ABSTRACT Perennialism is common among the higher plants, yet little is known about its inheritance. Previous genetic studies of the perennialism in Zea have yielded contradictory results. In this study, we take a reductionist approach by specifically focusing on one trait: regrowth (the plant’s ability to restart a new life cycle after senescence on the same body). To address this, six hybrids were made by reciprocally crossing perennial Zea diploperennis Iltis, Doebley & R. Guzman with inbred lines B73 and Mo17 and Rhee Flint, a heirloom variety, of Z. mays L. ssp. mays. All the F1 plants demonstrated several cycles of growth, flowering, senescence and regrowth into normal flowering plants, indicating a dominant effect of the Z. diploperennis alleles. The regrowability (i.e., the plants’ ability to regrow after senescence) was stably transmitted to progeny of the hybrids. Segregation ratios of regrowth in the F2 generations are consistent with the trait controlled by two dominant, complementary loci, but do not exclude the influence of other modifiers or environment. Genome-wide screening with genotyping-by-sequencing technology indicated two major regrowth loci, regrowth 1 (reg1) and regrowth 2 (reg2), were on chromosomes 2 and 7, respectively. These findings lay the foundation for further exploration of the molecular mechanism of regrowth in Z. diploperennis.

Importantly, our data indicate that there is no major barrier to transferring this trait into maize or other grass crops for perennial crop development with proper technology, which enhances sustainability of grain crop production in an environmentally friendly way.

Perennialism is the phenomenon that a plant can live for more than two years; the ability of doing so is termed perenniality. Plants typically have a life cycle of growth, reproduction (sexual and/or vegetative) and senescence. Annuals and biennials have only one such cycle in their life, leaving behind seeds, bulbs, tubers, etc. to initiate another life cycle. Perennials maintain juvenile meristematic tissues capable of regrowth after senescence to start a new life cycle on the same body. How perennials do so remains as a mystery. Subterranean stems (such as rhizomes), polycarpy and tuberous roots are often cited as the means by which plants achieve perenniality. However, none of these traits is absolutely required by perennials. For instance, bamboos are essentially monocarpic perennial that regrow from rhizomes. Many perennial temperate grasses, such as switchgrass (Haferkamp and Copeland 1984), cordgrass (Boe et al. 2009) and eastern gamagrass (Jackson and Dewald 1994), regrow from the crowns instead of rhizomes. On the other hand, some annual/biennial plants, such as radish (Raphanus sativus), grow tuberous roots.

Although perennialism is common among higher plants, the study of its genetics and molecular biology is sporadic. So far, the only published research in molecular mechanism of plant perennialism was conducted in Arabidopsis. Melzer et al. (2008) successfully mutated this annual herb to show some perennial habits, such as increased woody fiber in the stem, by down-regulating two flowering genes coding for MADS-box proteins, SUPPRESSOR OF OVEREXPRESSION OF CONSTANT 1 and FRUITFUL. Unfortunately, this woody mutant was sterile, and no follow-up research was reported. Perennial-related genes and quantitative loci (QTL) have been reported in other species. Major QTL controlling rhizome development, regrowth and tiller number have been mapped on sorghum linkage groups C (chromosome 1) and D (chromosome 4) (Paterson et al. 1995; Hu et al. 2003), which are homoeologous to regions of maize chromosomes 1, 4, 5 and 9, respectively (Wei et al. 2007). Hu at al. (2003) mapped two dominant, complementary QTL Rhz2 (Rhizomatousness 2) and Rhz3 that control rhizome production on rice (Oryza sativa) chromosomes 3 and
4 at the loci homeologous to the sorghum (Sorghum bicolor) QTL. Tuberous roots in a wild perennial mung bean (Vigna radiate ssp. sublobata) are conditioned by two dominant, complementary genes (Nguyen et al. 2012). However, after years of effort, these perennialism-related genes have yet to be cloned from any of the species despite that mapping data and complete genomic sequences of rice and sorghum are readily available. Therefore, no further research has been reported about these perennialism-related loci/genes.

Recently, Ryder et al. (2018) reported a set of 98 expressed contigs in Johnsongrass (Sorghum halepense) that are likely associated with rhizome development.

In the genus Zea L., most species, including maize (Z. mays ssp. mays), are annual. However, two closely related species, tetraploid Z. perennis [Hitchc.] Reeves and Mangelsdorf and diploid Z. diploperennis Ilits, Doebley & R. Guzman, are perennial. Perennialism appears to be a complex trait, strongly influenced by genetic and environmental factors. A perennial plant in one environment usually cannot survive in another due to the lack of the required adaptability. For example, Z. diploperennis, which is perennial in the highlands of Mexico, cannot survive the harsh winter in the American Midwest. The various criteria for what constitutes perennialism in Zea may have contributed to contradictory observations. Traits such as rhizome formation, evergreen stalks, and dormancy are important adaptive features that support the viability of various perennial plants in various environments. In this study, we take a reductionist approach and specifically focus on a plant’s regrowability (i.e., the ability to maintain some juvenile meristematic tissues after each life cycle that can initiate a new life cycle). Although this trait by itself is insufficient for functional perenniality, it appears to be an essential component of perennialism in Zea L. Here we report the results of our genetic analysis and genome-wide screening of the regrowth trait with genotyping-by-sequencing (GBS) technology.

MATERIALS AND METHODS

Plant materials and phenotyping

Zea diploperennis (PI 462368; Zd, hereafter in a cross combination) and Z. mays cv. Rhee Flint (PI 213764; RF, hereafter in a cross combination) were obtained from the USDA North Central Region Plant Introduction Station, Ames, IA. Maize B73 and Mo17 inbred lines were from the collection of Dr. Auger and are traceable back to the Maize Genetics Cooperation Stock Center, Urbana/Champaign, IL. Rhee Flint is small, fast-growing heirloom maize variety and usually has tillers, which affords serial plantings with an increased opportunity of a plant simultaneously flowering with Z. diploperennis. In our designations of F1s and their derivatives, the female parental is shown first. All the plants used in this study were grown in the greenhouse during the winter and

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in the field during the summer in Brookings, SD. Controlled pollinations were done by covering tassels and ears with paper bags before and after the pollination was made. In the greenhouse, plants were maintained with a 16 h-light/8 h-dark cycle and 20/16°C day/night temperature except that two-month old *Z. diploperennis* and its hybrid plants were treated with a 10 h light/14 h dark cycle for four weeks to induce the floral transition.

Plants were scored as regrowth if they produced shoots from the basal axillary buds after the original stalks finished flowering and senesced. Rhizome and tuber development were visually investigated on plants that were dug from the soil after senescence. Tiller number at tasseling was done using GoTaq Green Master Mix (Catalog# M7505, Promega, Madison, WI) at the following conditions: 95°C, 55°C, 72°C, 95°C, 55°C, 72°C, 95°C, 55°C, 72°C, 95°C, 55°C, 72°C, 95°C, 55°C, 72°C, 95°C, 55°C, 72°C, 95°C, 55°C, 72°C, 95°C, 55°C, 3 cycles of 95°C for 45 s, 55°C for 1 min and 72°C for 1 min. The annealing temperatures were determined using a touchdown PCR step starting from 65°C.

**Pcr assay**

DNA samples were isolated from young leaves using the CTAB procedure (Doyle and Doyle 1990) and used for PCR-based marker assay. Table 1 lists all the PCR primers used in this study. PCR assays were done using GoTaq Green Master Mix (Catalog# M7505, Promega, Madison, WI) at the following conditions: 95°C, 35 cycles of 95°C for 45 s, 55°C–62°C (primer dependent) for 1 min and 72°C for 1 min, and 72°C for 10 min. The annealing temperatures were determined using a 1-touchdown PCR step starting from 65°C.

**SNP discovery and locus mapping**

The GBS assay was conducted according to Elshire et al. (2011). The preparation and sequencing of the library were conducted by the University of Wisconsin Biotechnology Center (UW BRC). Generally, DNA samples were digested with *ApeK*I restriction enzyme (RE), and unique barcodes were annealed to each DNA fragments. A single-end 100 bp (1x100bp) sequencing run was carried out on an Illumina HiSeq 2500 platform. The raw data were pooled as a single fastq file and downloaded from UW BRC.

The TASSEL (Trait Analysis by Association, Evolution and Linkage) 3 pipeline was used under the guidance of TASSEL manual (Glaubitz et al. 2014) for the discovery of SNPs between *Z. diploperennis* and *Z. mays*. The barcoded sequence reads were collapsed into a set of unique sequence tags with counts. The tag count files were filtered for a minimum count threshold and merged into the master tag count file. The *B73* RefGen V4 reference genome sequence was downloaded from MaizeGDB and processed with Bowtie2 for alignment (Langmead and Salzberg 2012). Master tags were aligned to the *B73* reference genome to generate a “Tags On Physical Map” (TOPM) file, which contains the genomic position of each tag with the best unique alignment. The occupancies of tags for each taxon were observed from barcodes information in the original FASTQ files. Tag counts were stored in a “Tags by Taxa” (TBT) file. The TOPM and TBT files were used to call SNPs at the tag locations on the genome. The SNPs were first subjected to a two-step filtration in TASSEL 3 with minimum tag counts of 5, genotype mismatch rate of 0.1, minimum taxon coverage of 0.01, minimum site coverage of 0.2 and minimum minor allele frequency of 0.01. Fastq files containing sequences of chromosomes 1 to 10 were merged by FASTX_Toolkit and indexed. All commands for SNP discovery were executed in Ubuntu 16.04 LTS platform. Steps and codes used in TASSEL pipeline including example command lines and brief descriptions are listed in Supplementary Table S1.

The SNPs resulted from the first filtration were filtered again by manually removing sites that had missing data in more than 20% of the *B73*-Zd *F*₂ plants. For those SNPs that have missing data in less than 20% of the *B73*-Zd *F*₂ plants, the missing data were imputed by treating them as heterozygote since both alleles can be embodied and considered to be moderate. The SNPs from the 2nd filtration were used for QTL mapping.

To understand the relationship between the mapped QTL with the genetic factors revealed by the genetic analysis, the SNPs from the 3rd filtration were further filtered (the 3rd filtration) with χ² (P < 0.05). This is based on our hypothesis that, if a SNP is associated with a regrowth locus, the teosinte allele of the SNP should be carried by all the regrowable *F*_₃₅ but only by those threes non-regrowable *F*_₃₅. Therefore, for each regrowth-associated SNP, we expected, in the regrowable subpopulation, 33.3% plants to carry the homoygous Zd alleles (AA), 66.6% to have the Zd-B73 heterozygous allele combination (AB) and none to be with the B73 homoygous alleles (BB) and, in the non-regrowable subpopulation, 14% of the plants with AA, 28.6% with AB and 57.1% with BB. Altogether, a 2 χ² contingency table were generated with expected χ² 0.05. 4 = 9.49. Any SNPs with χ² < 9.49 were kept fitting the 9:7 segregation model.

The 4th SNP filtration was performed by collapsing immediately neighboring SNPs, up to a 100-bp range, that share the same haplotypes into one cluster and using the first SNP to represent the cluster. In the locus analysis with the chi-square imputation, such a cluster of SNPs was treated as one locus. Removing the redundant SNPs makes the locus analysis more precise because repeated SNP sites would affect calculating LOD (logarithm of the odds) scores and influence interval estimation.

The SNPs after the 2nd and the 4th filtrations were used for candidate locus/QTL estimation, respectively. The locus analysis was executed by a standard QTL procedure in R using the R/qtl package (version 1.40-8) (Broman et al. 2003) to better observe the contribution of each SNP and its neighbors. Position simulation was drawn with a maximum distance of 1.0 cm and an error probability of 1x10⁻⁴. The conditional genotype probability (calc.genopro), as well as simulated genotypes (sim.geno with n.draw = 32), were calculated. The “haldane” function was used to convert genetic distances into recombination fractions. Genome scan with a single locus model (scanone) was performed with a binary model using the expectation-maximization algorithm (Broman et al. 2003). A permutation test with 1000 replicates was performed in scanone to visualize the LOD thresholds. We determined a locus interval by selecting the first and last SNP sites with significant LOD value. Genes within the intervals were identified by searching the corresponding region on the Gramene website. The R codes used for candidate locus/QTL analyses in this study are listed in Supplementary Table S2.

### Table 1 PCR primers used in this study

| Primers           | Sequences               |
|-------------------|-------------------------|
| tb1MF             | 5’-AGTAGGCCATAGTACGTAC-3’ |
| tb1MR             | 5’-CTCTTTTACCGACCCCTACA-3’ |
| tb1Z2              | 5’-GTAATGCGAATGAGTACGTCA-3’ |
| tga1CF             | 5’-TGCTGCAAAAGATTACTGAT-3’ |
| tga1CR             | 5’-GTGGCCCTGTGATGAG-3’ |
| mmc0381F          | 5’-GCGACACGAGCCCAGG-3’ |
| mmc0381R          | 5’-GACCCGATGACCGAAG-3’ |
| gt1-ZF             | 5’-TGTAGCGGTGGTCCGATGACT-3’ |
| gt1-ZR             | 5’-TGTAGCGGTGGTCCGATGACT-3’ |
| gt1-MF             | 5’-GAAGCGAGCTGCTGAGATGAG-3’ |
| gt1-MR             | 5’-TGACTGTTGTTAGCGGTACT-3’ |

### Statistical analyses

For statistical analyses, all genotypes and phenotypes were transformed into numeric values. For phenotypes, the regrowth plants were scored as...
“1” and the non-regrowth plants were scored as “2”. For genotypes, the plants that were homozygous to the Z. diploperennis allele were scored as “1”; those that were homozygous to the B73 or Rhee Flint allele were scored as “2”; and those that were heterozygous were scored as “3”. When conducting locus analysis, genotype “1” was transformed to “AA”, “2” to “BB” and “3” to “AB”.

A chi square goodness-of-fit test was used to find the best-fit model or linkage in the genetic analysis and reveal candidate loci on chromosomes. To determine if TNT has any correlation with regrowth, a One-Way ANOVA of TNT by regrowth was performed in JMP (JMP 11.2.0).

**RESULTS AND DISCUSSION**

**The production and growth of the hybrids**

We made reciprocal crosses of Z. diploperennis with the following three maize lines: B73, Mo17 and Rhee Flint. The first F1 was made with Rhee Flint in a greenhouse. Rhee Flint is small, fast-growing and usually has tillers, which affords serial plantings with an increased opportunity for one or more regrowth genes, that the experimental environments were unfavorable for regrowth to happen, or that some plants needed more time to break up their dormancy. Shaver (1967) and Camara-Hernandez and Mangelsdorf (1981) observed that some of their F1 plants eventually regrew from basal axillary buds after a period of dormancy. Indeed, some of our F2 plants need about two months of dormancy before regrowth. This observation reinforces the view that even regrowth is a complex trait that is modified by genetics and environment.

TNT has been associated with perennialism in several studies (Camara-Hernandez and Mangelsdorf 1981; Doebley et al. 1997; Shaver 1964, 1967; Mangelsdorf et al. 1981; Camara-Hernandez and Mangelsdorf 1981) and we have not observed rhizomes in any of our F1 or the derived plants. When regrowth occurs, it is always from an axillary bud. Indeed, it is also our observation that the regrowth of Z. diploperennis is mainly from basal axillary buds, and only occasionally from rhizomes. The Z. perennis - 4X maize F1s made by R. A. Shaver also were all “weakly perennial” under the environmental conditions with few or no rhizomes (Emerson and Beadle 1930).

Other possible explanations for contrasting results are that the perennial teosinte plants used in those studies were polymorphic for one or more regrowth genes, that the experimental environments were unfavorable for regrowth to happen, or that some plants needed more time to break up their dormancy. Shaver (1967) and Camara-Hernandez and Mangelsdorf (1981) observed that some of their F1 plants eventually regrew from basal axillary buds after a period of dormancy. Indeed, some of our F2 plants need about two months of dormancy before regrowth. This observation reinforces the view that even regrowth is a complex trait that is modified by genetics and environment.

TNT has been associated with perennialism in several studies (Camara-Hernandez and Mangelsdorf 1981; Doebley et al. 1997; Shaver 1964, 1967; Westerbergh and Doebley 2004), therefore we investigated the relationship of TNT with regrowth in the Zd-RF F2s. One-way ANOVA of TNT by regrowth, however, revealed no significant difference of TNT (F = 0.897, P = 0.353) between the regrowth and the non-regrowth F2s. Indeed, we observed regrowth from several single-stalked hybrid derivatives (Figure 4A) and non-regrowth of some multi-stalked plants (Figure 4B). These results suggest that TNT is not essential to regrowth in Zea.

**The genetics of regrowth**

All our F1 plants regrew and underwent several life cycles alternating between the greenhouse and the field. This indicates that, with our materials and in our environment, regrowth is a dominant trait. Regrowable F1 hybrids of maize with perennial teosintes were previously obtained by Emerson (Emerson and Beadle 1930), Shaver (1964), Galinat (1981b), Camara-Hernandez and Mangelsdorf (1981) and Ting and Yu (1982). Srinivasan and Brewbaker (1999) suggested cytoplasm may contribute to perennialism, but our reciprocal F1s performed without difference from one another.

To analyze the genetics of regrowth further, 134 B73-Zd F2s (derived from several F1 plants where B73 was the female) and 159 Zd-RF F2s (derived from a single F1 plant where Zd was the female) were tested. Among the 134 B73-Zd F2s, 81 regrew and 53 did not (Table 2). Similarly, among the 159 Zd-RF F2s, 90 regrew after senescence and 69 did not (Table 3). One B73-Zd F3 population (Supplementary Table S4) and three Zd-RF F3 populations (Table 3), each of which was derived from a single regrowth F1 plant, were also evaluated for their regrowth.
A chi square ($\chi^2$) goodness-of-fit test suggests that both of the F2 populations and one Zd-RF F3 population (Zd-RF F3-5) we tested best fit a 9:7 regrowth to non-regrowth ratio (Table 4), and the B73-Zd F1 population and the remaining two Zd-RF F3 populations best fit a 3:1 ratio (Table 4). The simplest model that explains these results is that regrowth in the F1,8 and their derivatives is mainly controlled by two dominant, complementary regrowth (reg) loci. The two dominant, complementary gene model parallels what has been found in other species, such as rice (Hu et al. 2003), Johnsongrass (Paterson et al. 1995; Hu et al. 2003; Washburn et al. 2013), basin wildrye (Leymus cinereus) (Yun et al. 2014) and wild mung bean (Nguyen et al. 2012), for perennialism-related traits.

The Zd-RF F1 was also backcrossed to each parental line. All plants from the F1-to-Zd backcross regrew, showing dominant effect of the Zd alleles. However, only one of the 20 plants from the F1-to-RF backcross showed regrowth. Segregation of genomic fragments of Z. diploperennis in maize backcross derivatives is known to be highly distorted (Wang et al. 2012). Therefore, this observed 1:19 ratio may be due to distorted segregation of Z. diploperennis genomic fragment(s) carrying the allele(s) of one or both reg genes in the RF backcross derivative. Alternatively, other genetic models, such as one or three dominant complementary genes, two major genes with a few minor modifiers, or that regrowth is a complex trait controlled by many QTL, are not eliminated by this genetic analysis, but are less probable. Nevertheless, this could add difficulty to the effort of transferring the Z. diploperennis alleles to maize.
The number of regrowth plants observed in any generation might be understated, because some plants initially recorded as non-regrowth eventually regrew after about two months of dormancy. Therefore, some plants recorded as non-regrowth and discarded to open up greenhouse space may have possessed the ability to regrow. Furthermore, transplanting from the field to the greenhouse and vice versa is very stressful so that some regrowable plants may have been killed this way.

The F₂ and F₃ plants afforded an opportunity to investigate whether some factors previously implicated in perennialism may contribute to the regrowth trait. The rice rhizomatousness gene Rhz2 has been mapped to rice chromosomes 3 (Hu et al. 2003) and sorghum chromosome 1 (Paterson et al. 1995; Hu et al. 2003; Washburn et al. 2013), which are both homeologous to parts of maize chromosome 1 (Wei et al. 2007). Additionally, gt1 and id1, which have been implicated with perenniality in Zea (Shaver 1967), and tb1, which controls

Figure 3 Photos of the ears produced from a Zea mays cv Rhee Flint-Z. diploperennis F₁ plant in different seasons (the upper panel) and from F₂ in summer 2014 in greenhouse (the lower panel).

The F₂ and F₃ plants afforded an opportunity to investigate whether some factors previously implicated in perennialism may contribute to the regrowth trait. The rice rhizomatousness gene Rhz2 has been mapped to rice chromosomes 3 (Hu et al. 2003) and sorghum chromosome 1 (Paterson et al. 1995; Hu et al. 2003; Washburn et al. 2013), which are both homeologous to parts of maize chromosome 1 (Wei et al. 2007). Additionally, gt1 and id1, which have been implicated with perenniality in Zea (Shaver 1967), and tb1, which controls

Figure 4 Photos of Zea mays Mo17-Z. diploperennis F₂ plants, showing regrowth from the basal node of a single-stalked plant (A) or non-regrowth from a multi-stalked plant (B).
| Plant | PT | gtf | tbf | idf | Plant | PT | gtf | tbf | idf |
|-------|----|-----|-----|-----|-------|----|-----|-----|-----|
| Zea   | R  | 1   | 1   | 1   | BZ2-006-9 | R  | 1   | 1   | 2   |
| B73   | NR | 2   | 2   | 2   | BZ2-006-10 | R  | 1   | 3   | 2   |
| BZ2-001-1 | R | 1   | 3   | 2   |
| BZ2-001-2 | R  | 3   | 3   | 2   |
| BZ2-001-3 | R  | 1   | 3   | 2   |
| BZ2-001-4 | R  | 1   | 3   | 2   |
| BZ2-001-5 | R  | 1   | 2   | 2   |
| BZ2-002-1 | NR | 1   | 3   | 2   |
| BZ2-002-2 | NR | 1   | 2   | 2   |
| BZ2-002-3 | R  | 3   | 3   | 2   |
| BZ2-002-4 | R  | 3   | 2   | 1   |
| BZ2-002-5 | NR | 3   | 2   | 2   |
| BZ2-002-6 | NR | 3   | 2   | 2   |
| BZ2-002-7 | NR | 2   | 2   | 2   |
| BZ2-002-8 | R  | 1   | 1   | 2   |
| BZ2-002-9 | R  | 3   | 2   | 2   |
| BZ2-002-10 | R | 3   | 3   | 2   |
| BZ2-002-11 | NR | 1   | 2   | 2   |
| BZ2-002-12 | R  | 1   | 2   | 2   |
| BZ2-002-13 | R  | 3   | 3   | 2   |
| BZ2-002-14 | NR | 1   | 1   | 2   |
| BZ2-002-15 | R  | 1   | 3   | 2   |
| BZ2-002-16 | NR | 3   | 3   | 1   |
| BZ2-002-17 | NR | 3   | 2   | 2   |
| BZ2-002-18 | R  | 1   | 2   | 2   |
| BZ2-002-19 | R  | 1   | 2   | 2   |
| BZ2-002-20 | R  | 2   | 2   | 2   |
| BZ2-002-21 | R  | 1   | 2   | 2   |
| BZ2-002-22 | R  | 3   | 2   | 2   |
| BZ2-002-23 | R  | 3   | 2   | 1   |
| BZ2-002-24 | R  | 3   | 2   | 2   |
| BZ2-002-25 | NR | 1   | 2   | 2   |
| BZ2-004-1 | R  | 3   | 1   | 2   |
| BZ2-004-2 | R  | 1   | 3   | 2   |
| BZ2-004-3 | NR | 3   | 2   | 2   |
| BZ2-004-4 | R  | 1   | 3   | 2   |
| BZ2-004-5 | R  | 3   | 2   | 2   |
| BZ2-004-6 | R  | 3   | 1   | 2   |
| BZ2-006-1 | NR | 3   | 1   | 2   |
| BZ2-006-2 | NR | 1   | 1   | 2   |
| BZ2-006-3 | R  | 3   | 2   | 2   |
| BZ2-006-4 | R  | 3   | 2   | 2   |
| BZ2-006-5 | R  | 3   | 3   | 2   |
| BZ2-006-6 | NR | 3   | 3   | 2   |
| BZ2-006-7 | R  | 3   | 1   | 2   |
| BZ2-006-8 | R  | 3   | 1   | 2   |

**Bold:** used for SNP calling in GBS; 1: homozygous Zd allele; 2: homozygous B73 allele; 3: heterozygous; -: missing data; R: regrowth; NR: non-regrowth.
gt1 (Whipple et al. 2011), are all on chromosome 1 in Zea (Colasanti et al. 1998; Whipple et al. 2011). Therefore, we investigated the allele compositions of these three genes in the B73-Zd F2s (Table 2), and 26 Zd-RF F2 plants and the three Zd-RF F3 populations (Supplementary Table S5), and assayed their association with regrowth. Of the 134 regrowth hybrid derivatives we examined, 5, 33 and 115 were homozygous for the maize gt1, tb1 or id1 alleles, respectively (Table 2 and Supplementary Table S5). Zd-RF F2 family Zd-RF F3-5 is homozygous for the gt1 allele of Z. diploperennis (Supplementary Table S5) but segregates approximately 9:7 for regrowth and non-regrowth (Table 3). Therefore, our results are inconsistent with the model of Shaver (1967), and show that gt1 and id1 do not control regrowth in our F1s and their derivatives. Z. diploperennis’s gt1 allele may be helpful to regrowth because the majority of the plants that regrew had at least one copy, but it is not indispensable because some plants regrew without it.
Table 4 Results of the $\chi^2$ goodness-of-fit tests of three simple genetic models

| Generations   | Total | Observed | No. dominant genes (the expected R to NR ratio) and $P(\chi^2)^*$ |
|---------------|-------|----------|------------------------------------------------------------------|
|               |       |          | 1 (3:1) | 2 (9:7) | 3 (27:37) |
| B73-Zd F$_2$  | 134   | 81       | 53      | 0.0001  | 0.2964    | 0.0001    |
| B73-Zd F$_3$  | 72    | 52       | 20      | 0.5862  | 0.0063    | 0.0001    |
| Zd-RF F$_2$   | 160   | 12       | 968     | 0.0001  | 0.7499    | 0.0001    |
| Zd-RF F$_3$-3 | 15    | 12       | 3       | 0.6547  | 0.0639    | 0.3000    |
| Zd-RF F$_3$-5 | 16    | 9        | 7       | 0.0833  | 1.0000    | 0.2547    |
| Zd-RF F$_3$-9 | 16    | 13       | 3       | 0.5637  | 0.0438    | 0.0016    |

*The best fit models are in bold.

Interestingly, we observed no heterozygosity for $id1$ and very low heterozygosity for $tib$ in all the hybrid derivatives that were examined, regardless of regrowth (Tables 2; Supplementary Table S3). Of 134 B73-Zd F$_2$ plants, only 16 had the $Z.\ diploperennis$ $id1$ allele (Table 2). Similar phenomena were observed in the derivatives of the Zd-RF cross (Supplementary Table S5). It seems that the maize chromosome fragment that carries $id1$ was preferentially transmitted to the hybrid derivatives. Excess homozygosity of the maize $id1$ allele indicates some sort of selection. It could be that a deficiency or other rearrangement adjacent to the teosinte $id1$ allele causes it not to transmit efficiently, or it could be that the teosinte $id1$ or tightly linked allele(s) cause the plant to grow poorly or be sterile in our experimental conditions.

Identifying regrowth loci with genotyping by-sequencing assay

A genome-wide mining of single nucleotide polymorphisms (SNPs) was conducted in a randomly selected sub-population of 94 (55 regrowth and 39 non-regrowth) B73-Zd F$_2$ plants with GBS technology (Table 2). We conducted the GBS assays to identify QTL for the regrowth trait. To prepare for these assays, a total of 2,204,834 (85.14%) Illumina sequencing tags that passed routine quality control filtrations were aligned with the B73_v4 reference genome. A total of 714,158 SNPs, covering all ten chromosomes with a total of 37,925 and 10,432 SNPs on chromosome 1 and chromosome 2, respectively (Table 2). Similar phenomena were observed in the derivatives of the Zd-RF cross (Supplementary Table S5). It seems that the maize chromosome fragment that carries $id1$ was preferentially transmitted to the hybrid derivatives. Excess homozygosity of the maize $id1$ allele indicates some sort of selection. It could be that a deficiency or other rearrangement adjacent to the teosinte $id1$ allele causes it not to transmit efficiently, or it could be that the teosinte $id1$ or tightly linked allele(s) cause the plant to grow poorly or be sterile in our experimental conditions.

A permutation test of 1,000 with the $p$-value of 0.05 resulted in a significant LOD score of 5.23 (Figure 5A). This significance threshold revealed two major QTL with one at 33,041,409 bp (the nucleotide position in the B73_v4 reference genome sequence) on chromosome 2 with a LOD score of 5.46 and one at 4,284,633 bp on chromosome 7 with a LOD score of 5.53.

To test if the two strongest QTL correspond to the two dominant and complementary loci suggested by the genetic analysis, we applied a $\chi^2$ test with 9:7 allele segregation ratio model to the 10,431 SNPs to investigate if the observed and the expected genotypes are significantly different ($P \leq 0.05$). This step kept 946 SNPs (Table 5, 3rd filtration). Finally, to simplify the mapping effort, the 946 SNPs were filtered once more by collapsing immediately neighboring SNPs that share the same haplotypes into one cluster. This step resulted in 597 SNP cluster (Table 5, 4th filtration). Locus mapping with a threshold of LOD$_{95\%} = 4.17$ revealed two significant loci with one on chromosome 2 in the interval from 24,244,192 bp to 28,975,747 bp with the peak at 27,934,739 bp and another on chromosome 7 in the interval from 2,862,253 bp to 6,681,861 bp with the peak at 5,060,739 bp (Figure 5B). These two loci were mapped closely to the two major QTL indicated by the mapping without imputation. These results are consistent with the two-factor model, which warrants further investigation. To that end we are naming the factor underlying the chromosome 2 QTL regrowth 1 ($reg1$) and the factor underlying the chromosome 7 QTL as regrowth 2 ($reg2$).

Identifying regrowth loci with genotyping by-sequencing assay

The result is shown in Figure 5A. A total of 126 SNPs (104 real sites and 22 simulated sites) showed LOD scores higher than 3.00.

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Our LOD analysis located two minor peaks on chromosome 1 that are associated with regrowth (Figure 5B). We wanted to know if these two loci are related to $gt1$ and $id1$. The SNPs at the peak of these loci are at 82,273,951 bp and 177,235,112 bp, far away from $id1$ (around 243,201,405 bp) and $gt1$ (around 23,625,801 bp), respectively (Figure 6). These observations further indicate that $id1$ and $gt1$ are not related to regrowth. Also, previous studies reported that $Z.\ diploperennis$ carried perennialism-related $Pe$-d (Mary Eubanks, personal communication) and an evergreen gene on chromosome 4 (Whipple et al. 2011). However, our data could not support these observations since no SNP on chromosome 4 is significantly associated with regrowth (Figures 5 and 6).

Table 5 Numbers of SNPs revealed in each chromosome of the B73-ZD F$_2$ population after each filtering step

| Chr | Raw SNP number | 1st filter | 2nd filter | 3rd filter | 4th filter |
|-----|----------------|------------|------------|------------|------------|
| 1   | 109,543        | 5,751      | 1,628      | 82         | 51         |
| 2   | 85,283         | 4,966      | 1,476      | 120        | 77         |
| 3   | 81,625         | 4,708      | 1,200      | 120        | 75         |
| 4   | 75,832         | 3,376      | 942        | 112        | 82         |
| 5   | 77,314         | 4,409      | 1,197      | 198        | 111        |
| 6   | 58,195         | 2,938      | 761        | 87         | 49         |
| 7   | 62,280         | 3,108      | 877        | 144        | 98         |
| 8   | 57,748         | 3,210      | 877        | 20         | 16         |
| 9   | 57,231         | 2,982      | 741        | 29         | 16         |
| 10  | 49,107         | 2,477      | 732        | 34         | 22         |
| Total| 714,158        | 37,925     | 10,431     | 946        | 597        |
In summary, the results presented here indicate that regrowth in Zea is inherited dominantly in our experimental conditions. Both the genetic and GBS analyses support a model where the regrowth trait is mainly controlled by two major regrowth loci, reg1 and reg2 on chromosomes 2 and 7, respectively. Even so, the data do not eliminate more complex models. Identification and functional study of the candidate genes for reg1 and reg2 and their possible modifiers will initiate an understanding about the molecular mechanism of perenniality in Zea. We recognize that adaptability is very important for a plant to realize perennialism in a certain environment. However, this issue can be addressed separately after we understand molecularly how Z. diploperennis regrows.

Figure 5 Graphics showing LOD scores of the QTL mapping the Zea mays B73-Z. diploperennis F2 population without (A) or with (B) chi-square imputation. The 95% threshold lines (the parallel red dash lines) were calculated with 1,000 permutations. Significant QTL/loci are indicated by the location of the peak SNPs of the loci.

Figure 6 Genetic map of 30 representing SNPs and genes gt1, id1, and tb1. Each SNP represents a one-Mbp region except of SNP S2_ 27934739, which represent a SNP cluster.
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