Mutual control of intracellular localisation of the patterning proteins AtMYC1, GL1 and TRY/CPC in Arabidopsis

Martina Pesch*, Ilka Schultheiß*, Simona Digiuni, Joachim F. Uhrig and Martin Hülskamp‡

SUMMARY
Trichome and root hair patterning is governed by a gene regulatory network involving TTG1 and several homologous MYB and bHLH proteins. The bHLH proteins GL3 and EGL3 are core components that serve as a regulatory platform for the activation of downstream genes. In this study we show that a homologue of GL3 and EGL3, AtMYC1, can regulate the intracellular localisation of GL1 and TRY. AtMYC1 protein is predominantly localised in the cytoplasm and can relocate GL1 from the nucleus into the cytoplasm. Conversely, AtMYC1 can be recruited into the nucleus by TRY and CPC, concomitant with a strong accumulation of TRY and CPC in the nucleus. When AtMYC1 is targeted to the nucleus or cytoplasm by nuclear localisation or export signals (NLS or NES), respectively, the intracellular localisation of GL1 and TRY also changes accordingly. The biological significance of this intracellular localisation is suggested by the finding that the efficiency of rescue of trichome number is significantly altered in NES and NLS fusions as compared with wild-type AtMYC1. Genetic analysis of mutants and overexpression lines supports the hypothesis that AtMYC1 represses the activity of TRY and CPC.

INTRODUCTION
The initiation of Arabidopsis thaliana trichomes serves as an excellent model system with which to study how cells are specified in a temporal and spatially defined manner by a gene regulatory network (Balkunde et al., 2010; Tominaña-Wada et al., 2011; Grebe, 2012). In wild type, trichomes are formed at the leaf basis of young leaves without a recognisable positional reference to other leaf structures (Hülskamp et al., 1994), and the analysis of genetic mosaics ruled out the possibility that the selection is based on cell lineage (Larkin et al., 1996; Schnittger et al., 1999). It is therefore postulated that trichome patterning is governed by cell-cell interactions between initially equivalent cells (Ishida et al., 2008; Pesch and Hülskamp, 2009; Balkunde et al., 2010).

In the current models of trichome patterning, a regulatory network of positively and negatively acting genes explains how trichomes are selected in a field of competent protodermal cells. The positive regulators are represented by a group of three proteins: the WD40 protein TRANSPARENT TESTA GLABRA 1 (TTG1) (Koornneef et al., 1982; Hülskamp et al., 1994), and the basic helix-loop-helix (bHLH)-like transcription factor GLABRA 1 (GL1) (Oppenheimer et al., 1981; Galway et al., 1994; Walker et al., 1999); the R2R3 MYB-related transcription factor GLABRA 1 (GL1) (Oppenheimer et al., 1991); and the basic helix-loop-helix (bHLH)-like transcription factor GLABRA 3 (GL3) (Koornneef et al., 1982; Hülskamp et al., 1994; Payne et al., 2000). GL1 and GL3 act partially redundantly with their homologues MYB23 and EGL3, respectively (Kirik et al., 2001; Zhang et al., 2003; Kirik et al., 2005). The negative regulators are a class of seven homologous R3 single-repeat MYB genes that act in a partially redundant manner (Schellmann et al., 2002; Kirik et al., 2004a; Kirik et al., 2004b; Wang et al., 2007; Tominaña et al., 2008; Wang et al., 2008; Wester et al., 2009; Wang et al., 2010; Gan et al., 2011). Among these, TRIPTYCHON (TRY) and CAPRICE (CPC) show the strongest single-mutant phenotypes (Hulskamp et al., 1994; Wada et al., 1997; Schellmann et al., 2002). TTG1, an R2R3MYB and a bHLH protein form a trimeric activator complex, in which TTG1 and the MYB both bind to the bHLH protein (Payne et al., 2000; Zhang et al., 2003; Zimmermann et al., 2004; Kirik et al., 2005; Digiuni et al., 2008; Gao et al., 2008; Wang and Chen, 2008; Zhao et al., 2008). The inhibition of the activator complex is governed by the binding of an R3MYB to the bHLH protein, thereby replacing the R2R3MYB (Payne et al., 2000; Bernhardt et al., 2003; Esch et al., 2003). The immediate downstream gene of this machinery promoting the differentiation of trichome cells is GLABRA 2 (GL2) (Rerie et al., 1994).

The activators and inhibitors of trichome patterning are engaged in a gene regulatory network (Benitez et al., 2007; Benitez et al., 2008), from which two principles acting in parallel can be extracted (Pesch and Hülskamp, 2009). First, the activator-inhibitor model explains pattern formation by a feedback loop between the activators and the inhibitors, in which the activators activate the inhibitors, which in turn repress the activators. The inhibitors mediate the cell-cell communication by intercellular movement. Second, the activator depletion model explains patterning by the removal of the mobile activator TTG1 in neighbouring cells through trapping by GL3 in trichome initials (Bouyer et al., 2008; Balkunde et al., 2011).

In addition to GL3 and EGL3, the bHLH gene AtMYC1 has been shown to participate in the regulation of trichome patterning (Symonds et al., 2011; Zhao et al., 2012). Formally, AtMYC1 acts as a positive regulator of trichome initiation because trichome number is reduced in mutants (Symonds et al., 2011; Zhao et al., 2012). Promoter swap experiments showed that GL3/EGL3 can replace AtMYC1, whereas AtMYC1 cannot rescue gl3 egl3 mutants, suggesting a redundant but also a divergent function (Zhao et al., 2012). The AtMYC1 gene was initially isolated in a screen for Antirrhinum DELILA homologues in Arabidopsis (Urao et al., 1996). Expression analyses suggest that AtMYC1 functions downstream of the patterning genes but upstream of GL2. Chromatin immunoprecipitation experiments have shown that
GL3/GL1 bind to the AtMYC1 promoter chromatin (Morohashi and Grotewold, 2009), suggesting transcriptional regulation of AtMYC1 by GL1 and GL3. In the root hair system, AtMYC1 is controlled by WER and CPC in a similar manner to that described for GL3/EGL3 (Bernhardt et al., 2005; Bruex et al., 2012). Its function upstream of GL2 was inferred from the finding that GL2 expression is reduced in atmyc1 mutants (Zhoa et al., 2012). The AtMYC1 protein has been grouped in a phylogenetic tree together with GL3, EGL3 and TT8 in subgroup IIIf of Arabidopsis bHLH proteins (Heim et al., 2003). Yeast two-hybrid assays revealed that, in addition to GL3, AtMYC1 protein also interacts with most of the other patterning proteins including the above-mentioned members CPC, TRY, TTG1, GL1 and MYB23 (Zimmermann et al., 2004; Tominaga et al., 2008; Zhao et al., 2012). However, in contrast to GL3 and EGL3, the AtMYC1 protein appears to be unable to form homo- or heterodimers with GL3/EGL3 (Zhoa et al., 2012).

Here, we explore the molecular basis of AtMYC1 function. We show that GL2, TRY and CPC expression patterns are unchanged in atmyc1 mutants and overexpression lines. In contrast to GL3 and EGL3, AtMYC1 protein is located in the cytoplasm. When AtMYC1 is co-expressed with GL1, GL1 is relocated into the cytoplasm. Co-expression of AtMYC1 with TRY or CPC leads to the recruitment of AtMYC1 into the nucleus, as well as to transport of TRY/CPC from the cytoplasm into the nucleus. Our genetic analysis indicates that AtMYC1 inhibits the function of TRY/CPC.

MATERIALS AND METHODS

Plant lines, growth conditions, transformation of plant material and phenotypic analysis

Plants were grown on soil at 24°C with 16 hours light/day. Plant transformations were performed by the floral dip method (Clough and Bent, 1998). We used the Col-0 wild type, gl3-3 gl3-3 (GK 545D05; SALK 077439) and atmyc1-1 (Salk 057383) to transform 35S:GL3, 35S:EGL3 and 35S:AtMYC1 in the respective backgrounds. 5'-AtMYC1:AtMYC1, 35S:YFP-AtMYC1, 35S:YFP-AtMYC1 and 35S:YFP-NES-AtMYC1 were transformed in Col-0 plants. 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants. The respective T2 lines of 5'-TRY:GUS, 5'-CPC:GUS and 5'-GL2:GUS were transformed in Col-0 plants.
RNA extraction and real-time PCR
Real-time PCR reactions were performed using the SYBR Green Real-Time PCR Master Mix (Applied Biosystems). To quantify RNA expression levels, published primers pairs were used: CPC, TRY primers (Morohashi et al., 2007), TRY' primers (Zhao et al., 2008). Actin primers were used as an endogenous control as previously described (Zhao et al., 2008). Three biological replicates, each including two technical replicas, were measured. Relative RNA levels were calculated according to the comparative Ct (2^ΔΔct) method.

Histochemistry and microscopy
GUS activity was assayed as described (Sessions et al., 1999). Light and fluorescence microscopy were performed using a Leica DMRE microscope (Leica Application Suite Version 3.7.0. Scanning electron microscope.)

Protein extraction and western blot
Transformed leaf material was homogenised in liquid nitrogen using a mortar and pestle. Leaf powder was incubated with lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl pH 8, 0.1% SDS, 10 mM DTT, Roche Complete Protease Inhibitor Cocktail (1 tablet/40 ml)) for 30 minutes on ice, centrifuged at 10,000 g for 15 minutes at 4°C, the supernatant denaturated by addition of 6× concentrated protein sample buffer (0.375 M Tris HCl pH 6.8, 12% SDS, 60% glycerol, 0.6 M DTT, 0.06% Bromophenol Blue) and heated for 15 minutes at 95°C. Samples were analysed by western blot using mouse anti-GFP IgG (Sigma, 1:50,000), both in 5% milk powder in PBS (PBS with 0.1% Tween 20). For detection of protein bands the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and the ImageQuant LAS 4000 (GE Healthcare) detection system were used.

RESULTS

Genetic analysis of AtMYC1
As recently described for three alleles (Symonds et al., 2011; Zhao et al., 2012), we found a strong reduction in trichome number in all five atmyc1 alleles tested in the Col wild-type background. When comparing the trichome number for each on the first two leaf pairs, we found a similar degree of reduction in all alleles independent of the T-DNA insertion position (supplementary material Table S1, Fig. S1A). We focused our analysis on the atmyc1-1 allele (SALK-057388), which does not produce a full-length mRNA and is therefore considered a null allele (supplementary material Fig. S1D).

Analysis of the trichome number in atmyc1-1 egl3 (Salk-019114) and atmyc1-1 gl3-3 double mutants compared with wild type showed subtle but statistically significant differences (Table 1). Since egl3 and gl3-3 single mutants also exhibit a reduced trichome number, this phenotype is considered additive. When we examined trichome branch number in the single and double mutants, we found no significant genetic effect on each other (Table 1).

Overexpression of AtMYC1 under the control of the CaMV 35S promoter resulted in an ~2-fold increase in trichome number in the Col background (supplementary material Fig. S2; Tables 2, 3). This raised the question of whether 35S:AtMYC1 can rescue the gl3 eg3 phenotype. We compared 35S:GL3, 35S:EGL3 and 35S:AtMYC1

Table 1. Trichome and branch number in atmyc1-1 single and double mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Trichomes per leaf pair</th>
<th>Four-branched trichomes per leaf pair (%)</th>
<th>Two-branched trichomes per leaf pair (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/2</td>
<td>3/4</td>
<td>1/2</td>
</tr>
<tr>
<td>Col</td>
<td>56.2±4.8</td>
<td>180.4±19.9</td>
<td>11.3±4.7</td>
</tr>
<tr>
<td>atmyc1-1</td>
<td>23.6±2.5</td>
<td>64.4±7.4</td>
<td>8.5±5.3</td>
</tr>
<tr>
<td>gl3-3</td>
<td>47.2±3.9</td>
<td>145.5±14.7</td>
<td>0</td>
</tr>
<tr>
<td>atmyc1-1 gl3-3</td>
<td>17.1±2.4</td>
<td>62.4±9.4</td>
<td>0</td>
</tr>
<tr>
<td>eg3-3</td>
<td>52.5±4.5</td>
<td>139.5±19.5</td>
<td>7.9±3.1</td>
</tr>
<tr>
<td>atmyc1-1 eg3-3</td>
<td>16.8±1.2</td>
<td>45.8±3.1</td>
<td>7.9±2.9</td>
</tr>
</tbody>
</table>

atmyc1-1, gl3-3, egl3-3, atmyc1-1 gl3-3 and atmyc1-1 eg3-3 were tested for significant difference to Col on leaves 1/2 or 3/4. atmyc1-1 egl3-3 was compared with atmyc1-1 eg3-3 and atmyc1-1 gl3-3 was compared with atmyc1-1 gl3-3. All values were significantly different in a Student’s t-test with P<10^-7, or in the case of a comparison of eg3-3 and Col on leaves 1/2 with P=0.0158. The means.d. is shown in each case (n=20).
with respect to their ability to rescue the gls egl3 trichome phenotype (Fig. 1A). We found full rescue with 35S:GL3, partial rescue with 35S:EG3, and no rescue with 35S:AtMYC1. This indicates that, despite its function as an activator of trichome development, AtMYC1 cannot replace GL3/EG3 function. This is consistent with the finding by Zhao and co-workers that AtMYC1 expression under control of the GL3 and EGL3 promoters cannot rescue the gls egl3 double mutant (Zhao et al., 2012). Conversely, 35S:GL3 or 35S:EG3 caused substantial overproduction of trichomes in the atmyc1-1 mutant background (Fig. 1B), whereas 35S:AtMYC1 showed a moderate rescue. This indicates that GL3 and EGL3 do not require AtMYC1 to induce extra trichome formation.

**pTRY:GUS, pCPC:GUS and pGL2:GUS expression is not altered in atmyc1-1 mutants**

The finding that AtMYC1 cannot replace GL3 and EGL3 function prompted us to assess the ability of AtMYC1 to regulate downstream genes. We analysed the temporal and spatial expression of pTRY:GUS, pCPC:GUS and pGL2:GUS in atmyc1-1 mutants and the corresponding Col background. At all leaf developmental stages we found indistinguishable expression patterns for pTRY:GUS, pCPC:GUS and pGL2:GUS in atmyc1-1 mutants and wild type (Fig. 2A-F; supplementary material Fig. S3A-F). Also, overexpression of AtMYC1 did not alter the expression pattern of pTRY:GUS, pCPC:GUS or pGL2:GUS (Fig. 2G-I; supplementary material Fig. S3G-I). To test whether the expression levels of TRY, CPC or GL2 are changed in atmyc1-1 mutants or AtMYC1 overexpression lines, we employed qPCR (Table 4). Although overexpression of AtMYC1 had no substantial influence on the expression levels, a slight but significant reduction was seen for AtMYC1 try-JC compared with 35S:AtMYC1 try-JC (Fig. 2A-F; supplementary material Fig. S3A-F). Also, The finding that GL3 or CPC do not require AtMYC1, and AtMYC1 expression is consistent with the finding by Zhao and co-workers that AtMYC1 function. This is with respect to their ability to rescue the gls egl3 trichome phenotype (Fig. 1A). We found full rescue with 35S:GL3, partial rescue with 35S:EG3, and no rescue with 35S:AtMYC1. This indicates that, despite its function as an activator of trichome development, AtMYC1 cannot replace GL3/EG3 function. This is consistent with the finding by Zhao and co-workers that AtMYC1 expression under control of the GL3 and EGL3 promoters cannot rescue the gls egl3 double mutant (Zhao et al., 2012). Conversely, 35S:GL3 or 35S:EG3 caused substantial overproduction of trichomes in the atmyc1-1 mutant background (Fig. 1B), whereas 35S:AtMYC1 showed a moderate rescue. This indicates that GL3 and EGL3 do not require AtMYC1 to induce extra trichome formation.

**Interactions of AtMYC1 with other patterning proteins**

Using the yeast two-hybrid method, AtMYC1 has been reported to interact with various proteins involved in trichome patterning, including ETC3 (Tominaga et al., 2008), GL1 and TTG1 (Symonds et al., 2011), WER and TRY (Zhao et al., 2012). The interactions with GL1, WER and TRY were confirmed by bimolecular fluorescence complementation (BiFC) (Zhao et al., 2012). We aimed to confirm and extend the characterisation of these interactions. Our yeast two-hybrid screens confirmed these interactions and also revealed, as expected, that AtMYC1 interacts with CPC (supplementary material Fig. S4). We further show that single amino acid exchanges (R/D) in the MYB-R/B-like bHLH transcription factor interface of the MYB factors (Zimmermann et al., 2004) strongly reduce the interaction of GL3 with GL1, TRY and CPC. These amino acid exchanges in the respective MYB factors also eliminate the interaction with AtMYC1. These data suggest that binding of AtMYC1 with the MYB factors occurs by a similar mechanism to that employed with GL3.

To provide independent support of the interaction between AtMYC1 and the other patterning proteins we used the luminescence-based mammalian interactome (LUMIER) assay (Barrios-Rodilés et al., 2005). Protein fusions to protein A (ProtA) or to luciferase were expressed in human HEC293TN cells. We immunoprecipitated the ProtA-tagged protein with IgG beads and quantified the co-immunoprecipitated proteins by luciferase assay. This confirmed the interaction of AtMYC1 with GL1, TRY, CPC and TTG1 (Table 5).

**Table 2. Trichome number and cluster frequency in try atmyc1 and try 35S:AtMYC1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Trichome number per leaf pair</th>
<th>Cluster (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/2</td>
<td>3/4</td>
</tr>
<tr>
<td>Col</td>
<td>62.5±6.3</td>
<td>241.0±31.3</td>
</tr>
<tr>
<td>try-JC</td>
<td>55.5±6.1</td>
<td>188.8±23.2</td>
</tr>
<tr>
<td>atmyc1-1</td>
<td>26.0±3.3</td>
<td>104.8±16.0</td>
</tr>
<tr>
<td>atmyc1-1 try-JC</td>
<td>36.0±5.5</td>
<td>115.5±15.3</td>
</tr>
<tr>
<td>35S:AtMYC1</td>
<td>127.4±14.9</td>
<td>492.4±51.1</td>
</tr>
<tr>
<td>35S:AtMYC1 try-JC</td>
<td>96.2±19.1</td>
<td>388.9±71.2</td>
</tr>
</tbody>
</table>

Trichome numbers of try-JC, atmyc1-1 try-JC, 35S:AtMYC1 and 35S:AtMYC1 try-JC were tested for significant difference to Col on leaf 1/2 and leaf 3/4. 35S:AtMYC1 try-JC was compared with try-JC and 35S:AtMYC1, and atmyc1-1 try-JC was compared with try-JC. All values were significant (Student’s t-test, P<10^-5). The mean±s.d. is shown in each case (n=20).

**Table 3. Trichome number and cluster frequency in cpc atmyc1 and cpc 35S:AtMYC1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Background</th>
<th>Trichome number per leaf pair</th>
<th>Cluster (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1/2</td>
<td>3/4</td>
</tr>
<tr>
<td>Col</td>
<td>Col</td>
<td>53.6±6.0</td>
<td>187.1±16.9</td>
</tr>
<tr>
<td>WS</td>
<td>WS</td>
<td>91.7±12.4</td>
<td>336.9±37.5</td>
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<tr>
<td>cpc-1</td>
<td>WS</td>
<td>126.6±15.0</td>
<td>449.6±59.1</td>
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<tr>
<td>cpc-1</td>
<td>WS/ Col</td>
<td>111.6±15.5</td>
<td>516.6±106.2</td>
</tr>
<tr>
<td>atmyc1-1</td>
<td>Col</td>
<td>23.6±2.9</td>
<td>81.6±11.0</td>
</tr>
<tr>
<td>atmyc1-1</td>
<td>WS/ Col</td>
<td>47.2±6.7</td>
<td>163.3±22.9</td>
</tr>
<tr>
<td>atmyc1-1 cpc-1</td>
<td>WS/ Col</td>
<td>42.8±6.6</td>
<td>194.5±38.5</td>
</tr>
<tr>
<td>35S:AtMYC1</td>
<td>Col</td>
<td>114.1±15.6</td>
<td>375.0±61.6</td>
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<tr>
<td>35S:AtMYC1 cpc-1</td>
<td>WS/ Col</td>
<td>106.7±10.3</td>
<td>393.5±32.2</td>
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<tr>
<td>35S:AtMYC1 cpc-1</td>
<td>WS/ Col</td>
<td>197.9±21.6</td>
<td>614.3±111.8</td>
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Trichome numbers of cpc-1, atmyc1-1 try-JC, 35S:AtMYC1 and 35S:AtMYC1 cpc-1 were tested for significant difference to Col and WS on leaves 1/2 and 3/4. 35S:AtMYC1 cpc-1 was compared with 35S:AtMYC1 (WS/Col) and cpc-1 (WS/Col), and atmyc1-1 cpc-1 was compared with cpc-1 and atmyc1-1. All values were significant (Student’s t-test, P<0.007). The mean±s.d. is shown in each case (n=20).
AtMYC1 is localised predominantly in the cytoplasm

We used transient expression assays to determine the intracellular localisation of AtMYC1. YFP-AtMYC1 localisation was studied in Agrobacterium-transfected tobacco cells. In contrast to YFP-GL3 (Fig. 3C), we found YFP-AtMYC1 fluorescence predominantly in the cytoplasm (Fig. 3B,E). This was unexpected for a bona fide transcription factor. We therefore studied the localisation behaviour in more detail. First, we confirmed the integrity of the YFP-AtMYC1 fusion protein in a western blot using a GFP antibody (Fig. 3L). We then analysed the localisation of AtMYC1 in four additional cell types: Arabidopsis protoplasts (Fig. 3H,I), Arabidopsis cotyledons (Fig. 3F), onion cells (Fig. 3K) and leek epidermal cells (supplementary material Fig. S5). In all tested cell types, AtMYC1 was localised predominantly in the cytoplasm. By contrast, GL3 was localised, as expected, in the nucleus (Fig. 3G,J). This visual impression was confirmed by quantifying the percentage of nuclear and cytoplasmic fluorescence (Tables 6, 7). To test whether the localisation of AtMYC1 depends on the construction of the fusion proteins, we also studied the C-terminal fusion of YFP to AtMYC1 (AtMYC1-YFP). Again, we found a predominantly cytoplasmic localisation (Fig. 3I). Finally, we excluded the possibility that fusion to the YFP protein affects localisation by showing that an N-terminal fusion with RFP (RFP-AtMYC1) is also found predominantly in the cytoplasm (Fig. 4, Table 6).

Nucleocytoplastic shuttling is cross-regulated by AtMYC1, GL1, TRY and CPC

Our finding that AtMYC1 localisation differs from that of its MYB interaction partners raised the question of whether AtMYC1 can recruit them from the nucleus into the cytoplasm or vice versa. We tested this by co-expressing RFP-AtMYC1 and YFP-GL3 or YFP-TRY in Arabidopsis cotyledon cells (Fig. 4). YFP-GL3 is localised mainly in the nucleus. In cells co-expressing RFP-AtMYC1 and YFP-GL3 we found YFP-GL3 in the cytoplasm (Fig. 4; supplementary material Fig. S5). Surprisingly, co-expression of RFP-AtMYC1 and YFP-TRY led to the localisation of RFP-AtMYC1 in the nucleus (Fig. 4). This was confirmed by a quantitative analysis using the YFP and RFP tags reciprocally to exclude biases caused by the tags (Tables 6, 7). The quantification revealed that the co-expression of TRY and AtMYC1 also caused an accumulation of TRY in the nucleus. When expressed alone, between 40% and 67.5% of the fluorescent protein fused to TRY was found in the cytoplasm. When co-expressed with AtMYC1, TRY protein was almost exclusively found in the nucleus (95.1%, 92.9%, see Tables 6 and 7). When combining RFP-AtMYC1 and YFP-CPC, we found similar, although slightly less drastic, localisation behaviours as with TRY (Table 7). The relocalisation was also seen in leek cells, demonstrating that this recruitment is not dependent on cell type (supplementary material Fig. S5).

Upon co-expression of AtMYC1, GL1 and TRY, we found all three in the nucleus (supplementary material Fig. S5), suggesting that TRY can counteract the AtMYC1-dependent recruitment of GL1 into the cytoplasm.

To further corroborate these findings, we studied the localisation of the GL1-AtMYC1, TRY-AtMYC1 and CPC-AtMYC1 interactions using BiFC assays. The TRY-AtMYC1 and CPC-AtMYC1 interactions occurred predominantly in the nucleus, whereas the GL1-AtMYC1 interaction took place in the cytoplasm (supplementary material Fig. S6).

We used the nuclear transportation trap (NTT) assay (Ueki et al., 1998) to independently test the nuclear transport behaviour of AtMYC1 in a more stringent manner. In the NTT assay the protein under consideration is expressed as a translational fusion to the artificial transactivator LexAD (LexA DNA-binding domain combined with GAL4AD transactivation domain) fused to a nuclear export signal (NES) from the HIV Rev protein. The NES can trigger the nuclear export of proteins lacking a nuclear localisation signal (NLS). A functional NLS is sufficient to overcome the NES-mediated nuclear export resulting in activation of the LEUCINE2 (LEU2) reporter gene. Using the NTT assay we found that AtMYC1, in contrast to GL3 (Balkunde et al., 2011), could not activate the LEU2 reporter, indicating that it does not contain a functional NLS. Significantly, additional expression of TRY or CPC led to the activation of LEU2, whereas co-expression of GL1 did...
combinations of overexpression lines and mutants. The AtMYC1 in Overexpression of that the atmyc1 double mutant showed an increased cluster frequency, indicating interactions between biologically relevant. To test this, we analysed the genetic whether the interaction with GL1 and/or TRY and CPC is recruited by TRY or CPC to the nucleus. This raises the question of recruiting it from the nucleus to the cytoplasm, and AtMYC1 is molecular level, AtMYC1 modulates the behaviour of GL1 by is a positive regulator of trichome development. At the AtMYC1 supernumerary trichomes in P atmyc1 cpc overexpressed AtMYC1 is independent of the presence of TRY. The trichome number, but no change in cluster frequency as compared The reduction in trichome number in atmyc1-1 mutants (Table 2). Thus, extra trichome initiation by GL1 try mutants caused an increase in cluster frequency. Lines overexpressing both GL1 and AtMYC1 showed a substantial increase in trichome number (Fig. 6C). Frequently, we found large clusters in which the outermost trichomes appeared smaller than at the centre, suggesting that they had been initiated later in leaf development (Fig. 6E). Overexpression of AtMYC1 has a similar genetic effect as the absence of TRY in cpc try and 35S:GL1 try mutant lines (Schnittger et al., 1998; Schellmann et al., 2002), suggesting that AtMYC1 is negative regulator of TRY.

Genetic interactions of AtMYC1 and MYB factors

The localisation of TRY and GL1 by AtMYC1 not activate the reporter. These findings confirm the results of our transient assays and support the conclusion that TRY and CPC can trigger nuclear transport whereas GL1 cannot direct AtMYC1 into the nucleus (Fig. 5).

Table 4. Relative mRNA expression levels of GL2, TRY and CPC in Col, atmyc1-1 and 3SS:AtMYC1 backgrounds quantified by real-time PCR

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GL2</th>
<th>TRY</th>
<th>CPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>1.00±0.00</td>
<td>1.00±0.00</td>
<td>1.00±0.00</td>
</tr>
<tr>
<td>atmyc1-1</td>
<td>0.44±0.06</td>
<td>1.00±0.53</td>
<td>0.71±0.22</td>
</tr>
<tr>
<td>3SS:AtMYC1</td>
<td>1.01±0.34</td>
<td>0.98±0.13</td>
<td>1.05±0.35</td>
</tr>
</tbody>
</table>

Expression levels in atmyc1-1 and 3SS:AtMYC1 were normalised to wild-type (Col) expression. ACTIN expression was used as an endogenous control. The standard deviation of the three biological replicates is shown (n=3).

Genetic interactions of AtMYC1 and MYB factors

**Table 5. LUMIER assays to analyse the interaction of GL3 and AtMYC1 with different patterning proteins**

<table>
<thead>
<tr>
<th>Fusion</th>
<th>Luc-GL3</th>
<th>Luc-AtMYC1</th>
<th>Luc*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProtA-GL1</td>
<td>49.7±3.1</td>
<td>15.4±0.8</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>ProtA-TRY</td>
<td>52.2±5.2</td>
<td>64±13.2</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>ProtA-CPC</td>
<td>47.2±4.0</td>
<td>33.4±15.8</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>ProtA-TTG1</td>
<td>10.6±1.5</td>
<td>19.9±4.3</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>ProtA*</td>
<td>0.4±0.1</td>
<td>0.4±0.0</td>
<td>0.1±0.0</td>
</tr>
</tbody>
</table>

Interaction was assessed using GL3 and AtMYC1 Renilla reniformis luciferase (Luc) fusions and protein A (ProtA) fusions of the patterning proteins. The proteins were co-expressed in human cells (HEK293TN) and co-immunoprecipitated with IgG Dynabeads.

Data are means±s.d. (n=3).

*Empty vector without CDS fusion.
Data are mean±s.d. (n=3).

*Empty vector without CDS fusion.

Table 6. The percentage of nuclear localised RFP-AtMYC1, YFP-GL1 and YFP-TRY depends on co-expression partners

<table>
<thead>
<tr>
<th>Co-bombarded constructs</th>
<th>Nuclear localised protein (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFP-AtMYC1</td>
<td>GFP-YFP</td>
<td>4.6±2.7</td>
</tr>
<tr>
<td>RFP-AtMYC1</td>
<td>YFP-GL1Δ27</td>
<td>8.4±2.3</td>
</tr>
<tr>
<td>RFP-AtMYC1</td>
<td>YFP-TRY</td>
<td>90.3±8.4</td>
</tr>
<tr>
<td>RFP*</td>
<td>YFP-GL1Δ27</td>
<td>7.8±3.5</td>
</tr>
<tr>
<td>RFP*</td>
<td>YFP-TRY</td>
<td>4.6±2.7</td>
</tr>
</tbody>
</table>

Data are mean±s.d. (n=3).

*Empty vector without CDS fusion.

In a second set of experiments, we constitutively expressed YFP-AtMYC1, YFP-NES-AtMYC1 and YFP-NLS-AtMYC1 in atmyc1 mutants and assessed the rescue of trichome number. As individual transformants may show a wide range of phenotypes, we studied at least 50 T1 plants (Pesch and Hülskamp, 2011) in order to catch the transformants may show a wide range of phenotypes, we studied at least 50 T1 plants (Pesch and Hülskamp, 2011) in order to catch the transformants may show a wide range of phenotypes, we studied at least 50 T1 lines; Wilcoxon signed-rank test, P=0.02. Conversely, the 35S:YFP-NLS-AtMYC1 construct rescued the atmyc1-1 mutant significantly better (176.5±81.5; n=50 T1 lines; Wilcoxon signed-rank test, P=0.005). Together, these data show that changing the intracellular localisation of AtMYC1 changes its biological activity.

**DISCUSSION**

*AtMYC1* is one of the genes regulating trichome and root hair patterning in *Arabidopsis thaliana*. It belongs to the large family of...
bHLH factors in Arabidopsis, which contains many key regulators of plant development. A phylogenetic analysis of the bHLH domains of 133 bHLH proteins placed AtMYC1 in subgroup IIIf (Heim et al., 2003). In addition to AtMYC1, this subgroup contains three further members, GL3, EGL3 and TT8, which are all involved in TTG1-dependent pathways related to trichome, root hair and seed coat development and anthocyanin production (Broun, 2005; Koes et al., 2005; Ramsay and Glover, 2005; Lepiniec et al., 2006; Serna and Martin, 2006; Balkunde et al., 2010; Feller et al., 2011; Tominaga-Wada et al., 2011). In support of this phylogenetic classification, several observations have suggested that AtMYC1 acts redundantly with GL3 and EGL3 in the root hair patterning system (Bruex et al., 2012) and in the context of trichome patterning (Zhao et al., 2012). In addition, AtMYC1 shows interactions with other proteins, similar to those reported for GL3, EGL3 and TT8, in pulldown experiments (this study). Moreover, AtMYC1 was consistently found in the cytoplasm of cells from four different plant species (tobacco, onion, leek and Arabidopsis) with N- and C-terminal YFP fusions and with an RFP fusion. We confirmed our results with the yeast NTT system, which is specifically designed to test the nuclear transport of proteins. Thus, our results consistently show that AtMYC1 predominantly localises in the cytoplasm. Misllocalisation of AtMYC1 protein, either to the cytoplasm or to the nucleus, showed that the correct localisation of the protein is important for its regulatory function. It is interesting to note that AtMYC1 contains a monopartite NLS sequence at its N-terminus that is predicted to target the protein predominantly to the nucleus with a similar efficiency as that predicted for GL3 (supplementary material Table S4). This suggests that the AtMYC1 NLS sequence is masked under normal conditions either by protein folding or binding to additional factors, which are likely to be general factors as the cytoplasmic localisation was observed in various species and cell types.

Genetic interpretation of the nucleocytoplasmic shuttling of AtMYC1, GL1 and TRY/CPC

Our observations of the quantitative relocalisation of AtMYC1, TRY/CPC and GL1 are based on the overexpression of the respective proteins. Therefore, we do not know the relative distribution of these proteins under native conditions. Our attempts to study this in planta have thus far failed because AtMYC1, TRY or GL1 fusions with YFP or RFP were below the detection limit when expressed under the endogenous promoters. Nevertheless, it is evident that the four proteins have the property to balance each
other’s localisation. Given that GL1 and TRY/CPC are involved in the transcriptional regulation of other genes, it is likely that their relocalisation changes their activity: the recruitment of AtMYC1 by TRY/CPC and concomitant accumulation of TRY/CPC in the nucleus reflects the fact that AtMYC1 binding to TRY/CPC can modulate the balance between nuclear and cytoplasmic TRY/CPC protein. The \textit{in vivo} net effect is likely to be a suppression of both TRY and CPC activity. This is suggested by several genetic observations. If AtMYC1 represses TRY and/or CPC one would expect lines overexpressing \textit{AtMYC1} to share phenotypic aspects of \textit{try} and \textit{cpc} mutants. A repression of CPC activity is consistent with an increased trichome number in \textit{35S:AtMYC1} lines. A repression of TRY activity by AtMYC1 could explain the substantial increase in trichome number and the slightly increased production of trichome clusters in \textit{35S:AtMYC1 cpc} and the large trichome clusters in \textit{35S:AtMYC1 35S:GL1} lines because this phenotype is reminiscent of the \textit{35S:GL1 try} phenotype (Schnittger et al., 1998; Szymanski and Marks, 1998). Also, the decrease in trichome number by 50\% compared with the wild type in the \textit{atmyc1} mutant can be interpreted as being caused by enhanced activity of CPC or TRY. The recruitment of GL1 by AtMYC1 from the nucleus into the cytoplasm is likely to impair the activity of GL1.
However, such a repressive role of AtMYC1 on GL1 is not consistent with our genetic finding that the reduced trichome number phenotype of 35S:GL1 plants is rescued in a 35S:AtMYC1 background. This suggests that the impact of AtMYC1 on the localisation of GL1 has little biological relevance or that the impact on TRY/CPC overwrites this effect. The latter is consistent with the observation that the relocation of GL1 by AtMYC1 is counteracted in the presence of TRY protein.

**Integrating AtMYC1 into the patterning gene network**

How is AtMYC1 integrated in the gene regulatory network? A putative repressive function of AtMYC1 on TRY/CPC is difficult to functionally integrate into existing models. One attractive possibility for its function can be inferred from the finding that the AI5MYC1 promoter is a direct target of the GL1-GL3/EGL-TTG1 activator complex (Morohashi and Grotewold, 2009). This suggests that the activator complex, and therefore also GL1, might activate the expression of AtMYC1 as well as of TRY/CPC. On top of this regulation at the transcriptional level we propose that the protein activities of TRY/CPC and GL1 are coupled through AtMYC1. As AtMYC1 can recruit GL1 from the nucleus into the cytoplasm and TRY/CPC from the cytoplasm into the nucleus the regulatory effect should be antagonistic (Fig. 8). In our model, the concentration of GL1 in the nucleus is reduced in the presence of AtMYC1, whereas the concentration of TRY in the nucleus is increased. It is possible that, in addition to this cell-autonomous effect, a reduced level of cytoplasmic TRY/CPC would lead to reduced lateral movement and therefore to less repression in the neighbouring cells. Consistent with this scenario, the distance between trichomes is markedly increased in atmyc1 mutants as compared with wild type. This type of negative-feedback loop could serve not only to fine-tune the network but would also provide an additional mechanism to modulate lateral repression.

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**Competing interests statement**

The authors declare no competing financial interests.

**Author contributions**

M.P., I.S. and S.D. designed and performed experiments; J.F.U. designed experiments; and M.H. designed experiments and wrote the manuscript.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.094698/-/DC1

**References**


Fig. S1. Characterisation and complementation of the atmyc1-1 mutant with 5’-AtMYC1 (1963 bp) and AtMYC1 CDS. (A-C) Binocular images as z-stacks of leaf three from (A) atmyc1-1, (B) Col, (C) 5’-AtMYC1:AtMYC1 atmyc1-1 (T2 line #1) plants. (D) RT-PCR of cDNA extracted from 14-day-old rosette leaves from atmyc-1-1 (1), Col (2) and PCR on genomic DNA of Col (3).

Fig. S2. Effect of atmyc1-1 mutation or AtMYC1 overexpression on trichome production in different Arabidopsis thaliana trichome patterning mutant backgrounds. Leaf four of 16-day-old plants analysed by light microscopy (z-stack overlay of binocular images). Scale bars: 1 mm.
Fig. S3. Expression analysis of TRY, CPC and GL2 in young leaves of *atmyc1-1* and *35S:AtMYC1* mutants. (A-I) Expression of *pGL2:GUS* (A,D,G), *pTRY:GUS* (B,E,H) and *pCPC:GUS* (C,F,I) was analysed in Col (A-C), *atmyc1-1* (D-F) and *35S:AtMYC1* (G-I) in rosette leaf four of 10-day-old plants. GUS staining was performed for 16 hours.

Fig. S4. Yeast two-hybrid interactions of AtMYC1 with trichome patterning proteins. Yeast two-hybrid assays with Gal4-AD-GL3, Gal4-AD-AtMYC1 and Gal4-AD and Gal4-BD-GL1Δ27 (1), Gal4-BD-GL1Δ27-R97D (2), Gal4-BD-TRY (3), Gal4-BD-TRY-R58D (4), Gal4-BD-CPC (5), Gal4-BD-CPC-R63D (6), Gal4-BD-TTG1 (7) and Gal4-BD-TTG1Δ26 (8). The constructs were selected on media lacking leucine and tryptophan (data not shown). Interaction leads to expression of the reporter (*HIS3*), which enables growth on medium lacking histidine supplemented with 30 mM 3-aminotriazol (3-AT). 1Empty control vector without CDS fusion.
Fig. S5. Colocalisation studies of YFP-AtMYC1, RFP-TRY and CFP-GL1 in leek leaf cells. Leek cells were transiently transformed by particle bombardment and analysed by fluorescence microscopy. For each combination, the YFP, RFP and CFP channels are shown. Scale bars: 50 μm.

Fig. S6. BiFC analysis of GL1/AtMYC1, TRY/AtMYC1 and CPC/AtMYC1 interactions. For each combination, the RFP (left) and YFP (middle) channels and a merge (right) are shown. Note that interactions of TRY and CPC are found only in the nucleus, whereas the RFP signal is found in the cytoplasm and the nucleus.
Table S1. Trichome number of wild type and different *atmyc1* alleles

<table>
<thead>
<tr>
<th><em>atmyc1</em> allele</th>
<th>T-DNA position</th>
<th>Trichome number per leaf pair ‡ (mean±s.d.)</th>
<th>1/2</th>
<th>3/4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salk 006354*</td>
<td>5′-promoter</td>
<td></td>
<td>31.6±2.9</td>
<td>109.4±15.2</td>
</tr>
<tr>
<td>Salk 057388 <em>(atmyc1-I)</em></td>
<td>Intron 2</td>
<td></td>
<td>29.7±4.4</td>
<td>98.7±16.2</td>
</tr>
<tr>
<td>Gabi 621-F09*</td>
<td>Intron 2</td>
<td></td>
<td>34.4±4.7</td>
<td>107.6±12.8</td>
</tr>
<tr>
<td>Sail_277_H01*</td>
<td>Intron 3</td>
<td></td>
<td>28.6±2.1</td>
<td>93.5±15.2</td>
</tr>
<tr>
<td>Salk 056899*</td>
<td>Intron 6</td>
<td></td>
<td>29.8±4.0</td>
<td>100.1±11.4</td>
</tr>
</tbody>
</table>

‡*n*=20.

*All myc1 alleles were tested for significant differences to Col on either leaves 1/2 or leaves 3/4 using Student's *t*-test. All datasets were significantly different (P<10^-17).

---

Table S2. Trichome number and cluster frequency in *cpc-2 atmyc1* and *cpc-2 35S:AtMYC1*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Trichome number per leaf pair ‡ (mean±s.d.)</th>
<th>% cluster ‡ (mean±s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/2</td>
<td>3/4</td>
</tr>
<tr>
<td>Col</td>
<td>121.4±14.5</td>
<td>320.5±53.5</td>
</tr>
<tr>
<td><em>cpc-2</em></td>
<td>173.6±22.8</td>
<td>448.3±73.3</td>
</tr>
<tr>
<td><em>atmyc1-1</em></td>
<td>38.0±5.7</td>
<td>126.3±18.3</td>
</tr>
<tr>
<td><em>atmyc1-1cpc-2</em></td>
<td>71.2±11.9</td>
<td>215.7±31.1</td>
</tr>
<tr>
<td><em>35S:AtMYC1</em></td>
<td>178.8±19.3</td>
<td>572.5±50.4</td>
</tr>
<tr>
<td><em>35S:AtMYC1 cpc-2</em></td>
<td>257.0±31.4</td>
<td>741.2±89.9</td>
</tr>
</tbody>
</table>

‡*n*=20.

Trichome numbers of *cpc-2, atmyc1-1, atmyc1-1cpc-2, 35S:AtMYC1* and *35S:AtMYC1 cpc-2* were tested for significant difference to Col on leaf 1/2 and leaf 3/4. *35S:AtMYC1 cpc-2* was compared with *35S:AtMYC1* and *cpc-2*, and *atmyc1-1cpc-2* was compared with *atmyc1-1* and *cpc-2*. All values were significant (Student’s *t*-test, P<10^-6).
Table S3. Primers

RT-PCR  AtMYC1

- cBHLH12-for ATGTCTTTGACAATGGCTGATGGTGTAGAAG
- cBHLH12-rev TTAAAACAAATCAACCAATGACTTCTTCTAGCTTC

RT-PCR  ACTIN7

- MM 53ACT7FOR TGCTCTTCTCATTGCTATCCTTC
- MM 54ACT7REV CACGAACCAGATAAAGACAGAC

PCR-based site-directed mutagenesis of GL1, TRY and CPC cDNAs

- MP-GL1-Leu/Ala-for ATTCGTCTCCACAAGGCACTCGGCAATAGATG
- MP-GL1-Leu/Ala-rev AATGAGGTCTTCTTCTTGTTCAGTGAAATTGCC
- MP-GL1-Arg/Ala-for GATGGTCTTTGATAGCTAAAGCAGTACCGGGAAG
- MP-GL1-Arg/Asp-for GATGGTCTTTGATAGCTAAAGATGTACCGGGAAG
- MP-GL1-Arg/Asp-rev TATTGCCGAGGAGCTTGTGGAGACGAATAATG
- MP-TRY-Leu/Ala-for ATCTTTCGAATGTACAGAGCAGTCGGTGATAG
- MP-TRY-Leu/Ala-rev GAGATCTTCTTCTTGTTCAGTCATGTTGATAAAC
- MP-TRY-Arg/Ala-for GATTTGATAGCAGGAGCAGTTCCTGGAAGAC
- MP-TRY-Arg/Asp-for GATTTGATAGCAGGAGATGTTCCTGGAAGAC
- MP-TRY-Arg/Asp-rev CCACCTATCACCGACAAGTCTGTACATTCG
- MP-CPC-Leu/Ala-for CTCGGATGTATAAAGCAGTTGGCGACAG
- MP-CPC-Leu/Ala-rev AAATGAGATCTTCTTCTTCTTCTGACATCTTCAC
- MP-CPC-Arg/Ala-for GAGTTGATCGCCGGAGCAATCCCGGG
- MP-CPC-Arg/Asp-for GAGTTGATCGCCGGAGATATCCCGGG
- MP-CPC-Arg/Asp-rev CCACCTGTCGCCAACGAGTTTATACATC

PCR-based AtMYC1-w/o-Stop-pDONR201

Deletion of the stop codon in AtMYC1-pDONR201

- bhlh12-for-attB1 GGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCTTTGACAATGGCTGATGGTGTAGAAG
- MP-BHLH12-cDNA-ost-rev-attB2 GGGGACCACTTTGTACAAGAAGCTGGGTCTTTGACAATGGCTGATGGTGTAG

PCR-based NLS-AtMYC1-pDONR201 and NES-AtMYC1-pDONR201

Insertion of the BamHI and XhoI restriction sites in AtMYC1-pDONR201

- BHLH12-BamHI-XhoI-for GGGGACCAAGTTTGTACAAAAAAGCAGGCTCCATGTCTTTGACAATGGCTGATGG
- BHLH12-rev-attB1 TAAAGCTTTTAAAACAAATCACCAATGACTCTTC

Insertion of the NLS and NES in BamHI-XhoI-AtMYC1-pDONR201

- BamHI-NLS-XhoI-for gatecTCGAGCCTAAGAAGAAGAAGAGGTTGGAGGAc
- BamHI-NLS-XhoI-rev tcgatTCCTCCAAACCTTCTTCTTCTTCTTCTTCTGCTGCAAG

- BamHI-NES-XhoI-for gatecTCGAGCAGTAGCTTCTTACATGGGACTTGGAATTAACCAAGAC
- BamHI-NES-XhoI-rev tcgatTCCTCCAGTCTTTGTTAATATCAAGCTCAGCAGAAAACAAATGCAATGGCAG

PCR-based 5'-AtMYC1-pAM-PAT

- MP-5'-BHLH12-for-AscI NGGCCGCACCTGAAAACAACTAAATACAGTCAAGAC
- MP-5'-BHLH12-for-XhoI NCTCAGATCTCAGATATCGTGAGAAAGCTGACCTCAGT

PCR-based SalI-AtMYC1-HindIII-pGEM-T-easy

1. BamHI-AtMYC1-HindIII-pGEM-T-easy

- MP-BHLH12-BamHI-for TAGGATCCATGGCTGACATGGCTGATGG
- MP-BHLH12-HindIII-rev TAAAGCTTTTAAAACAAATCAACCAATGACTTCTC

2. Exchange of BamHI for SalI to create SalI-AtMYC1-HindIII-pGEM-T-easy

- MP-BamHImut-Sall-Linker gateggtcgcacc
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<th>Predicted bipartite NLS</th>
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*Scores of 8-10 predict an exclusively nuclear localized protein. Values of 7 or 8 indicate a partial nuclear localization. Scores between 3 and 5 predict localization in the nucleus and the cytoplasm, whereas values between 1 and 2 indicate a cytoplasmic localizing protein.

cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi).