Functional diversity of R3 single-repeat genes in trichome development

Katja Wester1, Simona Digiuni1, Florian Geier2, Jens Timmer3, Christian Fleck3 and Martin Hülskamp1,*

Trichome and root hair patterning are governed by a conserved cassette of bHLH and MYB factors, the WD40 protein TTG1, and six single-repeat MYB R3 factors that are thought to counteract them. In this work we focus on the single-repeat R3 factor ETC3 and show that its major role is in the regulation of trichome density in a redundant manner. Diversification of the ETC3 gene has occurred at the promoter level, as etc3 mutants can be rescued by expressing ETC3 under the control of the TRY or CPC promoter. ETC3 movement was detected between epidermal cells as well as between the epidermis and underlying tissues. Finally, we found marked differences in the ability of the single-repeat R3 factors to interfere with the dimersisation of GL1 and GL3 in a yeast three-hybrid system, with CPC being the most potent inhibitor followed by ETC1, TRY, ETC3 and ETC2. Mathematical analysis predicts that this behaviour has a major impact on protein mobility, suggesting a tight reverse correlation between inhibitory function and the diffusion/transport range of the inhibitors. This prediction is supported by a comparison of CPC and ETC3 mobility in egl3 gl3 double mutants and 35S:GL3 lines.

KEY WORDS: Trichomes, Patterning, Arabidopsis

INTRODUCTION
The differentiation of epidermal cell types in a spatially regulated manner is an excellent model system in which to study pattern formation in plants. Two apparently independent patterning systems have been recognised: one that controls the spatial distribution of stomata (Bergmann and Sack, 2007; Serna, 2005) and one that controls the spatial distribution of root hairs and trichomes on leaves (Ishida et al., 2008; Larkin et al., 2003; Pesch and Hulskamp, 2004; Schellmann et al., 2007). The latter patterning system is based on an evolutionarily conserved gene cassette consisting of three types of positive regulators comprising a WD40 protein, R2R3 MYB-type transcription factors and bHLH transcription factors, and negative regulators of the single-repeat R3 MYB transcription factor family. The WD40 protein is encoded by the single-copy gene TRANSPARENT TESTA GLABRA1 (TTG1) (Galway et al., 1994; Walker et al., 1999). The R2R3 MYB-type transcription factors are represented by a small gene family, with GLABRA1 and MYB23 functioning in trichome patterning and WEREWOLF in root hair patterning (Kirik et al., 2005; Kirik et al., 2001; Lee and Schiefelbein, 1999; Oppenheimer et al., 1991). The bHLH factors are GLABRA3 and ENHANCER OF GLABRA3 (EGL3), which act redundantly in trichome and root hair patterning (Bernhardt et al., 2005; Payne et al., 2000; Zhang et al., 2003). The single-repeat R3 MYB proteins act as negative regulators and have been grouped into a family of six genes (Wang et al., 2007). The CAPRICE (CPC) gene was initially identified as a major regulator of root hair development, whereas TRIPTYCHON (TRY) is predominantly required for trichome patterning (Kirik et al., 2004a; Kirik et al., 2004b; Schellmann et al., 2007; Wada et al., 1997). Subsequent analyses showed that CPC and TRY act redundantly in root hair and trichome development (Schellmann et al., 2007). Also, two other members of the family, ENHANCER OF TRY AND CPC 1 (ETC1) and ETC2, act redundantly with TRY and CPC (Esch et al., 2004; Kirik et al., 2004a; Kirik et al., 2004b). In addition, TRICHOMELESS1 (TCLI) has been shown to play an important role in trichome formation on the stem and the pedicels (Wang et al., 2007). In an initial report, the analysis of etc3 mutants focused on root hair development (Simon et al., 2007). More recently, further phenotypes caused by mutations in ETC3 were studied and CPL3 (CPC-LIKE-MYB3) was introduced as a second name for this gene (Tominaga et al., 2008). ETC3 seems to represent an unusual member of the single-repeat R3 MYB class, as various unusual phenotypes were reported for the mutant including an early flowering time phenotype, reduced trichome size and branching, longer hypocotyls and general plant growth changes (Tominaga et al., 2008). A role for ETC3 in trichome formation is controversial. Tominaga and co-workers reported a higher trichome density in the corresponding mutant and, in contrast to the other members of the single-repeat R3 MYBs, a conspicuous absence of trichome-specific expression (Tominaga et al., 2008). Wang and co-workers could not detect any difference in trichome number between etc3 single mutants and the corresponding wild type (Wang et al., 2008).

The positive regulators are thought to form a complex in which the R2R3 MYB factor and TTG1 bind to GL3 or EGL3, and the negative regulation by the R3 single-repeat MYBs is postulated to be governed by their competition with MYB factors for binding to GL3 and EGL3, thereby rendering the activator complex inactive (Bernhardt et al., 2003; Esch et al., 2003; Payne et al., 2000). Inhibition by direct binding to the GL1 promoter was suggested by ChIP experiments for TCL1 (Wang et al., 2007).

Although the same machinery is central to the spatial regulation of root hair and trichome patterning, the context is different. Whereas trichome formation on rosette leaves takes place without a recognisable reference to other leaf structures except for other trichomes (Hulskamp and Schnittger, 1998; Larkin et al., 1996), root hair patterning is strongly biased such that root hairs are normally found only in epidermal cells overlying a cleft between two underlying cortex cells (Berger et al., 1998; Dolan et al., 1994). For both systems, models that try to explain pattern formation are based on a feedback loop in which the positive regulators activate the...
negative regulators and the negative regulators inhibit the activators, with the negative regulators being able to move between cells (Digut et al., 2008; Larkin et al., 1996; Marks and Esch, 2003; Pesch and Hulskamp, 2004; Scheres, 2002). Recently, evidence for a parallel second patterning system has been reported in which the depletion of TTG1 around incipient trichomes contributes to pattern formation (Bouyer et al., 2008).

In this study we focus on the function of the single-repeat R3 MYB factors in trichome patterning with emphasis on ETC3. Initially, we reassess the published molecular and genetic data (Tominaga et al., 2008; Wang et al., 2008). We show that ETC3 is expressed in trichomes, that trichome density is increased in etc3 mutants and that this phenotype can be rescued by a PETC3:ETC3 construct. Our data do not support most of the additional phenotypes, such as the flowering time, the hypocotyl length and the leaf size and branching phenotypes previously reported. Next, we compared the molecular function of TRY, CPC, ETC1, ETC2 and ETC3. These factors differ in their binding strength to GL3 and, using the yeast three-hybrid system, we show that their capacity to compete with GL1 for binding to GL3 also differs. Marked differences were found, with CPC being the most potent inhibitor followed by ETC1, TRY, ETC3 and ETC2. We further show that ETC3 protein can travel between cells. Promoter-swap experiments revealed that transcriptional regulation, in particular, is important for the functional diversity. Mathematical analysis suggests that the mobility of the inhibitors depends on their affinity for GL3. This prediction is supported by a comparison of the mobility of CPC and ETC3 in egfl3 gl3 double mutants and 35S:GL3 lines.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

The following single and double mutants were used in this study and have been described previously: cpc-1 in WS ecotype (Wada et al., 1997), try-82 in Ler ecotype (Hulskamp et al., 1994), try-1c in Col ecotype (Larkin et al., 1999), etc1 in Col ecotype (Kirik et al., 2004a), etc2 in WS ecotype (Kirik et al., 2004b), etc3 in Col ecotype (Simon et al., 2007) and cpc try double mutant (Schellmann et al., 2002). Plants homozygous for multiple mutations were constructed by crossing single, double or triple mutants. The single and multiple mutants were confirmed by PCR. All MS plates contained 1% sucrose. The gl3 egfl3 double mutant was created by crossing the single mutants gl3 (Salk 118201) and egfl3 (Salk 019114). The GL3-overexpressing line was established by transformation of wild-type (Col) plants with the pAM-PAT p35S:GL3 construct.

*Agrobacterium*-mediated transformation of *Arabidopsis* plants was performed as described (Clough and Bent, 1998).

**Cytological methods**

GUS staining using X-glucuronide was performed as described (Vroemen et al., 1996). Forty-four-old plants from soil were used for GUS staining of leaves. The root expression analysis was performed with 5-day-old plants grown on MS plates. Pictures were taken with a light microscope equipped with DISKUS software (Carl H. Hilgers-Technisches Büro, Königswinter, Germany; version 4.30.19).

For staining cell walls, a propidium iodide solution (0.3 mg/ml) was used. Plant material was incubated for 5 minutes at room temperature and washed twice in water.

**Evaluation of root-hair numbers and stomata density**

For quantification of root hairs, plants were grown on MS plates in a vertical orientation for 5 days. Stomata numbers were determined on 3-week-old leaves stained with propidium iodide. Using a confocal laser-scanning microscope, a 1 mm² area of the fourth leaf was scanned for the statistical analysis.

**Confocal laser-scanning microscopy (CLSM)**

For CLSM, a Leica SP2 was used. Pictures were taken using the LCS software and images processed using Adobe Photoshop 6.0.

The quantitative comparison of YFP signals in neighbouring cells was based on full stacks that were subsequently merged to one plane. The resulting raw image was analysed using Leica Confocal Histogram Quantification software. In order to determine how much signal of the initially targeted cell arrives in the neighbouring cell, the signal intensity of the nuclei was compared.

**Nucleic acid analysis**

Plants carrying the T-DNA insertion in the ETC3 gene were identified by PCR on genomic DNA using primers ETC3-for (5′-ATGGATAACCATC-GAGTGAC-3′) and LBa1 (5′-TGGTCACTGAGTGGCCCATCG-3′).

ETC3 cDNA was amplified from Col wild-type cDNA by PCR using primers ETC3-XbaI-for (XbaI site added, 5′-TCTAGATGAATAAACAAGTTTTTGCAAAAGAATAGATGCACAGCTTACAAGAATAGATGAC-3′) and ETC3-SacI-rev (SacI site added, 5′-GAAGCTCT-CAATTGTTCATGACCCAAA-3′). The PCR product was subcloned into pBlueScript (pBS) with 5′ XbaI and 3′ SacI restriction sites downstream of GFP cDNA.

The ETC3 cDNA in pBS was amplified by PCR using primers ETC3-attB1 (5′-GGGGACAAGTTTGATCAAAAGACGACTTGTGGAAGTGCATAC-3′) and ETC3-attB2 (5′-GGGGACCGACTGAGACTGAC-3′). The PCR product was recombined in the DONR201 vector by the BP reaction (Invitrogen) and constitutes the ETC3 pEntry clone.

The GFP-ETC3 fusion fragment was amplified and recombined through BP in pDONR201 using primers GFP-attB1 (5′-GGGGACCAATGTTTGTAAGAGAGACCGAGAATAC-3′) and ETC3-attB2. The YFP-ETC3 pEntry was created by exchanging the GFP for YFP in the entry clone.

A 203 bp fragment upstream of the ETC3 start codon was used as the putative 5′ ETC3 promoter. The promoter fragment was amplified by PCR from genomic Col DNA using primers 5′-pETC3-for (5′-ATTCGGAATTCCTACATAAC-3′) and 5′-pETC3-rev (5′-GTCGACGCTACATAAC-3′). The PCR product was subcloned into Smal-cut pBluescript (pBS) by blunt-end ligation. The promoter was cloned into the pAM-PAT-GW p35S vector (GenBank AY436765) with 5′ EcoRI (EcoRI) and 3′ XhoI restriction sites, resulting in an exchange of the 35S promoter for the ETC3 promoter. For the CPC promoter, a 1091 bp fragment upstream of the start codon was used. For the TRY promoter, a 1.4 kb fragment upstream of the TRY start codon was used (Schellmann et al., 2002). Both fragments were cloned into the pAM-PAT-GW vector, exchanging the CPC or TRY for the 35S promoter.

The RUBISCO SMALL SUBUNIT 2B (RBCS-2B) promoter (pRHC2Sb) was cloned from the pK1573 plasmid (gift from the David Jackson laboratory, Cold Spring Harbor, NY, USA) into the pAM-PAT-GW vector, exchanging for the 35S promoter for the ETC3 promoter. For the CPC promoter, a 1091 bp fragment upstream of the start codon was used. For the TRY promoter, a 1.4 kb fragment upstream of the TRY start codon was used (Schellmann et al., 2002). Both fragments were cloned into the pAM-PAT-GW vector, exchanging the CPC or TRY for the 35S promoter.

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The GFP-ER tag cDNA was amplified from a subclone by PCR using primers GFP-ER-attB1 (5′-GGGGACAAGTTTGATCAAAAGACGACTTGTGGAAGTGCATAC-3′) and GFP-ER-attB2 (5′-GGGGACCGACTGAGACTGAC-3′). The PCR product was cloned into XhoI-cut pBS by blunt-end ligation. The promoter was cloned into the pAM-PAT-GW p35S vector (GenBank AY436765) with 5′ EcoRI (EcoRI) and 3′ XhoI restriction sites, resulting in an exchange of the 35S promoter for the ETC3 promoter. For the CPC promoter, a 1091 bp fragment upstream of the start codon was used. For the TRY promoter, a 1.4 kb fragment upstream of the TRY start codon was used (Schellmann et al., 2002). Both fragments were cloned into the pAM-PAT-GW vector, exchanging the CPC or TRY for the 35S promoter.

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ETC1, ETC2, ETC3, CPC and TRY with the YFP C-term vector. Protoplast isolation and transfection were performed as described (Spitzer et al., 2006).

**Yeast two-hybrid and three-hybrid assays**

All assays were performed in the Saccharomyces cerevisiae strain AH109. Transformation was performed as described (Gietz and Schiestl, 1995). For two-hybrid assays, the GAL4 DNA-BD plasmid pAS2-1 and GAL4 DNA-AD plasmid pAct from Clontech were used. GL3 was fused to the DNA-AD in pACT. GL1Δ (lacking 27 amino acids at the C-terminus), ETC1, ETC2, ETC3, CPC and TRY were fused to the DNA-BD in pAS. For analysing protein-protein interactions, yeasts were grown on media lacking histidine and supplemented with 5 mM 3-amino-1, 2, 4-triazole.

For three-hybrid assays, GL3 was fused to the DNA-AD in pAct. GL1Δ was fused to the GAL4 DNA-BD in the pBridge vector (Clontech). ETC1, ETC2, ETC3, CPC and TRY were fused downstream of the methionine-repressible promoter in pBridge. For analyzing competition between GL1 and one of the other inhibitor proteins for binding to GL3, yeasts were grown on plates lacking histidine. The plates contained different concentrations of methionine (0, 100, 250 or 500 μM) and were supplemented with 50 mM 3-amino-1, 2, 4-triazole.

**Transient expression**

Rosette leaves were co-bombarded with DNA-coated gold particles using the Biolistic PDS-1000/He system (Bio-Rad). Gold particles (1.0 μm) were coated with 400 ng of each DNA. Particles were bombarded into epidermal cells of rosette leaves with 900-psi rupture disks under a vacuum of 26 inches of Hg. The p35S:YFP-peroxisome (the targeted cell) with a rate constant λ. These assumptions lead to the following one-dimensional second-order differential equation for the steady state profile of \( U(X) \):

\[
D \frac{\partial^2 U(X)}{\partial X^2} = U(X)[\lambda + \beta G(X)], \quad (1a)
\]

\[
D \frac{\partial U(0)}{\partial X} = -\kappa, \quad (1b)
\]

\[
U(\infty) = 0. \quad (1c)
\]

The production of \( U \) is stated in the boundary condition 1b. Boundary condition 1c ensures that the profile of \( U \) declines to zero for \( X= \infty \), as the source of \( U \) at \( X=0 \) is the only source in the considered domain. In the following we assume that GL3 is spatially constant, i.e. \( G(X)= \) constant. The movement ability of the inhibitor is measured by its characteristic decay length (CDL), \( \alpha \), which is the distance from the source at which the inhibitor level drops to 1/e (37%) of its source level. We use a scaling approach in order to identify the effective parameters of the problem. The following substitutions for the concentrations \( U \) and \( G \) and the spatial coordinate \( X \) are introduced:

\[
u = U \frac{D}{\alpha_0 \lambda}, \quad g = G \frac{D}{\alpha_0 \lambda}, \quad x = X \alpha_0^{-1} .
\]

The factor \( \alpha_0\sqrt{D/\lambda} \) is the CDL of \( U \) for \( \beta=0 \). Using these substitutions gives the new dimensionless system:

\[
\frac{\partial^2 u(x)}{\partial x^2} = u(x) \left[ 1 + \frac{\alpha_0 \beta}{g} \right], \quad (2a)
\]

\[
\frac{\partial u(0)}{\partial x} = -\kappa / \lambda, \quad (2b)
\]

\[
U(\infty) = 0. \quad (2c)
\]

The solution of equation 2 is \( u(x)=e^{\lambda x}e^{-\kappa x/\lambda} \) where \( e=\kappa/(\lambda+\sqrt{\lambda^2+\kappa^2}) \) and \( v(\alpha_0 \beta) \) are the only effective parameters of the problem. Parameter \( \gamma \) has a convenient interpretation because it is the ratio of two time scales: the time it takes one unit of \( u \) to diffuse a distance of \( \alpha_0 \) and the time it takes one unit of \( u \) to bind to one unit of \( g \). The parameter \( \gamma \) can be interpreted as a measure for the relative binding affinity of \( u \) for \( g \). The relative CDL of the inhibitor as plotted in the inset of Fig. 5 is given by \( \alpha_0\sqrt{D/\lambda} \).

**RESULTS**

Isolation of the etc3 mutant

The ETC3 gene is the smallest of the six-member R3-type MYB-domain TRY/CPC-like gene group (Simon et al., 2007; Tominaga et al., 2008). The deduced 75-amino-acid ETC3 protein shows high homology to TRY and CPC (38% and 50% amino acid identity, respectively). Our comparison of the genomic and cDNA sequences showed that the ETC3 gene consists of three exons and two introns (Fig. 1A).

As a first step towards a functional analysis of the ETC3 gene, we isolated a T-DNA knockout line of the corresponding At4G01060 locus. The SALK line 094027 has an insertion after 354 bp in the second intron (Fig. 1A). RT-PCR analysis with primers in exons 1 and 2 before the insertion yielded no amplification product, indicating that the isolated mutant is a null (Fig. 1B).

We noticed that etc3 mutant plants exhibit a higher trichome density. On leaves one and two, as well as on leaves three and four, ~50% more trichomes were found as compared with the respective Columbia wild-type background (Table 1; Fig. 2A,B). In order to ensure that this phenotype is due to a mutation in the ETC3 gene, we expressed the ETC3 cDNA under the control of a 2023 bp upstream putative promoter fragment. This pETC3:ETC3 construct rescued the trichome phenotype completely (Table 1).
The expression of ETC3 was initially studied by RT-PCR. ETC3 was expressed in all analysed tissues ranging from very weak expression in the root to strong expression in flowers (Fig. 1C). This expression profile generally fits the expression analysis of Tominaga and co-workers (Tominaga et al., 2008). For a better spatial resolution we used the 2023 bp promoter fragment shown to rescue the trichome phenotype to drive the expression of the GUS marker gene (pETC3:GUS). Plant lines containing this construct showed GUS expression in the basal parts of leaves in young trichomes (Fig. 1D) and in stomata (Fig. 1E). Expression ceased during trichome differentiation and was absent in mature trichomes. Because Tominaga and co-workers were unable to detect ETC3 expression in trichomes (Tominaga et al., 2008), we reasoned that this might be due to differences in the promoter fragments. To test this possibility we created a pETC3:GUS construct using the same 2.4 kb promoter fragment as described by Tominaga et al. (Tominaga et al., 2008). Arabidopsis lines containing this construct revealed the same expression pattern as we found for the 2023 bp fragment, including expression in trichomes (see Fig. S1 in the supplementary material).

In roots, pETC3:GUS expression was only occasionally detected after more than 24 hours GUS staining and is therefore not considered further. However, RT-PCR revealed strong expression in try cpc double mutants (Fig. 1C). This indicates that ETC3 is only weakly expressed in roots owing to repression by the other inhibitors, similar to what has been found for TRY (Schellmann et al., 2002).

**Redundant function of ETC3 in trichome patterning**

To further investigate the role of ETC3 in trichome patterning we expressed a p35S:ETC3 construct in a wild-type background. As observed previously for all other members of the TRY/CPC group, overexpression of ETC3 resulted in a glabrous phenotype (Fig. 2C; Table 1).

A potential redundant functional overlap with the other four members of the TRY/CPC group was tested by creating double, triple and quadruple mutants (Table 2). As a first step, we compared the four possible double mutants of etc3 with the try/cpc group members. The single mutants fell into three phenotypic classes: try, which exhibited trichome clusters; cpc and etc2, which exhibited increased trichome number; and etc1, which was without a detectable trichome phenotype. The etc3 try double mutant displayed a weak synergistic phenotype. Trichome density was similar to that of etc3 mutants and cluster frequency was significantly increased compared with try mutants (Student’s t-test, P=0.006). The cpc etc3 double mutant showed a similar phenotype to the cpc single mutant. The etc3 etc1 double mutant showed a significant increase in trichomes (P=0.003) indicating that ETC1 acts redundantly with ETC3. By contrast, the etc2 etc3 double mutant exhibited a trichome density similar to that of the etc3 single mutant. However, a redundant function of ETC2 with ETC3 was uncovered in the etc1 etc2 etc3 triple mutant, in which a significantly higher trichome number was found as compared

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**Table 1. Trichome density on rosette leaves in etc3 mutants and in ETC3 overexpression lines**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of trichomes per leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First leaf pair</td>
</tr>
<tr>
<td>WT (Col)</td>
<td>16.6±2.2</td>
</tr>
<tr>
<td>etc3 (Col)</td>
<td>25.6±2.9</td>
</tr>
<tr>
<td>p35S:ETC3</td>
<td>0±0</td>
</tr>
<tr>
<td>5’ pETC3:ETC3 etc3</td>
<td>11.5±4.0</td>
</tr>
<tr>
<td>5’ pCPC:ETC3 etc3</td>
<td>11.4±2.8</td>
</tr>
<tr>
<td>5’ pTRY:ETC3 etc3</td>
<td>12.5±3.1</td>
</tr>
<tr>
<td>pRbcS:ETC3</td>
<td>5.4±2.8</td>
</tr>
<tr>
<td>pRbcS:CPC</td>
<td>3.9±3.0</td>
</tr>
</tbody>
</table>

For each line, leaf pairs from 45 plants were examined. WT, wild type.
with the etc1 etc3 double mutant (Fig. 2E; Table 2). A much stronger effect was found in the etc3 try cpc triple mutant, in which only a few large clusters of trichomes were found that contained more than 100 trichomes (Fig. 2F). A phenotypic enhancement was also observed for ectopic trichome formation on the hypocotyl (Fig. 2G,H). The phenotypic strength of this triple mutant was increased in the etc1 etc3 try cpc (Fig. 2J) and etc1 etc2 try cpc (Fig. 2I) quadruple mutants.

### Role of ETC3 in root hair and stomata development

Tominaga and co-workers noted a reduction of root hairs in the etc3 single mutant (Tominaga et al., 2008), whereas Wang and co-workers did not (Wang et al., 2008).

We analysed the root hair pattern with respect to the underlying cortex cells (Table 3), focusing on the etc3 mutant and cpc etc3 double mutant. In etc3 mutants the number of root hairs in trichoblast positions was significantly decreased (P<0.01). The cpc etc3 double mutant showed no difference compared with cpc, and the overexpression line p35S:ETC3 exhibited significantly more root hairs in the atrichoblast position (P<0.01) (Table 3).

Because of the strong and specific expression of ETC3 in stomata (Fig. 1E) we also analysed the stomata pattern in the leaf epidermis of etc3 mutants and in p35S:ETC3 lines. In addition, we tested the double mutant etc3 etc2 because ETC2 is the only other gene of the TRY/CPC group that shows strong expression in stomata. As reported by Tominaga and co-workers (Tominaga et al., 2008), we found no deviation from wild type in any of these lines (data not shown).

### ETC3 phenotypes unrelated to canonical TRY/CPC functions

Tominaga and co-workers reported several etc3 mutant phenotypes that are not observed for any of the other TRY/CPC group mutants. These include an early flowering phenotype under long-day conditions, a reduction of trichome size and branching, an increase in hypocotyl length, and drastic overall growth changes. Our combination of a null mutant and a genomic rescue of this mutant enabled us to reassess these unusual phenotypes.

The flowering time was studied under long-day conditions (16 hours light/8 hours dark) (see Table S1 in the supplementary material). Under these conditions, wild-type plants flowered after 27±1 days (n=20), whereas etc3 mutants flowered after 28±1 days (n=20). By that time, wild-type and etc3 plants had produced 13±1 and 15±1 rosette leaves, respectively. The same behaviour was found for plants kept under continuous light conditions. An early flowering phenotype was not observed.

In our hands, trichome size was indistinguishable in wild type and etc3 mutants (see Fig. S2 in the supplementary material). The marked reduction reported by Tominaga and co-workers (Tominaga et al., 2008) was not observed. Also, trichome branching was not affected in the etc3 mutant (see Table S2 in the supplementary material).

Hypocotyls were reported to almost double in length in etc3 mutants and to be shorter in 35S:ETC3 plants, which was correlated with changes in the epidermal cell shape (Tominaga et al., 2008). We could not detect any difference in hypocotyl length between wild type, etc3 and 35S:ETC3 mutants (see Fig. S3 in the supplementary material), nor any epidermal cell shape changes (data not shown).

### Table 2. Trichome numbers in etc3 double and triple mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Trichome initiation sites per leaf</th>
<th>Trichomes per leaf</th>
<th>Trichome clusters per leaf (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Col)</td>
<td>16.6±2.2</td>
<td>16.6±2.2</td>
<td>0</td>
</tr>
<tr>
<td>etc3 (Col)</td>
<td>25.6±2.9</td>
<td>25.6±2.9</td>
<td>0</td>
</tr>
<tr>
<td>etc1 (Col)</td>
<td>19.9±2.6</td>
<td>19.6±2.6</td>
<td>0.2</td>
</tr>
<tr>
<td>try (Col)</td>
<td>16.5±2.3</td>
<td>18.6±2.0</td>
<td>6.2</td>
</tr>
<tr>
<td>etc3 try</td>
<td>24.7±3.3</td>
<td>28.8±4.5</td>
<td>8.4</td>
</tr>
<tr>
<td>etc3 etc1</td>
<td>36.7±4.6</td>
<td>37.1±4.6</td>
<td>0.5</td>
</tr>
<tr>
<td>WT (WS)</td>
<td>19.8±2.5</td>
<td>19.8±2.5</td>
<td>0</td>
</tr>
<tr>
<td>cpc (WS)</td>
<td>27.7±3.7</td>
<td>27.7±3.7</td>
<td>0</td>
</tr>
<tr>
<td>etc2 (WS)</td>
<td>24.3±2.9</td>
<td>24.3±2.9</td>
<td>0</td>
</tr>
<tr>
<td>etc3 etc2</td>
<td>30.5±3.5</td>
<td>30.5±3.5</td>
<td>0</td>
</tr>
<tr>
<td>etc3 cpc</td>
<td>38.8±3.9</td>
<td>39.2±3.9</td>
<td>0.6</td>
</tr>
<tr>
<td>etc1 etc2</td>
<td>47.0±3.0</td>
<td>47.0±3.0</td>
<td>0</td>
</tr>
<tr>
<td>etc1 etc2 etc3</td>
<td>92.6±2.5</td>
<td>92.6±2.5</td>
<td>0</td>
</tr>
</tbody>
</table>

At least 20 plants for each line were used for the analysis.

WT, wild type; n.d., not determined.

### Table 3. Root hair formation in the leaf epidermis of etc3 mutants and ETC3 overexpression lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hair cells in epidermis (%)</th>
<th>Trichoblast position</th>
<th>Atrichoblast position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hair cells (%)</td>
<td>Non-hair cells (%)</td>
<td>Hair cells (%)</td>
</tr>
<tr>
<td>WT (Col)</td>
<td>41.2±1.7</td>
<td>93.5±9.6</td>
<td>6.5±9.6</td>
</tr>
<tr>
<td>WT (WS)</td>
<td>40.1±3.8</td>
<td>90.7±9.0</td>
<td>9.3±9.0</td>
</tr>
<tr>
<td>etc3 (WS)</td>
<td>27.3±1.5</td>
<td>73.9±15.9</td>
<td>26.1±15.9</td>
</tr>
<tr>
<td>cpc (WS)</td>
<td>11.8±1.7</td>
<td>83.8±11.7</td>
<td>16.2±11.1</td>
</tr>
<tr>
<td>etc3 cpc</td>
<td>9.6±1.4</td>
<td>84.3±11.1</td>
<td>15.7±11.1</td>
</tr>
<tr>
<td>p35S:ETC3</td>
<td>62.0±4.5</td>
<td>100±0</td>
<td>0±0</td>
</tr>
</tbody>
</table>

Five-day-old seedlings grown on MS plates were used. At least 20 roots were studied for each line.

WT, wild type.
When comparing overall plant growth we noted moderate differences between wild-type, etc3, 35S:ETC3 and pETC3:ETC3 plants with respect to rosette leaf size and height (see Fig. S4 in the supplementary material).

Together, these data suggest that most non-canonical phenotypes previously reported for the etc3 mutant are not caused by the mutation in the ETC3 gene.

**Expression of ETC3 protein under the TRY or CPC promoter can rescue etc3 mutants**

The overlapping redundancy of the five TRY/CPC genes raises the question of whether the relative differences in their importance for trichome patterning are due to differences in the regulation of their expression or in protein function. As a first step, we compared the ability of ETC3 to rescue the etc3 mutant under its own promoter, the TRY promoter and the CPC promoter. All rescue experiments described here were analysed in the first generation progeny (T1) using 45 plants. The three constructs rescued the etc3 phenotype equally well (Table 1). This indicates that the TRY, CPC and ETC3 promoters are interchangeable with respect to etc3 rescue.

This result prompted us to consider to what extent the expression of ETC3 under these three promoters can rescue the cpc try etc3 triple mutant. To our surprise, we found qualitatively different results for the three constructs. As expected, the pETC3:ETC3 construct rescued the cpc try etc3 mutant to the same extent as the try cpc mutant. When using the pTRY:ETC3 and pCPC:ETC3 constructs, we observed a wide range of rescue phenotypes and often plants were rescued back to the try cpc phenotype or even exhibited an over-rescued phenotype resembling that of try. The over-rescue phenotype was never observed in pETC3:ETC3 lines. We noted one striking difference between pTRY:ETC3 and pCPC:ETC3 with respect to the intermediate phenotypes. In pTRY:ETC3 we observed huge, separated trichome clusters that were typically devoid of trichomes in their middle region (Fig. 2K). The pCPC:ETC3 construct, by contrast, showed only a few clusters and these were smaller than in the try cpc mutants and trichomes were arranged irregularly at a high density (Fig. 2L). Although we do not understand the exact basis for these different patterns, these results strongly suggest that the qualitatively different phenotypes of try and cpc are due to differences in the regulation of their expression.

**Localisation and cell-to-cell movement of ETC3**

The localisation of the ETC3 protein was initially determined using a p35S:YFP-ETC3 construct. As shown in Fig. 3A, the protein was localised in the nucleus as well as in the cytoplasm. Expression under the control of its own promoter revealed a more differentiated pattern. We compared the expression and localisation of two constructs. The pETC3:GFP-ER construct was used as a control to visualise the expression pattern with a non-mobile GFP protein. In pETC3:GFP-ER lines, expression was found in single cells (Fig. 3C). The ubiquitous expression detected with the GUS lines was not observed, most likely because the sensitivity of the GFP signal was much lower than in the GUS lines. In pETC3:YFP-ETC3 lines, by contrast, cells around an incipient trichome showed a clear YFP signal. Strikingly, the YFP-ETC3 signal in surrounding cells was restricted to the nucleus, whereas in trichome initials YFP-ETC3 was found both in the cytoplasm and in the nucleus (Fig. 3B). This indicates that ETC3 protein moves from the trichome initial into the neighbouring epidermal cells.

We also demonstrated movement of ETC3 in two independent experiments. First, by expressing ETC3 in the subepidermis under the control of the RUBISCO promoter, in the construct pRbc:YFP-ETC3, in a wild-type background. If ETC3 were able to move into the epidermis one would expect a trichome reduction similar to that found in p35S:ETC3 lines. In these lines, a clear reduction of trichomes was observed (Fig. 3F; Table 1). Direct microscopic inspection revealed fluorescence in the epidermis suggesting that YFP-ETC3 protein or RNA has moved into the epidermis (Fig. 3E,F).

In a second experiment, p35S:GFP-ETC3 was transiently expressed in single leaf epidermal cells using the particle bombardment method. As shown in Fig. 3D, fluorescence was also found in cells around the cell expressing the construct. This movement was observed in 76% of all successfully transformed cells (n=100).

**Protein-protein interactions of ETC3**

The most important recognised protein-protein interaction of TRY and CPC during pattern formation is their binding to GL3. As shown in Fig. 4A, all five TRY/CPC group proteins showed strong binding to GL3 in yeast two-hybrid assays. This interaction was confirmed for TRY, CPC, ETC1, ETC2 and ETC3 with the BiFC system (Fig. 4B). As a control we used the truncated GL3 protein lacking the N-terminal 96 amino acids that was previously shown not to bind to GL1, TRY, CPC, ETC1, ETC2 or ETC3 in yeast two-hybrid assays (Kirik et al., 2005; Payne et al., 2000) (data not shown). The interaction of the TRY/CPC proteins with GL3 competes with the interaction of GL1 with GL3. We used this situation to study whether the TRY/CPC group proteins compete with GL1 with different efficiency using the yeast three-hybrid system. Growth at different concentrations of methionine was compared (Fig. 4C-F). At 500 μM methionine the methionine promoter is inactive (Fig. 4F). At 250 μM methionine CPC (4*) prevented GL1-GL3
interaction and ETC1 (1*) caused a clear growth reduction (Fig. 4E). At 100 μM methionine TRY (5*) prevented the GL1-GL3 interaction (Fig. 4D), and without methionine ETC3 (3*) could repress growth. ETC2 (2*) showed only a little growth reduction (Fig. 4C). Together, these data suggest that CPC is the most potent competitor followed by ETC1, TRY, ETC3 and ETC2.

As differences in the competition with the GL1-GL3 interaction reflect different binding affinities to GL3 we speculated that this might also affect the movement of the inhibitors. We took a theoretical approach to assess whether this idea is correct. We simulated a situation in which the inhibitor is produced locally and diffuses into the tissue. Along its path, the inhibitor is degraded at a constant rate. In addition, it is depleted owing to binding to GL3 with different binding affinities. We measured the movement ability of the inhibitor by its characteristic decay length (CDL), α, which is the distance over which the level of the inhibitor drops to 1/e (37%) of its source level. As shown in Fig. 5, a clear reduction in the movement ability of the inhibitor is predicted for increasing binding affinities. For example, if the relative binding affinity, γ, of the inhibitor of GL3 is increased from 1 to 10, its decay length is decreased from 71% to 30% relative to its CDL in the absence of any binding to GL3. Therefore, a strong binding affinity for GL3 is expected to result in a less mobile inhibitor, whereas a weak binding affinity should make the inhibitor more mobile as long as all other parameters are constant.

To experimentally test the prediction that higher binding affinity for GL3 decreases mobility, we compared the movement behaviour between single cells expressing YFP-CPC and YFP-ETC3 and their immediate neighbouring cells. In a first series of experiments we co-bombarded YFP-CPC or YFP-ETC3 with the peroxisome marker PTS in order to recognise the transformed cells. To evaluate the influence of GL3 on the movement behaviour we compared movement on rosette leaves of gl3 egl3 double mutants and a p35S:GL3-overexpression line (Table 4). Movement into neighbouring cells was detected in 74% and 66% of the targeted cells for YFP-ETC3 and YFP-CPC, respectively. Elevated GL3 levels significantly reduced the number of cells from which movement was observed. YFP-CPC movement was reduced to 22%, which corresponds to a reduction to 33% of the levels found in gl3 egl3 plants. As theoretically expected, YFP-ETC3 movement was much less affected by higher GL3 levels: 39% of the cells still showed movement, which corresponds to 53% of the levels found in gl3 egl3 plants.

In order to quantify the movement of YFP-CPC and YFP-ETC3 more directly, we measured the fluorescence intensity in the nuclei of the initially transformed cell (source, considered 100%) and the neighbouring cell (target). Again, the p35S:GL3 and gl3 egl3 plants were used for comparison. As a control we used YFP alone to

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**Table 4. Frequency of CPC and ETC3 movement into neighbouring cells**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Movement in gl3 egl3 (%)</th>
<th>Movement in p35S:GL3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p35S:YFP-ETC3</td>
<td>74</td>
<td>39</td>
</tr>
<tr>
<td>p35S:YFP-CPC</td>
<td>66</td>
<td>22</td>
</tr>
</tbody>
</table>

Movement was analysed for 50 transformed cells on cotyledons.
demonstrate that the two mutant backgrounds showed no general differences with respect to protein mobility (Table 5) (Student’s t-test, P=0.315). In this system we detected a subtle but significant difference between p35S:GL3 and gl3 egl3 plants for YFP-ETC3 movement (Table 5) (Student’s t-test, P<0.009). By contrast, the mobility of YFP-CPC was drastically reduced by ~50% in p35S:GL3 plants as compared with gl3 egl3 plants (Table 5) (Student’s t-test, P=0.000). The different movement behaviour of CPC and ETC3 in the two mutants was evident from the fact that neither showed a significantly different target/source ratio in gl3 egl3 mutants (Table 5) (Student’s t-test, P=0.129), but highly significant differences in p35S:GL3 plants (Table 5) (Student’s t-test, P=0.000). The robustness of all Student’s t-test results was confirmed using a non-parametric test according to Mann-Whitney (U-test). Together, the data indicate that the presence of GL3 reduces CPC mobility more efficiently than it reduces ETC3 mobility.

**DISCUSSION**

The presence of six close homologues of the R3 MYB transcription factor family and their involvement in root hair and trichome development raises two major questions. First, what is their relative contribution in the two processes and second, how are different functional adaptations realised?

## Functional diversity of R3 MYB transcription factors

Phylogenetic analysis groups TRY and ETC2 in one tree and CPC, TCL1, ETC1 and ETC3 in a second tree (Wang et al., 2007); within this tree, CPC is separated from TCL1 and a second branch containing ETC1 and ETC3. How does this evolutionary tree fit with their functional adaptations in root hair development and trichome formation?

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### Table 5. Nuclear signal ratio of YFP, YFP-CPC and YFP-ETC3 of neighbouring (target) to transiently transformed (source) cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>Target/source ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p35S:YFP-ETC3*</td>
<td>44.0±13.1</td>
</tr>
<tr>
<td>p35S:YFP-CPC*</td>
<td>39.2±8.8</td>
</tr>
<tr>
<td>p35S:YFP†</td>
<td>21.2±4.4</td>
</tr>
</tbody>
</table>

#### Target/source ratios (%) for YFP, YFP-CPC and YFP-ETC3 of neighbouring (target) to transiently transformed (source) cells using Student’s t-test results was confirmed using a non-parametric test according to Mann-Whitney (U-test). Together, the data indicate that the presence of GL3 reduces CPC mobility more efficiently than it reduces ETC3 mobility.

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### Expression or protein function: which is relevant for functional diversification?

Differences in transcriptional regulation are the most obvious reason for functional diversification. This is particularly evident for ETC2 and TCL1, neither of which is expressed in the root (Kirik et al., 2004b; Wang et al., 2007). In all other cases, the expression pattern is almost indistinguishable in root and shoot. The expression levels seem to vary in RT-PCR experiments; however, it is not possible to judge the relevance of this as the expression levels in the relevant cell types (trichoblast or atrichoblast/trichomes or epidermal cells) at the time when the pattern is established remain elusive. Also, our promoter-swap experiments suggest relevant differences at the promoter level, as etc3 try cpc triple mutants can be over-rescued when ETC3 is expressed under control of the CPC or TRY promoter.

Five of the six R3 MYB genes are involved in the regulation of root hair formation, although to different extents. CPC has the most prominent role and the corresponding mutants show a strong reduction of root hairs (Wada et al., 1997). A function for TRY and ETC1 was recognised in the respective double mutants with cpc; the try and etc1 single mutants are indistinguishable from wild type (Kirik et al., 2004a; Schellmann et al., 2002). A function of TCL1 in root hair formation was recognised in a cpc etc1 etc3 tcl1 mutant (Wang et al., 2008). A role of ETC3 in root hair formation was suggested by Tominaga et al. (Tominaga et al., 2008) and by our data presented here. However, such a role was not found in two previous studies (Simon et al., 2007; Wang et al., 2008), which might be explained by different growth conditions.

By contrast, all six R3 MYB genes play a role in trichome formation. This, however, does not simply constitute redundant action, as found for ETC1, but also functional diversification. TRY seems to be important for the local selection of trichome cells as suggested by a cluster phenotype in try mutants (Hulskamp et al., 1994). CPC, ETC2 and ETC3 seem to regulate the distance between trichomes as indicated by a higher trichome density in these mutants (Kirik et al., 2004b; Schellmann et al., 2002). Finally, TCL1 is important for organ-specific trichome regulation (Wang et al., 2007), although a role in pattern formation on leaves is also suggested by the tc1 cpc etc1 etc3 phenotype (Wang et al., 2008). The double and triple mutant analyses revealed plenty of redundancies between these three regulatory aspects. The etc3 try and etc2 try double mutants, for example, revealed an additional function of ETC3 and ETC2 in the local selection process (Kirik et al., 2004b). The triple mutant combination of etc1 and etc2 with the try cpc double mutant revealed a region specificity for ETC1 and ETC2 in the regulation of the trichome formation of petioles (Kirik et al., 2004a; Kirik et al., 2004b). Conversely, the synergistic enhancement of trichome formation on pedicels and stem in the tcl1 cpc double mutants indicates a redundancy between TCLI and CPC (Wang et al., 2007). Taken together, there is no obvious correlation between the functional diversification and evolutionary distances of these proteins.
Promoter-swap experiments also revealed examples demonstrating differences at the protein level. In the root system the rescue of the cpc mutant phenotype with CPC-promoter driven cDNAs of the R3 MYB family revealed clear differences, such that ETC1 rescued best, followed by ETC3, TRY and ETC2 (Simon et al., 2007). In the same experimental set-up, the cpc trichome phenotype was rescued equally well by all proteins except for TRY, which over- rescued. TCL1 seems to have diverged the most among the R3 MYB group at the protein level. Whereas overexpression of all the other genes causes supernumerary root hairs, TCL1 overexpression has no effect on root hair formation (Wang et al., 2007). A difference is also seen in the trichome system, such that TCL1 expression under the CPC promoter can rescue the cpc mutant phenotype but expression under the TRY promoter cannot rescue the trv mutant phenotype (Wang et al., 2007).

Protein properties: which aspects matter for patterning?

Currently, two mechanisms are proposed to explain how the R3 MYB proteins inhibit the activators. First, it is suggested that TCL1 can bind directly to the regulatory DNA regions thereby repressing their expression (Wang et al., 2007), although no evidence supporting such a mechanism is available for any of the other group members. Second, it is suggested that GL1-GL3 dimerisation is inhibited by binding of the inhibitors to GL3 (Esch et al., 2003). We demonstrated here that this property is shared by CPC, TRY, ETC1, ETC2 and ETC3. It is likely that TCL1 also shares this function as it can bind to GL3 (Wang et al., 2008).

Our finding that the five analysed inhibitors differed in their ability to interfere with the GL1-GL3 interaction raised the question of whether this might be relevant for their role in patterning. Theoretical models provide the key criteria to formulate the relevant parameters (Koch and Meinhardt, 1994): (1) the binding strength to GL3 or to DNA; (2) the diffusion/transport rates; and (3) the degradation rates. Our theoretical calculations suggest that if all other parameters are constant, movement of the inhibitors should be affected by their different binding strengths to GL3, such that ETC2 moves best and CPC least. Focusing on the movement of YFP-CPC and YFP-ETC3 between two cells, we provide two lines of evidence supporting the theoretical prediction. First, we show that the number of transiently transformed cells from which movement is observed depends on the presence of GL3 and that this affects CPC movement more than that of ETC3. In a second line of experiments we measured the fraction of YFP-CPC or YFP-ETC3 that had moved into the neighbouring cell, and again CPC mobility was increased in the absence of GL3, whereas ETC3 movement was not. It is important to note that these predictions cannot be simply extended to the whole patterning process and to the phenotypic differences between the mutants because a quantitative determination of the expression levels in trichomes, a quantitative comparison of the protein degradation rates and a quantitative evaluation of the transport rates between the cells are missing. Future steps towards a mechanistic understanding of the patterning systems will require not only an unvarnelling of the genetic and molecular network, but also a combination of quantitative experimental and theoretical approaches.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/9/1487/DC1

References


