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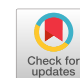
Research Article

Cloning of the *Coffea canephora SERK1* promoter and its molecular analysis during the cell-to-embryo transition



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A B S T R A C T

Background: Somatic embryogenesis receptor-like kinase 1 (SERK1) is a cell membrane receptor active in different plant tissues and involved in cell differentiation activities including somatic embryogenesis. The identification of promoter elements responsible for *SERK1* expression during the onset of somatic embryogenesis can be useful to understand the molecular regulation of the cell-to embryo transition, and these promoter elements represent biotechnological tools in plant organ tissue culture.
Results: A −1,620 bp DNA sequence located upstream of the *Coffea canephora SERK1* gene homologue (*CcSERK1*) was isolated, and then, different segments containing key response elements (REs) for somatic embryogenesis onset and development were fused to the *uidA* (encoding a β-glucuronidase, GUS) reporter gene to evaluate its expression in transgenic leaf explants. DNA segments of −1,620 and −1048 bp in length directed *uidA* expression with patterns in leaf explants similar to those occurring during somatic embryogenesis. When a −792-bp fragment was used, *uidA* expression disappeared only in leaf explants and pro-embryogenic mass but persisted in developing embryos. No *uidA* expression was detected in any embryogenic stage when a −618-bp fragment was used.
Conclusion: DNA deletions showed that a −1048-bp sequence located upstream of the *CcSERK1* gene is sufficient to direct gene expression during the onset and the development of *C. canephora* somatic embryogenesis. The DNA segment located between −1048 and −792 bp (containing BBM and WUS REs) is needed for gene expression before embryogenesis onset but not during embryo development. The promoter segment between −792 and −618 bp (including GATA, ARR1AT, and ANT REs) regulates gene expression in developing embryos.
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1. Introduction

Biotechnological approaches to improve the quality of coffee have focused on the manipulation of genes associated with specific agronomical traits [1] and the analysis of isolated plant promoters. Expression of coffee gene promoters in heterologous systems revealed that they are recognized by the host transcription machinery and could thus be used as biotechnological tools in plant breeding [2,3,4,5]. Frequently, gene promoter analysis involved the ectopic expression of reporter genes in *Nicotiana tabacum* and *Nicotiana benthamiana* species as heterologous validation systems. For example, Brandalise et al. [6] demonstrated that the promoter of a gene encoding a putative isoflavone reductase from *Coffea arabica* regulated leaf-specific gene expression of the *uidA* gene, and it was highly enhanced in response to fungal infections and mechanical injury. Ectopic expression of this reporter system in transgenic tobacco confirmed responsiveness of the putative promoter to abiotic stress. In another report, transgenic lines of tobacco transformed with *C. arabica* promoters of the CaWRKY1a and CaWRKY1b transcription factors, regulating the *uidA* gene (pW1a::GUS) as a reporter, showed differential responses of these promoters to plant growth regulators (PGRs), fungal infections, and leaf injury [7].

Other promoters related to metabolic pathways of coffee have also been studied. Satyanarayana et al. [8] demonstrated that a *Coffea canephora N*-methyltransferase (*NMT*) gene promoter was able to

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direct the expression of the *uidA* gene in transgenic *N. tabacum*. The promoter of a *C. canephora* dehydrin coding gene (*CcDH2*) was isolated, and analysis of its nucleotide sequence revealed the existence of regulatory sequence motifs probably involved in the control of *CcDH2* dehydrin expression during seed development [9]. Marraccini et al. [10] cloned a ribulose 1,5-bisphosphate carboxylase/oxygenase gene (*RBCS1*) promoter from *C. arabica* and found the presence of several putative DNA boxes with similarities to well-characterized light responsive elements; by using a nucleotide sequence of length 1 kb to direct the expression of the *uidA* gene, they showed that this coffee promoter could direct leaf-specific and light-regulated gene expression in transgenic tobacco. Promoters from the genes encoding 11S globulin and NMT were able to direct transgene expression in endosperm tissues and in secondary transgenic embryos of *C. canephora*, respectively [11]. These results demonstrate the incipient beginning of studies on gene promoters in coffee species and the diversity of research areas as well as the absence of studies with an aim to understand the regulation of gene expression during cell differentiation of the somatic embryogenesis process within the economically important species of the genus *Coffea*.

In vitro somatic embryogenesis of *C. canephora* has recently become a suitable model to study the molecular regulation of cell differentiation because it is highly efficient and reproducible [12] as well as the *C. canephora* genomic DNA sequence has been published [13]. Therefore, genes involved in the molecular regulation of cell differentiation can be studied using this model. These genes include those encoding the transcription factors WUSCHEL (*WUS*) [14], AGAMOUS-like 15 (*AGL15*) [15], LEAFY COTYLEDON2 (*LEC2*) [16], BABY BOOM (*BBM*) [17], ANTEGUMENTA (*ANT*) [18], and the kinase receptor somatic embryogenesis receptor-like kinase 1 (*SERK1*). Among these genes, *SERK* is a multimember family of cell membrane receptors involved in different processes such as defense responses [19,20,21], cell differentiation, tissue and organ development [22], brassinosteroid (BR) signaling [23], control of root development [24], anther development [25,26], abscission [27], and cell death regulation [28,29]. *SERK1* homologs have been described in many species, where they also function as molecular markers of embryogenic competence [30,31] and are essential for embryogenesis development [32], and their ectopic expression was found to increase embryogenic competence [30].

There is evidence suggesting that among the multiple regulatory sequences present in the promoters of the plant *SERK* genes, those for WUS, BBM, and ANT transcription factors are able to induce embryonic fate or increase embryo development when ectopically expressed in transformed tissues; Zuo et al. [14] demonstrated that *Arabidopsis* WUS maintains the identity of embryonic stem cells, and its overexpression promotes a vegetative-to-embryogenic transition. The ectopic expression of *BBM* homologs induces spontaneous somatic embryogenesis in *Arabidopsis thaliana* and *Brassica napus* seedlings but not in *N. tabacum*, thus indicating divergent BBM competence between species [33]. ANT is an AP2-like ethylene responsive transcription factor that is part of a molecular system that regulates cell division during ovule development and floral organ growth [34, 35], and coordinates patterning signals with cell proliferation and organ growth [36,37]. Overexpression of the *ANT* gene in transgenic *A. thaliana* and tobacco plants increases cell number in embryos and shoot organs because of an extended period of cell proliferation and organ growth [38].

The multiplicity of signals inducing *SERK1* expression reveals a complex system that integrates cell function and tissue developmental stages with cell differentiation competence. Identification of promoter elements responsible for *SERK1* expression during the onset or development of somatic embryogenesis can be useful to understand the molecular regulation of the cell-to embryo transition and could represent a biotechnological tool for the manipulation of cell differentiation and tissue regeneration *in vitro*. In this study, a −1620-bp genomic DNA sequence located immediately upstream of the *C. canephora SERK1* gene homolog was cloned by genome walking. This was followed by sequence deletions and the ability to direct the *uidA* reporter to analyze the requirement of specific response elements (REs) for the onset and development of somatic embryogenesis.

2. Materials and methods

2.1. Plant material and growth conditions

C. canephora var. robusta plantlets, kindly donated by Dr. Víctor M. Loyola-Vargas' laboratory from an *in vitro* propagated material, were clonally propagated *in vitro* at 25 ± 2°C under a 16/8-h photoperiod (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in maintenance medium [39] supplemented with 4-$mg \cdot L^{-1}$ thiamine-HCl, 10-$mg \cdot L^{-1}$ L-myoinositol, 25-$mg \cdot L^{-1}$ L-cysteine, 1-$mg \cdot L^{-1}$ nicotinic acid, 30-$g \cdot L^{-1}$ sucrose, and 0.25% (*w*/*v*) Gelrite, pH 5.8. Plantlet subculturing was done every 6 weeks by transplanting shoot inter-nodal segments to fresh maintenance medium *in vitro*. *Nicotiana benthamiana* seeds that were surface sterilized with 0.6% sodium hypochlorite and thoroughly rinsed with sterile water were germinated *in vitro*, and emerging seedlings were grown in a semisolid medium composed of the following, until transformation: 1X MS, 100-$mg \cdot L^{-1}$ myoinositol, 2-$mg \cdot L^{-1}$ glycine, vitamin mix (0.5-$mg \cdot L^{-1}$ nicotinic acid, 0.5-$mg \cdot L^{-1}$ pyridoxine, 0.1-$mg \cdot L^{-1}$ thiamine-HCl), 3% sucrose, 0.2% (*w*/*v*) Gelrite, pH 5.7.

2.2. C. canephora somatic embryogenesis

For somatic embryogenesis induction, selected *C. canephora* plantlets were preincubated *in vitro* in MS medium supplemented with 0.54-μM naphthaleneacetic acid (NAA; Sigma-Aldrich, Germany) and 2.32-μM Kinetin (KIN; Sigma-Aldrich, Germany). After a 14-day pretreatment, four fully developed leaves were excised from every plantlet, and then, five 0.8-cm diameter explants were cut from the middle part of every leaf, thereby avoiding the base and the apex. Five isolated disc explants were then cultivated in Yasuda liquid medium as modified by Quiroz-Figueroa et al. [12] in the presence of 5-μM benzyladenine (BA; Sigma-Aldrich, Germany) and then incubated at 25 ± 2°C in the dark in a shaker at 55 rpm. Five independent disc explants were cultivated in the absence of BA as the negative control. Each treatment was repeated at least three times (Fig. S1).

2.3. Isolation and cloning of the CcSERK1 promoter and construction of pCcSERK1 binary vectors

The *CcSERK1* promoter was isolated with the Universal Genome Walker™ Kit (Clontech, California, USA) according to manufacturer's instructions by using the previously cloned *CcSERK1* full-length cDNA as a reference template as given in Pérez-Pascual et al. [40]. Genomic DNA was isolated from young *C. canephora* leaves by the cetyltrimethylammonium bromide (CTAB) method [41] as indicated. The purity and quality of the DNA were analyzed using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, USA), and DNA integrity was analyzed by agarose gel electrophoresis (0.8%). PCR amplifications from the *Eco*RV, *Stu*I, *Pvu*I, and *Dra*I restriction enzyme libraries were performed with AP primers and the gene-specific primers GSP1 and GSP2 given in the kit (Table 1); these primers align with the beginning of the ORF of the *CcSERK1* cDNA in the following manner: 94°C for 25 s for the first PCR cycle, and then 72°C for 3 min for 7 cycles, followed by 94°C for 25 s, 67°C for 3 min for 32 cycles, and a final extension step at 67°C for 7 min; for nested PCR: 94°C for 25 s for the first cycle, and then, 72°C for 3 min for 7 cycles, followed by 94°C for 25 s, 67°C for 3 min for 22 cycles, and a final extension step at 67°C for 7 min. After PCR analysis, positive DNA fragments were purified (Fig. S2) and cloned into the pGEM®-T Easy

Table 1
Oligonucleotide primers used for the cloning of the *CcSERK1* promoter and the construction of pCcSERK1::GUS vectors.

Name	Sequence[2]	Amplicon/promoter size[1] (bp)	Details
AP1	5′GTAATACGACTCACTATAGGGC3′	- - -[3]	Adaptor primer 1 for genome walking
AP2	5′ACTATAGGGCACGCGTGGT3′	- - -	Adaptor primer 2 for genome walking
GSP1	5′AAAGCAGCATTACCCAGATCTACTCTT3′	- - -	Gene-specific primer 1 for genome walking
GSP2	5′GCAAAGCATCACCTTCCATGTTAGCAA3′	- - -	Gene-specific primer 2 for genome walking
pCcSERK1-KpnI-F1	5′CGG **GGTACC** CCAATTACAAAAATATGATGCTGCATGG3′	2072/1620	Forward primer for the synthesis of pCcSERK1::GUS-FL and prCcSERK1::Gus-aux(−)
pCcSERK1-KpnI-F2	5′CGG**GGTACC**CATAAGTGGTTTTTGAATGGTTGGTCC3′	1500/1048	Forward primer for pCcSERK1::GUS-1048 synthesis
pCcSERK1-KpnI-F3	5′CGG**GGTACC**AAAAATCGATAAAAAGTGCGTTCAAATT3′	1244/792	Forward primer for pCcSERK1::GUS-792 synthesis
pCcSERK1-KpnI-F4	5′CGG**GGTACC**CCTAACAAGATAATTGTTGTTGTTATCTC3′	1070/618	Forward primer for pCcSERK1::GUS-618 synthesis
pCcSERK1-NcoI-R1	5′CATG**CCATGG**CATCCTCAACTTCATTATCAGCAACAAA3′	*	Reverse primer for synthesis of all pCcSERK1::GUS versions

1 The amplicon size includes the 452-bp 5′UTR region of the *CcSERK1* promoter.
2 Letters in bold indicate the DNA sequence of the *Kpn*I and *Nco*I restriction sites, as indicated.
3 Dotted line indicates undefined amplicon size.
* Use of the pCcSERK1-NcoI-R1 primer produces different amplicon/promoter sizes according to the pCcSERK1-KpnI-forward primer pair used.

plasmid (Promega, Madison, WI, USA) by standard techniques. Identity of cloned fragments was achieved by previous PCR corroboration of amplicon sizes (Fig. S3) (PCR conditions as follows: initial denaturation step at 94°C for 2 min, then 94°C for 15 s, 55°C for 20 s, 72°C for 2 min, 30 cycles, and a final extension step at 72°C for 10 min) and sequencing of positive clones in double-strand DNA using universal M13 primers of pGEM®-T Easy vector (Davis Sequencing, Piscataway, N.J.).

pCcSERK1 binary vectors were constructed by replacing the 35S promoter that controls the expression of the *uidA* gene in the binary vector pCAMBIA1301 (http://www.cambia.org/daisy/cambia/585.html) with different versions of the *CcSERK1* promoter to generate pCcSERK1::GUS-FL (from +452 to −1620), pCcSERK1::GUS-1048 (from +452 to −1048), pCcSERK1::GUS-792 (from +452 to −792), pCcSERK1::GUS-618 (from +452 to −618), and pCcSERK1::GUS-aux (−) (DNA segment from +452 to −1620, where the four AuxRe were mutated during chemical synthesis, GenScript, https://www.genscript.com/) and were verified by DNA sequencing. *Kpn*I and *Nco*I restriction enzymes were used to excise the 35S promoter, and then, the different *CcSERK1* promoter fragments, previously amplified by PCR using *Kpn*I and *Nco*I-containing primers (Table 1), were ligated after enzymatic digestion to the *Kpn*I-*Nco*I linearized pCAMBIA1301 vector. PCR conditions were as follows: 94°C for 2 min, then 94°C for 15 s, 67°C for 30 s, 72°C for 2 min, and a final extension step at 72°C for 10 min. The identity of the different pCcSERK1::GUS and pCcSERK1::GUS-aux(−) constructs was corroborated by *Kpn*I and *Nco*I enzymatic digestion, PCR amplification under conditions described above (Fig. S4 and Fig. S5), and sequencing of positive clones.

2.4. Co-cultivation with Agrobacterium tumefaciens

The *A. tumefaciens* LBA4404 strain was transformed with each of the different pCcSERK1::GUS and pCcSERK1::GUS-aux(−) constructs. Positive transformation of *A. tumefaciens* colonies was corroborated by direct PCR amplification using *CcSERK1* promoter-specific primers (Table 1) (primer positions shown in Fig. S6). Leaf and stem cuttings from 6-week-old *N. benthamiana* plants grown *in vitro*, or embryogenic disc explants from 14-day pretreated *C. canephora* seedlings, were transiently transformed by co-cultivation with the LBA4404 strain of *A. tumefaciens* carrying the corresponding binary vectors [pCcSERK1::GUS-FL, pCcSERK1::GUS-1048, pCcSERK1::GUS-792, pCcSERK1::GUS-618, or pCcSERK1::GUS-aux(−)] by using standard co-cultivation protocols. Bacteria were eliminated by washing three times in MS liquid media containing 1-μg·mL^{-1} cefotaxim and 400-mg·L^{-1} timentin. Samples from *N. benthamiana* explants or *C. canephora* explants from different time periods (25, 35, 50, and 60 d after embryogenic induction [DAI]) were subjected to the GUS histochemical assay, as reported by Leroy et al. [42]. As a negative control, the corresponding *N. benthamiana* or *C. canephora* explants were transformed with the empty vector pCAMBIA1301.

2.5. Bioinformatics analysis of the CcSERK1 promoter

An analysis of putative cis-acting elements in the −1620-bp promoter region located upstream of the *CcSERK1* gene was performed in line with the PLACE (http://www.dna.affrc.go.jp/PLACE/), Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and PLANT PAN 2.0 (http://plantpan2.itps.ncku.edu.tw/) web tools.

The MapChart 2.30 graphical tool was used to generate a diagram describing the physical mapping of the *CcSERK1* promoter region in the *C. canephora* genome (Fig. S7).

Alignment of the DNA sequence of the *CcSERK1* promoter isolated in this work with the corresponding Coffee DNA genome sequence (Fig. S6) was done using the Multalin online program.

2.6. Effect of auxin addition on CcSERK1 promoter activity

N. benthamiana leaf and stem explants were transformed by co-cultivation with *A. tumefaciens* carrying either native or mutated versions of the *CcSERK1* promoter [pCcSERK1::GUS-FL or pCcSERK1::GUS-aux(−)]. In mutated versions, two nucleotide substitutions (TGACT→TaAaT, TGAAG→TaAAaT, TGACT→TaAaT, TGAGG→TaAaG) were introduced into the four cis auxin REs (AUXRETGA1GMGH3) [43] (Fig. S8). Transiently transformed explants of *N. benthamiana* were pre-incubated for 3 d in maintenance medium, and then, different concentrations of either NAA (0.1 and 0.54 μM) or 2,4-dichlorophenoxyacetic acid (2,4-D; 0.1 and 0.54 μM) were added. After different incubation periods (0, 12, 24, and 36 h), the explants were collected for GUS histochemical assay.

3. Results and discussion

3.1. Isolation and cloning of the C. canephora CcSERK1 promoter

Somatic embryogenesis receptor-like kinases are a family of plant flexible co-receptors with putative regulatory functions in cell differentiation, cell death, and immunity, as demonstrated by their gene expression patterns and mutant phenotypes [22]. This range of functions suggests a diversity of *cis* elements present in its promoter. In this work, the genome walking technology using specific primers (Table 1) and BLAST searches of the recently published genome sequence of *C. canephora* (http://coffee-genome.org/) allowed the amplification of a −2072-bp DNA fragment, which includes the first 452 bp of the *C. canephora* 5′-UTR cDNA [40] (Fig. S6).

```
|-1700---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---- -1601|
                                                                                        CCAATTACAA AAATATGATG
                                                                                        LEC1          AGL15

|-1600---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---- -1501|
CTGCATGGGA TGGGACATTT AATAGATCAA ATCTTTAACA CACATGTAAA TGTATGTCCT GACTATAATT ATTTGACCTT TTGCCTATTC GCAAATATTG
                              LEC1                                                                 LEC1

|-1500---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---- -1401|
GCTCTTTCAA GTGCAAAGTT GTACTACCAT TAGCTGTTTT TTAGTGTATT TTTGAAAAAT TTTATTGTAG CAGCGTATAC GAATTTTTGT TTTGAGATTT

|-1400---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---- -1301|
TTTTGAGTTG TATATATTTG TGTGTTATCG TAATATTGTA TTTGAAAAAC AGACAAATCT CAACGGAGTT GTCCAAAAAG AGACTTAATC ATTTTTAATG
                                                           LEC1

|-1300---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---- -1201|
ACCGCTGAGC CAAAACTCGG TGGTTAACTG TAATACTCAT GACTAAAAAT GAACGCTTGT CAAAATCAAG GGTTCAACAT TTCCTGAATC CCTAAATGAA
          LEC1                            AuxRE

|-1200---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---- -1101|
ACGGAAGATC CAAATTATGT ACAATGGATA CTAATTACGA CAAATATTAT AACTGCATCA TTTCACTACT TAAATATAGC TATCATTAAA AAATGGCAAT
          LEC1                               LEC1

|-1100---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---- -1001|
CTTTTTAGAG GAAGACTGGT TAATAATCAC TTGAAGTATT TCAAATGTTC TTCATAAGTG GTTTTTGAAT GGTTGGTCCG GAATTGCGTT CAGATACGTC
                                  AuxRE       LEC1

|-1000---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -901|
CAATTCAAGT TTGCTGTTCT TGTTGCATAA ATCAATTCGG CACAAGTTCC ATTGGCAACA ATGTGTAAAT TAATCCTAAA GGTTCAAGAA AAAAGTTAAA
LEC1                                            BBM                         WUSCHEL

|-900----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -801|
TCCATTGCAG ATATAGATGA ATCATACATT CAATATTTGA ATCAACTTGG CAAGAGATTC TTTGAATTTA GTTCGTCAAC AAGTTAGATC ACGATTGAAA

|-800----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -701|
AAAATTTTTA TATTCGATAA TTTTAAAAAT CGATAAAAAG TGCGTTCAAA TTCGATTCGA TAAATAAATA AGCCAAATTT ATACAAAATT TTAATCTAGT

|-700----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -601|
TAAAATAATC AAACAAATTT AAATACTAAA TTATTCGATT TGATTAGTCT CGTTCACACC CGAATTGGAT TGGAGAGGCC TAACAAGATA ATTGCTGTTG
                                                                 ANT

|-600----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------501|
TTATCTCAAC ATATCTCTCT GCCGCTAAAG GCAGTCAAAA TGCACAACTT TAGGTACACT TTTATTATGC ATTTATTACC ATCACACTCT AAAAGTAAAG

|-500----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -401|
AAAAAAGTTA GTGCTAATCA CATTTGTGCA TTTAATTACT TTGACTTAAC AAAATTGAGG TCATTAATAA AGAAGATGTA GTTATACTAT AAATCTTTAG
                                             AuxRE          AuxRE

|-400----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -301|
ATTTCCAAAA CCAAAATGAG AATTACCAGA GAGCTTGAAT GTAACAAAAG AGCACAAAAC AAAAACAATA AAAGAAAAGG AAACCGTGGT CTGTCAAGAC

|-300----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -201|
TGGAAATAAG GGAAAGCGCG AAGCTGTTTG GAAGTTAAAA AGAGCTTTCG GGCAGGTGTC CGGGCAACAA GCCCACCGTT CGGGCAACAC CCCTCCTCAG

|-200----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -101|
AAGCCCACTG CAGGGCGCAC ACACACGCCC AAAAAGACAA TAATCTCTCT CACACACTCA CGCACTCTCT CTCCCTCTCT CTGTGCAGCA AAGAAACTGA

|-100----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------- -1|
GTAGAGAGAG AGAGAACAGA GGAGAGGCTT CTAGTTTGGA GAGAGAAAAA GCGTTAGTAC AGTACAACAA ATACAATACT ACAATACAAT CCACATAGTC
                                                                         TATA

|+1------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----+100|
ATTACAGAAG TGAAAAGTTT GCAGGGTGAT AAAAAGGGTG CAACATTAAC AATGGAAAAA AAAGAAAAGC CCAGAAAAAT AAAAGGCCAC ATTTATCACC
TTACTGACTG AGGAAGCAAA AGAAAGAGAA AAAGATAGAA TCTTTGAGGG AAGAAAAGGG ATTTTTAGAA ATGGGTTTTT GGTAAAGTAC GGTTAAGTGA
TAAGATCAGG TTTTTTGGGT TTTATTTTTT TTTACTGTAG TTAATTAATG GTGGTTTTCT TTGGGTGTAA AATGGTGGTT TTTGGGGGAA ATGAGGGATA
GGGGTTTGCG TGTTTTGTTG TTAATGCTAC TTTTAAGTGT GTTTTATTTG GATCTGAGGG ATTAATTGTT TTGGAGCTGG TGGGTTAAGA AAATATACGT
TCTTATATAT GAATTTGTGG GGGTTTTGTT GCTGATAATG AAGTTGAGGA TG
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Fig. 1. Nucleotide sequence and *in silico* prediction of *cis* response elements present in the *CcSERK1* promoter. The −1620-bp DNA sequence of the *CcSERK1* promoter was analyzed with the PLACE, PLANT CARE, and PLANT PAN 2.0 online software. The initial Met ATG codon is underlined. The transcription start site is indicated by an arrow at position +1. BBM, WUSCHELL, and ANT REs are boxed with a double line. The four AuxREs are boxed with a bold line. Predicted TATA box is double underlined.

Physical mapping using the MapChart 2.30 graphical tool located the putative *CcSERK1* promoter within positions 5023122–5021051 of Chromosome 10 of the *C. canephora* genome sequence (http://coffee-genome.org/), upstream of the predicted *CcSERK1* (Cc10_g06160) translation initiation site (Fig. S7). Multiple sequence alignment of the *CcSERK1* promoter sequence and the corresponding genomic DNA revealed that there is 98% identical (Fig. S6).

Bioinformatics analysis with different online programs (PLACE, PLANT CARE, and PLANT PAN 2.0) identified REs within the *CcSERK1* promoter nucleotide sequence (Fig. 1). REs include those important for proper transcription initiation (CAAT and TATA boxes), REs for biotic (GT1GMSCAM4) and abiotic signals (GATABOX and GT1CORE), and REs for tissue specificity (OSE2ROOTNODULE and POLLEN1LELAT52). The DNA sequence of the *CcSERK1* promoter contains multiple REs for PGRs [auxins (AuxRE), cytokinins (ARR1AT as well as ARR2, 10, 11, and 14), ethylene (EIN3, ERELEE4, and ERF), gibberellins (GRAS, GAREAT, and MYBGAHV), and abscisic acid (ABA; AREB2 and ABF4)]. With regard to *cis* elements specific for homeotic regulators, the *CcSERK1* promoter contains single REs for BBM, WUS, and ANT but contains multiple REs for AGAMOUS and LEC1 (Table 2).

3.2. Functional analysis of the CcSERK1 promoter during somatic embryogenesis

To study *CcSERK1* promoter function during *C. canephora* somatic embryogenesis, embryogenic leaf explants were transformed with one of the pCcSERK1::GUS binary vectors (Fig. 2), and then, GUS activity was evaluated in time periods either before the embryogenesis onset (25–35 DAI) or during embryo development (35–60 DAI).

Embryogenic leaf explants transformed with pCcSERK1::GUS-FL and pCcSERK1::GUS-1048 displayed similar results. At 25 DAI, slight GUS activity was detected in central and secondary veins of the leaf explants and in the callus generated in the leaf borders. At 35 DAI, GUS activity increased considerably in central and secondary veins, and it was strongly detected in the pro-embryogenic mass surrounding explant edges. In later stages (50 and 60 DAI), GUS activity was strongly detected in globular and heart structures and decreased in torpedo and cotyledonal embryos (Fig. 3, Fig. 4). These GUS activity patterns resembled the *SERK1* gene expression patterns displayed in the embryo development of other species [44,45]. In embryogenic leaf explants transformed with pCcSERK1::GUS-792, GUS activity was not

Table 2
Cis response elements (REs) present in the *CcSERK1* promoter.

Cis element	Sequence	Position (chain)	Function	References
AGAMOUS-like 6,8, 10, 11, 13, 14, 17, 18;	caatggaaaaaaaAGAA Aagc	−388, 1328 (+)	AGAMOUS protein-binding site	[62]
AGAMOUS-like 15	CAA AAATATG CATTTaatag	−1585, 1613 (+)	**AGAMOUS-like 15 protein-binding site**	[63,64]
Aintegumenta (ANT)	gttcacaCCCGAat	−702 (+)	**Cell-division cycle protein**	[34,65,66,67]
AREB2; ABF4	ACACAtgt ACACTttt	−545, 1561, 5, 45 (+)	Abscisic acid responsive element	[68,69]
ARF; IAA22	TaCGCACaa	−1165 (+)	Auxin response factor (ARF)	[70]
ARR1AT	CGATT	−402, 634, 660, 665, 748, 809, 846, 1406 (+)	ARR1 participates in the cytokinin signaling pathway	[71,72]
AUXRETGA1GMGH3	TGACTaaa TGAAGtat TGAGGtca	−445, 459, 1069, 1261 (+)	Auxin response elements	[73]
BBM	cgGCAC Aagttccattggcaa	−963 (+)	**Binding site of the protein BABY BOOM**	[74]
CCA1	aacATATCtc cAGATAcgtc	−1010, 1406 (+)	Circadian clock associated 1	[75]
CDC5	accGCTGAgcc	−1300 (+)	Cell division cycle 5-like protein	[76]
ERELEE4	ATTTCAAA	−1063 (+)	ERE (ethylene responsive element)	[77,78,79]
GAREAT	TAACAag	−157, 323, 419, 1167, 1262, 1538 (+)	GARE (GA response element)	[80]
GT1CORE	GGTTAA	−190, 1083, 1279 (+)	Light-responsive element	[81,82]
LEC1	CCAAT CAAAT CCTAT CCATT CGAAT CTAAT	−1620, 1573, 1509, 1421, 1347, 1328, 1291, 1191, 1190, 1180, 1160, 1105, 1059, 1001, 969, 952, 943… (+)	**Protein LEAFY COTYLEDON 1 [LEC1].** Acts as a central regulator of embryogenesis. Required for the speciation of cotyledon identity and the completion of embryo maturation.	[71]
LS7ATPR1	ACGTCATAGA ACGACaaata AGGTCattaa	−443, 1164, 1287 (+)	Salicylic acid responsive element	[83,84,85,86]
OSE2ROOTNODULE	AAGAG	−1499 (+)	Organ-specific element (OSE) characteristic of the promoters activated in infected cells of root nodules	[87,88,89]
POLLEN1LELAT52	AGAAA	−441, 662, 702, 756, 896, 948, 1170, 1346 (+)	Specific expression in pollen	[90,91]
RAV1	tctCAACAtatc	−38, 93, 97, 219, 239, 597, 827, 843, 948, 1079, 1229, 1441, 1539 (+)	Ethylene-responsive transcription factor.	[92,93]
RHERPATEXPA7	TCACGa	−812	Right part of RHEs (Root Hair-specific cis-Elements)	[94]
WRKY71OS	TGAC	−459, 724, 1261, 1303, 1527, 1541 (+)	Transcriptional repressor of the gibberellin signaling pathway	[95,96]
W-box	TTGAC	−460, 1528 (+)	WRKY protein-binding site	[97,98,99,100]
WRKY	tcGTCAAcaa	−313, 569, 829 (+)	WRKY DNA-binding protein 61	[100]
WUSAT (WUS)	TTAATcc	−931 (+)	**Protein WUSCHEL.** Plays a central role during early embryogenesis, oogenesis, and flowering. Required to specify stem cell identity in meristems such as shoot apical meristem (SAM)	[101]
ATWOX13	ttTAATTact	−470 (+)	WUSCHEL-related homeobox 13. Transcription factor, which may be involved in developmental processes	[70]

REs shown in bold font are discussed in the text.

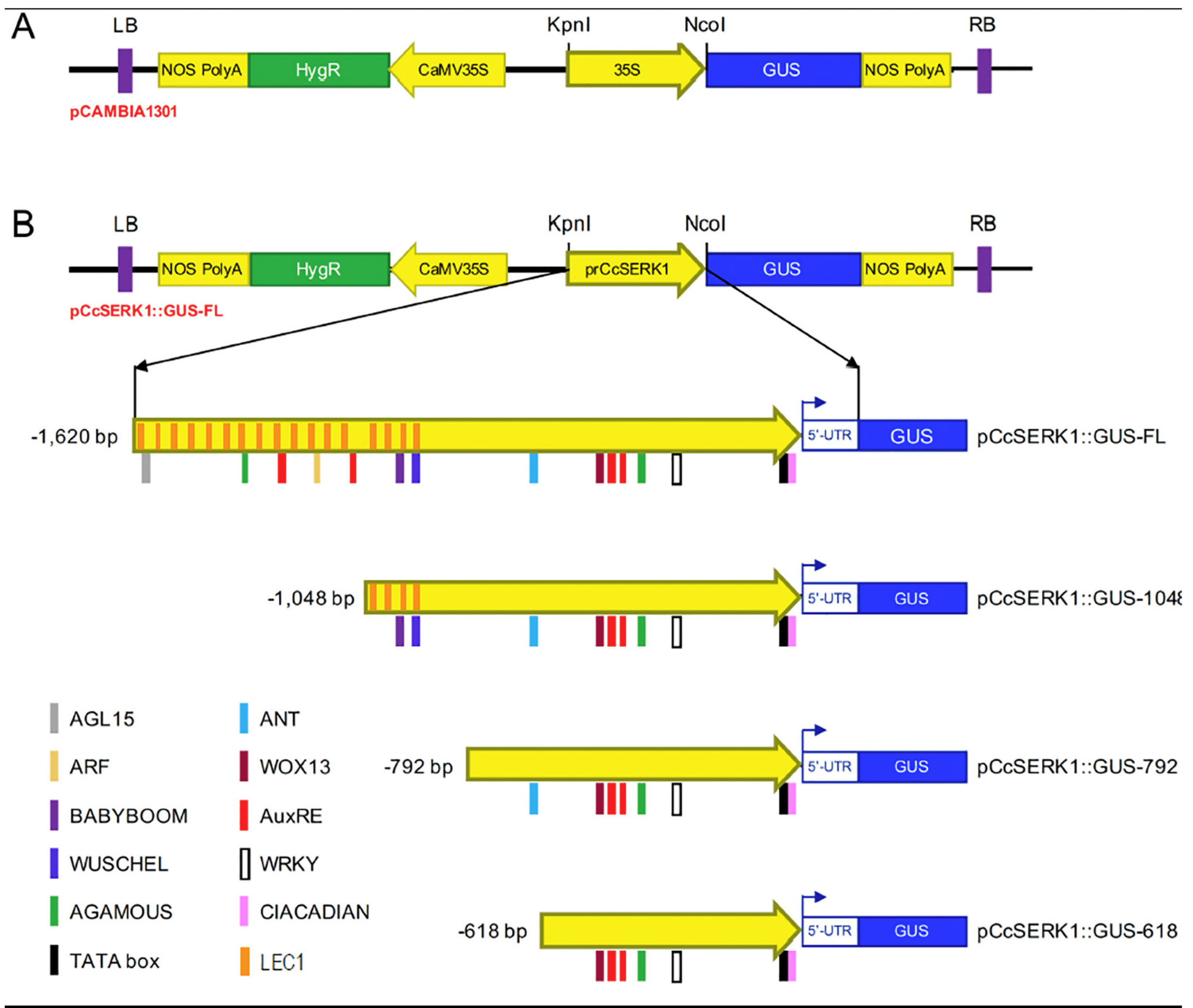

Fig. 2. Construction of expression vectors. A. pCAMBIA1301 vector taken as the positive control. B. DNA segments of different length from the *CcSERK1* promoter (−1620, −1048, −792, and −618) were linked to pCAMBIA1301, with the CaMV 35S promoter removed using PCR to obtain pCcSERK1::GUS-FL, pCcSERK1::GUS-1048, pCcSERK1::GUS-792, and pCcSERK1::GUS-618, respectively.

detected in central veins or pro-embryogenic mass when measured at 25 and 35 DAI; however, GUS activity was clearly detected in embryo structures when analyzed at 50 and 60 DAI. The activity patterns displayed by pCcSERK1::GUS-792 are identical to those observed with pCcSERK1::GUS-FL and pCcSERK1::GUS-1048 at these stages (Fig. 3, Fig. 4). When leaf explants were transformed with pCcSERK1::GUS-618, there was no significant GUS activity in any structure independent of the embryogenesis stage analyzed (Fig. 3, Fig. 4).

SERK1 promoters from other species have been used to study *SERK1* gene expression patterns through plant development. In *A. thaliana*, a 2-kb genomic DNA sequence located upstream of *AtSERK1* was fused to the *uidA* reporter gene to investigate *AtSERK1* expression *in planta*.It was demonstrated that *AtSERK1* is expressed in reproductive tissues and embryo tissues up to the heart stage, in different types of companion cells in the primary root tips, and the procambial cells of the inflorescence stem base [30,32]. A similar reporter system using 1500 bp of the *Medicago truncatula SERK1* promoter (*MtSERK1*) showed GUS activity in tissues with a high differentiation activity (meristems, junctions between different organs, and procambial cells). During the establishment and development of somatic embryogenesis, GUS activity was detected in the leaf explant edges and veins by 2 d after somatic embryogenesis onset, and then, it diminished until callus formation. When somatic embryos are formed, GUS activity was highly detected in the first embryo structures, up to the heart stage and then diminished later in the last stages [31].

3.3. Identification of cis elements in the CcSERK1 promoter

Among the different homeotic REs found in the *CcSERK1* promoter, AGL15 and WOX13 seemed to be dispensable for *uidA* expression in the *C. canephora* embryogenic stages analyzed. In other species, AGL15 seems to play an important role during somatic embryogenesis [46, 47]. In *A. thaliana,* AGL15 was identified as a SERK1-protein complex component [48]. In addition, analysis of the *MtSERK1* nucleotide sequence revealed the existence of one RE for AGL15; this result and the fact that both *SERK1* and *AGL15* homologs are induced after auxin treatment imply that they could have a close relationship in the embryogenesis signal transduction pathway [49,50,51]. However, the location of AGL15 (−1613 bp) and WOX13 (−468 bp) within regions that are dispensable for early or late expression of the *uidA* reporter suggests that they should not be involved in the control of *CcSERK1* expression during the development of *C. canephora* somatic embryogenesis.

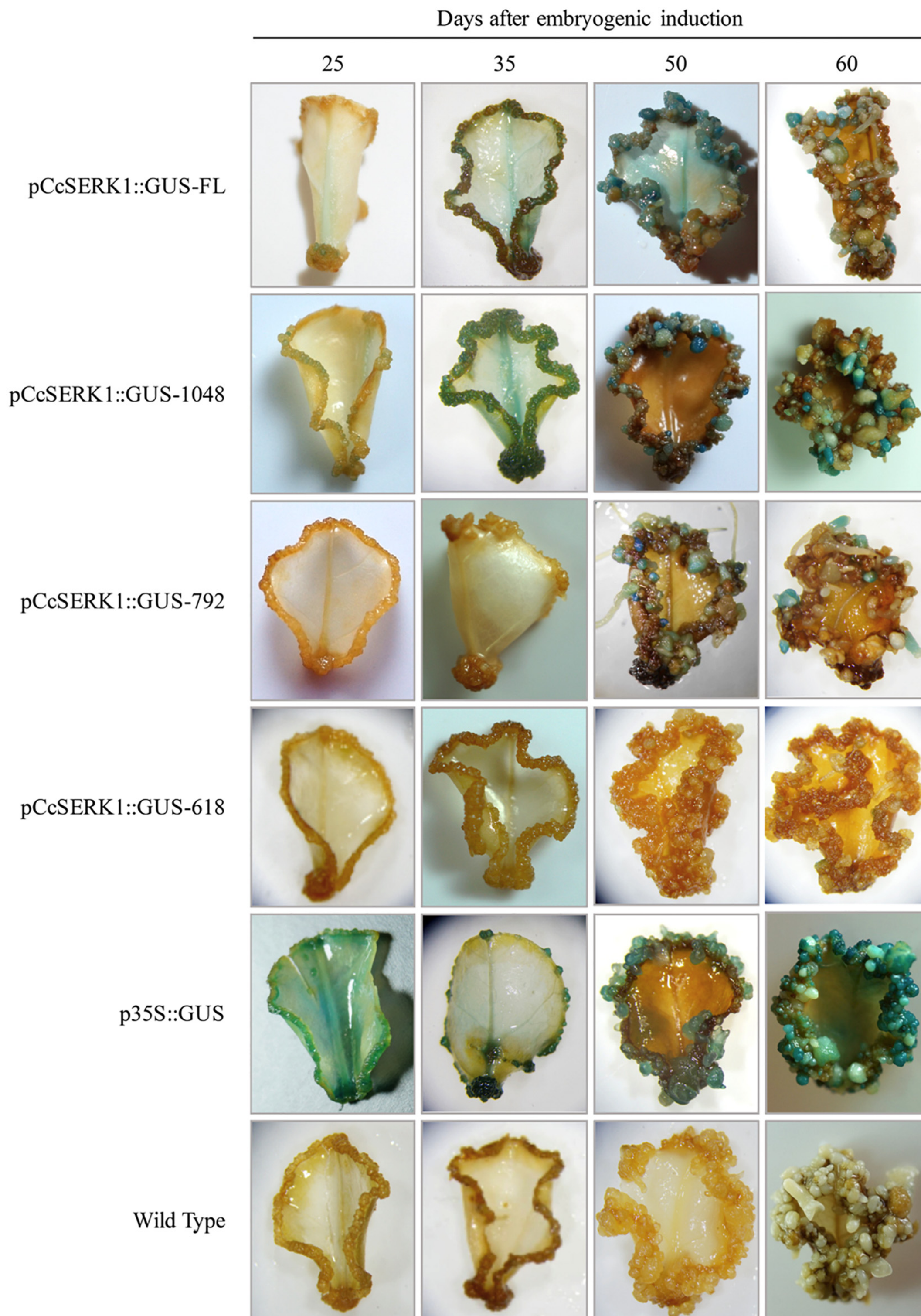

Fig. 3. Promoter activity in transformed embryogenic leaf explants of *C. canephora*. *uidA* reporter gene expression driven by different segments of the *CcSERK1* promoter or the 35S promoter was evaluated in transformed leaf explants collected at different stages of the *C. canephora* somatic embryogenesis process. *uidA* expression was evaluated in nontransformed leaf explants as the negative control. The experiment was repeated three times, and representative images are shown.

BBM (−963 bp) and WUS REs (−931 bp) are located within the −1048-bp and −972-bp region (Fig. 2); when this region was eliminated, GUS activity was eliminated from the central veins and the pro-embryogenic mass at early stages of embryogenesis, but it was still present in the different embryo structures that develop at late stages. These results indicate that either BBM or WUS or both REs are required for the early expression of *CcSERK1* during early embryogenesis, when the pro-embryogenic mass is differentiating, but they are not necessary at late stages when the embryogenesis process has been triggered and cell differentiation is leading to different embryo structures. Reports in the literature indicate that when BBM or WUS homologs are overexpressed ectopically, they promote a vegetative to embryogenic fate [14,17], which implies that they have a crucial function during the somatic to embryogenic cell differentiation.

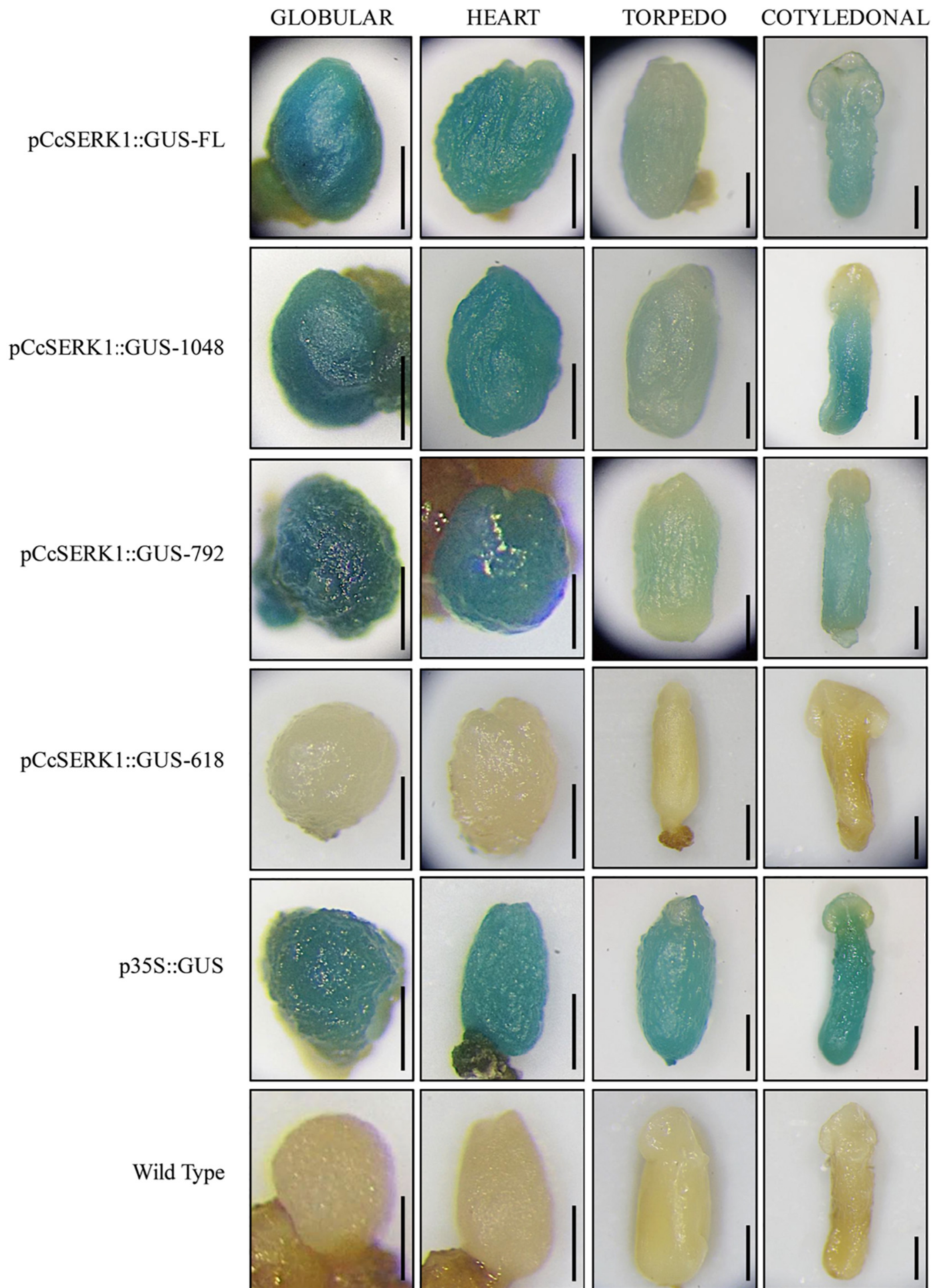

Fig. 4. Promoter activity in transformed embryo structures of *C. canephora*. *uidA* reporter gene expression in globular, heart, torpedo, and cotyledonal embryos present at 60 d after embryogenic induction, isolated from transformed leaf explants in which *uidA* gene expression was driven by different segments of the *CcSERK1* promoter or the 35S promoter. The images are representative of embryos collected from three independent transformation experiments.

GATA, ARR1AT, and ANT REs are present in the region between −618 and −792 bp of the *CcSERK1* promoter (Fig. 2), a region required for *uidA* reporter expression once embryo development has started but not before the embryogenesis onset. ANT is a critical factor maintaining the meristematic competence of cells during organogenesis [35]. It has been proposed that transcription factor-binding sites in the promoter regions of *SERK1* genes, but not those present in *SERK2* genes, evolved exclusively for its spatial expression during SE [52]; specifically, authors mentioned that the ANT transcription factor-binding site in the *AtSERK1* promoter is prominently more accessible than that in the *AtSERK2* promoter. Because the ANT binding site is also present in the regulatory sequence of the *MtSERK1* gene, they postulated that ANT transcription factors evolved toward a precise role in the acquisition of SE across

species. Results from the present work suggested that the ANT-binding site in the *CcSERK1* promoter is needed for *SERK1* expression only after embryo development has started.

On the other hand, even though *CcSERK1* promoter has multiple LEC1 REs distributed along the nucleotide sequence, most of them (13 out of 17 REs) are located between the −1620-bp and −1048-bp regions (Fig. 2), a region that seemed to be dispensable for the induction of the *uidA* gene before or after the embryogenesis onset. These results suggest that either LEC1 REs have a weak influence on the *CcSERK1* expression occurring at early embryogenesis stages or the remaining four LEC1 REs (Fig. 2) are sufficient to drive *CcSERK1* expression. This hypothesis is supported by the fact that *CcSERK1* overexpression repressed *LEC1* and induced *BBM* and *WUS* during the first 10 d of somatic embryogenesis in *C. canephora* [40]. In addition, even though the ectopic expression of *LEC1* confers embryonic characteristics to seedlings and is sufficient to induce somatic embryo development from vegetative cells [53], LEC1 is needed late in embryogenesis for the acquisition of desiccation tolerance, the accumulation of storage reserves [54], the specification of cotyledon identity, and the completion of embryo maturation [55].

The DNA sequence of the *CcSERK1* promoter also contains REs for ethylene, cytokinins, auxins, gibberellic acid, and ABA. The relationship between *SERK1* expression and PGR balance has been studied. Expression of *Ananas comosus AcSERK1* was induced in nonembryogenic calli by the addition of jasmonic acid (JA) and salicylic acid (SA) [56]. *Oryza sativa OsSERK1* was induced in leaves by the external addition of SA, JA, benzothiadiazole (BTH), and ABA. *Arabidopsis AtSERK1* was induced in different tissues in response to the external addition of 2,4-dichlorophenoxyacetic acid (2,4-D) [32]. In the *C. canephora* somatic embryogenesis model, embryogenic competence is achieved in donor plants by a 14-day preincubation in a medium supplemented with NAA and KIN. Although further embryogenic differentiation is induced by the addition of BA to the culture medium, no embryos are produced in the absence of the auxin pretreatment. In the present work, a functional evaluation of the auxin REs present in the *CcSERK1* promoter was evaluated in the *N. benthamiana* heterologous system. This system has been extensively used to validate heterologous promoters from different species including the genus *Coffea* [2,3,4,5,6,7]. Results demonstrated that the *CcSERK1* promoter is physiologically responsive to auxins because exposure to different concentrations of NAA or 2,4-D increased GUS staining in transgenic *N. benthamiana* leaves (Fig. 5) and stems (Fig. 6) explants that were transformed with the wild-type *CcSERK1* promoter (control plants) in a time- and dose-dependent manner; however, no *uidA* staining was detected when *N. benthamiana* explants exposed to auxins were transformed with the *CcSERK1* promoter in which the four AuxREs [57,58] were mutated (Fig. 5, Fig. 6). In this heterologous expression system, basal GUS activity was detected at time zero of the auxin addition in explants transformed with the wild-type *CcSERK1* promoter, but no GUS activity was detected when the mutated *CcSERK1* promoter was used. Thus, it is possible that the observed basal GUS activity was produced by the action of endogenous auxins in the explant.

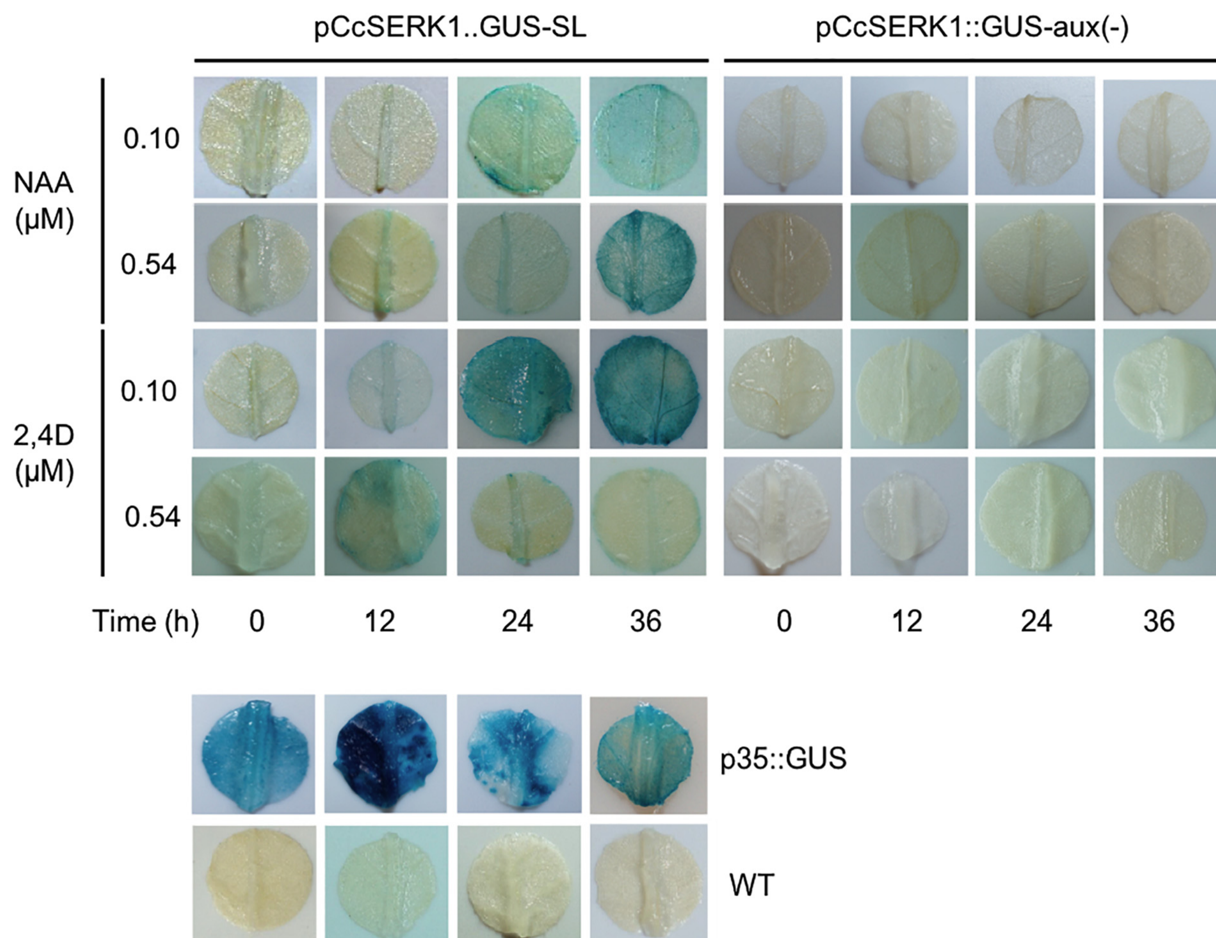

Fig. 5. Auxin regulation of the *CcSERK1* promoter activity in *N. benthamiana* leaves requires AuxREs. *uidA* gene expression driven by the native promoter (pCcSERK1::GUS-FL), the auxin-mutated *CcSERK1* promoter [pCcSERK1::GUS-aux(−)], or the 35S promoter (p35S::GUS) was evaluated in *N. benthamiana* transgenic leaf explants that were exposed to different concentrations of NAA and 2,4-D (0.1 or 0.54 μM) for different time periods (0, 12, 24, and 36 h). *uidA* gene expression was evaluated in wild-type leaf explants as the negative control. The experiments were repeated three times, and representative images are show.

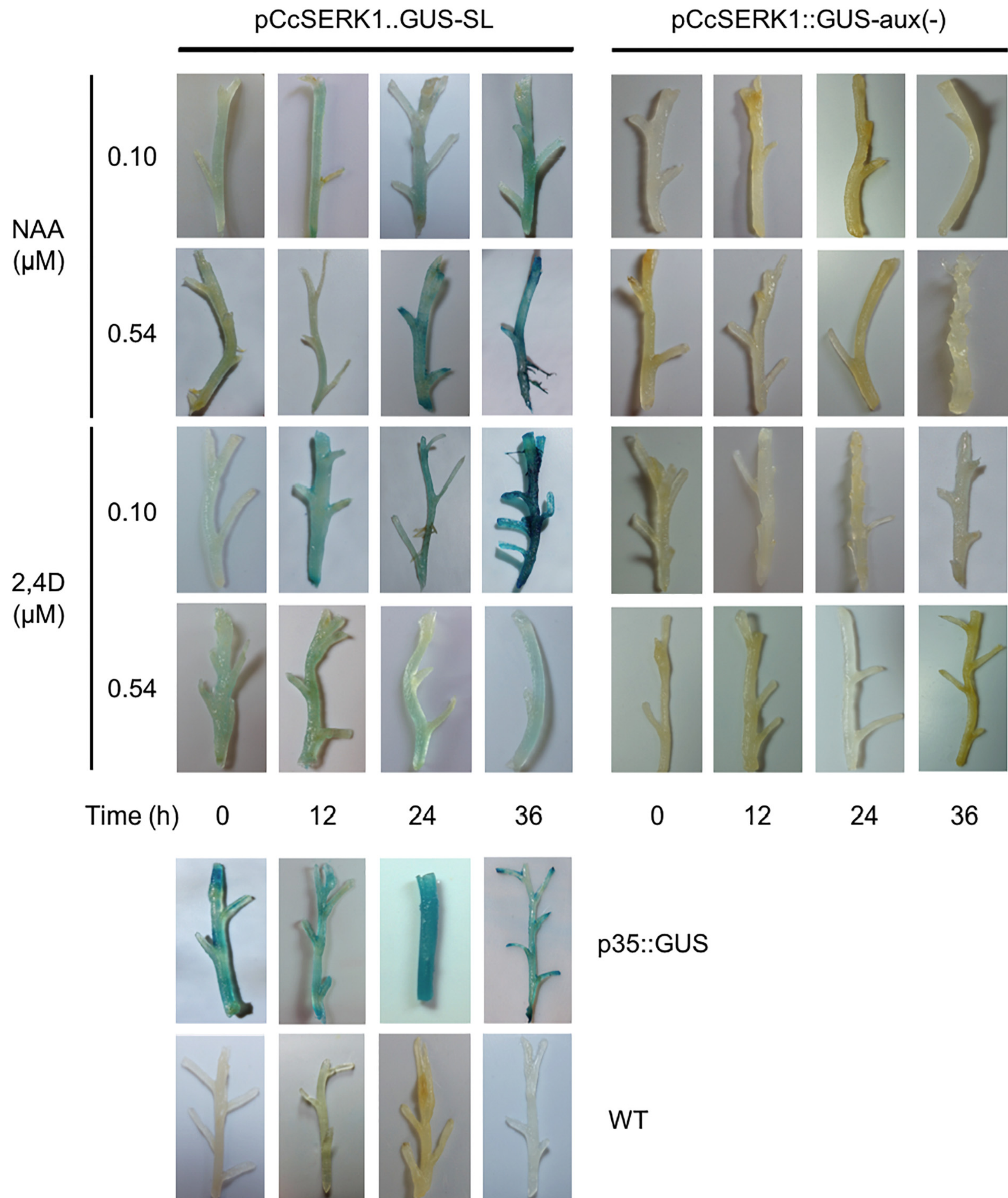

Fig. 6. Auxin regulation of the *CcSERK1* promoter activity in *N. benthamiana* stems requires AuxREs. *uidA* gene expression driven by the native promoter (pCcSERK1::GUS-FL), the auxin-mutated *CcSERK1* promoter [pCcSERK1::GUS-aux(−)], or the 35S promoter (p35S::GUS) was evaluated in *N. benthamiana* transgenic stem explants. Experiments were conducted as described in Fig. 5. The experiments were repeated three times, and representative images are shown.

Positive effects of cytokinins and auxins on *SERK1* expression during plant development have been demonstrated in other species [32]. Nolan et al. [31] found that the external addition of NAA increased *MtSERK1* expression, but the addition of BA alone had no effect; nonetheless, the simultaneous addition of NAA and BA caused a twofold increment of *MtSERK1* expression compared to auxin application. In *Cattleya maxima* (Lindl.), addition of thidiazuron (TDZ, a compound with cytokinin-like effects) increased the expression of *CmSERK1* in the tip and the wound of detached leaves, but the addition of NAA alone induced *CmSERK1* only in the leaf wound site [59]. In *Citrus sinensis* (Valencia sweet orange), *CitSERK1* is induced by NAA and 2,4-D during the first stages of embryogenic callus development; thereafter, expression of *CitSERK1* decreased, thus suggesting that *CitSERK1* expression is triggered by auxins, and it declined later when calli become insensitive to auxins [60]. In *Arabidopsis*, root treatment with 2,4-D enhanced the transcription of *AtSERK1* and produced a moderate increment in SERK1 protein only in the vascular axes; in hypocotyls, the addition of 2,4-D produced an increment of the cell size and

proliferation of the parenchymal pericycle [30]. In *Triticum aestivum*, addition of 2,4-D to leaf explants induced somatic embryogenesis and increased transcription levels of the *TaSERK1* homolog; the authors proposed that the 2,4-D-mediated induction of somatic embryogenesis in wheat is mediated by the SERK pathway [61].

4. Conclusion

Indirect evidence obtained in the present work demonstrated that the −1048-bp region of the *CcSERK1* promoter is functional and sufficient to direct gene expression during somatic embryogenesis, with patterns identical to those reported for other *SERK1* homologs. Interestingly, the region containing BBM and WUS REs is required for *CcSERK1* expression before the onset of the embryogenic differentiation but is not required once embryo structures are formed. By contrast, a DNA sequence containing the ANT RE controls the expression of *CcSERK1* in developing embryos but not before the cell-to-embryo differentiation. These findings suggest that *C. canephora* WUS, BBM, and ANT transcription factors orchestrate the time and spatial expression patterns of *SERK1* genes in somatic embryogenesis.

Identification of the promoter REs responsible for gene expression that occurs closely before the triggering of cell differentiation constitutes a valuable tool to evaluate the potential of *SERK1* as a molecular marker of embryogenesis. For example, the pCcSERK1::GUS reporter system can be used to precisely monitor *CcSERK1* expression during embryo organ differentiation. pCcSERK1::GUS can also be used as a reporter system in experiments designed to identify the external signal that induces *SERK1* expression, as well as to identify intermediates in the signal transduction cascade that are responsible for transducing the signal generated by SERK1 and its co-receptor(s).

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary material

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