Title

AGO1 and HSP90 buffer different genetic variants in Arabidopsis thaliana

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Article Summary
Argonaute 1 (AGO1), a key player in plant development, interacts with the chaperone HSP90 which buffers environmental and genetic variation. We found that AGO1 buffers environmental and genetic variation in the same traits; however, AGO1-dependent and HSP90-dependent loci do not overlap. Detailed analysis of a buffered locus found that a non-functional HUA2 allele decouples days to flowering and rosette leaf number in an AGO1-dependent manner, suggesting the AGO1-dependent buffering acts at the network level.

Abstract
Argonaute 1 (AGO1), the principal protein component of microRNA-mediated regulation, plays a key role in plant growth and development. AGO1 physically interacts with the chaperone HSP90, which buffers cryptic genetic variation in plants and animals. We sought to determine whether genetic perturbation of AGO1 in Arabidopsis thaliana would also reveal cryptic genetic variation, and if so, whether AGO1-dependent loci overlap with those dependent on HSP90. To address these questions, we introgressed a hypomorphic mutant allele of AGO1 into a set of mapping lines derived from the commonly used Arabidopsis strains Col-0 and Ler. Although we identified several cases in which AGO1 buffered genetic variation, none of the AGO1-dependent loci overlapped with those buffered by HSP90 for the traits assayed. We focused on one buffered locus where AGO1 perturbation uncoupled the traits days to flowering and rosette leaf number, which are otherwise closely correlated. Using a bulk segregant approach, we identified a non-functional Ler hua2 mutant allele as the causal AGO1-buffered polymorphism. Introduction of a non-functional hua2 allele into a Col-0 ago1 mutant background recapitulated the Ler-dependent ago1 phenotype, implying that coupling of these traits involves different molecular players in these closely related strains. Taken together, our findings demonstrate that even though AGO1 and HSP90 buffer genetic variation in the same traits, these robustness regulators interact epistatically with different genetic loci, suggesting that higher-order epistasis is uncommon.

Introduction
Genetic networks rely on various types of feedback loops, redundancy, and other mechanisms like chaperones and small RNAs to ensure phenotypic robustness in spite of environmental or genetic perturbations (Rutherford and Lindquist 1998; Queitsch et al. 2002; Masel and Siegal 2009; Whitacre 2012; Leme et al. 2013; Lachowiec et al. 2018; Zabinsky et al. 2019). Network disruptions decrease environmental and developmental robustness and, dependent on their nature, increase phenotypic variation in a trait or affect organismal phenotypes more broadly. For example, non-lethal perturbation of the essential chaperone HSP90 broadly increases phenotypic variation in plants, fungi, and animals, with many organismal traits affected in a background-specific manner (Rutherford and Lindquist 1998; Queitsch et al. 2002; Yeyati et al. 2007; Sangster et al. 2008b; a; Jarosz and Lindquist 2010; Rohner et al. 2013; Karras et al. 2017; Zabinsky et al. 2019). When fully functional, HSP90 chaperones a select but highly diverse group of client proteins, including many conserved kinases, receptors and transcription factors that play crucial roles in development (Schopf et al. 2017). When chaperone function is perturbed, client proteins encoding genetic variants may fail to mature or fold differently, leading to pathway failure or rewiring (Dorrity et al. 2018) and hence altered phenotypes (Zabinsky et al. 2019). The phenomenon that HSP90 keeps genetic variation phenotypically silent and HSP90 perturbation allows its expression has become known as phenotypic capacitance (Rutherford and Lindquist 1998; Masel and Siegal 2009) – a different term for epistasis (Zabinsky et al. 2019). In
contrast to the traditional definition of epistasis, which describes the non-reciprocal interaction of two loci, phenotypic capacitance is an epistasis phenomenon in which one locus, e.g., HSP90, interacts with many others. It is noteworthy that non-lethal HSP90 perturbation can increase phenotypic variation even in the absence of genetic variation, presumably because subtle, undetectable differences in microenvironment, developmental stage, or cell states lead to inhibition of different client proteins among individuals in a seemingly stochastic manner (Zabinsky et al. 2019). However, the impact of impaired developmental stability in isogenic individuals upon HSP90 perturbation can differ by as much as an order of magnitude from that observed among genetically divergent individuals (Sangster et al. 2008b).

Another important source of developmental and environmental robustness is post-transcriptional regulation by small RNAs. Small RNAs regulate the expression of their target genes in a sequence-specific manner. In plants, most endogenous post-transcriptional gene regulation is mediated by AGO1 loaded with microRNAs (miRNAs, MIR) (Axtell 2013; Bologna and Voinnet 2014). In animals, some microRNAs are known to buffer stochastic (Hilgers et al. 2010), environmental (Li et al. 2009), and genetic variation (Cassidy et al. 2013). MicroRNAs play major roles throughout plant development, including in the onset of flowering, an irreversible developmental transition of outsized effect on reproductive success and plant fitness (Dong et al. 2022). In particular, the MIR156 and MIR172 gene families are essential for fine-tuning expression of the complex gene network that determines the number of days until flowering is initiated and the number of rosette leaves at this developmental stage. Misregulation or mutation of their gene targets alters both traits in the crucifer model Arabidopsis thaliana (Aukerman and Sakai 2003; Yamaguchi et al. 2009; Wu et al. 2009).

In Arabidopsis, the traits days to flowering (i.e., flowering time, onset of flowering) and rosette leaf number are so closely linked that the traits are often used interchangeably. This close correlation reflects the need for sufficient vegetative tissue (i.e., rosette leaves) to produce the resources for flowering and seed development. Because of the irreversible nature of the transition from the vegetative to the reproductive stage, the coupling of these traits is crucial for plant fitness. Uncoupling of flowering time and rosette leaf number occurs in some early and late flowering time mutants (Pouteau et al. 2004) and in response to treatment with nitrogen dioxide (Takahashi and Morikawa 2014); however, the mechanistic underpinnings for this uncoupling remain unknown.

Studies in several organisms suggest that AGO proteins are chaperoned by HSP90. HSP90 physically interacts with AGO proteins in yeast (Wang et al. 2013; Okazaki et al. 2018), flies (Iwasaki et al. 2010; Miyoshi et al. 2010; Gangaraju et al. 2011), humans (Johnston et al. 2010; Gangaraju et al. 2011), Tetrahymena (Woehrer et al. 2015), and plants (Iki et al. 2010, 2012). Because miRNAs buffer environmental and genetic perturbations and AGO1 interacts with HSP90, we set out to investigate the extent to which AGO1 perturbation affects phenotypic variation in isogenic Arabidopsis seedlings and buffers genetic variation in divergent backgrounds, and AGO1-dependent loci overlap with HSP90-dependent loci. We find that AGO1 perturbation can significantly increase phenotypic variation in morphological and quantitative traits in isogenic seedlings. AGO1 perturbation also buffers the phenotypic effects of genetic variation between two divergent backgrounds. However, none of the AGO1-buffered loci overlapped with those buffered by HSP90, consistent with a prevalence of first-order epistatic
interactions relative to higher-order epistasis. Lastly, our detailed investigation of one such buffered locus reveals that the coupling of the fitness-relevant traits days to flowering and rosette leaf number relies on different molecular players in these commonly used strains of *Arabidopsis*.

**Results**

**Genetic perturbation of AGO1 increases phenotypic variation in isogenic *Arabidopsis* seedlings**

To determine if perturbation of AGO1 leads to increased phenotypic variation in isogenic seedlings, we examined several morphological and quantitative traits of two hypomorphic *ago1* mutants, *ago1*-46 (Smith *et al.* 2009), and *ago1*-27 (Morel 2002), the former being a less severe mutant than the latter. Ten-day old isogenic seedlings of *ago1*-46 and *ago1*-27 showed increased phenotypic variation in morphological traits such as lesions in cotyledons (Mason *et al.* 2016), rosette symmetry, and organ defects compared to isogenic wild-type seedlings ([Figure 1, Supplemental Table 1](#)). As expected, the more severe *ago1*-27 mutant showed more abnormal phenotypes than the less severe *ago1*-46 mutant. Next, we examined hypocotyl length in the dark, a quantitative trait that shows increased variation in response to HSP90 perturbation (Queitsch *et al.* 2002; Sangster *et al.* 2008b). Similar to our previous results (Queitsch *et al.* 2002; Sangster *et al.* 2008b), *ago1*-27 dark-grown seedlings showed a different mean value (p < 2.3e-16, Wilcoxon test) and significantly greater variance of hypocotyl length than wild-type seedlings (p = 0.0002, Levene’s test) ([Figure 1B, Supplemental Table 1](#)). The less severe *ago1*-46 seedlings also showed a different mean value (p-value = 3.6e-05, Wilcoxon test) and greater variance of hypocotyl length compared to wild-type seedlings (p-value = 0.00004, Levene’s test). Based on these results, AGO1 maintains phenotypic robustness and buffers developmental noise among isogenic seedlings in a manner similar to HSP90.

**AGO1 buffers genetic variation independent of HSP90**

We next tested whether AGO1 perturbation could reveal cryptic genetic variation and whether *AGO1*-dependent loci overlapped with those buffered by HSP90. To do so, we introgressed the hypomorphic *ago1*-27 allele into Col-0 lines with single chromosome substitutions from another, genetically divergent *Arabidopsis* strain, Landsberg erecta (Ler) (STAIRS, STepped Aligned Inbred Recombinant Strains, [Figure 2A, Supplemental Table 2](#)). STAIRS lines have been generated for chromosomes 1, 3 and 5 (Koumproglou *et al.* 2002). Since *AGO1* is located on chromosome 1, we excluded these STAIRS lines from our analysis. For chromosomes 3 and 5, we selected two STAIRS lines each (chr3; N9448 and N9459, chr5; N9472 and N9501). The introgressed lines were genotyped to confirm the integrity of the respective Ler segments.

We measured hypocotyl and root length, rosette diameter, and the closely correlated traits days to flowering and rosette leaf number across many individual plants per line using a randomized experimental design ([Supplemental Table 2](#)). We selected these traits because they are readily measurable and show evidence of HSP90-buffered variation in our previous studies of Col-0/Ler mapping lines (Sangster *et al.* 2008a; Sangster *et al.* 2008b). Specifically, three previously described HSP90-dependent loci within the Ler segments of the tested STAIRS lines affect the traits measured here (Sangster *et al.* 2008b; a).

*AGO1* perturbation may alter the contribution of a cryptic genetic variant to a quantitative trait in two ways: first, *AGO1* perturbation may reveal a genetic variant by increasing its contribution to
a trait; or second, AGO1 perturbation may conceal a genetic variant by increasing the relative contribution of others. Indeed, the phenomenon of revealing and concealing genetic variation has been previously observed for HSP90 perturbation across many traits in Arabidopsis recombinant inbred lines (Sangster et al. 2008a). In addition, genetic variation in the respective Ler segments may mask the phenotypic differences observed between Col-0 wild-type and the ago1-27 mutant that was generated in the Col-0 background (i.e., Ler segments may epistatically interact with ago1-27). We observed all three scenarios of epistasis (Figure 2B, C, Supplemental Figures 2,3). Despite the strong evidence that HSP90 facilitates AGO1 function in many organisms, including plants, no overlap of AGO1-dependent loci with HSP90-dependent loci was observed.

Perturbation of AGO1 uncouples flowering time and rosette leaf number in a background-specific manner

One AGO1-buffered locus showed dramatically different effects on the two closely correlated traits days to flowering and rosette leaf number (Figure 3A-C). Arabidopsis plants add about one rosette leaf per day until flowering is initiated. On average, Col-0 wild-type plants initiated flowering ~5 days later and added ~5 more leaves than the STAIRS line 9472 that carries a Ler segment on chromosome 5 (coordinates 1 – 9,479,000bp). This result was expected because the Ler segment in this STAIRS line encompasses the FLOWERING LOCUS C (FLC) gene (Figure 3D) which is non-functional in the Ler strain (Michaels et al. 2003; Liu et al. 2004). FLC is a strong repressor of flowering (Whittaker and Dean 2017). In the Col-0 background, ago1-27 plants initiated flowering ~9 days later and added ~2 more leaves, albeit the traits were less tightly correlated than in wild type (Figure 3C, compare blue and green dots). In stark contrast, in the STAIRS 9472 background, ago1-27 plants showed no change in the number of days to flowering; however, these plants showed dramatically fewer leaves at the onset of flowering, developing on average only ~4 leaves. In fact, the severity of the rosette leaf number phenotype of STAIRS9472;ago1-27 was comparable to that observed in loss-of-function early flowering mutants (Pouteau et al. 2004; Undurraga et al. 2012). In short, AGO1 perturbation in the STAIRS line specifically affected the trait rosette leaf number while not affecting the trait days to flowering.

The close correlation of the traits days to flowering and rosette leaf number traits relies on FLC in the Col-0 background

The Ler fragment in STAIRS9472 encompasses several known flowering time genes, including FLC which delays flowering by repressing the gene FLOWERING LOCUS T (FT). FLC expression is repressed when plants are exposed to cold temperatures for a prolonged period of time (i.e., vernalization or winter period), allowing FT expression and onset of flowering (Andrés and Coupland 2012; Whittaker and Dean 2017). Genetic variation in FLC and in FRIGIDA (FRI), a positive regulator of FLC, accounts for the vast majority of differences in flowering time across Arabidopsis strains (Shindo et al. 2005; Kim et al. 2009; Bloomer and Dean 2017). Many Arabidopsis strains do not require vernalization to initiate flowering because they carry FLC mutations, as is the case for Ler, or FRI mutations, as is the case for Col-0. The STAIRS9472 line carries the non-functional Ler FLC allele.

We wondered if the lack of functional FLC in STAIRS9472;ago1-27 contributed to its unusual phenotype. To test this possibility, we examined the consequences of repressing FLC through vernalization for both flowering time traits in Col-0 wild-type, STAIRS9472, Col-0 ago1-27 and
STAIRS9472; ago1-27 plants (Supplemental Figure 3). Vernalization did not erase the difference in rosette leaf number between Col-0 ago1-27 and STAIRS9472; ago1-27 plants, with the latter still showing significantly fewer leaves (p = 5.704e-12 Wilcoxon test). However, vernalization uncoupled both flowering time traits in an AGO1-dependent manner in the Col-0 background. Although vernalized ago1-27 plants initiated flowering ~5 days later than vernalized Col-0 wild-type plants, they added ~4 fewer leaves rather than more leaves. We conclude that the close association of days to flowering and rosette leaf number in the Col-0 background requires the presence of FLC and functional AGO1. Perturbation of AGO1 alone diminished the close correlation between both traits but did not reverse it. Ler and other natural FLC mutants must have rewired flowering time pathways such that the traits days to flowering and rosette leaf number remain closely correlated in the absence of functional FLC.

MIR156 polymorphisms are unlikely to cause AGO1-dependent phenotype
To identify the causal polymorphism(s) underlying the AGO1-dependent STAIRS9472 phenotype, we examined other genes within the Ler segment with functions in flowering time (Song et al. 2013, 2015; Spanudakis and Jackson 2014) and candidate polymorphisms between Col-0 and Ler (Nordborg et al. 2005; Borevitz et al. 2007; Ossowski et al. 2008) (Figure 3D). We measured expression of these candidate genes among the four genotypes Col-0 wild-type, STAIRS9472, Col-0 ago1-27 and STAIRS9472; ago1-27; for the three MIR156 genes (e, d, f), and MIR172b, we measured expression of major target genes (Ji et al. 2011). As expected, FLC expression was barely detectable in STAIRS9472 and STAIRS9472; ago1-27 plants (Figure 3D), consistent with the known disruption of FLC in Ler (Michaels et al. 2003; Liu et al. 2004). FLC expression increased in Col-0 ago1-27 plants relative to Col-0 wild-type plants, consistent with the late flowering phenotype of the former genotype. As a general trend, target genes of MIR156 increased in expression in the STAIRS ago1-27 background compared to target gene expression in the Col-0 ago1-27 background, suggesting that MIR156 may be less functional in the STAIRS line. MIR156 represses the expression of several SQUAMOSA PROMOTER BINDING LIKE (SPL) transcription factors (miR156-SPL module, Figures 3E, 5D) that regulate flowering by activating and repressing other transcription factors and miRNAs (Aukerman and Sakai 2003; Yamaguchi et al. 2009; Wu et al. 2009). Overexpression of MIR156 leads to delayed onset of flowering with many more rosette leaves (Wu et al. 2009; Xu et al. 2016), suggesting that less functional MIR156 may diminish rosette leaf number.

We searched for Ler-specific polymorphisms in the MIR156 genes in available genome assemblies and found a predicted single nucleotide polymorphism (SNP) within the loop of MIR156f. Resequencing of all three MIR156 genes confirmed this SNP and identified an additional deletion of 14 nucleotides near the base of the stem loop. As the MIR156 genes are highly conserved in the plant kingdom (Cuperus et al. 2011; Luo et al. 2013), we examined their natural variation among other Arabidopsis strains, sequencing an additional 55 strains. Of all sequenced strains, 42 carried the Ler-specific C-to-T SNP, one carried a C-to-G SNP, and 32 carried the 14-nt deletion (Supplemental Figure 2). The presence or absence of the deletion was highly correlated with the presence or absence of the SNP (R² = 0.3506, p = 0.0007, Pearson correlation test). To address whether either one or both Ler-specific MIR156f polymorphisms affect rosette leaf number, we tested for association with this trait across these accessions (phenotypic data from (Lempe et al. 2005)). No association was found. Although this result did
not rule out the MIR156f polymorphisms as the causative AGO1-dependent alleles, it made it less likely that these polymorphisms would explain the unusual STAIRS9472;ago1-27 phenotype.

Identifying the AGO-1 dependent Ler-specific polymorphism with a bulk segregant analysis.

To identify the Ler-specific variant(s) causing the observed trait uncoupling in STAIRS9472;ago1-27 plants, we used a classic bulk segregant analysis followed by high-throughput sequencing (Cuperus et al. 2010; Sun and Schneeberger 2015). To generate a segregating population for the tested alleles, we crossed STAIRS9472 with ago1-27, and allowed for selfing to generate F₂ seeds. F₂ plants were measured for days to flowering and the number of rosette leaves at this point (Figure 4A). From this F₂ experiment, we pooled plants based on phenotype, defining the STAIRS9472:ago1-27 phenotype as plants with 6 or fewer rosette leaves (Figures 3A-C, 4A), and isolated their DNA. We combined equal DNA amounts for 100 plants with the AGO1-dependent STAIRS9472 phenotype and 100 plants with higher numbers of rosette leaves. Using short-read sequencing, we aligned reads to the relevant chromosome 5 segment using SHOREmap (Sun and Schneeberger 2015), relying on the many known polymorphisms between Ler and Col-0 to distinguish Ler- and Col-0-specific reads. If successful, bulk segregant analysis will show increasing enrichment of homozygosity near the causal locus, with the causal locus at the center of a peak region (Salathia et al. 2007; Schneeberger et al. 2009; Cuperus et al. 2010; Sun and Schneeberger 2015). This mapping approach works best if variation at a single locus causes a segregating phenotype, and if phenotypes can be clearly distinguished from each another in order to pool samples with high confidence.

Although our phenotype of interest was quantitative in nature, i.e., a range of leaf numbers rather than an absence or presence of a feature, we observed a skew towards Ler alleles on chromosome 5 with a SHOREmap peak region at chr5:7,600,000 to chr5:7,800,000 (Figure 4B). Of the known flowering time-associated genes, only one fell in this peak region, HUA2 (AT5G23150). Some Ler backgrounds, including the STAIRS9742 line, carry a premature stop codon mutation in HUA2, likely disrupting function (Zapata et al., 2016). HUA2 function is less well characterized than that of other flowering time genes; however, hua2 mutants in a Col-0 background show reduced FLC levels and fewer rosette leaves at onset of flowering (Doyle et al. 2005). MIR156f did not reside in the peak region, consistent with the previously described lack of genotype-phenotype association.

To confirm that loss of functional HUA2 was responsible for the AGO1-dependent phenotype in STAIRS9472, we used a Col-0-derived hua2 mutant allele, hua2-4, and generated a double mutant hua2-4;ago1-27 in the Col-0 background. We predicted that this homozygous double mutant would exhibit the uncoupling of days to flowering and rosette leaf number traits observed in the STAIRS9472; ago1-27 line. Using a segregating F₂ population, we simultaneously measured days to flowering and rosette leaf number, and genotyped each plant (Figure 5A-C). The hua2-4 single mutant plants and the hua2-4;ago1-27 double mutant plants showed no significant difference in days to flowering but rosette leaf number was markedly reduced in double mutant plants, recapitulating our original finding with STAIRS9472:ago1-27 plants. The observed uncoupling of these traits was independent of FLC which is not disrupted in the hua2-4;ago1-27 double mutant. This result suggests that the Ler-specific, non-functional hua2 allele
may have arisen to compensate for the Ler-specific FLC disruption in order to maintain the close association of days to flowering and rosette leaf number traits.

**HUA2 effects on gene expression suggest SPL4 as a likely HUA2 target.**

To understand in more detail how HUA2 affects the complex flowering gene network, we conducted RNA-seq experiments examining wild-type Col-0, single mutants hua2-4 and ago1-27 and hua2-4;ago1-27 double mutant seedlings. As expected, ago1-27 mutants and Col-0 wild-type showed differential expression of many miRNA target genes (Supplemental Table 6). The expression of the known HUA2 targets FLC and FLOWERING LOCUS M (FLM, MAF1) was reduced in both the single hua2-4 mutant and the hua2-4;ago1-27 double mutant seedlings, excluding them as sources of the AGO1-dependent phenotype.

However, the comparison of ago1-27 and hua2-4;ago1-27 plants showed strong upregulation of SPL4 expression in the latter (Figure 5D), consistent with our finding that SPL4 was strongly upregulated STAIRS9472:ago1-27 (Figure 3E). Other important flowering time genes were also differentially expressed in hua2-4;ago1-27 double mutant plants, including the master regulator FT, LATE ELONGATED HYPOCOTYL (LHY), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), AGAMOUS-LIKE 8 (AGL8, FRUITFUL), and MIR159b (Figure 5D). These genes interact in complex ways to control the transition to flowering (Figure 5E). Because HUA2 is involved in mRNA processing and splicing (Chen and Meyerowitz 1999; Cheng et al. 2003; Janakirama 2013), we speculate that SPL4 may be one of its targets. SPL4 has three splice isoforms, and two of them (SPL4-2, SPL4-3) lack a miR156-binding site (Yang et al., 2012). Overexpression of SPL4-1, the splice form with the miR156 binding site, does not affect days to flowering but decreases rosette leaf number. In contrast, overexpression of SPL4-2 or SPL4-3 decreases days to flowering and reduces rosette leaf number (Yang et al. 2012). In a hua2:ago1 double mutant background the balance of SPL4 splice forms may be altered, which together with the absence of functional AGO1 disrupts the close correlation of days to flowering and rosette leaf number.

**Discussion**

Here we show that AGO1, the principal player in miRNA-mediated control of gene expression in plants, buffers micro-environmental variation and maintains developmental stability in isogenic Arabidopsis seedlings. Compared to wild-type Col-0 plants, ago1 mutant seedlings showed more lesions on cotyledons (Mason et al. 2016), more rosette symmetry defects and abnormal organs, and increased variation in hypocotyl length of dark-grown seedlings. Given the crucial role that miRNAs play in plant development, these results are not altogether surprising. MicroRNAs can impact developmental stability, i.e., the accuracy with which a given genotype produces a trait in a particular environment, in various ways (Hornstein and Shomron 2006; Voinnet 2009). For example, miRNAs can buffer gene expression noise as part of incoherent feedforward loops, in which a transcription factor will activate both the expression of a target gene X and a miRNA, with the latter repressing target gene X (Hornstein and Shomron 2006; Voinnet 2009). MicroRNAs enforce developmental patterning decisions through mutual exclusion and spatial or temporal restrictions in expression, e.g., by suppressing fate-associated transcription factors in neighboring cells or at a certain time in development (Hornstein and Shomron 2006; Voinnet 2009).
In plants, we previously reported increased variation for the same traits in isogenic seedlings upon perturbation of the chaperone HSP90 (Queitsch et al. 2002; Sangster et al. 2008b), consistent with the reported functional relationship of HSP90 and AGO1 (Iki et al. 2010, 2012; Iwasaki et al. 2010, 2015; Naruse et al. 2018). An exception was the peculiar environmentally-responsive lesions found on cotyledons in ago1-27 seedlings (Mason et al. 2016). These lesions are dead tissue due to an aberrant hypersensitive response driven by the plant defense hormone jasmonate (Mason et al. 2016). Double mutants of ago1-27 and coronatine insensitive1, the jasmonate receptor, show greatly decreased frequency of lesions. HSP90 perturbation significantly upregulates jasmonate signaling (Sangster et al. 2007). However, HSP90 single mutants produce far fewer seedlings with lesions than ago1-27 mutants, and double mutants show many more lesions than ago1-27 single mutants, inconsistent with simple epistasis. Thus, we previously suggested that AGO1 is a major, but largely HSP90-independent, factor in providing environmental robustness to plants.

In addition to maintaining developmental stability, HSP90 also buffers genetic variation in plants, fungi, and animals, including humans (Zabinsky et al. 2019). The hypothesized mechanism by which HSP90 overcomes the presence of genetic variation is the chaperone’s well-characterized function in protein folding and maturation (Sangster et al. 2004; Jarosz et al. 2010; Zabinsky et al. 2019). This hypothesis is supported by the reported differences among disease-associated protein variants chaperoned by HSP90 versus those chaperoned by HSP70 (Karras et al. 2017). HSP70-dependent variants affect protein folding and hence disease more severely, consistent with HSP70’s general role in protein folding compared to HSP90’s more selective role in protein maturation. Moreover, across thousands of humans, kinases that are HSP90 clients tend to carry more amino acid variants than non-client kinases, and these amino acid variants are predicted to be more damaging to protein folding (Lachowiec et al. 2015). HSP90-buffered variants in Ste12, a yeast transcription factor that governs mating and invasion, reside in two positions that are close to one another and alter charge and DNA binding, possibly because they alter Ste12 dimerization (Dorrity et al. 2018). HSP90-dependent Ste12 variants are not simply unstable, because while cells carrying them no longer mate, they are hyper-invasive, consistent with altered protein folding.

In contrast, it is harder to envision a simple, direct mechanism by which AGO1 overcomes the presence of genetic variation in either miRNAs or their targets, unless such buffering involves AGO1’s close relationship with HSP90 for the latter. Although we observed several instances in which AGO1 perturbation revealed and concealed genetic variation in the same traits in which we previously found HSP90-dependent variation (Sangster et al. 2008b), there was no overlap in the genetic loci buffered by AGO1 and HSP90. While this result was consistent with our study of the AGO1-dependent lesions (Mason et al. 2016), it raised anew the question as to how AGO1 may buffer genetic variation. In flies, proper expression of mir-9a, a miRNA acting on the transcription factor Senseless, buffers genomic variation (Cassidy et al. 2013). Reducing mir-9a regulation of Senseless leads to phenotypic variation in sensory cell fate in genetically diverse flies, with candidate causal variants in genes that belong to the Senseless-dependent proneural network governing sensory organ fate. In other words, in this case, AGO1-dependent buffering via mir-9a occurs at the network level, consistent with the mechanisms by which miRNAs buffer development stability and micro-environmental fluctuations.
To fully understand an instance of AGO1-dependent genetic variation, we focused on the uncoupling of the traits days to flowering and rosette leaf number in STAIRS9472. Both traits involve the mir156-SPL module and the key players *FLC* and *FRI* (Fig. 5D). We show that in the Col-0 background the coupling of these traits requires functional *FLC* and *AGO1*. In STAIRS9472, *FLC* is non-functional because the gene resides in the Ler-introgression segment. Without *FLC*, how does wild-type Ler couple days to flowering and rosette leaf number? Using bulk segregant analysis, we identified the non-functional *hua2* Ler-allele as the likely causal AGO1-dependent polymorphism. Indeed, we were able to recapitulate the uncoupling phenotype in the *hua2-4; ago1-27* double mutant in the Col-0 background.

We speculate that the non-functional *HUA2* allele arose in Ler to compensate for the background’s nonfunctional *FLC* allele and maintain the fitness-relevant coupling of both traits. Other Arabidopsis accessions with nonfunctional *FLC* genes must have found the same or other solutions to this fitness challenge. Our expression analysis offered some clues as to how *HUA2* may facilitate the close coupling of days to flowering and rosette leaf number (Figure 5D, E). Comparing gene expression in *ago1-27* and *ago1-27; hua2-4* plants, we found that the mir156-SPL module gene *SPL4* was highly upregulated in the double mutant. *SPL4* is expressed in three splice isoforms (*SPL4-1, SPL4-2, SPL4-3*) with only one, *SPL4-1*, regulated by miR156 (Yang et al., 2012). Overexpression of *SPL4-1* in transgenic plants does not alter days to flowering but reduces rosette leaf number. In contrast, overexpression of *SLP4-2* or *SPL4-3* decreases both days to flowering and reduces rosette leaf number (Yang et al. 2012). Because *HUA2* functions in mRNA processing and splicing (Chen and Meyerowitz 1999; Cheng et al. 2003; Janakirama 2013), *SPL4* may be one of its targets. Non-functional *HUA2* may lead to increased presence of the *SPL4-1* splice form, which is exacerbated when mir156-dependent suppression of *SPL4-1* fails in the *ago1-27; hua2-4* double mutant, disrupting the close correlation of days to flowering and rosette leaf number (Figure 5E). Thus, similar to the scenario in flies (Cassidy et al. 2013), AGO1 appears to buffer genetic variation via microRNA-dependent network connections in plants. Disruption of the miRNA-dependent network path in *ago1*-mutants can reveal genetic variants such as the non-functional *HUA2* allele in other paths controlling the same trait (Figure 5E).

Taken together, our study holds several important lessons. AGO1 buffers phenotypic variation in isogenic seedlings and genetic variation in genetically divergent ones. AGO1 does so independently of the chaperone HSP90 despite their close functional relationship, suggesting that epistasis is largely a first-order phenomenon. AGO1 and HSP90 are only partially inhibited in these studies because they are essential genes; this essentiality may impact these results. AGO1 suppresses the phenotypic consequences of genetic variation by enabling miRNA-dependent network paths rather than acting directly on variant-containing molecules. Lastly, we show that key pathways can involve different molecular players even in closely related strains of the same species. The uncoupling of highly correlated traits could be a useful tool for plant breeders who want to improve one trait without compromising another tightly coupled trait. Our study suggests miRNAs as good candidates for such targeted breeding and engineering efforts.
Plant Materials and Growth Conditions: The following parental lines were used: Col-0, ago1-27 in the Col-0 background, and STAIRS N9448, N9456, N9472, N9501 (Morel 2002; Koumproglou et al. 2002). ago1-27 plants were crossed into the STAIR lines and F2’s that carried the wild-type and ago1-27 allele in both Col-0 and the STAIRS backgrounds were isolated. Selected F2’s and their progeny were used to perform the described experiments. For the hypocotyl and root length assays, the plants were grown on MS media containing 0.0005% MES hydrate, 0.004% vitamin solution, 3% phytoagar, and 1% sucrose.

Genotyping of F2 plants: We used PCR to genotype the F2’s from each STAIRS – ago1-27 cross. PCR conditions for ago1-27 genotyping is as follows: 5’ at 94 °C, followed by 35 cycles at 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C. PCR product was then digested at 37°C with Bsp1286I, which cuts wild-type sequence. PCR conditions for MIR156F genotyping is as follows: 2’ at 95°C, followed by 35 cycles at 30 s at 94°C, 50 s at 57°C, 40 s at 72°C.

Hypocotyl and root length assays. Seeds from different genotypes were plated on agar plates (10 seeds/per plate, equally spaced). The plates were stacked in racks to ensure vertical position, wrapped in aluminum foil, and transferred to 4°C for five days to promote germination. They were then unwrapped, and exposed to light for two hours. After that, the plates were wrapped in aluminum foil again, to prevent further light exposure and were transferred to a 23°C tissue culture incubator for seven days. The plants were grown vertically. After that, the plates were taken out, and photographed. The photographs were used to measure the seedlings’ hypocotyls and roots using the ImageJ software (http://rsbweb.nih.gov/ij/).

Early morphology traits analysis. Seeds from the different genotypes were plated on agar (36 seeds/per plate). The plates were wrapped in aluminum foil and transferred to 4°C for five days. Plates were unwrapped and transferred to long days (LD) in 23°C tissue culture incubator for 10 days. The plants were grown horizontally. The plates were rotated every day to prevent biases due to location in the incubator. On the 10th day, the seedlings were scored for their morphological traits.

Flowering time experiments: Seeds from different genotypes were embedded in 1ml of 0.1% agar, and then stratified for five days at 4°C. They were sown on soil in 36-pot trays. Flowering time was measured by scoring both the number of rosette leaves and days to flowering when the primary inflorescence of the plant had reached a height of 1cm. Flowering time experiments were performed in long days (LD, 16 hours of light, 8 hours of dark), at 23°C.

Rosette diameter measurements: The diameter of the rosette was measured on the day that the primary inflorescence of the plant reached a height of 1cm.

Vernalization treatment: Seeds were stratified for five days at 4°C and then sown on soil. They were allowed to grow for five days at 23°C in LD or short days (SD) conditions and then transferred to 4°C for forty days, according to recommendations from Sung et al., 2006 (Sung et al. 2006).

Gene expression analysis. To determine the expression levels via qPCR, total RNA was isolated from the aerial parts of 14-day old plants at ZT16 using the SV Total Isolation System.
RNA quality was determined using a Nanodrop and only high-quality samples (A260/A230 > 1.8 and A260/A280 > 1.8) were used for subsequent qPCR experiments. To remove possible DNA contamination, RNA was treated with DNaseI (Ambion) for 60 minutes at 37˚C. We used the Transcriptor First Strand cDNA Synthesis Kit (Roche) for cDNA synthesis. The qPCR primers were designed using the Universal Probe Library Assay Design Center tool (Roche), and Primer3 (Untergasser et al. 2012). Specific amplification was confirmed before conducting the qPCR experiments. The qPCRs experiments were carried out in 96-well plates with a LightCycler480 (Roche) using SYBR green. The following program was used for the amplification: pre-denaturation for 5 min at 95˚C, followed by 35 cycles of denaturation for 15 s at 95˚C, annealing for 20 s at 55˚C, and elongation for 30 s at 72˚C. All qPCR experiments were carried out with two biological replicates (independent samples harvested on different days), and with three technical replicates per sample.

RNA-seq samples were prepared similarly as for qPCR, and then using the Illumina stranded Tru-seq kit following the standard protocol. Samples were sequenced using the Nextseq550 platform. We used TopHat (v2.1.2) to align RNA-seq reads to the TAIR10 genome annotation (Trapnell et al. 2009), htseq-count (v0.12.4) to calculate counts per gene (Anders et al. 2015), using a minimum map quality of 10 and Cuffdiff (v2.2.1) to generate FPKMs (Trapnell et al. 2013), and DESeq2 to identify differentially expressed genes among genotypes (Love et al. 2014).

**Sequencing of miR156F, D and E in diverse A. thaliana strains.** The genes MIR156F, MIR156D and MIR156E were amplified using the primers listed on Supplemental Table 7. PCR products were sequenced by the Sanger method. The sequences were aligned using T-coffee.

**Bulk segregant analysis – library preparation and sequencing:** Approximately 400 F2 plants were sown, and leaf samples of equal size were collected from 100 plants that resembled the STAIRS9472; ago1-27 phenotype (6 leaves or fewer at flowering) and 100 plants with a greater number of leaves. Individual plants were genotyped. In parallel, leaf samples were collected for all genotypes. DNA was extracted using CTAB extraction (Weigel and Glazebrook 2002) and quantified using the Qubit HS dsDNA assays. Libraries were quality checked on the Agilent 2100 bioanalyzer using a DNA 1000 chip (Agilent). Samples were pooled and libraries were generated using the Nextera sample kit according to the manufacturer’s instruction. DNA concentration of the amplified libraries was measured with the DNA 1000 kit as well as the DNA high-sensitivity kit for diluted libraries (both Agilent). Samples were sequenced on an Illumina Nextseq in a 75-bp paired-end run.

**Bulk segregant analysis – data analysis:** Using the function SHORE import, raw reads were trimmed or discarded based on quality values with a cutoff Phred score of +38. After correcting the paired-end alignments with an expected insert size of 300 bp, we applied SHORE consensus to identify variation among mutants and reference. We applied SHOREmap using the included Ler/Col-0 SNPs. Plot boost was applied to further define a mapping interval.

**Accession Numbers**
All RNA sequencing reads were deposited in the NCBI Sequence Read Archive under the BioProject accession PRJNA836875.
Acknowledgements

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Figure Legends:

Figure 1. Perturbation of AGO1 increases phenotypic variation among isogenic seedlings. A) Early seedling trait measures for wild-type (Col-0 WT), ago1-46, and ago1-27 seedlings. Ten-day-old seedlings were scored for three different morphological traits: Lesions, asymmetrical rosettes, and organ defects. The data represent two biological replicates (two replicates, n = 144 for ago1 mutants, and n = 216 for Col-0 WT, *p<0.05, ttest). (B) Hypocotyl mean length and variance differ between wild-type and ago1-mutant seedlings. Hypocotyl length was measured for seven-day old, dark-grown seedlings. ago1-27 mutant seedlings showed greater variance than Col-0 wild-type seedling in hypocotyl length (Levene’s test, p< 1.0E-03; n = 475 for ago1-27, n = 486 for Col-0 WT). Inset: boxplots of hypocotyl length means. Y-axis represents hypocotyl length (mm), **p< 1.0E-15, Mann-Whitney Wilcoxon test.

Figure 2. Perturbation of AGO1 buffers genetic variation. (A) Experimental design to examine the phenotypic consequences of genetic variation within the Stepped Aligned Inbred Recombinant Strains (STAIRS) in the context of the ago1-27 mutation. (B) Summary of examined quantitative traits with evidence for AGO1-dependent or Ler-dependent variation in each tested STAIRS line. AGO1 perturbation reveals a cryptic genetic variant if this variant’s contribution to a quantitative trait can be detected only in an ago1-mutant background. AGO1 perturbation conceals a genetic variant if this variant’s contribution to a quantitative trait can no longer be detected in an ago1-mutant background. Genetic variation in the respective Ler segments can epistatically interact (i.e., mask) the phenotypic differences observed between Col-0 wild-type seedlings and ago1-27 mutant seedlings in the Col-0 background. For STAIRS line N9472, 78 seedlings were measured for hypocotyl length in the dark, for STAIRS lines N9448, N9459, and N9501 100 seedlings were measured for this trait. At least 32 plants were measured for all other traits. See Supplemental Tables 2 and 3 for traits values and assessment of significance. (C) Two examples of AGO1-dependent and one example of Ler-dependent genetic variation are shown for three different traits. Blue, Col-0 WT; Yellow, STAIRS; Red, ago1-27 in the Col-0 background; Green, ago1-27 in a STAIRS background.

Figure 3. AGO1 perturbation uncouples the traits days to flowering and rosette leaf number. Plants for Col-0 WT, STAIRS9472, Col-0 ago1-27 and STAIRS9472; ago1-27 were grown in a random block design in long days, n = 30-36. Days to flowering were recorded and rosette leaf numbers at the onset of flowering were counted. Blue, Col-0 WT; Yellow, STAIRS9472; Red, ago1-27 in the Col-0 background; Green, ago1-27 in the STAIRS9472 background (A) Days to flowering. The ago-1 mutant flowered ~9 days later than Col-0 WT (p=5.51E-12, Mann-Whitney Wilcoxon test); no significant difference was found between STAIRS9472 and the ago-1 mutant in the STAIRS9472 background (p=0.4714, Mann-Whitney
Wilcoxon test). The Ler introgression in STAIRS9472 was epistatic to ago1-27 \( *p<0.0001 \), Mann-Whitney Wilcoxon test. (B) Rosette leaf number. Col-0 WT plants showed fewer leaves than ago1-27 mutant plants, consistent with the mutant’s late flowering phenotype. In the STAIRS 9472 background, ago1-27 mutant plants showed no change in the number of days to flowering; however, these plants developed significantly fewer leaves (\( p=3.45E-12 \), Mann-Whitney Wilcoxon test). (C) Scatter plot of the two measured traits days to flowering and rosette leaf number in the four tested genotypes. The traits were less well correlated in the ago-1 mutant in the Col-0 background (compare blue and green dots); however, the normally tight correlation was fully lost in the STAIRS background (compare red and yellow dots). (D) Known flowering time genes are residing within the Ler chromosome 5 region of the STAIRS9472 line. (E) Quantitative PCR measurements for candidate gene expression. TFL1 and SPL4 were significantly increased in expression in the STAIRS9472;ago1-27 background. 14-day old plant tissue was collected at ZT16 (Zeitgeber 16; 16 hours after dawn). Mean expression data represent two biological replicates, each with three technical replicates. Standard error is indicated. (* = \( p < 0.05 \), ** = \( p < 0.005 \), T-test).

**Figure 4: Bulk segregant analysis identifies the non-functional Ler hua2 allele as a candidate AGO1-dependent locus.** (A) F2 plants from ago1-27 x STAIRS9472 cross were grown in long days, phenotypes were recorded, and plants were genotyped for the ago1-27 allele. For bulk segregant analysis, we selected plants that were homozygous for the ago1-27 mutation and flowered with six or fewer leaves (\( n=100 \)), resembling the AGO1-dependent STAIRS9472 phenotype. Representative F2 ago1-27 plants at flowering are shown. Scale bar = 1cm. (B) Bulk segregant analysis. Red dots represent Ler allele frequencies on chromosome 5 (bp, x-axis). Allele frequencies (y-axis) were estimated as the fraction of reads supporting a Ler allele divided by the number of reads mapping to that locus. Dashed blue line represents sliding window-based allele frequencies as estimated by SHOREmap. Dashed black line represents window-based plot boost as estimated by SHOREmap. The Ler hua2-5 allele emerged as the candidate AGO1-dependent locus because Ler allele frequencies were highest at this locus compared with other regions on chromosome 5. (D) F2 plants homozygous for the ago1-27 mutation with six or fewer leaves at flowering were PCR genotyped for alleles at FLC, HUA2, and MIR156f loci. The near perfect enrichment of Ler hua2-5 allele validates the result of our bulk segregant analysis.

**Figure 5: The ago1-27; hua2-4 double mutant uncouples the traits days to flowering time and rosette leaf number in the Col-0 background.** An F2 population segregating for the ago1-27 and hua2-4 mutant alleles was grown in long days. Days to flowering were recorded and rosette leaf numbers at the onset of flowering were counted. Grey, Col-0 WT; white ago1-27 parent Red, ago1-27;HUA2+/- F2; Blue, hua2-4 parent; Green, ago1-27;hua2-4. See Supplemental Table 5 for further details. (A) Plants carrying a homozygous ago1-27 allele flowered \( ~8.6 \) days later than Col-0 WT with \( ~16 \) leaves. Plants carrying a homozygous hua2-4 allele initiated flowering \( ~4.5 \) days earlier than Col-0 WT. As observed for STAIRS9472;ago1-27, the hua2-4 mutant allele was epistatic to ago1-27. \( *p<0.0283, **p<1.0E-06 \), Mann-Whitney Wilcoxon test. The double mutant ago1-27;hua2-4 plants showed a similar mean value but greater trait variance. (B) The rosette leaf number phenotype of the double mutant ago1-27;hua2-4 plants resembles that of the STAIRS9472;ago1-27 line. ago1-27;hua2-4 plants flower with 5 leaves on average. \( *p<0.0283, **p<1.0E-06 \), Mann-Whitney Wilcoxon test, for both A
and B) (C) Scatter plot with rosette leaf number on the x-axis and days to flowering on the y-axis. Data are shown for F2 plants that are homozygous for the ago1-27 mutant allele and segregate for the hua2-4 mutant allele. (D) Known flowering time genes with differential expression in the ago1-27;hua2-4 double mutant as determined by RNA-seq. (E) Suggested intersection of miRNAs and HUA2 in flowering time pathways. Depicted in red is the proposed connection between HUA2 and SPL4 such that SPL4 expression is regulated by both mir156 and HUA2. In bold, genes that are overexpressed in the double mutant ago1-27, hua-2 relative to the single mutant ago1-27.

**Supplemental Figure 1.** Five phenotypes were measured for four STAIRS inbred lines, resulting in cases of revealing, concealing and epistatic phenotypic changes. The five phenotypes measured were days to flowering, rosette leaf number, rosette diameter, hypocotyl length and root length.

**Supplemental Figure 2.** (A) Landsberg, Col-0 and the inbred strain STAIRS9472 we sequenced across the MIR156F gene, supporting that there was both a SNP and a 14-nucleotide deletion in both the Landsberg and STAIRS9472 strains compared to Col-0. (B) Further sequencing across 55 A. thaliana strains. Of the sequenced strains, 42 carried the Ler-specific C-to-T SNP, one carried a C-to-G SNP, and 32 strains carried the 14-nt deletion.

**Supplemental Figure 3.** Seeds were stratified for five days at 4°C and then sown on soil. They were allowed to grow for five days at 23°C in LD or short days (SD) conditions and then transferred to 4°C for forty days to vernalize plants. (A) Rosette leaf number at flowering with vernalized plants in long day conditions. (B) Days to flowering with vernalized plants in long day conditions.

**References**


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Figure 1
**Figure 2**

### A

- **ST**epped **Aligned**
- **Inbred**
- **Recombinant**
- **Strains**

- Selfed twice
- Selected homozygous
  - STAIRS, ago1-27 lines
- Measured quantitative
  - traits
- Identified genetic
  - variation

**ago1-27 (Col-0)**

### B

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### C

- **ago1-27 reveals on Chr3**
  - Rosette diameter (mm)
  - Col-0 STAIRS Col-0 STAIRS
  - WT **ago1-27**

- **ago1-27 conceals on Chr3**
  - Rosette leaf number (LD)
  - Col-0 STAIRS Col-0 STAIRS
  - WT **ago1-27**

- **Ler epistatic to ago1-27 on Chr3**
  - Days to flowering (LD)
  - Col-0 STAIRS Col-0 STAIRS
  - WT **ago1-27**

*Note: The images include bar charts and graphs representing the data. The graphs are not described in detail, but they likely illustrate the statistical significance of the traits and the genetic interactions.*
**Figure 3**

A. Box plots showing days to flowering for different genotypes: Col-0 WT, ago1-27, STAIRS9472, and STAIRS9472;ago1-27.

B. Box plots showing rosette leaf number at flowering for the same genotypes.

C. Scatter plot correlating days to flowering with rosette leaf number for different genotypes.

D. Diagram of the STAIRS9472 Ler region on Chr5:1-9,479,000, highlighting miR156 targets.

E. Bar graph showing relative qPCR expression levels for SMZ, TFL1, ELF6, FLC, CO, SPL7, SPL3, SPL4, and SPL9 for Col-0 WT, ago1-27, STAIRS9472, and STAIRS9472;ago1-27.
Figure 4

A

![Image of plant development stages](image)

B

![Gene expression levels](chart)

C

![Allele frequency charts](charts)

**Known flowering time associated genes**

- HUA2
- PNY
- PR7
- LMI1
- TFL1
- ELF6
- MIR172b
- MYB33
- CHE
- FLC
- MIR156d
- EMF1
- MIR156e
- NFYA1/HAP2A
- FY
- ALG15
- CONSTANS
- FRL1
- TFL2
- HUA2
- PRR5
- FPF1
- MIR156f
- GID1C

**Chromosome 5 position**

1.0

0.5

0.4

0.3

0.2

0.1

0.0

Col-0 allele

Ler allele

Heterozygous

**Allele frequency (Ler)**

- **FLC** allele frequency (n=28)
- **HUA2** allele frequency (n=48)
- **MIR156f** allele frequency (n=99)

**SHOREmap 3.0 with “plot boost”**

51x coverage, n=100

**Figure 4**
Figure 5