Effects of ethephon on DNA methylation and gene expressions associated with shortened internodes in maize

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ABSTRACT
In this study, the molecular mechanism underlying ethephon-induced shortened internodes in maize was investigated using Zhengdan958 maize variety. The ethephon treatment was performed by spraying 225 mL/ha Ethephon 40% SL onto the foliage at the eight-expanded-leaves stage. The differentially expressed genes in the young internodes under ethephon treatment were identified through methylation-sensitive amplified fragment length polymorphism (MSAP), start codon targeted polymorphism (SCoT) and cDNA-amplified fragment length polymorphism (AFLP) analyses. MSAP results showed the methylation sites were widely distributed in both the ethephon-treated (at 27.8%) and control plants (30.1%). This suggested that ethephon treatment modified the methylation patterns; with 3.0% of the sites being hyper-methylated and 7.3% demethylated compared with the control. Based on SCoT analysis, 148 transcript derived fragments (TDFs) were obtained in the ethephon-treated plants. Among them, 38 were up-regulated (25.7%) and 47 down-regulated (31.8%). cDNA-AFLP analysis using 70 primer pairs identified 1635 TDFs in the ethephon-treated and the control plants. Of these, 600 and 564 TDFs were up- and down-regulated by the ethephon treatment, respectively. BLASTX analysis on 50 (randomly selected) differentially expressed TDFs divided them into several categories based on their putative biological functions: signal transduction (6%), resistance-related (14%), energy and metabolism (22%), transcription (4%), cell apoptosis (2%), unknown functional proteins (42%) and unknown genes (10%). Our results revealed that ethephon treatment could induce DNA methylation variation principally by increasing the demethylation tendency. This is suggested to play roles in stress-defence genes expression regulation and the differentially expressed genes could be associated with shortened internodes in maize.

KEYWORDS
Ethephon; maize; MSAP; SCoT; cDNA-AFLP; differential expression

Introduction
Maize (Zea mays L.) is one of the important food crops around the world [1–3]. Its role has become increasingly prominent in the food, feed and industrial processing sectors as a result of continuously rising world food demands and development of global economy [4,5]. This scenario calls for strategies to improve maize output. The increase of the total output in maize is reported to depend mainly on improvement of the yield per unit area, in which the planting density acts as the key parameter to determine the maize yield [6,7]. Increasing the planting density in maize, however, frequently results in increased maize stalk fragility and risk of falling over, which dramatically reduces the maize output [8,9]. Recently, lodging in the late growth stage have been reported a serious problem in maize production. Therefore, application of techniques in prevention and control of stalk lodging in maize are crucial in sustaining the yield formation under high planting densities. Through metabolism, ethephon can transform into ethylene and then exerts drastic effects in shortening the internodes in maize as well as other crops. Several studies have noted that application of ethephon can thicken and shorten the internodes, subsequently reducing stalk lodging and enhancing maize yield [10,11]. These studies have largely centred on the physiological and biochemical mechanisms of how ethephon regulates the internode characterization [3,7,12]. However, the molecular mechanism underlying the ethephon controlled internode shortening is still largely unknown.

The molecular analysis approach referred to as methylation-sensitive amplified fragment length polymorphism (MSAP) is established based on amplified fragment length polymorphism (AFLP). MSAP could effectively identify the epigenetic modifications in the genomic DNA that are widely present in animals and
plants to elucidate the effect of distinct internal and environmental cues on genomic stability and gene expression [13]. Meanwhile, used as an analysis approach, start codon targeted polymorphism (SCoT) is becoming a novel tool in detecting new molecular markers based on the short conserved region flanking the ATG start codon in plant genes [14–16]. cDNA-amplified fragment length polymorphism (cDNA-AFLP) is a powerful tool in identification of differentially expressed genes and has been widely used to determine the expression profiles under various stress conditions [17]. It shares the advantages of reliability, stability and high efficiency, and has been applied to a variety of plant gene expression studies [18,19].

In this study, the MSAP analysis was performed in order to understand the DNA methylation patterns regulated by ethephon, whereas SCoT and cDNA-AFLP were conducted to identify the gene expression in ethephon-treated and control plants in maize. Our results are a step towards an in-depth understanding of the internode shortening mechanism induced by ethephon and provide a theoretical basis for the control of stalk lodging in maize.

Materials and methods

Plant materials and treatments

Plants of maize (cv. Zhengdan 958) were planted in an open field at the Xinji Experimental Station of the Agricultural University of Hebei, Shijiazhuang, China in the 2013–2014 summer season. The Station (37°47′N, 115°17′E, 35 m altitude; consisting of typical loam soil of kaolinite mineralogy) is located in the middle of the Hebei Agricultural Plain. The site has a typical temperate continental arid climate, with a mean annual temperature of 12.5 °C and an average annual precipitation of 500 mm, most (80%) of which falls between July and September. Field experiments were laid out in randomized complete block design (RCBD) and replicated three times. Each 24-m² plot had 8 rows spaced at 0.5 m and plants spaced at 0.25 m in-row. Fertilization was achieved by applying CO(NH₂)₂, Ca(H₂PO₄)₂ and KCl for 255 kg/ha N, 90 kg/ha P and 150 kg/ha K, respectively. At the early internode initiation (8th leaf) stage, a uniform ethephon spraying at 225 mL/ha, using PGR Ethephon 40% SL (Shenzhen Yufull Industry Co. Ltd., Shenzhen, China) onto the maize foliage was carried out to set up the ethephon treatment group (using 192 seedlings). A control treatment group was set up by spraying water onto another 192 seedlings. All the other agronomic practices were kept standard and uniform for both treatment groups. Forty-eight hours after ethephon treatment, young internodes of 5 random plants from each treatment group (ethephon and control) were collected. All the samples were immediately frozen in liquid nitrogen and stored at −80 °C for further study.

RNA extraction and cDNA synthesis

TRIzol reagent was used to extract total RNA in the collected ethephon-treated and control internodes as described by the User Manual procedure. The quantity of the total RNA was determined based on the ultraviolet (UV) absorbance values measured by a spectrophotometer (OD₂₆₀/OD₂₈₀ ratios) and agarose gel electrophoresis. The cDNA was synthesized from the total RNA by using the HiFi-MMLV cDNA Kit (CWBio Company, Beijing, China) and the products were analysed by agarose gel electrophoresis.

DNA extraction and purification

The genomic DNA in internodes of the ethephon-treated and control plants was extracted according to a modified CTAB (cetyltrimethylammonium bromide) method. The extracted DNA was then treated with 10 mg/mL RNase to avoid contamination, and its quality was assessed by electrophoresis in 1% agarose gel.

MSAP analysis

The MSAP analysis was performed as follows: genomic DNA (750 ng) was incubated with EcoR I and Msp I/Hpa II for 7 h at 37 °C. Next, the adapters of EcoR I and Msp I/Hpa II, and the T₄ DNA ligase were added to the enzyme-treated DNA and the mixture was incubated for 13–16 h at 16 °C. Polymerase chain reactions (PCR) included the following components: 5.0 μL treated DNA, 1.6 μL deoxynucleoside triphosphates (dNTPs; 2.5 mmol/L), 0.6 μL E₀₀0, 0.6 μL H/M₀₀0, 0.2 μL Taq polymerase (5 U/μL), 2.0 μL 10× Taq Buffer, and 10 μL ddH₂O. The PCR programme, using TProfessional Thermocycler (Biometra, Analytik Jena Company, Göttingen, Germany), initiated at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and a final extension step at 72 °C 5 min. The selective amplification process was performed by using the following components: 5.0 μL pre-amplification products, 1.8 μL dNTPs (2.5 mmol/L), 0.8 μL EcoR I primers, 0.8 μL Hpa II / Msp I primers, 0.2 μL Taq polymerase (5 U/μL), 2.0 μL 10× Taq Buffer, and 9.4 μL ddH₂O. The PCR programme included the following conditions: 95 °C for 2 min, 13 cycles of 95 °C for 50 s, 65 °C (−0.7 °C/cycle) for 40 s, 72 °C for 1 min, 23 cycles of 95 °C for 50 s, 56 °C for 40 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. Gel (6% polyacrylamide) electrophoresis was used to identify the PCR products.
The differentially methylated fragments (DMF) identified in the ethephon-treated internodes were sliced out from the gel, put in 30 μL ddH2O and heated in a PCR analyzer to dissolve. The supernatants were subjected to a second PCR amplification and the PCR products were separated by electrophoresis in 1.5% agarose gel.

**ScoT analysis**

ScoT was performed based on PCR analysis. The PCR was conducted in 20 μL volumes containing 13.5 μL ddH2O, 2 μL 10 × PCR buffer, 1.6 μL dNTPs (2.5 mmol/L), 1 μL cDNA (60 ng/μL), 1.6 μL primer (10 μmol/L), and 0.3 μL Taq Plus DNA polymerase (5 U/μL). PCR was performed under the following conditions: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min 30 s and a final extension step at 72 °C for 5 min. The PCR products were separated in 1.5% agarose gel, stained with EB (ethidium bromide) and visualized under BIO-RAD Gel Doc XR. The identified amplification bands suggested to reveal high polymorphism were selectively sliced from the gel and reamplified (reproduced) for further analysis.

**cDNA-AFLP analysis**

cDNA-AFLP was performed according to the procedure described by Bachem et al. [17]. First, 750 ng of cDNA was incubated with EcoR I and Mse I for 4 h at 37 °C. The reactions were then conducted at a temperature of 65 °C for another 1 h. Next, the adapters of EcoR I and Mse I, and the T4 DNA-ligase were added and the reaction mixtures were subjected to further 13–16 h of incubation at 16 °C. The PCR programme was the same as that used in MSAP analysis.

**Sequence analysis**

The differentially identified transcript derived fragments (TDFs) by ScoT and cDNA-AFLP analyses were cloned in PGM-T vector and then transformed into an E. coli TOP10 strain (TIANGEN Pvt. Ltd. Co., Beijing, China) stored in LB Agar growth medium (COOLABER Science and Technology Pvt. Ltd, Beijing, China) at −70 °C. PCR analysis using primers (primer T7: TAATA CGACT CACTA TAGGG and primer SP6: ATTTA GGTGA CACTA TAG) was performed to confirm the positive clones. The sequencing for the positive clones was conducted by Sangong Biotech Company (Shanghai, China). Basic local alignment search program (BLAST) analyses were carried out to identity the putative biological roles of these differentially expressed genes (TDFs).

**Real-time PCR validation**

Internode total RNA in the ethephon-treated and control plants was extracted. cDNA was synthesized by using a Prime-Script RT-PCR Kit (TaKaRa). Quantitative real-time RT-PCR analysis was carried out in LightCycler® to evaluate the gene expression profiles using a SYBR® Premix DimerEraserTM (TaKaRa). In each PCR reaction, the components contained 10 μL SYBR® Premix DimerEraser (2 ×), 0.6 μL PCR forward primer (10 μmol/L), 0.6 μL PCR reverse primer (10 μmol/L), 2 μL cDNA (<100 ng) and 6.8 μL ddH2O. Thermal cycling conditions in the qPCR were set up as follows: an initial 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 20 s and 72 °C for 30 s, then a final melting curve step from 59 to 95 °C and ramp rate of 0.05 °C/s. A steady and constitutively expressed maize gene GAPDH (accession no. X07156), together with forward primer GAPDH-F: 5’-ACTGTG-GATGTCTCGGTGTTG-3’ and reverse primer GAPDH-R: 5’-CCTCGGAAGCAGCCTTAATAGC-3’, was used as

![Figure 1. Agarose gel electrophoresis results of DNA (a), RNA (b) and cDNA (c). M, DM2000 DNA marker (CWBI Company, Beijing, China); Lane 1, ethephon treatment; Lane 2, control group.](image-url)
Results and discussion

Detection of the quality of genomic DNA

The genomic DNA quality was determined based on the OD_{260}/OD_{280} ratios. The results indicated that the ratios of the genomic DNA extracted from the samples varied from 1.8 to 1.9, suggesting the genomic DNA shared good quality. The DNA integrity was further detected based on agarose gel electrophoresis. High brightness without RNA contamination proved that the extracted genomic DNA (Figure 1(a)) used for MSAP analysis was of high integrity.

Detection of the quality of extracted total RNA and the synthesized cDNA

The total RNA extracted from the internode growth meristems of the ethephon-treated and control plants was evaluated in a 1% agarose gel electrophoresis. The clear and high brightness in the bands of 28S and 18S indicated the total RNA was of good quality (Figure 1(b)). The OD_{260}/OD_{280} ratios varying from 2.11 and 2.13 in the RNA samples confirmed the good quality in these RNA samples. Based on transcription, cDNA was synthesized from the total RNA samples (Figure 1(c)). It was found that the reversely transcribed products mainly diffused from 150 to 2000 bp and could be used for the subsequent experiments.

DNA methylation patterns

Both Msp I and Hpa II can recognize the same sites of CCGG, but Hpa II is more sensitive than Msp I. According to the different sensitivity degree in methylation, the enzymatic amplification results can be divided into different types (Table 1) [21].

| Types | E/M bands | E/H bands | Methylation status of the cytosine |
|-------|-----------|-----------|-----------------------------------|
| I     | 1         | 1         | No methylation                    |
| II    | 1         | 0         | Double-strand DNA methylation of the internal |
| III   | 0         | 1         | Single-strand DNA methylation of the external |
| IV    | 0         | 0         | Double-strand DNA methylation of the external |

The DNA methylation level

Fifty-nine primer combinations were used to detect cytosine methylation frequency in the CCGG sequences in the internodes treated by ethephon and those in control conditions. Of these, 22 primer combinations (Table 2) that were shown to be highly polymorphic were used for DNA methylation analysis. Typical electrophoresis maps detected by MSAP are shown in Figure 2. In addition, the DNA methylation levels in the different samples are shown in Table 3. Based on the above results, the number of methylated ( hemi-methylated and fully methylated) DNA bands were determined, in which 157–170 bands were confirmed to be polymorphic (Type II + Type III + Type IV bands). They account for 27.8% to 30.2% of all bands. Among them, full-methylation was detected more frequently than hemi-methylation.

Effect of ethephon treatment on DNA methylation patterns

All possible banding patterns in the ethephon-treated and the control samples were compared to investigate the changes in cytosine methylation patterns. Thirteen different banding patterns were observed between the control and ethephon-induced conditions (Table 4). Of these, the patterns of A-1, A-2 and A-3 represented the monomorphic class in which the methylation pattern was similar in the control and the ethephon-induced treatments. The percentage of unchanged bands was 89.4%. In this study, the patterns of B-1, B-2, B-3 and B-4 indicated cytosine hyper-methylation patterns, whereas cytosine demethylation events induced by ethephon were represented by the patterns of C-1, C-2, C-3, C-4 and C-5. The percentage of the hyper-methylated bands under ethephon treatment was 3.0%, while the percentage of demethylated bands was 7.3%. Pattern D
currently was unable to distinguish the status of DNA methylation, with a percentage of only 0.3%. These patterns revealed a trend towards a decrease in the methylation level under ethephon treatment. MSAP technology has been confirmed to be an efficient, reliable detection tool for DNA methylation analysis and has been widely applied in rice, wheat, sorghum and other cereals in order to improve the plant resistance to diverse stresses [22]. DNA methylation is one of the major pathways of epigenetic modification that plays crucial roles in regulating the plant growth, development, organogenesis and the gene transcription [23,24]. Through methylation, the genomic DNA can be altered at a higher structural level without variation of the primary structure of DNA molecules in order to modify the gene expressions at the genome level.

In this study, the DNA methylation levels in the internodes of the ethephon-treated plants and the control plants were investigated. We found that the number of methylation and methylation-free loci was similar between the ethephon-treated and control plants. However, ethephon modified the DNA methylation status with preference for demethylation, which plays an important role in regulating the gene expression associated with the phenotypic changes such as the length of the internodes in maize.

Previous studies have revealed that the DNA methylation in maize is associated with the ability to respond to various abiotic stresses [25–29]. It has been observed

Table 3. DNA methylation levels of maize genome in ethephon treatment.

| Types | Ethephon treatment | Control (CK) |
|-------|--------------------|--------------|
| I     | 407                | 394          |
| II    | 92                 | 79           |
| III   | 53                 | 67           |
| IV    | 12                 | 24           |
| Total methylated bands (ratio) | 157 (27.8%) | 170 (30.2%) |
| Hemi-methylated bands (ratio) | 53 (9.4%) | 67 (11.9%) |
| Fully methylated bands (ratio) | 104 (18.4%) | 103 (18.3%) |

Note: Total methylated bands = type II + III + IV; fully methylated bands = type II + IV; hemi-methylated bands refers to type III.

Table 4. DNA methylation patterns of maize genome in ethephon treatment.

| Types | Ethephon treatment | Control (not ethephon treated) |
|-------|--------------------|-------------------------------|
|       | Number and frequency of types |                     |
| A     | M     | H     | M     | H     | M     | H     | ETH- CK |
| A-1   | 1     | 1     | 1     | 1     | 389   |
| A-2   | 1     | 0     | 1     | 0     | 72    |
| A-3   | 0     | 1     | 0     | 1     | 43    |
| B     | 1    | 1      |      | 1   | 17 (3.0%) |
| B-1   | 1     | 0     | 1     | 1     | 3     |
| B-2   | 0     | 1     | 1     | 1     | 2     |
| B-3   | 0     | 1     | 0     | 0     | 6     |
| B-4   | 0     | 0     | 1     | 0     | 6     |
| C     | 1    | 1      |      | 1      | 41 (7.3%) |
| C-1   | 1     | 1     | 0     | 1     | 1     |
| C-2   | 1     | 1     | 0     | 1     | 16    |
| C-3   | 1     | 1     | 0     | 0     | 1     |
| C-4   | 1     | 0     | 0     | 0     | 15    |
| C-5   | 0     | 1     | 0     | 0     | 8     |
| D     | 1     | 0     | 0     | 1     | 2 (0.30%) |

Note: the score of 1 and 0 represents the presence and absence of bands, respectively. The A banding patterns represent monomorphic class; B banding patterns represent hyper-methylation class; C banding patterns represent demethylation class; and D pattern could not identify any methylation type.
that these environmental perturbations could decrease the methylation level in the introns and improve the demethylation expression in leaves [25]. Thus, the demethylation patterns might be related to the expression of some stress-defence genes that are involved in plant adaptation to stress conditions [30]. In our present study, MSAP analysis showed that ethephon treatment influenced the DNA methylation changes in maize, with priority given to the demethylation process, which may be related to plant cell regulation of gene expression associated with stress defence. Further comprehensive investigation of the differentially expressed genes identified in our study is necessary to obtain a clearer understanding of the molecular basis underlying the effect of DNA methylation and demethylation on ethephon-controlled lodging resistance.

**Identification of the differentially expressed genes**

Forty-three SCoT primers (Supplementary Table 1S) and 70 cDNA-AFLP primers (Supplementary Tables 2S and 3S) were used to identify the differentially expressed genes in response to the ethephon treatment. Representative results obtained based on ScoT and cDNA-AFLP are shown in Figures 3 and 4, respectively. In total, 1783 bands were amplified, with 638 TDFs showing an up-regulated expression pattern, 611 TDFs exhibiting down-regulated expression and 534 TDFs expressing a similar pattern between the ethephon treatment and the control conditions. These TDFs accounted for 35.8%, 34.3% and 29.9% of the total TDF number (Table 5), respectively.

The SCoT analysis results revealed by agarose gel electrophoresis showed that the PCR products varied...
from 200 to 1000 bp. Totally, 148 bands (TDFs) were amplified, in which 38 TDFs (25.7%) were shown to be up-regulated, 47 TDFs (31.8%) to be down-regulated and 63 TDFs (42.5%) to be unchanged in expression.

The cDNA-AFLP analysis results based on agarose gel electrophoresis showed that the PCR products ranged from 100 to 400 bp. Totally, 1635 TDFs were generated, in which 600 TDFs (36.7%) were shown to be up-regulated, 564 TDFs (34.5%) to be down-regulated and 471 TDFs (28.8%) to have maintained similar expression levels. Only 50 of the TDFs that showed repeatability and were stable in replications were randomly selected and sequenced (Shangong Biotech Company, Shanghai, China). BLASTX analysis revealed that, of these, 24 fragments shared similarity with known functional proteins in maize or other plant species. Another 21 fragments showed homology to expressed sequence tags with unknown protein functions, whilst 5 search probes did not match anything in the database (Table 6). Based on the putative biological functions, these 50 differentially (up-regulated or down-regulated) expressed genes identified to be regulated by ethephon were classified into several categories, including those involved in energy and metabolism (22%), signal transduction (6%), transcription regulation (4%), resistance and defense (14%), cell apoptosis (2%), unknown functional proteins (42%) and unknown genes (10%) (Figure 5; Supplementary Data Table 4S).

Internode length and sheer strength of the stem are important factors contributing to plant resistance to lodging [3]. In grass species, including maize, internode length expansion is triggered by cell division and elongation of the intercalary meristems at the base of the growing internodes [31]. As maize stems develop, sclerenchyma and rind region parenchyma tissues develop thick and cell walls are lignified, contributing to the sheer strength of the stems. It is therefore hypothesized that genes involved in suppression of elongation of intercalary meristems at the base of the growing internodes and genes involved in cell wall remodelling and cell wall-related processes might have significant functions during defence response, including stalk lodging.

In the present study, we observed that the genes with known specific functions differentially regulated by ethephon included diverse functional group genes that are involved in modification of the physiological and biochemical processes, consequently resulting in the adjustment of plant internodes length, plant height, ear height and the centre of gravity of maize stalks. Thus, these genes may play a decisive role in controlling the internode elongation in response to ethephon application in maize. In crops, internode cell expansion or constriction responds continuously to various biotic and abiotic stresses which affect the growth of individual plants and the final crop production. Here, we report that stress-response genes, transcription regulation genes, metabolism-related genes and signal transduction genes are involved in arresting internode cell elongation in response to ethephon treatment.

The chalcone-flavonone isomerase (CHI) gene was identified to be differentially expressed under ethephon-treatment conditions in this study (TDF24). CHI, an enzyme that participates in flavonoid biosynthesis, has been confirmed to play important roles in plant growth and development, defence and UV protection. Previously, the studies by Moustafa and Wong [32] and Muir et al. [33] indicated that CHI is the first enzyme in flavone and anthocyanins synthesis and is critical in increasing the flavonoid contents. Dixon and Steel [34] discovered that CHI has an important positive effect on human health through its function on scavenging the free radicals and improving the activities of antioxidant enzymes and other biologically active substances. Thus, the expression level of CHI-encoding genes will affect the flavonoids metabolism and induce stress response in plants [35]. Wei et al. [31] discovered that Zm.722.1.A1_at, a putative homolog of the chalcone synthase gene, was upregulated 21.5-fold in response to ethephon treatment. Chalcone synthase, as the first enzyme in the pathway to flavonoids, could lead to increased auxin transport when upregulated [33]. Additionally, previous studies have revealed that chalcone synthase was identified as active in almost all maize tissues depending on the expression of different regulatory genes [36]. As also supported by Wei et al. [31], it is therefore correct to suggest that the phenotypic changes in internodes in response to ethephon treatment may emanate from the interactive effect of these significantly changed gene expression levels in the intercalary meristem cells.

In the present study, we also identified a differentially expressed gene (TDF23) encoding a pentatricopeptide repeat-containing protein (PPR) that significantly contributes to plant growth and development, RNA editing and processing, cell formation, signal transmission between the nucleus and organelles, cytoplasmic male sterility in fertility restoration, as well as the defence against biotic and abiotic perturbations. The study on OsPPR1 (a kind of PPR) in chloroplasts by Gothandam et al. [37] suggested that PPR, specified by a characteristic repeat motif consisting of a 35-amino acid tandem, is involved in various biological processes in plants. The

Table 5. Statistics of differentially expressed bands.

| Different induction of maize | Bands | Percentage (%) |
|-----------------------------|-------|----------------|
| Up-regulated expression     | 638   | 35.8           |
| Down-regulated expression   | 611   | 34.3           |
| Same expression             | 534   | 29.9           |

*Note:*|
OsPPR1 product was targeted to the chloroplast and caused albinism of the plants when its expression was suppressed. Bentolila et al. [38] found that RF-PPR592, an analogue of PPR, could restore the fertility, once it was expressed in the cytoplasmic-male sterile plants. It can be suggested here that the PPR gene may be involved in transcription regulation in the cell, growth and development processes, including cell-wall component synthesis proteins. Similarly, Wei et al. [31] discovered that a gene probe set Zm.7715.1.A1_1, putatively encoding the Wiskott-Aldrich syndrome proteins, was upregulated and was crucial in sending a signal to the cytoskeleton through an actin nucleating assembly that regulates the structure and dynamics of actin filament networks at the leading edge of a cell [39].

The DNA repair protein genes encoding glutamyl-tRNA synthetase and cytochrome P450 were also identified to be differentially expressed by ethephon in this study. It has been reported that this DNA repair protein gene is involved in cell’s responses to DNA injury. It can revive DNA structure and help it execute its original function again, so it is important for the survival of cells.

| TDF No. | Accession No. | Similar protein | E-value | Expression |
|---------|---------------|----------------|---------|------------|
| 1       | AQ239589.1    | Retroviral aspartyl protease | 2E−96   | —          |
| 2       | AQK92834.1    | Trans-cinnamate 4-monooxygenase | 0.062   | —          |
| 3       | AQ239567.1    | Retron transpon gna protein | 6E−46   | +          |
| 4       | AQ60305.1     | Vacular proton pump3 | 2E−131  | +          |
| 5       | AEI07934.1    | Xilon1 gag-pol polyprotein | 3E−97   | +          |
| 6       | XP__008454.472.1 | Succinate dehydrogenase iron-sulphur subunit 3 | 9E−19   | —          |
| 7       | WP__044807.154.1 | Calcium/sodium:proton antiporter, partial | 2E−41   | +          |
| 8       | T02955.1      | Probable cytochrome P450 monooxygenase-maize | 9E−104  | +          |
| 9       | WP__050806.364.1 | Glutamine–tRNA ligase | 6E−91   | +          |
| 10      | KFC17444.1    | Phosphoglycerate mutase family protein | 2E−80   | +          |
| 11      | WP__054897.003.1 | Exodeoxyribonuclease V subunit gamma | 5E−121  | +          |
| 12      | WP__005323.214.1 | Arabinosyltransferase | 5E−70   | —          |
| 13      | XP__011650.079.1 | THO complex subunit 7A-like | 1E−58   | —          |
| 14      | OCF37975.1    | Serine/threonine-protein kinase ATR | 8E−69   | +          |
| 15      | AHF27428.1    | Catalase, partial | 1E−74   | +          |
| 16      | WP__004932.192.1 | Exodeoxyribonuclease III | 8E−162  | +          |
| 17      | WP__023445.126.1 | Membrane protein | 2E−84   | +          |
| 18      | KYP12063.1    | Alkal1 1-monooxygenase | 1E−35   | —          |
| 19      | CSQ79953.1    | Flagellar biosynthesis protein FilIP | 2E−56   | —          |
| 20      | WP__056610.418.1 | Tnb-dependent receptor | 1E−23   | —          |
| 21      | NM__202.452.2 | DNA repair protein | 9E−65   | —          |
| 22      | WP__072118.649.1 | NADH-quinone oxidoreductase, partial | 4E−124  | —          |
| 23      | XP__004148.109.1 | Pentatricopeptide repeat-containing protein | 3E−24   | +          |
| 24      | XP__004146.009.1 | Chalcone-flavonone isomerase | 0.005   | +          |
| 25      | XP__008664.114.1 | Uncharacterized protein LOC103642672 | 5E−66   | —          |
| 26      | XP__008646.835.1 | Uncharacterized protein LOC103628405 | 2E−126  | —          |
| 27      | ACG30507.1    | Hypothetical protein | 0.012   | +          |
| 28      | XP__008671.509.1 | Uncharacterized protein LOC103648909 | 6E−34   | —          |
| 29      | XP__008650.680.1 | Uncharacterized protein LOC103631580 | 6E−35   | —          |
| 30      | AQK48963.1    | Hypothetical protein ZEAMMB73__Zm00001d048725 | 7E−25   | —          |
| 31      | AQK54545.1    | Hypothetical protein ZEAMMB73__Zm00001d051895 | 0.064   | —          |
| 32      | AGJ54793.1    | Hypothetical protein | 2E−64   | —          |
| 33      | XP__017326.817.1 | Uncharacterized protein LOC108267311 | 8.2     | +          |
| 34      | EHH17269.1    | Hypothetical protein EGK__13 631, partial | 1E−23   | +          |
| 35      | BAE33649.1    | Unnamed protein product | 6E−83   | +          |
| 36      | EMS54240.1    | Hypothetical protein TRIUR3__11 406 | 9E−16   | +          |
| 37      | EMS57213.1    | Hypothetical protein TRIUR3__30 242 | 2E−14   | —          |
| 38      | WP__068734.334.1 | Hypothetical protein | 1.7     | +          |
| 39      | ENF90130.1    | Hypothetical protein ECP030526013__2872 | 6E−21   | —          |
| 40      | XP__018860.511.1 | Uncharacterized protein LOC109022142, partial | 4E−19   | —          |
| 41      | AJB60002.1    | Hypothetical protein KU45__025 710 | 4E−54   | —          |
| 42      | WP__038127.569.1 | Hypothetical protein | 2E−85   | —          |
| 43      | XP__002460.972.1 | Hypothetical protein SORBIDRAFT__02g038550 | 0.65   | +          |
| 44      | KGN48778.1    | Hypothetical protein Csa_66500700 | 8E−156  | +          |
| 45      | XP__012699.226.1 | Uncharacterized protein LOC105913826 | 2E−73   | +          |

**Note:** +, up-regulation; —, down-regulation.
The glutamyl tRNA synthetase (TDF9) gene has been observed to be crucial in binding glutamate with tRNA. In addition, it also participates in 5-aminolevulinic acid (5-ALA) biosynthesis in the C5 pathway on which to enhance the plant resistance to abiotic stresses such as salt and frost [40–42]. Cytochrome P450 monooxygenase gene is involved in biosynthesis of different endogenous hormones and plays an important role in plant growth and development [43]. Wei et al. [31] showed that (Zm.390.1.S1.at) and (Zm.13 480.1.S1.at) mRNAs probe sets were significantly changed in maize internode elongation responses to ethephon treatment. Zm.390.1. S1.at, an ortholog of d3 (cytochrome P450) encoding an enzyme involved in an early step in the biosynthesis of gibberellic acid (GA) and probably the 13-hydroxylation step, was upregulated 3.30 times and has been confirmed as regulating dwarf plants and stems [44,45]. Zm.13 480.1.S1.at, an ortholog of OsGA20ox2 (SD1), encoding a gibberellin 20 oxidase, a key enzyme in the biosynthesis of GA that catalyses the three steps GA 53 → GA 44 → GA 19 → GA 20, was upregulated 3.75 times and has already been confirmed to control shorter leaves and stems [31,46]. Here, we speculate that the gene encoding cytochrome P450 regulates the hormone pathways and expressions related to internode elongation in response to ethephon treatment. Moreover, by modulating the expressions of DNA repair protein genes, the glutamine tRNA synthetase gene and the cytochrome P450 monooxygenase gene, ethephon inhibited plant internode elongation through regulation of the corresponding physiological and biochemical processes.

In addition, in this study, numerous TDFs were shown to be differentially expressed by ethephon treatment. Further comprehensive investigation is needed to reveal the molecular basis underpinning the ethephon controlled internode elongations as well as other biological processes of growth and development in maize.

**Real time PCR analysis**

To confirm the validity of the ScoT analysis results, we conducted real-time PCR analysis. Three up-regulated TDFs obtained in the ScoT analysis, including those encoding for cytochrome P450 monooxygenase (TDF8), glutamyl tRNA synthetase (TDF9) and DNA repair protein (TDF21) were randomly selected for the RT-PCR analysis (Figure 6). As highlighted earlier in this paper, we used the GAPDH gene as a reference with a steady level of expression. The quantitative real-time PCR results showed that the expression trends of these three genes

![Figure 5. Functional classification of gene fragments (TDFs).](image1)

![Figure 6. Real-time PCR analysis of differentially expressed fragments (randomly selected) to validate the ScoT analysis results.](image2)
were consistent with those obtained in the SCoT analysis.

Conclusions
In this study, we found that ethephon treatment significantly reduced plant height, ear height by suppressing internode cell elongation, increased stem diameter and increased stem tension, and thus, can effectively improve the lodging resistance of maize. This can be an effective technique to help maintain high maize plant densities and reduce losses due to lodging, which consequently sustain yield under these high plant density conditions.

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