

PvNAC1 and *PvNAC2* Are Associated with Leaf Senescence and Nitrogen Use Efficiency in Switchgrass

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Abstract Two full-length cDNAs encoding NAM, ATAF, and CUC (NAC)-family transcription factors (TFs) were isolated from two different cultivars of *Panicum virgatum* L. (switchgrass) and named *PvNAC1* and *PvNAC2*. Phylogenetic analysis of *PvNAC1* and *PvNAC2* grouped them with NAC proteins involved in senescence in annual plant species. Transcript profiling revealed that both *PvNAC1* and *PvNAC2* are induced during leaf senescence. Expression of a *PvNAC1*-green fluorescent protein (GFP) fusion in plant cells revealed a nuclear location of the protein, consistent with a role in transcriptional regulation. Expression of *PvNAC1* in an *Arabidopsis nap* stay-green mutant suppressed its senescence defect. Expression of *PvNAC1* in wild-type *Arabidopsis* triggered early leaf senescence and remobilization. Transcriptome analysis implicated leaf protein degradation and nitrogen recycling enzymes in NAC-dependent seed protein increase in *Arabidopsis*. Overexpression of *pvNAC2* in switchgrass resulted in increased aboveground biomass associated with increased transcript levels of key nitrogen metabolism genes in leaves and nitrate and ammonium transporter genes in roots. The results indicate that NAC TFs play conserved roles in leaf senescence in the plant kingdom not only in annual monocots

and dicots but also in perennial plants such as switchgrass. *PvNAC1* and *PvNAC2* hold promise for improving nutrient use efficiency in switchgrass through genetic manipulation.

Keywords Switchgrass · NAC · Senescence · Nitrogen remobilization/uptake

Introduction

Switchgrass (*Panicum virgatum* L.) is a perennial plant with water-efficient C4 photosynthesis that is native to the North American tall grass prairies and has been targeted for development as a bio-energy crop in the USA. Switchgrass has higher biomass yield than many annual crops and can be grown on marginal soils, which avoids competition with food crops for prime arable land [1–3]. There are two switchgrass ecotype classes, “upland” and “lowland,” which refer to latitude. Average annual biomass yields of switchgrass in the USA were estimated to be 12.9 and 8.7 tonnes/hectare for lowland and upland ecotypes, respectively [4].

Successful development of switchgrass for biofuel production will depend, in part, on agricultural sustainability including nutrient conservation in the soil-plant system [1, 5]. Like other perennial grasses, switchgrass plants remobilize a portion of some nutrients, including N, P, and K, from shoots to roots during yearly senescence [5]. Nonetheless, substantial amounts of soil nutrients are removed with the harvested shoot biomass. For example, total N removed with biomass varied from 31 to 63 kg N ha⁻¹ year⁻¹ in a one-cut fall harvest after senescence, compared to 90 to 144 kg ha⁻¹ year⁻¹ for a two-cut system with the first harvest prior to senescence, over 5 years of measurements [6]. Such nutrient withdrawal rates inevitably deplete soil N and necessitate the addition of fertilizer N to maintain switchgrass productivity. Synthesis and application of fertilizer N are energy-intensive and

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economically and environmentally costly. High N content in harvested biomass can be an additional liability because it yields NO_x compounds upon oxidation, which are potent atmospheric pollutants [7]. Therefore, decreasing the nutrient concentration in harvested switchgrass, with the exception of C, H, and O that are converted to biofuels, will help to conserve soil nutrients, reduce the need for fertilizers, and, thereby, reduce the cost and environmental impact of switchgrass production.

Senescence is the final stage of plant development, leading to the death of specific organs or the whole plant [8]. Leaf senescence is a regulated process and is important for remobilization of nutrients from leaves to other plant parts. For annual plants including many crop species, leaf senescence mobilizes nutrients for seed production. Small-grain cereals like rice, wheat, and barley mobilize up to 90 % of the nitrogen in vegetative organs to the grain [9, 10]. In perennial grasses like switchgrass, nutrients in leaves are remobilized during yearly senescence not only to the inflorescence for seed production but also to the root system for storage and later re-use. There is natural variation among switchgrass accessions for nitrogen remobilization efficiency, with a range of 20–61 % remobilization from shoots recorded in one set of experiments [5]. Nothing is known about the genetic regulation of nutrient remobilization in switchgrass and other perennial grasses at present. Such knowledge and the corresponding regulatory genes could pave the way to improved cultivars with increased nutrient remobilization and, consequently, increased nutrient use efficiency for biomass production.

Transcription factor (TF) genes have been implicated in the control of leaf senescence and nutrient remobilization to seeds in annual plant species. A NAC TF gene, *NAM-B1*, from wild emmer wheat was associated with accelerated senescence and increased grain zinc, iron, and protein content [11]. Repression of four homologs of *NAM-B1* in a hexaploid wheat, via RNA interference (RNAi), delayed plant senescence by more than 3 weeks and reduced grain protein, zinc, and iron content by more than 30 %, while flag leaves of RNAi plants retained more residual nitrogen, zinc, and iron than wild-type controls [11]. Rice *OsNAC5* was found to be induced earlier and to a greater extent in flag leaves and panicles of rice cultivars with higher seed protein content, indicating that *OsNAC5* may be involved in amino acid remobilization from green tissues to seeds [12]. Although there have been some microarray-based transcriptomic studies of senescence in *Arabidopsis* [13–15], *Populus* [16], wheat [17], and barley [18], little is known about processes that are regulated directly by NAC TFs. Recently, the effect of downregulation of *TtNAM-B1* on wheat flag-leaf gene expression during senescence (12 days after anthesis) was investigated using RNA-seq technology [19]. The list of putative *TtNAM-B1*-regulated genes included transporters, hormone-regulated genes, and other transcription factors.

Here, we report the identification and characterization of two NAC TF genes, *PvNAC1* and *PvNAC2*, from two different switchgrass cultivars, Summer VS16 and Alamo AP13, respectively. Phylogenetic analysis of *PvNAC1* and *PvNAC2* grouped them with NAC proteins involved in senescence in annual plant species. *PvNAC1* and *PvNAC2* transcript levels increased in switchgrass leaves during natural and artificial, dark-induced senescence. Expression of *PvNAC1* in an *Arabidopsis* stay-green mutant, *atnap*, suppressed the defective-senescence phenotype of the mutant. Expression of *PvNAC1* in wild-type *Arabidopsis* promoted leaf senescence and nitrogen remobilization. Transcriptome analysis linked *PvNAC1* expression in *Arabidopsis* to induction of protein and nitrogen-recycling genes and leaf protein degradation. Overexpression of *pvNAC2* in switchgrass increased nitrogen use efficiency and biomass production in switchgrass.

Results

Cloning and Phylogenetic Analysis of *PvNAC1* and *PvNAC2*

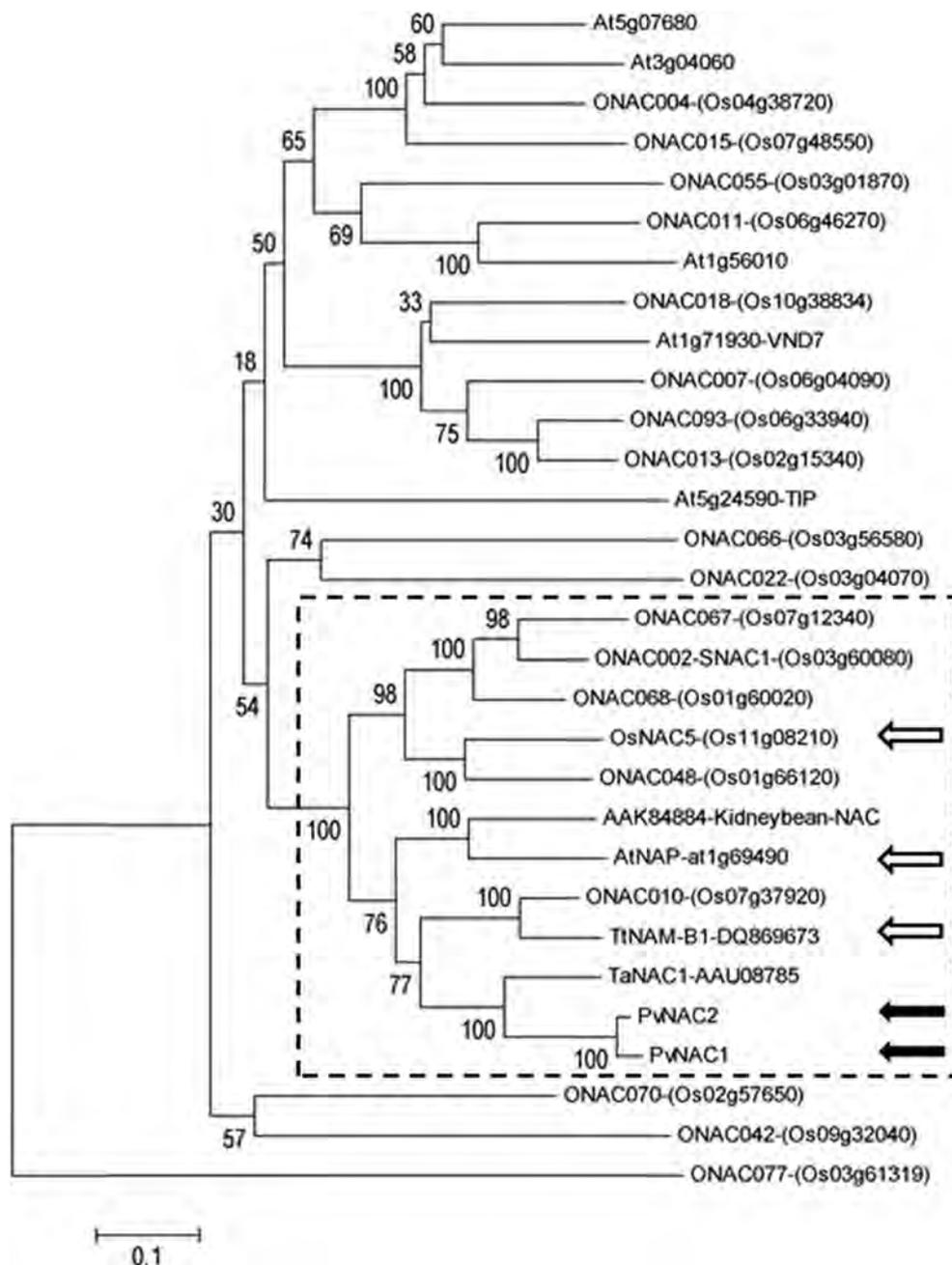
Fragments of several putative NAC TF cDNA were amplified from switchgrass cultivar Summer VS16 by 5'-Rapid Amplification of cDNA Ends (RACE) and 3'-RACE (see experimental procedures). The N-terminal portions of deduced putative NAC proteins were aligned with typical NAC TFs from other plant species, resulting in the identification of a switchgrass NAC protein with five characteristic N-terminal DNA binding motifs (A–E) that we called *PvNAC1* (Fig. S1) [20, 21]. The full-length *PvNAC1* coding sequence was found to be 1,104 bp (GenBank accession no. JN673545). Comparison of *PvNAC1* cDNA and genomic sequences, obtained via PCR-amplification, revealed two introns in the *PvNAC1* gene. Another NAC TF cDNA, with a coding sequence of 1113 bp, was amplified from switchgrass cultivar Alamo AP13 and named *PvNAC2*. The coding sequences of *PvNAC1* and *PvNAC2* share 95.2 % identity and their deduced proteins share 92 % identity, indicating that they may have similar functions in the two cultivars.

Phylogenetic analysis of various plant NAC TFs grouped *PvNAC1* and *PvNAC2* with *AtNAP*, *TtNAM-B1*, and *OsNAC5*, each of which have been implicated in senescence and/or nutrient remobilization [22, 11, 12] (Fig. 1). At the amino acid level, the complete *PvNAC1* protein shares 48.1 % identity with *AtNAP*, 37.9 % with *TtNAM-B1*, and 34.4 % with *OsNAC5*.

Expression of *PvNAC1* and *PvNAC2* in Leaves Is Induced During Natural Senescence and by Prolonged Darkness

Natural senescence in grasses begins in the oldest, most basal leaves of vegetative plants and proceeds to younger, higher

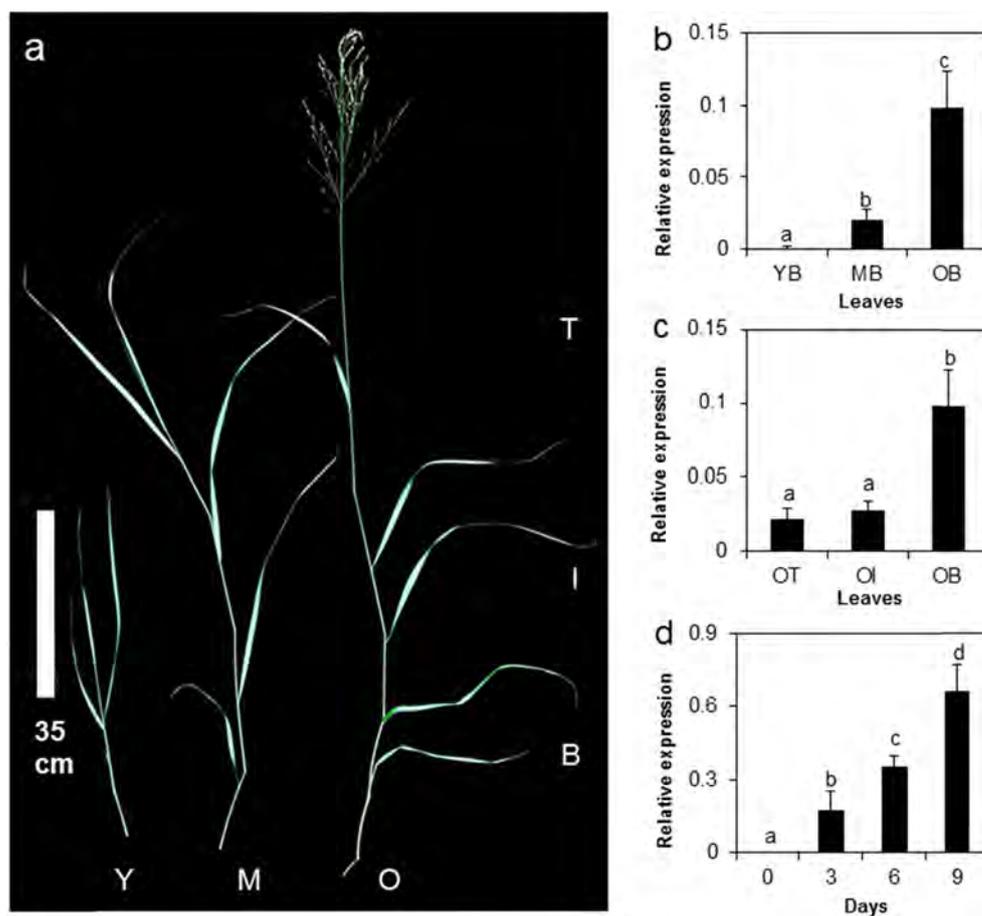
Fig. 1 Phylogenetic analysis of PvNAC1 and PvNAC2 with representative rice NACs as well as other NACs with known or indicative functions. The branch within the *dashed box* includes PvNAC1 and PvNAC2 (indicated by *solid arrows*) and NAC TFs related to senescence in *Arabidopsis* (AtNAP) [22] and nutrient remobilization in wheat (TtNAM-B1) [11] and rice (ONAC009/OsNAC5) [12] (indicated by *empty arrows*). Confidence levels are shown as percentages



leaves of the panicle as plants mature and transition to reproductive development [23]. Transcript levels of *PvNAC1* in bottom (B), intermediate (I), and top (T) leaves of young (Y) and middle-aged (M) and old (O) tillers of cultivar Summer were quantified by quantitative real-time (RT)-PCR (qRT-PCR) (Fig. 2a). *PvNAC1* transcript levels in bottom leaves were lowest in young tillers (YB), higher (19-fold) in late vegetative tillers (MB), and highest (91-fold) in reproductive tillers (OB; Fig. 2b). Within old, reproductive tillers, *PvNAC1* transcript levels were lowest in young leaves at the top of

tillers (OT), intermediate in intermediate leaves (OI), and highest in the oldest, bottom leaves (OB; Fig. 2c). In other words, *PvNAC1* expression increased with plant development and leaf age. Notably, *PvNAC1* transcript levels in leaves increased prior to visible signs of senescence (leaf yellowing). Finally, leaf *PvNAC1* transcript levels increased in response to prolonged darkness, which induced senescence/yellowing of detached leaves (Fig. 2d). *PvNAC2* expression in Alamo AP13 showed similar responses to leaf aging and dark incubation (Fig. S2). Taken together, these results are consistent

Fig. 2 Expression of *PvNAC1* in leaves of switchgrass genotype VS16. **a** Young (*Y*), middle-aged (*M*), and old (*O*) tillers and leaves at different positions (*T*, top; *I*, intermediate; and *B*, bottom). **b** Relative transcript levels of *PvNAC1* in the bottom leaves of young, middle-aged, and old tillers (YB, MB, and OB, respectively), determined by qRT-PCR. **c** Relative transcript levels of *PvNAC1* in the top, intermediate, and bottom leaves of old tillers (OT, OI, and OB). **d** Relative transcript levels of *PvNAC1* in intermediate leaves detached from middle-aged tillers and incubated at room temperature on wet paper towel in continuous darkness for 0, 3, 6, and 9 days. All transcript levels are expressed relative to that of the switchgrass *Ubiquitin* gene in each sample. Error bars indicate SD of three biological replicates. Different letters above columns in each figure indicate significant differences based on Tukey's test ($p < 0.05$)



with a possible role of *PvNAC1* and *PvNAC2* in the regulation of leaf senescence.

PvNAC1 Is Localized in the Nucleus

To determine the subcellular location of *PvNAC1*, a translational fusion of *PvNAC1* and green fluorescent protein (GFP) was constructed under the control of the constitutive CaMV-35S promoter, *p35S* (Fig. 3a). In particle-bombarded onion epidermal cells expressing the *PvNAC1*-GFP fusion protein, GFP fluorescence was detected only in nuclei (Fig. 3b–d), whereas in cells bombarded with *p35S*-GFP, green fluorescence was detected in multiple compartments including nuclei, plasma membrane, and cytoplasm (Fig. 3e–g). Nuclear localization of *PvNAC1* is consistent with a possible role of the protein in regulation of gene transcription.

Complementation of an *Arabidopsis Atnap* Mutant with *PvNAC1*

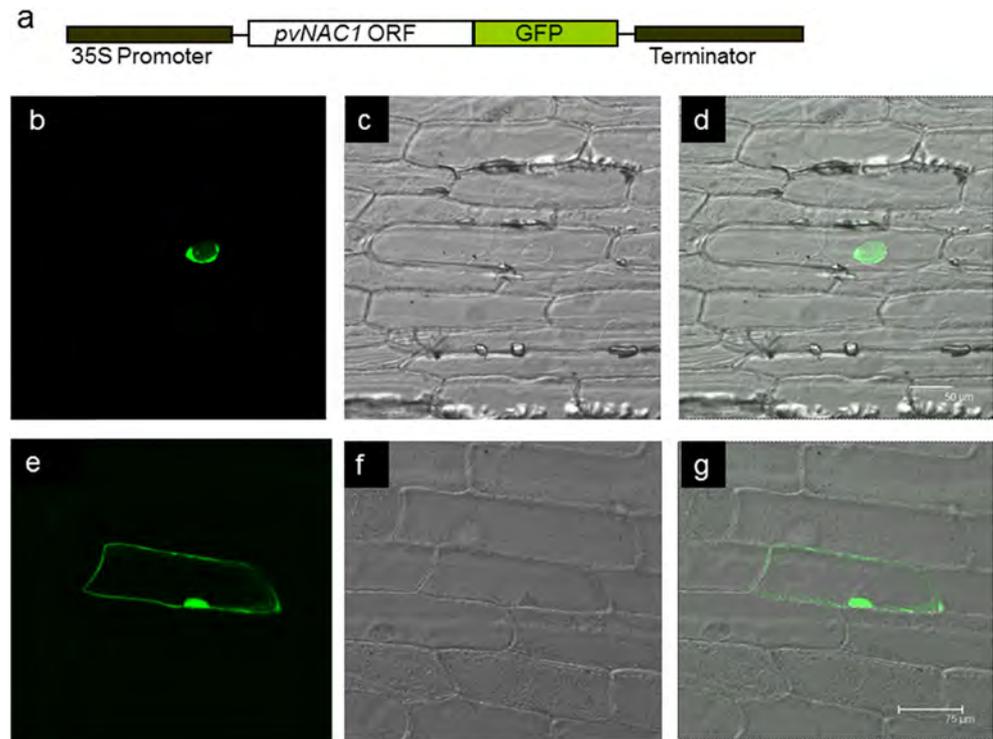
PvNAC1 and *PvNAC2* are on the same branch of the phylogenetic tree as *AtNAP* (Fig. 1). To test the function of *PvNAC1* *in planta*, an *Arabidopsis atnap* T-DNA insertion mutant was isolated (Fig. S3ab), and homozygous mutant

plants were transformed with constructs that contained *PvNAC1* or *GUS* (negative control) coding sequence under the control of 2 kb of the *Arabidopsis AtNAP* promoter sequence. Fully expanded, non-senescing leaves of wild type (WT) and *atnap* mutant plants were excised and incubated on a wet filter paper in total darkness at 22 °C for 5 days, which resulted in yellowing of WT leaves but not of *atnap* leaves, which remained green (Fig. 4a). In the same assay, leaves of *atnap* plants transformed with *PvNAC1* turned yellow, like the WT, while those transformed with *GUS* remained green (Fig. 4a). The chlorophyll concentration of WT and *PvNAC1*-expressing *atnap* were not significantly different (approx. 300 ng/g FW), while those of *atnap* and *GUS*-expressing *atnap* were more than threefold higher than the WT (approx. 1100 ng/g FW; Fig. 4b). Thus, *PvNAC1* restored the WT phenotype to *atnap*, indicating that the switchgrass protein *PvNAC1* can substitute for *AtNAP* and trigger leaf senescence.

Expression of *PvNAC1* Triggers Leaf Senescence and Nitrogen Translocation

PvNAC1 was cloned into vector *pER8* under the control of an estrogen-inducible transactivator [24] and transformed into

Fig. 3 Localization of PvNAC1 protein in plant cells. **a** Schematic representation of the PvNAC1-GFP fusion construct in vector *pAVA121*. **b–d** Nuclear localization of p35S::PvNAC1-GFP fusion protein detected in onion epidermal cells. **e–g** Subcellular localization of p35S::GFP as a control. **b, e** GFP fluorescence. **c, f** White-light micrographs. **d, g** Overlay of **b** and **c** and **e** and **f**, respectively



Arabidopsis wild type. Detached leaves of WT, *pER-PvNAC1*, and *pER-GFP* (negative control) transgenic plants were incubated on filter paper wetted with estradiol (EST) solution and kept under constant light for 5 days, which resulted in senescence (yellowing) of *pER-PvNAC1* leaves but not those of WT or *pER-GFP* (Fig. S4).

Estradiol treatment of whole plants had no effect on *pER-GFP* or WT controls but resulted in accelerated senescence (yellowing) of mature leaves on *pER-PvNAC1* transgenic plants (Fig. 5a). Interestingly, young leaves of *pER-PvNAC1* plants remained green after estradiol treatment. DMSO solution without estradiol had no effect on any of the plant genotypes. Mature leaves of estradiol-treated *pER-PvNAC1* plants had 53 % less chlorophyll (Fig. S5a) and 24 % less total nitrogen than DMSO-treated controls, after 5 days of treatment (Fig. 5b). *PvNAC1* was found to be expressed only in estradiol-treated *pER-PvNAC1* transgenic leaves (Fig. S5b). Thus, expression of *PvNAC1* was sufficient to trigger leaf senescence in whole plants, resulting in chlorophyll degradation and nitrogen translocation out of leaves. However, transient expression of *PvNAC1* for 5 days did not increase nitrogen concentration in mature seeds (Fig. S5c).

Role of PvNAC1 in Nitrogen Remobilization During Senescence

To understand how *PvNAC1* mediates leaf senescence and nitrogen remobilization when expressed in *Arabidopsis*, we carried out transcriptome analysis of *pER-PvNAC1* transgenic

Arabidopsis leaves, following estradiol-induced *PvNAC1* expression for 3, 6, 12, 72, and 120 h. *PvNAC1* transcript level peaked after 6 h of estradiol treatment, then declined gradually (Fig. S6a). Transcript levels of the senescence marker gene, *SAG12*, increased while chlorophyll concentration decreased significantly within 72 h of estradiol treatment, compared to the DMSO-treated control (Fig. S6bc). Six hours after the commencement of estradiol treatment, at the peak of *PvNAC1* expression (Fig. S6a), transcript levels of 248 genes had increased by at least twofold, and transcripts of 18 genes decreased at least twofold compared to the DMSO control (Table S1). After 72 h of *PvNAC1* expression, there were 921 genes upregulated and 1,151 genes downregulated by a factor of at least two (Table S1). To explore the biological processes regulated directly or indirectly by *PvNAC1*, MapMan analysis (Pathways: Regulation_overview) [25] was performed on transcriptome data. After 6 h of estradiol treatment, a total of 99 genes with at least twofold change in transcript level were assigned to multiple processes, but especially regulation of transcription with 22 genes, 19 of which were induced and 3 repressed; receptor kinase signaling with 18 genes all being induced; calcium signaling with 17 of 18 genes induced; hormone metabolism and signaling with 13 of 16 genes induced; protein degradation with 11 of 13 genes induced; and posttranslational protein modification with five of six genes induced (Fig. 6a). Transcript levels of 569 genes with a change of at least twofold by 72 h of *PvNAC1* expression, compared to DMSO treatment control, were assigned to a variety of regulatory processes (Fig. 6b). Transcriptional regulators

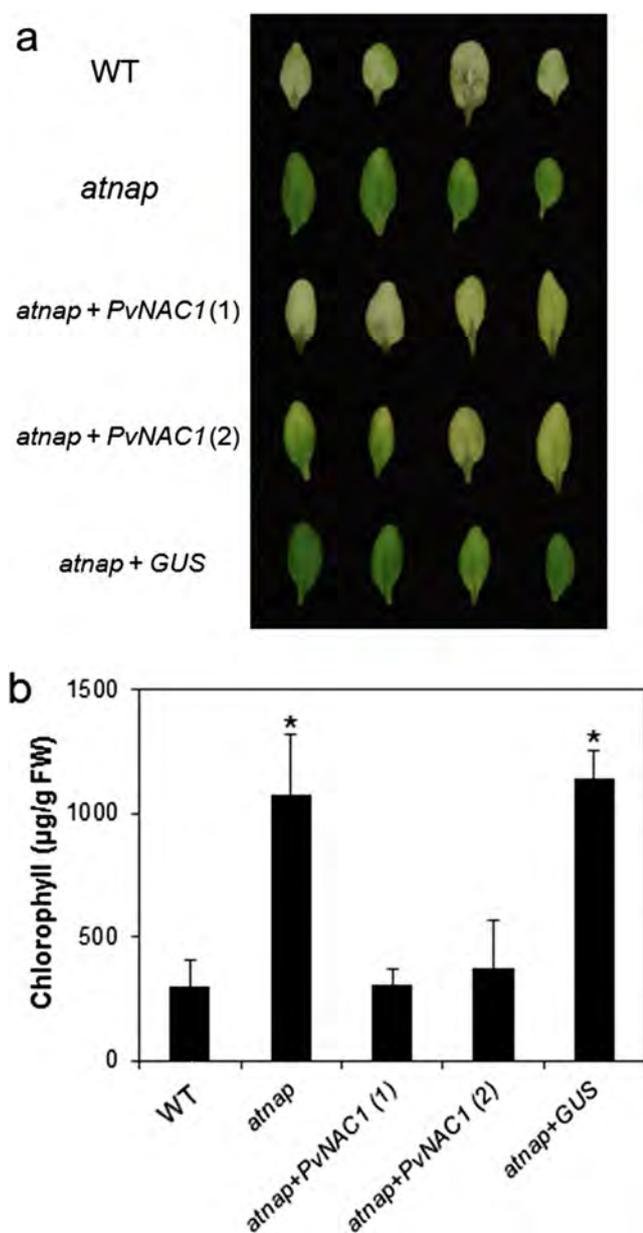


Fig. 4 *PvNAC1* suppression of the stay-green phenotype of the *Arabidopsis nap* mutant. **a** Leaves were detached from wild type (*WT*), *atnap* and *atnap* transformed with *PvNAC1* (two independent lines) or the *GUS* gene (as a negative control), and incubated in complete darkness for 5 days prior to photography. **b** Chlorophyll concentration in different leaves after 5 days of dark treatment, with abbreviations as in (a). Values are means \pm SD ($n=4$). Asterisk indicates significant difference at $p<0.01$ compared with WT, using the Student's *t* test

constituted the largest functional class of genes regulated by 6 and 72 h of *PvNAC1* expression. Interestingly, induced genes involved in protein degradation moved from the fifth most numerous set of regulated genes at 6 h to the second most numerous set by 72 h, perhaps reflecting a shift towards cellular deconstruction by that time. Consistent with this idea, chlorophyll concentration of plant leaves decreased by 12 % after 72 h of *PvNAC1* expression (Fig. S6c). In contrast to the

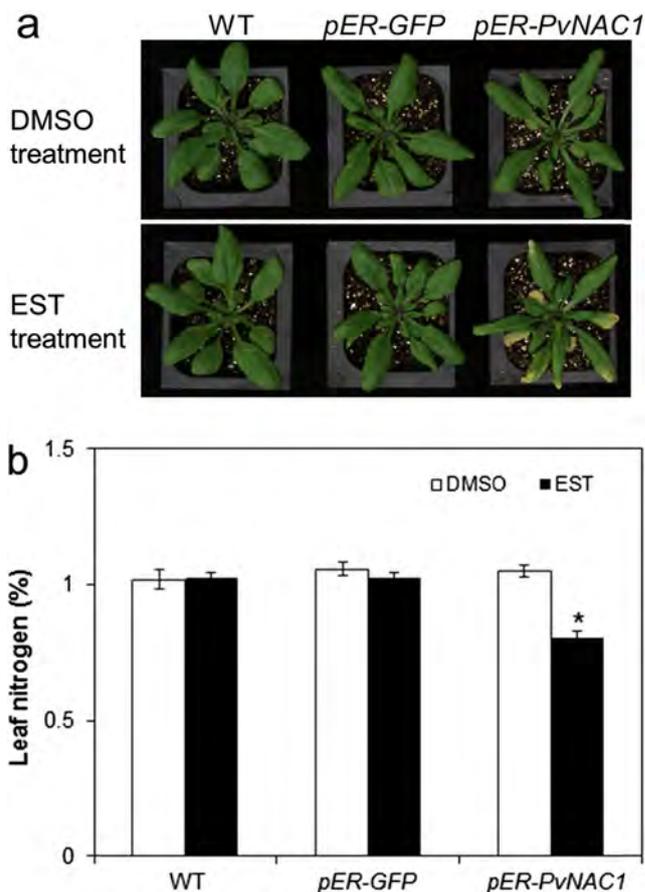


Fig. 5 Phenotypic effect of *PvNAC1* expression on *Arabidopsis*. **a** Phenotype of whole plants of WT, *pER-GFP*, and *pER-PvNAC1* transgenics after 5 days of treatment with 100 μM estradiol (EST, in 0.1 % DMSO) and control (0.1 % DMSO). Inflorescences were removed to reveal the leaf phenotypes. **b** Leaf nitrogen concentration in DMSO- or EST-treated mature leaves. Values are means \pm SD ($n=4$). Asterisk indicates significant difference at $p<0.01$ of EST-treated *pER-PvNAC1* compared with all other lines, using the Student's *t* test

protein degradation genes, the relative importance of genes for receptor kinase signaling and calcium signaling among those regulated following *PvNAC1* expression was higher at 6 h than at 72 h, indicating that signaling pathways played more central roles early after *PvNAC1* induction.

MapMan analysis of nitrogen and GABA (4-aminobutyrate) metabolism identified genes encoding two cytosolic glutamine synthetases (*GS1: GLN1.1* and *1.3*), a ferredoxin-dependent glutamate synthase (*Fd-GOGAT*) and a glutamate dehydrogenase (*GDH2*) that were upregulated following 72 h of *PvNAC1* induction. These enzymes have been implicated in N-recycling during leaf senescence in the past [26]. Further, qRT-PCR confirmed the role of *PvNAC1* in inducing expression of these genes (Fig. S7). Except *Fd-GOGAT*, which showed a 1.7-fold increase in expression, the expression levels of the other three genes were enhanced more than twofold by *PvNAC1* expression for 72 h.

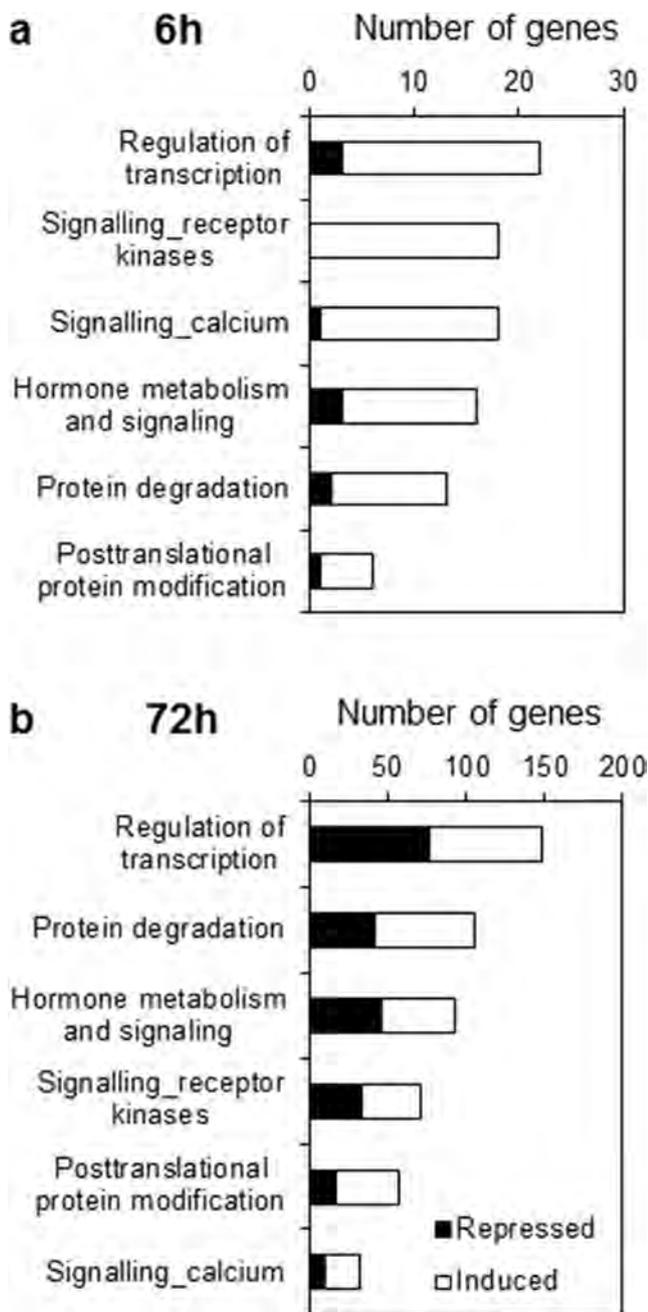


Fig. 6 Numbers of genes involved in major regulatory processes that responded to *PvNAC1* expression induced by estradiol (EST, 100 μ M in 0.1 % DMSO) for 6 h (a) and 72 h (b). Only genes for which transcript levels increased or decreased more than twofold compared to the control (0.1 % DMSO) were included

Overexpression of *PvNAC2* in Switchgrass Increases Aboveground Biomass and Nitrogen Content

An efficient switchgrass transformation protocol has been developed for cultivar Alamo [27] but not for cultivar Summer. Therefore, we transformed Alamo *PvNAC2*, driven by the *CaMV 35S* promoter, into the Alamo cultivar to investigate its physiological role in switchgrass. Together with a

transgenic negative control (Tc), three independent transgenic switchgrass lines (T20, T42, T54) were selected for physiological analysis based on their high levels of *pvNAC2* expression (Fig. S8a). Split tillers (vegetative clones) of different lines were grown continuously in a greenhouse for 6 months prior to harvesting of whole shoots. No accelerated yellowing of whole shoots was observed in transgenic plants during this 6-month period.

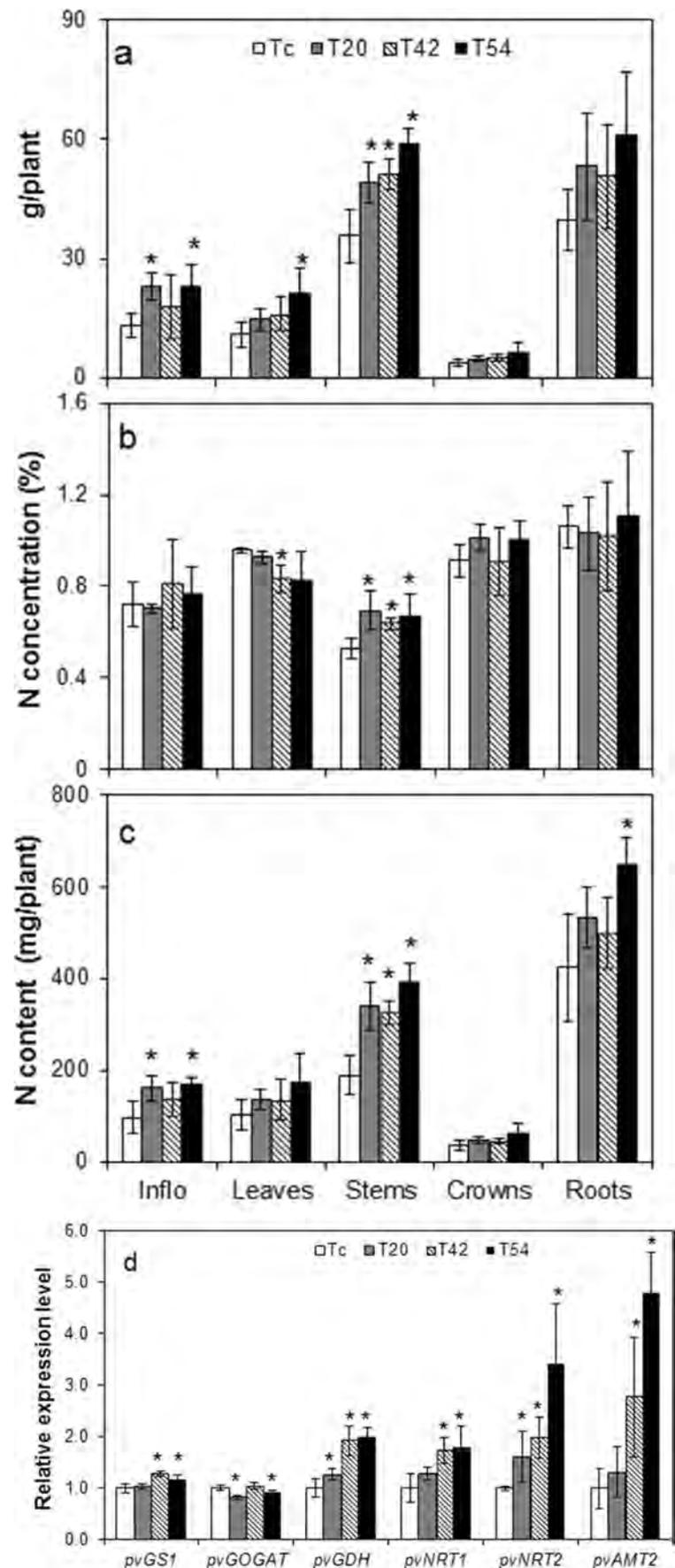
Inflorescences of T20 and T42, leaves of T54, and stems of all three transgenic lines exhibited significantly higher dry biomass than the control, resulting in the significantly higher total aboveground biomass (sum of leaves, stems, and inflorescences) in all lines, i.e., 87, 85, and 103 g/plant in T20, T42, and T54, respectively, compared to 60 g/plant in the control (Figs. 7a and S8b). In contrast, there were no statistical differences in biomass of underground organs (crown and roots) between transgenic and control plants (Figs. 7a and S8b). Nitrogen concentration of T42 leaves was significantly lower than that of Tc, while nitrogen concentration of all three transgenic stems was significantly higher. No significant difference was found for nitrogen concentration in inflorescences, crowns, and roots between transgenics and control (Fig. 7b). The nitrogen content was calculated by multiplying nitrogen concentration by biomass. The inflorescences of T20 and T54 and stems of all three transgenic lines showed significant higher nitrogen content than the control, while the nitrogen contents in leaves of transgenic and control plants were statistically similar (Fig. 7c). For underground organs, nitrogen content in T20 and T54 root was higher than that of control (Fig. 7c). We further calculated the aboveground and underground nitrogen content (Fig. S8c). All three transgenic lines showed significantly higher nitrogen contents in aboveground organs (sum of inflorescences, leaves, and stems), but only T54 showed significantly higher nitrogen content in underground organs.

To uncover possible mechanisms of increased biomass and nitrogen content in the shoots of *PvNAC2* overexpressor lines, we measured transcript levels of the nitrogen metabolism genes *PvGSI*, *PvGOGAT*, and *PvGDH* in leaves at an intermediate position on mature tillers from transgenic and control plants (c.f. Fig. 2a). Transcript levels of *PvGDH*, but not *PvGSI* or *PvGOGAT*, were consistently higher in all three *PvNAC2* overexpressor lines than in the control (Fig. 7d). Transcript levels of the nitrate and ammonium transporter genes, *PvNRT1*, *PvNRT2*, and *PvAMT2* in roots, were also measured and found to be significantly higher in *PvNAC2* overexpressor lines than in the control (Fig. 7d).

Discussion

We have identified two TFs of switchgrass, *PvNAC1* and *PvNAC2*, that appear to be positive regulators of leaf

Fig. 7 Overexpression of *PvNAC2* in switchgrass. **a** Biomass yield. **b** Nitrogen concentration. **c** Nitrogen content in inflorescences, leaves, stems, crowns, and roots in control and transgenic plants after 6 months of growth in greenhouse. **d** Relative transcript levels of putative nitrogen metabolism genes (*PvGS1*, *GOGAT*, and *GDH*) in leaves at intermediate position on mature tillers and genes of nitrate transporter (*PvNRT1*, *NRT2*) and ammonium transporter (*PvAMT2*) in roots of three independent transgenic lines (T20, T42, and T54), compared to those of control (Tc). Values are means±SD of three biological replicates. Asterisk indicates significant difference at $p < 0.05$ of transgenic lines compared with control, using the Student's *t* test



senescence and associated with nitrogen use efficiency, based on the following observations: the proteins are closely related to NAC TFs of other species that are known to be involved in leaf senescence and nutrient remobilization; expression of *PvNAC1* and *PvNAC2* increased with leaf age and was induced by prolonged darkness, concomitant with leaf senescence; PvNAC1 protein was localized to the nucleus; *PvNAC1* suppressed the stay-green phenotype of the *Arabidopsis atnap* mutant; inducible expression of *PvNAC1* in wild-type *Arabidopsis* triggered senescence and nitrogen remobilization in mature leaves; and constitutive expression of *pvNAC2* in switchgrass increased aboveground biomass, which was associated with upregulation of genes for nitrogen metabolism in leaves and nitrate and ammonium transport in roots.

Previous work revealed functional conservation among related NAC TFs of different annual species, including *Arabidopsis*, rice, and kidney bean. For instance, rice and kidney bean homologs of AtNAP suppressed the stay-green mutant phenotype of *Arabidopsis atnap* [22]. Using the same approach, we showed that PvNAC1 from switchgrass can substitute for AtNAP in *Arabidopsis* (Fig. 4). Thus, we demonstrated that conservation of NAC function in senescence extends beyond annual plants into perennial plants like switchgrass.

We found that ectopic expression of *PvNAC1* in wild-type *Arabidopsis* was sufficient to trigger controlled senescence in mature leaves of intact plants (Fig. 5). This implies that PvNAC1 and its homologs (such as TtNAM-B1) act early in the senescence developmental program [11]. In contrast, no senescence (yellowing) was observed in young leaves of estradiol-treated *pER-PvNAC1* transgenic *Arabidopsis* plants (Fig. 5), which may reflect an absence in young leaves of additional regulatory factors that are required for senescence or the presence of factors that interfere with PvNAC1 activity.

In contrast to *Arabidopsis*, switchgrass plants grown in our greenhouse and growth cabinets do not undergo whole-shoot senescence and as they do in nature. Instead, they continually produce new tillers while mature tillers mostly remain green. Overexpression of *PvNAC2* in switchgrass did not trigger premature shoot senescence in plants grown in the greenhouse, perhaps for the same reason that *PvNAC1* overexpression in young leaves of *Arabidopsis* did not trigger senescence there (see previous paragraph). Taken together, our experiments with *Arabidopsis* and switchgrass indicate that developmental and environmental signals influence NAC proteins, possibly via other transcription factors, to determine where and when senescence occurs in plant shoots.

Development of estradiol-inducible *pER-PvNAC1* transgenic lines of *Arabidopsis* allowed us to identify biological processes and potential gene targets of NAC TF activity. A relatively large number of TF genes responded rapidly (at 6 h) to *PvNAC1* induction (Fig. 7a and Table S1), which indicates

that PvNAC1 may be a master positive regulator of other TFs that in turn regulate the many processes associated with senescence. Among the latter are 105 genes involved in protein degradation, which represented the second largest group regulated by 72 h following *PvNAC1* expression (Fig. 7b). Sixty-four protein degradation-related genes were upregulated at least twofold 72 h after *PvNAC1* induction, nearly half of which (30) were also induced during natural senescence in *Arabidopsis* [13]. Natural senescence occurs more slowly and in a different developmental context than was the case in our experiments with vegetative plants.

Although *PvNAC1* transcript levels in *pER-PvNAC1* transgenic lines of *Arabidopsis* peaked after 6 h of estradiol treatment, there were no significant increases in transcript levels of genes for nitrogen metabolism, compared to the DMSO control, at that stage (Table S1). However, after 72 h of *PvNAC1* expression, four genes involved in ammonium recycling and metabolism, i.e., *GLN1.1*, *GLN1.3*, *Fd-GOGAT*, and *GDH2*, exhibited significantly higher transcript levels (Fig. S7). *GLN1.1* and *GLN1.3* encode isoforms of cytosolic glutamine synthetase (GS1). GS1 has been implicated in nitrogen remobilization during leaf senescence [26]. For example, maize mutants lacking specific GS1 isoforms accumulated large amounts of amino acids and ammonia in leaves below ears due to a dysfunction in nitrogen export [28]. Ferredoxin-dependent glutamate synthase (*Fd-GOGAT*) is known to play an important role in leaf photorespiratory ammonium assimilation [29]. Induction of *Fd-GOGAT* during *PvNAC1*-induced senescence in *Arabidopsis* leaves implicates it in nitrogen remobilization during this developmental process. Glutamate dehydrogenase (*GDH*) plays a central role liberating ammonium from amino acids following hydrolysis of protein reserves in seeds during germination or proteins in other organs during senescence [29]. The *Arabidopsis GDH2* gene was induced substantially 72 h after the onset of *PvNAC1* expression (Fig. S7). A similar level of induction of *GDH2* was found during natural senescence of *Arabidopsis* [13]. In view of the delay between *PvNAC1* induction and the accumulation of transcripts of genes involved in nitrogen mobilization, it is unlikely that PvNAC1 regulates these genes directly. However, some of the TFs induced within 6 h of *PvNAC1* expression may regulate nitrogen metabolism genes. In any case, the results presented here shine light on the network of genes and processes that respond to expression of senescence-related NAC TF genes.

Nitrogen use efficiency (NUE) in plants is determined by how well they take up inorganic nitrogen from the soil, assimilate nitrate and ammonium, and recycle organic nitrogen to harvested organs [30]. Although transient expression of *PvNAC1* in *pER-PvNAC1 Arabidopsis* plants at the bolting stage of early reproductive development resulted in nutrient remobilization from older leaves, as reflected by 24 % less nitrogen and 53 % less chlorophyll (Figs. 5b and S6a), no

increase in seed nitrogen concentration was found (Fig. S5c). Presumably, other sink tissues such as expanding leaves or the inflorescence competed with seeds for the remobilized leaf nitrogen. Alternatively, some nitrogen may have been lost from plants as gaseous nitrogen, such as NH_3 [31, 32].

NUE is an important target for switchgrass breeding as high NUE will help to minimize the need for fertilizer N, which will reduce biomass and biofuel production costs [1], and the environmental costs of fertilizer loss from agricultural systems. Although efficient uptake of N compounds from the soil to maximize biomass production will be an important part of NUE in switchgrass, high N content of the harvested biomass will not be, in contrast to crop and forage species where the objective is generally high protein (N) in food and feed. Remobilization of N from aboveground to belowground organs in switchgrass is a natural process that occurs during shoot senescence in autumn, which helps to conserve nitrogen in the plant for regrowth of the shoot in spring. There is substantial variation in N remobilization efficiency during shoot senescence between switchgrass ecotypes, which could be harnessed in breeding programs to conserve N [5]. The results presented here point to roles of PvNAC1 and PvNAC2 in leaf senescence in switchgrass and to the possibility of using these TFs to improve plant NUE. Overexpression of PvNAC2 in switchgrass increased aboveground biomass and nitrogen content under greenhouse conditions (Fig. S8bc). Stems of all three PvNAC2 overexpressor lines exhibited significantly higher biomass, nitrogen concentration, and content (Fig. 7abc). Because stems serve as a conduit for N movement between organs, the higher N concentration in stems of the PvNAC2 overexpressors compared to the control may have reflected an increase in N uptake from the soil or N remobilization from mature leaves. In fact, we found evidence for both, as discussed below.

Transient overexpression of PvNAC1 in *Arabidopsis* leaves and constitutive overexpression of PvNAC2 in switchgrass led to increased transcript levels of *GS1* and *GDH* (Figs. S7 and 7d), which also occurs during natural and dark-induced senescence [13, 15]. Remobilization of N compounds from leaves to other organs, mediated by *GS1*, *GDH*, and other enzymes, may explain the drop in N concentration in leaves expressing PvNACs (Figs. 5b and 7b). In the case of switchgrass constitutively expressing PvNAC2, the greater N content of shoots (Figs. 7c and S8c) indicates that more ammonium and/or nitrate was taken up from the soil by the roots and transported aboveground. Consistent with this, we found higher transcript levels of *PvNRT1*, *PvNRT2*, and *PvAMT2* in roots of the PvNAC2 transgenics (Fig. 7e). Increased nitrogen remobilization from leaves and/or increased N uptake by roots may explain the increased aboveground biomass of these plants compared to control (Figs. 7a and S8b).

In summary, we have identified and characterized two NAC TFs from two different ecotypes of switchgrass that have similar roles as positive regulators of senescence. Our

work demonstrates functional conservation among closely related NAC genes in annual and perennial plants. PvNAC1 and PvNAC2 show promise as tools to control senescence and nitrogen remobilization in shoots and, indirectly, nitrogen uptake in roots. Thus, they are interesting targets for improvement of NUE in switchgrass in the future.

Materials and Methods

Plant Materials and Growth Conditions

Wild-type switchgrass (*P. virgatum* L.) cultivars Summer (genotype VS16) and Alamo (genotype AP13) were used for gene cloning. Wild-type and transgenic switchgrass plants (Alamo background) were propagated asexually by tiller-splitting and grown in a greenhouse under standard conditions (temperature range, 25–29 °C) with a 16-h day from 0600 to 2200 hours. Single tiller splits (all of similar size) of transgenic and control plants were grown in 1-gal pots with Metro-Mix 830 soil for 6 months. Plants were watered every week using tap water and fertilized monthly with 250 ml of 0.1 % Peters Professional Peat-Lite 20-10-20 fertilizer solution (Scotts, USA) in which nitrogen was supplied as 5.72 mM NH_4NO_3 and 2.86 mM KNO_3 with a ratio of nitrate to ammonium of 60:40.

Arabidopsis ecotype Columbia-0 was used as wild type. An *atnap* (At1g69490) T-DNA insertion mutant (SALK_005010) was obtained from the Arabidopsis Biological Resource Center (Ohio State University). Homozygous mutant plants lines were confirmed as described previously [33]. *Arabidopsis* were grown in soil (Metro-Mix 350, Sun Gro Horticulture, Canada) in a controlled culture room at 23 °C with relative humidity of 55 % under long day (16-h light and 8-h dark) or 24-h light condition with white light illumination ($120 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

Isolation of Putative Switchgrass NAC cDNAs

Total RNA was extracted from switchgrass senescing leaves with yellow tips. cDNA was synthesized from 2 μg purified total RNA and used as template for 5'- and 3'-RACE (FirstChoice RLM-RACE Kit, Ambion, USA). The two nested degenerate primers for 5'-RACE were 5'-ACTCGT GCATGATCCAGTYKGT-3' (outer primer) and 5'-KCGGG SWGAAGAAGTACCACTC-3' (inner primer). The primers for 3'-RACE were 5'-TCGACCTCTACAAGTTCGAYCC-3' (outer primer) and 5'-GCGAGMAGGAGTGGTACTTCTT-3' (inner primer).

Sequence Alignment and Phylogenetic Analysis

RACE products were sequenced and the N-terminal portion of putative NAC protein sequences were aligned with several

known NAC sequences using ClustalW of MegAlign (DNASTAR, Madison, USA) to confirm the presence of the conserved motifs of the NAC family. The full-length sequences of representative rice and *Arabidopsis* NAC proteins were first aligned with PvNAC1 and PvNAC2 using ClustalW with the default parameter values. The alignment results were then used to produce the neighbor-joining phylogenetic tree using MEGA5 [34]. The bootstrap method, with 100 replications, was used to provide confidence levels (reported as a percentage) of branch points on the phylogenetic tree.

Expression Profiling of PvNAC1 and PvNAC2 in Switchgrass

Switchgrass tillers at the developmental stages, vegetative 3, elongation 5, and reproductive 4 [35], were classified as young, medium, and old tillers, respectively. Transcript levels of PvNAC1 were measured by qRT-PCR in the first leaves (numbered from bottom to top) on different tillers and in leaves at different positions (top, intermediate, and bottom) on old tillers. Intermediate leaves detached from middle-aged tillers were incubated on wet filter paper in continuous darkness to artificially induce senescence prior to qRT-PCR analysis. Transcript levels of PvNAC1 were expressed relative to the transcript level of switchgrass *Ubiquitin* (GenBank accession JQ425118) in each sample.

Subcellular Localization

The open reading frame of PvNAC1 was PCR-amplified with gene-specific primers containing Sall and PciI enzyme sites and cloned into vector *pAVA121* cut with XhoI and NcoI [36]. The resulting 35S:PvNAC1-GFP construct or the 35S:GFP control vector was transiently expressed in onion (*Allium cepa*) epidermal cells using particle bombardment [37]. Subcellular localization of the GFP signal was determined using a confocal laser scanning microscope (TCS SP2 AOBS; Leica).

Plasmid Constructs and Plant Transformation

Approximately 2.0 kb of *AtNAP* (At1g69490) promoter sequence (P_{NAP}) was amplified as described previously [22] to replace CaMV-35S promoter, via the *Pst*I and *Nco*I sites of *pCAMBIA3301* which contains *GUS* reporter gene driven by CaMV-35S promoter (*35S-GUS*) (<http://www.cambia.org>), thus resulting in P_{NAP} -*GUS* construct.

P_{NAP} -*NAC1* The open reading frame (ORF) of PvNAC1 was amplified by PCR using a pair of gene-specific primers, 5'-(*Bgl*III) TTAGATCTATGGCGGTAAGCTCTGC-3' (forward) and 5'-(*Bst*EII) TAGGTCACCCTAGTGTTTTTTCTTTTCATATTTGAATTTG-3' (reverse), and cloned into the P_{NAP} -*GUS* construct via the *Bgl*III and *Bst*EII restriction sites to replace *GUS* sequences.

pER -*NAC1* The PvNAC1 ORF was cloned via *Asc*I and *Spe*I sites into an estradiol-inducible vector *pER8* [24] and named pER -*NAC1*. The *pER8* vector containing *GFP* as reporter gene (pER -*GFP*) was used as a negative control vector.

35S-NAC2 The ORF of PvNAC2 was cloned into *pCAMBIA1305* (<http://www.cambia.org>) via the *Bgl*III and *Bst*EII restriction sites to replace *GUS* sequences.

Following the floral dip method [38], *Arabidopsis* homozygous mutant *atnap* was transformed with *Agrobacterium tumefaciens* strain C58 containing P_{NAP} -*GUS* or P_{NAP} -*NAC1* for complementation testing (COM); *Arabidopsis* wild type (Col-0) was transformed with pER -*GFP* or pER -*NAC1* for inducible overexpression (IOE).

Switchgrass cultivar Alamo was transformed with *A. tumefaciens* strain EHA105 containing *35S-NAC2* or *35S-GUS*, following an established protocol [27].

Dark Incubation of Detached *Arabidopsis* Leaves and Estrogen Treatment on Whole Plants

Leaves (number 5 or 6) from 4-week-old plants of P_{NAP} -*NAC1* complementation transgenic, WT, and *atnap* *Arabidopsis* were excised and placed on filter papers moisturized with distilled H₂O in Petri dishes with adaxial side facing up. The plates were kept in darkness at 22 °C for 5–7 days.

Estradiol treatments were conducted as previously described [24]. The detached leaves of *Arabidopsis* plants of pER -*NAC1* transgenics and wild type were placed on filter paper moisturized with 100 μM EST (in 0.1 % DMSO) or 0.1 % DMSO solution in Petri dishes with adaxial side facing up. The plates were kept under 120 μmol white light at 22 °C for 5–7 days.

Three-week-old pER -PvNAC1 and pER -*GFP* transgenic as well as WT plants which were grown in soil under continuous light (120 μmol m⁻² s⁻¹) were sprayed with 100 μM EST (in 0.1 % DMSO) or 0.1 % DMSO solution every 3 h to ensure continuous wetting of foliage area. The sprayed plants were kept under a transparent plastic dome for 120 h. The phenotype of plants was observed, and leaves were harvested for physiological and molecular analysis.

Measurement of Chlorophyll Concentration

Arabidopsis leaves were ground to powder in liquid nitrogen. Chlorophyll was extracted from powdered samples with 80 % acetone, and chlorophyll concentration was determined by measuring absorption at 663 and 646 nm [39].

Measurement of Nitrogen Concentration

Leaves or seeds from inducible overexpression transgenic *Arabidopsis* plants were dried at 37 °C for at least 3 days, then ground into a fine powder using a SPEX SamplePrep 6870 Freezer/Mill (Metuchen, New Jersey, USA).

After 6 months of growth, whole plants of 35S-*NAC2* transgenic switchgrass lines and a Tc were harvested. Above-ground biomass was separated into inflorescence (including seeds), leaves, and stems (including sheaths), while under-ground biomass was separated into roots and crowns (including rhizomes). Samples were dried at 65 °C for at least 2 days, weighed, and then ground into fine powder.

The concentration of total nitrogen in each sample was analyzed by Servi-Tech Laboratories (Amarillo, Texas, USA) using a combustion method [40] and expressed in percent (mg/100 mg). Nitrogen content in switchgrass samples was calculated by multiplying nitrogen concentration by biomass.

BLAST Search of Switchgrass Nitrogen Metabolism/Uptake Genes

The coding regions of maize genes for glutamine synthetase, *GS1.4* (GenBank accession X65929), glutamate dehydrogenase, *GDHI* (NM_00111831), glutamate synthase, *GOGAT* (NM_001112223), low-affinity nitrate transporter, *NRT1.2* (NM_001112455), high-affinity nitrate transporter, *NRT2.2* (NM_001279422), and ammonium transporter 2, *AMT2* (NM_001154311), were used to search a Switchgrass Unique Transcript Database (<http://switchgrassgenomics.noble.org>). The top query results with complete coding regions were selected as switchgrass homologs of *GS1*, *GDH*, *GOGAT*, *NRT1*, *NRT2*, and *AMT2* (Unitranscript IDs are listed in Table S3). Oligonucleotide primers for quantitative real-time PCR were designed based on conserved sequences in the switchgrass and maize homologs.

qRT-PCR

Transcript levels of *PvNAC1*, *PvNAC2*, and putative *GS1.4*, *GDHI*, and *GOGAT* in switchgrass leaves and *PvNAC1* in transgenic *Arabidopsis* plants were determined with a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). As described previously [41], each 5- μ L reaction included 2 μ L primers (0.5 μ M each primer), 2.5 μ L SYBR Green Master Mix reagent (Applied Biosystems), and 0.5 μ L 1:10 diluted cDNA from the reverse transcription step. The qRT-PCR data were analyzed using SDS 2.2.1 software (Applied Biosystems). The relative transcript levels were normalized to those of the constitutively expressed housekeeping gene, *Arabidopsis UBQ10* (At4g05320) or switchgrass *UBQ* (GenBank accession

JQ425118), using the equation $2^{-\Delta C_t}$, where C_t is the threshold cycle for each gene in every sample [42]. qRT-PCR was also conducted to confirm the microarray results on transcript changes of nitrogen metabolism genes due to *PvNAC1* expression. The qRT-PCR primers are listed in Table S4.

Microarray Analysis

Total RNA samples from estradiol (100 μ M, in 0.1 % DMSO)-treated IOE leaves (*pER-NAC1* transgenic) were subjected to Affymetrix microarray analysis. To exclude genes that respond to treatment of DMSO or estradiol, RNAs from estradiol-sprayed *pER-GFP* leaves were used as the control. RNA was isolated with TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol, then purified and concentrated using the RNeasy MinElute Cleanup Kit (Qiagen). Probe labeling, hybridization, and scanning were conducted by the Genomics/Microarray Facility at the Noble Foundation, according to the manufacturer's instructions for the GeneChip 3' IVT Express Kit (Affymetrix).

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Conflict of Interest The authors have no conflict of interest to declare.

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