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.

KEY WORDS: Arabidopsis thaliana, GL1, MYC1, TRY, Patterning, Trichome

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; Tominaga-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), the basic helix-loop-helix (bHLH)-like transcription factor GLABRA 2 (GL2) (Rerie et al., 2008), 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 (Kirk 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; Tominaga 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 (Hülskamp 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
GL2 was inferred from the finding that GL2 expression is reduced in atmyc1 mutants (Zha et al., 2012). The AtMYC1 protein has been grouped in a phylogenetic tree together with GL3, EGL3 and TT8 in subgroup III 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 (Zhao 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, gl3-3egl3-3 (GK 545D05; SALK 077439) and atmyc1-1 (Salk 057388) to transform 35S:GL3, 35S:EGL3 and 35S:AtMYC1 in the respective backgrounds. 5’-AtMYC1:AtMYC1, 35S:YFP-AtMYC1, 35S:YFP-NLS-AtMYC1 and 35S:YFP-NES-AtMYC1 were transformed in atmyc1-1 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 into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants. The respective T2 lines of 5’-TRY:GUS, 5’-CPC:GUS and 5’-GL2:GUS were transformed into Col-0 plants.
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±3.5</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±4.9</td>
<td>7.9±3.1</td>
</tr>
<tr>
<td>egl3-3</td>
<td>52.5±4.5</td>
<td>139.5±19.5</td>
<td>7.9±2.9</td>
</tr>
<tr>
<td>atmyc1-1 egl3-3</td>
<td>16.8±1.2</td>
<td>45.8±3.1</td>
<td>18.5±6.5</td>
</tr>
</tbody>
</table>

atmyc1-1, gl3-3, egl3-3, atmyc1-1 gl3-3 and atmyc1-1 egl3-3 were tested for significant difference to Col on leaves 1/2 or 3/4. atmyc1-1 egl3-3 was compared with atmyc1-1 egl3-3 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<10⁻², or in the case of a comparison of egl3-3 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 gl3 egl3 trichome phenotype (Fig. 1A). We found full rescue with 35S:GL3, partial rescue with 35S:EGL3, and no rescue with 35S:AtMYC1. This indicates that, despite its function as an activator of trichome development, AtMYC1 cannot replace GL3/EGL3 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 gl3 egl3 double mutant (Zhao et al., 2012). Conversely, 35S:GL3 or 35S:EGL3 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. 5A-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. 5G-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 CPC and GL2 in the atmyc1-1 mutant. The latter is consistent with the findings of a previous study (Zhao et al., 2012).

**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-Rodilles et al., 2005). Protein fusions to protein A (ProtA) or to luciferase were expressed in human HEK293TN 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>
<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></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>
</tr>
<tr>
<td>cpc-1</td>
<td>WS</td>
<td>126.6±15.0</td>
<td>449.6±59.1</td>
</tr>
<tr>
<td>atmyc1-1</td>
<td>WS/Col</td>
<td>111.6±15.5</td>
<td>516.6±106.2</td>
</tr>
<tr>
<td>try-JC</td>
<td>WS/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>35S:AtMYC1</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>
</tr>
<tr>
<td>35S:AtMYC1</td>
<td>WS/Col</td>
<td>106.7±10.3</td>
<td>393.5±32.2</td>
</tr>
<tr>
<td>35S:AtMYC1</td>
<td>cpc-1</td>
<td>197.9±21.6</td>
<td>614.3±111.8</td>
</tr>
</tbody>
</table>

Trichome numbers of cpc-1 (WS/Col), atmyc1-1 (WS/Col), 35S:AtMYC1 (WS/Col) and 35S:AtMYC1 cpc-1 (WS/Col) were tested for significant difference to Col and WS on leaves 1/2 and 3/4. 35S:AtMYC1 cpc-1 (WS/Col) 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 means ±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. Therefore, we conducted experiments to test 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). Then, we examined 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 evaluated 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).

Nucleocytoplasmic 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-GL1 or YFP-TRY in Arabidopsis cotyledon cells (Fig. 4). YFP-GL1 is localised mainly in the nucleus. In cells co-expressing RFP-AtMYC1 and YFP-GL1, we found YFP-GL1 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 determine 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 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...
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).

**Genetic interactions of AtMYC1 and MYB factors**

The reduction in trichome number in atmyc1-1 mutants and the supernumerary trichomes in 35S:AtMYC1 lines indicate that AtMYC1 is a positive regulator of trichome development. At the molecular level, AtMYC1 modulates the behaviour of GL1 by recruiting it from the nucleus to the cytoplasm, and AtMYC1 is recruited by TRY or CPC to the nucleus. This raises the question of whether the interaction with GL1 and/or TRY and CPC is biologically relevant. To test this, we analysed the genetic interactions between AtMYC1, GL1, CPC and TRY using combinations of overexpression lines and mutants. The try atmyc1 double mutant showed an increased cluster frequency, indicating that the atmyc1 mutant enhances the try cluster phenotype (Table 2). Overexpression of AtMYC1 in try mutants caused an increase in trichome number, but no change in cluster frequency as compared with try mutants (Table 2). Thus, extra trichome initiation by overexpressed AtMYC1 is independent of the presence of TRY. The atmyc1 cpc double mutants showed essentially additive phenotypes.

This experiment was performed with cpc1-1 in the Ws background (Table 3) and repeated with the cpc1-2 allele in the Col background in order to exclude the possibility that a quantitative trait locus (QTL) modifies the trichome patterning phenotype in the double mutant (supplementary material Table S2). Overexpression of AtMYC1 in cpc mutants caused a slight 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 a negative regulator of TRY.

**Targeting AtMYC1 into and out of the nucleus by NLS and NES fusions**

In order to test whether the intracellular localisation of AtMYC1 is biologically relevant, we created N-terminal fusions with an NLS (Kalderon et al., 1984) and with an NES (Wen et al., 1995; Matsushita et al., 2003). We first tested the intracellular localisation of the respective YFP-tagged fusion proteins by particle bombardment in leaf cells. As expected, YFP-NES-AtMYC1 was

### Table 4. Relative mRNA expression levels of GL2, TRY and CPC in Col, atmyc1-1 and 35S: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>35S:AtMYC1</td>
<td>1.01±0.34</td>
<td>0.98±0.13</td>
<td>1.05±0.35</td>
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</tbody>
</table>

Expression levels in atmyc1-1 and 35S: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).

*GL2 expression was significantly reduced in atmyc1-1 mutants (Student’s t-test, P<0.0004).

*CPC expression was significantly reduced in atmyc1-1 mutants (Student’s t-test, P<0.04).

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

<table>
<thead>
<tr>
<th>Protein</th>
<th>Luc-GL3 (%)</th>
<th>Luc-AtMYC1 (%)</th>
<th>Luc* (%)</th>
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<tbody>
<tr>
<td>ProtA-GL1</td>
<td>49.7±13.1</td>
<td>15.4±0.8</td>
<td>0.0±0.0</td>
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<tr>
<td>ProtA-TRY</td>
<td>52.2±5.2</td>
<td>64.4±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>
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<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.
found in the cytoplasm and YFP-NLS-AtMYC1 in the nucleus (Fig. 7). YFP-NLS-AtMYC1 and RFP-TRY co-expression resulted in an accumulation of both fusion proteins in the nucleus (Fig. 7). In cells co-expressing YFP-NES-AtMYC1 and RFP-TRY we found the RFP signal in the nucleus and the cytoplasm, rather than predominantly in the nucleus (Fig. 7). The co-expression of YFP-NLS-AtMYC1 or YFP-NES-AtMYC1 with CFP-GL1 led to an accumulation of CFP in the nucleus or cytoplasm, respectively (Fig. 7). Together, these data indicate that the mislocalisation of AtMYC1 triggers a change in the intracellular localisation of TRY and GL1.

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

### 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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RFP</td>
</tr>
<tr>
<td>RFP-AtMYC1 YFP</td>
<td>4.6±2.7</td>
</tr>
<tr>
<td>RFP-AtMYC1 YFP-GL1Δ27</td>
<td>8.4±7.3</td>
</tr>
<tr>
<td>RFP-AtMYC1 YFP-TRY</td>
<td>90.3±4.8</td>
</tr>
<tr>
<td>RFP* YFP-GL1Δ27</td>
<td>7.8±3.5</td>
</tr>
<tr>
<td>RFP* YFP-TRY</td>
<td>4.6±2.7</td>
</tr>
</tbody>
</table>

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

### DISCUSSION

AtMYC1 is one of the genes regulating trichome and root hair patterning in Arabidopsis thaliana. It belongs to the large family of atmyc1-1 lines we found an average of 151.3±92 trichomes on the first four leaves (n=50 T1 lines). Compared with 35S:YFP-AtMYC1, the 35S:YFP-NES-AtMYC1 construct exhibited significantly less efficient rescue of atmyc1-1 (112.1±44.5; n=51 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.

### Table 7. The percentage of nuclear localised YFP-AtMYC1, RFP-GL1 and RFP-TRY depends on co-expression partners

<table>
<thead>
<tr>
<th>Co-bombarded constructs</th>
<th>Nuclear localised protein (%)</th>
<th>YFP</th>
<th>RFP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YFP-AtMYC1 RFP</td>
<td>6.7±1.8</td>
<td>15.2±3.5</td>
<td>34</td>
</tr>
<tr>
<td>YFP-AtMYC1 RFP-GL1</td>
<td>6.5±3.5</td>
<td>7.3±3.2</td>
<td>25</td>
</tr>
<tr>
<td>YFP-AtMYC1 RFP-GL1Δ27</td>
<td>5.3±1.8</td>
<td>9.6±3.5</td>
<td>21</td>
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<tr>
<td>YFP-AtMYC1 RFP-TRY</td>
<td>97.4±8.9</td>
<td>92.9±8.8</td>
<td>21</td>
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<tr>
<td>YFP-AtMYC1 RFP-CPC</td>
<td>87.1±20.6</td>
<td>72.7±19.5</td>
<td>23</td>
</tr>
<tr>
<td>YFP* RFP</td>
<td>25.0±3.5</td>
<td>25.9±4.3</td>
<td>32</td>
</tr>
<tr>
<td>YFP* RFP-GL1Δ27</td>
<td>30.7±8.7</td>
<td>96.4±3.2</td>
<td>22</td>
</tr>
<tr>
<td>YFP* RFP-TRY</td>
<td>24.9±5.0</td>
<td>55.4±10.2</td>
<td>27</td>
</tr>
<tr>
<td>YFP* RFP-CPC</td>
<td>19.5±3.7</td>
<td>40.0±8.8</td>
<td>20</td>
</tr>
<tr>
<td>YFP* RFP</td>
<td>29.3±6.1</td>
<td>37.2±6.8</td>
<td>24</td>
</tr>
</tbody>
</table>

Data are means±s.d. (n=3).
*Empty vector without CDS fusion.
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 *Arabidopsis* cotyledons. For each combination, the RFP (left) and YFP (middle) channels and a merge (right) are shown. *Arabidopsis* leaves were transformed biolistically and analysed by confocal microscopy. Scale bars: 50 μm.

**Fig. 4.** Subcellular colocalisation of the AtMYC1 and MYB transcription factors in *Arabidopsis* cotyledons. For each combination, the RFP (left) and YFP (middle) channels and a merge (right) are shown. *Arabidopsis* leaves were transformed biolistically and analysed by confocal microscopy. Scale bars: 50 μm.

A possible molecular function can be deduced from the intracellular localisation of *AtMYC1* protein. In contrast to a previous study (Zhao et al., 2012), we found *AtMYC1* predominantly in the cytoplasm. As the *AtMYC1* CDS was successfully used for the rescue experiments and shows the expected protein-protein interactions in yeast and in pulldown assays, the *AtMYC1* sequence used in this study is fully functional. *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 *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 *AtMYC1* to share phenotypic aspects of *try* and *cpc* mutants. A repression of CPC activity is consistent with an increased trichome number in 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 35S:*AtMYC1* *cpc* and the large trichome clusters in 35S:*AtMYC1* 35S:*GL1* lines because this phenotype is reminiscent of the 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 *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.

Fig. 6. 35S:*AtMYC1* enhances the 35S:*GL1* phenotype. (A-C) Rosette leaves of (A) 35S:*GL1*, (B) *atmyc1*-1 35S:*GL1*, (C) 35S:*AtMYC1* 35S:*GL1*. (D,E) SEM of trichomes on wild-type Col (D) and 35S:*AtMYC1* 35S:*GL1* (E) leaves. Scale bars: 1 mm in A-C; 200 μm in D,E.

Fig. 7. Subcellular colocalisation of GL1 or TRY with NLS or NES fusions of AtMYC1. Single leek cells expressing the respective fusion proteins after transformation by particle bombardment. For each combination, the YFP (left), CFP (middle) and RFP (right) channels are shown. Scale bars: 50 μm.
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 relocalisation 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 AtMYC1 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 of TRY/CPC and GL1 are coupled through AtMYC1. As regulation at the transcriptional level we propose that the protein complex (Morohashi and Grotewold, 2009). This suggests that the impact of AtMYC1 on the localisation of TRY/CPC is likely to influence movement rates into neighbouring cells.

**Transcriptional Regulation**

![Diagram of transcriptional regulation](image)

Fig. 8. Model summarising the postulated effects of AtMYC1 on the intracellular localisation of GL1 and TRY/CPC. We postulate that AtMYC1, TRY/CPC and GL1 mutually control their relative concentrations in the nucleus and cytoplasm such that AtMYC1 can relocate TRY/CPC into the nucleus and GL1 out of the nucleus, whereas TRY can relocate cytoplasmic AtMYC1 into the nucleus. The model highlights two possible consequences of intracellular re-localisation. First, changes in the concentration of TRY, CPC and GL1 in the nucleus might be expected to affect transcriptional regulation by the TTG1-Gl-GL1 complex and by AtMYC1. Second, changes in the nuclear/cytoplasmic concentrations of TRY/CPC are likely to influence movement rates into neighbouring cells.

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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.orglookup/suppl/doi:10.1242/dev.094698/-/DC1

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Localisation of TRY and GL1 by AtMYC1


