Q^{Dtbn1}, an F-box gene affecting maize tassel branch number by a dominant model

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Summary

Tassel branch number (TBN) is one of the important agronomic traits that directly contribute to grain yield in maize (Zea mays L.), and identification of genes precisely regulating TBN in the parental lines is important for maize hybrid breeding. In this study, a quantitative trait nucleotide (QTN), Q^{Dtbn1}, related to tassel branch number was identified using a testcrossing association mapping population through association mapping with the Indels/SNPs in the 5'-UTR (untranslated region) of Zm00001d055358, which encodes a Kelch repeat-containing F-box protein. Q^{Dtbn1} was further confirmed to be associated with TBN by a dominant model using an F2 population, and over-expression of the candidate gene resulted in a decreasing of TBN, implying that Q^{Dtbn1} was governed by the candidate gene with a negative model. This makes Q^{Dtbn1} very useful in maize hybrid breeding. Q^{Dtbn1} could interact with a maize Skp1-like protein and a SnRK1 protein, and the SnRK1 could also interact with a SnRK2.8 protein. In addition, quantitative real-time PCR assay showed that five substrates of SnRK2 were down-regulated in the over-expressed plants. These imply that the SCF (Skp1/Cul1/F-box protein/Roc1) complex and ABA signal pathway might be involved in the modulation of TBN in maize.

Keywords: tassel branch number, F-box protein, dominant model, overexpression, maize.

Introduction

Single cross F1 hybrids are extensively used to take advantage of heterosis in maize production. Tassel branch number (TBN), a main trait related to architecture of tassel, is one of the important agronomic traits associated with yield (Duvick and Cassman, 1999). The relationship between TBN and maize yield is complex. On one hand, smaller tassels, such as TBN, are shown to be negatively associated with grain yield (Brown et al., 2011; Geraldi et al., 1985; Lambert and Johnson, 1978). On the other hand, to ensure high quality seed production, the ideal male parent should have a relatively large tassel that sheds enough pollens over a long period of time. Thus, identification and characterization of genes precisely controlling tassel traits is important for genetic improvement of maize yield.

As a quantitative trait, a number of quantitative trait loci (QTLs) for TBN have been mapped with different populations (Brown et al., 2011). A large F2 maize population constructed from the maize inbred lines, chang7–2 and 787, was used to map tassel architecture-related QTLs, and seven QTLs for TBN were identified (Chen et al., 2014). Using a maize backcross population, a QTL for inflorescence architecture was detected in bins 7.02 and 9.02 (Upadayayula et al., 2006a). Recently, using a BC1S1 population of 123 lines and a RIL population of 238 lines, some QTLs for TBN have been identified on chromosomes 2, 3, 5, 7, 8 and 10 (Chen et al., 2014). To circumvent the limitation of parents, researchers used other populations to identify QTLs or quantitative trait nucleotides (QTNs) for TBN. Yang et al. (2014) identified 30 QTNs for maize TBN using a genome-wide association study (GWAS) based on a nonparametric model. Using a combination of linkage mapping and GWAS, Wu et al. (2016) dissected some QTLs and QTNs for tassel traits and identified 503 candidate genes, which included 24 known genes that regulate tassel traits. A total of 19 QTLs associated with TBN were detected with an association population and doubled haploid lines (Wang et al., 2019). However, majority of these QTLs have small effects, and they cannot be easily and directly used in molecular breeding.

The development of plant inflorescence is complicate and governed by multiple genes and biological processes. Based on the genetic analysis of mutants, some key genes involved in the development of TBN have identified in maize. For instance, ramosa mutants, ramosa1 (ra1), ramosa2 (ra2) and ramosa3 (ra3) increased the number of maize tassel branch, and ramosa gene plays an important role in regulating meristem of inflorescence branch in maize (Claeys et al., 2019; Vollbrecht et al., 2005). thick tassel dwarf 1 (td1) and fasciiculated ear 2 (fe2) also showed the increase of tassel branch number and a large number of spikelets, and they are Arabidopsis gene CLAVATA1 (CLV1) and CLV2’s homologues, respectively (Bommert et al., 2005; Taguchi-Shiobara et al., 2001). In addition, mutants with reduced TBN in several genes, such as baren stalk1 (ba1) (Gallavotti et al., 2004), barren inflorescence2 (bif2) (McSteen et al., 2007; Skirpan et al., 2009), teosinte brachched 1 (tb1) (Wang et al., 2005), liguleless 2 (lg2) (Walsh et al., 1998), unbranched 2 (ub2), ub3 (Chuck et al., 2014) and ub4 (Li et al., 2019) have also been identified. These cloned genes will enable identification of favourable alleles, which can rapidly alter inflorescence architecture and be used in molecular breeding.
The ubiquitin-proteasome system (UPS) plays roles in the degradation of targeted proteins, and it is related to many cellular processes (Smalle and Vierstra, 2004; Sorokin et al., 2009). The E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases are involved in the ubiquitination reactions. F-box proteins are one of the major components of SCF (Skp1/Cul1/F-box protein/Roc1) complex, which belongs to a major type of E3 ubiquitin ligases (Ho et al., 2006). SnRK (SNF1-related protein kinase), the plant orthologue of yeast sucrose non-fermenting 1 (SNF1) and mammalian AMP-activated protein kinase (AMPK), plays important roles in plants (Baena-Gonzalez et al., 2007; Baena-González and Sheen, 2008; Nukarinen et al., 2016). Previous reports indicated that F-box proteins can interacted with SnRKs, and SnRKs were involved in the plant abiotic stress response via abscisic acid (ABA) responsive signal (Li et al., 2000; Mustilli et al., 2002; Umezawa et al., 2004; Yoshida et al., 2002). In rice, LARGER PANICLE (LP) encodes a kelch repeat-containing F-box protein, which could interact with rice SKp1-like proteins, and two mutants of this gene produced more inflorescence branches and grain yield (Li et al., 2011). In this study, a QTN for TBN was identified through association mapping in maize, and the candidate gene is a homologue of LP. Genetic study revealed that it negatively regulated TBN with a dominant model. Thus this QTN was designated as QDmtn1. QDmtn1 may regulate the downstream genes of SnRK2 through SnRK1, and participate in ABA signaling pathway. The utility of this QTN in precisely controlling TBN in maize hybrids and their parental lines was also discussed.

Results

Identification of a QTN controlling TBN via candidate gene association mapping with a testcrossing population

TBN was investigated in a testcrossing association mapping panel in two environments, Hainan and Wuhan, which had been used to identify the loci for fertility restoration to CMS-S (Feng et al., 2015). In a preliminary assay with t-test, a QTN (chr4:227,485,251 B73_RefGen4), designated as QDmtn1, was detected significantly associated with TBN in both environments (Figure S1). QDmtn1 located in the 5' UTR (untranslated region) of Zm00001d053338, which encodes an F-box/kelch-repeat protein. To validate and accurately identify the association site, part of this gene produced more inflorescence branches and grain yield. In this study, a QTN for TBN was identified through association mapping in maize, and the candidate gene is a homologue of LP. Genetic study revealed that it negatively regulated TBN with a dominant model. Thus this QTN was designated as QDmtn1. QDmtn1 may regulate the downstream genes of SnRK2 through SnRK1, and participate in ABA signaling pathway. The utility of this QTN in precisely controlling TBN in maize hybrids and their parental lines was also discussed.

Expression of QDmtn1 is significantly different in the tassels at the stages of V8, V10 and V12 between the maize inbreds B73 and HZ4

Expression pattern of QDmtn1 in different tissues from B73 and HZ4 was examined. The results showed that it was highly expressed in young tassels of the two inbred lines. In comparison with HZ4, expression level of QDmtn1 was significantly higher in the tassels of B73 at the stages of V8, V10 and V12. This is consistent with the fact that TBN of B73 was significantly lower than that in HZ4 (Figure 2), and QDmtn1 is a negative regulator of TBN.

The Indel 4 in the 5' UTR is important for the expression of QDmtn1

Promoter sequences in B73, HZ4 and two truncated sequences of the B73 promoter were cloned to construct promoter-activity vector (pGreenII0800). The two truncated sequences included B73 (ΔIndel4) (only truncated Indel 4) and B73.ΔIndel4 + SNP4) (truncated Indel 4 and SNP4) (Figure 3). According the result of LUC/REN (ratio of Firefly Luciferase (LUC) to Renilla luciferase (REN)) assay, the promoter activity of QDmtn1 in B73 was significantly higher than that in HZ4, this is agree with the result presented in the Figure 2. Compared with B73, the activity of the promoter with truncated Indel4 was significantly increased, while the activity of B73.ΔIndel4 + SNP4) was significantly decreased. This is also agreed with the fact that inbred lines harbouring alleles with short length of poly (dA:dT) at the locus of Indel4 had

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Therefore, the length of poly (dA:dT) at Indel4 is important to the expression of \( Q_{Dtbn1} \). However, whether the activity decreasing of B73 (D\(^{Indel4}\) + SNP4) was caused by SNP4 or by too short of the promoter needs further study.

Overexpression of \( Q_{Dtbn1} \) in maize confirmed its function on TBN

To further confirm the function of \( Q_{Dtbn1} \), it was over-expressed (OE) in a maize inbred line C01 (provided by Science and Technology Center, China National Seed Group Co. LTD). Three independent OE lines with significantly higher expression level of the candidate gene (Figure S5) were selected to investigate the agronomic traits in the T2 and T3 generations under different environments. Among the eight traits investigated, TBN showed significant difference between all the OE-plants with their negative controls in the generation of T2 in the spring of 2019 (Figure 4) (Figure S6). Significant difference was only detected in one or two OE-plants for the primary branch number, secondary branch number, plant height, stem diameter, kernel row number and kernel number per row. Therefore, only tassel traits were investigated in the subsequent field experiments.

In the autumn of 2019, the three tassel traits were investigated in the lines of the two generations. The values of TBN, primary branch and second branch number in the three OE-plants were significantly less than that observed in their negative controls in the generations of T2 and T3 with an exception of second branch number.

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**Figure 1** Candidate gene association analysis and gene structure of the candidate gene, Zm00001d053358 (a) associations of the ten SNPs/Indels with TBN in the two environments, Wuhan and Hainan. The dotted green line is the threshold of \( -\log_{10}(P\text{ Value}) > 2.30 \) (\( \alpha = 0.05 \)). (b) gene structure of Zm00001d053358. Positions of the two significant SNP/Indel identified in both of the environments are also show.

**Table 1** Performance of the four agronomic traits in the F\(_2\) population and their parents

| Traits\(^*\) | Parentsb | Individuals in the F\(_2\) populationc |
|---|---|---|
| | B73 (GG) | HZ4 (AA) | AA | AG | GG |
| TBN | 7.30 ± 1.03 | 12.9 ± 2.07** | 14.26 ± 4.07 A | 12.16 ± 3.70 B | 12.07 ± 4.07 B |
| | 6.72 ± 1.38 | 9.65 ± 3.55** | 9.78 ± 3.35 A | 8.41 ± 3.44 B | 8.39 ± 3.06 B |
| EH | 86.58 ± 10.45 | 53.5 ± 6.91** | 94.75 ± 19.27 a | 91.77 ± 19.85 a | 96.75 ± 17.87 a |
| | 55.92 ± 6.91 | 47.42 ± 6.10** | 70.21 ± 16.33 a | 72.03 ± 20.73 a | 73.45 ± 19.77 a |
| PH | 206.3 ± 34.47 | 123.9 ± 17.67** | 206.40 ± 26.29 a | 203.10 ± 27.24 a | 206.65 ± 28.63 a |
| | 148.04 ± 30.69 | 123 ± 17.70** | 194.72 ± 42.09 a | 192.36 ± 51.75 a | 198.61 ± 49.70 a |
| SD | 4.39 ± 0.44 | 2.71 ± 0.33** | 3.96 ± 0.64 a | 4.04 ± 0.56 a | 4.02 ± 0.65 a |
| | 3.88 ± 0.60 | 2.53 ± 0.47** | 3.63 ± 0.67 a | 3.77 ± 0.63 a | 3.80 ± 0.73 a |

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\( \text{TBN} \)-tassel branch number; EH-ear height (cm); PH-plant height (cm); SD-stem diameter (cm); the data of the traits are presented as mean ± standard deviation.

**\( *\)** means the difference between the two parents is significant at the level of \( P < 0.01 \). The data in the upper and lower rows of each trait is form the field trial in 2018 and 2020, respectively.

**\( \text{c} \)** Different letter with lowercase and uppercase behind the means indicated that the difference is at the level of \( P < 0.05 \) and \( P < 0.01 \), res.
number in OE-4 in the T3 generation (Figure 5). These results indicate that the candidate gene negatively controlled TBN in maize.

QDtbn1 could interact with a member of SCF complex system and SnRK1

Yeast two-hybrid (Y2H) screening was conducted to identify QDtbn1 interacting proteins. A cDNA library from maize panicle was used as the prey, and the full-length QDtbn1 was used as the bait. After screening 2 × 106 yeast transformants, five positive clones were obtained (Table S3). Interestingly, three out of the five clones were found to encode the maize SKP1-like protein 1. To verify these interactions, the full-length coding sequences of SKP1-like protein 1 were cloned into pGADT7, and the full-length QDtbn1 was cloned into pGBKKT7. The results revealed that QDtbn1 interacted with the one of the SKP1-like proteins (Zm00001d049230) (Figure 6a). To further validate the interaction, bimolecular-fluorescence complementation (BiFC) assay was conducted using maize protoplasts. GFP signal was detected in the over-expressed lines, while a gene related to ROS homeostasis, RNA processing and miRNA regulation was compared between the OE-plants and their control. Of them, Zm00001d025899 and Zm00001d052613 (homologous to XRN3), Zm00001d046645 and Zm00001d031680 (homologous to MO35), Zm00001d007426 (homologous to RBOH) were down-regulated in the over-expressed lines, while a gene related to ROS homeostasis (Zm00001d045538, homologous to FSD2) was unaffected (Figure 8).

Discussion

Tassel branch is of a complex trait, and multiple genes involved in this biological processes. Genes (ra1, ra2, ra3, fea2, td1, tb1, bd1 and so on) and a number of QTLs (about 124) for tassel branches identified with mutants or various populations have been greatly increased the understanding of its development in maize (Berke and Rocheford, 1999; Mickelson et al., 2002; Upadayayula et al., 2006a, b; Briggs et al., 2007; Chen et al., 2014, 2017; Yang et al. (2014; Wu et al., 2019; Yi et al., 2018). Particularly, maize has rich genetic diversity and rapid linkage disequilibrium attenuation. Maize inbred lines were ideal materials for genome-wide association analysis (Walbot, 2004), and this help to understand the genetic basis of important agronomic traits including TBN. In this study, only one QTN for TBN (QDtbn1) was identified with a testcrossing association mapping population. This indicates that the efficiency of GWAS with testcrossing population is very low, especially for QTNs with additive effects. In addition, the association analysis and the LUC/REN assay revealed the Indel4 with poly (da:dT) in the 5'-UTR is important for the expression of QDtbn1. Poly (da:dT) sequence is very high rigid and can inhibit nucleosome formation (Struhl and Segal, 2013). It was found that the number, length and integrity of poly (da:dT) sequences were related to the nucleosome deletion in promoter region (Hughes et al., 2012). Nucleosomes not only participate in the packaging of DNA, but also influence the initiation of transcription elements such as transcription factors and RNA polymerase II by their location in the genome, and then participate in gene transcription regulation. Thus it should have an important effect on gene transcriptional regulation (Iyer and Struhl, 1995), post transcriptional inhibition and other histone post translation modifications (Bi et al., 2004). In this study, over-expressed QDtbn1 in C01 confirmed that it negatively controlled TBN, and this also implied that TBN would be affected by expression level of QDtbn1.

QDtbn1 belongs to the S8 group of the F-box protein family (Jia et al., 2013), and this F-box gene is high homologous to the rice LARGER PANICLE (LP), which encoding a kelch repeat-containing F-box protein. In rice, LP could interact with SKP1 and formed a SCF complex, and OsCKX2, which encodes cytokinin oxidase/dehydrogenase, was down-regulated evidently in lp. This caused the mutants producing more inflorescence branches, especially the primary branches (Li et al., 2011). Extensive studies have demonstrated that cytokinin is essential in panicle formation (Zhang and Yuan, 2014). For instance, Grain number 1a (Gn1a) encodes cytokinin oxidase 2 (OsCKX2), which is responsible for cytokinin degradation. Accumulation of cytokinin in natural allelic variation of Glna leads to increased panicle branches and grain yield (Ashikari et al., 2005). In maize, QDtbn1 could interact with SKP1, implying that this F-box gene affecting TBN might be also via a SCF complex and CKX genes in maize. Gu et al. (2010) identified 13 ZmCKXs in maize, and OsCKX2 is homologous to ZmCKX5 (Zm000001d008862), OsCKX1 is homologous to six genes served as SnRK2 substrates and involved in ROS homeostasis, RNA processing and miRNA regulation was compared between the OE-plants and their control. Of them, Zm00001d025899 and Zm00001d052613 (homologous to XRN3), Zm00001d046645 and Zm00001d031680 (homologous to MO35), Zm00001d007426 (homologous to RBOH) were down-regulated in the over-expressed lines, while a gene related to ROS homeostasis (Zm00001d045538, homologous to FSD2) was unaffected (Figure 8).
ZmCKX1 (Zm00001d039520), ZmCKX7 (Zm00001d053578), and ZmCKX4 (Zm00001d043293). However, these ZmCKX genes were not significantly affected in the OE-plants (data not shown), thus the ZmCKXs maybe not involved in the Q\textsuperscript{Dtb1-1} mediated development of TBN. SnRK1 is related to glucose and ABA signaling pathways, and plays a central role in metabolic regulation (Halford and Hey, 2009). SnRK2 is a plant specific subfamily, and it plays a key role in plant response to abiotic stress and nutrient restriction in an ABA dependent and independent manner (Fuji and Zhu, 2009; Leung et al., 1997). In this study,
QDtbn1 was found to directly interact with a SnRK1 protein and a Skp1-like protein, and the SnRK1 could interact with a SnRK2.8 protein. In addition, five substrates of SnRK2 were found significantly decreased in the OE-plants. Belda-Palazon et al. (2020) found that in Arabidopsis lack of SnRK1 affects specific ABA responses, and snrk1a1 mutation clearly enhanced the ABA insensitivity of the snrk2d mutation. In Arabidopsis, SnRK2.8 could be induced by ABA in Arabidopsis (Wu et al., 2019), and a F-box gene, AtPP2-B11, could degrade SnRK2.3 to attenuated ABA signaling and the abiotic stress response (Cheng et al., 2017). These indicate that the ABA signal pathway would be involved in the QDtbn1 mediated TBN development in maize. Under optimal conditions, SnRK2s and PP2Cs (phosphatase 2Cs) are required for the formation of SnRK1 repressor complexes, which are important for normal growth in Arabidopsis by preventing it from interaction with the growth-promoting target of rapamycin (TOR). While under stress conditions, ABA causing disassembly of the complexes would release SnRKs, then trigger stress responses and inhibit growth (Belda-Palazon et al., 2020). It can be inferred that as a member of SCF, QDtbn1 could interact with the Skp1-like protein and form a SCF complex, then interact with and degrade SnRK1, and subsequently disassemble the SnRK1-SnRK2-PP2C complexes and release SnRK2s. Thus, over-expression of QDtbn1 would eventually lead to the decrease of TBN in maize.

Single cross F1 hybrids are extensively used in maize. The relationship between TBN and maize yield is complex (Duvick and Cassman, 1999). Since TBN is negatively associated with grain yield (Brown et al., 2011; Lambert and Johnson, 1978), the hybrids should have small tassels. However, it is a contrast to the request of the male parents in hybrids production. In this study, the F-box gene, QDtbn1 negatively regulated TBN via a dominant model. Thus utility of this gene would provide an opportunity to precisely control TBN in maize hybrids and theirs parents,

Figure 5  Performance of the three tassel traits in the OE-plants and their negative controls in the T2 or T3 generation growing in the autumn of 2019. a–c: Tassel traits investigated in the lines in the T2 generation. d–f: Tassel traits investigated in the lines in the T3 generation. Student t-test was performed between the negative controls and the three OE lines, respectively. Single and double asterisks represent significance difference determined by the Student’s t-test at P < 0.05 and P < 0.01, respectively. WT: negative control. OE-4/8/12: Over-expressed F-box gene line.
respectively. For instance, maize inbred lines with dominant (AA) and recessive alleles (aa) of $Q_{Dtbn1}$ could be selected and used as female and male parents, respectively. This would reduce TBN in the hybrids (Aa), and increase TBN in the male parent (aa), simultaneously (Figure 9).

**Materials and methods**

Plant materials, field experiments and trait measurements

**Population for GWAS**

The testcrossing population was constructed using maize inbred line Mo17 as female parent and an association panel including 513 diverse inbred lines as male parents in 2012 in the experimental station of Huazhong Agricultural University, Wuhan, China. The testcrossing hybrids with sufficient seeds were selected for GWAS. The inbred lines were genotyped with 556,809 SNPs, and the testcrossing hybrids were planted in three environments following a randomized complete block design with three replicates, and ten plants were grown in each one-row replicate. Field management was applied following local agronomic practice.

**Population for linkage analysis**

An F$_2$ population derived from a cross between maize inbred line HZ4 and B73 were grown in the experimental station of Huazhong Agricultural University, Wuhan, China in the spring of 2018 (230 individuals) and the autumn of 2020 (230 individuals). The two parents, HZ4 and B73, were grown following a randomized complete block design with three replicates, and ten plants were grown in each one-row plot. Field management was applied according to local agronomic practice.

**Field experiments and trait measurements for the overexpression lines**

Three OE lines (Figure S5) and their negative controls in the T2 and T3 generation were planted in the experimental station of Huazhong Agricultural University (Wuhan, China) in the spring and autumn of 2019, respectively. The field experiments were arranged according to a randomized complete block design with three replicates, and ten plants were planted in each one-row replicate. Field management was applied following local agronomic practice.

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**Figure 6** Interaction assay between $Q_{Dtbn1}$ with the Skp1-like protein. (a) Yeast two-hybrid assays between $Q_{Dtbn1}$ (F-box) and the SKP1 protein. 49230 is the abbreviation of Zm00001d049230. pGBK7-T53/pGAD77-T as the positive control, pGBK7-LAM/pGAD77-T as the negative control. (b) BiFC assay between $Q_{Dtbn1}$ (F-box) and the Skp1 protein. The first and second panel showing images of Nicotiana benthamiana leaves injected with construct expressing F-box-GFPYN (173) and Skp1-GFPYC (155) (Zm00001d049230). Bottom panel showing images of Nicotiana benthamiana leaves injected with construct expressing F-box-GFPYN (173) and YN155 vector.
agronomic practice. Plant height (PH, cm), ear height (EH, cm), stem diameter (SD, cm), primary branch number (PBN), secondary branch number (SBN), tassel branch number (TBN = PBN + SBN), tassel branch angle (TBA, °), kernel number per row (KNR) and kernel row number (KRN) were measured on five plants per row after genotyped.

Candidate gene association mapping and phylogenetic tree construction
Part of the 5'-UTR (~700 bp) of the candidate gene in the association panel (Feng et al., 2015) was amplified and sequenced. Candidate gene association mapping was conducted with 250 maize inbred lines, of which high quality sequences were obtained. A total of ten SNPs/Indels with minor allele frequency (MAF) > 0.10 were used in the analysis. The MLM model (Q + K) was selected for detection of SNPs significantly associated with TBN using the Tassel 3.0 program (Bradbury et al., 2007).

Phylogenetic tree was drawn with the neighbour-joining method using software MEGA 6 (https://www.megasoftware.net). The protein sequences were downloaded from the Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/) and the Rice Genome Annotation Project websites (http://rice.plantbiology.msu.edu/index.shtml).

RNA extraction and qPCR analysis
Ears, tassels and leaves were sampled from HZ4 and B73 at the stages with eight (V8), ten (V10) and twelve (V12) leaves, respectively. Root and leaf samples were collected from HZ4 and B73 at the one-leaf stage (V1). Three biological replicates were sampled for each tissue. All of the samples were immediately stored at −70°C until use. Total RNA was extracted using TRIZOL. Integrity and concentration of the RNA samples were measured with Agilent2010 (Agilent, Santa Clara, CA). First-strand cDNA was synthesized using TransScript® II One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) based on the manufacturer’s instructions. Primers were designed using the online tool Primer3.0, and the primer sequences are included in Table S2. The qPCR was performed in a 20 μL system for each sample with the Bio-Rad CFX Manager3.0 system (BIO-RAD, USA). The 20 μL qPCR mixture included 6 μL of diluted cDNA, 10 μL of 2×Sybr Green qPCR Mix, and 0.5 μL of each of the 10 μM primers. The amplification was performed as follows: 95 °C for 2 min followed by 40 cycles of 95 °C for 20 s, 62 °C for 20 s and 72 °C for 30 s. The maize Actin gene (LOC1000282267) was served as the internal control. The Ct value of each sample was calculated, and the relative expression level of each gene was calculated as 2−ΔΔCt. The qPCR was conducted with three technical replicates for each sample.

Vector construction and plant transformation
For overexpression vector construction, the coding sequence of QDtbn1 was amplified from B73 cDNA using specific primers (Table S2). The PCR product was cloned into the binary vector pCAMBIA1300, and then transformed into the maize inbred line C01 by Life Science and Technology Center, China National Seed Group Co., Ltd.

Y2H assay
The library was constructed using the purified total RNA from the tassel of B73. The “make your own mate and plate library system” (Clontech; no.630490) was used to generate the library. The full-length coding sequence of QDtbn1 was amplified from B73 cDNA using specific primers (Table S2). The PCR product was cloned into the binary vector pCAMBIA1300, and then transformed into the maize inbred line C01 by Life Science and Technology Center, China National Seed Group Co., Ltd.

Figure 7: Yeast two-hybrid assays between Zm00001d107867 (SnRK1), QDtbn1 (F-box), and Zm00001d034161 (SnRK2.8). 49230, 28733, and 34161 are the abbreviations of Zm00001d049230, Zm00001d107867, Zm00001d034161, respectively. pGBK7-T53/pGADT7-T as the positive control, and pGBK7-LAM/pGADT7-T as the negative control.
Ade-His-Leu-Trp dropout supplements (Clontech Mountain View, USA), and the positive clones were blued by X-a-Gal and ABA (Aureobasidin A) (Solarbio Beijing, China). Yeast strains co-transformed with pGBKT7-p53 and pGADT7-T or with pGBKT7-lam and pGADT7-T were used as positive control and negative control, respectively.

BiFC assay

The BiFC assay was carried out according to a previous report (Yuan and Xu, 2018). The full-length cDNAs of $Q_{\text{Dtbn1}}$ and Skp1-like genes potentially interacted with $Q_{\text{Dtbn1}}$ were amplified using the primer pairs listed in Table S2. To obtain fusion construct, $Q_{\text{Dtbn1}}$ was fused to pFGC-YN173, and the Skp1-like genes were fused to pFGC-YN155 by Gateway site-specific recombination. The vector pairs enable the expression of proteins of interest fused to the N-terminal 155 amino acids (nGFP) or to the C-terminal 86 amino acids (cGFP) of GFP (Shyu et al., 2008). Wild-type tobacco ($Nicotiana benthamiana$) plants were grown under a normal light regime (14 h of light, 10 h of darkness) at 25 °C and 70% relative humidity for 4–5 weeks. All BiFC vectors contain the target genes were transferred into the Agrobacterium tumefaciens strain GV3101. The obtained Agrobacterium strains were used to infiltrate tobacco leaves. Transformed Agrobacterium strain harbouring the constructs of target gene was grown for 2 day in a shaking incubator (200 rpm) at 28 °C in 5 mL of LB medium, supplemented with appropriate antibiotics. Transferred 1 mL of culture medium of Agrobacterium to 20 mL of LB medium containing corresponding antibiotics, which contains 15 mM acetosyringone, and cultured at 28 °C till OD600 = 0.5–0.6 (200 rpm). Then, centrifugated at 3913 g at room temperature for 10 min to collect the bacteria, and the Agrobacterium was suspended to OD600 = 1.0 with the infection solution (containing 10 mM MgCl2, 10 mM MES, 150 μM acetosyringone, pH = 5.6). Kept at room temperature for 2–3 h, and mixed two kinds of bacteria with different plasmids in equal volume, used a 1 mL needle to slightly open a small opening on the back of tobacco leaves (pay attention not to pierce), then injected the bacterial solution into the leaves with the needle from the wound of the leaves. Marked the water stained area of tobacco leaves with a marker.

Figure 8: Quantitative RT-PCR analyses on some genes served as SnRK2 substrates in the OE-plants and the negative controls. Student t-test was performed between the negative control and the three OE lines, respectively. Single and double asterisks represent significance difference determined by the Student's t-test at $P < 0.05$ and $P < 0.01$, respectively. WT: negative control. OE-4/8/12: independent lines over-expressed with $Q_{\text{Dtbn1}}$. 

$Q_{\text{Dtbn1}}$ negatively controlling TBN by a dominant model
isolated from etiolated leaves by digesting with an enzyme Renilla luciferase (REN). The maize mesophyll protoplasts were obtained in each group, and the ratio of LUC/REN was calculated. The primers used are listed in Table S2.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

Conceived and designed the experiments: B. Y.; Performed the experiments: X. Q., S. T.; X. D.; C. M., Analyzed the data: W. Z., population construction: Y. W. and J. Y.; Wrote the paper: X. Q. and B. Y.; All authors have read and approved the final manuscript.

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Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 The SNP in the 5’-UTR of Zm00001d053358 significantly associated with TBN in Wuhan and Hainan.

Figure S2 Phylogenetic tree of Zm00001d053358 in maize, rice, Arabidopsis thaliana. The neighbour-joining (NJ) phylogenetic tree was built by MEGA6 (https://www.megasoftware.net) based on the amino acid sequence alignment of the proteins.

Figure S3 Haplotypes based on the two significant Indel/SNP simultaneously identified in the two environments.

Figure S4 ANOVA and multiple comparisons of TBN among the five haplotypes with MAF > 0.05 in Wuhan and Hainan. Different letters represent significance difference at P < 0.05.

Figure S5 Expression level of the three independent OE lines of Zm00001d053358.

Figure S6 Performance of the tassel traits in the over-expressed plants and their controls.

Table S1 Ten SNPs/Indels with MAF (minor allele frequency) ≥0.1 identified in the 5'-UTR (−1450 bp to −580 bp) of the candidate gene from 250 inbred lines.

Table S2 Primers used in this study.

Table S3 Five putative QDtbn1-interacted clones detected by the Y2H assay.