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The Stress-Induced Soybean NAC Transcription Factor GmNAC81 Plays a Positive Role in Developmentally Programmed Leaf Senescence

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The onset of leaf senescence is a highly regulated developmental change that is controlled by both genetics and the environment. Senescence is triggered by massive transcriptional reprogramming, but functional information about its underlying regulatory mechanisms is limited. In the current investigation, we performed a functional analysis of the soybean (*Glycine max*) osmotic stress- and endoplasmic reticulum (ER) stress-induced NAC transcription factor GmNAC81 during natural leaf senescence using overexpression studies and reverse genetics. *GmNAC81*-overexpressing lines displayed accelerated flowering and leaf senescence but otherwise developed normally. The precocious leaf senescence of *GmNAC81*-overexpressing lines was associated with greater Chl loss, faster photosynthetic decay and higher expression of hydrolytic enzyme-encoding GmNAC81 target genes, including the vacuolar processing enzyme (VPE), an executioner of vacuole-triggered programmed cell death (PCD). Conversely, virus-induced gene silencing-mediated silencing of *GmNAC81* delayed leaf senescence and was associated with reductions in Chl loss, lipid peroxidation and the expression of GmNAC81 direct targets. Promoter–reporter studies revealed that the expression pattern of *GmNAC81* was associated with senescence in soybean leaves. Our data indicate that GmNAC81 is a positive regulator of age-dependent senescence and may integrate osmotic stress- and ER stress-induced PCD responses with natural leaf senescence through the GmNAC81/VPE regulatory circuit.

Keywords: Developmental regulation • GmNAC81 • *Glycine max* • Leaf senescence • NAC family • Transcription factor.

Abbreviations: DAG, days after germination; ER, endoplasmic reticulum; GmERD15, *Glycine max* early responsive to dehydration 15; GUS, β-glucuronidase; JA, jasmonic acid; MDA, malondialdehyde; MeJA, methyl jasmonate; NAC, no apical meristem (NAM), Arabidopsis ATAF1/2, and cup-shaped cotyledon (CUC); NRP/DCD, developmental cell death (DCD) domain-containing N-rich protein (NRP); PCD, programmed cell death; qRT–PCR, quantitative reverse transcription–PCR; SA, salicylic acid; SAG, senescence-associated gene; SoCSV, *Soybean chlorotic spot virus*; TBA, thiobarbituric acid; TF, transcription factor; VIGS, virus-induced gene silencing; VPE, vacuolar processing enzyme.

Introduction

Leaf senescence is a developmental process that begins with the transition of a photosynthetic leaf organ to an actively degenerating, nutrient-recycling leaf tissue. Senescence ensures the recycling of resources from senescent leaves to other plant organs to support growth and reproduction, and it becomes visible via leaf yellowing due to Chl loss. Leaf senescence is genetically linked to aging and involves the regulated expression of senescence-associated genes (SAGs; Yoshida 2003, Lim et al. 2007). Several SAGs also respond to environmental stressors and may integrate stress-induced regulatory cascades with developmental signals (Balazadeh et al. 2010). The massive changes in gene expression that accompany the onset of leaf senescence are probably controlled by a subset of senescence-associated transcription factors (TFs), but their potential functions and the regulatory cascades that they control are largely unknown. Recently, high-resolution temporal expression profiles revealed that a large fraction of NAC [for *no apical meristem* (*NAM*), Arabidopsis *ATAF1/2* and *cup-shaped cotyledon* (*CUC*)] TFs are differentially expressed during several stages of natural leaf senescence in Arabidopsis (Breeze et al. 2011), suggesting that they play a crucial role in the regulation of senescence; however, functional information is available for only a few family members (Guo and Gan 2006, Kim et al. 2009,

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Balazadeh et al. 2010, Yang et al. 2011, Lee et al. 2012, Wu et al. 2012).

The NAC domain-containing TFs make up a large family of plant-specific TFs that are involved in development and stress responses (Xie et al. 2000, Hegedus et al. 2003, Fujita et al. 2004, Lin and Wu 2004, Oh et al. 2005, Olsen et al. 2005, Guo and Gan, 2006, Nakashima et al. 2007, Kim et al. 2009, Zeller et al. 2009, Jensen et al. 2010, Nuruzzaman et al. 2010, Le et al. 2011). NAC TFs share a conserved domain at the N-terminus, designated the NAC domain, which is responsible for their DNA binding activity. The C-terminal region is more divergent in sequence and size and contains a potential transcriptional regulatory (TR) domain that has either activator or repressor functions (Xie et al. 2000, Duval et al. 2002, Ooka et al. 2003, Nakashima et al. 2012); it may sometimes possess protein binding activity and an additional transmembrane domain (Kim et al, 2007, Tran et al. 2009, Seo et al. 2010, Lee et al. 2014, Yang et al. 2014a, Yang et al. 2014b).

Although the NAC proteins represent a large fraction of the senescence-regulated TFs in several plants, biological functions have been assigned to only a few senescence-regulated NACs (Andersson et al. 2004, Guo et al. 2004, Lin and Wu 2004, Buchanan-Wollaston et al. 2005, Gregersen and Holm 2007, Balazadeh et al. 2008). In Arabidopsis, the *AtNAP* (*ANAC029*) gene, encoding a NAC domain-containing TF, is up-regulated during leaf senescence (Guo and Gan 2006). Leaf senescence occurs early in *AtNAP*-overexpressing plants, whereas the loss of *AtNAP* function delays its onset. The downstream targets of AtNAP include SAG113, a phosphatase 2C-encoding gene, which regulates ABA-mediated stomatal movement and water loss during leaf senescence (Zhang et al. 2012, Zhang and Gan 2012). *NAP* genes from rice and kidney bean, *OsNAP* and *PvNAP*, respectively, have been shown to restore the delayed leaf senescence caused by *atnap* null alleles, suggesting that the *AtNAP* gene may be a universal TF in the regulation of leaf senescence (Guo and Gan 2006). Consistent with these results, overexpression of *OsNAP* significantly promoted senescence, whereas knockdown of *OsNAP* produced a marked delay of senescence, confirming the role of this gene in the development of rice senescence (Liang et al. 2014). Therefore, the manipulation of *NAP* expression levels may be a novel strategy to control leaf senescence in economically relevant crops. In fact, reduced *OsNAP* expression leads to delayed leaf senescence and an extended grain-filling period, resulting in a 6.3% and 10.3% increase in the grain yield (Liang et al. 2014). The NAC family member ANAC092/AtNAC2/ORE1 has also been identified as a positive regulator of leaf senescence-induced cell death in Arabidopsis (Kim et al. 2009, Balazadeh et al. 2010, Balazadeh et al. 2011, Li et al. 2013, Kim et al. 2014). Microarray hybridizations identified 218 genes that are differentially expressed during senescence and are potential targets of ANAC092 (Balazadeh et al. 2010). Similarly, the ABA-responsive NAC gene *VND-INTERACTING2* (*VNI2*, *ANAC083*) can integrate ABA-mediated abiotic stress signals into leaf aging by regulating a subset of *COR* (cold-regulated) and *RD* (responsive to dehydration) genes (Yang et al. 2011). In addition, the NAC transcription factor NTL4 plays a role in promoting drought-induced senescence in Arabidopsis (Lee et al. 2012), whereas JUNGBRUNNEN1 (JUB1), a reactive oxygen species-responsive NAC TF, negatively regulates longevity in Arabidopsis (Wu et al. 2012). In rice, the NAC TF OsNAC5 has been associated with natural senescence (Sperotto et al. 2009), and, in soybean, at least two NAC genes, *GmNAC81* and *GmNAC30*, have been shown to be involved in stress-induced leaf senescence (Pinheiro et al. 2009, Faria et al. 2011, Reis et al. 2011, Mendes et al. 2013).

The soybean NAC TFs GmNAC81 (Glyma.12G022700, previously designated GmNAC6; Faria et al. 2011) and GmNAC30 (Glyma.05G195000) integrate signals from osmotic stress and endoplasmic reticulum (ER) stress into a programmed cell death (PCD) response (Irsigler et al. 2007, Costa et al. 2008). The stress-induced cell death pathway initiates signaling by inducing the TF *Glycine max* early responsive to dehydration 15 (GmERD15), which, in turn, activates the expression of the developmental cell death (DCD) domain-containing N-rich protein (NRP) by binding to its promoter (Alves et al. 2011). NRP/DCD proteins activate a signaling cascade that leads to the induction of *GmNAC81* and *GmNAC30* (Reis et al. 2011, Mendes et al. 2013). When ectopically expressed in soybean protoplasts and in *Nicotiana benthamiana* leaves, the osmotic stress- and ER stress-induced GmNAC81 and GmNAC30 proteins induce a cell death response bearing the hallmarks of leaf senescence and PCD, such as the induction of caspase 1-like activity, DNA fragmentation, Chl loss, protein degradation, enhanced lipid peroxidation and the induction of senescence-associated marker genes (Costa et al. 2008, Faria et al. 2011, Reis et al. 2011, Mendes et al. 2013).

GmNAC81 is nuclear localized, and GmNAC30 was identified because of its capacity to interact with GmNAC81 in the nuclei of stressed cells (Mendes et al. 2013). Consistent with having a role in PCD, GmNAC81 and GmNAC30 bind to and co-ordinately transactivate the promoters of hydrolytic enzymes in soybean protoplasts. One such direct downstream target gene, the vacuolar processing enzyme (VPE), exhibits caspase 1-like activity and is the executioner of vacuolar collapse-mediated cell death, a type of plant-specific PCD that is triggered by the vacuole and is induced during pathogenesis and plant development (Kinoshita et al. 1999, Hatsugai et al. 2004, Yamada et al. 2004, Hara-Nishimura et al. 2011). GmNAC30 is stress induced and works together with GmNAC81 to activate PCD by inducing VPE. Therefore, GmNAC81, GmNAC30 and VPE constitute a regulatory cascade that controls stress-induced senescence.

However, the expression of the components of this stress-induced senescence signaling pathway, such as GmNAC81, its partner GmNAC30 and the upstream regulator NRP/DCD, has also been associated with the onset and progression of natural leaf senescence (Carvalho et al. 2014). Likewise, the downstream target *VPE* is highly expressed during leaf senescence and has also been linked to developmental PCD (Nakaune et al. 2005, Carvalho et al. 2014), raising the possibility that the NRP–GmNAC81–VPE module might function in age-dependent leaf senescence, as well. In the present investigation, we examined the possible role of GmNAC81 in natural leaf senescence

through overexpression studies and reverse genetics. Our data indicate that GmNAC81 is a positive regulator of age-dependent leaf senescence and may connect the integrative PCD response to osmotic and ER stress with natural leaf senescence in soybean.

Results

Generation of transgenic lines that ectopically express *GmNAC81*

To examine the possible function of GmNAC81 in senescence under normal growth conditions, we generated several independent transgenic lines that expressed *GmNAC81* under the control of the 35S *Cauliflower mosaic virus* (CaMV) promoter and the polyadenylation signal of the nopaline synthase (*nos*) gene. The incorporation of the transgene was verified in the subsequent generations by PCR and segregation analysis; and the expression levels of the transgene were analyzed by quantitative reverse transcription–PCR (qRT–PCR). The independently transformed lines 35S::GmNAC81.1, 35S::GmNAC81.2 and 35S::GmNAC81.3 (**Supplementary Fig. S1A**), which were homozygous, accumulated higher levels of *GmNAC81* mRNA (**Supplementary Fig. S1B**) than the wild-type control, BR16, and were selected for further analysis. The selected transgenic lines expressed higher levels of *GmNAC81* transcripts throughout development, although to different extents (**Supplementary Fig. S1B**).

The growth-related phenotypes of *Gm0NAC81*-overexpressing lines

The developmental stages of the plants were scored using the Fehr and Caviness (1977) classification for the vegetative (V1–V11) and reproductive (R1–R8) phases. The wild-type and *GmNAC81*-overexpressing lines were grown in randomized blocks to compare phenotypic changes in growth and development. In general, the life cycle of the *GmNAC81*-overexpressing BR16 transgenic lines was accelerated, with a length in the range of 117–120 d, compared with the 122–125 d life cycle of the untransformed BR16 control (**Supplementary Fig. S2**). Apart from transgenic line 35S::GmNAC81-3, which had a similar number of nodes to the wild type, the other two *GmNAC81* overexpressors, 35S::GmNAC81-1 and 35S::GmNAC81-2, had significantly more nodes throughout development (**Fig. 1A**). This difference in the number of nodes did not alter growth; the height of the the transgenic lines did not differ significantly from that of the wild-type control (**Fig. 1B**), although there was a clear bias for a positive relationship between the number of nodes and size. During the vegetative phase, the growth parameters among the genotypes were indistinguishable; they displayed determinate stem growth with a deceleration in the height increase rate and in the quantity of nodes between the beginning of bloom (R1) and stem termination (ST) stages. Although growth apparently ceased after flowering, the shoot and root biomass of wild-type and *GmNAC81*-overexpressing lines continued to increase, reaching a plateau at 96 days after germination (DAG), when the seeds were full (R6) and maturation had begun (R7; **Fig. 1C, D**). The shoot and root biomass of the transgenic lines did not differ significantly from those of the wild-type control until 96 DAG, when the root biomass of the wild type became significantly greater than that of the transgenic lines. In contrast, the *GmNAC81*-overexpressing lines flowered earlier and progressed more quickly towards the stages of soybean reproductive development (R1–R8) than the wild-type control (**Fig. 2**). Although the beginning bloom (R1) stage could not be precisely distinguished from full bloom (R2), they started at 47 DAG for transgenic lines 35S::GmNAC81.2 and 35S::GmNAC81.3; at 53 DAG for the wild type; and at an intermediate point for the 35S::GmNAC81.1 line (**Fig. 2**). The acceleration of the early reproductive stages in the transgenic lines led to a faster progression to the subsequent R3 (beginning pod) and R4 (full pod) developmental stages compared with the wild type. Likewise, all the subsequent reproductive stages, from R5 (beginning seed), R6 (full seed) and R7 (beginning maturity) to full maturation (R8) occurred sooner in the *GmNAC81*-overexpressing lines, although to different extents. The reproductive phase of the 35S::GmNAC81-2 and 35S::GmNAC81-3 lines was more highly accelerated than that of 35S::GmNAC81-1. These phenotypes are probably associated with GmNAC81 expression levels rather than with a particular insertional event because three additional independently transformed lines, GmNAC81-12, GmNAC81-22 and GmNAC81-32, also displayed a similar precocious flowering phenotype and accelerated reproductive stages in comparison with the wild type (**Supplementary Fig. S3**).

GmNAC81-overexpressing lines have an early leaf senescence phenotype

Our results showed that at early stages of growth and development, the *GmNAC81*-overexpressing lines appeared normal (**Fig. 1**; **Supplementary Fig. S2**), but leaf senescence was remarkably precocious, as judged by the progression of leaf yellowing in *GmNAC81*-overexpressing lines compared with the wild-type control (**Supplementary Fig. S2**). Leaf yellowing occurred more quickly and was more severe in the GmNAC81-2 and GmNAC81-3 lines (**Fig. 3A, B**), which correlated with the levels of *GmNAC81* transcripts in the transgenic lines (**Supplementary Fig. S1B**). This premature leaf senescence phenotype also correlated fairly well with the Chl content (**Fig. 3C**) and was recapitulated in the subsequent generation R4 of additional independently transformed lines, GmNAC81-12, GmNAC81-22 and GmNAC81-32 (**Supplementary Fig. S4A–C**). Chl loss occurred earlier and was significantly more severe in *GmNAC81*-overexpressing lines compared with the wild-type parental BR16 plants, and slight differences were observed among the overexpressing lines. The leaf senescence phenotype was further examined by assessing senescence-associated lipid peroxidation via measuring the accumulation of thiobarbituric acid (TBA)-reactive compounds such as malondialdehyde (MDA) (**Fig. 3D; Supplementary S4D**). Compared with the untransformed BR16 line, the concentration of TBA-reactive compounds increased more rapidly in the transgenic lines and reached a significantly greater content at 103 DAG.

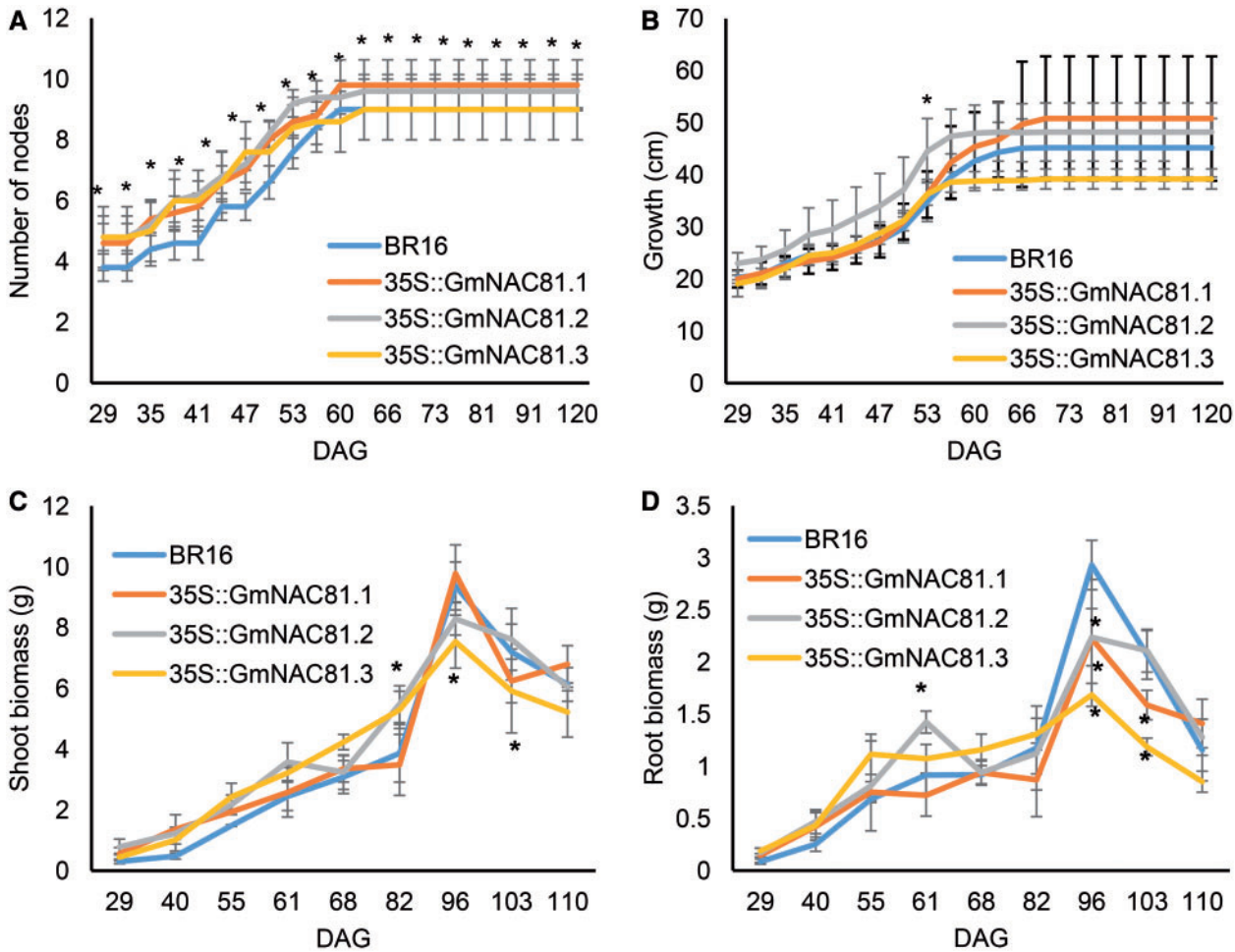

Fig. 1 Growth-related parameters of the transgenic lines and the wild-type cv. BR16 throughout development. The values at the indicated DAG represent the mean ± SD of five biological replicates. Asterisks indicate means that are significantly different from that of BR16 by Tukey's HSD ($P < 0.1$).

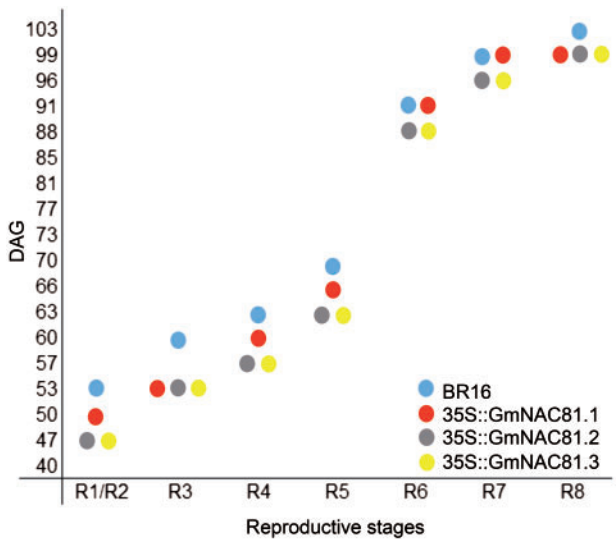

Fig. 2 The reproductive stages of BR16 and the transgenic lines. The dots represent the day after germination (DAG) required for the first plant of a given genotype ($n = 5$) to reach the indicated reproductive event.

As a complementary hallmark for leaf senescence, we also monitored photosynthetic activity throughout development. During the vegetative phase and until the R4 (full pod) stage, the rate of photosynthesis did not differ significantly between the wild type and *GmNAC81*-overexpressing lines (**Fig. 3E**). However, during the R5 stage (between days 75 and 82), photosynthetic CO_2 assimilation reached a plateau and was significantly higher in the *GmNAC81*-overexpressing lines than in the wild-type control. Nevertheless, after 82 d, during the progression of leaf senescence, CO_2 assimilation declined more drastically in the *GmNAC81*-overepressing lines than in the wild-type control, and at 103 d it was significantly lower in the GmNAC81-2 and GmNAC81-3 lines than in the wild type. The transpiration rate (E) and stomatal conductance (g_s) displayed the same trend as the photosynthetic rate (**Supplementary Fig. S5A, B**).

The evidence for an earlier reduction in photosynthesis in the overexpression lines was further supported by regression analysis of the data (**Supplementary Fig. S6**). Quadratic models were the best fit for the developmental timeline of all genotypes (BR16 R^2 = 0.85, 35S::GmNAC81.1 R^2 = 0.849,

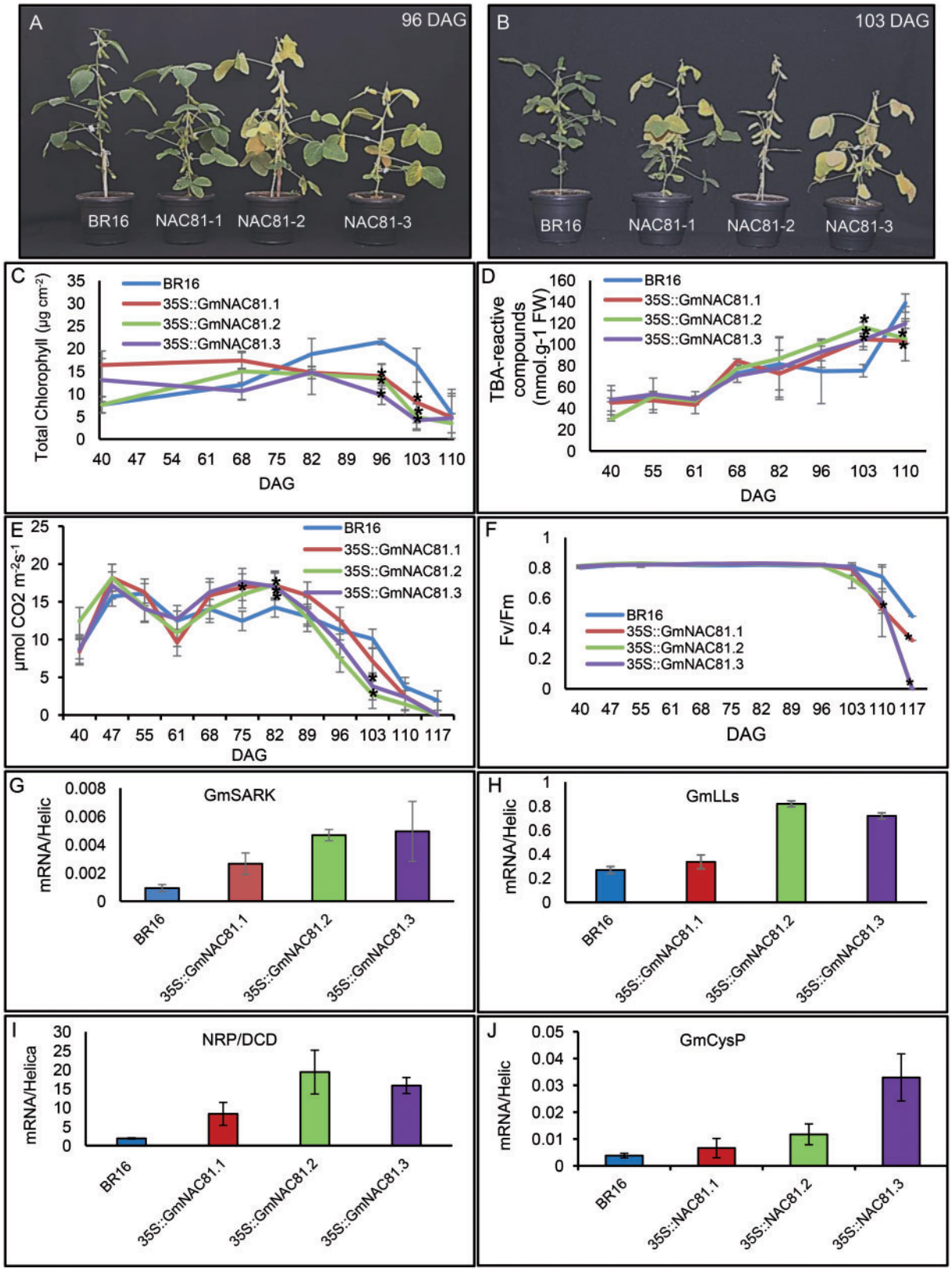

Fig. 3 *GmNAC81* overexpression accelerates leaf senescence in soybean. (A, B) Developmental performance of representative plants from the 35S::GmNAC81.1, 35S::GmNAC81.2 and 35S::GmNAC81.3 lines at 96 and 103 DAG. (C) Chl loss during the progression of leaf senescence. The total Chl content was measured over time in the leaves of the wild type and the 35S::GmNAC81 lines during senescence. (D) Lipid peroxidation in wild-type and 35S::GmNAC81 leaves. Lipid peroxidation in the leaves was monitored by determining the level of thiobarbituric acid

(continued)

35S::GmNAC81.2 R^2 = 0.92, 35S::GmNACo81-3 R^2 = 0.88) (**Supplementary Fig. S6A**). The confidence limits of both the maximum peak values and the areas under the curves revealed no differences in the photosynthetic rate among the genotypes (**Supplementary Fig. S6B, C**). However, when the analysis was restricted to the data on photosynthesis during later developmental stages (senescence), linear models were the best fit (BR16 R^2 = 0.8741, 35S::NAC81-1 R^2 = 0.9356, 35S::NAC81-2 R^2 = 0.9178, 35S::NAC81-3 R^2 = 0.9034), and these linear functions uncovered significant differences between BR16 and the *GmNAC81*-overexpressing lines (**Supplementary Fig. S6D**). The confidence limits of the slopes indicated that the 35S::GmNAC81 lines clearly had a greater decay rate than BR16, i.e. the 35S::GmNAC81 genotypes may senescence earlier than BR16.

We also observed that the respiration rate in the wild type and the *GmNAC81*-overexpressing lines paralleled the developmental timeline of the reproductive phase (**Supplementary Fig. S5C**). The respiration rate reached a plateau at 89 d in the transgenic lines, but it declined more rapidly in the *GmNAC81*-overexpressing lines than in wild-type untransformed BR16 control (**Supplementary Fig. S5C**). The premature leaf senescence phenotype of the *GmNAC81*-overexpressing lines was also supported by late-stage changes in the maximum photochemical efficiency of PSII in the dark-adapted state (F_v/F_m; **Fig. 3F**). A senescing leaf often loses its photosynthetic activity, as reflected by a decline in the F_v/F_m ratio. The F_v/F_m ratio started to decline at 96 d, and at 110 d the *GmNAC81*-overexpressing lines had significantly lower F_v/F_m ratios than did the wild type.

During gas exchange analyses, the CO_2 concentration was kept constant, such that the variations in the C_i/C_a ratio reflected variations in the internal CO_2 concentration (C_i). During all developmental stages, the C_i/C_a ratio of the *GmNAC81*-overexpressing lines was similar to that of the wild type (**Supplementary Fig. S5D**). Nevertheless, at later stages of development, the C_i/C_a ratio was slightly higher in the *GmNAC81*-overexpressing lines than in the wild type, indicating less CO_2 fixation in the transgenic lines. However, at 117 d, the C_i/C_a ratio fell significantly in the *GmNC81*-overexpressing lines. This drastic reduction of C_i/C_a in the transgenic lines at 117 d was due to the faster abscission of their leaves, which was consistent with the premature leaf senescence phenotype of the lines.

The photosynthetic activity of the soybean lines was also evaluated by measuring Chl fluorescence parameters including the electron transport rate (ETR), non-photochemical quenching (qN) and photochemical quenching (qP) (**Supplementary Fig. S7**). Photochemical processes, as measured by qP and ETR, progressively declined during development, whereas the energy dissipation, qN, did not change.

We further investigated the expression of senescence-associated marker genes, such as *GmSARK* (*Glycine max senescence-associated receptor-like kinase*) and *GmLL* (*lethal leaf-spot 1*; Li et al. 2006), *NRP*-B (Carvalho et al. 2014) and *GmCysP* (for *Cys protease 1*; Valente et al. 2009) at 82 d, when the rate of photosynthesis and the Chl content started to decline in the GmNAC81-overexpressing leaves, marking the onset of senescence. As shown in **Fig. 3G–J**, the accumulation of senescence-associated marker gene transcripts was significantly higher in the *GmNAC81*-overexpressing lines than in the BR16 wild-type control. Likewise, the transcript levels of senescence-associated marker genes were significantly higher in senescing leaves (90 DAG) of GmNAC81-12, GmNAC81-22 and GmNAC81-32 lines (**Supplementary Fig. S4E–G**) than in wild-type leaves. Taken together, these data confirmed the precocious leaf senescence phenotype of the transgenic plants at the molecular level.

Alterations in the endogenous hormone levels of the *GmNAC81*-overexpressing lines

Although leaf senescence is genetically programmed in an age-dependent manner, it can be triggered by environmental cues and is also positively and negatively regulated by various plant hormones. The phytohormones ABA, jasmonic acid (JA) and salicylic acid (SA) are positive regulators of senescence, and their endogenous levels increase during the senescence process (Zhang and Zhou 2013). We analyzed the expression of *GmNAC81* and its partner *GmNAC30* (Mendes et al. 2013) in response to these three hormones and to stress conditions that are known to trigger leaf senescence. *GmNAC35* (Glyma.06G114000, previously designated *GmNAC2*), which is closely related to *GmNAC30* but is not involved in leaf senescence, was also included in the assay as a control for stress-induced *NAC* gene expression (Pinheiro et al. 2009, Le et al. 2011). As an *ATAF1* homolog, *GmNAC35* is strongly induced by drought and moderately induced by NaCl, ABA, SA and methyljasmonate (MeJA), and it is quickly induced in response to cold (**Fig. 4**; Pinheiro et al. 2009). The hormones ABA, SA and MeJA, as well as stress treatments including drought, NaCl and cold, induced both *GmNAC30* and *GmNAC81* with similar kinetics although to different extents (**Fig. 4**). As downstream functional partners in NRP/DCD-mediated cell death signaling, *GmNAC81* and *GmNAC30* are co-ordinately up-regulated by exposure to different abiotic stresses. *GmNAC81* and

Fig. 3 Continued
(TBA)-reactive compounds and is expressed as the malondialdehyde (MDA) content. (E, F) Physiological measurements of *GmNAC81*-overexpressing lines throughout development. CO_2 assimilation (E) and the F_v/F_m ratio (F) of the third fully expanded trifoliate leaves of the WT and the 35S::GmNAC81 lines were measured with a LI-6400 infrared (IR) gas analyzer during the experimental period. For (C–F), asterisks indicate means that are significantly different from that of BR16 by a Tukey test ($P < 0.1$, $n = 5$) on the same DAG. (G–J) The expression of SAGs. Total RNA was isolated from the leaves of senescing wild-type (BR16) and 35S::GmNAC81 leaves, and the transcript levels of selected genes (as indicated) were quantified by real-time PCR using gene-specific primers. Gene expression was calculated using the $2^{-\Delta CT}$ method, and RNA helicase served as an endogenous control. The error bars indicate 95% confidence intervals based on *t*-tests ($P < 0.05$, $n = 3$).

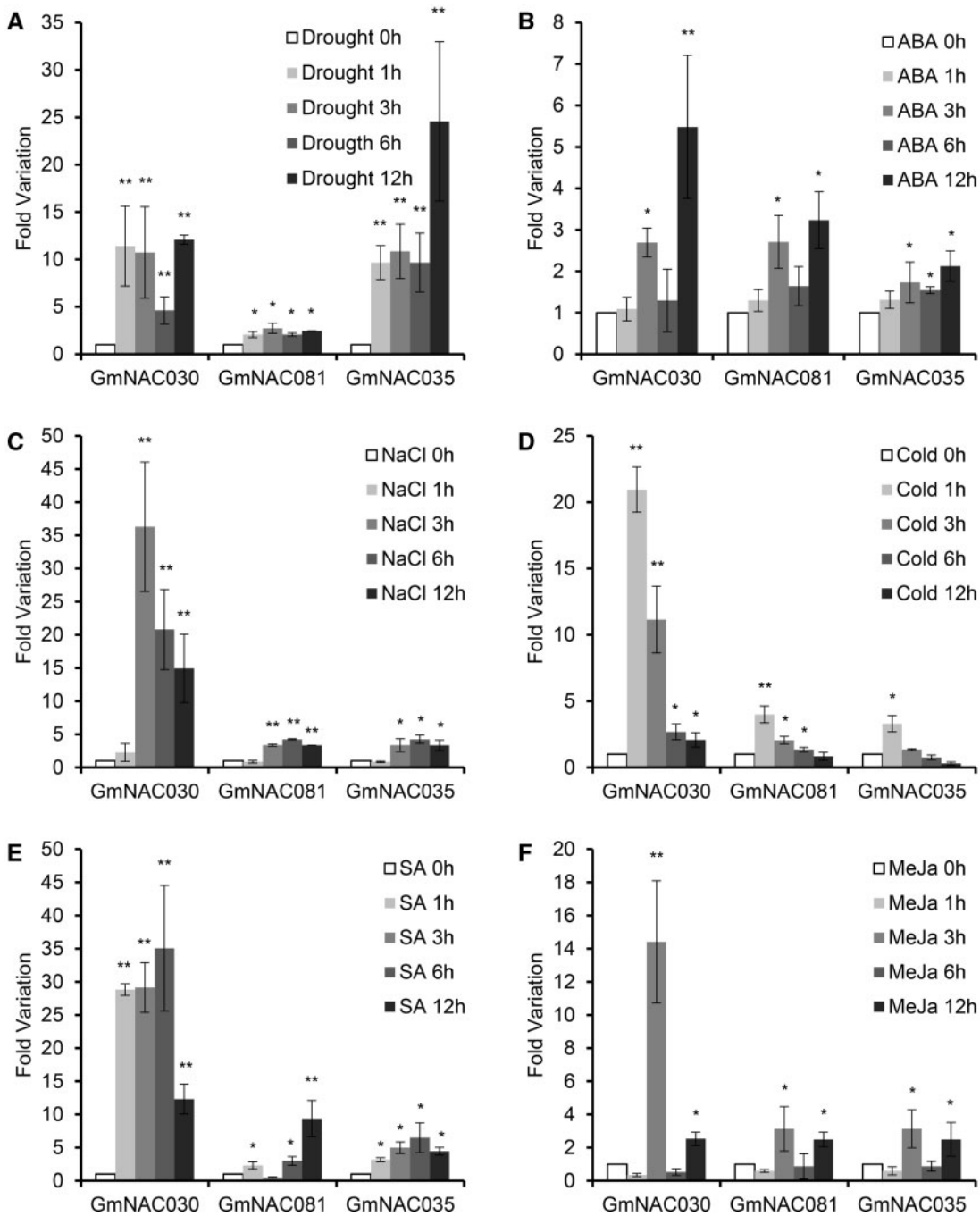

Fig. 4 The expression profiles of *GmNAC81* and *GmNAC30* after exposure to different environmental stimuli. A time course of *GmNAC30*, *GmNAC81* and *GmNAC35* induction after exposing soybean plants at the VC developmental stage to drought (A), ABA (B), NaCl (C), cold (D), salicylic acid (SA) (E) and methyl jasmonate (MeJa) (F) for the indicated length of time. The fold variation in gene expression (relative to the control treatments) was calculated using the $2^{-\Delta\Delta Ct}$ method with helicase as an endogenous control. The statistical method was performed by non-parametric multiple contrast tests and simultaneous confidence intervals. Level of significance: $^{*}P < 0.05$, $^{**}P < 0.01$. The means are from three biological replicates.

GmNAC30 were also induced by ABA, SA and MeJA, which are all inducers of leaf senescence.

To examine the relationship between *GmNAC81* overexpression and hormone inducers of senescence, we examined the endogenous levels of ABA, JA and SA in *GmNAC81*-overexpressing lines at the onset of leaf senescence (82 d under normal growth conditions). Although the JA levels in the *GmNAC81*-overexpressing lines did not differ from those in the wild type (**Fig. 5C**), the transgenic lines had significantly higher ABA levels and significantly lower SA levels than the wild-type, untransformed control lines (**Fig. 5A, B**). In the GmNAC81-1, GmNAC81-2 and GmNAC81-3 transgenic lines,

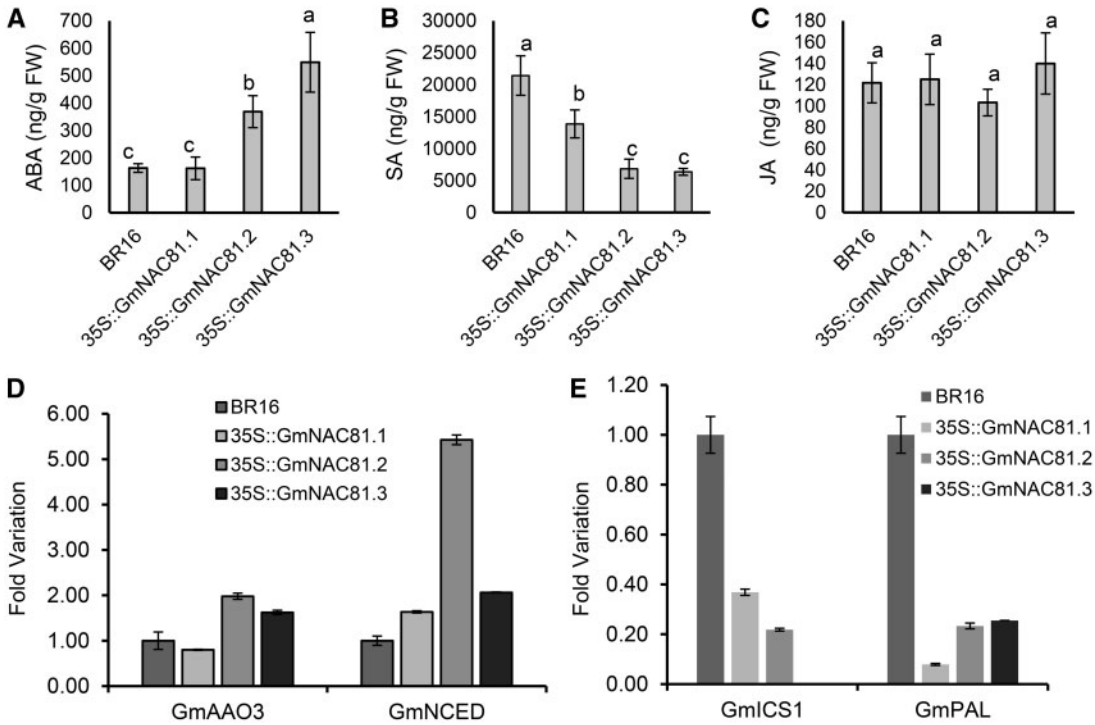

Fig. 5 The relative abundance of phytohormones in wild-type and 35S::GmNAC81 transgenic plants. (A–C) The relative contents of ABA (A), SA (B) and JA (C) were determined from extracts of the third trifoliate leaves at 82 DAG. The values represent the means ± SD of three replicates. Different letters indicate significantly different means between the genotypes (Tukey's test, $P < 0.1$). (D, E) Expression of ABA (D) and SA (E) biosynthetic enzymes. Total RNA was isolated from the leaves of senescing wild-type (BR16) and 35S::GmNAC81 leaves, and the transcript levels of selected genes (as indicated) were quantified by real-time PCR using gene-specific primers. The relative expression was quantified using the $2^{-\Delta\Delta Ct}$ method and using 18S rRNA as an endogenous control. The values are relative to the control treatment (wild-type), and error bars indicate the 95% confidence interval based on a *t*-test ($P < 0.05$, $n = 3$).

the extent of the increase in ABA levels and the decline in SA levels closely paralleled the levels of *GmNAC81* transcripts (compare **Fig. 5** with **Supplementary Fig. S1B**), suggesting that GmNAC81 may act as a positive regulator of ABA synthesis and a negative feedback regulator of SA synthesis.

We also examined the effect of GmNAC81 overexpression on the expression of enzymes involved in ABA and SA biosynthesis. Enhanced expression of the ABA biosynthetic enzymes 9-*cis*-epoxycarotenoid dioxygenase homolog (*GmNCED*) and Arabidopsis aldehyde oxidase 3 homolog (*GmAAO3*) was observed in *GmNAC81-2-* and *GmNAC81-3*-overexpressing senescing leaves as compared with wild-type senescing leaves, which paralleled the increased accumulation of ABA in these transgenic lines (**Fig. 5C**). These enzymes are involved in the ABA biosynthesis pathway and are induced by conditions that increase ABA levels (Seo and Koshiba 2002). In contrast, the expression of the SA biosynthetic enzymes isochorismate synthase 1 homolog (*GmICS1*) and phenylalanine ammonia-lyase 2 homolog (*GmPAL*) was lower in the senescing leaves of *GmNAC81-1-* and *GmNAC81-2*-overexpressig lines than in wild-type leaves, which correlated with the reduced accumulation of SA in the overexpressing lines (**Fig. 5D**). Likewise, during leaf senescence, GmPAL displayed lower expression in GmNAC81-3 leaves than in wild-type leaves. Very probably, the enhanced accumulation of GmNAC81 in transgenic lines may regulate ABA biosynthesis positively and impact SA biosynthesis negatively. Consistent with this hypothesis, enhanced accumulation of the molecular chaperone binding protein (BiP), which has been shown to regulate *GmNAC81* and *GmNAC30* expression negatively, leads to an increase in SA accumulation (Carvalho et al. 2014).

The temporal and organ-specific expression of *GmNAC81*

qRT–PCR analysis of leaves at different developmental stages revealed that *GmNAC81* is expressed at low levels in the V4 vegetative stage (29 DAG) and is induced during the reproductive phase and in senescing leaves (**Fig. 6A**). To examine the transcriptional expression of *GmNAC81*, we performed promoter–GUS (β-glucuronidase) assays in transgenic tobacco lines (**Fig. 6B**). The activity of the *GmNAC81* promoter was barely detectable in seedlings and in young, non-senescing leaves, but was highly expressed in senescing leaves. GUS activity was not detected in the leaves and seedlings of wild-type, non-transformed lines or in plants transformed with pCambia1381Z, a promoterless vector. Likewise, no GUS activity was detected in wild-type plants recovered from the segregating populations of pNAC81-GUS-transformed lines (WT*).

Transgenic plants expressing the *GmNAC81-GUS* fusion gene were examined for tissue-specific GUS activity. In senescing leaf discs, GUS staining was dispersed in the mesophyll, and in leaf transverse sectors, GUS activity was often observed

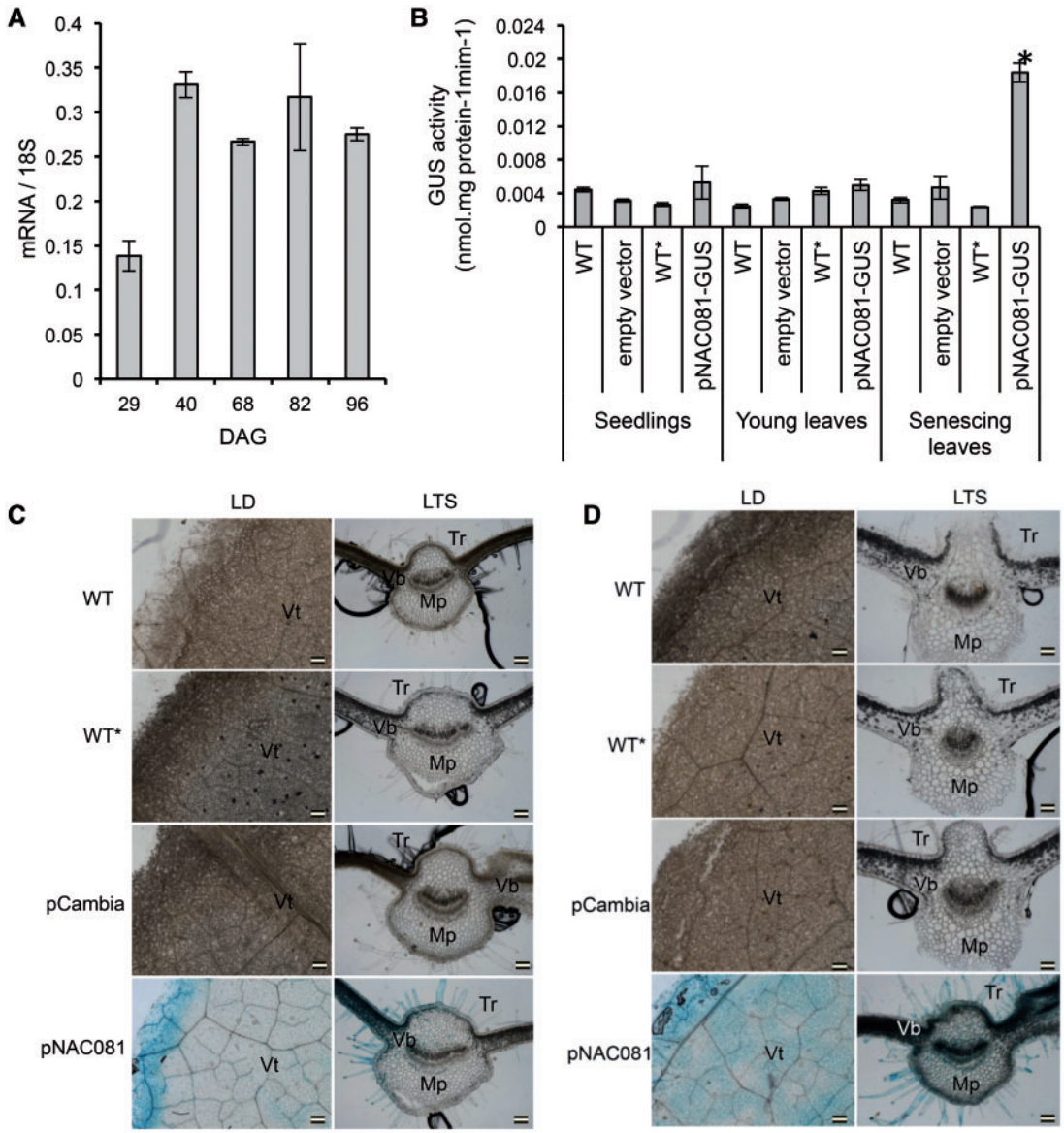

Fig. 6 The temporal and organ-specific expresssion of *GmNAC81*. (A) The accumulation of *GmNAC81* transcripts at different developmental stages. Total RNA was isolated from wild-type leaves at the indicated DAG, and the transcript levels of the indicated genes were determined via qRT–PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method, and 18S rRNA served as an endogenous control. The error bars indicate the 95% confidence interval based on a *t*-test ($P < 0.05$, $n = 3$). (B) The transcriptional activity of the *GmNAC81* promoter at different developmental phases in transgenic tobacco plants expressing the pNAC81-GUS fusion gene. Specific GUS activity was determined via fluorometric assays of total extracts from seedlings, non-senescing leaves and senescing leaves. The error bars indicate the 95% confidence interval based on a *t*-test ($P < 0.05$, $n = 3$). WT is the untransformed control and WT* is an additional control in which the pNAC81-GUS fusion gene had segregated out, leading to a wild-type genotype. 'Control' represents plants transformed with the promoter-less binary vector (empty vector). Asterisks indicate signicant differences from the respective controls by Tukey's HSD ($P < 0.05$). (C, D) Histochemical analysis of GUS staining in leaf discs (LDs) and leaf transverse sections (LTSs) of WT, WT* and tobacco leaves transformed with the empty vector (pCAMBIA) or with pGmNAC81-GUS.

within the trichomes, mesophyll and vascular tissue (Fig. 6D). Conversely, in non-senescing young leaves, GUS activity was not detected in leaf transverse sectors and was barely detectable in leaf discs (Fig. 6C). The GUS staining in the border of the leaf disc is probably due to the wounding-mediated activation of the *GmNAC81* promoter (Faria et al. 2011). In control assays, GUS activity was not detected in leaf discs or transverse sectors of senescing leaves from wild-type, WT* and control plants harboring a promoterless *GUS* gene (pCAMBIA; Fig. 6C, D). Collectively, these results may indicate that *GmNAC81* up-regulation in senescing leaves is under transcriptional control, and they argue favorably for a developmentally programmed function of GmNAC81.

Downstream events in NRP/DCD-mediated cell death signaling in *GmNAC81*-overexpressing lines

GmNAC81 has been characterized as a downstream component of stress-induced, NRP/DCD-dependent cell death

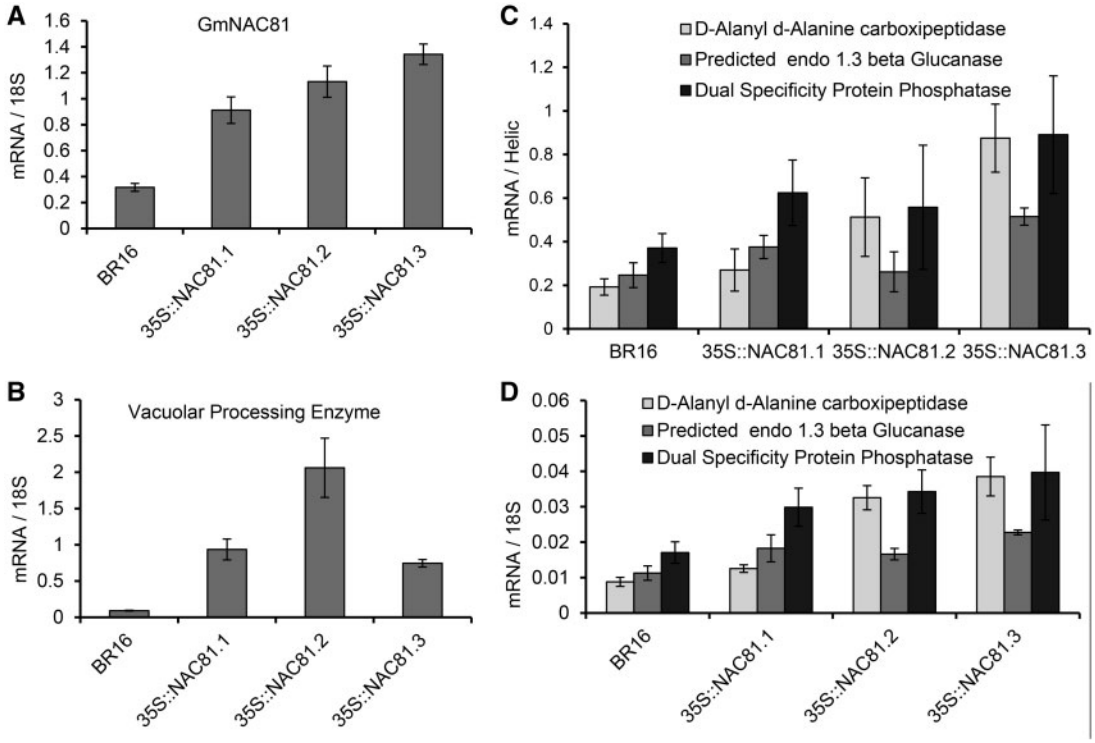

Fig. 7 Direct target genes of GmNAC81 are induced by *GmNAC81* overexpression. (A–C) Total RNA was isolated from wild-type and senescing leaves from independently transformed transgenic lines (35S::GmNAC81.1, 35S::GmNAC81.2 and 35S::GmNAC81.3), and the transcript levels of the indicated genes were determined by qRT–PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method, and 18S rRNA (A, B, D) or RNA helicase (C) were used as endogenous controls. The error bars indicate 95% confidence intervals based on *t*-tests ($P < 0.05$, $n = 3$). (A) The abundance of *GmNAC81* mRNA in 35S::GmNAC81 lines at 82 DAG. (B) The induction of VPE by *GmNAC081* overexpression. (C and D) The expression of GmNAC81 direct target genes in *GmNAC81*-overexpressing lines.

signaling. The stress-induced expression of NRP/DCDs leads to the induction of *GmNAC81*, which works with GmNAC30 to activate PCD by inducing the cell death executioner caspase1-like *VPE* (Mendes et al. 2013). VPE has been shown to trigger vacuolar collapse-mediated PCD during pathogenesis and development (Hatsugai et al. 2004, Nakaune et al. 2005). To examine if VPE could be a molecular link for the GmNAC81-mediated induction of leaf senescence, we assayed *VPE* expression in the *GmNAC81*-overexpressing lines at the onset of senescence (**Fig. 7A, B; Supplementary Fig. S4A, S4H**). In addition to *VPE*, we selected other GmNAC81 direct target genes, such as the predicted hydrolytic enzyme gene endo-1,3-β-glucanase, a dual specificity protein phosphatase, and D-alanyl-D-alanine carboxypeptidase (Mendes et al. 2013) for study. We monitored the expression of these genes in *GmNAC81*-overexpressing lines with qRT–PCR, using both helicase and 18S rRNA as endogenous controls (**Fig. 7C, D; Supplementary Fig. S4I**). As a direct target of the GmNAC81 TF, the expression of *VPE* was higher in the *GmNAC81*-overexpressing lines than in the wild-type, suggesting an underlying molecular mechanism for the GmNAC81-mediated induction of leaf senescence. Similarly, the transgenic lines accumulated higher levels of the other GmNAC81 target genes, confirming the functionality of the ectopically expressed GmNAC81 protein.

Reducing *GmNAC81* expression via virus-induced gene silencing (VIGS) delays leaf senescence

In addition to precocious leaf senescence, the *GmNAC81*-overexpressing lines flowered earlier and progressed more quickly through the reproductive phase. Therefore, we could not determine whether their premature leaf senescence was due to a direct role for the *GmNAC81* gene in senescence or a consequence of accelerated reproduction, which could have resulted from pleotropic effects of ectopically expressing *GmNAC81* with a constitutive promoter. To examine these possibilities, we used a VIGS vector derived from a soybean-infecting geminivirus, *Soybean chlorotic spot virus* (SoCSV, Coco et al. 2013), to silence the endogenous *GmNAC81* gene. Soybean radicles were inoculated via biolistics with pSoCSV-GmNAC81, which harbors the N-terminal portion of GmNAC81 as the silencing target, or with the empty VIGS vector pSoCSV as a control. Viral DNA accumulation in the inoculated leaves was detected via PCR at 28 days post-inoculation (DPI; **Fig. 8A**). The silencing efficiency of the vector was monitored by qRT–PCR in three plants that had been independently inoculated with pSoCSV-GmNAC81, all of which accumulated viral DNA at 28 DPI (**Fig. 8B**). During leaf senescence (at 105 d, R7 stage), compared with plants infected with the empty VIGS vector, *GmNAC81* expression was reduced by 82–86% in pSoCSV-NAC81-1 and pSoCSV-NAC81-2 plants and by 99% in pSoCSV-NAC81-3

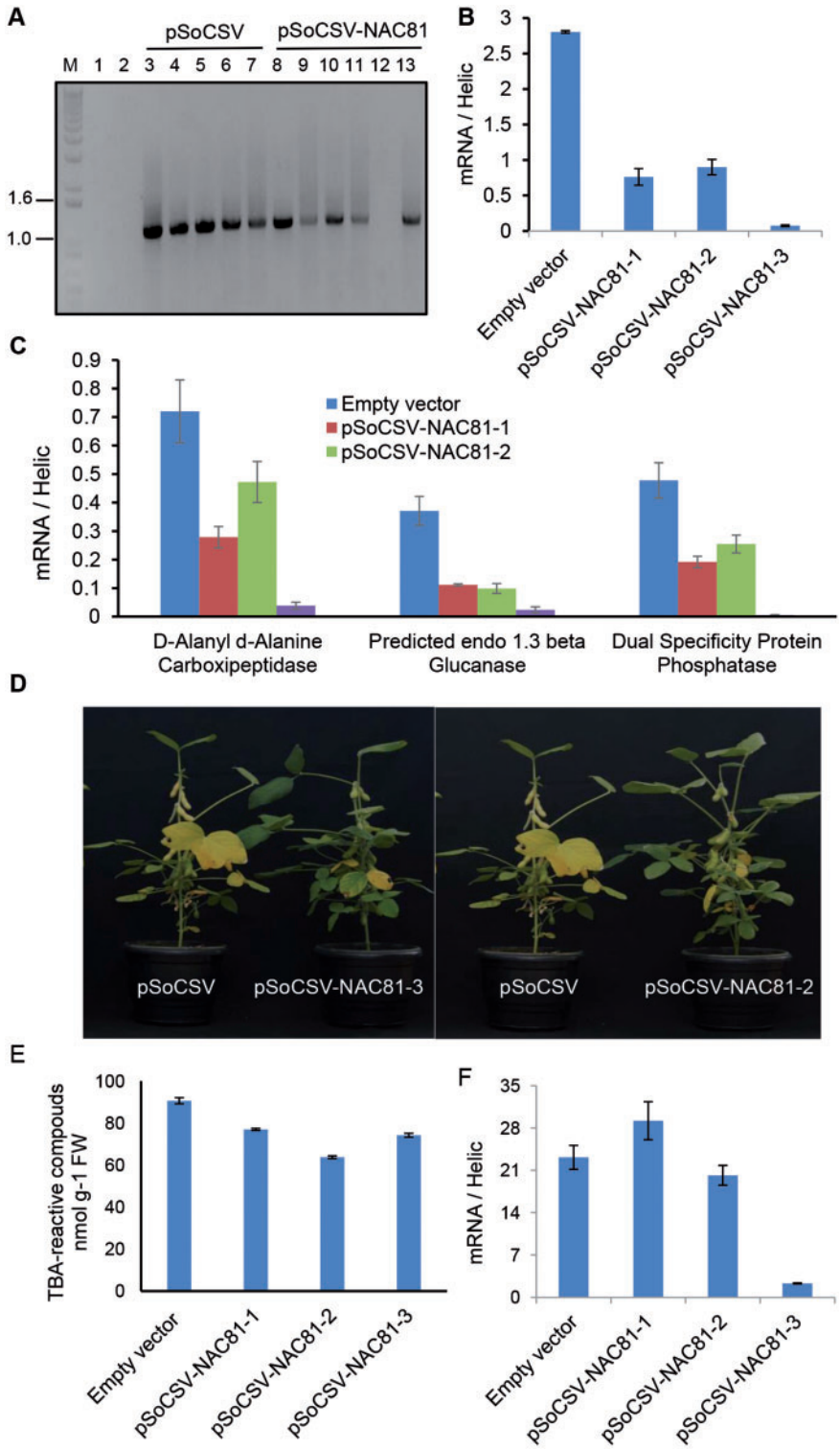

Fig. 8 Inactivation of *GmNAC81* function delays leaf senesncence. (A) Virus-induced gene silencing of *GmNAC81*. For the detection of viral DNA in infected plants, total DNA was isolated from uninfected leaves (lanes 1 and 2), from leaves inoculated with the empty VIGS vector + pSoCSV-DNAB (pSoCSV, lanes 7–12) and from leaves inoculated with pSoCSN-NAC81 + pSoCSV-DNAB (lanes 8–13) at 28 DPI as detected with viral DNA-specific primers. M indicates DNA markers (kb). (B) Reduced expression of *GmNAC81* in the infected leaves of plants that had been independently inoculated with pSoCSV-NAC81. Total RNA from the infected leaves was isolated, and the transcript levels of *GmNAC81* were determined by qRT–PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method, and RNA helicase was used as an endogenous control. The error bars indicate a 95% confidence interval based on a *t*-test ($P < 0.05$, $n = 3$). (C) mRNA abundance of GmNAC81 direct target genes in silenced leaves. The expression of the indicated genes was monitored by qRT–PCR as described in (B). (D) Delayed leaf senescence phenotype of

(continued)

leaves. The silencing of the *GmNAC81* gene led to the proportional repression of the hydrolytic enzyme-encoding target genes in the silenced lines (**Fig. 8C**). Both silenced and non-silenced plants appeared normal during the vegetative phase, and they flowered simultaneously. However, the silenced plants displayed a delayed leaf senescence phenotype (**Fig. 8D**). In fact, during senescence, at the R7 stage, the leaves of the silenced plants remained green, whereas leaf yellowing proceeded normally in the control, non-silenced plants (**Fig. 8D**). The delayed leaf senescence phenotype was further examined by measuring lipid peroxidation, which was lower in the silenced leaves than in non-silenced leaves that had been inoculated with the empty vector (**Fig. 8E**). In the VIGS-NAC81-3 plant, which was the most efficiently silenced, total inactivation of the *GmNAC81* gene led to the drastic repression of *VPE* (**Fig. 8F**), indicating that the GmNAC81/VPE regulatory cascade might at least partially underlie the molecular mechanism of GmNAC81 function in leaf senescence.

Discussion

GmNAC81 is involved in natural leaf senescence in soybean

Many reports have shown that leaf senescence is a highly regulated process that is accompanied by the massive reprogramming of gene expression via the action of transcriptional regulators (Guo et al. 2004, Buchanan-Wollaston et al. 2005). A few TFs belonging to the WRKY family and the NAC family have been assigned direct regulatory functions in developmental leaf senescence; these TFs include *WRKY53b* in Arabidopsis and soybean; *ORE1* (*ANAC092*), *ORS1*, *AtNAP* (*ANAC029*) and *NAC016* in Arabidopsis; *OsNAC5* and OsNAP in rice; *NAM-B1* in wheat; and *BeNAC1* in bamboo (Guo and Gan 2006, Uauy et al. 2006, Kim et al. 2009, Sperotto et al. 2009, Balazadeh et al. 2011, Chen et al. 2011, Kim et al. 2013, Meng et al. 2013, Liang et al. 2014), as well as the senescence-inhibiting regulators *NAC042*/*JUB1* and *NAC083*/*VNI2* of Arabidopsis (Yang et al. 2011, Wu et al. 2012). In addition to being developmentally regulated, leaf senescence is also regulated by environmental factors and stress signals. Despite longstanding interest in how developmental and environmental signals are integrated to control leaf senescence, the interplay between stress-induced and developmentally induced regulatory cascades remains poorly understood. In soybean, the only regulatory cascade known to integrate an environmental signal (light) into a natural leaf senescence program involves CRY2a, CIB1 and WRKY53b (Meng et al. 2013). Photoexcited CRY2a interacts with CIB1 to suppress its binding to *WRKY53b*, thereby antagonizing the pro-senescence function of CIB1. Here, we demonstrated that the soybean NAC transcription factor GmNAC81 is involved in age-dependent leaf senescence and, as discussed below, may integrate the osmotic stress- and ER stress-induced PCD response with a natural leaf senescence program thorough the GmNAC81/VPE regulatory module.

We have previously shown that GmNAC81 is a downstream component of the NRP/DCD-mediated cell death signaling pathway that promotes PCD and leaf senescence in response to ER stress and osmotic stress (Faria et al. 2011, Reis et al. 2011). Here, we show that the promoter activity and expression of *GmNAC81* are up-regulated during leaf senescence, suggesting that the function of GmNAC81 is closely correlated with the age-dependent leaf senescence process, as well. To substantiate this hypothesis, we provided several lines of evidence establishing that GmNAC81 plays a positive role in natural leaf senescence. First, we showed that overexpression of *GmNAC81* using the 35S promoter accelerates leaf senescence, which was monitored by measuring hallmarks of leaf senescence such as yellowing, Chl loss, MDA production, the rate of photosynthesis, the F_v/F_m ratio and the expression of senescence-associated marker genes. In fact, increased expression of *GmNAC81* accelerated and intensified leaf yellowing, Chl loss and MDA production. These phenotypes were associated with a larger, faster decline in the rate of photosynthesis, a greater F_v/F_m ratio at later stages of development and increased expression of the senescence marker genes *NRP-B* and *GmCysP* in the transgenic lines. Secondly, to some extent, the precocious senescence phenotype and transcriptional activity of GmNAC81 in the transgenic lines correlated with the expression level of *GmNAC81*. In the GmNAC81-2 and GmNAC81-3 lines, which accumulated higher transcript levels during senescence, senescence progressed more quickly and with more intense leaf yellowing, which was associated with lower Chl content, higher MDA production and faster photosynthetic decay (82 d). These changes led to a significantly lower rate of photosynthesis at later stages (103 DAG) in addition to higher expression of senescence-associated marker genes in the transgenic lines. Furthermore, the expression level of *GmNAC81* in the transgenic lines was also proportional to the up-regulation of the predicted endo-1,3-β-glucanase, the dual-specificity protein phosphatase and D-alanyl-D-alanine carboxypeptidase, which are genes targeted by GmNAC81 (Mendes et al. 2013). Finally, we showed that VIGS-mediated inactivation of *GmNAC81* causes delayed leaf senescence. The degree of *GmNAC81* silencing was negatively correlated with the expression of its direct target genes. During the R7 stage of development, we observed intense yellowing of the wild-type leaves, whereas the silenced leaves remained green. The delayed leaf senescence observed in

Fig. 8 Continued

GmNAC81-silenced plants. Representative images of plants at the R7 reproductive stage that had been infected with pSoCSV or pSoCSV-NAC81 in combination with pSoCSV-DNAB. (E) Lipid peroxidation in leaves infected with the indicated VIGS vector. Lipid peroxidation in leaves was monitored by determining the level of thiobarbituric acid (TBA)-reactive compounds and is expressed as the MDA content. The error bars indicate the 95% confidence interval based on a *t*-test ($P < 0.05$, $n = 3$). (F) The mRNA abundance of VPE in *GmNAC81*-silenced leaves. The expression of VPE was monitored by qRT–PCR. Gene expression was calculated using the $2^{-\Delta CT}$ method, and RNA helicase was used as an endogenous control. The error bars indicate the 95% confidence interval based on a *t*-test ($P < 0.05$, $n = 3$).

the silenced leaves was associated with higher Chl content, lower lipid peroxidation and lower expression of senescence-associated marker genes than in the wild-type, non-silenced leaves.

The *GmNAC81*-overexpressing lines also had shorter life cycles, which were very probably due to their earlier induction of flowering, leading to a more rapid progression through the reproductive phase. In the GmNAC81-2 and GmNAC81-3 lines, this phenotype clearly correlated with the increased accumulation of *GmNAC81* transcripts prior to flowering. In the GmNAC81-1 line, which flowered normally, the *GmNAC81* transcript levels were only slightly increased or were even repressed at the stages prior to flowering. Nevertheless, despite the precocious flowering phenotype of the GmNAC81-2 and GmNAC81-3 lines and the natural up-regulation of *GmNAC81* during the vegetative to reproductive phase transition (**Fig. 6**), our data do not allow us to assign a flowering function to the *GmNAC81* gene; because silencing *GmNAC81* did not delay flowering, as would be expected for a non-redundant gene with a direct role in flowering induction, The early flowering phenotype exhibited by the *GmNAC81*-overexpressing lines may instead reflect a pleiotropic effect of the ectopic expression of *GmNAC81*. The *GmNAC81*-silenced plants displayed delayed leaf senescence but otherwise exhibited normal development.

The GmNAC81/VPE regulatory cascade may couple stress-induced and age-dependent leaf senescence

The *GmNAC81* gene is a downstream component of the stress-induced, NRP/DCD-mediated cell death signaling pathway. Prolonged ER stress and osmotic stress induce the expression of the transcriptional activator GmERD15, which targets the NRP/DCD promoter (Alves et al. 2011). Up-regulation of NRP/DCD leads to the induction of *GmNAC81* and *GmNAC30*, which work together to activate the expression of *VPE*, the executioner of the stress-induced PCD program (Hara-Nishimura et al. 2011, Mendes et al. 2013). Therefore, GmNAC81- and GmNAC30-mediated activation of *VPE* expression links the activation of the stress-induced NRP/DCD-mediated signaling pathway to the PCD response. VPE has been shown to trigger vacuolar collapse-mediated PCD during pathogenesis and development (Hara-Nishimura et al. 2011). Here, we show that *GmNAC81* overexpression in transgenic lines causes premature leaf senescence and up-regulation of *VPE*, whereas silencing of the *GmNAC81* gene delays leaf senescence and down-regulates *VPE* expression, suggesting an underlying mechanism for the execution of the leaf senescence program. Collectively, these results suggest that GmNAC81 and VPE constitute a shared regulatory cascade for both stress-induced and natural leaf senescence in soybean. As further evidence for this hypothesis, our previous and current data show that during both age-dependent and environmentally induced leaf senescence, *VPE* and *GmNAC81* are co-ordinately up-regulated (Carvalho et al. 2004; **Fig. 6**).

In the *GmNAC81*-overexpressing lines, the expression level of the transgene was associated with the precocious senescence phenotype but was not proportionally correlated with *VPE* induction. In fact, compared with the other transgenic lines, the greater accumulation of *GmNAC81* transcripts in the GmNAC81-3 line did not result in increased *VPE* expression. Likewise, knocking down *GmNAC81* expression did not suppress *VPE* expression in the pSoCSV-NAC81-1 and pSoCSV-NAC81-2 VIGS plants but still delayed leaf senescence. Suppressed *VPE* expression was only observed in the pSoCSV-NAC81-3 VIGS plant, in which *GmNAC81* expression was completely abolished. These results have at least two implications. First, *VPE* may be controlled by additional transcriptional regulators and/or regulatory cascades that are not altered in the silenced plants or the transgenic lines. In line with this hypothesis, we have previously shown that GmNAC30 works with GmNAC81 to activate the *VPE* promoter fully (Mendes et al. 2013). The optimal stoichiometry of these two transcriptional regulators is likely to be a prerequisite for the maximal induction of the *VPE* promoter. Secondly, GmNAC81-mediated leaf senescence may be only partially controlled by the GmNAC81/VPE regulatory cascade. The increased accumulation of ABA in the overexpressing lines may be an additional factor that contributes to the precocious leaf senescence phenotype. During leaf senescence, genes associated with ABA synthesis and signaling are up-regulated, and the endogenous ABA level increases (Zhang and Zhou 2013). The increased ABA level during the progression of leaf senescence induces a series of SAGs, including SAG113, a Golgi-localized protein phosphatase of the PP2C family, which is a direct target of the senescence-promoting AtNAP transcription factor (Zhang and Gan 2012). The ABA–AtNAP–SAG113 regulatory module controls stomatal movement and water loss specifically in senescing leaves. In rice, the ortholog *OsNAP* has also been shown to function upstream of SAGs in ABA-induced leaf senescence, as a key component linking ABA signaling and leaf senescence (Liang et al. 2014). Although GmNAC81 may also regulate ABA synthesis (**Fig. 5**), several lines of evidence indicate that the molecular mechanism underlying GmNAC81 positive regulation of leaf senescence is distinct from the OsNAP- and AtNAP-mediated connection of ABA and leaf senescence. First, while *OsNAP* is induced exclusively by ABA, *GmNAC81* is generally up-regulated by a set of environmental stressors and hormones, including ABA, SA and MeJA, all involved in leaf senescence. Furthermore, unlike OsNAPs, which negatively regulate ABA synthesis, GmNAC81 may inversely modulate ABA synthesis as a positive regulator of the expression of ABA biosynthetic enzymes (**Fig. 5D**). Moreover, a phylogenetic analysis of senescence-associated NAC genes demonstrated that *GmNAC81* (in red) and NAPs (blue and asterisks) differ greatly from each other as they cluster separately in very distinct clades (**Supplementary Fig. S8**). Finally, while the NAPs target genes are known SAGs, such as Chl degradation genes and nutrient mobilization genes, the GmNAC81 target genes are hydrolytic enzymes most probably involved in PCD in addition to VPE, an executioner of the stress-induced and developmentally regulated plant-specific PCD, which is mediated by collapse of the vacuole (Kinoshita et al. 1999, Hatsugai et al. 2004, Yamada et al. 2004, Hara-Nishimura et al. 2011).

GmNAC81 overexpression also decreased SA levels during leaf senescence. Because *GmNAC81* itself is induced by SA, this observation suggests that GmNAC81 may regulate SA synthesis or accumulation via negative feedback. Consistent with this hypothesis, we have previously shown that overexpression of the ER-resident molecular chaperone BiP inhibits the expression and cell death activity of GmNAC81, causing an increase in SA level in senescing leaves (Reis et al. 2011, Carvalho et al. 2014). BiP also represses the expression of the upstream component NRP/DCD, thereby inhibiting the transduction of PCD signals derived from multiple types of stress (Reis et al. 2011) and from developmental triggers to regulate leaf senescence (Carvalho et al. 2014). Therefore, although the negative regulation of NRP/DCD and *GmNAC81* expression and activity induce SA accumulation, increased *GmNAC81* expression decreases SA levels. Collectively, these data suggest that the NRP/DCD–GmNAC81 signaling module may be a negative feedback regulator of SA synthesis or accumulation.

Materials and Methods

Plant expression cassettes and soybean transformation

The full-length *GmNAC81* open reading frame (ORF; GenBank accession No. AY974354) was amplified from leaf cDNA with primers NAC6XBAF (5′-ATCTCTAGACAACCATGGAAGACATG-3′) and NAC6KPNR (5′-CGCGGTACCTCAGTAGAAGTTTAG-3′), which contained *Xba*I and *Kpn*I restriction sites, respectively (underlined). The amplified fragment was digested with *Xba*I and *Kpn*I, and cloned into the corresponding sites of pUC19-35SAMVnos (Dias et al. 2006). The resulting vector, p35SNAC6, contained the full-length *GmNAC81* coding region under control of a tandem CaMV 35S promoter and an enhancer region from the *Alfalfa mosaic virus*, as well as the polyadenylation signal of the *nos* gene. Then, the *GmNAC81* expression cassette was released from p35SNAC6 and cloned into pAC321 (Rech et al. 2008) to yield pNAC6ahas, which harbors the *GmNAC81* expression cassette and the *ahas* gene, which confers tolerance to the herbicide imazapyr. Soybean (*Glycine max* cv. BR16) was transformed with pNAC6ahas, as previously described by Aragão et al. (2000). Primary transformants were selected by PCR. Segregation analyses of independently transformed soybean lines (35S:GmNAC81-1, 35S:GmNAC81-2 and 35S:GmNAC81-3) were performed using PCR.

PCR analysis of transgenic lines

Total DNA was isolated from 7-day-old leaves of transformed and untransformed soybean cv. BR16 using extraction buffer containing 2% CTAB (cetyltrimethyl ammonium bromide), 1.4 M NaCl, 100 mM Tris–HCl pH 8.0, 20 mM EDTA and 0.2% β-mercaptoethanol as previously described by Doyle and Doyle (1987). PCR was carried out on 50 ng of genomic DNA using primers GmNAC6 Forward 5′- AAAAAGCAGGCTTCACAATGGCAACCATGGAA-3′, and GmNAC6 Reverse 5′-AGAAAGCTGGGTCTTTCAGTAGAAGTTTAGAAT-3′ (0.5 nM each) and 0.25 U of GoTaq® DNA Polymerase (Promega) in a final volume of 50 µl. The mixture was heated at 94 °C for 3 min, and 35 cycles of PCR were conducted (45 s at 94 °C, 45 s at 53 °C and 60 s at 72 °C) with a final extension at 72 °C for 10 min. The reactions were performed using a PCR System 9700 (Life Technologies). The GmNAC81-specific primers amplify an 850 bp sequence from the GmNAC81 cDNA and span an intronic region (1,000 bp) in the genomic GmNAC81 sequence.

Plant materials and growth conditions

Seeds of the wild type (*G. max* cv. BR16) and transgenic soybean lines (35S::GmNAC81-1, 35S::GmNAC81-2 and 35S::GmNAC81-3) were germinated in organic soil (Bioplant), transferred to 3 liter pots containing a mixture of organic soil and soil (3 : 1 v/v) and grown in a greenhouse under natural light, relative humidity (65–85%) and temperature (15–35 °C) conditions with approximately equal day and night length. The genotypes were grown side-by-side in randomized blocks and were observed throughout their life cycles.

All physiological parameters were measured in the third trifoliate leaf. Analyses of gas exchange and pigment content were initiated 40 DAG, when the leaves had fully expanded and were photosynthetically active, and terminated with leaf abscission at 117 d. IRGA (infrared gas analyzer) analyses were performed weekly until senescence. Rulers were used to measure the heights of the stems on each of 3 d, when the plants were photographed, the number of nodes was examined and the developmental stage was scored. To measure biomass, the roots were separated from the shoots, weighed and kept in an incubator with forced ventilation at 60 °C until they reached a constant weight.

Virus-induced silencing of the *GmNAC81* gene

Total RNA was extracted from soybean leaves using TRIzol (Life Technologies) and treated with RNase-free DNase (Life Technologies). cDNA synthesis was performed using 3 µg of total RNA, oligo-dT(18) and M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendations. Approximately 1 µl of the cDNA was used to PCR-amplify a cDNA fragment of *GmNAC81* encoding the C-terminus, delimited by positions 486–889, using 0.2 mM dNTPs, the primers at concentrations of 0.4 µM each [gmNac6BglIIFwd, 5′-aaaagatctAACTTCGTTGAAAGTGCTGGAG-3′ and gmNac6BglIIRvs, 5′-aaaagatctAGAAGTTTAGAATATTGGTGGGAG-3′ both carrying a *Bgl*II site (underlined)], 5 µl of 10× High Fidelity Platinum Taq buffer (Promega) and 0.2 U of High Fidelity Platinum Taq (Promega) in a final volume of 50 µl. The amplification reaction was conducted with an initial denaturation step at 94 °C for 3 min; followed by 35 cycles of 94 °C for 45 s, 54 °C for 45 s and 68 °C for 45 s; and a final extension at 68 °C for 10 min. The amplified product was analyzed by electrophoresis on a 1% agarose gel stained with 0.1 µg ml^{-1} ethidium bromide, purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned into vector pCR2.1 (Life Technologies) using a TA Cloning® Kit according to the manufacturer's recommendations. The resulting clone pUFV2435 harbors the fragment encoding the GmNAC81 C-terminus flanked by *Bgl*II restriction sites. The GmNAC81 fragment was released from pUFV2435 by digesting with *Bgl*II, purified from the agarose gel and inserted into the multiple cloning site of the VIGS vector pUFV1713, which is also called pSoCSV because it was derived from the soybean-infecting geminivirus SoCSV (Coco et al. 2013). The resulting clone, pSoCSV-NAC81, is an infectious derivative of SoCSV DNA-A that harbors a 403 bp GmNAC81 fragment in place of the SoCSV coat protein gene. It and the rest of the SoCSV DNA-A sequence are located between two viral replication origins. Soybean radicles were inoculated via biolistics with 4 µg of pSoCSV-NAC81 or pSoCSV in combination with 4 µg of SoCSV DNA-B infectious clones to mediate the systemic movement of the VIGS vector as previously described (Santos et al. 2008). The accumulation of viral DNA was monitored by PCR, and the silencing of the *GmNAC81* gene was monitored by qRT–PCR.

Measurements of photosynthesis

Leaf gas exchange parameters and Chl *a* fluorescence were measured simultaneously using the aforementioned gas exchange system equipped with an integrated fluorescence chamber head (LI-6400-40, LI-COR Inc.). The net CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s) and internal CO_2 concentration (C_i) were measured on attached leaves under artificial PAR, i.e. 1,000 µmol photons m^{-2} s^{-1} at the leaf level and 400 µmol CO_2 mol^{-1} air. All measurements were performed under naturally fluctuating temperature and vapor pressure deficit conditions.

After measuring the gas exchange parameters, the steady-state fluorescence yield (F_s) was measured, after which a saturating white light pulse (8,000 µmol m^{-2} s^{-1}; 0.8 s) was applied to determine the light-adapted maximum fluorescence (F_m'). The actinic light was then turned off and far-red illumination (2 µmol m^{-2} s^{-1}) was used to measure the light-adapted initial fluorescence (F_0'). Using these parameters, the photochemical quenching coefficient (qP) and the capture efficiency of excitation energy by open PSII reaction centers

(F_v'/F_m') were estimated. The actual PSII photochemical efficiency (φ_{PSII}) and electron transport rate (ETR) were also calculated.

Chl content

Total Chl content was determined as described by Wellburn (1994). Three 0.7 cm leaf discs were incubated with 2.5 ml of $CaCO_3$-saturated dimethylsulfoxide (DMSO). Chl *a* and *b* absorb within a narrow region (maxima) in the blue (near 428 and 453 nm) and red (near 661 and 642 nm) spectral ranges. Isolated yellow carotenoids have broad absorption, with three maxima or shoulders in the blue spectral range between 400 and 500 nm. Therefore, after 16 h at room temperature, the absorbance of the extracts was determined spectrophotometrically at 480, 649.1 and 665.1 nm and is expressed as $\mu g\ cm^{-2}$.

Lipid peroxidation

The extent of leaf lipid peroxidation was estimated by measuring the MDA content as described by Cakmak and Horst (1991). Approximately 0.150 g of leaves were homogenized in 2 ml of 0.1% (v/v) trichloroacetic acid and then centrifuged at 12,000×*g* for 15 min. All steps were performed at 4 °C. An aliquot of the supernatant (0.5 ml) was added to 1.5 ml of 0.5% (v/v) TBA in 20% (v/v) trichloroacetic acid, and the samples were incubated at 90 °C for 20 min. The reaction was stopped by incubation on ice followed by centrifugation at 13,000×*g* for 4 min. The absorbance of the supernatant was measured at 532 nm and subtracted from the non-specific A_{600} value. The MDA concentration was calculated using the molar absorption coefficient of $155\ mM^{-1}\ cm^{-1}$ (Heath and Packer 1968).

Determination of ABA, SA and JA content

Six leaf discs were crushed in 200 µl of 10% (v/v) methanol and 1% (v/v) acetic acid using tugsten beads (25 Hz for 8 min). The plant extract was sonicated in a water bath for 1 h and then for five more additional pulses with a hand sonicator (ultrasonic homogenizer 4610 series, Cole-Parmer Instrument) at 30% power for 20 s on ice. The extract was purified by centrifugation for 10 min, and 5 ml of the supernatant was injected into an LC-MS system (ultra performance liquid chromatography, model 1200 infinity series, coupled to a quadrupole sequential mass spectrometer, model 6430, Agilent). The mobile phase was water with 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B) using a time gradient of 0/5, 11/60, 13/95, 17/95, 19/5 and 20/5. Hormone analyses were performed in the MRM (multiple reaction monitoring) mode, and the mass of the precursor ion (fragments established upon testing the fragmentation of each molecule) was determined for ABA (263/153), SA (137/93) and JA (209/59). The absolute quantity of each hormone was determined based on the calibration curves of their respective standards. The data were analyzed using the MassHunter package (Agilent) to obtain the area under the peaks and the retention time of each hormone in each sample. A defined peak for each analyzed hormone was obtained from soybean leaf extracts.

RNA extraction, cDNA synthesis and qRT–PCR

Total RNA was extracted from leaves at different developmental stages (V4–V5; R1–R7) using TRIzol (Life Technologies) and was treated with 2 U of RNase-free DNase (Life Technologies). The total RNA was quantified with a spectrophotometer (Evolution 60, Thermo Scientific) and examined by electrophoresis on a 1.5% (w/v) denaturing agarose gel stained with ethidium bromide. cDNA synthesis was performed using 3 µg of total RNA, 5 µM oligo-dT(18), 0.5 mM dNTPs and 1 U of M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendations.

All of the real-time PCR procedures, including tests, validations and experiments, were conducted according to the recommendations of Life Technologies. Real-time RT–PCRs were performed on an ABI 7500 instrument (Life Technologies) using cDNA from different developmental stages, gene-specific primers (**Supplementary Table S1**) and SYBR Green PCR Master Mix (Life Technologies). The amplification reactions were performed as follows: 10 min at 95 °C followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. The soybean RNA helicase gene (Irsigler et al. 2007) and 18S rRNA were used as endogenous controls to normalize the real-time RT–PCR values. Gene expression was quantified using the $2^{-\Delta Ct}$ (absolute quantification) method.

Statistical analyses

For the stress-induced expression data (**Fig. 4**), the statistical analysis was performed using the R package nparcomp, which provides access to rank-based methods for the non-parametric analysis of one-way layouts (Konietschke et al. 2014). It provides procedures performing multiple comparisons and computing simultaneous confidence intervals for the estimated effects.

All other statistical analysis was performed using R software with the ExpDes package (Ferreira et al. 2013). A split-plot analysis of variance (ANOVA) with a completely randomized design was used for the IRGA and developmental data experiments. The main plot was the genotype and the subplot was the time (DAG). A Tukey test with *P*-value <0.1 considered significant was used to compare multiple means. Regression analysis was performed for the photosynthesis data. Linear, quadratic and cubic models were tested for the best fit for R^2. The limits of the 95% confidence intervals of the regression coefficients were calculated with the *t*-values of the formulae of the linear models and were presented as the class stats in R. For the yield data, one-way ANOVA with Tukey test was performed, with a *P*-value <0.05 considered significant.

Phylogenetic analyses of senescence-associated NAC genes

The senescence-associated NAC genes were initially identified from *Arabidopsis thaliana*, soybean, rice, cotton, wheat and barley using the database of orthologous groups and functional annotation eggNOG 4.1 (Powell et al. 2013). The amino acid sequences of orthologous genes were recovered from TAIR (http://arabidopsis.org/) and Phytozome v10.3 databases. For phylogenetic analyses, the amino acid sequences were aligned using MUSCLE (Edgar 2004). Phylogenetic trees were constructed using Bayesian inference performed with MrBayes v3.2.2 (Ronquist and Huelsenbeck 2003) with mixed amino acid substitution model (Blosum). The analyses were carried out running 20,000,000 generations and excluding the first 5,000,000 generations as burn-in. The trees were visualized with Figtree v1.4 software (http://tree.bio.ed.ac.uk/software/figtree/).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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