UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
DEPARTAMENTO DE GENÉTICA
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SCI1 expression and its transcriptional regulation in the floral meristem and flowers of *Nicotiana tabacum*

Expressão de *SCI1* e sua regulação transcricional no meristema floral e nas flores de *Nicotiana tabacum*

Ribeirão Preto- SP

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Orientadora: Profª. Drª. Maria Helena de Souza Goldman

Co-orientadora: Profª. Drª. Maria Cristina da Silva Pranchevicius

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"*Eu sou porque nós somos*"

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GENERAL INDEX

ABSTRACT

CRUZ, Joelma de Oliveira. *SCI1* expression and its transcriptional regulation in the floral meristem and flowers of *Nicotiana tabacum*. [Ph.D. Thesis]. University of São Paulo, Medicin School of Ribeirão Preto, Department of Genetics; 2023.

The reproduction of angiosperms is strictly regulated by genetic pathways and environmental signals that result in the transition from the vegetative to the reproductive phase. The reproductive phase is marked by the floral meristem that results in the flower, a highly modified branch formed by the four floral whorls: sepals, petals, stamens, and pistil. The stamens and the pistil are responsible for producing male and female gametes, therefore, are of great importance in reproduction. Understanding the development of these whorls involves understanding the molecular mechanisms that guarantee correct development. To better understand the development of the pistil, our research group carried out the initial characterization of a gene that was preferentially expressed in the pistil of the *Nicotiana tabacum* flower and controls proliferation in this organ. This was named *SCI1* (*Stigma/style Cell-cycle Inhibitor 1*). The mechanism of action of *SCI1* has not yet been elucidated, and advances in investigations have revealed an extensive network of proteins with which *SCI1* interacts. The *SCI1* interactome allowed us to assume its involvement in RNA processing and cell cycle, basic processes for cell maintenance. The involvement of *SCI1* in these processes allowed us to raise the hypothesis that *SCI1* could express itself in other floral whorls and start to express itself in the initial stages of floral development. Therefore, this work aimed to determine where and when the *SCI1* gene starts its expression in *N. tabacum* flowers, relate *SCI1* expression to pistil development and analyze the expression of *SCI1* in the ovaries analyze the transcriptional regulation of *SCI1*. To analyze *SCI1* expression, in situ hybridization was used. It was observed that SCI1 starts its expression in the floral meristem and remains intensely expressed in the beginnings of floral whorls. Expression of *SCI1* in floral meristem and whorl primordia indicates its involvement in the development of all floral whorls. As the whorls are specified, the expression of *SCI1* is reduced, except in the pistil, the organ in which the last meristematic cells of the flower are located. *SCI1* mRNA was detected both in non-fused and already fused carpels. *SCI1* is also detected in the young ovary when the placenta is expanding. It is later detected in the primordia of ovules and ovules with their structures, integument funiculus, and nucellus established. In these structures, *SCI1* was detected in the funiculus and integument. In situ hybridization also

revealed co-expression of *SCI1* with the *NAG1*, *NtANT*, and *NtWUS* genes. *SCI1* co-expressed with *NtWUS* and *NAG1* in the floral meristem. *NtWUS* encodes a Homeobox transcription factor that regulates the proliferation of pluripotent cells in the floral meristem, and *NAG1* encodes a transcription factor responsible for the specification of the third and fourth floral whorls and the termination of meristematic cells. In stamens and carpels, *SCI1* is co-expressed with *NAG1* and *NtANT*. *SCI1* co-expresses with *NtANT* in ovule primordia, integument, and funiculus. *NtANT* encodes a transcription factor with the AP2 domain that positively regulates cell proliferation from the beginning of floral development and acts on the correct development of the pistil and ovules. The co-expression of *SCI1* with the transcription factors NtWUS, NAG1, and NtANT, added to the function described for *SCI1*, which involves controlling cell proliferation and controlling stigma and style development, reveals a possible regulation of *SCI1* by these transcription factors. These transcription factors interactions with cis-elements in the *SCI1* promoter were predicted *in silico* and confirmed in monohybrid assays (Yeast One Hybrid) with the portion of the *SCI1* promoter, called Frag1, which comprises 443pb above the initiation codon (ATG). Interactions were also confirmed via EMSA. With luciferase assay, it was possible to determine what effect the interaction of these transcription factors has on *SCI1* expression. Transcription factors NAG1 and NtANT promote activation of *SCI1* expression by binding to their respective cis-elements. While the interaction of NtWUS was demonstrated to reduce the expression of *SCI1*, how results were not expressive. The involvement of auxin in the regulation of *SCI1* was also predicted. It was demonstrated by the luciferase assay that the synthetic phytohormone NAA significantly increases the expression of *SCI1* when regulated by NtANT. The IIA phytohormone positively influenced *SCI1* expression when regulated by NtANT. The endogenous expression of the SCI1 protein was obtained through transgenic plants expressing the SCI1 protein in translational fusion to GFP under the control of its endogenous promoter. Thus, it was possible to determine the protein's location in the flower. Like mRNA, the SCI1 protein is detected from the floral meristem and in all young whorls. A centripetal protein reduction was observed as the whorls developed, but this reduction was not observed in the pistil until stage 2. Due to the size of the flower, observations at more advanced stages were impossible. It was also possible to observe the SCI1 protein in specialized tissues of the stigma and style, transmitting tissue of the style and secretory zone of the stigma and in the parenchyma. In the ovaries, the SCI1 protein was detected in the placenta and the ovules in their integument and funiculus. With these plants, it was possible to confirm the location of

SCI1 in the nucleus and nucleolus. The data set confirms the hypothesis that SCI1 begins to express itself in the floral meristem. Furthermore, the data also point to the expression of SCI1 in meristematic cells. Regulation of *SCI1* by NtWUS reinforces its relationship with meristematic cells in the floral meristem. While the presence of SCI1 in the pistil and its regulation by the transcription factors NAG1 and NtANT reinforce the involvement of SCI1 in the proliferation of meristematic cells still present in these organs.

Keywords: Floral meristem, floral development, meristematic cells, transcriptional regulation; cell proliferation

RESUMO

CRUZ, Joelma de Oliveira. Expressão de *SCI1* e sua regulação transcricional no meristema floral e nas flores de *Nicotiana tabacum*. [Tese de doutorado]. Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Departamento de Genética; 2023.

A reprodução das angiospermas é um processo estritamente regulado por vias genéticas e sinais ambientais que resultam na transição da fase vegetativa para fase reprodutiva. A fase reprodutiva é marcada pela presença do meristema floral que resulta na flor, um ramo altamente modificado formado pelos quatro verticilos florais: sépalas, pétalas, estames e pistilo. Os estames e o pistilo são os responsáveis pela produção dos gametas, masculino e femininos, respectivamente e por isso, de grande importância na reprodução. Entender o desenvolvimento desses verticilos passa por compreender os mecanismos moleculares envolvidos que garantem o correto desenvolvimento. Para melhor compreender o desenvolvimento do pistilo, em nosso grupo de pesquisa foi realizada a caracterização inicial de um gene que se mostrou preferencialmente expresso no pistilo da flor de *Nicotiana tabacum* e controla a proliferação nesse órgão. Este, foi denominado *SCI1* (*Stigma/style Cell-cycle Inhibitor 1*). O mecanismo de ação de *SCI1* ainda não foi elucidado e os avanços nas investigações tem revelado uma extensa rede de proteína com quais *SCI1* interage. O interactoma de *SCI1* permitiu assumir seu envolvimento no processamento de RNA e ciclo celular, processos básicos para a manutenção da célula. O envolvimento de *SCI1* nesses processos permitiu levantar a hipótese de que *SCI1* poderia se expressar em outros verticilos florais e começaria a se expressar em momentos iniciais do desenvolvimento floral. Portanto, este trabalho teve por objetivos determinar onde e quando o gene *SCI1* inicia sua expressão em flores de *N. tabacum*; relacionar a expressão de *SCI1* com o desenvolvimento do pistilo; analisar a expressão de *SCI1* nos ovários e analisar a regulação transcricional de *SCI1*. Para analisar a expressão de *SCI1* foi utilizada a hibridização in situ. Foi observado que *SCI1* inicia sua expressão no meristema floral e mantem-se expresso de forma intensa nos primórdios dos verticilos florais. A expressão de *SCI1* no meristema floral e primórdios dos verticilos indica seu envolvimento no desenvolvimento de todos os verticilos florais. À medida que os verticilos se especificam, a expressão de *SCI1* é reduzida, exceto no pistilo, órgão em que se localizam as últimas células meristemáticas da flor. O mRNA de *SCI1* foi detectado tanto nos carpelos não fusionados, quanto já fusionados. *SCI1* também foi detectado no ovário jovem quando a placenta está em expansão e posteriormente detectado nos

primórdios dos óvulos e óvulos com suas estruturas, funículos integumento e nucelo, já estabelecidos. Nessa estruturas, *SCI1* foi detectado no funículo e integumento. A hibridização *in situ* também revelou a co-expressão de *SCI1* com os genes N*AG1*, *NtAINTEGUMENTA* (*NtANT*) e NtWUSCHEL (*NtWUS*). *SCI1* co-expressa com *NtWUS* e *NAG1* no meristema floral. *NtWUS* codifica um fator de transcrição Homeobox que regula a proliferação de células pluripotentes no meristema floral e *NAG1* codifica um fator de transcrição responsável pela especificação do terceiro e quarto verticilos florais e pela terminação das células meristemáticas. Nos estames e carpelos *SCI1* co-expressa com *NAG1* e *NtANT*. *SCI1* co-expressa com *NtANT* nos primórdios dos óvulos, no integumento e funículo. *NtANT* codifica um fator de transcrição com domínio AP2 que regula positivamente a proliferação celular desde o início do desenvolvimento floral e atua no correto desenvolvimento do pistilo e óvulos. A co-expressão de *SCI1* com os fatores de transcrição NtWUS, NAG1 e NtANT, adicionada à função descrita para *SCI1* que envolve da controle da proliferação celular e controle do desenvolvimento do estigma e estilete revela uma possível regulação de *SCI1* por esses fatores de transcrição. As interações desses fatores de transcrição a cis-elementos presentes no promotor de *SCI1* foram preditas *in silico* e confirmadas em ensaios de mono híbrido (*Yeast One Hybrid*) com a porção do promotor de *SCI1*, denominada Frag1, que compreende 443pb acima do códon de iniciação (ATG). As interações também foram confirmadas através do EMSA. Com ensaio de luciferase foi possível determinar qual efeito a interação desses fatores de transcrição tem sobre a expressão de *SCI1*. Os fatores de transcrição NAG1 e NtANT promovem a ativação da expressão de *SCI1* ao de ligarem aos seus respectivos cis-elemento. Enquanto a interação de NtWUS demonstrou reduzir a expressão de *SCI1*, porém os resultados não foram expressivos. Também foi predito o envolvimento da auxina na regulação de *SCI1*. Foi demonstrado pelo ensaio de luciferase que o fitohormônio sintético ANA aumenta significativamente a expressão de *SCI1* quando regulado por NtANT. Já o fitohormônio AIA apresentou influência positiva sobre a expressão de *SCI1* quando regulado por NtANT. A expressão endógena da proteína SCI1 foi obtida através de plantas transgênicas expressando a proteína SCI1 em fusão traducional a GFP sob controle de seu promotor endógeno. Assim foi possível determinar a localização da proteína na flor. Como o mRNA, a proteína SCI1 é detectada desde o meristema floral e em todos os verticilos jovens. Foi observada redução da proteína de forma centrípeta à medida que os verticilos se desenvolviam, porém essa redução não foi observada no pistilo até o estádio 2. Devido ao tamanho da flor, não foram possíveis

observações em estádios mais avançado. Com essas plantas também foi possível observar a proteína SCI1 nos tecidos especializados do estigma e estilete, tecido transmissor do estilete e zona secretória do estigma, assim como também no parênquima. Nos ovários, a proteína SCI1 foi detectada na placenta e nos óvulos, em seu integumento e funículo. Com essas plantas foi possível confirmar a localização de SCI1 no núcleo e nucléolo. O conjunto de dados obtidos confirmam a hipótese e de *SCI1* começa a se expressar no meristema floral. Além do mais os dados ainda apontam a expressão de *SCI1* em células meristemáticas. A regulação de *SCI1* por NtWUS reforça a sua relação com células meristemáticas no meristema floral. Enquanto a presença de *SCI1* no pistilo e sua regulação pelos fatores de transcrição NAG1 e NtANT reforçam o envolvimento de *SCI1* na proliferação de células meristemáticas ainda presentes nesses órgãos.

Palavras-chave: Meristema floral, desenvolvimento floral, células meristemáticas, regulação transcricional, proliferação celular

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LIST OF ACRONYMS AND ABBREVIATIONS

AD - Activation Domain

ade – adenine

AG- AGAMOUS

AIL1- AINTEGUMENTA-LIKE 1

AIL3- AINTEGUMENTA-LIKE 3

AIL4- AINTEGUMENTA-LIKE 4

AIL5- AINTEGUMENTA-LIKE 5

AIL6- AINTEGUMENTA-LIKE 6

AIL7- AINTEGUMENTA-LIKE 7

AP1- APETALA1

AP2- APETALA2

AP3- APETALA3

ARF3- AUXIN RESPONSE FACTOR 3

ARF5- AUXIN RESPONSE FACTOR 5

BBM- BABY BOOM

BEL1- BELL1

bp – base pairs

BRM- BRAMA

cDNA – complementary DNA

CDS - Coding sequence

CLV1/ CLV2/ CLV3- CLAVATA

CRC- CRABS CLAW

CRF2- CYTOKININ RESPONSE FACTOR 2

CUC1/CUC2/ CUC3- CUP SHAPED COTYLEDON

dATP - Deoxyadenosine triphosphate

DEPC - Diethyl Pyrocarbonate

dH2O - deionized water

dNTP – Deoxyribonucleotide 5´-triphosphate

DRN- DORNROSCHEN

ETT- ETTIN

FIL- FILAMENTOUS *FLOWER*

g – gravitational force

GFP – Green Fluorescent Protein

IPTG - Isopropyl β-D-1-thiogalactopyranoside

LB - Luria-Bertani (culture medium)

LFY- LEAFY

LiAc – Lithium Acetate

LUG- LEUNIG

KAN- KANADI

KNU- KNUCKLES

MP-MONOPTERO

MS - Murashige and Skoog (culture medium)

NaCl – Sodium Chloride

NaOH - Sodium hydroxide

NCBI – National Center for Biotechnology Information

NPY- NAKED PINS IN YUC MUTANTS 1

NtANT- AINTEGUMENTA de *Nicotiana tabacum*

OD600 – Optical Density at 600 nm

PCR - Polymerase Chain Reaction

PEG3350 - Polyethylene Glycol 3350

PI- PISTILLATA

PID- PINOID

PIN1- PIN-FORMED1

RNAi – RNA interference

rpm – Rotations per minute

SD – Synthetic Dropout (culture medium)

SCI1- Stigma/style cell-cycle inhibitor 1

SEP1/SEP2/SEP3/SEP4- SEPALLATA1, SEPALLATA 2, SEPALLATA 3 and *SEPALLATA 4*

SEU- SEUSS

STM- SHOOTMERISTEMLESS

*SHP1/ SHP2- SHATTERPROOF1*and *SHATTERPROOF2*

SPT- SPATULA

STK- SEEDSTICK

STY1- STYLISH1

SYD- SPLAYED

REV- REVOLUTA

RT - Room temperature

TFL1- TERMINAL FLOWER 1

Trp – Tryptophan

UV – Ultraviolet

Ura – Uracil

w/v - wight/volume

WUS- WUSCHEL

Y1H – Yeast One-Hybrid

GENERAL INTRODUCTION

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Joelma O. Cruz, Greice Lubini, Fernanda M. Nogueira, Edward J. Strini, Vitor F. Pinoti, Pedro B. Ferreira, Vanessa Thomé, Andréa C. Quiapim, Maria Cristina S. Pranchevicius, M. Manuela R. Costa, Maria Helena S. Goldman ***SCI1*** **EXPRESSION IS ACTIVATED BY THE AINTEGUMENTA TRANSCRIPTION FACTOR AND POSITIVELY INFLUENCED BY AUXIN**

FLOWER DEVELOPMENT

Flower development results from a sequence of events and gene action that result in the establishment of the reproductive phase of the plant. The first event is the transition from the vegetative to the reproductive phase. This process involves an integrated collaboration of endogenous, exogenous, and genetic factors that converge in the activation of the floral meristem identity genes *LEAFY (LFY)* and *APETALA1(AP1*) (Lai et al., 2021; Weigel, 1995; Yamaguchi, 2021). The *LFY* and *AP1* genes act partially redundantly to specify the floral meristem (Weigel, 1995). And ensure the identity and maintenance of this meristem by preventing the expression of *TERMINAL FLOWER 1 (TFL1)* (Parcy et al., 2002; Serrano-Mislata et al., 2017).

The transition from the vegetative meristem to the reproductive meristem results in the inflorescence meristem that can, in theory, give rise to many lateral organs that are the floral meristems (Yanofsky, 1995; Zik & Irish, 2003). In angiosperms, the floral meristem is formed by three layers and three functional zones: central zone, peripheral zone, and organizational center. The central zone is formed by cells with pluripotent characteristics that divide less frequently. The cells of this zone that divide differentiate from the central zone, whicht gives rise to the outer whorls. The organizing center (OC) is where the population of pluripotent cells is maintained until the fourth floral whorl is specified (Jenik & Irish, 2000; Vijayraghavan et al., 2005).

The organizing center is where the expression of *WUSCHEL (WUS)* is located. (Laux et al., 1996). After specification of the floral meristem, this structure needs to coordinate cell divisions so that a population of cells remains with pluripotent, undifferentiated characteristics and another population differentiates, giving rise to floral whorls. This process is generally coordinated by the transcription factor of the Homeobox WOX family *WUS* and the genes of the *CLAVATA* family (*CLV1*), *CLV2*, and *CLV3*. These genes act through negative feedback to repress WUS expression (Mayer et al., 1998). However, the floral meristem is a determinate meristem; it emerges programmed to give rise to a specific number and certain floral whorls and then terminates the population of pluripotent cells (Irish, 2010). Therefore, in this framework, *WUS* repression requires additional regulatory processes.

LFY encodes a transcription factor unique to the plant kingdom (Yamaguchi, 2021) and is a central regulator of genes that act downstream and promote floral development. LFY activates AP1 in the young floral meristem and maintains its uniform expression throughout the floral primordium (Benlloch et al., 2011; Wagner et al., 1999). The LFY protein can be observed in the flower of Arabidopsis thaliana up to stage 3. At this stage, LFY binds to the cis-element present in the second intron of the *AGAMOUS* gene and promotes its activation in the center of the floral meristem (Parcy et al., 2002; Winter et al., 2015).

The termination of the floral meristem is regulated by the action of transcription factors LFY and AGAMOUS (AG) that repress *WUS* expression. LFY indirectly represses *WUS* because AG, activates a MADS-BOX transcription factor, that specifies the third and fourth whorl in the center of the flower (Bowman et al., 1989) and determines the termination of pluripotent cells (Lenhard et al., 2001; Liu et al., 2011; Xue et al., 2020). *AG* is activated by LFY and WUS that bind to elements present in the second intron of the gene, in the center of the floral meristem, where the androecium and gynoecium will form (Lohmann et al., 2001; Sloan et al., 2020). According to Lenhard et al. (2001), *AG* activation leads to *WUS* repression. For a long time, the molecular mechanisms for the inactivation of *WUS* remained unknown, as well as the knowledge of other factors that seemed necessary for the fine regulation and repression of *WUS*. It is now known that AG represses *WUS* by recruiting Polycomb Group Proteins that methylate histone H3 Lys-27 (Liu et al., 2011). It has also been demonstrated that PcG acts downstream to *AG* and probably with *KNUCKLES* (Kwaśniewska et al., 2021; Shang et al., 2021).

Despite the wide variety of shapes, floral whorls represent the basic structure of the flower (Irish, 2017). The flower is formed by a set of sepals, petals, stamens, and pistil (Yanofsky, 1995). It is the result of a coordinated gene regulation that culminates in the activation of the homeotic genes of the ABCDE model for the determination of floral whorls (Abraham-Juárez et al., 2020; Coen & Meyerowitz, 1991; Heijmans et al., 2012).

The ABCDE model explains the specification of floral organs based on the performance of protein complexes that determine the formation of each whorl (Bowman et al., 1989; Theissen & Melzer, 2007; Winter et al., 2002). The model is composed of the genes: class A, *APETALA1 (AP1)* and *APETALA2 (AP2)*; B, *APETALA3 (AP3)* and *PISTILLATA (PI)*; C, *AGAMOUS (AG)*; D, *SEEDSTICK (STK)*, *SHATTERPROOF1* and *SHATTERPROOF2 (SHP1*

and SHP2); and class E, is composed of *SEPALLATA1*, *SEPALLATA 2, SEPALLATA 3* and *SEPALLATA 4 (SEP1, SEP2, SEP3,*and *SEP4*) (Murai, 2013; Thomson et al., 2017; Wils & Kaufmann, 2017; Zahn et al., 2006). This developmental model is composed, except for *AP2*, of genes that encode transcription factors of the MADS-box family. These transcription factors bind to elements called CArG -box, consensus sequences generally represented by the generic forms: CC[A, T]6GG or CC[A, T]7G (Riechmann & Meyerowitz, 1997; Sieburth & Meyerowitz, 1997). The ABCDE model is called a floral quartet based on the interaction of their proteins (Theissen & Melzer, 2007; Zahn et al., 2005) and explains the specification of floral whorls as shown in the diagram below (Figure 1).

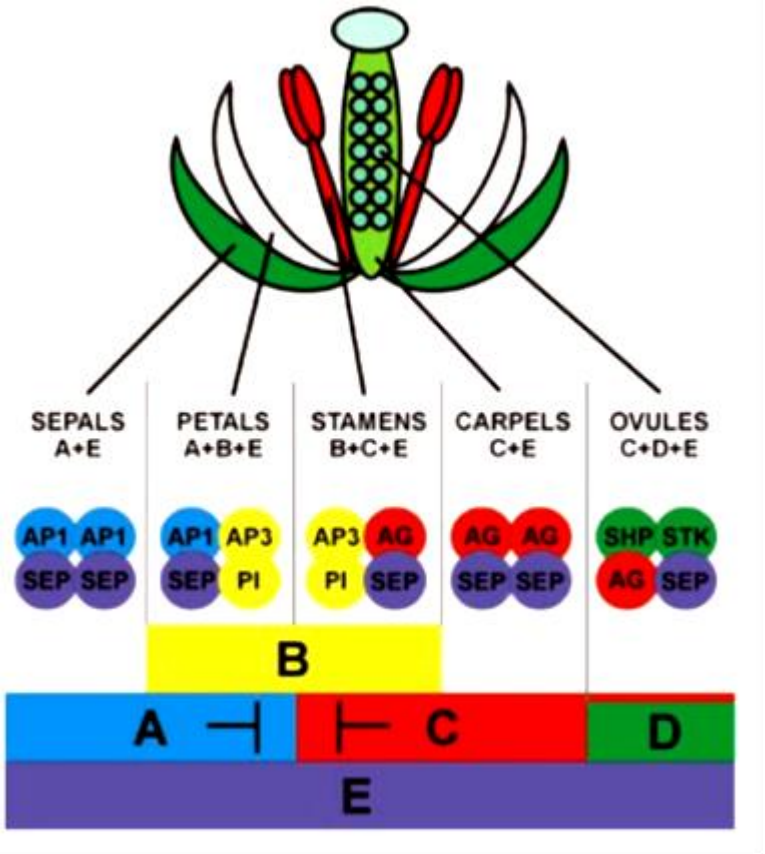

Figure 1. Revised ABCDE model. In *A. thaliana, APETALA1-(AP1)* A function genes form the sepals. The petals are formed by the B function genes *(APETALA3-AP3* and *PISTILATA-PI)* and the A function gene *AP1*. The B function genes and the C function gene *AGAMOUS (AG)* specify the stamens. The action of the C function gene specifies the pistil. The model expansion was necessary to include the D and E class genes. The D function includes the *SEEDSTICK (STK)* genes and *SHATTERPROOF1* and *SHATTERPROOF2 (SHP1 and SHP2).*

The E function genes, *SEPALLATA 1 (SEP1, SEP2, SEP3, and SEP4)*, are required for the correct function of the other classes - withdrawal from Dornelas and Dornelas (2005).

The process of flowering and flower development is also regulated by other transcription factors. The *AINTEGUMENTA* (*ANT*) and *AINTEGUMENTA-LIKE* genes are involved in several processes since the establishment of floral meristem until ovule development (Blásquez et al., 1997; Hu et al., 2020; Krizek, 2009; Schultz & Haugn', 1991). These transcription factors are part of the AINTEGUMENTA-LIKE/PLETHORA (AIL/PLT) clade of the Ethylene (Apetala 2/Ethylene Response Factor) family (Nole-Wilson et al., 2005). The genes *ANT, AINTEGUMENTA-LIKE 1 (AIL1), AINTEGUMENTA-LIKE 3 (AIL3), AINTEGUMENTA-LIKE 4 (AIL4), AINTEGUMENTA-LIK 5 (AIL5), AINTEGUMENTA-LIKE 6 (AIL6), AINTEGUMENTA-LIKE 7 (AIL7)* and *BABY BOOM (BBM)* compound the AIL/PLT clade in the model plant *A. thaliana* (Horstman et al., 2014; Nole-Wilson et al., 2005). Until now, the most understood during flower development are *ANT* and *AIL6*. These two transcription factors are related to meristem development, promotion petal, stamens, carpel, and correct establishment of ovules. ANT and AIL6 control specification of floral organs by regulating genes of ABCDE model. *ANT* and *AIL6* directly activate the APETALA 3 gene of class B and the AGAMOUS gene of class C at the stage of floral development where the sepals are specified (Krizek et al., 2000, 2021).

OVULE DEVELOPMENT

In angiosperms, the ovules develop inside the pistil. The pistil represents the structure formed by the fusion of two or more carpels, and one of its primary functions is to protect the ovules, which are essential for the reproduction of this group of plants (Galbiati et al., 2013; Kelley & Gasser, 2009).

Ovules are simple yet highly specialized structures. They are divided into three main parts: integument, funiculus, and nucellus. The integument grows to cover the nucellus and, in the end, maintains a small opening, the micropyle, through which the pollen tube has access to the nucellus and grows to the embryo sac (Endress, 2011). The funiculus holds the ovule attached to the placenta and acts as a conduit for delivering nutrients to the developing ovule and embryo. The funiculus can also influence the position of the micropyle. The nucellus is

where the megasporocyte develops and supports the subsequent events for developing the germline, the female gametophyte (Wilkinson et al., 2018).

In *Arabidopsis thaliana*, the ovules start as finger-shaped structures from the placenta. As the structures develop, the integuments grow asymmetrically, resulting in anisotropy, the curved shape characteristic of mature ovule (Qadir et al., 2021). The haploid female gametophyte is surrounded by diploid maternal tissue, and the integument forms the mature ovule. The female gametophyte is formed by an egg cell, two polar nuclei within the central cell, two synergid cells, and a group of 3 to 100 antipodal cells. The number of antipodal cells depends on the species (Endress, 2011). Figure 2 clearly represents the structures that make up the ovules in the species *A. thaliana*. In general, in eudicots, ovule development occurs similarly (Rudall, 2021). However, differences can be found in the size and number of ovules, thickness, degree of curvature, funiculus compliance, and sometimes histological differentiation (Endress, 2011; Rudall, 2021).

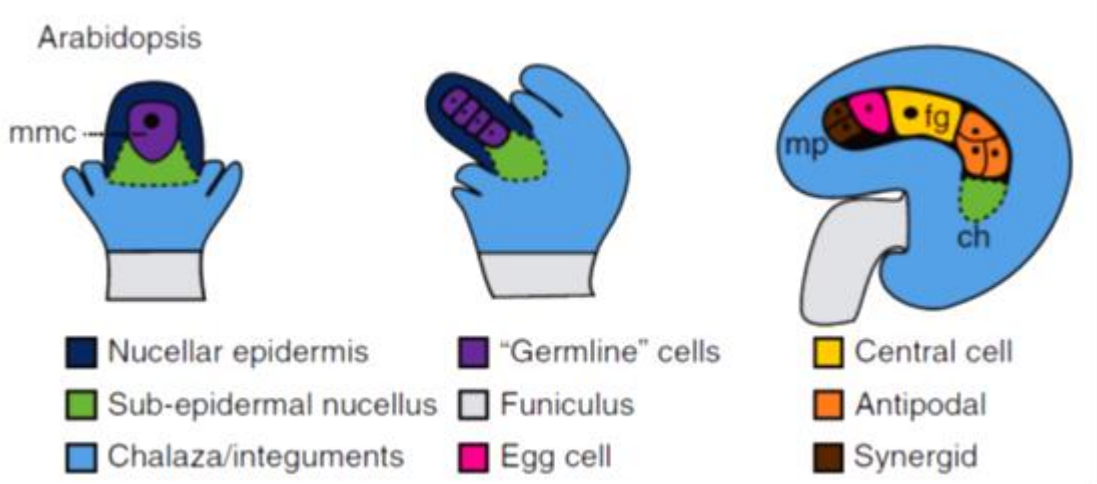

Figure 2. Schematic illustration of ovule development. The ovules begin their development from the placenta and establish the three functional regions: chalaza, which develops to form the integuments; funiculus, which connects the ovule to the placenta and mother plant; nucellus, where the embryo sac develops. The nucellus divides into epidermal and subepidermal cells. The mature ovule has the female gametophyte formed by an egg cell, two polar nuclei within the central cell, two synergid cells, and a group of 3 to 100 antipodal cells. Adapter from Wilkinson et al., 2018.

When thinking about the genetic pathways that control ovule development, the ABCDE model is essential in determining ovules' identity (Angenent & Colombo, 1996). The ovules are produced through the redundant activity of the transcription factors *SEEDSTICK (STK)* and *SHATTERPROOF (SHP1/SHP2),* which are expressed in the placenta (Favaro, 2003). However, the action of several other genes is necessary for correct development. Some of these genes seem to act redundantly, which is not surprising given the importance of ovules for plant reproduction.

ANT has been described to be associated with ovule initiation. In addition to failing to develop integuments, *ANT* mutants produce fewer ovules than wild-type plants (Elliott et al., 1996). Consequently, ovules of *ant* plants do not develop nucellus. The mechanisms associated with the absence of a nucellus in ovules that do not develop an integument remain poorly understood. ANT and other AIL/PLT proteins are related. *AIL6* appears to bind to the same cis-elements as ANT (Nole-Wilson & Krizek, 2000). Another aspect that seems necessary for the development of ovules is the determination of the correct spacing between them. The expression domains of the transcription factors *CUP SHAPED COTYLEDON2 (CUC1*), *CUC2 and CUC3* delimit the space for the individual development of each egg. Mutants for these genes produce fused ovules and eventually fused seeds (Gonçalves et al., 2015).

Once initiated the ovule development, the action of genes is necessary to establish the correct differentiation pattern of the three functional regions that make up the ovules. In recent years, several genes have been identified that appear to be necessary for ovule development. *ANT*, already mentioned above, has its domain and expression in the chalaza, the cell population that develops the internal and external integuments. Another identified gene, *WUS*, has its expression domain in the nucellus (Groβ-Hardt et al., 2002). In plants that mutate these genes, the chalaza does not develop into the integument, and they also fail to develop the embryo sac. However, no evidence exists that these genes interfere with each other's expression domain. The confinement of *WUS* expression in the nucellus is mediated by polarity-determining genes (Gasser & Skinner, 2019). *BELL1* mutants *(BEL1)* combined with *C3HDZ* expand *WUS* expression and ectopic ovule formation (Yamada et al., 2016).

Ovule primordia develop in the context of floral development, but they need to be redirected to differentiate and develop as ovules. The transcription factor AGAMOUS, MADS-Box class C is expressed in the primordia of ovules (Bowman et al., 1989). However, the action

of other transcription factors is necessary to distinguish the domain of ovules from the domain of carpels. This action is carried out by individual genes and genes acting in combination. Mutations in *BEL1* can lead to amorphous structures instead of integuments, even aberrant structures, and abortive nucellus. This variety of phenotypes can be explained due to the absence of *BEL1* in the chalaza, so tissue continues to grow without ovule identity (Gasser & Skinner 2019). Without identity, the structure initiates the priority path of development; in this case, the priority is to develop into carpels and, in sequence, the nucellus (Gasser & Skinner, 2019).

Loss of ovule identity is also observed in the combined *SHP1, SHP2,* and *STK* mutants. In these mutants, the integument is converted into a carpeloid organ (Brambilla et al., 2007). Likewise, partial loss of function of *SEP3* has also been observed to produce a similar phenotype. This phenotype suggests that *SEP3* may be required for *SHP1*, SHP2, and STK activity (Favaro, 2003).

AUXIN AND FLORAL DEVELOPMENT

Phytohormones are small molecules that originate from different metabolic pathways and play an essential role in plant development (Santner & Estelle, 2009). Collectively, phytohormones regulate several processes in the life cycle of plants, from developmental processes to responses to biotic and abiotic stress (Bencivenga et al., 2011). Therefore, it is not surprising to observe the action of phytohormones in processes involving floral development. In recent years, information on the role of phytohormones in floral development has accumulated. The role of auxin in flower formation is remarkable (Bencivenga et al., 2011).

Auxin is essential for initiating the floral meristem, organogenesis, and the termination of the floral meristem (Cucinotta et al., 2021). But there are large gaps in understanding effective mechanisms used for auxin to regulate these processes Chandler (2011).

Precise spatial and temporal localization of auxin is essential for initiating FM. Increased auxin concentration in IM due to increased biosynthesis and polar transport results in FM forming in the periphery of IM (Yadav et al., 2019). Polar auxin transport is a highly regulated process involving two genes*: PIN-FORMED1 (PIN1)* and *PINOID (PID*)

(Adamowski & Friml, 2015; Berkel et al., 2013;). *Pin1* mutants show a substantial reduction in polar auxin transport (Muday & DeLong 2001). PID, a protein serine-threonine kinase, interferes with the polar transport of auxin by modulating the localization of *PIN1*, acting directly on its phosphorylation (Michniewicz et al., 2007). The *pin1* mutants are clear evidence of the need for auxin transport. Flowers of these mutants do not develop sepals, and the petals show variable abnormalities. Exogenous application of auxin restores flower formation from the point of application in a *pin-formed1-1 (pin1-1)* mutant (Reinhardt et al., 2000). The phenotype observed in *pin1* mutants results from the failure of polar auxin transport (Cucinotta et al., 2021). The same phenotype can also be observed in the loss of function mutants of *NAKED PINS IN YUC MUTANTS 1(NPY), MAB4*. Both genes encode proteins that regulate PIN1 endocytosis (Furutani et al., 2014).

The spatial distribution of auxin in the floral meristem is an important signal instructing when cells should be exposed to maximum auxin at the sites where floral meristems form. In the inflorescence meristem, the location where the floral meristem will form has as its main characteristic the inhibition of the pluripotency of the cells in that region by reducing the expression of *STM*. In *mp* mutants and the triple mutants *mp arf3 arf4*, the *SHOOTMERISTEMLESS (STM)* transcripts are not reduced, resulting in complete loss of the floral primordium (Nole-Wilson et al., 2010). *AUXIN RESPONSE FACTOR 3 (ARF3)* or *ETTIN (ETT)* represses *STM* expression since the transcription factor is a direct target of this auxin response gene (Chung et al., 2019). *MONOPTERO (MP)* or *(ARF5)*, in turn, does not have *STM* as its direct target but regulates the expression of FILAMENTOUS *FLOWER (FIL*), which negatively regulates the expression of *STM*. In parallel, *ARF3, ARF4, and FIL* form a complex that recruits histone deacetylases to repress the target gene, *STM* (Chung et al., 2019).

MP acts as a central regulator of the auxin pathway during development. In floral development, this gene regulates local auxin concentration to maximum levels and transforms these auxin levels in floral primordia by regulating transcription factors. Two transcription factors that are initially regulated are *LFY* (Yamaguchi et al., 2013) and *AP1*, which confers the identity of the floral meristem (Dong et al., 2021). In general, *MP* works as an initiation point for floral development. This transcription factor increases by transcriptional regulation of the expression of *LFY, ANT*, and *AIL6* by two parallel and independent pathways. *ANT* and *AIL6* are direct targets of LFY. On the other hand, *LFY* is regulated by the positive feedback that keeps the auxin pathway on and ensures flowering (Yamaguchi et al., 2017). *MP* action also

involves controlling chromatin remodeling. MP transcriptionally regulates the *SWI/SNF ATPases BRAMA (BRM)* and *SPLAYED (SYD)* genes, which increase chromatin accessibility in floral primordia (Bezhani et al., 2007; Nishioka et al., 2020).

AUXIN AND DEVELOPMENT OF THE GYNOECIUM AND OVULE

The involvement of auxin in the gynoecium's development is like that observed in the lateral development of floral meristems in the meristems and inflorescence. It was postulated by Nemhauser et al. (2000) that an auxin gradient is necessary for the proper development of the gynoecium. These authors described that higher auxin levels lead to stigma and style development. Medium levels promote ovary differentiation, and lower levels promote gynophore development in the A. thaliana flower.

The auxin gradient model was explained by (Moubayidin & Østergaard, 2017). According to the authors, this model is refined by cytokinin, which acts antagonistically and establishes the apical-basal, mediolateral, and adaxial-abaxial domains. Likewise, the auxin levels determine the action of transcription factors involved in forming the gynoecium and responding to auxin. Among these transcription factors, ARF3/ETT is involved in forming the gynoecium. *arf3/ett* mutants show severe polarity defects. The mutants have reduced ovaries, excess proliferation in the stigma, and a longer gynophore (Simonini et al., 2016). The synergic action of ARF3/ETT with ARF4 to regulate the axial-abaxial polarity of the valves in the ovary of *A. thaliana* is known. This synergy leads to the activation of *KANADI (KAN). KAN* is part of a family of genes that coordinate the abaxial polarity of the carpels, and the loss of function of this gene leads to the formation of ovules on the outer face of the gynoecium (Pekker et al., 2005).

Other transcription factors are closely related to the auxin pathway and gynoecium development (Simonini & Østergaard, 2019). *CRABS CLAW (CRC)* controls auxin homeostasis in the medial portion of the pistil and maintains auxin at maximum levels for development in this region. In the biosynthesis pathway, *YUC4* and *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1(TAA1)* mediate local auxin synthesis and coordinate the growth of the top of the gynoecium (Stepanova et al., 2011). Positive regulators of auxin synthesis also have relevance in forming the gynoecium. Mutations in the transcription factors *SPATULA (SPT),*

STYLISH1 (STY1), LEUNIG (LUG), and *SEUSS (SEU)* produce a phenotype in the organ because they interfere with auxin synthesis and transport pathways (Moubayidin & Østergaard, 2014; Schuster et al., 2014). Inhibition of auxin transport causes several changes in gynoecium formation. Narrowing the valves and expanding the gynoecium may be associated with this inhibition. *PID1* mutants or flowers treated with auxin transport blockers do not correctly develop the gynoecium, resulting in a radial structure (Nemhauser et al., 2000). Similarly, it has been shown in rice that *OsPINOID* is required for stigma and ovule initiation and establishment (Xu et al., 2019).

The processes that involve the auxin pathways in the development of the gynoecium are also directly related to the development of ovules. ARF5 regulates the expression of genes such as *CUC1, CUC2, ANT, CYTOKININ RESPONSE FACTOR 2 (CRF2),* and *DORNROSCHEN (DRN)* (Cole et al., 2009; Yang & Tucker, 2021). These transcription factors, in addition to establishing the space for the development of each ovule (Kamiuchi et al., 2014) and in the development of the functional regions that make up these structures (Elliott et al., 1996). They act to regulate *PIN1* (Michniewicz et al., 2007) and, therefore, interfere with the concentration of auxin in the placenta, the site of egg formation. *arf5* mutants in Arabidopsis do not develop marginal meristem of carpels and placenta and consequently do not have ovules. The *arf3* mutants have shorter ovaries and fewer ovules compared to wild-type plants. Double mutating s*eu ant* results in a complete inability to form ovule due to the need for these transcription factors for effective auxin response.

The participation of auxin in the development of the gynoecium and ovules makes it an essential hormone for reproduction, as it acts in the transition from somatic to germinal lineage and, in addition, acts in the processes of megasporogenesis and formation of the female gametophyte. Again, *PIN1* regulates auxin transport and increases to maximum levels in the nucellus (Pagnussat et al., 2009; Wang et al., 2021). *ARF3/ETT* downregulates megaspore mother cell (MMC) identity. In the nucellus, the expression of this gene is finely regulated, and failures in this regulation result in supernumerary CMM, repressing germline specification (Su et al., 2017). During megagametogenesis, the auxin response is detected in the chalaza, which suggests an indirect pathway for its action in gametophyte specification. Auxin also assists in establishing the micropyle (Cucinotta et al., 2021).

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HYPOTHESIS

The presence of meristematic cells in reproductive tissues after *WUS* repression in floral meristem and pistil specification suggested that there is an upstream mechanism to these events that regulate the termination process of these cells and control proliferation. The hypothesis is that *SCI1* start to be express in the beginner of flower development and makes part of the regulation that controls the proliferation of meristematic cells, after the action of key transcription factors in this process, until the complete development of the flower.

Related to the first hypothesis, we hypothesized that *SCI1* controls the proliferation of the last pistil meristematic cells. Ovules are the last population of meristematic cells to be terminated in flower. *SCI1* is expected to be expressed in the specialized tissues of the pistil and later in the ovary during development. Furthermore, it is expected that *SCI1* is transcriptionally regulated by transcription factors important in the termination and proliferation of floral meristem and ovule development.

OBJECTIVES

This thesis aimed to determine the pattern of *SCI1* expression during *N. tabacum* flower development and analysis of its transcriptional regulation. As well as characterize the initial stages of flower development of this study model.

This work is divided into two parts. The first was to characterize the expression of *SCI1* from the moment the floral meristem appears and in the initial stages of flower development. For this purpose, the initial development of the *N. tabacum* flower was also characterized. Furthermore, in this part, the transcriptional regulation of *SCI1* by key transcription factors for the maintenance and termination of the floral meristem was also determined. The results of this part are presented in chapter 1 in the form of a previously published paper.

The second part sought to analyze and characterize the expression of *SCI1* and *NtANT* in flower, and ovary development. Furthermore, analyze the transcriptional regulation of *SCI1* by NtANT. Part of this objective was realized at Biosystems and Integrative Sciences Institute, Plant Functional Biology Center, University of Minho, Braga, Portugal, under supervision of Drª Maria Manuela Ribeiro Costa. The results of this part are presented in chapter 2 as a manuscript to be submitted.

SPECIFIC OBJECTIVES

- CHAPTER I
    - Analyze the expression of *SCI1* in the floral meristem and the initial stages of the flower of *N. tabacum*;
    - Characterize the initial stages of *N. tabacum* flower development;
    - Perform in silico analysis of the genomic sequence of *SCI1* to identify putative cis-elements for the interaction of transcription factors;
    - Choice of transcription factors to be tested;
    - Compare the spatial expression of *SCI1* with the carpel identity gene in *N. tabacum* (*NAG1*);
    - Compare the spatial expression *SCI1* with the meristematic cell maintenance gene *NtWUS*;
    - Test the binding of transcription factors NAG1 and NtWUS with the *SCI1* promoter;
    - Determine the effect of NAG1 and NtWUS interaction on *SCI1* expression;
    - Produce transgenic plants expressing the SCI1 protein fused to GFP at its carboxy-terminal end under the control of the endogenous promoter;
    - Analyze whether transgenic plants express SCI1-GFP similarly to the endogenous SCI1 gene.

- CHAPTER II
    - Identify the *NtANT* gene in *N. tabacum;*
    - Determine *NtANT* expression in *N. tabacum* flower;
    - Compare *SCI1* and *NtANT* expression during flower development *in N. tabacum*;
    - Determination of *NtANT* expression during ovule development in *N. tabacum* flowers;
    - Compare *SCI1* and *NtANT* expression during ovule development in *N. tabacum* flowers;
    - Test *NtANT* binding to the *SCI1* promoter;

- Determine the effect of NtANT interaction on *SCI1* expression;
- Analyze whether auxin interferes with *SCI1* expression during regulation of *SCI1* by NtANT.

CHAPTER I

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

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

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ABSTRACT

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 coexpressed 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

1. 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; Ó'Maoiléidigh 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* $SCI1^{Ri}$ silencing plants and *A. thaliana sci1* mutants presented elongated styles and increased stigmatic areas. These phenotypes resulted from increased cell number (DePaoli et al., 2011, 2014). Furthermore, $SCI1^{Ri}$ plants have accelerated cell differentiation in the stigma surface, consistent with a role for SCI1 in triggering differentiation through cell proliferation control (DePaoli et al., 2011). There is a relationship between *SCI1* and the auxin signaling pathway not yet understood. The *NtAux/IAA19*, *NtAux/IAA13*, and *NtARF8* expression levels were affected by *SCI1* expression, especially in plants overexpressing *SCI1* (DePaoli et al., 2012). In *A. thaliana*, the altered phenotypes of *sci1*, *yuc2yuc6*, and *npy1* mutants cannot be distinguished. Additionally, overexpression of *SCI1* in a *yuc2yuc6sci1* background restores the wild-type phenotype. These findings point to an overlap of *SCI1* and *YUCCA* genes in the auxin signaling pathway during pistil development in Arabidopsis (DePaoli et al., 2014).

Due to its key role in cell proliferation regulation and early expression in pistil development, we asked when *SCI1* starts to be expressed during flower development. In this study, we carried out detailed analyses of *SCI1* expression since flower meristem specification, by *in situ* hybridization detection of the endogenous transcript, and the expression driven by *SCI1* genomic sequence translationally fused to GFP in transgenic plants. *In silico* analyses of the *SCI1* genomic sequence indicated the presence of AG and WUS cis-regulatory elements. We demonstrated the co-expression of *SCI1* and *N. tabacum WUSCHEL* (*NtWUS*), as well as *SCI1* and *N. tabacum AG* (*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 (EMSA), and *in planta* luciferase activity assay for NAG1. We gathered evidence showing that *the TF NAG1 activates SCI1 expression*. 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.

2. MATERIALS AND METHODS

2.1 *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.

2.2 *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 Embedding Kit, Leica). Longitudinal sections of the floral axis, with a thickness of 2–3 µm, were obtained in a Leica® RM2265 rotation microtome, with the aid of a high-profile, disposable steel razor (LEICA 818), and stained with 0.05% (w/v) toluidine blue, pH 4.4. The slides were mounted in Entellan mounting medium (Merck), and the images acquired under a microscope (Zeiss – Axiolab), equipped with a camera (Zeiss – Axiocam Color). For SEM, flowers from SR1 plants were dissected under a Wild M7A or Leica 165 FC stereoscopic microscope. They were fixed in 1% (w/v) glutaraldehyde and 4% (w/v) formaldehyde pH 7.2, submitted to vacuum (negative pressure of 760 mmHg) for 24 h, and kept in fixative solution at 4°C until use. They were then dehydrated in an increasing ethanol series, dried to a critical point with a CPD 030 (BAL-TEC) in CO_2. Subsequently, the floral primordia were covered with a 15 nm gold layer in SCD 050 metallizer (BAL-TEC). The samples were analyzed under 10 kV voltage, and the electron micrographic record was performed using a scanning electron microscope JEOL 6060.

A laser confocal microscope Leica TCS SP5 (Leica Microsystems Heidelberg, Germany) with the Leica LAS-AF Lite software was used for detecting fluorescence microscopy of GFP (argon laser; wavelength for excitation 488 nm and emission in the lengths between 500 and 590 nm). The LSM 7MP multiphoton microscope was used for excitation of

thick samples, like floral buds (excitation using an 800 nm laser and detection in the spectrum between 500 and 540 nm). The autofluorescence of chloroplasts was detected in the range between 640 and 730 nm. Images were processed using the software Fiji-ImageJ 2.1.0/1.53c (Schindelin et al., 2012).

2.3 *In situ Hybridization*

In situ hybridization was performed using digoxigenin-UTP-labeled probes, as described by Javelle et al. (2011). For the synthesis of the probes, the coding sequences (CDS) of *NAG1*, *NtWUS*, and *SCI1* were amplified by RT-PCR (primers are listed in Supplementary Table 2) and cloned in 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).

2.4 *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 pABAi-GW vector upstream of the AUR1-C coding sequence. The constructs pFrag1-abaiGW (−515 to −73bp upstream of the ATG), pFrag2-abaiGW (−1197 to −706 upstream ATG), pFrag3-abaiGW (−1667 to −1178 upstream ATG), pIntron3-abaiGW (+1583 to +2069 bp third intron) were sequenced and introduced in the PJ69-4A yeast genome by homologous recombination. The resultant yeast transformants were tested for self-activation of the AUR1-C gene and used in the Y1H assays. The CDS of NAG1 and NtWUS were cloned in fusion with the GAL4 activation domain at the pDEST22 vector (ProQuest™ Two-Hybrid System with Gateway™ Technology) and introduced in the yeast cells containing each of the genomic fragments mentioned above. The transformants were cultured in synthetic defined media (SD/-Ura-Trp) with or without 150 ng/ml of AbA for 3–5 days at 30°C.

2.5 *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.

2.6 *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.

3. RESULTS

3.1 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 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 and 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 two ovary locules will develop (Figures 2D,E). 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 ovary 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 ovary 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.

3.2 *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 *SCI1prom: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 mature transgenic plants' leaves, stems, and roots.

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 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.

3.3 *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 software[1] using the *N. tabacum* database. In the analysis using a *p*-value $\leq$ 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 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, which are involved in the regulation of tissue boundaries and asymmetric cell division (Welch et al., 2007); LFY, a master regulator of flowering; and the AUXIN RESPONSE FACTOR 5 (ARF5). Interestingly, in two different positions of the *SCI1* genomic

sequence, there is an overlap between AIL1 and SOC1 putative binding sites. 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.

3.4 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 *SCII* 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 2I). *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.

3.5 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.

4. DISCUSSION

4.1 *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 $SCI1_{prom}$*: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.

4.2 *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, including AG binding sites. Putative AG binding sites were also found in the *SCI1* 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 CArG-Boxes (Huang et al., 1993), but in *N. tabacum*, one of the identified regulatory domains represents a perfect CArG-Box (Aerts et al., 2018). Through Y1H, we demonstrated that NAG1 binds to fragment 1 (−515 to −73) of the *SCI1* promoter (Figure 5C), which contains a perfect CArG-Box (TTTATACCATTTGGAGATA). We also demonstrated that NAG1 binds specifically to the *SCI1* sequence (Supplementary Figure 4) containing the above mentioned CArG-Box by EMSA (Figure 6B). The luciferase activity assay showed that NAG1 interaction with the *SCI1* promoter significantly increased the reporter gene's activity in relation to the control (Figure 7B). Taken together, these results demonstrate that the NAG1 TF binds to the regulatory region present in the *SCI1* promoter, activating its expression. Our data also reveal that NAG1 is necessary and sufficient to induce *SCI1* expression. Moreover, these results indicate that SCI1 acts downstream of NAG1, which, in addition to specifying the third and fourth floral whorls, is also responsible for the floral meristem termination (Mizukami and Ma, 1995; Wils and Kaufmann, 2017).In Arabidopsis, AG is involved in regulating several genes related to flower development, among them *KNUCKLES*, *CRABS CLAW*, *JAGGED*, *NOZZLE/SPOROCYTELESS*, *SHATTERPROOF2*, *SEP3*, *ARABIDOPSIS THALIANA HOMEOBOX GENE1*, *AP1*, *AP3*, and even *AG* itself (Ó'Maoiléidigh et al.,

2013). Gómez-Mena et al. (2005) reported that, at the beginning of floral organogenesis, AG regulates genes involved in the cell cycle, DNA repair, signal transduction, and maintenance of meristematic cells, among others. Additionally, a ChIP-seq experiment has identified almost 2000 AG targets, among which many transcriptional regulators and genes related to multiple cellular processes (Ó'Maoiléidigh et al., 2013). 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 carpelar 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.

5. 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.

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).

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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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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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FIGURE LEGENDS

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.

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 –5; 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.

Figure 3. Expression of SCI1-GFP in transgenic plants. Upper panel – Schematic representation of the construct used to produce SCI1-GFP transgenic plants. **(A–C)** Confocal microscopy of a crushed flower meristem (transgenic plant SCI1gen-GFP17.1). In the first focal

plane, a sepal is seen, and behind, the floral meristem. Scale bar: 100 μm. **(D–F)** Inflorescence apex containing three floral buds; observe the intense GFP fluorescence at the floral meristem (FM). Scale bar: 300 μm. **(G–I)** Stigma/style of transgenic plant SCI1gen-GFP108.3, basolateral longitudinal section of the upper part of the pistil; observe intense GFP fluorescence at the stylar transmitting tissue (STT), and the stigmatic secretory zone (SSZ); very weak GFP fluorescence at the parenchyma tissue. Scale bar: 150 μm. **(J–L)** Ovary section of transgenic plant SCI1gen-GFP5.1; observe the intense GFP fluorescence at the placenta (pl) and nucellus (nu); no GFP was detected at the megaspore region (m) and integument (in). Scale bar: 40 μm. **(M)** Schematic representations of the flower regions analyzed by confocal microscopy. Floral meristem (FM), parenchyma (p.), stigmatic secretory zone (SSZ), stylar transmitting tissue (STT), ovule (OV).

Figure 4. *In situ* hybridization with *SCI1*, *NAG1*, and *NtWUS* antisense probes. **(A–C)** *In situ* hybridization in very young floral buds with the indicated probes. Scale bars: 100 μm. **(D,E)** *In situ* hybridization in floral buds with *NAG1* antisense probe. Stages –8 and –5 of flower development, respectively. Scale bars: 100 μm. **(F–H)** *In situ* hybridization with *NtWUS* antisense probe. **(F,G)** Flower buds younger than stage –7. Scale bars: 100 μm. **(H)** Stage –7 of flower development. Scale bars: 50 μm. Floral meristem (FM), sepals (S), petals (P), anther (A), young ovary (YO). Negative controls of flower buds in equivalent developmental stages are shown in Supplementary Figure 1

Figure 5. NAG1 and NtWUS associated with *SCI1* promoter in the Y1H assay. **(A)** Schematic representation of the *SCI1* genomic region, with the representation of exons and introns, and the fragments considered for the Y1H bait constructs. **(B)** Schematic representation of the bait and prey constructs used in the Y1H assay. The indicated promoter fragments of *SCI1* were used to make the *SCI1-AUR1-C* baits. **(C)** Physical interactions of NAG1 and NtWUS with fragment 1 of *SCI1* promoter in Y1H assays. Yeast expression plasmids pDEST22-NAG1 or pDEST22-NtWUS were introduced into yeast cells (PJ69-4a) carrying the reporter gene AUR1-C under the control of the different fragments of *SCI1* promoter. Eight independent transformants were cultured in synthetic defined media (SD/-Ura-Trp) in the presence of 150 ng/ml of Aureobasidin A (AbA). The empty vector pDEST22 was included as a negative control (last row).

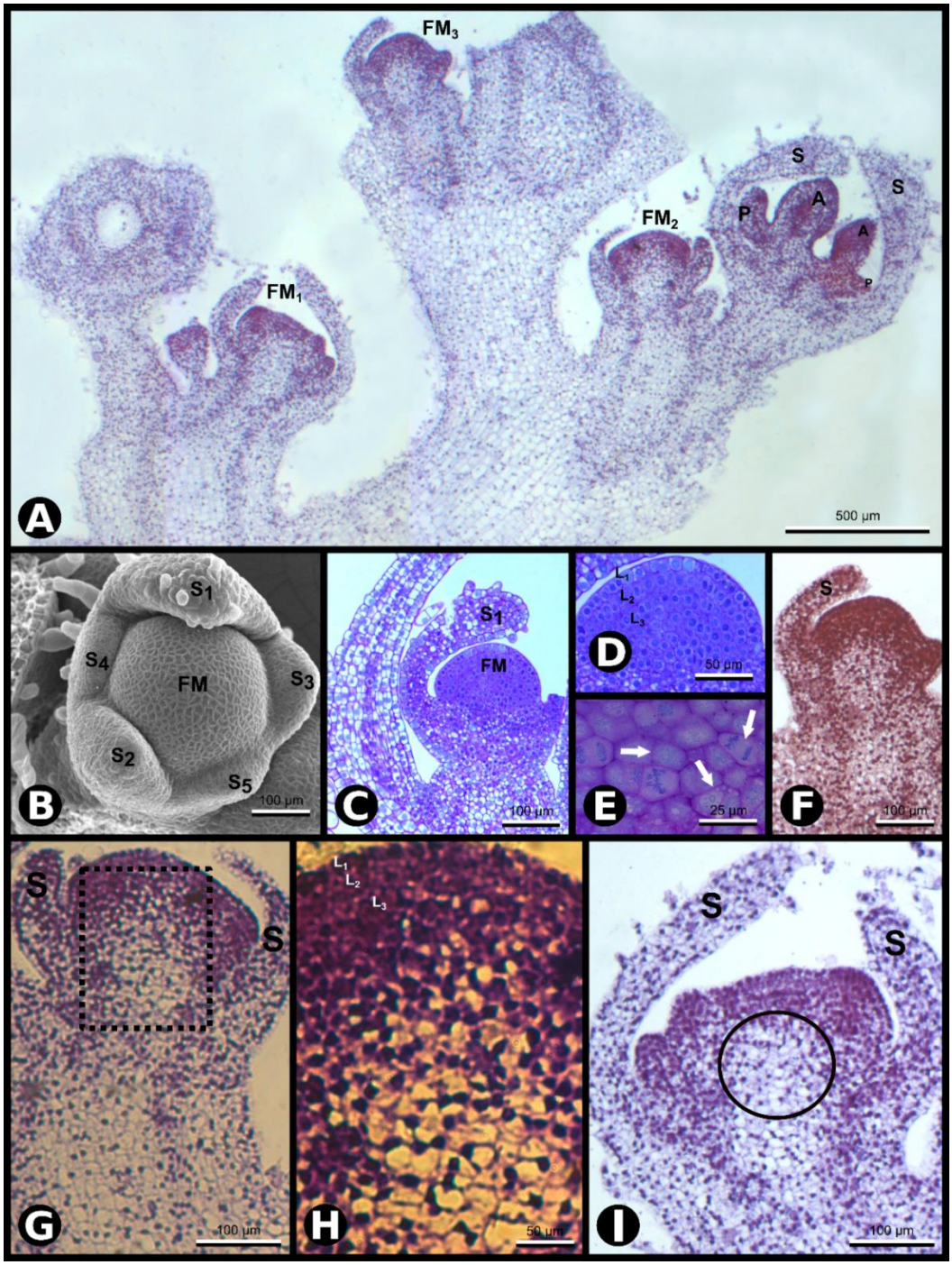
Figure 6. **(A)** Schematic representation of *SCI1* promoter (fragment 1) and the identified *cis*-acting elements for NAG1 (–285 to –267) and NtWUS (–212 to –203). **(B)** Electrophoretic mobility shift assay of NAG1 with the *SCI1* promoter sequence around position –285 to –267. Competitive EMSA using 77.5 fmols of the labeled probe; Lane 3 contains 5× unlabeled specific competitor; Lane 4 contains 10× unlabeled specific competitor. **(C)** Electrophoretic mobility shift assay of NtWUS with the *SCI1* promoter sequence around position –212 to –203. Competitive EMSA using 77.5 fmols of the labeled probe; Lane 3 contains 20× unlabeled specific competitor; Lane 4 contains 30× unlabeled specific competitor. Arrows indicate complexes between proteins and target sequences. The complete sequences of the EMSA probes are in Supplementary Figure 4. Poly-d(A-T) and Poly-d(I-C) are non-specific competitors.

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

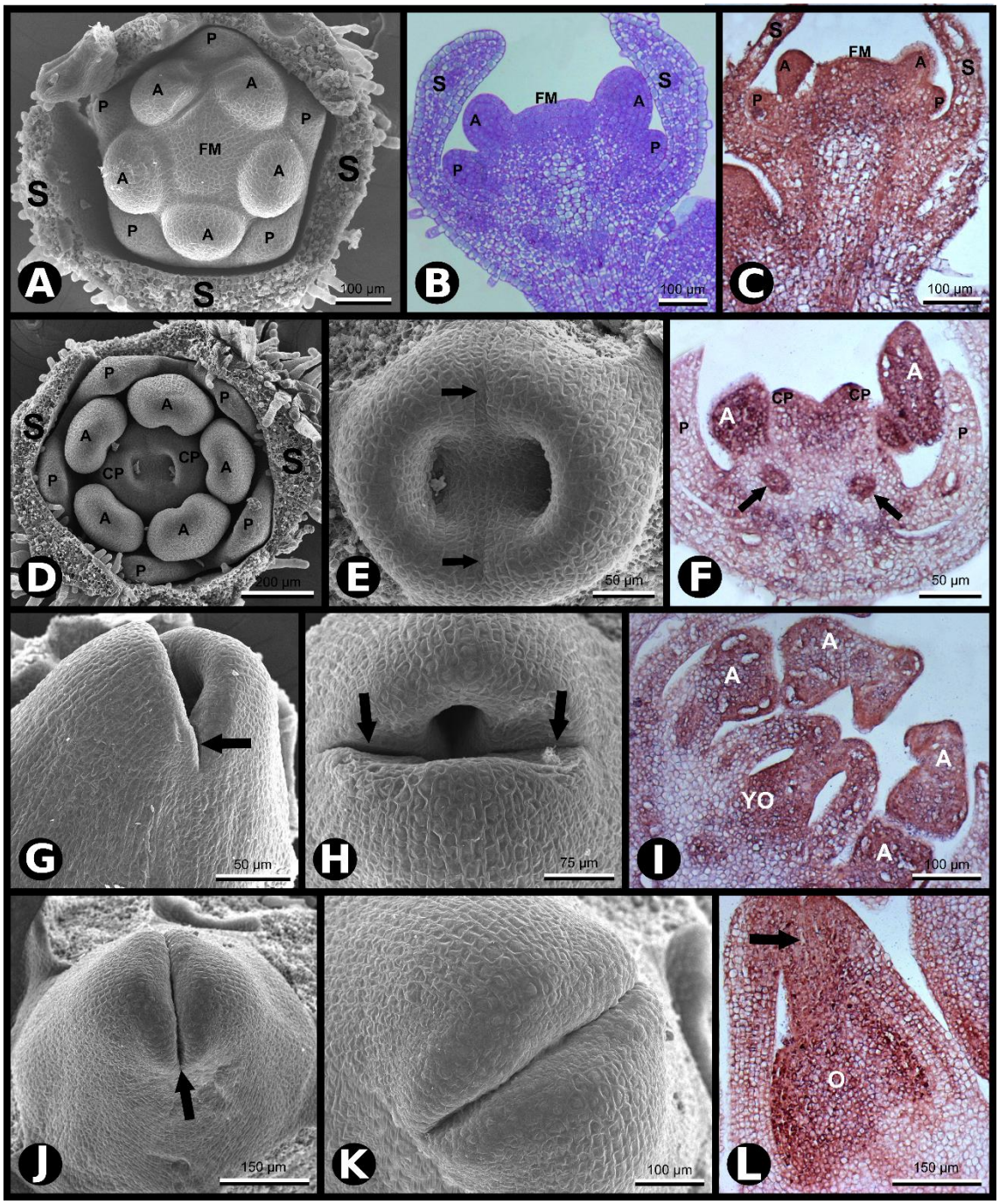
effector constructs used in the luciferase activity assay. **(B)** Luciferase activity as a result of the interaction of NAG1 and NtWUS with *SCI1* promoter sequences indicated. Relative activity is the ratio LUC/REN. The expression of *REN* was used as an internal control. Data represent the means of three biological replicates. **Significantly different from the control (empty vector) by Student's *t*-test ($p < 0.01$).

Figure 8. Proposed model for *SCI1* transcriptional regulation at the *N. tabacum* floral meristem. **(A, B)** *SCI1* expression is activated very early in development as soon as the floral meristem is specified. It is expressed in all proliferative cells, except in the organizing center (in red), where a high NtWUS protein level is expected. *SCI1* activation at the floral meristem depends on TF(s) not yet identified (AP1 and/or LFY are possibilities according *in silico* analyses). **(C, D)** Later in flower development, *NAG1* is expressed at the two inner whorls (stamens and carpels) and drives the activation of *SCI1*. Then, *SCI1* is expressed in the same cells as *NAG1*, in all meristematic cells of the flower, until style/stigma and ovules differentiation. Floral meristem (FM), sepals (S), petals (P), anther (A), carpels (C)

FIGURES


FM3
FM2
FM1
S
A
P
S
A
P
500 µm
A
S1
S4
FM
S3
S2
S5
100 µm
B
S1
FM
100 µm
C
L1
L2
L3
50 µm
D
25 µm
E
S
100 µm
F
S
S
100 µm
G
L1
L2
L3
50 µm
H
S
S
100 µm
I

1.


A
P
A
P
A
P
FM
A
A
S
S
P
A
P
S
100 µm
B
S
FM
S
A
A
P
P
100 µm
C
S
FM
A
A
S
P
P
100 µm
D
P
A
P
S
A
S
CP
CP
P
A
A
P
P
200 µm
E
50 µm
F
A
CP
CP
A
P
A
P
50 µm
G
50 µm
H
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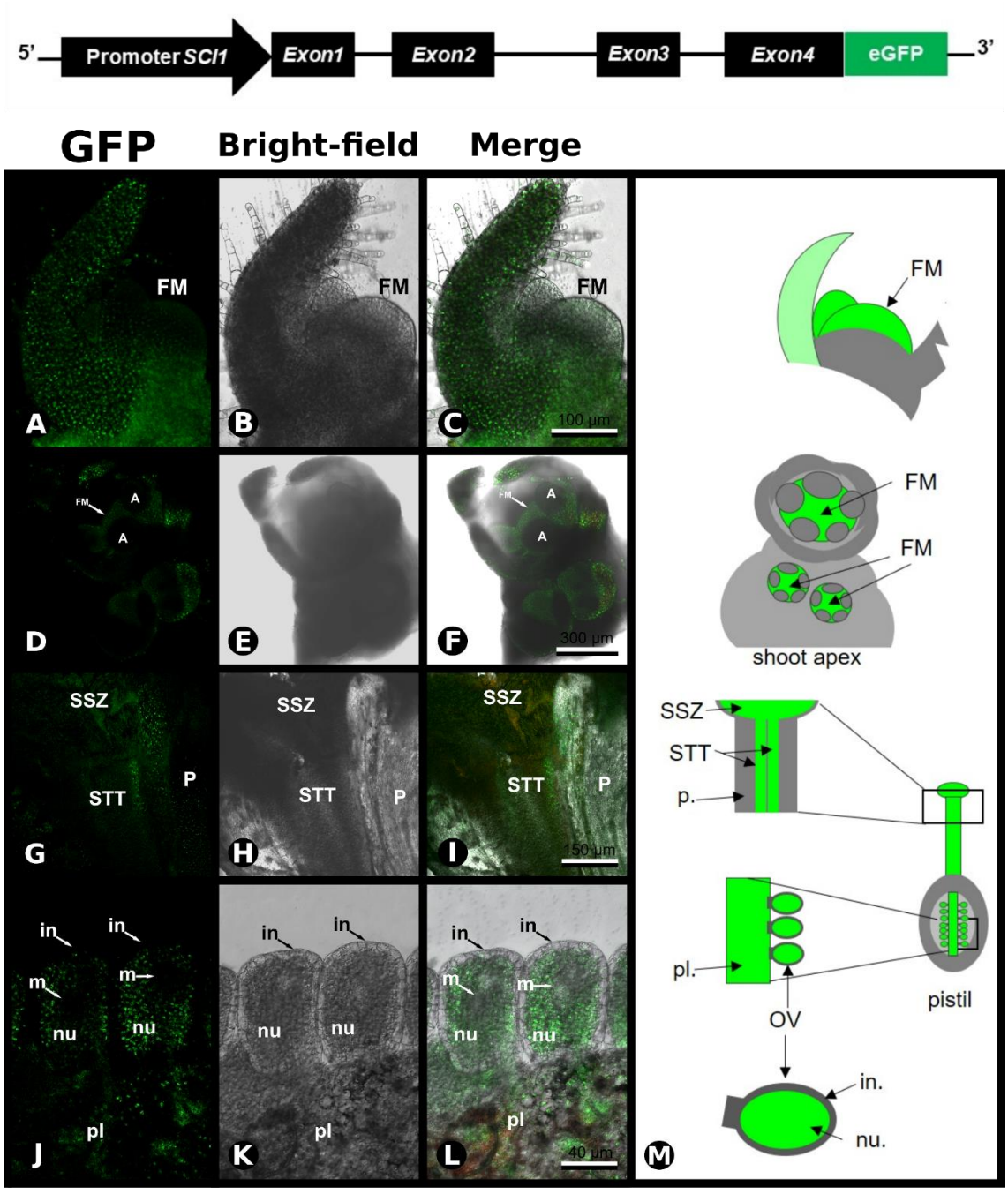
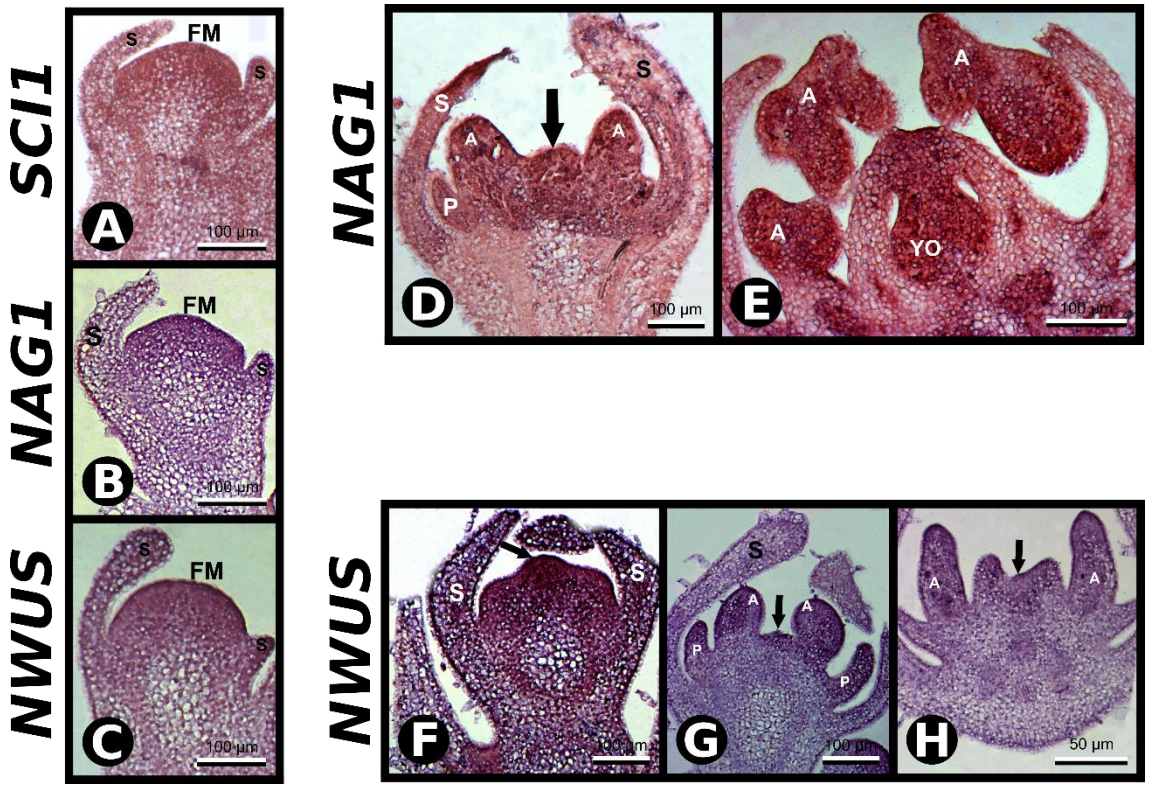
I
A
A
A
YO
A
100 µm
J
150 µm
K
100 µm
L
O
150 µm

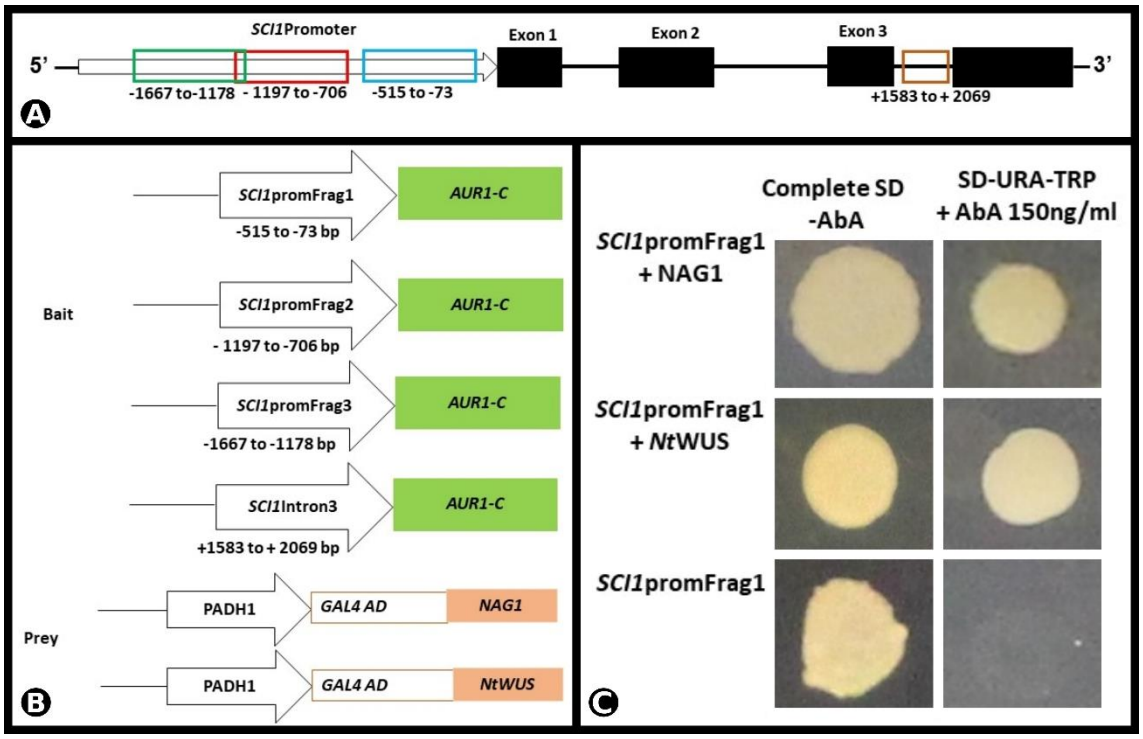
2.

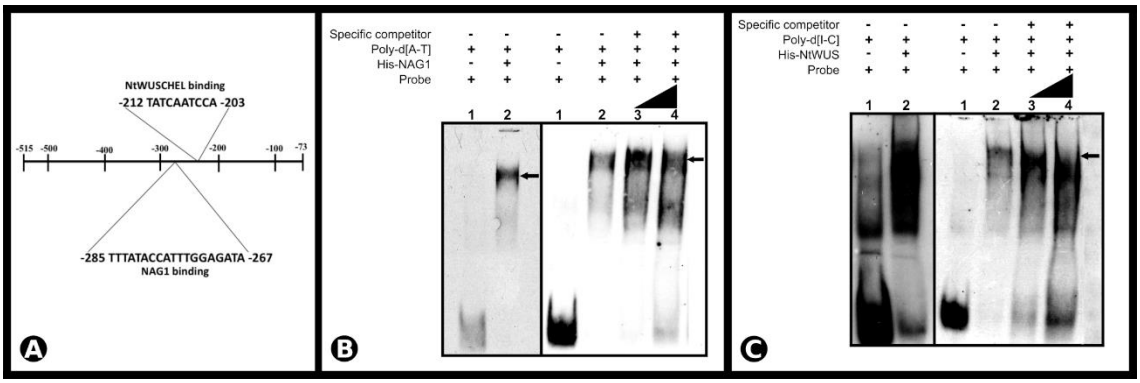

5'
Promoter SCI1
Exon1
Exon2
Exon3
Exon4
eGFP
3'
GFP
Bright-field
Merge
FM
A
B
C
100 µm
D
E
F
300 µm
SSZ
STT
P
G
H
I
150 µm
in
m
nu
pl
J
K
L
40 µm
shoot apex
STT
p.
pl.
OV
pistil
in.
nu.
M

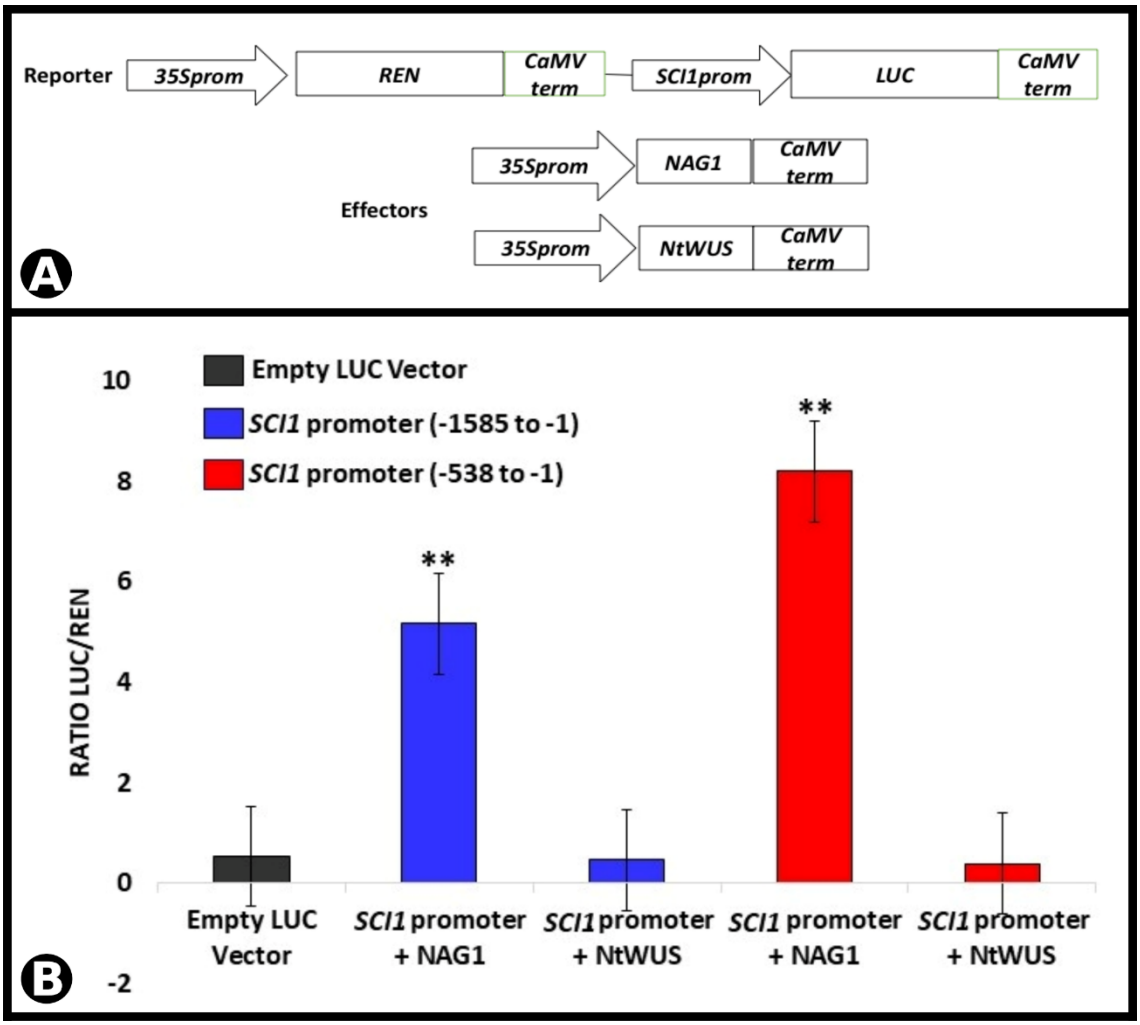
3.


SCI1
NAG1
NWUS
FM
S
A
P
YO
100 µm
50 µm

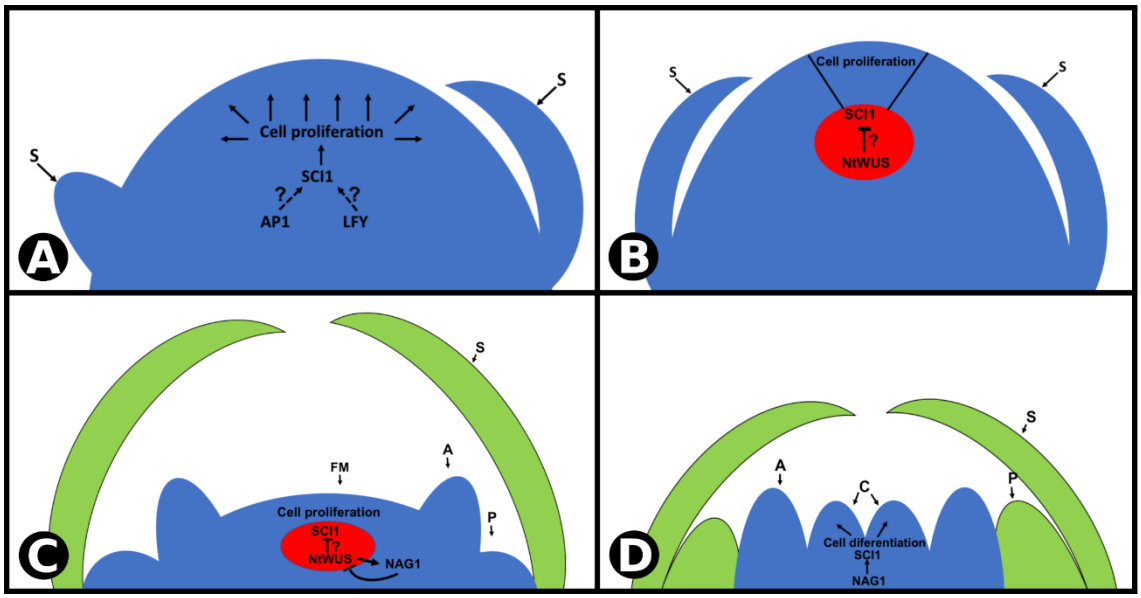
4.


SCI1Promoter
5'
3'
Exon 1
Exon 2
Exon 3
-1667 to-1178
- 1197 to -706
-515 to -73
+1583 to + 2069
A
SCI1promFrag1
-515 to -73 bp
SCI1promFrag2
- 1197 to -706 bp
SCI1promFrag3
-1667 to -1178 bp
SCI1Intron3
+1583 to + 2069 bp
AUR1-C
Bait
Prey
PADH1
GAL4 AD
NAG1
NtWUS
B
Complete SD
-AbA
SD-URA-TRP
+ AbA 150ng/ml
SCI1promFrag1
+ NAG1
SCI1promFrag1
+ NtWUS
SCI1promFrag1
C

6. 
NtWUSCHEL binding
-212 TATCAATCCA -203
-515 -500 -400 -300 -200 -100 -73
-285 TTTATACCATTTGGAGATA -267
NAG1 binding
A
Specific competitor
Poly-d[A-T]
His-NAG1
Probe
1 2 1 2 3 4
B
Specific competitor
Poly-d[I-C]
His-NtWUS
Probe
1 2 1 2 3 4
C


Reporter
35Sprom
REN
CaMV term
SCI1prom
LUC
CaMV term
Effectors
35Sprom
NAG1
CaMV term
35Sprom
NtWUS
CaMV term
A
Empty LUC Vector
SCI1 promoter (-1585 to -1)
SCI1 promoter (-538 to -1)
RATIO LUC/REN
10
8
6
4
2
0
-2
**
**
Empty LUC Vector
SCI1 promoter + NAG1
SCI1 promoter + NtWUS
SCI1 promoter + NAG1
SCI1 promoter + NtWUS
B

7.


A
S
S
Cell proliferation
SCI1
?
?
AP1
LFY
B
S
S
Cell proliferation
SCI1
T?
NtWUS
C
S
A
FM
P
Cell proliferation
SCI1
T?
NtWUS
NAG1
D
S
A
C
P
Cell diferentiation
SCI1
NAG1

8.

SUPPLEMENTAR FIGURE LEGENDS

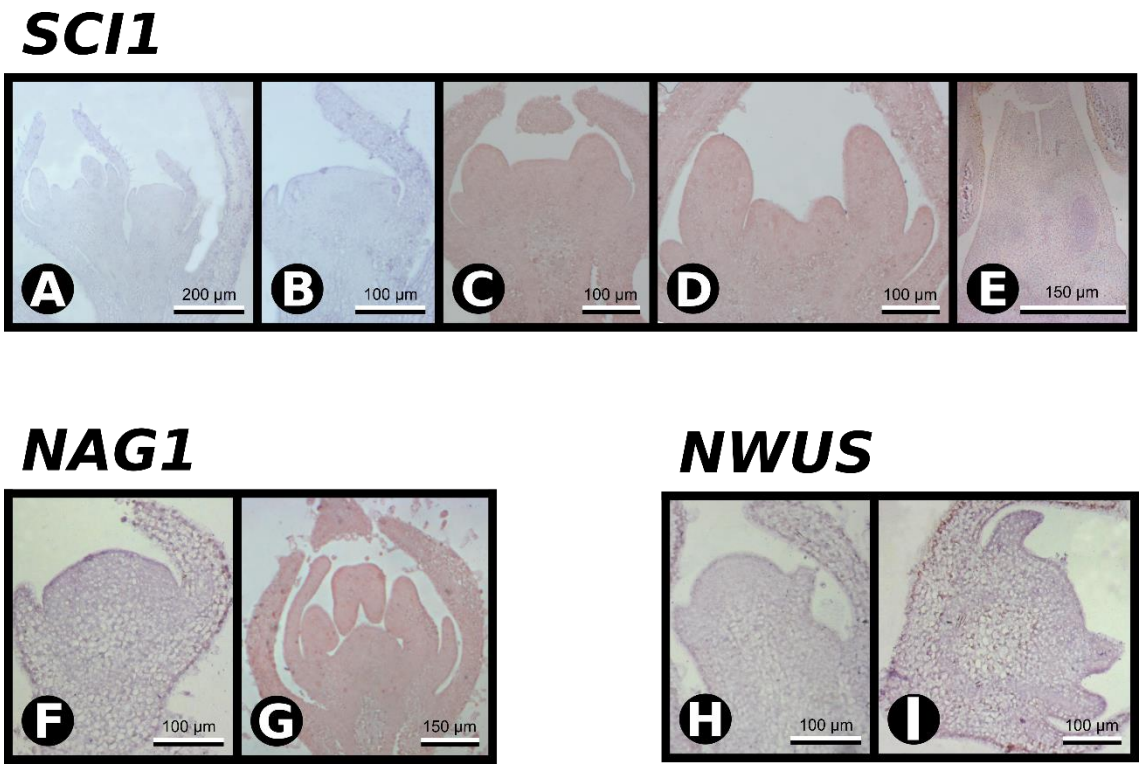
Supplementary Figure 1. Negative controls of *in situ* hybridizations (*SCI1* sense probe).

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.

SUPPLEMENTAR FIGURES



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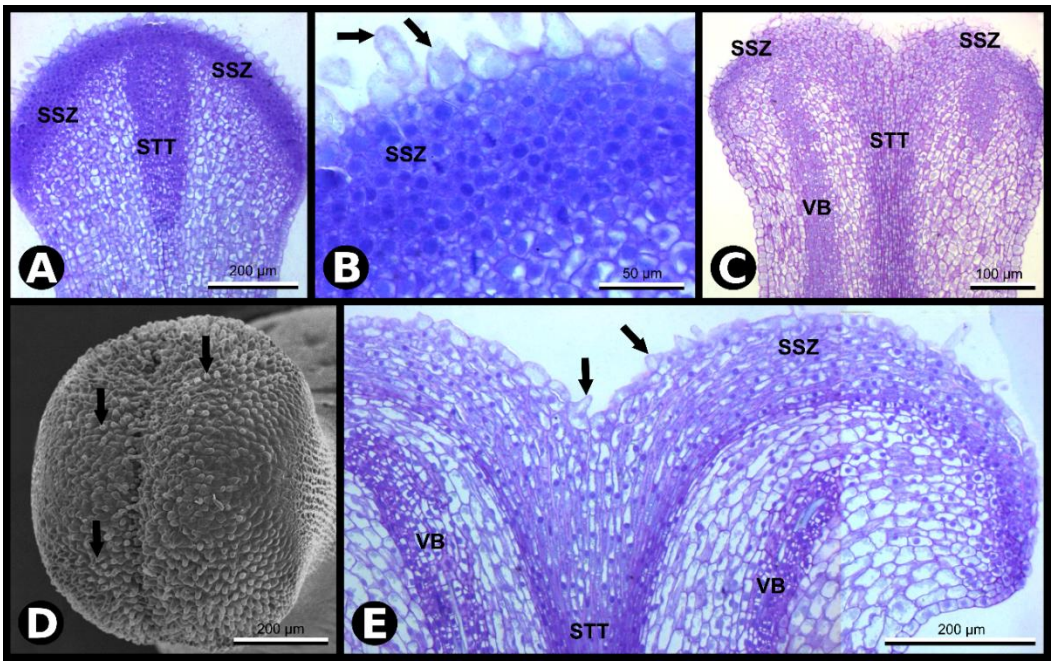

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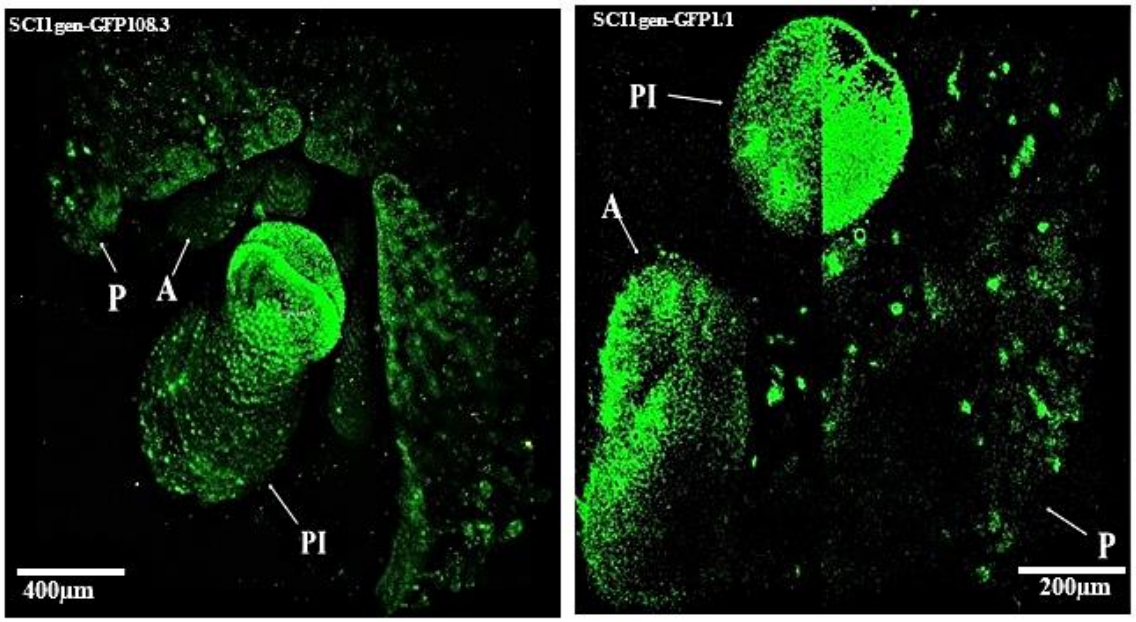

3.

Target NAG1

Prom*SCI1* 5′GCTTCGTAATATCT**TTTATACCATTTGGAGATA**GACTAACATAAGAG 3′
3′CGAAGCATTATAGAAAATATGGTAAACCTCTATCTGATTGTATTCTC 5′

Prom*SCI1* 5′GTAATAAATTCAAGACTAGGATAGTTAGGTTTACCAGATTTAAACAG 3′
3′CATTATTTAAGTTCTGATCC**TATCAATCCA**AATGGTCTAAATTTGTC 5′

Target NtWUS

4.

SUPPLEMENTARY TABLE LEGENDS

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

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

SUPPLEMENTARY TABLE

Supplementary table 1

#pattern name	Family	Transcription factor	sequence name	start	stop	strand	score	p-value	q-value	matched sequence
LOC107781702	E2F/DP	PREDICTED: transcription factor E2FC-like	Nicotianatabacum	-1	+14	+	5,64E+04	0,00996	0,441	AATGGGGAGCGATAA
LOC107774346	LFY	PREDICTED: floricaula/leafy homolog 2 isoform X2	Nicotianatabacum	+1216	+1234	-	8,10E+05	0,00619	0,275	AATGATCCACAGGATCAAA
LOC107773468	MIKC_MADS	Nicotiana tabacum cultivar SR1 sepallata3-like MADS-box (SEP3)	Nicotianatabacum	-812	-799	+	1,20E+05	0,00352	0,313	ACCAAAAAAGAAA
LOC107781702	E2F/DP	PREDICTED: transcription factor E2FC-like	Nicotianatabacum	+1685	+1699	-	9,02E+05	0,00317	0,28	CAGTTCCAGCCAAAA
LOC107761268	MIKC_MADS	Nicotiana tabacum cultivar SR1 apetala 1 protein (AP1)	Nicotianatabacum	-1032	-1020	-	1,23E+05	0,00232	0,201	CTTAAAATGGGA
LOC107783687	bHLH	PREDICTED: transcription factor bHLH130-like	Nicotianatabacum	+1270	+1280	-	1,46E+05	0,000944	0,0837	GACCACTTGCT
LOC107773801	ERF	PREDICTED: ethylene-responsive transcription factor 4-like	Nicotianatabacum	+17	+31	-	1,21E+05	0,000892	0,0791	TCTCCTCCGTCGTCT
LOC107816356	ERF	ethylene-responsive transcription factor 4	Nicotianatabacum	+20	+34	-	1,25E+05	0,000879	0,078	TCTTCTCCTCCGTCG
LOC107779587	HD-ZIP	PREDICTED: homeobox-leucine zipper protein ATHB-6-like isoform X1	Nicotianatabacum	-661	-650	-	1,33E+05	0,000802	0,0696	CCAATCATTCA
LOC107830873	ERF	PREDICTED: ethylene-responsive transcription factor 5-like	Nicotianatabacum	+11	+31	+	1,21E+05	0,000769	0,0681	ATAAGAAGACGACGGAGGAGA
LOC107778042	ERF	PREDICTED: ethylene-responsive transcription factor 4-like	Nicotianatabacum	+11	+31	+	1,21E+05	0,000769	0,0681	ATAAGAAGACGACGGAGGAGA
LOC107804504	ERF	PREDICTED: ethylene-responsive transcription factor 3-like	Nicotianatabacum	+16	+30	+	1,22E+05	0,000752	0,0667	AAGACGACGGAGGAG
LOC107761881	C2H2	PREDICTED: protein indeterminate-domain 2-like	Nicotianatabacum	+2078	+289	-	1,45E+05	0,000739	0,0316	AAGAACAGAGGC
LOC107761881	C2H2	PREDICTED: transcription factor IIIA-like isoform X1	Nicotianatabacum	+2168	+2179	-	1,45E+05	0,000739	0,0316	AAGAACAGAGGC
LOC107804908	ERF	PREDICTED: ethylene-responsive transcription factor ERF091-like	Nicotianatabacum	+17	+31	+	1,30E+05	0,000737	0,0654	AGACGACGGAGGAGA
LOC107807574	ERF	PREDICTED: ethylene-responsive transcription factor LEP-like	Nicotianatabacum	+19	+35	+	1,20E+05	0,000679	0,0602	ACGACGGAGGAGAAGAG
LOC107808166	ERF	PREDICTED: ethylene-responsive transcription factor CRF4-like	Nicotianatabacum	+8	+29	-	1,27E+05	0,000673	0,0596	TCCTCCGTCGTCTTCTTATCG
LOC107770237	ARF	Auxin response factor 5	Nicotianatabacum	+1678	+1695	+	5,45E+02	0,000668	0,0586	TTTTCTGTTTTGGCTGGAACT
LOC107802463	Dof	PREDICTED: dof zinc finger protein DOF1.5-like	Nicotianatabacum	+1967	+1976	+	1,15E+05	0,000649	0,0283	CAAAAAGGGC

LOC107815583	HD-ZIP	PREDICTED: homeobox-leucine zipper protein HAT22-like	Nicotianatabacum	-659	-649	-	1,38E+05	0,000635	0,0564	GCCAATCATT
LOC107777976	C2H2	PREDICTED: protein indeterminate-domain 2-like	Nicotianatabacum	+21	+39	-	1,32E+05	0,000622	0,0539	CTTCCTCTTCTCCTCCGTC
LOC107828273	ERF	PREDICTED: ethylene-responsive transcription factor ERF118-like	Nicotianatabacum	+11	+31	-	1,20E+01	0,000585	0,0517	TCTCCTCCGTCGTCTTCTTAT
LOC107794493	MYB	PREDICTED: transcription factor MYB86-like isoform X1	Nicotianatabacum	-1668	-1647	-	1,48E+05	0,000562	0,0497	GCACCTGATATGTTTGGTAGA
LOC107790642	MYB	PREDICTED: transcription factor MYB46-like	Nicotianatabacum	-1668	-1647	-	1,48E+05	0,000562	0,0496	GCACCTGATATGTTTGGTAGA
LOC107800804	ERF	PREDICTED: ethylene-responsive transcription factor ERF118-like	Nicotianatabacum	+17	+31	-	1,35E+05	0,000557	0,0494	TCTCCTCCGTCGTCT
LOC107796720	C2H2	PREDICTED: lysine-specific demethylase REF6 isoform X1	Nicotianatabacum	+211	+225	+	1,43E+05	0,000543	0,0463	AAGAAAAGGACAAAT
LOC107803851	MIKC_MADS	PREDICTED: floral homeotic protein PMADS 2	Nicotianatabacum	-812	-798	+	1,46E+04	0,000511	0,0437	ACCAAAAAAGAAAA
LOC107777142	AP2	AIL1 PREDICTED	Nicotianatabacum	+1678	+1697	+	1,48E+05	0,000482	0,0102	GAAGGAAAGAAAAGGACAAA
LOC107792698	BBR-BPC	PREDICTED: protein BASIC PENTACYSTEINE2-like	Nicotianatabacum	+195	+218	+	6,94E+04	0,000467	0,0198	GATGAAGAGCGAAGGAAAGAAAAG
LOC107768279	TALE	Nicotiana tabacum knotted 3 (kn3)	Nicotianatabacum	+20	+39	-	1,46E+03	0,000457	0,0394	CTTCCTCTTCTCCTCCGTCG
LOC107793226	C2H2	PREDICTED: lysine-specific demethylase REF6 isoform X1	Nicotianatabacum	+211	+227	+	1,51E+05	0,000432	0,0372	AAGAAAAGGACAAATCC
LOC107777199	GRAS	PREDICTED: DELLA protein GAI1-like	Nicotianatabacum	+212	+231	+	1,47E+02	0,000397	0,0169	AAGAAAAGGACAAATCCAAG
LOC107812533	Trihelix	PREDICTED: trihelix transcription factor ASR3-like isoform X1	Nicotianatabacum	-1717	-1699	-	1,42E+05	0,000387	0,0343	AGATAAAAATACCCTTAA
LOC107776514	MIKC_MADS	PREDICTED: MADS-box protein SOC1-like isoform X1	Nicotianatabacum	+205	+225	-	1,51E+05	0,000386	0,00819	TTTGTCCTTTTCTTTCCTTCG
LOC107778147	ERF	PREDICTED: ethylene-responsive transcription factor ERF086	Nicotianatabacum	+17	+31	-	1,45E+05	0,000364	0,0323	TCTCCTCCGTCGTCT
LOC107827424	MYB_related	PREDICTED: telomere repeat-binding factor 4	Nicotianatabacum	+896	+910	-	1,53E+05	0,000354	0,0314	AGGGAACCCTAATTC
LOC107804592	MYB_related	PREDICTED: telomere repeat-binding factor 4-like	Nicotianatabacum	+896	+910	-	1,53E+05	0,000354	0,0314	AGGGAACCCTAATTC
LOC107777142	AP2	AIL1 PREDICTED	Nicotianatabacum	+199	+220	+	1,53E+05	0,000339	0,00957	AAGAGCGAAGGAAAGAAAAG
LOC107777199	GRAS	PREDICTED: DELLA protein GAI1-like	Nicotianatabacum	+13	+32	+	1,49E+05	0,000329	0,0169	AAGAAGACGACGGAGGAGAA
LOC107802463	Dof	PREDICTED: dof zinc finger protein DOF1.5-like	Nicotianatabacum	-845	-836	+	1,20E+05	0,000324	0,0283	GAAAAAGTGC
LOC107759135	B3	PREDICTED: B3 domain-containing protein REM16-like	Nicotianatabacum	+1678	+1695	+	7,22E+05	0,000316	0,0277	TTTTCTGTTTTGGCTGGAACT
LOC107794045	C2H2	PREDICTED: zinc finger protein MAGPIE-like	Nicotianatabacum	+211	+230	+	1,56E+05	0,000298	0,0256	AAGAAAAGGACAAATCCAAG

LOC107777142	AP2	AIL1 PREDICTED	Nicotianatabacum	+632	+651	+	1,55E+05	0,000297	0,00957	AAGAAAAAGAAGAAAAATGA
LOC107803539	C2H2	PREDICTED: zinc finger protein JACKDAW-like	Nicotianatabacum	+211	+226	-	1,55E+05	0,000289	0,0247	GATTTGTCCTTTTCTT
LOC107809390	AP2	AIL1 PREDICTED	Nicotianatabacum	+1309	+1329	-	1,41E+05	0,000266	0,0236	GCACACATCTCCCTATGTATA
LOC107800699	C2H2	PREDICTED: protein indeterminate-domain 7-like	Nicotianatabacum	+211	+228	+	1,60E+05	0,000228	0,0198	AAGAAAAGGACAAATCCA
LOC107776514	MIKC_MADS	PREDICTED: MADS-box protein SOC1-like isoform X1	Nicotianatabacum	+627	+647	-	1,58E+05	0,000204	0,00576	TTTCTTCTTTTTCTTTTTTAT
LOC107789230	C2H2	PREDICTED: zinc finger protein JACKDAW-like isoform X1	Nicotianatabacum	+211	+227	-	1,63E+05	0,000184	0,0159	CTTGGATTTGTCCTTTTCTT
LOC107830371	ERF	PREDICTED: ethylene-responsive transcription factor ERF003-like	Nicotianatabacum	+14	+32	+	1,57E+05	0,000168	0,0149	AGAAGACGACGGAGGAGAA
LOC107776514	MIKC_MADS	PREDICTED: MADS-box protein SOC1-like isoform X1	Nicotianatabacum	+199	+219	-	1,61E+05	0,000159	0,00576	CTTTTCTTTCCTTCGCTCTTC
LOC107792698	BBR-BPC	PREDICTED: protein BASIC PENTACYSTEINE2-like	Nicotianatabacum	+193	+2016	+	1,05E+05	0,000117	0,00993	GGGATGAAGAGCGAAGGAAAGAAA
LOC107823205	Dof	PREDICTED: cyclic dof factor 3-like	Nicotianatabacum	-873	-842	+	1,58E+03	0,000111	0,00953	AAGCTCGAAAAAGTGCAAA
LOC107812294	Dof	PREDICTED: dof zinc finger protein DOF1.5-like	Nicotianatabacum	+626	+646	+	1,36E+05	0,000094	0,0269	ATAAAAAAGAAAAAGAAGAAA
LOC107823205	Dof	PREDICTED: cyclic dof factor 3-like	Nicotianatabacum	+206	+224	+	1,37E+05	0,000092	0,0395	AAGGAAAGAAAAGGACAAA
LOC107776514	MIKC_MADS	PREDICTED: MADS-box protein SOC1-like isoform X1	Nicotianatabacum	+628	+648	-	1,66E+05	0,0000865	0,00576	TTTTCTTCTTTTTCTTTTTTA
LOC107812294	Dof	PREDICTED: dof zinc finger protein DOF1.5-like	Nicotianatabacum	-875	-844	+	1,58E+05	0,0000848	0,00728	AAAAGCTCGAAAAAGTGCAAA
LOC107770058	Dof	PREDICTED: dof zinc finger protein DOF3.4-like	Nicotianatabacum	+624	+645	-	1,63E+05	0,0000835	0,00348	TCTTCTTTTTCTTTTTTATAT
LOC107828216	LBD	PREDICTED: protein LATERAL ORGAN BOUNDARIES-like	Nicotianatabacum	+18	+38	-	1,18E+05	0,000083	0,0736	TTCCTCTTCTCCTCCGTCGTC
LOC107780621	LBD	PREDICTED: protein LATERAL ORGAN BOUNDARIES-like	Nicotianatabacum	+18	+38	-	1,18E+05	0,000083	0,0736	TTCCTCTTCTCCTCCGTCGTC
LOC107771704	LBD	PREDICTED: protein LATERAL ORGAN BOUNDARIES-like	Nicotianatabacum	+18	+38	-	1,18E+05	0,000083	0,0736	TTCCTCTTCTCCTCCGTCGTC
LOC107770058	Dof	PREDICTED: dof zinc finger protein DOF2.4-like	Nicotianatabacum	-664	-644	-	1,63E+05	0,0000821	0,00348	TTTTCTTTTTCTCATTATTTT
LOC107799709	bHLH	PREDICTED: transcription factor bHLH130-like isoform X1	Nicotianatabacum	+1271	+1279	-	1,51E+05	0,000067	0,0596	ACCACTTGC
LOC107765238	Dof	PREDICTED: dof zinc finger protein DOF2.4-like	Nicotianatabacum	+629	+649	-	1,56E+05	0,0000624	0,00532	ATTTTTCTTCTTTTTCTTTTT
LOC107812294	Dof	PREDICTED: ethylene-responsive transcription factor ERF003-like	Nicotianatabacum	+204	+224	+	1,41E+05	0,000061	0,0262	CGAAGGAAAGAAAAGGACAAA

LOC107777142	AP2	AIL1 PREDICTED	Nicotianatabacum	+629	+648	+	1,74E+05	0,0000484	0,0041	AAAAAGAAAAAGAAGAAAAA
LOC107796712	WOX	PREDICTED: protein WUSCHEL	Nicotianatabacum	-661	-650	-	1,53E+05	0,000042	0,0366	CCAATCATTCA
LOC107765920	HD-ZIP	PREDICTED: homeobox-leucine zipper protein ATHB-12-like	Nicotianatabacum	-661	-648	+	1,51E+05	0,000041	0,0357	TGAATGATTGGCT
LOC107813200	AP2	Nicotiana tabacum ANT-like 6 protein (AIL6)	Nicotianatabacum	-581	-566	+	1,21E+04	0,0000262	0,233	CCTTGATTTTCGGGT
LOC107825059	ERF	PREDICTED: ethylene-responsive transcription factor ERF021-like	Nicotianatabacum	+16	+36	-	1,50E+05	0,000026	0,023	CCTCTTCTCCTCCGTCGTCT
LOC107824071	Nin-like	PREDICTED: protein NLP5-like	Nicotianatabacum	-99	-84	-	1,73E+05	0,0000247	0,00219	GCCGCAGCACGGGCC
LOC107776521	Dof	PREDICTED: dof zinc finger protein DOF5.6-like	Nicotianatabacum	+626	+646	+	1,65E+05	0,0000224	0,00191	ATAAAAAAGAAAAAGAAGAAA
LOC107826816	C2H2	PREDICTED: protein indeterminate-domain 5, chloroplastic-like	Nicotianatabacum	+211	+227	+	1,65E+05	0,000016	0,0138	AAGAAAAGGACAAATCC
LOC107809033	Dof	PREDICTED: dof zinc finger protein DOF1.7-like	Nicotianatabacum	+629	+649	+	1,67E+05	0,0000109	0,00093	AAAAAGAAAAAGAAGAAAAAT

Supplementary table 2.

Num	*Primer*	Sequence 5' - 3'
1	attB1compl-SCI1prom	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCATAA
2	attB2compl-SCI1exon4ss	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTTTTGAA
3	SCI1promNtomintern o-RV	GCACCTGATATGTTTGGTAG
4	BP-1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC
5	BP-2	GGGGACCACTTTGTACAAGAAAGCTGGGTC
6	Oligo_dTV	TTTTTTTTTTTTTTTTTTTTTTV
7	T7	TAATACGACTCACTATAGGG
8	attB1-NtWUS1-Fw	GCAGGCTTCGAAGGAGATAGAACCATGGAAGCTGCTCAACAAC AAAAC
9	attB2-NtWUS1-Rv (CS)	AAGCTGGGTCTTAAGGGGAATTAGGAGATCTG
10	M13-Fw	GTAAAACGACGGCCAGT
11	M13 Rv	CAGGAAACAGCTATGAC
12	SCI1->2Kb-FW	AGTATTATGCCGCTCAGCAATC
13	SCI1-2KB-FW2	GTGTGACTCATGGTGACATCT
14	SCI1-2KB-Rv2	TCTGATAGTGTGGACGCCAT
15	promNtomSCI1-Fw	GCCATAAGCTATAAGTTGGTCACCCCCAAC
16	promNtomSCI1-Rv	GACATTTGGATTTATGGGATTTGTGCTTCTCC
17	promSCI1(-1585) mutHindIII-Fw	GCACTTCAAGCTTATCAGTTATTTAC
18	promSCI1(-538) mutHindIII-Fw	GGTACGGAAGCTTGAATAACTAATTGAG
19	promSCI1_mutNcoI-Rv	CGCTCCCCATGGACTCGCACCTCT
20	SCI1p-N.to-Frag1-Fw	GCAGGCTTCGTAGCATACGATGCGGTTTC
21	SCI1p-N.to-Frag1-Rv	AAGCTGGGTCCTAGTTATGATAGCCGCAGCAC
22	SCI1p-N.to-Frag2-Fw	GCAGGCTTCCCTTCTGGAGGAAACTAATC
23	SCI1p-N.to-Frag2-Rv	AAGCTGGGTCCCTCACTGTAAGTACGATG
24	SCI1p-N.to-Frag3-Fw	GCAGGCTTCCTACCAAACATATCAGGTGC
25	SCI1p-N.to-Frag3-Rv	AAGCTGGGTCGATTAGTTTCCTCCAGAAGG
26	SCI1-3rdIntron-N.to-Fw	GCAGGCTTCCGCGTAGTTATTATCCTTCGCAC
27	SCI1-3rdIntron-N.to-Rv	AAGCTGGGTCCTGCTACTGCTACAGTCTAAC
28	NtAG-FW	AGCAGGCTTCATGGTGTTTCCTAATGAAGAATTTG
29	NtAG-RV	AAGCTGGGTCTCAGACAAGCTGGAGAGCAG
30	NAG1site SCI1-Fw	GCTTCGTAATATCTTTTATACCATTTGGAGATAGACTAACATAAG AG
31	NAG1site SCI1-Rv	CTCTTATGTTAGTCTATCTCCAAATGGTATAAAAGATATTACGAA GC
32	NtWUSsiteSCI-Fw	GACAAATTTAGACCATTTGGATTGATAGGATCAGAACTTAAATA ATG
33	NtWUSsiteSCI-Rv	CATTATTTAAGTTCTGATCCTATCAATCCAAATGGTCTAAATTTG TC

CHAPTER II

SCI1 EXPRESSION IS ACTIVATED BY THE AINTEGUMENTA TRANSCRIPTION FACTOR AND POSITIVELY INFLUENCED BY AUXIN

This chapter will be submitted as:

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SCI1 expression is activated by the AINTEGUMENTA transcription factor and positively influenced by auxin

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ABSTRACT

Understanding the molecular mechanisms that controls floral development is crucial to obtain knowledge about reproduction in angiosperms. *SCI1* (*Stigma/style cell-cycle inhibitor 1)*, a regulator of cell proliferation, is expressed since *Nicotiana tabacum* floral meristem specification and in all meristematic cells, affecting the final size of the pistil. Here we demonstrate that *SCI1* is co-expressed with *NtAINTEGUMENTA* (*NtANT*) during flower development. *In situ* hybridization experiments showed that *SCI1* and *NtANT* are co-expressed in the pistil-specialized tissues, like the stigmatic secretory zone, stylar transmitting tissue, the placenta inside the ovary and functional regions of ovules. Additionally, *SCI1* is detected in the ovule integument and funiculus. *In silico* analyses of the *SCI1* genomic sequence identified several putative *cis*-regulatory elements, among them for binding of the transcription factor NtANT, a member of the AP2/ERF family, which is involved in floral formation. Using yeast one-hybrid (Y1H), we showed that the transcription factor NtANT1 binds to the *SCI1* promoter in a cis-acting element located in the proximal region of the translational start codon (initial ATG). This interaction was confirmed by the electrophoretic mobility shift assay (EMSA). The luciferase activity assay demonstrated that *SCI1* expression is activated by the interaction of NtANT1, and its expression is enhanced by the addition of auxin (5µM NAA or 5µM IAA). Our results establish that *SCI1* is important during pistil development, that its expression is induced by NtANT1 and is stimulated by auxin. Taken together, our data corroborate SCI1 as a cell proliferation regulator during flower development, acting in the auxin signaling pathway.

Keywords: co-expression, dual luciferase, EMSA, flower development, transcriptional activator, Y2H

1. INTRODUCTION

In vascular plants, the ability to continually form new organs is due to regions formed by pluripotent cells, the meristems (Kaufmann et al., 2010a). Meristems are divided into two main zones: the central and peripheral zones. In the peripheral zone, cell proliferation provides cells for forming new organs (Doerner, 2001). In the central zone, cell proliferation is maintained to supply cells to the peripheral zone and to the central zone itself. The maintenance of pluripotent cells is a process carried out through negative feedback between the genes of the *CLAVATA* family and the *HOMEOBOX WUSCHEL* (*WUS*) (Laux et al., 1996; Schoof et al., 2000 Lenhard et al., 2001). Plants have two main meristems responsible for building the primary plant body, the shoot apical meristem (SAM) and the root apical meristem (RAM). The SAM generates leaf whorls, branches and stems (Wang et al., 2021). Endogenous and environmental signals promote the appearance of the inflorescence meristem (IM), which derives from the SAM, and, ultimately, gives rise to the floral meristems (Irish, 2010).

The floral meristem is marked by the expression of the identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Ferrándiz et al., 2000; Kaufmann et al., 2010b). The onset of *LFY* expression occurs in response to increased auxin levels at the site where the floral meristem forms in the IM due to auxin efflux, as well as its direct biosynthesis (Yamaguchi, 2021; Yamaguchi et al., 2013). The formation of floral meristems from the inflorescence meristem is also regulated by other transcription factors. The AINTEGUMENTA (ANT) and AINTEGUMENTA-LIKE 6 (AIL6) transcription factors act redundantly to promote the development of the floral meristem and formation of the first flower (Blásquez et al., 1997; Hu et al., 2020; Krizek, 2009; Schultz & Haughn, 1991). Double *ant ail6* mutants take longer to develop the first flower, as well as *lfy* mutants (Blásquez et al., 1997; Krizek, 2009; Yamaguchi et al., 2016). The expression of *LFY, AIL6* and *ANT* are necessary for the formation of floral primordia (Krizek, 2009). The floral meristem gives rise to the four whorls, sepals, petals, stamens, and pistil specified by the genes of the ABCDE model of floral development (Bowman et al., 1989; Irish, 2017). *ANT* is characterized as a key regulator of floral organ growth and mutations in this gene result in flowers with smaller organs, while its ectopic expression leads to the development of larger flowers later in development (Krizek, 1999; Mizukami & Fischer, 2000; Wang et al., 2022). Additionally, *ANT* and *AIL6* act together to promote the identity of petals, stamens, and carpels

(Elliott et al., 1996; Klucher et al., 1996; Krizek et al., 2000, Kuluev et al.,2015) regulating the number of organs, their positioning, identity and growth. *ANT* and *AIL6* directly activates the *APETALA 3* (*AP3*) and the *AGAMOUS* (*AG*) genes at the stage of floral development in which the sepals are specified (Krizek et al., 2000, 2021). *AP3* and *AG* are, respectively, the class B and class C genes from the ABCDE model of flower development (Irish, 2017). AG plays a central role in reproductive organs (stamens and carpels) development, as well as floral meristem (FM) determinacy (Das et al., 2009; Wynn et al., 2011). The switch from meristematic cell to organ cell (determinacy) is directed by AG through the regulation of the auxin biosynthetic gene YUCCA4 (YUC4) expression (Pelayo et al., 2021).

In the promotion of floral development, the *ANT* and *ANT-LIKE* genes also have a strict relationship with the auxin synthesis and transport pathways that act in the formation of floral meristems and floral development (Krizek et al., 2020). *ANT* is downregulated in *pin1* mutants during meristem development (Vernoux et al., 2000). *PIN1* encodes a serine-threonine kinase that controls auxin transport (Okadalat et al., 1991). During pistil development, *ANT* acts redundantly with *REVOLUTA (REV)* and *Auxin Response Factor 5* (*ARF5*) to promote the development of the meristematic region that will give rise to the ovary, placenta, and ovule (Cucinotta et al., 2021; Nole-Wilson et al., 2010).

Stigma/style cell-cycle inhibitor 1 (*SCI1)* was previously characterized as a gene preferentially expressed in the stigma and style that controls cell proliferation in the upper pistil of *Nicotiana tabacum* and *Arabidopsis thaliana* (DePaoli et al., 2011; DePaoli et al., 2014). *SCI1* RNAi silenced *N. tabacum* plants and *sci1 A. thaliana* mutant plants showed elongated styles and larger stigmatic area due to increased cell number (DePaoli et al., 2011; DePaoli et al., 2014). Furthermore, in *SCI1* RNAi plants pistil differentiation occurred earlier than that observed in wild-type plants, according to (DePaoli et al., 2011) this is consistent with the *SCI1* pathway accelerating differentiation through control of cell proliferation. Recently, we demonstrated that *SCI1* starts its expression in the floral meristem and continues to be expressed in meristematic cells present in developing floral whorls (Cruz et al., 2021). Therefore, the effects observed in mutant and silenced *SCI1* plants may be a cumulative result of *SCI1* action since floral meristem specification (Cruz et al., 2021). A relationship between *SCI1* and auxin pathways was also observed, but still poorly understood. (DePaoli et al., 2012) reported that the expression of the genes *NtAux/IAA19, NtAux/IAA13* and *NtARF8* have their levels affected by the expression of *SCI1*. In *Arabidopsis thaliana*, the phenotype of the *yuc2yuc6* mutant plants

was recovered through overexpression of *SCI1* (DePaoli et al., 2014). *In silico* analyzes of the genomic sequence of *SCI1* identified putative cis-regulatory elements for interaction of ANT, AIL 6 and several Auxin-Responsive Elements (ARFs) (Cruz et al., 2021). Therefore, we decided to investigate the relationship of ANT and auxin in the transcriptional regulation of *SCI1* gene expression. Here we demonstrate that *SCI1* and *NtANT1* (*N. tabacum* AINTEGUMENTA) are co-expressed in the same flower tissues and in several different developmental stages. We show by Y1H and EMSA that NtANT1 binds to specific sequences in *SCI1* promoter region. Dual luciferase activity essay revealed that NtANT1 activates *SCI1* expression, which is increased in the presence of auxin. Our results add important information concerning *SCI1* transcriptional regulation during flower development.

2. MATERIALS AND METHODS

2.1 *Plant material*

N. tabacum cv Petit Havana SR-1 and and *N. benthamiana* seeds were germinated in Carolina Soil® substrate and cultivated in a fitotron Weiss GallenKamp, at 22°C, 55% humidity, and light/dark cycle (12/12 h). *N. benthamiana* plants were kept in the fitotron and used for transient expression experiments when 8-12 weeks old. At this age, *N. tabacum* plants were transferred to the greenhouse, irrigated by automatic sprinkler every 12 hours, and grown under natural conditions in Ribeirão Preto – SP, Brazil (Latitude - 21º 10'24" S, Longitude - 47º 48'24" W).

2.2 *Identification and phylogenetic analysis of AIL genes in N. tabacum*

The amino acid sequences of the AILs from *A. thaliana* were obtained from The Arabidopsis Information Resource (TAIR) database http:// www.Arabidopsis.org/. To identify the AIL proteins in *N. tabacum*, 8 protein sequences from the AIL gene family were used to BLASTP Search against the sequence database of the *N. tabacum* in SolGenomics https://solgenomics.net/. The same process was used to find the AIL sequences in *Solanum lycopersicum*. To investigate the phylogenetic relationships of AIL protein sequences in the species *A. thaliana*, *N. tabacum,* and *S. lycopersicum*, were used to construct the phylogenetic tree. The phylogenetic trees were generated with the Maximum Likelihood method and the JTT matrix-based model, with 1000 Bootstrap Replications using MEGA11 (Kumar et al., 2008).

2.3 *In situ hybridization*

For *in situ* hybridization, flowers of *N. tabacum* were collected, and the stage of development was identified by size. The flowers were harvested and fixed in paraformaldehyde and dimethyl sulfoxide. The tissues were dehydrated by graduated ethanol series, replaced with xylene, and infiltrated in Paraplast plus (Sigma). Sections (10µm thick) to be directly compared were processed together. The *NtANT* and *SCI1* CDS were amplified (primers are described in

supplementary Table 3) and cloned into the TOPO® TA Cloning® Dual Promoter Kit vector. After cloning, the vectors were linearized with NotI enzyme, and T7 RNA polymerase was used for probe synthesis. Probe synthesis was performed using *SP6/T7 Transcription Kit from Roche Life Science*, following the manufacturer's specifications. *In situ* hybridizations were performed as described by Jackson (1991). The slides were photographed in a stereomicroscope Leica M80 and camera Leica DFC295.

2.4 *Production of recombinant NtANT1 protein*

To produce the recombinant NtANT1 protein, the CDS was amplified (primers are described in supplementary Table 3) and cloned into the pDEST17 expression vector (*Gateway ®)* to generate pDEST17-NtANT1. This construction was transformed into *Escherichia coli* BL21 (DE3)pLysE cells to produce the 6xHis-tag fused protein. The production of His-NtANT1 was obtained by self-induction by adding 0.2% lactose and 0.05% glucose to the medium. The culture was maintained under agitation at 220rpm at 37°C until an OD600 of 1.5 was obtained. The cultured cells were pelleted by centrifugation at 5000g for 15 minutes, and the cell pellet was resuspended in 40mM phosphate buffer pH 7.6. Cells were lysed by sonication with 6 pulses of 30 seconds and intervals of 1 minute between each pulse. The cell suspension was centrifuged at 10000g for 10 minutes, and the supernatant was collected. The His-NtANT1 protein was concentrated and purified by exclusion using *Pierce ™ protein concentrator EPS, 30K MWCO 2-6ml, 10pk,* and subsequently used for EMSA.

2.5 *Electrophoretic mobility shift assays (EMSA)*

Four primers of 44 nucleotides each (see supplementary Table 3), complementary two by two, and comprising the 2 putative NtANT cis-elements identified in *SCI1* promoter sequence were synthesized. The sequences were synthesized in sense and antisense orientations and subsequently annealed in a mix containing [TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 8.0); 10 picomoles forward sequence and 10 picomoles reverse sequence]. The mix was heated at 95°C for 10 minutes and then cooled on the bench to room temperature. Paired sequences were labeled with DIG-ddUTP using Terminal transferase Roche® and then used for

EMSA. Binding, electrophoresis, and detection assays were performed according to DIG Gel Shift Kit, 2nd Generation.

2.6 *Yeast One-Hybrid Assay (Y1H)*

To perform the Y1H assay, the *SCI1* promoter was amplified from genomic DNA in 3 different parts called Fragment 1 (-515 to -73bp), Fragment 2 (-1197 to -706bp), and Fragment 3 (- 1667 to -1178bp to), containing ~500 base pairs each. Each fragment was then cloned into the pABAI-GW vector. These constructs were used as baits in the Y1H test. The NtANT CDS was cloned into the pDEST22 vector (ProQuest™ Two-Hybrid System with Gateway™ Technology) and was used as prey in the assay. Both bait and prey were transformed into PJ69-4a yeast cells and cultured in SD/-URA-TRP with or without 150 ng/ml Aureobasidin A (AbA) for 3–5 days at 30°C.

2.7 *Dual Luciferase assay*

The *SCI1* promoter was amplified and cloned in two different sizes. The largest sequence contains 1585 base pairs, and the smallest sequence contains 538 base pairs of the proximal promoter. The sequences were cloned separately into the pGreenII 0800-LUC vector to create reporter constructs. The CDS of *NtANT* was cloned into the pK7WG2 Gateway ® vector for overexpression of the proteins and was used as assay effectors. Reporter and effector constructs were separately transformed into *Agrobacterium tumefaciens* GV3101, and transformed cells were confirmed by PCR. Inoculums were prepared with the GV3101 strain transformed with the reporter and effector constructs and maintained under growth for 24h. After 24h, the inoculum was pelleted, and the pellets were washed with MES buffer (10 mM MES and 10 mM MgCl2). The OD600 of each culture was checked, and then the infiltration solutions were prepared. For the preparation of the solutions, the OD600 was adjusted to 0.2, as described by Sawers et al. (2006). Cells from the pCB301-p19 *Agrobacterium* culture (Win & Kamoun, 2003) were added to the infiltration solutions at the OD600 described for the other two constructs. Acetoseringone was added to the infiltration solution at a concentration of 100 µg/ml. To the interactions tested with auxin, 5µM of NAA and 5µM of IAA were added for each phytohormone test (Kuluev et al., 2015). Leaves of one-month-old *N. benthamiana* plants

were infiltrated. After three days, the infiltrated leaves were collected, and for each sample, a mass of 5 milligrams of tissue was adjusted for analysis. The samples were macerated and resuspended in 300 microliters of Passive Lysis Buffer 1X (Promega, Madison, WI, United States) 5 microliters of the supernatant were collected, and Firefly (LUC) and Renilla (REN) were detected with dual luciferase assay reagents (Promega, Madison, WI, United States) using a 96-well Costar® plate. The ratio of LUC/REN and the data submitted to one-way ANOVA using GraphPad Prism 8.0.1.

3. RESULT

3.1 *Identification and phylogenetic analysis of the N. tabacum AIL family of transcription factors*

As the putative cis-acting elements for binding of the AINTEGUMENTA (ANT), AINTEGUMENTA-LIKE 2/BABY BOOM (AIL2/BBM) and AINTEGUMENTA-LIKE 6 (AIL6) transcription factors were previously found in *SCI1* promoter sequence (Cruz et al., 2021; Supplementary Figure 1), we decided to pursue a detailed analysis of the AINTEGUMENTA-LIKE (AIL) gene family in the *N. tabacum* genome. We performed a BlastP using the *A. thaliana* AIL protein sequences to search for the *N. tabacum* corresponding sequences. We also searched for the *Solanum lycopersicum* sequences, to obtain a comparison parameter with a diploid Solanaceae genome. A phylogenetic tree was constructed by the Maximum Likelihood method and the JTT matrix-based model using the full-length protein sequences of *A. thaliana* (8 sequences), *N. tabacum* (18 sequences), and *S. lycopersicum* (8 sequences) in MEGA11 with 1000 bootstrap replicates (Figure 1).

In the *N. tabacum* genome, as in the other two species, AIL proteins were classified into six clades: ANT, AIL1, AIL2, AIL3/4, AIL5, and AIL6/7 (Figure 1), with AIL3/4 and AIL6/7 being evolutionarily closely related and located in the same phylogenetic branch. A single ANT sequence exists in the Arabidopsis genome, while in Solanaceae there are two ANT genomic sequences, which were named ANT1 and ANT2. As *N. tabacum* is an allotetraploid, for each ANT sequence in *S. lycopersicum*, we found two sequences in tobacco (Supplementary Table 1), one corresponding to *N. sylvestris* and another to the *N. tomentosiformis* ancestral genomes (Figure 1; Supplementary Figure 2). Interestingly, the ANT clade also has additional members in other species like in *Populus trichocarpa* – 4 genes (Rigal et al., 2012), *Brassica napus* - 5 genes (Shen et al., 2020), and *Medicago truncatula* - 4 genes (Wang et al., 2022). In contrast, the seven Arabidopsis AIL genes each has only one corresponding gene in *N. tabacum*, while in clade AIL3/4 there is a single gene in the *S. lycopersicum* genome (Figure 1). Similarly, to what has been described in *M. trunculata* (Wang et al., 2022), the phylogenetic analysis also showed that NtANTs is more closely related to NtAIL1s, while NtAIL2 is clustered with NtAIL3/4, and NtAIL5 is closer to NtAIL6/7 (Figure 1).

To decide which ANT sequence to clone for further studies, we analyzed the expression of the two *N. tabacum ANT* genes, based on RNASeq data available in public databases (Sierro et al., 2014). The *NtANT1* gene shows higher expression levels than *NtANT2* in all the organs examined (Figure 2), especially in young flowers and stigmas/styles, the organs in which the *SCI1* gene is expressed. Therefore, we PCR-cloned the coding sequence (CDS) of *NtANT1* (LOC107809390), sequenced it and confirmed its identity, including the presence of the two AP2 domains (Supplementary Figure 2) characteristic of the AIL gene family (Kim et al., 2006).

3.2 *Co-expression of NtANT and SCI1 in young flowers*

To investigate the possible transcriptional regulation of *SCI1* by NtANT1, we studied the co-expression of these genes in young *N. tabacum* flowers using *in situ* hybridization. At stages -2 and -1, the two carpel primordia are fused, originating the ovary, and the style is elongating, while the stylar transmitting tissue and the stigma are forming (Koltunow et al., 1990). At stage -2, ovules are initiated in the placenta's medial portion; at stage -1, the ovule primordia become evident on the placenta's distal and proximal edges (Chang & Sun, 2020). In our *in situ* hybridization results, at stages -2 and -1 (Figures 3A-3B and 3C-3D, respectively), *SCI1* and *NtANT* are expressed in the ovule and placenta of the pistil and the sporogenous tissue of the anthers (Figure 3). Here, it should be mentioned that the antisense *NtANT1* probe used may also hybridize with *NtANT2* transcripts (Supplementary Figure 3). However, as *NtANT2* is just weakly expressed, we assume that most of the *in situ* hybridization signals correspond to *NtANT1* transcripts. In any case, we describe the *in situ* results as being from *NtANT*.

At stage -2, the carpel primordia are expanding, and *SCI1* and *NtANT* are detected in the placenta of the ovaries (Figures 3A-3B). At stage -1, the style is elongating, and the stigma appears (Figures 3C-3D). At this stage, the expression of *SCI1* and *NtANT* is detected in the carpels, now more elongated, and in the stigma that is forming (Figures 3C-3D). We observe that the expression of both genes is concentrated in the lateral portions of the placenta. In this region, ovule initiation occurs (Galbiati et al., 2013), but there is no expression of *SCI1* and *NtANT* in the ovary walls. Above the ovary, *SCI1* and *NtANT* are expressed in the style where cells are dividing. *SCI1* begins to be detected in the central region of the style - the stylar transmitting tissue (stt), and in the upper portion where the stigma is developing, as already

described by DePaoli et al. (2011). Co-expression of *NtANT* and *SCI1* is observed in these same tissues (Figures 3C-3D).

Clearly, there is a co-expression of *SCI1* and *NtANT* in the pistil during the early stages of development. It is already known that in late developmental stages, ANT can be detected in the ovules of the *A. thaliana* flower (Elliott et al., 1996). However, there is a lack of information on *SCI1* expression in more advanced stages of ovary development and during ovule formation. *In situ* hybridizations were performed to obtain information about the expression pattern of *SCI1* and *NtANT* at stages 1 to 5 of the *N. tabacum* flower. In Figure 4, we can observe the expression of *SCI1* and *NtANT* at these stages of flower development (for negative controls, see Supplementary Figure 4). At stage 1, the anthers are positioned at the same height as the pistil. The stigma has a round shape, but it is still not possible to observe the umbrella-like shape, typical of the *N. tabacum* stigma. At this stage (Figures 4A-4B), the expression of *SCI1* and *NtANT* continues in the same tissues as in stage -1 (Figure 3). However, here, there is no detection in the stylar transmitting tissue (stt), probably as a result of the section plane. These results corroborate what has already been described by Cruz et al. (2021) concerning *SCI1* expression that remains in cells with meristematic characteristics.

At stage 2, *SCI1* and *NtANT* expression are clear in the ovule primordia (Figures 4C-4D). At stage 3, both genes are expressed at ovule primordia, and a decrease in signal detection in the placenta tissue (Figures 4E-4F). These genes are also co-expressed in the funiculus and integument of ovules that are already at a more advanced stage of development. As the *N. tabacum* ovary does not have a uniform differentiation pattern, there are ovules at different stages of development along the ovary, with older ovules localized in the medial portion of the placenta (Chang & Sun, 2020). At stages 4 and 5 (Figures 4G-4H and 4I-4J, respectively), all observed ovules already present funiculus and integument and, therefore, are no longer primordia. At these stages, *SCI1* and *NtANT* continue to be co-expressed. It is important to note that the expression of *SCI1* and *NtANT* is concentrated in the ovules and no longer in the placenta, and there is no expression in the ovary walls. Taken together, *NtANT* and *SCI1* are co-expressed in the organs and developmental stages analyzed, supporting the proposal that NtANT is an important transcription factor regulating *SCI1* expression.

3.3 *SCI1 is a direct target of the AINTEGUMENTA transcription factor*

Based on the presence of 2 putative cis-acting elements for the NtANT transcription factor in *SCI1* 5' regulatory region (here denominated promoter) and its co-expression with *SCI1* in several floral tissues and developmental stages, we decided to investigate the binding of NtANT1 to *SCI1* DNA sequence. For this purpose, we performed the yeast one-hybrid (Y1H) experiment, using the Aureobasidin fungicide as selectable marker (for details see Material and Methods). The assay was performed with three independent fragments of the *SCI1* promoter region, produced in a previous work (Cruz et al., 2021), each containing approximately 500bp (fragment 1, from -515 to -73bp; fragment 2, from -1197 to -706bp; and fragment 3, from -1667 to -1178bp, considering the A base of the translation initiation codon as +1). The Y1H revealed that NtANT1 is capable of binding to fragment 1 (Figure 5), with is closer to the ATG codon, and contains two putative overlapping NtANT sites in positions -332 to -310 and -323 to -303bp (Figure 5 and Supplementary Table 2). NtANT1 did not bind to fragment 2 and fragment 3 (data not shown). It is important to mentioned that fragment 3 encloses a single putative site for NtANT (on position -1634 to -1614), which was not able to promote the binding of NtANT1 in Y1H. The consensus sequence for ANT binding was determined by Nole-Wilson et al. (2000) by *in vitro* assays. According to the authors, ANT binds with high affinity to the consensus sequence 5'-CAC[AG]N[AT]TNCCNANG-3' and also to similar sequences with different affinities. Considering only the p-value of the PlantRegMap analysis, the three putative ANT cis-elements are similar. Therefore, the lack of binding in Y1H to fragment 3 could have occurred due to different factors: 1) the ANT cis-element was positioned close to the end of the promoter sequence used at the assay, and other nearby sequences may be important to allow NtANT1 interaction with the DNA or to increase its affinity; 2) interaction with this single ANT binding site, rather than 2 overlapping sites (as present in fragment 1), may have a lower affinity for the transcription factor, especially in a heterologous system like yeast; 3) The binding of NtANT1 on the site present in fragment 3 may depend of its interaction with another transcription factor not present in yeast.

We also performed EMSA to investigate the *in vitro* binding of NtANT1 to both ANT cis-elements previously identify at the *SCI1* promoter sequence. The DNA sequences were synthesized and labeled with digoxigenin to produce probes 1 and 2 (Figures 6A-C). The His-NtANT1 tagged protein was produced in *E. coli* and purified by size exclusion chromatography. After incubation of the His-NtANT1 recombinant protein with each probe and electrophoresis,

we observed the mobility shift for both probes. The binding of His-NtANT1 generated two high bands (arrows in Figure 6C), consistent with the formation of ANT homodimers, as already described by Heflin (2019) in Arabidopsis flowers and also for other transcription factors of the AP2/ERF family (Nole-Wilson et al., 2000; Nole-Wilson et al., 2005; Horstman et al., 2015). These high bands decreased in intensity with the increasing concentration of specific unlabeled competitors, confirming the binding of His-NtANT1 to the two DNA sequences identified as possible cis-elements for ANT.

3.4 *NtANT1 is able to activate SCI1 expression*

Dual luciferase activity assay (Hellens et al., 2005) was conducted through transient expression in *N. benthamiana* leaves to understand the effect of NtANT1 on *SCI1* regulation. Two fragments of the *SCI1* promoter were cloned independently into the pGreenII 0800-LUC vector controlling firefly luciferase (LUC) expression (Figure 7A) (for more details see Materials and Methods). Each promoter gene construction was co-infiltrated with the vector expressing the NtANT1 transcription factor into *N. benthamiana* leaves. After 3 days, leaf extracts were produced, and luciferase activity analyzed. As shown in Figure 7B, NtANT1 is able to activate the *SCI1* promoter, especially with the smaller fragment (-538 to -1 bp). It is noteworthy that this is a DNA fragment that contains the two cis-elements for ANT binding, similar to the promoter fragment which produced a positive result in the Y1H experiment. However, the longer promoter fragment (-1585 to -1 bp) did not produce a significant activation of luciferase activity. It can be speculated that the longer fragment encompasses *cis*-elements for binding of repressors of *SCI1* expression in leaves. Regardless, it is evident that NtANT1 activates *SCI1* expression.

3.5 SCI1 activation by NtANT1 is positively influenced by auxin

It is known that ANT acts to induce gene expression in response to auxin (Yamaguchi et al., 2016; Krizek et al., 2020). Additionally, *SCI1* promoter sequence contains several putative cis-elements responsive to auxin (Supplementary Figure 1). Therefore, we decided to investigate if auxin could have an effect on NtANT1 activation of *SCI1* expression. To test this hypothesis, we performed the luciferase activity assay in the presence of naphthalene acetic acid (NAA), a

synthetic auxin, and indole acetic acid (IAA), the most common naturally occurring auxin. The experiment was executed only in the presence of the longer promoter fragment (-1585 to -1 bp) which comprises five putative ARF-like cis-acting elements. The results showed that NAA had a remarkable influence on the NtANT1 activation of luciferase activity under the control of the *SCI1* promoter (Figure 8A). IAA also had a positive effect on the activation produced by NtANT1 (Figure 8B), but to a lesser extent than the effect of NAA. Taken together, our results demonstrate the positive effect of auxin on the activation that NtANT1 promotes in *SCI1* expression.

4. DISCUSSION

DePaoli et al. (2011; 2014) demonstrated, through tobacco *SCI1* RNAi transgenic plants and sci1 Arabidopsis mutants, that *SCI1* plays a role in controlling cell proliferation in the upper pistil. Recently, we have shown through *in situ* hybridization and $SCI1_{prom}$::SCI1-GFP transgenic plants that *SCI1* is expressed since floral meristem specification and in the meristematic cells of all floral organ primordia (Cruz et al., 2021). Here we show, using *in situ* hybridization, that *SCI1* is expressed in the ovaries and their specialized tissues, placenta, and ovules (Figures 3 and 4). In the ovaries, the last population of meristematic cells will give rise to the ovules (Skinner et al., 2004). The expression of *SCI1* in the ovules suggests that it has an important role in their proper development. Although it has been reported that *sci1* mutant plants fail to develop siliques (DePaoli et al., 2014), the ovaries of mutant *A. thaliana* plants and *N. tabacum* overexpression and silencing transgenic plants were not analyzed, and the failure to develop siliques was attributed to the heterostyly of these plants. Another characteristic described in *A. thaliana sci1* mutant plants was the decrease in fertility with a smaller number of siliques (DePaoli et al., 2014). In *N. tabacum*, SCI1-RNAi plants also have decreased fertility with fewer seeds (DePaoli et al., 2011), a phenotype that was not investigated in detail. Detection of *SCI1* expression in ovules can now shed light on the phenotypes described above and assign a yet important role for *SCI1* in flower development and plant reproduction.

4.1 *Regulation of SCI1 by NtANT1*

We established that *SCI1* and *NtANT* are co-expressed in the placenta and ovules of *N. tabacum* (Figures 3 and 4). Previous analyzes identified *in silico* putative *cis*-regulatory elements for the interaction of NtANT with the *SCI1* promoter sequence (Supplementary Material Figure 1 and Table 2). We demonstrated that NtANT1 binds to the proximal ANT cis-element by Y1H, EMSA, and luciferase activity (Figures 5-7). We also showed that the binding of NtANT1 to the *SCI1* promoter has a clear activating effect inducing *SCI1* expression quantified by luciferase activity (Figure 7B). These results point to NtANT1 as a direct regulator of *SCI1* gene transcription and that *NtANT1* and *SCI1* participate, at least, in some common flower developmental pathway.

ANT is part of the large family of AP2 domain transcription factors (Krizek et al., 2021). According to data obtained from analyzes of *A. thaliana* mutants, ANT plays an important role in floral development ranging from the initiation of floral organ primordia to the development of the gynoecium (Mizukami et al., 2000), including ovule morphogenesis (Elliott et al., 1996). Krizek (2009) demonstrated through mutants that ANT promotes the growth and proper development of floral whorls. Mutant plants of *ant* present smaller organs and abnormalities in the pistil and ovules (Elliot et al., 1996). In arabidopsis and tobacco, ectopic expression of ANT results in larger flowers when they complete their development (Krizek,1999, Kuluev et al., 2015). Therefore, alterations in *ANT* expression levels clearly affect floral organ growth. Similarly, alterations in *SCI1* expression levels also affect growth, but to a lesser extent, corroborating the proposal that they participate in the same growth-controlling pathway. SCI1-RNAi plants exhibit larger stigmas with elongated styles, while *SCI1* overexpression plants present smaller stigmas (DePaoli et al., 2011). *sci1* arabidopsis mutants also displayed larger stigmas and elongated styles (DePaoli et al., 2014). Our interpretation of these phenotypes is that the last floral meristematic cells, which are located in the pistil, give rise to style, stigma, and ovules. After these structures are formed, no meristematic cells will be present, and floral organ growth will occur by cell expansion/elongation. Thus, a lower level of *SCI1* in meristematic cells results in more cell divisions, culminating in elongated styles and larger stigmas. It is possible to speculate that changes must also occur in the ovules, but this aspect has not yet been investigated.

In arabidopsis, ovule identity is primarily a result of the class D genes (SEEDSTICK, SHATTERPROOF1 and SHATTERPROOF2) of the ABCDE model (Angenent & Colombo, 1996). ANT is a transcription factor that acts downstream of the class D gens and is necessary for the correct integument formation and nucellus development. In *N. tabacum*, Rieu et al. (2005) described the expression of *NtANTL* in the ovary. NtANTL corresponds to the NtANT1 (of the present work) from the *N. sylvestris* ancestral genome of *N. tabacum*. Here, we demonstrate that *NtANT* is detected in specialized tissues of style (Figures 3B and 3D), as well as in the specialized tissues of the ovary, placenta, and ovules (Figures 3 and 4). In the ovules, *NtANT* was detected in the funiculus and integuments (Figures 4D, 4F, and 4H), and this expression occurs since the beginning of ovule development (Figures 3B and 3D; 4B). We also demonstrate that *SCI1* co-expresses with NtANT in the same regions during ovule

development. In Figure 4, at stages 2 and 3, the expression of *SCI1* and *NtANT* can be observed in ovules since the beginning of its formation, which occurs at stage 1.

SCI1 is a novel direct target of NtANT1 (Figures 5 and 6), not previously identified in plants, and this transcriptional regulation results in the activation of *SCI1* expression (Figure 7B). ANT, a positive regulator of cell proliferation, activates *SCI1* during pistil development in its specialized tissues. Recently, we showed that *SCI1* co-expresses with and is regulated by NAG1 (AGAMOUS of *N. tabacum*) (Cruz et al, 2021). AG is responsible for the determination of the last meristematic cells in the flower (Das et al., 2009; Wynn et al., 2011). It was proposed that *SCI1* is a regulator of cell proliferation in the floral meristematic cell, and that the phenotypes observed in *SCI1* mutants and transgenic plants result from the action of *SCI1* in those cells (Cruz et al, 2021). In *A. thaliana*, AG is regulated by ANT (Krizek, 2009; Krizek et al., 2021). *SCI1*, as a direct target of *Nt*ANT and NAG1, two important transcription factors for pistil and ovule development, reinforces our proposal of *SCI1* as a regulator of the proliferation of meristematic cells in the floral whorls in formation.

4.2 *SCI1 and the auxin signaling pathway*

Our data point to the involvement of auxin in the regulatory pathway of *NtANT1* and *SCI1*.The results show increased levels of luciferase activity under the control of *SCI1* promoter when NtANT1 binds to it in the presence of auxin. The relationship between *SCI1* and auxin was proposed by DePaoli et al. (2012; 2014). DePaoli et al. (2012) reported that overexpression of *SCI1* resulted in a significant induction of the auxin-responsive genes NtIAA19, NtIAA13, and NtARF8 in the style of *N. tabacum* flowers. The NtIAA13 gene was induced in both overexpression and silencing plants, but the inducing effect was clearly greater in overexpression plants. The induction in silencing plants can be explained by the presence of *SCI1*, which did not have its expression reduced to zero in silencing plants. Corroborating this statement, the silenced plants with a higher level of *SCI1* expression also have higher levels of NtIAA13 induction. This fact suggested a possible dose-dependent action of *SCI1* on NtIAA13. DePaoli et al. (2014) also described that *AtSCI1* overexpression was sufficient to revert the phenotype in *yuc2yuc6* and *npy1* arabidopsis mutants. This finding shows that *SCI1* acts downstream to these genes. However, like all regulations involving phytohormones (Lee et al.,

2019), the *SCI1* and auxin relationship is a process that seems to involve several pathways and is, therefore, still difficult to understand.

The function of ARFs (Auxin Response Factors) is to regulate the expression of genes responsive to auxin, of which one is *ANT* (Krizek, 2009). On the other hand, ANT transcriptionally regulates *PINFORMED1* (*PIN1*) and *PINOID* (*PID*) (Krizek et al.,2020). PIN1 and PID act in the transmembrane transport of auxin and are necessary for an auxin gradient, essential for the formation of gynoecium and ovules (Kuhn et al., 2019). If *SCI1* participates in the auxin signaling pathway, it would be expected to have auxin responsive elements in its genomic sequence. *In silico* analyses revealed putative *cis*-regulatory elements for the interaction of several ARFs in *SCI1* genomic sequence (Supplementary material Table 2, Cruz et al., 2021). Among these ARFs, we can mention ARF5-like and ARF8-like. ARF8 acts redundantly with ARF6 to control flower maturation (Nagpal et al., 2005; Ulmasov et al., 1999). In arabidopsis, ARF5/MP (MONOPTEROS) acts as a transcriptional activator and auxin receptor (Qadir et al., 2021). ARF5 is described to act as an essential integrator of the auxin signaling pathway by activating or transcriptionally controlling genes that act in the transport pathway of this phytohormone (Zhao et al., 2010). Interestingly, RNAseq data from *pid1* and *mp* arabidopsis mutants indicates that *SCI1* is somehow transcriptionally misregulated in these mutants despite not showing statistical significance (Larrieu et al., 2022). *pid1* and *mp* mutants fail to start organs in the reproductive phase (Zhao et al., 2010), and this is due to reduced polar auxin transport (Konishi et al., 2015; Okadalat et al., 1991).

Our data demonstrate upregulation of luciferase activity driven by the *SCI1* promoter in the presence of auxin (Figure 8), which probably occurs due to the *cis*-elements for ARFs present in its sequence. Remarkably, it is known that ARF5/MP upregulates the expression of LEAFY (LFY), ANT and AINTEGUMENTA-LIKE 6 (AIL6), to specify flower primordia and promote their outgrowth (Yamaguchi et al., 2013; Krizek et al., 2020). The fact that *SCI1* genomic sequence has *cis*-regulatory elements for ARF5, LFY, ANT and AIL6 (Supplementary Material Table 2) strengthens our proposal of SCI1 as an important auxin signaling player and a regulator of flower development, for which the action of ANT was demonstrated here.

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FIGURE LEGENDS

Figure 1. Phylogenetic tree analysis of AILs transcription factors from *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Solanum lycopersicum*. Maximum Likelihood method and JTT matrix-based model were used to construct a phylogenetic tree using full-length protein sequences of *A. thaliana* (8), *N. tabacum* (18), and *S. lycopersicum* (8) in MEGA11 with 1000 bootstrap replicates. *Sequence used by Rieu et al. (2005) and Kuluev et al. (2015). **Sequence used in this work.

Figure 2. Pattern expression of *ANT*s genes in different tissue in *N.tabacum*. * Sequence used by Rieu et al. (2005) and Kuluev et al. (2015). ** sequence used in this work.

Figure 3. *In situ* hybridization with *SCI1* and *NtANT* antisense probe. Different stages of *N. tabacum* flower development. (A- B) stage -2; (C-D) stage -1. (A and C) *SCI1* antisense probe. (B and D) *NtANT* antisense probe. petals-p, sepals-s, anther-A, ovary-ov, stylar transmitting tissue- stt, stigma -stg. Scale bar- 1000µm or 1mm.

Figure 4. *In situ* hybridization with *SCI1* and *NtANT* antisense probe. Different developmental stages of the *N. tabacum* ovary. (A- B) stage 1 (C-D) stage 2 (D-F) stage 3 (G-H) stage 4 (I-J) stage 5. (A, C, E, G, I) *SCI1* antisense probe. (B, D, F, H, J) *NtANT* antisense probe. ovary wall - ow, funiculus - f, integument - I, ovule primordium - op, placenta - pl. Scale bar - 1000µm or 1mm.

Figure 5. (A) Y1H assay. Schematic representation of the reporters and effectors constructs used in the Y1H assay. (B) Y1H assay, showing the interaction of NtANT to fragment 1 (-515bp to -73bp) of *SCI1* promoter.

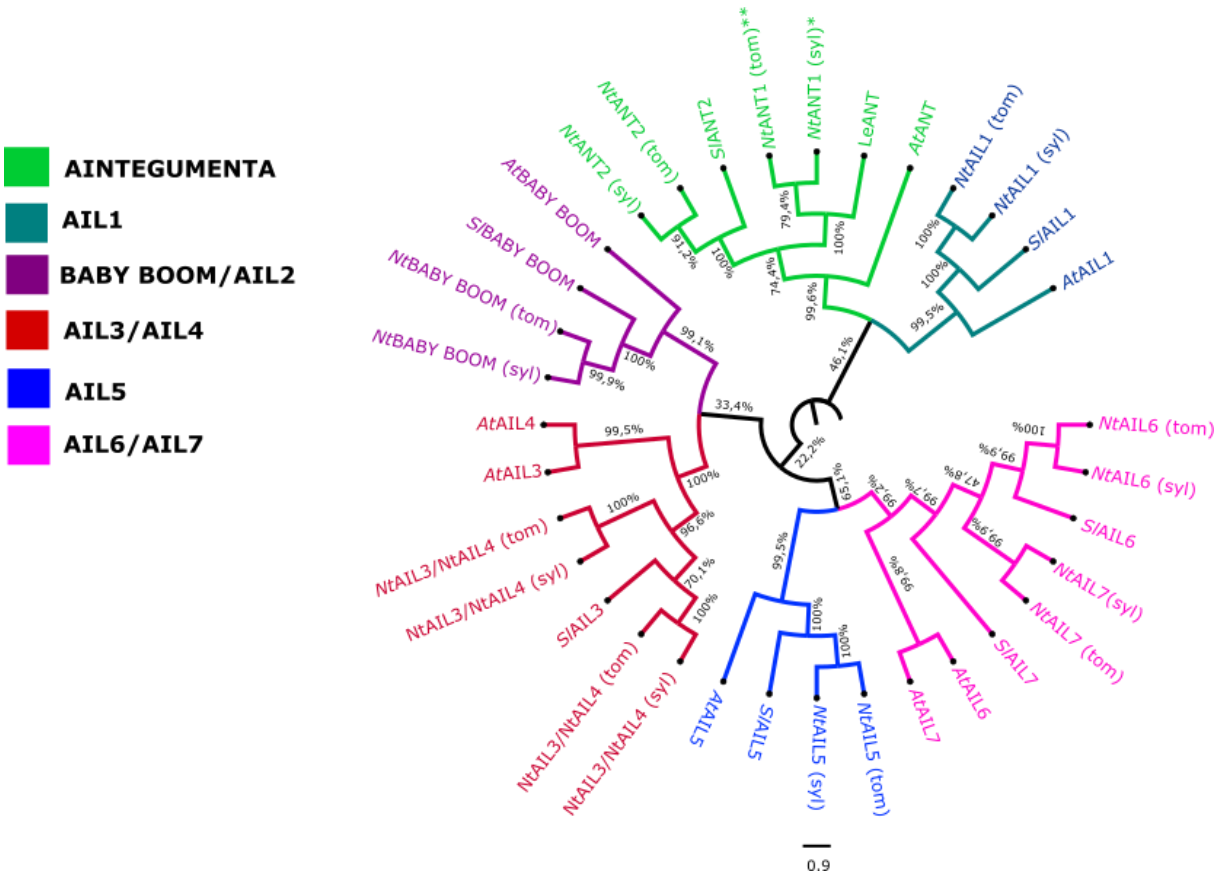
Figure 6. (A) Sequences at positions -331 to -302 in which NtANT interacts. (B) Sequences at positions -1613 to -1634 in which NtANT interacts. (C) Electrophoretic mobility shift assay (EMSA) of NtANT with the *SCI1* sequence at positions -331 to -302 and -1634 to -1613. Were used 77,5 fmols of probe. Well 1-5: sequence of position -331 to -302. Well 6-10: sequence of position -1634 to -1613. Competitive assay in wells 3 and 8: 20x of specific competitor; well 4 and 9, 40x specific competitor; well 5 and 10, 50x specific competitor. Arrows indicate

complexes between proteins and target molecules. Arrows indicate complexes between proteins and target molecules.

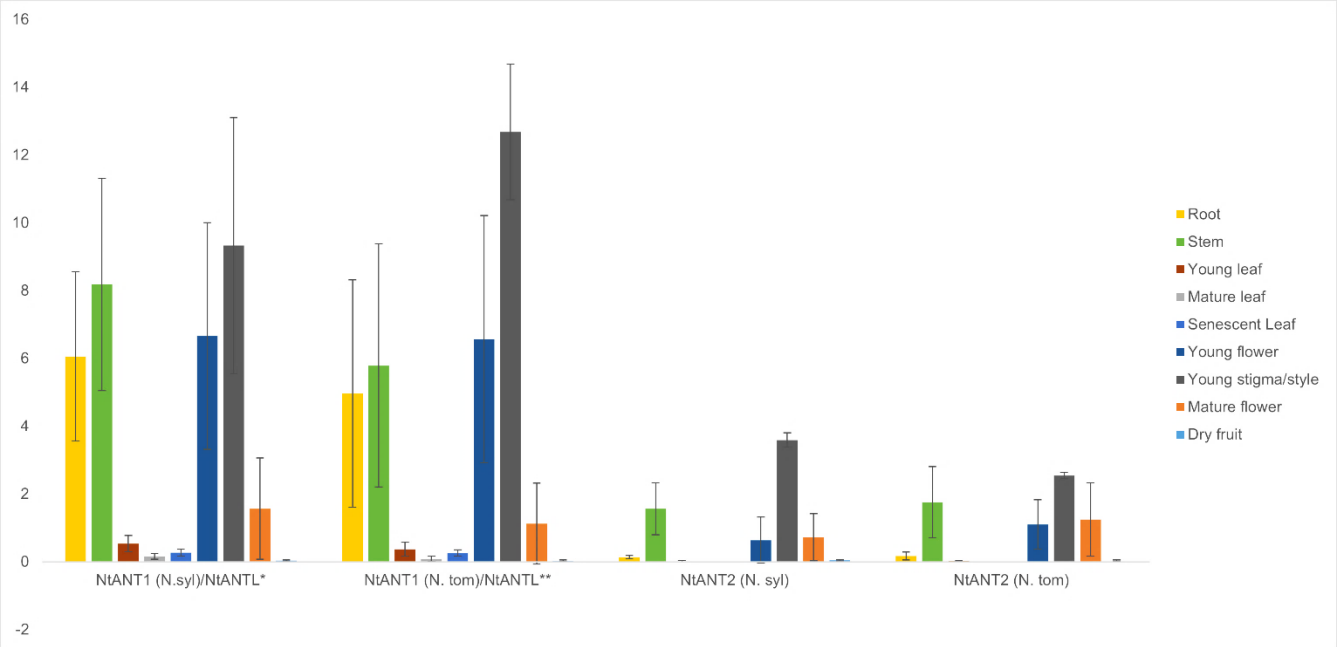
Figure 7. Luciferase activity assay performed by transient expression driven by the *SCI1* promoter fragments. (A)- Schematic representation of the reporter and effector constructions used in the luciferase activity assay. (B)- Transcriptional activation by *SCI1* promoter in *N. benthamiana* leaves through the interaction of NtANT with its sequence. Relative activity is the ratio LUC/REN. The expression of *REN* was used as an internal control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different from the control (empty vector and empty vector + transcription factor).

Figure 8. Luciferase activity assay performed by transient expression driven by the *SCI1* promoter fragments. (A) Transcriptional activation by *SCI1* promoter in *N. benthamiana* leaves through the interaction of NtANT with its sequence with its sequence with NAA. (B) Transcriptional activation by *SCI1* promoter in *N. benthamiana* leaves through the interaction of NtANT with its sequence with its sequence with IAA. Relative activity is the ratio LUC/REN. The expression of *REN* was used as an internal control. * $p < 0.05$, ** $p < 0.01$ significantly different from the control (empty vector and empty vector + transcription factor).

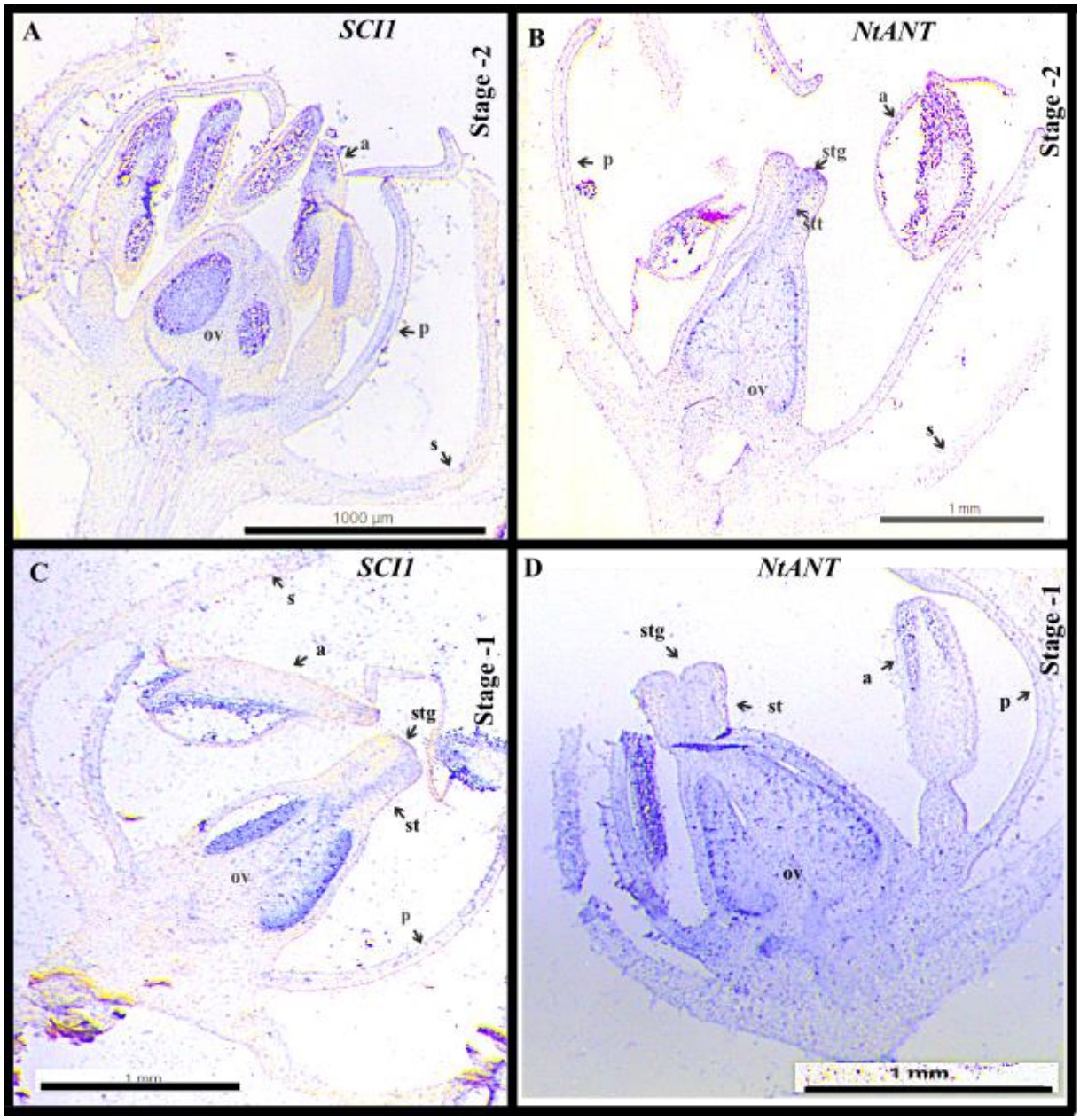
FIGURES


AINTEGUMENTA
AIL1
BABY BOOM/AIL2
AIL3/AIL4
AIL5
AIL6/AIL7
NtANT2 (tom)
NtANT2 (syl)
SlANT2
NtANT1 (tom)**
NtANT1 (syl)*
LeANT
AtANT
NtAIL1 (tom)
NtAIL1 (syl)
SlAIL1
AtAIL1
AtBABY BOOM
SlBABY BOOM
NtBABY BOOM (tom)
NtBABY BOOM (syl)
AtAIL4
AtAIL3
NtAIL3/NtAIL4 (tom)
NtAIL3/NtAIL4 (syl)
SlAIL3
NtAIL3/NtAIL4 (tom)
NtAIL3/NtAIL4 (syl)
AtAIL5
SlAIL5
NtAIL5 (syl)
NtAIL5 (tom)
AtAIL7
AtAIL6
SlAIL7
NtAIL7 (tom)
NtAIL7(syl)
SlAIL6
NtAIL6 (syl)
NtAIL6 (tom)
0.9

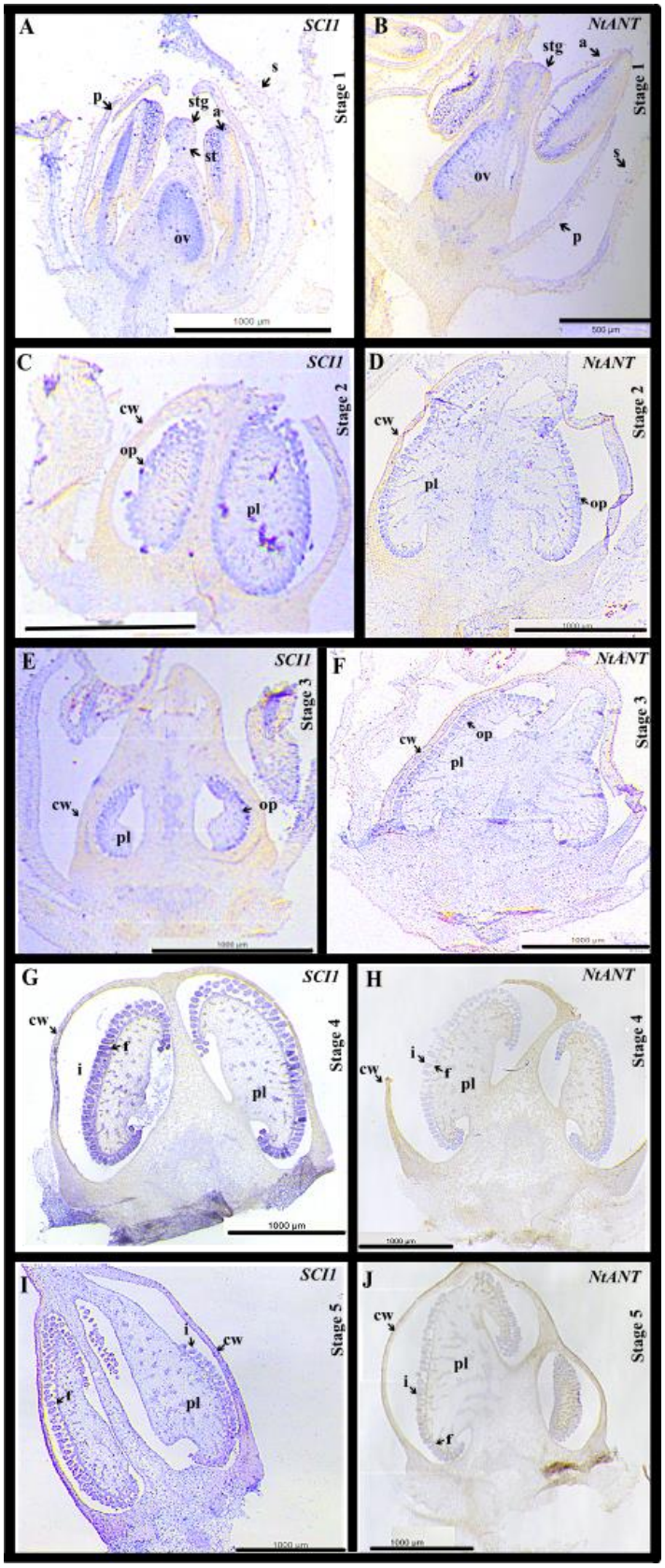
1.


16
14
12
10
8
6
4
2
0
-2
NtANT1 (N.syl)/NtANTL*
NtANT1 (N. tom)/NtANTL**
NtANT2 (N. syl)
NtANT2 (N. tom)
Root
Stem
Young leaf
Mature leaf
Senescent Leaf
Young flower
Young stigma/style
Mature flower
Dry fruit

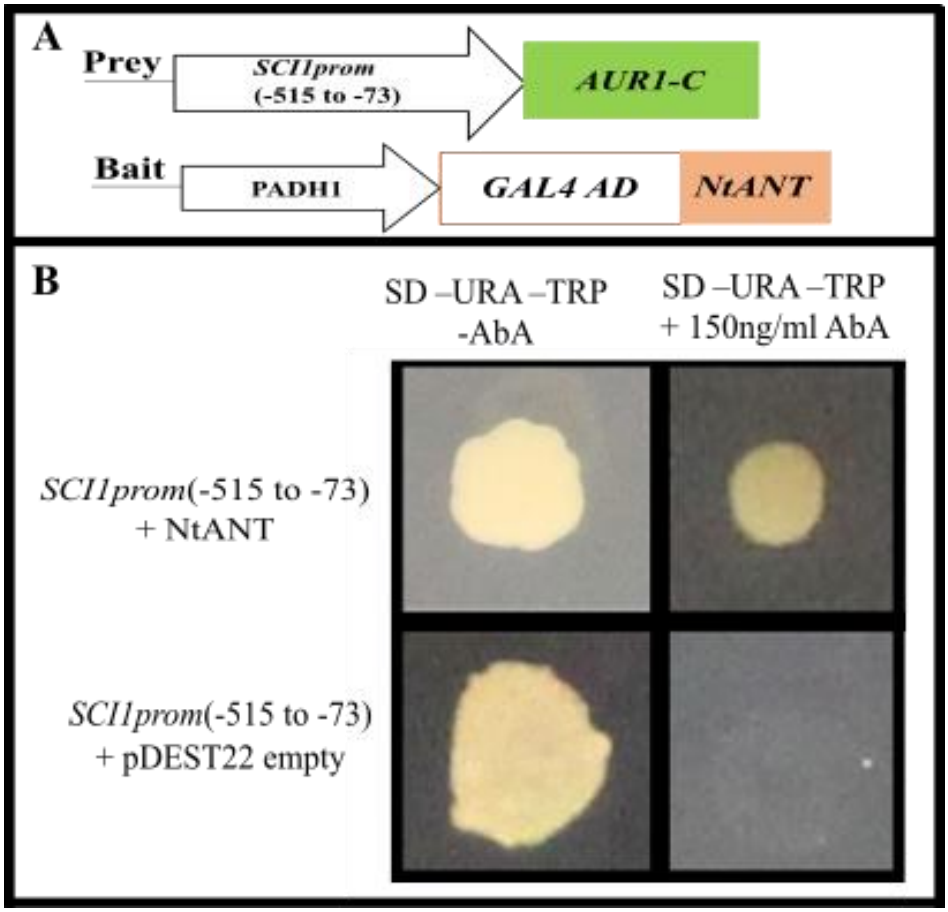
2.


A
SCI1
Stage -2
a
p
ov
s
1000 µm
B
NtANT
Stage -2
a
p
stg
stt
ov
s
1 mm
C
SCI1
Stage -1
s
a
stg
st
ov
p
1 mm
D
NtANT
Stage -1
stg
a
st
p
ov
1 mm

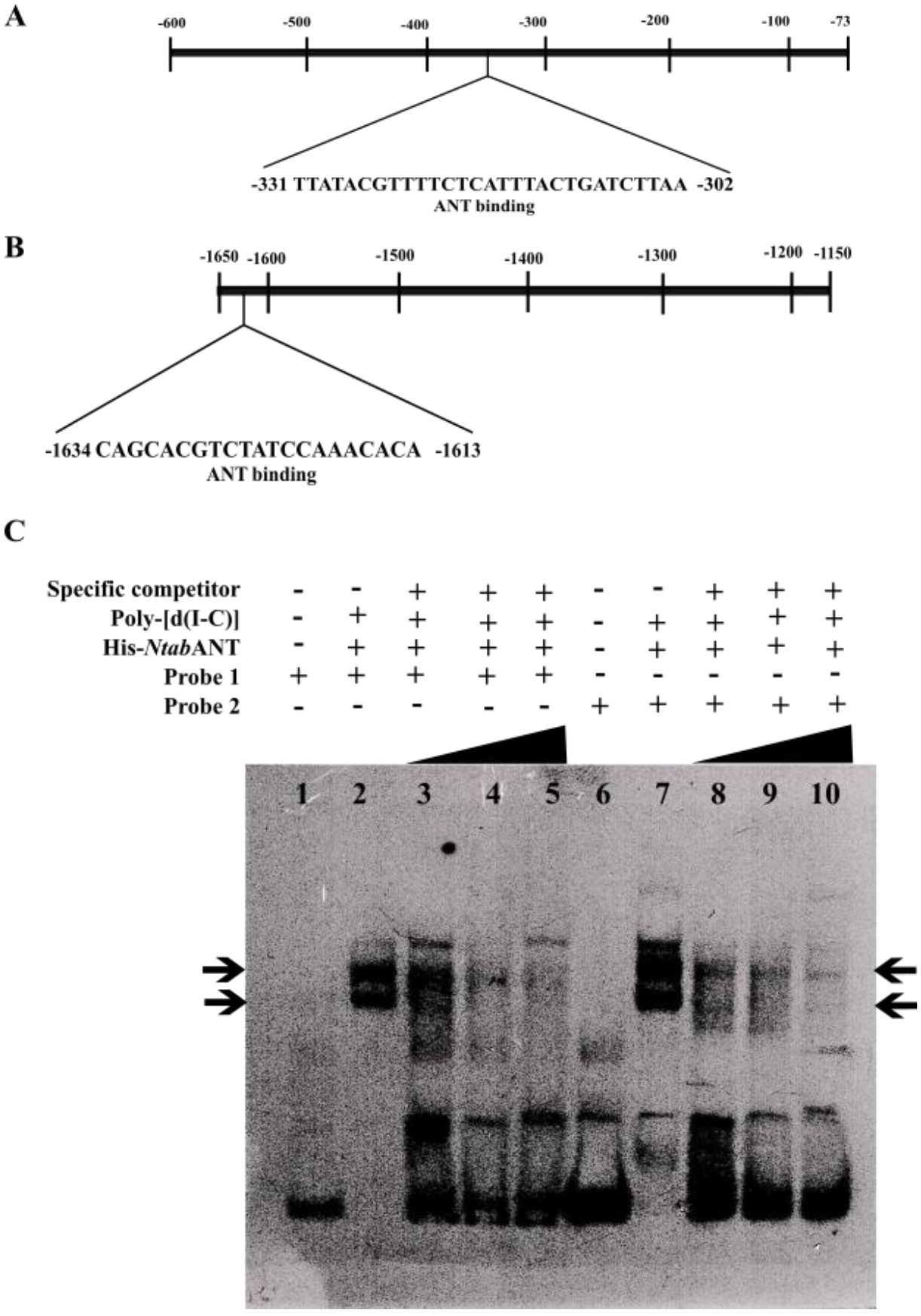
3.


A
SCI1
Stage 1
s
p
stg
a
st
ov
1000 µm
B
NtANT
Stage 1
stg
a
ov
s
p
500 µm
C
SCI1
Stage 2
cw
op
pl
D
NtANT
Stage 2
cw
pl
op
E
SCI1
Stage 3
cw
op
pl
F
NtANT
Stage 3
cw
op
pl
G
SCI1
Stage 4
cw
f
i
pl
1000 µm
H
NtANT
Stage 4
i
cw
f
pl
I
SCI1
Stage 5
i
cw
f
pl
J
NtANT
Stage 5
cw
pl
i
f

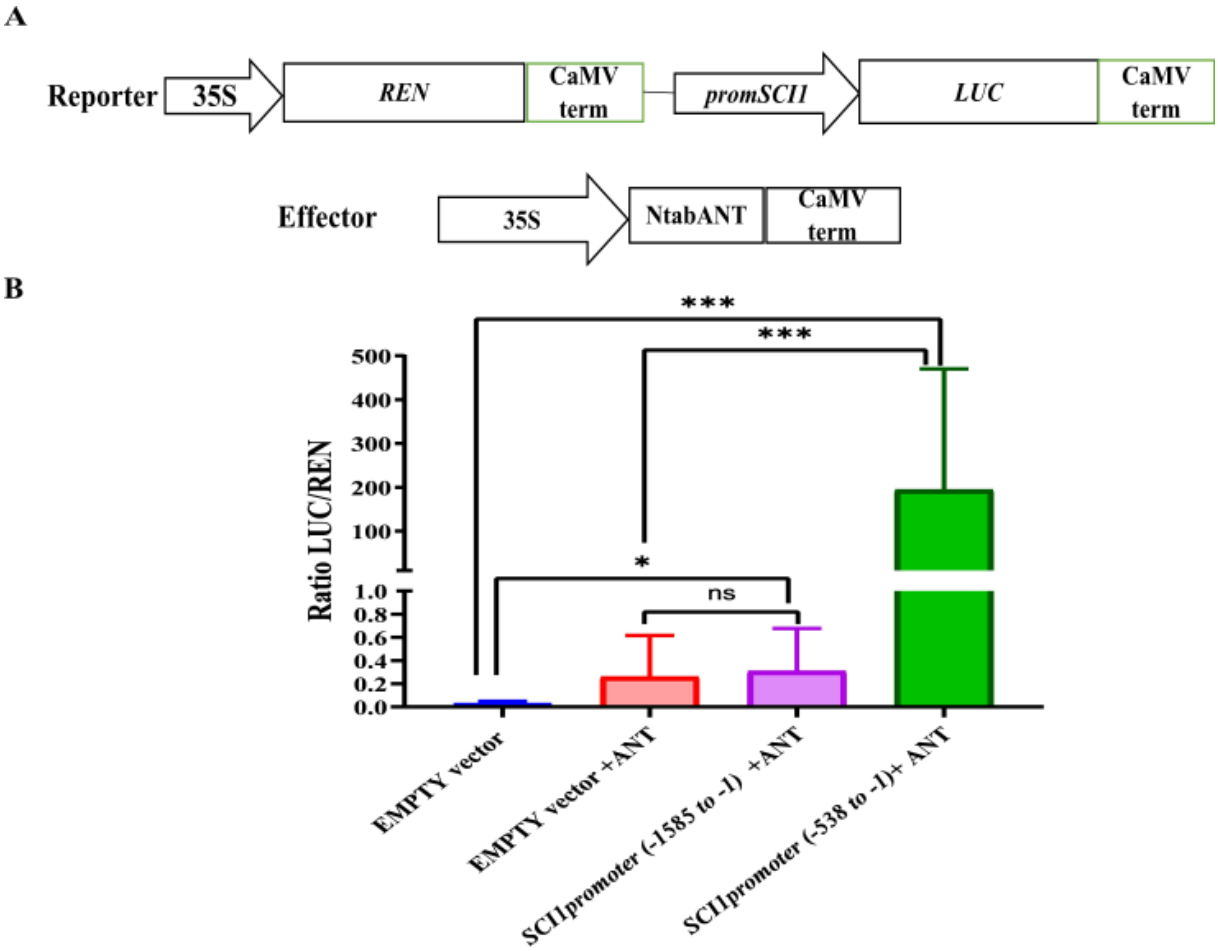
4.


A
Prey
SCI1prom
(-515 to -73)
AUR1-C
Bait
PADH1
GAL4 AD
NtANT
B
SD –URA –TRP
-AbA
SD –URA –TRP
+ 150ng/ml AbA
SCI1prom(-515 to -73)
+ NtANT
SCI1prom(-515 to -73)
+ pDEST22 empty

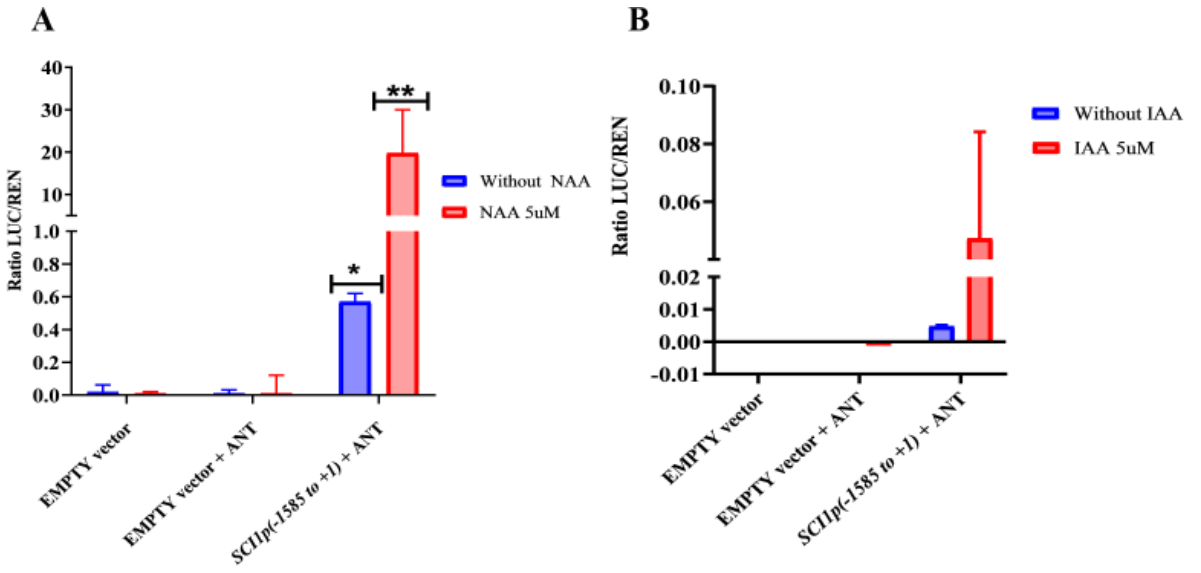
5.


A
-600
-500
-400
-300
-200
-100
-73
-331 TTATACGTTTTCTCATTTACTGATCTTAA -302
ANT binding
B
-1650
-1600
-1500
-1400
-1300
-1200
-1150
-1634 CAGCACGTCTATCCAAACACA -1613
ANT binding
C
Specific competitor
Poly-[d(I-C)]
His-NtabANT
Probe 1
Probe 2
1 2 3 4 5 6 7 8 9 10

6.


A
Reporter
35S
REN
CaMV term
promSCI1
LUC
CaMV term
Effector
35S
NtabANT
CaMV term
B
Ratio LUC/REN
500
400
300
200
100
1.0
0.8
0.6
0.4
0.2
0.0
***
***
*
ns
EMPTY vector
EMPTY vector +ANT
SCI1promoter (-1585 to -1) +ANT
SCI1promoter (-538 to -1)+ ANT

7.


A
Ratio LUC/REN
40
30
20
10
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Without NAA
NAA 5uM
EMPTY vector
EMPTY vector + ANT
SCI1p(-1585 to +1) + ANT
B
Ratio LUC/REN
0.10
0.08
0.06
0.02
0.01
0.00
-0.01
Without IAA
IAA 5uM
EMPTY vector
EMPTY vector + ANT
SCI1p(-1585 to +1) + ANT

8.

SUPPLEMENTARY FIGURE LEGENDS

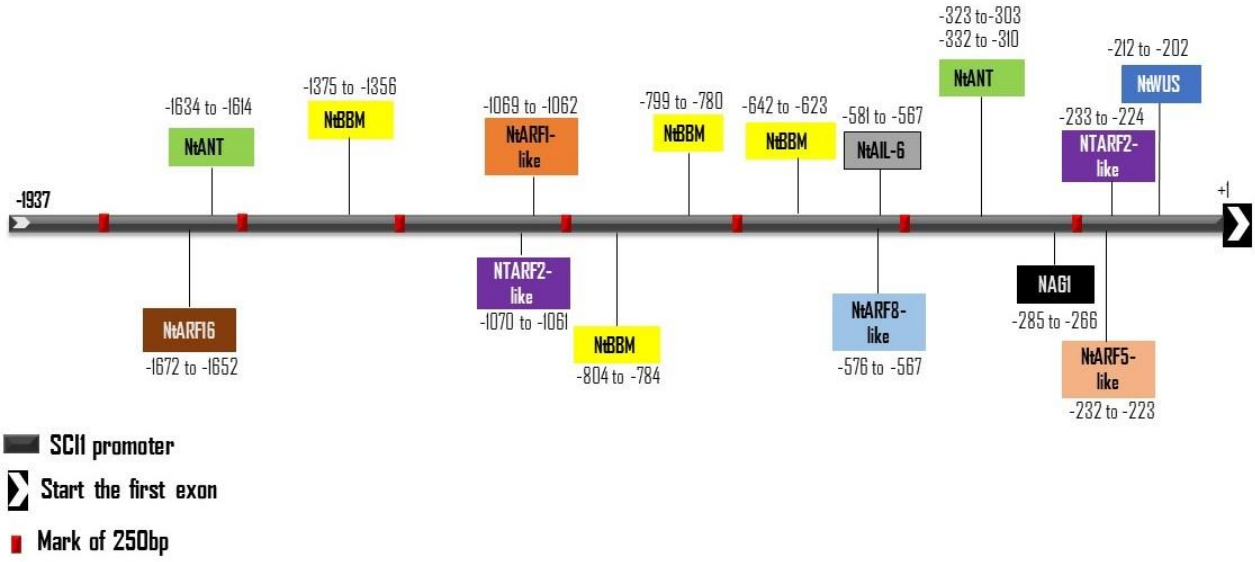
Supplementary Figure 1. Schematic representation of *SCI1* promoter and positions of cis-elements identified.

Supplementary figure 2. Sequence alignment of *ANT*s proteins in *A.thaliana* and *N.tabacum*. Red: similar aminoacids. Blue: identical aminoacids. AP2 domains are underling by the red line

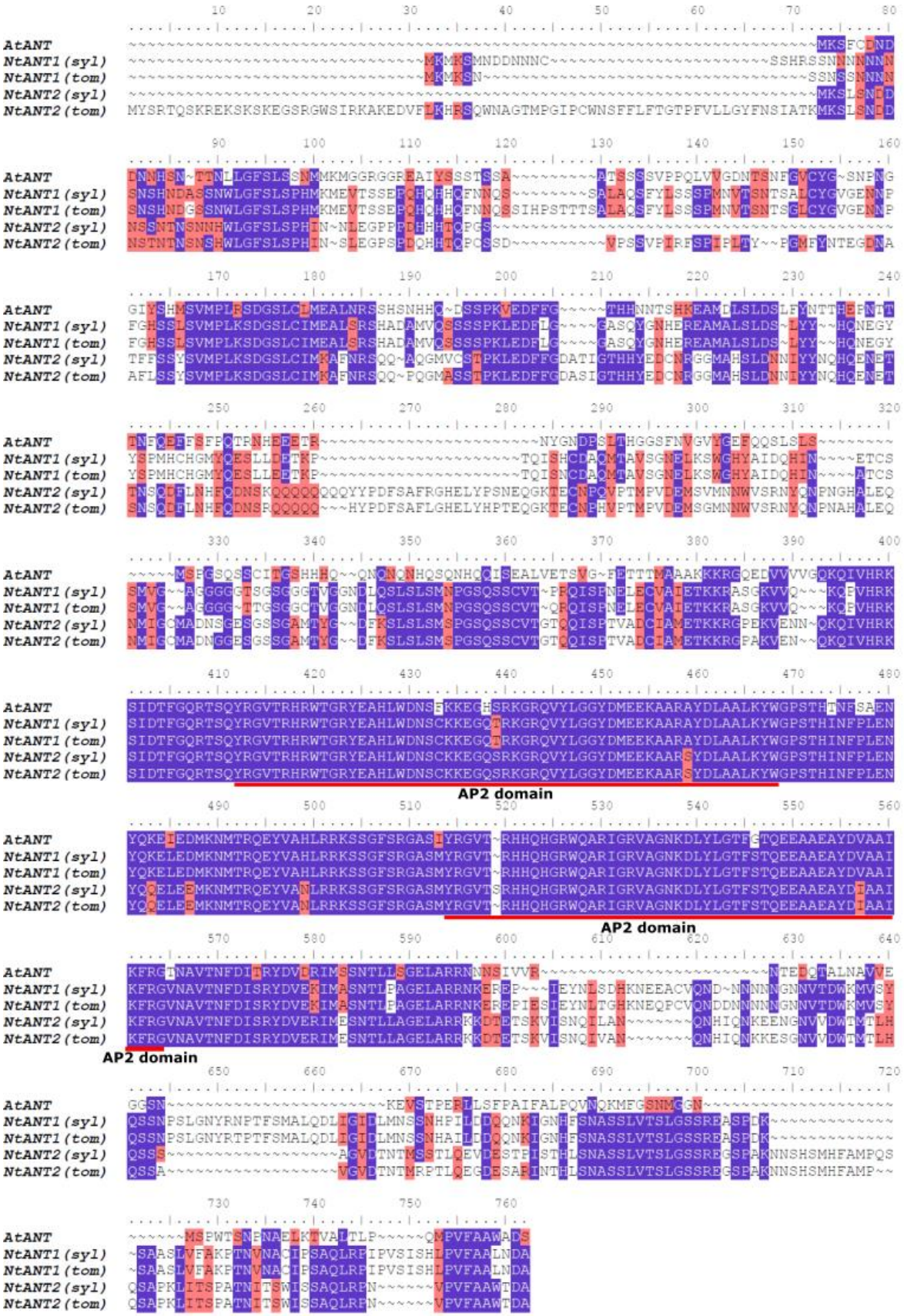
Supplementary figure 3. Alignment of mRNA *ANT* genes of the specie *N. tabacum*. Blue color represents identical nucleotides.

Supplementary figure 4. Controls of in situ hybridization. Different stages of the ovary of the *N. tabacum* flower development hybridized with sense probe. (A) stage -7; (B) stage -5; (c) stage-4; (D) stage 3; (E) stage 3; (F) stage 3. (C, E, and F) sense probe *NtANT*. (A, B, and D) sense probe *SCI1*. petals-p, sepals-s, anther-A, ovary-ov, style- st, stigma -stg, ovule primordium- op, placenta- pl.

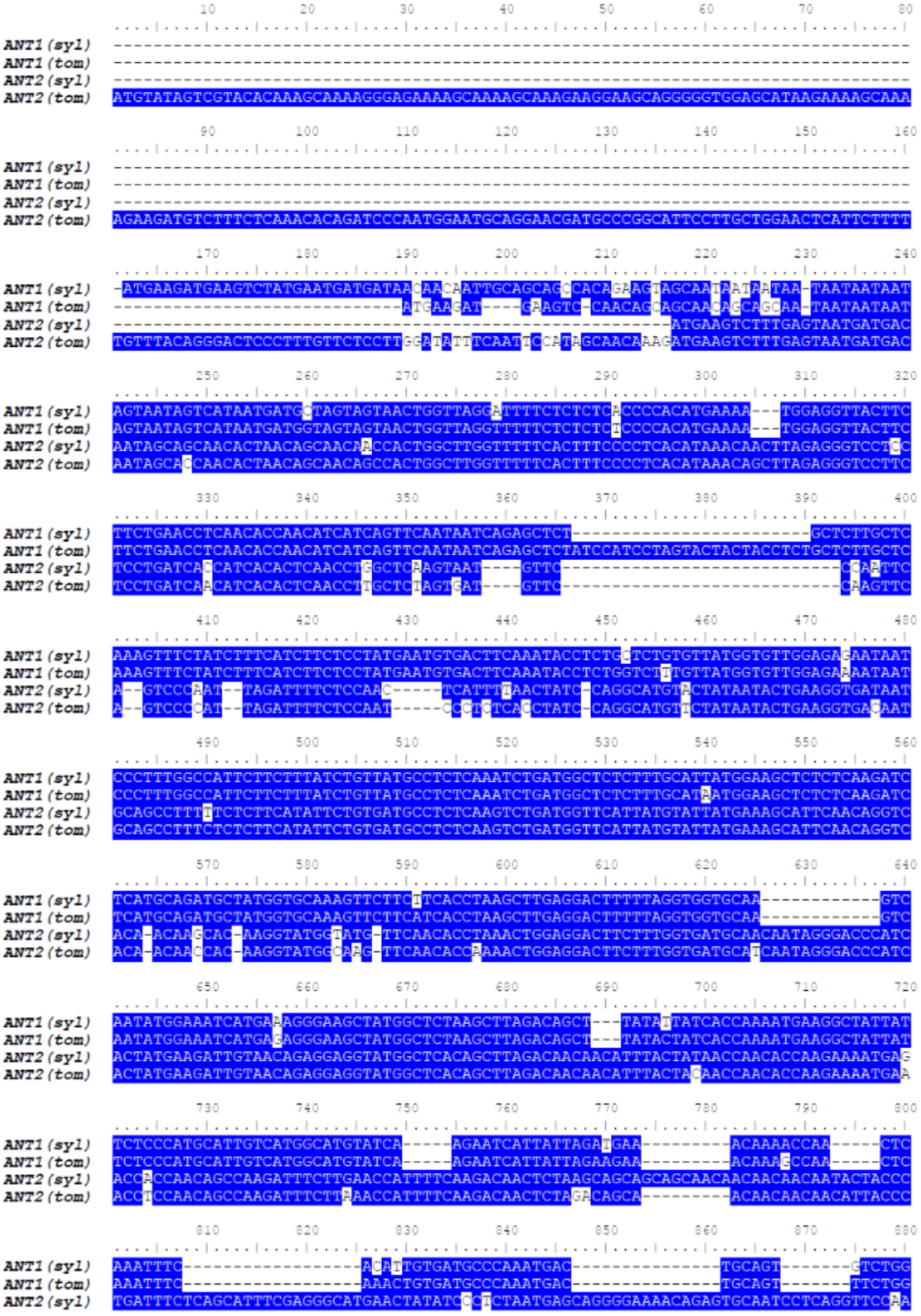
SUPPLEMENTARY FIGURES



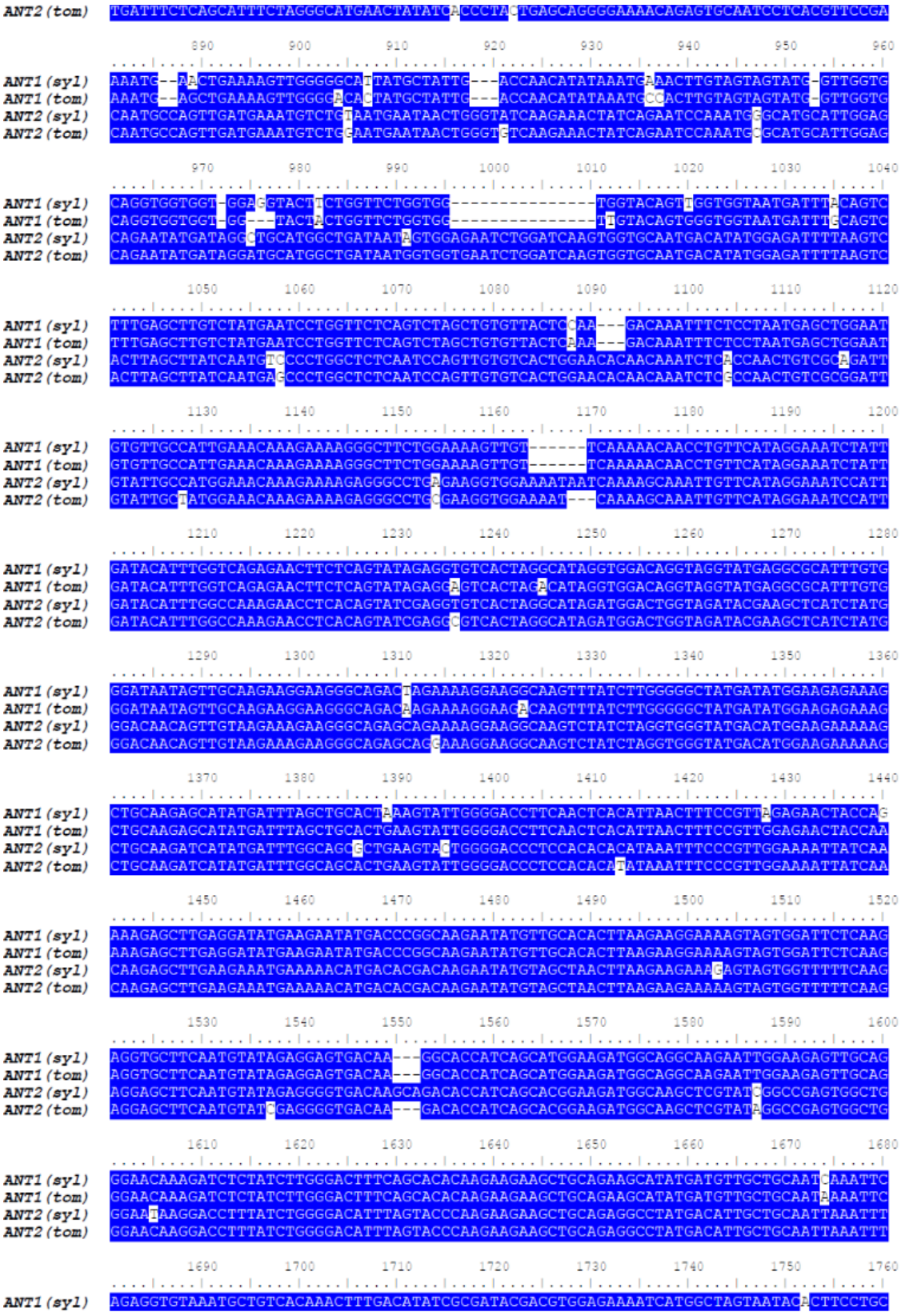
1.


AtANT
NtANT1(syl)
NtANT1(tom)
NtANT2(syl)
NtANT2(tom)
AP2 domain
AP2 domain
AP2 domain

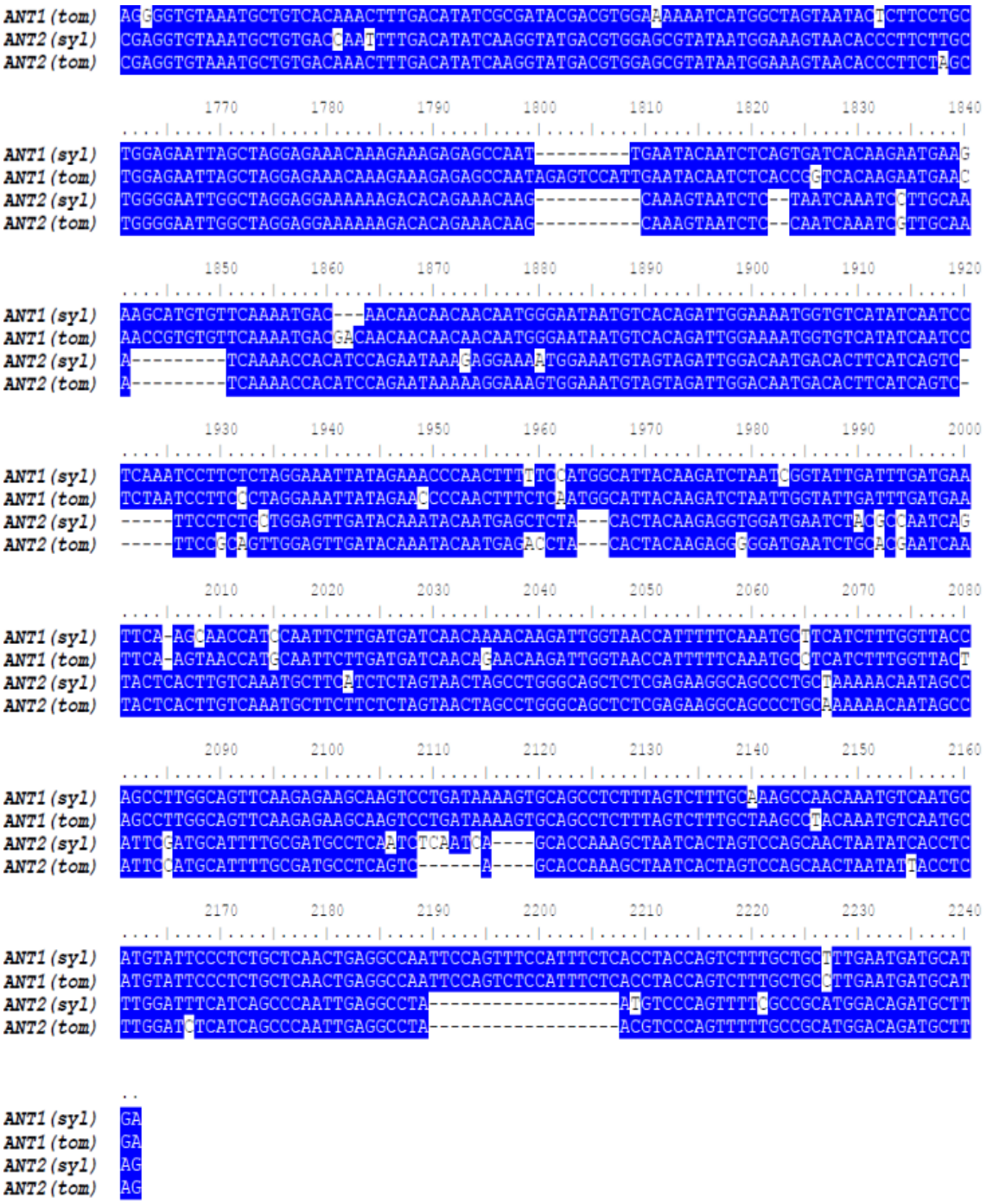
2.



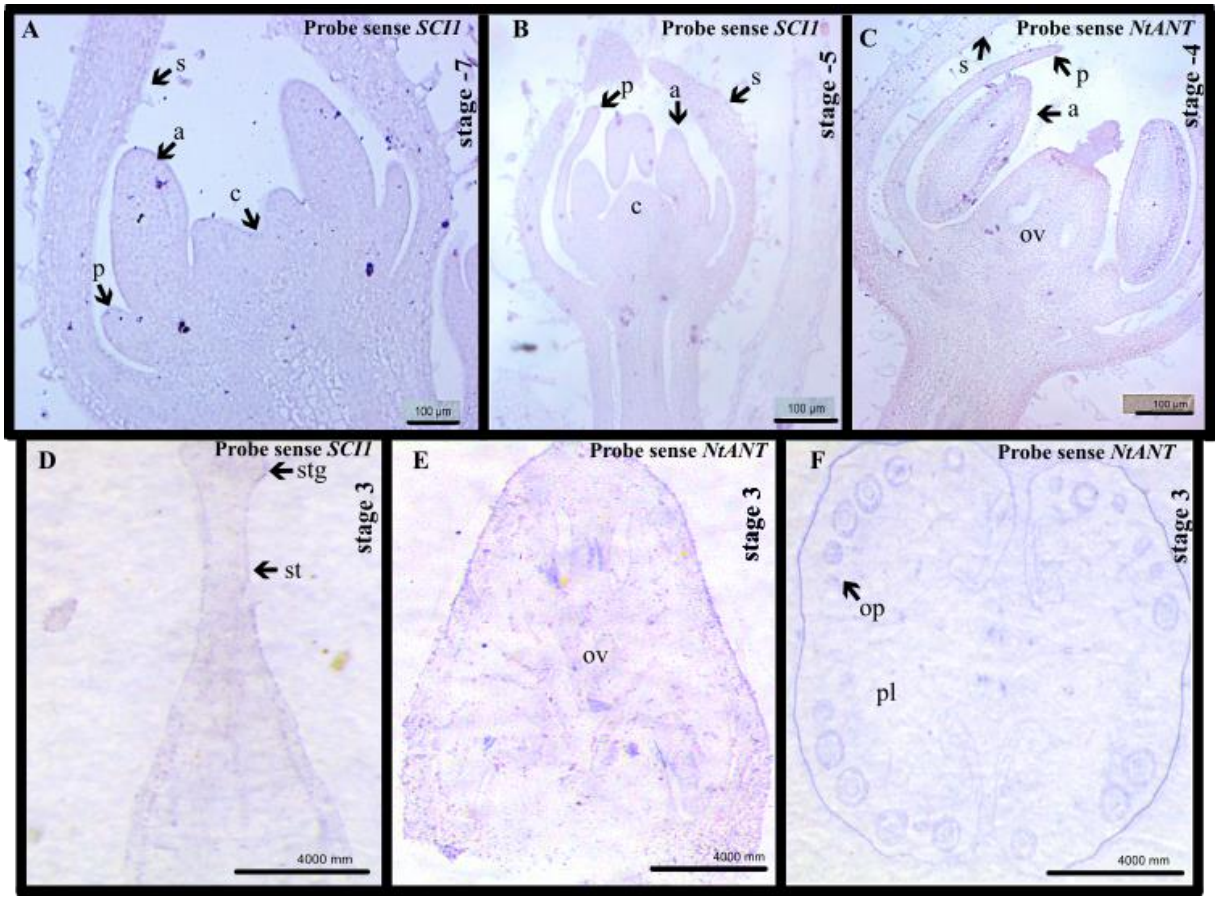
3.



Continue supplementary figure 3.



Continue supplementary figure 3.


A
Probe sense SCI1
s
a
c
p
stage -7
100 µm
B
Probe sense SCI1
p
a
s
c
stage -5
100 µm
C
Probe sense NtANT
s
p
a
ov
stage -4
100 µm
D
Probe sense SCI1
stg
st
stage 3
4000 mm
E
Probe sense NtANT
ov
stage 3
4000 mm
F
Probe sense NtANT
op
pl
stage 3
4000 mm

4.

SUPPLEMENTARY TABLE LEGENDS

Supplementary Table 1. List of *ANT* and *AIL* genes found in *A. thaliana*, *N. tabacum*, an, *S.lycopersicum*. * Sequence used by Rieu et al., 2005 and Kuluev et al., 2015; ** Sequence used at work; # identified by Ichihashi et al., 2014

Supplementary Table 2. List of cis-acting regulatory elements to interaction AP2 family and ARF transcription factors identified at *SCI1* promoter by PlantRegMap.

Supplementary Table 3. Primers were used to amplify and sequence the different DNA fragments and CDSs used in this work.

SUPPLEMTARY TABLES

Supplementary table 1.

Arabidopsis AIL Family member	**Arabidopsis Locus (gene Identifier)**	**Arabidopsis proteins AIL access numbers**	***S. lycopersicum* Family member**	***S. lycopersicum* LOC member**	***S. lycopersicum* proteins AIL access numbers**	***N. tabacum* Family member**	***N. tabacum* LOC numbers**	***N. tabacum* proteins AIL access numbers**
ANT	At4g37750	NP_195489.1	SlANT1/LeANT#	LOC101251772	NP_001287930	NtANT1(N.syl)/NtANTL*	LOC107782138	NP_001312262
						NtANT1(N.tom)/NtANTL**	LOC107809390	XP_016489488.1
			SlANT2	LOC101244632	XP_010316035.1	NtANT2 (N. Syl)	LOC107790283	XP_016467673.1
						NtANT2 (N. tom)	LOC107789316	XP_016466578.1
AIL1	At1g72570	NP_177401.2	SlAIL1	LOC101251371	XP_010318466.1	NtAIL1 (N. syl)	LOC107823845	XP_016506032.1
						NtAIL1 (N. tom)	LOC107816336	XP_016497518.1
AIL2/BBM/PLT4	At5g17430	NP_197245.2	SlAIL2	LOC101257035	XP_004250505.1	NtAIL2 (N. syl)	LOC107775621	XP_016450856.1
						NtAIL2 (N. tom)	LOC107777142	XP_016452614.1
AIL3/PTL1	At3g20840	NP_188720.2	SlAIL3	LOC101252050	XP_019066613.1	NtAIL3/NtAIL4.1 (N. syl)	LOC107801656	XP_016480504.1
						NtAIL3/NtAIL4.1 (N. tom)	LOC107822342	XP_016504355.1
						NtAIL3/NtAIL4.2 (N. syl)	LOC107783177	XP_016459641.1
						NtAIL3/NtAIL4.2 (N. tom)	LOC107810725	XP_016491022.1
AIL4/PTL2	At1g51190	NP_175530.2	SlAIL4	Not Found	Not Found	NtAIL3/NtAIL4.1 (N. syl)	LOC107801656	XP_016480504.1
						NtAIL3/NtAIL4.1 (N. tom)	LOC107822342	XP_016504355.1
						NtAIL3/NtAIL4.2 (N. syl)	LOC107783177	XP_016459641.1
						NtAIL3/NtAIL4.2 (N. tom)	LOC107810725	XP_016491022.1
AIL5/PTL5	At5g57390	NP_200549.2	SlAIL5	LOC101262024	XP_010323408.1	NtAIL5 (N. syl)	LOC104221008	XP_016485713.1
						NtAIL5 (N. tom)	LOC107808093	XP_016488066.1
AIL6/PTL3	At5g10510	NP_001331270.1	SlAIL6	LOC101259843	XP_004240254.1	NtAIL6 (N. syl)	LOC107816479	XP_016497688.1
						NtAIL6 (N. tom)	LOC107813200	XP_016493916.1
AIL7/PTL7	At5g65510	NP_001318882.1	SlAIL7	LOC101248536	XP_004250153.2	NtAIL7 (N. syl)	LOC107768174	XP_016442764.1
						NtAIL7 (N. tom)	LOC107768187	XP_016442778.1

* Sequence used by Rieu et al., 2005 and Kuluev et al., 2015; ** Sequence used at work; # sequence identified by Ichihashi et al., 2014

Supplementary table 2.

Motif	Family	Transcription Factor	Seq ID	Start	Stop	Strand	Score	p-value	q-value	Matched sequence
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+1154	+1173	-	10.0253	9.63e-05	0.0483	AAAAAAAAAAAC AATAGTGG
LOC107813200	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor AIL6	SCI1genomicanalyzes	+868	+882	+	10.25	8.4e-05	0.325	cttcgattgctgtgc
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+27	+46	+	10.443	7.8e-05	0.0472	ggaggaagaggaagcataa ga
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+72	+91	-	10.443	7.8e-05	0.0472	AGACACAAGAAA GGAAAAAA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-799	-780	+	10.8608	6.26e-05	0.0442	aatgaaaaataaaaaaatag
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+206	+225	+	14.8481	4.82e-06	0.0102	gaaggaaagaaaaggacaa aa
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+73	+92	-	11.6076	4.15e-05	0.032	AAGACACAAGAA AGGAAAAA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-642	-623	+	11.6329	4.09e-05	0.032	aataatgagaaaaagaaaa c
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+201	+220	+	11.8608	3.59e-05	0.032	agagcgaaggaaagaaaa gg
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+200	+219	+	15.2911	3.39e-06	0.00958	aagagcgaaggaaagaaa ag
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+636	+655	+	15.4557	2.97e-06	0.00958	aagaaaaagaagaaaaatg a
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-1375	-1356	+	12.1772	2.97e-05	0.0315	aataaaaaagaaaatataca
LOC107809390	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor ANT	SCI1genomicanalyzes	+1313	+1333	-	14.0698	2.66e-06	0.0236	GCACACATCTCC CTATGTATA
LOC107813200	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor AIL6	SCI1genomicanalyzes	-581	-567	+	12.125	2.62e-05	0.233	ccttgattttcgggt
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+631	+650	+	12.4051	2.59e-05	0.0314	taaaaaagaaaaagaagaa a
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-805	-784	+	13.2658	1.5e-05	0.0211	aaagaaaaatgaaaaataa a
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-804	-784	+	13.6582	1.15e-05	0.0195	agaaaaatgaaaaataaaa a
LOC107784366	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor TOE3 isoform X1	SCI1genomicanalyzes	-725	-716	+	13.2596	1.09e-05	0.0973	acatcgtact
LOC107813200	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor AIL6	SCI1genomicanalyzes	-1436	-1422	+	5.5	0.000991	1	catcgatgtgggcaa

LOC107784366	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor TOE3 isoform X1	SCI1genomicanalyzes	+63	+72	-	5.08654	0.00095	1	ACCTCGTGGA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+207	+226	+	4.24051	0.00094	0.126	aaggaaagaaaaggacaaat
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+67	+86	-	4.26582	0.000933	0.126	CAAGAAAGGAAAAAACCTCG
LOC107809390	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor ANT	SCI1genomicanalyzes	+2436	+2456	-	-3.68605	0.000887	1	TGTCAAGCTTTCCCTTGTTCC
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+196	+215	+	4.4557	0.000879	0.122	gatgaagagcgaaggaaaga
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-1136	-1349	+	4.48101	0.000872	0.122	aagaaaatatacaaaacaac
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+630	+649	+	4.49367	0.000868	0.122	ataaaaaagaaaaagaaga a
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+209	+228	+	4.51899	0.000861	0.122	ggaaagaaaaggacaaatcc
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+212	+231	+	4.59494	0.000841	0.122	aagaaaaggacaaatccaag
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-717	-698	+	4.64557	0.000828	0.122	cttacagtgaggactacaaa
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+2235	+2254	-	4.68354	0.000818	0.122	TCAAAGATGGAGGAATAAAG
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+286	+305	-	4.72152	0.000808	0.122	AAAACAAAAACAAAATTGT
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+628	+647	+	4.74684	0.000801	0.122	atataaaaaagaaaaagaag
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+198	+217	+	4.83544	0.000778	0.122	tgaagagcgaaggaaagaaa
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+31	+50	+	4.86076	0.000772	0.122	aagaggaagcataagagaaa
LOC107784366	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor TOE3 isoform X1	SCI1genomicanalyzes	+2485	+2494	+	5.65385	0.000729	1	gcctcggtca
LOC107784366	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor TOE3 isoform X1	SCI1genomicanalyzes	-511	-502	-	5.70192	0.000711	1	GCATCGTATG
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+2411	+2430	-	5.26582	0.000676	0.114	ATGACAAAGTCAGAGAATAA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+488	+507	+	5.32911	0.000661	0.114	tttgaaaagggaaagcaagt
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+70	+89	-	5.39241	0.000647	0.114	ACACAAGAAAGGAAAAAACC
LOC107813200	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor AIL6	SCI1genomicanalyzes	+179	+193	-	6.42188	0.000643	0.817	CTTTGATATTCTGGC
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+35	+54	+	5.70886	0.000581	0.105	ggaagcataagagaaattcg

LOC107784366	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor TOE3 isoform X1	SCI1genomicanalyzes	-1125	-1116	-	6.25962	0.000565	1	ACATGGGACA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-145	-126	+	5.81013	0.000561	0.103	tataactaaaaaaaactaca
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+635	+654	+	5.83544	0.000556	0.103	aaagaaaaagaagaaaaatg
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+490	+509	+	5.86076	0.000551	0.103	tgaaaagggaaagcaagtat
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+377	+396	-	6.20253	0.000488	0.0961	AGGCCCAAGTAAAGAACAAA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-186	-167	+	6.27848	0.000475	0.0957	aatggctaaaagaatataca
LOC107784366	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor TOE3 isoform X1	SCI1genomicanalyzes	-546	-537	+	6.70192	0.000465	1	ttctggtacg
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+632	+651	+	6.37975	0.000457	0.0945	aaaaaagaaaaagaagaaaa
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+1679	+1698	-	6.46835	0.000443	0.0938	CCAGCCAAAACAGAAAAAGA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+5	+24	+	6.49367	0.000439	0.0938	ggagcgataagaagacgacg
LOC107809390	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor ANT	SCI1genomicanalyzes	-323	-303	+	-0.930233	0.000434	0.642	tttctcatttactgatcttaa
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-644	-625	+	6.72152	0.000403	0.0898	aaaataatgagaaaaagaaa
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-1004	-985	-	6.96203	0.000368	0.0842	AAGAACATAAATACAATTGA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-1380	-1361	+	7	0.000362	0.0842	ttgagaataaaaaagaaaat
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-1095	-1076	+	7.01266	0.000361	0.0842	aaaaacataggtaatataca
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+2371	+2790	-	7.18987	0.000337	0.0839	AAAGATCTGAGAAGAACAAA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-5	+15	+	7.22785	0.000332	0.0839	agtaaATGgggagcgataag
LOC107813200	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor AIL6	SCI1genomicanalyzes	-1632	-1618	-	7.76562	0.00033	0.489	TTTGGATAGACGTGC
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+2435	+2454	+	7.3038	0.000322	0.0839	tggaacaagggaaagcttga
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+624	+643	+	7.36709	0.000314	0.0839	acacatataaaaaagaaaaa
LOC107809390	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor ANT	SCI1genomicanalyzes	-1634	-1614	+	0.27907	0.000312	0.553	cagcacgtctatccaaacaca
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-1378	-1359	+	7.40506	0.000309	0.0839	gagaataaaaaagaaaatat

LOC107809390	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor ANT	SCI1genomicanalyzes	-332	-310	+	0.313953	0.000309	0.553	ttatacgttttctcatttact
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-646	-626	+	7.43038	0.000306	0.0839	tcaaaataatgagaaaaaga
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+1159	+1178	-	7.44304	0.000305	0.0839	CATCAAAAAAAAAAAACAAT
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-68	-49	+	7.55696	0.000291	0.0839	ataaataaaaggaagacaac
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-65	-46	+	7.86076	0.000257	0.0838	aataaaaggaagacaacaat
LOC107809390	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor ANT	SCI1genomicanalyzes	+1315	+1335	-	1.09302	0.000248	0.553	GTGCACACATCTCCCTATGTA
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-1773	-1754	-	8.10127	0.000232	0.0788	TGTAGAGAAAATGATAAACA
LOC107813200	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor AIL6	SCI1genomicanalyzes	+2486	+2500	+	8.4375	0.000232	0.412	cctcggtcatcccac
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-826	-807	+	8.18987	0.000224	0.0788	aaaaacatagataaaccaaa
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+2073	+2092	-	8.22785	0.00022	0.0788	AGAACAGAGGCGAAAACAGT
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+2164	+2183	-	8.22785	0.00022	0.0788	AGAACAGAGGCGAAAACAGT
LOC107809390	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor ANT	SCI1genomicanalyzes	+238	+258	+	1.54651	0.000218	0.553	aagcacaaatcccataaatcc
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-1370	-1351	+	8.74684	0.000176	0.0709	aaaagaaaatatacaaaaca
LOC107813200	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor AIL6	SCI1genomicanalyzes	+1317	+1331	+	9.28125	0.000146	0.325	catagggagatgtgt
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	-814	-795	+	9.29114	0.000137	0.0581	aaaccaaaaaagaaaaatga
LOC107813200	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor AIL6	SCI1genomicanalyzes	-574	-560	+	9.45312	0.000133	0.325	tttcgggtatcgctc
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+1156	+1175	-	9.48101	0.000125	0.0559	CAAAAAAAAAAAACAATAGT
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+622	+641	+	9.89873	0.000103	0.0483	aaacacatataaaaaagaaa
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+25	+44	+	9.93671	0.000101	0.0483	gaggagaagaggaagcataa
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+634	+653	+	9.94937	0.0001	0.0483	aaaagaaaaagaagaaaaat
LOC107777142	AP2	PREDICTED: AP2-like ethylene-responsive transcription factor -BABY BOOM	SCI1genomicanalyzes	+207	+226	+	17.4177	4,840E-07	0.0041	aaaaagaaaaagaagaaaa

Motif	Family	Transcription Factor	Seq ID	Start	Stop	Strand	Score	p-value	q-value	Matched sequence
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	-1672	-1652	-	-3.6125	9.09e-05	0.266	CTGATATGTTTG GTAGAAAAG
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	+1681	+1702	+	5.45	6.68e-06	0.051	ttttctgttttggctggaact
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	-1706	-1686	+	3.675	1.16e-05	0.051	tttatctttttaacaagagaa
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	+1681	+1702	+	-13.9125	0.000987	0.787	tttttctgttttggctggaac
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	-151	-131	-	-13.6875	0.000942	0.787	TTTTTTTTAGTTA TAAAAAAA
LOC107787782	ARF	PREDICTED: auxin response factor 8-like	SCI1genomicanalyzes	+977	+985	+	7.47917	0.000876	1	cttgtcagt
LOC107787782	ARF	PREDICTED: auxin response factor 8-like	SCI1genomicanalyzes	-576	-567	+	7.48958	0.000867	1	attttcggg
LOC107798348	ARF	PREDICTED: auxin response factor 2-like	SCI1genomicanalyzes	+446	+455	-	7.71875	0.000834	1	CAGCAACAAC
LOC107787968	ARF	PREDICTED: auxin response factor 5-like	SCI1genomicanalyzes	-232	-223	+	6.51562	0.000776	1	agagacaaat
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	+290	+310	+	-12.6375	0.000756	0.737	tttttgttttgttttaaatt
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	+2091	+2071	+	-12.475	0.000731	0.737	tttaccttctttctcagaaaa
LOC107787782	ARF	PREDICTED: auxin response factor 8-like	SCI1genomicanalyzes	+1281	+1136	+	7.77083	0.000704	1	cttgttggt
LOC107798348	ARF	PREDICTED: auxin response factor 2-like	SCI1genomicanalyzes	+1619	+1610	-	8.07812	0.000701	1	CAGCAACAAA
LOC107787968	ARF	PREDICTED: auxin response factor 5-like	SCI1genomicanalyzes	-57	-48	+	6.89062	0.000672	1	gaagacaaca
LOC107787782	ARF	PREDICTED: auxin response factor 8-like	SCI1genomicanalyzes	+1961	+1969	+	7.84375	0.000669	1	cctgtcaga
LOC107798348	ARF	PREDICTED: auxin response factor 2-like	SCI1genomicanalyzes	-1070	-1061	+	8.1875	0.000655	1	aaccaacata
LOC107770412	ARF	PREDICTED: auxin response factor 1	SCI1genomicanalyzes	-1431	-1424	-	4.94186	0.000644	1	GCCCACAT
LOC107787968	ARF	PREDICTED: auxin response factor 5-like	SCI1genomicanalyzes	+1127	+1136	-	7.01562	0.00064	1	ACCAACAAGA
LOC107770412	ARF	PREDICTED: auxin response factor 1	SCI1genomicanalyzes	+1129	+1136	-	5.12791	0.000544	1	ACCAACAA
LOC107787782	ARF	PREDICTED: auxin response factor 8-like	SCI1genomicanalyzes	+144	+152	+	8.32292	0.000464	1	tgtgtctgc
LOC107770412	ARF	PREDICTED: auxin response factor 1	SCI1genomicanalyzes	-1069	-1062	+	5.38372	0.000403	1	accaacat
LOC107770412	ARF	PREDICTED: auxin response factor 1	SCI1genomicanalyzes	+145	+152	-	5.43023	0.000337	1	GCAGACAC
LOC107798348	ARF	PREDICTED: auxin response factor 2-like	SCI1genomicanalyzes	-233	-224	+	9.375	0.000328	0.966	aagagacaaa
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	+86	+106	-	-8.8125	0.000324	0.406	TTTGCAGATTTTG GAAGACAC

LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	-742	-722	+	-8.4	0.000295	0.406	tttacatgtgaagtagaacat
LOC107798348	ARF	PREDICTED: auxin response factor 2-like	SCI1genomicanalyzes	+1128	+1137	-	9.64062	0.000275	0.966	TACCAACAAG
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	-1630	-1610	-	-6.6125	0.000193	0.338	TTTATGTGTTTGGATAGACGT
LOC107787968	ARF	PREDICTED: auxin response factor 5-like	SCI1genomicanalyzes	+143	+152	-	9.82812	0.000171	1	GCAGACACAA
LOC107798348	ARF	PREDICTED: auxin response factor 2-like	SCI1genomicanalyzes	+144	+153	-	10.375	0.000157	0.966	AGCAGACACA
LOC107770237	ARF	Auxin response factor 16	SCI1genomicanalyzes	+1360	+1380	+	-5.2125	0.000137	0.299	gttactggtttggtgctagaa

Supplementary table 3.

Num	*Primer*	Sequence 5' ⟶ 3.'
1	BP-1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC
2	BP-2	GGGGACCACTTTGTACAAGAAAGCTGGGTC
3	Oligo_dTV	TTTTTTTTTTTTTTTTTTTTTTV
4	T7	TTAATACGACTCACTAT
5	M13-Fw	GTAAAACGACGGCCAGT
6	M13 Rv	CAGGAAACAGCTATGAC
7	SCI1p-N.to-Frag1-Fw	GCAGGCTTCGTAGCATACGATGCGGTTTC
8	SCI1p-N.to-Frag1-Rv	AAGCTGGGTCCTAGTTATGATAGCCGCAGCAC
9	SCI1p-N.to-Frag2-Fw	GCAGGCTTCCCTTCTGGAGGAAACTAATC
10	SCI1p-N.to-Frag2-Rv	AAGCTGGGTCCCTCACTGTAAGTACGATG
11	SCI1p-N.to-Frag3-Fw	GCAGGCTTCCTACCAAACATATCAGGTGC
12	SCI1p-N.to-Frag3-Rv	AAGCTGGGTCGATTAGTTTCCTCCAGAAGG
13	promSCI1_mutHindIII-Fw	GCACTTCAAGCTTATCAGTTATTTAC
14	promSCI1menor_mutHindIII-Fw	GGTACGGAAGCTTGAATAACTAATTGAG
15	promSCI1_mutNcoI-Rv	CGCTCCCCATGGACTCGCACCTCT
16	attB1compANT Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGATGAAGTCTATGAATGATGATAAC
17	attB2compANT Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATGCATCATTCAAAGCAGCAAAGAC
18	Sequence probe 1- Fw	CAAATTGTTATACGTTTTCTCATTTACTGATCTTAAGTGGCTTC
19	Sequence probe 1- Rv	GTTTAACAATATGCAAAAGAGTAAATGACTAGAATTCACCGAAG
20	Sequence probe 2- Fw	CTTATTATCAGTTTCAGCACGTCTATCCAAACACATAAATAGAT
21	Sequence probe 2- Rv	GAATAATAGTCAAAGTCGTGCAGATAGGTTTGTGTATTTATCTA
22	Ct11attB1FW	GCAGGCTTCACCATGGGGAGCGATAAGAAGAC
23	Ct11attB2RV	AAGCTGGGTCTTACTTTTTGATATTCCAAGCGTG

GENERAL CONCLUSIONS

The work was focused on determining when *SCI1* starts its expression and identifying the organs in which the expression is maintained during floral development.

The objective was to determine the expression profile of *SCI1* during floral development and to analyze its transcriptional regulation.

The thesis presents an anatomical description of the initial stages of the N. tabacum flower, from the formation of the floral meristem to stage -5. This description is correlated with the expression of *SCI1*, which starts in the floral meristem and is detected in all cell layers of the structure but shows reduced expression in the organizational center.

As the floral whorl develops, *SCI1* has its expression centripetalally reduced and remains expressing only in the pistil at stage -5. In more advanced stages of floral development, *SCI1* keeps expressing itself in the pistil, more precisely in the specialized tissues of the style and the specialized tissues of the ovary, placenta and ovule.

In floral meristem *SCI1* co-expresses with NtWUS and has its expression regulated by this transcription factor.

In the pistil up to stage -5, *SCI1* co-expresses with *NAG1* and is regulated by this transcription factor.

In more advanced stages, from stage -2, *SCI1* co-expresses with *NtANT* and keeps expressing itself with this transcription factor during ovule development. *SCI1* is also transcriptionally regulated and activated by NtANT.

The maintenance of *SCI1* expression in meristematic cells and its regulation by key transcription factors for meristematic cell proliferation point *SCI1* as a regulator of meristematic cell proliferation and allows us to propose that the phenotype described by DePaoli et al. (2011) is the result of the action of SCI1 in cells with meristematic characteristics during floral development.

FUNDING INFORMATION

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The work was also supported by UIDB/04046/2020 and UIDP/04046/2020, Centre grants from FCT, Portugal (to BioISI), and by the Joint Project "Edital 1024 – Iniciativas de Parceria Estratégica USP – Uminho."

APPENDIX

SCIENTIFIC PRODUCTION AND EXTRACURRICULAR ACTIVITIES (JULY 2018 – APRIL 2023)

Taught of mini-course entitled “Studying gene function in plants with molecular biology” at the XXIII Summer Course in Genetics, held at the Departamento de genética - Faculdade de Medicina de Ribeirão Preto at the Universidade de São Paulo, from January 15 to 26, 2018.

Participation of VII Simpósio Brasileiro de Genética Molecular de Plantas. Poster presentation: SCI1, an inhibitor of cell proliferation, is expressed at the floral meristem and is probably activated by WUSCHEL and AGAMOUS binding to its promoter In: VII Simpósio Brasil e Campos do Jordão. VII Simpósio Brasileiro de Genética Molecular de Plantas, 2019. v. VII.

Participation of Ciclo de Atividades de Formação Didático-Pedagógica no Ensino Superior, realized in 4, June 2019 at Ribeirão Preto.

Honorable mention for participating in the Postgraduate Poster Award in plant genetics at the 65th Brazilian Congress of Genetics. Poster: *SCI1* in Nicotiana tabacum flower meristem is regulate by the transcription factors WUSCHEL, AINTEGUMENTA-LIKE 6 and AGAMOUS.17 to 20, September 2019.

Participation of the 65th Brazilian Congress of Genetics. Poster presentation: *SCI1* in Nicotiana tabacum flower meristem is regulate by the transcription factors WUSCHEL, AINTEGUMENTA-LIKE 6 and AGAMOUS. In Hotel Monte Real Resort, Águas de Lindóia, SP. 17 to 20, September 2019.

Participation of I Simpósio de Novas Tecnologias Aplicadas à Agricultura Moderna realized on September 22, 23, and 24 from 2020.

Estágio PAE: Estágio na disciplina “RIB0103- Metodologia da Pesquisa Científica” para o curso de Informática Biomédica by supervision of professor Dr. Antonio Carlos Shimano. From July to November of 2020.

Participation of the 66th Brazilian Congress of Genetics. Oral presentation: SCI1 starts to be expressed in the onset of floral meristem and is regulated by AGAMOUS and *Nt*WUSCHEL. 13 to 16, September 2021.

Honorable mention in GENÉTICA 2021 – 66th Brazilian Congress of Genetics in PRÊMIO ALCIDES CARVALHO by oral presentation the work: *SCI1* starts to be expressed in the onset of floral meristem and is regulated by AGAMOUS and *Nt*WUSCHEL. 13 to 16, September 2021.

Participation of Minicourse CLAGeneC-Genômica aplicada à Conservação, in GENÉTICA 2021 - Brazilian Congress of Genetics. 13 to 16, September 2021.

Taught the minicourse entitled "Como estudar a função de genes em plantas" at the XXVI Curso de Verão em Genética, realizado no Departamento de Genética da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo, on January 27, 2021.

Worked as a volunteer monitor, from April to July 2021, in the Undergraduate discipline " Biotecnologia Vegetal (5920944)" of the Ciências Biológicas- Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto - FFCLRP-USP- Departamento de Biologia.

Presented a lecture entitled: Introdução a escrita acadêmica in the Undergraduate discipline "Biotecnologia Ambiental (5920946)" of the Ciências Biológicas- Faculdade de Filosofia Ciências e Letras de Ribeirão Preto - FFCLRP-USP- Departamento de Biologia in April 23, 2021.

Presented a lecture entitled: Introdução a escrita acadêmica in the Undergraduate discipline "Biotecnologia Vegetal (5920944)" of the Ciências Biológicas- Faculdade de Filosofia Ciências e Letras de Ribeirão Preto - FFCLRP-USP- Departamento de Biologia in April 19, 2021.

Participation of theoretical course "TECNOLOGIAS MOLECULARES E DESENVOLVIMENTO BIOTECNOLÓGICO: APLICAÇÕES EM SAÚDE, AMBIENTE E AGRICULTURA", realized in the distance modality by the Universidade Federal dos Vales do Jequitinhonha e Mucuri, in the period from March 17 to June 30, 2022.

Internship abroad with funding of Edital 1024/2019 – Iniciativas de Parceria Estratégica USP – Minho – Edital conjunto para apoio à pesquisa at Universidade do Minho, Braga,

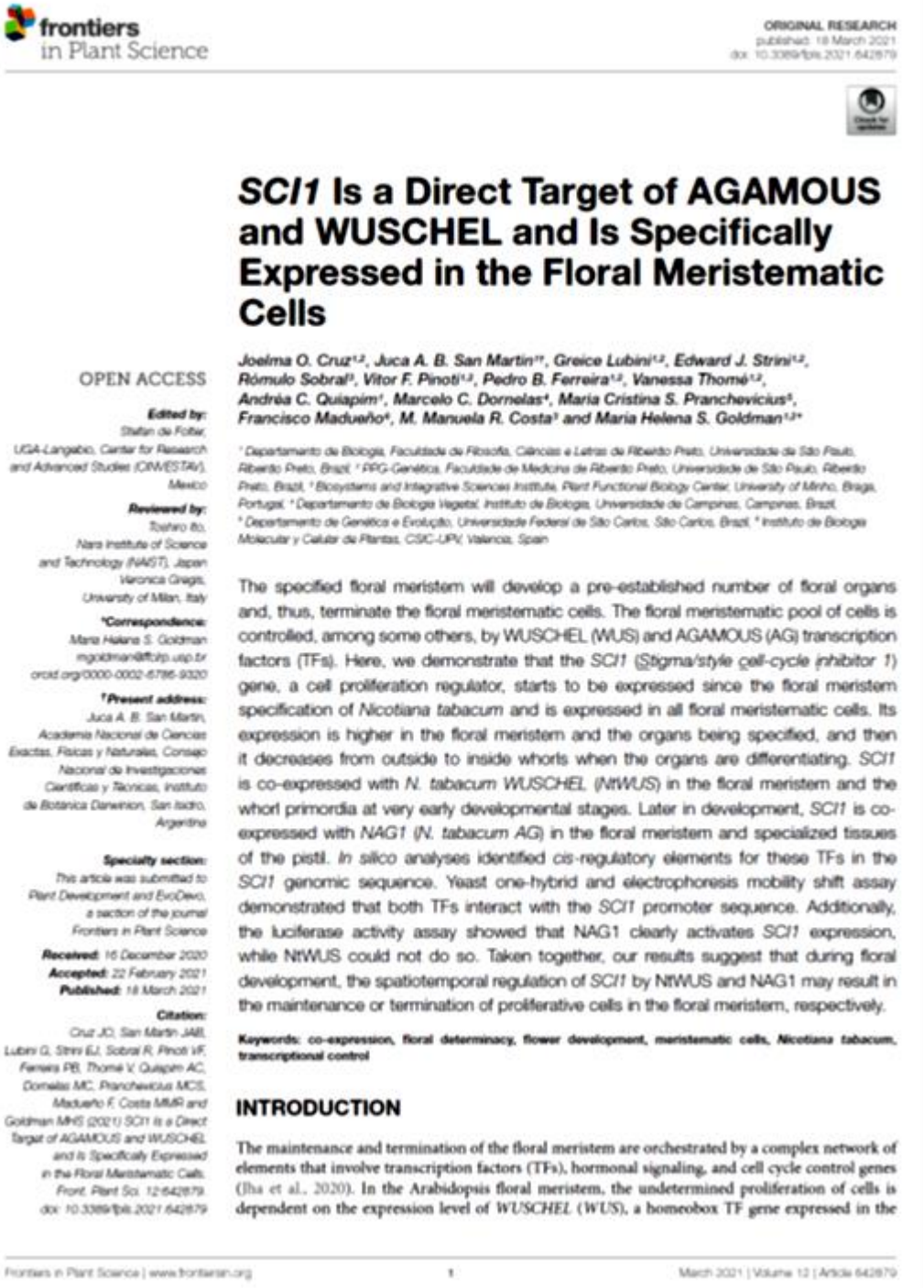
Portugal. Supervisor: Drª. Maria Manuela Ribeiro Costa. Project: Regulação da expressão gênica de *SCI1* pelos fatores de transcrição AGAMOUS e WUSCHEL. Edital 1024/2019 – Iniciativas de Parceria Estratégica USP – Minho – Edital conjunto para apoio à pesquisa. October 5 to December 12.

Participation of DS Bridges Program in 2022.

Honorable mention in recognition of outstanding participation in all DS Bridges Program activities in 2022.

SCIENTIFIC PRODUCTION

SCI1 is a direct target of AGAMOUS and WUSCHEL and is specifically expressed in the floral meristematic cell



frontiers in Plant Science

ORIGINAL RESEARCH
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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* (*S*tigma/style *c*ell-cycle *i*nhibitor *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

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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