Carpels in maize undergo programmed cell death in half of the flowers initiated in ears and in all flowers in tassels. The HD-ZIP I transcription factor gene GRASSY TILLERS1 (GT1) is one of only a few genes known to regulate this process. To identify additional regulators of carpel suppression, we performed a gt1 enhancer screen and found a genetic interaction between gt1 and ramosa3 (ra3). RA3 is a classic inflorescence meristem determinant gene that encodes a trehalose-6-phosphate (T6P) phosphatase (TPP). Dissection of floral development revealed that ra3 single mutants have partially derepressed carpels, whereas gt1;ra3 double mutants have completely derepressed carpels. Surprisingly, gt1 suppresses ra3 inflorescence branching, revealing a role for gt1 in meristem determinacy. Supporting these genetic interactions, GT1 and RA3 proteins colocalize to carpel nuclei in developing flowers. Global expression profiling revealed common genes misregulated in single and double mutant carpels, as well as in derepressed gt1 auxillary meristems. Indeed, we found that ra3 enhances gt1 vegetative branching, similar to the roles for the tassel pathway and GT1 homologs in the eudicots. This functional conservation over ~160 million years of evolution reveals ancient roles for GT1-like genes and the trehalose pathway in regulating auxillary meristem suppression, later recruited to mediate carpel suppression. Our findings expose hidden pleiotropy of classic maize genes and show how an ancient developmental program was redeployed to sculpt floral form.

V

aration in development drives variation in organismal form. One important process in floral development and evolution is growth suppression in floral organs (1, 2). A prominent form of this suppression exists in the grass family (Poaceae). Most grass flowers initiate both carpel and stamen primordia, but selective suppression of these primordia has led to immense diversity in floral sexuality (3). This diversity is critical for patterns of gene flow in natural populations and fertile flower production in nature and agriculture, and it facilitates controlled crossings in breeding programs (4–6). Despite these important consequences to both evolution and agriculture, only a handful of genes are known to regulate floral sexuality in the grasses.

Floral sexuality has long been studied in Zea mays (maize), in which programmed cell death suppresses carpel development in all tassel flowers and in one of the two flowers in each ear spikelet (7, 8). Among the few characterized suppression genes, most encode enzymes with roles in hormone metabolism and have pleiotropic effects on development and defense (9–15). This list includes several genes underlying the classic tasselseed (ts) mutants, which exhibit extensive tassel feminization beyond carpel suppression (9, 10, 12–14, 16). Most of the cloned ts mutants encode genes involved in jasmonic acid metabolism, whereas ts4 encodes a microRNA that targets Ts6, a developmental regulator with multiple roles in flower and inflorescence development (12, 14).

Most genes that affect carpel suppression simultaneously affect other traits that differentiate tassels from ears, such as stamen development, bract (glume) morphology, and inflorescence morphology (9–11, 16). GRASSY TILLERS1 (GT1) is an exception. While GT1 was defined by its role in regulating axillary branching, gt1 mutants have a weak carpel suppression phenotype in otherwise normal flowers and inflorescences (17). To find additional regulators of carpel suppression, we conducted an enhancer screen of gt1 mutants and found a genetic interaction with the classic inflorescence determinacy gene, RAMOSA3 (RA3) (18). Our results reveal surprising pleiotropy and interactions between gt1 and ra3, which together regulate carpel suppression, meristem determinacy, and axillary meristem suppression.

Results

The rapunzel (rz) Genes Suppress Carpels in Tassel and Ear Flowers

To identify genes that regulate carpel suppression with gt1, we conducted an ethyl methanesulfonate (EMS) enhancer screen, looking for mutants where carpel growth was derepressed in

Significance

Floral morphology is immensely diverse. One developmental process acting to shape this diversity is growth suppression. For example, grass flowers exhibit extreme diversity in floral sexuality, arising through differential suppression of stamens or carpels. The genes regulating this growth suppression and how they have evolved remain largely unknown. We discovered that two classic developmental genes with ancient roles in controlling vegetative branching were recruited to suppress carpel development in maize. Our results highlight the power of forward genetics to reveal unpredictable genetic interactions and hidden pleiotropy of developmental genes. More broadly, our findings illustrate how ancient gene functions are recruited to new developmental contexts in the evolution of plant form.

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tassels. Maize flowers typically initiate three stamens and three carpel primordia, with stamen development suppressed in ear flowers and carpel development suppressed in tassel flowers (Fig. 1 A and D). When carpel growth is not suppressed, gynoe-
cia, each consisting of three carpels, develop in tassel flowers, resulting in silks emerging from tassel spikelets (Fig. 1C). In gt1 single mutants, only 7% of scored tassel flowers developed long silks that emerged from spikelets (>0.2 cm, Fig. 1B and G). However, in the double mutants we identified in our screen, 100% of scored tassel flowers developed long silks (Fig. 1C and G). We called this enhanced mutant phenotype rapunzel (rzl), after the Grimm brothers’ fairy tale character with long hair. Two of these rzl mutants, gt1;rzl-3 and gt1;rzl-4, phenocopied one another and did not complement each other, indicating that rzl-3 and rzl-4 are allelic (Fig. 1C and I; SI Appendix, Fig. S1). Therefore, we focused many of our downstream analyses on either rzl-3 or rzl-4.

The gt1;rzl-3/4 floral phenotype was specific to carpels and manifested in both tassels and ears. In contrast to most ts mutants (9, 10, 12, 13), stamen development in gt1;rzl-3 and gt1;rzl-4 tassel flowers was not suppressed (Fig. 1; SI Appendix, Fig. S1 and Table S1). In normal ear spikelets, two flowers initiate, but only the upper flower completes development; the lower flower is suppressed (7). In gt1;rzl-3 ears, carpel growth was derepressed in lower flowers, resulting in two fertile flowers per spikelet (Fig. 1F and H; SI Appendix, Fig. S2). Thus, rzl-3/4 interacts with gt1 to disrupt carpel suppression while leaving other floral and inflorescence traits unaffected.

rzl-3 and rzl-4 are Alleles of the Trehalose-6-Phosphate Phosphatase Gene, RAMOSA3. To identify the rzl-3/4 gene, we used both bulked segregant analysis coupled with whole genome shotgun sequencing (BSA-seq) and fine mapping (19). Our BSA-seq results revealed a broad peak between 160 and 180 Mbp on chromosome seven that represented the rzl-4 mapping interval (Fig. 24). The narrow peaks on chromosomes three and nine are likely because of differences between B73 laboratory stocks and the reference genome (19, 20). The broad region of homo-
yzosity on chromosome one results from the introgression of gt1, which arose in A619 (17), into B73. Using fine mapping (19), we reduced the chromosome seven mapping interval to a ~230-kbp region containing nine genes (Fig. 2B), only one of which contained a canonical EMS single nucleotide polymor-
phism (SNP) predicted to negatively affect gene function (21).

This EMS SNP was predicted to change a single amino acid (Ala337Thr) in the trehalose-6-phosphate phosphatase (TPP) encoded by RAMOSA3 (RA3) (18). Ala337 is deeply conserved in TPP paralogs and contacts the active site in homology models (SI Appendix, Fig. S3). In addition, the substitution of a homologous amino acid in an RA3 paralog negatively affects protein function (22), suggesting that the rzl-4 SNP would impact RA3 function similarly. Because rzl-4 and rzl-3 were allelic, we sequenced RA3 in rzl-3 mutants and found an EMS mutation at a likely splice acceptor site S’ of exon 4. Transcripts from this ra3 allele had an in-frame deletion of two active-site amino acid codons (SI Appendix, Fig. S2). Thus, both gt1;rzl-3 and gt1;rzl-4 double mutants harbor alleles of ra3 predicted to negatively affect gene function, indicating that rzl-3 and rzl-4 encode alleles of ra3. From now on, we will refer to these alleles as ra3-rzl3 and ra3-rzl4.

gt1 Suppresses ra3 Tassel and Ear Branching. Usually, ra3 mutants have branched ears and increased tassel branching due to inde-
terminate meristems that produce many spikelets on long branches (18, 22). Therefore, we were surprised to find unbranched ears in gt1;ra3-rzl3/4 double mutants and in ra3-
ra3/4 single mutants heterozygous at gt1 (Fig. 1F). Given that gt1 is semidominant, the lack of ear branching in these mutants could have been caused by a second genetic interaction between gt1 and ra3, regulating meristem determinacy. The lack of ear branching may also have been the result of the genetic background (A619) used for EMS mutagenesis. Most characteriza-
tion of ra3 has been in the B73 genetic background (18, 22), and background modifiers affect the ra3 ear determin-
cy phenotype (18).

To test for a genetic interaction between gt1 and ra3 independ-
ent of any A619 modifiers, we made a gt1;ra3 double mutant with a third, well-characterized allele of ra3 (ra3-fea1) in the B73 genetic background (18, 22) (Fig. 2). gt1;ra3-fea1 double mutants recapitulated the rzl phenotype, with silks in
tassel flowers, as in our gt1;ra3–rzl3 and gt1;ra3–rzl4 mutants (Fig. 2F). ra3–fea1 single mutants had branched ears and increased tassel branching, as expected (18). However, most gt1;ra3–fea1 double mutants lacked ear branches and had fewer tassel branches than ra3 single mutants (Fig. 2C–H), indicating a second genetic interaction between gt1 and ra3, regulating meristem determinacy. Thus, RA3 and GT1 act to regulate both meristem determinacy in inflorescences and carpel suppression in flowers.

ra3 Mutants Have a Carpel Suppression Phenotype and RA3 Colocalizes with GT1 in Carpel Nuclei. ra3–rzl3 mutants (ra3–rzl3;gt1+/+) had significantly more derepressed carpels in tassel flowers than A619 individuals (Fig. 1G, P value < 0.001, χ² test, two degrees of freedom). This could be because of gt1 semidominance (17) or because ra3 single mutants have a weak floral phenotype. Indeed, there have been hints of likely background-dependent ra3 floral phenotypes (23). To investigate the ra3 floral phenotype further, we followed tassel flower development in mutants using scanning electron microscopy.

In wild-type flowers, the two silk carpels are first visible as a raised line of tissue called the gynoecial ridge. In ear flowers, these silk carpels grow to enclose the third, ovulate carpel and fuse to form the stigma, called the silk in maize (7). In tassel flowers, carpels initiate but undergo programmed cell death shortly after the initiation of the gynoecial ridge (8, 24) (Fig. 3A–D). In contrast, in gt1 single mutants, silk carpels continued to grow past the gynoecial ridge stage and formed a peak of tissue over the developing ovulate carpel (Fig. 3E–H). The tassel flowers of ra3 single mutants had carpels that resembled those of gt1 mutants: The two silk carpels formed a peak of tissue over the ovulate carpel but did not fuse laterally to enclose the ovulate carpel (Fig. 3I–L). These data indicate that carpel suppression is partially disrupted in both ra3 and gt1 single mutants.

The carpels in gt1;ra3 double mutant flowers continued to grow well past the gynoecial ridge stage, eventually fusing to form silks (Fig. 3 M–P; SI Appendix, Fig. S2). In addition, the gynoecia of gt1;ra3 double mutants closely resembled the gynoecia of wild-type ear florets, with two silk carpels and an ovulate carpel (Fig. 3 O and P; SI Appendix, Fig. S2). In contrast, other floral meristem determinacy mutants, such as Z. mays agamous1 (zag1), bearded ear (bde), Tasselseed6 (tse6), and Tasselsense (tsn) have a weakened carpel phenotype (25), but their floral meristems persist in inflorescences (26). We noted that GT1 and RA3 expression was disrupted in floral meristems of gt1;ra3 double mutants in both inflorescences and gynoecial ridges (Fig. 3L). We did not observe GT1 or RA3 expression in wild-type flowers (Fig. 3K). In contrast, the gynoecia of ra3 single mutants were weakly developed and had an indeterminate floral meristem (Fig. 3L). In the maize flower, the floral meristem remains indeterminate in inflorescences and carpel suppression in flowers.

ra3–gt1 Mutants Have a Carpel Suppression Phenotype and RA3 Suppresses GT1 in Carpels. In contrast, other floral meristem determinacy mutants, such as Z. mays agamous1 (zag1), bearded ear (bde), Tasselseed6 (tse6), and Tasselsense (tsn) have a weakened carpel phenotype (25), but their floral meristems persist in inflorescences (26). We noted that GT1 and RA3 expression was disrupted in floral meristems of gt1;ra3 double mutants in both inflorescences and gynoecial ridges (Fig. 3L). We did not observe GT1 or RA3 expression in wild-type flowers (Fig. 3K). In contrast, the gynoecia of ra3 single mutants were weakly developed and had an indeterminate floral meristem (Fig. 3L). In the maize flower, the floral meristem remains indeterminate in inflorescences and carpel suppression in flowers.
not detect GT1 protein in gt1 (B73) carpels, nor did we detect RA3 in ra3-fea1 carpels, which we used because ra3-fea1 is a known protein null allele (18). Importantly, both GT1 and RA3 were predominantly localized to the nuclei of subepidermal cells, where programmed cell death initiates in maize and its relatives in the Andropogoneae (8, 30). While RA3 likely acts non–cell autonomously in regulating meristem determinacy (18, 31), the GT1 and RA3 localization patterns in tassel flow­ers suggest that both act cell autonomously in regulating carpel suppression.

GT1 and RA3 Regulate Genes Predicted to Mediate Programmed Cell Death. To identify the effectors of carpel suppression down­stream of GT1 and RA3, we examined the transcriptional profiles of wild-type (A619), gt1, ra3, and gt1;ra3 tassels at two developmental timepoints: 1) prior to carpel suppression (pre­suppression, 0.8–1 cm tassels) and 2) during active carpel sup­pression (misexpression, 1–2 cm tassels). We reasoned that genes that were 1) misexpressed in gt1;ra3 mutant flowers and 2) changed over wild-type carpel development represented the best carpel suppression gene candidates. We performed differential expression analyses on our sequencing data (32) and focused on the 73 genes that satisfied both of these conditions and were highly differentially expressed at the misexpression stage (fold change > 2, Fig. 4 B and C; Dataset S1). Most genes in this carpel suppression gene set that were down­regulated in mutants were up-regulated over the course of development in A619 or vice versa (69/73 genes, or 95%; Fig. 4C). This pattern of regulation is consistent with a role for these genes in mediating the development of the rainbow phenotype. Notably, 62/73 genes (85%) were misexpressed in gt1 or ra3 single mutants, and 44/73 (60%) were misexpressed in gt1 and ra3 single mutants. This suggests that the carpel suppression phe­notype in gt1;ra3 double mutants is a matter of degree, not the result of an entirely different set of genes being misregulated in double versus single mutants.

Although the carpel suppression gene set was not enriched for specific gene ontology (GO) terms, it did contain several genes whose homologs in Arabidopsis thaliana (Arabidopsis) have roles in reactive oxygen species signaling, callose deposition, cell wall remodeling, and programmed cell death (Fig. 4C and Table 1). For example, homologs of the Arabidopsis NAM, ATAF and CUC (NAC)-family transcription factor genes KIRA1 (GRMZM2G081930, NACTF36) and NAC087...
(GRMZM2G181605, NACTF2) were up-regulated as carpel suppression commenced in A619 but expressed at lower levels in mutant tassel primordia versus A619 at the midsuppression stage (Fig. 4D) (3, 34). KIRA1 acts to regulate programmed cell death in stigmatic papillae (33). ANAC087, with ANAC046, initiates programmed cell death in the root cap and regulates chromatin degradation following programmed cell death (34). Two protease genes show a similar pattern of regulation: a cysteine protease gene (GRMZM2G456217) with an Arabidopsis homolog (CEP1) with roles in programmed cell death of xylem elements and the tapetum (35, 36) and an uncharacterized serine protease gene (GRMZM2G313321; see ref. 37). One of the genes that was down-regulated over development in A619 but highly up-regulated in mutant versus A619 tassels (GRMZM2G161233) likely encodes a pectin biosynthetic enzyme involved in cell wall remodeling (38). Cell wall remodeling genes are also differentially expressed between upper and lower floral meristems in maize ears, and pectin modification differs between upper and lower floral ear meristems and between B73 and gt1ra3 ear primordia (39). These and other examples (Table 1) are consistent with roles for GT1 and RA3 in carpel suppression and further point to genes that may be directly or indirectly regulated by GT1 and RA3.

Trehalose Levels Are Lower in gt1ra3 Mutant Tassels. Two ra3 paralogs, TPP7 (GRMZM2G083034) and TPP10 (GRMZM2G055150), were highly up-regulated during carpel suppression in A619 and strongly down-regulated in gt1ra3 mutants (Fig. 4C and Table 1). Although RA3’s enzymatic activity is not essential in regulating meristem determinacy, it is a catalytically active TPP enzyme (22). TPP enzymes catalyze the second (and last step) of the only trehalose biosynthesis pathway in plants; they dephosphorylate T6P to produce trehalose and a free phosphate group (22, 40). The misexpression of multiple TPP genes led us to measure sugar and metabolite levels at the carpel suppression stage in tassel primordia (1–1.5 cm in length) (Fig. 4D; SI Appendix, Fig. S4). Consistent with the down-regulation of TPP genes, trehalose was substantially lower in gt1ra3 mutants (Fig. 4D). However, T6P levels were not consistently higher in mutants versus A619. T6P is derived from the hexose phosphates glucose 6-phosphate (G6P) and uridine phosphate-glucose (UDPG) and likely signals sucrose status during plant growth and development (40–42). Similar to T6P and most metabolites, sucrose, G6P, and UDPG levels were also not significantly different between A619 and mutant tassels (Fig. 4D; SI Appendix, Fig. S4). This indicates that while trehalose biosynthesis is impacted in gt1ra3 double mutants, sucrose status, as signaled by T6P (40, 42), is not affected, at least not at the level of entire tassel primordia. We reasoned that T6P levels may be unchanged because of the homeostatic control of sucrose and T6P levels (41). In support, four T6P synthase genes (TPPs), including at least one encoding a likely catalytically active TPS (43, 44), were also down-regulated in gt1ra3 double mutants (Dataset S1). Similarly, trehalose metabolism gene expression and sugar accumulation differ between the upper and lower floral meristems in ear primordia (39). Taken together, these data suggest that the trehalose pathway is important in the regulation of carpel suppression.

ra3 Enhances Tilling in a gt1 Mutant Background. T6P signaling has roles in regulating bud outgrowth in eudicots (45, 46) and is associated with bud outgrowth in maize (47). Notably, 24 of

**Table 1. Putative carpel suppression genes that have been functionally characterized**

| Maize gene ID | Arabidopsis best hit | Functional annotation | Biological function | Citation |
|---------------|----------------------|-----------------------|---------------------|----------|
| GRMZM2G456217 | AT5G50260 | Cysteine protease | Programmed cell death | (35, 36) |
| GRMZM2G081930 | AT4G28530 (KIRA1) | NAC transcription factor 36 (NACTF36) | Programmed cell death | (33) |
| GRMZM2G181605 | AT1G18270 (ANAC087) | NAC transcription factor (NACTF2) | Programmed cell death | (34) |
| GRMZM2G313321 | AT1G36210 | Serine protease, vignain | Proteolysis | (37) |
| GRMZM2G085243 | AT1G16470 (PAB1) | Proteasome subunit PAB1 | Proteolysis | (37) |
| GRMZM2G145554 | AT4G18170 (WRKY28) | WRKY DNA-binding domain protein | Leaf senescence | (98) |
| GRMZM2G104299 | AT2G33620 | AT-hook motif nuclear-localized protein 10 | ABA response; senescence (maize) | (99, 100) |
| GRMZM5G858784 | AT1G30100 (NCED5) | Nine-cis-epoxycarotenoid dioxygenase 3 | ABA biosynthesis | (101) |
| GRMZM2G052902 | AT2G23460 (RAF2) | Serine/threonine-protein kinase HT1 | ABA signaling | (102) |
| GRMZM5G884349 | AT1G35530 (bZIP16) | bZIP transcription factor protein (bZIP32) | ABA and GA response; cell elongation | (103) |
| GRMZM2G000264 | AT5G62670 (AHAI1) | ATPase 4 plasma membrane-type | ABA response | (104) |
| GRMZM2G088289 | AT1G30190 (CBC1) | MAP kinase kinase kinase 55 | Stomatal opening | (105) |
| GRMZM2G001205 | AT5G58920 (ZAT12) | ZNF5, C2H2 zinc finger protein5 | ROS response; salt and osmotic stress | (106–108) |
| GRMZM2G152827 | AT1G4040 (PHT3;1) | Mitochondrial phosphate transporter 1 (MPT1) | ROS accumulation; salt stress | (109, 110) |
| GRMZM5G854625 | AT1G60940 (SNRK2-10) | SNF1-related protein kinase 2.10 | ROS homeostasis; osmotic stress | (111–115) |
| GRMZM2G080354 | AT5G10100 (TPPI) | tpp10; trehalose-6-phosphate phosphatase 10 | Trehalose metabolism | (116) |
| GRMZM2G055150 | AT2G2190 (TPPE) | tpp7; trehalose-6-phosphate phosphatase 7 | Trehalose metabolism | (117) |
| GRMZM2G090922 | AT1G61800 (GP2) | Glucose-6-phosphate/phosphate translocator | Response to sucrose | (118) |
| GRMZM2G077181 | AT3G57250 (SI2P) | Galactosyl-sucregalactosyltransferase 2 | Sugar metabolism | (119) |
| GRMZM2G376416 | AT1G02850 (BGLU11) | Beta-glucosidase 5 (GLU5) | Carbohydrate metabolism | (120) |
| GRMZM2G170049 | AT4G25560 (LAF1) | MYB domain protein 26 | Light signaling | (120, 121) |
| GRMZM2G006762 | AT5G18280 (APY2) | Putative apyrase family protein | Cell elongation | (122–124) |
| GRMZM2G079957 | AT1G04240 (IAA3; SHY2) | Auxin-responsive protein IAA2 | (125–127) |
| GRMZM2G161233 | AT3G23820 (GAE6) | UDP-glucuronate 4-epimerase 1 | Pectin biosynthesis; cell wall remodeling | (128) |
| GRMZM2G060824 | AT5G23630 (MIA;PDR2) | ATPase PDR2 | Callose deposition; meristem maintenance | (129) |
| GRMZM2G703857 | AT3G25070 (NCK1) | Rpm1-interacting protein 4 | Immunity; callose deposition | (130) |
| GRMZM2G099454 | AT1G12500 (CHI-B) | Basic endochitinase | Immunity | (130) |
| GRMZM2G046382 | AT1G3540 | 17.4 kDa class I heat shock protein (HSP9) | Heat acclimation | (131) |
| GRMZM2G078025 | AT1G15450 (CLBP-P) | Casein lytic proteinase B3 | Heat stress; plastid development | (132, 133) |
| GRMZM2G109618 | AT5G22880 (DMC1) | DMC1; disrupted meiotic CDNA homolog1 | Meiosis | (134) |

*Genes in the carpel suppression set of 73 genes with functional annotations, most of which are from Arabidopsis.

1 Gene IDs in bold differentially expressed in gt1 tiller buds (47). ABA, abscisic acid; CDNA, complementary DNA; GA, gibberellin; ROS, reactive oxygen species.
the 73 carpel suppression genes (~31%), including TPP7 and TPP10, were differentially expressed in gt1 versus wild-type tiller buds (47). A gene set enrichment analysis also revealed that genes differentially expressed in gt1 versus wild-type tiller buds (47) were enriched in the differentially expressed genes in our study (SI Appendix, Table S2), and RA3 and GT1 are bound and regulated by TEOSINTE BRANCHED1 (TBI), a conserved regulator of axillary bud suppression (47–49). These associations led us to ask whether ra3 is also involved in regulating axillary bud outgrowth. We counted tillers and measured their lengths in A619, gt1, ra3, and gt1;ra3 plants and found that gt1;ra3 double mutants produced more and longer tillers than gt1 single mutants (Fig. 5). In addition, these double mutants produced more ears than single gt1 mutants (SI Appendix, Fig. S5). Thus, RA3 also regulates vegetative branching in concert with GT1 and adds another developmental context in which both genes act to suppress growth.

Discussion
Organ repression is an important driving force in the evolution of floral diversity (1, 2). Here, we sought to identify the genes that regulate growth suppression in maize carpels and unexpectedly found the classic meristem determinacy gene, RA3 (18). We show that RA3 acts with GT1 in multiple developmental contexts: to regulate carpel suppression, meristem determinacy, and vegetative branching. Although it is catalytically active, RA3’s enzymatic activity is not essential for regulating meristem determinacy, and RA3 colocalizes with the transcriptional machinery in nuclear speckles in young ear primordia (22, 50). These data suggested that RA3 has an alternate “moonlighting” role in regulating transcription, potentially connected to T6P signaling (22, 50). Notably, we found a similar pattern of nuclear localization for both RA3 and GT1 in carpel primordia (Fig. 4A), suggesting RA3’s moonlighting role extends to carpel suppression. Although the levels of T6P and its precursors were not significantly different between wild-type and mutant tassels, trehalose levels were lower in gt1;ra3 tassels, consistent with the down-regulation of other TPP genes (Fig. 4 C and D). These data suggest that the trehalose pathway, potentially connected to RA3’s moonlighting role, has roles in regulating carpel suppression, as does it in a number of developmental processes (42).

The trehalose pathway and GT1-like genes have ancient roles in regulating vegetative branching. GT1-like transcription factors regulate axillary meristem growth suppression in Arabidopsis, maize, and Oryza sativa (rice) (17, 51, 52). In addition, high T6P and sucrose levels lead to increased axillary bud outgrowth in Arabidopsis and Pisum sativum (garden pea) (42, 45). In maize, high T6P levels are correlated with axillary bud outgrowth in gt1 and tb1 mutants (47). Notably, we found that two independent mutations in RA3 enhance gt1 vegetative branching (Fig. 5; SI Appendix, Fig. S5). Taken together, these data indicate that growth regulation by the trehalose pathway and GT1-like genes appeared before the divergence of monocots and eudicots (Fig. 6).

In contrast to this ancient role in vegetative branching, carpel suppression arose later and repeatedly in the grasses, for example, in the lineages leading to maize and barley (Hordeum vulgare) (3, 53, 54). Strikingly, floral organ suppression in barley is also mediated by a GT1 paralog, SIX-ROWED SPIKE1 (VRS1), and an RA3 homolog is down-regulated in barley floral organ suppression mutants (54–59). Critically, while VRS1 does have a particular role in barley carpel suppression, it also acts to suppress the growth of other floral organs in lateral spikelets, and barley carpel suppression does not involve programmed cell death (58, 59). These differences in GT1 and VRS1 function, together with the evolutionary history of carpel suppression in the grasses, suggest that RA3 and GT1 homologs were independently recruited to mediate grass carpel suppression (Fig. 6). This recruitment may have been mediated by changes in expression of GT1-like and trehalose metabolism genes (58, 60). Interestingly, in the eudicots, GT1-like genes have been independently recruited to mediate unisexual flower development in Diospyros kaki (persimmon; ref. 61), and a TPP gene occurs within the female-determining region of Vitis vinifera (grapevine; ref. 62). As in the grasses, floral unisexuality arose independently in the lineages leading to persimmon and grapevine, which are separated from each other by ~120 million years and from the grasses by ~160 million years of evolution (63). This striking convergent evolution suggests that GT1-like genes and TPPs were repeatedly deployed to mediate growth repression in the development of unisexual flowers.

In conclusion, we show that GT1-like and RA3-like genes control an ancient growth suppression program recruited to multiple developmental contexts. This reflects the iterative recruitment of an ancient branching program to suppress carpel primordia. Asterisks are roles for GT1 and RA3 we show here; citations for roles of TPP genes and T6P are in the text. IM, inflorescence meristem; SM, spikelet meristem.

Fig. 5. GT1 and RA3 act in concert to regulate axillary meristem suppression. (A–F) Tillering was enhanced in gt1;ra3 double mutants (57 gt1 and 64 gt1;ra3 individuals over two field seasons, P < 0.05, ANOVA, Tukey’s post hoc). gt1;ra3 double mutants produced both (E) more and (F) longer tillers. Tiller length in F is normalized to the height of the main culm.

Fig. 6. GT1-like gene and trehalose pathway-mediated regulation of AM growth has been recruited to suppress carpel primordia. Asterisks are roles for GT1 and RA3 we show here; citations for roles of TPP genes and T6P are in the text. IM, inflorescence meristem; SM, spikelet meristem.

GT1/TPP6:
- AM suppression
- carpel suppression
- floral organs

maize
rice
barley
wheat
garden pea
arabidopsis

GT1 homolog
T6P and TPPs

AM SM/IM
nature of plant development, in which most aerial organs are leaf homologs, produced by a shoot apical meristem established early in embryogenesis (64–66). This deep homology of plant organs suggests that there may be additional cases of developmental genes seemingly functioning in discrete contexts, but whose pleiotropy is masked. Indeed, thorough genetic and phenotypic analyses continue to expose pleiotropy of developmental regulators (67–71). Recent examples include WUSCHEL-like HOMEBOX9 (WOX9), which controls both inflorescence branching and embryo development (70), and THORN IDENTITY1, which controls thorn development and branching in citrus (71). Detailed dissection of gene function—alone, in the context of other genes, and in different developmental contexts—is likely to reveal that this pleiotropy is widespread.

Materials and Methods

Plant Material, Growth Conditions, and Phenotyping. We used the gt1-1 allele (17) for all our experiments with gt1, gt1-1 arose in A619 and was backcrossed five times with B73 to generate the B73 introgression lines used for BSA-seq and for phenotypic characterizations of the gt1;ra3 double mutants in B73. The ra3 alleles used here were either those that arose in our enhancer screen (ra3-fl, ra3-rzl4) in A619, or ra3-fea1 in B73 (18). Plants for the ra3-fl ra3-rzl4 BSA-seq, gt1-1;ra3-rzl3 tassel and ear mature pheno-
types, and gt1-1;ra3 tiller quantification were grown at the University of Mas-
sachusetts Amherst Crop and Animal Research and Education Farm in South Deerfield, MA (~42°29′N, 72°35′W). Plants for the gt1-1;ra3-fea1 tassel and ear branch counts were grown over two seasons at the UMass Amherst Crop and Animal Research and Education Farm and near Valle de Banderas, Mexico (~20°47′N, 105°15′W). Plants for scanning electron microscopy (SEM), metabolite measurements, and RNA sequencing (RNA-seq) were grown in the College of Natural Sciences and Education Greenhouse on the UMass Amherst campus under long day conditions (16 h light, 8 h dark) at 28 °C.

Flowers and spikelets for phenotyping were selected from the central sec-
tions of ears or from the central sections of the tassel central spike. For tassel flower phenotyping, 20 flowers from each of four individuals per genotype were examined. For ear spikelet phenotyping, 50 spikelets from each of five individuals per genotype were examined. For measuring silk length, silks from 12 flowers from each of three individuals per genotype were measured. Tassel branches from 68 B73, 67 gt1, 59 ra3, and 66 gt1 ra3 ra3 individuals and ear branches from 26 B73, 30 gt1, 54 ra3, and 65 gt1 ra3 individuals were counted over two field seasons. Tillers were measured from 57 gt1 and 64 gt1 ra3;ra3 indivi-
duals (in three planting blocks over the course of two field seasons). To account for differences in plant height, we normalized tiller length measure-
ments to the height of the main culm. Statistical significance was assessed using an ANOVA and Tukey’s test, or Student’s t test, as appropriate (72, 73).

Enhancer Screen and gt1-1;ra3-rzl3 BSA-seq. We performed an EMS mutagenesis
screen as in (74). Briefly, gt1-1 (A619) pollen was mutagenized with EMS and crossed onto gt1-1 (B73) ears. M1 progeny were selfed to generate M2 fami-
lies that were screened for enhanced silk length in tassels. We identified rzl4 and rzl3 in this screen.

To map rzl4, we crossed gt1-1;ra3-rzl4 (A619) individuals to gt1-1 (B73) indivi-
duals and selfed the F1 progeny to generate an F2 mapping population. In the F2 population, leaves from 238 gt1-1;ra3-rzl4 mutant individuals were selected for a pooled DNA extraction for BSA-seq, as described (19). Extracted genomic DNA was sequenced on an Illumina HiSeq. 2500 (paired-end reads, 70–150 bp) at Novogene for library construction and 20 Mbp Illumina paired-end RNA-seq Library Preparation and Sequencing. We prepared a total of 32 pools of three tassels per pool at two developmental stages (1–1.1 cm and 1.3–2.0 cm) for A619, gt1-1, rzl3, and rzl3 rzl4 plants for RNA-seq. Pools for gt1
and rzl3-rzl4 samples contained a mixture of individuals: either homoyogous wild-
type or heterozygous at rzl3-rzl4 or gt1, respectively. Total RNA was extracted from 96 individual tassels using TRIzol and the Qiagen RNeasy Plant Mini kit protocol. Tissue was collected in 1.7 mL RNase-free safe-lock tubes with ceramic grinding beads and immediately placed into liquid nitrogen. Samples were ground in a Qiagen TissueLyser II for 30 seconds, and 1.0 mL of TRIzol was added to each sample and mixed well by vortexing until all sample pow-
er was thoroughly mixed. Samples were incubated for five minutes at room temperature (RT), with frequent vortexing. Then, 0.2 mL of chloroform was added to each tube and vortexed for 15 s, followed by a 1-min incubation at RT and another 15-s vortex. Samples were centrifuged at 15,000 × g for 10 min to separate phases. After centrifugation, 200 μL of the aqueous layer was removed and added to 700 μL of 10% (v/v) trichloroacetic acid (TCA) in a tube. TCA pellet was prepared by adding 10 μL of 0.1 M Na-acetate con-
tained in 1.0 mL RLT buffer. RNA was then extracted from the supernatant using a Qiagen RNeasy Mini kit protocol, according to the manufacturer’s instructions. RNA quality was assessed on a 1% agarose gel run for 20 min at 120 V and quanti-
tied using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

Four pools were generated for each genotype and developmental stage by combining 1 μg of RNA from each of the three tassels. Total RNA from each sample was treated on-column with DNase I (NEB) for 15 min. RNA pools were sent to Novogene for library construction and 20 Mbp Illumina paired-end 150-bp sequencing for each pool.

RNA-seq Data Analysis. RNA-seq sequencing libraries were trimmed using Trimomatic (version 0.36.3) (87). These were then aligned to the Z. mays B73 v3 genome (76) and processed into BAM files using STAR (version 2.7.0) (88). Read counts were generated with the Rsubread package function feature-
Counts in R (89, 90). edgeR was used to construct principal component analysis plots of libraries (32). Because two of these libraries did not cluster with their corresponding replicates, we removed these libraries from further analysis (SI, Appendix, Table S4).

Pairwise differential expression was calculated between wild-type and each mutant using edgeR (32). GO term enrichment was calculated using topGO (32). GO term enrichment was calculated using topGO (32). GO term enrichment was calculated using topGO (32). GO term enrichment was calculated using topGO (32). GO term enrichment was calculated using topGO (32). GO term enrichment was calculated using topGO (32).

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Recruitment of an ancient branching program to suppress carpel development in maize flowers
Antibody Purification. GT1 (guinea pig) and RA3 (rabbit) antisera were used for western blots. Affinity purification to the c-terminal region of GT1 or to the N-terminal region of RA3 recombinant protein, using magnetic beads (Invitrogen), as described in (94). Validation of antibody was carried out by immunoblot following the protocol in (95), using total protein extract from wild-type and gt1 or ra3 mutants as negative controls.

Immunolocalizations. To perform triple whole-mount immunolocalizations, we used previously published protocols with minor modifications (50, 96). Tassels between 1 and 1.5 cm from ra3-fea1 (B73), gt1–1 (B73), and B73 were collected and prefixed at 4°C in 4% (wt/vol) paraformaldehyde-hyde and 2% (vol/vol) Tween-20 in phosphate-buffered saline (PBS) for 1 h, immersed in 6% agarose, sectioned at 75 μm using a vibratome (Leica), and collected in fixative solution for 2 h. Sections were washed and permeabilized and cell wall digestion (1% Driselase, Sigma-Aldrich; 0.5% cellulase, Sigma-Aldrich; 0.75% Pectolyase Y-23, Duchefa Biochemie) for 12 min at RT. Tissue was rinsed and incubated for 2 h in PBS and 2% Tween-20, rinsed two times, and blocked with 4% (wt/vol) bovine serum albumin (Sigma-Aldrich) for 1 h. The blocking solution was removed, and the tissue sections were incubated overnight at 4°C with anti-GT1 (1:200), anti-GT1 (1:75), and anti-guinea pig Alexa 488 (RA3), anti-mouse Alexa 568 (RNA POL II), and anti-guinea pig Alexa 647 (GT1)—counterstained with DAPI (Sigma), and mounted with Pro-Long Gold (Thermo Fisher Scientific). Immunolocalizations were repeated at least three times for each genotype. Images were acquired using a Zeiss LSM 780 confocal microscope.

Metabolic Measurements. Tassels between 1–1.4 cm were dissected from A619, gt1–1, ra3-rlz2;gt1–1;ra3–1ra3–rlz3 individuals and immediately frozen in liquid nitrogen. Eight tassels of each genotype were sampled in four waves over a 4-wk period. All individuals in a wave were sampled at 15 h 0 min to minimize variability. T6P and sugar metabolites were extracted from single tassels using a protocol from (41). T6P, other phosphorylated intermediates, and organic acids were measured by anion-exchange high performance liquid chromatography coupled to tandem mass spectrometry as described by (41) with modifications as described by (97). Sugars and sugar alcohols were measured as described by (98). Statistical significance was assessed using an ANOVA and Tukey’s test.

Data Availability. Raw sequencing data are available at the National Center for Biotechnology Information BioProject (RNA-seq: PRJNA657042; ra3-rlz4 BSA-seq: PRJNA656888); all data underlying the figures are available either in SI Appendix or in Datasets S1 and S2.

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