Supporting Information

File S1

Material and Methods

Nucleotide Variation in *tga1*. We augmented the sample of teosinte individuals used by Wang *et al.* (2005) for nucleotide diversity analysis by sequencing an additional 11 teosinte plants (Table S1) for the 1042 bp control region defined by Wang *et al.* (2005) which includes the first 18 bp of the ORF and 1024 bp upstream of the ORF. The primer sequences, PCR programs, and sequencing protocols are the same as those used by Wang *et al.* (2005). For heterozygous DNA samples, PCR fragments were cloned into TOPO®-TA vectors (Life Technologies) and multiple clones were sequenced to acquire the individual allele sequences without potential Taq polymerase incorporated errors. To determine the putative causative site underlying the glume architecture difference between maize and teosinte, we aligned the complete set of 16 maize and 20 teosinte sequences and visually inspected the alignment for fixed differences between maize and teosinte.

Screening for *tga1* Mutants and Identification of *neighbor of tga1* (*not1*). Efforts to obtain a loss-of-function allele of *tga1* were made by screening several maize mutant collections, including maize targeted mutagenesis (May et al. 2003), maize TILLING project (Till et al. 2004) and Pioneer Hi-Bred International's Trait Utility System for Corn (TUSC). We also performed informatics searchers of the databases for the RescueMu (Lunde et al. 2003) and UniformMu (Settles et al. 2007) projects. No *tga1* mutants were found in any of these collections, however, five *Mu* insertion events were identified in *neighbor of tga1* (*not1*), a gene closely related to *tga1* (Preston et al. 2012), from TUSC using a *Mu* terminal repeat primer (5'-agagaagccaacgccawcgcctcyatttcgtc-3') with a primer designed from *tga1* (5'agaaagcgtctggacgggcacaatc3').

Quantitative PCR. RNA extractions were performed, as previously described (Wang et al. 2005), on immature top ears that were 25mm in length. All gene expression comparisons were based on plants from the same nursery location and year. cDNA was produced as previously reported (Wang et al. 2005) and then used for quantitative PCR (RT-qPCR). RT-qPCR was performed on ABI Prism 7000 sequence detection system (Applied Biosystems) with Power SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Specific forward primers were used for *tga1* (5'-cagtgcagcaggttccatc-3') and *not1* (5'-cgcatcactcaccaaatcca-3') along with a common reverse primer (5'gaagcttatctcgccctcct3'). We sequenced PCR products from 20 independent clones for each primer set to confirm that they were specific to either *tga1* or *not1*. A maize actin gene (AY104722, GRMZM2G126010) was selected as a control, and amplified using the primers 5'- ccaaggccaacagagagaaa

-3’ and 5’- ccaaacggagaatagcatgag -3’ (Bomblies 2003). Gene expression quantification was performed using 10 biological replicates for each genetic stock.

Protoplast Transient Assays. The TGA1 N-terminal coding sequences (amino acids 1-103) from the maize, teosinte and ems alleles were fused to a GAL4-DNA Binding Domain (DBD) followed by the *Nopaline synthase (Nos) terminator* (maize-GD, teosinte-GD and ems-GD respectively). These fusion gene effectors were expressed using the rice *Actin1* promoter. An effector with the GAL4-DBD alone (GD) served as the negative control. The effector GD-IAA17(I), encodes a GAL4-DBD fused to the IAA17 protein domain I, which is an known repressor (Tiwari et al. 2004). This effector served as a positive control for trans-repression. The effector LD-VP16 encodes an *E. coli* LexA-DBD fused to the *Herpes simplex virus* VP16 activation domain (LaMarco and McKnight 1989). This effector serves as a positive control for activation. Both the GD-IAA17(I) and LD-VP16 effectors are under the control of the cauliflower mosaic virus 35S minimal promoter (-46bp). These two effectors were generous gifts from Dr. Tom J. Guilfoyle at the University of Missouri-Columbia.

Two reporters were constructed for the transient assays. The LG-Fluc reporter contains a firefly luciferase gene under the control of the cauliflower mosaic virus 35S minimal promoter (-46bp) with two LexA and two GAL4 binding sites upstream. The LexA and GAL4 binding sites upstream of the minimal promoter allowed the fusion gene effectors containing Gal4-DBD or LexA-DBD to bind and drive the expression of the firefly luciferase gene. The Actin-Rluc reporter contains a *Renilla* luciferase gene under rice *Actin1* promoter. This reporter was co-transformed into the protoplasts with the LG-luc reporter and served as an internal control. Using both firefly and *Renilla* luciferase enabled us to take advantage of using the dual-luciferase reporter assay system (Promega Corp., Madison, WI) to take two readings from the same samples.

Transient expression assays using maize mesophyll protoplasts were performed following a detailed protocol from the Sheen’s lab (http://molbio.mgh.harvard.edu/sheenweb/protocols_reg.html). Briefly, 2-3 x 10^5 freshly isolated protoplasts in 400 □l electroporation buffer were mixed with 50 □l of plasmids which include different combination of effectors and reporters. All the plasmids were prepared with EndoFree Plasmid Mega Kit (Qiagen) and each effector : reporter LG-Fluc : reporter Actin-Rluc mixture contained 25 □g : 15 □g : 5 □g, respectively. The protoplast-plasmid mixes were transferred in 0.5 ml cuvettes and electroporated with a Gene Pulser II Electroporation System (Bio-rad) set to 250 Volts for 1.5 msec. Three pulses spaced 20 sec apart were used for each sample. After electroporation, protoplasts were incubated for 18 hrs at 25 °C before harvesting for quantification. The harvested protoplasts were then lysed with CCLR (Cell Culture Lysis Reagent, Promega) and subjected to dual-luciferase assay using a Dual-Luciferase Reporter Assay System (Promega) following the manufacturer’s instruction. Six biological replicates were measured per effector/reporter mixture.

Heterologous Protein Expression and Western Blot Assays. Tagged TGA1 proteins were generated by cloning various full-length *tga1* cDNA alleles, and a *tga1* maize allele with an N-terminal coding region deletion, into the pET151/D-TOPO® vector (Invitrogen) and transforming into BL21 codonPlus (DE3)-RIRL cells (Stratagene). All of the proteins cloned into the pET151/D-TOPO® vector are expressed with a 6xHis-V5-tag derived from the vector. Overexpressed proteins were extracted using BugBuster® Master Mix (Navagen) and then solubilized from inclusion bodies and purified under denaturing condition (with 6M urea) using a His-bind purification Kit (Navagen). The purified proteins were then re-natured by dialyzing through serial refolding solution with decreasing urea concentration (4 M, 2 M, 1 M to 0 M). The refolding solution consists of 0.1 M Tris, pH 8.0, 0.4M L-Arginine, 0.1 mM ZnCl2, 5 mM DTT, 0,2 mM PMSF and plant protease inhibitor cocktail (Sigma). Dialysis was performed in 2 liter volume at 4 °C with at least 2 hours in between solution exchanges. The purity and integrity of the proteins were assessed by SDS-PAGE and western analysis.

A soluble tag-free maize-TGA1 was generated, but could only be retained at low concentrations (≤ 5 ng/ul). The maize full-length *tga1* cDNA was cloned into a pVP-GW vector (Singh et al, 2005), which can produce proteins with S-His-tag and enhanced solubility. Proteins were overexpressed in *E. coli*, and then extracted and purified using Ni-NTA Superflow Columns (Qiagen) following the manufacturer's instructions. After purification, the S-His-tag was cleaved off using AcTEV protease (Invitrogen). All proteins were diluted to low concentration (< 40 ng/µl) prior to the TEV protease reaction to prevent precipitation of TGA1 upon cleavage of the tag. Final purification of the tag-free TGA1 proteins was performed by passing them through Ni-NTA Superflow Columns.

Western blot assays were performed on immature ear tissue 25mm in length using the anti-TGA1 antibody as previously described (Wang et al. 2005). The mini gel for the western blot in Figure 1B was run at 150 Volts for 80 min. The blot was probed with anti-TGA1 first, and then stripped and re-probed with anti-ACTIN to confirm equal loading. A large gel that was run at 150 Volts for 18 hours, was used for the western blot in Figure 1C to resolve TGA1 and NOT1. Immature *not1-Mu2* ears were used for the western blot in Figure 5B.

PCR-Assisted Binding Site Selection. PCR-assisted binding site selections were performed via Electrophoretic Mobility Shift Assays (EMSAs) as described previously (Tang and Perry 2003). A 76-mer oligo (5'-actcgaggaattcggcaccccgggt$(n)_{26}$tggatccggagagctcccaacgcgt -3') containing a core of 26 randomized nucleotides was synthesized and then converted to double-stranded DNA by PCR using two primers that corresponding to the non-degenerate ends of the

76-mer (5'-actcgaggaattcgctaccccgggt -3' and 5'- acgcgttgggagctctccggatcca -3'). The double-stranded 76-mer population was labeled with [α-^{32}P]dATP and purified on a 8% polyacrylamide gel for use as an EMSA probe.

EMSA were performed as reported previously (Wang et al. 2004) with slight modifications. Approximately 100 ng of maize-TGA1 or teosinte-TGA1 was pre-incubated in 1× binding buffer (50 mm Tris–HCl, pH 7.5, 50 mm NaCl, 1 mM ZnCl2, 1 mM DTT, 10% glycerol, 0.1 μg μl^{-1} poly(dI-dC), 0.5 μg μl^{-1} BSA) at room temperature for 10 min before adding 10^4-10^5 cpm of the radiolabeled DNA fragments. Then, the entire reaction mix (20 μl) was incubated for an additional 10 min and analyzed on a 5% PAGE gel containing 0.5 x TBE and 2.5% glycerol. The gel was dried and exposed to a PhosphorImager screen (Amersham Biosciences). The regions corresponding to shifted bands were excised and electroeluted. Then, an aliquot from each elutant was used for PCR amplification and radiolabeling to generate new probes. The new probes were used for the next round of binding and EMSA and this procedure was repeated for 5 rounds. Finally, the enriched TGA1 bound oligonucleotides were cloned into pCR2.1-TOPO® cloning vector (Invitrogen) and at least 40 clones from each shifted band were sequenced and aligned to obtain the consensus sequence.

EMSA Testing *in vitro* Interaction Between maize-TGA1 and *not1* Promoter. An 825 bp of promoter segment of *not1-maize* as well as 480 bp of 5' end sequence of *not1-teosinte* were isolated using the GenomeWalker Universal Kit (Clontech, Mountain View, CA) following the manufacture's instructions. A DNA fragment corresponding to the *not1* regulatory region (from –389 to –343) and containing a GTAC motif (–372 to –361) was generated using two partially complementary oligonucleotides that were extend by PCR. The two oligonucleotides were as follows: oligonucleotide 1, 5'- ttgctacagtcgcaactgtctgtgtctgcaaaGTACgactgct -3', and oligonucleotide 2, 5'- ccggaactgggggtggagtgggagcagtcaGTACtttgcagacaca -3'. A mutated version of the DNA fragment was created using oligonucleotide 3 (same as oligonucleotide 1 except that the **G**TAC was changed to **C**TAC) and oligonucleotide 4 (same as oligonucleotide 2 except that except that the **G**TAC was changed to **C**TAC). The wild-type and mutant DNA fragments were labeled with [α-^{32}P]dATP and EMSA performed as described above.

Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays were performed to verify *in vivo* binding of TGA1 to DNA fragments containing the GTAC motif from the *not1* regulatory region. Immature ear primordia (1-5 cm in length) from *not1-Mu2* plants were isolated and sliced to pieces, with at least one dimension less than 5 mm, and then fixed in 1% formaldehyde. The experimental procedure was done as described previously (Gendrel et al. 2002; Wang et al. 2002). An anti-TGA1 polyclonal antibody was used for chromatin immunoprecipitation. DNA populations recovered from ChIP were then used as templates for

qPCR with a maize actin gene as the control. Primers specific to *not1* promoter region are 5'- acaggtgcacagcacaacat -3' and 5'- agcagcagccaacaaagatt -3'. qPCR was performed as described above using 7 biological replicates.

***tga1-RNAi* Plants Generation and Phenotyping.** An RNAi vector that targets *tga1* was constructed by generating a hairpin loop with an inverted exon3 sequence (amplified using the primers 5'-GAGAGTCCATATCACATCACTCACC-3' and 5'-CACTAAAGCCAGTATCTCCCTACCAGATCGTC-3') link to intron2-exon3 of *tga1* (5'-AGTCTGTACTTGGTTCGAATACATCTAC-3' and 5'-CACTAAAGCCAGTATCTCCCT-3'). The hairpin structure was driven with a maize ubiquitin promoter (Christensen and Quail 1996) and transformed into Hi-II maize using the Agrobacterium-mediated method. Maize transformation was done at the Plant Transformation Facility at Iowa State University (http://agron-www.agron.iastate.edu/ptf/index.aspx).

Different transgenic events of *tga1-RNAi* were recovered and crossed to W22. Then, 80 progeny from each cross were grown out and subjected to a BASTA painting assay. Briefly, a 1% solution of glufosinate (BASTA) solution with 0.01% Tween was painted on the tip of an expanded leaf when plants were at 8-10 leaf stage. Four days after the BASTA application, the plants were scored for resistance or susceptibility to the herbicide. The correlation between RNAi transgene presence and BASTA resistance in plants were further validated by western blot (Figure S4). Western blots using anti-TGA1 were performed with four BASTA resistant and four susceptible plants for each event using young ear tissue. Consistent with expectation, TGA1 was not detected, or had a weak signal, in all ear samples from BASTA resistant plants, while samples from BASTA susceptible plants produced a strong signal with western blot analysis.

Thirty BASTA resistant and 30 susceptible plants, from segregating F_2 families derived from a cross between BATSA resistant T_0 plants with W22, were phenotyped for plant and ear architecture traits. These include lateral branch number, length of the uppermost primary lateral branch, blade length of the first husk leaf of the top ear, number of nodes with prop roots, glume length, the weight of 50 kernels, ear diameter, and ear length. To measure glume length, the kernels were removed from the middle of the ears and then the cobs were broken to expose the glumes for measurement. Eight glumes were measured from each ear and the average value calculated. T-tests were used to evaluate whether the trait values were significantly different between plants with/without the *tga1-RNAi* transgene. Segregating families from four independent transgenic events were assayed.

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