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The regulation of the SARK promoter activity by hormones and environmental signals

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ABSTRACT

The *Senescence Associated Receptor Protein Kinase* (P_{SARK}) promoter, fused to *isopentenyltransferase* (*IPT*) gene has been shown to promote drought tolerance in crops. We dissected P_{SARK} in order to understand the various elements associated with its activation and suppression. The activity of P_{SARK} was higher in mature and early senescing leaves, and abiotic stress induced its activity in mature leaves. Bioinformatics analysis suggests the interactions of multiple *cis*-acting elements in the control of P_{SARK} activity. In vitro gel shift assays and yeast one hybrid system revealed interactions of P_{SARK} with transcription factors related to abscisic acid and cytokinin response. Deletion analysis of P_{SARK}, fused to *GUS*-reporter gene was used to identify specific regions regulating transcription under senescence or during drought stress. Effects of exogenous hormonal treatments were characterized in entire plants and in leaf disk assays, and regions responsive to various hormones were defined. Our results indicate a complex interaction of plant hormones and additional factors modulating P_{SARK} activity under stress resulting in a transient induction of expression.

1. Introduction

A complex interplay of DNA and protein interactions activates plant promoters defining the timing and extent of gene expression. From a biotechnological standpoint, these properties are of paramount importance to achieve success in genetic engineering. Most of the genes engineered into crops to improve abiotic stress tolerance were driven by constitutive promoters [1]. Although some of these attempts have been effective in the production of transgenic plants with enhanced stress tolerance, the constitutive expression of candidate genes is not always desirable because of pleiotropic effects on growth and development of the transgenic plants under control conditions. These aspects become even more relevant with the manipulation of key regulatory genes such as transcription factors or enzymes mediating the synthesis of plant growth regulators (reviewed by [1]). A solution to this problem is the use of stress-inducible promoters allowing turning on transgene expression at certain stages of development and during abiotic stress episodes. However the design of such promoters is complicated [2] and only a small number of stress-inducible promoters are available and effective [3].

Water stress can induce early leaf senescence and leaf abscission, an adaptive survival mechanism aiming to decrease plant canopy size and reduce water loss [4]. Senescence-associated processes are highly regulated [4–6] and result in the remobilization of nutrients and carbohydrates from the senescing organs (source) to the developing grains/fruits (sink) (reviewed by [6]). Elevated cytokinin (CK) levels can inhibit plant senescence. Several studies have reported that senescence could be delayed in transgenic plants expressing *isopentenyltransferase* (*IPT*), a gene involved in CK biosynthesis, using different promoters ([7] and references therein). Moreover, the expression of *IPT* under the control of the promoter of *SARK* (P_{SARK}, Senescence Associated Receptor Protein Kinase), a maturation- and drought-induced promoter, was shown to confer drought tolerance in several dicot and monocot plant species [8–12].

The *SARK* gene was isolated from bean (*Phaseolus vulgaris* cv. Bulgarian) and was shown to be expressed during the later stages of leaf maturation, prior to the development of senescence symptoms in the plant [13]. The main advantage that P_{SARK} had over other promoters is the moderate levels of CK produced and the

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fine-tuning of the expression maintaining a balance between the positive effects of plant hormones for acquiring stress tolerance and the negative effects on growth and development that are associated with increased CK amounts (reviewed by [1]).

In this current study, we describe the functional analysis of P_{SARK}. A series of transgenic tobacco lines carrying partial promoter regions, fused to the *GUS*-reporter gene was generated. Expression analysis was performed in response to natural senescence, abiotic stresses and hormonal treatments, aiming to identify regions in the promoter involved in the activation or repression of transcription.

2. Material and methods

2.1. Plasmid construction

The binary vector *pBI121*, carrying a T-DNA containing the *GUS* gene (GenBank accession number AF485783) was used for all plasmid constructs after the removal of *CaMV35S* promoter. The promoter sequence used was exactly the same used for the P_{SARK}:IPT construct (Supplemental Fig. S1). Deletion constructs were designed using promoter clones as template. The fragments were produced by PCR using oligonucleotide primers including a *Hind*III restriction site at the 5′ end of the forward primer and a *Bam*HI restriction site at the 3′ end of the reverse primers (Table S1). For the 3′-deletion series, chimeric promoters composed of the truncated promoter region followed by the *CaMV35S*-46 minimal promoter (to supplement a putative lack of a TATA box) were used. Two additional constructs, S8 having a deletion in the middle of the P_{SARK}, and B2 containing two single nucleotide mutations in two MYB1AT *cis*-elements, were generated by modified Splicing by Overlap Extension (SOEing) PCR [14]. The promoter region of all constructs was confirmed by sequencing. The *CaMV35S*-46 minimal promoter was fused to the *GUS* reporter gene and used as a negative control. To identify *cis*-regulatory elements in the promoter, *cis*-element search programs at PLACE (http://www.dna.affrc.go.jp/PLACE/info.html, PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and NSITE (http://linux1.softberry.com/cgi-bin/programs/promoter/nsite.pl) were used.

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2.2. Plant transformation

To produce stable transformants, the vectors were electroporated into the *Agrobacterium tumefasciens* EHA 105 strain, which was then used for transformation of tobacco (*Nicotiana tabacum* cv. SR1) plants by the leaf disc transformation method [15]. Kanamycin-resistant transgenic plants were selected on medium containing 100 mg L^{-1} kanamycin and 400 mg L^{-1} cefotaxime, shoots were rooted in rooting medium containing 100 mg L^{-1} kanamycin. Each individual transformant was checked for T-DNA insertion by PCR analysis. Promoter regions of at least one tobacco line of each construct were sequenced to ensure correct insertion and that no rearrangements occurred in the promoter-*GUS* region.

2.3. Plant growth conditions

Experiments were performed in T_0, T_1 and T_2 plants. T_0 plants were transferred to 1 L pots containing soil (Metro-Mix 200, Sun Gro) in controlled greenhouse (1200 μmol of photons m^{-2} s^{-1}, 16 h photoperiod, 28–30 °C). T_1 and T_2 seeds were germinated on media containing 50 mg L^{-1} kanamycin. Seedlings resistant to kanamycin were transferred to 4 L pots containing a mixture of 50% sand and 50% peat. Plants were irrigated three times a day with a total of 1 L of fertilized water containing N 155.49 mg L^{-1}, P 15.4 mg L^{-1}, K 173 mg L^{-1}, Ca 126.5 mg L^{-1}, Mg 41.3 mg L^{-1}, S 54 mg L^{-1}, Fe 0.95 mg L^{-1}, Cu 0.037 mg L^{-1}, Mn 0.15 mg L^{-1}, Mo 0.03 mg L^{-1} and Zn 0.03 mg L^{-1}. T_0 and T_1 transgenic plants were evaluated for the natural senescence experiments. Leaf discs from the 6th leaf (from top to bottom) were collected at flowering and immediately frozen in liquid nitrogen for protein extraction and fluorometric determination of GUS activity. For drought experiments, watering was stopped when the plants were at the 6th leaf stage of growth or at flowering time (as indicated) and discs were collected daily from the 5th leaf. In experiments were CK was sprayed to the leaves, 24 h after water was withheld, the 5th leaf of the plants (T_0 and T_2 generations) was sprayed with 10 μM 6-benzylaminopurine, 0.1% Tween 20 or double distilled water, 0.1% Tween 20. After 24 h, leaf discs were collected for GUS activity analysis. For the hormone treatment experiments, discs from the 5th leaf of flowering tobacco plants were collected in petri dishes containing distilled water. The discs were acclimated for 1–2 h and transferred to water or to different solutions containing: abscisic acid (ABA) (100 nM, 100 μM), or 6-benzylaminopurine (5 μM, 100 μM) and incubated in the dark at 30 °C. Individual discs were collected every 24 h and immediately frozen in liquid nitrogen for protein extraction and fluorometric GUS activity analysis. For the evaluation of young plants, kanamycin-selected T_2 seedlings were transfer to 1 L pots containing soil (Metro-Mix 200, Sun Gro) and grown as described above. Twenty days after transfer, drought, salt (50 mM NaCl – daily irrigation), or cold (4 °C) conditions were applied for 4 days. Plants were then collected and GUS activity was evaluated by histochemical analysis. Leaf discs from completely expanded leaves were transferred to petri dishes containing water or NaCl 50 mM (under 12 h:12 h light/dark regime) and evaluated daily for 5 days.

2.4. Histochemical and fluorometric determination of GUS activity

For histochemical staining of GUS, fresh samples were subjected to the X-Gluc solution [16]. After overnight incubation at 37 °C, stained samples were bleached by ethanol series. GUS activity was analyzed quantitatively in tissue extracted from 27 mm^2 leaf discs (in 300 μL of 50 mM sodium phosphate buffer pH 7, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% sodium N-laurosylsarcosine, 0.1% Triton X-100) using 4-methyl umbelliferyl glucuronide (4-MUG) as the substrate in the β-Glucuronidase Fluorescent Reporter Gene Activity Detection Kit (Sigma, USA), 10 μL of protein extract was added to 10 μL of substrate and incubated for 60 min. Ten microliters of the reaction was then transferred to a reading plate and the reaction was stopped by the addition of 200 μL of sodium carbonate 200 mM. The protein concentration of tissue homogenates was estimated by the Bradford assay [17].

2.5. Gene expression analysis

For rice plants, samples of the last fully exposed leaf, from transgenic P_{SARK}*::IPT* or wild type plants were collected throughout their entire life-cycle, in order to analyze the P_{SARK} expression patterns [12]. For analyzing the expression of *Os03g0315400* (Myb-like gene) and *Os03g0224200* (ARR1-like gene), flag leaf samples from P_{SARK}*::IPT* or wild type plants were collected during the drought treatments. Total RNA was extracted from plant tissue using RNeasy®Mini kit (Qiagen, Valencia, CA) and quantified using a Nanodrop ND-1000 spectrophotometer. First strand cDNA was synthesized from 1 μg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Quantitative RT-PCR was performed on the StepOnePlus™ (Applied Biosystems, Foster City, CA), using SYBR® GREEN. The gene-specific primer pairs are described in Table S1. The $2^{-\Delta\Delta}CT$ method [18] was used to

normalize and calibrate transcript values relative to the endogenous rice transcription elongation factor (*TEF*) gene as described in Peleg et al. [12].

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A similar procedure was used for tobacco plants. Samples of middle leaves from P_{SARK}::*IPT*/P_{SARK}::*GUS*, P_{SARK}::*IPT*/*S8*::*GUS*, P_{SARK}::*GUS*, *S8*:*GUS* or wild-type plants before and under drought conditions were collected, RNA was extracted and cDNA was synthesized as described above. Expression levels of *GUS* and *AB032540* were analyzed by RT-PCR. The values were normalized and calibrated to the endogenous *GAPDH* gene.

GUS expression in different organs of transgenic P_{SARK}::*GUS* plants was evaluated by semi-quantitative RT-PCR. Samples of root, stem, flower and leaves from P_{SARK}::*GUS* tobacco plants at flowering stage during drought conditions were collected, RNA was extracted and cDNA was synthesized as described above. The PCR reaction was performed using the same primers described early for *GUS* and *GAPDH*. The amplicons were visualized in 1% agarose gel.

2.6. Yeast one hybrid assays

Cis-elements, MYB1AT and ARR1AT (primers described in supplementary Table S1) were inserted as concatamers (three units) into the bait vector PHIS2. Full length cDNA of *MYB*- and *ARR*-like genes were amplified from tobacco (*AB032540*, *AB028649.1*) and rice cDNA (*AK120551*, *AK111899*) and cloned into the vector PGADT7-Rec2. Yeast one hybrid was performed following Matchmaker™ One-Hybrid Library Construction & Screening Kit (BD Biosciences Clontech, Palo Alto, CA, USA).

2.7. Gel shift assays

Detection of sequence specific DNA binding proteins was performed using the DIG Gel Shift Kit (Roche Applied Science, Indianapolis, IN, USA). Nuclear proteins were extracted from middle leaves of transgenic P_{SARK}::*IPT* tobacco plants (lines 4–24) [10] and from flag leaf from transgenic P_{SARK}::*IPT* rice plants (line 5 T) under drought conditions. DNA probes for ARR1AT461 and CPBCSPOR455 were obtained using primers described in Table S1.

3. Results and discussion

3.1. P_{SARK} activity pattern

The *SARK* gene was initially characterized as expressed during the onset of senescence in *P. vulgaris* [13]. In order to gain more information on its activation pattern and the factors affecting it, we generated transgenic tobacco plants expressing P_{SARK} fused to the *GUS* reporter gene (Fig. 1). The transgenic P_{SARK}::*GUS* plants displayed increased GUS activity during leaf maturation and early senescence (Fig. 1A), confirming previous results reported in bean [13]. At the flowering stage, expression was also detected in the stem, roots and at lower levels in the flowers (Supplemental Fig. S2), further supporting that P_{SARK} is active in all major plant organs [12]. The expression of P_{SARK}::*GUS* in the leaves was significantly higher at the flowering stage as compared to six-leaf stage plants, grown under control conditions (Fig. 1E). A similar age-related P_{SARK} activity was also confirmed in rice plants by analyzing the expression of *IPT* gene driven by P_{SARK} [12]. Almost no expression was observed in the leaves at tillering stage (i.e. vegetative growth) and a significant increase in expression was detected at the pre-anthesis stage (i.e. reproductive phase) (Fig. 1F). Therefore, the age- related behavior of P_{SARK} is conserved in monocots and dicots. Leaf senescence is an age-dependent programmed process with substantial degradation of macromolecules and subsequent mobilization to seeds in annual plants (reviewed by [19]). We have shown previously that P_{SARK} induced the expression of *IPT* in response to water deficit stress [10,12]. In agreement, increased expression of *GUS* was observed under drought conditions in the middle leaves of flowering transgenic P_{SARK}::*GUS* tobacco plants (Figs. 1C and D). The P_{SARK} responsiveness to stress was dependent on the plant

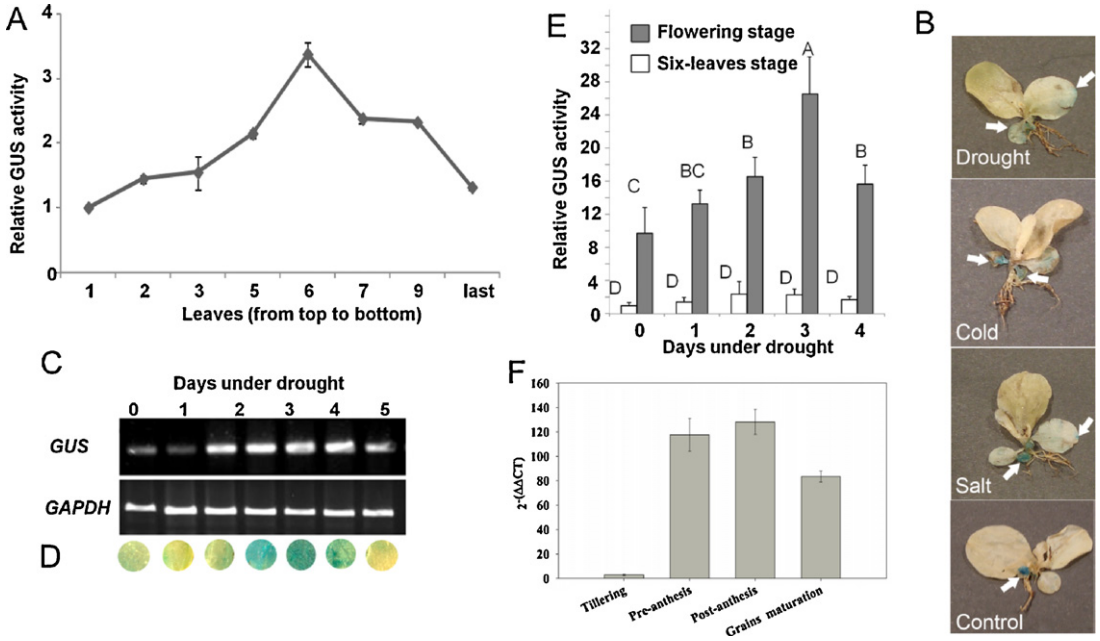

Fig. 1. *SARK* promoter responsiveness to senescence and abiotic stress. (A) GUS activity in flowering tobacco leaves along the stem (leaf 1 is the youngest). (B) Histochemical staining of GUS activity in 25 days old plants exposed to drought, salt or cold for 4 days. Arrows indicate regions of expression in cotyledonary leaves and at the first true leaves. (C) Expression of *GUS* gene and (D) GUS activity in leaf discs of tobacco middle leaves at flowering stage under drought; (E) relative GUS activity in middle leaves under drought, comparing plants at their sixth-leaf stage and plants at the flowering stage. Values are the mean ± SD ($n = 5$). Same letters indicate no significant differences at $P \leq 0.05$ by Tukey test; (F) relative *IPT* expression (qPCR) in transgenic P_{SARK}::*IPT* rice plants at different developmental stages. Values calculated and normalized using transcription elongation factor gene as internal control. Values are the mean ± SD ($n = 6$).

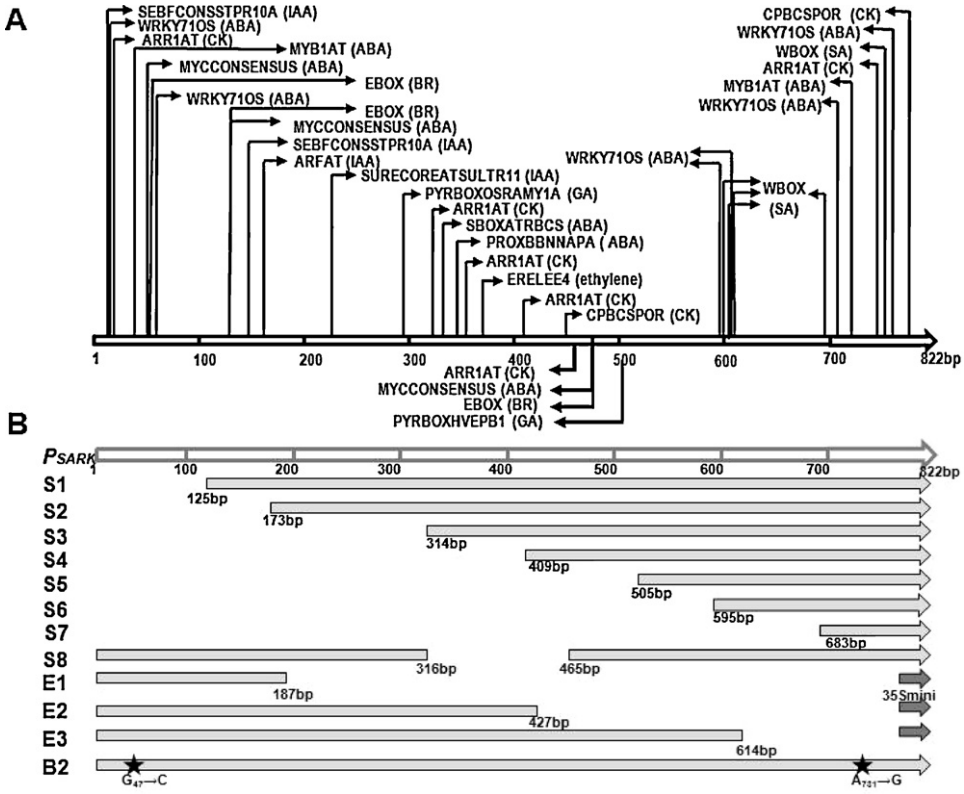

Fig. 2. The *SARK* promoter. (A) Putative *cis*-elements possibly involved in the response to hormones. (B) Schematic diagram of the deletion derivatives used to express *GUS* in transformed tobacco. The dark gray arrows represent the 35S minimum promoter.

development stage. At the flowering stage increased GUS activity was observed in middle leaves in response to drought, but not in the middle leaves of younger plants (6-leaf stage). Moreover, total GUS activity was lower in 6-leaf stage plants than at flowering plants (Fig. 1E), and P_{SARK} was not efficient in driving *GUS* expression in response to drought, salt or cold stress in 25-day old seedlings (Fig. 1B). After 4 days of irrigation with 50 mM NaCl, only weak GUS activity was observed in the first two true leaves and after 4 days under low temperature (4 °C) only a slight increase in GUS activity was observed.

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3.2. Cis-acting element analysis of the P_{SARK} promoter region

To characterize *cis*-acting elements in P_{SARK}, a motif search was carried out for the 822 bp DNA fragment previously isolated from bean, using the PLACE database [20], the NSITE program (http://www.softberry.com/) and the PlantCARE database [21]. Several regulatory motifs that are potentially related to phytohormones, such as ABA, ethylene, auxin (IAA), CK, gibberellins (GA), salicylic acid (SA) and brassinosteroids (BR) were identified in the *SARK* promoter sequence (Fig. 2A). The sequence also contains 11 putative CAAT boxes, from which 4 are CCAAT box (enhancers). In addition, the sequence contains regulatory motifs related to environmental cues, such as 14 putative DOFCORE, which are related to C:N partition response; 21 CACT, the key component of Mem1 (mesophyll expression module1) [22]; two GT1-box motifs related to salt response [23]; one ATHB-2 (homeobox) related to shade avoidance [24], one ANAERO1CONSENSUS and one ANAERO3CONSENSUS, both related to anaerobic response [25], three MYC consensus related to cold response, three BIHD1 related to disease response [26]; and 8 *cis*-elements that are specific for root elongation zones [27] (Supplemental Fig. S3). The complexity of P_{SARK} regulation is highlighted by the high number and diversity of the putative *cis*-elements predicted. Molecular details of how these motifs interact to bring out combinatorial regulation of the promoter are unknown. Complexity may be even higher considering that some of the proteins that bind to *cis*-elements may activate promoters synergistically.

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3.3. Deletion analysis of the P_{SARK}

In an attempt to identify functional *cis*-elements that could be involved in the regulation of P_{SARK} activation and its response to environmental stress, senescence and hormones, we produced a series of 5′ (S1 to S7) and 3′ (E1 to E3) deletion derivatives from P_{SARK} and an additional derivative lacking the middle part of the promoter (S8). These derivatives were fused to *GUS* (Fig. 2B) and used for stable transformation of tobacco plants. To evaluate the regions related to senescence responsiveness, GUS activity was measured in the youngest expanded and the middle mature leaves of the transgenic plants at the flowering stage. The highest level of GUS activity was found in the full-length P_{SARK} construct (Fig. 3). GUS activity decreased in order from E3 to E1 and from S1 to S7, except for S3. These results indicated that the majority of the elements included in the 822 bp are required for the full responsiveness of the promoter to senescence. The S3 construct (removal of 314 bp from 5′) reduced GUS activity by 30%, but the removal of 173 bp from 5′ in S2 reduced the GUS activity by more than 70% (Fig. 3). These results may suggest that the region between 173 and 314 bp contains *cis*-elements that reduced the promoter's induction only in the absence of the first 125 bp. *Cis*-elements also interact with components of the chromatin remodeling machinery in an integrated state

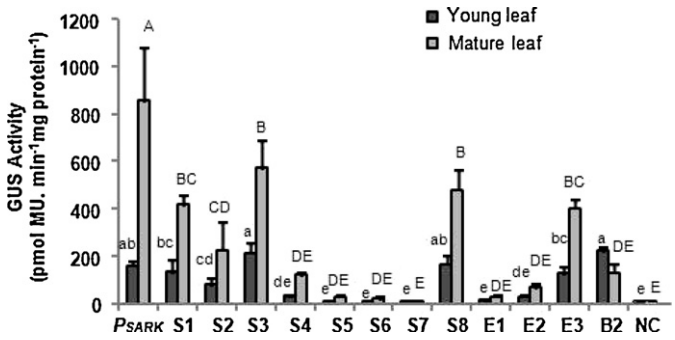

Fig. 3. *GUS* activity in young and mature leaves of flowering transgenic tobacco plants carrying a series of deletion derivatives of P_{SARK}::*GUS*. NC, negative control *CaMV35S*-46 minimal promoter fused to the *GUS* reporter gene. Same letters indicate no significant differences at $P \leq 0.05$ by Tukey test, capital letters compare mature leaves and small letters compare young leaves. Values are the mean ± SD ($n = 5$).

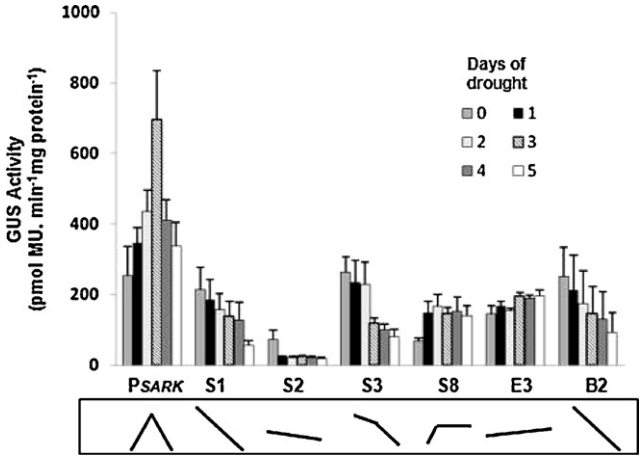

Fig. 4. GUS activity in the middle leaves of flowering transgenic tobacco plants carrying a series of deletion derivatives of the *SARK* promoter in a GUS reporter construction under drought. Values are the mean ± SD ($n = 5$). The schematic diagrams below the *x*-axis represent the patterns of changes in GUS activity during the 6 days of drought treatment.

[2], therefore changes in the motif location and distance from other *cis*-elements may affect the promoter's expression. As seen for the entire promoter, the level of expression in all the deletion constructs was lower in the young leaves, but the expression patterns were similar to that seen in mature leaves.

In response to natural senescence, the plants carrying S1 (removal of 125 bp from 5′) and E3 (removal of 208 bp from 3′) displayed a 50% reduction in GUS activity as compared to the full-promoter (Fig. 3). These two regions have in common at least 7 distinct putative *cis*-elements, among them two MYB1AT sites (47 and 731 bp) (Fig. 2A), which have been shown to be associated to ABA responsive genes, such as *rd22* (*RESPONSIVE TO DESSICATION 22*) [28]. Interestingly, S8 lines, harboring a construct carrying these two MYB1AT elements but lacking the central region of the promoter (bps 316–465), displayed a relatively high expression level (Fig. 3). The S8 deleted region contains among others, three ABA-related putative *cis* elements and one ERELEE4, which has been associated to ethylene response (Fig. 2), that could be required for full response. Removal of either the first 505 bp from the 5′ (S4) or the last 395 pb (E2) reduced the GUS activity to levels similar to the negative control (*CaMV35S*-46 minimal promoter) (Fig. 3). In order to test the effect of these MYB elements, we produced another derivative (B2) bearing two point mutations ($G_{47} \rightarrow C$ and $A_{731} \rightarrow G$) on the two MYB1AT elements present in the P_{SARK} promoter (Fig. 2B). Interestingly, the point mutations caused a reduction of 75% of the promoter activity in the mature leaf, but no effect was observed in the youngest expanded leaf (Fig. 3). These results suggested that at least one MYB1AT element was required for promoter activation under natural senescence, but the presence of only one of these elements was not sufficient for full induction.

To study the stress response of the P_{SARK} *cis*-elements, the transgenic plants containing the different P_{SARK} derivatives were subjected to drought at the flowering stage and the 5th leaf was evaluated. Under drought, the importance of each promoter segment was even more apparent. The data indicated that the complex regulation required all segments of the isolated promoter, and that there were no unnecessary segments, i.e., full activation was only attained in the presence of the full promoter. Plants containing the full promoter reached the highest activity on the 3rd day after the start of the stress, showing a 3-fold increase in GUS activity as compared to the activity before drought. After the 3rd day the promoter's activity returned to control levels (Figs. 1D and 4). The pattern of expression of plants containing the deletion derivatives did not show the induction of GUS activity in response to drought stress (Fig. 4). Only S8 plants showed significant increase in GUS activity upon stress, but the maximum value was similar to that of the P_{SARK} plants before drought. S1, S3 and B2 presented a similar pattern, with a reduction in expression as the stress continued. No similar reduction in the activity was observed in the other large deletion derivative harboring plants, as shown in the diagram in Fig. 4. The similarity among S1, S3 and B2 expression patterns would suggest that the 5′ MYB1AT *cis*-element is required for promoter activation.

In an effort to confirm that the MYB1AT was an active *cis*-element in P_{SARK}, we evaluated the binding between the MYB1AT sequence found in P_{SARK} and *Myb*-like proteins from tobacco and rice in vitro in a yeast one hybrid assay. One *Myb*-like protein from tobacco, AB032540 (GI:27529845), as well as one, *Os03g0315400* (AK120551) from rice (Fig. 5A and B) were able to bind to MYB1AT. Other *Myb*-like proteins were tested but no specific binding to the *cis*-element could be detected (Fig. 5A2 shows AB028649.1). The expression of the genes encoding the proteins that interacted in vitro with the MYB1AT *cis*-elements were evaluated by qPCR in wild-type tobacco and rice under a drought treatment (Figs. 5C and D). Both genes showed increased expression in response to drought. Similarly to the P_{SARK} activity, the expression of these two Myb-like genes was reduced after the initial stress (4 and 2 days for tobacco and rice, respectively).

Artificial senescence was induced in leaf discs from plants carrying the P_{SARK}::*GUS* derivatives. In plants expressing the complete P_{SARK}::*GUS* plants, GUS activity increased up to the 4th day and decreased thereafter (Fig. 6A). In general, the GUS activity in the plants harboring the P_{SARK} derivatives was lower than that seen in the plants harboring the complete P_{SARK}::*GUS* (Fig. 6A). A two-way hierarchical clustering formed two main groups, one containing plants with the full P_{SARK}, S8, S1, E3, B2 and S3, showing increased GUS activity, and a second group comprising S2 and E2 plants, with marginal or no activity, respectively (Fig. 6A).

Senescence and plant responses to stress are processes highly regulated by hormone homeostasis [4,29]. CK and ethylene have been shown to play roles in the regulation of plant senescence. Several putative *cis*-elements related to hormone responsiveness were identified in P_{SARK} by in silico analysis (Fig. 2A). The effect of different plant hormones on the *SARK* promoter was studied in leaf discs from plants expressing P_{SARK}::*GUS* and the different P_{SARK}::*GUS* derivatives (Fig. 6). The addition of ABA increased the GUS activity in day 1 with highest activity in day 3 (Fig. 6B). Plant ABA levels increase under stress conditions, such as drought and high-salinity [30,31] and excess of ABA may also inhibit CK biosynthesis, at least in Arabidopsis [32]. Additionally, genes that are associated with senescence have been shown to be induced by ABA [33]. Two main groups were formed by two way hierarchical

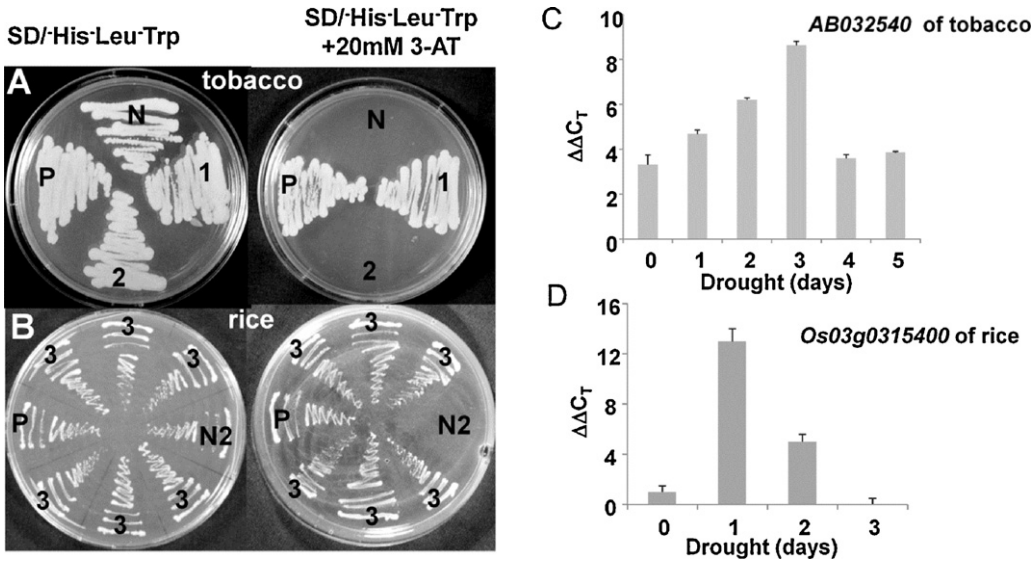

Fig. 5. Candidates interacting with the MYB1ATC *cis*-element. Yeast-one hybrid assay for (A) tobacco proteins; (P) positive control (p53HIS2 + pGAD-53); (N) negative control (p53HIS2 + pGAD-AB032540); (1) pGAD-AB032540 + pHIS2-MYB1AT; (2) pGAD-AB028649.1 + pHIS2-MYB1AT; and (B) rice proteins; (N2) negative control (p53HIS2 + pGAD-Os03g0315400); (3) pGAD-Os03g0315400 + pHIS2-MYB1AT; (C) relative expression (qPCR) of *AB032540* (Myb-like protein) in middle leaves of tobacco plants at flowering stage under drought; (D) *Os03g0315400* (Myb-like protein) in flag leaves of wild type rice under drought. Values were calculated and normalized using *GAPDH* and the transcription elongation factor gene as internal controls for tobacco and rice, respectively.

cluster analyses of the promoter derivatives in ABA exposed leaf disks, one grouped P_{SARK}, S8, B2, E3 and S3 plants, and the other grouped S1, S2 and E2 plants. Interestingly, leaf discs from S1 plants did not show responsiveness to ABA (Fig. 6B). This area (first 125 bp from 5′) contains some putative *cis*-elements related to ABA response, such as MYB1AT and WRKY71OS. The response of E3 and B2 to ABA was lesser from that of P_{SARK}, suggesting that they also harbor *cis*-elements required for full ABA responsiveness. E3 lack the last 208 bp, containing WRKY71OS and MYB1AT, in addition to several CCAAT BOX1 elements. B2, containing point mutations in the two MYB1AT *cis* elements, displayed less GUS activity than E3. Thus, it suggests that the reduction in GUS activity was due to removal of the two MYB1AT *cis*-elements. Although the MYB1AT elements appeared to play a role in the regulation of

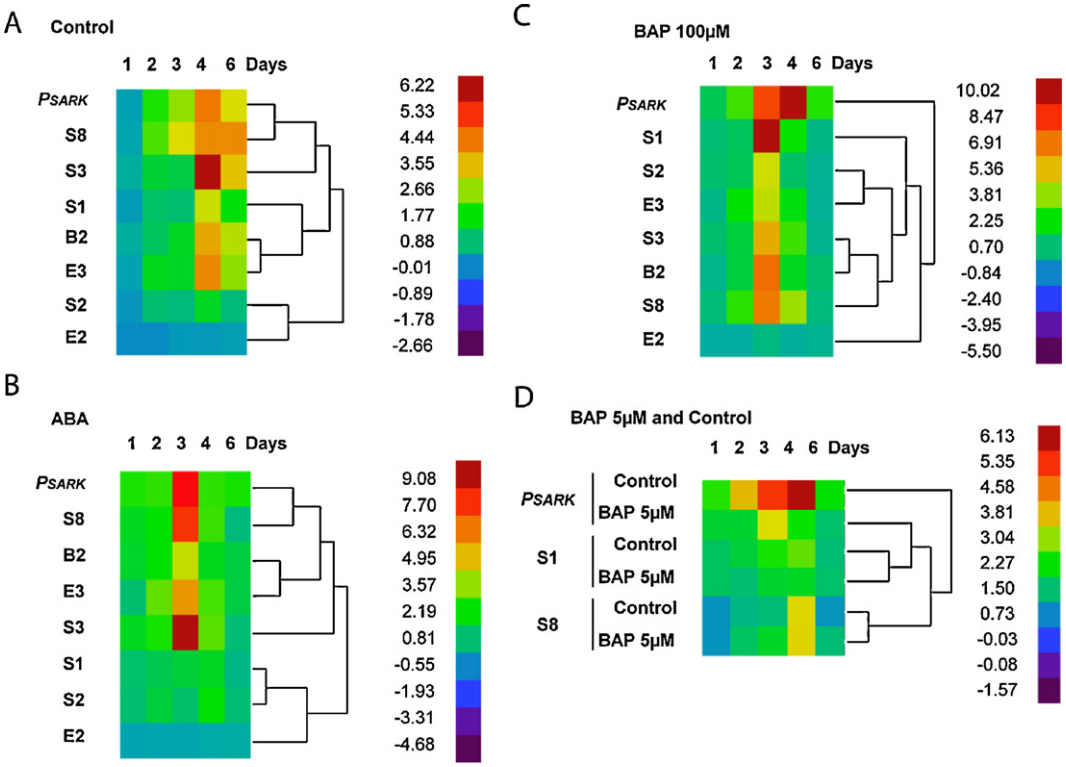

Fig. 6. *SARK* promoter responsiveness to hormones. Two way hierarchical cluster analysis of GUS activity (pmol MU min^{-1} mg protein^{-1}) in leaf discs from the 5th leaf of flowering transgenic tobacco plants containing P_{SARK} deletion derivatives, exposed to dark and different hormones for 6 days ($n = 7$). Plants in (A) control (H_2O), (B) 100 μM abscisic acid; (C) 100 μM benzylamino purine; (D) 5 μM benzylamino purine ($n = 5$). The patterns of expression of several additional promoter fragments: S4, S5, S6 and S7, were very low and similar to that of E2. For clarity, these were not included in the clustering analysis.

the promoter, they were not sufficient to induce promoter activity during senescence and/or drought (Figs. 3 and 4). The requirement of MYB1AT elements for the full response of the promoter to ABA (Fig. 6B) together with the ability of the MYB1ATs to bind, albeit in vitro, to specific stress-induced MYB-like proteins from tobacco and rice (Fig. 5), support a possible role in signaling via ABA.

Plant CK levels are reduced during natural senescence [34,35], and the foliar application of CK delays plant senescence [36]. Recently, it has been suggested that ABA/CK ratios in the xylem sap play a role in stress signaling [37], and a reduction in ABA levels was observed after water stress in rice flag leaves expressing P_{SARK}::*IPT* [12]. Nevertheless, a reduction in CK levels is not always associated with an increase in ABA. We expected that the application of CK would delay *GUS* expression in leaf discs expressing P_{SARK}::*GUS*. When 6-benzylamino purine (BAP, a synthetic CK) was added to the leaf discs at high concentration (100 μM), an increase in GUS activity was observed (Fig. 6C). Notably, increases in ABA levels have been observed in CK-overexpressing *35S::ZOG1* (*zeatin O-glucosytransferase*) plants [38], suggesting that high CK may increase ABA content. On the other hand, lower BAP concentrations (5 μM) on a subset of lines (P_{SARK}, S1 and S8) induced a reduction in *GUS* activity driven by P_{SARK} and S1 (Fig. 6D) as expected when CK delays senescence. Altogether, these results would suggest the existence of a fine-tuning mechanism maintaining the ABA:CK ratio, as proposed before [32]. In S8 plants the CK effect was abolished, i.e., no difference was observed between low CK and control treatments. This result suggests that the specific region removed from the promoter (between 316 bp and 465 bp) in this derivative is associated with CK response either directly or indirectly (Fig. 6D).

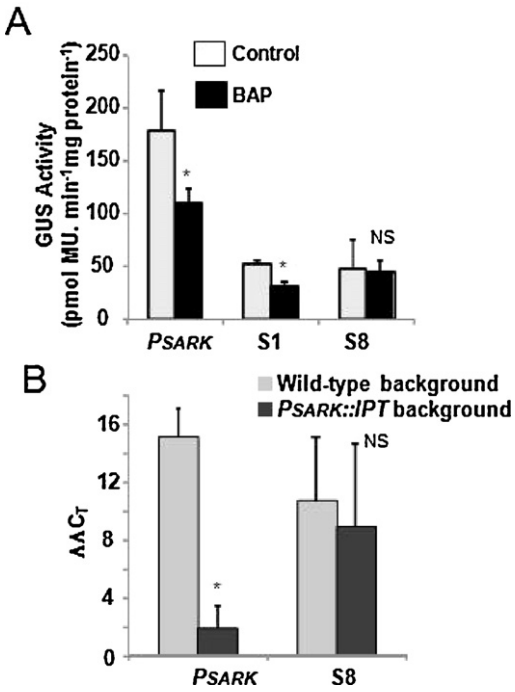

Fig. 7. *SARK* promoter responsiveness to cytokinin. (A) GUS activity in middle leaves of transgenic tobacco flowering plants under drought after 24 h of exposure to 10 μM benzylamino purine ($n = 6$); (B) relative expression (quantitative-PCR) of *GUS* (driven by *PSARK* or S8 derivative) in wild-type and P_{SARK}::*IPT* plants at the flowering stage. Values were calculated and normalized using *GAPDH* as internal control. Asterisks identify statistical difference ($P \leq 0.05$) between treatments for each genotype, NS = no significant difference.

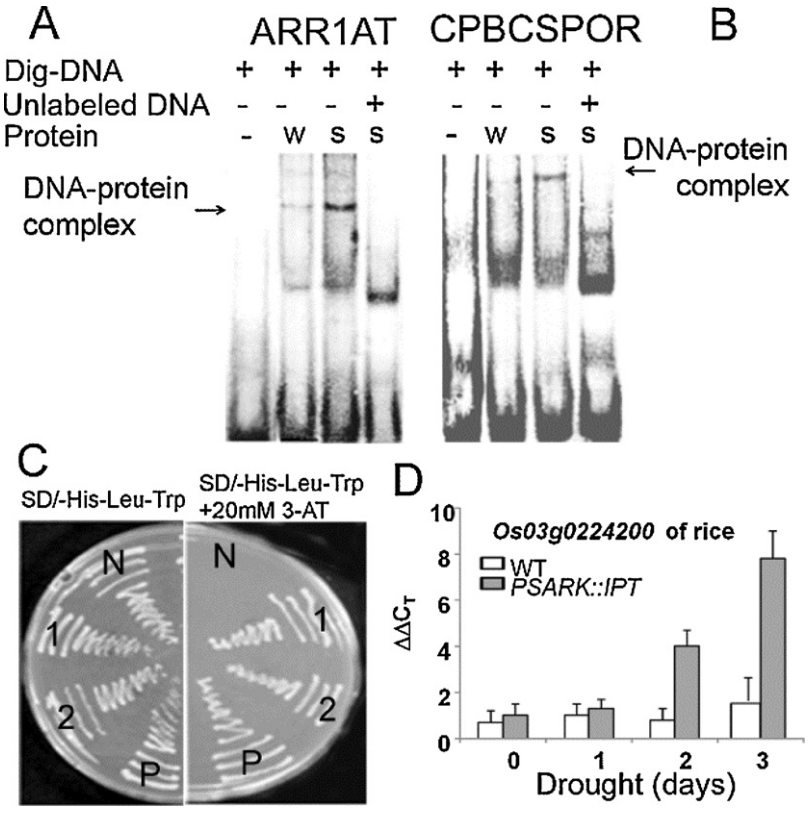

Figure 8. Properties of binding complexes formed with (A) ARR1AT and (B) CPBCSPOR *cis*-elements in nuclear extracts from rice leaves containing P_{SARK}::*IPT* gene, under well-watered, (W) and drought stress (S). (C) Yeast-one hybrid assay for the *cis*-element ARR1AT, (P) positive control (pHIS2-53 + pGAD-53); (N1) negative control (pHIS2-53 + pGAD-*Os03g0224200*); (1–2) different colonies containing Phis2-ARR1AT and Pgad-*Os03g0224200*. (D) Relative expression (q-PCR) of *Os03g0224200* in flag leaves of wild-type and transgenic P_{SARK}::*IPT* rice plants under drought. Values were calculated and normalized using the transcription elongation factor gene as internal control.

Further support for the role of the middle segment of P_{SARK} (deleted in line S8) came from a CK (BAP) spray experiment under drought. Middle leaves of P_{SARK}::*GUS* plants subjected to drought at the flowering stage and sprayed with BAP, displayed a reduction in GUS activity, as compared to control leaves (sprayed only with water) after 24 h (Fig. 7A). Reduction in GUS activity was also detected upon the application of CK to plants harboring the S1 deletion construct, but not in S8 plants (Fig. 7A). Moreover, when the transgene P_{SARK}::*GUS* was transferred to the background of P_{SARK}::*IPT* tobacco plants, a strong reduction in *GUS* expression was observed (Fig. 7B), possibly because of an increase in endogenous CK levels. However, no difference in expression was observed if *GUS* was driven by S8 (Fig. 7B). The deleted DNA segment of S8 contains 4 ARR1AT *cis*-elements (Fig. 2A), that were reported to be associated to CK response [39,40], and a CPBCSPOR *cis*-element identified as a CK enhancer [41]. Both elements bound in vitro to nuclear proteins extracted from flag leaves of transgenic P_{SARK}::*IPT* rice plants under drought (Fig. 8A and B). Similar results were obtained using nuclear proteins from middle leaves of P_{SARK}::*IPT* tobacco plants under drought (data not shown). Elements related to other hormones were also identified in this region, such as ERELEE4 (ethylene related), SBOX and PROXBBNNAPA (ABA related), as well as *cis*-elements associated to N:C relationships (DOFCORE). The presence of these elements may explain the reduction in GUS activity seen under stress when the region was deleted.

It is expected that after extended stress, the induction of *IPT* would trigger an increase in some negative regulators of the CK response [40]. Using the yeast one-hybrid assay we identified one putative ARR transcription factor from rice, *Os03g0224200* (AK111899) able to bind the ARR1AT from the *SARK* promoter (Fig. 8C). The expression of *Os03g0224200* was evaluated in wild type and transgenic P_{SARK}::*IPT* rice plants under drought. In wild type plants *Os03g0224200* expression was independent from the treatments, however increased expression was observed in the transgenic plants as the drought treatment progressed, suggesting a stress-induced CK-mediated induction in the P_{SARK}::*IPT* plants (Fig. 8D). This protein could then bind to the *SARK* promoter and reduce expression of *IPT*, causing a fine-tuning feedback inhibition of the CK levels. Moreover, a very low *GUS* expression was observed in the P_{SARK}::*IPT* background when *GUS* was driven by P_{SARK}, but not if the region containing these *cis*-elements was removed (derivative S8) (Fig. 7B). This region of the promoter may be an important element enabling the successful use of P_{SARK} for the expression of *IPT*, since it would provide a mechanism for maintaining steady state CK concentrations in the transgenic plants higher than normal levels during stress, but not high enough to impinge in developmental processes. The delay of stress-induced senescence while maintaining sink strength in the leaves prevents the alteration of normal processes of nutrient and sugar remobilization to the newer plant organs and seeds. As a consequence, the expression of P_{SARK}::*IPT* enables drought resistance but does not abolish normal grain and seed filling in transgenic P_{SARK}::*IPT* rice and peanut plants [8,12].

4. Conclusions

Despite the identification of regions that are associated with the induction and repression of P_{SARK} activity by hormones, the complexity of the promoter's response remains unsolved. A reduction in the promoter's activity was obtained with every deletion construct evaluated, suggesting that all the *cis*-elements positioned along the entire promoter are required for full activity. However, it is plausible that the positions of the elements, their mutual interaction (via transcription factors or chromatin remodeling factors recruited by them) are the factors regulating P_{SARK} activity. P_{SARK} proved to be a useful promoter in driving *IPT* expression, with the concomitant biosynthesis of CK, to mitigate water deficit stress in different crops. The *cis*-element interacting factors affected by different hormones enable cross-talk between signaling pathways, contributing to establish a new hormonal balance in the transgenic plants under stress.

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