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MADS-box encoding gene *Tunicate1* positively controls maize yield by increasing leaf number above the ear

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The leaves above the ear serve as a major source of carbohydrates for grain filling in maize. However, increasing the number of leaves above the ear to strengthen the source and improve maize yield remains challenging in modern maize breeding. Here, we clone the causative gene of the quantitative trait locus (QTL) associated with the number of leaves above the ear. The causative gene is the previously reported MADS-box domain-encoding gene *Tunicate1* (*Tu1*), which is responsible for the phenotype of pod corn or *Tunicate* maize. We show that *Tu1* can substantially increase the leaf number above the ear while maintaining the source–sink balance. A distal upstream 5-base pair (bp) insertion of *Tu1* originating from a popcorn landrace enhances its transcription, coregulates its plastochron activators and repressors, and increases the number of leaves above the ear. Field tests demonstrate that the 5-bp insertion of *Tu1* can increase grain yields by 11.4% and 9.5% under regular and dense planting conditions, respectively. The discovery of this favorable *Tu1* allele from landraces suggests that landraces represent a valuable resource for high-yield breeding of maize.

The optimization of crop yield from limited land areas is crucial in addressing the growing global demand for food[1]. Enhancing the photosynthetic capacity (source) is an effective measure to improve yield per unit area. The photosynthetic capacity of maize is closely linked to the number of leaves and stage of plant development. Leaves located above the main ear are relatively younger and more metabolically active compared to those below the ear, which are susceptible to shading and senescence[2,3]. Additionally, the leaves above the ear primarily contribute to the provision of carbohydrates for grain filling of the ear, whereas the leaves below the ear primarily sustain the stem and roots[4]. Therefore, the number of leaves above the ear in maize is an important factor in determining the source strength, source–sink balance and overall yield of the plant. A proper increase in the number of leaves above the ear can increase the source strength, leading to improved photosynthetic capacity and greater accumulation of assimilates in the ear. This, in turn, leads to a larger ear size and increased grain yield.

Maize has undergone significant changes in source strength depending on the number of leaves above the ear during the course of its domestication and improvement (Fig. 1a). Teosinte generally produces ears on most leaf axils. Compared with their wild ancestor, maize landraces typically present a more limited number of leaves above the ear. Conversely, maize inbred lines are often characterized by more leaves situated above the ear. Hence, maize inbred lines typically possess a stronger source than teosinte and landraces. However, the genetic basis underlying this reshaping of leaf number above the ear to increase the source strength during maize domestication and improvement remains poorly understood.

The shoot apical meristem (SAM) initiates early during embryogenesis to form a series of leaves through sequential initiation from

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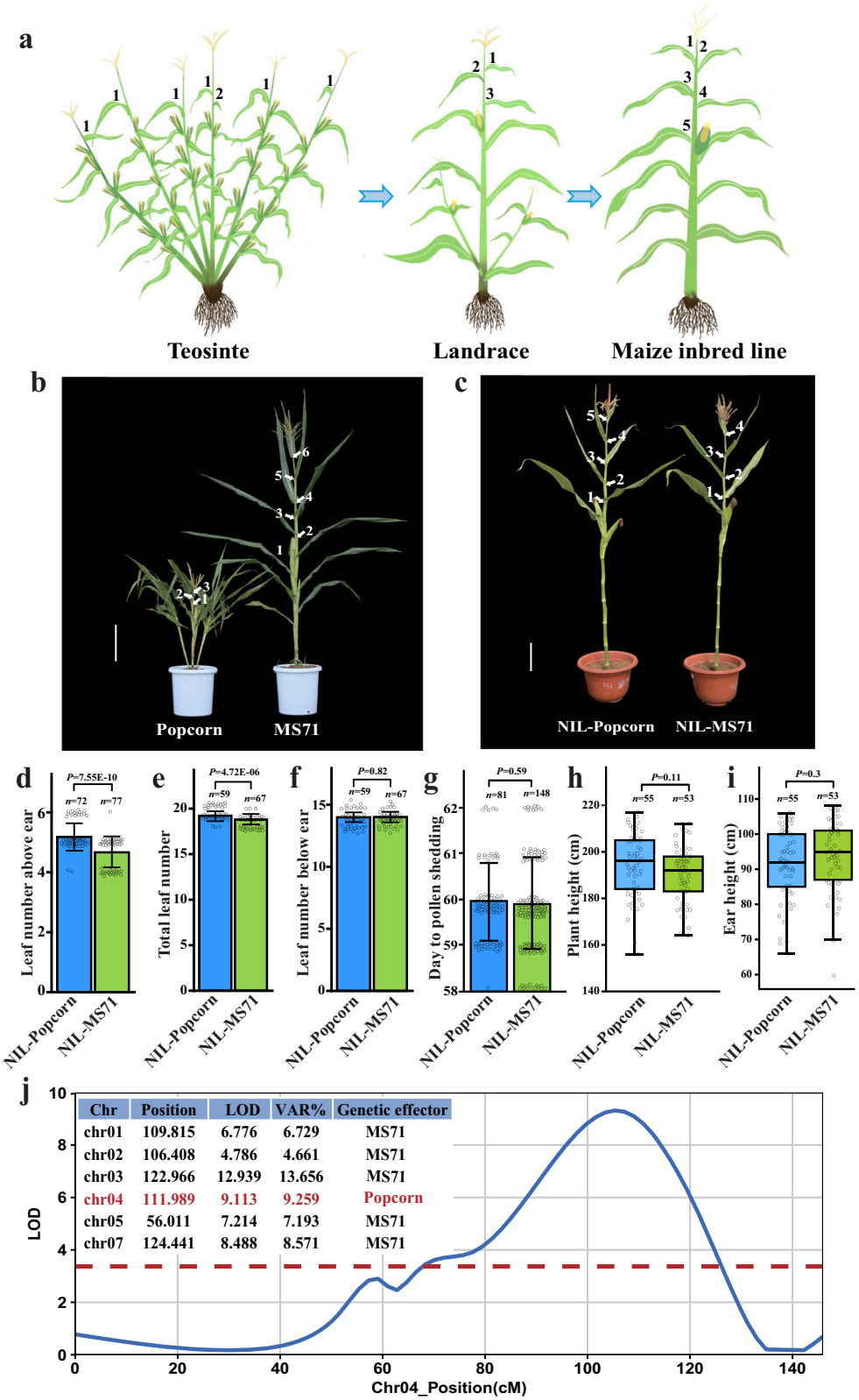

Chr	Position	LOD	VAR%	Genetic effector
chr01	109.815	6.776	6.729	MS71
chr02	106.408	4.786	4.661	MS71
chr03	122.966	12.939	13.656	MS71
chr04	111.989	9.113	9.259	Popcorn
chr05	56.011	7.214	7.193	MS71
chr07	124.441	8.488	8.571	MS71

the leaf primordia until floral transition occurs[5,6]. The number of leaves produced is determined by both the onset or rate of leaf initiation (plastochron) and the duration of the vegetative phase, which is controlled by the timing of floral transition[7]. A number of genes involved in these processes have been identified. For example, *paused* (*psd*) mutants in *Arabidopsis* exhibit a delay in leaf initiation, leading to fewer leaves than those of the wild type[8]. Conversely, the *Knotted1* (*Kn1*) gene suppresses leaf initiation and preserves meristem identity in maize[9]. The *PLASTOCHRON1* (*PLA1*)[10,11], *PLA2*[12], and *PLA3*[13] genes in rice, *TERMINAL EAR1* (*TE1*)[14], *WEE1*[15] and *VIVIPAROUS8*[16] in maize, *ALTERED MERISTEM PROGRAM1* (*AMP1*)[17] in *Arabidopsis*, and *MANY-NODED DWARF* genes in barley regulate the plastochron, and loss of function leads to an increase in leaf number[18–21]. The SBP-box gene *unbranched 2* (*ub2*) regulates the rate of leaf initiation, and loss of function results in

Fig. 1 | Phenotypes of maize *Lna1*. **a** Leaves located above the ear provide the main source of carbohydrates for grain filling in maize. The number of leaves above the ear increased during maize domestication, diversification, and improvement. Teosinte typically grows only one to two leaves above the ear, whereas maize landraces have two to three leaves, and maize inbred lines often have five to six leaves above the ear. The maize source was greatly increased during maize domestication and improvement. **b** Leaf number above the primary ear in Popcorn (left) and MS71 (right). Popcorn generally has 3–4 leaves above the ear, whereas MS71 often has 5–6 leaves above the ear. **c** Leaf number above the ear in NIL-Popcorn (left) and NIL-MS71 (right). Scale bar, 30 cm. Comparisons of the leaf number above the ear (**d**), total leaf number (**e**), leaf number below the ear (**f**), flowering time (**g**), plant height (**h**) and ear height (**i**) between NIL-Popcorn and NIL-MS71. **j** QTL mapping identified 6 QTLs for leaf number above the ear in an F_2 population derived from a cross between the maize landrace, Popcorn, and the maize inbred line MS71. Among these QTLs, only a major one, *Lna1*, on chromosome 4, harbors a genetic effect from the Popcorn allele that is greater than that of MS71. The arrows represent leaves. The figure shows the sample size (n) and P values for each group. P values were calculated using two-tailed Student's t tests; the data are presented as the means ± SD. Boxplots show the mean (horizontal line), the 25th and 75th percentiles (colored box), and the minimum and maximum percentiles (whiskers). Source data are provided as a Source Data file.

an increase in leaf number[22]. Similarly, *Big embryo1* (*Bige1*) is involved in lateral organ initiation, and loss-of-function mutations of the gene can accelerate leaf initiation in maize[23]. Furthermore, the MADS-box transcription factors *ZmMADS1* and *ZmMADS3*[24,25], the auxin efflux transporter *PIN1a*[26], and the meristem size regulator *Thick Tassel Dwarf1* (*TD1*) have all been linked to maize leaf number[27].

As the timing of floral transition is a determining factor in the duration of the vegetative phase, leaf number is often used as an indicator of flowering time in maize. However, flowering time is a critical factor in crop adaptation and can influence numerous other agronomic traits, making it an unsuitable approach to improve leaf number for increased grain yield while also delaying flowering time. To date, no exploitable natural loci that enhance maize grain yield by increasing the leaf number above the ear without influencing flowering time have been identified.

In this study, we clone the gene underlying a major QTL associated with leaf number above the ear. We show that the causative gene (*Tunicate1*) (*Tu1*) encodes a MADS-box protein[28]. An upstream 5-bp (AGAAG) insertion in the promoter of *Tu1* enhances its transcription, regulating plastochron activators such as *TD1*, *PIN1a* and *yabby1* and repressors such as *ub2*, *Bige1*, *Kn1*, *ZmMADS1*, *ZmMADS3* and *WEE1* to moderately increase the number of leaves above the ear, while keeping the majority of other traits unchanged. The favorable 5-bp insertion allele is originally derived from teosinte. Its ability to improve maize yield while maintaining the source–sink balance indicates its application value in maize breeding.

Results

Lna1 is a major QTL associated with leaf number above the ear in maize

To discover the molecular genetic basis underlying the leaf number above the ear in maize, a landrace called Strawberry Popcorn (Popcorn hereafter) was crossed with a maize nested association mapping population (NAM) parental inbred line, MS71, to construct an F_2 population (Fig. 1). Popcorn produced only 3–4 leaves above the ear, whereas MS71 produced 5 or 6 leaves (Fig. 1b). Genome-wide QTL analysis revealed six QTLs for leaf number above the ear located on chromosomes 1, 2, 3, 4, 5 and 7 in this F_2 population. Among these identified QTLs, the one located on the long arm of chromosome 4, which was named *Leaf number above the ear 1* (*Lna1*), was found to be the only QTL with a genetic effect originating from the Popcorn parent, accounting for 9.3% of the total phenotypic variation (Fig. 1j).

To precisely quantify the genetic effect of *Lna1*, a pair of near-isogenic lines (NILs) were then generated (see "Methods"). Compared with the NIL carrying the homozygous MS71 *Lna1* allele (NIL-MS71), the NIL with the homozygous Popcorn *Lna1* allele (NIL-Popcorn) had more leaves above the ear (Fig. 1 c, d). The total leaf number of NIL-Popcorn was greater than that of NIL-MS71, with no significant difference in the leaf number below the ear (Fig. 1e, f). This increase in the leaf number above the ear resulted in an increase in the total leaf number in NIL-Popcorn. Although leaf number is typically strongly correlated with flowering time in maize, our results revealed that there was no significant difference in flowering time between NIL-MS71 and NIL-Popcorn (Fig. 1g). Similarly, the plant height and ear height did not differ significantly between the two NILs (Fig. 1h, i). Furthermore, the length of each internode above the ear was measured, and it was found that the 1st−4th internodes (from top to bottom) of NIL-Popcorn were shorter than those of NIL-MS71, whereas the 5th internode was similar in length between the two NILs (Supplementary Fig. 1). These results suggested that *Lna1* can significantly increase the number of leaves above the ear while keeping most other traits unchanged.

High-resolution mapping of the *Lna1* locus in maize

Genetic linkage analysis of the $F_{2:3}$ population revealed that *Lna1* was located between two markers, M1 and M2, on the long arm of chromosome 4 (Fig. 2a). To further fine map the *Lna1* locus, a large population with 11,914 individuals was constructed from 17 representative F_7 recombinant plant lines, and a modified progeny testing strategy was employed (see Methods and Supplementary Fig. 2)[29]. Using 12 simple sequence repeat (SSR) and insertion/deletion (Indel) markers, the causal gene of *Lna1* was finally narrowed down within a 195-kilo base pair (kb) genomic fragment from 181,861,347 to 182,056,761 bp on the basis of the B73 genome sequence (V4; http://www.maizegdb.org), which was flanked by the markers M9 and M11 (Fig. 2b, Supplementary Fig. 2). Sequence annotation revealed that only the first exon of one gene (*Zm00001d052180*) was present in this region (Fig. 2c). *Zm00001d052180* is known to be responsible for the phenotype of the *Tunicate1* (*Tu1*) mutant, i.e., pod corn or *Tunicate* maize[30]. Thus, *Zm00001d052180* was considered the candidate causative gene of *Lna1*. To avoid confusion, we keep the previous name *Tunicate1* (*Tu1*) in this study.

Tu1 contains eight exons and seven introns (Fig. 2c), encoding a MADS-box protein with 228 amino acid (aa) residues. Phylogenetic analysis revealed that this MADS-domain protein clusters within the MIKC-type MADS group and is specifically associated with the Arabidopsis SHORT VEGETATIVE PHASE (SVP) protein[31] (Supplementary Fig. 3). A sequence comparison between MS71 and Popcorn revealed that only one exon of *Tu1* in the fine-mapped interval had no variation, but many single nucleotide polymorphisms (SNPs) and insertions/deletions between the two parental lines were detected upstream of the gene within this 195-kb interval. To determine which variant was causative of leaf number above the ear, we performed association mapping in the NAM populations, which consisted of approximately 5000 individuals[32,33]. A total of 475 variants were identified in the 195-kb fragment on the basis of whole-genome sequencing data from 26 NAM parents (https://nam-genomes.org)[34]. Association mapping revealed that the strongest signal correlated with the number of leaves above the ear ($P = 1.8 \times 10^{-5}$), which was located at a 5-bp insertion (AGAAG) in the distal region positioned −194 kb upstream of *Tu1* (Fig. 2d). This region was also reported as a chromatin accessibility landscape as determined by the Assay for Transposase-Accessible Chromatin using Sequencing (ATAC-seq) data from a public repository (Supplementary Fig. 4). In addition, this region was identified to have a H3K27me3-HiChIP loop for long-distance interaction with *Tu1*, and H3K27me3 functions as a transcriptional silencing element (Supplementary Fig. 5). As other variants had signals below the threshold

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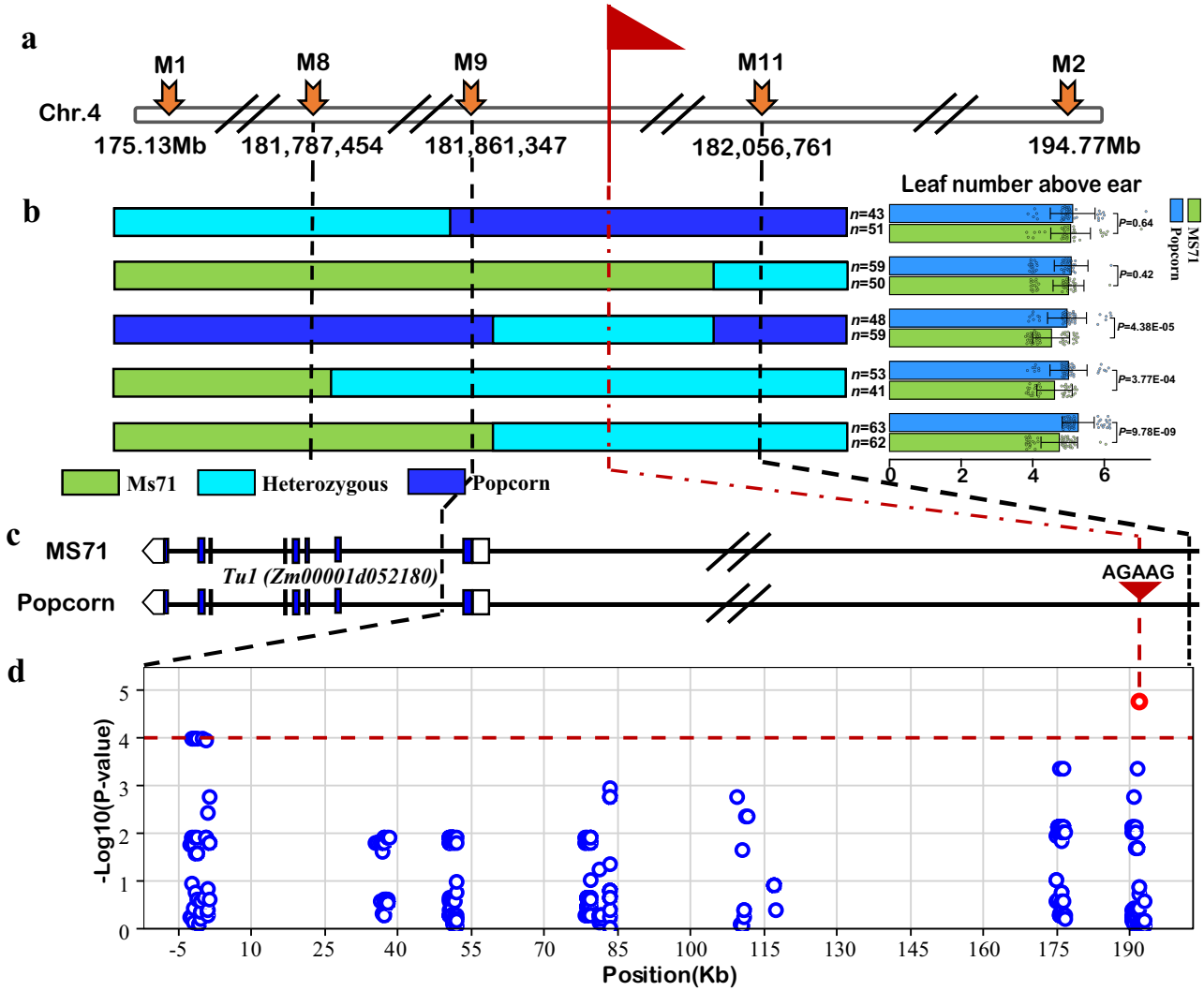

Fig. 2 | High-resolution mapping of the *Lna1* locus. a–c Fine-mapping narrowed down the location of *Lna1* within a 195-kb segment between M9 and M11 on chromosome 4. The blue, green and cyan bars represent the homozygous Popcorn, MS71 and heterozygous genotypes, respectively. Red flag, *Lna1*. Two-tailed Student's *t* test; the data are shown as the mean ± SD. **d** Association mapping with a mixed linear model revealed that a 5-bp insertion in Popcorn was strongly associated with the number of leaves above the ear. The horizontal red dashed line represents the significance threshold at the $P = 0.05$ level with Bonferroni correction for 475 tests. The position of marker M9 was set as position "0". Source data are provided as a Source Data file.

($P = 1.0 \times 10^{-4}$), the 5-bp insertion with the strongest signal in Popcorn compared with MS71 was then identified as the causal variant of *Lna1*. These results suggest that *Tu1* is the causal gene for the number of leaves above the ear in maize.

The effect of the *Tu1* gene on the number of leaves above the ear was confirmed through transgenic analysis

To determine the role of the *Tu1* (*Zm00001d052180*) gene, which corresponds to *Lna1*, in maize leaf number above the ear, genetic transformations were conducted (Fig. 3). A construct controlled by the *Ubiquitin* promoter (*Tu1-OE*) was introduced into the maize inbred line LH244, resulting in three independent transgenic events (Fig. 3a). Compared with those of the control plants, the leaf number above the ear and the total number of leaves significantly ($P < 2.5 \times 10^{-5}$) increased in the transgenic plants that overexpressed the *Tu1* gene from the three events (Fig. 3d–f). Like the natural *Tu1* allele, ectopic overexpression of this MADS-box gene also induced large spikelets on the tassel and long glumes covering grain on ear in this study (Supplementary Fig. 6). Maize transformation via CRISPR/Cas9 was subsequently performed with a single cutting target in the coding sequence (CDS) of the *Zm00001d052180* gene. Three independent edited events (*Tu1-CR1*, *Tu1-CR2*, and *Tu1-CR3*) with loss of function in the *Tu1* gene were obtained (Fig. 3b). The edited plants carried deletions in the first exon of *Tu1*, which introduced a frameshift mutation and resulted in early termination of translation (Supplementary Fig. 7). These edited plants were semidwarf and had a significantly ($P < 2 \times 10^{-30}$) lower number of leaves above the ear, averaging only 3–5 leaves, than the control plants, with an average of 6.5 leaves above the ear (Fig. 3b, i–m). These transgenic results confirm that the *Tu1* gene, corresponding to *Lna1*, controls the leaf number above the ear in maize.

We investigated the plastochron number in the SAMs of both overexpressing and knockout-edited maize plants by scanning electron microscopy (SEM) and light microscopy. At 30 days after planting (DAP), the overexpression plants presented 19 plastochrons (Supplementary Fig. 8a, e, f), whereas the knockout-edited plants presented 15 plastochrons (Supplementary Fig. 8b, g, h). By 35 DAP, the plastochron number had increased to a maximum of 21 in the overexpression plants and 16 in the knockout-edited plants. Inflorescence primordia had emerged on the SAMs of the overexpression plants by this time, whereas they had not yet appeared in the knockout-edited plants

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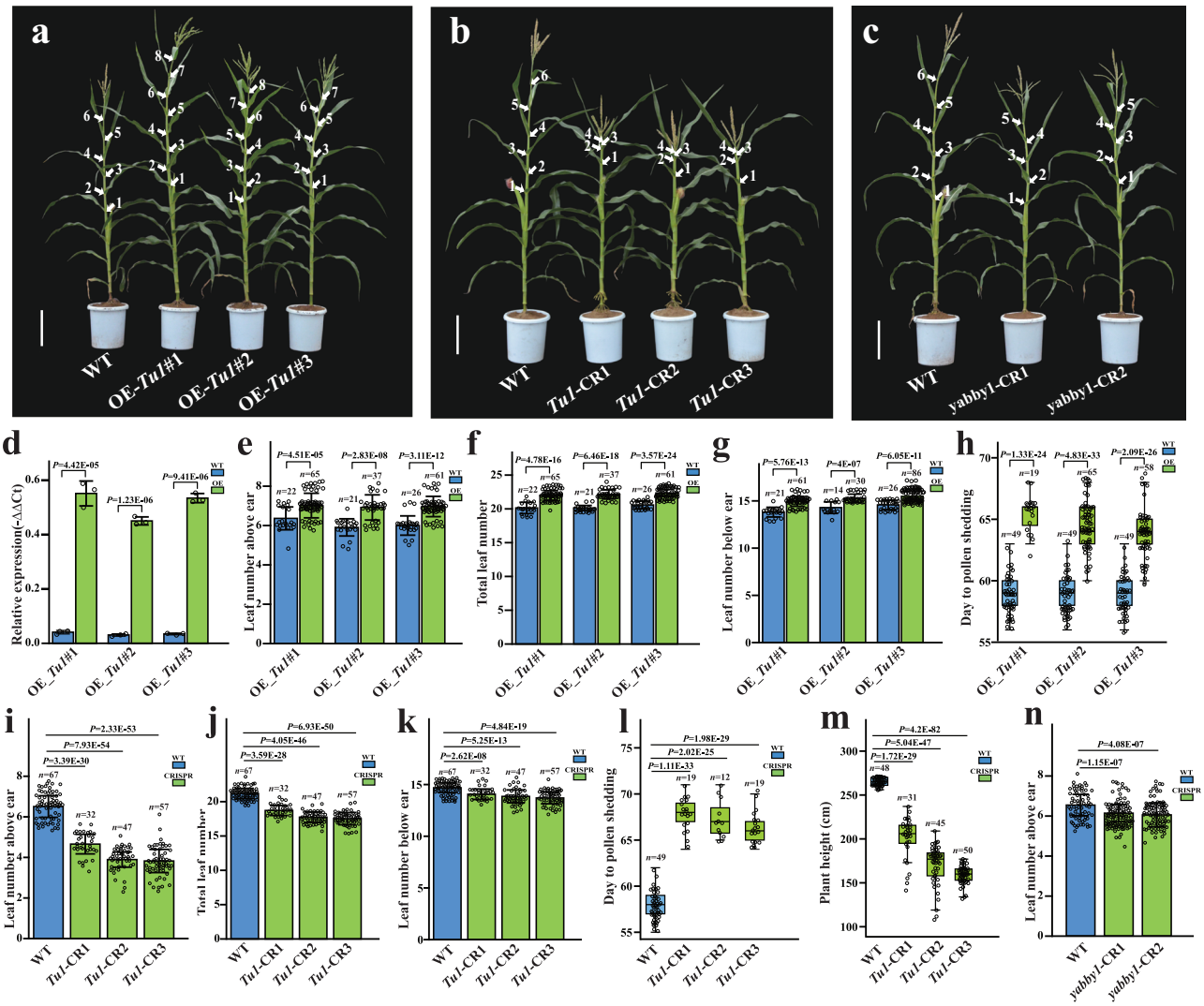

Fig. 3 | Transformation of *Tu1*. **a** Plants expressing *Tu1* under the control of the *ubiquitin* promoter developed more leaves above the primary ear. Scale bar, 30 cm. **b** Compared with the control (WT) plants, the *Tu1* CRISPR-edited plants whose single cutting target was in the coding sequence of *Tu1* showed a significant decrease in leaf number. **c** CRISPR-edited *yabby1* plants developed fewer leaves above the primary ear. The white arrow indicates the leaves above the primary ear. **d** Compared with nontransgenic control (WT) plants, overexpression transgenic (*OE-Tu1*) plants exhibited a substantial increase in *Tu1* transcript levels. The data are shown as the means ± SD ($n = 3$). Compared with the control (WT) plants, the *OE-Tu1* plants developed significantly greater leaf numbers above the primary ear (**e**), total leaf numbers (**f**), leaf numbers below the primary ear (**g**), and days to pollen shedding (**h**). Compared with nontransgenic control plants, CRISPR-edited plants developed significantly decreased leaf numbers above the primary ear (**i**), total leaf number (**j**), leaf number below the primary ear (**k**), and plant height (**m**), excluding days to pollen shedding (**l**). **n** Leaf number above the primary ear was significantly decreased in the *yabby1* knockout plants relative to the control plants. The figure shows the sample size (n) and P values for each group. P values were calculated using two-tailed Student's t tests; the data are presented as the mean ± SD. Boxplots show the mean (horizontal line), the 25th and 75th percentiles (colored box), and the minimum and maximum percentiles (whiskers). Source data are provided as a Source Data file.

(Supplementary Fig. 8c, d). These findings indicate that the number of leaves in maize is determined by the plastochron number before the emergence of floral primordia in the SAM, and the SAMs in the over-expression plants remained more active than those in the knockout-edited plants.

A 5-bp insertion moderately enhances *Tu1* expression and slightly increases the number of leaves above the ear

To determine how the 5-bp insertion in the distal region upstream of *Tu1* regulates its transcription, we performed real-time RT–PCR in SAMs from NIL-Ms71 and NIL-Popcorn at stages V3-V5. RT–PCR revealed that the expression level of *Tu1* was obviously greater in NIL-Popcorn with the 5-bp insertion than in NIL-MS71 (Fig. 4a). To further test the effect of this 5-bp insertion on *Tu1* expression, we performed a dual-luciferase (LUC) transcriptional activation assay in maize protoplasts (Fig. 4b). The constructs harboring the LUC reporter gene driven by the distal promoter segments of MS71 and Popcorn were subsequently transformed into protoplasts. Compared with that in the MS71 fragment, luciferase activity in the Popcorn segment was greater. Furthermore, when the 5-bp insertion was deleted from the Popcorn segment, the luciferase activity was clearly repressed (Fig. 4b). These results indicated that the 5-bp insertion increased *Tu1* expression in the Popcorn plants. Therefore, both association mapping and LUC transient assay analysis consistently revealed that the 5-bp insertion (AGAAG) in the distal promoter region was causative to the *Tu1* gene.

To elucidate the regulatory role of *Tu1* in determining leaf number above the ear, a comprehensive investigation into leaf number-related traits, including leaf numbers above/below the ear, date to pollen shedding (DTP), and the node of ear emergence (equivalent to leaf number below the ear), is imperative (Fig. 1d–i, and Fig. 3e–l). A

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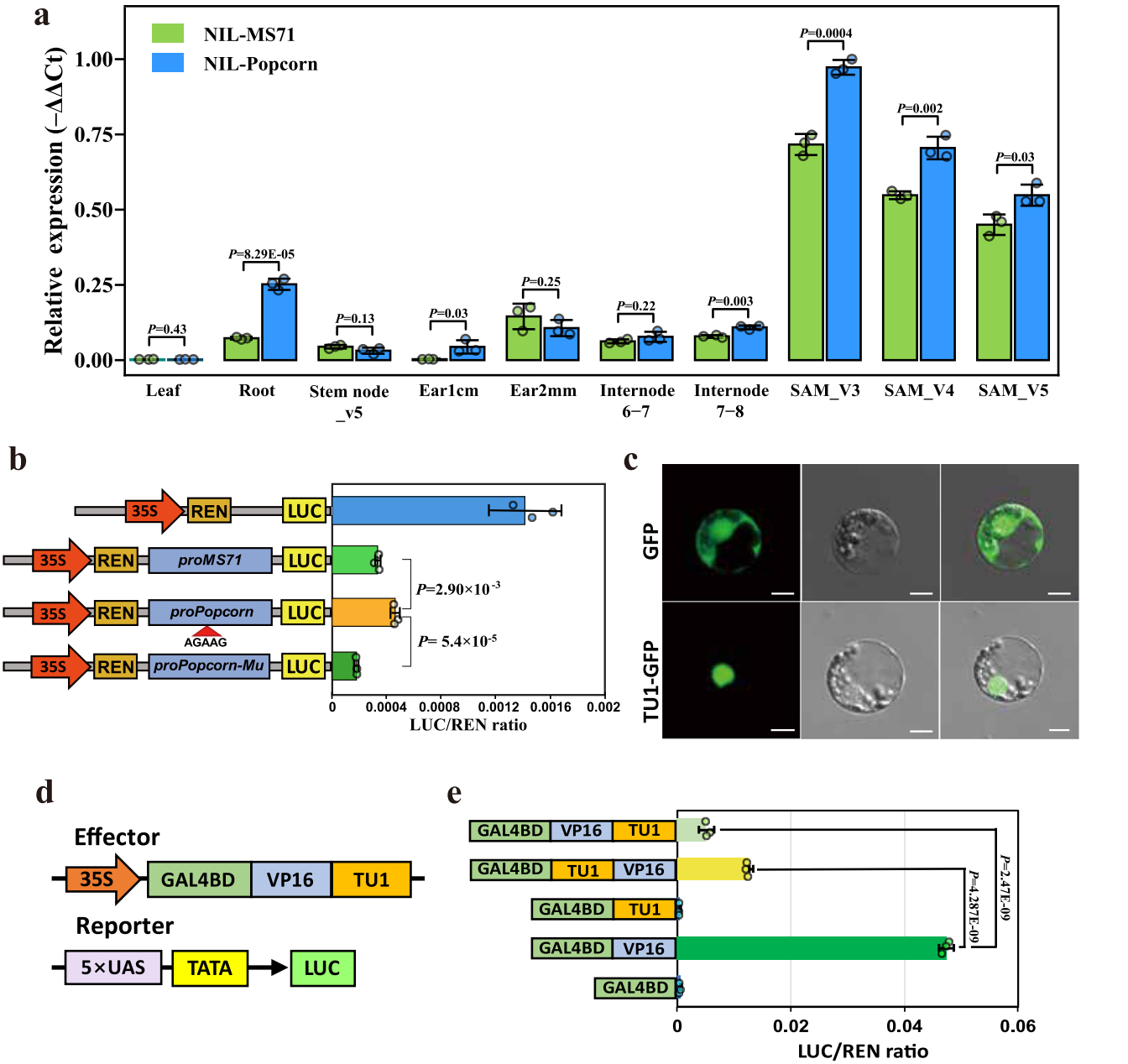

Fig. 4 | Gene function analysis of *Tu1* in maize. a Transcription levels of *Tu1* in the leaf, stem node, root, 1 cm ear, 2 mm ear, internode, and shoot apical meristem (SAM) at different stages (v3, v4 and v5). **b** A transient luciferase reporter gene assay was conducted in maize mesophyll protoplasts, in which the luciferase (LUC) gene was driven by a promoter sequence without the 5-bp insertion from NIL-MS71 and a promoter sequence with the 5-bp insertion from NIL-Popcorn. Additionally, a mutant reporter with a deletion of the 5-bp insertion in the NIL-Popcorn promoter was constructed to assess its effect. An empty vector was used as a control. Scale bar, 10 μm. **c** Subcellular localization of the TU1–GFP fusion protein in maize mesophyll protoplasts. **d**, **e** Dual-luciferase transient activity assays indicated that TU1 acted as a transcriptional repressor. Compared with the control protein GAL4DB–VP16–TU1, the GAL4DB–VP16–TU1 fusion protein strongly down-regulated luciferase activity. *P* values are shown in the figure, two-tailed Student's *t* test; the data are shown as the mean ± SD ($n = 3$). Source data are provided as a Source Data file.

comparison of these traits across the overexpression and edited transgenic plants with those of the nontransgenic controls revealed robust patterns. The transgenic overexpression plants presented a significant increase in both the number of leaves above and below the ear, leading to a considerable increase in the total leaf number (Fig. 3e–g). The average number of nodes associated with ear emergence in these overexpression plants showed an increase by one internode in comparison to the control plants (Fig. 3g). Additionally, the average DTP in the overexpression plants was significantly delayed in contrast to that in the control plants (Fig. 3h).

Conversely, the edited transgenic plants presented a marked decrease in leaf number above/below the ear, resulting in a significant reduction in total leaf number (Fig. 3i–k). Compared with those in the control plants, the average number of nodes associated with ear emergence in the edited plants decreased by nearly one internode (Fig. 3k). However, the average DTP in the edited plants was significantly delayed (Fig. 3l), resembling that in the overexpression plants. These findings underscore the role of *Tu1* in controlling leaf number above and below the ear, influencing both the node of ear emergence and DTP in the transgenic plants.

In contrast, the plant with a 5-bp insertion in the promoter of *Tu1*, which induced moderately increased expression, regulated the total leaf number without influencing the nodes associated with ear emergence and DTP (Fig. 1f, g). Thus, the number of leaves below the ear of the plant with the 5-bp insertion did not differ, and the number of leaves above the ear was controlled (Fig. 1d, f). Both the overexpression and edited knockout plants (overrepression), along with the plants featuring the 5-bp insertion, consistently demonstrated that *Tu1* regulates the total leaf number without conflicts. Unlike the overexpression and overrepression plants, those with the 5-bp insertion and moderately enhanced expression of *Tu1* did not alter the node where the ear occurs or flowering time, indicating that changes in these factors depend on *Tu1*'s expression levels. Therefore, the control exerted by *Tu1* on leaf number above the ear is attributed to the unaltered node of ear emergence and flowering time, a result of the moderately increased expression in the maize plant with the 5-bp insertion in the promoter of *Tu1*.

TU1 might function as a transcriptional repressor

The expression levels of the *Tu1* gene were low in the leaves, stem nodes, internodes, roots, and 1-cm or 2-mm ears but were significantly greater in the SAM (Fig. 4a). The accumulation of *Tu1* transcripts in the SAM decreased gradually from the V3 to V5 developmental phases, with higher transcript abundance in the NIL-Popcorn than in the NIL-MS71, regardless of developmental stage (Fig. 4a).

The TU1 protein is a MADS-box transcription factor (Supplementary Fig. 3). To determine the subcellular localization of TU1, a construct encoding a TU1-GFP fusion protein was introduced into maize leaf protoplasts, and the fluorescent signals for TU1-GFP were detected only in the nuclei, in contrast to free GFP (Fig. 4c). To examine the self-activating activity of TU1, a yeast two-hybrid assay was performed, which revealed that the TU1 and BD (GAL4 DNA-binding domain) fusion proteins did not activate the expression of the reporter gene, suggesting that TU1 lacks transcriptional activation activity (Supplementary Fig. 9). To confirm this, transcriptional activity assays were conducted by generating chimeric proteins in which TU1 was fused with the DNA-binding domain from the yeast GAL4 transcription factor (GAL4-DB) and the activation domain from the herpes simplex virus protein 16 (VP16) (Fig. 4d). The reporter constructed for these assays consisted of the luciferase reporter gene driven by a synthetic promoter that comprises five copies of the GAL4 upstream activating sequence (UAS) and a TATA box (Fig. 4d). While GAL4BD-VP16 strongly activated luciferase expression (as indicated by high luciferase activity), the GAL4BD-VP16- TU1 chimeric protein dramatically repressed luciferase activity from the same reporter (Fig. 4e). These results indicate that TU1 might act as a transcriptional repressor of its downstream genes.

The gene regulatory network of TU1 in terms of leaf number in maize

To understand how TU1 controls downstream genes involved in leaf number, RNA-seq was performed on the SAMs of a transgenic gene-edited plant (*Tu1-CR1*) with a 2-bp deletion mutation inducing the loss of function of TU1 and a nontransgenic control plant (LH244). The analysis revealed 1,715 and 2,574 differentially expressed genes (DEGs) whose expression was upregulated and downregulated, respectively, in the edited plants compared with the control plants (Fig. 5a, Supplementary Data 1). We subsequently conducted a gene ontology (GO) analysis on the DEGs identified through RNA-seq (http://systemsbiology.cau.edu.cn/agriGOv2/)[35]. The analysis highlighted five of the top 20 enriched GO terms associated with biological processes, including organonitrogen compound metabolic process, organonitrogen compound biosynthetic process, nitrogen compound metabolic process, cellular nitrogen compound biosynthetic process, and cellular nitrogen compound metabolic process (Supplementary Data 2). These results suggest that TU1 governs organonitrogen and nitrogen biosynthesis, as well as metabolic processes, in the development of SAM. Leaves sequentially grow out from the leaf primordium in the SAM until floral transition. The leaf number in maize is determined by both the plastochron and the time of floral primordia emergence. A careful search of the RNA-seq data revealed 9 DEGs, namely, *TD1*[27], *ub2*[22], *Bige1*[23], *WEE1*[15], *yabby1*, *PIN1a*[26] and *Kn1*[9], which are related primarily to plastochron, meristem development and the timing of floral primordia emergence (Supplementary Data 3). The MADS-box genes *ZmMADS1* and *ZmMADS3* function in floral activation and leaf development[24,25]. TU1 repressed the expression of *WEE1* and *ZmMADS1*, whereas it upregulated the transcription of *TD1*, *ub2*, *Bige1*, *ub2*, *yabby1*, *PIN1a* and *Kn1*. The relative expression levels of all of these DEGs were confirmed by real-time qPCR (Supplementary Fig. 10). As the function of *yabby1* has not been identified in maize, we generated plants with a loss-of-function mutation in the *yabby1* gene via the CRISPR/Cas9 editing system. Two edited events carried deletions in the CDS, introducing frameshift mutations and resulting in early translation stops (Supplementary Fig. 11). Compared with the nontransgenic control plants, the edited plants from these two events presented fewer leaves above the ear (Fig. 3c, n).

We reanalyzed a previously reported maize single-cell RNA-seq dataset, focusing on the SAM and following the reported method[36]. *Tu1* is expressed across 20 distinct cell types, with obvious expression observed in leaf primordia cells (Supplementary Fig. 12), which is consistent with the transcriptional pattern based on in situ hybridization (Supplementary Fig. 8i). Among the nine chosen downstream target genes, excluding *ZmMADS3*, which was undetected in this single-cell RNA-seq dataset, we identified correlated expression patterns in these leaf primordium cells, providing robust evidence of their association with TU1 in the regulation of leaf number.

We next performed DAP-seq for TU1 (Supplementary Data 4). This analysis revealed 52,806 TU1 binding peaks in the regulatory regions of 20,821 genes, with a $GG(A/T)_6CC$ motif being predominantly enriched in these binding sites (Fig. 5b). This motif was found to be congruent with the binding site of previously identified MADS-box transcription factors[37,38]. The *ZmMADS1* gene contains the TU1 binding site "$GG(A/T)_6CC$" in its promoter region (Fig. 5c). The expression level of the *ZmMADS1* gene was suppressed by TU1. Additionally, the region with the $GG(A/T)_6CC$ motif in the *ZmMADS1* promoter showed high chromatin accessibility (Supplementary Fig. 13). Luciferase activity assays and electrophoretic mobility shift assays (EMSAs) confirmed that TU1 can directly bind to the motifs in the *ZmMADS1* promoter and subsequently repress its expression (Fig. 5d-f).

These results indicated that TU1 can directly bind to the *ZmMADS1* promoter, leading to direct repression of its expression. Moreover, TU1 indirectly upregulated *TD1*, *ZmMADS3*, *PIN1a*, *Kn1*, *Bige1*, *ub2* and *yabby1* and indirectly downregulated *WEE1* (Fig. 5g). The genes *TD1* (Supplementary Fig. 14), *PIN1a*, and *yabby1* have a positive influence on the number of leaves, whereas the genes *WEE1*, *ZmMADS1*, *ZmMADS3*, *Kn1*, *ub2*, and *Bige1* have a negative effect on this characteristic (Fig. 5g). As a result, TU1 modulates the effects of these genes and mildly enhances the total leaf number in maize.

The *Lna1* locus was under selection in landraces during maize diversification

To investigate whether the *Lna1* locus was under selection in maize, we performed large-scale sequencing of a 1.4-kb promoter fragment harboring a 5-bp insertion/deletion from 136 accessions, including 42 teosinte lines, 37 landraces and 57 common maize inbred lines (Supplementary Data 5). The majority of the accessions did not possess the 5-bp insertion, with the exception of 4.76% (2 lines) of teosinte, 48.65% (18 lines) of landraces, and 5.26% (3 lines) of maize inbred lines (Fig. 6a). This result suggested that the Popcorn *Lna1* allele

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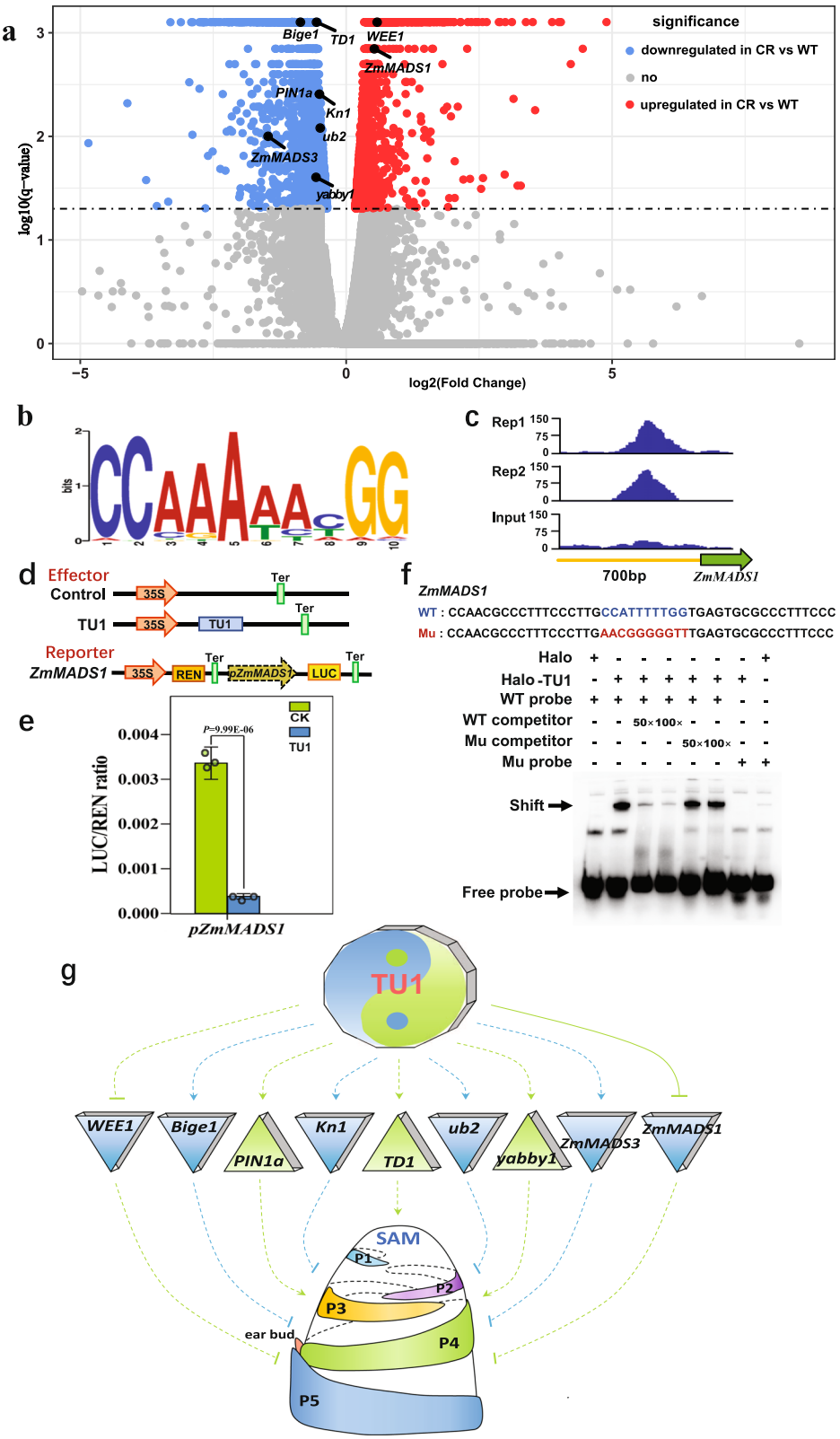

accumulated during the domestication of maize from teosinte to landrace but was gradually lost during the improvement of maize. Furthermore, phylogenetic analysis was performed on all of the accessions (23 lines) with the 5-bp insertion, and the results revealed that the teosinte lines and maize lines with the 5-bp insertion (AGAAG) shared a single common ancestor (Fig. 6b). We then compared the nucleotide diversity among teosinte lines, landraces, and maize inbred lines. The teosinte lines presented the greatest degree of DNA diversity, which decreased in the maize inbred lines, whereas the landraces presented the lowest level of diversity. The 1.4-kb fragment was divided into three segments (1–600 bp, 601–1000 bp, and 1001–1400 bp). We subsequently performed Tajima's D tests on the three segments from these accessions. Tajima's D test significantly rejected the neutral null hypothesis (Tajima's D = −1.8303, $P < 0.05$) only for the second

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Fig. 5 | Gene regulatory network of TU1. a RNA sequencing (RNA-seq) was performed to compare transgenic edited (*Tu1-CR1*) and nontransgenic plants, and the results were visualized via a volcano plot. The horizontal dashed line on the plot represents the threshold of significance ($q = 0.05$) for the differentially expressed genes. The blue and red points represent genes whose expression was significantly downregulated and upregulated, respectively, in the transgenic *Tu1* edited plants compared with the nontransgenic plants. DNA affinity purification sequencing (DAP-seq) analysis identified the motif of the TU1 protein (**b**) and detected a binding peak in the promoter of the *ZmMADS1* gene (**c**). **d** Schematic representation of effectors and reporter constructs for dual-luciferase transient expression assays. The reporter was designed to place LUC under the control of the *ZmMADS1* promoter with a mini 35S promoter. **e** LUC activity was significantly repressed by the overexpression of TU1 (effector) in the construct. These results suggest that TU1 directly represses the transcription of *ZmMADS1*. Two-tailed Student's *t* test; the data are shown as the mean ± SD ($n = 3$). **f** Electrophoretic mobility shift assays (EMSAs) were performed using fragments of the *ZmMADS1* promoter containing the motif. The EMSAs were conducted with at least one of the following reagents: HaloTag, Halo-TU1 protein, a biotin-labeled probe, a competitor without a biotin label, and a competitor with the mutated motif and without a biotin label. The specificity of binding was tested with competitors. The wild-type competitors dramatically decreased binding to the probes, whereas the mutated competitors had no effect on binding. +, present; −, absent. The motif is marked in blue in the sequence. **g** TU1 functions as a balancer in the gene regulatory network for the number of leaves above the ear; downregulates plastochron repressors *WEE1* and *ZmMADS1*; simultaneously upregulates plastochron repressors such as *Bige1*, *Kn1*, *ZmMADS3* and *ub2*; and activators include *PIN1a*, *TD1* and *yabby1*. The plastochron in the shoot apical meristem is accelerated, and the leaf number is ultimately increased in maize. The green and blue triangles represent plastochron activators and repressors, respectively. SAM shoot apical meristem; arrow bar, upregulation, T bar downregulation, P1-5, plastochrons. The solid and dashed lines represent direct and indirect regulation, respectively. The green and blue lines represent the final positive and negative effects on leaf number, respectively. The position of the ear bud is shown. Source data are provided as a Source Data file.

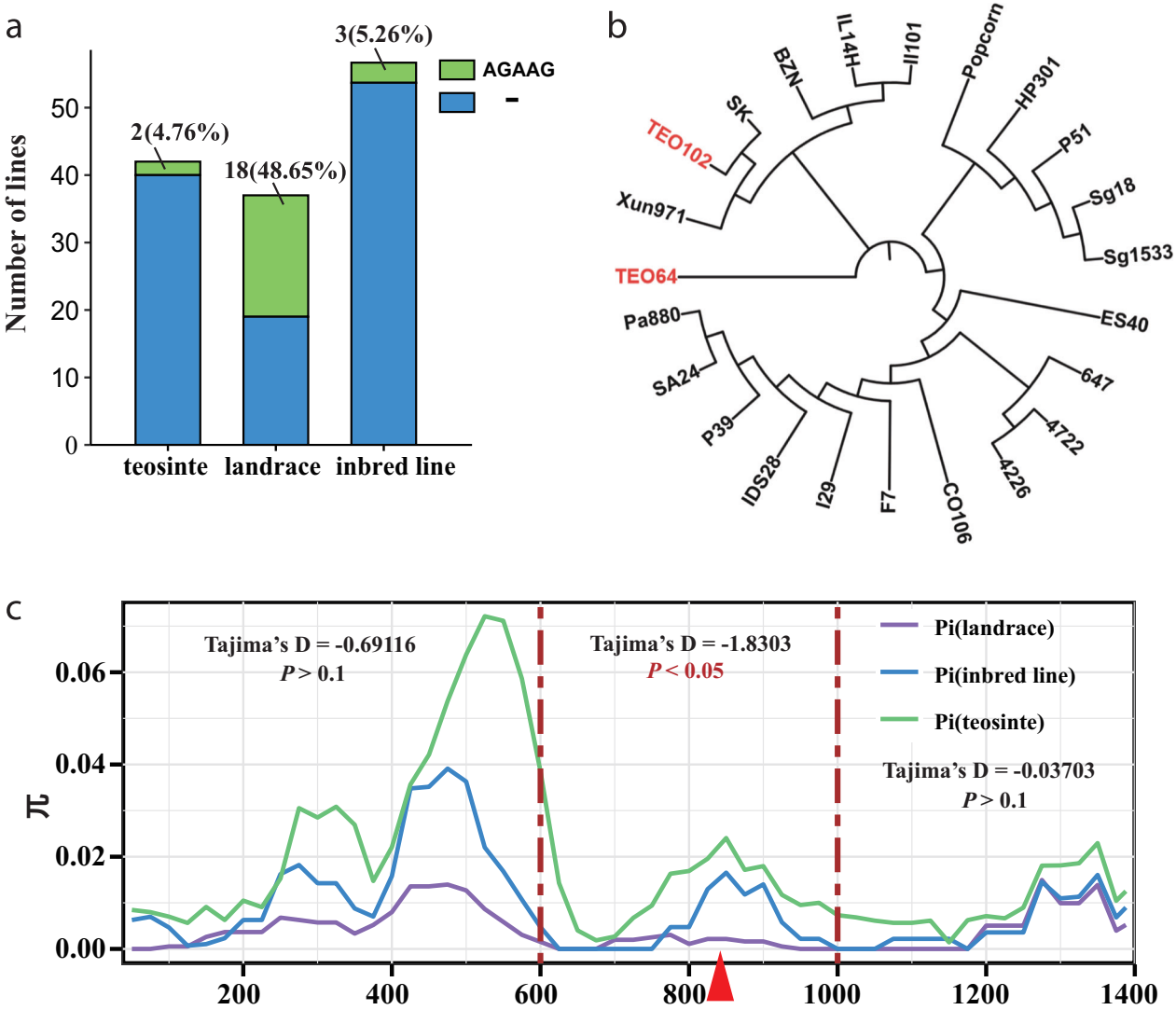

Fig. 6 | The *Lna1* locus was under selection. a Proportion of lines with a 5-bp insertion in teosinte, landrace and maize inbred lines. **b** Phylogenetic analysis of maize and teosinte lines with the 5-bp insertion. The maize lines with 5-bp insertions share a single common ancestor. The teosinte lines with the 5-bp insertion are highlighted in red. **c** DNA diversity analysis for *Tu1* from a 1.4-kb promoter fragment harboring the 5-bp insertion. Significant selection signals (Tajima's D = −1.8303, $P < 0.05$) were present only in the promoter fragment (601−−1000 bp) with the 5-bp insertion in landraces. However, no selection was present in other sequenced promoter regions (200−600 bp and 1001−1400 bp) in landraces. Tajima's D and *P* values for the 1.4-kb fragment are shown for the landraces. Nonsignificant selection signals were detected in the maize inbred lines above the 1.4-kb promoter fragment of *Tu1*. The red triangle on the *x*-axis represents the 5 bp insertion present in the promoter of *Tu1*. Source data are provided as a Source Data file.

segment (601–1000 bp), with a 5-bp insertion in the landraces, whereas no significant Tajima's D signals were detected in the teosinte or maize inbred lines in any sequenced region (Fig. 6c). These findings indicate that the *Lna1* locus underwent selection in landraces during maize diversification.

Popcorn *Lna1* allele enhances maize yield by increasing the number of leaves above the ear

The results of the sequence analysis revealed that only two out of 42 teosinte lines and 21 out of 94 maize lines carried the 5-bp insertion. The majority of the maize lines with the insertion are landraces. These findings indicate that the 5-bp insertion is a rare variant in teosinte that mostly accumulated in landraces, but has been largely lost during maize improvement.

Despite being almost completely lost during maize improvement, the 5-bp variant in the *Lna1* locus, which is known to increase the number of leaves above the ear, has the potential to be exploited for optimizing leaf architecture, particularly in densely planted modern breeding programs. To test this possibility, we conducted a yield test from three sets of NIL-MS71 and NIL-Popcorn lines planted at normal (8000 plants ha^{-1}, Fig. 7a–c) and high (16,000 plants ha^{-1}, Fig. 7d–f) densities (see "Methods"). The results at a regular planting density revealed that the single ear weight, grain weight per ear, and hundred-grain weight improved by an average of 10.2%, 11.4%, and 2.6%, respectively, in NIL-Popcorn relative to NIL-MS71. When the NILs were planted at double the density, the single ear weight, grain weight per ear, and hundred-grain weight improved by an average of 10.8%, 9.5%, and 5.5%, respectively, in NIL-Popcorn compared with NIL-MS71. These findings indicate that the Popcorn *Lna1* allele can increase both the number of leaves above the ear and the yield potential in both normal- and high-density fields (Fig. 7g, h). As such, the Popcorn *Lna1* allele can be utilized to improve plant architecture and increase maize yield in both regular and densely planted fields.

Discussion

The source strength and balance between the source and sink play critical roles in determining the final yield of crops. The term "source" refers to the photosynthetic capacity of the leaves, which serves as the main source of carbohydrates for the ear. In maize, leaves located above the ear are more metabolically active, contributing a greater amount of carbohydrates to the ear compared to leaves located below the ear. A proper increase in the number of leaves above the ear strengthens the source strength of maize plants, thereby improving the final yield. However, an excessive number of leaves above the ear can disrupt the source–sink balance, leading to greater plant stature, delayed flowering and grain filling, and lower yields in maize. How to increase the source strength while maintaining the source–sink balance remains a challenge in maize breeding.

In this study, we identified a favorable allele with a 5-bp insertion in the *Lna1* locus from a landrace called Strawberry Popcorn. This favorable allele of *Lna1* has a positive effect on the number of leaves above the ear, with no effect on flowering time or plant stature (Fig. 1). Our field studies have shown that, compared with maize plants without the 5-bp insertion in the *Lna1* locus, maize plants with this favorable allele of *Lna1* significantly improved the final yield at regular and doubled high planting densities. On the one hand, TU1 regulates plastochron activators such as *TD1*, *PIN1a* and *yabby1* and repressors such as *WEE1*, *ub2*, *Bige1*, and *Kn1*. The overall effect of this regulation slightly favors positive impacts on leaf number, leading to a limited increase in the number of leaves above the ear. On the other hand, TU1 modulates the expression of floral genes, including *ZmMADS1* and *ZmMADS3* (Supplementary Data 3). *ZmMADS1* functions as a flowering activator. The flowering time gene *ZmMADS1* was predicted to bind to the site close to this 5-bp insertion through PlantRegMap (http://plantregmap.gao-lab.org/index.php). In addition, *ZmMADS1* is directly repressed by TU1. ZmMADS1 and TU1 might mutually repress each other in terms of transcription. Thus, the complex regulatory network involving TU1 may fine-tune the timing of flowering. Hence, the *Tu1* gene can strengthen the source strength while maintaining the source–sink balance, resulting in an increase in maize yield. This finding suggests that the favorable allele of the *Lna1* locus from Popcorn has high potential for improving maize yield in maize breeding.

In this study, NIL plants carrying a 5-bp insertion in the *Tu1* promoter, which led to moderately increased expression of *Tu1*, did not show changes in either the node where the ear formed or the flowering time. However, these plants presented an increased total leaf number compared with the NIL plants lacking the insertion. As a consequence, the number of leaves above the ear increased. The number of leaves below the ear is generally strongly correlated with flowering time in maize[39], suggesting that flowering time determines the node of ear formation. While TU1 regulates flowering time-related genes such as *ZmMADS1* and *ZmMADS3*, the moderate changes in their expression levels induced by TU1 were insufficient to alter the flowering time. Thus, both the flowering time and the ear node remained consistent between NIL plants with and without the 5-bp insertion in *Tu1*. Moreover, TU1 moderately regulates genes associated with plastochron activators and repressors, including *yabby1*, *WEE1*, *PIN1a*, *ub2*, *TD1*, *Kn1*, and *Bige1*, leading to a slight increase in plastochron number. Consequently, the NIL plants with the 5-bp insertion in *Tu1* showed an increased leaf number above the ear.

MADS genes are classified into types I and II, with plant type II MADS genes playing pivotal roles in various plant developmental processes[40]. Plant type II MADS proteins, also known as MIKC-type MADS proteins, encompass the MADS (M), Intervening (I), Keratin-like (K), and C-terminal (C) domains. Phylogenetic tree analysis revealed that TU1 clustered with the group of Arabidopsis MIKC-type SVP proteins (Supplementary Fig. 3a). Additionally, MIKC domains were identified in seven TU1 orthologous proteins from maize, rice, wheat, barley, sorghum, foxtail millet, and Arabidopsis (Supplementary Fig. 3b). The MADS and K domains displayed high conservation across these seven species, whereas lower conservation was observed in the I- and C-terminal domains (Supplementary Fig. 3b). Our transcriptional assays in this study indicated that TU1 functions as a transcriptional repressor (Fig. 4e and Supplementary Fig. 9). Consistent with our findings, ChIP-seq data demonstrated that Arabidopsis SVP also functions as a transcriptional repressor[41]. New ChIP-seq studies support that MIKC-type proteins, such as TU1, can act as transcriptional repressors as well as activators.

Maize was initially domesticated from its wild ancestor, teosinte, and then underwent diversification into landraces and further development into improved inbred lines. During this process, the diversity of maize gradually decreased. Teosinte exhibits a high level of DNA diversity, and maize landraces retain a substantial amount of diversity from teosinte. In contrast, the maize inbred lines exhibit a sharp reduction in diversity. Compared with other crop species, maize landraces exhibit lower differentiation from their progenitor teosinte[42]. The narrow diversity of maize inbred lines has become a major obstacle to further enhancing maize yield from hybrids. In this study, the favorable allele of the 5-bp insertion in the *Lna1* locus was found to have originated from teosinte and was significantly enriched in maize landraces, whereas it was nearly absent in maize inbred lines. Teosinte harbors a wealth of advantageous alleles in the wild, and the domestication process may have led to the loss of many of these alleles in maize inbred lines. However, these alleles are largely preserved in maize landraces. Given the lower prevalence of unfavorable traits in maize landraces than in teosinte, maize landraces offer a readily usable resource in breeding programs to increase the diversity of maize inbred lines with limited genetic diversity. Consequently, maize landraces represent a highly valuable resource for future maize breeding to improve maize yield.

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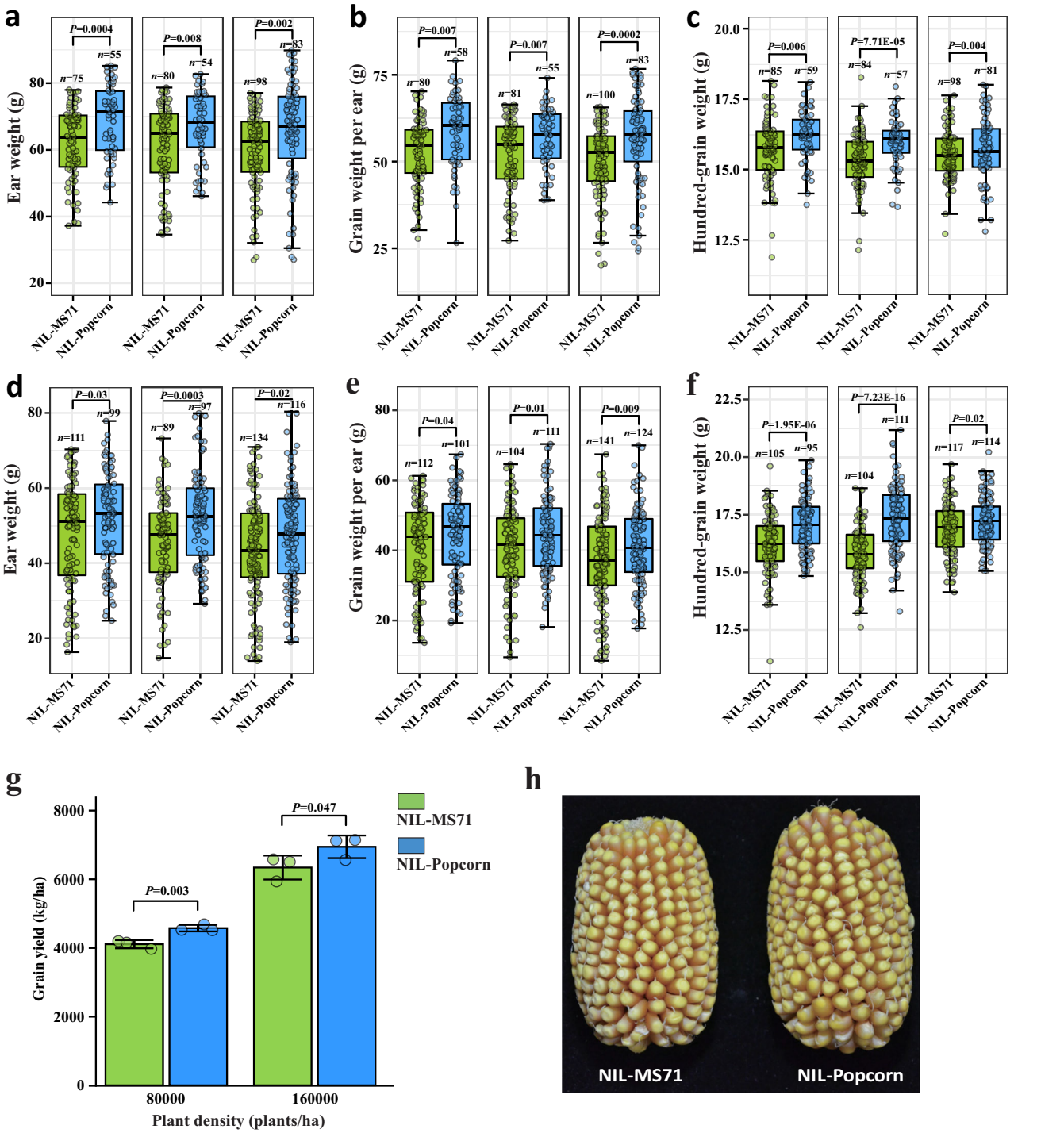

Fig. 7 | Yield test between NIL-MS71 and NIL-popcorn. The popcorn *Lna1* allele significantly increased maize grain yield, including ear weight, grain weight per ear, and hundred-grain weight, among three sets of NILs in both regularly planted fields (8,000 plants per hectare, **a**–**c**) and densely planted fields (16,000 plants per hectare, **d**–**f**). **h** The ear phenotypes of the NILs are shown. **g** The final estimated yield potential was compared between the NILs in regularly planted (8000 plants ha^{-1}) and high-density planted fields (16,000 plants ha^{-1}). The figure shows the sample size (*n*) and *P* values for each group. *P* values were calculated using (**a**–**f**) two-tailed Student's *t* tests and (**g**) one-tailed Student's *t* tests; the data are presented as the mean ± SD. Boxplots show the mean (horizontal line), the 25th and 75th percentiles (colored box), and the minimum and maximum percentiles (whiskers). Source data are provided as a Source Data file.

Methods

Plant materials

The maize landrace (Strawberry Popcorn; Ames 14282) was crossed with the maize inbred line (MS71; PI 587137) from the NAM population to generate an F_2 population comprising 240 individuals. QTL mapping was conducted in 2015 on both the F_2 and F_3 populations, which were planted during summer and winter in Beijing (40°08′ N, 116°11′ E) and Hainan (18°09′ N, 108°56′ E), respectively. Between 2015 and 2020, plants from the F_2 and F_3 populations, as well as the plant materials used for fine mapping, were grown at a spacing of 25 cm from neighboring plants with a row-to-row distance of 50 cm at the experimental stations of China Agricultural University in Hainan and Beijing. To

construct near-isogenic lines, we selfed a heterogeneous inbred family (HIF, F_7) that contained heterozygous genotypes at the *Lna1* locus and homozygous fragments at other loci, resulting in the generation of NIL-Popcorn and NIL-MS71 (F_8). To identify whether *Lna1* enhances maize yield, three sets of NIL plants were planted at normal (25-cm plant-to-plant distance and 50-cm row-to-row distance) and high-density (12.5-cm plant-to-plant distance and 50-cm row-to-row distance) locations in Hainan in 2019. Each NIL was planted in 8 rows with 15/30 plants in each row. The fields were supplied with 120 kg ha^{-1} N, 90 kg ha^{-1} P, and 90 kg ha^{-1} K.

QTL mapping

The F_2 population comprising 240 lines was genotyped using 218 SSR markers, which were evenly distributed across the 10 maize chromosomes. We subsequently constructed a genetic map, which spanned 1614.69 centimorgans (cMs) and had an average genetic distance of 7.41 cM between pairs of neighboring markers. The phenotypic data and genetic map were then input into R/qtl[43] for QTL detection using a multiple-QTL mapping method. We initiated simple interval mapping using the Haley–Knott regression method with the R/qtl function scanone, and a significance threshold of $P = 0.05$ was established for each trait via 1,000 permutations. We subsequently refined the positions of the QTLs with logarithm of odds (LOD) scores above the threshold via the R/qtl function refineqtl. Next, we scanned additional QTLs with the function addqtl on the basis of the refined QTLs. Upon detecting an additional significant QTL with an LOD score above the threshold, we added it to the model and refined the positions of all of the QTLs again. These steps were repeated until no significant QTL was added. Once all QTL positions had been refined, we finally assessed the genetic effect and significance of each QTL using drop-one-QTL analysis in the full model.

Fine mapping of the *Lna1* locus

To fine map the major QTL responsible for leaf number above the ear, *Lna1*, a large population consisting of approximately 12,000 F_7 individuals (Supplementary Fig. 2), was developed. This population was derived from a single residual heterozygous line (RHL, F_5), which carried a heterozygous genomic fragment at the *Lna1* locus and homozygous genotypes at other loci. The population was screened with 12 markers to identify 17 representative recombination types (Supplementary Fig. 2). Descendant populations from the selfing of these recombinant plants, all carrying heterozygous/homozygous fragments within the *Lna1* target region, were used to examine the correlation between genotypes and leaf number above the ear. A linear regression model was used to determine the correlation, and a significant *P*-value indicated the presence of *Lna1* in the heterozygous segments, whereas *Lna1* was mapped to the homozygous segments in the presence of a nonsignificant *P*-value. Using this modified progeny test, the *Lna1* locus was narrowed down to the region between two markers, M9 and M11. The sequences of the primers used in the fine-mapping process are listed in Supplementary Data 6.

Plant transformation

The CDS of *Tu1* of MS71 was inserted into the binary vector pBECXUN under the control of the ubiquitin promoter. The resulting construct was then transformed into the maize inbred line LH244 via a protocol based on Hill[44]. Cas9 is driven by the *OsU3* promoter from rice, and a gRNA targeting a site in the first exon of the *Tu1* gene was designed using CRISPR-P software and subsequently introduced into the CRISPR/Cas9 binary vector. All of these constructs were then introduced into the maize inbred line LH244. Three *Tu1*-overexpressing (T_0) events and three homozygous *Tu1*-CRISPR (T_0) gene-editing events were obtained. The T_0 transgenic plants were self-crossed to create homozygous T_1 plants for the investigation of leaf number above the ear, with LH244 used as the control.

Association mapping

To conduct association mapping analysis for leaf number above the ear in NAM populations consisting of approximately 5,000 individuals[32,33], we identified 475 variants from a 195-kb upstream fragment of the *Tu1* gene on the basis of the genomes of 26 NAM parents. We performed association mapping testing using a mixed linear model implemented in TASSLE5[45]. To correct for multiple testing, we used the Bonferroni correction. The significance threshold ($\alpha' = 0.0001$) was calculated via the following equation:

$$\alpha' \approx \alpha / n \quad (1)$$

where α is the nominal significance threshold ($\alpha = 0.05$) and n is the number of variants ($n = 475$).

RNA sequencing

Tu1-CRISPR and control plants at the v5 stage with similar growth vigor were simultaneously collected. Shoot apices were dissected at the base of P2 or P3 leaves using a stereomicroscope and rapidly frozen in liquid nitrogen. Approximately 20 shoot apices were pooled per biological replicate, and three biological replicates were collected for each genotype. Total RNA samples were extracted from the shoot apices and sequenced using a HiSeq-2500 System (Illumina), resulting in 50 Gb of raw sequencing data. The raw RNA-seq reads were analyzed using a standard RNA-seq pipeline[46]. Specifically, the raw reads were trimmed using Trimmomatic[47], cleaned by fastq_clean[48], and aligned to the maize B73 reference genome (v4) using STAR[49]. Gene expression was then calculated using the fragments per kilobase of exon per million fragments mapped (FPKM) method with Cufflinks and Cuffdiff2[50]. Differentially expressed (DE) genes between the transgenic and control groups were identified on the basis of their corrected *P* values (*q* values).

DNA affinity purification sequencing

A genomic DNA library was created by modifying a previously reported protocol[51,52]. In brief, genomic DNA was extracted from V5 shoot apices and fragmented into 200 bp fragments. The resulting fragments were ligated with a truncated Illumina TruSeq adaptor to generate the library. The *Tu1* coding sequence was fused with a HaloTag and expressed in the wheat germ extract system in two independent experiments. The resulting HaloTagged TU1 was immobilized onto Magne HaloTag beads, which were then incubated with the genomic DNA library (300 ng) for 1 h and washed. The washed beads, which contained bound genomic DNA fragments, were tagged with dual-indexed multiplexing barcodes through 15 cycles of PCR amplification. The resulting libraries from the two independent replicates were pooled and sequenced on the Illumina NovoSeq 6000 platform. Input DNA libraries were also prepared using the same protocol to control the background noise.

The raw reads from each replicate and input DNA library were processed by trimming adapter sequences and low-quality bases using fastp[53]. The resulting clean reads were mapped to the maize B73 reference genome (v4) using Bowtie2 v2.35[54]. The mapped reads were then filtered with SAMtools 1.9[55] to restrict the reads that aligned to multiple positions with the following parameters: -h -q 30 -F 4 -F 256. Peak calling was conducted using MACS2 v2.2.7.1[56] with a cutoff *q* value of 0.05, using the input DNA library as the control. The final list of candidate peaks was generated by identifying significant overlapping peaks from the two replicates. The bam files were converted to bigwig files and visualized in the Integrative Genome Browser. The most enriched motif for these overlapping peaks was determined using the MEME suite v5.5.1[57].

Subcellular localization

The CDS of *Tu1* was merged with green fluorescent protein (GFP) to create the 35Spro::TU1-GFP construct, which was regulated by the cauliflower mosaic virus (CaMV) 35S promoter. The 35Spro::TU1-GFP construct was subsequently transferred into maize MS71 leaf protoplasts, and the subcellular GFP signal was analyzed using an Olympus FV1000 laser scanning microscope with a 488 nm laser line.

Protoplast transient expression assay

Transient assays were conducted in maize leaf protoplasts to assess the impact of different parental sequences in the distal regulatory regions of *Tu1* on gene expression. The ~500-bp fragments containing the 5-bp (AGAAG) insertion/deletion variation from MS71 (MS71-Pro::LUC) and Popcorn (Popcorn-Pro::LUC) were amplified, and site-specific mutations targeting this 5-bp insertion were introduced using specific primers through standard oligonucleotide-directed mutagenesis techniques. These segments were integrated into the LUC vector (pGreenII 0800-LUC), which consisted of a *Renilla reniformis* reporter gene (REN) controlled by the cauliflower mosaic virus (CaMV) 35S promoter and a firefly luciferase reporter gene (LUC) controlled by a customized promoter. These constructs were then introduced into etiolated maize MS71 mesophyll protoplasts at the seedling stage. Approximately 10 μg of the reporter construct was mixed with the newly isolated protoplasts in PEG transfer solution for 18 min at room temperature before being returned to WI medium. After incubation at 25 °C for 17 h, the transformed protoplasts were harvested by centrifugation, lysed in Passive Lysis Buffer (PLB, Promega), and analyzed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Three to six biological replicates of each construct were carried out, and all of the assays were replicated three times.

To examine the impact of TU1 on the expression of two potential target genes, we then introduced the promoter fragment upstream of *ZmMADS1* (1,786 bp) with a 35S mini-promoter into the pGreenII-0800-LUC vector to construct another set of reporters. The complete coding sequence of *Tu1* introduced into the pGreenII 62-SK vector under the control of the 35S CaMV promoter was constructed as the effector construct. The appropriate sets of reporter and effector constructs were cointroduced into maize leaf protoplasts, and a relative reporter with the empty effector pGreenII 62-SK was used as the control. Luciferase activity was assessed via the same method described above.

Real-time PCR

Total RNAs were extracted from various tissues, including the tassel, ear (2 mm, 1 cm), bud, leaf, root, node, root, and SAM (v3, v4, v5) of the NIL plants, using an RNA Extraction Kit (Aidlab). First-strand cDNA was synthesized from 1 μg of total RNA via TransScript-Uni cDNA Synthesis SuperMix (TransGen Biotech). Quantitative PCR (qPCR) was performed using TB GreenTM Premix Ex TaqTM II (Takara), using the maize housekeeping gene *GADPH1* as the internal control. qPCR was carried out on a CFX ConnectTM Real-time System (Bio-Rad) with three technical replicates and three biological replicates. The final relative transcript levels were determined using the ΔΔCT (DDCT) relative quantification method[58].

Transcriptional activity assay

To evaluate the transcriptional activity of the TU1 protein, we conducted a transcriptional activity assay using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech). The full-length and two truncated coding sequences of *Tu1* were subsequently cloned and inserted into the pGBKT7 vector to fuse TU1 with the DNA-binding domain of GAL4 (GAL4-BD). As a positive control, the transcription factor ZmCCT was fused with GAL4-BD. The resulting constructs were subsequently transformed into the yeast strain AH109 according to the manufacturer's instructions. The colonies were then spotted onto yeast synthetic drop-out media that lacked Trp or Trp, Ade, and His.

To further evaluate the transcriptional activity of the TU1 protein, we conducted a dual-luciferase transient expression assay in maize leaf protoplasts. The *Tu1* coding sequence was cloned and inserted into a vector to fuse TU1 with GAL4-DB and VP16, constructing the effector construct GAL4DB-VP16-TU1. To generate the reporter construct, we introduced a promoter with a 5×GAL4 UAS sequence and a TATA box into pGreenII 0800-LUC. We cotransformed the reporter and effector constructs into maize leaf protoplasts and used an empty effector construct as a control.

DNA diversity analysis

To analyze DNA diversity, we amplified two segments flanking the 5-bp deletion/insertion break point, each containing a forward 814-bp sequence and a downstream 612-bp sequence, from a global maize population consisting of 57 inbred lines, 37 landraces, and 42 teosinte accessions (Supplementary Data 5). The resulting PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and then sequenced on an ABI 3730 sequencer. The obtained sequences were aligned using ClustalW to construct a nucleotide alignment matrix. This matrix was imported into DnaSPv5.1 to calculate nucleotide diversity (π) with a sliding window of 100 bp and a step size of 25 bp[59], with sites containing gaps being excluded. Additionally, Tajima's D tests were calculated using DnaSPv5.1.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The RNA-seq and DAP-seq data were deposited at the National Center for Biotechnology Information (NCBI) under BioProject accession number PRJNA960713. Source data are provided with this paper.

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Author contributions

Z.L. designed the study. Y. L., J. W., S. Z., Q. H., Q. W., Y. S., H. L., J. L., Y. S. and X. F. performed the research. Y.L. and Z.L. analyzed the data. Y.L. and Z.L. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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