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ENHANCER of *TRY* and *CPC 2* (*ETC2*) reveals redundancy in the region-specific control of trichome development of *Arabidopsis*

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Abstract

An evolutionarily conserved set of proteins consisting of MYB and bHLH transcription factors and a WD40 domain protein is known to act in concert to control various developmental processes including trichome and root hair development. Their function is difficult to assess because most of them belong to multigene families and appear to act in a redundant fashion. In this study we identified an enhancer of the two root hair and trichome patterning mutants *triptychon* (*try*) and *caprice* (*cpc*), *enhancer of try and cpc2* (*etc2*). The *ETC2* gene shows high sequence similarity to the single-repeat MYB genes *CPC* and *TRY*. Overexpression results in the suppression of trichomes and overproduction of root hairs similarly as observed for *TRY* and *CPC* suggesting that ETC2 has similar biochemical properties. The *etc2* single mutant shows an increase in trichome number on leaves and petiols. Double and triple mutant analysis indicates that the *ETC2* gene acts redundant with *TRY* and *CPC* in trichome patterning.

Introduction

In *Arabidopsis* processes as diverse as trichome initiation, root-hair initiation, anthocyanin biosynthesis and the production of seed coat mucilage are controlled by a suit of evolutionary conserved group of regulators: MYB-related transcriptions factors (Oppenheimer *et al*., 1991; Lee and Schiefelbein, 1999), bHLH-like transcription factors (Payne *et al.,* 2000; Bernhardt *et al.,* 2003), a WD40 protein (Walker *et al.,* 1999) and small single-repeat MYB-like proteins (Wada *et al.,* 1997; Schellmann *et al.,* 2002). All these processes are controlled by the WD40 factor TRANSPARENT TESTA GLABRA1 (TTG1) (Koornneef, 1981; Zhang *et al.,* 2003). The specificity is narrowed down by at east three bHLH-like and at least five MYB-related transcription factors (Zhang *et al.,* 2003). These act in a partially redundant manner, which is indicated by the observations that the corresponding single mutants affect only a subset of the processes and that additional functions are only revealed in certain double mutants.

Trichome and root hair patterning involve additional factors that mediate the cell-cell interactions necessary to determine, which cells differentiate to trichomes or root hairs and which develop to normal epidermal cells. Initially, the *CAPRICE* (*CPC*) gene was identified as a root-hair specific patterning gene and *TRIPTYCHON* (*TRY*) as a trichome specific patterning gene (Hülskamp *et al*., 1994; Wada *et al*., 1997). The *try cpc* double mutant revealed that they act redundantly to suppress trichome cell fate in the shoot and non-root hair development in the root: trichomes are initiated in large clusters and no root hairs are formed (Schellmann *et al*., 2002).

The current models explaining trichome and root hair patterning postulate that *TTG1*, the MYB-related transcription factors *GLABRA1* (*GL1*) (shoot trichomes) and *WEREWOLF* (*WER*) (root-hairs), and *GLABRA3* (*GL3*)/*ENHANCER OF GL3* (*EGL3*) promote trichome development in the shoot and non-root hair development in the root (Larkin *et al*., 2003; Hulskamp, 2004). Their expression in trichomes and non-root hair cells promotes *CPC* and *TRY* expression and movement of these through the plasmodesmata mediates lateral repression of the respective cell type fates.

Although all current data are in principle consistent with such a model the strength of the single and double mutant phenotypes is in particular for trichomes weaker as theoretically expected. In the absence of all negative regulators one would expect that all epidermal cells should develop into trichomes. This is not the case. The size of trichome clusters is drastically increased from 2 to 3 trichomes/cluster in *try* mutants and up to 40 trichomes per cluster in *try cpc* double mutants. If in addition another *TRY*/*CPC* homolog, *ETC1* is absent, large areas of the leaf are covered with trichomes, however, substantial leaf portions remain free of trichomes (Kirik *et al*., 2004).

We therefore, reasoned that additional negative regulators are involved and tested the possibility that other *TRY*/*CPC*/*ETC1*-like genes are additionally involved in lateral inhibition. The *TRY*, *CPC* and *ETC1* genes encode proteins of 106, 94 and 84 amino acids, respectively, that possess a single R3 MYB repeat and do not have any recognisable activation domain (Wada *et al*., 1997; Schellmann *et al*., 2002; Kirik *et al*., 2004). The Arabidopsis genome encodes a group of five MYB proteins that are highly homologous to *TRY*, *CPC* and *ETC1*. Here we show that one of them, which we named *ENHANCER* of *TRY*, and *CPC*2 (*ETC2)* shares an overlapping function with *TRY* and *CPC*. Interestingly, the strength of the patterning phenotype was not generally increased but additional trichomes were found at the leaf margin in the *etc2 try cpc* triple mutant.

Results

The analysis of the Arabidopsis genome sequence revealed a distinct group of five genes with high sequence similarity to *TRY* and *CPC*. This group is characterised by a single R3 type MYB-domain and the absence of any recognizable activation domain (Figure 1). To test whether the other genes are functionally redundant to *TRY* or *CPC* we used a reverse genetic approach to study their functions. This study is focused on the analysis of the *ETC2* gene (At2g30420). The comparison of the cDNA and genomic sequences revealed that the *ETC2* gene has three exons and two introns. Using 5′RACE we mapped the transcription start of the *ETC2* 80 bp upstream of the start codon. The deduced ETC2 protein has 112 amino acids and shows high homology to CPC and TRY (44% and 58% amino acids identity, respectively; Figure 1).

Overexpression of ETC2 inhibits trichome development and induces root-hair development

That *TRY* and *CPC* act as negative regulators of trichome and non-root hair development was supported by the observations that their overexpression under the control of the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter

Figure 1. Amino acid sequence comparison of the Arabidopsis single-MYB domain proteins sharing high homology with TRY and CPC. Amino acids identical with the ETC2 protein are shown in white letters in a black background, similar amino acids are highlighted in light grey.

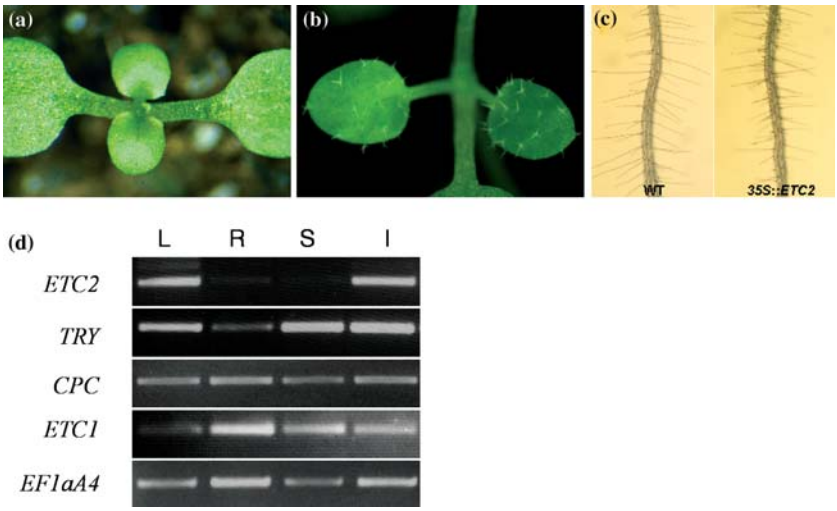

Figure 2. Overexpression phenotype of *ETC2* and comparative RT-PCR analysis of the *ETC2*, *TRY*, *CPC* and *ETC1* expression. (a) glabrous leaves of the *35S::ETC2* plant; (b) wild-type leaves; (c) root-hair development in wild-type and *35S::ETC2*; (d) comparative RT-PCR analysis of *ETC2*, *TRY*, *CPC* and *ETC1* expression in leaves (L), roots (R), stem (S) and inflorescence (I). Constitutively expressed translation elongation factor *EF1αA4* was used for RT-PCR control.

resulted in the complete absence of trichomes and the formation of extra root-hairs (Wada *et al.*, 1997; Schellmann *et al.*, 2002). To test whether ETC2 can adopt a similar function as suggested by the high sequence similarity to CPC and TRY we created a *35S::ETC2* construct using the *ETC2* cDNA and transformed it into plants. Among the 42 independent *35S::ETC2* plants we found 22 transgenic lines with reduced number of leaf trichomes and 16 completely glabrous plants (Figure 2a). The analysis of root-hair patterning in these plants revealed severe patterning defects. We found that *35S::ETC2* plants have increased number of root-hairs resulting from ectopic root-hairs in the N (non-hair) position (Figure 2c, Table 1). These data demonstrate that the ETC2 protein has similar biochemical functions as TRY and CPC.

ETC2 gene expression is developmentally regulated

To study the expression pattern of the *ETC2* gene we created a fusion of the 5′ promoter region (−1577 to −35 bp fragment) of *ETC2* and the *GUS* reporter gene. Transgenic plants containing the *ETC2::GUS* reporter construct exhibit strong expression in stomatal guard mother cells and young stomata (Figure 3a and e). Initially

Table 1. Effect of *ETC2* on the production and position of root-hair cells.

Genotype	Root-hair cells (% of total root epidermal cells)	Root-hair cells in H position (% of total cells in H position)	Root-hair cells in N position (% of total cells in N position)
Wild-type (Ws)	41.1 ± 6.9	98.3 ± 1.9	2.5 ± 3.2
etc2	39.0 ± 1.4	95.0 ± 1.9	0.8 ± 1.7
try	40.0 ± 1.9	98.3 ± 1.9	2.5 ± 3.2
try etc2	37.2 ± 3.9	91.7 ± 1.9	0.8 ± 1.7
cpc	14.6 ± 2.9	24.2 ± 6.9	0.8 ± 1.7
cpc etc2	10.4 ± 3.1	17.5 ± 3.2	0.8 ± 1.7
try cpc	0	0	0
try cpc etc2	0	0	0
35S::ETC2	57.2 ± 10.8	100	25.0 ± 11.7

ETC2::GUS is expressed ubiquitously in developing leaves (Figure 3b and c). Fully developed leaves did not show any *ETC2::GUS* expression (Figure 3f). *ETC2::GUS* expression was also found in trichomes of young (Figure 3b and c) but not old leaves (Figure 3f). Expression was also found in papilla cells of the stigma (Figure 3d). No expression was detected in the roots of the *ETC2::GUS* plants (Figure 3g).

To compare the relative expression levels of the *ETC2* gene with the trichome inhibitor genes *TRY*, *CPC* and *ETC1* we performed RT-PCR analysis using different plants organs. We found relatively low *ETC2* gene expression levels in roots and stems compared to young leaves and inflorescences (Figure 2d). *TRY* gene expression is also relatively low in roots which is consistent with the minor role of *TRY* in root epidermal patterning (Schellmann *et al.*, 2002). In contrast, *CPC* and *ETC1* mRNA levels are not substantially different plant organs analysed (Figure 2d). Together the data suggest that *ETC2* in contrast to *CPC* and *ETC1* is likely to be not involved in root development and is important during shoot development.

Leaves of the etc2-1 mutant have slightly increased trichome number

To assess the function of the *ETC2* gene we identified a T-DNA mutant allele, *etc2-1*, in the Wisconsin T-DNA collection. The *etc2* plants have a T-DNA insertion in the second intron (Figure 4a). Using RT-PCR no *ETC2* mRNA could be detected in the *etc2* mutant indicating that transcription of the *ETC2* gene is disrupted by the T-DNA insertion (Figure 4b).

The strong expression of the *ETC2::GUS* reporter construct in guard mother cells and young guard cells points to a function in stomata development. To test this, we analysed the stomata density, stomatal index and morphology and arrangement of the guard cell complex in the *etc2* mutants and plants overexpressing *ETC2* (Table 2). We have not detected any significant difference to wild-type. Also triple mutant *try cpc etc2* did not show aberrant stomata phenotype (Table 2).

Papilla cells appeared to be normal in morphology and function as plants were fully fertile.

We also analysed the root-hair pattern in *etc2* mutants because it had been previously shown that *TRY* is involved in root-hair patterning despite low expression in roots. The frequency of root-hairs at the H-position (position over the cleft of two cortex cells where root-hairs are normally formed) and the N-position (position over cortex cells where root-hairs are normally not formed) were determined. We could not detect any changes in the single *etc2* mutant nor in double mutants with *try* or *cpc* (Figure 4e, Table 1).

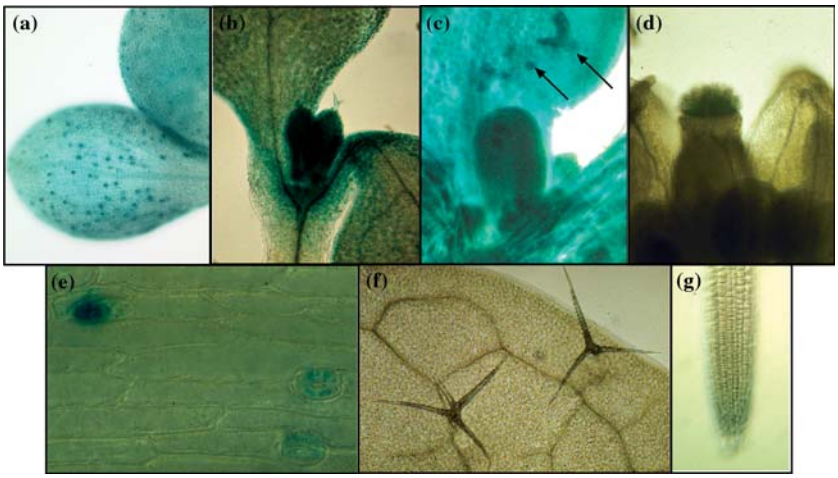

Figure 3. Expression analysis of the *ETC2* gene. Activity of the *ETC2::GUS* reporter in *Arabidopsis*: (a) cotyledons of the young seedling; (b) young leaves and trichomes; (c) developing trichomes (arrows); (d) papillae cells of stigma; (e) stomata guard mother cells and stomata on the hypocotyl of 7 day old seedlings; (f) mature leaves and trichomes; (g) root tip.

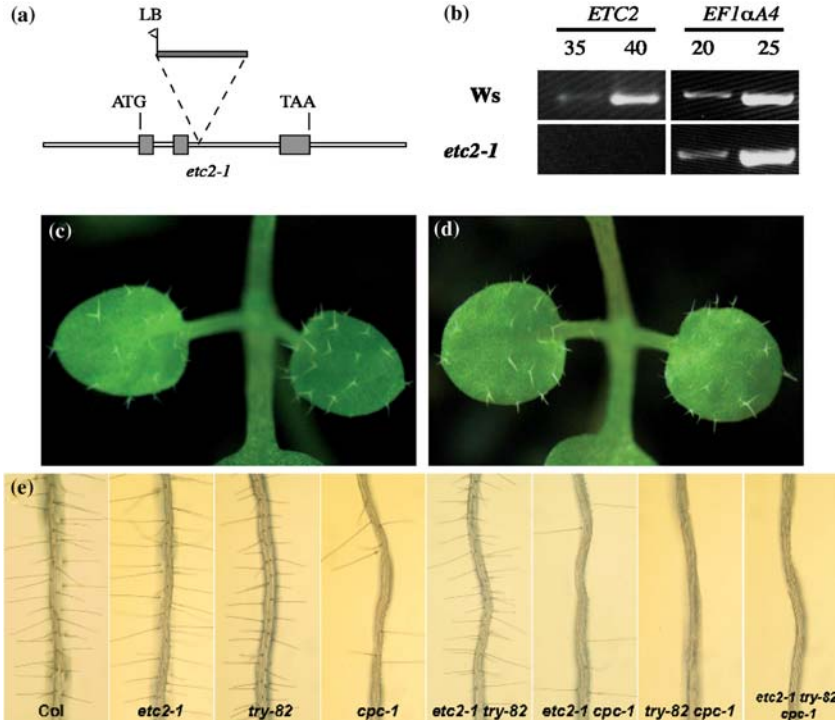

Figure 4. Characterisation of the *etc2-1* T-DNA mutant. (a) Schematic presentation of the *ETC2* gene and the T-DNA insertion in the *etc2-1* line; (b) Expression of the *ETC2* transcript in the Wassilewskaya wild-type (Ws) and the *etc2-1* mutant. 35 and 40 cycles were used in semi-quantitative RT-PCR. The expression of the *EF1αA4* was used as a control applying 20 and 25 amplification cycles; (c) Wild-type leaves; (d) *etc2-1* leaves; (e) root hair pattern in the *etc2-1* mutant and in double and triple mutants with *try* and *cpc*.

Trichome morphology in the *etc2* mutant was indistinguishable from wild-type (Figure 4c and d). Leaves of the *etc2* mutant have on average 25% more trichomes then wild-type leaves ($P < 0.001$) (Table 3). Similar slight but significant increase in trichome number was observed in the *etc2 cpc* double mutant. Leaves of the *etc2 cpc* double mutants have 18% more trichomes ($P < 0.001$) then of the *cpc* mutant. These data indicate that *ETC2* acts redundantly with *CPC* during trichome patterning.

The ETC2 gene has a region specific redundant function with TRY and CPC in the inhibition of trichome development

To test whether *TRY* and *CPC* act redundantly with *ETC2* we created double and triple mutant combinations of *etc2* with *try* and *cpc*. Examination of the root hair pattern did not reveal any significant effect of the *etc2* mutation in the *try* or *cpc* background (Table 1; data not shown). However, the *etc2 try cpc* triple mutant displayed

Table 2. Stomatal density on cotyledons of the *etc2* mutant, *try cpc etc2* triple mutant and *ETC2* overexpressing line.

Genotype (ecotype)	Stomata per mm^2	Stomatal index (%)	Clusters per mm^2
WS	48.8 ± 4.9	12.7	0
etc2	47.2 ± 4.3	12.6	0
Col	50.7 ± 4.8	17.2	0
35S::ETC2	51.8 ± 4.8	19.6	0
try cpc etc2	48.0 ± 4.2	15.1	0
try cpc	52.3 ± 4.2	17.3	0

Table 3. Effect of the *etc2* mutation on trichome production[a]

Genotype	Number of trichomes per leaf	Frequency of trichome clusters (%)	Number of trichomes per leaf petiol
WT (Ws)	43 ± 5	0	1.9 ± 1.1
etc2	54 ± 6	0	7.6 ± 1.3
cpc	74 ± 11	0	6.5 ± 1.5
etc2 cpc	87 ± 13	0.2	10.2 ± 1.5
WT (Ler)	14 ± 2.0	0	0.1 ± 0.2
try	11 ± 1.8	6.5	0.02 ± 0.1
try etc2	56 ± 12	9.7	5 ± 1.9
try cpc	n.d	94	11 ± 3
try cpc etc2	n.d	97	20 ± 6

[a]At least 20 leaves and 40 petiols of the first leaf pair were examined for each line.
n.d: not determined.

increased trichome development at the edges of leaves and on petioles (Figure 5b). To verify that the observed phenotype is due to the *etc2* mutation we have transformed the *etc2 try cpc* plants with the *ETC2* genomic fragment including 1639 bp sequence 5′ of the *ETC2* start codon and 485 bp sequence 3′ after the stop codon. This construct rescues the loss of *etc2* aspect of the *etc2 try cpc* plants and restored the *try cpc* phenotype (Figure 5c). This confirms that the *ETC2* gene functions as an inhibitor during trichome patterning.

ETC2 protein interacts with GL3 in yeast two-hybrid assay

Based on genetic and protein–protein interactions it was suggested that an activator complex consisting of TTG1, GL3 and GL1 proteins promote trichome initiation and the TRY protein suppress the activity of this complex by competing with the GL1 protein for GL3 binding (Payne *et al.*, 2000; Esch *et al.*, 2003). To test whether ETC2 and CPC proteins can also interact with the GL3 protein we performed a yeast two-hybrid analysis. We found that similar to TRY (Esch *et al.*, 2003), ETC2 and CPC proteins interact with the GL3 protein (Figure 6). Thus, ETC2, TRY and CPC proteins are likely to act in a biochemically similar manner by counteracting the activity of the proposed activator complex.

Discussion

Sequencing of the Arabidopsis genome revealed the existence of a large number of multi-gene families (ArabidopsisGenomeInitiative, 2000). Gene families have evolved by gene or whole genome duplications followed by functional diversification. Gene family members that have not completely diverged functions and show functional redundancy, represent intermediate stages of such a diversification (Thomas, 1993; Cooke *et al.*, 1997). It is recognized as a common phenomenon that in contrast to the 'housekeeping' genes in particular the developmental genes are members of multi-gene families (Byrne *et al.*, 2002; Kumaran *et al.*, 2002; Vroemen *et al.*, 2003). It is assumed that the duplication of regulatory genes

Figure 5. Ectopic trichomes on the *try cpc etc2* leaves and petioles. (a) *try cpc* leaves and petioles; (b) *try cpc etc2* leaf. Note increased trichome development at the leaf edges and petioles (arrow); (c) Leaf of the *try cpc etc2* mutant rescued with the genomic fragment containing the *ETC2* gene.

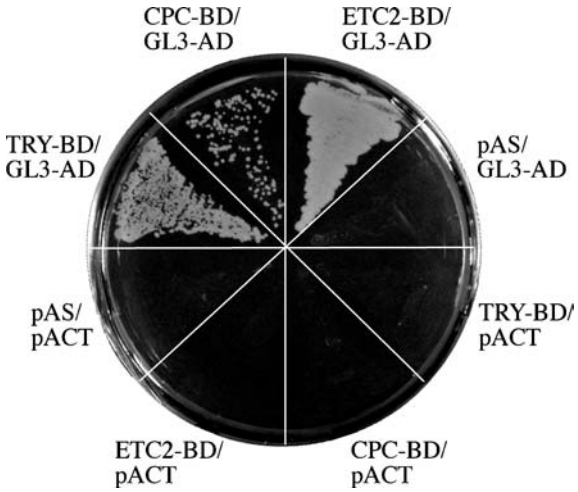

Figure 6. Interactions of the ETC2, TRY, CPC proteins with the GL3 protein in yeast two-hybrid assays. Growth on the histidine-deficient medium indicates interaction of the ETC2, TRY and CPC proteins with the GL3 protein. Yeasts containing plasmids pACT and pAS without fused proteins represent negative control. AD, GAL4 transcriptional activation domain, BD, GAL4 DNA binding domain.

enables the acquisition of new roles that can have adaptive advantages.

A particularly interesting gene family in this context is the MYB-transcription factor family that consists of more than 100 members (Stracke *et al*., 2001). Based on the homology outside the DNA-binding MYB domain the Arabidopsis *R2R3 MYB* gene family was divided into groups that are postulated to have similar functions (Kranz *et al*., 1998). Using a reverse genetic approach T-DNA and transposon insertions were found in 36 distinct *MYB* genes (Meissner *et al*., 1999). None of the mutants showed a discernable mutant phenotype suggesting that many *MYB* genes may share redundant functions.

Redundancy of MYB genes in epidermal differentiation

Two groups of apparently antagonizing MYB genes are involved in the epidermal differentiation of roots and shoots. The two homologous genes *GL1* and *WER* act as positive regulators of trichomes and non-root hair cells, respectively (Oppenheimer *et al*., 1991; Lee and Schiefelbein, 1999). Although GL1 and WER proteins are functionally equivalent, they are not redundant in their function (Lee and Schiefelbein, 2001). These two genes are antagonized by the three small single-repeat MYB-like proteins TRY, CPC and ETC1 (Lee and Schiefelbein, 2002; Schellmann *et al*., 2002; Kirik *et al*., 2004) that belong to a group of five homologous proteins. As judged by overexpression studies these genes share the same biochemical properties. The phenotype of the 35S::*ETC2* line suggests that the ETC2 protein is capable of altering trichome and root hair specification pathways. This is similar to the effect of the 35S::*CPC*, the 35S::*TRY* and the 35S::*ETC1*.

The single mutant phenotypes initially suggested that *TRY* and *CPC* act specifically in the shoot and the root, however, the double mutant revealed that they are both important in both patterning systems (Schellmann *et al*., 2002). The function of *ETC1* could only be observed in the *cpc* and *try* mutant background (Kirik *et al*., 2004). Our analysis of the *ETC2* gene revealed another level of redundancy. In contrast to *TRY* and *CPC* it does not appear to play a role in root-hair development as it is neither expressed in roots nor does it show a phenotype in the single, double or triple mutants. *ETC2* is rather important for the initiation of trichomes. All mutants identified to date affect the patterning of trichomes on the leaf in either of two aspects (Larkin *et al*., 2003; Hulskamp, 2004): (1) the trichome density; (2) the formation of clusters. This is well reflected in the phenotypes of *try* and *cpc* mutants. Mutations in *try* result in trichome clusters and *cpc* mutants exhibit an increased number of trichomes per leaf (Hülskamp *et al*., 1994; Schellmann *et al*., 2002). The *try cpc* double mutant produces large trichome clusters with up to 40 trichomes (Schellmann *et al*., 2002). The *etc2* single mutants show increased trichome numbers and double and triple mutant combinations revealed redundant function with *TRY* and *CPC*. Additional trichome formation is found at the leaf margin and the petiole. This redundancy in the region-specific trichome initiation reveals a new function of *TRY* and *CPC* in the repression of leaf edge and petiole trichomes that is not necessarily linked to their role in patterning on the leaf surface.

The expression pattern of the *ETC2::GUS* reporter suggests functions other than only trichome inhibition. We did not observe any non-trichome phenotype in the single *etc2* mutant indicating that other possible functions of the *ETC2* in, for instance, stomata development may also be masked by functional redundancy.

Materials and methods

Plant materials

The wild-type strain used in this work was Wassilewskaja (Ws). The *etc2-1* mutant was isolated from a Wisconsin T-DNA population. The *try-82* and *cpc-1* alleles were described previously (Hülskamp *et al*., 1994; Wada *et al*., 1997). Double and triple mutants of *try* and *cpc* with *etc2-1* were identified in the F2 progeny using PCR to identify the homozygous *etc2-1* plants. Selected double and triple mutants were checked and documented in the F3. Agrobacterium mediated transformation of *Arabidopsis* plants was done as previously described (Clough and Bent, 1998).

Evaluation of stomatal density and root-hair production

Adaxial surface of cotyledons was used for counting of stomata and pavement cells. Light microscopy was used to determine stomatal density and stomatal indices. Five plants for each line were taken for analysis. On each plant five separate fields of 0.26 mm^2 were analysed.

Root-hair cell production was determined from at least 20 five-day-old seedling roots for each strain, as previously described (Lee and Schiefelbein, 2002). An epidermal cell was scored as a root-hair cell if any protrusion was visible. Root epidermal cells were deemed to be located in the N position if they were positioned outside a periclinal cortical cell wall, whereas cells in the H position were located outside an anticlinal wall between adjacent cortical cells.

Nucleic acid analyses

5′ RACE was carried out with the GIBCO BRL 5′RACE kit using TH-RT (5′-GTTCTACTTT TATGATTGAAAGC) as GSP1 primer and nested primer TH-as2 (5′-ACTAGTCTTTACAATT TTAGATTTTCTTGGAG).

For GUS analysis of the *ETC2* expression, 1543 bp upstream fragment (−1638 to −35 bp 5′to the start codon) was PCR amplified using THprom-s2 (5′-CCCGGGCCATTAAGCCCTG TTTCCT) and THprom-as1 (5′-AGTCGACAG AGAAACTGAAAATGTAGAG) as primers and sequenced. A −1577 to −35 bp *HindIII-SalI* fragment was subcloned in front of the *GUS* marker gene in the vector pGUS1. A *HindIII-XmaI* fragment containing the *ETC2* promoter::*GUS* fusion was further subcloned into the *HindIII* and *XmaI* sites of pBAR-B.

For creating the *35S::ETC2* construct the *ETC2* cDNA was PCR amplified from the root cDNA library with TH-s2 primer (5′-TCTAGAGTAGTTATGGATAATACCAACC-GT) and TH-as2 primer. Error-free cDNA clone was used to create the *35S::ETC2* construct.

For *ETC2* expression analysis in the *etc2* mutant, RNA isolated from two-weeks old seedlings was used for semi-quantitative RT-PCR analysis, which was essentially performed as described previously (Kirik *et al*., 2002) using TH-s2 and TH-as2 primers. For comparative RT-PCR analysis ETC1, ETC2, TRY and CPC gene-specific primers were used for amplification using young leaves, roots, stems and inflorescence as RNA source. *ETC2* c DNA was amplified using 40 amplification cycles, for *ETC1*, *CPC* and *TRY* 35 amplification cycles were used. As a control, the ubiquitously expressed Arabidopsis *EF1αA4* gene (GenBank accession number X16432) was amplified using 20 amplification cycles.

Selection for plants carrying a T-DNA insertion in the *ETC2* gene was performed by PCR on genomic DNA using THW-s1 (5′-ACA GGTGAACTCCTTTGTCCGTCATATCA) and primer for the inserted T-DNA JL-202 (5′-CATT TTATAATAACGCTGCGGACATCTAC). Homozygous for T-DNA insertion plants were identified by PCR with the TH-s2 and TH-as2 primers.

For genomic rescue of the *try cpc etc2* mutants, a 3104 bp genomic fragment was amplified by PCR using *Pfu* DNA polymerase and primers THprom-s2 and TH-e2 (5′-TCCCGGGATGGGT AAAGAAATTACAG). Selected by sequencing genomic fragment without nucleotide changes was cloned into pBAR-B vector for plant transformation.

Cytological methods

GUS staining was performed as described previously (Vroemen *et al*., 1996). Images were processed using Adobe Photoshop 6.0 (Adobe Systems Inc., Mountain View, CA), and Aldus Freehand 10.0 (Aldus Corp., Seattle, WA) software.

Yeast two-hybrid assay

Fusions with the GAL4 activation domain and GAL4 DNA-binding domain were performed in the pACT and pAS plasmids (Clontech). For detection of protein-protein interactions yeasts were grown on a medium lacking histidine supplemented with 3 mM 3-Amino-1,2,4-triazole. TRY, CPC and ETC2 full length proteins were fused to the GAL4 DNA-binding domain in the pAS vector. GL3 protein was fused with the GAL4 activation domain in the pACT plasmid.

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