Analysis of differential expression of genes induced by ethephon in elongating internodes of maize plants

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Abstract   Plant growth regulators (PGRs) are commonly used in cereal cropping systems to restrict plant height and control lodging. Ethephon has been reported to shorten internodes and increase grain yield of maize. To analyze the transcriptomic profiles of maize internode elongation following ethephon treatment, differentially expressed genes were compared between the treatment and control samples of inbred line Zong 31 using the Affymetrix Maize Genome Array. According to the microarray data, 326 probe sets showed significant change in expression. Further research revealed that the most remarkable effects of ethephon on maize internodes elongation occurred during a 48 h period, when 89 differentially expressed genes were detected. There were dramatic change in transcript levels at 24 h and six Auxin transport genes and four gibberellin biosynthesis pathway genes were differentially expressed in Zong 31 in response to ethephon treatment. In summary, we showed that gaseous ethylene release is involved in internode meristem cell elongation through the regulation of plant hormone signaling in maize. This work provides a platform for studies in which candidate genes will be functionally tested for involvement in internode elongation.

Keywords   maize, ethephon, internode elongation, microarray, qPCR

1   Introduction

Lodging (root and stalk lodging) is one of the major problems in maize production worldwide, impacting greatly on field management, grain quality, and mechanized harvesting and accounts for annual yield losses of 5%–25%[1,2]. Chemical control is an effective measure to reduce plant height and prevent lodging in maize[3]. Plant growth regulators are involved in various aspects of chemical control to promote, retard or inhibit growth, and are commonly applied in agriculture to control lodging in cereal crops[4]. These exogenously applied chemical compounds reduce elongation and lead to dwarfing by promoting biosynthesis of ethylen or inhibiting production of gibberellins (GA). Ethephon application results in the release the gaseous plant hormone ethylene, which can trigger myriad physiological and morphological responses in cereal crops. Previous studies have shown that ethylene released after applying ethephon can reduce plant height and control lodging by decreasing the internode length[3,5–7].

Ethylene has multiple and diverse roles in plant physiology, including the triple response, inhibition of growth, stress response, fruit ripening, leaf/petal/fruit senescence, leaf abscission, promotion of stem elongation in deep water rice, sex determination, breakdown of seed dormancy/promotion of seed germination and adventitious root formation[8–11]. During the past decade, molecular genetic studies on a model dicotyledonous plant (*Arabidopsis*) and a monocotyledonous plant (rice) have revealed a linear signal transduction pathway from signal perception to transduction and identified the signal components involved in the ethylene biosynthesis pathway[12–15]. For example, the physical interaction between the receptors (ETR1, ERS1, ETR2, ERS2 and EIN4) and CTR1 in the absence of ethylene keeps downstream signaling compo-

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nents EIN2 and EIN3 inactive, and ethylene insensitive EIN2 and EIN3 are known to be tightly controlled by a proteasome-dependent regulatory module[16,17]. The regulation of this signaling pathway involves complex regulatory mechanisms involving regulation by protein turnover, potential posphorylation of EIN3 through MAP kinase cascades, transcriptional activation of the receptors and EIN3 binding F-box proteins and EIN5/exoribonuclease (XRN4)-mediated control of *EBF* mRNA levels[18]. MKK9-MPK3/6, the mitogen-activated protein kinase cascades, are probably also involved in ethylene signaling and/or ethylene biosynthesis[19,20]. Additionally, as a plant hormone, ethylene is known to differentially control internode elongation by interacting with other plant hormones such as auxin (indole-3-acetic acid, IAA), GA and abscisic acid (ABA), and some molecular elements have been identified in *Arabidopsis* or rice[3–7,21–26]. Nonetheless, to the best of our knowledge, the signal transduction cascade and molecular mechanisms associated with ethylene biosynthesis in maize internode elongation have not been characterized in detail.

Microarray technology has been established as a sensitive and convenient tool for rapid analysis of plant gene expression to characterize different processes of plant growth and development[27–29]. Research on signal transduction pathways activated during plant responses is often initiated from microarray data analyses[30]. Recently, increasing information on microarray analysis of plant hormones that are differentially regulated during their own biosynthesis and through signal transduction pathways has been obtained[31–35]. However, no report on large-scale differential gene expression analysis of ethephon treatment affecting internode elongation in maize has been published. Considering the importance of morphological improvements in plant architecture for maize production, understanding the molecular mechanisms controlling internode elongation and development is essential[36,37].

In the present study, we found phenotypic responses of maize internodes to ethephon, through released ethylene, included inhibition of longitudinal cell growth and promotion of lateral growth. Microarray data revealed that the greatest stabilization effect of ethephon on maize internode elongation occurs during the first 48 h, with the peak in differentially-expressed genes in maize internodes occurring at 24 h. Furthermore, six auxin transport genes and four GA biosynthetic pathway genes are differentially expressed in maize (*Zea mays* L.) inbred line Zong 31 (one of the most widely used for maize breeding in China, developed and contributed by National Maize Improvement Center of China, China Agricultural University) by ethephon treatment. Therefore, we postulated that ethylene gas release is involved in internode meristem cell elongation by regulation of the plant hormone signaling crosstalk in maize. This might provide a basis for further study of the molecular mechanisms of the phenotypic responses of maize internodes to ethephon.

2 Materials and methods

2.1 Plant materials and ethephon treatment

Seeds of maize (*Zea mays*) inbred line Zong 31 were sown in a greenhouse (28°C day/20°C night; 16 h photoperiod; 40% RH (relative humidity)) at China Agricultural University, Beijing, in December 2009. At the nine-leaf growth stage where leaf collars were visible, ethephon (270 g·hm^{-2} of 600 mg·L^{-1}) was evenly sprayed on maize foliage with 200 seedlings as the treatment group and water sprayed on another 200 seedlings as the control group. All other agronomic practices were applied uniformly to both groups.

2.2 Phenotypic and physiological characterizations analysis

To test internode phenotypic divergence between the exogenous ethephon treatment group and the control group, at 0, 24 and 48 h, intercalary meristems (within 2 cm below the node) of the twelfth internode were collected. Three plants were collected from each group. Nine days after treatment, paraffin sections in the longitudinal and vertical planes of stems were prepared from control and treated seedlings.

To quantify the phenotypic variation, three independent samples (15 maize seedlings in each group) from the treatment and control groups were monitored and analyzed. Statistical analysis was performed using Student's *t*-test.

2.3 RNA isolation and Affymetric GeneChip hybridization

Total RNA was extracted from the harvested seedlings (mixed sample from elongating internodes of three maize plants) and subjected to different treatments according to the Trizol reagent (Invitrogen, Beijing, China) protocols. GeneChip Maize Genome Arrays were used for differential gene expression analysis. The microarray consisted of 17555 probe sets for about 14850 *Zea mays* transcripts, representing 13339 genes. The standard wash and double-stain protocols were applied using a fluidics station (Affymetrix GeneChip Fluidics Station 450). The arrays were scanned on a GeneChip scanner (Affymetrix GeneChip Scanner 3000). Each sample included three biological replicates to determine both the reproducibility and quality of the chip hybridization.

The raw data sets (CEL) and the normalized expression data sets have been deposited in the Gene Expression Omnibus (GSE56062) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo).

2.4 Microarray data processing and analysis

First, the scanned arrays were analyzed using Affymetrix GCOS 1.4 software to generate raw data (estimated probe

intensity values) and saved as CEL files. Next, DNA-Chip Analyzer 2008 software was used to normalize all 15 array sets[38]. The Significance Analysis of Microarrays software was used for screening differentially-expressed genes (http://www.dchip.org). A gene was considered to be expressed when it had detection call P in all three replicates, with its detection $P < 0.05$. We used Gene-Spring GX 11 software to analyze the correlation coefficients between every pair of arrays, all correlation coefficients were greater than 0.9. Genes showing statistically significant differential expression of transcriptional changes were identified when two conditions were satisfied: (1) more than twofold upregulation or downregulation after ethephon treatment; (2) a change with a $P < 0.05$. Negative or positive values represent downward or upward regulation, respectively. All probe sets (CapitalBio Corporation) were subjected to the statistically enriched Gene Ontology analysis using the AgriGO online service[31] (http://bioinfo.cau.edu.cn/agriGO); the hormone signaling pathways associated with the differentially-expressed genes were identified on the basis of a background distribution from the whole chip using Kyoto Encyclopedia of Genes and Genomes (KEGG; version 43) and grouped into functional categories and metabolic pathways using the Molecule Annotation System MAS 3.0 (http://bioinfo.capitalbio.com/mas3) of CapitalBio Corporation. Microsoft Excel and Access were used to extract and manage microarray data.

2.5   Real-time quantitative reverse transcription polymerase chain reaction analysis

Total RNA samples from maize internodes for GeneChip experiment (three biological replicates) were used for real-time polymerase chain reaction (PCR) assays in order to ensure the reliability and repeatability of the results. Genomic DNA contamination was eliminated by treating the RNA samples with DNase I (TaKaRa, Dalian, Liaoning, China). Then, the total RNA was used to synthesize cDNA in a reverse transcription reaction using random primers (Promega, Madison, WI, USA). The cDNA samples were diluted to 4 ng·μL^{-1}. The gene-specific primers (Appendix A, Table S1) were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/input.htm). As a positive control, 18S rRNA was used to normalize the data. Three biological replicates were assayed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a 7500 Real Time PCR System machine (Applied Biosystems) according to the manufacturer's protocols. The amplification reactions were performed under the following PCR conditions: one cycle at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s, a final extension at 72°C for 7 min, and holding at 4°C. The average Ct values of three different samples were calculated for different genes and references. The relative changes in expression were calculated using the relative $2^{-\Delta\Delta C_T}$ method[39].

3   Results

3.1   Phenotypic responses to ethephon treatment

As shown in Fig. 1a and Fig. 1b, the lengths of the eleventh, twelfth and thirteenth internode of the ethephon-treated plants were significantly shorter than in the control plants (Appendix A, Table S2). This change in internode elongation of Zong 31 in response to exogenous ethephon treatment was related to the morphology of intercalary meristems cells and cytological observations revealed that ethylene release inhibited the longitudinal growth of cells and promoted lateral growth. The cross section and the longitudinal sections showed that the ethephon-treated internodes had shorter and wider cells, and greater vascular density (Figs. 1c–1g), with the average cell length (Appendix A, Table S3) decreasing from 68.9 to 32.6 μm, and the average width increased from 92.6 to 103 μm, respectively.

3.2   Differentially-expressed genes involved in internode elongation by ethephon treatment

Ethephon triggered reduced maize internode length probably by decreasing the longitudinal growth of cells. Previous studies have shown consistent results in maize, wheat and barley[3–7]. Accordingly, we selected three time points (0, 24 and 48 h) to investigate the differential expression of genes at the transcription level, and at every point setting, three biological samples were collected independently for analysis. The Affymetrix *Zea mays* whole genome chip was designed mainly from the representative maize inbred genotypes B73, Ohio 43 and W22. We first tested probe level between the treatments and the control for Zong 31. The average percentage of 17555 probes varied from 72.8% to 76.1%, indicating that the polymorphic probes will probably not significantly impact our results (Appendix A, Table S4). Of the tested probes, 326 genes showed significant changes in expression in response to ethephon after 24 h; 190 were significantly upregulated and 136 were significantly downregulated, accounting for 58.3% and 41.7% of the total number of differentially-expressed genes, respectively. After 48 h of treatment, 89 differentially-expressed genes were detected; 55 were significantly upregulated and 34 were significantly downregulated, accounting for 61.8% and 38.2% of the differentially-expressed probes, respectively (Table 1). Furthermore, the number of differentially-expressed probes sets identified in the ethephon treatment at 48 h was smaller than at 24 h. Specifically, most of the differentially-expressed genes were regulated by two to 5-fold, while a few were
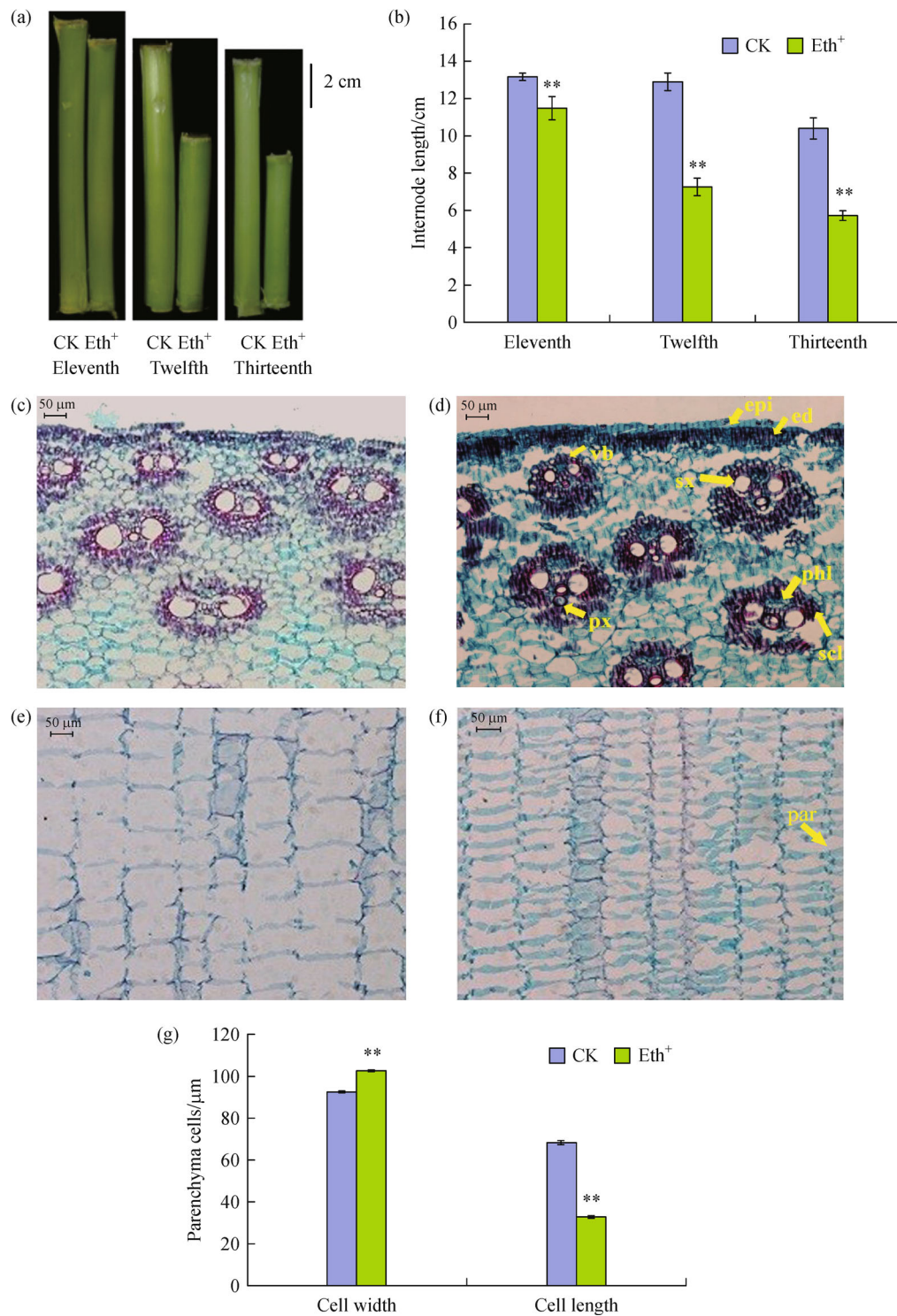
Fig. 1 Morphological characterization 9 d after ethephon treatment (Eth+) and control (CK). (a) Phenotypic characterization of the eleventh, twelfth and thirteenth nodes of maize cv. Zong 31 plants; control (CK) and ethephon treatment (Eth+). Scale bar = 2 cm; (b) comparison of the length of the eleventh, twelfth and thirteenth nodes of Zong 31, at least 30 plants were analyzed. Asterisks indicate significant differences between control (CK) and ethephon treatment (Eth+) (**, $P < 0.01$; Student's *t*-test). Values indicate mean±SD; (c–d) paraffin-cut cross section (× 10) of the eighth nodes of Zong 31 at the mature stage after control (c) and ethephon treatment (d). Dark coloration indicates the presence of syringyl lignin units. epi, epidermis; ed, exodermis; vb, vascular bundle; sx, secondary xylem; px, protoxylem; phl, phloem; scl, sclerenchyma. Scale bar = 50 μm; (e–f) paraffin-cut longitudinal section (× 10) of eighth nodes of Zong 31 with control (e) and ethephon treatment (f), respectively, at the mature stage. par, parenchyma cell. Scale bar = 50 μm; (g) length and width of parenchyma cells in paraffin-cut longitudinal section (e–f). Three slides of each sample were observed, and more than 100 cells were measured for each slide. Asterisks indicate significant differences between control (CK) and ethephon treatment (Eth+). Values are means±SD ($n > 300$). Student's *t*-test was used to calculate the P value (**, $P < 0.01$).

Table 1 Percent of differentially-expressed probe sets with fold change by ethephon treatment

Treatment	Category	Number of probe sets	Fold change/%			
			2–3	3–5	5–10	10–50
24 h Eth vs. 24 h CK	Upregulated	190	51.05	28.95	12.11	7.89
	Downregulated	136	69.85	22.06	5.15	2.94
48 h Eth vs. 48 h CK	Upregulated	55	50.91	38.18	7.27	3.64
	Downregulated	34	50.00	41.18	5.88	2.94

regulated by five to 10-fold or even up to 50-fold, which was similar to the trend observed after 24 h of treatment. These findings indicate that exogenous ethephon had differentially induced changes in expression of internode genes at differing time points.

To further investigate the similarities between the differentially-expressed genes caused by ethephon at different time points, we performed a hierarchical cluster analysis (Fig. 2a). The genes showing changes in expression at 24 and 48 h showed similar expression patterns, which were different from those of genes showing changes in expression at 0 h. This suggests that the changes in

transcript levels at 0 h may represent responses of maize internodes to small environmental changes during growth and development. However, the significant differences between 24 and 48 h were caused by ethephon. Further analysis revealed that the differentially-expressed genes showed greater reproducibility and consistency at 24 h than at 48 h. This indicates that the maximal effect of ethephon on maize internode elongation occurred within 48 h, and the peak number of differentially-expressed genes occurred at 24 h.

To present the differences between the genes differentially expressed at various time points, we constructed a
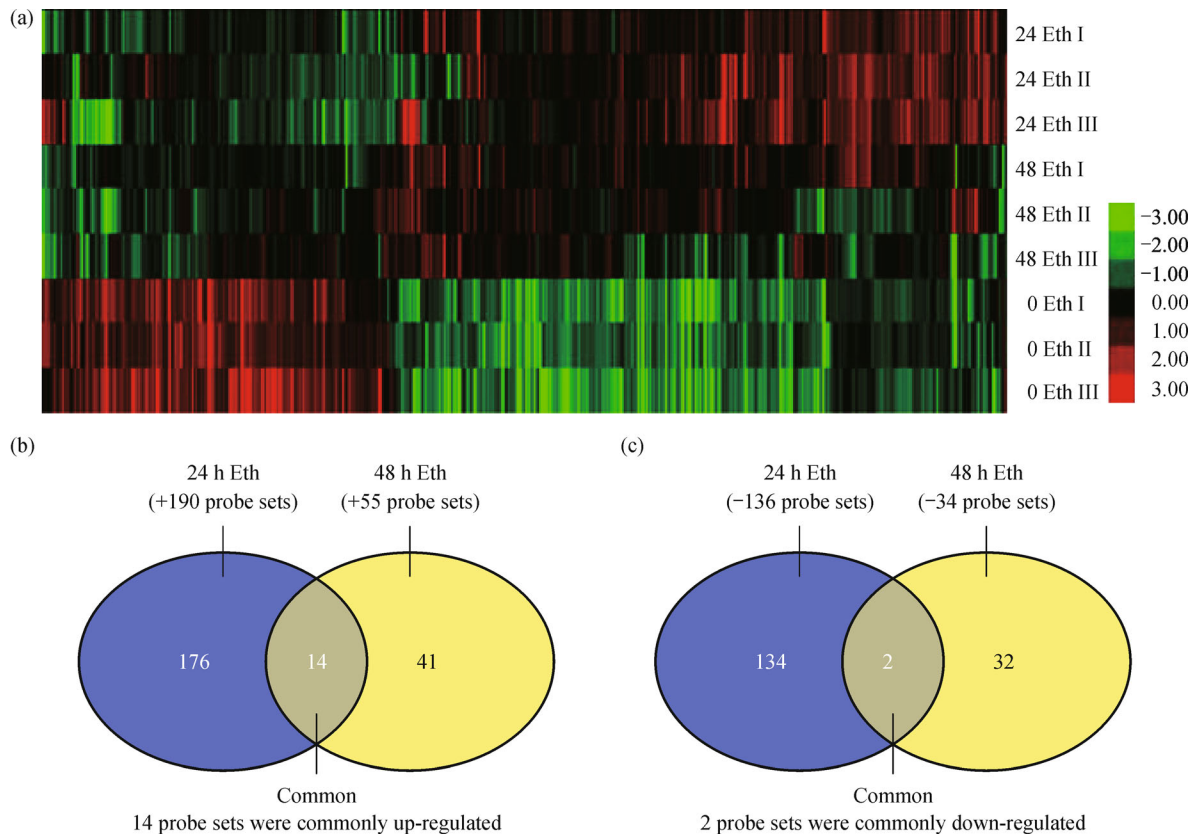

Fig. 2 Hierarchical cluster analysis and Venn diagrams of differentially-expressed genes in response to ethephon treatment. (a) Hierarchical cluster analysis of differentially-expressed genes in response to ethephon treatment. 0 Eth, ethephon treatment 0 h; 24 Eth, ethephon treatment 24 h; 48 Eth, ethephon treatment 48 h. I, II, III indicate biological replicates; (b) the upregulated probe sets for ethephon treatment after 24 and 48 h; (c) the downregulated probe sets for ethephon treatment after 24 and 48 h.

Venn diagram (Fig. 2b, Fig. 2c) which showed that, of the upregulated genes, 190 probe sets were detected at 24 h, and 55 probe sets were detected at 48 h. Further analyses revealed that 14 probe sets were sustained and had stable upregulation at 24 and 48 h (Fig. 2b). Of the down-regulated genes, 136 probe sets were detected 24 h, and 34 were detected at 48 h. Further analyses showed two probe sets were persistently downregulated at 24 and 48 h (Fig. 2c). These 16 probe sets were continuously expressed throughout the ethephon treatment, which was consistent with results of the hierarchical cluster analysis, suggesting that these probe sets may be potentially important in Zong 31 internode cell elongation.

3.3 Gene ontology analysis of the differentially-expressed genes

GO ontology is widely applied to understand the biological significance of differential gene expression data obtained using microarrays. To evaluate the potential functions of these differentially-expressed genes after 24 h in response to ethephon, we performed GO category enrichment analysis for 326 probe sets using the AgriGO online service (http://bioinfo.cau.edu.cn/agriGO). In the Affymetrix maize microarray, these 326 genes are represented by 326 probe sets. As shown in Table 2 and Table 3, 75.3% of 190 upregulated probe sets and 69.9% of 136 down-

regulated probe sets with GO term annotations were identified.

The GO categories (Table 2) and results of enrichment analysis for the 190 upregulated probe sets, which contained 13162 annotated reference items on the whole genome chip, were classified into 16 functional categories. A total of 143 probe sets with GO term annotations were identified ($P < 0.05$). Further analyses revealed that the most significantly enriched GO term of the biological processes was cell wall macromolecule catabolic process (GO:0016998, 8.90×10^{-6}). Other GO terms were also enriched, such as the cell wall macromolecule metabolic process (GO:0044036, 5.70×10^{-5}), cellular amino acid derivative metabolic processes (GO:0006575, 0.002), and the multi-organism process (GO:0051704, 0.044). In the cellular component, there were 22 probe sets each with GO term annotations for cytoplasmic membrane-bound vesicle (GO:0016023, 0.0018), membrane-bound vesicle (GO:0031988, 0.0018), vesicle (GO:0031982, 0.002), and cytoplasmic vesicle (GO:0031410, 0.002). The functions of these probe sets in plants included gibberellin metabolic process, ethylene binding, ethylene-mediated signaling pathway, electron transport, hydrolase activity, peroxidase activity, lignin biosynthetic process, stilbene biosynthetic process, amino acid metabolic process, oxidoreductase activity and chloroplast binding. For molecular functions, there were nine, nine, seven, six

Table 2 Gene ontology analysis of upregulated probe sets responsive to ethephon treatment

GO ID	GO term	Query item	Background item	*P* value
Biological process				
GO:0016998	Cell wall macromolecule catabolic process	5	22	8.90×10^{-6}
GO:0044036	Cell wall macromolecule metabolic process	5	34	5.70×10^{-5}
GO:0006575	Cellular amino acid derivative metabolic process	9	251	0.0020
GO:0051704	Multi-organism process	5	174	0.0440
Cellular component				
GO:0016023	Cytoplasmic membrane-bound vesicle	22	1027	0.0018
GO:0031988	Membrane-bound vesicle	22	1027	0.0018
GO:0031982	Vesicle	22	1035	0.0020
GO:0031410	Cytoplasmic vesicle	22	1035	0.0020
Molecular function				
GO:0050825	Ice binding	9	135	2.50×10^{-5}
GO:0050824	Water binding	9	135	2.50×10^{-5}
GO:0016798	Hydrolase activity, acting on glycosyl bonds	7	153	0.0017
GO:0004553	Hydrolyzing O-glycosyl compounds	6	130	0.0035
GO:0005506	Iron ion binding	8	264	0.0091
GO:0003677	DNA binding	15	752	0.0180
GO:0020037	Heme binding	5	142	0.0210
GO:0046906	Tetrapyrrole binding	5	157	0.0310
Total		143 (75.3%)	13162	

probe sets respectively with GO term annotations for ice binding (GO:0050825, 2.50×10^{-5}), water binding (GO:0050824, 2.50×10^{-5}), hydrolase activity, action on glycosyl bonds (GO:0016798, 0.0017) and hydrolyzing O-glycosyl compounds (GO:0004553, 0.0035), respectively. Their functions included cell wall macromolecule catabolic process, cation binding, carbohydrate metabolic process and rRNA N-glycosylase activity. There were 8, 15, 5, and 5 probe sets with GO term annotations for iron ion binding (GO:0005506, 0.0091), DNA binding (GO:0003677, 0.018), heme binding (GO:0020037, 0.021), and tetrapyrrole binding (GO:0046906, 0.031), respectively. The functions of these probe sets in plant included iron ion transport, iron ion binding, electron transport, gibberellin metabolic process, peroxidase activity, oxygen transporter activity, oxygen transport, transcription factor activity, regulation of transcription, defense response, ethylene binding, protein histidine kinase, gibberellin metabolic process, heme binding and cytoplasmic membrane-bound vesicle formation.

One hundred and thirty-six probe sets were downregulated according to GO categories and enrichment analysis, which contained 13162 annotated reference items on the whole genome chip. These were classified into 10 functional categories, including only biological process and cellular component, and a total of 95 probe sets with GO term annotations were identified (Table 3). Further analyses revealed that the most significant enriched GO term was RNA metabolic process (GO:0016070, 0.0015) in biological processes. Also there were other GO terms enriched, including regulation of transcription, DNA-dependent (GO:0006355, 0.033), nucleic acid metabolic process (GO:0006139, 0.037), regulation of RNA metabolic process (GO:0051252, 0.044) and regulation of biological process (GO:0050789, 0.049). The functions of these probe sets in plants included signal transduction

(phosphorelay), chromatin assembly or disassembly, structural constituent of chromatin, transcription factor activity, DNA topological changes, pyrimidine nucleotide biosynthetic process, mitotic cell cycle spindle assembly checkpoint, cell division, salicylic acid-mediated signaling pathway and jasmonic acid (JA) mediated signaling pathway. For the cellular component, there were 14 probe sets each with GO term annotations for cytoplasmic membrane-bound vesicle (GO:0016023, 0.016), membrane-bound vesicle (GO:0031988, 0.016), vesicle (GO:0031982, 0.017), and cytoplasmic vesicle (GO:0031410, 0.017). The functions of these probe sets in plants include gibberellin metabolic process, ethylene binding, ethylene-mediated signaling pathway, cytoplasmic membrane-bound vesicle, metal ion binding, and electron transport. In addition, 10 probe sets GO terms included chloroplast (GO:0009507, 0.045); their functions in plants include hydrolase activity, unfolded protein binding, embryonic development, chloroplast organization, DNA topological change and response to chlorate.

The results of GO analysis indicated that the differentially-expressed genes were enriched ($P < 0.05$) in biological process, cellular component, and molecular function classifications. Notably, cell wall macromolecule catabolic process (GO:0016998, 8.90×10^{-6}) was significantly enriched among the 190 upregulated probe sets and RNA metabolic process (GO:0016070, 0.0015) was significantly enriched among the 136 downregulated probe sets. Also many genes were predicted to be involved in amino acid metabolic process, lignin biosynthetic process, transcription factor activity, defense response, metal ion binding, electron transport and IAA/GA metabolic processes. Ethylene/jasmonic acid-mediated signaling genes were also preferentially differentially expressed, indicating that various factors in cellular size have a specific function in the maize internode elongation process induced by

Table 3 Gene ontology analysis of down-regulated probe sets responsive to ethephon treatment

GO ID	GO term	Query item	Background item	P value
Biological process				
GO:0016070	RNA metabolic process	13	693	0.0015
GO:0006355	Regulation of transcription, DNA-dependent	7	419	0.0330
GO:0006139	Nucleic acid metabolic process	16	1373	0.0370
GO:0051252	Regulation of RNA metabolic process	7	446	0.0440
GO:0050789	Regulation of biological process	16	1421	0.0490
Cellular component				
GO:0016023	Cytoplasmic membrane-bound vesicle	14	1027	0.0160
GO:0031988	Membrane-bound vesicle	14	1027	0.0160
GO:0031982	Vesicle	14	1035	0.0170
GO:0031410	Cytoplasmic vesicle	14	1035	0.0170
GO:0009507	Chloroplast	10	749	0.0450
Total		95 (69.9%)	13162	

ethephon. Plant organ growth is a prerequisite for both cell elongation and division[40,41], as well as for maize internode elongation and changes in cell morphology. The elongation and division of the meristem cells is largely regulated by the feedback regulation mechanism of small molecular compounds and biological macromolecules involved in cell metabolism. Overall, the analysis implies that exogenous ethephon treatment may change cell metabolism through a mechanism involving a network of small molecular compounds and biological macromolecules. The functional categories of differentially-expressed genes associated with cell elongation and division by ethephon treatment in maize internodes were further analyzed on the basis of the results of GO term annotations and gene classifications (Table 4 and Table 5).

3.3.1   Cell and cell wall component synthesis proteins

Further analysis of the functional categories of differentially-expressed genes indicated that six probe sets related to cell and cell wall component synthesis changed significantly. Of these, probe sets *Zm.7715.1.A1_at* (Wiscott-Aldrich syndrome, C-terminal) and *Zm.17188.1.A1_*at (Arabinogalactan protein) were upregulated more than 10-fold, and *Zm.16805.3.S1_at* (Chitinase) and *Zm.2147.1.A1_s_at* (Glycine-rich cell wall structural protein) were upregulated more than twofold. Cell and cell wall biogenesis is a complex process involving the action of many protein families directly involved in the synthesis of cell and cell polysaccharides and the rearrangement of polymers[42]. *ZmGRP3*[43] and *ZmGRP4*[44] have been reported to be root specific, with the highest expression level in the meristematic and elongation regions, as putative signal peptides become insoluble within the cell wall. As for two downregulated probe sets, one gene was associated with a cell cycle checkpoint protein MAD2 (*Zm.16475.1.S1_at*) homolog, which is related to the microtubules of internode cells. The other gene (*Zm.6789.1.A1_at)* was the cell division cycle protein 20. Furthermore, the cell wall of maize stalks represents a major energy storage compartment as most of the solar energy captured by plants is photosynthetically converted into chemical energy locked into the cell wall polymers: cellulose, hemicellulose, and lignin[42]. These are important factors that can directly affect cell elongation and extension, suggesting that ethephon treatment may influence differentially-expressed gene responses affecting internode cell polysaccharides and the rearrangement of polymers.

3.3.2   Stress-responsive genes

There were nine probe sets that changed significantly related to the stress response functional category. Of these, five probe sets (*Zm.10149.1.A1_at*, *Zm.94.1.S1_at*, *Zm.17335.1.S1_at*, *Zm.2193.1.A1_at* and *Zm.1449.2.*

S1_a_at) were induced strongly during drought stress and were upregulated more than twofold in response to ethephon[29]. These are putatively homologs of *dehydrin COR410*, *Dof2*, *stachyose synthase*, *glutathione transferase 20* and *NaCl stress protein 1* in *Arabidopsis*, respectively. The four downregulated probe sets, *Zm. 283.1.S1_at* included genes involved in production of defensive chemicals, putatively encoding *tryptophan synthase* and *bx 1* (benzoxazinless 1) were downregulated more than 12-fold. The other three probe sets (*Zm.2488.1. A1_at*, *Zm.16643.1.S1_at*, and *Zm.5799.1.A1_at*), which are putative homologs of *CTP synthase*, *peroxidase 27* and *gamma-tocopherol methyltransferase*, were downregulated two to 5-fold. These genes, preferentially expressed in response to internal and external stimuli, can trigger a wide range of developmental processes in plants. For example, *Zm.5799.1.A1_at* upregulation enhances antioxidants in maize[45], implying that these are excellent candidates for involvement in the biosynthetic processes and cellular organization of internodes.

3.3.3   Transcription factors

Transcription factors act as master regulators of cellular processes for modifying complex traits in crop plants. Twenty two transcription factor genes were detected and predicted to be excellent candidates for involvement in the response to ethephon. The expression of 10 probe sets was upregulated more than twofold, with *Zm.82.1.S1_at* (b1) more than 15-fold and *Zm.10147.1.A1_at* (NAC1 transcription factor) and *Zm.18537.1.S1_at* (leucine zipper protein) more than threefold. The other six probe sets (*Zm.217.1.S1_at*, *Zm.94.1.S1_at*, *Zm.4529.1.A1_a_at, Zm.12864.1.S1_at*, *Zm.3709.1.S1_at* and *Zm.275.1. S1_a_at*),which are the putative homologs of *MAP kinase 4, Dof 2, protease inhibitor, nuclear transcription factor, RNA recognition motif containing protein* and *ethylene receptor 2,* were upregulated twofold. Among the 12 downregulated probe sets, five probe sets (*Zm.9708.1. A1_at*, *Zm.325.1.A1_at*, *Zm.14450.1.A1_at*, *Zm.4871.1. A1_at* and *Zm.6485.1.A1_at*) were downregulated three to 5-fold, including the putative homologs of *bhlh84-DNA binding protein, RR6-response regulator, SBP-domain protein 5, growth-regulating factor 1, general regulatory factor 2*. The other seven probe sets (*Zm.1066.3.A1_at*, *Zm.6732.3.A1_at*, *Zm.19219.1.A1_at*, *Zm.326.1.S1_at*, *Zm.5439.1.S1_at*, *Zm.11846.1.A1_at*, *Zm.289.1.S1_at*), which are the putative homologs of *SBP-domain containing protein, HMG1/2-like protein, RNA binding protein, RR7, serine/threonine-protein kinase 12, SBP-domain protein 7, Rop6 protein*, respectively, were downregulated twofold. In previous studies, most attention was directed to the MYB and NAC transcription factors, however, transcription factors belonging to other families might also be involved in regulating cell wall biogenesis. In this

Table 4 Differentially-expressed cell component, stress-responsive, and transcription factor genes in the internode elongation response to ethephon treatment

Probe set ID	Changing fold	Gene ID	Description
Cell and cell wall component synthesis proteins			
Zm.7715.1.A1_at	18.16	GRMZM2G001661	Wiscott-Aldrich syndrome protein, C-terminal
Zm.17188.1.A1_at	9.87	GRMZM2G043035	Polypeptide: Arabinogalactan protein
Zm.16805.3.S1_at	4.50	GRMZM2G099454	Enzyme: Chitinase
Zm.2147.1.A1_s_at	2.68	GRMZM2G109959	Glycine-rich cell wall structural protein
Zm.16475.1.S1_at	0.33	GRMZM2G047143	Cell cycle checkpoint protein MAD2
Zm.6789.1.A1_at	0.27	GRMZM2G063192	Cell division cycle protein 20
Stress-responsive genes			
Zm.10149.1.A1_at	2.77	GRMZM2G147014	Dehydrin COR410
Zm.94.1.S1_at	2.49	GRMZM2G009406	Dof 2 (DNA binding with one finger 2)
Zm.17335.1.S1_at	2.42	GRMZM2G340656	Stachyose synthase
Zm.2193.1.A1_at	2.29	GRMZM2G434541	Glutathione transferase 20
Zm.1449.2.S1_a_at	2.00	GRMZM2G015605	NAC1 (NaCl stress protein1)
Zm.2488.1.A1_at	0.43	GRMZM2G153058	CTP synthase family protein
Zm.16643.1.S1_at	0.36	GRMZM2G320269	Peroxidase 27 (peroxidase superfamily protein)
Zm.5799.1.A1_at	0.31	GRMZM2G035213	Gamma-tocopherol methyl transferase
Zm.283.1.S1_at	0.08	GRMZM2G085381	bx1 (benzoxazinless1, tryptophan synthase)
Transcription factors			
Zm.82.1.S1_at	15.07	GRMZM2G172795	b1 (colored plant1, DNA binding domain)
Zm.10147.1.A1_at	6.80	GRMZM2G347043	NAC1 transcription factor(nactf49)
Zm.18537.1.S1_at	5.95	GRMZM2G117164	homeobox-leucine zipper protein ATHB-6
Zm.10253.1.A1_at	3.88	GRMZM2G134334	Multiple stress-responsive zinc-finger protein ISAP1
Zm.217.1.S1_at	2.75	GRMZM2G127141	MAP kinase 4
Zm.94.1.S1_at	2.49	GRMZM2G009406	Dof 2 (DNA binding with one finger 2)
Zm.4529.1.A1_a_at	2.17	GRMZM2G065557	Protease inhibitor/seed storage/LTP family
Zm.12864.1.S1_at	2.12	GRMZM5G857944	Nuclear transcription factor Y subunit A-3
Zm.3709.1.S1_at	2.08	GRMZM2G114325	RNA recognition motif containing protein
Zm.275.1.S1_a_at	2.07	GRMZM2G420801	Ethylene receptor homolog (etr2,Histidine kinase)
Zm.1066.3.A1_at	0.48	GRMZM2G067624	SBP-domain containing protein (sbp29)
Zm.6732.3.A1_at	0.47	GRMZM2G162284	HMG1/2-like protein(high mobility group)
Zm.19219.1.A1_at	0.45	GRMZM2G134398	RNA binding protein (NusB family)
Zm.326.1.S1_at	0.43	GRMZM2G096171	RR7 (response regulator 7)
Zm.5439.1.S1_at	0.43	GRMZM2G107689	serine/threonine-protein kinase 12
Zm.11846.1.A1_at	0.40	GRMZM2G460544	SBP-domain protein 7
Zm.289.1.S1_at	0.40	GRMZM2G176217	Rop6 protein (rhoptry protein)
Zm.6485.1.A1_at	0.32	GRMZM2G078641	General regulatory factor2 (14-3-3 protein)
Zm.4871.1.A1_at	0.30	GRMZM2G178261	Growth-regulating factor 1
Zm.14450.1.A1_at	0.28	GRMZM2G160917	SBP-domain protein 5
Zm.325.1.A1_at	0.26	GRMZM2G040736	RR6 (type-A response regulator)
Zm.9708.1.A1_at	0.17	GRMZM2G159456	bhlh84, DNA binding protein

Note: Changes≥2 fold indicate differentially upregulated genes; Change≤0.5 fold indicated differentially downregulated genes.

study, *ethylene receptor 2*, *SBP-domain containing protein* were shown to up or downregulate the internode development process involved in cell growth following ethephon treatment, indicating that some of these regulatory

Table 5 Differentially-expressed transporter, metabolic pathway, plant hormone synthesis and signal transduction genes in response to ethephon treatment

Probe Set ID	Changing fold	Gene ID	Description	Pathway
Transporters				
Zm.6759.1.A1_at	10.85	GRMZM2G472167	Peptide transporter PTR2	Oligopeptide transport
Zm.9753.1.A1_at	4.54	GRMZM2G318213	Grx_C15-glutaredoxin subgroup III	Electron transport
Zm.390.1.S1_at	3.30	GRMZM2G093195	d3 (dwarf plant 3, cytochrome P450 protein)	Electron transport
Zm.16578.1.A1_s_at	3.22	GRMZM2G126582	Aquaporin NIP-type	Transport
Zm.485.1.A1_at	2.60	GRMZM2G067402	Non-symbiotic hemoglobin	Oxygen transport
Zm.714.1.A1_at	2.60	GRMZM2G010868	PLT2 (phospholipid transfer protein homolog 2)	Lipid transport
Zm.4529.1.A1_a_at	2.17	GRMZM2G065557	Lipid binding protein	Lipid transport
Zm.13789.1.A1_at	2.10	GRMZM2G403007	Nonspecific lipid-transfer protein	Lipid transport
Zm.171.1.A2_at	0.41	GRMZM2G155021	Eukaryotic porin	Anion transport
Zm.4845.1.S1_at	0.18	GRMZM2G131525	Peroxidase (syntaxin-related protein)	Intracellular protein transport
Metabolic pathway related gene				
Zm.722.1.A1_at	21.51	GRMZM2G422750	c2 (colorless2, chalcone synthase)	Chalcone biosynthesis
Zm.354.1.S1_at	6.78	GRMZM2G114642	gl1 (glossy1, phospholipid transfer protein)	Metabolism
Zm.2869.1.A1_at	3.88	GRMZM2G093175	Asparagine synthetase	Asparagine biosynthesis
Zm.17335.1.S1_at	2.42	GRMZM2G340656	Stachyose synthase	Carbohydrate metabolism
Zm.17569.1.S1_at	2.31	GRMZM2G106263	Hydroxymethylglutaryl-CoA synthase	Steroid biosynthesis
Zm.13251.1.A1_at	0.49	GRMZM2G138060	su1 (sugary1, isoamylase)	Carbohydrate metabolism
Zm.17147.1.A1_at	0.47	GRMZM2G068506	Glucose-1-phosphate adenylyltransferase	Glycogen biosynthesis
Zm.2488.1.A1_at	0.43	GRMZM2G153058	CTP synthase	Pyrimidine nucleotide metabolism
Zm.5799.1.A1_at	0.31	GRMZM2G035213	Gamma-tocopherol methyltransferase	Vitamin E biosynthesis
Zm.283.1.S1_at	0.08	GRMZM2G085381	bx1 (benzoxazinless 1, tryptophan synthase)	Aromatic biosynthesis, Tryptophan biosynthesis
Plant hormone synthesis and signal transduction gene				
Zm.722.1.A1_at	21.51	GRMZM2G422750	c2 (colorless 2, chalcone synthase)	IAA (indole-3-acetic acid)
Zm.11809.1.A1_at	13.84	GRMZM2G380432	Phosphate-responsive 1 family protein	ABA (abscisic acid)
Zm.10147.1.A1_at	6.80	GRMZM2G347043	NAC1 transcription factor (nactf49)	IAA
Zm.7548.1.A1_at	5.35	GRMZM2G087192	Opr5 (12-oxo-phytodienoic acid reductase 5)	JA (jasmonic acid)
Zm.19276.1.A1_at	4.92	GRMZM2G315321	Ethylene-responsive element binding protein 2	Ethylene
Zm.463.1.A1_at	3.66	GRMZM2G014392	vp14 (viviparous 14, retinal pigment epithelial membrane protein)	ABA
Zm.13480.1.S1_at	3.57	GRMZM2G060940	GA20ox (gibberellin 20 oxidase)	GA (gibberellin)
Zm.390.1.S1_at	3.30	GRMZM2G093195	d3 (dwarf plant 3, cytochrome P450 protein)	GA
Zm.217.1.S1_at	2.75	GRMZM2G127141	MAP kinase 4	JA, IAA
Zm.275.1.S1_a_at	2.07	GRMZM2G420801	Ethylene receptor homolog (etr2, Histidine kinase)	Ethylene
Zm.5439.1.S1_at	0.43	GRMZM2G107689	serine/threonine-protein kinase 12	IAA
Zm.326.1.S1_at	0.43	GRMZM2G096171	RR7 (response regulator 7)	CTK (cytokinin)
Zm.12687.1.S1_a_at	0.40	GRMZM2G171702	Auxin efflux carrier component 1c	IAA
Zm.6485.1.A1_at	0.32	GRMZM2G078641	General regulatory factor 2 (14-3-3 protein)	GA
Zm.4871.1.A1_at	0.30	GRMZM2G178261	Growth-regulating factor 1	GA
Zm.325.1.A1_at	0.26	GRMZM2G040736	RR6 (type-A response regulator)	CTK
Zm.283.1.S1_at	0.08	GRMZM2G085381	bx1 (benzoxazinless1, tryptophan synthase)	IAA

Note: Changes≥2 fold indicate differentially upregulated genes; Change≤0.5 fold indicate differentially downregulated genes.

functions might well be fulfilled by some of the other transcription factor family members.

3.3.4   Transporters

Transporters are essential proteins for plants in various cellular processes, involved in enzyme function, cell elongation, signal transduction and maintenance of the plasma membrane potential[46]. Ten probe sets in in the transporters functional category changed significantly. Of these, eight probe sets were upregulated more than twofold and *Zm.6759.1.A1_at* (*Peptide transporter 2*) was upregulated more than 10-fold. The other seven probe sets (*Zm.9753.1.A1_at*, *Zm.390.1.S1_at*, *Zm.16578.1.A1_s_at*, *Zm.485.1.A1_at*, *Zm.714.1.A1_at*, *Zm.4529.1.A1_a_at* and *Zm.13789.1.A1_at*) were upregulated two to 5-fold, and were the putative homologs of *glutaredoxin subgroup III*, *d3*, *aquaporin NIP-type*, *non-symbiotic hemoglobin*, *PLT2*, *Lipid binding protein* and n*onspecific lipid-transfer protein*. Downregulated probe sets (*Zm.171.1.A2_at* and *Zm.4845.1.S1_at*), which are the putative homologs of e*ukaryotic porin* and *peroxidase*, respectively. Ion transport proteins regulate the ion balance inside and outside of cells; lipid transport proteins transfer phospholipids across membranes; water channel proteins remarkably increased the membrane water permeability. Transporter genes exist in all tissues of maize, and the expression of 11 probe sets changed during the internode development process after ethephon treatment, indicating that some of these regulatory functions may also involve in cell elongation and division in maize.

3.3.5   Metabolic pathway genes

Ten probe sets in the metabolic pathways functional category changed significantly, and the results of KEGG analysis were consistent with GO classification. Of these, five genes were upregulated more than twofold. *Zm.722.1. A1_at* (c2), which putatively encodes *chalcone synthase,* was upregulated more than 20-fold. The other four probe sets (*Zm.354.1.S1_at*, *Zm.2869.1.A1_at*, *Zm.17335.1. S1_at* and *Zm.17569.1.S1_at*) were upregulated two to 6-fold, and are the putative homologs of *phospholipid transfer protein*, *asparagine synthetase*, *stachyose synthase*, *hydroxymethylglutaryl-CoA synthase*. Four downregulated probe sets (*Zm.17147.1.A1_at*, *Zm.2488.1.A1_at*, *Zm.5799.1.A1_at*, *Zm.283.1.S1_at*) encode *glucose-1-phosphate adenylyltransferase*, *CTP synthase*, *gamma-tocopherol methyltransferase* and *tryptophan synthase*, respectively. These findings indicate that changes in metabolic pathways are interrelated. The significantly upregulated chalcone synthase may be affected by the uptake and translocation activities of other transporters in maize, which can facilitate the

homeostasis of nutrient ions in plant cells. However, further research is required to clarify the details of this regulatory mechanism.

3.3.6   Plant hormone synthesis and signal transduction genes

Plant hormones regulate a wide range of development processes, including seed germination, leaf expansion, stem elongation, flowering, and fruit and seed development[47]. Among the functional categories of differentially-expressed genes, 17 probe sets were involved in plant hormone synthesis and signal transduction (Table 5). Of these, 10 were upregulated more than twofold. *Zm.722.1. A1_at* (c2), which encodes ochalcone synthase, was upregulated more than 20-fold. *Zm.11809.1.A1_at*, which encodes a phosphate-responsive protein, was upregulated more than 13-fold. Two probe sets (*Zm.10147.1.A1_at*, *Zm.7548.1.A1_at*) that were upregulated by 5-fold are the putative homologs of *NAC1 transcription factor* and *Opr5*, respectively. Six probe sets (*Zm.19276.1.A1_at*, *Zm.463.1. A1_at*, *Zm.13480.1.S1_at*, *Zm.390.1.S1_at*, *Zm.217.1. S1_at* and *Zm.275.1.S1_a_at*) that were upregulated two to 5-fold, are the putative homologs of *ethylene-responsive element binding protein 2*, *vp14*, *GA20ox*, *d3*, *MAP kinase 4* and *ethylene receptor homolog*, respectively. Seven probe sets (*Zm.5439.1.S1_at*, *Zm.326.1.S1_at*, *Zm.12687. 1.S1_a_at*, *Zm.6485.1.A1_at*, *Zm.4871.1.A1_at*, *Zm.325.1. A1_at*, *Zm.283.1.S1_at*) were downregulated two to 5-fold and putatively encode *serine/threonine-protein kinase 12*, *RR7*, *auxin efflux carrier component*, *general regulatory factor 2*, *growth-regulating factor 1*, *RR6* and *bx1*, respectively. Further analyzed show that six probe sets (*Zm.722.1.A1_at*, *Zm.10147.1.A1_at*, *Zm.217.1.S1_at*, *Zm.5439.1.S1_at*, *Zm.12687.1.S1_a_at* and *Zm.283.1. A1_at*) were involved in IAA synthesis, four probe sets (*Zm.13480.1.S1_at*, *Zm.390.1.S1_at*, *Zm.6485.1.A1_at* and *Zm.4871.1.A1_at*) were related to GA signal transduction, two probe sets (*Zm.19276.1.A1_at* and *Zm.275.1. S1_a_at*) were related to ethylene synthesis and signal transduction; two probe sets (*Zm.11809.1.A1_at* and *Zm.463.1.A1_at*) were related to ABA signal transduction. The results from previous studies of dwarf mutants indicated that plant hormones, such as GAs, IAA, are important for the control of plant height[48]. The microarray data show that Gas and IAA synthesis and signal transduction genes were markedly up or downregulated by ethephon treatment, suggesting that these genes may be important in the internode elongation response to ethylene released in maize plants from ethephon. The findings for genes related to ABA, cytokinin and jasmonic acid imply that crosstalk between ethylene and other plant hormones occur concurrently and closely regulates the internode elongation process in maize, and that exogenous ethephon

application might cause changes in hormones that inhibits internode elongation.

3.4 Verification of microarray data by quantitative real-time PCR

We conducted real-time PCR analysis for 16 randomly selected genes to confirm the validity of the microarray data. A set of 16 differentially-expressed genes were chosen, representing three types of response observed in the microarray analysis: upregulation, downregulation and no expression in control or ethephon treatments. 18S rRNA was used as a reference with a steady level of expression (Primers used are shown in Appendix A, Table S1).

The quantitative real-time PCR results showed that the expression trends of these genes were largely consistent with those of the microarray analysis. However, given the speed of maize internode absorption of released ethylene and the greater sensitivity of real-time PCR[49–51], the variation of differentially-expressed genes in real-time PCR was considered more significant than those in the microarray analysis (Table 6). For some genes, quantitative real-time PCR suggested a larger difference in expression levels. For example, the transcript encoding a putative *Opr5* (*12-oxo-phytodienoic acid reductase5*) showed a 5.35-fold upregulation 24 h after ethephon treatment in the microarray analysis (*Zm.7548.1.A1_at*), but the quantitative real-time PCR result showed 64.3-fold difference at the same time.

4 Discussion

4.1 Internode phenotypes showed significant difference in response to ethephon treatment

We characterized the phenotypic differences in plants of maize inbred line Zong 31 with and without exogenous ethephon treatment (Fig. 1a, Fig. 1b), and the results were consistent with previous research[3,6,23]. The characterization of cell morphology by analysis of paraffin sections from the two group revealed that the internode cells of the ethephon treated group had a higher content of syringyl lignin units, and thinner and relatively wider parenchymatous tissue cells than the control group (Fig. 1e, Fig. 1f, and Fig. 1g), which validated the substantial differences between internode elongation in the control and ethephon treated maize.

4.2 Timing of differentially-expressed genes in response to ethephon treatment

All plant growth regulator processes are dynamic. In our results, with the passage of time, the number and intensity of the differentially-expressed genes 48 h after ethephon treatment might become reduced, which could result from a relatively lower amounts of ethylene released at the cellular level compared to that at 24 h, and thus give rise to a more limited effect on differential expression of transcriptional response (Table 1, Fig. 2a). In the Venn diagram (Fig. 2b, Fig. 2c), a relatively small number of

Table 6 Verification of microarray results by qPCR

Probe set ID	Gene ID	Description	GeneChip		Qpcr	
			24 h	48 h	24 h	48 h
Zm.217.1.S1_at	GRMZM2G127141	MAP kinase 4	2.75	3.16	3.81	9.22
Zm.82.1.S1_at	GRMZM2G172795	b1 (colored plant1, DNA binding domain)	15.07	2.84	85.12	12.84
Zm.10147.1.A1_at	GRMZM2G347043	NAC1 transcription factor (nactf49)	6.80	4.54	21.83	18.17
Zm.7548.1.A1_at	GRMZM2G087192	Opr5 (12-oxo-phytodienoic acid reductase 5)	5.35	3.18	64.28	5.33
Zm.275.1.S1_a_at	GRMZM2G420801	Ethylene receptor homolog (etr2, histidine kinase)	2.07	1.43	6.50	2.93
Zm.11809.1.A1_at	GRMZM2G380432	Phosphate-responsive 1 family protein	13.84	7.84	2.94	7.01
Zm.17569.1.S1_at	GRMZM2G106263	Hydroxymethylglutaryl-CoA synthase	2.31	1.91	1.01	1.18
Zm.390.1.S1_at	GRMZM2G093195	d3 (dwarf plant 3, cytochrome P450 protein)	3.30	–	8.07	4.37
Zm.2147.1.A1_s_at	GRMZM2G109959	Glycine-rich cell wall structural protein	2.68	–	15.94	1.07
Zm.16643.1.S1_at	GRMZM2G320269	Peroxidase 27 (peroxidase superfamily protein)	0.36	0.74	1.31	2.99
Zm.326.1.S1_at	GRMZM2G096171	RR7 (response regulator 7)	0.43	0.72	0.17	0.18
Zm.171.1.A2_at	GRMZM2G155021	Eukaryotic porin	0.41	0.89	0.88	0.94
Zm.12687.1.S1_a_at	GRMZM2G171702	Auxin efflux carrier component 1c	0.40	0.62	0.15	0.21
Zm.16475.1.S1_at	GRMZM2G047143	Cell cycle checkpoint protein MAD 2	0.33	0.50	0.64	0.94
Zm.325.1.A1_at	GRMZM2G040736	RR6 (type-A response regulator)	0.26	1.17	0.19	0.87
Zm.283.1.S1_at	GRMZM2G085381	bx1 (benzoxazinless 1, tryptophan synthase)	0.08	1.08	0.11	0.52

Note: Dashes indicate undetected. Data in bold represents significant difference at $P < 0.01$ in the consecutive comparison of time points.

probe sets (16) were regulated in common between 24 and 48 h. Thus, with the decrease of ethylene released, the intensity of expression of a few probe sets remained steady and level in a certain time effects (Fig. 2b, Fig. 2c). Therefore, it can be speculated that the probe sets regulated in common were absolutely necessary for response of maize internode elongation to ethephon treatment.

4.3 Internode cell elongation and expression of related differentially-expressed genes in response to ethephon treatment

Internode number and length are two crucial factors affecting plant height. In maize, internode elongation is attributed to the cell division and elongation of intercalary meristems at the base of growing internodes[36,37]. As maize stems develop, sclerenchyma and the rind region parenchyma tissues develop thick, lignified cell walls[52]. Genes involved in cell and cell wall-related processes might also function in cell growth and cell wall remodeling during a defense response[53]. Our results show that the differentially-expressed genes significantly change cell and cell wall component synthesis, transporters, stress-responsive and metabolic pathway genes, and transcription factors.

The functional category, cell and cell wall component synthesis proteins, contained 6 differentially-expressed genes, a probe set (*Zm.7715.1.A1_at*) that putatively encode the homologs of *Wiscott-Aldrich syndrome proteins* was significantly upregulated, which sends a signal to the cytoskeleton through the Arp2/3 complex, an actin-nucleating assembly that regulates the structure and dynamics of actin filament networks at the leading edge of a cell[54]. *Zm.6789.1.A1_at*, which ecodes a putative cell division cycle protein 20 related to cell division, was significantly downregulated; another probe set (*Zm.17188.1.A1_at*) encodes a putative arabinogalactan protein which was significantly upregulated, and which is also involved in cell division during pollen germination and pollen tube growth[55]. In indica rice, a homolog of an arabinogalactan protein gene *OSIAGP*, performs a similar function to *ATAGP23* in *Arabidopsis*, and regulates pollen tube growth in the dark[56]. *Zm.2147.1.A1_s_at* encodes a putative glycine-rich cell wall structural protein involved in cell wall component synthesis. It is noteworthy that a similar expression trend in response to ethephon treatment was obtained by quantitative real-time PCR, which showed that *Zm.2147.1.A1_s_at* was upregulated 2.68-fold in the microarray data. Previous studies have shown that *ZmGRP* has a putative RNA binding motif[57], and is expressed in the epidermal cells of embryo, scutellar tissue and young leaf, and is induced by ABA, water stress and wounding in leaves[58]. The expression of a related gene, *ZmGRP3,* was root-specific, with the highest expression level in the meristematic and elongation regions[43]. Based on these analyses, we confidently hypothesize that *Zm.2147.1.*

A1_s_at may have a similar mode of action in the intercalary meristem of maize internodes. *Zm.16475.1. S1_at* was the putative homolog of a *cell cycle checkpoint protein MAD2*. Previous studies showed that there is a link between MAD2 and cell cycle regulatory proteins in the initiation of anaphase[59]. Combined immunolocalization of MAD2 and a recently cloned maize CENPC homolog indicates that MAD2 localizes to an outer domain of the prometaphase kinetochore, and MAD2 staining show that microtubule attachment has a major role in the mitotic spindle checkpoint but the meiotic spindle checkpoint may rely more heavily on sensing the amount of tension at the kinetochore[60]. Our study found that *Zm.16475.1.S1_at* was downregulated 0.33-fold in quantitative real-time PCR which also agreed with the statistical analysis of microarray data, suggesting that cell cycle checkpoint protein MAD2 might potentially function in maize internode elongation in responses to ethephon treatment[59–62].

In crops, internode cell elongation responds continuously to various biotic and abiotic stresses, which affect the growth of individual plants and the final crop production. With the exception of cell and cell wall component synthesis proteins, the GeneChip results showed that stress-responsive genes, transcription factor, transporters, metabolic pathway-related genes, plant hormone synthesis and signal transduction genes also participate in internode cell elongation process in responses to ethephon treatment. Nine probe sets with stress-responsive genes changed significantly. Among them, *Zm.283.1.S1_at*, representing the putative homolog of the maize *bx1,* which catalyzes the conversion of indole-3-glycerol phosphate to indole and also serves as the penultimate intermediate in the formation of tryptophan by tryptophan synthase in secondary metabolism, was downregulated 0.08-fold[63]. Additionally, 22 annotated transcription factor genes were detected (Table 4), suggesting that various transcriptional regulatory mechanisms play important functions in responses to ethephon treatment in intercalary meristems of maize internodes. Among the total differentially-regulated genes were 10 probe sets that were annotated as transporters which changed significantly, which suggests that various regulatory pathways are involved in the cell elongation and cell division responses to ethephon treatment in maize internodes. For example, *Zm.6759.1.A1_at*, which represents the putative homolog of the peptide transporter *PTR2* in *Arabidopsis*, was upregulated 10.9-fold, and was identified as a flowering and seed development related gene[64]. Recent reports show that the homolog gene of the peptide transporter *AtPTR1* was localized to the plasma membrane and was shown to expressed in vascular tissue, and to mediate transport of the dipeptide phytotoxin phaseolotoxin[65]. In addition, it has been reported that salicylic acid and methyl jasmonate can regulate the expression of *AtPTR3* and the mutant has increased susceptibility to virulent bacterial pathogens and contains elevated levels of reactive oxygen species[66]. According to

microarray data, 10 of the sequences homologous to probe sets that changed significantly were putatively participated in metabolic pathways, which suggested that various regulatory metabolic and biosynthesis pathways change in responses to ethephon treatment in maize internodes. For example, *c2* (*Zm.722.1.A1_at*), representing the putative homolog of the chalcone synthase gene, was significantly upregulated 21.5-fold. Chalcone synthase, the first enzyme in the pathway to flavonoids and anthocyanins, is upregulated that could lead to increased auxin transport[67]. This suggests that flavonoids might act as endogenous auxin transport inhibitors in plants[68]. 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[69], including the pericarp, the aleurone layer of the endosperm tassels[70], and vegetative organs such as ear husks and leaf sheaths[71]. Based on the above analysis, phenotypic change in internodes in response to ethephon treatment may result from the interactive effect of these significantly changed genes in intercalary meristem cell level.

4.4 Possible roles of plant hormone pathway and expression in internode cell elongation responses to ethephon treatment

It is well known that plant hormones play a crucial role in plant height regulation[13,71–74]. Meristem cell elongation and expansion contribute significantly to the growth and morphogenesis of maize internodes, which usually undergo substantial enlargement when they differentiate. The extent of elongation depends on the cell type and is often regulated by environmental conditions and endogenous hormones.

In this study, three genes (the probe set of *Zm.275.1. S1_a_at*, *Zm.19276.1.A1_at* and *Zm.7548.1.A1_at*) were annotated and related to the ethylene pathway. *Zm.275.1. S1_a_at*, a homolog of *Arabidopsis ER-associated receptor* and perceives ethylene gas, binds to ethylene and is proposed to inhibit receptor function[10,75]. *Zm.19276.1. A1_at,* the putative homolog of several *Arabidopsis* EREBP transcription factors, was upregulated 4.92-fold and is known to be the immediate target of EIN3/EIL1, which can bind to a primary ethylene response element in the promoters of EREBP genes[75]. *Zm.19276.1.A1_at,* upregulated 4.92-fold, is the putative homolog of several *Arabidopsis* EREBP transcription factors, and is known to be the immediate target of EIN3/EIL1, which can bind to a primary ethylene response element in the promoters of EREBP genes[76]. *Zm.7548.1.A1_at* (*Opr5*) putatively, which encodes a jasmonate-mediated gene related to cyclopentanones, was upregulated 5.35-fold and functions as a plant growth regulator in various developmental processes such as root elongation, senescence, anther dehiscence and tuber formation[77], which are also involved in the ethylene signal transduction pathway.

Two genes (*Zm.11809.1.A1_at*, *Zm.463.1.A1_at*) were related to ABA synthesis and signal transduction. It has been reported that the function of the ABA signaling pathway is significantly enriched in response to various biotic and abiotic stresses, seed germination and stomata regulation in various crops[78], but there are, however, few reports of the function of ABA in relation to plant height or internode elongation. Compared with ABA, although also known as the stress hormone, ethylene has been reported to mediate submergence-induced shoot elongation in rice, which is the initial regulator of fast underwater elongation[79,80]. During submergence, low oxygen stresses promote ethylene synthesis in submerged tissues, and elevated GA/IAA and lower ABA levels lead to increases in cell division and cell elongation activities of internodes growth in deep water rice[14]. These research findings are completely contrary to our study of maize. This is understandable, as the two crops belong to different species and differ in the evolution of primary process in plants and the developmental responses to their associated physical environments. We speculate that the molecular basis of the internode elongation responses to an ethephon treatment would be different between crop species as different as maize and deep water rice.

Cytokinins are plant-specific hormones that regulate cell division and are the most thoroughly studied hormones involved in maintaining meristem activity[81], and increased cytokinin production is associated with the formation of larger vegetative meristems[82]. Based on this study, two probe sets (*Zm.325.1.A1_at*, *Zm.326.1.S1_at*) that encode a putative response regulator receiver, identified as ARR homolog genes negatively influencing meristem size, were significantly downregulated[83]. The results from previous studies of dwarf mutants indicated that GA plays significant roles in the plant height decision, and plants defective in GA biosynthesis show typical GA-deficient phenotypes, such as dwarfism, small, dark-green leaves, inhibited cell growth and defective flowering[84,85]. In maize, several genes have been reported that encode the enzymes involved in GA biosynthesis, and mutants for all of these genes show dramatically reduced plant height[85]. *D8* is a negative GA-responsive regulator in maize[86], and its orthologs in *Arabidopsis* (*GAI*)[87] and *wheat* (*Rht*)[88] share a conserved DELLA domain that is important for the recognition of GAs. *ZmGA3ox2* (*D18*), an ortholog of *OsGA3ox2*, encoding a GA3β-hydroxylase, has a similar expression pattern to *OsGA3ox2*, which has been confirmed as controlling the elongation of the vegetative shoot[89]. Here we showed that mRNAs corresponding to four probe sets (*Zm.13480.1.S1_at* and *Zm.390.1.S1_at*) were significantly changed in the maize internode elongation responses to ethephon treatment. *Zm.13480.1.S1_at*, which was upregulated 3.75 times, is an ortholog of *OsGA20ox2* (*SD1*) encoding a gibberellin 20 oxidase, a key enzyme in the biosynthesis of GA that catalyzes the three steps GA 53 → GA 44 → GA 19 → GA 20, which

has been confirmed as controlling shorter leaves and stems[90]. *Zm.390.1.S1_at* was upregulated 3.30 time, and is an ortholog of *d3* (cytochrome P450) encoding an early step in the biosynthesis of GA, probably the 13-hydroxylation step, which has been confirmed as regulating shorter plants and stems[91,92]. *Zm.6485.1.A1_at* was downregulated and was identified as a 14-3-3 family protein, which is a post-translational regulator involved in the regulation of endogenous amounts of GA biosynthetic pathway[93]. *Zm.4871.1.A1_at* was downregulated and was identified as growth-regulating factor protein containing two conserved regions: the QLQ (Gln, Leu, Gln) and WRC (Trp, Arg, Cys) domains, which show a pleiotropic phenotypes in *Arabidopsis* and have regulatory roles in stem elongation, curly leaves and delayed flowing[94]. The results indicated that these probe sets involved in GA biosynthetic pathway are likely to lead to a shorter cell phenotype in response to ethylene release, resulting in the reduced maize internode elongation following ethephon treatment.

IAA has also been reported to regulate plant height and cell growth[95]. The exogenous application of IAA activates cell division in pericycle cells, resulting in the formation of additional lateral roots[96]. In the present data, six probe sets were detected and related to the Aux/IAA signaling pathway. For example, *Zm.10147.1.A1_at* (*NAC transcription factor 49*) was significantly upregulated 6.8-fold, and is a putative homolog of an *Arabidopsis* transcription activator *NAC1*, consisting of an N-terminal conserved NAC-domain that binds to DNA and a C-terminal activation domain, which may form a helix-turn-helix structure that specifically binds target DNA. Previous studies have shown that gene *NAC1* was induced by auxin and mediates auxin signaling to promote lateral root development[97]. Moreover, NAC genes may be critical in adjusting the development of the shoot apical meristem and the differentiation of flower organs, which may participate in incorporating responses to environmental and endogenous stimuli into the process of plant lateral root development[98]. The maize *ZmSIMK1* gene (*Zm.217.1.S1_at*) which was significantly upregulated 2.75-fold, encodes mitogen-activated protein kinase 1, which can phosphorylate specific effector proteins leading to activation of cellular responses. *ZmSIMK1* has a positive effect in *Arabidopsis* germination and growth[99]. *Zm.5439.1.S1_at* was significantly downregulated 0.43-fold, and encodes serine/threonine-protein kinase 12, which is the putative homolog of SNF1-related protein kinase. This kinase was first found in yeast (from where the name originated) and is highly conserved, with pivotal roles in plant growth and metabolic responses to cellular stress[100]. *Zm.12687.1.S1_a_at* (*ZmPIN1d*), which was significantly downregulated 0.4-fold, encodes a PIN-form protein. *ZmPIN1d* was reported to be a PIN auxin efflux carrier family member that is localized in the postembryonic root and expressed in maize root apexes,

epidermis, stem and central cylinder[101,102], and marks the transition from the vegetative to the reproductive development in the shoot apical meristem and inflorescence meristem. Therefore, we propose that polar auxin transport decreased in response to released ethylene in maize may trigger a wide range of developmental processes from crosstalk with ethephon treatment, such as inhibiting cell elongation at internodes meristem. c2 (the probe set of *Zm.722.1.A1_at*) represents a putative homolog of chalcone synthase. *Zm.283.1.S1_at* was upregulated 0.08-fold, and encodes maize tryptophan synthase bx1, another metabolic pathway related gene. In our previous study, the endogenous auxin (IAA) and gibberellin (GA4) contents of maize internodes were significantly decreased, abscisic acid (ABA) content was significantly increased, but the ratio of GA4/ABA was significantly decreased[103,104]. In combination, this suggests that ethylene release, inducing crosstalk between plant hormone signaling pathways, is one of the explanations for internode shortening and thickening of maize. Moreover, the morphological and microarray data presented here support the suggestion that the potential components of six auxin transport genes and four GA biosynthesis pathway genes play an important role in primarily targeting elongation processes, including upregulation of *GA20ox2* and *D3*, downregulation of *General regulatory factor 2* and *Growth-regulating factor 1* in the GA signaling pathway, c2, upregulation of *MAP kinase 4* and *NAC1*, *bx1*, and downregulation of *Auxin efflux carrier component 1c*, *serine/threonine-protein kinase 12* in the Aux/IAA signaling pathway.

Based on a described network of plant hormone signaling pathways[13,68,73,74,105,106] and the putative components of plant hormone responses identified in this research, a putative schematic of the cellular plant hormone signaling pathways in response to ethephon treatment was proposed. The blue probe sets represents the putative cell and cell wall component genes identified in this research. The small arrows indicate up or down-regulation of the mRNAs corresponding to the probe sets in response to ethephon treatment. Arrows and t-bars represent positive and negative effects, respectively. Solid lines indicate effects that occur through direct interaction, whereas dotted lines indicate effects that occur through indirect interaction.

5 Conclusions

The microarray data showed that more genes are involved in internode elongation 24 h after ethephon treatment, and many genes in plant hormone signaling pathways were markedly upregulated or downregulated. We also conducted a comparative analysis by qPCR, suggesting that these genes may be important in maize internode responses to gaseous ethylene release. Based on a described network

of plant hormone signaling pathways[13,73,74,105,106], a putative schematic of the cellular response to ethephon treatment is proposed which incorporates most of the significantly change probe sets identified in maize internodes (Fig. 3). Although little is known about how ethylene triggers such a wide array of diverse responses during the internode elongation process in maize, our results provide a basis for the selection and functional analysis of candidate genes involved in inhibition of internode elongation by ethephon treatment. These include six auxin transport genes and four GA biosynthesis pathway genes, and a gene (the probe set of *Zm.13480.1. S1_at*), an ortholog gene of *OsGA20ox2* (*SD1*) with an unknown function during internode elongation in response to induced ethylene release in maize. Further functional analyses of the differentially-expressed genes identified in our results should provide more information and eventually a better understanding of the reduction in plant height and prevention of lodging in maize by ethylene.

Supplementary materials The online version of this article at http://dx. doi.org/10.15302/J-FASE-2016103 contains supplementary materials (Appendix A)

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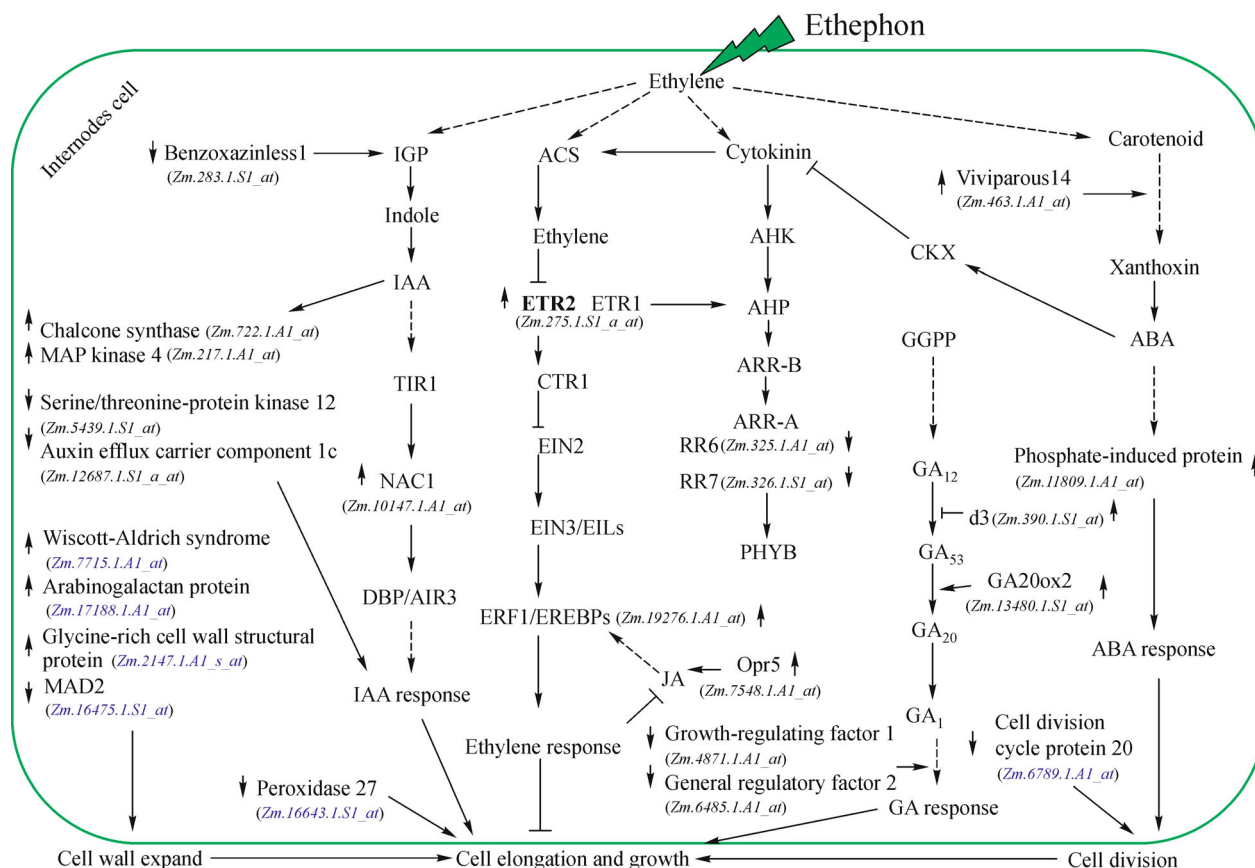

Fig. 3 A putative schematic of the differentially-expressed internode cell genes involved in plant hormone crosstalk in response to ethephon treatment in maize plants. MAD 2, mitotic arrest deficient 2; IGP, indole-3-glycerol phosphate; IAA, indole-3-acetic acid; TIR1, transport inhibitor response 1; NAC1, NaCl stress protein1; DBP, DNA binding protein; AIR3, auxin-induced in root cultures 3; ACS, 1-aminocyclopropane-1-carboxylate synthase; ETR1 (2), ethylene receptor 1 (2); CTR1, constitutive triple response 1; EIN2 (3), ethylene insensitive 2 (3); EILs, ethylene insensitive 3-like; ERF1, ethylene response factor 1; EREBPs: ethylene-responsive element binding protein; AHK, *Arabidopsis* histidine kinases; AHP, *Arabidopsis* histidine-phosphotransfer proteins; ARR-A (B), *Arabidopsis* response regulator-A (B); RR6 (7), response regulator 6 (7); PHYB, phytochromes B; Opr5, 12-oxo-phytodienoic acid reductase 5; CKX, cytokinin oxidase; GGPP, geranylgeranyl pyrophosphate; GA, gibberellin; ABA, abscisic acid; d3, dwarf plant 3; GA20ox2, gibberellin 20 oxidase 2.

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