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Article Addendum

E2F and retinoblastoma related proteins may regulate *GL1* expression in developing Arabidopsis trichomes

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This is an addendum to our recent paper published in *The Plant Journal* (52:352–61). The major findings were: (1) trichomes on the leaves of *gl3-sst sim* double mutants developed as large multicellular clusters whereas wild type trichomes are composed of single cells; (2) ectopic *CYCD3;1* expression in *gl3-sst* trichomes also resulted in trichome cluster formation; and (3) that *GL1* expression is prolonged in the *gl3-sst sim* trichome clusters. This addendum shows that ectopic *CYCD3;1* expression in *gl3-sst* also enhanced *GL1* expression. An analysis of the *GL1* promoter found two overlapping potential E2F binding sites in a region of the promoter known to be essential for *GL1* function. This finding indicates that *GL1* may be directly regulated by the activity of a CYCD3/CDKA complex that phosphorylates E2F-RB bound to the *GL1* promoter.

Introduction

The development of Arabidopsis trichomes is being used as a model to address basic biological questions concerning control of cell fate and differentiation.[1-4] On the leaves, trichomes are composed of large unicellular structures containing a stalk and three to four branches. These are the first cells to terminally differentiate on young leaf primordia, and, like many differentiated plant cells, they undergo endoreduplication (excess DNA replication without cell division). Over the past twenty years a core of transcription factors have been identified that are required for the trichome cell fate. These include *GLABROUS1* (*GL1*; a R2R3 MYB), *TRANSPARANT TESTA GLABRA1* (*TTG1*; a WD40 repeat containing protein) and the semi-redundant pair of genes *GLABRA3* and *ENHANCER OF GLABRA3* (*GL3* and *EGL3*; bHLH proteins).[5-8] Mutations in any of the three classes of genes result in a loss of trichome initiation on most plant epidermal surfaces. Several studies indicate that these factors interact, and likely form a transcriptional complex that regulates genes required for trichome formation.[6,8,9]

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Whereas double *gl3 egl3* mutants lack trichomes, plants deficient in *GL3* have more slender less branched trichomes that exhibit reduced endoreduplication (8-16C vs. 32-64C).[8,10] Interestingly, a special mutant allele of *GL3* called *gl3-shapeshifter* (*gl3-sst*) has been identified that induces enlarged variably shaped and branched trichomes that exhibit extremely enhanced endoreduplication (over 200C).[9,11] These mutants also develop fewer trichomes. The mutant *gl3-sst* allele contains a single base pair change that converts a leucine to a phenylalanine residue in the region of the GL3 peptide that interacts with GL1. Yeast two hybrid analyses have shown that the substitution results in decreased interaction between GL1 and gl3-sst polypeptides [9]. A model explaining the *gl3-sst* phenotype posits that the reduced interaction between GL1 and gl3-sst results in fewer cells acquiring a threshold level of activator complex needed to activate genes required for commitment to the trichome fate. The extra endoreduplication and cell expansion of those few trichomes that do develop are thought to be due to the inability of the altered GL1/gl3-sst containing complex to activate genes needed to limit trichome growth during early phases of trichome differentiation. Indeed, it has been found that the expression of *TRIPTYCHON* (*TRY*), which functions to limit trichome expansion and endoreduplication, is decreased in *gl3-sst* trichomes.[12,13]

A genetic screen was conducted to identify additional genes that might play a role in the *gl3-sst* phenotype. This screen resulted in the identification of a mutant that exhibited large clusters of trichome cells with apparent meristematic activity.[11] The clusters were shown to be derived from individual trichome precursors that repeatedly underwent anticlinal and periclinal cell divisions. Outcrossing of the mutant to a wild type plant resulted in an F2 population containing a new mutant phenotype. This mutant exhibited trichomes that appeared somewhat normal, but on closer inspection were found to be composed of multiple cells. This phenotype was identical to that described for the *SIAMESE* (*SIM*) mutant.[14] Allele testing confirmed that the mutants were allelic. *SIM* has been cloned and found to encode a protein related to proteins that inhibit cyclin D activity.[15] Further, it was shown that SIM protein interacts with both CYCDs and Cyclin Dependent Kinase A (CDKA).

CYCDs are thought to be important for both G_1 to S phase and G_2 to M phase transitions.[16] In wild type trichomes, it has been shown that ectopic expression of *CYCD3;1* induces the

formation of *sim*-like multicellular trichomes that have fairly normal morphology.[17] To test if the clusters found in *gl3-sst sim* could be due to extra CYCD activity, *CYCD3;1* was ectopically expressed via the *GLABRA2* promoter (*GL2*; drives high level of expression in trichomes[18]) in the *gl3-sst* mutants.[11] The resulting plants formed trichomes that roughly phenocopied those seen on *gl3-sst sim* double mutants. However, there were differences. For example, the trichomes on *gl3-sst CYCD3;1* plants exhibited a delay in cell division and the resulting clusters contained more branched trichomes than seen in *gl3-sst sim* clusters.

GL1 also appears to play a role in the formation of the clusters. *GL1* is normally expressed throughout young leaf primordia and then is up regulated in developing trichomes (Fig. 1A).[19] A zone of *GL1* expression in young leaves persists but abruptly disappears once a leaf reaches a certain size (Fig. 1B). In addition, *GL1* expression normally shuts down as trichomes develop. In *gl3-sst sim*, high *GL1* expression was found to persist in mature trichome clusters.[11] This high level of *GL1* expression required the loss of SIM activity as *gl3-sst* trichomes showed more normal levels of *GL1* expression. For this addendum we wished to explore the status of *GL1* expression in *gl3-sst CYCD3;1* trichomes. The key question was whether or not altering the cell cycle through ectopic *CYC3;1* expression can alter *GL1* expression.

In Marks et al., (2007) real time qPCR was used to compare levels of *GL1* expression between young shoot apexes, where *GL1* expression is highest in wild type (see Fig. 1A), to young developing leaves where *GL1* expression rapidly declines (Fig. 1B). This analysis showed that *GL1* expression decreased over a 1000-fold from apex to leaf. Similar results were obtained for the individual *gl3-sst* and *sim* mutants. However, as stated above similar levels of *GL1* expression were found in the apexes and young leaves of *gl3-sst sim* plants. In the *gl3-sst sim* leaves, most of the *GL1* mRNA was likely derived from the trichome clusters because almost all of the GUS staining in *GL1*::GUS containing *gl3-sst sim* plants was seen in the trichome clusters. The real time qPCR experiment has been repeated to compare *GL1* mRNA levels in the apexes and young leaves of wild type, *gl3-sst sim*, and *gl3-sst GL2::CYCD3;1* plants.

New Findings

As shown in Table 1, *GL1* expression in the apex tissue was similar in all three genotypes. And as previously shown, *GL1* expression falls dramatically in wild type young leaf tissue (declined by 490 fold), but holds fairly steady in *gl3-sst sim* (declined by only 1.5 fold). The expression in *gl3-sst GL2::CYCD3;1* young leaves was reduced by 19.6 fold. This level of reduction is much less than seen for wild type or in the previous analysis of *gl3-sst* alone. Thus, the increase in expression is likely due to elevated *CYCD3;1* expression. This result suggests that there is a linkage between progression through the cell cycle and *GL1* expression in developing trichomes.

E2F and Gene Expression in Trichomes

One of the key targets of CYCD/CDKA activity is the *RETNOBLASTOMA RELATED* (*RBR*) protein.[20,21] RBR binds to the transcription factor E2F to repress its activity. Phosphorylation of RBR by CDKA reduces the interaction between RBR and E2F.[16] After the release of of E2F from phosphorylated RBR, E2F is free to activate the expression of target genes, many of which have roles in DNA replication.[22] The interaction between E2F and RBR also can result in direct inactivation of the expression of gene targets.[23] This is thought to occur when E2F recruits RBR to target genes

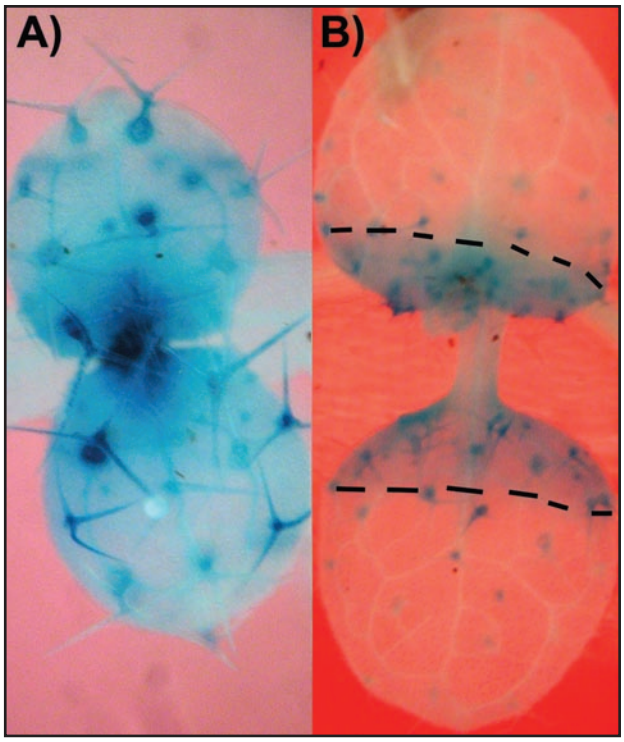

Figure 1. *GL1*::GUS expression in young Arabidopsis shoots. (A) *GL1*::GUS expression in a young Arabidopsis seedling. Intense staining is seen in the trichomes and apex region, whereas lighter more diffuse staining is seen throughout the leaf surface. (B) Slightly older seedling showing abrupt loss of diffuse surface staining and reduced expression in trichomes. The *GL1*::GUS construct is described in ref. 19 and is available from the ABRC stock center (CDC-364 plasmid or CS8850 seeds).

Table 1 Relative levels of *GL1* and *HSP70* expression[4]

Gene	Tissue	Relative Quantity[1]	Shoot over Leaf
GL1	Col shoot[2]	1.000 ± 0.063	490
GL1	Col leaf[3]	$2.04 \times 10^{-3} \pm 0.045 \times 10^{-3}$	
GL1	*sstsim* shoot	1.586 ± 0.093	1.5
GL1	*sstsim* leaf	1.043 ± 0.109	
GL1	*sst cycD3;1* shoot	2.666 ± 0.701	19.6
GL1	*sst cycD3;1* leaf	0.136 ± 0.011	
HSP70[5]	Col shoot[2]	1.000 ± 0.018	1.09
HSP70	Col leaf	0.915 ± 0.026	
HSP70	*sstsim* shoot	1.195 ± 0.099	1.2
HSP70	*sstsim* leaf	1.028 ± 0.051	
HSP70	*sst cycD3;1* shoot	1.578 ± 0.096	1.85
HSP70	*sst cycD3;1* leaf	0.852 ± 0.082	

[1]All quantities were normalized to the level of expression obtained for Col shoot. *GL1* and *HSP70* values were normalized separately. Mean ± standard deviation values are shown derived from 2 replicates of each tissue. [2]All young shoot tissue contained just the apex and emerging leaves that lacked petioles. [3]Third leaves were dissected from seedlings just at the stage when the leaves were beginning to develop petioles. [4]All reaction conditions and primers are described in Marks et al., 2007. [5]The *HSP70* gene was used an internal control of mRNA integrity, as previous analyses have shown that the expression of this gene varies little from tissue to tissue under normal growth conditions.

where interactions between RBR and histone deacetylases can induce localized heterochromatin formation to silence gene expression. There is some evidence that E2F can play a role in trichome gene expression. Ramirez-Parra and Gutierrez (2007) have shown via GUS reporter constructs that the *FASCIATA1* (*FAS1*) gene, which encodes a component of the Chromatin Assembly Factor 1, is expressed in trichomes. Further, they were able to show that mutations in one of two E2F binding sites in the *FAS1* promoter enhanced gene expression, whereas mutations the nearby second E2F binding site repressed gene expression.[24]

GL1 Expression and the Cell Cycle

In developing trichomes, *GL1* expression is greatest during the same interval of time that endoreduplication occurs.[10,19] This is a period during which CYCD/CDKA activity would be involved in promoting this activity. Thus, if *GL1* expression is directly modulated by E2F, then the rise and fall in CDKA activity during endoreduplication could play a role in *GL1* expression. This could occur either through direct up regulation via E2F activation or via repression through chromatin remodeling.

In a previous analysis, promoter sequences responsible for *GL1* expression and function were identified.[19] Interestingly, a 153 bp interval starting 1028 bp downstream of the stop codon was essential for *GL1* gene function. Genomic fragments lacking this region failed to restore trichome development in *gl1* mutants. An analysis of the sequences 1000 bp upstream of the start codon and over 2800 bp downstream of the stop codon revealed three potential E2F binding sites. One of these is in the large second intron of the gene. This site is likely not responsible for regulating *GL1* expression as this site is not present in the *GL1*::GUS construct that revealed reduced activity in mature wild type trichomes and high *GL1* expression in *gl3-sst sim* clusters. However, at the 3' end of the important 153 bp interval there are two overlapping potential E2F binding sites present in opposite orientations (TTTGCCGG and ATTCCCGG; motifs described in ref. 22). These sites were present in the *GL1*:: GUS reporter used to study *GL1* expression in wild type and *gl3-sst sim* backgrounds.[11] The presence of these potential E2F binding sites in the precise region defined to be important for *GL1* expression supports the possibility that *GL1* expression is directly tied to the cell cycle. Future experiments to test this region directly for E2F binding and to assess its chromatin conformation during trichome development will aid in strengthening the connection between *GL1* expression and the cell cycle.

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