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Identification of a genetic network for an ecologically relevant behavioural phenotype in *Drosophila melanogaster*

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Abstract

Pupation site choice of *Drosophila* third-instar larvae is critical for the survival of individuals, as pupae are exposed to various biotic and abiotic dangers while immobilized during the 3–4 days of metamorphosis. This singular behavioural choice is sensitive to both environmental and genetic factors. Here, we developed a high-throughput phenotyping approach to assay the variation in pupation height in *Drosophila melanogaster*, while controlling for possibly confounding factors. We find substantial variation of mean pupation height among sampled natural stocks and we show that the *Drosophila* Genetic Reference Panel (DGRP) reflects this variation. Using the DGRP stocks for genome-wide association (GWA) mapping, 16 loci involved in determining pupation height could be resolved. The candidate genes in these loci are enriched for high expression in the larval central nervous system. A genetic network could be constructed from the candidate loci, which places *scribble* (*scrib*) at the centre, plus other genes known to be involved in nervous system development, such as *Epidermal growth factor receptor* (*Egfr*) and *p53*. Using gene disruption lines, we could functionally validate several of the initially identified loci, as well as additional loci predicted from network analysis. Our study shows that the combination of high-throughput phenotyping with a genetic analysis of variation captured from the wild can be used to approach the genetic dissection of an environmentally relevant behavioural phenotype.

KEYWORDS
automated phenotyping, *Drosophila melanogaster*, gene network, GWA mapping, pupation behaviour

1 | INTRODUCTION

The pupal stage is a life history stage found in holometabolous insects undergoing transformation between larval and adult stages (Jones & Reiter, 1975; Price, 1970), including *Drosophila*. The choice of pupation site is known to directly influence the probability of successful eclosion of adult flies (Bauer & Sokolowski, 1985; Joshi & Mueller, 1993; Rodriguez, Sokolowski, & Shore, 1992), as pupae are exposed to many biotic and abiotic dangers while immobilized during 3–4 days of metamorphosis. Pupation height, defined as the vertical distance between larval pupation site and food surface in a vial, can be considered as a measure of pupation site

choice under laboratory conditions. The suitable location selected for pupation can immediately impact survival in various environmental conditions (Riedl, Riedl, Mackay, & Sokolowski, 2007). For instance, in a humid environment, animals pupating close to the food are less likely to survive, with increased risk of microbial attack or being drowned as other larvae churn up and liquefy the substrate (Markow, 1979; Rodriguez et al., 1992). The pupation site choices in *Drosophila melanogaster* were revealed to be under stabilizing selection, with significantly decreased viabilities for individuals pupating both close and far from the medium surface (Joshi & Mueller, 1993).

The pupation site choices in a number of *Drosophila* species have been extensively investigated (Erezyilmaz & Stern, 2013; Markow, 1979; Vandal, Shivanna, & Modagi, 2012; Vandal, Siddalingamurthy, & Shivanna, 2008), and substantial variation has been found both within and between species (Erezyilmaz & Stern, 2013; Markow, 1979; Vandal et al., 2008). Several environmental factors, such as temperature (Schnebel & Grossfield, 1992), light (Markow, 1981; Schnebel & Grossfield, 1986), humidity (Casares, Carracedo, & García-Florez, 1997; Sokal, Ehrlich, Hunter, & Schlager, 1960) and food medium (Harini, 2013; Hodge & Caslaw, 1998), have been shown to contribute to the variation of pupation site choice in *Drosophila*. Biotic factors were also identified, including sex (Casares & Carracedo, 1987), larval development time (Welbergen & Sokolowski, 1994) and larval density in the vial (Joshi & Mueller, 1993; Sokolowski & Hansell, 1983).

Artificial selection experiments on pupation height in several *Drosophila* species revealed a quick response in both directions (i.e., low and high pupation) of selection, with significant divergence being observed after only a few generations of selection (Casares & Carracedo, 1986; Garcia-Flores, Casares, & Carracedo, 1989; Singh & Pandey, 1993). The rapid response of artificial selection resulted in realized heritability estimates ranging from 0.13 to 0.18. Genetic association studies were conducted to explore the genetic basis of pupation site choice in *Drosophila* and were consistent with it being a complex behavioural trait with a polygenic basis (Bauer & Sokolowski, 1985; Erezyilmaz & Stern, 2013; Riedl et al., 2007; Sokolowski & Bauer, 1989). The genetic contributions were mostly found on the autosomes, but which chromosome contributed most to the explained variation remained disputed (Bauer & Sokolowski, 1985; Sokolowski & Bauer, 1989). Using a set of 76 recombinant inbred strains for a quantitative trait locus (QTL) study, Riedl et al. (2007) identified a major QTL at position 56A01-C11 on chromosome 2R, which includes at least 39 annotated genes.

Natural populations of *D. melanogaster* harbour a wealth of genetic variation, which has been proven to constitute a powerful resource to detect the genetic architecture for a wide range of phenotypes (Huang et al., 2014; King, Macdonald, & Long, 2012; MacKay et al., 2012). The *Drosophila* Genetic Reference Panel (DGRP), a population consisting of more than 200 highly inbred *D. melanogaster* strains derived from the Raleigh, US population (Huang et al., 2014; MacKay et al., 2012), has been successfully exploited to identify the association between a broad range of phenotypes and their underlying genetic basis, including several behavioural traits (Lee et al., 2017; Rohde, Gaertner, Ward, Sørensen, & Mackay, 2017; Shorter et al., 2015; Xue, Wang, & Zhu, 2017). The highly inbred nature of these DGRP strains allows accurate association mapping, through repeatedly sampling for individuals with the same genotype. Together with the well-resolved richness in genetic polymorphisms and rapid decay in linkage disequilibrium (LD) in these strains (Huang et al., 2014), these make the DGRP an excellent panel to study the genetic basis of pupation height choice in *D. melanogaster* at a fine-scale resolution.

The efficient and accurate measurement of phenotypes is often a limiting factor in QTL studies, particularly for behavioural traits where variation may be impacted by large numbers of interacting loci. Automated or semi-automated measurements can solve this problem. We have previously described an automated procedure for pupal case size measurements (Reeves & Tautz, 2017). Here, we used the same general approach as a means for the high-throughput accurate measurement of pupation height, while attempting to control for nongenetic confounding factors. We systematically assessed the pupation height choice patterns in both a range of natural global stocks and 198 DGRP inbred strains. We also examined the potential influence of several biotic factors, including sex, *Wolbachia* infection and parental effects. We explored the genetic heritability of pupation height and dissect the contributions of genetic variants via a GWA analysis. This allowed us to identify 16 significant genetic loci associated with pupation height variation across DGRP strains, of which we experimentally evaluated 7 candidates in gene disruption lines. Furthermore, we also analysed both gene expression data and genetic interaction data, in order to identify a genetic network involved in pupation height choice in *D. melanogaster*.

2 | MATERIALS AND METHODS

2.1 | *Drosophila* strains

The list of wild-type strains, DGRP inbred lines, deficiency and transposon insertion stocks, and their progenitor lines used in this study and the detailed information is provided in Table S4. Flies were reared under standard culture conditions (cornmeal–molasses–agar medium, 24°C, 55%–75% relative humidity, 12-hr light/dark cycle). A HOBO® data logger was placed in the incubator to monitor and record the environmental changes, that is, temperature, light and humidity, across all the experimental periods.

2.2 | Automated phenotyping of pupation height

A previously established automated pupal case length detection pipeline was adopted and modified for the automatic screening of pupation height measurements (Reeves & Tautz, 2017). In brief, standard food was dispensed into 28.5-mm-diameter and 95-mm-height vials (Genesee Scientific), and the food height (defined as the

distance from the surface of the food to the bottom of the vial) for each vial was manually measured and recorded. Once the food vials had fully cooled, 10.1 cm × 10.5 cm squares of overhead projector film (Nobo, plain paper copier film, 33638237) were slid into each vial lining their entire vertical wall. Approximately 10 healthy female flies (15 for inbred stocks) and 5 healthy male flies were introduced into each vial, for which a custom-printed semitransparent label (GA International Inc.), including a unique barcode, was affixed to the outside of each vial. Adult flies were removed from the vials after 1–2 days, and vials were kept under the same incubation condition (see above) for another 8–9 days to allow them to reach pupation stage. In general, by the 10th day after the parents were initially introduced, the majority of offspring in the vials were present as pupae attached to the transparent film. The film was gently moved out from each vial, the food from the lower part was scraped away, and any larvae or pupae at white puparium stage (P1) were removed. The film was then placed into a pre-made plastic frame, which holds the film flat for further photographing using bottom illumination in a light-tight box. Batches of the resulting images were then introduced into the image analysis procedure.

The open-source image analysis software CELLPROFILER (v2.1.0) (Carpenter et al., 2006) was applied for the simultaneous recognition of pupae and measurements of a variety of attributes (Dataset S1), with a customized pipeline slightly modified from Reeves and Tautz (2017). In brief, any shape significantly distinct from the background was first identified without size restriction (module "identify primary object"). The identified objects composed of multiple juxtaposed pupae were disentangled into distinct pupae (module "untangle worms"). The resulting pupae were then shrunk and re-delineated based on boundary changes in pixel intensity (module "identify secondary objects"). Finally, a specific confidence level was assigned for each pupa based on its size attribute. The digital outlines of pupae were overlaid onto a cropped version of the original image for easy visualization (Figure 1). A manual check on 40 randomly selected films showed that the CELLPROFILER pipeline can successfully identify 96% of true pupae (sensitivity), with an accuracy of 81% for identified putative pupae. To further improve the detection accuracy, an additional refinement criterion was defined based on the size attributes of "true" pupae from manual curation. Applying the new criteria, the accuracy for pupae detection was improved to around 99.85%, with only a negligible fraction (<0.7%) loss of true-positive results.

In addition, a 1 euro cent coin (16.25 mm diameter) was included in each image, for the control of camera coordinate changes and the conversion of measurements from pixels to millimetres. The pupation height for each pupa was calculated as the subtraction of the vial food height from the vertical coordinate measurement (CELLPROFILER parameter: "Areashape_Y"). Overlaid images and files with a variety of attribute measurements were imported into a FileMaker database (v14, FileMaker Inc.). The quality filtering of pupae and related analysis were conducted with the tools implemented in the database. A more detailed description on the pupae detection pipeline can be found in File S1.

2.3 | Treatment of confounding factors

Pupal density in the vial is a biotic factor that could affect the pupation site selection preference of third-instar larvae (Joshi & Mueller, 1993; Sokolowski & Hansell, 1983). Here, density was controlled by limiting the number of parents used per vial and restricting the number of nights they remained before being cleared (see above). Further, to reduce the possible bias from including vials containing very low number of individuals, only vials with a pupal density of a minimum of 15 were considered as reliable, and a measurement for each stock should include at least 6 such reliable vial measurements. All of the tested stocks exhibited a similar positive correlation between individual density and pupation height estimate (File S1). The following equations were used to correct the influence of individual density in the vial on the mean estimate of pupation height in a first step:

$$\text{Average regression slope of pupae density against pupation height: } \beta = \mu\left(\beta_i\right) \tag{1}$$

β_i: regression slope of density against pupation height mean vial for strain i.

$$\text{Pupation height corrected for individual density} = O - \left(D - M\right) * \beta \tag{2}$$

O: pupation height vial mean to be corrected; D: automated estimate of individual density in the vial to be corrected; M: average vial density across whole experiment (set as 70); β: average slope of regression of density against pupation height mean vial (set as 0.145).

In a second step, to correct the influence from the change of incubator humidity and other cryptic abiotic factors, two wild-type stocks representing two extreme sides on pupation height (S-317 and S-314) were included and measured in each round of experiments for the phenotyping measurements of DGRP inbred stocks. The correction on incubation environment change (after correction of density) was achieved with the following equation:

$$\text{Pupation height corrected for incubator environment change} = ODC - \left[\left(H - H\mu\right) + \left(L - L\mu\right)\right] / 2 \tag{3}$$

ODC: pupation height of target DGRP stock from original measurement; H: pupation height measurement of high stock (S-314) for current round of experiment; $H\mu$: average pupation height measurement of high stock (S-314) across all rounds of experiments; L: pupation height measurement of low stock (S-317) for current round of experiment; $L\mu$: average pupation height measurement of low stock (S-317) across all rounds of experiments.

2.4 | Automatic measurement of pupal case length

The measurement of pupal case length followed the procedure described in Reeves and Tautz (2017). In brief, the pupal case length

FIGURE 1 Automated measurement of pupation height. (a) The larvae in the vial crawl up the wall for pupation. The vial includes a plastic sheet that lines the wall. This is taken out after a sufficient number of pupae are attached, and it is photographed (b). The image analysis software CELLPROFILER is then used to identify the outlines of the pupae (c) and to identify single ones that can be reliably measured (marked in blue). After optimizing the system, we achieve accuracy for pupae detection of around 99.85%, with only a small fraction of loss of true-positive results (<0.7%) [Colour figure can be viewed at wileyonlinelibrary.com]
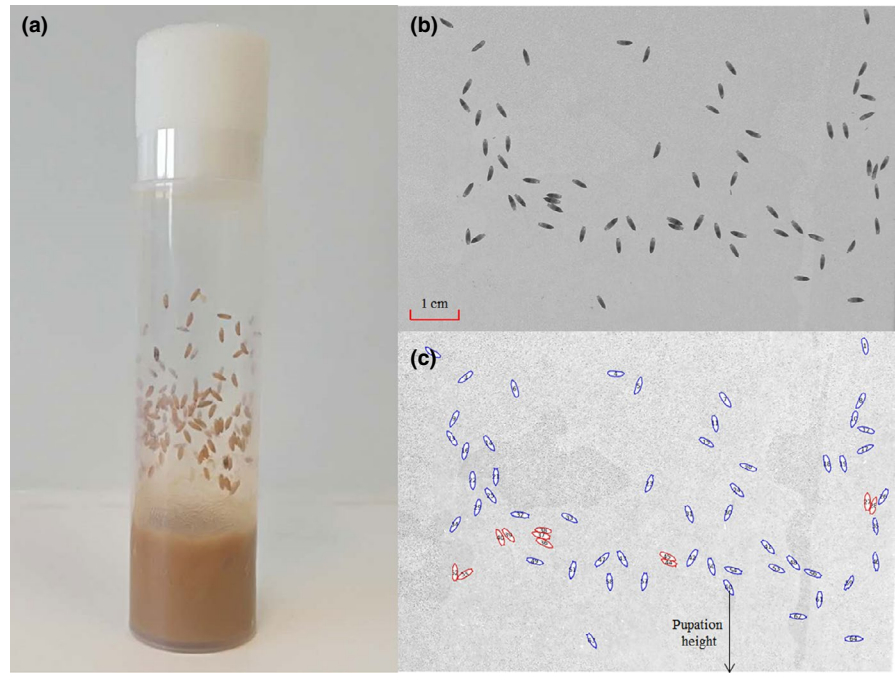
is defined as the length of the major axis of the ellipse that has the same normalized second central moments as the region of identified pupae, measured with the "Areashape_MajorAxisLength" index in CELLPROFILER. As the pupal case length measurements are relatively robust to the pupal density in the vial and the minor change of incubator humidity (File S1), the measurement for pupal case length was not corrected for these factors.

2.5 | Wolbachia infection, sex and maternal/paternal effect test

The *Wolbachia* infection status data of DGRP strains were directly obtained from Huang et al. (2014). Two different approaches were applied to test whether there is any effect on pupation site status from *Wolbachia* infection: (a) one indirect way applied here was to compare the differences of pupation height between *Wolbachia*-infected stocks and *Wolbachia*-uninfected stocks. (b) Another direct experimental way was to compare the differences of pupation height between *Wolbachia*-infected stocks and the same stocks for which the *Wolbachia* was removed using tetracycline treatment. Three DGRP randomly chosen inbred lines with *Wolbachia* infection were used to create *Wolbachia*-free stocks through two generations of tetracycline treatment (by adding an appropriate volume of 100 mg/ml of tetracycline suspended in 99% ethanol to the surface of the solid prepared food) and then reared for at least another two generations with standard food to avoid any detrimental parental effects (Zeh, Bonilla, Adrian, Mesfin, & Zeh, 2012). Meanwhile, the flies from the same three strains were also reared with standard food across the experiment as controls. Pupation height between the three *Wolbachia*-infected and the corresponding *Wolbachia*-free lines was then measured and compared via a

Wilcoxon rank-sum test. In addition, the same procedure was applied to three randomly selected DGRP *Wolbachia*-uninfected lines to exclude the possibility that tetracycline treatment could have an influence on pupation height. The *Wolbachia* infection status of the strains with and without exposure to tetracycline retreatment was experimentally verified. Genomic DNA from these stocks was extracted individually using DNeasy Blood and Tissue Kit (Qiagen), and the purity and concentration of the resulting DNA were measured with NanoDrop ND-1000 Spectrophotometer (Thermo Fisher). A diagnostic PCR to test for the presence of the *Wolbachia* wsp gene was done using the primers wsp81F (5′-tggtccaaaatgt-gagaaac-3′) and wsp691r (5′-aaaattaaacgctactcca-3′) (Richardson et al., 2012). The conditions for this diagnostic reaction were 35 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 1 min. The expected PCR product length is around 630 bp. A standard (1%) agarose gel electrophoresis was used to test for the presence of the PCR product, with the broad-range Quick DNA Marker (NEB #N0303) as loading ladder.

In our previously published data set (Reeves & Tautz, 2017), we had also conducted a pupal sexing by moving single pupae into vials and calling the sex after their eclosion. This was not done in the present study; hence, we used the results of the previous study to determine possible sex effects. In brief, >4,000 pupae picked from 728 vials were sexed (corresponding to 2,340 female pupae and 1,935 male pupae), and their pupation site coordinates were measured and recorded. Since this earlier study had not recorded the level of the food surface, the pupation height for each sexed pupae was calculated as the deviation from the corresponding vial to average pupation height, and then, the average deviation between two sexes was compared.

A reciprocal crossing approach was used to detect whether any maternal, for example, genetic effect from mitochondria, or paternal

effects for pupation site selection exist. Two pairs of high and low pupation height DGRP inbred lines (DGRP-486 and DGRP-383; DGRP-228 and DGRP_318) were selected for this analysis. The pupation height of F1 offspring from reciprocal crossing, that is, virgin females from high stock crossing with males from low stock, and vice versa, was measured and compared with the phenotype of their parental stocks.

2.6 | Estimates of heritability

The broad-sense heritability (H^2) was estimated with the variance components of a linear model of the form: Phenotype = Population mean + Line effect + error (Schmidt et al., 2017). Total phenotypic variance was estimated as Genetic Variance + Environmental Variance, and the H^2 was thus estimated as $G_v/(G_v + E_v)$. This was implemented in IBM SPSS Statistics (version 22) (SPSS Inc, 2007), with pupation height as the dependent variable and DGRP strain names as a random factor.

The narrow-sense heritability was estimated as the proportion of variance in a phenotype explained by all available genetic variants used for mapping, an estimate that is often called "SNP heritability" (Visscher, Hill, & Wray, 2008; Wray et al., 2013). In practice, a genetic relationship matrix (GRM) between pairs of inbred strains from all the DGRP annotated genetic variants was built by using GEMMA (version 0.96) (Zhou & Stephens, 2012), and then, the narrow-sense heritability (denoted as PVE) was calculated based on the above GRM with the univariate linear mixed model (Zhou & Stephens, 2012) implemented in GEMMA.

2.7 | Principal component analysis (PCA) and GWA analysis

The genetic variant information and major genomic inversion status were retrieved from DGRP freeze 2 (Huang et al., 2014). Genetic variants with missing values above 20% and minor allele frequency below 5% were excluded from further analysis, with 1,903,028 genetic variants (on average ~14 genetic variants per kb) passing the stated criteria. To assess the possible influence of population structure on the pupation site selection, the PCA module from PLINK v1.90 (Purcell et al., 2007) was used to identify top principal components (PCs) from the filtered genetic variant data. The projection length of each strain on top 20 PCs was used to test the influence of cryptic population structure on pupation site selection.

The linear regression model implemented in PLINK was initially used to perform association analysis for the above filtered genetic variants without correction of population structure. In order to correct any potential influence from cryptic population structure, a linear mixed model using the FASTLMM (Lippert et al., 2011) program (version 0.2.32) was also applied for GWA analysis. By using FASTLMM, another two GWAs were conducted for without and with the incorporation of pupal case length.

A nominal p-value threshold (p-value = 1×10^{-5}), commonly used in *Drosophila* GWA studies (Dembeck et al., 2015; Lee et al., 2017), was applied to identify candidates for genetic variants associating with pupation height (note that this is not meant to be a significance cut-off). The R package "QQMAN" (Turner, 2014) was exploited for the visualization of GWA results in a Manhattan plot and QQ plot. The standardized effect size for each genetic variant was calculated as one-half the difference between marker classes divided by the overall phenotypic standard deviation (Harbison, McCoy, & Mackay, 2013). The associating genes for each genetic variant were annotated by SnpEff (Cingolani et al., 2012) with default parameters. In brief, all the protein-coding genes within 5 kb up/downstream of the target genetic variant were taken as associating genes.

The genotypic linkage disequilibrium (LD) for each pair of significant genetic variants was tested by calculating the squared correlation estimator r^2 with PLINK (Purcell et al., 2007). Moreover, the r^2 values for each genetic variant and all other genetic variants were also computed. A significant genetic region (QTL) was defined by the position of the most distant downstream and upstream genetic variants showing a minimum r^2 of .8 to the significant genetic variants. All the associating genes as determined above, together with the genes within the LD regions, were considered as candidate genes for further analysis.

2.8 | Expression and genetic interaction network analysis

The gene expression profiling data (downloaded in December of 2017) from *Drosophila* modENCODE project (Brown et al., 2014) were used for expression analysis. These were generated by measuring the genome-wide gene expression for 5 tissues in third-instar larvae stages, including central nervous system (CNS), digestive system, fat body, image disc and saliva glands. The expression level for each gene within each tissue was measured in units of RPKM. The fractions of genes with expression (RPKM > 0), as well as the gene expression levels of both GWA candidate genes and network-predicted genes (as stated below), were compared with those of total annotated protein-coding genes (Fisher's exact test for the comparison of the fractions of expressed genes; Wilcoxon rank-sum test for the comparison of gene expression levels).

The genetic interaction database was directly downloaded (in May of 2018) from Flybase V6.19 (Attrill et al., 2016). A network for edges was either a direct connection between candidate genes or bridged by only one gene not among the candidate gene list was extracted. The significance of the size of the largest cluster (a set of tightly connected genes) among the subnetworks was tested by a randomization test in which we randomly extracted subnetworks for 1,000 times with the same number of input genes. The p-value was determined by dividing the number of instances where the size of the largest cluster (identified with igraph v1.2.4.1) (Csardi & Nepusz, 2006) exceeds the observed largest size by the total number of randomizations (Zhou et al., 2016). Additionally, an extended network

was also retrieved by adding the first-neighbour interacting genes of candidate genes to the above-identified subnetwork. In order to correct for the gene length bias, gene ontology (GO) enrichment analysis on the network genes was performed with GOSeq (v1.38.0) (Young, Wakefield, Smyth, & Oshlack, 2010). Only significant GO BP (biological process) terms after multiple testing correction (FDR ≤ 0.01) are shown in this study. Cytoscape (v3.7.0) (Shannon et al., 2003) was used for the visualization of these subnetworks.

2.9 | Functional validation experiments

Functional validation experiments to test the phenotypic effects of candidate genes were conducted on two types of gene disruption mutagenesis (with disruption in the gene region of at least 1 gene for each locus) based on their stock availability: (a) two tested loci were disrupted by transposon (*Minos* or *PBac*) insertions (Bellen et al., 2011); (b) five tested loci were disrupted through DNA segment deletions. The detailed information about the gene disruption stocks and their progenitors can be found in Table S4C.

Both transposon insertion strains are homozygous insertion complete-viable. All the DNA segment deletion (deficiency) lines are homozygous complete-lethal. For each test, at least 8 vials (15 females and 5 males introduced to each vial) were set up for each insertion/deficiency and its progenitor stock, and their pupation heights were then measured and compared. Based on the gene disruption type and selection marker on the balancer chromosome, the functional validation experiments were conducted as following (Figure S8):

1. Transposon insertion lines: the pupation height status of the transposon insertion lines and their corresponding progenitor lines were directly measured and compared.
2. DNA segment deletion lines with detectable marker at pupal stage: three out of five deficiency stocks are segregating balancer chromosomes with *Tubby* (*Tb*) as visible marker (short rounded pupae) (Table S4C). The pupation site choices of the background stock (BG line) and F1 generation of the crossing of each segment deletion stock (virgin females) and its BG stock (males) were measured with the phenotyping pipeline. The pupation height statuses of hemideletion individuals without *Tb* (no presence of balancer chromosome) were compared with those from BG stocks. The absence of *Tb* for individual pupae was determined by their pupal case length (>73 pixels for Areashape_major_len from the output of CELLPROFILER), on the basis of the apparent distinction between individual pupae with and without *Tb*. Moreover, a manual check was done to further separate ambiguous individuals.
3. DNA segment deletion lines without detectable marker at pupal stage: Two deficiency lines are segregating balancer chromosomes with no detectable marker at pupal stage (*curly wing* or *stubble* marker is visible at adult stage only) (Table S4C). Virgin female individuals from these insertion or deficiency stocks were crossed with male individuals from BG lines to generate a F1

generation. Virgin female individuals without screening markers (*curly wing* or *stubble*) at adult stage from the F1 generation were selected and backcrossed with males from BG lines. The pupation height status of the F2 generation was measured with the phenotyping system and compared with that of their progenitor stocks. Note that the detected significances of the phenotyping effect of candidate genes from this approach are likely to be underestimated, as in theory only half of the individuals in the experiment group contain the gene semideletion.

Moreover, phenotypic effects of additional four genes, that is, epidermal growth factor receptor (*Egfr*), E2F transcription factor 1 (*E2f1*), Ras oncogene at 85D (*Ras85D*) and *p53*, in the above-predicted network were further checked, via direct comparisons of pupation height status between the co-isogenic progenitor stock and transposon disruption of each target gene (Table S4D). For gene *p53*, two different transposon insertion strains (with disruption at different gene regions) were tested. The experimental verification scheme follows the protocol as described in the above text.

3 | RESULTS

3.1 | Establishment of an automated phenotyping pipeline

The acquisition of phenotype data from a large number of individuals is a prerequisite for high-resolution genetic mapping studies. Instead of using manual measurement-based approach from previous studies on pupation height (Bauer & Sokolowski, 1985; Erezyilmaz & Stern, 2013; Riedl et al., 2007; Sokolowski & Bauer, 1989), we adapted an image analysis-based phenotyping pipeline (Reeves & Tautz, 2017). This pipeline was initially developed for the high-throughput measurement of pupal case length and was shown to have the capability for the automatic detection of pupae with a high precision (Reeves & Tautz, 2017). We modified it for the purpose of the automated measurement of pupation height, defined as the distance from the vertical coordinate of pupation site (pupal centre) to the food surface in the vial in millimetre (mm). Figure 1 shows an example of the automated measurement of pupation height. The detailed experimental setup and the entire phenotyping procedure are provided in File S1.

Density of individuals within the vial is a common major environmental covariate of many *Drosophila* traits, including pupation site choice (Joshi & Mueller, 1993; Sokolowski & Hansell, 1983). In the present study, individual density variation was controlled in an indirect manner through limiting the number of parents used per vial (10 females for wild-type strains, and 15 females for inbred strains) and restricting the number of nights they remained before being removed (1–2 nights). Still, some variation was apparent that needed to be addressed. Based on a set of test experiments, we defined a minimal sampling rule of at least 15 measured pupae per vial and at least six replicate vials per strain. We found that almost

all stocks exhibited a uniformly positive relationship between individual density and pupation height, allowing us to use an average slope (0.145) across all tested stocks to correct the mean estimate of pupation height in all vials (File S1). However, note that by using such a single correction factor, we ignore the possibility of strain-specific effects, which leads to somewhat increased noise in the data.

3.2 | Wild-type variation is captured by the DGRP stocks

Two distinct sets of strains were used to explore the variance in pupation height choice. The first set consisted of 14 natural wild-type *D. melanogaster* strains, collected from different parts of the world (Table S4A). The second set was from the DGRP (Huang et al., 2014) and included 198 lines (Table S4B). In order to correct for environmental factors, especially cryptic differences in humidity (Casares et al., 1997; Sokal et al., 1960), two wild-type stocks (S-317 and S-314) representing two extremes of pupation height from the first strain set were continually remeasured to act as controls throughout all experiments (File S1). The estimates of pupation height for the strains were corrected based on the average pupation height of the two control stocks across all rounds of experiments. Similar as above, we note that this procedure ignores the possibility of strain-specific effects, that is, is expected to increase the noise. In the following, all presented measurements of pupation height on DGRP lines incorporate the correction for environmental factors in common with the control vials.

Figure 2 shows the profiles of corrected pupation height from the wild-type and DGRP sets of strains. On average, 7.8 vial measurements (530 individual pupae) and 8.2 vial measurements (335 individual pupae) were examined for each strain from global and DGRP inbred stocks, respectively. We observe large variation of pupation height among strains, ranging from pupation height of only 15 mm above the food medium up to the very highest possible position adjacent to the plug surface of the vial (50 mm). The wild-type lines showed no obvious geographical clustering of pupation heights. The spread of pupation height among the DGRP stocks exceeds that of the wild-type stocks, suggesting that they may capture at least a major part of the existing variation in *D. melanogaster*.

3.3 | Sexual dimorphism and parental effects

Sexual dimorphism, the condition where sexes from the same species exhibit different characteristics for morphological or behavioural traits, is a commonly observed phenomenon (Berner, Sládek, Holt, Niskanen, & Ruff, 2017). Regarding the pupation site choice in *Drosophila*, a controversy on the existence of sexual dimorphism has persisted for several decades. Early studies have reported no sexual dimorphism (Markow, 1979; Sokolowski & Bauer, 1989; Welbergen

& Sokolowski, 1994), while the results from other studies showed that males pupate significantly higher than females (Casares & Carracedo, 1987; Riedl et al., 2007).

To address the sexual dimorphism question, a distinct data set incorporating both pupation height and sex information of >4,000 randomly selected (2,340 females and 1,935 males) individuals from 728 vials generated from the 4-way pedigree data set reported by Reeves and Tautz (2017) was analysed. Since this earlier study had not recorded the level of the food surface, the pupation height for each sexed pupae was calculated as the deviation from the corresponding vial to average pupation height (Figure 3a) (Reeves & Tautz, 2017). As shown in Figure 3b, there was a significant difference in pupation height between males and females (Wilcoxon rank-sum test: p-value = 1.5×10^{-7}), with male individuals pupating on average around 2 mm higher than females. This result is roughly consistent with the observed sexual dimorphism reported in two previous studies (Casares & Carracedo, 1987; Riedl et al., 2007).

A parental bias, by which the phenotype of an individual depends more upon the mother's or father's phenotype or genotype, can be observed for some traits (Reik & Walter, 2001). This can be caused by the inheritance of genetic material in the cytoplasm (e.g., mitochondria, *Wolbachia* bacteria), sex chromosomes or imprinted gene regions. Previous studies on this aspect for pupation site selection provided two opposing views, one suggesting the pupation site choice in *Drosophila* fits a simple additive model of inheritance without any parental bias (Sokolowski & Bauer, 1989), while the other found a significant maternal effect on pupation site selection (Garcia-Flores et al., 1989; Singh & Pandey, 1993). To address this question, two pairs of DGRP inbred lines with each pair representing two extremes of pupation height were randomly chosen and were reciprocally crossed to test for parental biases. As shown in Figure 3c,d, the pupation heights for offspring from both directions lie between their parental stocks, with no significant differences (Wilcoxon rank-sum test: p-values .11 and .17) on pupation height choice in reciprocal crosses. This finding supports the additive model of inheritance on pupation site selection in *D. melanogaster* (Sokolowski & Bauer, 1989).

Wolbachia pipientis is a maternally transmitted endosymbiotic bacterium that infects around 53% of DGRP strains (Huang et al., 2014). It was reported to have a significant effect on some behavioural traits, for example, acute and chronic resistance to oxidative stress (Huang et al., 2014). Two different approaches were used to explore a possible effect of *Wolbachia* infection on pupation height. First, the statistical analysis on the pupation height between all tested strains with infected and noninfected strains exhibited no significant difference of pupation height (Wilcoxon rank-sum test: p-value = .29, Table S1). Second, the experimental phenotypic comparison between pupation height of three randomly selected DGRP strains with *Wolbachia* infection and those after the removal of *Wolbachia* using tetracycline treatment showed no significant statistical difference on pupation height choice for all tested strains (Figure S1). Accordingly, the *Wolbachia* infection on DGRP strains

FIGURE 2 Distribution of pupation height for different strains. (a) Natural global stocks and (b) DGRP inbred strains (b). At least 6 vial measurements (vial pupae density ≥ 15) were conducted for each stock. On average, 7.8 vial measurements (530 individual pupae) and 8.2 vial measurements (335 individual pupae) were examined for each strain from the wild-type and DGRP inbred stocks, respectively
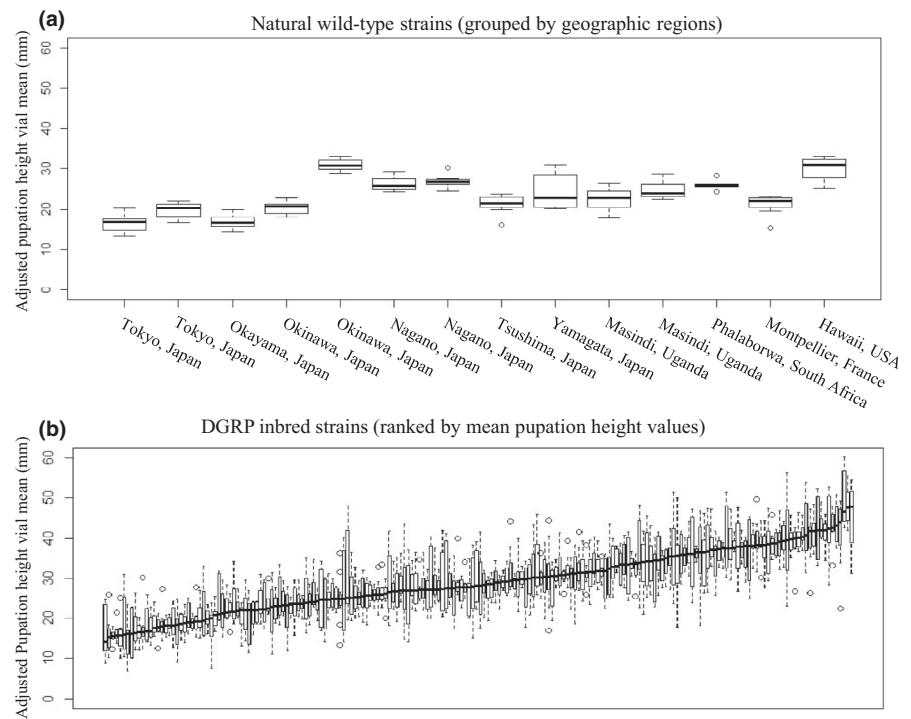
was not incorporated in the association analysis below, as both the indirect and direct evidences described above revealed no strong effect on pupation height choice.

3.4 | Heritability and chromosome effects of pupation height

The broad-sense heritability (H^2) was estimated by determining the proportion of total variance in the mean strain height measurements compared to the average within each strain (Schmidt et al., 2017). The narrow-sense heritability (h^2) was estimated here as "SNP heritability" (Wray et al., 2013), that is, the estimate of the proportion of phenotypic variance explained by all available SNPs (or genetic variants) in the DGRP stocks.

All estimates are shown in Table 1. Values of H^2 of 0.64 (0.70 for wild-type strains) and h^2 of 0.46 (SE: 0.2) based on the estimates from DGRP inbred stocks imply higher heritability than from previous estimates within this species (Casares & Carracedo, 1986; Garcia-Flores et al., 1989; Singh & Pandey, 1993).

Partitioning the variance by chromosome reveals that all chromosomes, except the 4th, contribute a substantial part to the variance of pupation height (Figure S2). The minimal contribution from chromosome 4 can be ascribed to the limited number of genetic variants within this chromosome. In line with previous reports (Bauer & Sokolowski, 1985; Sokolowski & Bauer, 1989), we find a somewhat higher contribution of autosomes to the variance of pupation height and also a slightly larger effect from chromosome 2 compared to chromosome 3 (Bauer & Sokolowski, 1985). These observations also confirmed the polygenic nature of

pupation height choice in *D. melanogaster* (Bauer & Sokolowski, 1985; Erezyilmaz & Stern, 2013; Riedl et al., 2007; Sokolowski & Bauer, 1989).

3.5 | GWA analysis

The GWA analysis was based on the genetic variants of DGRP freeze 2 (Huang et al., 2014); variants with missing values above 20% and minor allele frequency below 5% were excluded from further analysis. A few possible covariates were first assessed, in order to optimize the model used for the GWA analysis.

To investigate whether any cryptic population structure could contribute to the observed variation in pupation site choice of inbred stocks, PLINK (Purcell et al., 2007) was used to identify major PCs of genetic variants in the DGRP strains. Only one major cluster was found in these stocks based on PC analysis (Huang et al., 2014), and there were no obvious clusters of strains that have different pupation height status on the first two principal axes (Figure S3). Moreover, only three out of the top 20 PCs showed significant (Pearson's correlation test, p-value < .05) correlations with pupation height. Though all these correlation coefficients are very low (ranging from 0.002 to 0.245, Figure S4), the slight correlations seem more likely to come from a few outlier individuals.

Given their driving force on population divergence and speciation (Hoffmann & Rieseberg, 2008), major genomic inversions in DGRP strains (Huang et al., 2014) might contribute to the observed population structure and the association between population structure and pupation height. The systematic test for the correlations between genomic inversion status in DGRP strains and the first
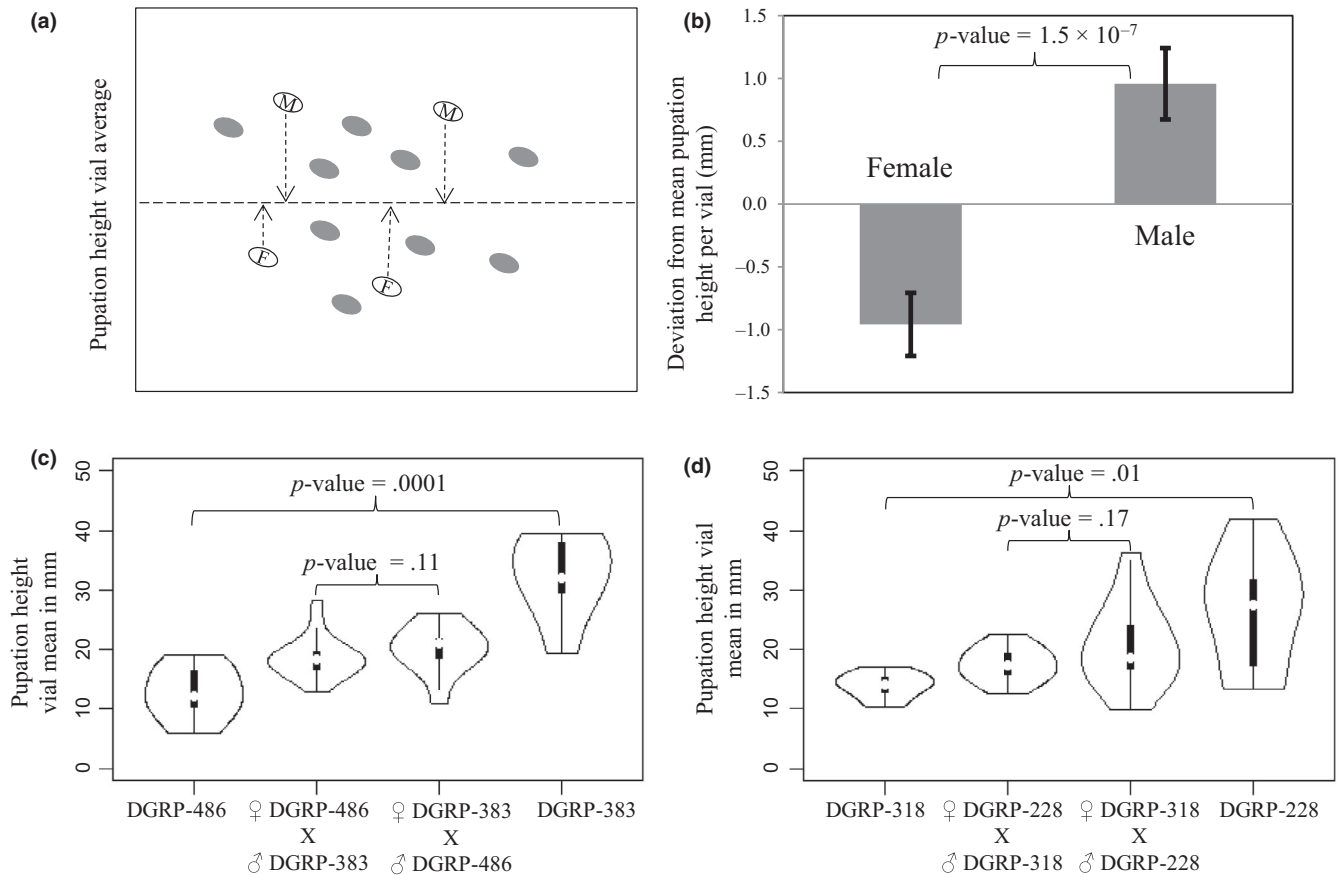
FIGURE 3 Sexual dimorphism and parental bias for pupation height in *Drosophila melanogaster*. (a) shows a scheme with pupae on the film surface and explains how the deviation on pupation height of sexed individuals was compared to the vial's mean value; (b) shows the distribution of the deviation of pupation height from vial mean in mm for sexed pupae. The error bars indicate the standard error of mean values. Two pairs of DGRP inbred lines, (c) DGRP-486 and DGRP-383, (d) DGRP_318 and DGRP-228, each pair representing two extremes of pupation height choice were reciprocally crossed to test for significant phenotypic differences with the parental stocks (shown as violin plots). Each significance *p*-value was obtained from a Wilcoxon rank-sum test

Heritability	Data set	Method	Heritability estimate
H^2	Wild-type strains[a]	IBM SPSS (Variance component)	0.70
H^2	DGRP inbred strains[b]	IBM SPSS (Variance component)	0.64
h^2	DGRP inbred strains[b]	GEMMA univariate linear mixed model	0.46 ± 0.20 *SE*

TABLE 1 Statistics for the estimation of the heritability of pupation height choice

Abbreviations: *SD*, standard deviation; *SE*, standard error.

[a]Number of stocks = 14; Number of vials measured = 109; Average number of replicates per strain = 7.8 ± 0.6 *SD*; Average number of measured pupae per vial = 69 ± 24 *SD*.

[b]Number of stocks = 198; Number of vials measured = 1,627; Average number of replicates per strain = 8.2 ± 1.6 *SD*; Average number of measured pupae per vial = 40 ± 17 *SD*.

two PCs of genomic variation showed significant effects only from In(2L)t and In(3R)Mo (Table S2), indicating their possible roles in population subdivisions (Hoffmann & Rieseberg, 2008). However, there is no significant association between pupation height and all tested genomic inversions, including In(2L)t and In(3R)Mo (Table S2). Consequently, genomic inversions were not included in the following association mapping analysis.

The original DGRP lines were constructed such that population structure effects should be minimized, but some genetic relatedness leading to cryptic population structure might still exist (Mackay & Huang, 2018). In order to correct any major influence from the cryptic population structure that we identified above, a linear mixed model using the FASTLMM (Lippert et al., 2011) program (version 0.2.32) was applied. We find a strong correlation between GWAs with and

without correction of population structure, confirming the minimized population structure effects within DGRP lines (Figure S5). Still, for further analysis, we use the GWA with correction of population structure (Figure 4).

A previous study has shown a possible role of larval size on their pupation site choice (Vandal et al., 2012). Here, we use pupal case length as a proxy of larval size (Reeves & Tautz, 2017). We find that there is indeed a weak, but significant negative correlation between pupal case length and pupation height, though only at vial level (Figure S6a, Pearson's correlation test, correlation coefficient r value = −.14, p-value = 3.1×10^{-8}), but not at strain level (Figure S6b, Pearson's correlation test, correlation coefficient r value = −.06, p-value = .41). Moreover, a GWA was performed using pupation height as phenotype and pupal case length as covariate, to compare with the GWA using pupation height as a phenotype without any covariate. This analysis revealed an extremely strong correlation between p-values of GWA with and without pupal case length as a covariate (Figure S6c, Pearson correlation test, correlation coefficient r value = .99, p-value < 2.2×10^{-16}). When applying a nominal GWA p-value threshold of 1×10^{-5} (defined below), we found that these two GWAs shared most of the identified significantly associated genetic variants (Figure S6d). Based on these analyses, we conclude that the identified candidate genetic variants on pupation height are mostly independent from its association with pupal case length. Therefore, pupal case length was not included in the GWA for further analysis.

3.6 | Candidate genes from the GWA analysis

A nominal GWA p-value threshold of 1×10^{-5} (Dembeck et al., 2015; Lee et al., 2017) was set to detect significant genetic variants associated with pupation height. At this cut-off, we found 16 significant genetic variants (13 SNPs and 3 indels) associated with pupation height in the DGRP strains, corresponding to 38 associating genes that locate within 5 kb up/downstream (default setting in SnpEff (Cingolani et al., 2012)) of these genetic variants (Table 2). A moderate negative correlation was observed between the minor allele frequencies (MAFs) and their GWA association p-values of

these genetic variants (Pearson's correlation test, r value = −.50, p-value = .05), indicating a tendency that gene variants with high MAFs are more likely captured by GWA test (Table 2 and Figure S7).

To identify additional candidate genes associated with the variants, we examined the long-range linkage disequilibrium (LD) between pairs of detected candidate variants and with other genetic variants found in the DGRP strains. No significant linkage between physically distant (≥1 Mb, with $r^2 \geq .8$) genetic variants was found, suggesting the associations are not confounded by long-range LD. LD blocks were then calculated for each significant genetic variant with a commonly used threshold $r^2 = .8$ (Pallares, Harr, Turner, & Tautz, 2014), and 6 significant LD blocks were found with average block size of 6.96 kb (Table S3). No pairs of identified significant variants were found in the same LD block. This finding is in line with the observation of a rapid decay of LD in the panel strains reported in the original DGRP resource reference (Huang et al., 2014). Combining the additional genes identified in the above LD blocks, we identified in total 49 candidate genes potentially associated with pupation height variation in *D. melanogaster*. None of these identified candidate genes overlaps with the previously reported pupation height association QTL at 56A01-C11 in Riedl et al. (2007). Given the relatively small number of DGRP lines ($n = 198$ in our study), false positive or negative discovery associations may exist from our GWA and LD analysis, while still identifying candidate genes for further experimental follow-up (Ayroles et al., 2015; Turner & Miller, 2012).

3.7 | Phenotype confirmations

The advantage of *Drosophila* as a model system is that one can use mutant alleles that have been constructed in a common coisogenic background to test whether different alleles in genes implicated by the GWA analyses indeed affect pupation site choice. Despite the potentially low effect of each candidate locus, an observable phenotypic change would still be expected, given the great sample size from our automatic phenotyping system. Gene disruption lines were available for 7 candidate loci (with disruption
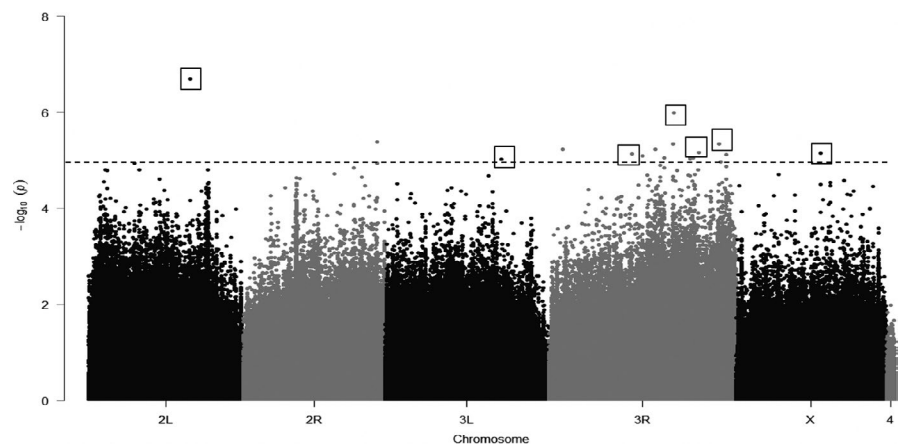
FIGURE 4 Manhattan plot for GWA results on pupation height with the correction for population structure. The p-values in −log10 transformation are shown in y-axis. The dashed horizontal line marks the significance p-value (10^{-5}) threshold used in this study. The genetic variants in rectangles are the ones applied for experimental verification in Figure 5

in the gene region of at least 1 gene for each locus) within the 16 identified associated loci. Two constitute transposon insertion mutations (Bellen et al., 2011), and five constitute small deficiencies (Table S4C). Experimental tests involve replicated phenotypic comparisons between the co-isogenic progenitor stock and homozygous or heterozygous disruptions of the target genes (see Methods).

An overview of the measured pupation height differences compared to the respective progenitor stocks is provided in Figure 5. Five out of 7 tested candidate loci showed a significant difference, two with an increased height and three with a decreased one. Both of the two transposon insertion stocks that are homozygous viable (*Smrter - Smr*, *scrib*) show a decreased pupation height. One of these two insertion stocks (*scrib*) reached the statistical significance (*p*-value < .05), while the other one (*Smr*) did not (*p*-value = .21).

The other stock that shows no significant overall change (Arl6IP1/nonA-I/Fas1) is a deletion stock that is homozygous lethal, that is, the fact that we did not find an effect on pupation height in hemizygous state does not rule out the possibility that other alleles would show it. Two other homozygous deletion lethal strains (including the candidate genes *CG7029* and *Oatp74D/Edin*) do show a significant influence on pupal height status, implying a possible haplo-insufficient or dominant effect. Two further homozygous lethal strains which could only be tested under conditions where 50% of the pupae should be hemizygotes (see Methods for details) (corresponding to the two candidate loci *CG15270* and *CG7567* from DNA segment deletion disruption) show significant effects as well, potentially indicating a particularly strong haplo-insufficient or dominant effect. However, each of the deletions affects multiple genes (Table S4C), leaving it an open question as to whether another gene in the region causes the effect.

3.8 | Expression and network analysis

Pupation follows the third-instar larval (L3) stage in *Drosophila*. Hence, one can ask whether genes involved in pupation site choice are more likely to have a substantial expression at this stage, especially in the central nervous system (CNS), given that this is a behavioural phenotype and such a CNS enrichment is also known from another behavioural phenotype (Ayroles et al., 2015). To test this hypothesis, the gene expression profiling data from the *Drosophila*

TABLE 2 GWA results on pupation height

Genetic variant	Minor allele frequency	Association *p*-value	Associating genes[a] (location)	Lesion types/effect in genetic stocks tested	Tester (BDSC ID)	Progenitor (BDSC ID)
2L_15093153_SNP	0.33	2.02E−07	**CG15270** (Intron)	DEL/*	25163	5905
3R_18609357_SNP	0.46	1.02E−06	**CG7029** (Intron)	DEL/*	7671	6326
2R_20001939_INS	0.09	4.21E−06	CG3253 (5′UTR); CG4049, CG3257 (Upstream)	–	–	–
3R_25409303_SNP	0.39	4.57E−06	**CG7567** (Downstream)	DEL/**	8925	5905
3R_18560721_SNP	0.10	4.67E−06	Dcr-1 (Exon); Takl2 (Upstream); CG45049 (Downstream)	–	–	–
3R_15859480_SNP	0.26	5.85E−06	CG4936 (Intron), trem (Upstream), CG4854, CG10889, CG17193 (Downstream)	–	–	–
3R_2131013_SNP	0.05	5.96E−06	Osi16 (Exon); CG31560 (Upstream); Osi14, Osi15, CG31556 (Downstream)	–	–	–
3R_22370558_SNP	0.18	6.88E−06	**scrib** (Intron)	INS/*	17791	6326
X_12597625_SNP	0.06	7.20E−06	**Smr** (Intron); CG15725 (Upstream)	INS/n.s.	26090	5905
3R_12465747_SNP	0.06	7.33E−06	**Arl6IP1**, **nonA-I** (Upstream); **fas1** (Downstream)	DEL/n.s.	7737	6326
3R_26421068_SNP	0.45	7.58E−06	CG2246 (Intron), CG31019 (Upstream)	–	–	–
3R_13952996_DEL	0.08	8.07E−06	stripe (Exon)	–	–	–
3R_21494031_SNP	0.13	8.91E−06	Lgr3 (Intron); RASSF8 (Downstream)	–	–	–
3R_17249156_DEL	0.26	8.98E−06	lbl (Intron)	–	–	–
3R_21109500_SNP	0.05	9.26E−06	CG31300 (Exon), CG10514, CG31104, CG13658 (Upstream), CG11892 (Downstream)	–	–	–
3L_17493227_SNP	0.08	9.40E−06	**Oatp74D** (Intron); **edin** (Downstream)	DEL/*	7611	6326

Note: DEL = deletion line, INS = transposon insertion line, **: *p*-value < .01; *: *p*-value < .05; n.s.: *p*-value > .05; BDSC: Bloomington *Drosophila* Stock Centre; Highlighted in bold are the candidates applied for phenotype confirmations. Additional associated genes from based LD blocks are described in Table S3.

[a]Associated genes are defined as those locate within 5 kb up/downstream of target genetic variant.
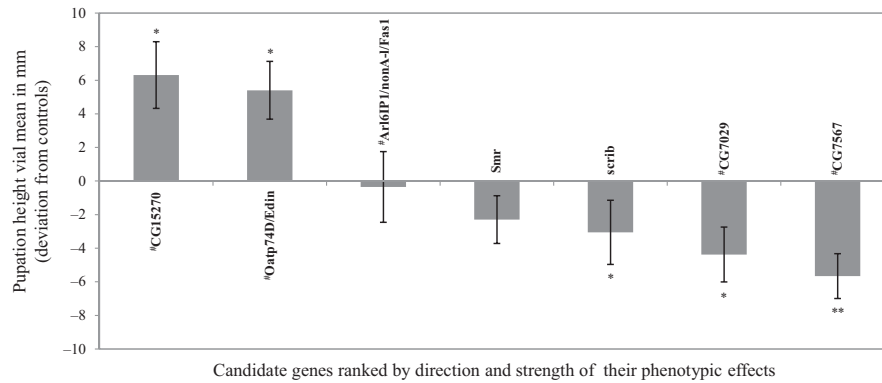
FIGURE 5 Phenotypic effects of candidate genes from gene disruption tests. The phenotypic effect was measurement as the deviation of pupation height of stocks with gene disruption compared with that from progenitor stocks via Wilcoxon rank-sum test. The error bars show the standard error of mean values. **: *p*-value < .01; *: *p*-value < .05; Stocks marked with "#" were small deficiency stock tested as heterozygotes; the remaining two stocks are transposon insertion stocks tested as homozygotes

modENCODE project (Brown et al., 2014) were explored, which measure the genome-wide gene expression for five tissues in L3 stages in *D. melanogaster*, including CNS, digestive system, fat body, imaginal disc and salivary glands. Larger fractions of expressed genes (RPKM > 0) for the identified candidate gene data set were found for most of the tested tissues in L3 stages (including CNS, digestive system and fat body), compared with the expression profiling of total annotated protein-coding genes as controls, but only at moderate *p*-value levels (Figure S9a). We also find that expression levels of candidate genes compared to all the annotated protein-coding genes are significantly higher in the CNS (Wilcoxon rank-sum test, *p*-value = .05), but not other tissues (Figure S9b). This observation is consistent with the above prediction that the identified candidate genes for pupation site choice are enriched in the CNS of third-instar larvae, where they could have a direct influence on behaviour.

For 13 out of 49 (27%) genes in the 16 candidate loci, their genetic interactions have been documented in Flybase v6.19 (http://www.flybase.org) (Attrill et al., 2016). We used this information to construct a computationally predicted network of genetically interacting genes, allowing one intermediate gene (i.e., a noncandidate gene connecting two candidate genes). This analysis revealed a connected subnetwork of 5 candidate genes from the GWA analyses and 4 computationally recruited intermediate genes (Figure 6a). The probability that this connected subnetwork would have arisen when the same number of genes is randomly sampled is very low ($p < 2.2 \times 10^{-16}$). Based on the availability of gene disruption stocks, the phenotypic influence of one intermediate gene (i.e., *Ras85D*) in the above network was experimentally tested and showed a significant effect on pupation height (Table 3). Therefore, this network appears to represent a core network controlling the pupation site choice in *D. melanogaster*.

To further substantiate this inference, we predicted that the genes that are directly connected to GWA candidate genes may also be involved in the pupation height choice phenotype. Hence, we used the genetic interaction information to construct another computationally predicted network (Figure 6b), including both the above

core subnetwork and the first-degree interacting genes of identified candidate genes within the core subnetwork. The phenotypic effects of three randomly selected first-degree interacting genes (*Egfr*, *E2F transcription factor 1 - E2f1* and *p53*) in the above network were then analysed, via direct comparisons of pupation height status between the co-isogenic progenitor stock and the corresponding gene disruption lines. All these three genes showed indeed a significant phenotypic effect on pupation height (*p*-value < .05), and the results were consistent for the disruption of different alleles for the same gene (i.e., *p53*, see Table 3). Gene ontology enrichment analysis of the genes in the network showed significant enrichment for genes associated with cellular and developmental processes, including larvae development and neuron development (Table S5). Therefore, the above network may represent a core network controlling the pupation height choice in *D. melanogaster* via the development of neuronal connections.

4 | DISCUSSION

Pupation site choice is an ecology-related behavioural trait that is critical to the fitness of holometabolous insects, including *Drosophila* (Joshi & Mueller, 1993; Rodriguez et al., 1992; Sokolowski, 1985). Previous artificial selection experiments on pupation height in *Drosophila* revealed that this trait is heritable (Garcia-Flores et al., 1989; Markow, 1979). However, the genetic basis of pupation height remained largely unexplored, mainly due to the lack of large numbers of reliable measurements. To tackle this issue, we developed a semi-automated phenotyping pipeline to reduce the variance of pupation height measurements, via repeated measurements for a given *Drosophila* strain in a high-throughput manner.

Applying the semi-automated phenotyping pipeline, we found a substantial variation on pupation height for both a collection of natural stocks and DGRP inbred lines (Figure 2). It is noteworthy that the natural stocks show a smaller range than the DGRP stocks,

FIGURE 6 Gene interaction subnetwork connecting identified candidate genes. (a) Gene interaction subnetwork that connects 5 candidate genes (circles) through other 4 intermediate genes (rectangles); (b) gene interaction subnetwork that includes both the above subnetwork and the first-degree interacting genes of candidate genes. Highlighted in red are the ones for which the phenotypic effects were experimentally tested

implying that the DGRP stocks might capture most of the relevant polymorphisms for this trait, at least if genetic and phenotypic variance are coupled. Hence, the genes inferred from the analysis of these stocks potentially constitute a core component for the genetic underpinning of this behavioural trait.

The existence of sexual dimorphism on pupation height in *Drosophila* was confirmed, with the observation that male individuals pupate on average ~2 mm higher than females (Figure 3). The difference in pupation site choice between sexes may be due to their distinct developmental timing, as females generally pupate later than males, and later larvae tend to pupate lower, possibly due to a response to diminishing levels of humidity inside the vials (Casares

& Carracedo, 1987). Note that the sex difference in pupation height could affect our GWA analysis and the heritability estimate, if the sexes were not equally distributed among the sampled pupae, or if there is a genotype * sex interaction; hence, this can be considered as another possible source of noise. The crossing experiments and tests on *Wolbachia* infection influence showed no major biased parental effects on pupation height (Figure 3), suggesting an additive model of inheritance on pupation site selection in *D. melanogaster* (Sokolowski & Bauer, 1989).

Our present study allows a more refined systematic exploration of heritability of pupation height in *D. melanogaster* using alternative methods to the existing artificial selection-based ones

TABLE 3 Phenotypic effects of core network genes

Tester gene	Pupation height GWA p-value[a]	Expression in L3 tissues (RPKM)[b]					Gene disruption region	Pupation height effect size (mm)[c]	Tester (BDSC ID)[d]	Progenitor (BDSC ID)[d]
		CNS	Imaginal discs	Digestive system	Fat body	Salivary gland				
scrib[e]	6.88×10^{-6}	15	19	14	2	3	CDS	−3.06 (SEM: 1.15)*	17791	6326
Egfr	1.75×10^{-4}	8	28	6	12	2	5'UTR	−14.28 (SEM: 3.77)***	20767	6599
E2f1	2.73×10^{-3}	102	36	27	11	9	5'UTR	−13.96 (SEM: 2.04)***	22140	6599
p53	7.29×10^{-4}	1	3	2	0	1	Intron	−17.82 (SEM: 2.78)***	17550	6599
							CDS	−18.67 (SEM: 1.78)***	20906	6599
Ras85D	2.75×10^{-2}	133	67	104	16	21	3'UTR	−15.87 (SEM: 1.90)***	15028	6599

Note: Statistical significance indications: ***: p-value < .001; **: p-value < .01; *: p-value < .05; otherwise p-value > .05.

Abbreviation: SEM, standard error of mean.

[a]The GWA p-value was defined as the lowest p-value from all the genetic variants within the gene and the 5 kb up/downstream of target gene.

[b]The gene expression profiling data were from Drosophila modENCODE project (Brown et al., 2014).

[c]The phenotypic effect size was defined as the deviation of pupation height of gene disruption stocks, compared with progenitor stocks.

[d]BDSC: Bloomington Drosophila Stock Center.

[e]This gene is also a significant associated gene from GWA results.

(Garcia-Flores et al., 1989; Markow, 1979). Values with H^2 of 0.64 (0.70 for natural stocks) and h^2 of 0.46 (SE: 0.2) based on the estimates from DGRP inbred stocks (Table 1) imply higher heritability than that (realized heritability h^2 = 0.13–0.18) from previous estimates within this species (Garcia-Flores et al., 1989; Markow, 1979). The apparent inconsistency between these studies likely stems from the difference in methods to estimate realized heritability compared with H^2 and h^2 as defined above. Where methodically more comparable estimators were also applied to DGRP strains, it can be seen that pupation height choice is similar to the values estimated for other behaviour traits, for example, taste sensitivity to sugars (H^2 = 0.63) (Uchizono & Tanimura, 2017), sensitivity to lead toxicity (H^2 = 0.76) (Zhou et al., 2016), aggression (H^2 = 0.69) (Shorter et al., 2015), DDT resistance (H^2 = 0.8) (Schmidt et al., 2017) and adult foraging behaviour (h^2 = 0.52) (Lee et al., 2017).

The substantial phenotypic variation on pupation height, as well as the richness of genetic variation in DGRP inbred lines, provides the possibility to dissect the genetic architecture of this behaviour trait at a fine scale. GWA analysis returns 16 significant associating genetic loci (corresponding to 49 candidate genes) from all major chromosomes (Table 2). Expression analysis of these candidate genes shows their enrichment in CNS of third-instar larvae, suggesting the role of controlling pupation height choice through the central nervous system (Figure S9).

The phenotypic effects on pupation height from several candidate genes were experimental confirmed with gene disruption assays (Figure 5). These include a potential cation transport function (CG15270), transmembrane transporter activity (Oatp74D), a scaffolding protein regulating apicobasal polarity (scrib) and genes of as yet unclear function (CG7567 and CG7029). scrib was found to be widespread expressed in olfactory organs and the central nervous system (Ganguly, Mackay, & Anholt, 2003), and has been reported to be associated with several behavioural traits in D. melanogaster, including olfactory behaviour (Ganguly et al., 2003), adult foraging (Lee et al., 2017) and sleep (Harbison et al., 2013).

More intriguingly, we predicted a genetic interaction subnetwork based on the connections of candidate genes, with the well-studied gene scrib as a hub gene (Figure 6). The phenotypic effects on pupation height of four randomly selected genes from the predicted network were further experimentally confirmed (Table 3). These include another well-studied gene Egfr, which is the transmembrane tyrosine kinase receptor for signalling ligands in the TGFα family and was also found to function in neuronal development and behavioural traits in Drosophila (King, Eddison, Kaun, & Heberlein, 2014; Potdar & Sheeba, 2013). Ras85D encodes a protein that acts downstream of several cell signals, most notably of receptor tyrosine kinases (RTKs), and has been reported to be involved in pupal size determination (Li, Li, Jin, Chen, & Ma, 2017). Another component of the network is p53, which is a general regulator of the cell cycle, but which has also been found to be involved in central nervous system development in Drosophila (Bauer et al., 2007) and behavioural traits, such as the entrainment

of the circadian rhythm in mice (Hamada, Niki, & Ishida, 2014). Furthermore, both gene expression analysis and GO functional enrichment analysis on the total gene set from the subnetwork revealed their significant association with third-instar larvae development/pupal morphogenesis and neuro-related functions (Table S5). Thus, we propose this *scrib*-centred subnetwork involved in the development of the nervous system to be at least part of a core network that also govern pupation height choice in *D. melanogaster*. Interestingly, although *scrib* is inferred as central gene, the actual phenotypic effect of the gene disruption line is not very high (Figure 5). This suggests that the transposon insertion of this stock has only a weak effect on the phenotype. Note that amorphic alleles of *scrib* are pupal lethal.

5 | CONCLUSIONS

In contrast to a previous QTL study on pupation site choice (Riedl et al., 2007), we do not simply find one major locus, but show a polygenic architecture that underlies variation in the pupation height in *D. melanogaster*. All of the major chromosome arms contribute to the trait, suggesting a broad distribution of influencing genes across the genome. Using a nominal GWA threshold *p*-value lower than 10^{-5}, we find 16 loci (corresponding to 49 candidate genes), of which >70% could be verified by gene disruption experiments. Most of these candidate genes are enriched in CNS of third-instar larvae, indicating their involvement in nervous system development. Our results predict a *scrib*-centred subnetwork as a core network that might potentially govern pupation height choice in *D. melanogaster* through controlling neuronal connections during larval development.

AUTHOR CONTRIBUTIONS

D.T., G.R. and W.Z. jointly conceived and planned the experiments. W.Z. carried out the experiments and analysed the results, with the support from G.R. All these three authors contributed to the final version of the manuscript. D.T. supervised the project.

DATA AVAILABILITY STATEMENT

All the *Drosophila melanogaster* strains used in this study are publically available through either the Bloomington *Drosophila* Stock Centre (https://bdsc.indiana.edu/) or the EHIME *Drosophila* stock centre (https://kyotofly.kit.jp/cgi-bin/ehime/index.cgi). The detailed information about the wild-type, DGRP inbred lines and gene disruption stocks, is provided in Table S4. The primers used for *Wolbachia* infection

detection are listed in the above text of this section. Supplementary data sets on the CELLPROFILER analysis code and raw data on individual-level pupal measurement can be accessed through DRYAD: https://datadryad.org/stash/dataset/doi:10.5061/dryad.2rbnzs7hk.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.