Plant Science 243 (2016) 23–34

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Ectopic expression of a maize hybrid up-regulated gene, *ErbB*-3 binding Protein 1 (*ZmEBP1*), increases organ size by promoting cell proliferation in *Arabidopsis*

Tianya Wang[a,b,1], Zhipeng Sui[a,b,1], Xinye Liu[a,b], Yangyang Li[a,b], Hongjian Li[a,b], Jiewen Xing[a,b], Fangwei Song[a,b], Yirong Zhang[c], Qixin Sun[a,b], Zhongfu Ni[a,b,*]

[a] *State Key Laboratory for Agrobiotechnology, Key Laboratory of Crop Heterosis and Utilization (MOE), Beijing Key Laboratory of Crop Genetic Improvement, China Agricultural University, Beijing 100193, China*
[b] *National Plant Gene Research Centre (Beijing), Beijing 100193, China*
[c] *National Maize Improvement Centre of China, China Agricultural University, Beijing 100193, China*

ARTICLE INFO

Article history:
Received 21 July 2015
Received in revised form 2 November 2015
Accepted 4 November 2015
Available online 14 November 2015

Keywords:
Zea mays
Heterosis
Gene expression
ZmEBP1
Cell number

ABSTRACT

The alteration of gene expression in hybrids may be an important factor promoting phenotypic variation and plasticity. To provide insight into the underlying molecular basis of maize heterosis in terms of the kernel number per ear, we established DGE profiles for the immature ears of maize hybrid Zong3/87-1 and its parental lines at the floral organ differentiation stage. Among 4,337 identified differentially expressed genes, 4,021 (92%) exhibited nonadditive expression patterns in the hybrid. Notably, the maize homolog of *Arabidopsis EBP1*, designated *ZmEBP1*, displayed an overdominant expression pattern in the Zong3/87-1 hybrid. Moreover, the results of qRT-PCR revealed that the *ZmEBP1* gene was upregulated in the immature ears of the reciprocal hybrids Zong3/87-1 and 87-1/Zong3 at different developmental stages. Additionally, ectopic expression of *ZmEBP1* in *Arabidopsis* increased organ size, which was mainly attributed to an increase in cell numbers, rather than cell size. Considering all of these findings together, we speculate that upregulation of *ZmEBP1* in maize hybrids may accelerate cell proliferation and promote ear development.

1. Introduction

Heterosis is a phenomenon in which the progenies of diverse varieties of a species or crosses among species exhibit increases in size, growth rates, or other parameters, as compared with both parents [1]. Despite the importance of heterosis and successful application, the molecular basis of this phenomenon remains elusive. Theoretically, all of the genes present in hybrids are inherited from their two parental lines, and no novel genes are generated, but the hybrid performs differently from its parents. Several studies have shown that the alteration of gene expression in hybrids may be responsible for hybrid vigor [2–5]. With the advent of genomic methods for global analysis, the gene expression profiles of different organs or tissues in hybrids and their parents have been analyzed, and varying numbers of genes have been shown to exhibit nonadditive patterns [6–19]. Additionally, the functions of some differentially expressed genes between hybrids and their parental lines have been characterized, which may contribute to the observed heterosis [20–22].

At the cellular level, the crucial effect of heterosis, resulting in a larger plant size of hybrids relative to that of their parents, is manifested primarily by increases in cell number, rather than cell size [23,24]. Two recent studies have provided molecular evidence of the relationship of cell proliferation with heterotic maize growth. Guo et al. [25] have found that the expression level of maize *Cell Number Regulator* (*ZmCNR*), an ortholog of *fruit weight* 2.2 (*f w2.2*) in tomato, is negatively correlated with tissue growth activity and hybrid vigor. Remarkably, silencing endogenous *CNR2* results in a more vigorous growth habit due to an increase in cell number [25]. Li et al. [20] have reported that *Zea mays Auxin Response Factor 25 (ZmARF25)*, which encodes a heterosis-associated *ARF* transcription factor in maize, decreases organ size by affecting cell proliferation in *Arabidopsis* [20]. Considering all of these data together, it can

* Corresponding author at: State Key Laboratory for Agrobiotechnology, Key Laboratory of Crop Heterosis and Utilization (MOE), Beijing Key Laboratory of Crop Genetic Improvement, China Agricultural University, Beijing 100193, China.
E-mail address: nizf@cau.edu.cn (Z. Ni).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.plantsci.2015.11.002
0168-9452/

be concluded that cell proliferation may play important roles in heterosis.

EBP1 acts as a regulator of cell growth by accelerating cell proliferation [26]. Increased *EBP1* expression enlarges organ size by enhancing cell number and influencing cell size, whereas decreased *EBP1* expression causes a reduction in organ growth. In addition, EBP1 is required for full activation of cell cycle genes in *Solanum tuberosum* and *Arabidopsis thaliana* [26]. In the present study, we established DGE profiles for the immature ears of the maize hybrid Zong3/87-1 and its parental lines at the floral organ differentiation stage. Among the 4337 identified differentially expressed genes, the maize homolog of *Arabidopsis EBP1*, designated *ZmEBP1*, was upregulated in the hybrid Zong3/87-1. Moreover, ectopic expression of *ZmEBP1* in *Arabidopsis* led to increased organ size, indicating that *ZmEBP1* may play an important role in maize yield heterosis.

2. Materials and methods

2.1. Plant materials and growth conditions

The maize (*Zea mays*) inbred lines Zong3 and 87-1 and two hybrids of these lines, Zong3/87-1 and 87-1/Zong3, were grown at the Shang Zhuang Experimental Station of China Agricultural University. Reciprocal crosses and self-pollination were performed as follows: ears were bagged before silking, and when the silk had grown to 2–3 cm in length, the ears were cut 2 cm from the top, and the tassels were bagged. Pollination was conducted the next day using appropriate pollen [27]. Ears were manually collected at four developmental stages according to the characteristics of the plants combined with microscopic observations [28]. At least three different ears for each genotype were collected to obtain three biological replicates and were immediately frozen in liquid nitrogen.

Arabidopsis thaliana ecotype Columbia (Col-0) was used in this study. Seeds were sterilized in 50% bleach with 0.04% Triton X-100 for 15 min and washed five times with sterilized water. The sterilized seeds were planted in Murashige and Skoog (MS) medium and vernalized in darkness at 4 °C for 3 days before the plate was transferred to a culture room at 22 °C under a 16-h-light/8-h-dark photoperiod. For morphological examination of aerial parts, seedlings at 7–10 days after germination were transferred to soil containing a mixture of soil and vermiculite (2:1), then placed in a growth chamber at 22 °C under a 16-h-light/8-h-dark photoperiod.

2.2. Field experiments

Field experiments to obtain phenotypic measurements were conducted in 2010 at three locations: the experimental station at Shanxi Agricultural University, the experimental station at Shandong Hua Liang Seeds Industry Co., Ltd., and the Shang Zhuang Experimental Station of China Agricultural University. The parental inbred lines Zong3 and 87-1, together with their reciprocal hybrids Zong3/87-1 and 87-1/Zong3, were arranged in randomized blocks with three replications and a single row plot per event. The row length was set as 5 m to achieve corresponding densities of 4.5 plants per square meter. Regular field management was maintained. Yield component data, including the kernel number per ear (KNE), kernel row number (KRN), and kernel number per row (KNR), were collected from 10 representative mature ears from each row.

2.3. DNA and RNA isolation

Total DNA was extracted via the cetyltrimethylammonium bromide (CTAB) method [29], with minor modifications. Total RNA was extracted using a standard TRIzol RNA isolation protocol (Invitrogen, USA) and then treated with RNase-free DNaseI (Promega, USA) to remove genomic DNA. The quantity and quality of the total RNA were verified by electrophoresis in a 1% agarose gel, and the concentration of RNA was measured using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies).

2.4. Digital gene expression (DGE)

The preparation of tag libraries for immature maize ears during floral organ differentiation for each genotype (Zong3/8-1, Zong3 and 87-1) was performed using an Illumina gene expression sample preparation kit, according to previously reported methods [30]. Briefly, oligo (dT) was used to synthesize first- and second-strand cDNA, and the 5′ ends of the tags were generated by one of two endonucleases: NlaIII or DpnII. The Illumina adaptor 1 was then ligated to the sticky 5′ end of the digested bead-bound cDNA fragments. After removing 3′ fragments with magnetic beads via precipitation, the Illumina adaptor 2 was ligated to the 3′ ends of the tags, producing tags with different adaptors at the two ends, to form a tag library for linear PCR amplification. The denaturized single-chain molecules were fixed onto the Illumina sequencing flowcell, and each molecule was amplified into a single-molecule cluster-sequencing template through in situ amplification. Finally, the four types of nucleotides were labeled with four colors and added to perform sequencing by synthesis (SBS) [31]. Each tunnel generates millions of raw reads with a sequencing length of 35 bp.

Prior to mapping to the reference database, all sequences were filtered to trim the 3′ adaptor sequences and filter empty tags (reads with only 3′ adaptor sequences and no tags) and low-quality tags containing Ns. Additionally, tags that were too long or too short were removed. A virtual library containing all possible CATG +17 base-length sequences from the maize genome database (AGPv2, release 5b.60) [32] was used. All clean tags were mapped to the maize B73 reference. Clean tags that could be mapped to multiple genes equally and those that contained more than one mismatch were filtered. The expression level of each gene was estimated based on the frequency of clean tags and then normalized to the TPM value (the number of transcripts per million clean tags) [33]. The Bio-conductor package "DEGseq" [34] was used for differential expression analysis with the "Fisher Exact Test" method. Genes showing absolute Log2Ratio values $\geq$0.95 and adjusted p values (FDR) <0.05 were considered to be differentially expressed genes. The functional classifications of the 4,021 nonadditive expressed genes were analyzed based on the MapMan ontology defined by Thimm et al. [35].

2.5. Quantitative real-time PCR

First-strand cDNA synthesis was performed using 2 μg of DNase-digested total RNA with an oligo (dT) 15 primer according to the manufacturer's protocol for first-strand synthesis (TaKaRa, Japan). Quantitative real-time PCR (qRT-PCR) using SYBR Green PCR master mix (TaKaRa, Japan) was performed with the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The specific primers used for qRT-PCR analysis are listed in Table S1, among which the PCR efficiency of four primer pairs was determined from standard curve experiments and ranged between 94.45% and 99.62% (Fig. S1). The PCR conditions consisted of an initial step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. To assess the specificity of the amplification products, a melting curve was generated at the end of each run and verified through agarose gel electrophoresis of the PCR products. All reactions were run in triplicate, and each reaction ended with a melting curve and amplification curve analysis to confirm the amplification specificity. Ct values were determined using CFX96™ software with the default settings. Differences between the Ct values of the target genes and *Actin* were calculated as