Published in final edited form as:
New Phytol. 2011 July ; 191(1): 251–263. doi:10.1111/j.1469-8137.2011.03656.x.

Differential regulation of a MYB transcription factor is correlated with transgenerational epigenetic inheritance of trichome density in *Mimulus guttatus*

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Summary

- Epigenetic inheritance, transgenerational transmission of traits not proximally determined by DNA sequence, has been linked to transmission of chromatin modifications and gene regulation, which are known to be sensitive to environmental factors. *Mimulus guttatus* increases trichome (plant hair) density in response to simulated herbivore damage. Increased density is expressed in progeny even if progeny do not experience damage. To better understand epigenetic inheritance of trichome production, we tested the hypothesis that candidate gene expression states are inherited in response to parental damage.
- Using *M. guttatus* recombinant inbred lines, offspring of leaf-damaged and control plants were raised without damage. Relative expression of candidate trichome development genes was measured in offspring. Line and parental damage effects on trichome density were measured. Associations between gene expression, trichome density, and response to parental damage were determined.
- We identified *M. guttatus MYB MIXTA-like 8* as a possible negative regulator of trichome development. We found that parental leaf damage induces down-regulation of *MYB MIXTA-like 8* in progeny, which is associated with epigenetically inherited increased trichome density.
- Our results link epigenetic transmission of an ecologically important trait with differential gene expression states – providing insight into a mechanism underlying environmentally induced ‘soft inheritance’.

Keywords

maternal effects; *Mimulus guttatus* (yellow monkeyflower); *MYB* transcription factors; phenotypic plasticity; soft inheritance; transgenerational epigenetic inheritance; trichome density; wound response

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Supporting Information
Additional supporting information may be found in the online version of this article.

Introduction

Heritable variation is usually attributed to DNA sequence variants that are stable, causative, and derived from random mutations (Lynch & Walsh, 1998). However, epigenetic inheritance has now been shown in over a hundred different cases involving bacteria, protists, fungi, plants, and animals (reviewed in Jablonka & Raz, 2009). Epigenetic inheritance, also referred to as cross-generational phenotypic plasticity, is the transmission of phenotypes that are not proximally determined by DNA. In the narrowest sense, epigenetic inheritance results from the transfer of modified chromatin states (e.g. DNA methylation, histone modification, and associations with nonhistone proteins) through the gametes of sexually reproducing organisms (Lippman *et al*., 2003, 2004; Rangwala *et al*., 2006; Rassoulzadegan *et al*., 2006; Richards, 2006; Chandler, 2007; Jablonka & Raz, 2009; Daxinger & Whitelaw, 2010; Richards *et al*., 2010). In a broader sense, epigenetic inheritance also includes maternal environmental effects (Rossiter, 1996; Jablonka & Lamb, 2008). As our understanding of mechanisms underlying both narrow-sense epigenetic inheritance and the transmission of maternal effects expands, the line between the two becomes quite blurred (Richards *et al*., 2010). For instance, maternal environment may result in transmission of small molecules, including small RNAs, to progeny, which in turn, may function to establish or maintain chromatin modifications (Matzke & Birchler, 2005). Both definitions of epigenetic inheritance will include phenomena that affect phenotypes across generations by altering gene expression profiles.

A growing number of studies have demonstrated epigenetic inheritance of environmentally induced phenotypes (Agrawal *et al*., 1999; Anway *et al*., 2005; Benyshek *et al*., 2006; Cropley *et al*., 2006; Crews *et al*., 2007). This so-called ‘soft inheritance’, in which the environment can alter a malleable hereditary material, is fundamentally different from DNA-based inheritance (Mayr, 1980, 1982; Richards, 2006). Mechanisms of ‘soft inheritance’ may evolve when environmental conditions experienced by parents and offspring are correlated and it is advantageous to provide information about the environment across generations (Bossdorf *et al*., 2008). *Mimulus guttatus* (yellow monkeyflower) exhibits epigenetic inheritance of trichome density, a putative defense trait, in response to leaf damage (Holeski, 2007). Trichomes are plant hairs that serve a variety of defensive and physiological functions. In *M. guttatus* they are straight, unicellular, mostly glandular, and often secrete a sticky and potentially noxious fluid. Leaf trichomes have been shown to reduce insect herbivory in a number of species (Levin, 1973; Agren & Schemske, 1993; Mauricio, 1998; Romeis *et al*., 1999). They interfere with insect movement and feeding, and the fluids they secrete may trap, poison, or repel herbivores (Elle & Hare, 2000). Constitutive production of trichomes is variable both within and among populations of *M. guttatus*, and simulated insect damage to leaves induces a within-generation increase in the production of trichomes. This induced state can be transmitted to offspring and expressed even if those offspring do not experience damage (Holeski, 2007).

Trichomes are a model trait for the study of plant cellular differentiation, development, and patterning (Szymanski *et al*., 2000; Larkin *et al*., 2003; Hülskamp, 2004; Serna & Martin, 2006; Ishida *et al*., 2008; Zhao *et al*., 2008). Consequently, there is detailed information on genes and developmental pathways involved in trichome development from two distantly related core eudicots, *Antirrhinum majus* (asterid) and *Arabidopsis thaliana* (rosid). In *Antirrhinum*, four closely related paralogous transcription factors, *MIXTA, MYB MIXTA LIKE 1 (AmMYBML1), AmMYBML2*, and *AmMYBML3*, have been implicated in the promotion of trichome development. Overexpression of these genes leads to increased trichome density, as well as trichome development from epidermal cells that would otherwise not produce trichomes (Glover *et al*., 1998; Perez-Rodriguez *et al*., 2005; Baumann *et al*., 2007; Jaffe *et al*., 2007). In addition, *MIXTA*-like homologs from

Arabidopsis and cotton have been shown to be both negative and positive regulators of trichome differentiation (Jakoby *et al*., 2008; Machado *et al*., 2009; Gilding & Marks, 2010). *MIXTA*-like genes therefore appear to affect trichome development across major core eudicot lineages. An alternate pathway has been identified in *Arabidopsis*, where a known transcriptional activation/repression complex consisting of multiple proteins likely acts as a regulator of *GLABRA2 (GL2)* expression, and *GL2*, when activated, promotes trichome development (Rerie *et al*., 1994; Szymanski *et al*., 1998, 2000; Fyvie *et al*., 2000; Ohashi *et al*., 2002; Schiefelbein, 2003; Pesch & Hulskamp, 2004; Serna, 2004; Ishida *et al*., 2007, 2008; Simon *et al*., 2007; Wang *et al*., 2007; Wang & Chen, 2008; Zhao *et al*., 2008). Interestingly, this *GL2*-dependent trichome initiation pathway appears to be conserved in non*Arabidopsis* rosid species, including cotton (Suo *et al*., 2003; Wang *et al*., 2004; Humphries *et al*., 2005; Guan *et al*., 2008), but appears to be absent from *Antirrhinum* and other asterid species (Payne *et al*., 1999; Ramsay & Glover, 2005; Serna & Martin, 2006).

In this study, we examine associations between epigenetic inheritance of damage-induced trichome production and expression of candidate trichome development genes in *M. guttatus* (asterid). We begin by identifying *M. guttatus* orthologs of *MIXTA*-like genes and *GL2*. Because of their widely conserved role in regulating epidermal outgrowth, including trichome differentiation, *MIXTA*-like homologs are excellent candidate genes for identifying underlying developmental genetic pathways involved in trichome production and differentiation in *M. guttatus*. In addition, if a *GL2*-dependent pathway functions to initiate trichome development in *M. guttatus*, any differences in the expression of upstream regulators of the *M. guttatus GL2* homolog will be reflected in the relative expression levels of *GL2* itself. Therefore, *GL2*, because of its downstream position in the *Arabidopsis* trichome development pathway, is a good candidate gene for assessing the effect of this putative pathway on trichome induction in *M. guttatus*.

Here we identify eight *MIXTA*-like homologs and a single *GL2* ortholog in *M. guttatus*. We measure the associations between trichome density, parental damage, and expression of *MIXTA*-like and *GL2* candidate genes using a panel of recombinant inbred lines (RILs) developed from two disparate natural populations of *M. guttatus*. This panel exhibits an average increase in trichome density of undamaged offspring when parental plants experience damage, that is, epigenetic inheritance of induction, genetic variation in epigenetic inheritance of induction (Holeski, 2007), and variation in constitutive trichome production. By measuring six different *M. guttatus* lines (four RILs and the two parental lines from which they were derived), we evaluate interactions between genetic and epigenetic variation at the phenotypic level, and compare associations between epigenetic variation, genetic variation, and gene expression. This represents a system in which the basis of environmentally induced, heritable epigenetic variation can be studied in conjunction with the effect of genetic variation in an ecologically relevant trait.

Materials and Methods

Plant materials

Mimulus guttatus DC. is native to western North America, ranging from Mexico to Alaska. It typically inhabits wet areas such as stream banks, but local populations differ extensively in morphology, life history, selfing rate, and microhabitat (Hall & Willis, 2006; Hall *et al*., 2006; Wu *et al*., 2007). This experiment was performed on six lines of *M. guttatus*: four RILs and the parental lines from which they were derived. The parental lines were collected from two natural populations, Iron Mountain (IM) and Point Reyes National Seashore (PR). IM is an annual population in the Cascade Mountains of central Oregon; plants are short-lived (1–2 months from germination to senescence), experience little insect herbivory, and typically produce few or no trichomes. The IM parent was from a specific line, IM767,

synthesized by 10 generations of single seed descent (Willis, 1999; Holeski, 2007). PR is a perennial, low-elevation, stream-side population in the fog belt of coastal northern California. Plants from the PR population generally experience heavy herbivory and individuals exhibit high leaf trichome densities (Holeski, 2007). The PR parent was not inbred. However, a single F1 individual from the PR × IM cross was self-fertilized to generate 1000 F2 individuals, each of which founded a distinct recombinant lineage. These lines were propagated through single-seed descent for eight subsequent generations to create a panel of RILs (Holeski, 2007). Owing to line loss, *c*. 400 RILs remain in the F8 generation. The four RILs used in this experiment (RILs 11, 50, 85, and 94) were selected to represent a range of constitutive trichome densities and transgenerational responses to leaf damage (Fig. 1).

To create the parental generation, six to eight plants were grown from each line (PR, IM767, RILs 11, 50, 85 and 94). Half of the plants from each line received damage and half did not. For plants receiving damage, we punched two holes of *c*. 6 mm diameter in each leaf as soon as the subsequent leaf pair expanded. This treatment commenced on the second pair of leaves and continued until seed collection. Leaves that were too small to sustain two hole punches received only one. Each plant was manually self-pollinated to produce seed for the experimental generation.

To create the experimental generation, 40 plants were grown from each line, half from seeds of damaged parents and half from undamaged parents. Within each parental damage treatment, seeds were derived from three to four different parental plants. No plants in the experimental generation received damage. When the sixth leaf pair expanded, both leaves from the fifth leaf pair were removed and measured for trichome density. For each leaf, we counted all trichomes visible on the underside of the leaf, in a 6-mm-diameter field of view bracketed by the right, distal-most leaf margin and the right side of the primary leaf vein, using an Olympus SZX7 dissecting scope (Holeski, 2007). The fifth pair of leaves were then frozen in liquid nitrogen and stored at −80°C for RNA extraction. Throughout, plants were grown under controlled glasshouse conditions as described in Arathi & Kelly (2004).

Candidate genes

We used drafts of the *M. guttatus* genome sequence to identify *M. guttatus* homologs of *GL2* and *MIXTA* genes. *Arabidopsis GL2*, and *Antirrhinum MIXTA*, *MYBML1*, *MYBML2* and *MYBML3* cDNA sequences (Supporting Information, Table S1 for GenBank accessions) were used as query sequences in tBLASTx searches (Altschul *et al*., 1997) against versions of the *M. guttatus* draft genome. Matches with *E* values $< 1 \times 10^{-50}$ were considered putative *GL2* and *MIXTA*, *MYBML1*, *MYBML2* and *MYBML3* orthologs, and included in further phylogenetic analyses. In addition, six housekeeping reference genes were identified from the *M. guttatus* draft genome sequence: ubiquitin (Ubq5), actin (Act), 18S nuclear ribosomal RNA (18S), 26S nuclear ribosomal RNA (26S), ribosomal binding protein L2 (L2), and elongation factor 1α (EF1α).

Housekeeping genes were identified by querying the draft genome sequence with the accessions listed later. Intron/exon boundaries for each gene identified in tBLASTx searches were estimated by comparison to cDNA sequences of *Arabidopsis GL2*, *Antirrhinum MIXTA*, *MYBML1*, *MYBML2*, *MYBML3*, *Arabidopsis* Ubq5 (At3g62250), *Solanum tuberosum* Act (X55749), *S. tuberosum* 18S (X67238), *Campanula trachelium* 26S, *Arabidopsis* L2 (At4g36130), and *Arabidopsis* EF1α (At1g07920). Intron/exon boundaries were later confirmed for candidate *GL2* and *MIXTA*-like genes by direct sequencing from *M. guttatus* cDNA.

To identify those *GL2*-like and *MIXTA*-like genes from the *M. guttatus* draft genome that are closest homologs to *GL2* from *Arabidopsis* and *MIXTA, MYBML1, MYBML2* and *MYBML3* from *Antirrhinum*, all putative candidate genes isolated from the *M. guttatus* draft genome were included in phylogenetic analyses with additional genes from each gene family. *Arabidopsis GL2* belongs to lineage IV of the HD-Zip gene family (Ariel *et al*., 2007). The nine putative *GL2* orthologs identified in the *M. guttatus* draft genome, and *MgGL2* confirmation sequences from lines IM62 (the line from which the *M. guttatus* genome sequence is derived), PR and IM767 (see the Methods section and Table S1), were aligned to all *Arabidopsis* HD-Zip IV genes (*GL2, HDG1-12, ANL2, ATML1, FWA* and *PDF2*), as well as *HDP1* from *Helianthus annuus*, *GhHOX1* from *Gossipium hirsutum*, *VvGL2like* from *Vitis vinifera*, *OCL1* and *OCL5* from *Zea mays*, and *Roc1* and *Roc2* from *Oryza sativa*. cDNA sequences were aligned using MUSCLE (Edgar, 2004a,b), and then manually adjusted in MacClade 4 (Maddison & Maddison, 2003). Highly variable regions, for which alignment could not be confidently assessed, were removed from further analyses.

Antirrhinum MIXTA, MYBML1, 2 and *3* belong to the R2R3 lineage of the MYB transcription factor superfamily (Chen *et al*., 2006; Serna & Martin, 2006). The 15 putative *MIXTA*-like orthologs identified in the *M. guttatus* draft genome were aligned to *Antirrhinum MIXTA, MYBML1, 2* and *3, AtMYB16* and *AtMYB106*, considered to be the *Arabidopsis* orthologs to *MIXTA, MYBML1, 2* and *3*, 11 additional *Arabidopsis* R2R3 MYB genes that form a clade with *AtMYB16* and *AtMYB106* (Chen *et al*., 2006), *MYB1* from *Petunia hybrida*, *MYB*-like from *Solanum lycopersicon*, *MYBML2* genes from *Thalictrum dioicum*, *T. filamentosum*, and *T. thalictroides*, *MYB25* from *Gossypium hirsutum*, and *Arabidpsis GL1* as an outgroup. cDNA sequences were aligned as described earlier, and highly variable regions, for which alignment could not be confidently assessed, were removed from further analyses. Following the identification of a *MYB MIXTA*-like clade (Fig. 2a), an alignment was generated for additional phylogenetic analyses restricted to just the *MYB MIXTA*-like clade. This additional alignment included all genes from the *MYB MIXTA*-like clade as well as confirmation sequences for *M. guttatus MYBML1, 2, 3, 5, 6, 7, 8* and *9* from lines IM62, PR and IM767 (see the Methods section and Table S1). This second analysis was undertaken in order to better resolve relationships among genes in the *MYB MIXTA*-like clade; by restricting the analysis to a subset of R2R3 MYB transcription factors, alignment could be confidently assessed over a larger extent of coding sequence. Alignment of cDNA sequences for phylogenetic analysis of the *MYB MIXTA*-like clade was generated as described earlier.

Gene trees were estimated using maximum likelihood (ML) and Bayesian phylogenetic methods. ML trees were estimated using GARLI (Genetic Algorithm for Rapid Likelihood Inference, ver. 0.951) (Zwickl, 2006), implementing the GTR + I + Γ model of molecular evolution. Node support under ML was assessed by conducting ML heuristic searches on 1000 bootstrap replicates of the data in GARLI. Bayesian analysis was performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001), implementing the GTR + I + Γ model of molecular evolution, running four Markov chains for one million to three million generations with default heating values. Two independent runs were performed, sampling trees every 1000 generations. At completion, the two runs were checked for convergence, and the first 25% of saved trees were discarded as initial burn-in. The remaining trees were combined to calculate posterior probabilities of node support in a 50% majority-rule consensus tree. Bayesian trees were viewed and rooted in FigTree v1.3. The expanded R2R3 MYB gene tree was rooted with *Arabidopsis GL1* (Fig. 2a), and the *MYB MIXTA*-like clade phylogeny was rooted with *AtMYB107, AtMYB9, AtMYB39, AtMYB53, AtMYB92*, and *AtMYB93* (Fig. 2b), consistent with the results of the expanded R2R3 MYB phylogenetic analysis. The *GL2* gene tree (Fig. 3) was rooted with *Arabidopsis HDG8, 9*, and *10*, consistent with the phylogeny of HD-Zip IV genes in Ariel *et al*. (2007).

Candidate genes sequence confirmation

The cDNA sequences for all *M. guttatus* candidate genes, *MgMYBML1, 2, 3, 5, 6, 7, 8, 9*, and *MgGL2* (Figs 2, 3), were confirmed in *M. guttatus* IM62 (the line from which the *M. guttatus* genome sequence is derived), and in *M. guttatus* PR and IM767 plants. Determining the cDNA sequence for each candidate gene in the parental lines used to generate our RILs allowed us to develop gene-specific primers for quantitative real-time PCR that would specifically hybridize to coding regions that are conserved between parental lines.

RNA was extracted from leaves and shoot apical meristems of one IM62 plant, six PR plants and two IM767 plants using Tri-reagent following manufacturer's instructions (Applied Biosystems/Ambion, Austin, TX, USA). RNA was DNAse-treated using Turbo DNA-free following manufacturer's instructions (Applied Biosystems/Ambion). DNAse-treated RNA was used as a template for cDNA synthesis using the iScript cDNA synthesis kit following manufacturer's instructions (Bio-Rad). Based on the inferred genome-estimated cDNA sequences, primers were designed to amplify near full-length cDNA of each candidate gene, *MgMYBML1, 2, 3, 5, 6, 7, 8, 9* and *MgGL2*, from *M. guttatus* lines IM62, PR and IM767 (Table S1, gene IDs; Table S2, primer seqs). cDNA from each plant/tissue was used as a template for PCR amplification of candidate genes. PCR was performed using the Expand High Fidelity PCR System, following manufacturer's instructions (Roche) using a gradient of annealing temperature from 48 to 62°C with 40 cycles. PCR products of the expected size were cloned into the pCR 4-TOPO TA cloning vector following manufacturer's instructions (Invitrogen). Plasmid DNA was isolated from three to five clones per PCR reaction for sequencing using the Plasmid Mini Kit following manufacturer's instructions (Qiagen).

Quantitative reverse-transcription PCR (qRT-PCR)

Gene-specific primer pairs for qRT-PCR were designed using Primer3 (v. 0.4) software to amplify short segments (100–300 bp) of each candidate gene and five reference genes. For candidate genes, primers were designed to distinguish among *M. guttatus* paralogs and to hybridize without mismatch to candidate gene sequences derived from both parental lines PR and IM767. In the case of reference genes, we allowed primers that could amplify more than one paralog in a gene family, as our reference genes are found in multiple copy number in the *M. guttatus* genome. For each qRT-PCR primer pair (Table S2), gene-specific amplification was confirmed using cDNA derived from leaf tissue of PR and IM767 individuals as a PCR template. Following the qRT-PCR protocol described later, amplicons from each qRT-PCR primer pair were purified using ExoSAP-IT (USB, Cleveland, OH, USA) following the manufacturer's instructions and sequenced directly using the qRT-PCR primers as forward and reverse sequencing primers. The results of this analysis confirm gene-specific priming during qRT-PCR, and additionally confirm that all of our candidate genes are expressed in *M. guttatus* leaves.

Optimal annealing temperatures and primer efficiencies were determined for each qRT-PCR primer pair. Following the qRT-PCR protocol described later, cDNA derived from PR and IM767 leaf tissue was used as a template in triplicate PCR reactions with a gradient of annealing temperatures from 52 to 62°C. The optimal annealing temperatures were determined based on the rate of amplification, repeatability among triplicates, and the amplification of a single template, as evidenced by a clean, single-peak melting curve. Using the optimal annealing temperature established for each qRT-PCR primer pair, cDNA derived from PR and IM767 leaf tissue as a template, and the qRT-PCR protocol described later, we determined efficiencies for each qRT-PCR primer pair using critical threshold *(c(T))* values obtained from a series of four 10-fold dilutions (range 1 : 10 to 1 : 10 000) with three technical replicates per dilution. Opticon2 was used to fit a line to a plot of *c(T)* cycle

vs log(relative quantity). The corresponding qRT-PCR efficiency was calculated according to the equation $E = 10^{[-1/\text{slope}]}$ (Rasmussen, 2000).

Before qRT-PCR of candidate genes, we performed outlier analysis of our six reference genes to determine the two with the most consistent expression levels across lines and treatments in this study. Following the qRT-PCR protocol described later, Ubq5, Act, 18S, 26S, L2 and EF1α were amplified in triplicate across a subset of our samples representing different genetic lines and damage treatment. *c(T)* values were subject to outlier analysis as implemented in geNorm (Vandesompele *et al*., 2002). The two most stably expressed reference genes (Ubq5 and EF1α) were then used to normalize qRT-PCR expression of candidate genes via geometric averaging.

For each line/damage treatment category, six out of 20 plants producing trichome densities closest to the mean for their category were chosen for gene expression analysis. RNA was extracted from fifth node leaves (described earlier). RNA was DNAse-treated as described earlier. One microgram of DNAse-treated RNA was used as a template for cDNA synthesis using the iScript cDNA synthesis kit as described above. Twelve DNAse-treated RNA samples were chosen at random for additional cDNA synthesis reactions performed without reverse transcriptase to serve as −RT controls. Following the qRT-PCR protocol described later, we attempted to amplify actin (qRT-PCR primer pair for actin does not span intron sequence) from all cDNA and −RT samples in tandem. Amplification from cDNA but not −RT controls confirmed successful cDNA synthesis and absence of DNA contamination in our samples.

We performed qRT-PCR for reference genes, EF1α and UBQ5, and nine candidate genes, *MgMBYML1, 2, 3, 5, 6, 7, 8, 9* and *MgGL2*, in triplicate on all 72 study samples (six lines, two treatments per line, six biological replicates per treatment). Each qRT-PCR reaction included 0.6 units DyNAzyme II Hot Start Taq, 1 × DyNAzyme II Hot Start buffer (Finnzymes, Espoo, Finland), 0.5 μM each gene-specific primer, 0.8 mM dNTPs (Qiagen), and 1 μl 5× SYBR Green (Applied Biosystems, Foster City, CA, USA). qRT-PCR reactions were run on a DNA Engine, Opticon2 using optimal annealing temperatures as determined earlier. Samples were arrayed across five 96-well PCR plates in a complete block design with two blocks. Within blocks, plates were amplified back-to-back using the same master mix, and triplicates from 12 samples (two samples per line) were split across plates in order to assess plate-level effects. For each line, parental damage and control samples were amplified on the same plate. Each block encompassed tissue from three individuals from each line and damage treatment category.

For each gene, the critical threshold was set manually to the same value for all plates, and successful amplification of the intended template was assessed via examination of melting curves. The resultant *c(T)* values were assessed via a general linear model (GLM), where *c(T)* values for a particular gene were modeled as a function of line and PCR plate. The coefficient corresponding to each PCR plate was subtracted from the *c(T)* values from that plate in order to remove any plate effect before further analysis. For each gene, *c(T)* values were converted into relative quantities using the delta *c(T)* method. Specifically, the minimum *c(T)* value was subtracted from each *c(T)* value to yield the delta *c(T)* value for each sample. The efficiency specific to the primer pair for that gene was raised to −delta *c(T)* to yield relative quantity. Samples that failed to amplify were given a relative quantity of 0. Triplicates were averaged to yield a single relative quantity per sample. For each sample, the relative quantity for each candidate gene was divided by the geometric average of the relative quantities of the two most stably expressed housekeeping genes, Ubq5 and EF1α (Vandesompele *et al*., 2002). For each gene, the resulting normalized values were rescaled via division by the maximum value. This set expression of the highest-transcript

sample equal to 1 for ease of interpretation. All subsequent analyses were performed using R (R Development Core Team, 2008).

Results

Epigenetic inheritance of trichome production

Consistent with previous studies (Holeski, 2007), we find there is epigenetic inheritance of increased trichome production in the offspring of parents with damaged leaves. Furthermore, there is genetic variation among lines in epigenetic inheritance of trichome density following parental damage. Trichome density measurements from the fifth leaf pair in our experimental generation were right-skewed, including many values of zero. Therefore, we log(count + 1)-transformed values before analysis. Means and standard deviations of log-transformed trichome density for each line and parental treatment are shown in Fig. 1 and Table S3. An ANOVA predicting log-transformed trichome density as a function of line, parental treatment, and line × treatment interaction showed significant effects of all factors on trichome density (all $P < 0.0005$; Table S4). ANOVAs performed on each line separately revealed a significant effect of parental damage on trichome density in the experimental generation in PR ($F_{1,37} = 5.89$, $P = 0.02$) and RILs 11 ($F_{1,37} = 30.97$, $P < 0.000$), 50 ($F_{1,38} = 27.99$, $P < 0.000$), and 94 ($F_{1,38} = 11.10$, $P = 0.002$). There was no significant effect of parental damage on trichome density in the experimental generation in IM767 or RIL 85 (Fig. 1).

Identification of *GL2* and *MIXTA*-like homologs in *M. guttatus*

To identify potential regulators of trichome development in *M. guttatus*, we sought to identify orthologs of genes known to regulate trichome production or differentiation in *Antirrhinum* and *Arabidopsis*. Using similarity searches of the draft *M. guttatus* genome, followed by assessment of gene trees, we identified eight close *MYBML* homologs (*MgMYBML1, 2, 3, 5, 6, 7, 8* and *9*; Fig. 2) and a single ortholog of *GL2* (*MgGL2*, Fig. 3), in the *M. guttatus* genome. We treated all nine genes as candidates. History of gene duplication/loss in the *MYB MIXTA*-like clade is complex. Although our results lack strong support for most relationships, it appears that paralogs *MgMYBML1, 3, 5* and *6* are together orthologous to *Antirrhinum MIXTA* and *MYBML1*, and that *MgMYBML7* and *8* are together orthologous to *Antirrhinum MYBMY2* and *3*. *MgMYBML2* and *9* form a third *MYBML* lineage within the *MYB MIXTA*-like clade, but it appears that the *Antirrhinum* ortholog(s) to *MgMYBML2* and *9* have either been lost from, or not yet identified in, *Antirrhinum* (Fig. 2b).

We sequence-confirmed near full-length coding sequence for each of our nine candidate genes from individuals representing IM62, PR and IM767 lines (Table S1). Genes isolated from each line form monophyletic groups with the corresponding candidate gene identified in the *M. guttatus* draft genome, confirming isolation of correct candidate genes (Figs 2b, 3). Notably, *MgMYBML1* identified from the draft genome sequence has a 2 bp indel resulting in a premature stop codon. Although this may be a sequencing artifact in the draft genome, we were unable to amplify *MgMYBML1* from IM62 cDNA, but were able to amplify *MgMYBML1* from the PR and IM767 lines, and in these lines the 2 bp indel is absent. This result suggests that there may be population-level variation in functional copies of *MgMYBML1* and potentially other *MgMYBML* genes.

Expression of *MgMYBML8* is correlated with trichome density

To determine whether expression levels of our candidate genes are associated with variation in trichome production resulting from line and parental treatment effects, we employed multiple linear regression analysis. Expression levels for all candidate genes were right-

skewed, with many values equal to 0 (i.e. failure to amplify) or close to 0.001. We thus consider 0.001 to be our detection limit. We addressed this by applying a (log(value + 0.001)) + 4) transformation to all expression data before analysis. The addition of 4 to all data points brought the minimum value to 0 for ease of interpretation. A multiple linear regression predicting trichome density as a function of expression of the nine candidate genes identified the transcript abundances most closely associated with trichome density. We found a significant negative effect of *MgMYBML8* expression on trichome density (Table 1). We followed this analysis with a general linear model predicting trichome density as a function of line, with expression of all candidate genes included as covariates. *MgMYBML8* retained a significant negative effect on log-transformed trichome density ($b = -0.22$, $t(57) = -3.10$, $P = 0.003$). We recovered the same result for *MgMYBML8* when failure to amplify was scored as missing data unless consistent failure was observed for all technical replicates (data not shown). These results identify *MgMBYML8* as a potential negative regulator of trichome production or differentiation in *M. guttatus*.

Epigenetic inheritance of gene expression in response to parental damage

Considering the fact that damage induces epigenetic inheritance of trichome production and that *MgMYBML8* is a candidate negative regulator of trichome development in *M. guttatus*, we sought to determine whether *MgMYBML8* expression states were also epigenetically inherited in response to parental damage. We used ANOVA to test for an effect of line, parental damage, and line × damage interaction on expression of *MgMYBML8*. The effects of both line and line × damage interaction were significant (Table 2). When only those lines that showed a significant effect of parental damage on trichome density (PR and RILs 11, 50 and 94) were included, ANOVA revealed significant down-regulation of *MgMYBML8* in response to parental damage ($F_{1,40} = 7.25$, $P = 0.01$; Fig. 4). When only those lines that did not show a significant effect of parental damage on trichome density (IM767 and RIL 85) were included, ANOVA revealed no significant difference in *MgMYBML8* expression (data not shown). These results indicate that just as there is genetic variation among the RILs in epigenetic inheritance of trichome number, there is correlated variation in the response of *MgMYBML8* expression to parental leaf wounding (Fig. S1).

Discussion

These experiments link epigenetic inheritance of increased trichome density to down-regulation of *MgMYBML8* (Table 2, Fig. 4). Parental damage reduces expression of this gene, but not other candidate genes, in the experimental (offspring) generation, and expression level of *MgMYBML8* correlates with trichome density. More generally, we confirm epigenetic inheritance of increased trichome density, previously reported by Holeski (2007), whereby plants with damaged parents produce more trichomes (Fig. 1, Table S4). The significant line × treatment interaction on trichome density demonstrates genetic variation in the transgenerational response to damage. One parental line (IM767) shows no evidence of induction whereas three RILs exhibit transgressive segregation (higher induction than either parental line). Finally, the significant effect of line on trichome density indicates genetic variation in trichome production, independent of any damage response (Table S4).

Association between *MgMYBML8* expression and trichome density

MgMYBML8 is implicated in *M. guttatus* trichome development by a significantly negative association between expression and trichome density. No other candidate *MgMYBML* or *MgGL2* genes showed a significant association with trichome density. However, as a result of restricted tissue sampling, relatively small sample sizes and collinearity between

MgMYBML levels of expression, we cannot exclude the possibility that additional *MgMYBML* genes are also involved in the trichome pathway.

MgMYBML8 expression is negatively associated with trichome density in *M. guttatus*. From our data, and from functional knowledge of *MgMYBML8* homologs in other species, we cannot conclude that down-regulation of *MgMYBL8* itself causes increased trichome production, or rule out that *MgMYBML8* is instead involved downstream in a trichome developmental program. *MYB MIXTA-like* genes have been shown to promote trichome initiation and other aspects of cell differentiation in *Antirrhinum* (Glover *et al*., 1998; Payne *et al*., 1999; Perez-Rodriguez *et al*., 2005; Jaffe *et al*., 2007). *MYB106*, one of only two *Arabidopsis* paralogs found in the *MYB MIXTA*-like clade (Fig. 2), has been shown to function as both a positive and negative regulator of trichome differentiation, but not trichome initiation (Jakoby *et al*., 2008; Gilding & Marks, 2010). We know of no other asterid *MYB MIXTA*-like transcription factors associated with negative regulation of cell outgrowth or differentiation, but it is interesting to note that single *MYB* genes (e.g. *MYB106* in *Arabidopsis*; Gilding & Marks, 2010) and close *MYB* paralogs (e.g. *RADIALIS* and *DIVARICATA* in *Antirrhinum*; Almeida *et al*., 1997; Galego & Almeida, 2002) have evolved to both positively and negatively regulate the same developmental program – trichome differentiation and dorsal flower identity, respectively. *MgMYBML8* may negatively regulate trichome development through initiation at early stages and/or differentiation at later stages of development. The precise role of *MgMYBML8* during trichome development will not be known until *MgMYBML8* expression has been directly manipulated in *M. guttatus* and trichome phenotypes determined.

We find no evidence for a *GL2*-dependent trichome pathway in *M. guttatus. GL2* is a positive regulator of trichome development in rosid species for which it has been characterized (Rerie *et al*., 1994; Suo *et al*., 2003; Wang *et al*., 2004; Humphries *et al*., 2005; Guan *et al*., 2008). Our results suggest that *MgGL2* does not function in regulating trichome outgrowth. This is concordant with other negative results for a *GL2*-dependent trichome pathway in asterids (Payne *et al*., 1999; Ramsay & Glover, 2005; Serna & Martin, 2006).

Differential expression of *MgMYBML8* associated with both genetic variation and parental environment

Expression of *MgMYBML8* is additionally associated with both line and damage × line interaction. The significant effect of line indicates genetic variation in overall expression of *MgMYBML8*. The significant line × damage interaction indicates genetic variation in the epigenetic response of *MgMYBML8* expression to parental damage. Considering only lines that significantly increase trichome density with damage, *MgMYBML8* is significantly down-regulated in response to parental damage (Fig. 4). While we have not directly manipulated *MgMYBML8* expression and observed a response in trichome production, our data suggest that *MgMYBML8* functions as a negative regulator of trichome production or differentiation in a developmental pathway that is influenced by both genetic and epigenetic inheritance of trichome density.

If future studies indicate that *MgMYBML8* is involved in the initiation of trichomes, and not a downstream program of trichome differentiation, it will then be critical to assess whether *MgMYBML8 cis* regulatory DNA sequences are the locus of epigenetic modification in response to parental damage. Differential methylation has been associated with nonDNA sequence-based inheritance of plant phenotypes (Jacobsen & Meyerowitz, 1997; Cubas *et al*., 1999; Chandler *et al*., 2000; Kalisz & Purugganan, 2004; Manning *et al*., 2006), and differential methylation is known to result from environmental stresses and to be transmitted from parent to offspring (Kakutani *et al*., 1999; Vaughn *et al*., 2007; Johannes *et al*., 2009;

Verhoeven *et al*., 2010). In the panel of *M. guttatus* RILs used for this study, epigenetic inheritance of traits induced by parental damage can be transmitted a minimum of two generations. In addition, treatment of offspring seedlings from damaged parents with the demethylating agent 5-azacytidine can eliminate epigenetically transmitted phenotypes. Although these lines of evidence are quite preliminary (A. Scoville & J. Kelly, unpublished), not yet having been subject to replication experiments, they suggest that epigenetically inherited trichome production in this system is the result of cross-generational transmission of DNA methylation states, and not to maternal loading of nutrients or other small molecules. Therefore, differences in patterns and/or abundance of DNA methylation at the *MgMYBML8* locus, or loci upstream from *MgMYBML8* but effecting *MgMYBML8* expression in *M. guttatus*, may be responsible for transgenerational epigenetic inheritance of trichome density in response to parental damage. Alternatively, the epigenetic mechanism may involve inheritance of histone modifications or siRNA that affect *MgMYBL8* expression, or expression of upstream regulators of *MgMYBML8*. Direct regulators of *MIXTA*-like genes are not yet known. Finally, we have not conclusively ruled out mechanisms consistent with broader-sense epigenetic inheritance such as damage-induced alteration to genetic feedback loops that lead to a bistable phenotypic switch (Becskei *et al*., 2001; Jablonka & Lamb, 2008).

Evolutionary implications

Epigenetic inheritance of environmentally induced traits challenges two cornerstones of modern biology: that heritable variation is the result of differences in nucleotide sequence and that changes in inherited information occur only at random, without direction from the environment towards specific phenotypes (Kalisz & Purugganan, 2004; Rapp & Wendel, 2005; Richards, 2006; Bonduriansky & Day, 2009). It is currently unclear how often epigenetic modifications affect functionally important traits in natural populations. Most examples of epigenetic inheritance involve extreme phenotypic alterations, agricultural or model organisms, and unrealistic ecological contexts (Kalisz & Purugganan, 2004; Bossdorf *et al*., 2008). Examples specific to environmentally induced traits primarily involve toxins that induce detrimental phenotypes (Anway *et al*., 2005; Benyshek *et al*., 2006; Newbold *et al*., 2006), or treatments that directly alter epigenetic states, such as exposure to 5-azacytidine (Sano *et al*., 1990; Fields, 1994; Fields *et al*., 2005; Bossdorf *et al*., 2010). By contrast, we demonstrate epigenetic transmission of a putative herbivore defense in response to simulated herbivore damage. Transgenerational induction of high trichome density could confer a selective advantage if offspring are likely to experience the same herbivore pressure as their parents. This type of advantage is likely to be particularly important in plants and other sessile organisms, which cannot cope with environmental heterogeneity via changes in behavior (Jablonka *et al*., 1995; Jablonka & Raz, 2009).

Interactions between genetic and epigenetic inheritance remain essentially unexplored (Bossdorf *et al*., 2008; Johannes *et al*., 2008). It is usually difficult to distinguish stable epialleles from epigenetic modifications that are immediately regenerated each generation contingent on DNA sequence variation (Richards, 2008). Our panel of RILs is ideal for investigating the relationship between epigenetic and genetic variance. We demonstrate a consistent, repeatable epigenetic response and genetic variation in that response. This indicates the potential for epigenetic inheritance of an induced defense to evolve. Elucidating the mechanism for epigenetic inheritance, including associations with *MgMYBML8* expression, will allow us to eventually determine whether genetic variation in epigenetic inheritance originates with variation in the ability to form, transmit, or respond to the epigenetic signal created by parental damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank members of KU-EEB Genetics discussion group for useful comments on early versions of this manuscript. This work was supported by the National Science Foundation (grant IOS-0951254 to J.K.K., L.C.H., and A.G.S.), and The University of Kansas General Research Fund. A.G.S. acknowledges the support of an Institutional Research and Academic Career Development Award postdoctoral fellowship from a National Institutes of Health grant (GM063651) to M.L. Michaelis.

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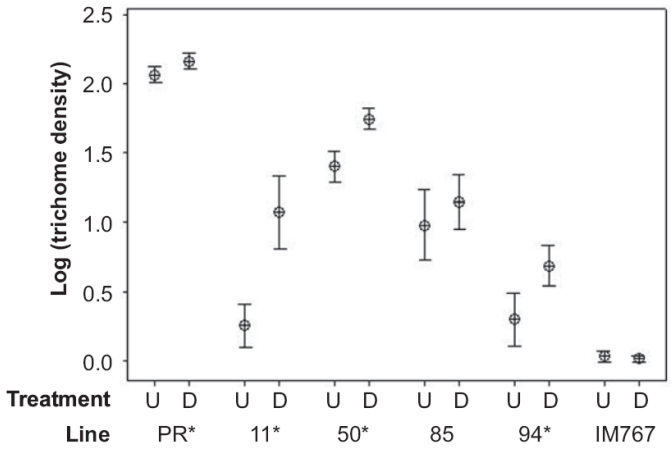

Fig. 1.

Genetic and epigenetic variation in trichome density. Log-transformed trichome density is shown for each genetic line and damage treatment (U, parent undamaged; D, parent damaged). Bars indicate the 95% confidence interval and asterisks indicate genetic lines that show a significant transgenerational epigenetic response in trichome density to parental damage.

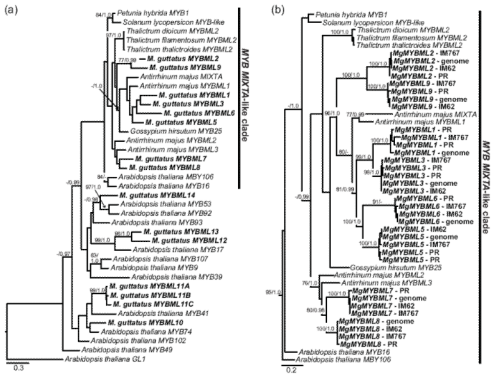

Fig. 2.

Evolutionary relationships among *MYB* and *MYB MIXTA*-like genes. (a) Maximum likelihood (ML) tree showing the relationship of all *Mimulus guttatus MYB* genes (shown in bold) with similarity to *Antirrhinum majus MIXTA*, *MYBML1*, *MYBML2* and *MYBML3* (*E* values $< 1 \times 10^{-50}$) identified from draft *M. guttatus* genome sequence. A clade containing candidate *M. guttatus MYB MIXTA*-like orthologs is indicated. (b) ML tree showing the relationships within the *MYB MIXTA*-like clade from (a). Each genome sequence-identified *MgMYBML* candidate gene (labeled –genome) was sequence-confirmed in the *M. guttatus* IM62 line (the line used to generate an *M. guttatus* genome sequence), and lines PR and IM767 (the parental lines used to generate recombinant inbred lines (RILs) used in this study). ML bootstrap percentages ≥ 75/Bayesian posterior probabilities ≥ 0.95 are shown above branches. Sequence identifiers can be found in Supporting Information, Table S1.

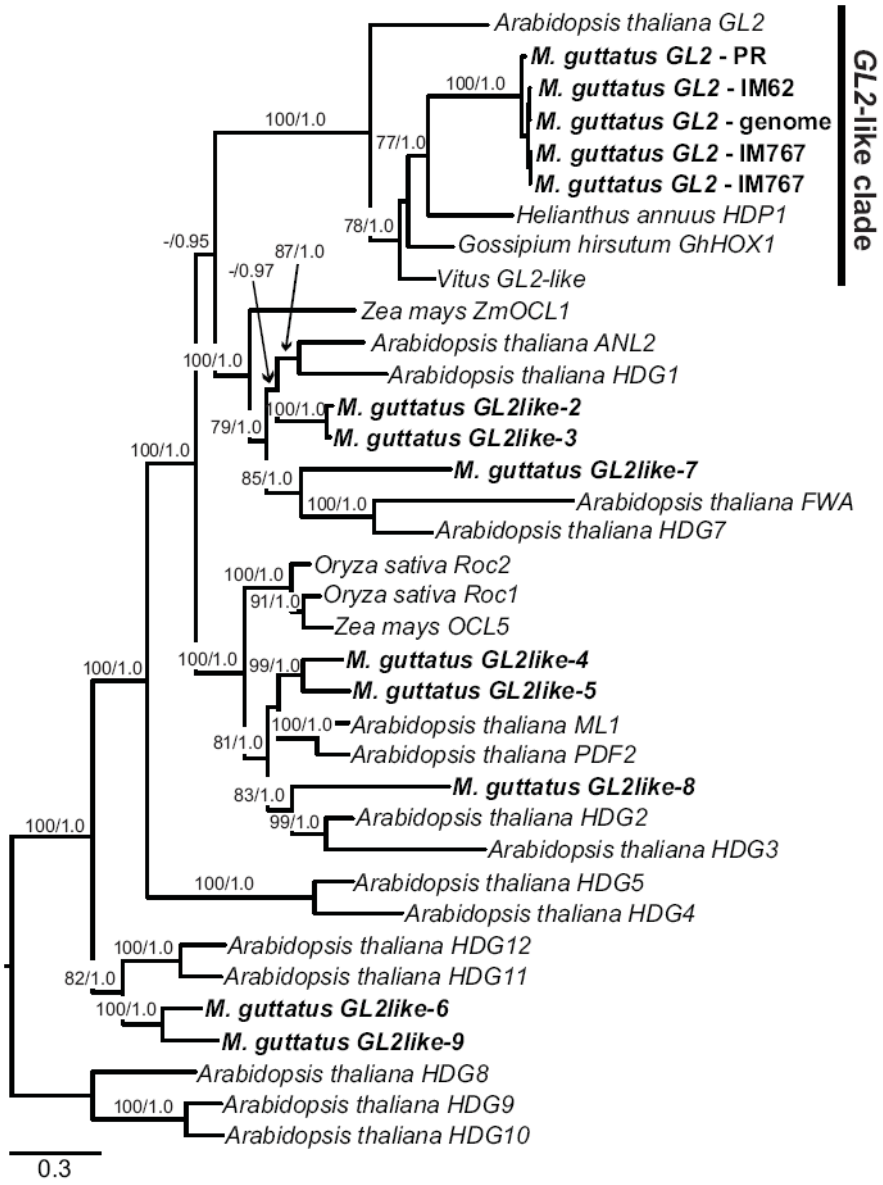

Fig. 3.

Evolutionary relationships among *GL2*-like genes. Maximum likelihood (ML) tree showing the relationship of all *Mimulus guttatus GL2*-like genes (shown in bold) with similarity to *Arabidopsis GL2* (*E* values < 1×10^{-50}) identified from draft *M. guttatus* genome sequence. A clade containing the candidate *M. guttatus GL2* ortholog is indicated. The genome sequence-identified *MgGL2* candidate gene (labeled – genome), was sequence-confirmed in the *M. guttatus* IM62 line (the line used to generate an *M. guttatus* genome sequence), and lines PR and IM767 (the parental lines used to generate recombinant inbred lines (RILs) used in this study). ML bootstrap percentages ≥ 75/Bayesian posterior probabilities ≥ 0.95 are shown above branches. Sequence identifiers can be found in Supporting Information, Table S1.

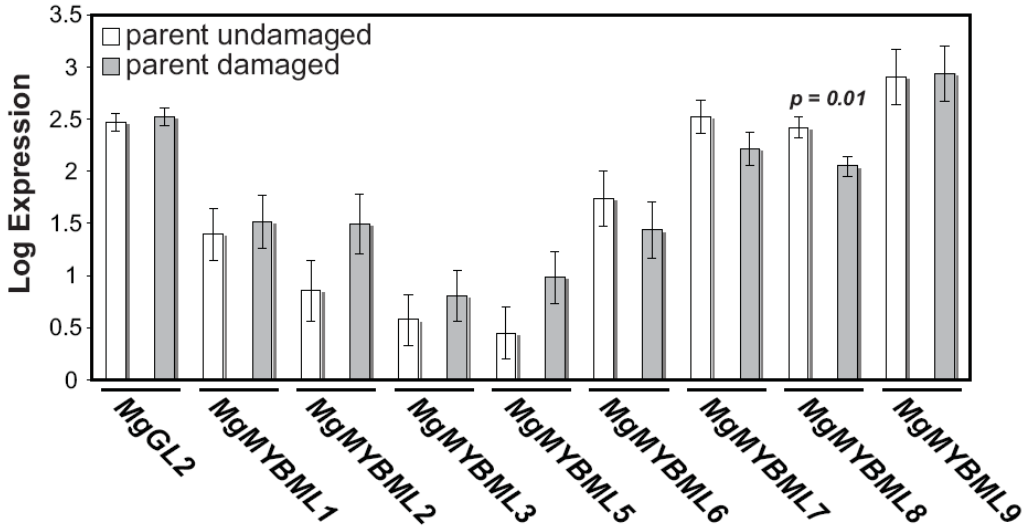

Fig. 4.

Candidate gene expression in response to parental damage. Least-squared means for relative expression of *MgGL2*, and *MgMYBML1, 2, 3, 5, 6, 7, 8, 9* in leaf tissue of plants from undamaged (white bars) and damaged (gray bars) parents. Values were obtained from an ANOVA predicting expression as a function of line, damage, and line × damage interaction, performed on data from lines that exhibit a significant increase in trichome density in response to damage (Point Reyes National Seashore (PR) and recombinant inbred lines (RILs) 11, 50, and 85). Bars represent a single standard error.

Table 1

Results from multiple regression predicting trichome density as a function of candidate gene expression

Predictor	Coefficient (SE)	*T*	*P*
Constant	2.26 (0.48)	4.72	0.0000
log*MgGL2*	−0.01 (0.15)	−0.08	0.9384
log*MgMYBML1*	−0.15 (0.09)	−1.62	0.1115
log*MgMYBML2*	−0.02 (0.06)	−0.37	0.7156
log*MgMYBML3*	0.02 (0.08)	0.24	0.8125
log*MgMYBML5*	0.03 (0.06)	0.50	0.6188
log*MgMYBML6*	−0.12 (0.06)	−1.90	0.0622
log*MgMYBML7*	0.05 (0.10)	0.51	0.6152
log*MgMYBML8*	−0.46 (0.12)	−3.88	**0.0003**
log*MgMYBML9*	0.06 (0.08)	0.70	0.4870

$R^2 = 0.32$, $F_{9,62} = 3.286$, $P = 0.002$

Table 2

ANOVA predicting log-transformed expression of *MgMYBML8* as a function of genetic line, parental damage treatment, and line × treatment interaction

Source	DF	*F*	*P*
Line	5	23.49	**0.0000**
Damage	1	1.71	0.1959
Line × damage	5	5.27	**0.0004**
Residuals	60		