**tassel-less1 Encodes a Boron Channel Protein Required for Inflorescence Development in Maize**

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**Introduction**

It has long been known that the micronutrient boron is essential for vascular plant growth and development, and increasing evidence has shown that it is also essential, or at least beneficial, for a variety of bacterial and animal species (Gauch and Dugger 1954, Dell and Huang 1997, Blevis and Lukaszewski 1998, Goldbach and Wimmer 2007, Tanaka and Fujiwara 2008). This is especially significant in agriculture as boron deficiencies have been reported in at least 80 countries in >130 different crop species (Shorrocks 1997). Symptoms of boron deficiency in plants are far ranging and can be exhibited as a lack of apical dominance, abnormal flower development and fruit/seed set, brittle leaves and stunted plant growth (Blevins and Lukaszewski 1998, Tanaka and Fujiwara 2008, Lordkaew et al. 2011). Boron toxicity is also an issue in some regions of the world including South Australia, the Middle East, the southern coast of Peru, etc. where marine evaporates and marine argillaceous sediment are prevalent (Nable et al. 1997).

The roles boron plays in plant development appear to be varied and complex, as evidenced by the array of phenotypes seen in deficient plants. One of the best understood roles of boron is its involvement in the cross-linking of two rhamnogalacturonan II (RGII) monomers (O’Neill et al. 2004). RGII is a component of pectin, which is important in cell wall structure (O’Neill et al. 2001, O’Neill et al. 2004). This role may therefore explain why boron-deficient plants can have brittle leaves (Blevins and Lukaszewski 1998). Boron has also been shown to be important in plasma membranes. The occurrence of boron deficiency has been associated with aberrant ion fluxes and altered membrane potential resulting from decreased ATPase activity (Cakmak and Römheld 1997). Although the molecular mechanism underlying these observations is not well understood, it is possible that the ability of boron to bind membrane compounds containing cis-diol groups, such as glycoproteins and glycolipids, is involved in membrane structure and, therefore, its function (Cakmak and Römheld 1997). Boron has also been implicated in a number of other plant processes including photosynthesis, metabolism, sugar translocation, nitrogen fixation and others (Gauch and Dugger 1953, Gauch and Dugger 1954, Cakmak and Römheld 1997, Dell and Huang 1997, Blevins and Lukaszewski 1998).

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**tassel-less1** (tls1) is a classical maize (*Zea mays*) inflorescence mutant. Homozygous mutant plants have no tassels or very small tassels, and ear development is also impaired. Using a positional cloning approach, ZmNIP3;1 (a NOD26-like intrinsic protein) was identified as the candidate gene for tls1. The ZmNIP3;1 gene is completely deleted in the tls1 mutant genome. Two Mutator-insertional TUSC alleles of ZmNIP3;1 exhibited tls1-like phenotypes, and allelism tests confirmed that the tls1 gene encodes ZmNIP3;1. Transgenic plants with an RNA interference (RNAi) construct to down-regulate ZmNIP3;1 also showed tls1-like phenotypes, further demonstrating that TLS1 is ZmNIP3;1. Sequence analysis suggests that ZmNIP3;1 is a boron channel protein. Foliar application of boron could rescue the tls1 phenotypes and restore the normal tassel and ear development. Gene expression analysis indicated that in comparison with that of the wild type or tls1 plants treated with boron, the transition from the vegetative to reproductive phase or the development of the floral meristem is impaired in the shoot apical meristem of the tls1 mutant plants. It is concluded that the tls1 mutant phenotypes are caused by impaired boron transport, and boron is essential for inflorescence development in maize.

**Keywords:** Boron • Boron channel protein • Tassel • Tassel-less1 • Zea mays • ZmNIP3;1.

**Abbreviations:** BAC, bacterial artificial chromosome; bd1, branched silkless1; blf2, barren inflorescence2; HMW, high molecular weight; MPSS, massively parallel signature sequencing; NIP, NOD26-like intrinsic protein; RFP, red fluorescent protein; RGII, rhamnogalacturonan II; RNAi, RNA interference; SAM, shoot apical meristem; SNP, single nucleotide polymorphism; tls1, tassel-less1; TUSC, Trait Utility System for Corn.
Perhaps the most important process in which boron plays a role is plant reproduction. Reproductive development can be affected at several different stages as a result of boron deficiency, including inflorescence formation, pollen development and germination, pollen tube growth, and seed and fruit set (Dell and Huang 1997). In addition, the requirement of a plant for boron is generally much higher at this stage than during vegetative growth (Gauch and Dugger 1954). Consistent with this notion, a recent study in maize (Zea mays) showed that plants grown in boron-deficient conditions have depressed grain yield while there is no significant difference in the dry weight of vegetative tissues (Lordkaew et al. 2011). Interestingly, it has been demonstrated that the application of boron increases yield in a number of different species including almond (Nyomora et al. 1997), apple (Wójcik et al. 1999), alfalfa (Dordas 2006), sour cherry (Hanson et al. 2001) and soybean (Schon and Blevins 1990). Despite these important implications, very little is known about the exact role boron plays in reproductive development.

Here, we report the characterization of the *tassel-less1* (*tls1*) mutant. *tls1* is a classical maize inflorescence mutant that was identified as early as 1926 (Woodworth 1926) and, more recently, characterized and mapped (Albertsen et al. 1993). Through a map-based cloning approach, it was determined that TLS1 encodes a NOD26-like intrinsic protein (NIP), ZmNIP3;1. This protein most closely matches boron channel proteins from Arabidopsis and rice. Treating *tls1* plants with a foliar boron spray was able to rescue the phenotype, confirming that TLS1 is related to boron transport and demonstrating that boron is essential for inflorescence development.

### Results

**tls1** plants are defective in reproductive and vegetative growth

The *tls1* mutant has been described previously (Albertsen et al. 1993). In contrast to wild-type plants which display full, well-developed tassels (Fig. 1A), *tls1* mutants exhibit a variety of phenotypes ranging from small tassels (Fig. 1B) to plants that completely lack a tassel (Fig. 1C). Ear development is also impaired in *tls1* mutants. Severe clones can exhibit multiple husks (Fig. 1D) containing very small ears or no ears at all (Fig. 1E). Less severe *tls1* mutants can develop more normal looking ears, albeit smaller than those of the wild type (Fig. 1E). Although visually indistinguishable at early stages of development, mature plant leaves are also affected. The leaves of mutant plants generally appear narrower, but are also thicker, stiffer and more brittle in texture (Fig. 1F). Severe mutants are also normally shorter than less severe mutants and wild-type plants (not shown).

**Candidate gene identification and tls1 reference allele characterization**

A map-based cloning approach was used to identify the gene responsible for the *tls1* phenotype. Seventy-five F2 individuals from *tls1 × Mo17* were phenotyped and genotyped with 15 single nucleotide polymorphism (SNP) markers across the long arm of chromosome 1, which were chosen based on previous mapping results (Albertsen et al. 1993). The identified flanking markers, PHM5484-22 (222,430,393 bp, B73Ref_v2) and PHM10765-46 (225,871,091 bp, B73Ref_v2) were subsequently used to genotype approximately 3,000 F2 individuals to identify recombinants (Fig. 2). The recombinant plants were scored for the *tls1* phenotype, self-pollinated, and approximately 175 ears were harvested. The phenotype of each recombinant was determined or confirmed by progeny test. With the use of additional markers and the recombinant lines, the *tls1* interval was delimited to a five overlapping bacterial artificial chromosome (BAC) interval between markers c0375b06_10 and c0260e13_35 (Fig. 2). This region has a very low level of polymorphism between *tls1* and Mo17, making it difficult to narrow down the interval any further. All 10 annotated genes within this region were sequenced in *tls1* and Mo17, and, one of them, annotated as NOD26-like integral membrane protein/ aquaporin/ZmNIP3;1 (hereafter referred to as ZmNIP3;1), was unamplifiable in mutant individuals. As no major variations were found in the other genes, ZmNIP3;1 became the apparent candidate gene for *tls1*.

Since ZmNIP3;1 was unable to be PCR amplified in mutant individuals, this gene is either completely missing in *tls1* or has major variations between the *tls1* and Mo17 alleles. In order to understand the nature of the mutation, a BAC library was generated from homozygous *tls1* plants and BAC clones spanning the *tls1* interval were sequenced. It was determined that although much of the *tls1* BAC sequence aligned to the B73 reference genome sequences and the flanking markers, c0375b06_10 and c0260e13_35, were present in the *tls1* BAC sequence, there was a large syntenic break between the B73 reference and the *tls1* mutant. This break included highly repetitive sequence that appeared to be unique to *tls1* in comparison with the B73 reference, as well as reference sequence that was absent in the *tls1* mutant. Although the exact sizes of the insertions and deletions could not be determined accurately due to the overall highly repetitive nature of the region, it was clear that the missing reference sequence included the ZmNIP3;1 candidate gene. Therefore, the ZmNIP3;1 gene is completely deleted in *tls1*, which may cause the *tls1* mutant phenotype.

**Validation of the tls1 candidate gene**

To validate ZmNIP3;1 as the candidate gene for *tls1*, two Trait Utility System for Corn (TUSC) lines (Meeley and Briggs 1995), P177F10, containing a Mutator (Mu) insertion in the promoter region of ZmNIP3;1, and P30DS, containing an insertion in exon 2, were identified (Fig. 3A). Individual plants which were homozygous for the Mu insertion exhibited the *tls1* phenotype, with no or small tassels. To confirm the candidate gene further, an allelism test was conducted by crossing plants heterozygous for the Mu insertion to heterozygous *tls1* plants. The resulting
progeny were phenotyped and analyzed by PCR for the presence of a Mu insertion in ZmNIP3;1 and for the presence of at least one copy of the wild-type ZmNIP3;1 allele (Fig. 3B). It was found that the vast majority of plants which contained one copy of the TUSC allele and one copy of the tls1 reference allele exhibited the tls1 phenotype, while the other plants were wild type (Table 1; Fig. 3C), indicating that ZmNIP3;1 and tls1 are allelic. The small number of discrepancies was attributed to misclassification as a result of the incomplete penetrance of the phenotype and the fact that the gene-specific primers used to amplify the wild type ZmNIP3;1 allele do not amplify efficiently in the TUSC line, P177F10. Overall though, the results of the allelism test validate that ZmNIP3;1 is the causative gene for the tls1 mutant phenotype.

As an additional validation of the ZmNIP3;1 candidate gene, transgenic RNA interference (RNAi) plants were produced in order to test whether knocking-down ZmNIP3;1 expression can result in the tls1 phenotype. Since the most obvious
phenotypes in tls1 are in the tassel and ear, a constitutive promoter (maize ubiquitin gene promoter) driving transgene expression was expected to target these two tissues. Twenty individual T0 transgenic plants were generated, which contained single or multiple copies of the transgene. All of these transgenic events were evaluated for their tassel phenotypes. Among these transgenic events, a spectrum of tassel sizes, ranging from tassel-less, to very small or nearly normal tassel sizes, was observed (Fig. 4; Supplementary Table S2). Six transgenic plants that were tassel-less or had very small tassels, had mostly 3–4 copies of the transgene, and tended to have a relatively higher level of transgene expression. Transgenic plants that had larger or near normal tassel growth mostly contained a single copy of the transgene and had no or a very low level of transgene expression (Supplementary Table S2). The level of native ZmNIP3;1 expression in the leaves was too low to determine the efficacy of RNAi silencing of the endogenous gene (data not shown). However, it is likely that higher transgene expression is more effective in silencing endogenous gene expression, resulting in the tassel-less phenotype.

Besides the tassel phenotype, ear growth of the transgenic plants was also affected. The effect on ear growth was largely consistent with the effects on tassel growth. That is, those plants that had a normal tassel also produced a normal ear, whereas those that were tassel-less or had severe tassel

**Table 1** Genotypes and phenotypes of progeny from crosses between the tls1 reference mutant allele and two TUSC alleles

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<td>tls1 phenotype</td>
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<td>Wild-type phenotype</td>
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^a Individuals containing one copy of the Mu-insertional TUSC allele and one copy of tls1 reference allele of ZmNIP3;1 (no amplification of ZmNIP3;1).

^b Individuals containing at least one intact copy of ZmNIP3;1.

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**Fig. 3** Allelism test for candidate gene validation. (A) Locations of Mutator insertion sites in the two TUSC alleles. (B) Diagram of allelism test strategy and expected results. (C) Phenotypes of a wild-type plant and a tls1 plant from the allelism test.
reduction also had no ear or impaired ear growth such as no silk (Supplementary Table S2).

**ZmNIP3;1 is involved in boron transport**

Sequence analysis revealed that the ZmNIP3;1 protein in maize is highly similar to AtNIP5;1 from Arabidopsis and OsNIP3;1 from rice (Fig. 5). All of these proteins are members of the class II NOD26-like intrinsic protein (NIP) family (Liu et al. 2009). NIP II proteins can be permeable to formamide, boric acid, glycerol, urea, arsenite and other large solutes (Wallace and Roberts 2005, Bhattacharjee et al. 2008, Bienert et al. 2008, Liu et al. 2009). Of particular interest, the closely related proteins, AtNIP5;1 and OsNIP3;1, have been implicated in the transport of boron (Takano et al. 2006, Hanaoka and Fujiwara 2007). The root, leaf and flower development of the Arabidopsis nip5;1 mutant is affected by boron limitation (Takano et al. 2006). It is possible that ZmNIP3;1 in maize may also be involved in boron transport. Consistent with this notion, it has been shown that plants grown in boron-deficient conditions exhibit a phenotype that is very reminiscent of tls1 mutants (Lordkaew et al. 2011).

To test the hypothesis that ZmNIP3;1 is involved in boron transport and the tls1 mutant phenotype is due to impaired transport of boron, wild-type and tls1 plants were treated with a foliar boron spray from V2 to V6 and the resulting phenotypes were observed. Wild-type plants treated with the boron spray exhibited no significant differences in comparison with the untreated wild-type plants (data not shown). However, the difference between the treated and untreated tls1 plants was striking. Visually, untreated mutant plants had very small tassels with short branches, or completely lacked a tassel (Fig. 6B), while the mutant plants that were treated with boron had full tassels with many long branches (Fig. 6C) and appeared very similar to the wild type (Fig. 6A). In addition, the ears of mutant plants that were treated with boron appeared similar in size to wild-type ears (Fig. 6D). Quantitatively, although the number of tassel branches was significantly different between wild-type and the treated mutant plants, the mean number of tassel branches in the treated mutant was much closer to the wild-type level than that of the untreated mutant (Table 2; Fig. 6E). The length of the tassel branches was actually significantly greater in the treated mutant plants than in the wild type (Table 2; Fig. 6F), and there was no significant difference in ear length between the sprayed mutant and the wild-type plants (Table 2; Fig. 6G). These results show that boron application to tls1 plants can rescue the mutant phenotype, confirming that ZmNIP3;1 is involved in boron transport and the tls1 phenotype is due to impaired transport of boron. Directly or indirectly, boron plays a crucial role for inflorescence development in maize. Indeed, the soil boron level affects the expression of the tls1 mutant phenotypes, with low boron levels associated with more severe tls1 phenotypes (A. Durbak and P. McSteen, personal communication). Nevertheless, differences in soil boron levels cannot fully explain the variations in tls1 phenotypes, as such variations in expression could be observed even under uniform soil conditions in the greenhouse.

**Fig. 5** Protein sequence alignment of ZmNIP3;1 with its most closely related proteins from rice (OsNIP3;1) and Arabidopsis (AtNIP5;1). ZmNIP3;1 and OsNIP3;1, 88.8% similar and 84.3% identical. ZmNIP3;1 and AtNIP5;1, 76.6% similar and 67.3% identical.
Fig. 6 Effects of boron application on tls1 mutant phenotypes. (A) Wild-type tassel. (B) A tassel of a tls1 plant not treated with boron. (C) A tassel of a tls1 plant treated with boron. (D) Comparison of immature ears from a wild-type plant (WT) and a tls1 plant that was treated with boron (tls1 + B). (E–G) Histograms with the distribution of tassel branch count (E), tassel branch length (F) and ear length (G) of wild-type (WT, 10 individuals), tls1 mutants (29 individuals) and tls1 mutants treated with boron (26 individuals).
Therefore, tls1 represents an example of incomplete penetrance.

To determine if the boron levels in the seeds from the boron-treated tls1 plants could have a maternal effect on the expression of the tls1 phenotype in later generations, the recovered tls1 mutant plants were self-pollinated and progeny were grown out to observe their phenotypes. Half of the plants were untreated and the other half were treated with boron spray as described above. The untreated progeny exhibited the tls1 mutant phenotype, while the tassel phenotype of the boron-treated progeny was similar to that of wild-type plants (data not shown). This indicates that the recovery of the phenotype is not heritable and boron application is necessary at each generation to rescue the phenotype.

Expression analysis

To examine the temporal and spatial expression pattern of the tls1 gene, ZmNIP3;1 was queried in a massively parallel signature sequence (MPSS) database consisting of libraries of 17 bp sequence tags from cDNAs isolated from >200 diverse maize tissues and developmental stages (Muszynski et al. 2006). While ZmNIP3;1 is expressed in floral tissues, most notably in the silks, it is also expressed in other tissue types throughout the plant, albeit at a low level in most tissues (Fig. 7). This may indicate that ZmNIP3;1 functions in multiple stages of plant development in addition to inflorescence development. This notion is consistent with the observation that tls1 plants exhibit aberrations in both vegetative and reproductive growth (Fig. 1) (Albertsen et al. 1993).

An RNA-seq experiment was conducted to identify genes, as well as developmental and biochemical pathways affected by the tls1 mutation. Leaf and the shoot apical meristem (SAM) tissues were taken from V5 tls1 plants, wild-type siblings and tls1 plants that had been treated with the foliar boron spray as described previously. Analysis of the results from the leaf tissue indicated that gene expression was only altered as a result of boron spray treatment, i.e. no notable differences in expression patterns between tls1 mutant plants and the wild-type sibling. However, two interesting classes of genes were observed when examining the results of gene expression in the SAM: genes that were highly expressed in the wild-type and the sprayed mutant plants but expressed at a low level in the untreated tls1 plants (Supplementary Fig. S1A) and genes that were strongly expressed in the tls1 mutant but expressed at a low level in wild-type and treated mutant plants (Supplementary Fig. S1B). A total of 368 genes were expressed at higher levels in the SAM of wild-type and boron-treated tls1 plants than in that of the untreated tls1 mutant plants, with a maximal obtainable fold change between any of the three genotypes (effect size) of at least three (Supplementary Fig. S1A; Supplementary Table S3). Interestingly, five of the genes within the top 10 effect sizes were found to be MADS-box genes involved in inflorescence development (Ambrose et al. 2000, Thompson et al. 2009, Ciaffi et al. 2011, Zhang et al. 2012). It was also found that genes involved in auxin pathways were enriched in this gene set, including barren inflorescence2 (bif2), which regulates axillary meristem development in maize inflorescence.
Discussion

In the current study, we report the cloning of the maize mutant tls1, and determined that the deletion of ZmNIP3;1, a putative boron channel protein, in the tls1 genome is responsible for the mutant phenotypes. Boron application could rescue the tls1 mutant phenotypes, demonstrating that boron is essential for inflorescence development in maize. It has long been known that boron is an essential micronutrient for plants (Warington 1923). In boron-limiting conditions, plants can exhibit a variety of symptoms including stunted plant growth, brittle leaves and shoots, impaired inflorescence development, and poor fruit and seed set. Because of the number of different phenotypes displayed by boron-deprived plants, the exact role that boron plays has been difficult to elucidate. Despite the uncertainties, it is known for many diverse plant species that the boron requirement is much higher in reproductive stages (Gauch and Dugger 1954, Shorrocks 1997, Blevins and Lukaszewski 1998, Lordkaew et al. 2011). These notions were supported by the current study. It is interesting to note that the expression analysis indicated that ZmNIP3;1 is expressed most strongly in the silks (Fig. 7), which may be indicative of an exceptionally strong requirement for boron in this tissue type. If, in other species, analogous tissue types express a ZmNIP3;1 homolog most strongly as well, this may explain why treating plants with boron increases seed and fruit yield and quality (Schon and Blevins 1990, Hanson 1991, Nyomora et al. 1997, Wójcik et al. 1999, Perica et al. 2001, Dordas 2006).

In Arabidopsis, two NIP II proteins have been identified which have been implicated in boron transport. AtNIP5;1 is important for boron uptake during boron-limiting conditions. It could also facilitate the transport of water (Tanaka et al. 2006). AtNIP6;1 is also involved in boron transport but is impermeable to water (Tanaka et al. 2008). Interestingly, these two boron transporters were found to be expressed in different tissue types; AtNIP5;1 was expressed in roots while AtNIP6;1 was expressed predominantly in nodal regions of shoots, especially the phloem region of vascular tissues (Tanaka et al. 2006, Tanaka et al. 2008). In the present study, it was found that ZmNIP3;1 is involved in boron transport (Fig. 6) and is expressed in multiple tissue types (Fig. 7). These analyses, together with the lack of a close maize homolog of AtNIP5;1, may suggest that ZmNIP3;1 is functionally equivalent to AtNIP5;1 and AtNIP6;1 from Arabidopsis. Indeed, a phylogenetic analysis of NIPs in plants indicated that both AtNIP5;1 and AtNIP6;1 are closely related to ZmNIP3;1 (Liu et al. 2009).

In this study, an RNA-seq experiment was conducted to identify genes and biological processes affected by the tls1 mutation to help determine the role boron plays in inflorescence development. Rather than identifying one main pathway, many genes involved in inflorescence development were induced in the SAM of boron-treated tls1 plants as compared with the untreated mutant (Supplementary Table S3). In addition to several MADS-box genes involved in inflorescence development (Ambrose et al. 2000, Thompson et al. 2009, Ciaffi et al. 2011, Zhang et al. 2012), other important inflorescence genes were also induced including: bif2, involved in auxin transport (Wu and McSteen 2007); the photoperiod proteins, CONSTANS and FLOWERING LOCUS T (Turck et al. 2008); and branched silkless1, a transcription factor involved in spikelet meristem identity (Chuck et al. 2002), among others. On the other hand, genes involved in vegetative processes, such as photosynthesis, are expressed at higher levels in the SAM of untreated tls1 plants as compared with those of boron-treated tls1 and wild-type plants (Supplementary Table S4). It seems that the transition from vegetative to reproductive phase or the development of the floral meristem is impaired in the SAM of the tls1 mutant plants.

Although the exact nature of the involvement of boron in inflorescence development cannot yet be determined, the current study may have important agricultural implications. In the present study, it was shown that ZmNIP3;1 can be down-regulated transgenically and that the level of RNAi transgene expression may correlate with the severity of the tls1 phenotype (Fig. 4; Supplementary Table S2). Down-regulation of ZmNIP3;1 in a tissue-specific manner may result in plants that lack tassels but have fewer pleiotropic effects associated with tls1 plants, specifically plants with normal ears. This tassel suppression would probably result in the reallocation to the ear of resources that would normally be needed for tassel development (Duncan et al. 1967, Hunter et al. 1969). These plants would be expected to perform better under normal and stress conditions, such as nitrogen or water stress. They may also benefit from reduced shading from tassels (i.e. increased rate of photosynthesis) normally experienced by plants (Duncan et al. 1967, Hunter et al. 1969). In addition, since foliar boron applications could rescue the tls1 mutant phenotypes, this scheme could be employed to maintain tls1 lines in a seed production setting.

Materials and Methods

Plant material and map-based cloning of tls1

The tls1 mutant was obtained from Marc Albertsen (Albertsen et al. 1993). An F2 mapping population was generated by...
crossing homozygous tassel-less (tls1) plants to the maize inbred line Mo17. Based on previous mapping results (Albertsen et al. 1993), 75 F2 individuals were genotyped with 15 SNP markers distributed across the long arm of chromosome 1 to identify markers flanking tls1. The two flanking markers, PHM5484-22 and PHM10765-46, were then used to screen approximately 3,000 F2 individuals to identify recombinants. Recombinant individuals were self-pollinated whenever possible and approximately 175 F3 ears were harvested. The F3 families were grown and phenotyped to confirm the phenotype of each F2 line. Tissues from single F2 plants or pools of eight F3 individuals from each family were used for DNA isolation and genotyping. Additional markers were developed from the tls1 interval and fine mapping was carried out as described (Jiang et al. 2012). All marker and primer information is listed in Supplementary Table S1.

Allelism test for candidate gene validation

To validate the tls1 candidate gene, two lines with a Mu insertion in ZmNIP3;1 were identified from the TUSC population (Meeley and Briggs 1995). Heterozygous TUSC individuals were crossed to heterozygous tls1 individuals for an allelism test (Fig. 3B). The progeny were classified as being either positive or negative for a Mu insertion as well as being with or without the wild-type ZmNIP3;1 allele. The presence of a Mu insertion was determined by PCR using a Mu-specific primer in combination with a ZmNIP3;1-specific primer, and the presence of the wild-type ZmNIP3;1 allele was determined by PCR using gene-specific primers (Fig. 3B). Gene-specific primers to test for the presence of the wild-type ZmNIP3;1 allele were designed such that they did not amplify ZmNIP3;1 efficiently in the TUSC background or a Mu insertion would prevent amplification. All primers are listed in Supplementary Table S1.

BAC library construction and screening

Leaves from approximately 200 etiolated tls1 seedlings were harvested and fast-frozen. High molecular weight (HMW) DNA was isolated as described (Luo and Wing 2003), with some modifications. The resulting agarose-embedded HMW DNA was partially digested with HindIII and a BAC library was constructed on the pCC1BAC vector, following the manufacturer’s recommendations (Epigen). A total of 73,728 recombinant colonies were picked in 384-well plates and grided at high density in a Hybond-N+ charged nylon membrane. Clones were screened and identified by hybridization using immunological detection of PCR digoxigenin (DIG)-labeled probes (Roche) according to the manufacturer’s protocol. Positive BACs were retrieved and confirmed by direct plus/minus colony PCR. Probes specific for the tls1 region were labeled from PCR fragments amplified using the primers listed in Supplementary Table S1.

BAC de novo sequencing

BAC DNA from positive clones was purified using the Qiagen Large Construct system. Sequencing random shear sublibraries were constructed on pBluescript SK+ following standard procedures (Song et al. 2001). Plasmids were amplified directly from 70 μl arrayed single-colony cultures using Templiphi (GE Healthcare Life Sciences) and sequenced in an ABI 3730XL DNA sequencer using ABI PRISM BigDye (Applied BioSystems). Base calling, quality assessment, assembly and validation were performed using the phred, phrap and expgap software (Ewing and Green 1998).

Phenotype rescue with boron spray

Wild-type and homozygous tls1 mutant individuals from the F2 mapping population were selfed to increase the seed, and the resulting F3 progeny were used for this analysis. Half of the individuals from each genotype were left untreated, while the other half were treated once a week from V2 to V6 with a foliar boron spray containing 1.2 g l−1 of a soluble boron powder consisting of 62% B2O3 and 20.5% elemental boron (www.jrjohnson.com, SKU: 19-0031). The boron solution was applied to the plants via a spray bottle until liquid was present on the upper surface of all the leaves. Recovered mutant plants were self-pollinated and seed was collected for a progeny test in which half the plants were untreated and the other half were treated with boron spray in the manner described above.

RNA-seq experiment and data analysis

Plants from the F2 mapping population of tls1 crossed to Mo17 were used for gene expression analysis. Twelve plants each of the tls1 mutant, the wild type and the tls1 mutant treated with boron spray were grown to the V5 stage. The treated tls1 mutant plants were sprayed from V2 until the time of sampling as described above. Three tissue types were sampled: the youngest fully expanded leaf, immature leaves (not fully expanded) and the SAM. At sampling time, for both leaf tissue types, three individuals from each genotype/treatment type were pooled together, resulting in four biological replicates. For the SAM, all 12 individuals for each genotype/treatment were sampled together in order to obtain enough tissue for analysis. All tissue was sampled and immediately frozen on dry ice. The tissue was then used for RNA-seq and further analysis.

Total RNAs were isolated from frozen maize (Z. mays) leaf and floral tissues by use of the Qiagen RNeasy kit for total RNA isolation (Qiagen). Sequencing libraries from the resulting total RNAs were prepared using the TruSeq mRNA-Seq kit and protocol from Illumina, Inc. and sequenced on the Illumina HiSeq 2000 system with Illumina TruSeq SBS v3 reagents. On average, 10 million 50 bp sequences were generated for each sample. The resulting sequences were trimmed based on quality scores and bowtie aligned (Langmead et al. 2009) to a DuPont Pioneer proprietary maize gene set and normalized to RPKtM (Mortazavi et al. 2008).

The generated RPKtM data matrix was visualized and analyzed in GeneData Analyst software. Specifically, two algorithms in the Analyst were applied: effect size to identify differentially
expressed genes because of lack of replicates, and K-mean clustering to identify genes with specific expression patterns. Function enrichment analysis was performed with Gene Ontology Fisher’s exact test in the Analyst and Pathway Studio (Ariadne Genomics, Inc.) to identify significantly over-represented gene groups (biological processes or metabolic/signal transduction pathways).

**T-DNA constructs and maize transformation**

T-DNA constructs and plant transformation Gateway technology (Invitrogen) were used for vector construction. An RNAi vector was made to silence the ZmNIP3;1 expression. The vector was designed such that a ZmNIP3;1/ADH1 intron/ZmNIP3;1 (reverse complementary) hairpin composed of 1,678 bp of the ZmNIP3;1 transcript (the sequence is given in the Supplementary data) was integrated between the maize ubiquitin promoter (ProZmUBI) and the Sorghum bicolor gamma kafarin (GKAF) terminator and co-integrated with JT vectors using Gateway technology as previously described (Unger et al. 2001, Cigan et al. 2005). The plasmid also contained a red transfer protein, PromHvLTP2 (Opsahl-Sorteberg et al. 2004) to transform maize embryos of GS3xGaspe Flint from DuPont Pioneer germplasm, a cross between the Hi-II and Gaspe Flint. Independent transgenic events were generated, and the copy number of transgene insertion was determined based upon the DNA copy number of the RFP vector. For real-time PCR analysis to measure transgene expression, a Taqman reverse transcription kit (Applied Biosystems) was used as described (Guo et al. 2010). The PCR primers used for transgene expression are listed in Supplementary Table S1. Transgene expression levels of ZmNIP3;1 were measured relative to the endogenous reference elF4g, a maize eukaryotic initiation factor gene (GenBank accession No. NP_568534) with the ΔCt method as described by the manufacturer.

**Supplementary data**

Supplementary data are available at PCP online.

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**Disclosures**

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**References**


