

# Comparative expression profiling in meristems of inbred-hybrid triplets of maize based on morphological investigations of heterosis for plant height

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**Abstract** Heterosis, the superior performance of hybrids as compared to their parental mean is an agronomically important phenomenon well-described morphologically. However, little is known about its molecular basis. We investigated four genetically unrelated maize (*Zea mays* L.) inbred lines and their F<sub>1</sub> crosses both at the phenotype and transcriptome level, focusing on plant height (PHT) component traits. Substantial mid-parent heterosis (MPH) was found for all parent-hybrid triplets for PHT in the range of 37.9–56.4% in the field and 11.1–39.5% under controlled greenhouse conditions. Analyses of heterosis for number and length of internodes showed two to three times higher MPH in the field as compared to the greenhouse. All three traits exhibited high heritabilities, highest for PHT 95–98%. Two methods for gene expression quantification were applied. High-density cDNA uni-gene microarrays containing 11,827 ESTs were utilized for the selection of differentially expressed genes related to heterosis for PHT. For the four triplets with eight possible parent-hybrid comparisons we identified 434 consistently differentially

expressed genes with a  $p \leq 0.05$ . Microarray results were used to verify the dominance/overdominance hypothesis. In our study, more than 50% genes showed overdominance, 26% partial dominance, 12.6% complete dominance and 10.2% additive gene action. Moreover, more consistently differentially expressed genes were detected in related triplets, sharing one parent, than in unrelated triplets. Quantitative RT-PCR was applied in order to validate microarray results. The role of the differentially expressed genes in relation to heterosis for PHT is discussed.

**Keywords** Gene expression profiling · Hybrid vigour · Maize · Microarray · Mid- · high-parent heterosis

## Abbreviations

|         |   |
|---------|---|
| Act     | Actin   |
| ANOVA   | Analysis of variance                                  |
| Cy3     | Cyanine 3   |
| Cy5     | Cyanine 5   |
| CHL P   | Geranyl-geranyl reductase                             |
| FDR     | False discovery rate                                  |
| GASR 1  | Gibberellin-stimulated transcript 1-like rice protein |
| GO      | Gene ontology assignment                              |
| HPH     | High-parent heterosis                                 |
| INT     | Internode length                                      |
| MAc1    | Maize actin 1 gene                                    |
| MPH     | Mid-parent heterosis                                  |
| NOI     | Number of internodes                                  |
| PHT     | Plant height  |
| qRT-PCR | Quantitative real-time polymerase chain reaction      |
| SOTA    | self-organizing tree algorithm                        |

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## Introduction

Heterosis (Shull 1908) is defined as the superiority of  $F_1$  hybrids over their inbred parents. Hybrid vigour is expressed by increased biomass, growth rate, fertility, resistance to diseases and insects as well as tolerance to abiotic factors (Birchler et al. 2003). Heterosis can be defined as mid-parent heterosis (MPH), the difference between the hybrid and the mean of both parents or high-parent heterosis (HPH), the difference between the hybrid and the parent with the highest trait value (Lamkey and Edwards 1998). Three hypotheses try to explain heterosis: (1) the dominance hypothesis: masking of harmful recessive alleles by superior dominant alleles in the heterozygous hybrid (Davenport 1908; Bruce 1910; Jones 1917), (2) the overdominance hypothesis: superior phenotypic performance of the heterozygote as compared to both homozygous genotypes (Hull 1945; Crow 1948) and (3) the epistasis hypothesis: interaction of favourable alleles at different loci contributed by the two homozygotes (Williams 1959; Li et al. 2001; Meyer et al. 2004). Maize (*Zea mays* L.) has been the first and most important crop plant used to study heterosis.

Plant height (PHT) is an excellent model character to study heterosis, as heterosis for PHT is substantial and can exceed 70% in maize (Becker 1993). PHT is easy to determine, highly heritable (Lübberstedt et al. 1997, 1998), and closely correlated with important agronomical traits, like biomass production and forage yield (Lübberstedt et al. 1998; Niklas and Enquist 2000). Moreover, significant correlations with PHT were found for number of leaves, grain yield and flowering time (Troyer and Larkins 1985). PHT is determined by complex interaction of many genes (Zsbori et al. 2002). Early morphological investigations suggested that 90% of heterosis for PHT is due to an increased cell number, while 10% is due to an increase in cell size (Kiesselbach 1922).

Genes influencing PHT have been isolated (Jacobs 1997; Yamauchi et al. 2004), including rice *Dwarf 1* (*d1*), the constitutive GA response gene *spy* in *Arabidopsis*, as well as the GA-deficient mutant genes in maize *d1-3*, *d5*, *D8/9* and *An1* (Ogawa et al. 1999; Olszewski et al. 2002). The simple dominant dwarfing mutation *D8/9* results in plants with significantly reduced internode length (INT) but unchanged internode number, not responding to exogenous GA application (Harberd and Freeling 1989; Milach et al. 2002). The *d1* mutation in rice is characterized by a defect in the signalling pathway mediated by a G protein (Ashikari et al. 1999). However, it is unknown

whether these genes are involved in the phenomenon heterosis for PHT. So far, heterosis research focussed on classical or DNA marker-based analyses, such as QTL mapping (Stuber et al. 1992). The genetic distance between parents of hybrids has been used as indicator for the extent of heterosis expected for a given pair of inbred lines. However, this connection is loose for lines from different heterotic groups (Melchinger 1999).

More recently, new tools provided by plant genomics such as microarrays allow simultaneous analysis of the steady state mRNA level of thousands of genes in parallel in order to understand complicated processes involved in the organization of organisms and their response to external factors. Expression profiling based on microarrays generates global gene expression patterns for different developmental stages, different tissue types, or environmental factors (Schnable et al. 2004). Microarrays have been successfully used in *Arabidopsis*, to determine expression profiles in various organs, to identify common signalling pathways and conserved genes or to compare specific tissues between *Arabidopsis* and other species (Desprez et al. 1998; Ruan et al. 1998; Horvath et al. 2003; Lee et al. 2004). In many applications on maize DNA arrays were utilized to determine gene expression levels in developing embryos (Lee et al. 2002), to check genes response to UV radiation (Casati and Walbot 2003) and to characterize SCMV resistance genes (Shi et al. 2005). The success of such experiments strongly depends on design, experimental protocol and data analysis methods employed ([http://www.mged.org/Workgroups/MIAME/miame\\_checklist.html](http://www.mged.org/Workgroups/MIAME/miame_checklist.html)). For studying heterosis based on a complex experimental design, Keller et al. (2005) suggested a mixed model approach recently.

The microarray-based expression profiling results in identification of expression patterns across sets of genes, while the discovery of individual candidate genes requires verification by another quantitative method, for instance quantitative real-time polymerase chain reaction (qRT-PCR). This allows direct measurements of the amount of target gene template in a linear range of the amplification reaction (Schmittgen and Zakrajsek 2000). Nowadays qRT is one of the most reliable and sensitive techniques being utilized for microarray data validation, permitting for measurements of mRNA expression levels, DNA copy number, transgene copy number and expression analyses (Ginzinger 2002).

The objective of this study was to (1) identify those stages and parts of maize plants explaining most of the

PHT differences between inbred lines and their hybrids, (2) identify differentially expressed genes in relation to heterosis for PHT, (3) determine the consistency of expression patterns between inbred parent-hybrid triplets also in view of differing degrees of relatedness of triplets, and to (4) validate consistently differentially expressed genes between hybrids and their parental inbreds by real-time (qRT) PCR and (5) relate gene expression patterns to the dominance and overdominance hypothesis. The implications of these findings in view of hybrid breeding are discussed.

## Materials and methods

### Materials and morphological analyses

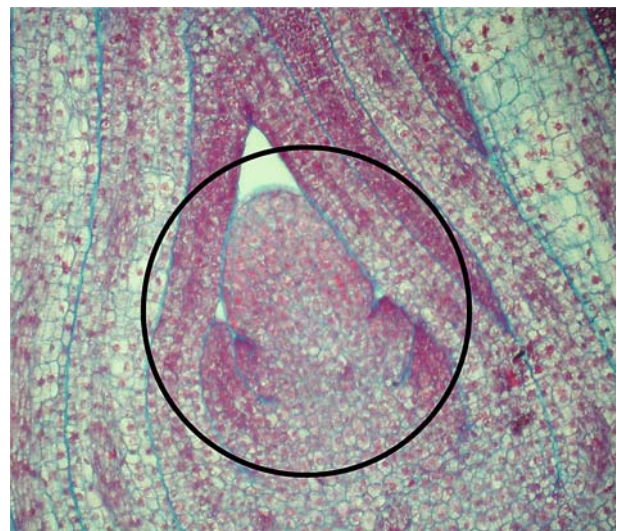
Maize (*Z. mays*) inbred lines 002, 005 (European flints), 250 (Iowa Stiff Stalk dent), 301 (Lancaster dent), their inter-pool (002  $\times$  301, 005  $\times$  250, 250  $\times$  002, 301  $\times$  005) and intra-pool hybrids (002  $\times$  005, 250  $\times$  301) were obtained from Prof. Dr. A.E. Melchinger, University of Hohenheim, Germany. In our study, we will use the term triplet (trip) for both parental inbreds and their hybrid: trip1 = 002, 301 and 002  $\times$  301; trip2 = 005, 250 and 005  $\times$  250; trip3 = 250, 002 and 250  $\times$  002; trip4 = 301, 005 and 301  $\times$  005; trip5 = 002, 005 and 002  $\times$  005 and trip6 = 250, 301 and 250  $\times$  301. Plants were grown in two greenhouse experiments at the Technical University of Munich, Freising at  $\sim 24^{\circ}\text{C}$  during the day and  $\sim 18^{\circ}\text{C}$  at night until maturity (BBCH uniform decimal growth stage scale 65–69 for maize: end of flowering, Lancashire et al. 1991) in a split plot design with the six hybrids and four inbreds in separate blocks, where hybrid versus inbred was the main plot factor and the genotypes the sub plot factor with two replications. Field experiments were carried out in Freising and Pulling, Germany, with three replications each. Both greenhouse and field experiments were carried out in summer 2004 and 2005. PHT, Number of internodes (NOI) and INT were measured on three representative plants per replication in the field and five representative plants per plot in the greenhouse, numbering internodes from top to bottom (internode 1 = tassel). Only above ground internodes were measured. Width of internodes was excluded from further analyses due to low heritabilities in the first year of this study.

### Microarrays: sample preparation

The four above described inbred lines and their inter-pool hybrids (25–40 plants per genotype) were grown

in the greenhouse at the Research Centre Flakkebjerg, Denmark in two seasons with two replications per season (winter 2003, summer 2004), at  $24^{\circ}\text{C}$  during the day (16 h) and  $18^{\circ}\text{C}$  at night temperature for  $\sim 21$ –23 days (BBCH uniform decimal growth stage scale for maize 14–15:4–5 leaves unfolded) in a randomized complete block design. Upper stem meristems (Fig. 1) were harvested with a sterile scalpel using a stereomicroscope and placed into 1.5 ml Eppendorf tubes, subsequently quick-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Meristems from the same genotypes from one replication were pooled to obtain sufficient mRNA.

mRNA was isolated using DynaBeads oligo(dT)<sub>25</sub> (Dyna Biotech, Oslo, Norway), reverse transcription was performed with SuperScript II (Invitrogen GmbH, Karlsruhe, Germany) and second strand synthesis by Klenow DNA polymerase I (Fermentas Life Sciences, St. Leon-Rot, Germany) on Dynabeads with incorporation of aa-dUTP's. Samples were labelled with Cyanine 3 (Cy3) and Cyanine 5 (Cy5) (Amersham Pharmacia, Piscataway, NJ, USA) and unincorporated dyes were purified with QiaQuick PCR purification kit (QiaGen, Hilden, Germany) according to the manufacturer's recommendations. The amount of labelled product was measured spectrophotometrically in a 50  $\mu\text{l}$  quartz cuvette (Cy3–550 nm, Cy5–650 nm). Sixty pmol of Cy3/Cy5 labelled cDNA, respectively, was applied to the microarray (Gregersen et al. 2005).



**Fig. 1** Maize meristem at developmental stage 14–15 (BBCH). Sample was fixed using the formaldehyde-glacial acetic acid-ethanol (FAA) method and stained with alcian blue and safranin dyes. The apical meristem (circle) was used for RNA isolation

## Expression profiling

Samples were hybridized to cDNA unigene microarrays produced by the laboratory of Prof. Dr. P. Schnable (Iowa University, USA), containing 11,827 maize ESTs clustered into 9,841 unigenes, which account for 20% of the about 50,000 maize genes (<http://www.plantgenomics.iastate.edu/maizechip/>) (Shi et al. 2005). The unigene EST collection used for preparing these microarrays was derived from 15 EST libraries, from a variety of organs, tissues and treatments. A total amount of 56 microarray hybridizations (hybrid versus parent and parent versus parent) were carried out with four biological dye-swapped replications. More labour was put on hybrid versus parent comparisons as this better estimates heterosis. Microarrays were scanned using the arrayWoRx microarray scanner (BioChipReader, Applied Precision, WA, USA). Quantification was done in Array Vision software (Version 8.0, Imaging Research Inc., St. Catharines, Ont., Canada). The spot grids were manually aligned with the spots for each slide. Details on experimental data are available through EMBL-EBI ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) with accession number E-MEXP-764.

## Quantitative RT-PCR

Total RNA from apical meristem (four biological replications) was isolated using TRIzol reagent (Invitrogen GmbH). RNA clean-up was performed by the RNeasy mini kit (QiaGen) following the manufacturer's instructions. After checking the quality of RNA on 1.2% formaldehyde agarose gels, RNA quantification was done by spectrophotometry. Sequence specific primers for real-time (RT) PCR were designed using Primer Express<sup>TM</sup> software, Version 1.5 (Applied Biosystems, Foster City, CA, USA) (Table 1).

Quantitative RT (qRT)-PCR experiments were conducted with One-Step QuantiTect SYBR<sup>®</sup> Green RT-PCR Kit (Qiagen) on a 7300 RT-PCR System

(Applied Biosystems) under the following conditions: 50°C for 30 min, 95°C for 15 min and 45 cycles of 94°C for 30 s, 58°C for 15 s and 72°C for 30 s with 100 ng RNA in total volumes of 25 µl. Four biological and three technical replications were used for every gene in order to precisely quantify the transcript abundance. Two biological replications were grown in Freising, another two in Denmark in the controlled greenhouse conditions. To identify primer-dimers and unspecific PCR products dissociation curve analyses were implemented. An endogenous reference sequence was deduced from the MAC1 gene (EMBL-EBI Accession No. J01238) using primers Act for and Act rev (Table 1). Actins are among the most commonly used references for quantification experiments (Thellin et al. 1999; Schmittgen and Zakrajsek 2000; Kim et al. 2003; Brunner et al. 2004; Abruzzo et al. 2005).

## Data analysis

The morphological data were analysed in PLABSTAT Version 2F (a computer program for statistical analysis of plant breeding experiments), developed at the University of Hohenheim (<http://www.uni-hohenheim.de/~ipspwww/soft.html>). Genotype means were computed per location based on a split-plot analysis of variance. Subsequently, two-factorial analysis of variance (ANOVA) with genotypes (G) and locations (O) as random factors was applied to the means per location and genotype. Estimates for heritabilities on plot base, variance components and coefficients of correlation were calculated by PLABSTAT. MPH was calculated from absolute values as:  $MPH = \frac{(F_1 - MP)}{MP} \times 100$ , where  $F_1$  is the mean of hybrid performance,  $MP = (P_1 + P_2)/2$  is the average of inbred parent means. High-parent heterosis (HPH) was calculated as following:  $HPH = \frac{(F_1 - HP)}{HP} \times 100$ , where HP is the mean of the higher parent (Betrán et al. 2003).

Loess regression was performed to normalize data regarding scale and dye effects and centring was

**Table 1** Primer sequences for qRT-PCR experiments

| Gene name  | Primer sequence (5'–3')  | Annealing temperature |
|--|--|-----------------------|
| Maize actin 1                                    | For: TCC TGA CAC TGA AGT ACC CGA TTG<br>Rev: CGT TGT AGA AGG TGT GAT GCC AGT T | 58°C                  |
| Putative defensin                                | For: GCA AGC GGA TCT GCT AGC T<br>Rev: CAG ACG GAC ACG CAC GTA C               | 58°C                  |
| Geranyl-geranyl reductase                        | For: CAT CGA TAC AAA CAG GCA GCA<br>Rev: AAT TTA GGC CAA CAT GCG TG            | 58°C                  |
| Gibberellin-stimulated transcript 1 like protein | For: GAC TCT GGA TCG GCG GAT<br>Rev: CCC TCT CAC TCT GGT GCA CA                | 58°C                  |

Sequence specific primers for a reference and three target genes



accomplished to normalize between arrays (Stekel 2003). A mixed model was used to model log-data for each spot. Genotype, dye, season, replicate and genotype  $\times$  season interaction were treated as fixed, the array being the only random effect. Thus the recovery of inter-array information is possible. Estimates for the genotypes as well as estimates for hybrid versus parent contrasts and estimates of MPH were determined. If the genotype  $\times$  season interaction was significant (after  $p$ -value adjustment) the main effects of genotype and season were removed from the model and the above-mentioned estimates were calculated separately for each season.  $p$ -values of effects or linear contrasts of effects were adjusted with the false discovery rate (FDR)-method (5%) (Benjamini and Hochberg 1995). All calculations were performed with the SAS System for Windows, Version 9.1.

Genes with no genotype  $\times$  season interaction and with  $p$ -values for estimates  $\leq 0.05$  were chosen for further quantitative analyses as significantly differentially expressed. Blast analysis was performed in TIGR Unique Gene Indices (<http://tigrblast.tigr.org/tgi/>), from *Arabidopsis thaliana*, barley (*Hordeum vulgare*), maize (*Z. mays*), rice (*Oryza sativa*), rye (*Secale cereale*) and wheat (*Triticum aestivum*), with a cut-off  $e$ -value of 10 (Ros et al. 2004). The self-organizing tree algorithm (SOTA) for gene clustering was employed according to TIGR MultiExperimentViewer software (Version 3.1).

The degree of dominance with regard to expression profiles was calculated for all differentially expressed genes as in Tanksley (1993), with  $d/a = (\text{hybrid} - 0.5(\text{parent 1} + \text{parent 2})) / (\text{parent 1} - \text{parent 2})$  and aligned as in Stuber et al. (1987), where the  $d/a$  ratio  $-0.2$  to  $0.2$  determined additive gene action,  $0.21$ – $0.8$  and  $-0.21$  to  $-0.8$  partial dominance,  $0.81$ – $1.2$  and  $-0.81$  to  $-1.2$  dominance and  $>1.2$  and  $<-1.2$  overdominance.

In our study, qRT was used for detection of the changes in hybrid expression relative to the inbred sample. RT-PCR efficiencies ( $E = 10^{(-1/\text{slope})} - 1$ ), were derived from calibration data of serially diluted RNA and relative expression rates of the target genes and were calculated as follows:

$$\text{rel.expression} = \frac{(1 + E_{\text{target}})^{\Delta C_{\text{ttarget}}}}{(1 + E_{\text{ref}})^{\Delta C_{\text{tref}}}},$$

where  $E_{\text{target}}$  is the PCR efficiency for the target gene and  $E_{\text{ref}}$  is the PCR efficiency for the endogenous reference.  $\Delta C_{\text{ttarget}}$  and  $\Delta C_{\text{tref}}$  values were determined as described by Dilger et al. (2003). The influence of genotypes on the expression of the endogenous reference gene was evaluated by one-way ANOVA ( $p < 0.01$ ).

## Results

### Morphological analyses

Morphological characters were studied to identify those parts of the plant explaining most of the PHT difference between inbred parents and their hybrids employed in this study, in order to choose the most appropriate plant stage and tissue for expression profiling experiments.

Heritabilities were high and ranged from ~84 to 97% for PHT, 94–99% for NOI and 71–97% for INT3–5, 8 and 9, but were lower for INT1, 2, 6 and 7. Therefore, INT1, 2, 6 and 7 were not analysed in more detail (data not shown).

Hybrid PHT was significantly higher ( $p < 0.05$ ) in the field as compared to the greenhouse, whereas inbred lines were substantially lower in the field as compared to the greenhouse (Table 2). Both hybrids and inbreds had significantly more internodes in the field as compared to those grown in the greenhouse, but length of internodes (INT3, 4, 5, 8, 9) showed a tendency to decrease with internode number in inbred lines in both environments. Hybrids had longer internodes in the field as compared to the greenhouse, except of INT9 (Table 2). The average MPH for PHT in the field was 48.5%, and 28.2% in the greenhouse. Average MPH for NOI in the field was 7.8%, and 2.5% in the greenhouse. For internode length the average MPH was highest for INT9, with 150.3% in the field and 59.7% in the greenhouse, followed by INT8, with 100.4% (field) and 44.6% in the greenhouse. The same pattern was observed for every single triplet except of trip2 for INT8 in the greenhouse. For INT3–5 the MPH value did not exceed 31.7% in the field and 29.1% in the greenhouse. Significant MPH estimates ( $p < 0.01$ ) for greenhouse were found for PHT, NOI, INT4 and INT8, for INT5 at  $p < 0.05$ . In field experiments MPH for PHT was significant at the level of  $p < 0.05$  and for NOI on  $p < 0.1$ . Average HPH for PHT was more than twice as high in the field (42.2%) as compared with the greenhouse (16.5%), while HPH for NOI was 0.6% for field and negative for the greenhouse  $-5.8\%$ . Average HPH for length of internodes was highest for INT9 in the field (89.9%) and for INT8 (27.2%) in the greenhouse (Table 2). Significant estimates for HPH (greenhouse) ( $p < 0.01$ ) were obtained for PHT, NOI, INT4, at  $p < 0.05$  for INT5 and for INT8, and at  $p < 0.1$  for INT3. In the field HPH was only significant for PHT ( $p < 0.05$ ). However, interpretation of the significance of results should be done with care, since tests on MPH and HPH were performed on only six triplets.

**Table 2** Morphological traits: plant height (*PHT*), number of internodes (*NOI*) and length of selected internodes (*INT*)

|                | Field  |        |        |        |        |        | Greenhouse |        |        |        |        |        | Average field ± SE | Average greenhouse ± SE |
|----------------|--------|--------|--------|--------|--------|--------|------------|--------|--------|--------|--------|--------|--------------------|-------------------------|
|                | Trip1  | Trip2  | Trip3  | Trip4  | Trip5  | Trip6  | Trip1      | Trip2  | Trip3  | Trip4  | Trip5  | Trip6  |                    |                         |
| PHT            |        |        |        |        |        |        |            |        |        |        |        |        |                    |                         |
| P <sub>1</sub> | 158.87 | 145.53 | 161.50 | 173.14 | 158.87 | 161.50 | 148.81     | 155.22 | 182.79 | 212.71 | 148.81 | 182.79 | 159.7 ± 3.22       | 174.9 ± 4.88            |
| P <sub>2</sub> | 173.14 | 161.50 | 158.87 | 145.53 | 145.53 | 173.14 | 212.71     | 182.79 | 148.81 | 155.22 | 155.22 | 212.71 |                    |                         |
| F <sub>1</sub> | 239.11 | 239.58 | 238.68 | 247.63 | 212.42 | 246.00 | 238.90     | 224.97 | 221.96 | 242.96 | 193.99 | 218.77 | 237.2 ± 3.12       | 223.6 ± 6.82            |
| MPH%           | 44.03  | 56.06  | 49.00  | 55.41  | 39.57  | 47.02  | 32.16      | 33.11  | 33.87  | 32.07  | 27.61  | 10.63  | 48.5               | 28.2                    |
| HPH%           | 38.10  | 48.35  | 47.79  | 43.02  | 33.71  | 42.08  | 12.31      | 23.08  | 21.43  | 14.22  | 24.98  | 2.85   | 42.2               | 16.5                    |
| NOI            |        |        |        |        |        |        |            |        |        |        |        |        |                    |                         |
| P <sub>1</sub> | 10.82  | 11.37  | 13.73  | 13.27  | 10.82  | 13.73  | 10.14      | 11.05  | 13.86  | 12.70  | 10.14  | 13.86  | 12.3 ± 0.21        | 11.9 ± 0.12             |
| P <sub>2</sub> | 13.27  | 13.73  | 10.82  | 11.37  | 11.37  | 13.27  | 12.70      | 13.86  | 10.14  | 11.05  | 11.05  | 12.70  |                    |                         |
| F <sub>1</sub> | 12.86  | 14.35  | 13.57  | 13.19  | 11.29  | 14.33  | 11.69      | 13.43  | 12.27  | 12.19  | 10.52  | 13.35  | 13.3 ± 0.15        | 12.2 ± 0.09             |
| MPH%           | 6.77   | 14.34  | 10.55  | 7.06   | 1.76   | 6.15   | 2.36       | 7.83   | 2.25   | 2.65   | -0.71  | 0.53   | 7.8                | 2.5                     |
| HPH%           | -3.09  | 4.52   | -1.17  | -0.60  | -0.70  | 4.37   | -7.95      | -3.10  | -11.47 | -4.02  | -4.80  | -3.68  | 0.6                | -5.8                    |
| INT3           |        |        |        |        |        |        |            |        |        |        |        |        |                    |                         |
| P <sub>1</sub> | 19.13  | 15.46  | 14.76  | 16.80  | 19.13  | 14.76  | 18.09      | 14.15  | 13.93  | 19.98  | 18.09  | 13.93  | 16.5 ± 0.37        | 16.5 ± 0.94             |
| P <sub>2</sub> | 16.80  | 14.76  | 19.13  | 15.46  | 15.46  | 16.80  | 19.98      | 13.93  | 18.09  | 14.15  | 14.15  | 19.98  |                    |                         |
| F <sub>1</sub> | 20.51  | 18.62  | 19.10  | 21.00  | 22.58  | 20.52  | 23.36      | 17.44  | 19.77  | 19.92  | 21.11  | 17.51  | 20.4 ± 0.60        | 19.9 ± 0.66             |
| MPH%           | 14.16  | 23.23  | 12.71  | 30.19  | 30.56  | 30.04  | 22.72      | 24.22  | 23.49  | 16.73  | 30.96  | 3.27   | 23.5               | 20.2                    |
| HPH%           | 7.21   | 20.44  | -0.17  | 25.00  | 18.03  | 22.14  | 16.92      | 23.25  | 9.29   | -0.30  | 16.69  | -12.36 | 15.4               | 8.9                     |
| INT4           |        |        |        |        |        |        |            |        |        |        |        |        |                    |                         |
| P <sub>1</sub> | 17.79  | 14.48  | 14.82  | 16.08  | 17.79  | 14.82  | 16.33      | 12.32  | 14.55  | 19.21  | 16.33  | 14.55  | 15.8 ± 0.40        | 15.6 ± 0.70             |
| P <sub>2</sub> | 16.08  | 14.82  | 17.79  | 14.48  | 14.48  | 16.08  | 19.21      | 14.55  | 16.33  | 12.32  | 12.32  | 19.21  |                    |                         |
| F <sub>1</sub> | 21.37  | 19.27  | 20.74  | 20.05  | 21.24  | 20.36  | 25.15      | 18.99  | 20.57  | 19.77  | 18.30  | 17.79  | 20.5 ± 0.53        | 20.1 ± 0.64             |
| MPH%           | 26.19  | 31.54  | 27.20  | 31.21  | 31.64  | 31.78  | 41.53      | 41.35  | 33.23  | 25.40  | 27.75  | 5.39   | 30.1               | 29.1                    |
| HPH%           | 20.12  | 30.03  | 16.58  | 24.69  | 19.39  | 26.62  | 30.92      | 30.52  | 25.96  | 2.92   | 12.06  | -7.39  | 22.9               | 15.8                    |
| INT5           |        |        |        |        |        |        |            |        |        |        |        |        |                    |                         |
| P <sub>1</sub> | 18.63  | 13.94  | 15.07  | 15.27  | 18.63  | 15.07  | 15.79      | 13.02  | 15.24  | 17.67  | 15.79  | 15.24  | 15.73 ± 0.40       | 15.43 ± 0.61            |
| P <sub>2</sub> | 15.27  | 15.07  | 18.63  | 13.94  | 13.94  | 15.27  | 17.67      | 15.24  | 15.79  | 13.02  | 13.02  | 17.67  |                    |                         |
| F <sub>1</sub> | 21.73  | 18.80  | 21.00  | 20.40  | 22.07  | 20.09  | 21.83      | 18.24  | 19.13  | 17.34  | 18.74  | 16.55  | 20.68 ± 0.45       | 18.64 ± 0.70            |
| MPH%           | 28.14  | 29.61  | 24.63  | 39.68  | 35.52  | 32.43  | 30.48      | 29.09  | 23.30  | 13.00  | 30.09  | 0.58   | 31.7               | 21.1                    |
| HPH%           | 16.59  | 24.75  | 12.72  | 33.60  | 18.46  | 31.57  | 23.54      | 19.69  | 21.15  | -1.87  | 18.68  | -6.34  | 22.95              | 12.5                    |
| INT8           |        |        |        |        |        |        |            |        |        |        |        |        |                    |                         |
| P <sub>1</sub> | 6.34   | 7.81   | 12.32  | 11.57  | 6.34   | 12.32  | 9.36       | 12.31  | 15.25  | 14.49  | 9.36   | 15.25  | 9.51 ± 0.82        | 11.83 ± 0.78            |
| P <sub>2</sub> | 11.57  | 12.32  | 6.34   | 7.81   | 7.81   | 11.57  | 14.49      | 15.25  | 9.36   | 12.31  | 12.31  | 14.49  |                    |                         |
| F <sub>1</sub> | 20.54  | 20.94  | 21.05  | 20.26  | 12.17  | 18.91  | 20.20      | 18.96  | 18.86  | 19.98  | 15.47  | 17.14  | 18.98 ± 0.84       | 18.44 ± 0.75            |
| MPH%           | 129.37 | 108.04 | 125.62 | 109.08 | 72.01  | 58.31  | 69.39      | 37.59  | 53.27  | 49.10  | 42.78  | 15.27  | 100.41             | 44.57                   |
| HPH%           | 77.53  | 69.97  | 70.86  | 75.11  | 55.83  | 53.49  | 39.41      | 24.33  | 23.67  | 37.89  | 25.67  | 12.39  | 67.13              | 27.23                   |
| INT9           |        |        |        |        |        |        |            |        |        |        |        |        |                    |                         |
| P <sub>1</sub> | 2.70   | 4.59   | 8.48   | 8.7    | 2.70   | 8.48   | 4.23       | 7.74   | 14.09  | 13.19  | 4.23   | 14.09  | 6.12 ± 0.70        | 9.82 ± 0.65             |
| P <sub>2</sub> | 8.70   | 8.48   | 2.70   | 4.59   | 4.59   | 8.70   | 13.19      | 14.09  | 4.23   | 7.74   | 7.74   | 13.19  |                    |                         |
| F <sub>1</sub> | 14.71  | 17.84  | 16.53  | 17.49  | 7.96   | 16.6   | 15.61      | 17.50  | 16.21  | 19.59  | 7.99   | 16.79  | 15.2 ± 0.84        | 15.62 ± 0.68            |
| MPH%           | 158.07 | 172.99 | 195.71 | 163.21 | 118.38 | 93.25  | 78.30      | 60.33  | 76.97  | 87.20  | 32.50  | 23.09  | 150.3              | 59.73                   |
| HPH%           | 69.08  | 110.38 | 94.93  | 101.03 | 73.42  | 90.80  | 18.35      | 24.20  | 15.05  | 48.52  | 3.23   | 19.16  | 89.94              | 21.42                   |

Mean values were calculated for  $n = 32$  plants in the field and  $n = 40$  plants in the greenhouse in both years for PHT, NOI and INT, in independent field and greenhouse experiments in 2004 and 2005  $\pm$  SE. *P<sub>1</sub>* Parent 1, *P<sub>2</sub>* Parent 2, *F<sub>1</sub>* Hybrid. *MPH* Mid-parent heterosis, *HPH* High-parent heterosis, *trip* Triplet (1: 002\_002  $\times$  301\_301, 2: 005\_005  $\times$  250\_250, 3: 250\_250  $\times$  002\_002, 4: 301\_301  $\times$  005\_005, 5: 002\_002  $\times$  005\_005, 6: 250\_250  $\times$  301\_301)

Consistent correlations for hybrids in the field (Table 3) were found for PHT with NOI (0.825,  $p < 0.05$ ) and for PHT with INT8 and INT9, respectively (0.881,  $p < 0.05$ ; 0.946,  $p < 0.01$ ). In the greenhouse, PHT was also significantly correlated with INT8 (0.922,  $p < 0.01$ ) and INT9 (0.862,  $p < 0.05$ ). In addition, consistent correlations were found between NOI and INT3, 5 and 9 in the field, and between selected internodes in the field as well as in the greenhouse

(field: INT3 and INT8, INT4 and INT5, INT8 and INT9; greenhouse: INT 3 and INT5, INT4 and INT5).

cDNA microarray-based expression profiling of inbred parent: hybrid triplets

Plant height showed the closest correlation with the lowermost internodes (INT8, INT9) in our morphological experiment. Moreover, the highest levels for

**Table 3** Correlations between plant height (*PHT*) and number (*NOI*) and length of internodes (*INT*) (for hybrids)

| F    | GH      |        |         |         |        |         |        |
|------|---------|--------|---------|---------|--------|---------|--------|
|      | PHT     | NOI    | INT3    | INT4    | INT5   | INT8    | INT9   |
| PHT  |         | 0.398  | 0.132   | 0.573   | 0.185  | 0.922** | 0.862* |
| NOI  | 0.825*  |        | −0.785  | −0.245  | −0.469 | 0.328   | 0.761  |
| INT3 | −0.584  | 0.819* |         | 0.783   | 0.832* | 0.212   | −0.373 |
| INT4 | −0.502  | −0.713 | 0.594   |         | 0.897* | 0.677   | 0.149  |
| INT5 | −0.588  | 0.844* | 0.694   | 0.968** |        | 0.366   | −0.244 |
| INT8 | 0.881*  | 0.790  | −0.830* | −0.458  | −0.549 |         | 0.788  |
| INT9 | 0.946** | 0.908* | −0.790  | −0.682  | −0.753 | 0.934** |        |

GH greenhouse, F Field

\*Significant at  $p < 0.05$

\*\*Significant at  $p < 0.01$

PHT heterosis were found for these lowermost internodes, which are being formed earliest during plant development. Due to this fact and because of the well-known difficulty in selecting an appropriate stage of development for expression profiling experiments we decided to focus on the apical meristem (terminal spikelet stage), where internodes have already been formed. Plants for these experiments were grown under controlled greenhouse conditions, to avoid biotic or abiotic stress as far as possible.

Out of 37,516 differentially expressed gene-triplet combinations (summarized over the four triplets), without significant genotype  $\times$  environment interactions, 51.2% showed overdominant gene action for mRNA expression levels, with 50.3% up- and 49.7% down-regulated genes in the hybrid as compared to the average of both parent lines. 26% showed a partial dominant expression pattern with 50.2% genes up- and 49.8% down-regulated and 12.6% had a dominant expression pattern, with 49.6% up- and 50.4% down-regulated genes. A 10.2% of all genes displayed additive gene action (Table 4). The analysis was performed on the whole set of differentially expressed genes to give a general impression on how hybrids perform compared to their parental inbreds with regard to mode of inheritance.

Up to 99 genes were significantly differentially expressed in individual hybrid–inbred comparisons. For all eight hybrid–inbred comparisons in this study, 434 genes were significantly differentially expressed at a significance level of  $p \leq 0.05$  (Table 5, Supplement data), which accounted for ~0.6% of all 75,515 gene-triplet combinations considered in individual experiments without significant genotype  $\times$  environment interaction. Across all comparisons about 75% of genes were up-regulated in a hybrid. A 93.1% of the 434 differentially expressed genes showed less than

**Table 4** Dominance/additivity ratios

| Effect            | (%)  | <i>d/a</i> ratio      | Number of genes | Percentage of up- / down regulated genes |
|-------------------|------|-----------------------|-----------------|--|
| Additivity        | 10.2 | −0.2 to 0.2           | 3,830           | –  |
| Partial dominance | 26   | −0.8 to −0.2; 0.2–0.8 | 9,754           | 50.2/49.8                                |
| Dominance         | 12.6 | −1.2 to −0.8; 0.8–1.2 | 4,727           | 49.6/50.4                                |
| Overdominance     | 51.2 | <−1.2 to >1.2         | 19,205          | 50.3/49.7                                |
| Total             | 100  |                       | 37,516          |  |

*D/a* ratios were calculated for all genes summarized over the four interpool—triplets, therefore the amount of up/down regulated genes has to be divided by four to calculate the average amount of spots per triplet

twofold differential expression between hybrids versus parental lines, 3.9% between two and threefold, 2.6% between three and fourfold, and 0.2% more than fourfold altered expression levels. For the eight hybrid–inbred comparisons sharing the same parent, 0, 15, 21 and 27 genes were differentially expressed in common for inbreds 002, 005, 250 and 301, respectively, the majority for the two dent lines.

When comparing the four triplet pairs sharing one parent, 107 genes were differentially expressed in common (average: 27 genes/triplet pair), while for those two triplet pairs with no shared parental line, 35 genes were in common (average: 18 genes/triplet pair) (Table 6).

In the eight hybrid-inbred comparisons, 99 genes were significantly differentially expressed in at least two comparisons. Gene Ontology assignment (GO) was applied to classify these 99 genes with regard to their molecular function ([http://www.tigr.org/tigr-scripts/tgi/GO\\_browser.pl?species=maize&gi\\_dir=zmg](http://www.tigr.org/tigr-scripts/tgi/GO_browser.pl?species=maize&gi_dir=zmg)). Due to the fact that one gene can have more than one biological function, the number of gene assignments increased to 126. Out of those, 63 were GO described, while another 63 had no gene ontology description. A 38.1% of the GO described genes were associated with catalytic activity, 33.3% with binding activity, 12.7% belonged to the molecular function unknown category, 6.3% were associated with structural molecule activity, 3.2% with transporter and translation regulator activity and 1.6% with signal transduction and antioxidant activity (Fig. 2). Similar results, differing slightly in percentages were found for each hybrid-inbred comparison. In order to normalize the GO results to the maize gene set with available molecular function assignment ([http://www.tigr.org/tigr-scripts/tgi/GO\\_browser.pl?species=maize&gi\\_dir=zmg](http://www.tigr.org/tigr-scripts/tgi/GO_browser.pl?species=maize&gi_dir=zmg)), the Fisher exact test was performed. Since the  $p$ -value was 0.85, the classification into different GO

**Table 5** Significantly differentially expressed genes from microarray experiments (without genotype\*environment interaction)

The uni-gene microarray contained 12,032 ESTs. Genes with significant genotype\*environment interaction were excluded from the analysis

| Triplet | Hybrid genotype versus inbred genotype | *Genes without interaction | Genes significantly differentially expressed ( $p \leq 0.05$ ) | Genes sign. diff. exp. up-regulated (in a hybrid) | Genes sign. diff. exp. up-regulated (%) |
|---------|--|----------------------------|--|---|---|
| 1       | 002 × 301 vs. 002                      | 9,506                      | 0  | 0   | 0                                       |
|         | 002 × 301 vs. 301                      | 9,271                      | 80   | 61  | 76.2                                    |
| 2       | 005 × 250 vs. 005                      | 9,504                      | 65   | 47  | 72.3                                    |
|         | 005 × 250 vs. 250                      | 9,495                      | 59   | 44  | 74.6                                    |
| 3       | 250 × 002 vs. 250                      | 9,496                      | 83   | 69  | 83.1                                    |
|         | 250 × 002 vs. 002                      | 9,503                      | 7  | 4   | 66.7                                    |
| 4       | 301 × 005 vs. 301                      | 9,273                      | 99   | 72  | 72.7                                    |
|         | 301 × 005 vs. 005                      | 9,467                      | 41   | 32  | 78.0                                    |
|         | Total                                  | 75,515                     | 434  | 329   | 74.8                                    |

categories in our study was not different from the above-mentioned GO classification in maize. Additionally, we performed the SOTA analysis on 99 common genes in order to classify them according to their expression patterns. Genes were clustered into 11 sub-clusters and their molecular functions confirmed the patterns from GO description assignment analysis (data not shown).

#### Validation of cDNA microarray expression profiling data by qRT-PCR

To confirm the differential expression of genes identified by expression profiling we conducted the qRT-PCR analysis on four biological replications (grown at two locations: Denmark and Freising). In order to accurately quantify expression levels for the genes of interest, the endogenous maize actin 1 gene was used as a reference. By one-way ANOVA ( $p < 0.01$ ) we tested the influence of external factors (in our case various genotypes) on the gene expression levels. The levels of actin in total RNA remained steady ( $p = 0.77$ ), resulting in cycle thresholds (Ct) for qRT experiments of 22.1–22.3 for various genotypes. This

**Table 6** Common differentially expressed genes among related and unrelated triplets

| Triplets    | Triplets | Germplasm type (F-flint, D-dent) | Genes in common |
|-------------|----------|----------------------------------|-----------------|
| Non-related | 1–2      | F1D2/F2D1                        | 13              |
|             | 3–4      | D1F1/D2F2                        | 22              |
|             | Average  |                                  | 17.5            |
| Related     | 1–3      | F1D2/D1F1                        | 17              |
|             | 1–4      | F1D2/D2F2                        | 29              |
|             | 2–3      | F2D1/D1F1                        | 32              |
|             | 2–4      | F2D1/D2F2                        | 29              |
|             | Average  |                                  | 26.8            |

Triplet 1: 002\_002 × 301\_301; 2: 005\_005 × 250\_250; 3: 250\_250 × 002\_002; 4: 301\_301 × 005\_005; F: flint, D: dent. First inbred denotes the female parent (example: F1D2, F1 flint 1 female, D2 dent 2 male) 002-flint 1, 005-flint 2, 250-dent 1, 301-dent 2

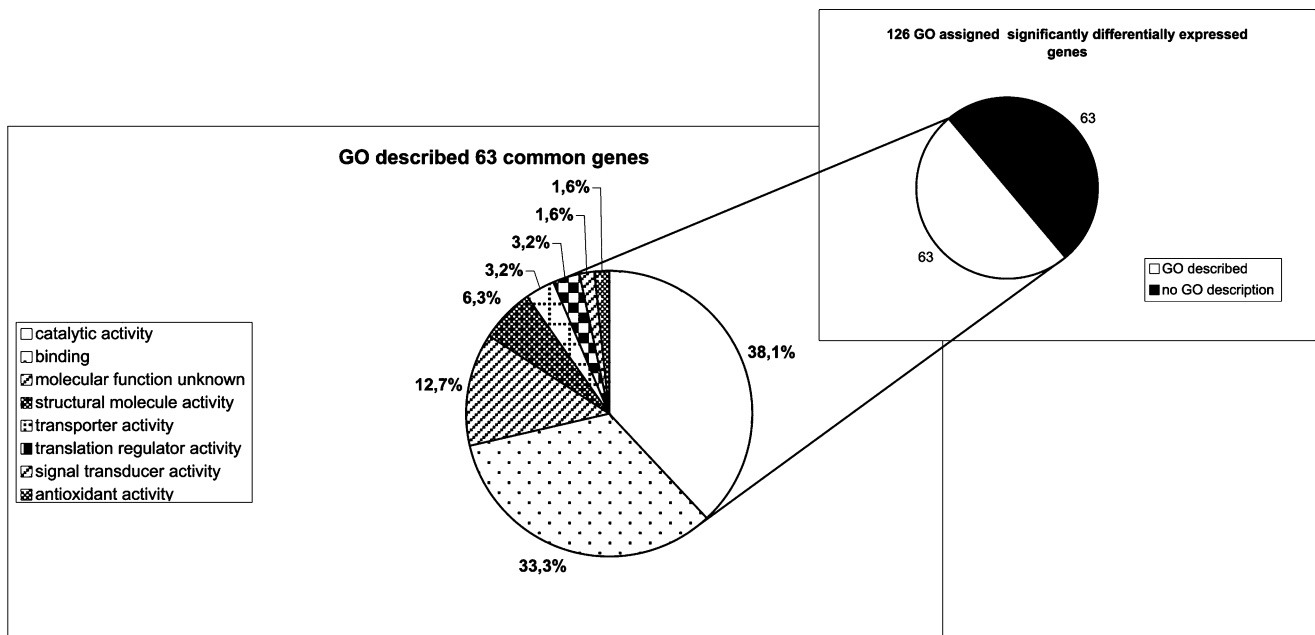
supports the reliability of the maize actin 1 gene as endogenous qRT-PCR control.

Two genes putatively involved in gibberellin biosynthesis and one in jasmonate signalling pathways were chosen for the qRT-PCR experiments. The choice of genes for validation was mainly based on their putative function (homology), relationship to genes affecting PHT and on high, statistically significant fold changes ( $p \leq 0.05$ ) (Table 7).

Differential expression of the gibberellin-stimulated transcript 1 like rice protein (GASR 1) homologue was confirmed by qRT-PCR with a fold change of 10.1 (average from four biological replications) as compared to 2.2 ( $p = 0.002$ ) folds from microarray experiments. The putative plant defensin gene was validated with a fold-change of 2,243.1 by qRT (average from four biological replications) as compared to 3.7 ( $p = 0.04$ ) from microarrays. The geranyl-geranyl reductase gene with a fold-change of 3.7 ( $p = 0.05$ ) from microarray experiments was not confirmed to be significantly differentially expressed when averaging fold-changes from all four replications (1.5 by qRT). However, a significant 2.3-fold-change was detected in one of the four replications (Freising). In all three cases genes were upregulated in hybrids in both microarray and qRT experiments.

Coefficients of correlation ( $R^2$ ) to evaluate the quality of the standard curve for reference and target genes were between 0.98 and 0.99 for all target genes as well for the reference gene, while PCR efficiencies ranged from 1.0 to 1.5 (Table 8). The gibberellin gene was stably expressed across different greenhouse environments (Denmark, Freising), differing only by 0.9 or 1.0-fold changes between environments for inbreds and hybrids, respectively. The defensin gene varied significantly (20.2-folds) between environments for inbreds but not for hybrids (0.5). The environmental variation for geranyl-geranyl reductase was significant both for inbreds (2.4) and for hybrids (2.5) (Table 8).





**Fig. 2** Gene Ontology (GO) description for 63 genes significantly differentially expressed in at least two hybrid-parent line comparisons

## Discussion

### Morphological analyses as basis for tissue selection for quantitative experiments

Owing to the genetic complexity of heterosis formation the choice of the appropriate sample (developmental stage, plant part) for molecular analyses is arguable. When looking at PHT components like internode number and length of individual internodes in our morphological studies, high heritabilities and close correlation to PHT were found for the lowermost internodes (INT8, INT9) in both field and greenhouse experiments. However, no specific internode across four triplets was found to explain most of the PHT heterosis in both environments. Highly significant heritabilities for PHT, number of internodes and for the length of selected internodes detected in our study are in agreement with other studies (Schön et al. 1993), thus confirming the suitability of these traits for heterosis exploration. Since lowermost internodes are formed early in plant development at the terminal spikelet stage of the apical meristem, this tissue was chosen for expression profiling experiments. We assumed that at this stage of meristem development, molecular processes affecting PHT might have already been initiated. Furthermore, maize meristems older than around 3 weeks start turning into floral organ formation (data not shown). In *Arabidopsis*, heterosis for rosette diameter was determined 19 days after

sowing and size differences were established as early as during seedling development (Meyer et al. 2004). Because our aim was to focus on the non-stress related component of heterosis we chose controlled greenhouse conditions for expression profiling analyses.

Significantly higher PHT for hybrids but lower for the inbreds found in the field versus greenhouse experiments led to a substantially increased MPH for PHT in the field. We speculate that increased PHT heterosis in the field is mainly due to a higher sensitivity of inbreds as compared to hybrids against abiotic stresses, such as low-spring temperatures in the field. Environmental stress has been shown to cause dwarfism (Zsubori et al. 2002). This is in agreement with poor performance of tropical maize inbreds under severe stresses reported by Betrán et al. (2003), as well as different response of inbreds and hybrids to abiotic stress (Tollenaar et al. 2004). Abiotic stress like for instance low temperatures, present at early stages of plant growth, could delay stretching of internodes in sensitive genotypes in this period, thus reducing the overall PHT, as reported by Sari-Gorla et al. (1999) and Sowiński et al. (2005). This is manifested in our experiment through the sensitivity and thus poor performance of inbred lines, which results in high differences in MPH for INT8 and INT9 in the field as compared to the greenhouse.

In conclusion, PHT heterosis seems to be due to two major components: (1) stress dependent heterosis acting through a comparatively high sensitivity of inbreds

**Table 7** Sequence homologies for selected differentially expressed genes

| Gene ID             | Genotype                 | <i>P</i>   | Microarrays<br>fold of<br>change | GO                            | TIGR description (homology)   |
|---------------------|--------------------------|------------|----------------------------------|-------------------------------|---|
| <b>614020G04.y1</b> | <b>301 × 005 vs. 301</b> | 0.04205    | 3.7                              | Unclassified                  | <i>Weakly similar to putative plant defensin SPI1B {Picea abies}, partial (41%)</i>   |
|                     | 250 × 002 vs. 250        | 9.07E-05   | 3.6                              |                               |   |
|                     | 005 × 250 vs. 250        | 0.0003     | 3.3                              |                               |   |
| MEST59-G12.T3       | 005 × 250 vs. 250        | 0.0006     | 2                                | Unclassified                  | Weakly similar to UPIQ8L698 (Q8L698)<br>Defensin precursor, partial (82%)   |
|                     | 250 × 002 vs. 250        | 0.0003     | 1.9                              |                               |   |
| <b>496026A12</b>    | <b>250 × 002 vs. 250</b> | 0.0500     | 3.7                              | Catalytic activity            | Similar to UPIQ9ZS34 (Q9ZS34)<br>Geranylgeranyl reductase, partial(88%)   |
| MEST19-G09.T3       | 002 × 301 vs. 301        | 4.93E-08   | 1.8                              | Molecular function<br>unknown | Similar to UPIQ49960 (O49960) Polyphenol<br>oxidase, partial (92%)  |
| MEST43-B01.T3       | 301 × 005 vs. 301        | 6.5071E-10 | 2                                |                               |   |
|                     | 002 × 301 vs. 301        | 7.34E-05   | 2.5                              | Unclassified                  | Similar to GB AAA49498.1 213613 QULPROT<br>protamine {Coturnix japonica;}, partial (44%)  |
| MEST283-D07         | 005 × 250 vs. 250        | 0.0032     | 3.5                              | Unclassified                  | Similar to GP 5690382 gb A Pkn10 {Myxococcus<br>xanthus}, partial(2%)   |
|                     | 301 × 005 vs. 301        | 0.0285     | 2.8                              |                               |   |
| 707020F12           | 005 × 250 vs. 250        | 1.33E-08   | 1.9                              | Unclassified                  | Similar to UPIQ9FYV0 (Q9FYV0) LLS1<br>protein, partial (14%)/similar to UPIQ7XC03<br>(Q7XC03) Putative chlorophyll synthase       |
| <b>605005D02.y2</b> | <b>005 × 250 vs. 250</b> | 0.0015     | 2.2                              | Molecular function<br>unknown | similar to PIR JE0159 JE0159 gibberellin-<br>stimulated transcript 1 like protein—rice<br>{ <i>Oryza sativa</i> ;}, partial (87%) |
| 614074F08.y3        | 002 × 301 vs. 301        | 0.0004     | 3.4                              | Unclassified                  | <i>Similar to (Q8H5X6) Putative NADH<br/>dehydrogenase, partial (3%)</i>  |

A limited number of genes were selected from microarray-based expression profiling experiments. In bold, genes chosen for qRT-PCR validation based on a fold change, *p*-value and putative homology

against stress and (2) stress-independent heterosis also present under “optimal” growing conditions.

#### Differentially expressed genes involved in PHT heterosis

Four hundred and thirty-four genes were detected as significantly differentially expressed between inter-pool hybrids versus parental inbreds with 99 genes common between at least two comparisons. Those genes were annotated according to the Gene Ontology assignment with regard to their molecular function. The largest group (38.1%) contained genes classified into catalytic activity. Through the reduction of macromolecules into smaller units chemical energy required for maintenance of living cells is provided, which in the

case of up-regulation in hybrids may speed their growth and development. The second largest group (33.3%) included genes with binding activities, providing specific binding of the vast majority of molecules necessary for metabolic processes, and the third group was of unknown molecular function (12.7%). For some genes classified into this category interesting homologous sequences that might explain hybrid vigour were found, for example, a gene with similarity to a gibberellin-stimulated transcript 1 like protein. Almost 75% of the 434 genes were up-regulated in a hybrid, while only 25% were down-regulated.

For the real-time RT-PCR validation of expression profiling experiments genes with putative relationship to PHT were chosen from the subset of 99 common consistently expressed genes were chosen. Genes coding for

**Table 8** qRT-PCR results. Comparison of microarray and qRT-PCR results

| Target gene   | Fold of change   |   |  |   |                                      |
|---|--|---|--|---|--------------------------------------|
|   | Fold of change<br>(microarrays), four<br>biological replications,<br>average | Fold of change (qRT),<br>four biological<br>replications, average | Fold of change<br>(environment)<br>inbreds/hybrids | Coefficients of<br>determination ( <i>R</i> )<br>target/reference | PCR efficiencies<br>target/reference |
| <i>Gibberellin-stimulated<br/>transcript 1 like protein</i> | 2.2  | 10.1  | 0.9/1.0  | 0.98/0.98   | 1.2/1.3                              |
| <i>Putative plant defensin</i>                              | 3.7  | 2243.1  | 20.2/0.5   | 0.99/0.99   | 1.3/1.2                              |
| <i>Geranyl-geranyl reductase</i>                            | 3.7  | 1.5   | 2.4/2.5  | 0.99/0.98   | 1.0/1.5                              |

the GASR1 and a geranyl-geranyl reductase (CHL P) belong to the pathway for gibberellin (diterpens) biosynthesis (Jacobs 1997). Gibberellins regulate development and growth and were found in higher concentrations in hybrids as compared to either parental inbred, which might involve them in regulation of hybrid vigour in maize (Rood et al. 1983). The GASR1 gene was confirmed to be 10-times higher expressed in a hybrid by qRT (pool of four biological replications from two environments, triplet 005  $\times$  250 vs. 250) as compared to twofold in microarray experiments and was both environmentally stable and repeatable. Geranyl-geranyl reductase catalyses the reduction of geranyl-geranyl diphosphate to phytol diphosphate, which takes part in porphyrin and chlorophyll metabolism. Reduced activity of geranylgeranyl reductase causes loss of chlorophyll and tocopherols required in photosynthetic reactions (Tanaka et al. 1999). The defensins are involved in the jasmonic acid signalling pathway and jasmonates, “non-traditional plant hormones” are key regulators in developmental, physiological, defence and signalling network processes (Creelman and Mullet 1997; Lincoln and Zeiger 1998). The expression of putative defensin was confirmed with a fold change of 2,243 as compared to 3.7 in microarray-based expression profiling experiments, whereas the absolute expression of parental inbred was nearly close to zero. Geranyl-geranyl reductase gene with the fold of change 1.5 was not confirmed to be differentially expressed as average from four biological replications, however in one replication it was significant, 2.3. Both genes (defensin and geranyl-geranyl reductase) showed differences between environments (Freising and Denmark), either for hybrids, inbreds or for both, which might result from different light, temperature and other regimes at both locations, even though plants were grown in the controlled greenhouse conditions. The qRT experiment generally confirmed up-regulation of all three genes in a hybrid as found with microarray experiments.

The very high fold-change differences between both techniques for the defensin gene might be explained by (1) the close to zero expression of the selected defensin gene in inbreds resulting in large variation for ratios for expression levels between hybrids and inbreds, (2) limited sensitivity of microarray technique to detect high differences between samples, (3) any kind of not observed infection of plants in the greenhouse, (4) putative simultaneous detection of different gene family members by microarrays and (5) incomplete primer specificity, since primers were designed based on public unigene sequences but not sequences from the genotypes studied. The first reason seems to be most likely, causing high variability in results from

both techniques, although we cannot rule out the other explanations.

The lack of validation of geranyl-geranyl reductase gene could be explained by the fact that (1) the probability of finding differential expression for genes at the significance threshold ( $p = 0.05$ ) is most difficult due to a comparatively high risk of the type I statistical error, or (2) the finding of differential expression for CHL P was a false positive result in microarray experiments. Generally the range of expression in qRT-PCR analyses is much greater than in the corresponding microarray experiments (Rajeevan et al. 2001). This variability might be due to the difference in sensitivity of both techniques, but also to cross-hybridizations with other gene family members for the microarray technique (Jenson et al. 2003; Chen et al. 2005). An overall good agreement of microarray and quantitative RT-PCR results exceeding 70% was reported by Rajeevan et al. (2001) and Jenson et al. (2003). The utilization of microarrays as selection tool for genes involved in PHT heterosis followed by more sensitive and accurate quantification seems to be reasonable in order to obtain an authentic picture of gene expression patterns.

#### Molecular basis of heterosis

Three main models, dominance (partial-dominance), overdominance and epistasis hypothesis are being discussed as explanation for hybrid vigour in plants. If heterosis is due to dominance, high-performing inbreds exceeding hybrids are theoretically possible, in contrast to overdominance (Tsaftaris 1995). The molecular basis of heterosis is, therefore, of pivotal interest for plant breeders in terms of long-term breeding strategies. We employed maize unigene microarray data to study the mode of inheritance for PHT heterosis at the transcript level. Out of 37,516 genes differentially expressed between inter-pool hybrids versus parental inbreds, more than half exhibited overdominant gene action, 26% partial dominant, 12.6% dominant and 10.2% additive effects with regard to heterosis. Within each group, the number of genes up- and down-regulated in hybrids was about 50%. Auger et al. (2005), based on their own and others reports indicated the non-additive gene expression in diploid and triploid hybrids of maize to play the predominant role. Rood et al. (1983) and Stuber et al. (1992) observed overdominance as major factor for heterosis for PHT and internode length in maize. In contrary, Swanson-Wagner et al. (2006) in their recent studies observed only a limited percentage of genes inherited non-additively, within which over- and underdominance played the minor role as compared with dominance. By using pairwise

comparison tests, in contrary to our *d/a* ratio calculations, the authors described significant patterns, among the genes where at least one genotype of the pair showed significant differential gene expression. However, the authors could not distinguish ~80% of the genes from additivity, what might be one of the reasons of the inconsistency of our results with their work. Additionally, different inbred lines and harvesting time were applied in their studies. Xiao et al. (1995) conducting a quantitative study on rice claimed dominance to be the main effect. Additionally, Yu et al. (1997), Li et al. (2001) and Luo et al. (2001) suggested epistasis and epistasis in combination with overdominance, respectively, to play a main role in generation of heterosis for grain yield components in rice.

Our findings from expression profiling experiments are in better agreement with the overdominance as compared to the dominance hypothesis. However, it is unclear yet, how the mode of gene action at the transcript level translates into heterosis for morphological characters.

Hybrid breeders are highly interested in the question, if and how hybrid vigour can be predicted by the use of molecular tools to obtain maximum efficiency in hybrid breeding programs, in order to identify the most promising hybrids. Hybrid breeding is characterized by the labour and cost intensive development of inbred lines, including tests on per se performance and different tests to determine the general and specific combining ability of these lines. Molecular markers were utilized (SSR, RFLP and RAPD) to predict correlations of genetic distance with hybrid performance in tropical and temperate maize lines (Lanza et al. 1997; Melchinger 1999; Benchimol et al. 2000). Melchinger (1999) observed significant correlations of parental genetic distance with  $F_1$  performance and with MPH in maize for yield between related (intragroup) crosses, but at a much lower level between intergroup crosses. We found similar patterns at the expression profiling level, where higher number of differentially expressed genes were in common in related (same parental contributor) as compared to unrelated triplets. Moreover, none of the genes was differentially expressed in all triplets. Thus, no obvious key genes controlling PHT heterosis in maize emerged from our study. We, therefore, conclude that each triplet possesses its own specific expression pattern, making the prediction of high-performing inbred parent combinations by expression profiling unlikely.

In conclusion, this study presents significant findings of heterosis in maize for next to the ground internodes, significantly positively correlated with PHT. Consistently

higher PHT of hybrids in stressed versus non-stressed environmental conditions gives an evidence for good acclimatization of  $F_1$  population plants to less than optimal growing conditions. We point at important differences in inbreds and hybrids architecture in distinct environments, which results in high -MPH values. Furthermore, expression profiling experiments in association with qRT-PCR resulted in successful identification of two heterosis-related candidate genes, demonstrates the potential of this combination of techniques in a gene discovery. Subsequent transformations or research on mutants might confirm or reject the participation of these genes in hybrid vigour formation. Moreover, differential gene expression in meristems at terminal spikelet stage shows a linkage to the morphology of the maize plants. The predominance of the greater phenotypic expression of a heterozygote than either homozygote (overdominance) was additionally observed.

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