Participation of the Arabidopsis bHLH Factor GL3 in Trichome Initiation Regulatory Events1[W][OA]

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The development of trichomes (leaf hairs) from pluripotent epidermal cells in Arabidopsis (Arabidopsis thaliana) provides a powerful system to investigate the regulatory motifs involved in plant cell differentiation. We show here that trichome initiation is triggered within 4 h of the induction of the GLABRA3 (GL3) basic helix-loop-helix transcription factor. Within this developmental window, GL3 binds to the promoters of at least three genes previously implicated in the development and patterning of trichomes (GL2, CAPRICE, and ENHANCER OF TRIPTYCHON AND CAPRICE1) and activates their transcription. The in vivo binding of GL3 to the promoters of these genes requires the presence of the R2R3-MYB factor GL1, supporting a model in which a GL3-GL1 complex is part of the trichome initiation enhanceosome. In contrast, GL3 is recruited to its own promoter in a GL1-independent manner and this results in decreased GL3 expression, suggesting the presence of a GL3 negative autoregulatory loop. In support of genetic analyses indicating that ENHANCER OF GL3 (EGL3) is partially redundant with GL3, we show that EGL3 shares some direct targets with GL3. However, our results suggest that GL3 and EGL3 work independently of each other. Taken together, our results provide a regulatory framework to understand early events of epidermal cell differentiation.

Position-dependent cell fate determination and pattern formation are unique aspects of the development of plant structures. The establishment of single-celled leaf hairs (trichomes) from pluripotent epidermal (protoplermal) cells provides a powerful system to determine the genetic networks and positional signals involved in cell fate determination (Szymanski et al., 2000; Larkin et al., 2003; Schellmann and Hulskamp, 2005; Serna and Martin, 2006). In a developing Arabidopsis (Arabidopsis thaliana) leaf, mature trichomes are present at the tip of the young leaf and there is a gradient of younger trichomes toward the base of the leaf. Trichome development is characterized by six phases (Szymanski and Marks, 1998), starting with the radial expansion of an epidermal cell (trichome initial) and concluding with the formation of a mature trichome. The mature trichome is characterized by the presence of a stalk with two to four branches and an average DNA content of 32C (Hulskamp et al., 1994). Over 30 genes controlling various aspects of trichome initiation, spacing, size, and morphology have been cloned (Schellmann and Hulskamp, 2005). Trichome initiation is regulated by the combinatorial action of the R2R3-MYB GLABRA1 (GL1) or ATMYB23 together with the basic helix-loop-helix (bHLH) GL3 or ENHANCER OF GL3 (EGL3) transcription factors (Oppenheimer et al., 1991; Payne et al., 2000; Kirik et al., 2001; Esch et al., 2003; Schiefelbein, 2003; Zhang et al., 2003; Kirik et al., 2005). While gl1 mutants are mostly glabrous, mutations in gl3 have only a modest effect, primarily affecting branching, DNA endoreduplication, and trichoblast size (Hulskamp et al., 1994; Payne et al., 2000). In contrast, egl3 plants have no obvious trichome defect, but gl3 egl3 double mutants show a complete glabrous phenotype (Zhang et al., 2003). Expression of both GL3 and EGL3 is low in the developing epidermis of young leaves, increases in initiating and young trichomes, and drops in mature trichomes and pavement cells of mature leaves (Zhang et al., 2003). This is similar to GL1 and consistent with their participation in the selection of protodermal cells to the trichome pathway (Larkin et al., 1993). In addition to GL1 and GL3/EGL3, trichome initiation also requires the presence of TRANSPARENT TESTA GLABRA1 (TTG1), a WD repeat-containing protein (Walker et al., 1999) that physically interacts with GL3 and EGL3 (Zhang et al., 2003).
Many of the factors that participate in trichome initiation have additional regulatory functions. \textit{TTG1}, for example, is involved in trichome specification but is also required for the accumulation of anthocyanin and proanthocyanidin flavonoid-derived pigments in leaves and seed coats, respectively; the accumulation of seed coat mucilage; and the establishment of nonhair cell files (atrichoblasts) in the root (Walker et al., 1999). While neither \textit{gl3} nor \textit{egl3} single mutants display an obvious root hair phenotype, \textit{gl3 egl3} double mutants show the ectopic accumulation of root hairs in nonhair files characteristic of \textit{ttg1} mutants (Zhang et al., 2003). For the specification of nonhair cell file in the root, the R2R3-MYB function is provided by \textit{WEREWOLF} (WER; Lee and Schiefelbein, 1999), which is functionally equivalent to \textit{GL1} (Lee and Schiefelbein, 2001). The regulation of leaf anthocyanin accumulation is specified by the \textit{PAP1} and \textit{PAP2} R2R3-MYB factors (Borevitz et al., 2000) and seed coat proanthocyanidin pigment formation by the R2R3-MYB factor TT2 (Nesi et al., 2001). Pigment formation requires EGL3 and, to a lesser extent, GL3 (Zhang et al., 2003). The TT8 bHLH factor (Nesi et al., 2000) controls flavonoid biosynthetic genes through its interaction with TT2 or PAP1/PAP2 (Baudry et al., 2004). The picture that emerges from these and other studies is one in which combinations of different R2R3-MYB, bHLH, and WD factors contribute to the specification of diverse cell types in Arabidopsis and other plants (Ramsay and Glover, 2005).

Several morphogenetic transformations follow the establishment of a trichome initial. Among the genes involved in trichome expansion and shape determination is \textit{GL2}, encoding a homeodomain Zip transcription factor (Rerie et al., 1994). Based on expression studies, \textit{GL2} was proposed to be a target of \textit{GL1-TTG1} (Szymanski et al., 1998), responsible for input integration of the patterning machinery into cellular differentiation (Schellmann and Hulskamp, 2005). Besides the participation in trichome morphogenesis, \textit{GL2} is also required for mucilage deposition, root hair formation, and accumulation of seed oils (Shen et al., 2006). In roots, \textit{GL2} directly represses a phospholipase gene, implicating \textit{GL2} as a regulator of a phospholipid-signaling pathway (Ohashi et al., 2003). Overexpression experiments also suggested an involvement of \textit{GL2} in the regulation of trichome spacing (Ohashi et al., 2002).

In contrast with root hair formation, where cortical cells provide positional cues to the overlaying protodermal cells dictating whether they will differentiate into a hair file, the control of trichome patterning is likely to occur by a self-organizing system (Larkin et al., 1996). This involves lateral inhibition with feedback regulation for either the initiation of trichome formation or the establishment of nonhair files in the root (Schellmann et al., 2002). At least four partially redundant single-repeat R3 MYB proteins, CAPRICE (CPC; Wada et al., 1997), TRIPYTCHON (TRY; Hulskamp et al., 1994), and ENHANCER OF TRY and CPC1 (ETC1) and ETC2 (Kirik et al., 2004), play central roles in this lateral inhibition by targeting specific components of the MYB/bHLH/TTG1 regulatory complex, making it nonfunctional (Schellmann et al., 2002). Consistent with the model, TRY and CPC can physically interact with GL3 and EGL3 (Zhang et al., 2003). CPC, regulated by WER (Koshino-Kimura et al., 2005), moves from atrichoblast to trichoblast cells in the root (Kurata et al., 2005). The phenotype of \textit{cpc} and \textit{try} single and double mutants suggests TRY might be more important in short-range inhibition, while CPC would have a more distant effect (Schellmann et al., 2002). The expression of the TRY, CPC, ETC1, and ETC2 inhibitory proteins in trichome initials and in mature trichomes (Schellmann et al., 2002; Kirik et al., 2004) poses the problem of how the MYB/bHLH/TTG1 complex can function to promote a trichome. To explain this conundrum, models have been proposed in which the expression of the inhibitors is tightly controlled by the activation complex, and these models invoke the ability of the inhibitory proteins to move to adjacent cells, resulting in, at first, equivalent cells, competing by mutual lateral inhibition for the ability to accumulate sufficient quantity of the MYB/bHLH/TTG1 complex to trigger trichome initiation (Schellmann et al., 2002; Schiefelbein, 2003).

The findings described above, obtained primarily through the careful analysis of mutants, have provided a powerful conceptual framework to study trichome and root hair patterning. However, the immediate targets of the MYB/bHLH/TTG1 complex and the regulatory circuitry responsible for triggering trichome formation remain unknown. Using a combination of posttranslationally controlled GL3- and EGL3-glucocorticoid receptor fusions (GL3-GR and EGL3-GR), experiments were directed at testing three specific hypotheses: (1) expression of GL3/EGL3 is sufficient to trigger trichome initiation; (2) some or all known trichome initiation genes are direct targets of the GL3/EGL3 transcription factors; and (3) the regulatory function of the bHLH factors is dependent on GL1. Our results show that 4 h of GL3/EGL3 induction is sufficient to trigger trichome initiation, and that some, but not all, of the trichome initiation genes are direct targets of GL3/EGL3. Our results also uncover two GL3 regulatory mechanisms, one of which is GL1 dependent and the other GL1 independent. We also exposed an unexpected binding of GL3 to its own promoter, suggesting the potential for a GL3 autoregulatory feedback. Together, these findings provide novel insights into the regulatory motifs participating in the initiation of trichome formation.

\section*{RESULTS}

\subsection*{Coordinated Induction of Trichome Initiation}

In a wild-type Arabidopsis plant, trichome formation occurs sequentially from the tip to the base of the leaf as development progresses, making it difficult to explore the events specifically associated with trichome initiation. Because GL3/EGL3 participate both in early trichome initiation as well as during later stages (e.g.
pressed (Payne et al., 2000). Initially, we utilized the GL3-GR construct, containing the GL3 pro-
moter and the GL3 3′ region, which recapitulates the in vivo GL3 expression pattern (Zhang et al., 2003). The gl3 egl3 pGL3::GL3-GR plants are glabrous (Supplemental Table S1; Fig. 1A, mock), unless treated with dexamethasone (DEX), a synthetic ligand for the GR. When grown in the presence of DEX, gl3 egl3 pGL3::GL3-GR plants developed trichomes within a few days (Fig. 1).

To start dissecting the earliest events associated with the differentiation of epidermal cells into trichomes, we examined whether inducing GL3 function for 4 h was sufficient to trigger the trichome initiation pathway. Towards this goal, gl3 egl3 pGL3::GL3-GR plants were grown for 16 d in plates without DEX, transferred to DEX-containing media for 4 or 24 h, and then moved again to media without ligand and observed after a week. Under these conditions, 4 h of DEX treatment was sufficient to induce the formation of a discrete number of trichomes, only in leaves that were at early stages of development when the seedlings were transferred to the inductive media. Trichomes form largely along the margins of the leaf, and they are unbranched (Fig. 1B). When the DEX treatment was extended to 24 h, there was no evident increase in the total number of trichomes when compared with plants treated for just 4 h, yet more trichomes showed a branched phenotype, with primarily two branches (Fig. 1C). When the DEX treatment was further extended to 7 d, more trichomes formed; trichomes in new leaves remained largely unbranched, while trichomes in older leaves showed the two-branches phenotypes (Fig. 1D). No trichomes were observed in mock-treated plants at any of the times, indicating that there is not sufficient GL3-GR to promote trichome formation (Fig. 1A). We observed very similar results when the plants were sprayed with DEX once rather than transferred to DEX-containing media, although the DEX spraying results in slightly more trichomes formed (data not shown).

Several genes have been suggested to participate in the early stages of trichome initiation (Schellmann and Hulskamp, 2005). Thus, we investigated whether 4 h of DEX treatment of gl3 egl3 pGL3::GL3-GR plants resulted in the activation of any of these genes. Quantitative reverse-transcriptase (qRT)-PCR experiments were performed on total RNA obtained from green tissues of seedlings induced for 4 h with DEX and compared with RNA obtained from mock-treated plants. Interestingly, of all of the trichome initiation genes tested (GL2, TRY, CPC, ETC1, and ETC2), only GL2 showed a very modest, yet significant, induction under these conditions (data not shown).

To determine whether the modest effects on the expression of these genes was a consequence of the low expression levels of the native pGL3::GL3-GR transgene, and thus only a few cells being competent to enter the trichome pathway, we carried out similar experiments using gl3 egl3 p35S::GL3-GR plants. As we observed with gl3 egl3 pGL3::GL3-GR plants, gl3 egl3 p35S::GL3-GR plants are glabrous (Supplemental Table S2,
Fig. 1E), unless induced with DEX. A 4 h induction of DEX is again sufficient to trigger trichome formation (Fig. 1F). In 4 h DEX-induced gl3egl3p35S::GL3-GR plants, more trichomes were observed than in the corresponding gl3egl3pGL3::GL3-GR plants (compare Fig. 1, B and F). In gl3egl3p35S::GL3-GR plants, trichomes remain unbranched in new leaves, while they show some branching in older leaves (Fig. 1F). A similar situation was observed after 24 h (Fig. 1G) and 7 d of DEX treatment (Fig. 1H). The different branch numbers in younger and older leaves could simply be a consequence of the former having been exposed to DEX for a shorter period of time than the latter. No trichomes were observed in gl3egl3p35S::GL3-GR plants mock-treated for 7 d (Fig. 1E).

As was previously determined for p35S::R-GR plants (Lloyd et al., 1994), trichome initiation can be detected 24 h after DEX induction (see Fig. 2, arrows). Indeed, in gl3egl3p35S::GL3-GR plants, many trichomes form simultaneously without strictly following the ordered apical/basal pattern associated with developing leaves (Szymanski et al., 2000). Taken together, these results indicate that, within 4 h, GL3 is capable of activating/repressing genes required for trichome initiation, while increased (as provided by the 35S promoter) or sustained (as found after 24 h of DEX treatment) GL3 expression is necessary for trichome branching.

Trichome Development Genes Are among the GL3 Immediate Direct Targets

In contrast to what we established for gl3egl3pGL3::GL3-GR plants, GL2, CPC, and ETC1 are robustly induced in gl3egl3p35S::GL3-GR plants within 4 h of DEX treatment, as shown by qRT-PCR (Fig. 3A). Under these conditions, no induction of TRY was observed. To determine whether GL2, CPC, and ETC1 are among the immediate GL3 targets, we exploited the posttranslational regulation of GL3-GR by DEX to distinguish between indirect downstream target genes (expression sensitive to the protein synthesis inhibitor cycloheximide [CHX]) and immediate direct target genes (expression insensitive to CHX, Sablowski and Meyerowitz, 1998). The induction by DEX of GL2 and ETC1, and to a lesser extent of CPC, persisted even when the plants were treated with CHX and DEX (Fig. 3A), indicating these genes are most likely immediate direct targets of GL3.

To confirm the in vivo recruitment of GL3 to the promoters of GL2, CPC, and ETC1, chromatin immunoprecipitation (ChiP) experiments were performed using antibodies against GR (αGR) in the presence and absence of 4 h of DEX treatment in gl3egl3p35S::GL3-GR plants. ChiP results show a significant in vivo enrichment of GL3-GR at the promoter regions of all three genes in the presence of DEX compared with mock-treated plants (Fig. 3B), yet not to TRY, consistent with the expression results (Fig. 3A). In some cases, a low level of GL3-GR binding is observed in mock-treated plants, suggesting perhaps a small amount of GL3-GR proteins enters the nucleus in the absence of the DEX ligand, although clearly not sufficient to complement the gl3egl3 mutant phenotype (Fig. 1). From these experiments, we conclude that GL3 directly activates GL2, CPC, and ETC1 expression and that this activation occurs at the early stages of trichome initiation.

GL3 Autoregulates Its Own Expression

Models that attempt to explain trichome pattern formation often involve the self activation of the regulators (Meinhardt and Gierer, 2000; Schellmann and Hulskamp, 2005). This prompted us to investigate whether GL3 may regulate its own transcription. Thus, we explored the expression of GL3 in gl3egl3pGL3::GL3-GR plants by qRT-PCR in the presence and absence of DEX. After 4 h of induction with DEX, the accumulation of the GL3 mRNA was significantly decreased compared to mock-treated plants (Fig. 4A, pGL3::GL3-GR). Consistent with a role of GL3 in repressing its own transcription, the gl3-1 allele present in the gl3egl3 plants is
expressed at higher levels compared to the GL3 wild-type allele (Supplemental Fig. S1B). Thus, the level of GL3 expression in the DEX induction experiments corresponds to the sum of the endogenous gl3-1 and the GL3-GR mRNAs. To further confirm the possibility that GL3 might repress its own transcription, we investigated the expression of the gl3-1 allele in gl3 eg3 p35S::GL3-GR plants, 4 h after DEX induction, using primers that distinguish between gl3-1 and GL3-GR. Again, DEX treatment resulted in a modest, yet significant, reduction in the steady-state levels of GL3 mRNA accumulation (Fig. 4A, p35S::GL3-GR). These results suggest the possibility of a negative feedback loop modulating GL3 expression.

To determine whether GL3 is directly involved in this feedback regulation, expression analyses were repeated in the presence of: (1) DEX and (2) DEX and CHX. As is often the case for immediate early genes in developmental processes (Edwards and Mahadevan, 1992), CHX significantly enhanced GL3 mRNA accumulation, likely reflecting increased transcript stability (data not shown). To correct for this, we normalized the qRT-PCR results to the levels of mRNA detected in plants treated just with CHX. The qRT-PCR experiments showed GL3 mRNA reduction observed after DEX treatment in gl3 eg3 pGL3::GL3-GR plants remains in the presence of CHX (Fig. 4A). However, when similar CHX and DEX treatments were carried out in the gl3 eg3 p35S::GL3-GR plants, the expression of the gl3-1 allele was restored to the levels present in plants treated with CHX alone (Fig. 4A). Taken together, these results provide strong evidence for a model involving a negative feedback loop.
autoregulation of GL3 but conflicting evidence as to whether this involves direct interaction of GL3 with its own promoter.

To unequivocally determine whether GL3 binds its own promoter in vivo, we performed ChIP experiments on gl3 egl3 p35S::GL3-GR plants mock or DEX treated for 4 h. A robust binding of GL3-GR to the GL3 promoter (furnished by the gl3-1 allele) is observed only in the presence of DEX (Fig. 4, B and D), and not in mock-treated plants (Fig. 4, B and M). We interpret these results to indicate that GL3 can bind to its own promoter and that the overall result of this binding is a reduction in GL3 expression.

Overlapping Functions of GL3 and EGL3

GL3 and EGL3 not only encode closely related bHLH transcription factors, but also cooperate in the control of trichome formation (Zhang et al., 2003). As a first step toward establishing whether these two proteins participate in trichome formation using similar mechanisms, we transformed gl3 egl3 plants with a p35S::EGL3-GR construct. As we established for p35S::GL3-GR, EGL3-GR plants remained glabrous in the absence of DEX (Supplemental Table S3; Fig. 5A, mock), and not in mock-treated plants (Fig. 4, B and M). We interpret these results to indicate that GL3 can bind to its own promoter and that the overall result of this binding is a reduction in GL3 expression.

GL1-Dependent and GL1-Independent GL3 Recruitment to DNA

The physical interaction between GL3 and GL1 (Zhang et al., 2003) was proposed to be required for the regulation of trichome gene expression (Larkin et al., 2003; Marks and Esch, 2003; Schellmann and Hulskamp, 2005). We were therefore curious to determine whether GL1 was required for the tethering of GL3 to the GL3, CPC, and GL2 promoters. Toward this goal, we expressed the previously described pGL3::GL3-YFP construct (Bernhardt et al., 2005), where YFP corresponds to the yellow fluorescent protein, in gl3 egl3 and gl1 plants. We reasoned that, by using pGL3::GL3-YFP plants rather than DEX-induced pGL3::GL3-GR plants, we would be able to capture trichomes at all possible developmental stages, rather than at a single

Figure 4. GL3 expression is controlled by an autoregulatory feedback regulatory loop. A, qRT-PCR of mRNA obtained from green tissues of 21-d-old gl3 egl3 pGL3::GL3-GR (pGL3::GL3-GR) or gl3 egl3 p35S::GL3-GR (p35S::GL3-GR) treated for 4 h with DEX (black) or with DEX and CHX (hatched). The bars indicate the relative expression to mock- or CHX-treated plants, respectively. Relative expression was determined in triplicate measurements, and error bars indicate the SDs. For pGL3::GL3-GR, primers were designed to recognize both the endogenous GL3 transcript (from the gl3-1 allele) and the GL3-GR mRNA. For p35S::GL3-GR, primers recognized specifically the endogenous GL3 mRNA (from gl3-1). B, Semiquantitative PCR of ChIP experiments carried out with chromatin obtained from green tissues of 20-d-old gl3 egl3 p35S::GL3-GR plants mock treated (M) or treated with DEX (D) for 4 h. Input corresponds to the chromatin prior to immunoprecipitation, aGR to the material recovered after immunoprecipitation with antibodies against GR. PCRs were performed on three 4-fold serial dilutions of the ChIP-ed material, represented by the black slope on the top. The graph on the left indicates the position of the PCR fragment relative to the transcription start site (indicated by an arrow) of the GL3 gene.
narrow developmental window furnished by the coordinate trichome formation provided at any given time following DEX treatment.

pGL3::GL3-YFP complements the gl3 egl3 mutant phenotype, yet results in some trichome clusters (Supplemental Table S4; Fig. 6A). ChIP experiments were performed in both plants using antibodies against GFP (which cross-react with YFP). Interestingly, the in vivo recruitment of GL3 to the CPC or GL2 promoters is not observed in gl1 mutants (Fig. 6B), supporting the model that the formation of a GL3/GL1 complex is necessary for the GL3 recruitment to these promoters. In contrast, GL3 binds its own promoter in vivo independently of GL1, because the binding is still present in gl1 mutant plants (Fig. 6B).

While we could not observe GL3-GR recruitment to the TRY promoter in p35S::GL3-GR (Fig. 3) or pGL3::GL3-GR (data not shown) plants induced with DEX for 4 h, GL3-YFP is clearly recruited to the promoter of this gene. Interestingly, however, the binding of GL3 to TRY is independent of GL1 (Fig. 6B, TRY). The main difference between the GL3-GR and the GL3-YFP experiments is that in the former, we assayed DNA binding 4 h after the induction of trichome initiation, whereas in the latter, experiments were performed using plants with trichomes at all possible developmental stages (Fig. 6A). Thus, GL3 binding to the TRY promoter might be associated with later aspects of GL3 function. Expression analyses by qRT-PCR support the GL1-dependent and GL1-independent GL3 regulatory activity described above, as the activation of transcription of CPC, ETC1, and GL2 in p35S::GL3-GR plants requires the presence of a functional GL1 allele (data not shown). While GL3-GR does not induce TRY, TRY expression is significantly reduced in gl3 egl3 plants compared to wild-type plants (data not shown). Taken together, these results indicate GL3 functions by both GL1-dependent and GL1-independent mechanisms.

DISCUSSION

Arabidopsis trichomes provide a powerful system to study plant epidermal cell differentiation and the bHLH transcription factors GL3/EGL3 have central functions in this process. We show here that a short period of GL3 or EGL3 induction is sufficient to trigger the trichome initiation pathways. Using ChIP, we established that among the early regulatory events associated with trichome initiation is the recruitment of GL3 to the promoters of several previously described trichome genes, including GL2, CPC, and ETC1. We demonstrate that these genes are immediate direct targets of GL3 and that GL3 directly regulates its own expression. We also established that EGL3 is tethered to the GL2 promoter and that this happens even in the absence of GL3 function. These findings establish some of the earliest regulatory events associated with the initial differentiation of protodermal cells to trichomes.

While our results suggest that 4 h of GL3 or EGL3 induction is sufficient to trigger the initiation of trichome formation, we cannot formally rule out the possibility that, once taken up by the plants during the 4 h treatment, DEX persists and continues to modulate the nuclear localization of GL3 or EGL3. Western analyses have so far been unsuccessful in detecting GL3-GR in the nucleus, perhaps because of the low level of GL3-GR accumulation, even under p35S. However, several observations, including the very rapid recruitment of the GL3/EGL3 regulators to various gene promoters and the activation of key trichome inducers (such as GL2) suggest that, within 4 h of GL3/EGL3 induction, significant regulatory events occur that might be sufficient to irreversibly change the fate of epidermal cells into the trichome pathway.
Previous studies implicated GL1 and TTG1 in GL2 regulation in the trichome developmental pathway (Szymanski et al., 1998). Our results show GL3 binds in vivo to and activates GL2 transcription within 4 h of induction. Because this induction is observed even in the absence of de novo protein synthesis (Fig. 3A), we conclude that GL2 is an immediate early direct target of GL3 (Fig. 7). In agreement with previous findings, this activity of GL3 is dependent on the presence of functional GL1 (Fig. 6), supporting the model of a GL3-GL1-TTG1 complex responsible for GL2 activation and subsequent trichome initiation.

In addition to putative GL1 binding sites (Szymanski et al., 1998), the GL2 promoter contains multiple E boxes, cis-regulatory elements likely responsible for the binding of GL3 or related bHLH factors. The recruitment of GL3 to the GL2 promoter happens in the absence of EGL3, suggesting that the physical interaction between GL3 and EGL3 (Zhang et al., 2003) is not required for the GL3 DNA-binding activity. Indeed, the region involved in GL3-EGL3 interaction is likely to be the same region that mediates GL3 homodimerization (Zhang et al., 2003), located at the very end of the protein, C terminal to the bHLH. This region is conserved among the R-like subgroup of bHLH factors (Feller et al., 2006). EGL3 is also recruited in vivo to the GL2 promoter, even in the absence of a functional GL3 protein (Fig. 5B). To our knowledge, our results provide some of the first direct in vivo evidence that R/GL3/EGL3-like transcription factors can be recruited to the promoters of genes they regulate (Fig. 7).

GL3 also binds to and controls the activation of CPC and ETC1 early during trichome initiation in a fashion that is dependent on GL1 (Fig. 3). In agreement with epistasis analyses suggesting these proteins function upstream of GL2, our results show they are also immediate direct targets of GL3 (Fig. 3). CPC and ETC1 encode single MYB-repeat proteins proposed to compete with GL1 for the interaction with GL3/EGL3, and, accordingly, both GL3 and EGL3 physically interact with CPC (Zhang et al., 2003). Similar to GL2, ETC1 and CPC promoters contain multiple candidate GL3/EGL3- and GL1-binding sites. Indeed, previous studies demonstrated direct binding of WER to the CPC promoter (Koshino-Kimura et al., 2005).

However, our studies failed to detect either TRY activation or binding of GL3 to TRY immediately (within 4 h) following GL3 induction (Fig. 3). Moreover, we did not detect binding by EGL3-GR to TRY 4 h after DEX treatment (Fig. 5B). Nevertheless, our results indicate that TRY is regulated by GL3/EGL3, as evidenced by the significantly reduced TRY mRNA levels in gl3 egl3 compared to wild-type plants (data not shown) and by the in vivo recruitment of GL3-YFP to the TRY promoter in gl3 egl3 plants harboring the pGL3::GL3-YFP construct, which display trichomes at various developmental stages (Fig. 6). The finding that GL3-YFP binds the TRY promoter is of significance, as it demonstrates that our inability to detect GL3-GR binding to TRY is not a consequence of probing for an incorrect promoter fragment in our ChIP experiments. Our results, suggesting differential regulation of TRY and CPC, complement functional studies that propose similar, yet distinct activities for these two small MYB proteins in trichome patterning (Schellmann et al., 2002). While try mutants accumulate frequent trichome

Figure 6. GL1-dependent and GL1-independent recruitment of GL3 to target promoters. A, Complementation of the trichome phenotype of gl3 egl3 mutants by pGL3::GL3-YFP (left), but not of gl1 (center), which remain glabrous compared to the wild-type Landsberg erecta (right). Scale bar = 1 mm. B, Semi-quantitative PCR of ChIP experiments carried out with chromatin obtained from green tissues of 20-d-old gl3 egl3 pGL3::GL3-YFP (left) or gl1 pGL3::GL3-YFP (right) plants. PCRs were performed on three 4-fold serial dilutions of the ChIP-ed material, represented by the black slope on the top.
Figure 7. Transcriptional regulatory interactions controlling trichome development. Activators are connected to their targets by arrows, proteins are indicated as circles in the same color as the corresponding genes. Question marks indicate identities that need yet to be established. Dotted arrows indicate the predicted movement of the small single MYB-repeat proteins TRY, CPC, and ETC1 from one cell to another. A, Two identical epidermal cells prior to either one adopting a trichome identity. B, The cell on the left has entered the trichome pathway while the cell on the right will remain a pavement cell.

clusters, cpc mutants display no clusters but approximately 2-fold increased trichome numbers. In addition, try mutants, but not cpc, display increased DNA content as a consequence of additional rounds of endoreduplication. Finally, the targets of CPC and TRY are likely to be different, based on the ability of p35S::R to rescue only the trichome phenotype of p35S::CPC plants but not of p35S::TRY (Schellmann et al., 2002). Our results indicating a delayed activation of TRY by GL3/EGL3 are consistent with a later function of TRY, CPC, and ETC1 (e.g. CPC, ETC1). The ability of the repressors to diffuse to adjacent cells, activity that has been shown for CPC in roots (Wada et al., 2002), together with the self activation of the positive regulators would result in small differences in the concentration of the active complexes, breaking equivalence and resulting in specific cells being selected for the trichome pathway (Fig. 7). However, two not mutually exclusive explanations may reconcile such a model with our own results. It is possible that another trichome regulator working either upstream of GL3 or with GL3 (e.g. GL1 or TTG1) is self activated and the observed GL3 auto-repression occurs temporally later, once the selection of trichome initials has occurred. An alternative explanation is that the negative autoregulation of GL3 we observed occurs in cells destined not to become trichomes, which are known to express reduced levels of GL3 (Zhang et al., 2003; Fig. 7). Perhaps a negative autoregulation of GL3 in nontrichome cells confounds a positive GL3 autoregulation in trichome initials just by the sheer larger number of the former. Experiments are currently under way to establish whether both positive and negative autoregulatory loops govern GL3 expression in trichome and nontrichome cells, respectively.

In conclusion, our results provide evidence that the GL3/EGL3 bHLH factors control early events in the differentiation of epidermal cells into leaf hairs by directly binding the promoters of a set of genes that, when mutated, affect trichome patterning. Among these genes, only GL2 is a positive regulator of trichome initiation, while CPC, TRY, and ETC1 are more likely involved in establishing leaf trichome patterns. It will be of significance to identify additional GL3/EGL3 direct targets to determine whether the activation of GL2 by these bHLH factors is sufficient to trigger initiation into this developmental pathway.

MATERIALS AND METHODS

Plant Culture

Arabidopsis (Arabidopsis thaliana) plants were grown on soil with 100 μm Basta (Liberty, AgrEvo; gl3egl3 p35S::GL3-GR) or Murashige and Skoog (MS) media supplemented with 50 μm kanamycin (gl3egl3 pGL3::GL3-YFP, gl1 pGL3::GL3-YFP) at 22°C under a photoperiod of 16 h of light and 8 h dark, unless otherwise indicated.

Description of GL3-GR and EGL3-GR Fusion Constructs

To construct the GL3-GR translational fusion clones, pD2L-2 (Payne et al., 2000) was modified as described (Bernhardt et al., 2005) to provide a GL3 promoter is independent of GL1, suggesting at least two distinct mechanisms by which GL3 can regulate gene expression. At first glance, the presence of a GL3 negative autoregulatory loop appears to be in conflict with models attempting to explain how, from a field of initially equivalent epidermal cells, trichome initials are selected (Meinhardt and Gierer, 1974, 2000). According to this model, the activators (GL3/GL1) stimulate their own expression and that of the negative regulators (e.g. CPC, ETC1). The ability of the repressors to diffuse to adjacent cells, activity that has been shown for CPC in roots (Wada et al., 2002), together with the self activation of the positive regulators would result in small differences in the concentration of the active complexes, breaking equivalence and resulting in specific cells being selected for the trichome pathway (Fig. 7). However, two not mutually exclusive explanations may reconcile such a model with our own results. It is possible that another trichome regulator working either upstream of GL3 or with GL3 (e.g. GL1 or TTG1) is self activated and the observed GL3 auto-repression occurs temporally later, once the selection of trichome initials has occurred. An alternative explanation is that the negative autoregulation of GL3 we observed occurs in cells destined not to become trichomes, which are known to express reduced levels of GL3 (Zhang et al., 2003; Fig. 7). Perhaps a negative autoregulation of GL3 in nontrichome cells confounds a positive GL3 autoregulation in trichome initials just by the sheer larger number of the former. Experiments are currently under way to establish whether both positive and negative autoregulatory loops govern GL3 expression in trichome and nontrichome cells, respectively.

In conclusion, our results provide evidence that the GL3/EGL3 bHLH factors control early events in the differentiation of epidermal cells into leaf hairs by directly binding the promoters of a set of genes that, when mutated, affect trichome patterning. Among these genes, only GL2 is a positive regulator of trichome initiation, while CPC, TRY, and ETC1 are more likely involved in establishing leaf trichome patterns. It will be of significance to identify additional GL3/EGL3 direct targets to determine whether the activation of GL2 by these bHLH factors is sufficient to trigger initiation into this developmental pathway.
promoter and coding region genomic clone (pGL3−GL3) with the stop codon replaced and named pScl and Safi restriction sites. The GR-coding region was amplified from pGR (Lloyd et al., 1994) with primers 5′-GGGAGCTCGGGAGGAGGAGAAGCTCGAAAAACAAAG-3′ and 5′-TCTAGAGTCGACCTACTTTTGTGAACAG-3′ and ligated in frame to the GL3 3′ end using the SacI and SfiI restriction sites. A BamHI fragment from this vector, containing the entire pGL3−GL3−GR fusion, was subcloned into the BgII site of the pDONR vector pAL47 (Lloyd and Davis, 1994) to make pGL3−GL3−GR containing GL3−GR under native control. A fragment containing the entire fusion of the start GL3 codon to the GR stop codon, plus Gateway (Invitrogen) recombination sequences on both ends, was PCR amplified from pGL3−GL3−GR with 5′-aattb1-atggctacggagggaaaagcagc-3′ and 5′-aattb2-CTGTCGAAATTTTTAACAAAG-3′ and recombined into the Gateway site of the vector pDONor221 (Invitrogen) to create pCHWL5GL3GR. pCHWL5GL3GR was recombined with pB7WG2 (Karimi et al., 2002) to make p35S−GL3−GR containing GL3−GR under CaMV35S control. The EGL3 cDNA was PCR amplified with primers 5′-agactagTGGCAGAACCAAGAAAGACAGG-3′ and 5′-agactagCATATCCTGGCGACCCCTTTTGACACATG-3′, and cloned into a TOPO-TA vector (Invitrogen) and subsequently cloned into the SpeI site of pBKS (Stratagene). The GR coding region was amplified by PCR with primers 5′-tactagCGAAAAGACGAAAAAGAAATGAAACG-3′ and 5′-ggggccgctCTAGATTCGAGTGGAAACAAAGAAAAAAATCAAAGGG-3′, and cloned into the SpeI and XhoI sites of pBKS containing previously cloned EGL3 cDNA. EGL3−GR was PCR amplified with primers 5′-aactagTCGGACCCCGAAACAGACG-3′ and 5′-ggggccgctCTAGATTGTCGCTGAAACCAAGGGTTTGGC-3′, cloned into pE nENTR, and then recombined into the Gateway site of pGW2 (http://bio2.ipc.shimane-u.ac.jp/PGWBS/INDEX.HTM) to construct p35S−EGL3−GR.

Optical and Scanning Electron Microscopy

The g3 gl3 pGL3−GL3−GR seedlings were grown on MS medium for 16 d and transferred onto MS medium containing 30 μM DEX or 2% ethanol as mock. After 4 h, 24 h, or 7 d of DEX induction, seedlings were transferred back onto plain MS medium. Optical images were taken with a Nikon SMZ800 dissecting microscope. The g3 gl3 plants carrying p35S−GL3−GR were grown on soil under constant light at 22°C for 16 d when the third and fourth leaves became visible. To induce trichomes, 20 μM DEX or 2% ethanol as mock was sprayed onto the plants once. Seedlings were collected at multiple time points between 0 and 72 h after the DEX treatment. For scanning electron microscopy (SEM) experiments, plant samples were prepared and visualized essentially as described (Payne et al., 2000) with minor modifications. Critical-dried specimens were coated with platinum palladium in a Cressington 208 sputter coater and then visualized with a Zeiss Supra 40 VP SEM.

Gene Expression Analyses

For the g3 gl3 pGL3−GL3−GR, 14-d-old seedlings were transferred from plain MS media to MS media containing 30 μM DEX or 2% ethanol (mock). For the p35S−GL3−GL3−GR, 16-d-old seedlings growing on soil were sprayed with 20 μM DEX, 100 μM CHX, and 20 μM DEX + 100 μM CHX. Tissues were collected after treatment and frozen immediately in N2(l). For RT-PCR experiments, green tissues from 30 to 40 seedlings were used for each RNA extraction following the Trial reagent protocol. The RNA was further purified using Qiagen RNeasy following the manufacturer’s instructions. Real-time PCR is performed using SYBR Green chemistry (Applied Biosystems) on a 7500 Real-Time PCR system (Applied Biosystems). Primers for PCR were designed to generate unique 100- to 200-bp fragments. For normalization, we used ACT2 (At3g18780) or At1g13320, which is reported to be an appropriate reference gene (Czechowski et al., 2004). Real-time PCR of test samples and the reference gene were performed using a standard dilution series for each transcript. Relative expression levels between the GL3−GR or the GL3−YFP transgenes and the endogenous g3−l mutant allele were determined by RT-PCR using GL3 intron-flanking primers followed by sequencing of the entire PCR product. Alternatively, the RT-PCR product was cloned and 25 to 50 clones were sequenced. The g3−l allele is characterized by the presence of a T at position 1,132 with respect to the transcript start site, while the wild-type GL3 allele contains at that position a C. The relative abundance of the wild-type GL3 and mutant g3−l transcripts was estimated from the relative peak heights in the electropherograms and by the counting the clones containing C or T (or their complementary when reading the other strand), respectively (Supplemental Fig. S1A).

ChiP Experiments

Green tissues from 3-week-old plants grown on soil were washed in distilled water and immersed in buffer A (0.4 M Suc, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% formaldehyde) under vacuum for 20 min. Glycine was added to 0.1 M and incubation was continued for an additional 10 min. The plants were washed in distilled water and frozen in N2(l). Approximately 60 mg of tissue were ground for each immunoprecipitation. The tissue was resuspended in 0.1 mL Lysis buffer (50 mM HEPES, pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate) and plant proteinase inhibitor cocktail (Sigma). DNA was sheared by sonication to approximately 300- to 1,000-bp fragments with a main peak of 500 bp. Sonication (Sonic Research & Materials) was performed on ice with an amplitude of 10% using 5 × 15-s pulses (5 s between bursts). After preclearing with 40 μL of salmon sperm DNA/Protein A-agarose beads (Upstate) for 120 min at 4°C, immunoprecipitations were performed overnight at 4°C with either 2 μg of IgG, 1 μg of anti-GR antibody (PA1-516; Affinity BioReagents), or 1 μg of anti-GFP antibody (ab290; Abcam). After incubation, beads were washed two times with LNDT buffer (0.25% Nonidet P40, 1% deoxycholate, 1 mM EDTA) and two times with Tris-EDTA buffer. The washed beads and input fraction were resuspended in elution buffer (1% SDS, 0.1 M NaHCO3) with 1 mg/mL proteinase K and incubated overnight at 65°C. After cross-link reversal of the immunoprecipitated and Input DNA (set aside from the sonication step), the DNA was purified using the PCR Purification kit (Qagen). Semiquantitative PCRs were performed under standard PCR conditions (55–38 cycles). DNA was detected using agarose gel electrophoresis and quantified by EthBr staining.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression level of transgenes and endogenous g3−l allele.

Supplemental Table S1. Transgenic lines generated for this study.

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