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SCI1, the first member of the tissue-specific inhibitors of CDK (TIC) class, is probably connected to the auxin signaling pathway

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The recent finding of a tissue-specific cell cycle regulator (SCI1) that inhibits cell proliferation/differentiation in the upper pistil points to an unanticipated way of controlling plant morphogenesis. The similarity between the SCI1 RNAi-silenced plants and some auxin-related phenotypes suggested that SCI1 could be involved in the auxin signaling pathway. To address this hypothesis, we analyzed the expression of three auxin-related genes in transgenic plants in which SCI1 was silenced and overexpressed. The results showed that the expression levels of the auxin-related genes largely correlated with the SCI1 expression level. Additionally, we analyzed the Arabidopsis SCI1 upstream regulatory region and found putative cis-acting elements also present in the *AtCYCB1;1 AtYUC1, AtYUC2* and *AtYUC4* URRs, suggesting a cell cycle- and auxin-related transcriptional regulation. Based on our previous and the current studies, we propose SCI1 as a signal transducer engaging auxin signaling and cell division/differentiation.

A major challenge in plant development is to elucidate how the molecular machinery present in each cell/tissue type correlates with the developmental program of plant organs. To help in addressing this question, we applied reverse genetics to unravel the role of a previous unknown protein, SCI1 (stigma/style cell-cycle inhibitor 1;[1]). The transcript corresponding to the *SCI1* gene was identified in a suppression subtractive hybridization library (DePaoli et al., unpublished) as preferentially expressed in the stigma/style tissues. *In silico* analysis of the SCI1 putative protein sequence revealed the presence of several putative domains; however, there was no clue to its gene function. After the expression analysis and careful examination of RNAi-silenced and overexpression plants, $SCI1^{Ri}$ and $SCI1^{OE}$ respectively, the unique alteration observed was an increased stigma/style size in the $SCI1^{Ri}$ plants and the opposite phenotype, decreased stigma size, in the $SCI1^{OE}$ plants. Interestingly, these changes in size were a consequence of an altered cell number in a restricted group of cells. Our results showed that SCI1, a previously unknown protein, has tissue-specific functions and negatively regulates the cell cycle in planta.

SCI1 is a Cell Cycle Regulator Distinct from the Previously Characterized Plant CDK Inhibitors

Different aspects of plant development have been studied to uncover the molecular network behind organ growth and development, culminating with the discovery of different classes of genes, such as those involved in cell division control. To achieve this control, the cell recognizes different signals from a variety of pathways, which are transduced by cell cycle regulators and result in the activation or inhibition of the cell cycle. Plant CKI/KRPs (CDK Inhibitors/Kip-related protein), which have similarity to the mammalian Kip/Cip inhibitors, directly interact with CDK and/or cyclin proteins and constitute the major negative regulators of CDK/cyclin complexes to control cell cycle in plants.[2,3] Despite its effect in inhibiting the cell cycle, SCI1 protein has no sequence similarity to the CKI/KRPs, even when their 9 conserved motifs or the C-terminal signature are either searched or aligned independently. SCI1 has only a limited but recognizable similarity with SIM (SIAMESE;[4]), a new class of CDK inhibitors, in a short region which coincides with the region of similarity between SIM and CKI/KRPs. Functionally, SCI1 inhibits cell division in a tissue-specific manner and does not alter cell size. On the other hand, the CDK inhibitors studied so far, including SIM, affect different plant organs. Taken together, we propose that, in addition to the two classes of CDK inhibitors already known in plants: (a) *CKI/KRPs* (CDK inhibitors/Kip-related proteins) and (b) *SIMs* (SIAMESE and SIAMESE-related proteins), both broadly expressed in plant tissues and with a direct change of CDK activity; there is a third class: (c) *TICs* (Tissue-specific inhibitors of CDK), which show tissue specificity and

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probably interact with the cell cycle machinery, regulating CDK activity either directly or indirectly. Thus, SCI1 is the first member of a new class of CDK inhibitors, the TIC class.

Does SCI Transduce Signals Engaging Auxin Signaling and Cell Division/Differentiation?

The initial carpel is a small group of cells that are able and committed to divide and differentiate to give rise to the fully developed pistil. Such developmental processes include mainly hormone-mediated organogenesis associated with cell division and differentiation. Therefore, alterations of final organ size are often one of the consequences of modified phytohormone signaling.[5] Taking in consideration that the SCI1^{Ri} tobacco stigma/style phenotype[1] resembles those where either auxin synthesis or transport was disturbed,[6-9] we investigated the possibility that some auxin-responsive genes might be influenced by SCI1 levels.

The Auxin/Indole-3-Acetic Acid (Aux/IAA) and auxin response factor (ARF) families of transcription factors are key regulators with well-characterized roles in auxin responses.[10,11] The Aux/IAA proteins dimerize with ARFs to control auxin-dependent gene transcription. In high intracellular auxin concentrations, the Aux/IAA are degraded, releasing the ARF proteins, which regulate auxin-dependent gene transcription through their ability to bind auxin responsive elements (AREs). Recent reports have shown that cell cycle regulators, as the ICK2/KRP2 member of the CKI/KRP family, are stabilized and/or degraded by auxin, demonstrating that cell division, cell differentiation and auxin signaling have molecular intersections.[12,13]

We analyzed the expression pattern of three different auxin-responsive genes: *NtAux/IAA19* (accession number GQ272333), *NtAux/IAA13* (GQ272334) and *NtARF8* (GQ272332) (**Fig. 1**), in *Nicotiana tabacum* stigmas/styles. These genes were chosen based on the ability of their Arabidopsis orthologs to change multiple auxin responses (*Aux/IAA19*,[14]), to impair auxin-regulated development (*Aux/IAA13*,[15]), and to participate in the control of flower development (*ARF8*,[16]). For this experiment, we chose three transgenic plants that displayed the lowest (SCI1^{Ri}) and the highest (SCI1^{OE}) *SCI1* mRNA levels (**Fig. 1A**;[1]). Note that *SCI1* expression on SCI1^{OE} plants is shown on a logarithmic scale and, therefore, despite their apparent proximity there is a considerable difference in their expression levels. Our results show (**Fig. 1B-D**) that the three auxin-responsive genes analyzed are significantly induced in the *SCI1* overexpression plants (Student's T-test; $p < 0.05$). However, it seems that there is a limit to which extent SCI1 can induce each of the auxin-responsive genes, as the highest expression level for each of these genes was not achieved in the plant with the highest *SCI1* transcript level (SCI1^{OE}3.1.1). Above a certain *SCI1* transcript level the induction effect is reduced, a response usually observed with increasing hormonal concentrations (a maximum in the dose-response curve), as previously described for auxin.[17] On the other hand, among these three genes tested only *NtAux/IAA19* transcript levels were consistently modified in the *SCI1* RNAi-silenced plants. As auxin can act on a cell- and tissue-specific basis and *SCI1* can only be silenced in the stigma/style cells in which it is usually expressed (stigmatic secretory zone – SSZ and stylar transmitting tissue - STT), we should consider that the effect on the auxin-responsive genes will be diluted (normalized) in expression analyses performed in whole stigma/style organs.

The Arabidopsis auxin insensitive mutant *msg2–1*, which encodes the Aux/IAA19 protein, is defective in the induction of *Aux/IAA4*, *DFL1* (DWARF IN LIGHT 1) and *SAUR(small auxin up RNA)-AC1* genes after hormone treatment.[14] These

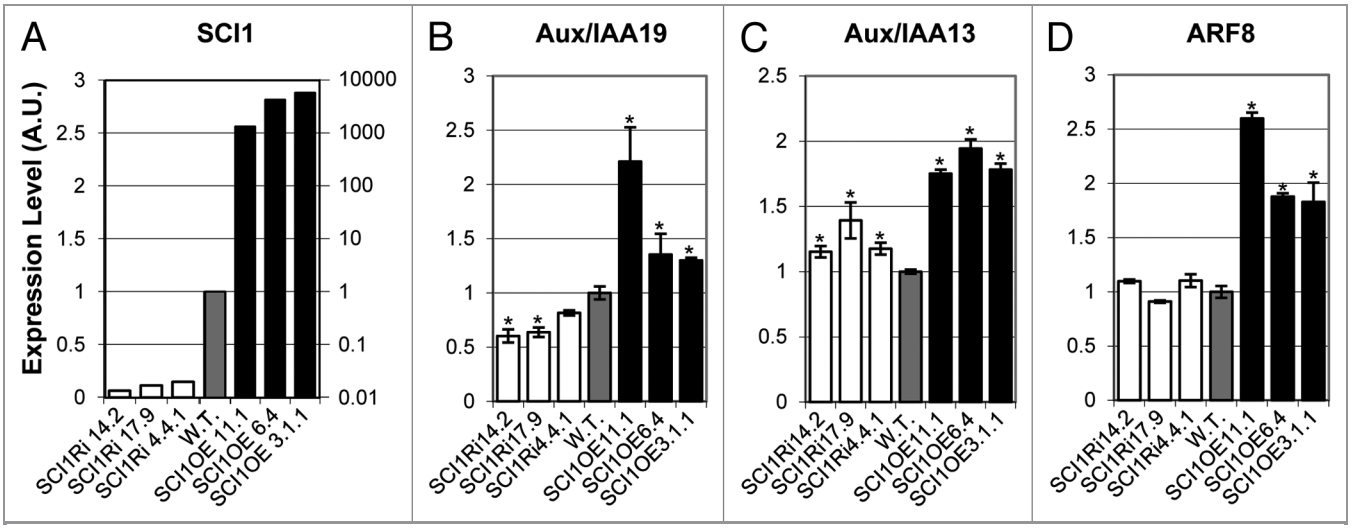

Figure 1. Transcript levels of SCI1 and three auxin-regulated genes in stage 4 stigmas/styles of tobacco wild-type (SR-1) and transgenic SCI1^{Ri} and SCI1^{OE} plants, determined by qRT-PCR. (A) *SCI1*, (B) *NtAux/IAA19*, (C) *NtAux/IAA13* and (D) *NtARF8* genes. The arbitrary units (A.U.) correspond to the calculated log of the number of copies of the mentioned gene normalized by the ubiquitin gene in each plant divided by the same calculation for the wild-type plant (wild-type value divided by the wild-type value is equal to 1). Note that the expression levels of the *NtAux/IAA19*, *NtAux/IAA13* and *NtARF8* genes were determined in an increasing amount of *SCI1* expression: three SCI1^{Ri}, wild-type and three SCI1^{OE} plants. (Methods are as in DePaoli et al.[1]). The statistically significant differences between each plant vs. the wild-type by Student's T-test are indicated (*$p < 0.05$).

genes belong, respectively, to the Aux/IAA, GH3 and SAUR classes of early auxin responsive genes, which are directly and quickly influenced by auxin treatment at the transcriptional level. Thus, the altered expression of these genes in the *msg2–1* mutant invokes Aux/IAA19 in the primary steps of auxin response. *NtAux/IAA19* was upregulated in SCI1^{OE} plants and down-regulated in SCI1^{Ri} plants, with these changes paralleling *SCI1* expression levels (**Fig. 1B**). The fact that the down- and upregulation of *SCI1* levels can, respectively, down- and upregulate the *NtAux/IAA19* mRNA levels, indicates that *SCI1* is positively correlated with *NtAux/IAA19* gene expression.

NtAux/IAA13 was induced on SCI1^{Ri} and SCI1^{OE} transgenic plants (**Fig. 1C**), but it is clearly more induced on SCI1^{OE} plants. It is interesting to note that Arabidopsis plants overexpressing *IAA13* grew more slowly than wild type,[18] what is consistent with the delayed stigma/style growth/development observed in SCI1^{OE} plants.[1]

The Arabidopsis *ARF8* overexpression resulted in repression of tissue elongation.[19] The fact that *NtARF8* is overexpressed in SCI1^{OE} plants, which shows decreased stigma size, supports the SCI1 link with auxin signaling. Arabidopsis *arf8* mutants show larger petals than wild type, which is caused by an increase in cell number and cell expansion.[20] In our tobacco SCI1^{Ri} plants, larger stigmas/styles occur as the result of an increased number of cells with approximately the same size.[1] The decreased *SCI1* transcript levels in SCI1^{Ri} plants did not influence *NtARF8* expression in our analysis with whole stigmas/styles. It is possible that the effect on *NtARF8* transcript level on the SSZ and STT may have been counterbalanced by the inclusion of stigma/style cells in which *SCI1* is not expressed, as already suggested above.

Despite the interesting results described above, it is not yet clear how *SCI1* expression can influence the transcript levels of auxin-responsive genes. It probably occurs indirectly through a feedback mechanism in the auxin signaling pathway.

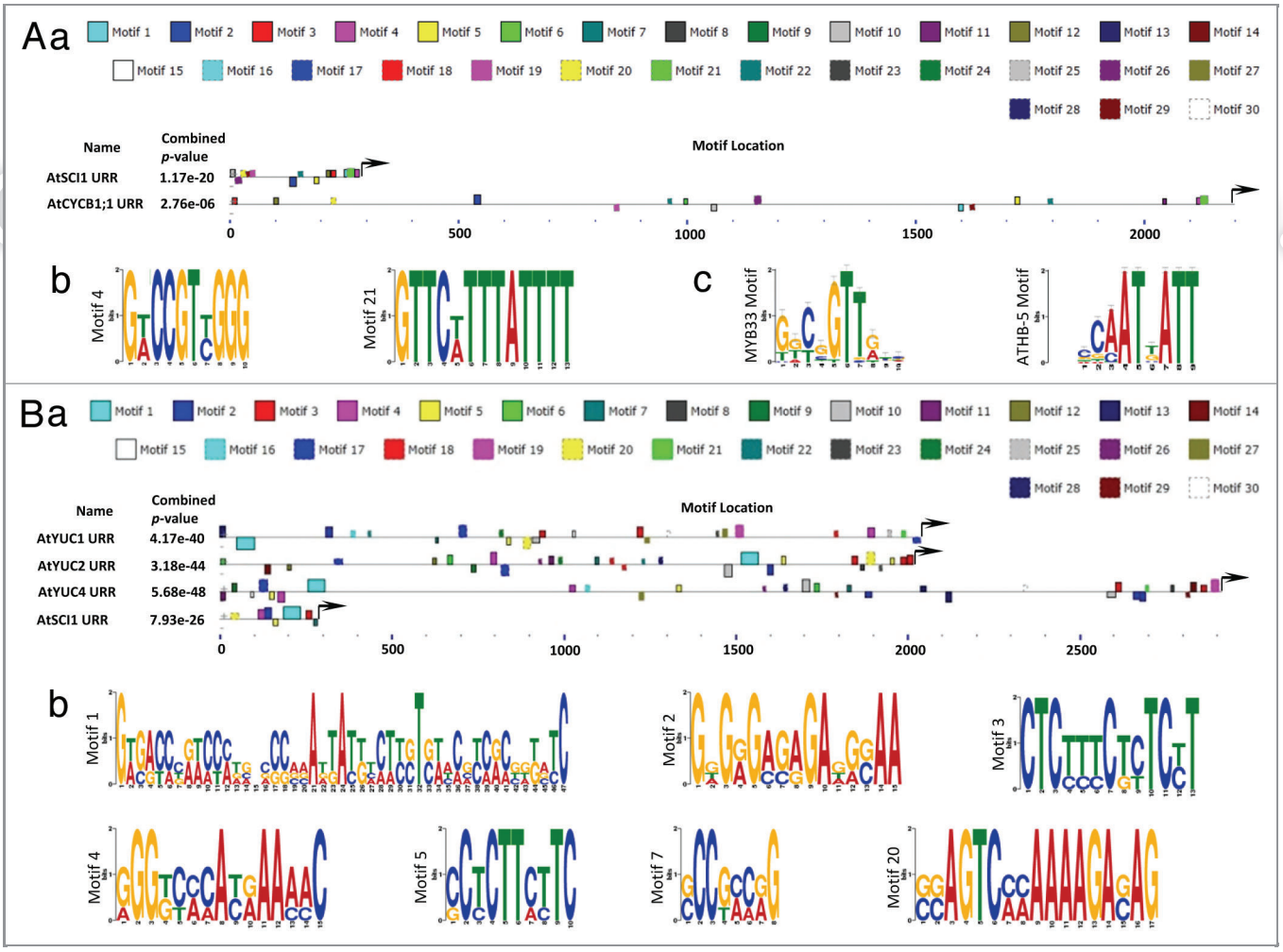

Figure 2. In silico analysis of the URR of the *AtSCI1* vs *AtCYCB1;1*, *AtYUC1*, *AtYUC2* and *AtYUC4*. The URR of the *AtSCI1* (287bp) was compared with the URR of the (A) *AtCYCB1;1* (2.2kb) and (B) *AtYUC1* (2.0kb), *AtYUC2* (2.0kb) and *AtYUC4* (2.9kb) simultaneously. (a) Non-overlapping sites are shown, in color scale, with a p-value better than 0.0001. The searches were limited up to 30 domains with at least 6bp long and at any occurrence (one or more times in the same URR). The height of the motif "block" is proportional to -log(p-value), truncated at the height for a motif with a p-value of 1e-10. The black arrows indicate the direction and start point of the open reading frame (ATG, position +1). The (b) detailed motifs and (c) TF-binding sites were found using MEME[21] and TOMTOM[22] softwares, respectively. For further information, access meme.nbcr.net.

The Arabidopsis *SCI1* Upstream Regulatory Region Contains Putative Cis-Acting Elements for Cell Cycle and Auxin Regulation

To have a better insight on how *SCI1* is related to cell proliferation/differentiation and auxin signaling at the transcriptional level, we performed a search for conserved DNA-binding domains using the MEME tools[21,22] in the upstream regulatory region (URR) of the Arabidopsis *AtSCI1* (At1g79200), *AtCYCB1;1* (At4g37490), *AtYUC1* (At4g32540), *AtYUC2* (At4g13260) and *AtYUC4* (At5g11320) genes. The B-type cyclins are critical for the proper timing of cell entry into mitosis and their expression is regulated at both the transcriptional and post-translational levels.[23] The YUCCA gene family is responsible for tissue-specific auxin synthesis.[24] We chose these three members of the YUCCA family based on their expression pattern: in SAM (shoot apical meristem) and pistils ([6]; and BAR[25]).

The short 202bp URR of the *AtCYCB1;1* gene is sufficient to drive cell cycle-regulated expression of a reporter gene, showing that cell cycle-regulatory elements are present in this region.[23] In this short URR, there are a 13bp (GTTC[AT]TTTATTTT) and a 10bp (G[TA]CCGT[TC]GGG) consensus sequences (**Fig. 2Aa/b**) that are binding sites for master transcription factors (TFs), as the ATHB5[26] (At5g65310; **Fig. 2Ac**) and AtMYB33[27] (At5g06100; **Fig. 2Ac**), respectively. The ATHB5 mediates growth inhibition[28] and AtMYB33 activates *AtCYCB1;1* gene expression, regulating growth. AtMYB33 is also known to be involved in cell differentiation and hormone signaling.[29] Both cis-acting elements were identified on the 287bp *AtSCI1* URR (positions -29 and -15) with a *p-value* of 1.79e-07 and 2.87e-06, respectively (**Fig. 2A**), suggesting that cell cycle-related TFs could be involved in controlling *SCI1* expression at the transcriptional level.

The short *AtSCI1* URR has significant similarity to 7 domains (1, 2, 3, 4, 5, 7 and 20) also found in the YUC1, YUC2 and YUC4 genes (**Fig. 2B**). All these consensus sequences bind to different plant TFs like HAT5/HMG-I:Y/ATHB5[26,30,31] (Domain 1), HMG1/ID1[31,32] (Domain 2), PEND[33,34] (Domain 5) and MNB1A[35] (Domain 20). The relevance of these predictions was also evaluated by the expression patterns of *AtSCI1* and those YUCCA genes. Having a pistil-restricted expression pattern, AtSCI1 has an incomplete overlap with YUC1, YUC2 and YUC4 genes, which display a broader expression pattern (SAM and pistils). However, it is interesting to note that the four genes respond equally to many different cellular signals, as determined by Genevestigator,[36] suggesting that some conserved transcriptional machinery is recruited to these promoters. Taken together, the existence of 7 common URR domains among these genes and their similar expression patterns are significant and support the idea that these genes share related regulatory networks. As a negative control, we compared the GIBBERELLIN 3-OXIDASE 1 (At1g15550) and GIBBERELLIN 2-OXIDASE 2 (At1g30040) URRs (both also expressed at SAM and flowers, as determined by BAR[25]) to the *AtSCI1* URR. Within the standard 1kb URRs considered for the GIBBERELLIN-OXIDASE genes, there were no common significant domains among them. Thus, we believe the common domains found within the *AtSCI1* and YUCCA URRs are not at random. Overall, the presence of these putative cis-acting elements on the *AtSCI1* URR suggests that auxin-related TFs may also regulate *SCI1* expression.

Final Remarks

The link between hormone signaling and the regulation of the cell cycle machinery has become more evident in the last years.[37] However, the unraveling of a cell cycle regulator that acts tissue specifically is new and unanticipated.[1] It supports the idea that tissue-specific players, in addition to the more general cell cycle machinery, are necessary to finely regulate plant morphogenesis. Therefore, we can propose the existence of proteins with similar functions in other plant tissues/organs. Based on the expression analyses of auxin-responsive genes, on the similarities between SCI1 transgenic plants and the auxin-related phenotypes, and on the presence of the above mentioned cis-acting regulatory elements, it is reasonable to suggest that SCI1 acts as a signal transducer connecting the auxin signaling pathway and the cell proliferation/differentiation control in the upper pistil (**Fig. 3**). To our knowledge, SCI1 would be the first molecule to provide a connection between cell division/differentiation machinery and auxin signaling in pistils. We believe that future genetic and molecular studies will strengthen SCI1 association with the auxin pathway and extend our knowledge on hormone signaling and cell

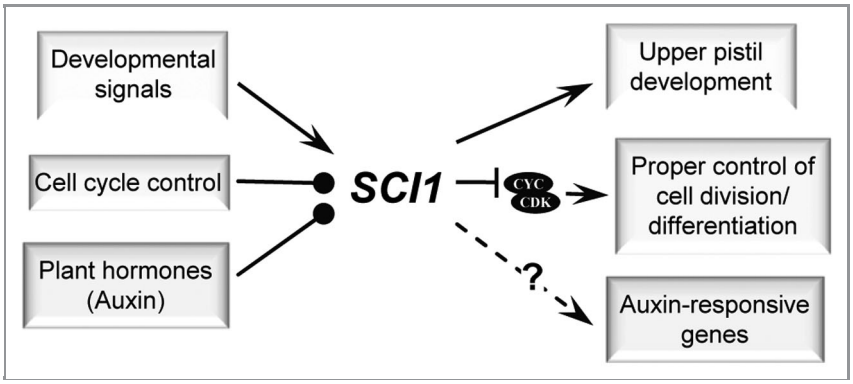

Figure 3. Proposed model for SCI1 as a signal transducer in the auxin signaling pathway and the cell proliferation/differentiation control during pistil development. Pistil developmental signals switch on SCI1 expression which is potentially regulated by auxin- and cell cycle-dependent factors at the transcriptional level. SCI1 controls cell proliferation/differentiation in specific tissues, promoting proper pistil development.[1] This control is very probably exerted by SCI1 interaction with cyclins and/or CDKs, which causes the inhibition of the cell cycle. Conversely, SCI1 influences the transcript levels of auxin-responsive genes, probably through a feedback mechanism in the auxin signaling pathway. The SCI1 inhibition on cell proliferation helps to provide the fine-tuning of the auxin response to cell proliferation/differentiation in stigmas/styles. (Arrow bars, induction, promotion; ball ended bars, induction or repression; interrupted bar, inhibition; dotted line, correlation, probably through an unknown factor (?).

proliferation control in a tissue specific manner. Unraveling the cross talk between these processes has invaluable importance to understand specific paths in plant reproduction as well as to answer basic questions in plant developmental biology.

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