Erratum

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We apologise to the authors and readers for this mistake.

Functional diversification of *MYB23* and *GL1* genes in trichome morphogenesis and initiation

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Summary

The functional diversification of duplicated genes is one of the driving forces in evolution. To understand the molecular mechanisms of gene diversification, we studied the functional relationship of the two *Arabidopsis* paralogous MYB-related genes *GL1* and *MYB23*. We show that *MYB23* controls trichome branching and trichome initiation at leaf edges. The latter is controlled redundantly together with *GL1*. We show that the two proteins are functionally equivalent during trichome initiation but not during trichome branching. RT-PCR and reporter construct analysis revealed spatial, temporal and genetic differences in transcriptional regulation of the *GL1* and *MYB23* genes. Presented data indicate that the diversification of *GL1* and *MYB23* gene functions occurred at the level of cis-regulatory sequences with respect to trichome initiation, and that, in parallel, the diversification with respect to regulation of trichome branching also involved changes in respective proteins.

Key words: Trichomes, Cell shape, Redundancy, GLABRA1 (GL1)

Introduction

One important evolutionary mechanism that creates new gene functions is the duplication and functional diversification of genes. In principle, diversification of duplicated genes can take place at the level of transcriptional regulation or by a diversification of protein function. The relative contribution of these two possibilities to diversification of duplicated genes is an important issue in evolutional biology.

A particularly attractive solution to addressing the mechanisms of gene diversification in evolution is provided by the large family of MYB-related R2R3 transcription factors in plants (Jin and Martin, 1999; Stracke et al., 2001). It is estimated that this family has rapidly evolved within the last 500 million years after the divergence of the vascular plants from the bryophytes (Rabinowicz et al., 1999). It has been suggested that the amplification of the R2R3 MYB genes occurred in conjunction with the development of new plant-specific cellular functions (Martin and Paz-Ares, 1997) and that the role of MYB genes is to provide plasticity to plant metabolism and development (Romero et al., 1998). This view is supported by the findings that members of the R2R3 MYB-family are involved in many different biological processes, including the trichome and root-hair differentiation (Lee and Schiefelbein, 1999; Oppenheimer et al., 1991), cell-shape determination (Noda et al., 1994), regulation of leaf form (Waites et al., 1998), control of secondary metabolism (Mol et al., 1998), pathogen response (Yang and Klessig, 1996), drought stress response (Urao et al., 1993), protection from UV radiation (Jin et al., 2000) and hormone signalling (Gubler et al., 1995).

A particularly well-suited model for studying the diversification of duplicated genes is the sub-class of the three paralogous R2R3 MYB genes, *WEREWOLF* (*WER*), *GLABRA1* (*GL1*) and *AtMYB23 (MYB23)*. *WER* and *GL1* act in two different functionally non-overlapping developmental processes. *WER* is important for root-hair development and *GL1* for trichome development on aerial organs (Lee and Schiefelbein, 1999; Oppenheimer et al., 1991). WER and GL1 proteins with 57% of sequence identity are functionally equivalent and changes in the cis-regulatory sequences completely account for the functional diversification of the two proteins (Lee and Schiefelbein, 2001). Overexpression studies and the analysis of the expression pattern suggested that *MYB23* could have a similar function to *GL1* (Kirik et al., 2001). The identification of two *myb23* mutants enabled us now to study the function of *MYB23* and its functional relationship to *GL1*. The *myb23* single mutants exhibit reduced trichome branching but no obvious effect in trichome initiation. The *gl1 myb23* double mutants, however, are devoid of trichomes at the leaf edges, which are not effected in the *gl1* single mutants indicating a functional redundancy of *GL1* and *MYB23*. Promoter and protein-coding region swap experiments showed that the two proteins are functionally equivalent with respect to the regulation of trichome initiation, but not with respect to trichome branching. This indicates that changes in the regulation of trichome initiation are evolved at the level of

the *cis*-regulatory regions and that diversification with respect to the regulation of branching evolved at the level of cis-regulatory regions, as well as at the level of altered protein function.

Materials and methods

Plant materials and growth conditions

Plants were grown at 24°C with 16 hours of light per day. The isolation of the *gl1*, *ttg1*, *gl2*, *gl3, cpc* and *try* mutant alleles used in this study has been described: *gl1-1* and *gl3-1* (in Landsberg background) (Koornneef et al., 1982); *ttg1-1* (in Landsberg background) (Koornneef, 1981); *cpc-1* (in Wassilewskija background) (Wada et al., 1997); and *try-JC* (in the Columbia background) (Larkin et al., 1999). *gl2-4AA* allele was kindly provided by ZIGIA, MPIZ Cologne. The *myb23-1* mutant was isolated from the SALK T-DNA collection (Columbia ecotype), the *myb23-2* mutant was isolated from a Wisconsin T-DNA population (Wassilewskija ecotype). Lines homozygous for multiple mutations and/or transgenes were constructed by crossing single mutant or transgenic plants, examining the F2 progeny for putative mutant phenotypes, and confirming the desired genotype in subsequent generations by backcrossing to single mutants and/or PCR-based tests.

Microscopy

Trichomes were analyzed on the first two leaves of soil-grown plants.

The histochemical analysis of plants containing the *GUS* reporter constructs was performed essentially as described previously (Vroemen et al., 1996).

The DISCUS software package (Carl H. Hilgers-Technisches Büro, Königswinter, Germany) was used to measure the fluorescence intensity of DAPI-stained nuclei. The relative fluorescence units (RFU) were calibrated with wild type trichome nuclei that were previously reported to have an average of 32C (Schnittger et al., 1998; Szymanski and Marks, 1998). By defining the average fluorescence intensity of the wild-type trichomes as 32 RFU our RFU values should roughly correspond to C values (Walker et al., 2000). As a control for this procedure, *gl3* (16C) and *try* (64C) mutants were included.

Molecular biology methods

RNA was isolated from rosettes of 2-week-old plants and subjected to RT-PCR, which was essentially performed as described previously (Kirik et al., 2002). *MYB23* gene-specific primers were used for RT-PCR (do-s2, 5′-AGAAGAATGAGAATGACAAGAG; and do-1, 5′-TACGTCAGTTGGTGTTGCGTGGAC). Amplifications of the translation elongation factor EF1αA4 cDNA (primers EF1α-UP: 5′-ATGCCCCAGGACATCGTGATTTCAT and EF1α-RP: 5′-TTGG-CGGCACCCTTAGCTGGATCA) were used as a control.

The *MYB23::cMYB23* was constructed by fusing the 2032 bp 5′ regulatory region (Kirik et al., 2001) with the *MYB23* cDNA and cloning the fusion in the pGPTV-BAR vector (Becker et al., 1992).

The *GL1* regulatory sequences used in these experiments are identical to the ones published previously (Lee and Schiefelbein, 2001) and include a 1.4 kb 5″ fragment and a 1.8 kb 3′ fragment. The *MYB23* regulatory sequences include a 3.1 kb 5′ fragment and a 1.0 kb 3′ fragment. Constructs encoding the GL1 and MYB23 proteins include the entire transcriptional unit of each gene from the start to the stop codons.

In the *GL2::GL3* construct a 2.1 kb 5′ *GL2* regulatory region (Szymanski et al., 1998) drives the expression of the full-length *GL3* cDNA. Details of the transgene constructs are available upon request.

In situ RNA hybridization

RNA in situ hybridisation was essentially performed as described previously (Larkin et al., 1993). Antisense and sense strand digoxigenin-labelled RNA probes were derived from the 450 bp 3′ fragment of the MYB23 cDNA that includes 180 bp of the 3′ UTR. This cDNA region does not comprise the conserved MYB-domain coding sequence and corresponds to the region with the lowest sequence similarity to other related MYB genes.

Yeast two-hybrid assay

Fusions with the GAL4 activation domain and GAL4 DNA-binding domain were performed in the pACT and pAS plasmids (Clontech). TRY, GL3 and N-terminal truncation of GL3 (GL3-96 aa) were fused to the GAL4 activation domain in the pACT plasmid. For the GL1 and MYB23 fusions with GAL4 DNA-binding domain in the pAS vector, we used truncated fragments missing 27 amino acids and 25 amino acids at the C terminus respectively. All used constructs and empty vectors did not show any self activation in yeasts. The interaction strength was determined by measuring the activity of the *lacZ* reporter gene using the ONPG assay (Clontech).

Results

The *myb23* mutant has a trichome morphogenesis defect

Previous studies have indicated that the *MYB23* gene is involved in the regulation of trichome development (Kirik et al., 2001). In order to study the function of the *MYB23* gene, we have screened the available T-DNA insertion lines. Two alleles of the *myb23* mutant were isolated. The *myb23-1* allele was isolated from the SALK T-DNA collection (Columbia ecotype) and has an insertion in the second intron of the gene (Fig. 1A). The *myb23-2* allele was isolated from the Wisconsin T-DNA insertion library (Wassilewskija ecotype) and has a T-DNA insertion 4 bp after the stop codon. RT-PCR analysis revealed that the *MYB23* expression is not detectable in the *myb23-1* allele and it is strongly reduced in the *myb23-2* allele (Fig. 1B).

When compared with the corresponding wild types the *myb23-1* and *myb23-2* alleles displayed reduced trichome branching (Fig. 1C,D; Table 1). To prove that this phenotype is specific for the mutations in *MYB23* we transformed *myb23-1* plants with the *MYB23::MYB23* construct containing the MYB23 protein-coding region, placed under the control of a 3.1 kb fragment 5′ to a start codon and a 1.0 kb fragment 3′ to the stop codon. This construct rescued the number of trichome branches to wild-type levels showing that the *MYB23* gene is required for proper trichome branching (Table 1).

MYB23 and *GL1* redundantly control trichome initiation at leaf edges

As overexpression of the *MYB23* gene causes ectopic trichome initiation (Kirik et al., 2001), we also analyzed epidermal patterning in shoots. Both *myb23-1* and *myb23-2* alleles displayed wild-type pattern of the trichomes, indicating that the *MYB23* gene does not have an essential function in trichome patterning.

The MYB23 and GL1 proteins share 63% of identical amino acid residues, which suggests that these two genes may have redundant or overlapping functions (Kirik et al., 2001). Plants that harbour a null *gl1-1* mutant allele, resulted from a deletion of the entire GL1 protein-coding region, are not completely glabrous, a small number of trichomes develop at the edges of late rosette leaves and petioles (Koornneef et al., 1982; Oppenheimer et al., 1991) (Fig. 2A). We did not find any changes in trichome production at the leaf edges of the *myb23* mutants (data not shown). To test whether *MYB23* function at

the edges of leaves and petioles is masked by genetic redundancy, we created the *gl1 myb23-2* double mutant. The double mutant plants were completely glabrous (Fig. 2A). The introduction of the *MYB23::MYB23* construct in the double mutant resulted in a rescue of the leaf edge and petiole trichomes (Fig. 2B). Thus, the *gl1 myb23-2* double mutant uncovers the redundancy of *MYB23* and *GL1* in trichome initiation at the edges and petioles of the rosette leaves.

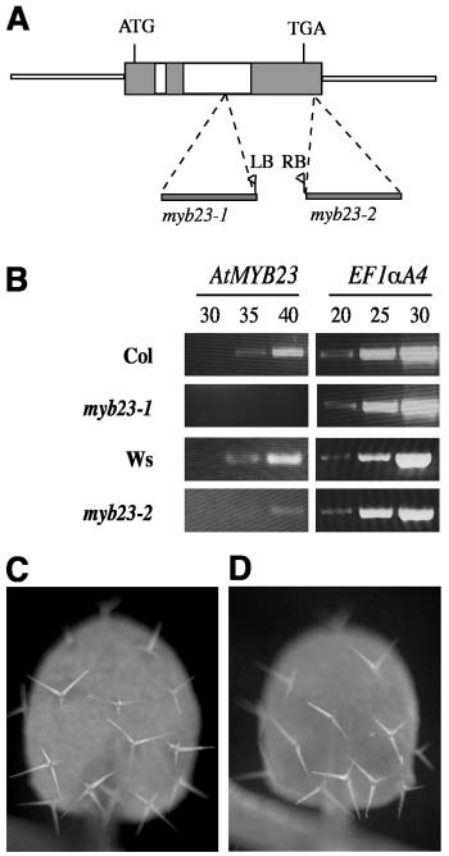

Fig. 1. Molecular analysis and trichome phenotype of *myb23* alleles. (A) Schematic illustration of the T-DNA insertions positions in the *myb23-1* and *myb23-2* alleles. (B) RT-PCR was conducted using *MYB23* gene-specific primers for 30, 35 and 40 cycles. After 40 cycles, a weak band is visible in the *myb23-2* mutant. Control RT-PCR was carried out with primers specific for the translation elongation factor *EF1αA4*. (C) Three- and four-branched trichomes on wild-type (Col) leaf. (D) Two- and three-branched trichomes on the *myb23-1* leaf.

MYB23 and GL1 proteins are functionally equivalent during trichome initiation

High sequence similarity of the *MYB23* and *GL1* genes and similar expression patterns (Kirik et al., 2001) raised the question of why *gl1* mutants are not rescued by the wild-type copy of *MYB23*. To what extend are differences in the transcriptional control or in the protein functions responsible for the redundancy? To address these questions, we exchanged the regulatory and protein-coding regions of the *GL1* and *MYB23* genes.

The finding that the above-described *MYB23::MYB23* construct rescues the *myb23* mutant phenotype indicates that the 3.1 kb of 5′ regulatory sequence and 1 kb of 3′ sequence contain all regulatory sequences necessary for normal *MYB23* gene function. For the *GL1* gene, we used 1.4 kb of the 5′ regulatory region and 1.8 kb of the 3′ region that was previously used in a complementation construct (Lee and Schiefelbein, 2001). As a reference for the subsequent experiments, the *GL1::GL1* construct and the *MYB23::MYB23* constructs were introduced into the *gl1* and the *gl1 myb23-2* backgrounds. Whereas the *GL1::GL1* construct rescued completely the *gl1* mutant phenotype, the *MYB23::MYB23* construct showed no effect in *gl1* mutants but induced leaf edge and petiole trichomes development in the double mutant (Fig. 2B). To test whether MYB23 protein is functionally equivalent to the GL1 protein, we transformed *gl1* mutant and the *gl1 myb23-2* double mutant with the *GL1::MYB23* and the *MYB23::GL1* constructs. The *GL1::MYB23 gl1* and *GL1::MYB23 gl1 myb23-2* transgenic plants showed a wild-type trichome number and pattern (Fig. 2B), indicating that the MYB23 protein posses the same biochemical activities necessary for trichome initiation as the GL1 protein. No effect of the *MYB23:GL1* construct was found in *gl1* plants but *gl1 myb23-2* plants were rescued back to the *gl1* phenotype (Fig. 2B). Together, these data demonstrate that during

Table 1. Trichome branching in wild-type, mutants and transgenic *Arabidopsis* lines

	Trichome branch points*									
Genotype (ecotype)	0	1	2	3	4	5	6	7	8	Number†
Wild type (Ws)	0	30	70	0	0	0	0	0	0	659
myb23-2	2	82	16	0	0	0	0	0	0	600
Wild type (Col)	0	0	84	16	0	0	0	0	0	497
myb23-1	2	35	63	0	0	0	0	0	0	532
MYB23::MYB23 myb23-1	0	0	87	12	1	0	0	0	0	603
MYB23::GL1 myb23-1	0	19	71	10	0	0	0	0	0	500
GL1::MYB23 myb23-1	3	40	56	1	0	0	0	0	0	700
GL1::GL1 myb23-1	3	38	59	0	0	0	0	0	0	232
try	0	0	2	23	46	25	3	1	0	290
try myb23-1	0	14	57	25	4	0	0	0	0	410
gl3	50	50	0	0	0	0	0	0	0	151
gl3 myb23-2	54	46	0	0	0	0	0	0	0	136
GL2::GL3	0	0	5	17	34	25	11	5	1	263
GL2::MYB23	0	1	77	22	0	0	0	0	0	295

*Percentage of trichomes with the indicated number of branch points on the first pair of leaves (one branch point indicates a trichome with two branches). For *GL2::GL3* and *GL2::MYB23* data were collected from 12 and 11 independent transgenic lines, respectively.

†Number of trichomes counted for each strain.

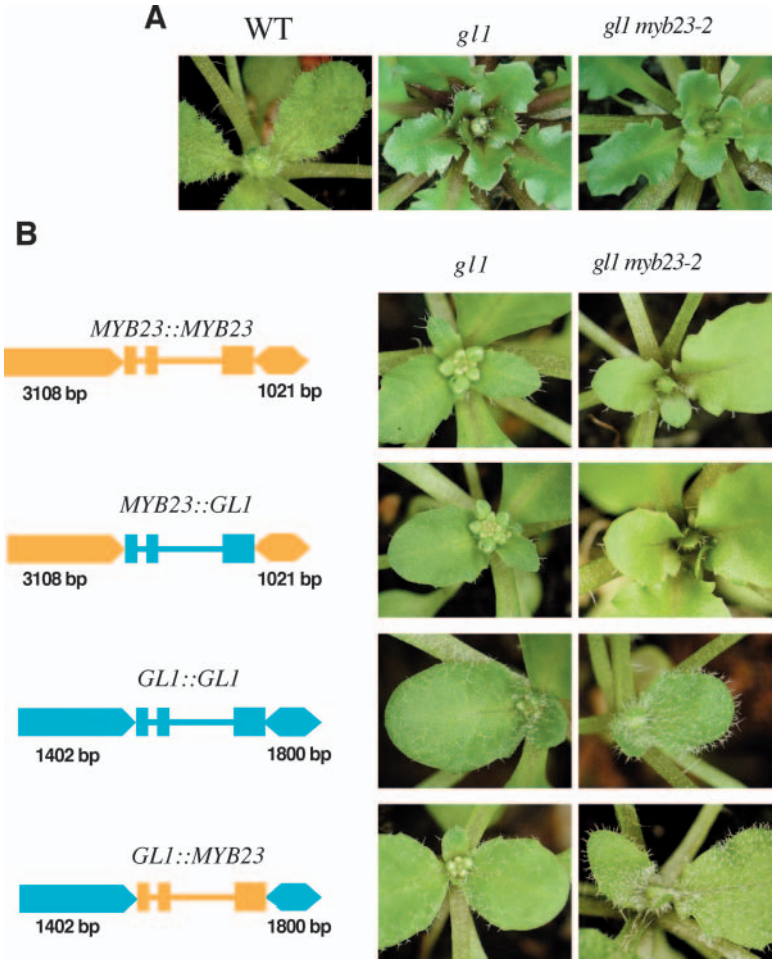

Fig. 2. MYB23 and GL1 proteins are functionally equivalent during trichome initiation. (A) Redundant function of *MYB23* and *GL1* during trichome initiation. Trichomes at the leaf edges and petioles present in *gl1* are absent in the *gl1 myb23-2* double mutant. (B) Cis-regulatory sequences, but not protein-coding regions, specify the functions of the *MYB23* and *GL1* genes in trichome initiation. Schematic presentations of the constructs used for rescue experiment are depicted on the left. Nucleotide numbers indicate the length of the used 5′ and 3′ flanking sequences before the start and after the stop codon respectively. Phenotypes of the *gl1* mutant and *gl1 myb23-2* double mutant transformed with the corresponding construct are shown on the right.

trichome initiation the GL1 and MYB23 proteins are functionally interchangeable and difference in function of these genes during trichome patterning is due to differences in the transcriptional regulation.

Diversification of the *MYB23* and *GL1* genes with respect to branch regulation occurs at both the transcriptional regulation level and protein function

Reduced trichome branching of the *myb23* mutants demonstrates that *MYB23* function is necessary for the proper trichome cell morphogenesis. Can GL1 protein substitute this morphogenetic function of the MYB23 protein? We tested this by introducing the *MYB23::GL1* construct into *myb23-1* plants. The resulting transgenic plants showed only weak rescue of the branching phenotype (Table 1). As *MYB23::MYB23* showed full rescue of trichome branching it is conceivable that GL1 protein is less active than MYB23 in promoting trichome branching.

To study the importance of the transcriptional regulation in functional specification of the *GL1* and *MYB23* genes, we introduced the *GL1::MYB23* construct into the *myb23-1* mutant background. These plants displayed no rescue of trichome branching (Table 1), indicating that transcriptional control is pertinent for the regulation of branching by *MYB23*. Taken together, the functional diversification of the *MYB23* and *GL1* genes with respect to branching occurs at the level of both the transcriptional regulation and protein function.

Differences in the transcriptional regulation of *MYB23* and *GL1* during leaf development

Although the initial studies of the expression of *MYB23* suggested that it is expressed similarly as *GL1* (Kirik et al., 2001), the above-described studies suggest differences in transcriptional regulation during early stages of trichome development.

To follow the expression of *GL1* and *MYB23* during trichome development, we used *GL1::GUS* and *MYB23::GUS* reporter lines (Kirik et al., 2001; Larkin et al., 1993). The *GL1::GUS* reporter line was shown previously to adequately reflect the transcription of the *GL1* gene (Larkin et al., 1993). For *MYB23*, we narrowed the relevant promoter region down to a 1.9 kb region that was previously used to analyze the expression of the *AtMYB23* gene (Kirik et al., 2001). We used this fragment to drive the expression of the *MYB23* cDNA and found that this fragment is sufficient to rescue the *myb23* mutant phenotype in the *gl1 myb23* background (data not shown). This shows that the *MYB23::GUS* reporter provides a pertinent proxy of the *MYB23* gene expression.

A comparison of the reporter expression *MYB23::GUS* and *GL1::GUS* in wild type revealed marked differences in the spatial and temporal pattern. Initially, *GL1::GUS* is expressed ubiquitously in the young leaf primordia and becomes more prominent in developing trichome cells (Fig. 3B) (Larkin et al., 1993). This expression ceases when trichomes begin to initiate branches. By contrast, using the same staining conditions we did not find a ubiquitous expression of *MYB23::GUS* in young leaf primordia. Expression is confined to developing trichome cells where it persists at high levels throughout all stages of trichome development (Fig. 3B).

To further analyze the transcriptional regulation of the *GL1* and *MYB23* genes, we compared their expression in different mutants affected in trichome initiation (Hulskamp et al., 1999; Szymanski et al., 2000). In *try* and *cpc* mutants, which display increased trichome nest frequency and trichome number, respectively, we did not detect expression changes using RT-PCR (Fig. 3A). In *gl1* and *ttg1* mutants, which show strongly reduced trichome production, *MYB23* mRNA was not detectable. In *gl3* mutants, which have less trichomes compared with wild type, we found that amount of the *MYB23* RNA was strongly reduced (Fig. 3A). By contrast, *GL1* expression was not changed in *ttg1* and *gl3* mutants. These results show that *MYB23* expression correlates with the

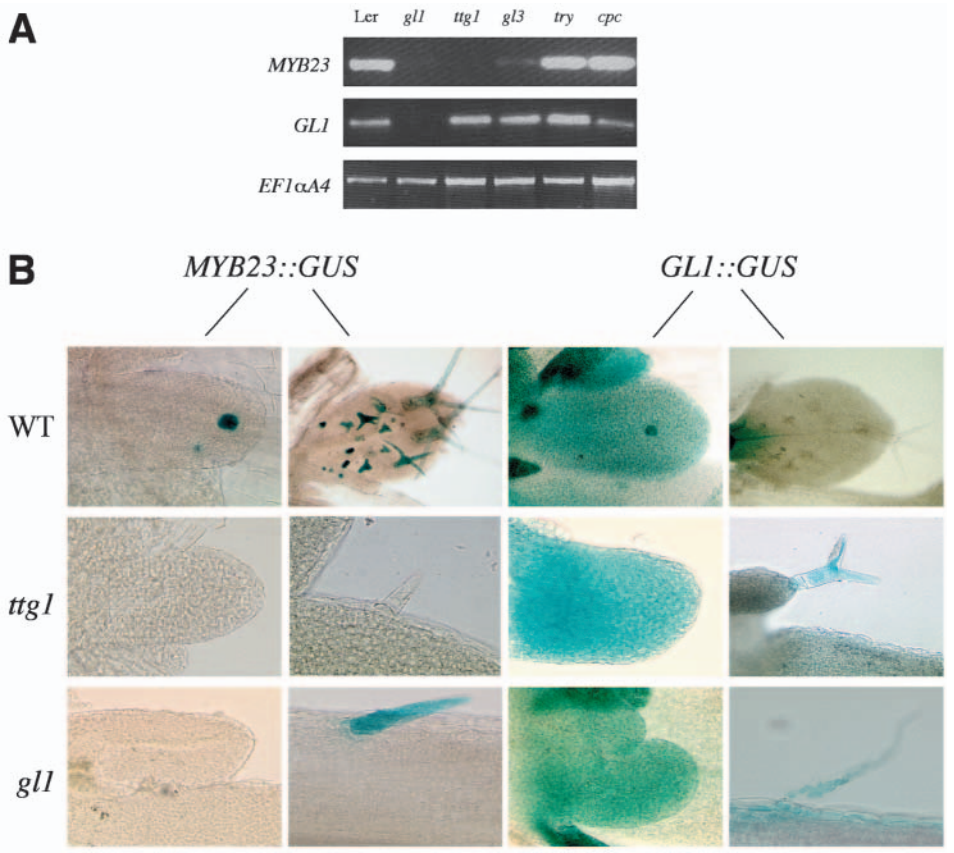

Fig. 3. Comparison of the *MYB23* and *GL1* expression during leaf development. (A) RT-PCR using *MYB23* gene-specific primers (35 cycles, top row) or using *GL1* gene-specific primers (35 cycles, middle row). RT-PCR using primers for translation elongation factor *EF1αA4* (20 cycles) were used as a cDNA control. The *gl1-1* allele has a deletion of the entire protein-coding region, resulting in the absence of *GL1* mRNA (Oppenheimer et al., 1991). (B) Comparison of *MYB23::GUS* and *GL1::GUS* expression (blue) in leaves of wild type (WT), *ttg1* and *gl1* mutants.

presence of trichomes on the leaves whereas *GL1* expression is independent of them.

Comparable results were obtained using the respective promoter::GUS fusions. The ubiquitous expression of the *GL1::GUS* reporter is not affected in *ttg1* and *gl1* mutants (Fig. 3B). Expression of both the *GL1::GUS* and *MYB23::GUS* reporters was not affected in the *gl3* mutant (data not shown), suggesting that reduced expression of *MYB23* detected by RT-PCR is due to reduced number of trichomes in this mutant. As *MYB23* is not initially ubiquitously expressed, we focused on the rare trichomes that occasionally formed at the leaf edges of *gl1* and *ttg1* mutants. In the *gl1* mutant, both reporter constructs were active in trichomes. Rare trichomes in the *ttg1* mutant showed expression of the *GL1::GUS* but not *MYB23::GUS* reporter, indicating that *MYB23* gene transcription depends on *TTG1* activity, whereas the *GL1* gene transcription is not *TTG1* dependent.

To verify the expression pattern of the *MYB23::GUS* reporter, we localized MYB23 transcript by in situ hybridization (Fig. 4). We detected a strong hybridization signal in developing trichomes of young leaf primordia (Fig. 4A) that was also persistent in the older trichomes (Fig. 4B). In contrast to the *GL1* mRNA in situ localization reported by Larkin et al. (Larkin et al., 1993), we did not find any epidermis-specific accumulation of the *MYB23* transcript, which is not confined to developing trichome precursors.

Genetic interactions of *MYB23* with other trichome genes

To further dissect the function of *MYB23* in trichome development, we created double mutants of *myb23* with *gl2*, *try*, *gl3* and *cpc*.

In *gl2-4AA* mutants, the majority of trichomes fail to grow out and only trichomes at the leaf edges develop and branch. In the *gl2-4AA myb23-2* double mutant, leaf edge trichomes do not grow out (Fig. 5H,I). Thus *myb23* mutants enhance the morphogenesis defect in the *gl2* mutant. Conversely, overexpression of *MYB23* in trichomes using strong *GL2* promoter partially rescued the *gl2* mutant phenotype (Fig. 5J-M). This result was surprising as *GL2* is thought to act downstream of *GL1* and therefore was expected to also act downstream of *MYB23*. Both observations together suggest that *MYB23* has a function in trichome cell morphogenesis at the same genetic level as *GL2*.

The *try myb23-1* double mutant showed an intermediate branching phenotype. When compared with *try* (average 5.0 branches) and *myb23-1* (average 2.6 branches) mutants, the double mutant exhibits in average 3.4 branches (Fig. 5E,F; Table 1). As the increased trichome branching in *try* mutants is correlated with an increased ploidy levels (64C DNA content versus 32C in wild type) (Hülskamp et al., 1994) (Fig. 6), we analyzed whether *MYB23* function is also required for the extra DNA endoreduplication rounds in the *try* trichomes. Both *myb23* alleles showed ploidy levels similar to wild type (Fig. 6). However, the ploidy level of the *try myb23-1* double mutant displayed an intermediate value (average is 44C) and differed significantly ($P<0.001$) from the *try* mutant (average is 67C) and from the *myb23* mutant ($P<0.005$; average is 34C). Thus, although the single mutant phenotypes suggest that regulation of branch number by the *MYB23* gene is independent from the regulation of DNA endoreduplication, the *try myb23* double mutant phenotype revealed that *MYB23* function becomes limiting in the regulation of DNA endoreduplication when nuclei undergo additional endoreduplication cycles.

The same two processes affected in the *try* mutant – cell morphogenesis and DNA endoreduplication in trichomes – are also impaired in the *gl3* mutant. *gl3* trichomes are smaller, underbranched and have reduced nuclear DNA content (Hülskamp et al., 1994). The *gl3* branching phenotype appears to be epistatic in the *gl3 myb23-2* double mutant (Fig. 5B,C;

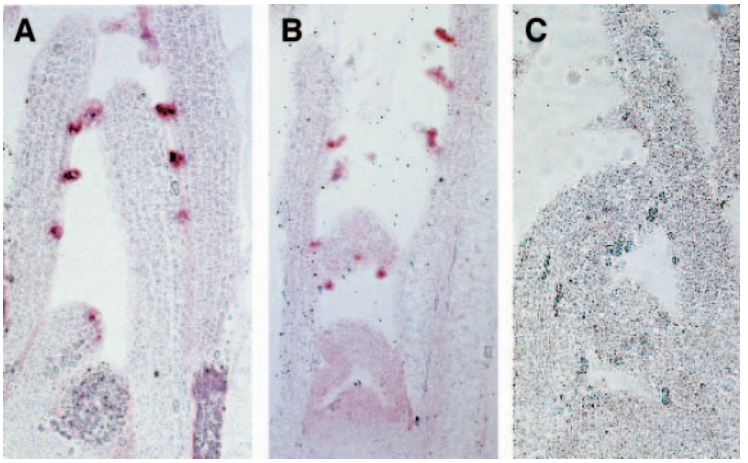

Fig. 4. Distribution of the *MYB23* mRNA in the shoot. (A,B) Longitudinal section through a vegetative apex hybridized with a *MYB23*-specific antisense probe. (C) Control hybridization with the labelled *MYB23* sense strand probe.

Table 1), suggesting that the *MYB23* and *GL3* may genes act in the same pathway.

To further elucidate the role of *MYB23* and *GL3* in trichome branch formation, we overexpressed the two genes under the control of the *GL2* promoter, which drives a strong trichome-specific expression in leaves (Szymanski et al., 1998). Plants transformed with the *GL2::GL3* construct exhibited enlarged, strongly over-branched trichomes with up to 10 branches (Fig. 5G; Table 1). The trichome nuclear size was drastically increased (Fig. 5G) with a ploidy level of on average 110C, suggesting that those nuclei have undergone six endoreduplication cycles, two more than wild-type trichome nuclei (Fig. 6).

Arabidopsis plants transformed with the *GL2::MYB23* construct did not exhibit any changes in trichome size, branching and DNA endoreduplication level (average is 31.6 C; n=66), indicating that the wild-type level of MYB23 is not a limiting factor for endoreduplication cycles and branch initiation in wild-type plants. To test whether the inhibitory activity of *TRY* may suppress the possible effect of the *GL2::MYB23* construct, we introduced this construct in the *try* mutant background. Overexpression of *MYB23* under the *GL2* promoter in *try* background resulted in ectopic trichome development but did not have any significant effect on the trichome branching and DNA content (*try GL2::MYB23*: 57C, n=64; *try*: 64C, n=65; Student's t-test: P=0.16).

Taken together, our data show that *MYB23* is required for proper branch formation but, in contrast to *GL3*, increased MYB23 levels do not trigger additional branch formation. This suggests that the MYB23 concentration in trichomes is not limiting for induction of trichome branches.

Protein-protein interactions of MYB23 with other trichome patterning proteins

The functional equivalence of the GL1 and MYB23 proteins during trichome initiation suggests that these proteins have similar biochemical properties. The GL1 protein directly interacts with GL3, GL3 binds to the WD-40 protein TTG1 but GL1 does not interact with TTG1. It has been suggested that these three proteins form the activator complex (Payne et al., 2000; Szymanski et al., 2000). To test whether MYB23 interacts with the GL3 protein, we made fusion constructs of the *GL3* cDNA with the GAL4 activation domain (AD) and *MYB23* cDNA with the GAL4 DNA binding domain (DB). Yeast two hybrid assays revealed an interaction between GL3 and MYB23 (Fig.

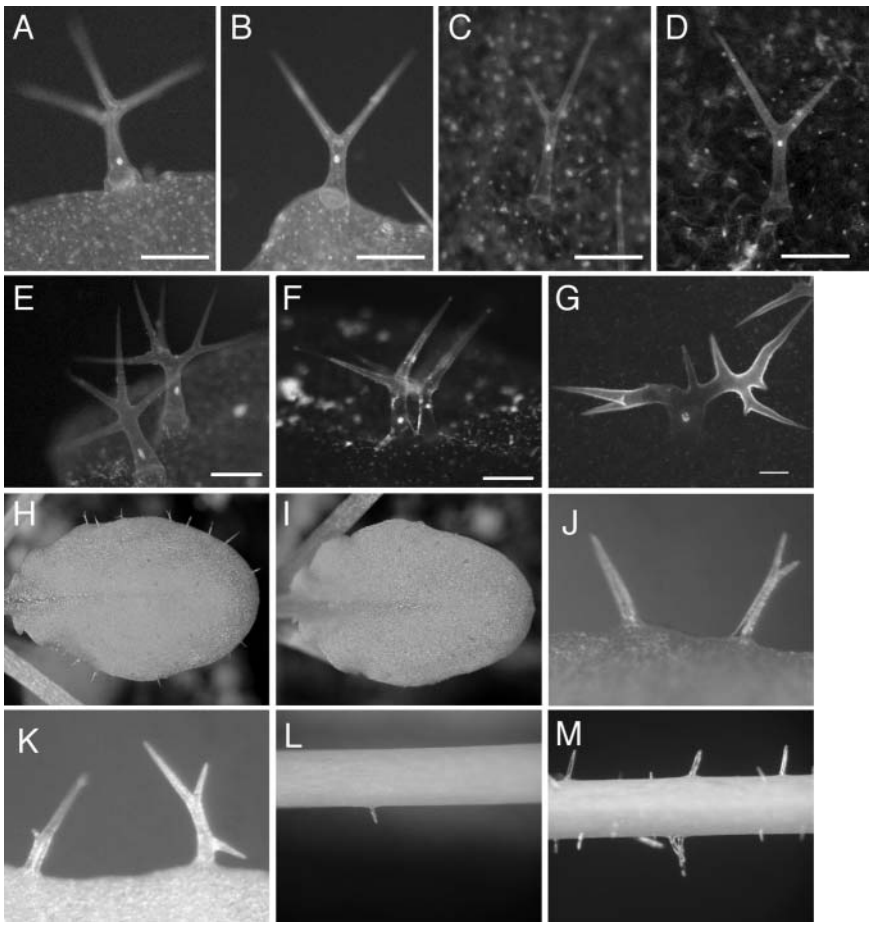

Fig. 5. Genetic analysis of the *MYB23* gene functions during trichome differentiation. (A-G) DAPI-stained trichomes on the leaves of wild-type (Wassilewskija) (A), *myb23-2* (B), *gl3* (C), *myb23-2 gl3* (D), *try* (E), *try myb23-1* (F) and *GL2::GL3* (G) plants. (H) Trichomes on the *gl2* mutant leaf surface. (I) Trichomes on the *gl2 myb23-2* leaf. There is no trichome outgrowth at the leaf edge. (J) Trichomes at the leaf edges of the *gl2*. (K) Trichome at the leaf edges of *GL2::MYB23 gl2* plants. (L) *gl2* stem trichomes. (M) Stem trichomes in *GL2::MYB23 gl2* plants. Scale bars: 100 μm in A-G.

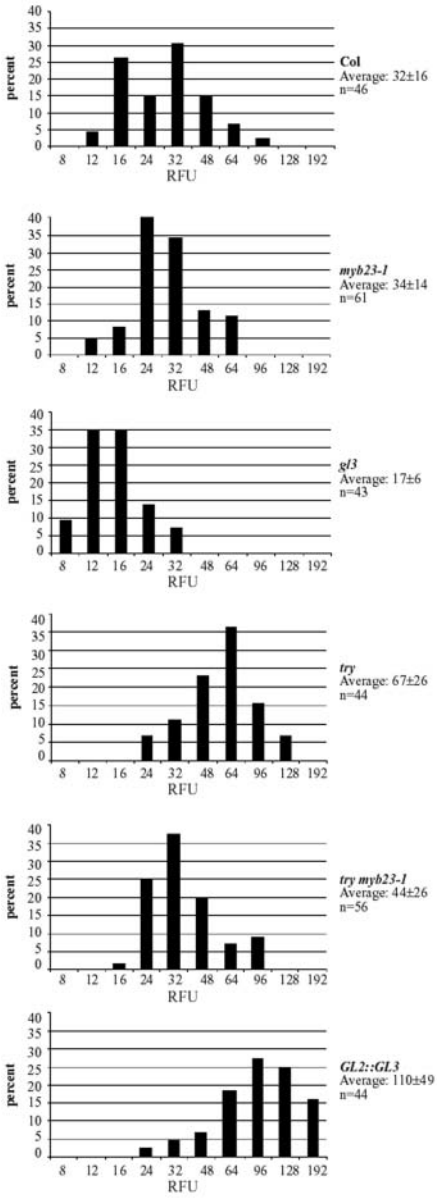

Fig. 6. Relative DNA content of DAPI stained trichomes. Fluorescence intensity was normalized to the mean fluorescence of wild-type trichomes (see Material and methods). RFU, relative fluorescence units; percent, indicates the fraction of the nuclei measured for each line.

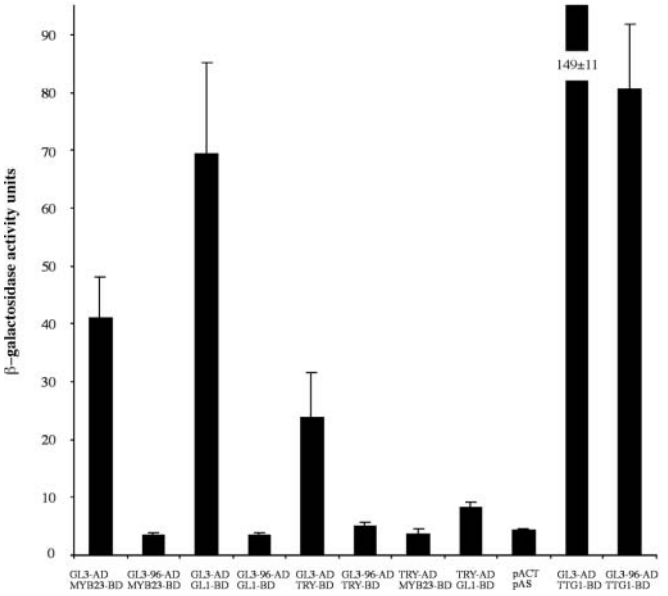

Fig. 7. Protein interactions between MYB23, GL1, TTG1, TRY and GL3 in yeast two-hybrid assays. *lacZ* reporter activity in yeast cells for the pair-wise combination of plasmids is shown. The activity of the negative control (yeast containing plasmids pACT and pAS) represents the background level in yeast cells. Interaction between GL3, GL3-96 and TTG1 demonstrates that the 96 amino acids N-terminal truncated version of GL3 is capable of interaction with TTG1 and is consistent with previous data (Payne et al., 2000). Error bars show standard deviation. AD, GAL4 transcriptional activation domain; BD, GAL4 DNA binding domain.

7) but not with TTG1 (data not shown). Payne et al. (Payne et al., 2000) demonstrated that GL3 interacts with GL1 through the N-terminal end of the GL3 protein. We found that the truncation of 96 amino acids from the N-terminal end of the GL3 protein also abolished the interaction between GL3 and MYB23 (Fig. 7). These results suggest that MYB23 interacts with the same GL3 protein domain as GL1.

Since *try* mutant trichomes are increased in size and in trichome branch number, *TRY* acts as a negative regulator of trichome branching. To address the mechanism of the inhibitory role of TRY in trichome branching we tested the interactions of the TRY protein with GL3, MYB23 and GL1 (Fig. 7). Our interaction assay did not reveal an interaction between TRY and MYB23 and between TRY and GL1. We found an interaction between TRY and GL3 proteins that was abolished when the N-terminally truncated version of GL3 was used in assay, indicating that TRY, GL1 and MYB23 may bind to the same region of GL3. This suggests that MYB23 and TRY proteins compete for binding to GL3 similar as it was shown for GL1 (Esch et al., 2003). As the binding strength of TRY and GL3 is lower than that of MYB23 and GL3 – as revealed in yeast two-hybrid studies – effective competition of TRY with GL3 would require that either the concentration of TRY or the combined concentration of all the TRY-like genes is higher than that of MYB23.

Discussion

What molecular mechanisms fuel the evolution of plant form? In principle, evolutionary changes are accompanied by functional diversification of regulatory genes and may occur through changes of protein function or by changes of the regulation of genes. In particular the latter has been favoured because the extreme morphological differences of organisms do not correspond to the low divergence of proteins. In fact, many examples are known where protein function is conserved over large evolutionary distances (Doebley and Lukens, 1998).

Functional specification of paralogous genes provides a valuable experimental system with which to study the molecular mechanisms of gene diversification. In this study, we have investigated the functional diversification of *MYB23* and *GL1*. They share a sequence identity of 63% over the entire

protein and 92% in the actual MYB domain. *MYB23* and *GL1* are located on chromosomes III and V in the regions that originated from chromosomal duplication (The Arabidopsis Genome Initiative, 2000). It is therefore likely that the two genes are derived from gene duplication. Our genetic analysis revealed that *MYB23* and *GL1* have a partially redundant function with respect to trichome initiation, and that *MYB23* has an additional role in the regulation of trichome branching. The analysis of their functional specification by promoter and protein-coding region swap experiments, expression studies and protein-protein interaction studies gave insights in the functional diversification of the two genes.

Functional divergence of *cis*-regulatory sequences of *GL1* and *MYB23*

In contrast to *GL1* and *WER* genes, which have completely non-overlapping expression domains and unrelated functions (Lee and Schiefelbein, 2001), *GL1* and *MYB23* are both expressed in trichomes and regulate their development. Our swapping experiments, however, revealed that their actual regulation is significantly different and not interchangable. In this study, we have not specifically addressed the regulatory role of introns, which have recently been shown to play a role in GL1 regulation (Wang et al., 2004). However, as *GL1::MYB23* and *GL1::GL1* or *MYB23::MYB23* and *MYB23::GL1* constructs rescued the corresponding mutants equally well, it is unlikely that the coding region and the introns carry any information relevant for the regulatory differences between *GL1* and *MYB23*.

A comparison of their temporal and spatial expression revealed two differences. First, *GL1* but not *MYB23* is initially expressed ubiquitously in young leaves; and second, *MYB23* is expressed throughout all stages of trichome development, whereas *GL1* expression ceases long before trichome development is completed. Both aspects can be correlated with their respective functions. The initial ubiquitous expression of *GL1* is thought to be important for early pattern formation (Hulskamp, 2004; Larkin et al., 2003). It is postulated that the initial ubiquitous expression of *GL1* and other positive regulators of trichome initiation triggers a patterning mechanism that leads to a regular pattern of differentiated cells in which the positive regulators, including *GL1* are upregulated. Consistent with the finding that *MYB23* is not expressed in these initial stages, *MYB23* has only a subtle role in trichome initiation and the expression of *GL1* under the *MYB23* promoter cannot rescue *gl1* mutants.

In addition, the extended expression of *MYB23* in developing trichomes is consistent with the primary role of *MYB23* gene in trichome branching. This extended expression of *MYB23* is functionally relevant, as trichome branching in *myb23* mutants is not rescued when *MYB23* is expressed under the control of the *GL1* promoter.

Some of these regulatory changes can be correlated with different responses to known trichome patterning genes. Although *GL1* expression is independent from *GL3*, *GL1* and *TTG1*, the expression of *MYB23* in leaf trichomes is regulated by *TTG1* but not by *GL3* and *GL1*.

Functional divergence of GL1 and MYB23 proteins

Despite the relative low sequence identity of GL1 and MYB23, the two proteins can functionally replace each other during trichome initiation. Trichome initiation on the whole leaf surface was rescued, with the *GL1* promoter driving either MYB23 or GL1 protein; trichome initiation at the leaf edges and petioles of the *gl1 myb23* double mutant was rescued with the *MYB23* promoter driving either GL1 or MYB23 proteins.

In accordance with the ability of the MYB23 and GL1 proteins to rescue the trichome initiation phenotype of each mutant, yeast two-hybrid assay showed that MYB23 protein interactions with known trichome patterning proteins are similar to those found with GL1. Therefore it came as a surprise that protein functions are not fully conserved during trichome branching. When *GL1* is expressed under the *MYB23* promoter, trichome branching was not completely rescued, indicating that GL1 cannot fully replace the protein function of MYB23 in this developmental context. This raises the question of whether the regulation of trichome branching by *MYB23* involves the same downstream genes as during trichome patterning. Although the current data do not allow the determination of this, some observations suggest that this is the case. Notably, mutations in *GL3* and *TTG1* not only lead to patterning defects but also to reduced branching; mutations in *TRY*, a gene that inhibits trichome initiation, also result in increased branching. A conceivable scenario is that, similar as postulated in the context of pattern formation, TTG1 and GL3 form a branch-promoting complex together with MYB23 and that TRY counteracts this by competing with MYB23 for the binding with GL3. One possible explanation for the difference between GL1 and MYB23 proteins in this context is that MYB23 protein may endow the trimeric complex with higher activity in the promotion of cell growth and DNA endoreduplication.

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