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ORIGINAL PAPER

Correlation between parental transcriptome and field data for the characterization of heterosis in *Zea mays* L.

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Abstract Heterosis is widely exploited in plant breeding, although its molecular basis is still not fully understood. For the characterization of this phenomenon and the development of transcriptome-based methods to predict hybrid performance (HP), we applied a microarray (46k) analysis of 21 European maize (*Zea mays* L.), 14 dent and 7 flint parental inbred lines. Expression profiles of the parental inbreds at the seedling stage were correlated with grain yield (GY) and grain dry matter content (GDMC) of 98 flint × dent factorial crosses at six locations. We observed highly significant correlations of the parental expression levels of certain differentially expressed genes with heterosis and HP for GY and also with HP for GDMC. This strong correlation provided first evidence toward a prediction potential of the genes and their expression levels. The identified gene set based on the parental transcriptome data revealed functional characteristics of HP and heterosis. Gene ontology (GO) analyses were performed to compare genes correlated with their expression pattern to HP for GY and GDMC, respectively. Between these gene groups, mostly different functional classes of genes were found to be enriched or underrepresented. The phenomenon of heterosis was characterized by the over- and underrepresentation of specific GO terms among heterosis-correlated genes.

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Introduction

Heterosis or hybrid vigor is the superior performance of hybrids compared to their homozygous parental inbred lines (Shull 1908; East 1936). It is either defined as the superiority of the hybrid progeny over the average parental performance [mid-parent heterosis (MPH)] or over the better performing parent (better parent heterosis). Field trials to assess hybrid performance (HP) are time consuming and expensive. Production and testing of all possible crosses among a large number of inbred lines is not feasible in a breeding program and, consequently, only a small fraction of them is field-evaluated. Thus, prediction of MPH and HP of hybrids is of great interest for plant breeders.

In hybrid breeding usually the concept of heterotic patterns is applied, where the breeding material is assigned to genetically divergent heterotic pools. These pools are kept genetically isolated to avoid gene flow between them, which is expected to reduce the inter-pool diversity. Inter-pool hybrids show in general a higher MPH and HP for GY

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than intra-pool hybrids (Melchinger 1999). Prediction of HP and MPH of inter-pool hybrids has great potential to improve the efficiency of hybrid breeding programs. Promising results were obtained in maize by employing different statistical models like BLUP (best linear unbiased prediction) (Bernardo 1996) or other linear models, using phenotypic and pedigree data as well as selected DNA markers (Vuylsteke et al. 2000; Schrag et al. 2006, 2007, 2009, 2010). Moreover, metabolic data were shown to have a potential for the prediction of HP (Steinfath et al. 2010) and the characterization of heterosis (Römisch-Margl et al. 2010). However, the prediction efficiency needs to be further improved.

In maize, many expression studies in inbred lines and their hybrids have been performed to obtain a better understanding of the molecular basis of heterosis (Guo et al. 2006; Meyer et al. 2007; Stupar and Springer 2006; Swanson-Wagner et al. 2006; Uzarowska et al. 2006; Hoecker et al. 2008; Stupar et al. 2008). The proportion of observed additive versus non-additive gene expression varied in hybrids, but in general the majority of differentially expressed genes showed an additive expression pattern. Additive behavior is expected, if solely allelic *cis*-regulatory differences are responsible for expression regulation in hybrids (Wittkopp et al. 2004) and allele specific expression analyses revealed that *cis*-regulatory differences of the inbred lines B73 and Mo17 can explain the expression profiles in their hybrids to a considerable extend (Stupar and Springer 2006). Since *cis*-regulatory elements affect the transcriptome substantially and expression variation is the basis for phenotypic variation, the expression profiles of parental inbred lines should be related to the performance of the hybrids they constitute. Supportive to this hypothesis, the percentage of differentially expressed genes between parental inbred lines was highly correlated with heterosis of 16 maize hybrids (Guo et al. 2006). The potential to predict heterosis based on parental gene expression profiles was evaluated in a companion paper (Frisch et al. 2010). In the present study we analyzed the functional information within a gene set highly correlated to HP and MPH for grain yield (GY) and grain dry matter content (GDMC).

For understanding heterosis, identification of genes associated with its expression is important. So far, the identity and genetic function of specific genes associated with heterosis of different traits is mostly unknown. Only a few studies identified differentially expressed genes in hybrids, possibly involved in heterotic effects (Meyer et al. 2007; Hoecker et al. 2008). In the current investigation we used gene ontology (GO) terms (Gene Ontology Consortium 2000) for the characterization of MPH and HP-correlated genes. GO terms provide a dynamic and controlled annotation system organized in three different functional categories, biological process (BP), molecular function (MF) and cellular component (CC), arranged in a hierarchical structure. For the biological interpretation of genes associated with MPH or HP, the examination of enriched or underrepresented GO terms in these functional gene groups in relation to all analyzed genes may suggest functional pathways or mechanisms substantially involved in the expression of these complex phenotypes. An algorithm, called "weight", scoring the enrichment or underrepresentation of GO terms by integrating the hierarchical GO topology into the score was developed by Alexa et al. (2006). This algorithm aims to identify, in a bottom-up order, more specific enriched or underrepresented GO terms instead of the more general GO terms in the hierarchy, related to them. Once a significant GO category is identified, all genes associated with it are down-weighted in the following analysis of its ancestral, more general categories.

The objectives of our study were to (1) design a hybridization scheme for an efficient comparison of parental inbreds of inter-pool crosses in microarray analyses, (2) examine for which genes the transcript abundance is correlated to MPH and HP for GY and GDMC in maize, (3) perform GO analyses to functionally compare the two gene groups correlated in their parental expression level to HP for GY and GDMC, and (4) functionally characterize the gene group correlated with MPH for GY.

Materials and methods

Field data

In this study 21 diverse (7 flint and 14 dent) parental maize inbred lines from the breeding program of the University of Hohenheim (Germany) were examined. Four of the seven flint lines had an European flint background, the remaining three had a flint/Lancaster background. The dent lines comprised eight lines with Iowa Stiff Stalk Synthetic and six with an Iodent background. The parental lines were crossed in a 7 × 14 factorial mating scheme resulting in 98 hybrids. The inbred lines were evaluated in 2003–2004 at a total of five locations and the hybrids in 2003 at six locations in Germany with diverse agroecological conditions. The intergroup hybrids showed varying degree of MPH for GY, ranging from 70 to 127% (Schrag et al. 2006). All field trials were conducted using two-row plots, adjacent α-design and two to three replications. The HP of the crosses was recorded for GDMC in percent at harvest and for GY in Mg ha^{-1} adjusted to 155 g kg^{-1} grain moisture. GDMC of the inbreds and hybrids ranged from 64.9 to 75.88% and 67.42 to 72.63%, respectively GY of the inbreds and hybrids ranged from 64.06 to 75.88 Mg ha^{-1} and 97.01 to 117.75 Mg ha^{-1}, respectively (Schrag et al. 2006).

RNA-probe synthesis for microarray experiments

One seedling of each 21 maize inbred lines were grown simultaneously in a climate chamber (Percival Scientific Inc., Perry, USA) under regulated growth conditions (25°C 16 h day, 21°C 8 h night, 70% air humidity). This procedure was repeated five times with randomized plate positions leading to five biological replicates. Since we aimed for the identification of genotype-dependent expression differences in inbred lines, the five biological replicates were pooled before target labeling and hybridization (Kendziorski et al. 2005). The whole 7-day-old seedlings were used. The pooled tissue was frozen in liquid nitrogen and finely pounded. The total RNA was isolated, precipitated with LiCl (4 M) and purified with the "NucleoSpin RNA Clean-up Kit" (Macherey–Nagel, Düren, Germany). The quality of the total RNA was controlled by agarose gel electrophoreses and used to synthesize aminoallyl-labeled RNA (aaRNA) following the "Amino Allyl MessageAmp II aRNA Amplification Kit" protocol (Applied Biosystems/Ambion, Austin, USA). Synthesised aaRNA was coupled with fluorescence dyes Cy3 or Cy5 (GE Healthcare, Chalfont St. Giles, UK). The RNeasy MinElute Kit (Qiagen, Hilden, Germany) was used for purification and removal of unbound dye.

Microarrays (46k)

The 46k arrays from the maize oligonucleotide array project (University of Arizona, USA) comprising 43,381 gene-oriented (in total 46,128 features) 70-mer oligonucleotides (ESTs, cDNA, genomic sequences) printed on a glass-slide were used for hybridization analyses. The oligonucleotides on the array were obtained from expression data of 16 diverse maize tissues. RNA labeling and hybridizations were performed according to the protocol of the maize oligonucleotide array project (http://www.maizearray.org). The microarrays were scanned (AppliedPrecision ArrayWorx Scanner, Applied Precision Inc., USA) and data were evaluated using the Software GenePix Pro 4.0 (Molecular Devices, Sunnyvale, USA). An experimental interwoven loop design, based on the assumptions of Kerr and Churchill (2001), was developed aiming to yield in a preferably low average variance among the hybridizations, especially between intergroup hybridizations. The hybridizations followed a specific scheme, in which the dye used for each genotype was alternated to reduce systematic bias. Background adjustment of the arrays was performed using the LIMMA package (version 2.9) from Bioconductor (Gentleman et al. 2004) in *R*. Normalization involved a two-step process consisting of print-tip group loss (within-array normalization) and between-array scale normalization.

Differentially expressed and trait-correlated genes

Differentially expressed genes were determined using a modified *F* test, implemented in Limma package, with a false discovery rate of 0.01. Genes were considered to be differentially expressed with an expression fold-change between each pair of parental inbred lines greater than 1.3 and an expression level (log2) of at least 8.0. Blank and negative controls from the array, which were located in all blocks of the array, were used to confirm the stability of the experiment. For validation experiments by qRT–PCR the iCycler iQ detection system (BIORAD, München, Germany) and the qPCR MasterMix Plus for SYBR Green I with fluorescein, 5 mM $MgCl_2$ (Reference: RT-SN2X-03 + NRFL, Eurogentec, Seraing, Belgium) were used. Quantitative RT–PCR was conducted like described by Meyer et al. (2007). The sequence of the gene specific primer pairs were shown in Tables S1, S2 (Online Resource 1).

The average gene expression of two parental inbred lines (mid-parent expression) and MPH were calculated for the subsequent correlation analysis. Correlation coefficients of mid-parent gene expression (log2-transformed) with MPH or HP for GY and HP for GDMC were computed. We tested correlation coefficients (r) for significance using Pearson product–moment correlation in *R*, to detect corresponding associated genes. For multiple test correction, the P value was adjusted with the false discovery rate of 0.01. For the characterization of heterosis, r between the MPH for GY and the mid-parent expression levels was calculated, but this analysis was not carried out for GDMC, as this trait had a very low level of MPH.

GO analyses

To assess the biological functionality of the trait-correlated genes ($P < 0.01$), analyses were performed to test for over-represented and underrepresented GO terms among these gene groups, in comparison to all expressed genes. The GO annotation (maizearray.org_Version4) was obtained from the University of Arizona (http://www.maizearray.org) and the used GO terms were listed in Table S3 (Online Resource 2). The GO analyses were performed in *R* using the package topGO (version 1.10.1) including the weight algorithm (Alexa et al. 2006). The analyses were based on Fisher's exact test. For the elimination of genes, which might be present on the array twice or more often, genes with a TC-annotation (maizearray.org_Version4) already observed in a more significant correlated gene were excluded from the GO analyses. Also genes without a GO-annotation were excluded.

With this GO analysis we compared the GO terms enriched or underrepresented among the genes either

significantly ($P < 0.01$) correlated to HP for GY or GDMC. For the characterization of heterosis and the identification of heterosis-associated gene functions, GO terms enriched or underrepresented among the genes correlated to MPH and among the genes correlated only to MPH (excluding genes correlated also to HP of GY) were identified.

Results

Design of a microarray hybridization scheme for inter-group crosses

A direct comparison of all 7 × 14 inter-group combinations among the 21 parental lines was not feasible, due to a restricted number of microarray hybridizations which could be performed. In total 63 hybridizations were realized in an interwoven loop, designed to keep the distance between flint and dent lines as small as possible. The resulting maximum distance between every flint and dent line accounted for either one or two hybridizations. The loop was designed in a way that each dent line was sampled five times and each flint line eight times. In addition to every direct connection between dent and flint lines, multiple indirect connections of three or more hybridizations were realized. The average variance of the whole experimental design was 0.5. The average variance of the experimental design part concerning the intra-group combinations was 0.64; for the part concerning the inter-group combinations it was 0.45. Thus, the design allows for the highest precision in the assessment of inter-group crosses expression data (Fig. 1).

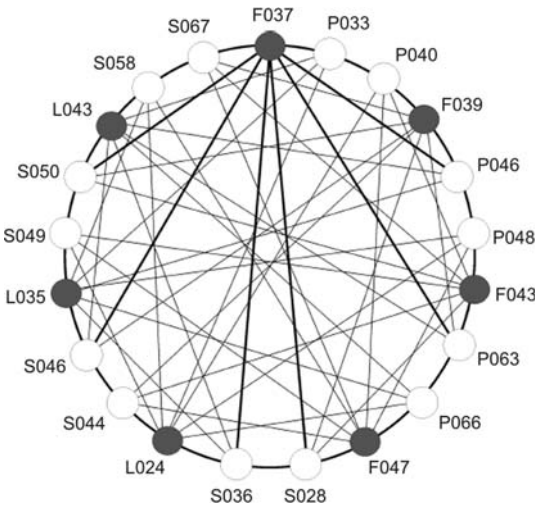

Fig. 1 Interwoven loop design of the microarray experiment. The *gray* and *white circles* indicate the 7 flint and 14 dent inbred lines, respectively. The *lines* represent the hybridizations, and the *bold lines* illustrate the general scheme of the interwoven hybridizations

Genes correlated to HP or/and MPH

The 46k array comprised 46,128 spots. After the exclusion of blank and control spots, 43,381 gene-oriented oligos remained. In our microarray hybridizations, 24,498 (56.5%) of these gene-oriented spots showed a reliable signal in at least one inbred line. Based on the *F* test a total of 12,288 (28.3%) of the genes showed differential expression between at least one pair of inbred lines. Validation experiments by qRT-PCR for two genes in eight different lines were essentially in accordance with the microarray gene expression data (Online Resource l).

Correlation analyses revealed genes, the mid-parent expression of which correlated significantly ($P < 0.01$), either negatively or positively, with HP for GY or GDMC, or MPH for GY. The mid-parent expression level of 2,317 genes showed significant correlation with HP for GDMC. Of these 2,317 genes, 969 showed significantly negative correlation (r) ranging from −0.3103 to −0.763, and 1,348 genes with a positive r ranging from 0.3102 to 0.648. Of 1694 genes, which were correlated with HP for GY, 758 genes were negatively ($-0.6319 < r < -0.3191$) and 936 genes were positively ($0.3189 < r < 0.6478$) correlated. The expression profile of 545 genes showed a significant r ($P < 0.01$) with HP for both traits. The r-values for all genes in this intersecting group were in opposite directions, i.e., if a gene was positively correlated to HP for GY, it was negatively correlated to HP for GDMC and vice versa. The expression profile of 1,999 genes revealed a significant ($P < 0.01$) correlation with MPH for GY, from which 887 genes were negatively ($-0.6733 < r < -0.3143$) correlated and 1,112 genes showed a positive correlation ($0.3143 < r < 0.6794$). Of these genes, 899 were correlated to MPH as well as to HP for GY, whereas 311 genes were, additionally to MPH for GY, also correlated with HP for GDMC. The common genes for HP and MPH for GY had r with the same sign for both measurements. The direction of r of the genes correlated to HP for GDMC and MPH for GY was not consistent. To all three measurements, 226 genes were correlated (Fig. 2). A list of all correlated genes is available in Online Resource 2.

Functional comparison of genes correlated with HP for GY and grain dry matter concentration

The genes correlated to HP for GY as well as for GDMC showed significantly overrepresented GO terms ($P < 0.03$) in all three categories of GOs, biological process (BP), molecular function (MF) and cellular component (CC). In all three categories no overlapping GO terms were found to be enriched for the HP-correlated genes of GY and GDMC. An exception was found in the category BP where two functional processes—the "regulation of glycolysis" and

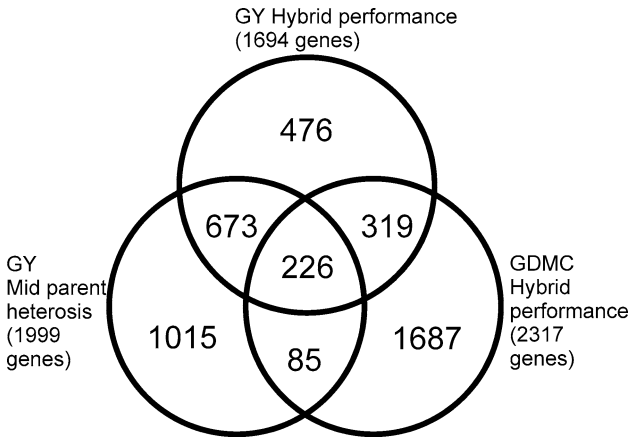

Fig. 2 *Venn diagram* of trait-correlated genes. The number of genes whose mid-parent expression level is correlated to hybrid performance (HP) for grain yield (GY) and grain dry matter concentration (GDMC) and also the genes correlated to mid-parent heterosis (MPH) for GY, with a *P* value less than 0.01 are shown

cell wall modification related processes—were enriched in both sets of trait specific HP-correlated genes (Figs. 3, 4).

Overrepresented GOs for GY included diverse BPs belonging to metabolic, regulative and stress/stimulus-responsive pathways. The most significant BP for GY was "DNA replication initiation" (*P* value = 0.0049). The GY-related enriched metabolic pathways were involved in nitrogen (cyanide metabolic process) and carbohydrate (trehalose metabolic process) metabolism, amino acid (cysteine biosynthetic process from serine) and amino acid derivative biosynthesis (chalcone biosynthetic process), lipid catabolism (phospholipid catabolic process), "regulation of glycolysis", "homeostatic regulation of membrane potentials", and stress or/and stimulus-responsive BPs including response to desiccation and nitrosative stress (detoxification of nitrogen compound). More enriched processes were the "growth related cell wall modification" and "protein folding" (Fig. 3).

In comparison to GY, for GDMC more GO terms concerning BPs, MF and CC were significantly enriched ($P < 0.03$). Enriched BPs for HP for GDMC included amino acid derivative biosynthetic processes (lignin biosynthetic process), amino acid catabolic processes (tryptophan catabolic process), biosynthetic (*S*-adenosylmethionine biosynthetic process, spermidine biosynthesis), and regulative biosynthetic processes (regulation of flavonoid biosynthetic process), and responses to stimuli and/or

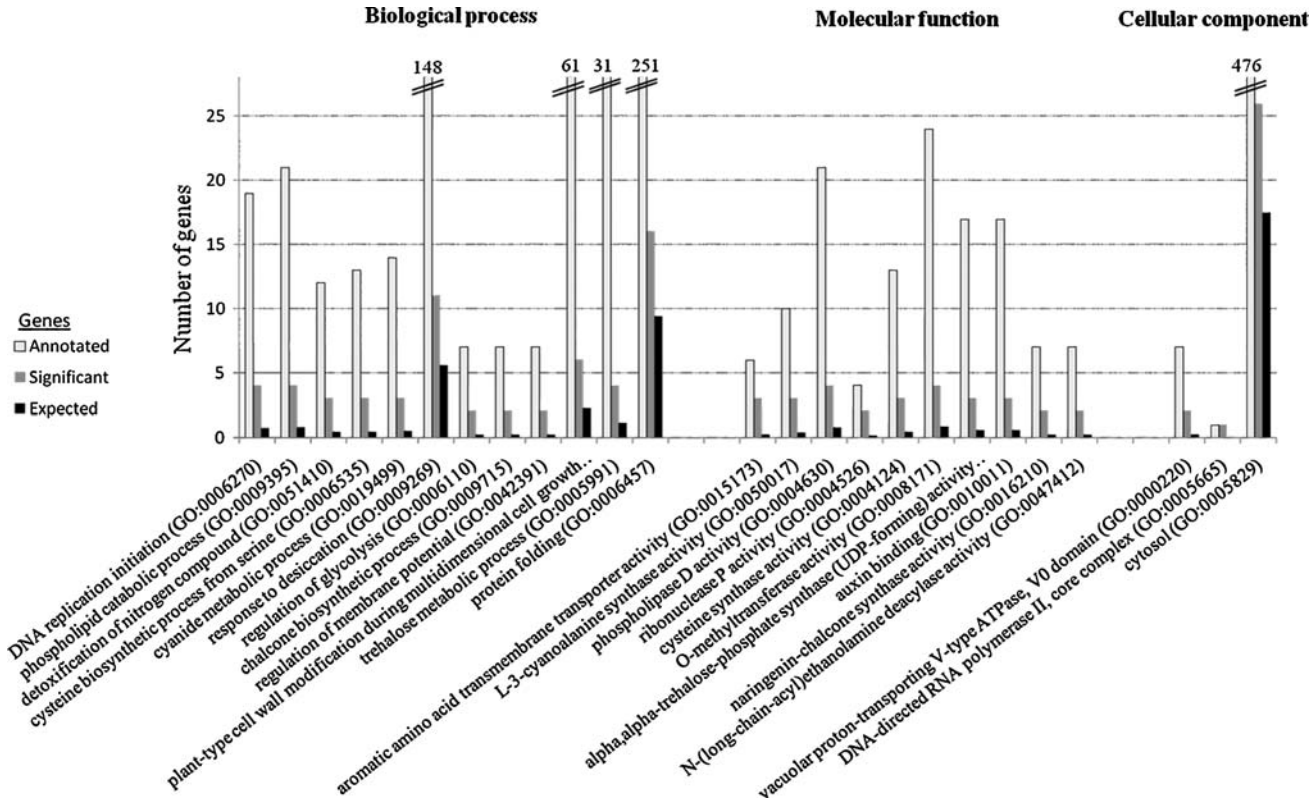

Fig. 3 Overrepresented GO terms among genes correlated to HP for GY, belonging to the indicated GO categories. Only GO terms significantly ($P < 0.03$) enriched are shown and sorted by significance, with the most significant term on the *left side* of each category. Each GO term is represented by the number of all GO-related annotated genes in the dataset (annotated), the number of GO-related genes significantly correlated to the trait (significant) and the number of genes expected, if no enrichment would occur (expected)

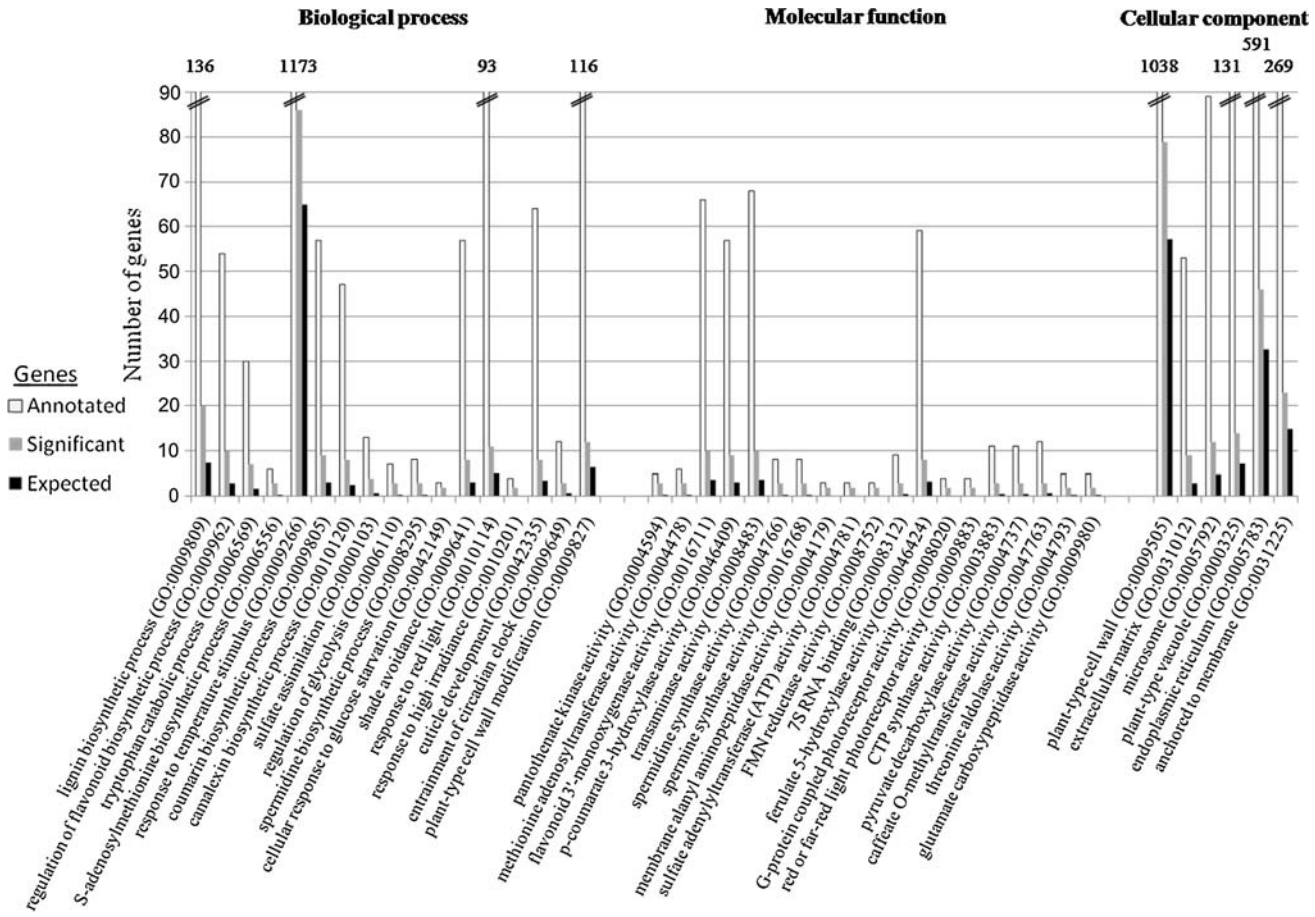

Fig. 4 Overrepresented GO terms among genes correlated to HP for GDMC, belonging to the indicated GO categories. Only GO terms significantly ($P < 0.03$) enriched are shown and sorted by significance, with the most significant term on the left side of each category. Each GO term is represented by the number of all GO-related annotated genes in the dataset (annotated), the number of GO-related genes significantly correlated to the trait (significant) and the number of genes expected, if no enrichment would occur (expected)

stress. The latter included responses to temperature (responses to temperature stimulus), light (shade avoidance, responses to red light, response to high irradiance), and nutrient levels (cellular response to glucose starvation). The metabolic processes inside the enriched BPs were involved in aromatic compound biosynthesis (coumarin biosynthetic process, camalexin biosynthetic process), and "sulfate assimilation". The most significant enriched BP in the group of GDMC-associated genes was the "lignin biosynthetic process" ($P = 5.7e{-}05$) (Fig. 4).

The enriched gene-MFs were reflecting the enriched BPs for each trait. A comparison of the enriched CCs, describing the main subcellular localizations of the HP-correlated genes, revealed more extracellular and cell periphery-associated localizations for GDMC and exclusively areas inside the cell without direct contact to the cell-periphery for GY (Figs. 3, 4).

Of the 3466 genes, which correlated significantly ($P < 0.01$) to HP for GY or GDMC, 545 genes showed a significant correlation with HP for both traits (Fig. 2). Processes significantly enriched among these 545 common genes were involved in lipid/lipopolysaccharide biosynthesis (triglyceride biosynthetic process, lipopolysaccharide biosynthetic process), growth-related processes including cell wall modifications, meiose regulation (synapsis), and response to light stimuli affecting development (photomorphogenesis). The most significantly enriched BP ($P = 0.003$) was the "regulation of glycolysis" (Table 1).

The analysis of possible certain underrepresented BPs among the genes correlated to GY, GDMC and of the intersected genes, correlated to HP of both traits revealed different GO terms of the category BP underrepresented for both traits but also certain GOs underrepresented for GY as well as for GDMC, for example the "negative regulation of gene expression" and "cell fate specification" observed for GY which is a child ontology term of the "cell fate commitment" observed for GDMC. The "negative regulation of gene expression" was the most significant underrepresented GO term for both traits. Considering only GY, processes concerning CC organization (protein complex assembly),

Table 1 Overrepresented GO terms (BPs) of common genes correlated to HP for GY and GDMC simultaneously (intersection)

	Overrepresented GO term	Genes (no.)	*P* value
Intersection	Regulation of glycolysis (GO:0006110)	2	0.003
	Plant-type cell wall loosening (GO:0009828)	4	0.005
	Plant-type cell wall modification during multidimensional cell growth (GO:0009831)	4	0.006
	Triacylglycerol biosynthetic process (GO:0019432)	2	0.011
	Synapsis (GO:0007129)	1	0.024
	Lipopolysaccharide biosynthetic process (GO:0009103)	1	0.024
	Photomorphogenesis (GO:0009640)	6	0.028

GO terms (BP) significantly ($P < 0.03$) enriched among genes correlated to HP ($P < 0.01$) for GY and GDMC (intersection). The number of genes belonging to each BP [Genes (no.)] and *P* values (weight algorithm) indicating the significance of the enrichment are given

developmental processes (cell fate specification, seed development), and defense responses (systemic acquired resistance) were underrepresented (Table 2).

For GDMC in addition to "cell fate commitment" and "negative regulation of gene expression", developmental processes (floral whorl development, pattern specification process, anatomical structure morphogenesis, trichome branching), cellular transport processes (vesicle-mediated transport), CC organization processes (cytoskeleton organization) and the response to cold-stress (cold acclimation) were significantly underrepresented among the genes correlated to HP for GDMC (Table 2).

Interestingly, within the intersected genes underrepresentation of "negative regulation of gene expression" was not observed, because different genes, belonging to this GO group were correlated to HP for each trait. Instead, "carbohydrate metabolic processes" and "negative regulation of metabolic processes" were underrepresented (Table 2).

Functional characterization of heterosis

As for the analysis of HP for GY, an enrichment of "DNA replication initiation" as the most significant enriched GO term of genes significantly correlated ($P < 0.01$) to MPH for GY was again evident. Furthermore, membrane lipid associated processes were enriched with pathways involved in biosynthesis (phosphatidylcholine biosynthetic process, choline biosynthetic process) and catabolism (phospholipid

Table 2 Underrepresented BPs of genes correlated to HP for GY, GDMC or both traits (intersection)

	Underrepresented GO term	Genes (no.)	*P* value
Intersection	Carbohydrate metabolic process (GO:0005975)	7	0.022
	Negative regulation of metabolic process (GO:0009892)	1	0.024
GY	Negative regulation of gene expression (GO:0010629)	10	0.0017
	Protein complex assembly (GO:0006461)	2	0.0144
	Cell fate specification (GO:0001708)	19	0.0158
	Seed development (GO:0048316)	7	0.0188
	(Salicylic acid-dependent) systemic acquired resistance (GO:0009627)	16	0.0265
GDMC	Negative regulation of gene expression (GO:0010629)	3	0.00085
	Biopolymer metabolic process (GO:0043283)	163	0.002
	Floral whorl development (GO:0048438)	12	0.00386
	Cell fate commitment (GO:0045165)	10	0.00403
	Cytoskeleton organization and biogenesis (GO:0007010)	9	0.00419
	Pattern specification process (GO:0007389)	13	0.00718
	Alkene metabolic process (GO:0043449)	3	0.00822
	Vesicle-mediated transport (GO:0016192)	4	0.00866
	Anatomical structure formation (GO:0048646)	7	0.00903
	Cold acclimation (GO:0009631)	1	0.00967
	Trichome branching (GO:0010091)	2	0.01289

GO terms (BP) significantly ($P < 0.03$) underrepresented among genes correlated to HP ($P < 0.01$) for GY, GDMC and both traits (intersection). The number of genes belonging to each BP [Genes (no.)] and *P* values (weight algorithm) indicating the significance of the underrepresentation are given

Table 3 Overrepresented and underrepresented GO terms (BPs) of genes correlated to MPH for GY

	GO term	Genes (no.)	*P* value
Overrepresented	DNA replication initiation (GO:0006270)	6	0.00028
	Choline biosynthetic process (GO:0042425)	3	0.00127
	Tetrahydrofolate metabolic process (GO:0046653)	2	0.00774
	Phosphatidylcholine biosynthetic process (GO:0006656)	4	0.01213
	Hexokinase-dependent signaling (GO:0009747)	3	0.01257
	Phospholipid catabolic process (GO:0009395)	4	0.02099
	ATP-dependent proteolysis (GO:0006510)	9	0.02905
Underrepresented	Cell fate specification (GO:0001708)	6	0.00051
	Cell division (GO:0051301)	9	0.00158
	Transmembrane receptor protein tyrosine kinase signaling pathway (GO:0007169)	9	0.00161
	Meristem organization (GO:0009933)	6	0.00266
	Stomatal complex development (GO:0010374)	2	0.00277
	Pattern specification process (GO:0007389)	9	0.00383
	Protein amino acid phosphorylation (GO:0006468)	24	0.0042
	Negative regulation of gene expression (GO:0010629)	6	0.00821
	Leaf development (GO:0048366)	18	0.01101
	Anthocyanin biosynthetic process (GO:0009718)	1	0.01198
	Cell adhesion (GO:0007155)	2	0.01252
	Cellular protein complex assembly (GO:0043623)	5	0.0178
	Transcription, DNA-dependent (GO:0006351)	26	0.02215
	Response to UV-B (GO:0010224)	13	0.02544
	Regulation of meristem development (GO:0048509)	4	0.02856

GO terms (BP) significantly ($P < 0.03$) over- or underrepresented among genes correlated to MPH ($P < 0.01$) for GY. The number of genes belonging to each BP among the genes correlated to MPH for GY [Genes (no.)] and *P* values (weight algorithm) indicating the significance of the over- or underrepresentation are given

catabolic process) of membranes. Furthermore, signal transduction (hexokinase-dependent signaling), proteolytic (ATP-dependent proteolysis), and tetrahydrofolate metabolic processes were enriched (Table 3).

Underrepresented gene functions for MPH of GY comprised functions involved in cellular developmental processes (cell fate specification) and multi-cellular developmental processes of diverse tissues like meristems (meristem structural organization, regulation of meristem development), and leafs (stomatal complex development, leaf development). Also processes in transcription and in the more general regulation of gene expression (negative regulation of gene expression) were underrepresented. Other underrepresented but very interesting functional groups comprised signal transduction (transmembrane receptor protein tyrosine kinase signaling pathway), cell division, and post-translational protein modifications (protein amino acid phosporylation) (Table 3).

Overrepresented categories among MPH-only correlated genes, in which genes also associated with HP for GY were excluded, maintained the same membrane lipid biosynthetic processes (choline biosynthetic process, phosphatidylcholine biosynthetic process), hexose-mediated signaling, DNA replication initiation and tetrahydrofolate metabolic process. In addition, response processes to stimuli like high light intensity and to copper ions, porphyrin biosynthesis (heme biosynthetic process) and an amino acid derivative biosynthetic process (spermidine biosynthetic process) were enriched. By exclusion of HP-correlated genes, "choline biosynthetic process" became the most significantly enriched GO term ($P = 0.00031$) (Table 4).

In comparison to the underrepresented genes of all MPH-correlated genes here some new BPs appeared. The most interesting gene functions, due to their growth-associated functions are involved in the response to certain stimuli, like gibberellin, salicylic acid, and sucrose (Table 4). The analysis of underrepresented MFs of genes correlated to only MPH for GY revealed the group "transcription regulator activity" as the only group, which was significantly ($P < 0.03$) underrepresented. This group contained 29 genes coding for transcription factors.

Discussion

Extensive differential gene expression in maize inbreds and hybrids has been documented in the past by several studies (reviewed by Hochholdinger and Hoecker 2007).

Table 4 Overrepresented and underrepresented GO terms of genes correlated exclusively to MPH for GY

	GO term	Genes (no.)	*P* value
Overrepresented	Choline biosynthetic process (GO:0042425)	3	0.00031
	Tetrahydrofolate metabolic process (GO:0046653)	2	0.003
	Response to copper ion (GO:0046688)	4	0.00882
	Heme biosynthetic process (GO:0006783)	2	0.01406
	Response to high light intensity (GO:0009644)	8	0.01824
	Phosphatidylcholine biosynthetic process (GO:0006656)	3	0.01855
	DNA replication initiation (GO:0006270)	3	0.02152
	Hexose mediated signaling (GO:0009757)	3	0.02152
	Spermidine biosynthetic process (GO:0008295)	2	0.02516
Underrepresented	Cell division (GO:0051301)	2	0.00017
	Pattern specification process (GO:0007389)	3	0.00054
	Shoot morphogenesis (GO:0010016)	8	0.00321
	Leaf development (GO:0048366)	8	0.00537
	Meristem development (GO:0048507)	5	0.00613
	Cell fate commitment (GO:0045165)	4	0.00738
	Regulation of RNA metabolic process (GO:0051252)	12	0.00768
	Response to gibberellin stimulus (GO:0009739)	3	0.00843
	Response to salicylic acid stimulus (GO:0009751)	9	0.00908
	Negative regulation of cellular process (GO:0048523)	10	0.01095
	Stomatal complex development (GO:0010374)	1	0.01534
	Response to sucrose stimulus (GO:0009744)	2	0.016
	Cell cycle process (GO:0022402)	3	0.01661
	RNA metabolic process (GO:0016070)	21	0.0185
	Protein amino acid phosphorylation (GO:0006468)	15	0.02068
	Response to UV (GO:0009411)	10	0.02382
	Positive regulation of developmental process (GO:0051094)	2	0.02501
	Circadian rhythm (GO:0007623)	1	0.0293
	Reproductive process in a multicellular organism (GO:0048609)	1	0.02934

GO terms (BP) significantly ($P < 0.03$) over- or underrepresented among genes correlated to MPH ($P < 0.01$) for GY exclusively, without genes intersecting with HP for GY. The number of genes belonging to each BP among the genes correlated only to MPH for GY [Genes (no.)] and *P* values (weight algorithm) indicating the significance of the over- or underrepresentation are given

This variation in transcript accumulation is likely to contribute to phenotypic variation and heterosis (Song and Messing 2003; Guo et al. 2004; Springer and Stupar 2007). In support of this hypothesis, Stupar et al. (2008) reported a positive correlation between genetic distance and extent of differential gene expression for five stiff stalk and non-stiff stalk genotypes. Moreover, Guo et al. (2006) demonstrated a positive correlation between the percentage of inter-parental differentially expressed genes and yield heterosis in maize. Further, the PCA based on the transcriptional profiles of seedlings reported in our companion paper (Frisch et al. 2010) clearly separated the heterotic groups of the 21 inbred lines used in this study. The relationship of transcriptional variation with MPH indicated that the transcriptional constitution of inbred lines has prediction potential for the performance of the hybrids they constitute. The genes involved in this relationship should also provide information about the molecular mechanism of heterosis.

In the present study, the observed mid-parent expression levels of certain genes showed a high correlation with HP and MPH for GY, and HP for GDMC (Fig. 2), based on genome-wide transcriptional profiles of inbreds and field data of inbreds and hybrids showing a variable degree of heterosis. In our companion paper (Frisch et al. 2010) a strong correlation between transcriptome-based distance and heterosis and HP was observed. The predictive power of the inbred line transcriptional profiles described here was reported by Frisch et al. (2010). The present study targets on the characterization of HP-associated gene functions for GY and GDMC and on the phenomenon of heterosis.

The assumption that specific genes might be associated and involved in the expression of HP or heterosis is supported by many studies in which quantitative trait loci

(QTLs) were related to certain traits (Stuber et al. 1992; Xiao et al. 1995; Lu et al. 2003). The computed mid-parent expression level used for correlation analysis gave us a defined transcriptional value, based on the differential gene expression levels of the parental inbred lines. This mid-parent expression level equals the hybrid expression level provided there is additive expression in the hybrid. Stupar et al. (2008) demonstrated for most inter-parental differentially expressed genes an additive expression pattern in hybrids and assumed that heterosis is predominantly influenced by additive rather than non-additive hybrid expression patterns. Guo et al. (2006) observed that the proportion of allelic additively expressed genes in maize was positively correlated to hybrid yield and heterosis, whereas no correlation was observed with the amount of over- and underexpression of specific genes. Springer and Stupar (2007) proposed that widespread differential *cis*-regulation between even closely related maize lines is caused, at least in part, by variable repetitive sequences between genes which were found to influence the gene expression of adjacent genes (Stam et al. 2002; Clark et al. 2006). Based on these observations they assumed a model with an optimal expression range. Expression levels outside this optimal range would probably affect the phenotype and may result in a loss of fitness. Mid-parent expression levels in hybrids would fall with a higher probability than their parents into this optimal range and cause a positive effect on the phenotype (Springer and Stupar 2007). Supportive to this model, the results of our correlation analysis revealed a number of genes which displayed mid-parent gene expression levels either significantly correlated to HP for GDMC, or HP or MPH for GY. Further, some of these genes correlated to two or even all three measurements (Fig. 2). The observation that various genes showed opposite sign of *r* for HP of GY and GDMC indicate opposing regulatory mechanisms to be operative in the expression of GDMC and GY and suggest that pleiotropic regulatory genes explain the negative correlation observed between the two traits (J. Fu, unpublished data). In the maize grain, protein concentration and GY are also negatively correlated traits (McDermitt and Loomis 1981), most likely caused by the higher glucose costs for the synthesis of proteins compared to the synthesis of carbohydrates (Mason and D'Croz-Mason 2002). Concerning the relation of GDMC and GY earlier seed maturation and desiccation might increase GDMC at harvest at the cost of a pre-terminated grain filling process reducing GY.

The GO analyses of genes correlated to HP or MPH revealed either over- or underrepresented functional classes in comparison to the average distribution of all expressed genes. Overrepresented functional classes indicated BPs with a wide range of genes associated to HP or MPH. In contrast, underrepresented GO terms pointed to processes wherein only a few specific genes of a pathway contribute to the expression of a trait. That specific genes may cause heterosis was indicated by tomato introgression lines in which single small chromosomal segments from wild tomatoes species caused heterosis (Semel et al. 2006). Many of the so far identified QTLs encoded transcription factors or genes involved in signal transduction cascades. Such genes often code for proteins which interact with other proteins in regulatory complexes, where the stochiometric relationship of its components highly affect the efficiency due to dosage-dependency (Birchler et al. 2001).

Both, GY and GDMC are traits with a complex background. The expression of such traits is influenced by interactions of numerous physiological processes and environmental effects during the whole life cycle of the plant (Tollenaar et al. 2004). Our functional analysis of genes correlated with HP for GY and GDMC revealed enrichment of different BPs except one (Figs. 3, 4). This observation strengthens the assumption that different biological and physiological processes are involved in the phenotypic expression of HP for the two analyzed traits. For GY the most significantly enriched BP, "DNA replication initiation", might be associated with GY due to its role in cell proliferation. Increased cell proliferation was shown to cause heterotic expression in early maize embryos (Meyer et al. 2007). For GDMC the most significantly enriched process was the "lignin biosynthetic process" (Fig. 4). Strikingly, a reduction of dry mater yield (15–20%) in brown midrib 3 (bm3) isolines with lowered lignin levels has been observed (Inoue and Kasuga 1989).

The characterization of heterosis was performed by the exclusion of intersecting genes from MPH and HP-correlated genes. These intersecting genes had *r* values with the same sign, meaning genes having a positive *r* with HP were also positively correlated to MPH and the same was true for the genes with negative *r*. Based on this observation and that some other genes were correlated to MPH only, we propose that genes having same direction of contribution to HP as well as to MPH might be associated with a general positive effect on HP for GY, whereas the genes correlated to only MPH and not to HP might be more specifically involved in heterosis and might be more likely contributing to the superiority of the hybrids over the parents.

The analysis of overrepresented BPs of these exclusively MPH-correlated genes revealed the biosynthetic process of phosphatidylcholine and its component choline significantly enriched (Table 4). Phosphatidylcholine is a major structural element of eukaryotic membranes (Harwood 1980) and is assumed to play a major role in plant adaption to stresses (Tasseva et al. 2004), like cold (Sikorska and Kacperska-Palacz 1980; Kinney et al. 1987) and salt (Pical et al. 1999), due to its property of maintaining cell structure and function (Tasseva et al. 2004). Spermidine, also an

overrepresented biosynthetic process, is a polyamine playing a protective role during salt stress as a free radical scavenger, and as a factor stabilizing cell membranes and maintaining the cellular ionic balance (Jiménez-Bremont et al. 2007). Stress adaption capability is most likely a factor contributing to heterosis (Guo et al. 2006). Tetrahydrofolate metabolism, another significantly enriched process, is involved, amongst others, in photorespiration (Jabrin et al. 2003). This finding is in line with higher respiration rates of hybrid corn seedlings compared to the parental lines during early stages of germination (Sarkissian et al. 1964). The "biosynthesis of heme", also enriched among heterosis-correlated genes, efficiently regulates the transcription of a cytochrome *c* gene within the mitochondria (Guarente and Mason 1983). Cytochromes are considered to be associated to heterosis (Sarkissian and Srivastava 1971) and cytochrome *c* oxidase was shown to be highly active in hybrid mitochondria (Sarkissian and McDaniel 1967). An overrepresented process involved in signaling and signal transduction was "hexose mediated signaling". Hexose signaling was demonstated to regulate gene expression of photosynthetic-associated genes (Koch 1996) and plant defense related genes (Herbers et al. 1996). Moreover, sugar-induced signals also interact with other sensing and signaling pathways (Smeekens and Rook 1997). Another interesting group that got enriched was the "response to high light intensity", and it was shown by Meyer et al. (2004) that the heterosis effect in *Arabidopsis* increased in the vegetative phase in several genotypes due to high light intensities. The underrepresented BPs in the group of only MPH-correlated genes comprised developmental processes of a wide range of plant tissues, like meristem, leaf, and shoot. Also cellular developmental processes were significantly underrepresented (Table 4). Interestingly, transcription factors constitute the only significantly underrepresented group among all MFs. This finding indicates that only few specific genes of this class are correlated to heterosis. Due to their dosage-dependency these genes might have a great impact on quantitative traits (Birchler et al. 2001) as demonstrated by single gene heterosis in *Arabidopsis* (Rédei 1962), caused by a transcription factor (Kim et al. 2002) and a signal transduction kinase (Shpak et al. 2004).

In the present study we identified functional groups and genes in maize highly correlated to HP and MPH for GDMC and GY as well as to heterosis. The appearance of genes and biological processes, which were formerly shown to be characteristic for the respective trait, indicates that our approach is well suitable for their characterization. Because the analyses were based on calculated mid-parental expression values, it will be interesting to prove the concept of a *cis*-regulated, additive contribution to heterosis by testing the genuine expression values in F1 hybrids. In future experiments the genes identified in this study might provide starting points to unravel the molecular mechanisms involved in the expression of heterosis.

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