Genotype, Age, Tissue, and Environment Regulate the Structural Outcome of Glucosinolate Activation¹[W][OA]

Adam M. Wentzell and Daniel J. Kliebenstein*

Genetics Graduate Group and Department of Plant Sciences, University of California, Davis, Davis, California 95616

Glucosinolates are the inert storage form of a two-part phytochemical defense system in which the enzyme myrosinase generates an unstable intermediate that rapidly rearranges into the biologically active product. This rearrangement step generates simple nitriles, epinphionitriles, or isothiocyanates, depending on the structure of the parent glucosinolate and the presence of proteins that promote specific structural outcomes. Glucosinolate accumulation and myrosinase activity differ by plant age and tissue type and respond to environmental stimuli such as planting density and herbivory; however, the influence of these factors on the structural outcome of the rearrangement step remains unknown. We show that the structural outcome of glucosinolate activation is controlled by interactions among plant age, planting density, and natural genetic variation in Arabidopsis (Arabidopsis thaliana) rosette leaves using six well-studied accessions. We identified a similarly complex interaction between tissue type and the natural genetic variation present within these accessions. This raises questions about the relative importance of these novel levels of regulation in the evolution of plant defense. Using mutants in the structural specifier and glucosinolate activation genes identified previously in Arabidopsis rosette leaves, we demonstrate the requirement for additional myrosinases and structural specifiers controlling these processes in the roots and seedlings. Finally, we present evidence for a novel EPITHIOSPECIFIER PROTEIN-independent, simple nitrile-specifying activity that promotes the formation of simple nitriles but not epinphionitriles from all glucosinolates tested.

Plants synthesize thousands of secondary metabolites, including glucosinolates, alkaloids, terpenoids, and phenylpropanoids. These diverse chemicals often serve as the primary medium for plant-ecosystem interactions, attracting pollinators and seed dispersers and providing defense against herbivores and pathogens (Wink, 1988). Many secondary metabolites have demonstrated defense functions as growth inhibitors, toxins, or feeding deterrents for myriad herbivores and pathogens, ranging from phloem-feeding aphids to leaf-chewing lepidopteran larvae (Burrow et al., 2006b; Kim and Jander, 2007). However, adapted herbivores have evolved strategies to detoxify or avoid plant defense systems and often use these phytochemicals to locate appropriate plant hosts. For example, several insect species specialize on glucosinolate-containing plants and use particular glucosinolate structures as cues for host recognition, feeding, and oviposition (Giamoustaris and Mithen, 1995; Griffiths et al., 2001; Moyes and Raybould, 2001; Miles et al., 2005). These opposing selective pressures imposed by generalist and specialist herbivores likely favor the structural diversification of defensive metabolites, as plants seek to avoid detection by adapted specialists yet maintain effective defenses against polyphagous herbivores (Wink, 2003; Lankau, 2007).

Population density can impact secondary metabolite defenses as a consequence of specialist herbivores using these metabolites to locate their hosts. A single isolated plant may not produce sufficient volatile metabolite to attract specialist herbivores and therefore would benefit from an emphasis on direct defenses. However, the combined volatiles of numerous plants producing the same metabolites will increase the risk of attracting specialist herbivores, thus favoring structures that are weaker attractants despite providing less effective defense against polyphagous insects (Fig. 1C). As such, plastic regulation of defensive metabolite structures in response to conspecific density could be advantageous when the defensive metabolite is also a volatile host recognition cue for adapted herbivores (Lankau, 2007).

Extrinsic factors controlling herbivore populations may also influence secondary metabolite diversity. For instance, seasonal changes can alter the herbivore community over the course of plant development (Wolda, 1988); therefore, selection might favor defensive metabolite systems capable of regulating structural variation over an individual plant’s life (Fig. 1A). Because most insect populations are composed of multiple feeding guilds, they may drive metabolic defenses to respond to differences in insects’ preferred feeding tissues (Burrow et al., 2006b; Kim and Jander, 2007). If metabolites that...

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* Corresponding author; e-mail kliebenstein@ucdavis.edu.

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provide defense against one guild are ineffective against herbivores with different feeding preferences, defensive metabolite systems capable of changing structural variation across tissues would be favored (Fig. 1B). Here, we focus on the structural outcome of glucosinolate activation in Arabidopsis (*Arabidopsis thaliana*) as a model secondary metabolite system to test whether structural diversity is modulated in response to development, tissue, and population density, consistent with the above hypotheses.

Glucosinolates are structurally diverse metabolites produced throughout the Brassicaceae, including the genetic model plant Arabidopsis (Fahey et al., 2001; Wittstock and Halkier, 2002). Glucosinolates are the inert storage form of a two-part defense system in which the biologically active structures are catabolically produced by the enzyme myrosinase (Louda and Mole, 1991; Bones and Rossiter, 1996). Structural variation in intact glucosinolates is achieved through the use of different amino acid precursors and sequential modification of the side chains. Natural genetic variation in the biosynthetic enzymes within Arabidopsis leads to diverse chemical mixtures influencing insect herbivory (Kliebenstein et al., 2001a, 2001b, 2001c). The accumulation and structural diversity of intact glucosinolates are regulated by plant development and tissue type (Petersen et al., 2002; Reichelt et al., 2002; Brown et al., 2003) and a suite of biotic and abiotic factors (Leach et al., 1999; Agrawal et al., 2002; Kliebenstein et al., 2002; Bidart-Bouzat et al., 2005; Mewis et al., 2006; Kim and Jander, 2007; Reifenrath and Muller, 2007).

Further structural diversity is introduced into the glucosinolate system during catabolic glucosinolate activation. The enzyme myrosinase initiates glucosinolate activation by hydrolyzing the thio-Glc bond, generating an unstable intermediate that rapidly rearranges into the biologically active structures. This intermediate will spontaneously rearrange into isothiocyanate structures. Structural specifiers alter this rearrangement in planta by enhancing the formation of specific structures. For example, ESP promotes the formation of epithionitriles and simple nitriles, whereas ESM1 blocks the formation of simple nitrile and epithionitrile structures. Only glucosinolates with a terminal double bond can form all three structures.
the presence of structural specifier proteins, such as EPITHIOSPECIFIER PROTEIN (ESP) and EPITHIOSPECIFIER MODIFIER1 (ESM1; Fig. 1D; Bernardi et al., 2000; Foo et al., 2000; Lambrix et al., 2001; Zabala et al., 2005; Burow et al., 2006a; Zhang et al., 2006). Structural outcomes are often reported as the percentage of a particular structure relative to all of the products formed from the parent glucosinolate (Lambrix et al., 2001; Burow et al., 2006a, 2006b, 2007; Zhang et al., 2006). This allows the analysis of biochemical processes that generate a particular structure with minimal influence from any variation in myrosinase activity or intact glucosinolate accumulation. In spite of the biological importance of the structural outcome of glucosinolate activation, it remains to be determined how this process is regulated during development and in different tissues.

In this study, we test the role that plant age, growth conditions, and tissue types play in modulating metabolite diversity using the structural outcome of glucosinolate activation in Arabidopsis as our model. We demonstrate that there is quantitative age-dependent regulation of structural outcomes that is modulated by planting density and that exhibits natural genetic variation using six well-characterized accessions. We also detected differential regulation of structural outcomes between roots, flowers, and seedlings. As previous research focused on the structural outcome of glucosinolate activation in mature rosettes, we used previously described mutants in rosette myrosinases and structural specifier genes to demonstrate that glucosinolate activation and the control of structural outcomes in roots and seedlings use distinct myrosinases and structural specifiers, which remain to be identified. This analysis of control over structural variation in the glucosinolate system should aid future studies designed to test whether this variation optimizes plant defenses in response to heterogeneous herbivore populations across different individual and community scales.

RESULTS

Does the Control of Structural Outcomes Show Age-Dependent Regulation?

Previous research on the control of structural rearrangement following glucosinolate activation in Arabidopsis has focused primarily on rosette leaves at a single stage in plant development. To determine whether the structural outcome of glucosinolate activation depends on the age of the plant, we determined the proportion of glucosinolate products in each structural class at weekly intervals in rosette leaves in six accessions.

Developmental Trajectory of Simple Nitrile Production in Columbia

Over the course of development in the common reference accession Columbia (Col-0), we observed significant changes in the proportion of simple nitrile structures produced. Both exogenous allyl glucosinolate and endogenous 4-methylsulfinylbutyl glucosinolate showed a 3-fold increase in simple nitrile proportions from 3 weeks after germination to a maximum at 8 weeks (Fig. 2A; Table I). The absolute magnitude of simple nitrile proportions for the two substrates was not identical, but similar differences in structural outcomes using diverse substrates have been observed previously (Lambrix et al., 2001; Zhang et al., 2006) and may indicate distinct rearrangement kinetics depending on parent glucosinolate structure. The simple nitrile proportions observed from weeks 4 through 8 are significantly higher than those in the controls using Sinapis alba myrosinase with allyl glucosinolate as the substrate.
The Developmental Trajectory of Epithionitrile Production

In addition to simple nitriles, the accessions Cvi, Landsberg erecta (Ler), and Sha possess ESP activity leading to the formation of epithionitrile structures from allyl glucosinolate. We tested whether the proportion of epithionitriles produced in these three accessions also depends on plant age (Fig. 3A; Table II). From a low at 3 weeks after germination, epithionitrile proportions increased gradually throughout development until it was the predominant structure produced following allyl or but-3-enyl glucosinolate activation (Fig. 3A; Table II, Supplemental Fig. S1A; Supplemental Table S1). In contrast to simple nitriles, the developmental trajectories for epithionitrile proportions did not vary significantly between Cvi, Ler, and Sha (Table II; accession × week). The proportion of isothiocyanates formed was affected by variation in both plant age and natural genetic diversity, with similar patterns between endogenous and exogenous glucosinolates (Table II; Fig. 3A; Supplemental Fig. S1A). The directionality of the changes agrees with the concept that isothiocyanate production is the default outcome of glucosinolate hydrolysis and is reduced as substrate is diverted into simple nitrile and epithionitrile structures (Fig. 3A).

Structural Outcomes and Rosette Leaf Age

The analysis of developmental effects on structural outcomes of glucosinolate activation could be complicated by changes controlled by the ages of individual leaves within a rosette. To test for a relationship between leaf age and structural outcomes, a leaf was sampled from the innermost, middle, and outermost whorls of the rosettes of 8-week-old plants. The leaf sampled from the innermost whorl was still expanding, while the oldest leaf showed signs of senescence. Most accessions showed no significant variation in endogenous or exogenous glucosinolate structural outcomes between these three leaf cohorts (Supplemental Fig. S2). This shows that variation in the structural outcomes of glucosinolate activation between leaf cohorts within the rosette cannot explain the observed developmental trajectories (Fig. 3A). In particular, the youngest leaves of Cvi, Ler, and Sha showed high proportions of epithionitrile structures, while young plants showed low epithionitrile proportions, suggesting that plant age is the main determinant of the structural outcome of glucosinolate activation in our analysis (Fig. 3A; Supplemental Fig. S2). The only accession showing variation between differently aged leaves within a rosette was Cvi (Supplemental Fig. S2; P = 0.034 and 0.022, respectively).

Does the Developmental Trajectory of Structural Outcomes Depend on Planting Density?

As Arabidopsis in the wild can grow as isolated plants or in more dense populations, the structural outcome of glucosinolate activation might be regulated by the presence of neighboring plants, using either direct signals from the neighboring plants or indirect competition for space, light, or nutrients. To test this, structural outcomes were assayed in plants grown at high and low densities (Fig. 3; Table II).

Density Modulates Simple Nitrile Production in Col-0

Within the reference accession, Col-0, the structural outcome of glucosinolate activation depends strongly on planting density and plant age (Table I). When grown at high density, simple nitrile proportions in Col-0 were highest at 2 weeks and declined with age. In contrast, simple nitrile proportions in plants grown

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**Table I. Simple nitrile proportions in Col-0 depend on age and growth conditions**

| Term            | df  | F Value | P > F | df  | F Value | P > F |
|-----------------|-----|---------|-------|-----|---------|-------|
| Week            | 7/15| 10.68   | <0.001| 1/4 | 13.13   | 0.022 |
| Density         | 5/15| 1.41    | 0.270 | 1/4 | 1.96    | 0.144 |

aWeek is the age of the plants in weeks. Density denotes whether the lines were grown at high or low density. bDegrees of freedom are given as numerator df/denominator df.
at low density started low and increased gradually over time (Table I; Fig. 2B). This suggests that Arabidopsis can modulate structural outcomes in response to planting density. Future experiments are required to identify the critical aspects of planting density that control this plasticity, such as increased competition for nutrients or the physical presence of neighbors.

Genetic Variation in Developmental Trajectories in Response to Planting Density

We next tested whether the observed response to planting density in Col-0 is found in all six of these accessions. In general, all accessions showed higher simple nitrile proportions in young plants at high density compared with the low-density planting (Table II; Fig. 3). Interestingly, there was a statistically significant effect of the accession (ESP) × density term, indicating that there are genetic differences among these accessions in their response to planting density (Table II). This interaction is most obvious in the accession Bay-0, in which strong increases in simple nitrile proportions were observed in older plants at high density in comparison with the other accessions (Fig. 3, Bay-0 versus Col-0).

Epithionitrile proportions were also affected by planting density, with high density accelerating the age-dependent increase in exogenous and endogenous epithionitrile proportions in both Cvi and Sha (Tables I and II; Figs. 2 and 3; Supplemental Fig. S1; Supplemental Tables S1 and S2). In contrast, simple nitrile proportions decline over time in these accessions at elevated density. The observation of distinct trajectories for simple nitrile and epithionitrile proportions also supports the idea that simple nitrile formation following glucosinolate activation is not an exclusively ESP-mediated process.

Structural Outcomes and the Onset of Bolting

We noted flowering times for each accession to investigate a possible connection between the regulation of the structural outcome of glucosinolate activation in rosette leaves and the transition to reproductive growth. Under our growth conditions, bolting in Cvi was accelerated at high density, with bolting at 6 and 7 weeks in the high- and low-density plantings, respectively. In contrast, bolting was delayed by 1 week at high density in Bay-0 and Sha, with bolting at 8 and 7 weeks in high and low density. Mr-0, Ler, and Col-0 bolted after the conclusion of this experiment. We observed no relationship between structural outcomes and the onset of bolting in Cvi, Sha, and Bay-0. For example, in Bay-0, bolting was delayed by high density, but the age-dependent increase in simple nitrile proportion was accelerated (Fig. 3). Furthermore, Cvi and Sha showed similar responses to density throughout development for both simple nitrile and epithionitrile proportions, despite the different effects of density on flowering time (Fig. 3). This suggests that the developmental regulation of structural outcomes is not a secondary effect of the transition to flowering.

Do Structural Outcomes Vary Among Tissues?

The preferences of different insect guilds for specific tissues could lead to tissue-specific regulation of the structural outcome of glucosinolate activation. To determine whether structural outcomes vary between plant tissues, we compared rosettes, flowers, cauline leaves, seedlings, and roots in these accessions.

Structural Outcomes in Cauline Leaves and Florets

Flowers showed a pronounced decrease in epithionitrile proportions relative to the rosettes. All three accessions containing functional ESP went from 80% to 90% epithionitrile products in the rosettes to less than 40% in flowers (Fig. 4). The observed declines in epithionitrile production in flowers were balanced by increased proportions of isothiocyanate structures. In contrast, simple nitrile proportions in flowers were higher than those in rosettes (Fig. 4). These observations are consistent with those from other studies (21, 22), which showed a decrease in simple nitrile and a concomitant increase in isothiocyanate structures in flowers relative to rosettes in Arabidopsis, but not in other species, such as Allium cepa. The higher proportion of simple nitriles relative to isothiocyanates in flowers is consistent with the hypothesis that the simple nitriles are formed from the activation of the glucosinolates present in these tissues. The lower proportion of epithionitriles in flowers is consistent with the hypothesis that the epithionitriles are formed from the activation of the glucosinolates present in these tissues.
contrast, cauline leaf epithionitrile proportions were statistically indistinguishable from those in concurrently sampled rosette tissue. Cvi was the lone exception, with increased epithionitrile proportions in the cauline leaves (Fig. 5; \( P < 0.041 \)). Of these six accessions, only Bay-0 showed a significant decrease in simple nitrile proportions between rosette and cauline leaves and floral organs (Fig. 4, rosettes versus cauline leaves \( P < 0.002 \), cauline leaves versus flowers \( P < 0.001 \)). Endogenous glucosinolate activation in Col-0, Cvi, and Sha exhibited similar patterns of structural outcomes as observed for allyl glucosinolate (Supplemental Fig. S3). Thus, the structural outcome of glucosinolate activation is differentially regulated between flowers and leaves in Arabidopsis, which may indicate that simple nitriles and epithionitriles are less effective at providing defense against insects that attack Arabidopsis flowers.

**Structural Outcomes in Seedlings**

Arabidopsis seedlings have a different glucosinolate composition than mature tissues, and we proceeded to test whether they also produce different structures following glucosinolate activation (Petersen et al., 2002; Brown et al., 2003). All accessions had high simple nitrile proportions at 3 and 7 d after germination, with higher levels of simple nitrile structures at 7 d than at any age of rosette leaf (Fig. 3). Simple nitrile proportions had a statistically significant accession (ESP) \( \times \) day interaction, indicating differences in the temporal control of simple nitrile formation in seedlings, with some accessions showing a rapid increase in simple nitrile proportions and others showing little to no change (Fig. 5A; Table III). Similar to the rosette, ESP did not affect simple nitrile proportions in seedlings (Table III).

Epithionitrile proportions were low in 3-d-old seedlings and increased from 3 to 7 d in Ler and Cvi but not in Sha (Fig. 5B; Table III). Endogenous glucosinolate structural outcomes exhibited the same trends observed for allyl glucosinolate in Col-0, Cvi, and Sha seedlings, although the magnitude of simple nitrile proportions for 4-methylsulfinylbutyl glucosinolate was considerably lower (Supplemental Fig. S4). Col-0 and Ler seedlings tested using the exogenous benzyl...
Glucosinolate showed the same trend of high simple nitrile proportions at 3 and 7 d (data not shown). These data suggest that juvenile Arabidopsis seedlings tend to form more simple nitrile structures relative to mature rosettes. It is possible that this may play a role in the turnover of endogenous glucosinolates during seed germination (Petersen et al., 2002), as the less toxic simple nitrile structures are more readily metabolized.

Glucosinolate Activation in Roots

This assay revealed very high proportions of simple nitriles in the roots of 2-week-old plants in all accessions, with no detectable epithionitriles and isothiocyanates forming the remainder of the observed products (Fig. 6). This was not an artifact of the Murashige and Skoog (MS) medium, as the observed simple nitrile proportions in roots were at least 10-fold higher than those observed in control reactions using S. alba myrosinase with added MS medium. The absence of epithionitrile structures further supports the observation that simple nitrile production is distinct from ESP-mediated processes. The structural outcome of glucosinolate activation in Col-0 and Ler root tissue was also tested using benzyl glucosinolate as the substrate (Fig. 6B). No significant difference in simple nitrile proportions was observed for this substrate relative to allyl glucosinolate ($P = 0.220$ for Ler and 0.072 for Col-0). These data show that Arabidopsis roots have a different structural outcome relative to the rosettes, supporting the concept that each tissue may be optimized for the particular challenges it confronts.

Do Known Structural Specifier Genes Control Structural Outcomes in Roots and Seedlings?

All previous genetic and biochemical analyses of the structural outcome of glucosinolate activation in Arabidopsis focused on mature rosette tissue. However, the previous data show that each tissue produces a different mixture of glucosinolate activation products. Therefore, we tested the known rosette leaf structural specifier genes and myrosinases for a role in glucosinolate activation in root and seedling tissues.

**ESP-Independent Simple Nitrile Formation**

To test directly whether simple nitrile formation is independent of ESP, we assayed the seedlings and roots of an ESP knockout line and a 35S::ESP over-expression line (Fig. 7). Simple nitrile proportions in the ESP knockout line did not differ significantly from those of wild-type Col-0 in seedlings or roots for either the endogenous or exogenous glucosinolates, demonstrating that ESP is not required for this activity (Fig. 7; Supplemental Fig. S5). This was further supported by simple nitrile production in seedlings and roots of the accession Mr-0, which contains a large deletion in the ESP open reading frame (Fig. 5; Lambrix et al., 2001). The 35S::ESP line, which increases the production of simple nitriles from endogenous 4-methylsulfinylbutyl glucosinolate in Arabidopsis rosettes, produced high levels of epithionitriles from allyl glucosinolate in seedlings and roots, showing that ESP can function in these tissues. Boiled root controls showed less than 2% simple nitriles, demonstrating that the root-specific simple nitrile structural specifier activity is heat labile and likely attributable to unknown structural specifier proteins.

**Structural Outcomes in ESM1 Mutants**

ESM1 is another glucosinolate structural specifier gene, which inhibits simple nitrile formation in rosettes with functional ESM1 (Zhang et al., 2006). ESM1 knockout seedlings had elevated simple nitriles relative to wild-type Col-0 seedlings, demonstrating that ESM1 is involved in the control of simple nitrile production.
tive to the parental Col-0 at 7 d after germination ($P = 0.004$) but not at 3 d, indicating that ESM1 does not affect simple nitrile proportions until after day 3 (Fig. 7A; Supplemental Fig. S5). In contrast, simple nitrile proportions were unchanged in the roots of the ESM1 knockout (Fig. 7C). The ESM1 overexpression line was created in a recombinant inbred line, CS1995, derived from a cross between Col-0 and Ler (Zhang et al., 2006). No significant changes in simple nitrile or epithionitrile proportions were detected between CS1995 and 35S::ESM1 lines in either seedlings or roots (Fig. 7; Supplemental Fig. S5). This contrasts with the rosettes, in which both the ESM1 knockout and 35S lines alter simple nitrile proportions (Zhang et al., 2006). Thus, ESM1 does not inhibit the unidentified simple nitrile specifier activity in Col-0 seedlings or roots.

Glucosinolate Activation and Structural Outcomes in TGG1/2 Mutants

The final known components of glucosinolate activation in Arabidopsis rosettes are the myrosinase iso-enzymes TGG1 and TGG2 (Barth and Jander, 2006). To test whether these myrosinases are responsible for glucosinolate activation in seedlings and roots, glucosinolate activation products were assayed in the TGG1/2 double knockout line (Fig. 7). Although the activation assay is not a quantitative measurement of myrosinase activity, nearly wild-type levels of activation products were detectable in seedlings and roots of the double knockout line, whereas no activation products were detectable in 5-week-old rosette leaves of this line (data not shown). Furthermore, the TGG1/2 mutant had no change in structural outcomes of glucosinolate activation in roots or 3-d-old seedlings and had only a slight but significant decline in simple nitrile production at 7 d (Fig. 7; Supplemental Fig. S5; $P = 0.031$).

DISCUSSION

The variable biological activities of the different glucosinolate activation product structures, and evidence of complex regulatory patterns in glucosinolate accumulation and myrosinase activity (Petersen et al., 2002; Brown et al., 2003; Barth and Jander, 2006), led us to hypothesize that structural outcomes may be subject to diverse developmental and environmental regulatory inputs. To test this, we assayed the structural outcome of glucosinolate activation in rosette leaves throughout development in six accessions grown at two different planting densities and in seedlings, flowers, cauline leaves, and roots. Our results reveal previously unknown levels of regulation in structural outcomes, dependent on plant age, tissue, and planting density. Microarray transcript profiling has shown that genes involved in glucosinolate activation and controlling structural outcomes respond to a variety of stimuli, including herbivory, fertilizer, and light regimen (Reymond et al., 2004; Hirai et al., 2005; Maruyama-Nakashita et al., 2006; Dombrecht et al., 2007). However, it was not tested directly whether this gene regulation results in age-, tissue-, or environment-
dependent regulation of structural outcomes. Recently, the leaf myrosinases and ESP were also shown to have distinct patterns of regulation between specific plant tissues that varied among Arabidopsis accessions (Barth and Jander, 2006; Burow et al., 2007). Assuming that the primary role of the glucosinolate system is plant defense, regulation of structural outcomes might allow plants to optimize their defenses in response to changing risks of herbivory.

Optimization of Herbivory Defense

Striking differences in the structural outcome of glucosinolate activation were observed over the course of plant development and among tissues tested. All accessions produced primarily isothiocyanates in the flowers and high levels of simple nitriles in the roots and seedlings (Figs. 5–7). This suggests important differences in the defensive needs of these tissues, possibly as a consequence of the distinct herbivore communities they confront. Isothiocyanates may provide better defense against pollen-feeding insects in flowers, whereas simple nitriles seem to be preferred in the roots. Additionally, the age of the plant modulates structural outcomes in rosette leaves (Figs. 2 and 3), possibly in response to seasonal changes in the herbivore community. This age-dependent regulation was affected by planting density, because crowding appeared to increase simple nitrile and epithionitrile proportions, particularly in younger plants. Since isothiocyanates are potent attractants and oviposition cues for specialized herbivores, the distinct trajectories observed at different planting densities may reflect the changing risk of attack by polyphagous and specialist herbivores. Detection and oviposition by specialists can lead to sustained herbivory by the developing insect larvae, and young plants may be unable to tolerate this damage. As such, a single isolated plant, which is not producing enough total volatiles to be at risk of detection, may prefer the more attractive but also more effective herbivory-deterring isothiocyanate structures. In contrast, younger plants at high densities might emphasize less attractive nitrile structures to avoid detection, despite reduced defensive potential toward generalist herbivores. Now that the patterns of regulation have been described, the stage is set for detailed studies to directly test these ecological hypotheses and investigate the regulatory networks involved.

Interactions of Natural Genetic Variation and Development

Significant natural variation was identified among the six Arabidopsis accessions tested in tissue-, age-, and density-dependent regulation of the structural

Figure 6. Structural outcomes in roots. The structural outcome of glucosinolate activation was assayed using allyl glucosinolate in the roots of 2-week-old plants. A, Representative gas chromatography-flame ionization detection chromatogram of glucosinolate activation products in Sha roots. No epithionitrile products were observed in the roots of any accession. B, Percentages of simple nitrile (dark shading) and isothiocyanate (white) are shown as averages ± se. Structural outcomes were also assayed in Col-0 and Ler roots using exogenous benzyl glucosinolate (Benzyl GS) as the substrate. The accessions producing significantly lower levels of simple nitriles relative to Col-0 are indicated by asterisks.
outcomes of glucosinolate activation. Nearly every tissue or condition tested showed evidence for control by natural genetic variation (Figs. 3–7; Tables II and III). These changes in structural outcomes could be mediated by the differential regulation of a single gene. This is clearly supported by the observed tissue-specific regulation of \( \text{ESP} \), which is tightly correlated with the production of epithionitriles (Burow et al., 2007). In contrast, interactions between natural variation and the regulation of simple nitrile proportions suggest that this may be a modular system, such that natural variation can affect the percentage of simple nitriles in one tissue without affecting other tissues or conditions. This is supported by the observation that structural specifier genes discovered in rosette leaves do not play a significant role in simple nitrile formation in roots and seedlings (Fig. 7). Further support for modularity comes from the observation that structural outcomes in CS1995 roots are identical to those in the Col-0 parent, yet in shoots, CS1995 is identical to its Ler parent and distinct from Col-0 (Fig. 7). Such modularity could be mediated by members of a gene family each acting in a limited set of tissues, a result of an evolutionary process called subfunctionalization (Hughes, 1994; Lynch and Force, 2000; Fraser et al., 2002). There are numerous Arabidopsis genes homologous with \( \text{ESP} \), \( \text{ESM1} \), and the myrosinase binding proteins, all with different tissue-specific expression patterns (Capella et al., 2001; Zhang et al., 2002; Barth and Jander, 2006). However, the homologs of \( \text{ESP} \) and \( \text{ESM1} \) have no demonstrated function, so further experiments are required to test their importance in the tissue-specific regulation of structural outcomes.

Identification of a Novel Simple Nitrile Specifier Activity

To date, \( \text{ESP} \) is the only known structural specifier protein in plants capable of increasing the production of epithionitriles from alkenyl glucosinolates and simple nitriles from all other substrates (Tookey, 1973; Bernardi et al., 2000; Foo et al., 2000; Lambrix et al., 2001; Zabala et al., 2005; Burow et al., 2006a, 2007). Our
study identified one or more novel simple nitrile specifier activities that are genetically and biochemically distinct from ESP. The strongest evidence came from root and seedling simple nitrile production in the absence of a functional ESP in Mr-0, which has a naturally occurring deletion (Lambrix et al., 2001), and the T-DNA knockout of ESP in Col-0 (Figs. 6–8). All accessions containing functional ESP produced simple nitriles in the roots, not epiphenonitriles, as would be expected if ESP were mediating structural outcomes (Fig. 6). Detection of epiphenonitriles in roots of the ESP overexpression lines confirmed that epiphenonitrile production is possible in Arabidopsis roots (Fig. 7C). We also observed high proportions of simple nitriles at various ages in shoot tissues of all accessions, which was statistically independent of ESP functionality (Figs. 3 and 6; Tables II and III). Combined, this evidence strongly supports the existence of a novel simple nitrile structural specifier activity that exclusively promotes the formation of simple nitriles, but not epiphenonitriles, regardless of the parent glucosinolate structure.

CONCLUSION

We have described several patterns of age- and tissue-dependent regulation of the structural outcome of glucosinolate activation and interactions with natural variation, setting the stage for detailed molecular studies to resolve the basis of this variation. These novel regulatory inputs indicate that Arabidopsis regulates the mixture of activation product structures over tissue, development, and planting density. However, the ecological roles of many of these defensive metabolites remain undefined, requiring further experiments to understand the ecological significance of these results. In particular, the biological role of simple nitrile activation products remains unclear, as simple nitrile structures are generally less toxic and provide less effective defense against polyphagous herbivores when compared directly with the corresponding isothiocyanates (Nastruzzi et al., 2000; Jander et al., 2001; Lambrix et al., 2001; Barth and Jander, 2006; Zhang et al., 2006). We hypothesize that increased proportions of simple nitrile and epiphenonitrile products reduce the risk of detection by adapted herbivores or provide indirect defense by attracting predators and parasites of adapted herbivores. The use of knockout and overexpression mutants with altered regulation of structural outcomes of glucosinolate activation will clarify the ecological significance of these results.

MATERIALS AND METHODS

Glucosinolate Activation Assay

The structural outcome of glucosinolate activation was assayed using a modified version of the previously published protocol (Lambrix et al., 2001). Briefly, 100 mg of Arabidopsis (Arabidopsis thaliana) tissue was harvested, weighed, and immediately crushed in an 8-mL reaction vial containing 1 mL of MES acid buffer at pH 6.0 and 0.4 μmol of allyl glucosinolate. Since the accessions and tissues used in this study vary in their endogenous glucosinolate content (Kliebenstein et al., 2001c; Petersen et al., 2002; Brown et al., 2003; Wentzell et al., 2007), exogenous allyl glucosinolate enabled comparisons of structural outcomes using a common substrate. For the flowers and roots and some of the earlier time points in the developmental trajectory, tissue was limiting. Analysis of samples ranging from 20 to 200 mg showed that the tissue mass sampled did not significantly affect structural outcomes (data not shown). Upon complete tissue homogenization, the reaction vial was capped and incubated for 5 min. The reaction was stopped and glucosinolate activation products were extracted with 4 mL of dichloromethane. The organic phase was removed, dried, and concentrated to 200 μL for gas chromatography analysis using an Agilent HP 5890 apparatus with a flame ionization detector (Lambrix et al., 2001). Peak identities were confirmed using a gas chromatograph-mass spectral detector (Agilent HP 6890 and Agilent 5973N MSD), by comparison with published mass spectra (Spencer and Daxenbichler, 1980).

Structural outcomes are reported as percentages of simple nitrile, epiphenonitrile, or isothiocyanate products for a particular glucosinolate. By dividing the absolute amount of a particular structure by the sum of all possible products, the effects of myrosinase activity and differences in the biosynthesis detected or accumulation of the endogenous substrates are canceled, since they affect both the numerator and denominator equally. This assay is not a quantitative measure of total myrosinase activity, both because three of the accessions used in this study produce allyl glucosinolate and, therefore, the substrate concentration is not constant and because this assay reaches saturation for some samples.

Commercially available myrosinase purified from Sinapis alba (Sigma-Aldrich catalog no. T4528) was assayed for structural outcomes in the absence of plant tissues using allyl glucosinolate as the substrate. To control for abiotic factors influencing nonenzymatic production of simple nitrile and epiphenonitrile product structures, activation was assayed in the presence of MS medium and soil with and without S. alba myrosinase (Tookey, 1973; Foo et al., 2000). No glucosinolate activation was observed without myrosinase. The soil and MS medium controls with added myrosinase showed 3% and 6% simple nitrile products and no epiphenonitriles.

Plant Material

Six accessions of Arabidopsis were analyzed to test for natural genetic variation in the regulation of structural outcomes of glucosinolate activation. The accessions Bay-0, Col-0, Cvi, Ler, and Sha were selected because they are the parents of three large recombinant inbred line populations (Lister and Dean, 1993; Alonso-Blanco et al., 1998; Loudet et al., 2002) and have been characterized for their glucosinolate content and glucosinolate activation in rosette leaves (Kliebenstein et al., 2001c; Lambrix et al., 2001; Wentzell et al., 2007). The accessions Ler, Cvi, and Sha all possess functional ESP and produce predominantly epiphenonitriles and simple nitriles following glucosinolate activation in 4-week-old rosette leaves, whereas Bay-0, Col-0, and Mr-0 lack functional ESP and produce primarily isothiocyanates (Lambrix et al., 2001). Mr-0 has a large deletion in the open reading frame of ESP, creating a natural knockout, whereas the loss of expression and a splice site defect are the causal polymorphisms in Col-0 and Bay-0 (Lambrix et al., 2001). Seeds were obtained from the Arabidopsis Biological Resource Center (ABRC).

Mr-0, Cvi, and Sha produce allyl glucosinolate, while Cvi and Sha also accumulate but-3-ynyl glucosinolate in rosette leaves (Kliebenstein et al., 2001c; Wentzell et al., 2007), and all three but-3-ynyl activation products were detectable. Col-0 produces allyl and but-3-ynyl butyl glucosinolate in the rosettes, and its simple nitrile and isothiocyanate products were detected (Kliebenstein et al., 2001c). Under these conditions, the products from endogenous 3-hydroxypropyl glucosinolate in Bay-0 and Ler could not be quantified reliably (Kliebenstein et al., 2001c; Wentzell et al., 2007). Activation products from endogenous glucosinolates in the roots were below the limit of detection in all accessions.

Growth Conditions for the Analysis of Age- and Planting Density-Dependent Regulation

Rearrangement outcomes were surveyed at weekly intervals in the rosette leaves of plants grown alone and with numerous conspecific neighbors. Plants were maintained at 25°C in a controlled-environment chamber with a 12-h light cycle under 150 μE fluorescent lighting. Seeds were cold stratified at 4°C for 48 h, then germinated and grown in Premier ProMix potting soil. Plants
were watered and fertilized by subirrigation at weekly intervals. The low-density treatment consisted of a single plant per 49-cm² pot. The high-density plants were maintained at approximately 100 plants per 49-cm² pot, ensuring extensive contact and crowding between adjacent plants throughout the experiment. The high-density plants were maintained in the same chamber and grown at the same time as the low-density plants. Rosette leaves were assayed beginning at 2 and 3 weeks for the high- and low-density treatments, respectively.

Pots were removed from the growth chamber, and rosette tissue was harvested in three independent biological replicates sampled from separate pots for each time point, accession, and planting density. All replicates were grown in independent randomized complete blocks. At weeks 3 and 4 in the low-density treatment and at weeks 2 through 5 at high density, rosette leaves from multiple plants were pooled to obtain sufficient tissue for each replicate. At least three leaves were collected from each individual included in every replicate for all pooled samples. Trajectories were ended after 7 weeks for Ler and after 8 weeks for Col-0 and Mr-0 in the low-density treatment, and the high-density treatment of Ler was not included due to limited germination. Wounded plants were never returned to the growth chamber, and at no point during this experiment were the plants subjected to visible pests.

To test for an association between structural outcomes and leaf age, three leaves were sampled from 8-week-old plants of each accession and assayed individually. These plants were maintained at low density under conditions identical to the developmental trajectory and never subjected to visible pests. The youngest leaf was sampled from the innermost whorl and not yet fully expanded. A healthy, fully expanded leaf was sampled from the middle whorl of the plant. The oldest leaf was sampled from the outermost whorl and was beginning to senesce, with approximately 10% of the leaf area yellow. This was repeated using four plants of each accession grown simultaneously.

Growth Conditions for the Analysis of Structural Outcomes in Different Tissues

Structural outcomes were assayed in the flowers and cauline leaves using four plants of each accession grown at low densities in 49-cm² pots and maintained in the same chambers and conditions as the developmental trajectory and free of pests throughout their life cycle. When the primary inflorescence reached 20 cm in height, all flowers from a single plant were collected and pooled to obtain sufficient tissue. Cauleine and rosette leaves from these plants were assayed simultaneously for these comparisons. This provided four independent biological replicates per tissue per accession.

Root analysis required the growth of each accession on vertically oriented plates of MS medium (Gibco-BRL). Seeds were surface sterilized and cold stratified to ensure synchronous germination and maintained at 20°C under 16/8 light/dark cycles. Root tissue from approximately 100 plants was pooled for each replicate, and three independent biological replicates taken from separate plates were assayed for each accession. Care was taken to exclude shoot tissue and any MS medium. Structural outcomes were also assayed using exogenous benzyl glucosinolate in Ler and Col-0. To control for the possibility that the presence of root chemical or ion concentrations were altering structural outcomes, boiled roots were incubated for 5 min with 50 μM α-sulforaphane and then assayed for structural outcomes.

For seedlings, each accession was germinated and grown on MS medium under conditions identical to those for the roots. At 3 and 7 d after germination, seedlings were assayed for structural outcomes. Approximately 50 seedlings were pooled for each sample, with three independent biological replicates sampled from different plates for each accession and time point. Care was taken to sample the aerial portions of the seedlings. All plates were free of pests and contamination by visual examination.

Analysis of Structural Outcomes in Known Mutants

We obtained previously described mutants in ESP, ESM1, and the myrosinases TGG1 and TGG2, the known glucosinolate activation and structural specifier genes identified in the rosettes, to determine whether they also function in roots and seedlings (Lambrix et al., 2001; Barth and Jander, 2006; Zhang et al., 2006). The confirmed ESP knockout line (Salk_010349) and the ESP overexpression line are in the Col-0 background (Lambrix et al., 2001; Burrow et al., 2006a; Zhang et al., 2006). The ESM1 knockout line (Salk_043148) is in the Col-0 background (Zhang et al., 2006). The ESP overexpression line was created in a Col-0 × Ler recombinant inbred line (CS1995) that has a predominantly Col-0 genetic background with the functional Ler allele of ESP and the nonfunctional Ler allele of ESM1 (Zhang et al., 2006). To test the principal myrosinases in Arabidopsis rosette tissues, we obtained the TGG1/2 double knockout, also in the Col-0 background (Barth and Jander, 2006). Each mutant and control wild-type plant was assayed in three biologically independent replicates for each tissue as described above.

Developmental Trajectory Data Analysis

Although all three structural outcomes are contingent on one another, they each describe biochemically distinct outcomes, with potentially different regulatory patterns; thus, each was analyzed separately. Sinigrin nitrite and isothiocyanate proportions were analyzed using a general linear mixed model, with density (high or low) and ESP genotype (functional or nonfunctional) as fixed factors, accession as a random factor nested within ESP, and age (in weeks) as a repeated measure to account for sampling these genotypes multiple times throughout development. Since epithionitrilre formation was confined exclusively to accessions with functional ESP, analysis of epithionitrile proportions was restricted to Cvi, Ler, and Sha, and ESP genotype was dropped from the model. All interactions between factors were also tested (Table II).

Since wounding can alter structural outcomes, sampled plants were discarded after each assay. Thus, repeated sampling occurred at the level of accession rather than in individual plants. For each trait, we also investigated whether endogenous allylglucosinolate accumulation and subsequent formation of tissue from these plants sampled affected structural outcomes by including each as a covariate in preliminary analyses. Neither had significant effects for any trait; thus, both were dropped from the final models.

As a result of excluding the high-density treatment of the accession Ler, and because the high-density treatment had enough tissue to be sampled starting at 2 weeks, as opposed to 3 weeks for the low density treatment, these data sets were unbalanced. Additionally, structural proportions were approximately log-normally distributed. To account for these departures from standard assumptions, the trajectory data were analyzed using the GLIMMIX procedure in SAS. This procedure minimizes the potential biases introduced by unbalanced and nonnormal data by using restricted penalized likelihood to fit the model and estimate random effects (Wolfgang and O’Connell, 1993).

To determine whether age, density, and their interaction have similar effects on exogenous and endogenous glucosinolate structural outcomes in Col-0, Cvi, and Sha, data for each substrate were analyzed within an accession according to the following model: proportion = age + density + age × density. The results obtained for each substrate were compared within each accession to determine whether the significance and directionality of each term were the same. Finally, to determine whether structural outcomes varied with leaf age within individual plants, differently aged leaves within a single rosette were compared within each accession using paired t tests. All post hoc comparisons were performed using Tukey adjustments.

Analysis of Structural Outcomes in Different Tissues and Mutants

Differences in structural outcomes between flowers, cauline leaves, and rosette leaves within an accession were analyzed using paired t tests within an accession. Root structural outcome data were compared between accessions, and within Col-0 and Ler between the benzyl and allyl glucosinolate substrates, using two-tailed heteroscedastic t tests, which allow for unequal sample variances. Post hoc comparisons were performed using Tukey adjustments.

Seedling structural outcome data were also analyzed using the GLIMMIX procedure. Each structural outcome was analyzed separately for the effects of days after germination, accession, ESP genotype (either functional or nonfunctional), and all possible interactions (Table III). Accession was nested within ESP genotype for all three nitrite and isothiocyanate proportions. For epithionitrile proportions, only the data from Cvi, Ler, and Sha were analyzed, and ESP genotype was removed from the model. Days were treated as repeated measures of the accessions. Post hoc comparisons were performed using Tukey adjustments.

Mutants in the known structural specifier and myrosinase genes identified in the rosettes were tested for the consequences on structural outcomes using two-tailed heteroscedastic t tests. ESP overexpression and ESP knockout lines were compared with Col-0, as were the ESM1 knockout and the TGG1/2 double knockout lines. The ESM1 overexpression line was compared with the recombinant inbred line CS1995.
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Endogenous glucosinolate structural outcomes in Cvi and Sha as a function of age and growth conditions.

Supplemental Figure S2. Leaf age-dependent changes in structural outcomes.

Supplemental Figure S3. Changes in endogenous glucosinolate structural outcomes in flowers and cauline leaves.

Supplemental Figure S4. Endogenous glucosinolate structural outcomes in seedlings of Col-0, Cvi, and Sha.

Supplemental Figure S5. Endogenous glucosinolate structural outcomes in seedlings of glucosinolate activation and structural specifiers mutants.

Supplemental Table S1. Structural outcomes in Cvi depend on age and growth conditions.

Supplemental Table S2. Structural outcomes in Sha depend on age and growth conditions.

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