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ORIGINAL PAPER

ZmGrp3: identification of a novel marker for root initiation in maize and development of a robust assay to quantify allele-specific contribution to gene expression in hybrids

Katrin Woll · Angela Dressel · Hajime Sakai · Hans-Peter Piepho · Frank Hochholdinger

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Abstract This study comprises a comprehensive gene expression analysis of the root tip specific maize gene *ZmGrp3*. In the first part of this paper expression of *ZmGrp3* was studied in maize inbred lines. First, RNA in situ hybridization experiments confined the expression of *ZmGrp3* to the columella and the epidermis of all embryonic and postembryonic root types. Second, Northern-blot analyses of the maize root initiation mutants *rtcs* and *lrt1* revealed that the *ZmGrp3* gene is not expressed prior to root initiation, thus providing a novel marker for this developmental process. Finally, a comprehensive expression profiling in 42 tissues via the Lynx MPSS system revealed almost exclusive expression of *ZmGrp3* in maize roots. In the second part of this survey, *ZmGrp3* expression was assayed in maize hybrids. In this context, a novel approach to quantify allele-specific contribution to gene expression in maize hybrids was developed. This assay combines RT–PCR amplification of polymorphisms between two alleles and subsequent quantification of allele-specific gene expression via a combination of didesoxyterminator assays and capillary electrophoresis. Allelic expression of the *ZmGrp3* gene in six reciprocal hybrids generated from three *ZmGrp3* alleles was analyzed via a new statistical mixed model approach.

Genbank Accessions of *ZmGrp3* alleles Allele UH002: DQ267623 · Allele UH250: DQ267624 · Allele UH301: DQ267625

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K. Woll · A. Dressel · F. Hochholdinger (✉)
Center for Plant Molecular Biology (ZMBP),
Department of General Genetics,
Eberhard-Karls-University Tuebingen,
Auf der Morgenstelle 28, Tuebingen 72076, Germany
e-mail: frank.hochholdinger@zmbp.uni-tuebingen.de

H. Sakai
DuPont Crop Genetics Research, Wilmington,
DE 19880-353, USA

H.-P. Piepho
Bioinformatics Unit, Institution for Crop Production and Grassland Research, University of Hohenheim,
Stuttgart 70599, Germany

Introduction

The rootstock of maize is composed of embryonically preformed primary and seminal roots, an extensive postembryonic shoot-borne root system and a highly branched lateral root system present on all root-types (Hochholdinger et al. 2004a, b). This complex root stock architecture provides a maximal absorbing surface and is a functional prerequisite for the mediation and regulation of nutrition and water uptake of the maize plant.

The genetic analysis of maize root formation recently revealed a number of mutants (Hochholdinger et al. 2004a, b) including *lrt1* (Hochholdinger and Feix 1998a) and *rtcs* (Hetz et al. 1996) that were affected prior to the initiation of lateral and shoot-borne roots, respectively. Despite the availability of a growing number of specific mutants affected in maize root development, little is known about the molecular basis of root formation in maize (Hochholdinger et al.

2005a). The *rth1* gene, which encodes for a homolog of the exocyst subunit *sec3* which regulates root hair formation, is the first root mutant gene from maize that has been cloned in a forward genetic approach (Wen et al. 2005). In addition, various high throughput screening techniques including microarray (Woll et al. 2005; Poroyko et al. 2005) and proteomic (Hochholdinger et al. 2004c, 2005b; Sauer et al. 2006) studies, but also traditional subtractive approaches, have identified genes that are differentially expressed between various genotypes or developmental stages of root formation. Among these, three root-specific glycine-rich, putative cell wall proteins have been cloned in maize and were designated *ZmGrp3* (Goddemeier et al. 1998), *ZmGrp4* (Matsuyama et al. 1999) and *Zm123A* (Ponce et al. 2000). These genes display no sequence homology; however, the proteins encoded by these genes are characterized by a high glycine content. Moreover, a few orthologous genes from maize known to be involved in Arabidopsis root formation such as *scr* (Lim et al. 2000) or *aux1* (Hochholdinger et al. 2000) have been cloned. Nevertheless, in maize only a few markers for specific developmental events in roots are available (e.g. Hochholdinger et al. 2000; Lim et al. 2000; Zimmermann and Werr 2005).

Northern-blot or in situ hybridization experiments that are typically used to monitor gene expression cannot discriminate between the contributions of different alleles to the expression of a gene. Therefore, currently only little is known concerning allelic variation of gene expression at the transcript level in maize hybrids (Guo et al. 2004). Such analyses will become more important in the future since allelic diversity is considered a crucial component for phenotypic variation (Buckler and Thornsberry 2002) as observed in heterotic hybrid plants (Birchler et al. 2003). To our knowledge, only one study has been conducted in maize hybrids applying a combination of RT–PCR and WAVE dHPLC to discriminate between the allelic transcript levels in young unpollinated ears (Guo et al. 2004).

This study represents a comprehensive gene expression analysis of the *ZmGrp3* gene of maize. In the first part of this study, expression of the *ZmGrp3* gene was analyzed in maize inbred lines. It was demonstrated that the *ZmGrp3* gene is exclusively expressed in roots after their initiation and that it displays a tissue specific expression pattern that is restricted to the root tips, thus providing a novel marker for this developmental process. In the second part of the manuscript, expression of the *ZmGrp3* gene was analyzed in maize hybrids. For this purpose a novel assay for the determination of allele-specific gene expression in combination with a rigorous data analysis tool was developed. This assay might be an interesting tool for future allele-specific gene expression studies of candidate genes related to the phenomenon of heterosis.

Materials and methods

Plant material and growth conditions

Experiments were conducted with the maize inbred line B73 that was developed from Iowa Stiff Stalk Synthetic (BSSS), the maize mutants *lrt1* (Hochholdinger and Feix 1998a) and *rtcs* (Hetz et al. 1996) and their homozygous wild-type siblings. For allelic variation studies of gene expression in maize hybrids, the German inbred line UH002 from the flint pool [National listing of plant varieties (NLPV), AC: M7830, European flint], the inbred lines UH250 (NLPV, AC: M9005, Iowa Stiff Stalk Synthetic) and UH301 (NLPV, AC: M8652, Iodent) from the dent pool and the six possible reciprocal hybrid combinations derived from these three parental inbred lines were used. These inbred lines were generated by the breeding program of the University of Hohenheim, Germany. Seeds were surface-sterilized and germinated in the paper roll test as previously described (Woll et al. 2005) in beakers with tap water at 28°C, 60% humidity and 16 h light, 8 h dark regime. Plant material for the Northern-blot analyses of the various mutants was harvested 10 days after germination (DAG) when the phenotypes of the root mutations were unambiguously assessable. Plant material for allelic expression variation studies was harvested 3.5 DAG, hence before lateral root emergence.

Northern-blot analysis

RNA was extracted from several tissues using the RNeasy Plant Mini RNA extraction-kit (Qiagen, Hilden, Germany). Total RNA (20 µg) was electrophoretically separated on 1% formaldehyde agarose gels and blotted on a Hybond N^+ membrane (Amersham, Buckinghamshire, UK) with 20× SSC over night according to the manufacturer. The RNA was cross-linked to the membrane at 0.12 J/cm^2. Hybridization was performed at 50°C O/N in a buffer containing 55% formamide, 50 mM Tris–HCl, pH 7.5, 1× SSC, 0.1% Na-pyrophosphate, 1% SDS, 10% dextran sulfate and 200 µg/ml herring sperm DNA using a PCR amplified 418 bp probe of the 3′ UTR of the *ZmGrp3* gene (position 766–1,184 of Genbank AC Y07781) labeled

by random priming. After hybridization the filter was washed in 1× SSC, 1% SDS at 50°C and 0.2× SSC at room temperature each for 30 min.

In situ hybridization experiments

Maize primary roots, coleoptilar nodes and embryos were fixed in 4% paraformaldehyde for 12 h at 4°C. After dehydration in a graded ethanol series and subsequent clearing in a graded histoclear series (National Diagnostics, Yorkshire, UK) the specimens were embedded in paraffin and 10–12 μm sections were prepared using a Leica RM2145 microtome (Leica, Jena, Germany). Samples were deparaffinized using histoclear and dehydrated in an ethanol series. Hybridization was performed with 166 bp digoxigenin-labeled RNA sense and antisense probes of the 3′ UTR of *ZmGrp3* (position 791–957 of Genbank AC Y07781) cloned into the pIS vector (Hochholdinger and Feix 1998b) in both orientations using the T7 primer for in vitro transcription according to the manufacturer (Boehringer Mannheim, Germany). After 2 h of prehybridization in 1× SPB buffer [50% formamide, 1.25× salts (10× salts: 3 M NaCl, 0.1 M Tris–HCl, pH 6.8, 0.1 M sodium phosphate, pH 6.8, 50 mM EDTA), 12.5% dextransulfate, 1.25 mg/ml tRNA, 0.625 mg/ml polyA$^+$, 1.25× Denhardt's solution] hybridization took place at 45°C for about 16 h. Slides were washed afterwards (5× SSC/50% formamide) and treated with RNaseA (10 μg/ml) at 37°C for 30 min and washed several times with NTE (0.5 M NaCl, 10 mM Tris–HCl, pH 8.0, 0.5 mM EDTA). The immunological detection was conducted by incubating the specimens with NBT/BCIP according to the manufacturer. Samples were analyzed under a Zeiss-Axioskop HBO 100 W/2 microscope (Zeiss, Jena, Germany) and results were documented with an Olympus SC35, Type12 camera (Olympus, Hamburg, Germany).

Survey of massively parallel signature sequencing database (MPSS)

The Lynx–MPSS technology allows for the quantification of 17 bp sequences in populations of 2×10^5 to 2×10^6 cDNAs. These 17 bp signature sequences almost always correspond to unique cDNAs, thus allowing for the quantification of the abundance of a particular cDNA in a sample representing a particular organ and developmental stage in a defined genetic background (Christensen et al. 2003). In our experiments we surveyed gene expression in B73 background.

Chromosomal mapping of the ZmGrp3 with oat–maize addition lines

Genomic DNA of oat–maize addition (OMA) lines obtained as a gift from Ron Phillips (University of Minnesota) were used as described in Kynast et al. (2001) to map the *ZmGrp3* gene to a maize chromosome. A standard PCR with the primers *ZmGrp3-fw*: 5′AACTCGCTCACTGGCAAT 3′ and *ZmGrp3-rv*: 5′ AGCTAGTCAATAGTCACATTCAA 3′ at an annealing temperature of 60°C was conducted with Thermo Taq Polymerase (ABgene).

Determination of allelic contribution to gene expression in maize hybrids

Genomic DNA of six different hybrids (UH002 × UH250, UH250 × UH002, UH301 × UH002, UH002 × UH301, UH250 × UH301 and UH301 × UH250) was extracted according to a CTAB protocol (Saghai-Maroof et al. 1984), total RNA of roots 3.5 DAG was isolated with the Qiagen RNeasy Plant Mini RNA extraction kit (Qiagen). Genomic DNA was removed from RNA preparations with the RNase free DNase set (Qiagen). cDNA was synthesized with superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer. Sequence polymorphisms of the *ZmGrp3* gene between the different inbred lines were detected by PCR amplification, sequencing and alignment of at least four genomic *ZmGrp3* sequences isolated from independent plant samples for each of the three inbred lines. Full-length cDNA clones of the *ZmGrp3* alleles UH002 (Genbank AC: DQ267623), UH250 (Genbank AC: DQ267624) and UH301 (Genbank AC: DQ267625) were generated via 5′ and 3′ RACE experiments with the Generacer kit (Invitrogen) as described by the manufacturer.

Detection of allele-specific gene expression was achieved in a two-step process. First, a 299 bp region of the 3′ UTR of the *ZmGrp3* gene which contained polymorphisms that allow for the discrimination between the three different inbred lines was amplified from cDNA via PCR (Primers: Grp-5′: 5′ TGATGCTACCGGTGGTG 3′; Grp-3′: 5′ GACTGACACT AACGCCGAG 3′) with Thermo Taq Polymerase (ABgene, Epsom, UK) at an annealing temperature of 60°C in 30 cycles. These primers annealed in conserved regions of the *ZmGrp3* gene, thus allowing for amplification of all three alleles of the *ZmGrp3* gene analyzed in this study. Therefore, amplification of each of the two alleles in a hybrid was expected to correlate with the abundance of its transcripts. The number of 30

cycles was previously determined to ensure that the amplification is below the saturation level of the PCR reaction. Single band PCR products which resulted from this reaction and which contained polymorphisms between the different inbred lines were then subjected to a didesoxyterminator based quantification of the allelic contribution to gene expression using the ABI Prism SNaPshot™ Multiplex Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer. The primers SNP1: 5′ GATCCGGACCCAGAGCC 3′ and SNP2: 5′ ACTAACGCCGAGCGCTCC 3′ were selected because the adjacent nucleotide following the 3′ end of these primers was polymorphic in the three UH alleles analyzed in this study. Thus, the SNP primers were extended by only one nucleotide, which corresponded to the two respective alleles in the hybrid. A ddATP was added to the 3′ end of primer SNP1 in alleles UH002 and UH250, while a ddGTP was added to the 3′ end of this primer in allele UH301. Therefore, primer SNP1 allowed for the discrimination between the *ZmGrp3* alleles in the four hybrids: UH301 × UH250, UH250 × UH301, UH002 × UH301 and UH301 × UH002. A ddGTP was added at the 3′ end of primer SNP2 during primer extension in allele UH002 while a ddATP was added to the 3′ end of SNP2 in allele UH250. Thus, primer SNP2 was used to distinguish between the two *ZmGrp3* alleles in the hybrids UH002 × UH250 and UH250 × UH002. The ddNTPs were fluorescently labeled and could therefore be visualized in an ABI 310 capillary sequencer (Applied Biosystems). The peak areas of the two alleles in a hybrid which represented their relative expression levels were quantified after sequencing using *Image Pro-Express* software (Media Cybernetics Inc., Silver Spring, MD, USA) and their ratios were determined. For each hybrid, two independent RNA samples were isolated. Each biological RNA replicate was then converted in three independent reactions into cDNA. Thus, for each hybrid six cDNAs were analyzed. To exclude dye effects, three independent biological replicates of genomic DNA for each of the six hybrids were isolated and subjected to the same PCR amplification (26 cycles) including subsequent allelic expression quantification procedure as the corresponding RNA/cDNA samples. Per definition genomic DNA of each of the two alleles should be present in equal amounts in heterozygous diploid hybrids for nuclear genes. The actually measured ratio of the genomic DNA of the two alleles in a hybrid was used as a correction factor for the cDNA samples to quantify accurate allelic expression ratios of the *ZmGrp3* gene in the hybrids.

Statistical analysis of allele-specific gene expression analysis

RNA and genomic DNA measurements were logarithmically transformed prior to analysis. For the RNA data, we fitted a mixed model of the form

$$y_{iajk}=\mu_{ia}+r_{iaj}+e_{iajk},$$

where y_{iajk} is the log-transformed value of the ath allele in the ith hybrid as recorded for the jth biological replicate and kth RNA sub-sample, μ_{ia} is the mean of the ath allele in the ith hybrid, r_{iaj} is the random effect of the jth biological replicate for the ith hybrid and ath allele and e_{iajk} is a random sub-sampling effect corresponding to y_{iajk}. Random effects r_{iaj} of two different alleles on the same biological replicate were assumed to have the compound symmetry variance–covariance structure. Random effects e_{iajk} of two different alleles on the same RNA sub-sample were assumed to have the compound symmetry variance–covariance structure.

For the genomic DNA data we fitted the model

$$x_{iaj}=\phi_{ia}+r_{iaj},$$

where x_{iaj} is the log-transformed value of the ath allele in the ith hybrid as recorded for the jth biological replicate, ϕ_{ia} is the mean of the ath allele in the ith hybrid, r_{iaj} is the random effect of the jth replicate for the ith hybrid and ath allele. Random effects r_{iaj} of two different alleles on the same biological replicate were assumed to have the compound symmetry variance–covariance structure.

The RNA data and genomic DNA data were analyzed jointly using an additional factor identifying the two types of data. The variance–covariance structures were assumed heterogeneous for the two types of data. The random effect r_{iaj} for the RNA data was fitted by using a suitable dummy variable. The joint analysis yielded mean estimates on the log-scale. Assuming normality, these can be interpreted as median estimates. Back-transformation yielded estimates of the median on the original scale (Connolly and Wachendorf 2001). The median estimates were used to estimate

$$\text{Normalized ratio} = \frac{\text{Median}(\text{RNA}-\textit{allele}1)/\text{Median}(\text{RNA}-\textit{allele}2)}{\text{Median}(\text{DNA}-\textit{allele}1)/\text{Median}(\text{DNA}-\textit{allele}2)}.$$

On the log-scale, this corresponds to the linear contrast

$$L = \mu_{i1} - \mu_{i2} - \phi_{i1} + \phi_{i2},$$

where $a = 1$ for the allele1 channel and $a = 2$ for the allele2 channel. This contrast was tested based on the fitted mixed linear model on the log-scale using the method of Kenward and Roger (1997).

Results

In situ hybridization experiments confine ZmGrp3 expression to the columella and the epidermis of root tips

Northern-blot analyses demonstrated the expression of the *ZmGrp3* gene in all tested root-types (Goddemeier et al. 1998). However, these analyses did not define the tissue specific expression pattern of *ZmGrp3*. Thus, in situ hybridization experiments with a specific DIG-labeled 166 bp RNA probe from the 3′ UTR of *ZmGrp3* were performed to localize the exact cell-type specific expression pattern of *ZmGrp3* in different root-types of the maize inbred line B73. Hybridization signals were observed in all analyzed root-types including primary roots 7 days after germination (Fig. 1a), the embryonic radicle 30 days after pollination (Fig. 1b, c), postembryonically formed lateral roots in 6 days old primary roots (Fig. 1d–f) and shoot-borne crown roots 5 (Fig. 1g, i) and 7 DAG (Fig. 1h). Hence, *ZmGrp3* is active in all roots irrespective of their embryonic or postembryonic descent. The expression pattern of the *ZmGrp3* gene is similar in all analyzed root-types with a strong expression in the epidermis of the root tips and weak expression in the columella of these roots. Thus, the *ZmGrp3* represents a cell-type specific marker of gene expression in root tips.

ZmGrp3 is expressed only after root initiation

The root initiation mutants *rtcs* (Hetz et al. 1996) and *lrt1* (Hochholdinger and Feix 1998a) were used to study the temporal expression pattern of *ZmGrp3* in roots. Both mutants are affected before the initiation, i.e. the first cell division of the respective roots. While the mutant *rtcs* is devoid of shoot-borne root initiation, the mutant *lrt1* does not initiate lateral roots on the primary root. Northern-blot experiments demonstrated that the *ZmGrp3* was solely expressed in primary and crown roots but not in leaves, mesocotyl and young unpollinated ears of the inbred line B73 (Fig. 2). In

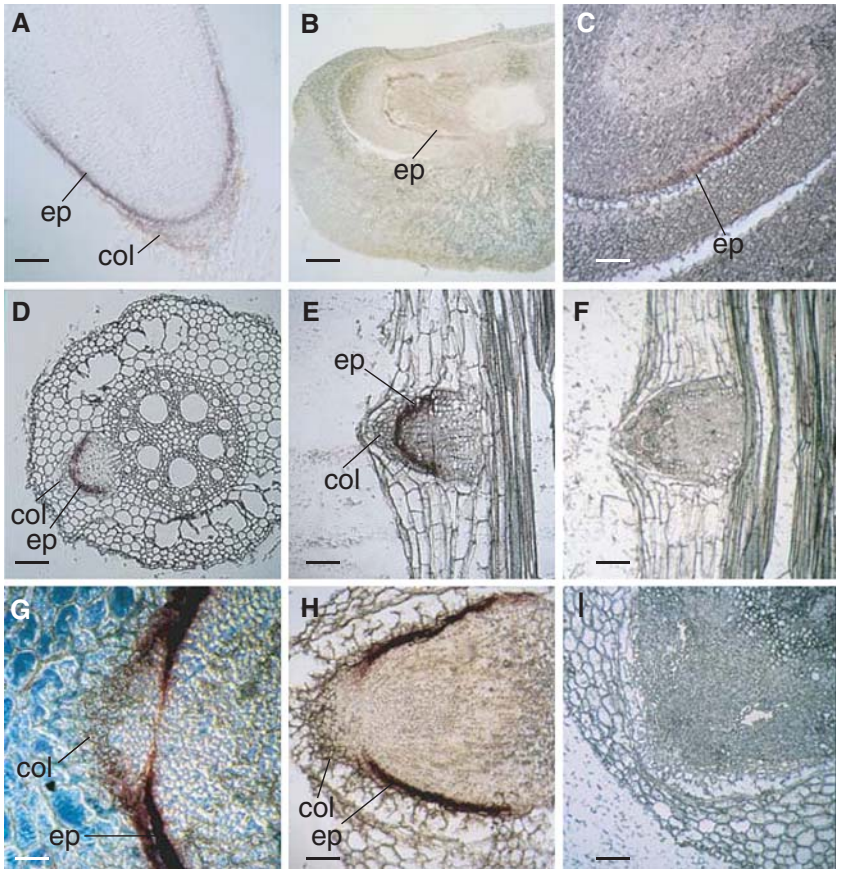

Fig. 1 In situ hybridization of roots of the maize inbred line B73 with a DIG-labeled *ZmGrp3* RNA probe. **a** Primary root tip, 7 DAG, antisense probe. **b–c** Embryo longitudinal section 30 DAP, antisense probe at different magnifications. **d–f** Lateral root emerging from a primary root 6 DAG. **d** Cross-section, antisense probe. **e** Longitudinal section, antisense probe. **f** Longitudinal section, sense probe. **g–i** Crown root emerging from a coleoptilar node, cross-section. **g** Antisense probe 5 DAG. **h** Antisense probe 7 DAG. **i** Sense probe 5 DAG. *DAP* days after pollination, *DAG* days after germination, *ep* epidermis, *col* columella. Bars = 300 μm in **a**, **d–f**, **h** and **i**, 30 μm in **b** and **g**, 10 μm in **c**

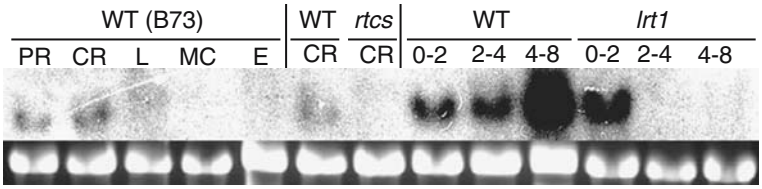

Fig. 2 Northern-blot hybridization with *ZmGrp3* RNA probe. Expression pattern in different tissues of wild-type (B73) and the root mutants *rtcs* and *lrt1*. *WT* wild-type, *PR* primary root, *CR* coleoptilar node, *L* leaf, *MC* mesocotyl (all 10 DAG), *E* immature ear, shortly before flowering. *Numbers* in the right part of the panel below WT and *lrt1* indicate the longitudinal parts of the primary root from the root tip (in cm). Ethidium bromide stained 26S rRNA bands are given below each blot as a loading control

order to analyze the longitudinal expression of *ZmGrp3* and to investigate if the gene was already expressed prior to lateral root initiation, RNA of the mutant *lrt1* and its corresponding homozygous wild-type was isolated from consecutive 2–4 cm sections of 10 days old primary roots (Fig. 2). At this developmental stage, lateral roots were already visible in wild-type primary roots while no lateral roots were initiated in the mutant *lrt1*. Sections 0–2 cm represent the root tip region. In wild-type primary roots hybridization signals were found in all sections along the primary root with increasing signal intensity towards the more differentiated root zone, which displayed the highest number of lateral roots. In the mutant *lrt1*, a signal was only detected in the tip of the primary root but not in the subsequent parts of the primary root. Since the mutant *lrt1* does not initiate lateral roots this indicates that *ZmGrp3* is only expressed after the initiation of this root-type. Similarly, RNA was isolated from coleoptilar nodes of the mutant *rtcs*, which does not initiate any shoot-borne roots, and its corresponding wild-type siblings 10 DAG. *ZmGrp3* expression was detected in wild-type coleoptilar nodes, but not in coleoptilar nodes of the mutant *rtcs* (Fig. 2). These results indicate that *ZmGrp3* expression can only be detected after the initiation of lateral and crown-roots.

Comprehensive gene expression study of the ZmGrp3 gene via the Lynx MPPS system confines expression almost exclusively to roots

Northern-blot analyses (Fig. 2) indicated that *ZmGrp3* is preferentially expressed in roots of the inbred line B73. To gain a comprehensive insight into the spatial and temporal expression of *ZmGrp3* the massively parallel signature sequencing database (MPSS) at DuPont (Wilmington, DE, USA) was surveyed (methods). MPSS profiling is based on the sequencing and deposition of approximately 10^6 short signature sequences per cDNA library (Brenner et al. 2000). Thus, MPSS databases allow for the sensitive detection of gene expression in a wide variety of tissues and developmental stages. The abundance of the *ZmGrp3* transcript was quantified in 42 cDNA libraries, which were all obtained from B73 plants. This extensive gene expression study confirmed that *ZmGrp3* is almost exclusively expressed in root tissues. The relative expression level of *ZmGrp3* is indicated by the number of *ZmGrp3* transcripts among one million transcripts in a defined tissue (ppm). *ZmGrp3* was expressed in root tissues in the range of approximately 50–700 ppm including embryonic roots older than 21 days after pollination. The highest expression in non-root tissue was detected in the coleoptilar node which, however, already started to initiate shoot-borne roots 14 DAG. Only residual (<30 ppm) or no expression was detected in all other non-root tissues (Fig. 3) indicating a very specific expression of *ZmGrp3* in maize roots. For the classification of the developmental stages of maize please refer to: http://www.extension.iastate.edu/pages/hancock/agriculture/corn/corn_develop

The ZmGrp3 gene maps to chromosome 5

Oat–maize addition lines are a valuable tool to map genes to one of the ten maize chromosomes. These lines carry one pair of homologous chromosomes of maize added to the entire chromosome complement of hexaploid oats in a doubled haploid, i.e. a 100% homozygous condition (Kynast et al. 2001). PCR amplification of the *ZmGrp3* gene with genomic DNA isolated from the oat and maize parental control lines and the ten different oat–maize addition lines as template mapped the *ZmGrp3* gene to maize chromosome 5 (Fig. 4).

Development of a novel assay to determine allelic contribution to ZmGrp3 expression in various maize hybrids

In inbred lines of maize two identical alleles of a gene are transcribed. In contrast, in F_1-hybrids most genes

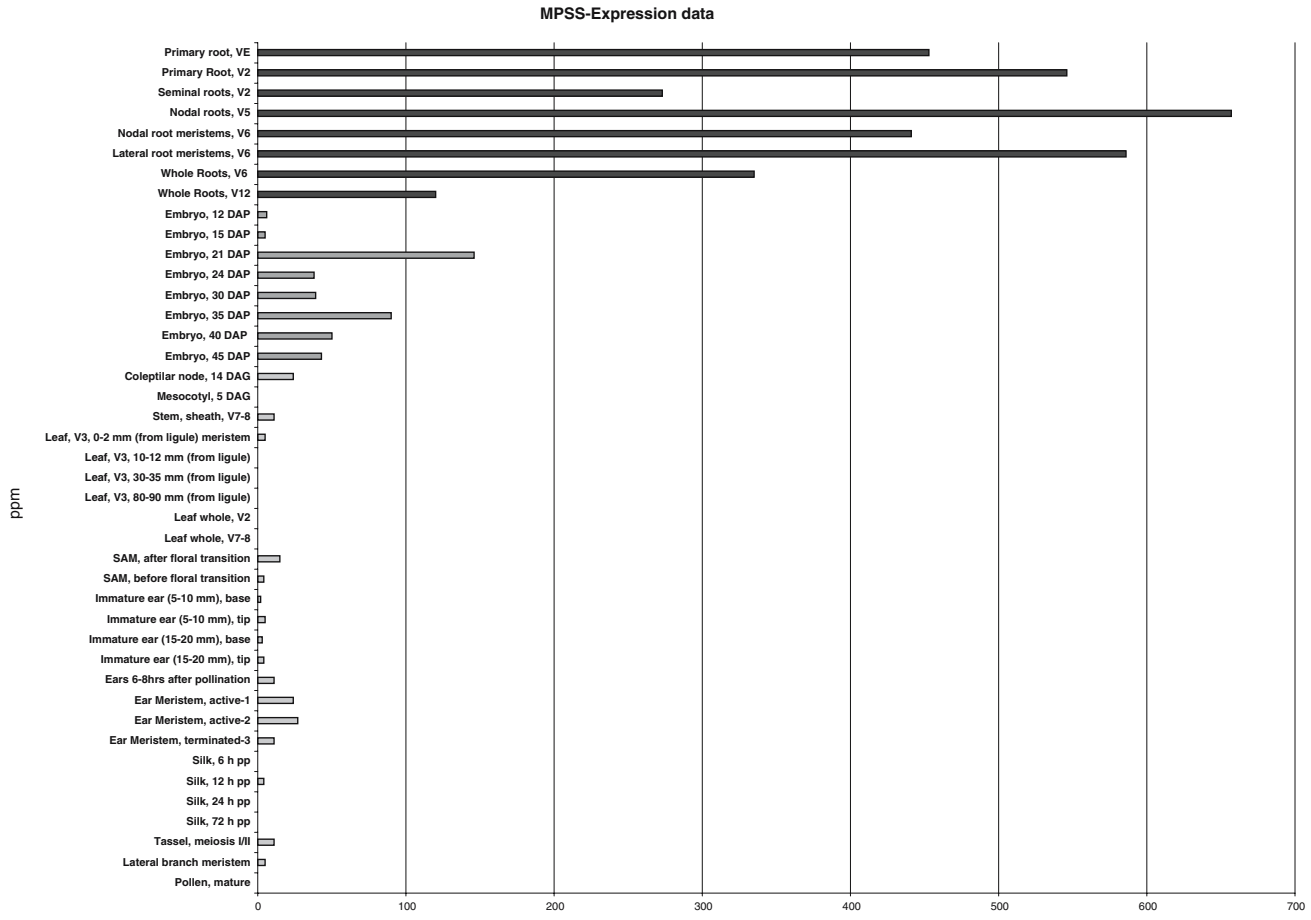

Fig. 3 Relative expression levels (in ppm) of the *ZmGrp3* gene in different B73 tissues and developmental stages obtained via the Lynx–MPSS system. *VE*, *V2–V12* vegetative stages of maize development. *VE* germination and emergence stage, *V2–V12* developmental stages according to the number of leaves, *DAP* days after pollination, *DAG* days after germination, *SAM* shoot apical meristem, *pp* post pollination

are represented by two distinct alleles that could be distinctly transcribed. Thus, in order to complement the expression data of *ZmGrp3* obtained via in situ hybridization, Northern-blot and MPSS experiments in inbred lines, allele-specific contribution to the expression of *ZmGrp3* in maize hybrids were quantified. Currently only little is known about the allelic contribution to gene expression in maize hybrids (Guo et al. 2004). Therefore, a novel approach that allows for the quantification of allelic contribution to gene expression was developed (Fig. 5). This approach combines the RT–PCR amplification of a polymorphic region in cDNA of maize hybrids and the subsequent quantification of the transcripts of two alleles in a didesoxyterminator assay combined with capillary electrophoresis. *ZmGrp3* specific oligonucleotide primers for RT–PCR were designed in conserved regions of the gene that flanked polymorphisms between the different analyzed inbred lines. This secured the amplification of both alleles in a hybrid according to their actual expression ratio. The *ZmGrp3* specific oligonucleotides were tested on cDNA and genomic DNA of inbred lines and hybrids. As expected, amplification of inbred line DNA yielded one peak in capillary electrophoresis (data not shown), while amplification of hybrid DNA always resulted in two peaks representing the two different alleles of a gene in a hybrid. We chose 3.5-day-old primary roots germinated under standardized conditions in distilled water of six reciprocal hybrids, UH002 × UH250, UH250 × UH002, UH002 × UH301, UH301 × UH002, UH250 × UH310 and UH301 × UH250, that were generated from the German inbred lines UH002, UH250 and UH301. At this early developmental stage the primary root has not yet initiated lateral roots hence confining expression of the *ZmGrp3* gene to the primary root tip which is histologically similar between the different hybrids (Hoecker, personal communication). Per

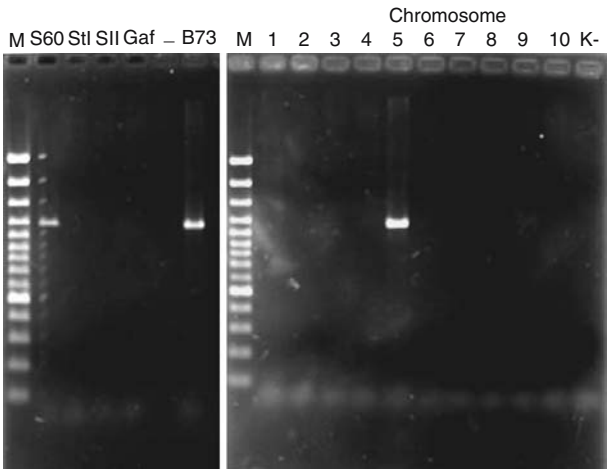

Fig. 4 Chromosomal mapping of the *ZmGrp3* gene via oat–maize addition lines. *Left panel* Control reactions. *ZmGrp3* PCR amplification product is present in the parental maize line Seneca 60, and the control inbred line B73. *Right panel* PCR amplification product is only present in oat–maize addition line where chromosome 5 of maize was subjoined. *M* 100 bp plus ladder (Fermentas), *S60* Seneca 60 (maize parent line), *StI* Starter I, *SII* Sun II, *Gaf* Gaf Park (all parental oat lines), *K*– water control, 1–10 oat–maize addition lines of the respective maize chromosomes

definition DNA of both alleles of a nuclear encoded gene is present in equal quantities in the genome. Therefore, genomic DNA was used as a standard to calibrate the allele-specific gene expression data (methods) and to eliminate dye effects caused by the different fluorescent labeling of the four ddNTPs. That *ZmGrp3* is most likely a nuclear encoded single copy gene has been demonstrated by mapping the gene on chromosome 5. The reliability of the genomic DNA ratios for calibrating the cDNA expression data was demonstrated by low CVs (%) in three independent biological replicates (online Supplement S1). Allele-specific expression data was generated from two independently isolated RNA samples for each hybrid and subsequent synthesis of three independent cDNA samples for each of the two RNA samples. The ratio of the two *ZmGrp3* alleles in the different hybrids determined in 3.5 days old maize seedling roots ranged between 0.9 and 1.38 (Table 1). For statistical data analysis we suggest a mixed model approach that accounts for various additive effects (methods). Data analysis revealed that the ratio of the two alleles was not significantly different from the 1:1 ratio in any of the analyzed hybrids indicating equal contribution of the two alleles to gene expression (Table 1). The most deviant ratio (1.38) was found for UH250 × UH002, with an associated *P* value of 0.19 (Table 1).

Discussion

Despite the availability of a growing number of specific mutants affected in maize root development (Wen and Schnable 1994; Hetz et al. 1996; Hochholdinger and Feix 1998a; Hochholdinger et al. 2001; Woll et al. 2005), little is known about the molecular basis of root formation in maize. Thus far only one gene that leads to a specific root mutant phenotype when disrupted has been cloned (Wen et al. 2005) and a number of genes and proteins differentially expressed in various root genotypes or developmental stages have been identified (e.g. Woll et al. 2005; Hochholdinger et al. 2004c,

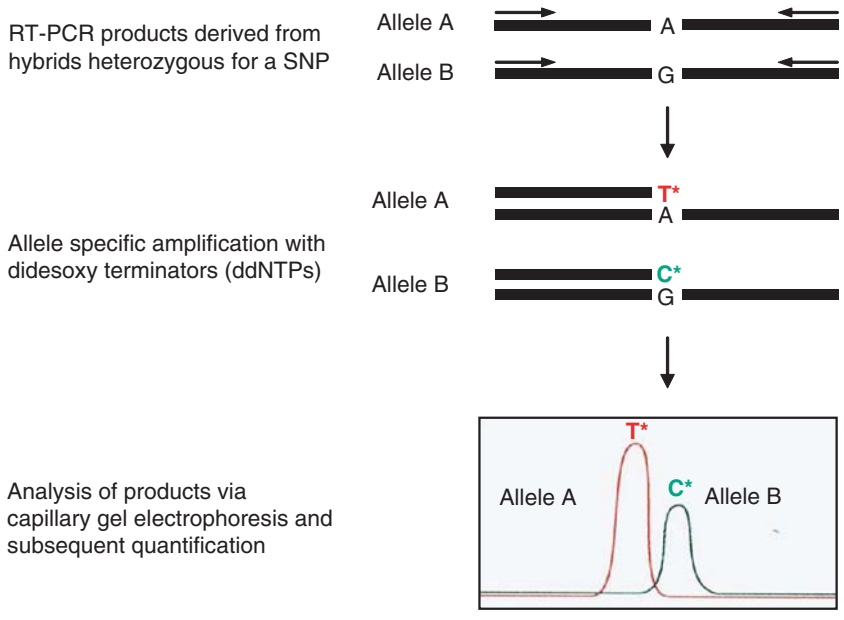

Fig. 5 Principle of the quantification of allele-specific gene expression via a combination of RT–PCR of a polymorphic region between two alleles and subsequent quantification via a didesoxyterminator assay and capillary electrophoresis

Table 1 Estimates of normalized ratio of the relative contribution of different alleles to the expression of *ZmGrp3* (original scale) in maize hybrids and test of null hypothesis ratio equals unity

Hybrid	Normalized ratio allele A:allele B	95% Confidence limits		*df*[a]	*P* value[a,b]
		Lower	Upper		
UH002 × UH301	1.22	0.73	2.02	8.47	0.40
UH301 × UH002	1.14	0.69	1.89	8.47	0.57
UH250 × UH301	1.11	0.67	1.85	8.47	0.64
UH301 × UH250	0.90	0.54	1.50	8.47	0.66
UH002 × UH250	1.07	0.64	1.77	8.47	0.78
UH250 × UH002	1.38	0.83	2.29	8.47	0.19

[a] The Kenward–Roger method was used to approximate the degree of freedom (*df*)

[b] Test of H_{01}: ratio = 1 on original scale based on test of H_{02}: $\mu_{i1} - \mu_{i2} - \phi_{i1} + \phi_{i2} = 0$ on log-scale

2005b; Sauer et al. 2006). Recently, three root-specific glycine-rich, putative cell wall proteins have been cloned in maize and were designated *ZmGrp3* (Goddemeier et al. 1998), *ZmGrp4* (Matsuyama et al. 1999) and *Zm123A* (Ponce et al. 2000). While in situ hybridization experiments demonstrated that *ZmGrp4* (Matsuyama et al. 1999) and *Zm123A* (Ponce et al. 2000) are strongly expressed in the lateral root cap and weakly in the root epidermis, in situ hybridization experiments in this study revealed that *ZmGrp3* also shows specific expression in the epidermis of the root tip and the columella. However, in contrast to *ZmGrp4* (Matsuyama et al. 1999) and *Zm123A* (Ponce et al. 2000), the *ZmGrp3* gene is strongly expressed in the epidermis and only weakly in the columella. The complementary expression patterns of *ZmGrp4* and *Zm123A* versus *ZmGrp3* might imply a subtle cooperative action of these glycine-rich proteins in the development of the maize root tip. In maize so far only a few markers for specific developmental events in roots are available (e.g. Hochholdinger et al. 2000; Lim et al. 2000; Zimmermann and Werr 2005). Thus, *ZmGrp3*, which is expressed only after the initiation of root primordia as demonstrated with the lateral and shoot-borne root initiation mutants *lrt1* (Hochholdinger and Feix 1998a) and *rtcs* (Hetz et al. 1996) is a novel marker for root initiation in maize. Interestingly, the *ZmGrp3* gene not only reveals a root tip specific expression pattern, but its expression is almost exclusively confined to roots as confirmed via Lynx-MPSS analyses. Thus, the promoter of *ZmGrp3* could be a valuable vehicle for the specific expression of genes of agronomic relevance in roots. Allele-specific analysis of gene expression in maize became available only recently. Guo et al. (2004) introduced a novel approach that combined RT–PCR with WAVE dHPLC chromatography. Since WAVE dHPLC is not available in every institution we suggest an alternative approach that is based on an allele-specific didesoxyterminator assay and subsequent transcript quantification via a capillary sequencer. The accuracy of this assay was tested by analyzing the allelic ratio of genomic DNA of hybrids in which both alleles of the nuclear *ZmGrp3* gene should be present in equimolar amounts. Since dye effects of the fluorescent ddNTP labels cannot be excluded, the ratios obtained from genomic DNA were used to calibrate the allelic ratios of the *ZmGrp3* transcripts. The robustness of this calibration approach was demonstrated by low CV (%) values in three independent biological replicates (see online Supplement S1). We introduced a mixed model data analysis approach which considered all relevant error effects on gene expression. This is important since simple *t* testing tends to overestimate expression differences which do not reflect actual biological differences but rather natural variation. Specifically, it is necessary to account for errors in estimates of the normalized genomic DNA ratio. For example, the combination UH250 × UH002 with an allelic ratio of 1.38 was significantly different from 1 in a simple *t* test ($P < 0.05$), while no allelic gene expression difference was detected when analyzing this dataset with our mixed model approach. Techniques that allow for the quantification of allele-specific gene expression differences will become more important in the future since allelic diversity is a crucial component for phenotypic variation (Buckler and Thornsberry 2002). The phenomenon of heterosis which describes the superior yield or vigor of highly heterozygous hybrid plants over homozygous plants can also be attributed to gene expression differences caused by the presence of different alleles in many loci of the maize plant (Birchler et al. 2003). Maize, in particular, is a suitable model for such analyses since it displays a high degree of heterosis and also a high degree of sequence polymorphisms between different genetic backgrounds (Buckler and Thornsberry 2002). Recently, it has been demonstrated that the phenomenon of heterosis can already be detected during early

root development in maize hybrids (Hoecker et al. 2006). Thus, the assay developed in this study for the detection of allele-specific gene expression might be helpful in the future to analyze candidate genes for heterosis. Mapping of the *ZmGrp3* gene to chromosome 5 and thus the demonstration that it is a nuclear encoded gene was instrumental since one prerequisite for using genomic DNA for calibration purposes was that both alleles of *ZmGrp3* were present in a 1:1 ratio. Further, fine mapping of *ZmGrp3* on chromosome 5 might help to correlate this gene with root related QTLs that become more and more important to dissect root traits of maize that are controlled by various interacting loci (Salvi and Tuberosa 2005).

In summary, *ZmGrp3* represents a novel root tip specific marker of root initiation in maize which will be helpful for the further molecular dissection of root development in maize. Moreover, almost exclusive expression of *ZmGrp3* in roots makes its promoter a potential vehicle for the specific expression of transgenes with agronomic relevance in roots. Last but not the least, a novel approach to quantify allele-specific gene expression is presented that might be helpful for the allele-specific gene expression analysis of candidate genes that are related to the phenomenon of heterosis.

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