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# Cloning and characterization of an up-regulated GA 20-oxidase gene in hybrid maize

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**Abstract**

Previous studies revealed that GA content and metabolism are positively correlated with a faster shoot growth rate of hybrid, and recently, genes participating in both GA biosynthesis and GA response pathways were also found to be differentially expressed between wheat hybrid and its parental inbreds. In this study, an up-regulated GA 20-oxidase gene in a maize hybrid, designated *ZmGA20,* was cloned. *ZmGA20* contains an open reading frame (ORF) encoding 391 amino acid residues. BLASTX searches in GenBank revealed that the *ZmGA20* is homologous to the sequences of GA20ox proteins from different species, and analysis also indicated that *ZmGA20* had typical features of GA 20-oxidase proteins, including a "LPWKET" sequence. Semi-quantitative RT-PCR analysis showed that *ZmGA20* was expressed in different tissues and/or organs. The expression level of *ZmGA20* in the hybrid was higher than that in two parents (in roots, leaves, stems and embryo, and ears). The abundance of *ZmGA20* transcript was equal to that of the highly expressed parents, which provided molecular evidence for the observed GA content heterosis in maize hybrids.


*Keywords:* GA 20-oxidase; Gene expression; Maize; Heterosis

## 1. Introduction

Heterosis or hybrid vigour was defined as the better performance of hybrid plants over its parental inbreds in terms of viability, growth and productivity. Hybrid cultivars have been used in many crop plants and have made significant contribution to the world food supply [1]. However, molecular basis of heterosis is still poorly understood. Recent studies suggested that differential or nonadditive gene expression in the hybrid might contribute to the heterosis, and many differentially expressed genes have been identified [2–6]. However, the casual relationship between differentially expressed genes and the observed heterosis is still an area for further investigation. Since all the genes in the hybrids are derived from their parental inbreds, the phenotypic differences between the hybrids and their parents, or heterosis, could be best explained by the spatio-temporal differences in gene expression [3].

Gibberellins (GAs) are natural tetracyclic diterpenoid carboxylic acids. GAs play important roles in plant development, including in seed germination, stem elongation and flower formation. It has been reported that GAs are also related to the heterosis [7–11]. In maize [12–15], poplar [9], and sorghum [16,17], the endogenous GA content is elevated in the hybrids, and slower-growing inbreds that contain lower levels of endogenous GAs may be particularly promoted by exogenous $GA_3$. These results suggested that the increased endogenous GA content might contribute to the increased plant growth in the hybrids [16,17].

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Up to date, the biosynthetic pathway of active GA has been well understood, in which GA 20-oxidase (GA20ox) has been shown to be important in regulating the levels of active GAs [18]. The function of GA20ox is to convert C-20 from a methyl group to an aldehyde by sequential oxidations, and then to remove this C atom and produce C19-GAs (GA9 and GA20) [19]. GA20ox belongs to a large class of Fe-containing enzymes found in plants and fungi that share a common mechanism [18,20]. The cDNA sequence encoding GA20ox has been isolated from pumpkin [19], Arabidopsis [21], spinach [22], tomato [23], watermelon [24], rice [25] and apple [26]. The expression of *GA20ox* gene was regulated by photoperiod and GA in a type of feedback regulation, and is also dependent on organs or developmental stages [27]. In addition, overexpression of *GA20ox* gene in Arabidopsis increases GA production and stimulates plant growth, which confirmed that GA 20-oxidase activity is a limiting factor in both processes [28,29]. Recently, it has been shown that *GA20ox* and *GA3ox* genes were up-regulated in a wheat hybrid, whereas *GA2ox* gene was down-regulated in hybrid, which, in turn, could increase bioactive GAs contents in hybrid [30]. In this study, a hybrid up-regulated GA 20-oxidase gene, designated *ZmGA20,* was identified in maize, and its expression patterns in different tissues were determined.

## 2. Materials and methods

### 2.1. Plant materials

One highly heterotic maize hybrid, Nongda 108, and its parental inbred lines, 178 and Huang C, were used in this study. For expression analysis, young leaves (not fully expanded), stems and roots of the seedling were harvested from greenhouse-grown plants; and immature ears (3–5 cm) were harvested after the tips of the husks emerged from the leaf sheath, and silks were excluded. The embryos of 15 days after pollination (the inbred lines were derived by self-pollinating) were also collected.

### 2.2. Preparation of total RNA and cDNA synthesis

RNA was isolated and purified using TRIzol reagent, following the manufacturer's instructions. Two micrograms of total RNA from each sample were used for the first-strand cDNA synthesis in 20 μl reactions containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 mM dNTPs, 200 U M-MLV reverse transcriptase (Promega, USA) and 50 pmol oligonucleotides T15. Reverse transcription was performed at 37 °C for 60 min with a final denaturation at 95 °C for 5 min.

Table 1
Comparison of *ZmGA20* with GA 20-oxidase from other plant species.

| Species | GenBank No. | Identity (%) |
|---|---|---|
| *Triticum aestivum* Wga20 | BAA21480 | 72 |
| *Triticum aestivum* S44B | CAA74330 | 72 |
| *Triticum aestivum* S39A | CAA74331 | 71 |
| *Triticum aestivum* S37E | CAA74332 | 71 |
| *Lolium perenne* | AAG43043 | 72 |
| *Oryza sativa* | AAB48329 | 71 |
| *Arabidopsis thaliana* GA5 | BAB11250 | 56 |
| *Nicotiana tabacum* | BAA31689 | 61 |
| *Lactuca sativa* | BAA37127 | 58 |
| *Solanum dulcamara* | CAB82616 | 61 |
| *Phaseolus vulgaris* | AAC49756 | 60 |
| *Lycopersicon esculentum* | AAD15755 | 60 |
| *Pisum sativum* | AAC49721 | 59 |
| *Spinacia oleracea* | AAC49211 | 55 |

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1       CAGTTACAGCGGCCTCCTCCTCTGCTTCCTCCCTCCTCCTCCTCACCATGGTGCTGGCTGCGCACGATCCCCCTC
                                                    M  V  L  A  A  H  D  P  P
76      CCCTTGTGTTCGACGCTGCCCGCCTGAGCGGCCTCTCCGACATCCCGCAGCAGTTCATCTGGCCGGCGGACGAGA
        P  L  V  F  D  A  A  R  L  S  G  L  S  D  I  P  Q  Q  F  I  W  P  A  D  E
151     GCCCCACCCCGGACTCCGCCGAGGAGCTGGCCGTGCCGCTCATCGACCTCTCCGGGGACGCCGCCGAGGTGGTCC
        S  P  T  P  D  S  A  E  E  L  A  V  P  L  I  D  L  S  G  D  A  A  E  V  V
226     GGCAGGTCCGGCGCGCCTGCGACCTGCACGGCTTCTTCCAGGTGGTGGGGCACGGCATCGACGCGGCGCTGACGG
        R  Q  V  R  R  A  C  D  L  H  G  F  F  Q  V  V  G  H  G  I  D  A  A  L  T
301     CGGAGGCCCACCGCTGCATGGACGCCTTCTTCACGCTGCCGCTCCCGGACAAGCAGCGCGCGCAGCGCCGCCAGG
        A  E  A  H  R  C  M  D  A  F  F  T  L  P  L  P  D  K  Q  R  A  Q  R  R  Q
376     GGGACAGCTGCGGCTACGCCAGCAGCTTCACGGGCCGGTTCGCGTCCAAGCTGCCCTGGAAGGAGACGCTGTCGT
        G  D  S  C  G  Y  A  S  S  F  T  G  R  F  A  S  K  L  P  W  K  E  T  L  S
451     TCCGCTACACCGACGACGACGACGGCGACAAGTCCAAGGACGTCGTGGCGTCCTACTTCGTGGACAAGCTGGGCG
        F  R  Y  T  D  D  D  D  G  D  K  S  K  D  V  V  A  S  Y  F  V  D  K  L  G
526     AGGGGTACCGGCACCACGGGGAGGTGTACGGGCGCTACTGCTCTGAGATGAGCCGTCTGTCGCTGGAGCTCATGG
        E  G  Y  R  H  H  G  E  V  Y  G  R  Y  C  S  E  M  S  R  L  S  L  E  L  M
601     AGGTGCTAGGCGAGAGCCTGGGCGTGGGCCGCCGCCACTTCCGCCGCTTCTTCCAGGGGAACGACTCCATCATGC
        E  V  L  G  E  S  L  G  V  G  R  R  H  F  R  R  F  F  Q  G  N  D  S  I  M
676     GCCTCAACTACTACCCGCCGTGCCAGCGGCCCTACGACACGCTGGGCACGGGGCCGCATTGCGACCCCACGTCGC
        R  L  N  Y  Y  P  P  C  Q  R  P  Y  D  T  L  G  T  G  P  H  C  D  P  T  S
751     TCACCATCCTGCACCAGGACGACGTGGGCGGACTCCAGGTGTTCGACGCCGCCACGCTCGCGTGGCGCTCCGTCA
        L  T  I  L  H  Q  D  D  V  G  G  L  Q  V  F  D  A  A  T  L  A  W  R  S  V
826     GGCCCCGCCCGGGCGCCTTCGTCGTCAACATCGGCGACACCTTCATGGCGCTCTCCAACGGGCGCTACAGGAGCT
        R  P  R  P  G  A  F  V  V  N  I  G  D  T  F  M  A  L  S  N  G  R  Y  R  S
901     GCCTCCACCGCGCCGTCGTCAACAGCCGGGTGGCACGCCGCTCGCTCGCCTTCTTCCTGTGCCCGGAGATGGACA
        C  L  H  R  A  V  V  N  S  R  V  A  R  R  S  L  A  F  F  L  C  P  E  M  D
976     AGGTGGTCAGGCCGCCCAAGGAGCTGGTGGACGACGCCAACCCGAGGGCGTACCCGGACTTCACGTGGAGGACGC
        K  V  V  R  P  P  K  E  L  V  D  D  A  N  P  R  A  Y  P  D  F  T  W  R  T
1051    TGCTGGACTTCACCATGAGGCACTACAGGTCGGACATGAGGACGCTCGAGGCCTTCTCCAACTGGCTCAGCACCC
        L  L  D  F  T  M  R  H  Y  R  S  D  M  R  T  L  E  A  F  S  N  W  L  S  T
1126    GTAGCAATGGCGGACAGCACCTGCTGGAGAAGAAGTAGGCATGC
        R  S  N  G  G  Q  H  L  L  E  K  K  *
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Fig. 1. The cDNA sequence and deduced amino acid sequence of *ZmGA20*.

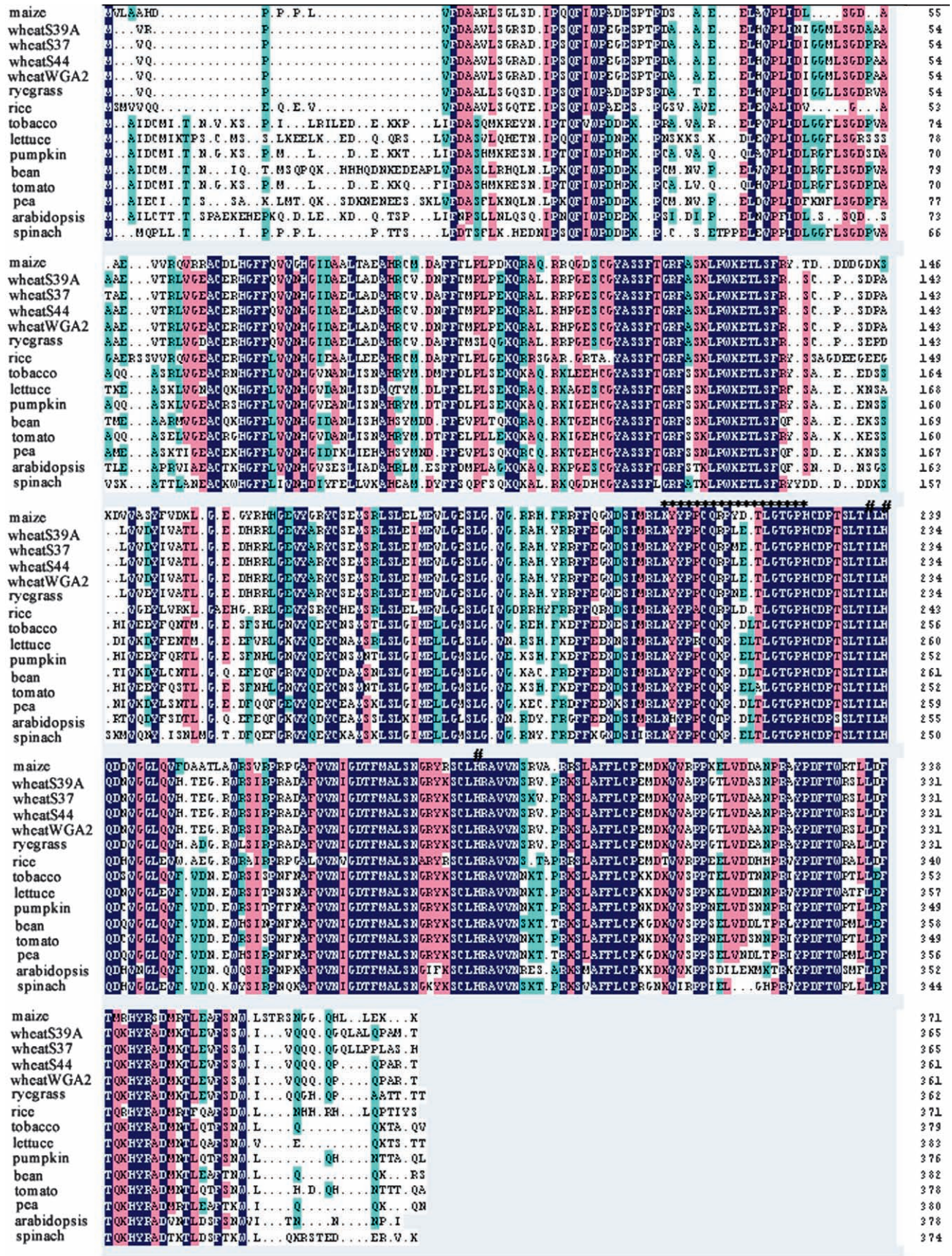


Fig. 2. The alignment of amino acid sequences from maize GA20ox and other GA20ox proteins. The identical residues are shaded. # represents the $Fe^{2+}$ binding domain; * represents the 2-oxoglutarate binding domain.

### 2.3. In silico cloning of ZmGA20

The cDNA sequence (BAA21480) encoding wheat *GA20ox* gene was selected for BLAST searches in the NCBI EST database. One maize EST (gi:21208729) was found to have high similarity to the wheat *GA20ox* cDNA. Specific primers were designed as follows: *ZmGA20*-OL:5′-CAGTTGCAGCGGCCTCCTCCTCTG-3′; *ZmGA20*-OR: 5′-GCATGCCTACTTCTTCTCCAGCAGGTG-3′. PCR was carried out using aliquots of 2 μl of the obtained cDNA and 125 pmol of specific primers in a 20 μl reaction volume containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 1U *Taq* polymerase. The PCR conditions were 5 min at 94 °C, 40 cycles of 30 s at 95 °C, 40 s at 68 °C, and 2 min at 72 °C, followed by a final extension of 10 min at 72 °C. PCR products were separated on 1% agarose gels, and the single specific band of PCR product was obtained and cloned into the pGEM-Teasy vector (Promega) for sequencing.

Sequence analysis was performed using the software DNAMAN (Version 3.0, Lynnon BioSoft). BLAST search was completed at NCBI (http://www.ncbi.nlm.nih.gov), and protein prediction was performed at CBI (http://www.cbi.pku.edu.cn).

### 2.4. Semi-quantitative RT-PCR

The *ZmGA20* gene specific primers were designed using DNAMAN software as follows. *ZmGA20*-RL:5′-AAGGAG ACGCTGTCGTTCCGCTACAC-3′; *ZmGA20*-RR:5′-GGA TGGTGAGCGACGTGGGGTCGCAATG-3′. A 350 bp β-actin gene fragment was amplified as an internal control using the primer pair 5′-CAGCAACTGGGATGATA TGG-3′ and 5′-ATTTCGCTTTCAGCAGTGGT-3′. Identity of PCR products was verified by sequencing. Three RT-PCR replications were conducted using independently isolated total RNAs with the following thermal cycling parameters: 94 °C for 30 s, 68 °C for 1 min, and 72 °C for 1 min. Various numbers of PCR cycles were tested to ensure that the reactions had not reached the plateau. For quantification, the intensity of the PCR bands was estimated with FluorChemTM 5500 software.

## 3. Results and discussion

### 3.1. Isolation and cloning of ZmGA20

In order to obtain *ZmGA20* cDNA sequence, the *GA20ox* sequence from wheat (BAA21480) was selected for BLASTN searches against the NCBI EST database, and a total of 30 maize ESTs with high nucleotide similarity were obtained, among which one EST (gi:21208729) was found to have a putative open reading frame (ORF). Based on this EST, gene-specific primers were designed to amplify the corresponding sequence from maize, and the amplified cDNA was cloned and sequenced. This 1169 bp cDNA fragment contains a 1173 bp open reading frame that encodes 391 amino acids, with an ATG initiation codon at nucleotide position 48 and a TGA stop codon at nucleotide 1160 (Fig. 1).

### 3.2. Sequence analysis of ZmGA20 protein

BLASTX searches in GenBank revealed that the deduced amino acid sequence of ZmGA20 protein is homologous to a group of GA20ox proteins from different species, including *Triticum aestivum, Lolium perenne, Oryza sativa, Arabidopsis thaliana, Nicotiana tabacum, Lactuca sativa, Solanum dulcamara, Phaseolus vulgaris, Lycopersicon esculentum, Pisum sativum* and *Spinacia oleracea*, with the similarity ranging from 55% to 72% (Table 1).

Further analysis indicated that *ZmGA20* protein has many features known in other GA20ox proteins, including a sequence "NYYPPCQRP", two histidine residues and a "LPWKET" domain. Among them, the sequence "NYYPPCQRP" (positions 207–215; Fig. 2) has been considered to bind the common cosubstrate 2-oxoglutarate; the two histidine residues (His-224 and -280) are involved in the binding of $Fe^{2+}$; and the domain "LPWKET" (position 127–132) is considered to be involved in the binding of the GA substrate. Also, ZmGA20 protein has the conserved regions of typical 2-oxoglutarate-dependent dioxygenases. In addition, the sequence "LPWKET"(positions 127–132) is highly conserved in the GA 20-oxidase proteins across different species (Fig. 2), but is not in other 2-oxoglutarate-dependent dioxygenases such as GA 3b-hydroxylase.

### 3.3. Phylogenetic analysis

A phylogenetic tree was constructed based on *ZmGA20* obtained in this study and on 11 known plant *GA20ox* genes (Fig. 3). These 12 sequences are clearly separated into

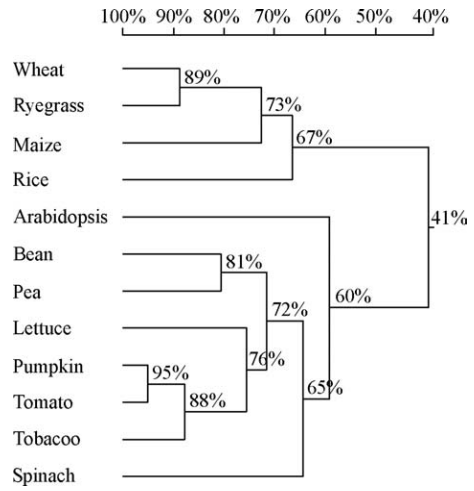


Fig. 3. The phylogenetic tree of GA20 proteins. The numbers on the branches indicate the degree of shared sequence similarity.

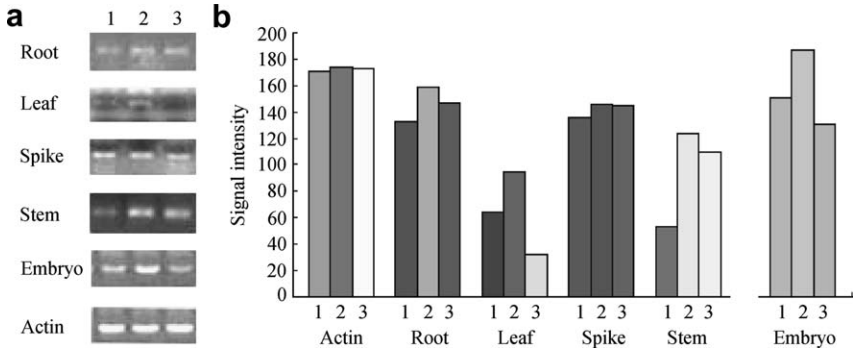


Fig. 4. Differential expression of *ZmGA20* between maize hybrid and its two parents in various tissues. Lanes 1–3 represent the female parent 178, hybrid Nongda 108, and male parent Huang C, respectively. (a) The RT-PCR result and (b) is the quantification of the expression by scanning the agarose gel with a FluorChem™ (Alpha Innotech). Signal intensity of bands was analyzed using FluorChem™ 5500 software.

two classes, where the sequences from monocots including maize, wheat, rice and ryegrass are grouped into one class, and the sequences from dicots are grouped into the other class. The amino acid sequences of GA20ox from monocots share only 40% identity to those from dicots.

### *3.4. Differential expression of ZmGA20 between maize hybrid $F_1$ and two parents*

A previous study suggested that differential gene expression might contribute to heterosis in plant [3]. In our study, we selected one commercial maize hybrid NongDa 108 and its parental inbred lines (178 and Huang C) for the analysis of *ZmGA20* gene expression patterns in maize hybrid and its parents by semi-quantitative RT-PCR. The results indicated that *ZmGA20* was expressed in roots, leaves, stems, ears and embryo of the hybrid and in its two parents, but the expression level in the hybrid was higher than that in the two parents in all the tissues we examined except for ears (Fig. 4).

It has been reported that overexpression of *AtGA20ox* in Arabidopsis plants leads to a 2- to 3-fold increase in the level of GA4, and the increased level of bioactive GA is positively correlated with biomass accumulation, lignin formation, and the rate of photosynthesis [29]. In this study, we found a higher *ZmGA20* expression level in leaves, stems and embryo in the hybrid than that in the two parents; therefore, we assume that the up-regulation of *ZmGA20* could also increase the level of bioactive GA in the hybrid, which might further lead to the enhanced growth of leaves and stems and a greater biomass production in the hybrid. However, further studies are needed to understand the relationship between the up-regulated expression of *ZmGA20* and heterosis in maize growth.

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