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RESEARCH ARTICLE

Rapid, nonparallel genomic evolution of *Brassica rapa* (field mustard) under experimental drought

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Abstract

While we know that climate change can potentially cause rapid phenotypic evolution, our understanding of the genetic basis and degree of genetic parallelism of rapid evolutionary responses to climate change is limited. In this study, we combined the resurrection approach with an evolve-and-resequence design to examine genome-wide evolutionary changes following drought. We exposed genetically similar replicate populations of the annual plant *Brassica rapa* derived from a field population in southern California to four generations of experimental drought or watered conditions in a greenhouse. Genome-wide sequencing of ancestral and descendant population pools identified hundreds of SNPs that showed evidence of rapidly evolving in response to drought. Several of these were in stress response genes, and two were identified in a prior study of drought response in this species. However, almost all genetic changes were unique among experimental populations, indicating that the evolutionary changes were largely nonparallel, despite the fact that genetically similar replicates of the same founder population had experienced controlled and consistent selection regimes. This nonparallelism of evolution at the genetic level is potentially because of polygenetic adaptation allowing for multiple different genetic routes to similar phenotypic outcomes. Our findings help to elucidate the relationship between rapid phenotypic and genomic evolution and shed light on the degree of parallelism and predictability of genomic evolution to environmental change.

KEYWORDS
Brassica rapa, drought adaptation, experimental evolution, parallel evolution, rapid evolution, resurrection approach

1 | INTRODUCTION

Rapid evolution to environmental conditions has been documented by a number of studies (Franks et al., 2014; Hendry & Kinnison, 1999). However, the genomic basis of rapid evolution is less often characterized yet is fundamentally important as it can allow identification of adaptive loci and possible genetic pathways that increase fitness (Franks & Hoffmann, 2012). Studies that explore the genomic basis of rapid adaptation often find changes in many polymorphic loci. For example, adaptation to climate has been tied to subtle shifts in allele frequencies across many loci in the thale cress *A. thaliana* (Hancock et al., 2011), the stickleback *G. aculeatus* (Roberts Kingman et al., 2021), and the mouse *M. musculus* (Ferris et al., 2021). These examples suggest that rapid adaptation may often occur through changes in standing variation (Messer & Petrov, 2013; Pritchard et al., 2010).

While progress has been made toward understanding the genomic basis of rapid adaptation, less is known about the consistency of genomic shifts underlying rapid evolution (Blount et al., 2018; Lobkovsky & Koonin, 2012). If rapid evolution is driven by selection, genetically similar populations under the same environment are likely to evolve similar outcomes (parallel evolution). However,

similar populations may evolve unique outcomes to the same environment if evolutionary responses are stochastic or genetic drift is strong (nonparallel evolution). The degree to which evolutionary shifts are parallel typically depends on the biological level explored, with greater parallelism generally found at higher levels (Blount et al., 2018). At the highest level, evolutionary changes in fitness is likely to occur in parallel if selection and genetic variation are present (Bailey et al., 2015). At the level of phenotypic traits, parallel evolution is also common (Colosimo et al., 2005; Endler, 1980; Hamann et al., 2018; James et al., 2021; Losos et al., 1997; Smith & Rausher, 2011). In contrast, at the genetic level, nonparallel evolution is often observed (Blount et al., 2018; Franks et al., 2016; Hancock et al., 2011; James et al., 2021). This may be because similar functional effects can be achieved through different genes for quantitative or polygenetic traits (Blount et al., 2018; Tenaillon et al., 2012; Van Etten et al., 2020). However, these examples of nonparallel genomic evolution often compare natural populations that may start with differences in allele frequencies and are influenced by multiple different environmental effects in different locations, making it difficult to determine if responses to similar selection regimes in similar populations would be parallel and predictable or divergent and stochastic. For these reasons, the consistency of rapid genomic evolution from standing variation is an active area of research in evolution and ecology (Blount et al., 2018; Lobkovsky & Koonin, 2012).

One approach that has substantial advantages for the study of parallelism in evolution is experimental evolution (Blount et al., 2018). During experimental evolution, populations are exposed to environmental conditions established by the researcher, which allows population sizes and agents of selection to be manipulated to explore specific questions (Kawecki et al., 2012). Experimental evolution can also be combined with the resurrection approach where ancestors and descendants are grown concurrently (Franks et al., 2018) and an evolve and resequence (E&R) design where ancestor and descendant individuals within populations are sequenced as a pool (Schlötterer et al., 2015; Turner et al., 2011). E&R designs that pool individuals of ancestral and descendant populations are cost-effective (Schlötterer et al., 2014) and have been used across a variety of systems (Long et al., 2015; Schlötterer et al., 2015). Using an E&R design to collect genomic-wide polymorphism data can identify specific loci underlying rapid adaptation from standing variation (Schlötterer et al., 2015), which can be missed in other studies that use genomic information to look for signatures of selection. While experimental evolution, the resurrection approach and E&R designs are thus highly appropriate for examining the genetic basis and degree of parallelism in evolutionary responses to climate change, they have rarely been combined for this purpose.

One system that is highly amenable to the study of evolutionary responses to climate change is populations of the annual plant *Brassica rapa* in the Mediterranean climate region in southern California. Prior work in this system has found that multiple years of drought, which shortened the growing season, led to rapid adaptive evolution of drought escape through earlier flowering in two natural populations (Franks, 2011; Franks et al., 2007; Hamann et al., 2018). Although these two populations evolved in parallel phenotypically, the genetic basis of the evolutionary changes appeared largely divergent and nonparallel. Specifically, only 2.5% of the evolutionary genetic changes occurred in common between the two populations, with the vast majority of genetic changes unique and nonparallel (Franks et al., 2016). In addition, evolution of transcript expression was also mostly unique between these populations (Hamann et al., 2021). However, it is unclear if the lack of parallelism at the genetic level observed between these two populations indicates true stochasticity in evolutionary responses or if the differences were due to differences in genetic composition or environmental conditions between sites.

To build on this work from the natural populations, we used lines derived from ancestral seeds of these populations to perform an experimental evolution study in which populations could be replicated and treatments experimentally controlled. The first results of this study focused on phenotypic evolution in response to experimental drought or watered experimental regimes (Johnson et al., 2022). This experiment, like the studies of natural populations, found that drought caused rapid evolution of earlier flowering time, greater specific leaf area, and increased seed mass under drought, consistent with adaptive drought escape (Johnson et al., 2022). This work also found evidence for parallel evolution at the phenotypic level, with evolution of flowering time, seed mass, and population trajectories in principal component space largely congruent among replicate populations under drought (Johnson et al., 2022).

Here, we extend the results of this experimental evolution study and examine the genetic basis of evolutionary changes and the degree of parallelism at the genetic level in these experimental populations. We assessed SNP frequency changes between ancestral, drought-descendant, and watered-descendant populations, and compared genetic diversity and genetic composition among replicates and experimental regimes. Because replicate populations were founded from the same natural population and responded to drought in parallel at the phenotypic level (Johnson et al., 2022), we expected some consistent evolutionary responses in SNPs at the genetic level. However, because dozens of genes contribute to flowering and stress responses in *B. rapa* (Osborn et al., 1997) and *A. thaliana* (Flowers et al., 2009), and prior studies suggested nonparallel genomic responses to drought between natural *B. rapa* populations (Franks et al., 2016; Hamann et al., 2021), we also expected that evolution of SNPs could be mostly nonparallel among replicate populations under drought. To uncover the genetic basis of evolutionary changes, we assessed the function of genes with evolved SNPs using existing annotations and investigated enrichment in gene function categories. Since evolutionary changes in drought-related traits has been shown in previous natural (Franks, 2011; Franks et al., 2007; Hamann et al., 2018) and experimental populations (Johnson et al., 2022), we expected to find enrichment of evolved SNPs associated with flowering and stress responses in response to our experimental drought. We also

compared SNP changes in this experimental study to those in natural populations under drought (Franks et al., 2016) to determine if natural and experimental drought caused consistent genetic responses. Overall, this study helps to elucidate the genomic basis of adaption to drought in *B. rapa*, and more broadly to assess consistency of genomic responses to selection.

2 | MATERIALS AND METHODS

2.1 | Study system

Brassica rapa L. (Brassicaceae), is an annual, ruderal plant commonly known as field mustard. *B. rapa* has been sequenced and annotated (Zhang et al., 2018). *B. rapa* is closely related to *A. thaliana* (Cheng et al., 2014; Franks et al., 2015; Wang et al., 2011), but is self-incompatible and reproduces only by outcrossing. The *B. rapa* population we used in this study originated from the San Joaquin Marsh Reserve in Orange County, CA in 1997 (Franks et al., 2007), and seeds were collected after several years of greater than average precipitation. This Arboretum population (hereafter ARB) was shown to have evolved earlier flowering following natural multiyear drought that occurred from 1999 to 2004 and that shortened the growing season each year (Franks et al., 2007). Seeds from around 100 maternal lines have been grown under unstressed conditions and crossed randomly several times since 1997 in order to maintain a viable seed stock.

2.2 | Experimental design

We combined experimental evolution with the resurrection approach and an evolve-and-resequence (E&R) design to directly assess genomic evolution to experimental drought. The design is discussed thoroughly in Johnson et al., 2022. Briefly, we constructed 24 experimental populations, each with one seed from 73 maternal lines that are refreshed descendants of the ARB *B. rapa* population (Franks et al., 2007). Experimental populations, which at this point were seeds, were randomly assigned to a *regime* (ancestral, drought, watered) with eight replicates of each. Drought and watered regime populations were then grown under experimental drought or watered conditions, respectively, for four generations, while ancestors were stored as seeds at 4°C during this time. Flowers were hand pollinated within experimental populations, and seeds were collected from all individuals and pooled by replicate population so that random selection of seeds for the next generation was proportional to individual fitness and allowed natural selection to occur. After four generations of experimental evolution, 73 individuals from each drought and watered regime population, as well as the 73 seeds from each ancestor regime population in storage, were grown concurrently under the same conditions as our watered regime. This served as a refresher generation, which is commonly used in resurrection studies to reduce maternal and storage condition effects on phenotypic traits, but which is not necessary for evaluating evolutionary change in allele frequencies (Franks et al., 2018). After the refresher generation, we grew a subsequent generation and measured functional traits and fitness to assess phenotypic evolutionary changes (discussed in Johnson et al., 2022).

2.3 | DNA extraction and library prep

During the refresher generation (described above), we collected tissue for DNA extraction from all drought and watered replicate populations as well as two randomly selected ancestor replicate populations. We collected tissue for sequencing during the refresher generation rather than the subsequent test generation to maximize the number of individuals in each population that we sampled, because half of the maternal lines were grown in the following generation. In addition, sampling after the refresher generation is necessary for reducing maternal effects on phenotypic traits but is not necessary for assessing evolutionary changes in allele frequencies (Franks et al., 2018), which was the focus of this analysis. We only sequenced two ancestor populations because ancestor populations were stored as seeds since they were drawn from the same gene pool. We harvested a flower bud from all bolting individuals in a replicate population once approximately 70 out of the 73 individuals had bolted. Sampling occurred for drought replicate populations on day 27, for watered replicate populations on day 34, and for the two ancestor replicate populations on day 40 after germination. We pooled and lysed buds by population using a FastPrep machine and Lysing Matrix M (FastPrep, MP Bio). We extracted DNA from the 18 samples (DNeasy Plant Mini Kit, Qiagen) and quantified DNA with a Qubit florometer to ensure successful extraction. We fragmented 55 μL of 20 ng/μl DNA extract for each sample using a Bioruptor Pico sonicator (Diagenode) with eight 15:90 s cycles to achieve fragments of approximately 350 base pairs. These settings were selected based on trial runs. For each sample, we prepared a DNA library with the Illumina TruSeq DNA PCR-Free Low Throughput Library Prep Kit and a unique index adapter from the Illumina TruSeq DNA Single Indexes Set A kit. For each library, we performed quality control with the Agilent 2100 Bioanalyzer and quantified concentration with qPCR. We then pooled equimolar amounts of each library, resulting in 108 μl of a 4 nM pooled library. Whole genome shotgun sequencing was performed by the New York Genome Center using NovaSeq S1 flow cell 300 cycle with 150 base paired-end reads.

2.4 | Read processing

We trimmed reads with Trimmomatic (Bolger et al., 2014) using a custom script based on previous studies (Feiner et al., 2017; Franks et al., 2016) and a modified list of TruSeq2 adapters. This script dropped leading bases below a quality score of three, trimmed read ends when the four base pair rolling quality score

fell below 20, and dropped reads under 36 base pairs. We then aligned paired end reads to the *B. rapa* reference genome version 3.0 (Zhang et al., 2018) using the BWA-MEM algorithm (Li & Durbin, 2009). We used default parameters with RGID, RGLB, RGPL, RGPU, and RGSM specified to add read group information. After mapping paired end reads to the reference genome, we sorted and merged technical replicates of each library sequenced on different flow cells using "sort" and "merge" in SAM_{TOOLS}, and then generated a BAM file for each resulting library with SAM_{TOOLS} sort (Li et al., 2009). We also merged libraries belonging to the same regime to generate a single BAM file representing ancestor, drought, and watered regimes. This resulted in 19 BAM files for downstream analyses, representing each of eight drought replicate populations, eight watered replicate populations, the drought regime (pool of all drought replicate populations), the watered regime (pool of all watered replicate populations), and the ancestor regime (pool of both ancestor replicate populations). We indexed each BAM file using the "index" function in SAM_{TOOLS}, calculated alignment metrics with "flagstat" in SAM_{TOOLS}, and checked for errors with Picard ValidateSamFile (Institute, B, 2017). We used the "mpileup" function in SAM_{TOOLS} (Li et al., 2009) to generate a multipileup file for each population library as well as a library representing each regime. We synchronized reads with QS > 20 for each multipileup file using the "mpileup2synch.jar" function in PoPoolation2 (Kofler, Pandey, & Schlötterer, 2011). Population libraries averaged 96.2 million paired end reads with 93.35% surviving trimming and 98.45% of survivors mapping to the reference. Population libraries averaged 70× coverage and ranged from 26× to 122× (Table S1). Most population libraries reached the recommended criteria for SNP discovery with pooled-sequencing designs (Schlötterer et al., 2014).

2.5 | Nucleotide diversity of experimental regimes

We inspected nucleotide diversity mean and standard error of across the genome for each regime library with Tajima's pi using the "Variance-sliding.pl" function of the PoPoolation package (Kofler, Orozco-terWengel, et al., 2011) and the parameters specified in Table S2.

2.6 | Differentiation among replicate populations

To explore potential evolutionary differentiation across experimental populations, we performed principal component analysis on allele frequencies for the two ancestral populations, eight drought populations, and eight watered populations. Variable loci were identified from libraries using the "snp-frequency-diff.pl" function of the PoPoolation2 package (Kofler, Pandey, & Schlötterer, 2011) and parameters outlined in Table S3. We then performed principal component analysis with the "prcomp" function in R version 4.0.5 (R Core Team, 2021).

2.7 | Identification of SNP frequency shifts

To assess genomic evolutionary responses from standing genetic variation, we used two approaches. First, we used a hierarchical linear model to assess consistent SNP frequency shifts among experimental populations. Specifically, we implemented the auxiliary model using BayPass (equation 9 in Gautier, 2015) to run a user-defined linear model with two custom binary covariates on the 18 population libraries (two ancestor, eight drought, eight watered). This model used SNP frequencies at each locus to assess the probability of SNP frequency shift while accounting for genetic covariance among experimental populations (Gautier, 2015), thus controlling for population structure and allowing substantial power to identify parallel SNP frequency changes by considering all populations in one model. Our model consisted of two covariates, (1) ancestor versus descendant replicate populations (time covariate) and (2) drought versus watered replicate populations (treatment covariate). We used default BayPass settings except for d0yij set to 35 based on recommendations in the BayPass manual, and isingbeta set to 0.9 to reduce the correlated signatures of nearby loci. The model calculated a Bayes factor (BF) for each covariate at each locus, which quantified the probability of association between the SNP frequencies of each covariate relative to the overall mean SNP frequency at each locus. Because this model considers all libraries simultaneously, BFs assess parallel evolution to conditions in the greenhouse (time covariate) and to drought specifically (treatment covariate). We consider BF above 10 as strong evidence and those above 20 as decisive evidence for parallel evolution at a locus (Kass & Raftery, 1995).

Since the BayPass model, by design, would likely fail to detect SNPs that changed in one or few replicate populations, we also assessed genomic shifts by comparing SNP frequencies between each individual drought replicate to the ancestor regime. Because we used the resurrection approach, SNP shifts identified between drought replicate populations and the ancestor regime represent a direct evolutionary shift but may not necessarily have been caused by drought (Franks et al., 2018). We avoid comparing drought replicate populations to watered replicate populations because this would not be a direct evolutionary comparison, and so nonparallel evolution in both drought and watered replicate populations could result in identification of substantial false positives. To assess shifts in SNP frequencies under experimental drought, we performed Fisher's exact tests (FETs) (Fisher, 1922) using the "fisher-test.pl" command in PoPoolation2 to assess SNP frequencies in each drought replicate relative to each of the ancestor regime using the parameters in Table S4 (Kofler, Pandey, & Schlötterer, 2011). Because the resulting *p*-values were inflated relative to a uniform distribution after Bonferroni correction, we "genomic controlled" *p*-values by this inflation factor and used these corrected *p*-values. To obtain genomic controlled *p*-values, we calculated the ratio between the median *p*-value observed in the data versus a uniform distribution and divided each test statistic by this ratio (Devlin & Roeder, 1999). SNPs with a significant

FET were considered to have evolved from the ancestor state. We also ran FETs on SNP frequencies of the two ancestor replicates that comprised our ancestor regime and identified only two different SNPs across ancestor replicates, suggestive of little sampling effect across ancestor replicates.

To assess consistency of evolutionary responses with those to natural drought, we tested whether genes that evolved under our experimental drought overlapped with genes that evolved in this population under natural drought. Because the Franks et al., 2016 study, which identified differentiated genes to natural drought, used version 1.18 of the *B. rapa* reference genome, we first aligned the v3.0 genome to the v1.5 genome with minimap2 and used "paftools.js liftover" of minimap2 to lift v1.5 protein coding sequences to the v3.0 reference build (Li, 2018). We used "bedtools intersect" (Quinlan & Hall, 2010) to identify SNPs from the BayPass model with BF > 10 that fell within coding sequences of differentiated genes reported in Franks et al., 2016.

2.8 | Gene enrichment

Because drought replicate populations demonstrated evolutionary responses in flowering and drought response traits, we evaluated a priori hypotheses for allele frequency shifts in flowering and stress genes. To generate a list of *B. rapa* stress coding regions, we used the list of *Arabidopsis* stress response genes provided by the STIFDB2 database (Naika et al., 2013), identified corresponding *B. rapa* orthologues using a list from (Qi et al., 2021), and lifted coding sequences in these genes using a list provided with the *B. rapa* v1.5 reference genome on BRAD (Cheng et al., 2011). To generate a list of *B. rapa* flowering coding regions, we used the list of *Arabidopsis* flowering genes from the Flowering Interactive Database (Bouché et al., 2016), identified *B. rapa* orthologs using the list from (Qi et al., 2021), and lifted coding sequences in these genes using the list from BRAD (Cheng et al., 2011). We used BEDTools intersect (Quinlan & Hall, 2010) to count the number of (1) SNPs without a frequency shift identified respective to the time covariate of the BayPass model and (2) SNPs with BF > 10 respective to the time covariate of the BayPass model that fell in each coding sequence list (stress and flowering). We only counted SNPs respective to the time covariate of the BayPass model as no SNP frequency changes were identified relative to the treatment covariate. We repeated this BEDTools intersect procedure using the number of (1) SNPs without frequency changes present in the synchronized mpileup file for ancestors and drought descendant population libraries and (2) the number of significant SNP frequency shifts identified by FETs. To test whether SNPs with frequency shifts were overrepresented in coding sequences of stress genes, we evaluated the proportion of SNP with frequency shifts in stress coding regions versus the number of SNPs without frequency shifts in stress gene coding regions with one-tailed FETs (Fisher, 1922). We did not run this analysis for flowering genes because BEDTools intersect identified only one SNP shift within a flowering gene coding sequence that represented an allele shift.

3 | RESULTS

3.1 | Genomic evolution to experimental conditions

Genetic diversity was similar among regimes, with average measures of nucleotide diversity (π) equal to 0.0105 ± 0.0040 in ancestral, 0.0104 ± 0.0040 in drought, and 0.0104 ± 0.0040 in watered regimes (Figure S1), indicating that there were not substantial changes in genetic diversity following the experimental regimes. Multivariate analysis of SNP frequencies using principal components indicated that the watered populations remained similar in genetic composition to the ancestral population, with the ancestor and all watered populations clustering all together (Figure 1). However, there was more divergence in the drought populations, with some of the drought populations clustering with the ancestral and watered populations and some occupying different regions of multivariate genetic space (Figure 1). This indicates that the drought treatment caused genetic changes in drought populations, and that these changes varied among population replicates.

We analysed the genetic basis of evolutionary changes and the consistency of these changes using Bayesian (BayPass) and contingency test (Fisher's exact test; FET) analyses. The Bayesian analysis, which is designed to detect consistent shifts in allele frequencies among descendant populations in a treatment compared to ancestral populations, identified 472 SNPs with strong (BF > 10) and 54

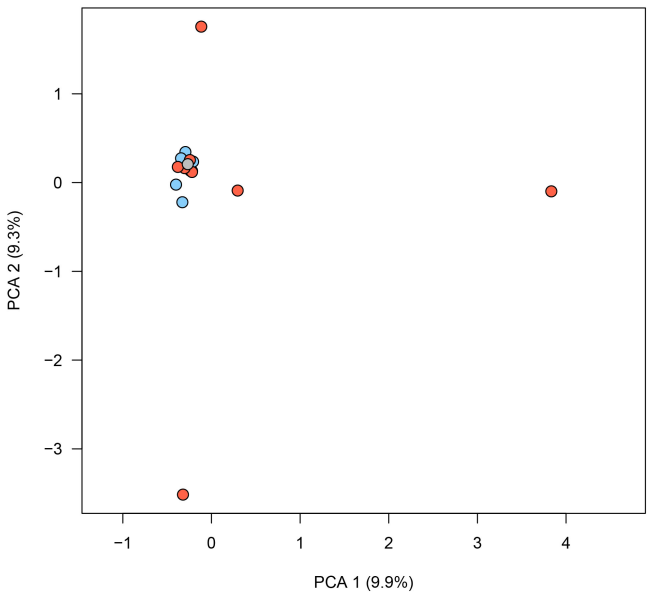

FIGURE 1 Principal component analysis of SNP frequency shifts. We performed principal component analysis on SNP frequencies across the ancestor regime, eight drought replicate populations and eight watered replicate populations. The ancestor regime is shown in grey, drought replicate populations in red, and watered replicate populations in blue. We observed several drought replicate populations diverged in multivariate genetic space, suggesting unique genomic differentiation across some drought replicate populations

SNPs with decisive (BF > 20) evidence for evolutionary shifts in frequency (Figure 2a). All SNP shifts were identified respective to the time covariate, which compared ancestors and descendants. Of these identified SNPs, 57 with BF > 10 and 11 with BF > 20 (Table 1) represent alleles within coding sequences.

In contrast to the Bayesian analysis, the contingency table analysis (FETs) can be used to assess evolutionary shifts in SNP frequencies in each individual drought replicate population compared to ancestors. This analysis identified 913 SNPs with frequency shifts between individual drought replicate populations and the ancestor regime (Figure 2b). These shifts were almost completely nonparallel, with 911 unique to individual replicate populations and only one locus, which was in the unannotated gene *Bra004959*, containing shifts in two drought replicate populations. Most SNP shifts fell in noncoding regions, with 147 SNPs within exons spanning 72 different genes. Like evolution at the SNP level, evolution at the gene level was also largely nonparallel, with 71 genes unique to individual replicate populations and only *Bra004959* containing an allele shift in multiple replicate populations under drought.

Of the genes that evolved in response to drought in this experiment, there was very little overlap with genes that evolved in our founder population (Arboretum) and another proximate population (Back Bay) under natural drought (Franks et al., 2016). Specifically, only one gene from each analysis (*Bra007979* from the BayPass model and *Bra006363* from FETs) overlapped with those that differentiated in the founder population under drought, and neither has an annotation related to drought. Thus, the experimental drought did not appear to cause similar genetic changes as natural drought caused in the founder population of this species.

3.2 | Genomic shifts in stress response and flowering genes

For the genes that evolved under experimental drought, we examined functions based on existing annotations and assessed whether there was enrichment in functional categories. For consistent SNP frequency changes identified by our Bayesian model we did not

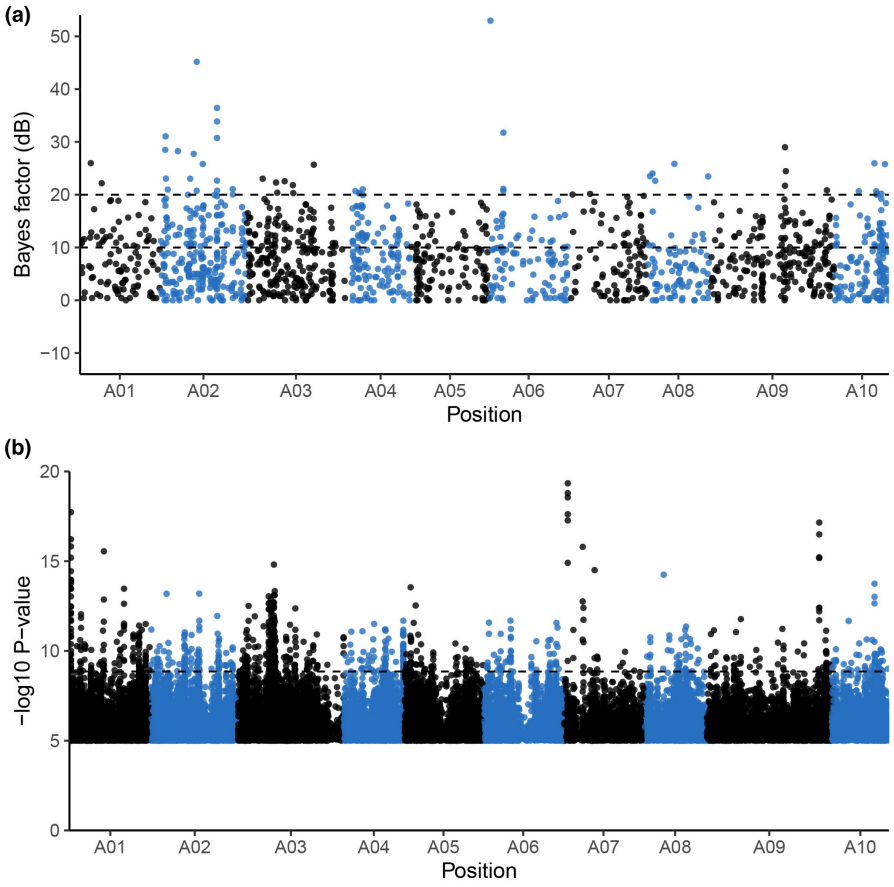

FIGURE 2 Loci with evidence for SNP frequency shifts. Shown are loci with SNP frequency shifts identified between ancestor and descendant replicate populations with two approaches. (a) Shown are Bayes factors (units in decibans; dB) for SNP frequency changes identified with a BayPass custom linear model. Strong (dB > 10) and decisive (dB > 20) significance thresholds are shown with dotted lines. All points plotted are respective to the time covariate, which assessed frequency shifts between ancestors and descendants. Points respective to the treatment covariate were excluded for clarity, as none reached either significance threshold. (b) Shown are significance values for SNP frequency changes calculated with Fisher's exact tests for each drought replicate population relative to the ancestor regime. Sites below a $-\log_{10}$ *p*-value of five are not plotted for clarity, and the corrected significance threshold is shown with a dotted line

TABLE 1 Genes with evidence for consistent evolutionary shifts

Gene	Chr	Position	BF	Arabidopsis homologue	Gene name	Annotation
Bra039661	A06	473033	53.0	AT1G53210	*AtNCL*	Sodium/calcium exchanger protein
Bra029365	A02	20524128	33.9	AT5G23260		MADS box protein
Bra040238	A06	5203204	31.8	AT3G05990	*LLR3*	Leucine-rich repeat family protein
Bra028560	A02	1595621	28.5	AT5G10870	*AtCM2*	Encodes chorismate mutase AtCM2
Bra008174	A02	12008876	27.7	AT1G75020	*LPAT4*	Lysophosphatidyl acyltransferase 4
Bra030923	A08	686441	23.5	AT1G53730	*SRF6*	STRUBBELIG-receptor family 6
Bra007979	A02	10712570	23.0			None
Bra014189	A08	2626040	22.6	AT1G49450		Transducin/WD40 repeat-like superfamily protein
Bra000437	A03	10657699	22.3	AT2G46980		Encodes ASY3, a coiled-coil domain protein required for meiosis
Bra008883	A10	8903168	20.7			None
Bra000587	A03	11110776	20.4	AT2G20950		Phospholipase-like protein (PEARLI 4) family protein

Note: Shown are genes with decisive evidence for evolutionary shifts identified by the BayPass model, which tests for consistent SNP frequency shifts (1) between ancestors and descendant replicates (time covariate) and (2) between drought and watered replicate populations (treatment covariate). This model identified 51 SNPs with decisive evidence (Bayes factor > 20) for consistent SNP frequency shifts between ancestor and descendant replicate populations during experimental evolution. Eleven of these SNPs fell within coding regions, thus providing evidence for evolution in the associated genes. These 11 genes are annotated here, in order of descending confidence quantified with Bayes factors (BF).

find any SNP frequency shifts in flowering genes and did not detect enrichment of SNP frequency shifts in stress genes ($p = 0.631$). However, several SNPs with consistent shifts fell in stress response and even found a differentiated SNP with a flowering-related annotation (Table 2). For example, the SNP with the highest BF in the BayPass model fell within *AtNCL*, a gene that encodes a Na^{+}-Ca^{2+} exchanger-like protein that functions in flowering (Li et al., 2016) and salt stress (Wang et al., 2012) signalling. Another stress gene with an allele shift that the BayPass model identified is *AIW2*, which is expressed in response to salt stress and abscisic acid application (Bray, 2004; Wang et al., 2020). Additional stress genes with an allele identified by BayPass include *AtCM2* and *AtNFXL1*, suggesting consistent evolutionary shifts in these genes. We did not detect enrichment of FET identified differentiated SNPs in coding sequences of stress genes ($p = 0.989$) or flowering genes ($p = 0.834$). However, we identified an allele shift in genes related to stress (*JAO2*) and flowering (*APPR5*), each of which were identified in one drought replicate population.

4 | DISCUSSION

We combined the resurrection approach with an evolve and resequence (E&R) protocol to examine the genomic basis of rapid evolution of *B. rapa* under experimental drought and watered conditions. We identify several SNPs with evidence for consistent shifts among drought and watered experimental populations, suggesting some degree of genomic parallelism to selection imposed by experimental conditions. However, the vast majority of SNP shifts occurred in unique replicate populations, indicating the documented evolution in drought escape traits (Johnson et al., 2022) is largely underlain by nonparallel changes at the genetic level, potentially due to polygenic adaptation. We also identified several allele frequency shifts in genes related to stress response and flowering, which may underlie the phenotypic shifts previously observed during experimental evolution (Johnson et al., 2022). These results help shed light on the genomic basis of rapid evolution as well as the degree and nature of parallelism in genomic and phenotypic evolution under natural and experimental conditions.

4.1 | Parallel genomic responses to environment

The Bayesian analysis of SNP frequency shifts done using the BayPass model detected signatures of parallel evolution across some SNPs, all of which were shared by all descendant populations and were not drought specific. Eleven of these SNPs that meet our criteria of decisive evidence for adaptation fell in coding sequences, suggesting parallel responses in these genes (Table 1). A wide range of parallel outcomes haven observed across species and traits at phenotypic and genomic levels, but studies exploring polygenic traits over short timescales often find a small amount of parallel evolution (Blount et al., 2018). Our founder population showed a small but greater than expected at random number of differentiated genes under drought (11 out of 434) were shared with another natural population (Franks et al., 2016), and a similar pattern has been observed in *M. musculus* across eastern and western climates (Ferris et al., 2021) and in response to serpentine soil in *Arabidopsis arenosa* (Konečná et al., 2022). Traits

TABLE 2 Flowering and stress response genes with evidence for evolution

Gene	Chr	Position	Sig	Arabidopsis homologue	Gene name	Annotation
Bra039661	A06	473033	53.0	AT1G53210	*AtNCL*	Encodes a Na^+/Ca^{2+} exchanger-like protein that participates in maintenance of Ca^{2+} homeostasis
Bra028560	A02	1 595621	28.5	AT5G10870	*AtCM2*	Encodes chorismate mutase AtCM2
Bra014189	A08	2 626040	22.6	AT1G49450	*AIW2*	Transducin/WD40 repeat-like superfamily protein
Bra040361	A01	23915232	18.6	AT3G07100	*SEC24a/ERMO2*	Encodes SEC24a/ERMO2, required for endoplasmic reticulum (ER) morphology
Bra029367	A02	20515514	16.1	AT5G23340		RNI-like superfamily protein
Bra031704	A09	30817879	15.0	AT1G10170	*AtNFXL1*	Encodes AtNFXL1, which negatively regulates trichothecene phytotoxin-induced defence response
Bra001273	A03	14689065	13.9	AT3G07870	*FBX92*	F-box containing protein, expression correlated to leaf size and cell proliferation
Bra033282	A10	532341	10.5	AT1G01140	*CIPK9/PKS6/SNF1*	Encodes a CBL-interacting protein kinase with similarity to SOS2
Bra036517	A09	2 522950	10.8	AT5G24470	*APRR5*	Encodes regulator that affects circadian-associated biological events including flowering
Bra016293	A08	15320937	10.8	AT1G26850		S-andelosyl-L-methionine-dependent methyltransferase superfamily protein
Bra039293	A04	16247775	10.7	AT2G46060		Transmembrane protein-like protein
Bra011083	A01	3 888819	10.6	AT4G29100	*BHLH68*	Helix–loop–helix DNA binding superfamily protein
Bra011083	A01	3 888831	10.3	AT4G29100	*BHLH68*	Helix–loop–helix DNA binding superfamily protein
Bra000894	A03	12889350	10.0	AT1G61580	*ARP2*	Encodes protein involved in detoxification of polycyclic aromatic hydrocarbons
Bra028752	A02	781956	9.5	AT5G05600	*JAO2*	Transmembrane protein-like protein
Bra026130	A06	5 114828	9.4	AT1G15430		Hypothetical protein
Bra038127	A05	10 605077	9.0	AT1G53500	*AATRHM2/MUM4/RHM2*	Encodes putative NDP-L-rhamnose synthase, an enzyme involved in seed coat mucilage cell development
Bra006303	A03	2 887873	9.0	AT5G15490	*UDG3*	Encodes UDP-glucose dehydrogenase gene

Note: Shown are stress and flowering genes for which we identified an allele shift. The first nine rows list allele shifts between ancestors and descendants identified with a custom BayPass model, while the remaining 10 list those identified between a drought replicate and ancestors with Fisher's exact tests (FETs). We report Bayes Factors for SNPs identified with the BayPass model and $-\log_{10} p$-values for those identified with FETs in the significance column (Sig). One flowering gene (*Bra036517*) was identified, while the remainder are stress response genes.

that underlie adaptation to climate in mice and field mustard are polygenic, which may explain the lack of parallel evolution given that there are multiple genomic pathways to adaptation in these systems.

While the BayPass model detected some SNPs that showed parallel evolution among descendant populations, the fact that these shifts were identified across all descendant populations and not specifically under drought is interesting and highlights the importance of proper experimental controls to conclude that evolution is in response to a particular agent of selection. In the case of this experiment, drought and watered replicate populations each evolved earlier flowering and higher specific leaf area (Johnson et al., 2022). This is consistent with observations in other studies, which often find selection for earlier flowering time when plants are grown under experimental conditions (Austen et al., 2017). However, several differences between drought and watered experimental populations at the phenotypic level offer support for selection imposed by drought. Drought descendants demonstrated greater shifts towards earlier flowering and higher specific leaf area relative to watered descendants, both of which are consistent with evolution of a drought escape strategy in response to drought (Johnson et al., 2022). Plants under drought also experienced stronger selection for flowering time and specific leaf area than plants under the watered treatment (Johnson et al., 2022), in support of earlier flowering as a response to drought. Within this context, we suggest that genomic shifts in drought replicate populations were likely at least partially driven by drought, but that the BayPass model may have failed to identify SNP frequency shifts related to drought if they occurred in the same direction across drought and watered experimental populations.

4.2 | Nonparallel genomic responses to environmental conditions

Because the BayPass model would only identify SNPs with consistent shifts and would, therefore, not detect nonparallel evolution that differed among replicates, we also compared SNP frequencies between the ancestor regime and each drought replicate population, which would indicate evolutionary changes that occurred within each replicate population. This approach detected many SNPs with evidence for evolution within each drought replicate population, indicating substantial genomic evolution in response to drought. Furthermore, these results indicate that genomic evolution among drought replicate populations occurred in nonparallel at SNP and gene levels, as 911 out of 913 SNP shifts occurred at unique loci and 71 out of the 72 genes that these loci mapped to were unique to one drought replicate population. The only allele identified in multiple drought replicate populations fell within the gene *Bra004959*, a protein kinase domain containing gene without an annotation. This nonparallel evolution is somewhat surprising given that the BayPass model identified several parallel SNP frequency shifts, and given that drought replicate populations evolved in parallel at the phenotypic level (Johnson et al., 2022). However, mostly nonparallel evolution matches results in the founder population under natural drought, as only 2.5% of genes (11 out of 434) differentiated in parallel with a second population (Franks et al., 2016). Our results also compare similarly to climate adaption in mice, which reported mostly nonparallel SNPs underlying adaptation between populations, even though more SNPs were shared than expected by chance (Ferris et al., 2021). Studies in plants have likewise shown this pattern with paired dune and headland ecotypes of *Senecio lautus* (James et al., 2021) and replicated populations of *A. arenosa* (Konečná et al., 2022) showing mostly nonparallel genomic evolution even though phenotypic evolution occurred in parallel.

While our results are similar to these previous studies, our study furthers the understanding of genomic evolution because nonparallel genomic evolution could not be explained by differences in environmental conditions or standing genetic variation, which often differ among populations used to assess parallel evolution in natural settings (Blount et al., 2018; Colosimo et al., 2005; Franks et al., 2016; James et al., 2021; Smith & Rausher, 2011; Van Etten et al., 2020). Under our experimental environment, environmental variation across our replicate populations would have been much less compared to natural population replicates. While all of our drought populations evolved earlier flowering under our greenhouse drought regime we still observed mostly nonparallel genomic evolution. Our discovery of nonparallel genomic evolution under our highly controlled and largely consistent environment support results from other studies suggesting that genomic evolution to similar selection regimes can be stochastic to a degree and lead to different evolutionary shifts across replicates.

The variety of SNPs that shifted across different drought replicate populations is consistent with polygenic adaptation to drought, where many loci contribute to a trait and populations may evolve through changes in different underlying SNPs and genes (Messer & Petrov, 2013; Pritchard et al., 2010; Stern & Orgogozo, 2009). Principal component analysis on SNP frequencies was also consistent with nonparallel genomic evolution across drought replicate populations. While the ancestor regime, most watered replicate populations, and several drought replicate populations clustered together, several drought replicate populations appear to have unique changes in SNP frequencies based on the first two principal component axes (Figure 1). While stochastic processes and drift undoubtedly explains much of the nonparallel frequency shifts across replicate populations, there are several lines of evidence that nonparallel adaptive evolution also occurred. First, previous work found a high degree of parallel evolution at the phenotypic level among drought replicates, which would be unlikely if drift were the only influence on our experiment (Johnson et al., 2022). Second, nucleotide diversity (π) was similar across regimes and across individual drought replicate populations (Table S5), which suggests that descendant replicates had not lost significant diversity due to drift, although undoubtedly many allele frequencies changes within populations occurred stochastically. Third, we used fitness data from the subsequent test generation to simulate random seed selection during experimental evolution to approximate effective population size of

replicates under each watering regime from reproductive variance. This estimated effective population size as comparable to the actual population size under drought and moderately larger than the actual population size for populations under the watered regime (Table S6). Together, this evidence suggests that natural selection, played an important role in the nonparallel evolutionary responses among replicates.

Other studies that have explored responses to selection often find results consistent with polygenic adaptation. For example, studies that find continued evolutionary shifts over many generations often conclude that standing variation in many loci underlies adaptation (Barton & Keightley, 2002; Hill & Caballero, 1992). While polygenic adaption may indicate a population has sufficient variation to respond to selection, this genomic flexibility may also reduce the predictability of genomic evolution to selection as many adaptive pathways are available (Bailey et al., 2015). In this study, evolution of replicate populations under the same experimental drought occurred in parallel at the phenotypic level (Johnson et al., 2022), but genomic shifts occurred differently across replicate populations. This nonparallel genomic evolution to experimental conditions suggests that predicting genomic shifts underlying polygenic adaptation may be difficult even under controlled selection environments, let alone under complicated selection regimes in nature.

4.3 | The genomic basis of evolutionary shifts

Several SNP shifts that we identified mapped to genes involved in stress responses, although we did not detect enrichment in stress response genes. Notably, the BayPass model identified consistent allele shifts among replicates in *AtNCL*, *AtNFXL2*, and *AIW2*, each of which have functions consistent with the evolution of a rapid growth strategy that we observed under experimental drought and watered conditions, and in *AtNCL*, a Na^+/Ca^{2+} exchanger protein that may function in Ca^{2+} homeostasis or signalling related to stress response. Specifically, we observed evolution of higher specific leaf area under both drought and watered regimes, and this shift was more pronounced in response to drought (Johnson et al., 2022). Because faster growth and cell division results in thinner leaves, higher specific leaf area is indicative of faster growth. The functions of each of these three genes relate to growth. *AtCM2* is associated with cell proliferation and larger leaf size (Baute et al., 2017), *AtNFXL1* is associated with growth under stress (Lisso et al., 2006), and *AIW2* is part of the water-stress response controlled by abscisic acid (Bray, 2004). The functions of these genes suggest that allele shifts may underlie the evolution of higher specific leaf area we observed under both drought and watered regimes (Johnson et al., 2022). Additionally, *AIW2* (Bray, 2004) and *AtNCL*'s family of proteins (Shao et al., 2008) have also been linked to the drought response. It is notable that several of these genes have annotations tied to drought yet were not identified respective to drought specifically by our Bayesian model. Again, this lack of drought-associated SNPs could be indicative of variation in differentiated SNPs across replicates (polygenic adaptation) that made treatment-specific detection more difficult. Furthermore, watered regime populations in our experiment also showed a shift towards earlier flowering. While this shift was smaller than for drought populations, if some SNPs involved in drought adaptation also evolved in the same direction under the watered regime these sites likewise would be difficult to detect with our design. For this reason, we acknowledge that some differentiated SNPs we detected among all descendants many underlie adaptation to our drought regime.

Several SNP shifts identified in unique drought replicates also occurred in stress or flowering genes. One drought replicate population had an allele shift in *JAO2*, which may also have contributed to the evolution of specific leaf area through altering the growth-defence trade-off, as it encodes for a protein that negatively regulates Jasmonate-mediated defence signalling (Caarls et al., 2017; Smirnova et al., 2017). However, evolution in *JAO2* was only detected in one replicate population, potentially due to variable genetic mechanisms of achieving rapid growth. The only allele shift identified in a flowering gene occurred in *APPR5*, a member of *Arabidopsis* pseudo-response regulator genes involved in regulation of circadian rhythm (Makino et al., 2002). Studies using mutation of *APPR5* have shown that this gene influences circadian-related genes and flowering time (Yamamoto et al., 2003). While exploring the annotations of evolved stress genes, we also identified that *AtNCL*, a gene identified with an allele shift identified with the BayPass model, has an additional role in flowering signalling but was not included in the list of flowering genes that we used for gene enrichment analysis (Li et al., 2016).

Prior studies consistently found that drought caused the evolution of earlier flowering time in natural (Franks et al., 2007; Hamann et al., 2018) and experimental (Johnson et al., 2022) populations of *B. rapa*. However, our study found very little evidence for evolutionary shifts in any known flowering time genes in response to experimental drought. We also checked if any SNPs in key flowering regulators (*SOC1*, *FLC*, *FT*, *CO*, *GI*, *FRI*, and *FCA*) were significant before genomic correction for inflation, in case the correction was overly conservative. This assessment revealed one additional candidate SNP, which occurred in one of the *B. rapa* orthologs of *SOC1*. While *SOC1* plays a central role in regulating flowering (Immink et al., 2012), this shift did not likely play a prominent role in evolution of earlier flowering in this experiment as it occurred in only one replicate and lost significance after genomic correction.

Because 83.9% of the 913 SNPs that we identified with Fisher's exact tests were not in coding regions, evolution of earlier flowering may have been achieved through changes in regulatory rather than coding regions. In the population used to found this experiment, expression of *GATA8*, which promotes the flowering regulator *SOC1*, evolved in response to natural drought (Hamann et al., 2021). Although we inspected *GATA8* and found no SNP shifts, shifts in regulators including *GATA8* were not included in the list of flowering genes we used, and could have played a role in evolution of earlier flowering. Other studies have also found large numbers of differentiated SNPs in noncoding, presumably regulatory sequences suggesting an important role of regulatory responses to climate

selection in *M. musculus* (Ferris et al., 2021) and *D. melanogaster* (Burke et al., 2010). Thus, we acknowledge that evolution of earlier flowering was likely achieved predominantly through mechanisms other than allele shifts in coding sequences.

4.4 | Using the evolve and resequence design to explore genomic evolution

One notable aspect of our E&R design is that we leveraged responses among replicate populations to assess the parallelism of genomic responses to drought. Although E&R designs are rarely used to assess parallelism of genomic shifts, our results suggest that pooled sequencing can be a viable method to explore the degree of parallelism in rapid evolution to selection (Czech et al., 2022).

While our experimental design allowed us to explore genomic shifts underlying adaptation to drought, the specific SNP frequency shifts we observed differed from those in the founder population under natural drought. This is notable as research in evolutionary ecology often concerns the degree to which evolutionary responses of populations may be predicted, especially concerning persistence versus local extinction (Lobkovsky & Koonin, 2012; Shaw, 2019). We not only observed evolution of earlier flowering and higher fitness under experimental drought (Johnson et al., 2022) and in the founder population under natural drought (Franks et al., 2007; Hamann et al., 2018) but also found evolution of higher specific leaf area that was not detected in the field. This may suggest that experimental evolution may be effective to anticipate responses to selection in fitness and traits under strong and direct selection. However, we observed unique responses at SNP and gene levels, with only two genes evolving in both the founder and experimental populations under drought, neither of which had an annotation tied to stress or drought. Therefore, while our experimental results did match those seen in the field for fitness and flowering time, this was not the case for genomic shifts underlying adaptation. More work that compares evolution in replicates under experimental evolution designed to mimic selection to evolution in natural populations is necessary to understand the use and limits to using experimental environments to anticipate evolution in nature. However, our results suggest that it will be challenging to anticipate the genomic responses that underlie rapid adaptation of polygenic traits.

5 | CONCLUSIONS

We used an E&R design to identify SNP frequency changes that underlie rapid evolution in experimental replicates of *B. rapa*. We detected evidence for parallel evolution in some SNPs, but these evolutionary changes were shared among all descendants and not unique to drought. We also identified SNPs that evolved specifically under drought, which occurred in nonparallel among replicates and showed little overlap with the founder population under natural drought, suggesting polygenic adaptation. Most SNPs with frequency changes were outside coding regions, indicating a possible role of noncoding regulatory regions in drought adaptation. Broadly, our results highlight that genomic evolution to drought occurred from standing variation, but mostly nonparallel genomic shifts suggest that genomic responses to selection are difficult to predict for polygenic traits, even under an experimental environment.

AUTHOR CONTRIBUTIONS

S.J. carried out the experiment with seed material that S.F. provided. S.J. and S.F. designed the experiment and S.J. and S.T. analysed data. All authors contributed to manuscript preparation.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Raw reads are located in the SRA under BioProject ID PRJNA882576 and scripts and other data files have been archived in the Dryad repository at https://doi.org/10.5061/dryad.n5tb2rbzx.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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