic (Fig. 2, Table S1). Some compounds were tissue-specific, such as gluconasturtiin, glucoerucin and n-hexyl-GSL isomers that were found only in primary reproductive tissues, i.e., anthers and pistils (Fig. 2).

Significant differences in the total amount of constitutive GSLs were observed according to the tissue and maturity stage ($F_{4,55} = 14.25$, $p < 0.001$, Fig. 3). GSLs were more concentrated in reproductive than in vegetative organs, with a 13.2-14.9 times higher total amount in the former (Fig. 3, see Fig. S1a,b and Tables S2 and S3 for analyses of glucosinolate categories and individual compounds). Within reproductive organs and all maturity stages confounded, perianths were less concentrated than anthers and pistils (Fig. 3) for five of the twelve GSLs studied (Fig. S2a,b).

No variation was observed with age in the mean total amount of constitutive GSLs in reproductive organs ($F_{2,61} = 0.961$, $p = 0.388$, Fig. 3). However, the total amount of constitutive GSLs showed an allocation shift with age when maturity stages were compared. Indeed, in intermediate flower buds, pistils were the most GSL-rich, followed by anthers then perianths, whereas no significant differences were found in old buds, and in flowers, anthers were the most GSL-rich while no difference was found between perianths and pistils (Fig. 3 and Fig. S1a,b, Tables S2 and S3). Significant variations were also found when maturity stages were compared per reproductive tissue. For perianths, the amount was highest in old buds, followed by flowers and intermediate buds. For anthers, the amount was highest in flowers, with buds of different maturity stages not differing from each other. The opposite was observed in pistils, with a higher amount in buds than in flowers (Fig. 4 and Fig. S3a,b).

Mechanical damage induced significant systemic variations in the total GSL amount ($F_{9,124} = 2.31$, $p < 0.001$, Fig. 5). These consisted in an increased amount in pistils of intermediate flower buds, and an almost significant decrease in amount in anthers of the same

intermediate buds. Strikingly, this increased amount in pistils of intermediate buds was observed for eleven of the twelve studied GSLs (Fig. S4a,b).

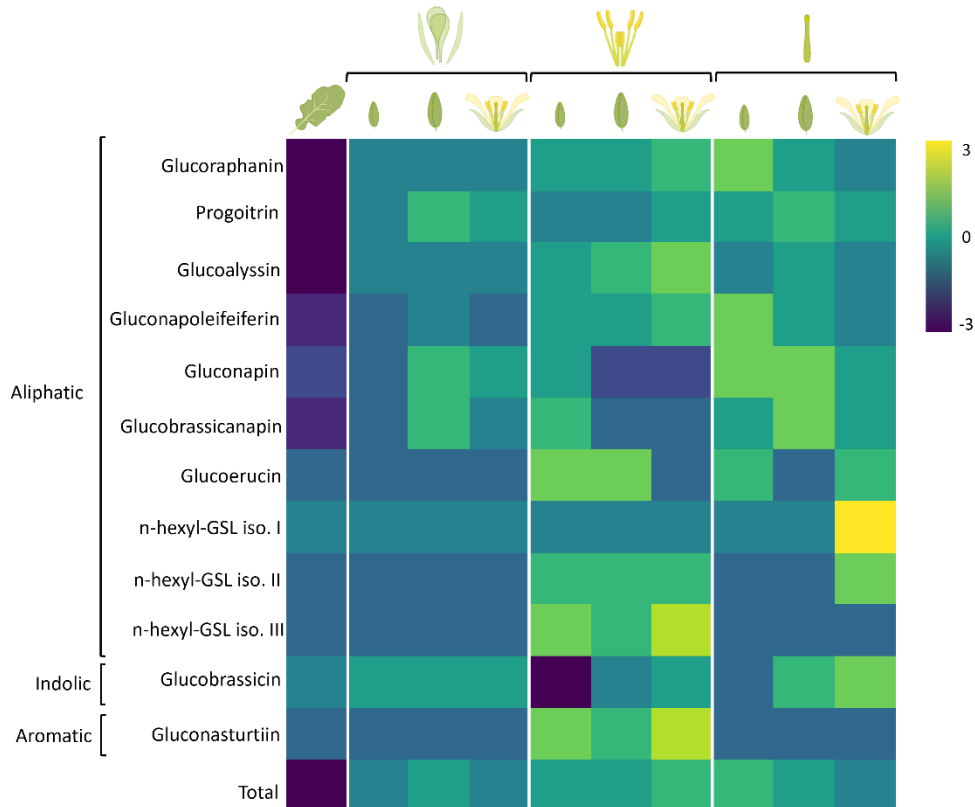


Figure 2: Twelve glucosinolates (GSLs) are constitutively present in inflorescences of *Brassica napus*, with some compounds being tissue- and stage-specific. Heatmap of mean normalized amounts of constitutive GSLs (top right: amounts autoscaled by compound, from low (dark blue) to high (yellow)) according to the tissue and maturity stage (see Fig. 1 for explanation of symbols).

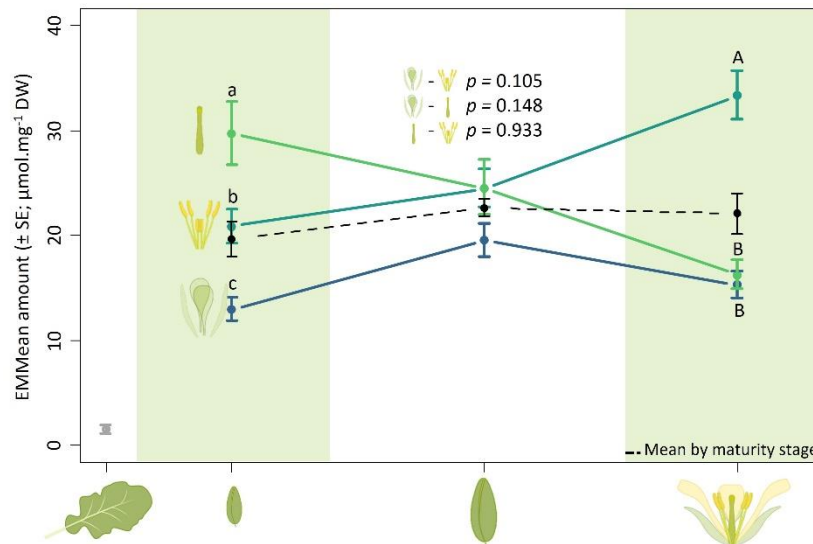


Figure 3: The allocation of constitutive GSLs depends on the plant tissue and changes with the bud maturity stage. Letters indicate significant differences between reproductive tissues for each maturity stage (see Fig. 1 for explanation of symbols). $n = 8$ per treatment with 10 plants per replicate, except for pistils of intermediate and old buds where $n = 4$ with 20 plants per replicate.

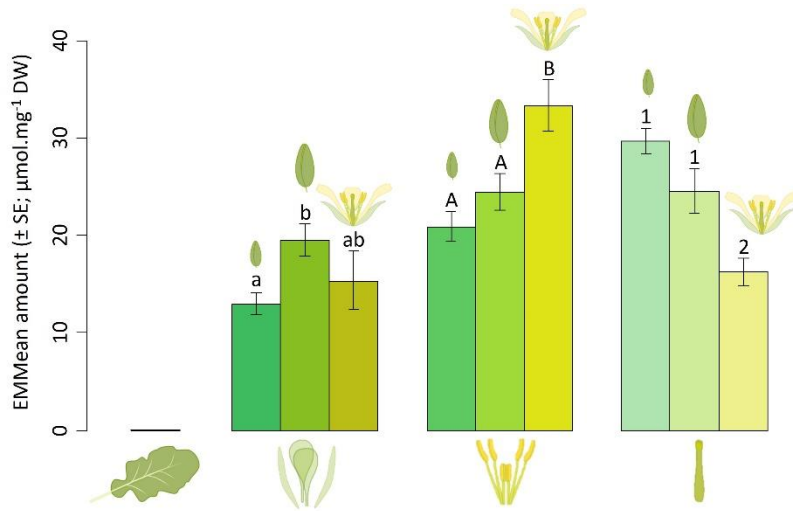
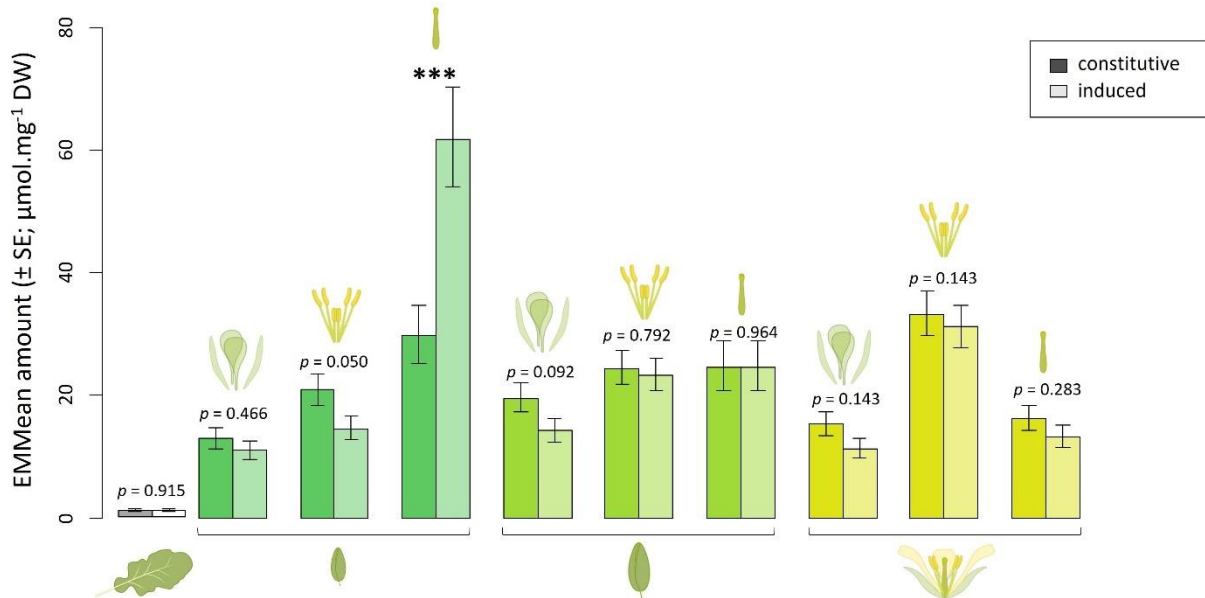


Figure 4: Perianths are more defended in old flower buds, while among primary reproductive tissues anthers are more defended in open flowers and pistils are more defended in flower buds. EMMean amount of total constitutive GSLs according to the tissue and maturity stage (see Fig. 1 for explanation of symbols). Letters and numbers indicate significant differences between maturity stages for each reproductive tissue. $n = 8$ per treatment with 10 plants per replicate, except for pistils of intermediate and old buds where $n = 4$ with 20 plants



per replicate.

Figure 5: Mechanical damage induces defenses only in pistils of intermediate flower buds. EMMean amount of total GSLs according to the tissue and maturity stage (see Fig. 1 for explanation of symbols). Asterisks indicate significant differences ($*p < 0.05$, $**p < 0.01$ and $***p < 0.001$). $n = 8$ per treatment with 10 plants per replicate, except for pistils of intermediate and old buds where $n = 4$ with 20 plants per replicate.

4. Discussion

The aim of the present study was to assess whether the age-specific defense allocation in reproductive tissues of an annual brassicaceous plant follows predictions of the ODT. At the more general level, our results show that reproductive organs contain on average 14 times higher amounts of constitutive GSLs than the youngest leaf. This is consistent with our first prediction that reproductive organs should be more defended due to a higher fitness value and vulnerability, and in agreement with previous studies [24,28,50–52]. Moreover, several aliphatic and aromatic GSLs were found exclusively in reproductive organs. Since aliphatic and aromatic GSLs are known to produce noxious isothiocyanates that are important defenses against chewing herbivores [53–56], this supports the hypothesis of a higher investment in defenses in reproductive organs.

Given that different reproductive tissues exhibit a high degree of specialization [57], with distinct transcriptomes and metabolomes [58,59] as well as unique interactions with pollinators and herbivores [5,32,60], tissue-specific defense allocation can be expected. In line with predictions of the ODT, we found that constitutive GSL amounts differed between reproductive tissues. Indeed, all maturity stages confounded, amounts in primary tissues (i.e., anthers and pistils), which directly contribute to reproduction, were higher than in accessory tissues (i.e., perianths). Moreover, all tissue-specific aliphatic and aromatic GSLs were found in primary tissues. These results are in line with previous fine scale-analyses on *Acanthus mollis* (Acanthaceae) and *Iris gracilipes* (Iridaceae) that also found the highest amount of defensive compounds in pistils and anthers [27,34]. GSLs that are specifically expressed in anthers and pistils might contribute to solving the defense-attraction dilemma of reproductive tissues [28], with an expected dual ecological function of protecting against herbivores while being neutral or helpful for pollinators [61]. However, although a growing number of studies have reported the presence of specialized metabolites in primary reproductive tissues [62,63], our understanding of their effects on antagonists and mutualists is still very fragmentary.

Organ ontogeny is known as a key source of variation in defense levels [25,35,36], these being driven by intrinsic and extrinsic factors such as developmental constraints [64,65], allocation costs [1,66] and temporal changes in selective pressures imposed by herbivores [67,68]. Here we found no overall change in the level of defenses between the different flower maturity stages, but a significant shift in defense allocation among primary tissues, with pistils being highly defended in intermediate flower buds and anthers highly defended in open flowers (both tissues being equally defended in old buds). These results suggest a trade-off in

the allocation of constitutive defenses between pistils and anthers, which would support the main assumption of the ODT that the total defense level is constrained due to metabolic costs associated with defense production [13–15]. However, they do not support the prediction that defense levels should decrease with age in perianths, and only partly support the prediction that among primary tissues, gamete-limited pistils should be more defended than gamete-unlimited anthers. While prioritizing the defense of pistils in intermediate buds remains to be explained, it can be hypothesized that in open flowers, the probability of being attacked is higher for anthers than pistils, hence the adaptiveness of prioritizing anthers defense. Indeed, anthers are exposed to pollen feeders from which they must be protected. However, although high levels of defense in anthers are advantageous against non-pollinating pollen feeders (reviewed in Rivest & Forrest (2020) [69]), they could be detrimental to pollinators [70,71] thus for the plant's fitness. It could be argued that a nutrient-rich nectar can counterbalance the negative effect of anther defenses on pollinators [72–76], but further research is needed to assess whether this happens here.

In contrast to constitutive defenses, we found that the pattern of systemically-induced GSLs did not agree with the one predicted by the ODT, i.e., that induced defenses should be allocated to structures of low value or probability of attack [16,17]. Indeed, we found a systemic induction of GSLs only in the pistil of intermediate flower buds, which was paralleled by a decrease in anthers of the same organs. Strikingly, this induction occurred for most of the individual GSLs and despite the fact that pistils of intermediate buds were already the most defended tissues constitutively. Such induction in high-value tissues is not expected by the ODT, although it has already been documented in a few cases [24,28,77,78]. It has been shown that in knockout mutants of glucosinolate transporter-encoding genes of *Arabidopsis thaliana*, wounding did not induce an increase in aliphatic glucosinolates in young leaves, whereas it induced de novo biosynthesis of indolic glucosinolates [79,80]. Thus, it could be hypothesized that the increase in glucosinolates we observed after mechanical damage in pistils is related both to (re)allocation of aliphatics from another biosynthesis site (e.g., the mature leaves) by transporters, and to de novo local biosynthesis for indolics. In addition, it should be noted that defense inductions observed here may have differed with other types of damage since they likely depend both on the nature of the damage (e.g., mechanical wounding, damage inflicted by herbivores or pathogens) and damage location (e.g. belowground or aboveground, vegetative or reproductive organs) [17,25,81,82]. To conclude, we found that defense patterns of reproductive tissues of a short-lived plant are partly in line with predictions from the ODT. However, the likelihood of attack among

different organs and tissues, as well as the benefit to defend them and the fitness consequences associated, remains to be assessed. As often, theoretical considerations prevailed here and these should be challenged to ensure robust predictions. Overall, our results still demonstrate the need to consider individual tissues rather than whole inflorescences when dealing with defense allocation, as quantification in whole inflorescences could hide tissue-specific patterns. They also show that, as for vegetative organs, variations in defense allocation with age also arise in reproductive organs and these depend on the tissue considered. How such variations influence biotic interactions and plant fitness remains to be studied

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Author contributions

M.H., A.M.C., S.F. and L.B. conceived the ideas and designed the methodology; N.M. and L.B. collected the data; M.H. and L.B. analyzed the data; L.B. wrote the first draft of the manuscript and all authors contributed to revisions. All authors gave final approval for publication.

Data availability

Data will be available on Figshare upon acceptance of the paper.

Competing interests

The authors declare no conflict of interest.

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Figure captions

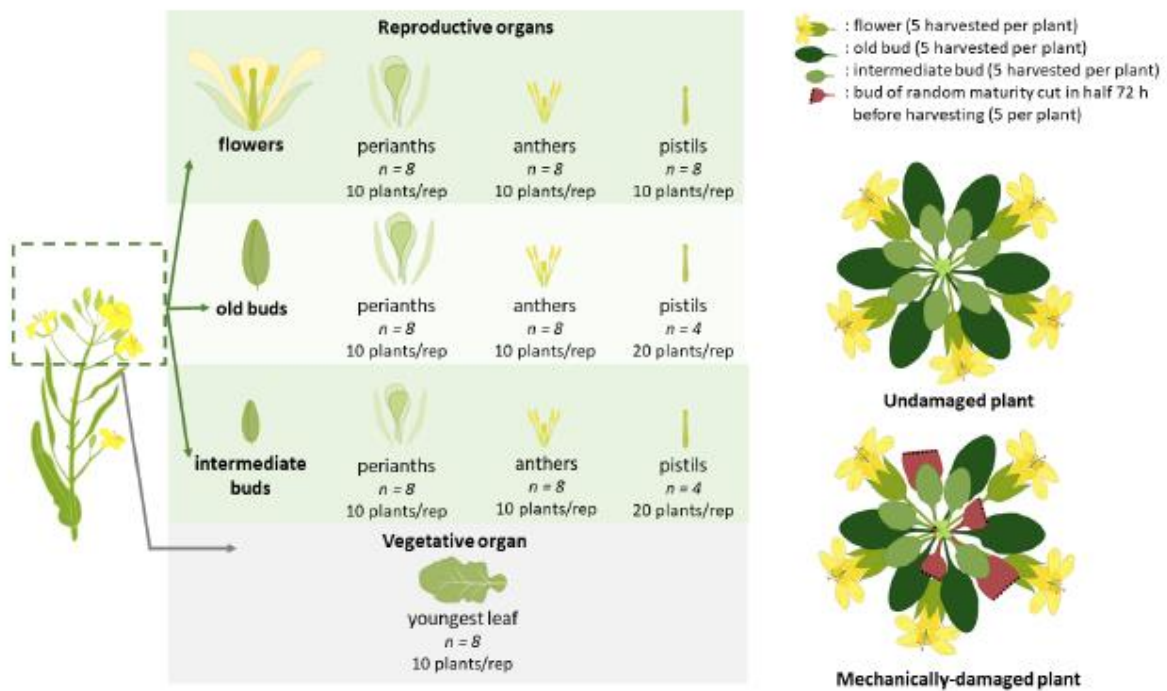


Figure 1: Detailed experimental design

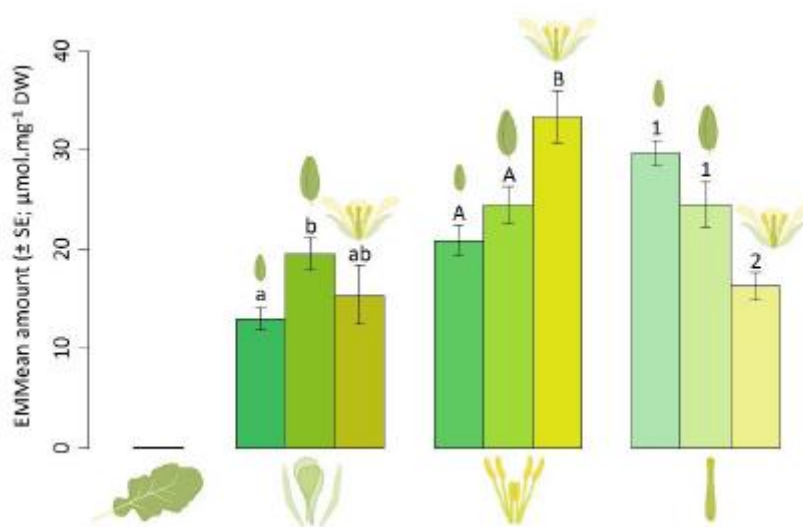


Figure 2: Twelve glucosinolates (GSLs) are constitutively present in inflorescences of *Brassica napus*, with some compounds being tissue- and stage-specific. Heatmap of mean normalized amounts of constitutive GSLs (top right: amounts autoscaled by compound, from low (dark blue) to high (yellow)) according to the tissue and maturity stage (see Fig. 1 for explanation of symbols).

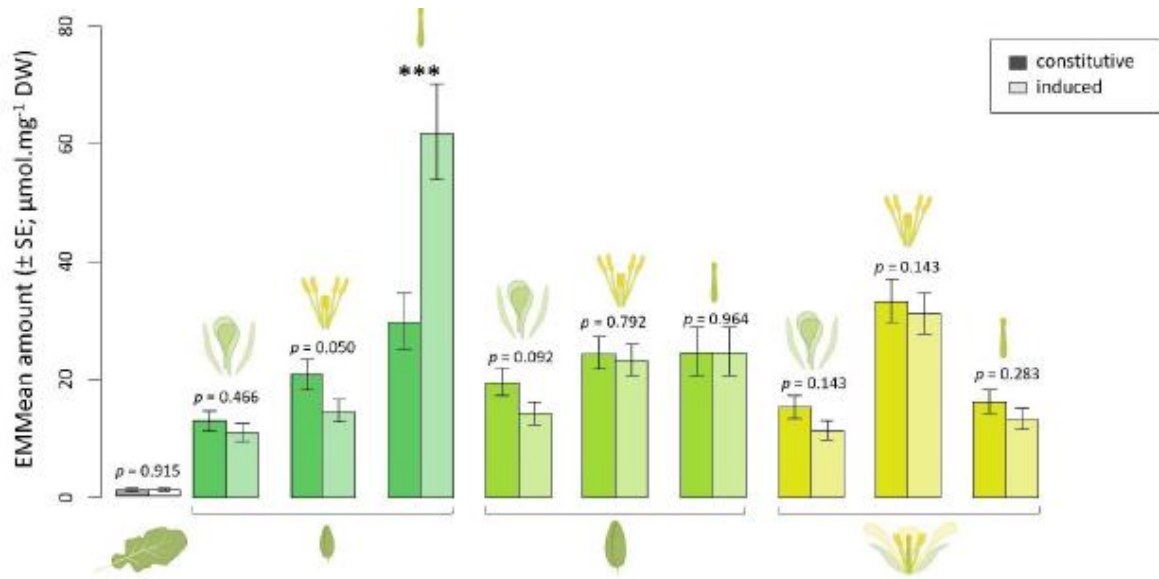


Figure 3: The allocation of constitutive GSLs depends on the plant tissue and changes with the bud maturity stage. Letters indicate significant differences between reproductive tissues for each maturity stage (see Fig. 1 for explanation of symbols). $n = 8$ per treatment with 10 plants per replicate, except for pistils of intermediate and old buds where $n = 4$ with 20 plants per replicate.

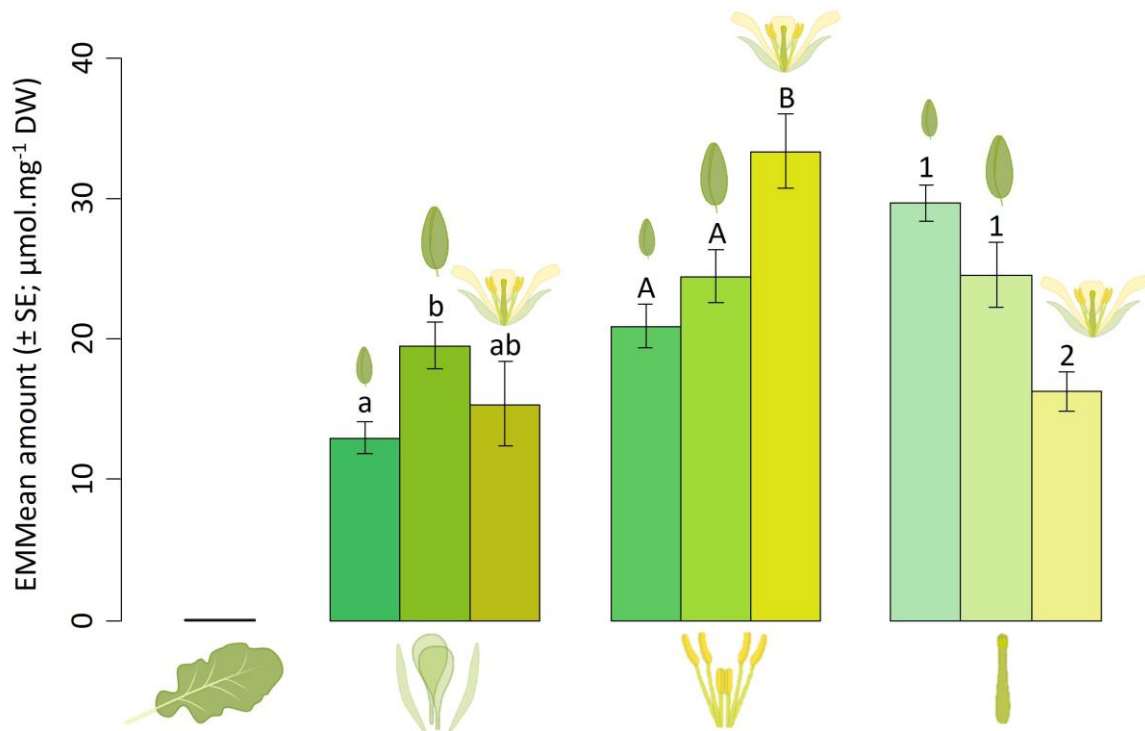


Figure 4: Perianths are more defended in old flower buds, while among primary reproductive tissues anthers are more defended in open flowers and pistils are more defended in flower buds. EMMean amount of total constitutive GSLs according to the tissue and maturity stage (see Fig. 1 for explanation of symbols). Letters and numbers indicate significant differences between maturity stages for each reproductive tissue. $n = 8$ per treatment with 10 plants per replicate, except for pistils of intermediate and old buds where $n = 4$ with 20 plants per replicate.

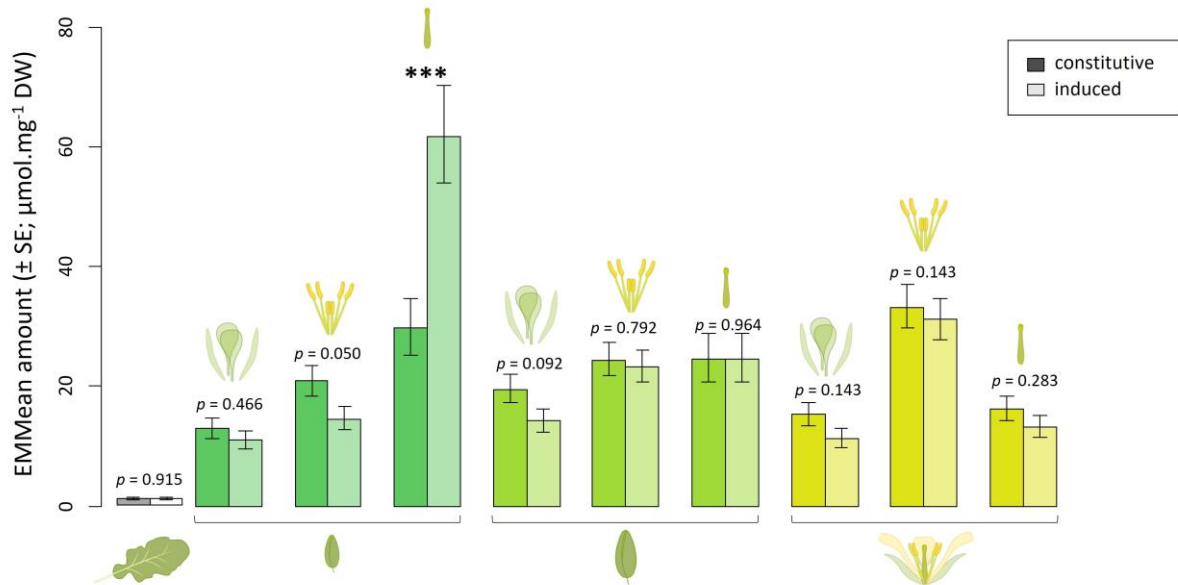


Figure 5: Mechanical damage induces defenses only in pistils of intermediate flower buds. EM Mean amount of total GSLs according to the tissue and maturity stage (see Fig. 1 for explanation of symbols). Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). $n = 8$ per treatment with 10 plants per replicate, except for pistils of intermediate and old buds where $n = 4$ with 20 plants per replicate.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Defense allocation within reproductive tissues is partly in line with Optimal Defense Theory
- Reproductive tissues are more defended than vegetative tissues
- Within reproductive tissues, tissue-specific defense allocation occurs
- Defense allocation evolves with age depending on the tissue considered