SCI1 Is a Direct Target of AGAMOUS and WUSCHEL and Is Specifically Expressed in the Floral Meristematic Cells

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The specified floral meristem will develop a pre-established number of floral organs and, thus, terminate the floral meristematic cells. The floral meristematic pool of cells is controlled, among some others, by WUSCHEL (WUS) and AGAMOUS (AG) transcription factors (TFs). Here, we demonstrate that the SCI1 (Stigma/style cell-cycle inhibitor 1) gene, a cell proliferation regulator, starts to be expressed since the floral meristem specification of Nicotiana tabacum and is expressed in all floral meristematic cells. Its expression is higher in the floral meristem and the organs being specified, and then it decreases from outside to inside whorls when the organs are differentiating. SCI1 is co-expressed with N. tabacum WUSCHEL (NtWUS) in the floral meristem and the whorl primordia at very early developmental stages. Later in development, SCI1 is co-expressed with NAG1 (N. tabacum AG) in the floral meristem and specialized tissues of the pistil. In silico analyses identified cis-regulatory elements for these TFs in the SCI1 genomic sequence. Yeast one-hybrid and electrophoresis mobility shift assay demonstrated that both TFs interact with the SCI1 promoter sequence. Additionally, the luciferase activity assay showed that NAG1 clearly activates SCI1 expression, while NtWUS could not do so. Taken together, our results suggest that during floral development, the spatiotemporal regulation of SCI1 by NtWUS and NAG1 may result in the maintenance or termination of proliferative cells in the floral meristem, respectively.

Keywords: co-expression, floral determinacy, flower development, meristematic cells, Nicotiana tabacum, transcriptional control

INTRODUCTION

The maintenance and termination of the floral meristem are orchestrated by a complex network of elements that involve transcription factors (TFs), hormonal signaling, and cell cycle control genes (Jha et al., 2020). In the Arabidopsis floral meristem, the undetermined proliferation of cells is dependent on the expression level of WUSCHEL (WUS), a homeobox TF gene expressed in the
organizing center (OC) (Laux et al., 1996). The OC is composed of pluripotent cells maintained until the specification of the four floral whorls: sepals, petals, stamens, and carpels (Sharma et al., 2003). During the early stages of floral development, the feedback between WUS-CLAVATA (CLV) sustains the homeostasis of the floral meristem (Zhou et al., 2015). WUS is transcribed in the OC, and the protein migrates to the outer layers of the floral meristem, where it activates CLV3 (Yadav et al., 2013). WUS blocks cell differentiation by inactivating genes, such as the ARR7/ARR15 (ARABIDOPSIS RESPONSE REGULATOR) that are mediators of the auxin control of cytokinin signaling (Leibfried et al., 2005; Zhao et al., 2010). Another example is the role of WUS in inhibiting genes in the auxin biosynthesis pathway (Mano and Nemoto, 2012), preventing cell differentiation (Yadav et al., 2013). The interaction between these pathways guarantees the balance between proliferation and differentiation, which is essential for the correct formation of the flower (Sun et al., 2009).

In the floral meristem, pluripotent cells do not multiply indefinitely as in the shoot apical meristem. The expression of WUS is down regulated during the specification of the fourth whorl in the center of the floral meristem (Mayer et al., 1998). The specification of carpels is established by AGAMOUS (AG), a MADS-box TF (Bowman et al., 1989; O’Maoléidigh et al., 2014). AG is also responsible for terminating the proliferation of undetermined cells in the center of the floral meristem (Lenhard et al., 2001). AG expression is activated due to the cooperation between LEAFY (LFY) and WUS TFs that bind to the second AG intron (Lohmann et al., 2001). Once activated, AG suppresses WUS expression by recruiting the CURLY LEAF protein (CLF) that is part of the polycomb repressive complex 2 (PRC2) and adds a tri-methylation to lysine 27 in histone 3 (H3K27me3), thus inactivating WUS expression (Liu et al., 2011). AG also activates the expression of KNUCKLES (KNU), which encodes a C2H2-zinc finger TF that directly represses WUS expression at stage 6 of Arabidopsis thaliana flower development (Sun et al., 2009), putting an end to undifferentiated proliferation. Although widely studied, there are still gaps in the knowledge involving the signaling processes downstream of WUS and AG that ultimately result in the termination of the pool of meristematic cells and differentiation.

Stigma/style cell-cycle inhibitor 1 (SCI1) was previously described as a gene preferentially expressed in stigma/style that controls cell proliferation in the upper pistil of N. tabacum and A. thaliana (DePaoli et al., 2011, 2014). N. tabacum SCI1 expression starts in situ hybridizations were performed using N. tabacum WUSCHEL (NAG1), the N. tabacum orthologs of WUS (Li et al., 2018; Zhou et al., 2018) and AG (Kempin et al., 1993), respectively, in the flower meristem. The binding of these two TFs to the SCI1 promoter sequence was confirmed by yeast one-hybrid (Y1H) and electrophoresis mobility shift assay (EMS), and in planta luciferase activity assay for NAG1. We gathered evidence showing that SCI1 expression is activated by the TF NAG1. As SCI1 is a regulator of cell proliferation/differentiation, we discuss the hypothesis of SCI1 being an effector of the termination process of the floral meristem of N. tabacum, which is under the control of NAG1.

RESULTS

SCI1 Starts to Be Expressed at the Specification of the Floral Meristem and Maintains Its Expression in Proliferative Cells

Our previous work demonstrated that the SCI1 gene is highly expressed at the very early developmental stages of N. tabacum pistils (DePaoli et al., 2011). Therefore, we became interested in studying when SCI1 expression starts. For this purpose, in situ hybridizations were performed using SCI1 antisense transcripts as a probe in histological sections of the inflorescence apex. The results demonstrate that SCI1 is expressed since floral meristem initiation and emergence (Figure 1A).

To have a detailed understanding of the N. tabacum flower meristem development and establish a parallel with SCI1 expression, we also implemented anatomical and histological analyses in conjunction with the in situ hybridization experiments. At the very early stages of development (stage −9, here defined based on the earlier stage described by Koltunow et al. (1990) as stage −7), five sepal primordia arise sequentially at the edges of the floral meristem, as documented by scanning electron microscopy (SEM) (Figure 1B). The floral meristem, seen at the center, is organized in three cell layers (L1, L2, and L3), easily distinguished in anatomical sections observed by bright field microscopy (Figures 1C,D). Several cell divisions are clearly visible at the meristematic cell layers (Figure 1E). The sepal primordia arise asynchronously and clockwise in divergent angles of approximately 144 degrees relative to the previous
FIGURE 1 | *SCI1* expression during *Nicotiana tabacum* early floral development. (A) *In situ* hybridization of inflorescence apex with *SCI1* antisense probe. Four floral buds are observed. Scale bar: 500 µm. (B) Scanning electron microscopy (SEM) showing the asynchronous emergence of the sepals (S1–S5) in a flower meristem and sepal S1 with trichomes (stage –9, here defined). Scale bar: 100 µm. (C) Bright-field microscopy showing a longitudinal section of a very young flower bud at stage –9. Scale bar: 100 µm. (D) A higher magnification view of the flower bud in C, in which the three meristematic cell layers (L1, L2, and L3) are seen. Scale bar: 50 µm. (E) A higher magnification view of the flower meristem shown in (D), in which cell divisions are visible (arrows). Scale bar: 25 µm. (F–I) *In situ* hybridization with *SCI1* antisense probe of very young flower buds, even before stage –7, the youngest developmental stage defined by Koltunow et al. (1990). (F) Flower meristem with emerging sepals (stage –10, here defined). This is a higher magnification of FM3 from (A). Scale bar: 100 µm. (G) Flower meristem with sepal primordia (stage –9, here defined). This is a higher magnification of FM2 from (A). Scale bar: 100 µm. (H) A higher magnification view of the marked area in (G). The meristematic cell layers (L1, L2, and L3) are identifiable. Scale bar: 50 µm. (I) Flower meristem with emerging petals and anthers, at stage –8 (here defined). This is a higher magnification of FM1 from (A). Scale bar: 100 µm. Floral meristem (FM), sepals (S). Compare the image shown in (F) with the images (G–I) and observe the reduced *SCI1* expression in the OC [encircled in (I)]. Negative controls of flower buds in equivalent developmental stages are shown in Supplementary Figure 1.
primordium (Figure 1B). After reaching a specific size, the sepal primordia show trichomes at the abaxial side (Figures 1B,C). At this very early stage, \(SCI1\) is expressed at the emergence of sepal primordia (Figures 1F,G) and at high levels in all cell layers of the central floral meristem (Figures 1F–I). When the sepals are specified, \(SCI1\) expression decreases in the region below the third layer, the area described as the OC (Figures 1G–I).

At developmental stage −8 (here defined), petal and stamen primordia upraise synchronously and almost simultaneously from the floral meristem, while sepals grow toward each other. At this stage, cells of the petal primordia, stamen primordia, and the central floral meristem have characteristics of meristematic cells (Chang and Sun, 2020), in contrast to the sepal cells that are differentiating (Figure 2A). At this stage, the floral meristem is restricted to the area in which the carpel primordia will form (Figure 2B). The five-petal primordia emerge in alternate positions in relation to the sepal primordia. Similarly, the five stamen primordia arise in alternate places with regard to the petal primordia. Therefore, the stamen primordia are positioned in the same direction as the sepal primordia. Despite the almost simultaneous upraise of petal and stamen primordia, the latter seem to grow faster than the petals at this stage. During flower development, \(SCI1\) expression is temporally and spatially regulated (Figures 1, 2). At advanced stage −8, \(SCI1\) is highly expressed in the petal and stamen primordia, as well as in the remaining central floral meristem (Figure 2C). Still, its expression has already decreased at the differentiating sepals.

Later in development (stage −7), the two carpel primordia emerge, and their invaginations are clearly seen where the expression has already decreased at the differentiating sepals. In \(N.\ tabacum\), the carpels fuse postgenitally, and it is possible to observe the carpel primordia growing toward each other to converge at the top, while the base is already connected (Figure 2E). At an equivalent stage, Chang and Sun (2020) identified cell divisions in the L1 layer and deeper layers of the \(N.\ tabacum\) carpels. At stage −7, the \(SCI1\) signal is much weaker in sepals and petals that are differentiating (Figure 2F). Meanwhile, specified anthers are developing, and \(SCI1\) is strongly detected in this whorl (Figure 2F). In the carpel primordia, the \(SCI1\) signal is intense, especially in the upper part where fusion occurs. Within the carpels, \(SCI1\) is also expressed in the ovule locules (Figure 2F), a region with meristematic cells that will develop into ovules, suggesting a function for \(SCI1\) in ovule development.

Development progresses (stage −6), and carpel primordia are growing toward each other (Figures 2G,H) and will fuse, where cells will continue to divide to give rise to the style and stigma (Figures 2J,K). At this developmental stage, \(SCI1\) expression is obvious in anthers and carpels (Figure 2I), while already very weak at sepals and petals.

At stage −5, carpels are already fused at the top of the ovary (Figures 2J–L). The fused carpels show high \(SCI1\) expression (Figure 2L), which is clearly visible on the top, where cell divisions will give rise to the style and stigma. The presence of \(SCI1\) transcripts is evident in the inner part of the developing style, which will become the stylar transmitting tissue (STT), and is also very clear in the ovule locules (Figure 2L).

As development progresses (stage −2), it is possible to observe intense cell proliferation along the carpel fusion line (Supplementary Figure 2). The stigma lobules are already established, the upper surface cells start differentiating as stigmatic papilla while, internally, the stigmatic secretory zone (SSZ) and STT are differentiating along the fusion line (Supplementary Figure 2). The intense cell proliferation continues along the fusion line, resembling a “volcano eruption,” resulting in the folding of the stigma to the “umbrella-like” structure, typical of the \(N.\ tabacum\) flower. These inner proliferating tissues (SSZ and STT) are the sites of \(SCI1\) expression, as we showed previously (DePaoli et al., 2011).

According to our results, \(SCI1\) is expressed since flower meristem specification and at all floral whorl primordia; its expression is restricted to cells with proliferative capacity and decreases during later developmental stages toward differentiation.

The Genomic Sequence of \(SCI1\) Drives Expression Specifically to the Floral Meristem and Its Proliferative Cells

To study the transcriptional control of \(SCI1\) expression, we have produced 17 independent transgenic plants containing the genomic sequence of \(SCI1\) (∼4.5 kb) in translational fusion with GFP (Figure 3). The \(SCI1\) genomic sequence contains a 1.9 kb sequence upstream of the initial ATG codon (here designated as \(SCI1\) promoter), the four exons, and three introns of the \(N.\ tabacum\) gene, from which the stop codon was removed for the translational GFP fusion. In all transgenic plants containing \(SCI1\) prom:SCI1-GFP, it was possible to detect SCI1-GFP at the floral meristems by confocal fluorescence microscopy. No GFP fluorescence was detected at the shoot apical meristem and root meristem, as well as in leaves, stems, and roots of mature transgenic plants. As seen using in situ hybridization for \(SCI1\) endogenous expression, SCI1-GFP was detected in all floral meristem cells (Figures 3A–F). Figures 3D–F show an inflorescence apex, with two young floral meristems and one floral bud at a later developmental stage. It is visible that the GFP fluorescence is limited to the cells of the young floral meristems and to the most recent (younger) developing primordia of the floral bud (Figures 3D–F), while its detection is already decreased in the more developed and differentiated external organs (Figures 3D–F). Therefore, during floral development, \(SCI1\) is always expressed at higher levels at the central portion of the flower, with decreasing levels in the outer whorls as they develop. At stage −3, SCI1-GFP is detected at petals, anthers, and pistil (Supplementary Figure 3A). At stage −2, SCI1-GFP is observed in the same whorls, but fluorescence is considerably reduced in petals (Supplementary Figure 3B).

In the carpels, SCI1-GFP was detected in the STT, in the SSZ, and, in much lower intensity/quantity, in the parenchyma tissue of the style (Figures 3G–I). Within the ovaries, SCI1-GFP was observed in the placenta and nucellus, while no GFP was noticed in the ovule integument and megaspore region, or very weakly detectable (Figures 3J–L). The results obtained
FIGURE 2 | SCI1 expression during later stages of floral development (continuation of the stages shown in Figure 1). (A) SEM of a flower bud in which petals and anthers are emerging (advanced stage –8). Scale bar: 100 µm. (B) Bright-field microscopy showing a longitudinal section of a flower bud in a developmental stage equivalent to the one shown in (A). Scale bar: 100 µm. (C) In situ hybridization of a flower bud (advanced stage –8) with SCI1 antisense probe. Scale bar: 100 µm. (D) SEM of a flower bud at stage –7 (as defined by Koltunow et al., 1990), in which carpels are emerging. Scale bar: 200 µm. (E) A higher magnification view of the flower bud shown in (D), in which the fusion lines are visible (arrows). Scale bar: 50 µm. (F) In situ hybridization of a flower bud at stage –7–6, with SCI1 antisense probe. Arrows point to ovary locules. Scale bar: 50 µm. (G,H) SEM of flower buds at stage –6; carpels fused at the base and not yet fused at the top. Scale bars: 50 µm (G) and 75 µm (H). (I) In situ hybridization of a flower bud at late stage –6, with SCI1 antisense probe. Scale bar: 100 µm. (J,K) SEM of flower buds at stage –6; carpels already fused at the top; the fusion region is a site of intense cell proliferation. Scale bars: 150 µm (J) and 100 µm (K). (L) In situ hybridization with SCI1 antisense probe of a flower bud at late stage –5; style beginning to form (arrow). Scale bar: 150 µm. Floral meristem (FM), sepals (S), petals (P), anther (A), carpels (C), carpel primordia (CP), ovary (O), young ovary (YO). Negative controls of flower buds in equivalent developmental stages are shown in Supplementary Figure 1.

with the SCI1-GFP protein in transgenic plants reproduce the endogenous SCI1 mRNA localization, as demonstrated by in situ hybridizations in wild-type plants. Therefore, we conclude that the SCI1 genomic sequence contains the necessary and sufficient cis-acting elements for the proper transcriptional regulation of SCI1 expression. Taken together, our results demonstrate that SCI1 is exclusively expressed at the floral meristem and in the proliferative cells of the floral organ primordia.
Cis-Acting Elements Identified in the SCI1 Genomic Sequence

The SCI1 genomic sequence considered in this work comprises 4455 bp, including 1937 bp upstream of the initial ATG codon (here denominated as SCI1 promoter), four exons, and three introns. The nucleotide A of the start codon was considered a +1 position. This genomic sequence was analyzed in the PlantRegMap software1 using the N. tabacum database. In the analysis using a p-value ≤ 1e-5 threshold, putative cis-acting regulatory elements were identified for binding of different TFs belonging to several families (for details, see Supplementary Table 1). Among the putative TFs to regulate SCI1 expression

1http://plantregmap.cbi.pku.edu.cn/binding_site_prediction.php
with cis-elements upstream of the initial ATG were: APETALA1 (AP1), which contributes to the establishment of the floral meristem; SEPALATTA3 (SEP3), involved in the specification of floral whorls; WUS and AINTEGUMENTA-like 6 (AIL-6), related to the control of cell proliferation and differentiation. Downstream of the initial ATG, sites for the following putative TFs were found: E2F/DP, related to the cell cycle; LATERAL ORGAN BOUNDARIES (LOB), with an essential role in plant growth and development; SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1)-like, a central regulator of flowering time; AINTEGUMENTA-like AIL1, that binds to the promoter of key cell cycle genes; JACKDAW and MAGPIE, OVEREXPRESSION OF CO1 (SOC1)-like, a central regulator of cell proliferation and differentiation. Moreover, considering a p-value ≤ 1e-3 threshold, we found three putative binding sites for AG in the SCI1 promoter region. Two additional putative binding sites for WUS were identified, one at SCI1 promoter, around 200 bp upstream the initial ATG, and another on the third intron. The regulatory elements identified in the SCI1 genomic sequence point to involvement in the cell proliferation regulatory pathway, as well as in the regulation of flowering and flower development.

**SCI1 Is Co-expressed With AGAMOUS (NAG1) e NtWUS in the Floral Meristematic Cells**

As putative cis-acting elements for AG and WUS binding were found in the SCI1 genomic sequence, and the SCI1 expression pattern is similar to both of these genes (Kempin et al., 1993; Zhou et al., 2018), we performed in situ hybridization experiments to detect their transcripts in histological sections of the same flowers used for SCI1 probes (Figure 4). At stage −8, NAG1 transcripts were observed in stamen primordia and the center of the floral meristem (Figure 4D). At a later developmental stage, in which the carpelar leaves are fused (stage −5), NAG1 is expressed in the ovary and in the cells that will give rise to the style (Figure 4E), as previously described (Kempin et al., 1993). The same expression pattern was observed for SCI1 transcripts (Figure 21). NtWUS is expressed at the full extension of the floral meristem when only the sepal primordia are observed (Figure 4F). At a later developmental stage, NtWUS transcripts were detected in the primordia of petals and anthers, as well as in the remaining floral meristem (Figure 4G). At stage −7, NtWUS is weakly expressed in the stamen and carpel primordia (Figure 4H). Our results for NtWUS expression corroborate those previously described by Zhou et al. (2018). The co-expression of these genes is clear when equivalent developmental stages are compared. NtWUS, SCI1, and NAG1 are co-expressed mainly at the central floral meristem and primordia of the floral organs. At the same time, the co-expression of SCI1 and NAG1 is more evident at the floral meristem, carpelar leaves, and ovary locules. The co-expression of SCI1 with NtWUS and NAG1, essential for floral meristem maintenance, reproductive organs specification, and floral meristem termination, respectively, points toward interconnection of these genes in the cell proliferation control at the floral meristem.

**SCI1 Is a Direct Target of NAG1 and NtWUS Transcription Factors**

To investigate if SCI1 expression is regulated by the TFs NAG1 and NtWUS, we performed a Y1H experiment. Three different fragments of the SCI1 promoter, as well as a fragment of the third intron (for details, see section “Materials and Methods”), were used as bait (Figures 5A,B). The Y1H results reveal that both NAG1 and NtWUS bind to promSCI1Frag1 and allow the growth of colonies on the SD/-Ura/-Trp with 150 ng/ml Aureobasidin A (AbA) plates (Figure 5C) or 175 ng/ml AbA (data not shown). NAG1 and NtWUS do not bind to any of the other three genomic fragments tested in the Y1H assays (data not shown).

Non-radioactive EMSA was used to demonstrate the binding of NAG1 and NtWUS with the respective putative binding sequences identified in the SCI1 genomic promoter (for details of the double-strand oligonucleotides used, see Supplementary Figure 4). Both His-NAG1 and His-NtWUS, produced in Escherichia coli, caused mobility shifts when incubated with the corresponding ds-oligonucleotide (Figures 6B,C). Additionally, the EMSA analysis of the competition assay with increasing amounts of the unlabeled specific competitors exhibited a decrease of each DNA/TF complex and an increase of the free probe (Figures 6B,C). Our results demonstrate that NAG1 and NtWUS can bind to the sequences identified in the SCI1 proximal promoter (Figure 6A) and may indicate that both NAG1 and NtWUS TFs regulate the SCI1 gene expression.

To confirm the binding of the TFs to the promoter sequence and assess their effects on SCI1 expression, transient dual-luciferase assays were performed in planta. Two SCI1 promoter fragments (for details, see section “Materials and Methods”), controlling the firefly luciferase expression (LUC), were tested for transactivation with NAG1 and NtWUS (Figure 7A). The results obtained for NAG1 had demonstrated a significant increase (p < 0.01) in luciferase activity in comparison with control (Figure 7B) when both fragments were used. On the other hand, NtWUS was not able to activate luciferase activity under the control of both SCI1 promoter fragments (Figure 7B). As this assay was performed in leaf cells in which SCI1 promoter is not expressed, only activation would be assessed. Then, it is still possible that NtWUS represses SCI1 expression or depends on other TFs to induce it. Our results clearly show an activation effect of NAG1 in SCI1 gene expression.

**DISCUSSION**

**Does WUSCHEL Regulate SCI1 Expression at the Early Floral Meristem?**

Our previous results have demonstrated that SCI1 regulates cell proliferation in pistils of N. tabacum (DePaoli et al., 2011).
and A. thaliana (DePaoli et al., 2014). Here, we demonstrated, through in situ hybridizations (Figures 1, 2) and analysis of SCI1prom:SCI1-GFP transgenic plants (Figure 3), that SCI1 starts its expression in the young floral meristem and maintains it in the meristematic undifferentiated cells of all floral whorls. Additionally, we showed that SCI1 is co-expressed with NtWUS in the floral meristem (Figure 4). The WUS expression in the N. tabacum floral meristem is broader than in the A. thaliana floral meristem. In A. thaliana, WUS expression is confined to the OC (Mayer et al., 1998; Adibi et al., 2016). However, WUS protein migrates from the OC, where it is found at the highest level, into adjacent cells via cell–cell movement and activates CLV3 transcription (Yadav et al., 2011; Perales et al., 2016; Rodriguez et al., 2016). Therefore, the WUS protein acts more broadly, in a concentration-dependent manner, to spatially regulate transcription and maintain the homeostasis of the flower meristem (Perales et al., 2016). Despite the co-expression of SCI1 and NtWUS in the floral meristem, SCI1 expression is reduced in the OC (Figures 1G–I). So, we can speculate that high levels of WUS in the OC may suppress SCI1 expression. It is known that WUS represses the expression of genes that can negatively influence the proliferation and indeterminate nature of meristematic cells (Ikeda et al., 2009). Examples of these genes are ARR5, ARR6, ARR7, and ARR15, which act in a negative feedback manner to regulate cytokinin signaling and inhibit cell proliferation in the meristem (Leibfried et al., 2005). Considering that SCI1 is a regulator/inhibitor of cell proliferation (DePaoli et al., 2011, 2014), its repression at the OC would be necessary to maintain the homeostasis of pluripotent cells.

As demonstrated by Y1H and EMSA (Figures 5C, 6C), SCI1 is a direct target of NtWUS, and both genes are co-expressed in many cells of the young floral meristems. NtWUS may be responsible for the early activation of SCI1 expression but cannot do it alone, as shown in the luciferase activity assay. Additionally, WUS is also expressed at the shoot apical meristem in different plant species (Laux et al., 1996; Mayer et al., 1998), whereas SCI1 is only expressed in the floral meristem (several Figures shown here). Therefore, SCI1 activation may be coordinated by NtWUS with some floral meristem identity gene, which is supported by the fact that putative cis-acting elements for LFY and AP1 were found in the SCI1 promoter. Future experiments will be necessary to determine the contribution of NtWUS is the regulation of SCI1 expression.

**NAG1 Binds to the SCI1 Promoter and Activates Its Expression**

Stigma/style cell-cycle inhibitor 1 and NAG1 are co-expressed in cells of the floral meristem and young floral buds (Figure 4), suggesting SCI1 as a possible target of NAG1. In silico analyses of the SCI1 genomic sequence have identified several putative cis-acting regulatory elements, among which, AG binding
Putative AG binding sites were also found in the SCII genomic sequence of the orthologs in *A. thaliana* (At1g79200), *Solanum lycopersicum* (Solyc05g008750.2), *Solanum tuberosum* (PGSC0003DMG400030526), *Oryza sativa* (LOC_Os02g07420), *Zea mays* (GRMZM2G010754), and *Glycine max* (08G16700). Most of the identified sequences correspond to non-canonical
A schematic representation of the reporter and effector constructs used in the luciferase activity assay. (A) Luciferase activity as a result of the interaction of NAG1 and NtWUS with SCI1 promoter sequences indicated. Relative activity is the ratio LUC/REN. The expression of REN was used as an internal control. Data represent the means of three biological replicates. **Significantly different from the control (empty vector) by Student’s t-test (p < 0.01).

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FIGURE 7 | Luciferase activity assay performed by transient expression in *N. benthamiana* leaves driven by the SCI1 promoter fragments. (A) Schematic representation of the reporter and effector constructs used in the luciferase activity assay. (B) Luciferase activity as a result of the interaction of NAG1 and NtWUS with SCI1 promoter sequences indicated. Relative activity is the ratio LUC/REN. The expression of REN was used as an internal control. Data represent the means of three biological replicates. **Significantly different from the control (empty vector) by Student’s t-test (p < 0.01).
However, SCI1 was not previously identified as an AG target, and this is novel information provided by our work.

In *N. tabacum*, after carpel specification, populations of meristematic cells are still maintained in different portions of the pistil, such as: in the fusion zone of the carpellar leaves, where subsequent style elongation and stigma development will occur; in placental primordium; placenta; and ovule primordia (Chang and Sun, 2020). As observed by *in situ* hybridization (Figures 1, 2) and SCI1*prom*:SCI1-GFP transgenic plants (Figure 3), SCI1 is expressed in these tissues while cells proliferate, and its expression decreases when the female organ completes its development. Additionally, SCI1 is expressed in the same pistil tissues in which NAG1 is highly expressed (Figure 4). Mizukami and Ma (1995) have established that AG functions, in specifying the reproductive organs and determining the floral meristem, are separate and dependent on different levels of AG expression. They have demonstrated that the termination of the floral meristem requires high concentrations of AG. AG is a target of PERIANTHIA (PAN), which can increase AG expression (Wynn et al., 2014), and this direct regulation is involved in floral stem cell termination (Das et al., 2009). Additionally, the AG-dependent dose termination of the floral meristem was also observed in the double mutants of the PAN and SEUSS (SEU) genes (Wynn et al., 2014). Despite what is already known, there are still gaps in the downstream processes regulated by AG that culminate in floral meristem termination.

Pistils are the last organs to be specified during flower development, and this occurs as a result of a feedback loop between WUS and AG (Lohmann et al., 2001). These genes act to maintain and terminate, respectively, the pluripotent cells in flower meristem. SCI1, as a target of both TFs, opens a way to understand the pluripotent cell homeostasis. A proposed model for SCI1 activation and its role in regulating cell proliferation/differentiation is shown in Figure 8. As soon as floral meristem is specified, SCI1 expression is induced by a TF(s) not yet identified, possibly LFY and/or AP1 (Figure 8A), based on the *in silico* analysis of SCI1 promoter. It remains to be established which TF(s) is (are) responsible for the early activation of SCI1 as soon as the floral meristem is specified (Figure 1).

At a later stage of floral meristem development, when sepals are being specified, SCI1 expression is reduced on the OC (Figure 8B), which may allow the maintenance of the pluripotent cells. At stage −7, SCI1 expression at the OC is still low, and NAG1 expression is activated. Based on what is known from Arabidopsis, we speculate that NtWUS may be responsible for repressing SCI1 expression on the OC and for activating NAG1 expression. Then, at a later stage, NAG1 may inactivate NtWUS in a negative feedback manner (Figure 8C). At stage −6, NAG1 takes over SCI1 activation, and the fourth whorl is specified. Development progresses, and pistil cells divide to give rise to style, stigma, and ovules. At this point, pluripotent cells are no longer available, the last pistil tissues differentiate, and the flower meristem is terminated.

**Final Remarks**

*Stigma/style cell-cycle inhibitor 1* is strictly expressed in floral meristematic cells, is activated by NAG1, and is expressed in the same pistil cells with high NAG1 expression. Considering...
that SCI1 is a regulator of cell proliferation as demonstrated by the phenotypes of the *N. tabacum* transgenic plants (DePaoli et al., 2011) and the *A. thaliana* mutants (DePaoli et al., 2014), we suggest that SCI1 may act as an effector of the floral meristem termination, performing the fine-tuning of cell proliferation and differentiation. The fact that a SEP3 binding site was also found in SCI1 promoter (Supplementary Table 1) and that tetramerization of AG and SEP3 is essential for floral meristem determinacy in Arabidopsis (Hugouvieux et al., 2018; Xu et al., 2019) strengthen our proposal. The identification of SCI1, a cell proliferation regulator, as a novel target of WUS and AG, contributes significantly to the understanding of flower meristem development.

**MATERIALS AND METHODS**

**Plant Material**

*Nicotiana tabacum* cv Petit Havana SR-1 seeds were surface sterilized, germinated *in vitro*, and grown in Murashige and Skoog medium at controlled conditions (26°C temperature and photoperiod of 16 h light and 8 h dark). Leaf disks from *in vitro* grown plants were used for transformation via *Agrobacterium tumefaciens* with the gene construct SCI1 prom:SCI1-GFP. Wild-type SR1 and transgenic plants were cultivated in a greenhouse in the city of Ribeirão Preto – SP, Brazil (latitude ~21°10’24”S, longitude ~47°48’24” W), with daily irrigations by automatic sprinkler, every 12 h.

**Microscopy Analyses**

For bright field microscopy, the samples were fixed in 4% (w/v) paraformaldehyde and 4% (v/v) DMSO, dehydrated in a growing ethanol series, and included in base acrylic resin (Historesin, Austria) with the Leica LAS-AF Lite software (Leica Microsystems, Heidelberg, Germany) with the Leica LAS-AF Lite software. For protein purification, the bacteria cells were lysed for 5 h at 30°C. In these conditions, it was possible to recover satisfactory levels of the desired proteins in the soluble fraction. For protein purification, the bacteria cells were lysed for 5 h at 30°C. In these conditions, it was possible to recover satisfactory levels of the desired proteins in the soluble fraction. For protein purification, the bacteria cells were lysed for 5 h at 30°C. In these conditions, it was possible to recover satisfactory levels of the desired proteins in the soluble fraction. For protein purification, the bacteria cells were lysed for 5 h at 30°C. In these conditions, it was possible to recover satisfactory levels of the desired proteins in the soluble fraction. For protein purification, the bacteria cells were lysed for 5 h at 30°C. In these conditions, it was possible to recover satisfactory levels of the desired proteins in the soluble fraction. For protein purification, the bacteria cells were lysed for 5 h at 30°C. In these conditions, it was possible to recover satisfactory levels of the desired proteins in the soluble fraction. For protein purification, the bacteria cells were lysed for 5 h at 30°C. In these conditions, it was possible to recover satisfactory levels of the desired proteins in the soluble fraction.

**Yeast One-Hybrid Assay**

The Y1H assay was performed using Clontech’s Matchmaker Gold Yeast One-Hybrid (Y1H) Library Screening kit. Three different fragments of the SCI1 promoter and the third intron of the SCI1 gene were amplified (primers are listed in Supplementary Table 2) and cloned into the TOPO® TA Cloning® Dual Promoter Kit vector. The probe synthesis was performed using the SP6/T7 Transcription Kit from Roche Life Science following the manufacturer’s specifications. The slides were photographed in a microscope (Zeiss – Axiolab) equipped with a camera (Zeiss – Axiocam Color).

**Production of Recombinant NAG1 and NtWUS Proteins and Electrophoretic Mobility Shift Assays**

To produce the recombinant proteins NAG1 and NtWUS, each CDS was amplified (primers are listed in Supplementary Table 2) and cloned into the expression vector pDEST17 (Gateway®) in fusion with a HIS-tag. The CDS in the constructs were sequenced before use. These constructs were transformed into *E. coli* BL-21 (DE3) CodonPlus-RP. The production of 6xHis-NAG1 was induced with 0.5 mM IPTG for 8 h at 20°C, and of 6xHis-NtWUS was induced with 0.1 mM IPTG for 5 h at 30°C. In these conditions, it was possible to recover satisfactory levels of the desired proteins in the soluble fraction. For protein purification, the bacteria cells were lysed in 150 mM Tris–HCl pH 8 buffer, 150 mM NaCl, 10 mM
imidazole, 1 mg/ml lysozyme, and cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche; 11836153001) and sonicated four times using 20-s periods. Soluble proteins were purified using Ni Sepharose® High Performance (Sigma; GE17-5268-01) and used for EMSA.

The double-strand probes (Supplementary Figure 4) used in EMSA were labeled with DIG-ddUTP using Roche® Terminal transferase. The binding and electrophoresis assays were performed according to the DIG Gel Shift kit, 2nd Generation.

Luciferase Assay

The SCI1 promoter was amplified in two different fragments, a long one (−1585 to −1) and a small fragment close to the ATG (−538 to −1). Both fragments were inserted in the pGreenII 0800-LUC vector (Hellens et al., 2005), which has two different luciferase genes: the Renilla luciferase gene used as an internal control of the transient expression and the firefly luciferase gene under the control of the SCI1 promoter sequences in this study (reporter construct). The NAG1 and NtWUS CDS were cloned into the vector pK7WG2 (Karimi et al., 2002) for protein expression under the 35S promoter control and used as effectors. All gene constructs were sequenced before use. The reporter and effector constructs were introduced separately into A. tumefaciens strain GV3101. Different combinations of reporter and effector constructs were agroinfiltrated in Nicotiana benthamiana leaves for transient expression. The activity assay was performed 3 days after agroinfiltration. Firefly (LUC) and Renilla (REN) luciferases were detected with the dual luciferase assay reagents (Promega, Madison, WI, United States) using Costar® 96-well plate. The promoter activity was calculated using the LUC/REN ratio. The data were analyzed by Student's t-test.

DATA AVAILABILITY STATEMENT

Sequence data from this article is available at the National Center for Biotechnology site under the following accession numbers: SCI1 (LOC107802286), NtWUS (LOC107812471), and NAG1 (LOC107812878).

AUTHOR CONTRIBUTIONS

All authors have contributed to the intellectual content of this manuscript and have met the following three requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.642879/full#supplementary-material

Supplementary Figure 1 | Negative controls of in situ hybridizations (SCI1 sense probe).

Supplementary Figure 2 | Anatomical and histological analyses of Nicotiana tabacum floral development (stage −2 to stage 1).

Supplementary Figure 3 | Multiphoton microscope images of young flower buds of SCI1prom:SCI1-GFP transgenic N. tabacum plants.

Supplementary Figure 4 | Sequences synthesized for EMSA with NAG1 and NtWUS.

Supplementary Table 1 | List of cis-acting regulatory elements identified at SCI1 genomic sequence by PlantRegMap.

Supplementary Table 2 | Primers used to amplify and sequence the different DNA fragments and CDSs used in this work.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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