Genome-wide association studies (GWAS) have become a standard approach for exploring the genetic basis of phenotypic variation. However, correlation is not causation, and only a tiny fraction of all associations have been experimentally confirmed. One practical problem is that a peak of association does not always pinpoint a causal gene, but may instead be tagging multiple causal variants. In this study, we reanalyze a previously reported peak associated with flowering time traits in Swedish Arabidopsis thaliana population. The peak appeared to pinpoint the AOP2/AOP3 cluster of glucosinolate biosynthesis genes, which is known to be responsible for natural variation in herbivore resistance. Here we propose an alternative hypothesis, by demonstrating that the AOP2/AOP3 flowering association can be wholly accounted for by allelic variation in two flanking genes with clear roles in regulating flowering: NDX1, a regulator of the main flowering time controller FLC, and GA1, which plays a central role in gibberellin synthesis and is required for flowering under some conditions. In other words, we propose that the AOP2/AOP3 flowering-time association may be yet another example of a spurious, “synthetic” association, arising from trying to fit a single-locus model in the presence of two statistically associated causative loci. We conclude that caution is needed when using GWAS for fine-mapping.

Heredity (2021) 127:245–252; https://doi.org/10.1038/s41437-021-00456-3

INTRODUCTION

Genome-wide association studies (GWAS) have become an essential tool for studying the genetics of natural variation. In addition to its tremendous impact on human genetics, GWAS is being applied routinely to a wide range of species, and massive numbers of genotype-phenotype associations have been revealed (Atwell et al. 2010; Flint and Eskin 2012; MacArthur et al. 2017). However, only for a tiny subset do we have any idea of why the association exists, i.e., the molecular mechanism (Gallagher and Chen-Plotkin 2018). There are many reasons for this, but an important one is that peaks do not always pinpoint the causal genes (Hormozdiari et al. 2014; Tam et al. 2019). In settings where the environment cannot be controlled, spurious associations may simply arise because of environmental confounding, but our focus here is on confounding by the genetic background, and in particular on genetic background effects that are not sufficiently diffuse to be readily be removed by approximate methods like kinship or Principal Component corrections (Atwell et al. 2010; Vilhjálmsson and Nordborg 2013). Such effects may arise whenever there is linkage disequilibrium between non-trivial allelic effects (Platt et al. 2010a; Dickson et al. 2010), and is a well-known problem for fine-mapping when there is allelic heterogeneity. In Arabidopsis thaliana, examples include multiple functional alleles of FRIGIDA (FRI) (Atwell et al. 2010), and DELAY OF GERMINATION1 (DOG1) (Kerdaffrec et al. 2016). Less clear is how frequently spurious associations arise from genetic heterogeneity, i.e., from alleles for different genes affecting the same trait, which is inherently less likely given the need for strong associations (linkage disequilibrium) between the causative alleles.

In this paper we discuss what we believe to be an example of this: causal allelic variation at two different genes, separated by roughly 120 kb, inducing a spurious peak of association in a third gene located between the causal loci. The phenotype in question is flowering time, one of the most intensely studied traits in A. thaliana, and the example is of special interest in that the two putatively causal loci are well-known flowering time regulators, whereas the putatively spurious association involves a highly polymorphic and adaptively important gene with a demonstrated role in glucosinolate metabolism and defense against herbivory, but which could well have organism-wide pleiotropic effects. Our intention is emphatically not to resolve causality in this particular case, because this would require experimental data that we do not have. Rather, we aim to re-open the discussion, and underscore the need for experimental confirmation. However, most importantly, we seek to draw attention to the general problem, which is that GWAS may be positively misleading (Platt et al. 2010b), especially when used for fine-mapping.

RESULTS AND ANALYSIS

GWAS for flowering time suggested a role for AOP2

Flowering time is an adaptively and agriculturally important trait that has been intensively studied in A. thaliana. Thanks to decades of functional work, the pathways involved in flowering time...
regulation (and their interaction with the environment) are extremely well understood (Koonerreef et al. 1998; Srikanta and Schmid 2011; Andréis and Coupland 2012). Known flowering-time regulators are also highly variable in nature, and GWAS for flowering time typical reveal a variety of known loci (Brachi et al. 2010; Atwell et al. 2010; Li et al. 2010; Sasaki et al. 2015; 1001 Genomes Consortium 2016; Zan and Carlberg 2019).

A major source of natural variation for flowering is a variety of loss-of-function alleles of FRI, which regulates FLOWERING LOCUS C (FLC), a key regulator of flowering time, crucial for helping plants flower in the right season by “remembering” exposure to cold winter temperatures using a fascinating epigenetic mechanism (Whittaker and Dean 2017). Indeed, FRI was first identified through natural variation (Johanson et al. 2000), but, despite explaining a considerable fraction of the variation for flowering time, has proven difficult to map using GWAS, mostly because it has such high allelic heterogeneity (Atwell et al. 2010). However, several GWAS identified a strong peak roughly 1 Mb from FRI, a peak which stood out because it was not obviously associated with a known flowering time gene (Brachi et al. 2010; Atwell et al. 2010; Li et al. 2010; Zan and Carlberg 2019). Instead, this peak appeared to pinpoint the highly variable and adaptively important ALKENYL HYDROXALKYL PRODUCING (AOP) cluster (Atwell et al. 2010; Kerwin et al. 2011; Jensen et al. 2015; Katz et al. 2020), containing three tandemly duplicated genes involved in the synthesis of glucosinolates, secondary metabolites that play important role in defense against herbivory (Kliebenstein et al. 2001). In the Swedish population, using SNPs from whole-genome sequencing, the strongest association was found for the SNP at chr4:1355057, 961 bp upstream of AOP2, and strongly correlated both with flowering time and FLC expression (Figs. 1 and S1). Here we focus on this association, and return to associations in other populations in the Discussion section.

Functional variation at AOP2 does not affect flowering time

Although transgene experiments had shown that AOP2 (but not AOP3) could affect flowering time (Kerwin et al. 2011; Jensen et al. 2015), pleiotropic effects on flowering time are common (Chong and Stinchcombe 2019), and we were not convinced that this was the explanation for the AOP2 peak. To investigate this further, we first explored the functional AOP2 variants tagged by chr4:1355057 (Fig. 1) to see if we could confirm a causal connection.

Kliebenstein et al. (2011) showed that day-length dependent flowering responses could change when AOP2 is introduced in the reference line Col-0, which carries a natural non-functional AOP2 allele due to a 5 bp deletion causing a frameshift and leading to accumulation of different glucosinolates (Kliebenstein et al. 2001). Jensen et al. (2015) showed that A. lyrata AOP2 delays flowering when overexpressed in Col-0, whereas overexpressed AOP3 does not. They suggested that delayed flowering results from an interaction between the glucosinolate and flowering pathways. Based on these results, we hypothesized that A. thaliana lines carrying functional AOP2 alleles should flower later than lines carrying the loss-of-function alleles. AOP2 has multiple alleles inducing frameshift in addition to the Col-0 allele (Neal et al. 2010). In the Swedish population, we identified five frameshift alleles, including the Col-0 type (Fig. 2A; Table S1).

We assessed the functional effect of these five indels directly using mass spectrometry (Fig. 2B). AOP2 converts 3-methylsulfonylpropyl and 4-methylsulfonylbutyl glucosinolate to 2-propenyl and 3-butenyl glucosinolate, respectively (Kliebenstein et al. 2001). Lines having any indel in the second exon (a to c in Fig. 2A) or the Col-0 indel (d) in the third exon did not accumulate 2-propenyl and 3-butenyl glucosinolates, although transcripts were detected in all cases (Fig. 2B; Table S2). The fifth insertion (e) did not affect 2-propenyl and 3-butenyl glucosinolate accumulation significantly.

Using these data, we then tested whether AOP2 functionality is associated with flowering time variation. Contrary to this hypothesis, AOP2 functionality is significantly associated neither with chr4:1355057 nor flowering time. For example, all 105 lines carrying the reference allele at chr4:1355057 show substantial AOP2 expression, and 19 lines of them have indels that disrupt function (Table S1), but this functionality showed only a very weak effect on flowering time (Fig. 2C; p value = 0.07)—in stark contrast with the very strong association between chr4:1355057 and flowering time (Fig. 1C; p value = 1.85e−09; MAC = 0.2). There is a weak correlation between AOP2 expression and flowering (Fig. S1B), but it seems unlikely that this relationship reflects causality, when functional allele variation does not. In conclusion, although it has been demonstrated that AOP2 can affect flowering time (Kerwin et al. 2011; Jensen et al. 2015), the considerable functional AOP2 variation observed in the Swedish population is not significantly correlated with flowering time, suggesting that the highly significant association between chr4:1355057 and flowering time arises for other reasons.

The AOP2 peak tags a diverged allele of NDX1

In order to identify potential causal variants, we dissected the local haplotype structure surrounding chr4:1355057 using principal component analysis (PCA) (Fig. 3A). Consistent with the fact that the Swedish population has a strong north-south population structure (Long et al. 2013), the first two principal components were correlated with the latitude of origin (PC1 r² = 0.28; PC2 r² = 0.25). However, the third principal component was not correlated with global structure, but rather identified an extended haplotype carried by 20 of the 51 lines that also carried the non-reference chr4:1355057 allele (Fig. 3A). This cluster was also found when the analysis was carried out only for the southern Swedish population (the northern subset is too small for meaningful analysis). The haplotype (denoted chr4:1355057b) contained three genes upstream of AOP2, including NDX1 (AT4G03090; chr4:1366053..1371237)—a known regulator of FLC that binds to the promoter region of COOLAIR, the antisense transcript of FLC, and inhibits the degradation of FLC by stabilizing the R-loop (Sun et al. 2013) (Fig. 3B).

Furthermore, the chr4:1355057b haplotype is perfectly associated with a highly diverged NDX1 allele (Fig. 3C). The non-synonymous sequence divergence between this allele and the reference allele is close to 1%, and two amino acid changes are in the NDX8 domain that is critical for the function (Sun et al. 2013) (Fig. S2). Multinomial lines confirmed that NDX1, unlike neighboring genes, has a significant effect on flowering (Fig. S3).

Multilocus GWAS including NDX1 reveals a new association near GA1

These observations suggested that the flowering time association peak centered on chr4:1355057 could partly be due allelic variation at NDX1 (Fig. 4A). To explore this further, we performed GWAS while including the chr4:1355057b haplotype as a cofactor to regress out the effect of the NDX1 polymorphism (Fig. 4B). Doing so did not eliminate the significant peak on chromosome 4 (suggesting that NDX1 is not the only causal variant), but moved it over 100 Kbp in the opposite direction of NDX1. The “new” peak was quite broad and flat, but the second strongest association (chr4:1236543; −log10p-value = 11.85; MAC = 15) was 1.1 Kbp downstream of another well-known flowering regulator, GIBBERELLIC ACID REQUIRING 1 (GA1) Chr4:1237671..1244822 that is essential for gibberellic acid biosynthesis (Sun and Kamiya 1994). Gibberellins plays a crucial role in the transition to flowering through regulation of LEAFY (LFY) and FLOWERING TIME LOCUS T (FT) (Blazquez et al. 1998; Porri et al. 2012), and loss of function mutants of GA1 cannot flower under certain conditions (Reeves and Coupland 2001).
Previous GWAS and linkage mapping studies have suggested that allelic variation at $\text{GA1}$ plays a role in flowering time variation, and the association is known to be sensitive to population structure correction (Brachi et al. 2010). These kinds of problems are often caused by extensive linkage disequilibrium, the existence of which is evident (Fig. S4). This can also be seen by carrying out a third GWAS, now with the chr4:1236543 ($\text{GA1}$) SNP as a cofactor, because this causes an increase in the height of the AOP peak demonstrating that these peaks are indeed not independent (Fig. 4C).

Polymorphisms at $\text{GA1}$ and $\text{NDX1}$ jointly explain the AOP association

Finally, we asked whether allelic variants at $\text{GA1}$ and $\text{NDX1}$ were jointly sufficient to explain the peak centered on the AOP2/AOP3 glucosinolate biosynthesis cluster can alternatively be explained using a two-locus model, where the causal variants are in two flanking genes directly involved in the regulation of flowering: $\text{GA1}$, essential for gibberellin synthesis, and $\text{NDX1}$, a regulator of $\text{FLC}$. Under this interpretation, the AOP2 peak is a spurious association (Fig. 5), an artifact of incorrectly fitting a single-locus model in the presence of two causative loci (Platt et al. 2010a; Dickson et al. 2010; Atwell et al. 2010). The problem is analogous to the problem of “ghost QTLs” in classical linkage mapping (Haley and Knott 1992; Martínez and Curnow 1992).

We emphasize that neither of these two models (single-locus AOP2 or two-locus $\text{NDX1}$ and $\text{GA1}$), has been experimentally confirmed. It is clear that all three genes discussed here can affect flowering time when deleted, but this tells us little about the natural allelic variants. Merely knocking genes out is not sufficient for a trait like flowering time, which has been shown to be highly “omnigenic” (Boyle et al. 2017) in the sense that random knock-out mutations are

**DISCUSSION**

In this paper we have presented an alternative interpretation for a published GWAS peak, a potential example of genetic confounding. We demonstrate that a reproducible association between flowering time (and $\text{FLC}$ expression) and SNPs in the AOP2/AOP3 glucosinolate biosynthesis cluster can alternatively be explained using a two-locus model, where the causal variants are in two flanking genes directly involved in the regulation of flowering: $\text{GA1}$, essential for gibberellin synthesis, and $\text{NDX1}$, a regulator of $\text{FLC}$. Under this interpretation, the AOP2 peak is a spurious association (Fig. 5), an artifact of incorrectly fitting a single-locus model in the presence of two causative loci (Platt et al. 2010a; Dickson et al. 2010; Atwell et al. 2010). The problem is analogous to the problem of “ghost QTLs” in classical linkage mapping (Haley and Knott 1992; Martínez and Curnow 1992).

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**Fig. 1** GWAS for flowering time revealed a peak centered on the chromosome 4 AOP cluster. A Genome-wide Manhattan plot for flowering time at 10 °C in 132 Swedish lines, with SNP reported in Atwell et al. (2010) highlighted in blue (chr4: 1330749), and the strongest association in red (chr4: 1355057). A linear model without structure correction was used (see Methods). B Zoom-in on the peak, with gene annotation. C Violin plot showing the difference in flowering time between lines carrying major and minor alleles at chr4:1355057 ($p = 1.85 \times 10^{-09}$ by two-tailed Welch’s $t$-test).

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as likely as a priori candidates to affect it (Chong and Stinchcombe 2019). Because direct gene replacement is not feasible in Arabidopsis, experimental testing of these two models would thus entail knocking out the native allele at multiple loci in multiple genetic backgrounds, and replacing it with cloned native alleles, using on the order of 50 independent transgenic lines per construct to account for position effects (Li et al. 2014). In late-flowering Arabidopsis, with an essentially annual life-cycle, this would be a multi-year project requiring considerable resources.

That said, we believe that our two-locus model (involving two known flowering regulators, one of which directly interacts with FLC) is a more likely explanation for the association seen here than the single-locus model (involving glucosinolate production). We say this primarily because, although flowering time is clearly “omnigenic” in the sense of presenting a large mutation target, GWAS results for flowering time (like many other phenotypes in Arabidopsis) have generally shown a strong over-representation for genes in known pathways (Atwell et al. 2010; Sasaki et al. 2015). This is in sharp contrast to human genetics, where GWAS results have generally been extremely difficult to interpret (Boyle et al. 2017). A plausible explanation for this difference is that much of the variation in Arabidopsis is adaptive (Atwell et al. 2010).

Indeed it may well be the case that selection is indirectly responsible for the AOP2 flowering time association. The role of the AOP cluster in defense against herbivory is well established (Kliebenstein et al. 2001), and it is tempting to speculate that strong selection on glucosinolate variation could have contributed to the complex haplotype structure in the region—leading to associations between SNPs in AOP2 and functional variants in nearby flowering regulators simply through random hitchhiking (Maynard Smith and Haigh 1974). It should be noted that while a two-locus model appears to be required to explain the AOP2 flowering-time association seen in the Swedish population, the association seen in two other samples (Atwell et al. 2010; Li et al. 2010), can simply be explained by extremely strong linkage disequilibrium between AOP2 variants and the diverged NDX1 haplotype described above (Fig. S9). Spurious flowering time associations due to regional selection on other traits has also been suggested for maize (Larsson et al. 2013).

To conclude, while we may never know which (if any) of the models proposed here is correct, there is no doubt that spurious associations like this do exist, and may complicate interpretation of mapping results (Huang et al. 2010; Atwell et al. 2010; Larsson et al. 2013; Hormozdiari et al. 2014; Kerdaffrec et al. 2016). Although representing a difficult model-selection problem, better methods for systematically identifying such associations could be a very cost-efficient way of getting more information out of GWAS results.

MATERIALS AND METHODS

Data sets
We used published Arabidopsis data sets containing genotypes (Long et al. 2013), RNA-seq transcriptome data (Dubin et al. 2015), as well as flowering time phenotypes (Sasaki et al. 2015, 2018) for the Swedish population. All plants were grown under a constant 10 °C (132 lines) in 16 h day length condition. For RNA seq analysis, RNA from whole rosettes were collected at 11–12 h after dawn at nine-leaf stage (Dubin et al. 2015). We obtained other phenotype data, including FLC expression (Atwell et al. 2010) and flowering time under Sweden spring condition in 2008 (Li et al. 2010), from AraPheno (Seren et al. 2017). All the data sources are listed in Table S3.

Plant materials
We grew loss-of function mutants of Atg403050 (SALK_001655), At4g03080 (SALK_051383), At4g03100 (SALK_082878), and NDX1 (WiscD- sLox344A04) (Sun et al. 2013) with the wild type under a constant 21 °C in 16 h day length condition.

Statistical analysis
GWAS. We performed GWAS using LIMIX version 3.0.4 (Lippert et al. 2014) with full genome SNPs in a Swedish population (n = 132) (Long et al. 2013)
and 250 K SNP chip genotypes in RegMap panel (Horton et al. 2012). We used a linear regression model (LM) for GWAS without correction of population structure (Figs. 1, 4, S1, S5, and S6) because several of the variants in the chromosome 4 region are strongly correlated with population structure in the Swedish population, rendering fine-mapping impossible because of lack of power. Note that our primary interest is not the genome-wide significance of the region, but rather identifying potential causal SNPs within it. It has previously been observed that kinship correction can obscure causality locally (Kerdaffrec et al. 2016). We used a linear mixed model (LMM) for GWAS to correct population structure with a kinship matrix representing genetic relatedness (IBS) (Yu et al. 2006; Kang et al. 2008) (Fig. S9).

Subsequently, we performed multilocus GWAS to dissect chromosome 4 peak using a multiple linear regression model without population structure correction in the Swedish population (Figs. 4, S5, and S6), and LMM for global population (Fig. S9). As described in figures, SNPs or the allele (chr4:1355057b) were taken as cofactors to be regressed out from the phenotypic variation. PCA. We used the entire Swedish population (259 lines; Long et al. 2013) to analyze local genetic structure around the AOP cluster. SNPs in the 60 kb region around chr4:1355057 were extracted and analyzed using the prcomp function in R (https://www.r-project.org).

Haplotype analysis. For the analysis (Fig. S7), we used SNPs covering GA1 and NDX1 regions, including the 30 Kbp upstream of GA1 and downstream of NDX1, from a pre-imputation version of the Regional Mapping Project SNP panel, including 1307 global lines (Horton et al. 2012). These SNPs were used as the input into fastPHASE version 1.4.8 (Scheet and Stephens 2006), which was run using the default settings as described in Li et al. (2014).

Genotyping NDX1 and AOP2
For population samples (n = 259), we predicted amino acid sequences of NDX1 and AOP2 using genome data, including SNPs and short indels (Long et al. 2013). NDX1 sequences of Col-0 and chr4:1355057b alleles were also confirmed by Sanger sequencing after cloning the 7.8 Kbp region with forward primer 5′-CTGGTAAATACTGTGTGTAGACAATTCT-3′ and reverse primer 5′-TCGATGTTTGACGGCAAAGGATGAAG-3′. Line 6180 (TÄL 07; latitude 62.6322 longitude 17.6906) was chosen to represent chr4:1355057b alleles. We confirmed all predicted chr4:1355057b allele-specific SNPs by the Sanger sequencing.

Measurement of expression levels
For population samples, we extracted FLC and AOP2 expressions from RNA-seq data of leaf tissue under 10 °C constant temperature (Dubin et al. 2015). For mutants, we extracted total RNA from aerial parts of nine-leaf stage seedlings collected at 8 h after dawn using RNeasy mini kit (Qiagen) with DNase treatment (Thermo Fisher Scientific). We used the SuperScript III First-Strand Synthesis System (Invitrogen) for cDNA synthesis. We performed qRT-PCR using the LightCycler 96 system (Roche) with FastStart Essential DNA Green Master (Roche).
SAND (AT2G28390) was used for a control to normalize the transcript abundance (Czechowski et al. 2005) using the ddCT method. The primer sequences were SAND: 5′-AACTCTATGCAGCATTTGATCCACT-3′ and 5′-TGATTGCATATCTTTATCGCCATC-3′, and FLC: 5′-TGAGAACAAAAGTAGCCGACAAAG-3′ and 5′-ATGCGTCACAGAGAACAGAAAGC-3′.

Assessment of AOP2’s functionality
Glucosinolate extraction from plant tissue. We extracted glucosinolates from whole rosettes at nine-leaf stage grown under 21 °C, 16 h light condition, as follows. Frozen 10 mg samples in liquid nitrogen in 2 ml Eppendorf tubes were stored at −80 °C. Precooled 1 ml 90% methanol (90% MeOH/10% 10 mM ammonium bicarbonate in H2O; −20 °C) was added by the final methanol concentration remaining above 78%. Tissue was disrupted by adding two small stainless beads bearings and agitating with a tissue lyser (TissueLyser II, Qiagen) for 10 min at 20 rev/s. The samples were shaken for a further 60 min (70 rev/s) in the cold. After centrifuging at 13,000 rpm, the supernatant was transferred to a fresh tube and the pellet was discarded.

Measurement. We measured glucosinolates according to a previous study (Liang et al. 2018). Briefly, 2 µl of each sample was injected into a SeQuant ZIC-pHILIC HPLC column (Merck, 100 × 2.1 mm; 5 µm), and the respective guard column operated with an Ultimate 3000 HPLC system (Dionex, Thermo Fisher Scientific) at a flow rate of 100 µl/min. The HPLC was directly coupled via electrospray ionization in the negative ion mode (2.8 kV) to a TSQ Quantiva mass spectrometer (Thermo Fisher Scientific). A linear gradient (A: 95% acetonitrile 5%, 10 mM aqueous ammonium acetate; B: 5 mM aqueous ammonium acetate) starting with 5% B and ramping up

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**Fig. 4 Multilocus GWAS suggests genetic heterogeneity.** Zoom-in plots of multilocus GWAS surrounding chr4:1355057 with violin plots illustrating how much of the variation is explained by each model. Arrows on the Manhattan plots indicate SNPs used for the cofactors. A The original association identified an association in AOP2 (chr4:1355057, magenta) that explained 33% of the flowering time variation. B Multilocus GWAS using a diverged NDX1 haplotype (chr4:1355057b, orange) that explained 29% of the variation revealed an association near GA1 (chr4:1326543, dark blue). C Multilocus GWAS using GA1 peak that explained 11% of the variation. D Multilocus GWAS using both NDX1 and GA1 as co-factors fully explained the original peak (explaining 45% of the variation).
Fig. 5  Summary of our results. We consider three bi-allelic SNPs, in (or near) GA1, AOP2, and NDX1, respectively. Because of strong linkage disequilibrium, the minor NDX1 allele is only found on haplotypes with the major GA1 and minor AOP2 allele, which means there are only five haplotypes-three are missing. The figure shows the flowering time distribution and observed frequency for each of these haplotypes. Our model is that the minor GA1 and NDX1 alleles are tagging early-flowering alleles at these two loci, and that the minor AOP2 allele is associated with early flowering because it is the best single locus that tags both of these loci. Colors in the single-locus model correspond to those in the two-locus model.

to 45% B in 9 min was used for separation. Chromatograms were interpreted using TraceFinder (Thermo Fisher Scientific) and manually validated. The following transitions were used for relative quantitation: 3-hydroxypropyl glucosinolate m/z 376 → m/z 97; m/z 376 → m/z 259, 2-propenyl glucosinolate m/z 358 → m/z 97; m/z 358 → m/z 75, 4-hydroxybutyl glucosinolate m/z 390 → m/z 259; m/z 390 → m/z 75 and 3-(methylsulfanyl)propyl glucosinolate m/z 422 → m/z 259; m/z 422 → m/z 97; m/z 422 → m/z 75.

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ACKNOWLEDGEMENTS
The authors would like to thank Caroline Dean for sharing knowledge and seeds of the AINDF1 mutant. We also thank Ümit Selen for technical support with the data analysis, and Daniel J. Kliebenstein and Haijun Liu for comments and helpful discussions. The VBCF Metabolomics Facility is supported by the City of Vienna through the Vienna Business Agency.

COMPETING INTERESTS
The authors declare no competing interest.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41437-021-00456-3.
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