Wheat NAC transcription factor NAC5-1 is a positive regulator of senescence

Evans, Catherine; Mogg, Sophie Louise; Soraru, Charline; Wallington, Emma; Coates, Juliet; Borrill, Philippa

DOI:
10.1002/pld3.620

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Evans, C, Mogg, SL, Soraru, C, Wallington, E, Coates, J & Borrill, P 2024, 'Wheat NAC transcription factor NAC5-1 is a positive regulator of senescence', *Plant Direct*, vol. 8, no. 7, e620. https://doi.org/10.1002/pld3.620

Received: 2 February 2024 | Revised: 4 June 2024 | Accepted: 10 June 2024

DOI: 10.1002/pld3.620

RESEARCH ARTICLE

Wheat NAC transcription factor *NAC5-1* is a positive regulator of senescence

Catherine Evans[1,2] | Sophie Louise Mogg[3] | Charline Soraru[4] | Emma Wallington[4] | Juliet Coates[2] | Philippa Borrill[1]

[1]Department of Crop Genetics, John Innes Centre, Norwich, UK

[2]School of Biosciences, University of Birmingham, Birmingham, UK

[3]School of Biological Sciences, University of Manchester, Manchester, UK

[4]NIAB, Cambridge, UK

Correspondence
Philippa Borrill, Department of Crop Genetics, John Innes Centre, Norwich, NR4 7UH, UK.
Email: philippa.borrill@jic.ac.uk

Funding information
This work was supported by the UK Biotechnology and Biological Sciences Research Council (BBSRC) through the Institute Strategic Programmes Delivering Sustainable Wheat (DSW) (BB/X011003/1) and Building Robustness in Crops (BRiC) (BB/X01102X/1). Transgenic wheat materials were funded by the BBSRC Bioinformatics and Biological Resources Fund through the Community Resource for Wheat and Rice Transformation grant BB/R014876/1 to EW. CE was funded through a BBSRC Midlands Integrative Biosciences Training Partnership (MIBTP) iCASE Studentship in collaboration with RAGT Seeds Ltd (BB/M01116X/1). PB also acknowledges funding from the Rank Prize New Lecturer Award and a Royal Society Research Grant (RGS\R1\191163). This research was also supported by the NBI Research Computing group through HPC resources and the University of Birmingham's BlueBEAR HPC resources.

Abstract

Wheat (*Triticum aestivum* L.) is an important source of both calories and protein in global diets, but there is a trade-off between grain yield and protein content. The timing of leaf senescence could mediate this trade-off as it is associated with both declines in photosynthesis and nitrogen remobilization from leaves to grain. NAC transcription factors play key roles in regulating senescence timing. In rice, *OsNAC5* expression is correlated with increased protein content and upregulated in senescing leaves, but the role of the wheat ortholog in senescence had not been characterized. We verified that *NAC5-1* is the ortholog of *OsNAC5* and that it is expressed in senescing flag leaves in wheat. To characterize *NAC5-1*, we combined missense mutations in *NAC5-A1* and *NAC5-B1* from a TILLING mutant population and overexpressed *NAC5-A1* in wheat. Mutation in *NAC5-1* was associated with delayed onset of flag leaf senescence, while overexpression of *NAC5-A1* was associated with slightly earlier onset of leaf senescence. DAP-seq was performed to locate transcription factor binding sites of *NAC5-1*. Analysis of DAP-seq and comparison with other studies identified putative downstream target genes of *NAC5-1* which could be associated with senescence. This work showed that *NAC5-1* is a positive transcriptional regulator of leaf senescence in wheat. Further research is needed to test the effect of *NAC5-1* on yield and protein content in field trials, to assess the potential to exploit this senescence regulator to develop high-yielding wheat while maintaining grain protein content.

KEYWORDS
DAP-seq, NAC transcription factor, senescence, wheat

1 | INTRODUCTION

Wheat (*T. aestivum* L.) is the fourth most-produced crop globally and production is under pressure from an increasing global population (FAO, 2020). Wheat grain protein is important for both baking quality, as it correlates positively with water absorption and loaf volume (Fradgley et al., 2022), and nutritional value, as wheat contributes 20% of the protein in human diets globally (Cauvain, 2012; FAO, 2020). For example, in the UK, criteria for grain to be sold as a 'Group 1' breadmaking wheat include a minimum of 13% grain

protein content (AHDB, 2023). However, there is a genetic trade-off between yield and protein content, such that past selection for yield has tended to decrease wheat grain protein content (Maeoka et al., 2020; Simmonds, 1995). Novel approaches are needed to understand this trade-off, in order to improve yield while maintaining grain protein content. In addition, the development of high-protein wheat varieties could ultimately reduce the use of nitrogenous fertilizer currently used to increase wheat grain protein content, which is responsible for nitrous oxide emissions and damage to aquatic environments (FAO, 2020; Yang et al., 2017).

Senescence is the final developmental stage of a wheat plant (Davies & Gan, 2012). The rate of photosynthesis declines during leaf senescence, and delayed leaf senescence correlates with increased yield in cereal crops (Buchanan-Wollaston, 1997; Gregersen et al., 2008; Kichey et al., 2007). However, senescence is required to allow nitrogen remobilization from leaves to the grain, which accounts for 65–90% of final grain protein content (Bogard et al., 2010; Kichey et al., 2007). Therefore, the timing of leaf senescence is proposed to mediate the trade-off between grain protein content and yield (Thomas & Ougham, 2014). A better understanding of the regulation of leaf senescence may enable the production of higher-yielding wheat without a decrease in grain protein content.

The timing of leaf senescence is regulated by a network of transcription factors (Schippers, 2015). In wheat, NAC transcription factor *NAM-1* promotes leaf senescence and grain protein content: introgression of a functional copy of *NAM-B1* led to earlier leaf and peduncle senescence and increased grain protein content in both tetraploid wheat (*Triticum turgidum ssp. durum* [Desf.] Husn) and hexaploid wheat *(T. aestivum*) (Uauy et al., 2006; Uauy et al., 2006). Delayed leaf senescence and reduced grain protein content were observed in hexaploid RNAi lines with reduced expression of *NAM-A1*, *NAM-D1*, and paralogs *NAM-B2* and *NAM-D2* (Uauy et al., 2006). Similarly, TILLING lines with mutation of either *NAM-A1* in tetraploid wheat (Distelfeld et al., 2012; Pearce et al., 2014) or *NAM-A1* and *NAM-D1* in hexaploid wheat (Avni et al., 2014) and barley near-isogenic lines lacking *HvNAM-1* (Jukanti & Fischer, 2008) displayed delayed senescence and decreased protein content. Mutation of *NAM-A2* and *NAM-B2* in tetraploid wheat delayed flag leaf and peduncle senescence, indicating that *NAM-2*, the paralog of *NAM-1*, also promotes earlier senescence (Borrill et al., 2019). Since the discovery of *NAM-1*, a few other NAC transcription factors have been shown to affect senescence timing in wheat or barley (Christiansen et al., 2016; Harrington, 2019; Zhao et al., 2015), although there are many NAC transcription factors in the wheat and barley genomes that remain to be functionally characterized (Borrill, Harrington, & Uauy, 2017; Murozuka et al., 2018; Vranic et al., 2022).

In rice (*O. sativa* L.) NAC transcription factors also act as key regulators of the balance between senescence and grain protein content. The NAC transcription factor *OsNAC5/NAC071/ONAC009* (*Os11g0184900*) increases in expression during flag leaf senescence and a high-grain-protein cultivar showed higher expression of *OsNAC5* than a low-grain-protein cultivar, in *O. sativa* ssp. *japonica* and separately in *O. sativa* ssp. *indica* (Sharma et al., 2019; Sperotto et al., 2009). Recombinant inbred lines were developed from *O. sativa* ssp. *indica* cultivars with a range of protein contents; these displayed a correlation between increased grain protein content and increased *OsNAC5* expression in leaves and panicles (Sharma et al., 2019). A T-DNA insertion line with enhanced expression of *OsNAC5* accumulated higher iron and magnesium concentrations in grains, and lower concentrations in leaves (Wairich et al., 2023). These findings suggest that *OsNAC5* may be involved in regulating nitrogen and metal ion remobilization during leaf senescence in rice (Ricachenevsky et al., 2013). *OsNAC5* has also been associated with ABA-dependent salt, drought, and cold stress tolerance (Jeong et al., 2013; Song et al., 2011; Takasaki et al., 2010).

The ortholog of *OsNAC5* in hexaploid wheat, *NAC5-1*, has been reported as the triad *TraesCS4A02G219700*, *TraesCS4B02G098200*, and *TraesCS4D02G094400* (Lv et al., 2020). Increased expression of *TraesCS4A02G219700* (*NAC5-A1*, referred to in the cited paper as *TaNAC071-A*), either via transgenic overexpression or a natural promoter insertion allele, was associated with seedling drought tolerance (Mao et al., 2022). This indicates that *TraesCS4A02G219700* shares a role with its ortholog *OsNAC5* in promoting drought tolerance. However, whether *NAC5-A1* shares a role in regulating senescence was not studied. Here we test whether the wheat ortholog of *OsNAC5*, *NAC5-1*, plays a role in senescence regulation using TILLING mutants, overexpression lines, and identification of downstream target genes via DNA affinity purification (DAP-seq).

2 | MATERIALS AND METHODS

2.1 | Verification of *OsNAC5* orthologs in wheat

To visualize the orthology of *OsNAC5*, multiple alignments were generated with Clustal Omega 1.2.2 (Madeira et al., 2022). Alignments were visualized using MView, and by generating a consensus tree with the Neighbor-Joining method and 100 bootstrap replicates, in Geneious software (Biomatters, 2022; Madeira et al., 2022). The consensus tree included peptide sequences of *OsNAC5* (Os11t0184900-01 and Os11t0184900-02), the 15 BLASTP hits of *OsNAC5* with the lowest E-value from *T. aestivum* cv. Chinese Spring (IWGSC RefSeq v1.1) (Appels et al., 2018) and *O. sativa* ssp. *japonica* cv. Nipponbare (IRGSP-1.0) (Kawahara et al., 2013; Sakai et al., 2013), and an ortholog of *OsNAC5* in *Physcomitrium patens* (Pp3c13_10800V3_2) as an out-group (Nakashima et al., 2012). The BLASTP search was carried out against the Ensembl Plants database (Yates et al., 2022). Five conserved NAC subdomains assigned to *OsNAC5* were annotated (Kikuchi et al., 2000).

2.2 | Gene expression data

To assess the pattern of *NAC5-1* expression during flag leaf senescence, transcript levels in wheat flag leaves from 3 to 26 days after

anthesis (DAA) were extracted from RNA-seq data (Borrill et al., 2019). Read counts in transcripts per million (tpm) were extracted via the Wheat Expression Browser and plotted for *NAC5-A1*, *NAC5-B1*, and *NAC5-D1* (Borrill, Ramirez-Gonzalez, & Uauy, 2016).

2.3 | Selection of TILLING mutant lines

Lines with missense mutations in *NAC5-A1* and *NAC5-B1* were selected from the *T. turgidum ssp. durum* cv. Kronos TILLING population (Krasileva et al., 2017) (Table 1). Line K2546, with a missense mutation in subdomain iii of the NAC domain of *NAC5-A1*, was crossed with line K2036 and independently with line K3328, with missense mutations in subdomain iv of the NAC domain of *NAC5-B1* (Figure S1). Two backcrosses were carried out with non-mutagenized Kronos to reduce the background mutation load. Plants were genotyped at each generation with KASP genotyping as described in the next section. Homozygous double and single mutants were obtained.

2.4 | KASP genotyping

For genotyping of mutations in *NAC5-A1* and *NAC5-B1* in Kronos TILLING lines, homoeolog-specific KASP (Kompetitive Allele-Specific PCR, LGC Biosearch Technologies) primers were designed using Polymarker (Ramirez-Gonzalez et al., 2015a). A HEX tag was added to the wild-type allele (WT) primer and a FAM tag to the mutant allele (MUT) primer. Genotyping was carried out at each generation using PACE mix (3CR Bioscience) and KASP markers (Table S1) as described in (Ramirez-Gonzalez et al., 2015b).

2.5 | Wheat transformation

The coding sequence for *NAC5-A1* (*TraesCS4A02G219700.1*) was synthesized by GENEWIZ (Azenta). PCR using Q5 High-Fidelity DNA polymerase (NEB), following the manufacturer's instructions, was used to add a ribosome-binding site immediately upstream of the methionine start codon along with an in-frame 5' 3xFLAG tag (primers in Table S1). The PCR product was cloned into the gateway entry vector pCR8 and checked using Sanger sequencing. The *NAC5-A1* sequence was then recombined into the Gateway-compatible binary vector pSC4-Act-R1R2 in an LR Clonase reaction (ThermoFisher) to create pMSH30, checked by restriction digest and Sanger sequencing then transferred by electrotransformation to *A.tumefaciens* strain EHA105. Plasmids were reisolated from Agrobacterium cultures and verified by restriction digest prior to use in wheat transformation experiments (Bates et al., 2017). *NAC5-A1* was expressed *in planta* from the rice Actin promoter (McElroy et al., 1990). Figure S2 shows the pMSH130 T-DNA region.

Wheat transformation experiments were set up with the spring wheat cultivar Fielder. Immature seeds were harvested 14–20 days after anthesis and immature wheat embryos were isolated and then co-cultivated with *A.tumefaciens* for 2 days in the dark (Ishida et al., 2015). Subsequent removal of the embryonic axis and tissue culture with selection agent G418 was performed as previously described (Risacher et al., 2009). Plantlets were hardened off following transfer to Jiffy-7 pellets (LBS horticulture) and genotyped prior to potting in compost.

2.6 | Copy number assay

Copy number assay was carried out for the marker gene *nptII* against single-copy gene *GAMYB*. Primers and Taqman probes in Table S1 were used at a final concentration of 200 nM, otherwise as described in (Milner et al., 2018).

2.7 | Selection of transgenic lines for *NAC5-A1* overexpression

For the selection of transgenic lines, T_0 plants were grown in a controlled environment and a copy number assay was carried out. Five independent transformants with a single construct insertion ("single-copy") and 25 with more than four construct insertions ("multi-copy") were obtained (Table S2). T_1 progeny of five single-copy transformants, six multi-copy transformants, and two non-transformed controls were grown in the glasshouse.

TABLE 1 Missense TILLING mutations selected in *NAC5-A1* and *NAC5-B1*. Gene ID from *Triticum aestivum* RefSeq v1.1 (Appels et al., 2018). Variant ID comprises the wheat line containing the mutation and the chromosome position of the mutation. Alleles of the transcript and amino acid changes are shown. Subdomain shows where in five conserved NAC subdomains the mutation sits. PSSM score indicates the likelihood of a missense mutation in this position of the NAC domain across all NAC transcription factors.

Gene name	Gene ID	Variant ID	Alleles	Amino acid change	Subdomain of NAC domain	PSSM score
NAC5-A1	*TraesCS4A02G219700*	Kronos2546[a]	G/A	G93E	iii	−8
NAC5-B1	*TraesCS4B02G098200*	Kronos2036. Chr4B.103056075	G/A	A111T	iv	−8
NAC5-B1	*TraesCS4B02G098200*	Kronos3328. Chr4B.103056044	G/A	G121D	iv	−4

[a]Variant ID not annotated for *NAC5-A1* in RefSeq v1.1 as mutation was initially identified in a previous gene annotation (TGACv1) (Clavijo et al., 2017).

2.8 | qPCR

For quantitative PCR (qPCR), 100 mg leaf tissue samples from 2- to 3-week-old wheat plants of T_1 transgenic lines were snap-frozen in liquid N_2 and ground with a micro-pestle pre-chilled in liquid N_2. RNA was extracted using TRI-reagent (Invitrogen), RNA was treated with DNAse 1 (ThermoFisher), and cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen), according to manufacturers' instructions. Primers were designed to amplify *NAC5-A1*, all homoeologs of *NAC5-1*, and the transgenic construct (Table S1). Using SYBR Green master mix and QuantStudio 5 (Applied Biosystems), qPCR was run for 5 min at 95 °C; 45 cycles of 10 sec at 95 °C, 15 sec at 60 °C and 20 sec at 72 °C; and a melt curve from 60 °C to 95 °C. Fold change in transcript level was calculated using the Pfaffl method incorporating primer efficiencies, with *ACT2* as the reference gene, normalized against the average of single-copy samples (Pfaffl, 2001; Tenea et al., 2011).

For preliminary qPCR, pooled samples of .5 cm leaf tissue from all plants of one line were collected in the same tube, and qPCR data were analyzed by the ΔΔCt method.

2.9 | Plant phenotyping

Plant growth conditions are summarized in Table S3. *NAC5-1* TILLING lines in cv. Kronos at the BC_2F_3 generation and *NAC5-A1* transgenic lines in cv. Fielder at the T_2 generation were phenotyped.

Senescence traits were measured for the primary tiller of each plant. Heading was scored as the date of complete ear emergence from the flag leaf sheath. Flag leaf chlorophyll content was assessed with a SPAD spectrometer, averaging eight points on the leaf surface (SPAD-502, Konica Minolta). The SPAD spectrometer measures the optical density difference of red (650 nm) and near-infrared (940 nm) wavelengths. In wheat, SPAD values show a strong correlation ($r^2 = .8–.9$) with leaf chlorophyll content estimated from chemical extraction (Uddling et al., 2007).

SPAD readings were made at heading, 6–8 days after heading, then every 7 days until the SPAD value decreased below 10 (Table S3). A threshold of 10 was used as SPAD values below 10 were found to be less reproducible. Days from heading to a SPAD value of 30 was calculated from the time-course of SPAD values in days after heading using linear interpolation, the same underlying method as previously used to calculate time to a specific leaf senescence score (Chapman et al., 2021). "25% flag leaf senescence" was scored as 25% leaf yellowing and "100% peduncle senescence" as yellowing of the full circumference of the peduncle (Borrill et al., 2019).

Tiller number and height of the primary tiller were measured at maturity. Grain mass, grain number, thousand-grain weight, average grain length, grain width, and grain area were measured with a Marvin seed analyzer (Marvintech). Grain protein content was measured with a Near Infrared Spectrometer (Perten DA7250), normalized for moisture content.

Boxplots were plotted with R package ggplot2 (Wickham, 2016). Lines show the median, box inter-quartile range, and whiskers the most extreme point within 1.5* inter-quartile range from the box. Each time-point of SPAD time-courses, and all traits in *NAC5-A1* overexpression lines, were analyzed by a pairwise Wilcoxon test comparing against the control line. Remaining traits in *NAC5-1* TILLING lines were analyzed by ANOVA and post-hoc Tukey tests with the formula "Trait ~ Row + Block + Genotype". "Block" represents the block of the randomized complete block design, while "Row" represents the row from center to edge of glasshouse bench, added as a covariate to account for lights positioned in the center of the bench.

2.10 | DAP-seq

Coding sequences of NAC transcription factors *NAC5-A1*, *NAC5-B1*, *NAM-A1*, *NAM-B1*, *NAM-D1*, *NAM-A2*, *NAM-B2*, and *NAM-D2* (Gene IDs in Table S4) were codon-optimized to reduce GC content using ATGme (Daniel et al., 2015) whilst selecting codons common in wheat to facilitate translation in an in vitro wheat germ system (Alexaki et al., 2019; Clarke & Clark, 2008). Constructs were synthesized in a Gateway-compatible entry vector by Twist Bioscience. Constructs were cloned into the vector pIX-HALO (obtained from *Arabidopsis* Biological Resource Center) in an LR Clonase reaction (ThermoFisher) and checked using Sanger sequencing by GENEWIZ (Azenta). A total of 100 mg samples of flag leaf tissue from *Triticum aestivum* cv. Cadenza at 7 days after heading (DAH) and 14 DAH were collected in liquid N_2, freeze-dried overnight, and homogenized in a Geno/Grinder (Cole-Palmer). Genomic DNA was extracted using a DNA Plant Mini Kit (Qiagen).

DAP-seq was carried out according to (Bartlett et al., 2017), with the following alterations. DNA was sonicated with a BioRuptor Plus (Diagenode) for 21 cycles at 30s on: 30s off, in 200 µl aliquots at 20 ng/µl, to achieve fragment sizes of 200-400 bp (confirmed by Tapestation). DNA was precipitated for 12 h at −70°C. Precipitated DNA was pooled, with equal proportions from 7 DAH to 14 DAH. Adapter ligation and library preparation were carried out using the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB), and adapter ligation was checked using qPCR. Proteins were expressed with TNT SP6 High-Yield Wheat Germ Protein Expression System (Promega) and reactions were incubated for 12 h at 25 °C with 10 µg pIX-HALO expression clone. HaloTag beads (Promega) were aliquoted with 2x buffer volume. Three technical replicates were prepared per transcription factor, and two or three replicates passing quality control were sequenced using Illumina 150 bp paired-end reads. Input DNA controls consisted of 2% DNA library, omitting bead-binding steps, and pIX-HALO controls consisted of DAP-seq carried out using the empty pIX-HALO vector. Primers used to verify cloning and adapter ligation are in Table S1.

2.11 | DAP-seq data analysis

DAP-seq data was analyzed following the pipeline in Klasfeld et al. (2022). Reads were trimmed with Trimmomatic (v0.39), mapped to

T. aestivum RefSeq v1.1 (Appels et al., 2018) with bowtie2 (v2.4.1), and filtered with samtools (v1.10) view for MAPQ>30 and to remove unmapped reads and secondary alignments. Duplicates were removed with samtools markdup. Two or three technical replicates of the DAP-seq sample preparation were pooled for peak calling, to maximize read depth. Peaks were called with MACS2 (v2.2.7.1) from pooled reads against pIX-HALO controls, to account for the possibility of binding sites of the HaloTag peptide in the wheat genome. A greenscreen mask to remove artifactual peaks, which can arise from amplification, sequencing, and mapping biases, was prepared using three input DNA controls with merge_distance = 50,000, according to Klasfeld et al. (2022). The greenscreen contained 228 regions and masked 85 transcripts. Peaks were filtered with q-value < 1×10^{-10} or q-value < .01, and the greenscreen mask. Peak sets with q-value < .01 and greenscreen mask were used for further analyses.

For datasets with >30 peaks, a de novo motif search was carried out against peak sequences with RSAT peak-motif (http://rsat.eead.csic.es/plants/peak-motifs_form.cgi), and motifs were compared against the Jaspar database (core nonredundant plants) (http://jaspar2016.genereg.net/). Candidate target genes were identified as the closest high confidence (HC) gene to each peak with bedtools (v2.29.2) closest.

The following sets of candidate target genes were obtained from published data:

1. Candidate target genes of *NAC5-1*, *NAM-1*, and *NAM-2* homoeologs from a GENIE3 gene regulatory network (Ramírez-González et al., 2018). Genes were extracted from the top 1 million connections in the network.
2. Candidate target genes of *OsNAC5* identified by both ChIP-seq, and RNA-seq of *OsNAC5* overexpression lines (Chung et al., 2018). Wheat orthologs of the rice genes were identified with Ensembl Biomart (Kinsella et al., 2011).
3. Candidate target genes of *NAC5-A1* identified by both DAP-seq and RNA-seq of *NAC5-A1* overexpression lines (Mao et al., 2022). Genes were split into upregulated (up) and downregulated (down) genes in *NAC5-A1* overexpression lines compared to wild-type, under well-watered conditions.

The overlap between sets of candidate target genes was assessed using a jaccard test (Chung et al., 2019). The jaccard statistic computes the intersection divided by the union of gene sets. An expected value for the jaccard statistic under the null hypothesis that gene sets are independent was computed based on the set of all high-confidence genes in *T. aestivum* RefSeq v1.1 (Appels et al., 2018). Upset plots were generated with UpSetR (Conway et al., 2017).

2.12 | Accession numbers

Raw data for the DAP-seq can be obtained through BioProject ID PRJEB72016 on the European Nucleotide Archive.

Scripts are available at Github https://github.com/Borrill-Lab/NAC5-1SenescenceDAPseq

The following genes were referred to in this study (Table S4).

3 | RESULTS

3.1 | *NAC5-1* is the ortholog of *OsNAC5* and shows increased expression during flag leaf senescence

The triad of hexaploid wheat genes *TraesCS4A02G219700*, *TraesCS4B02G098200*, and *TraesCS4D02G094400* has previously been noted as the ortholog of *OsNAC5/ONAC071* (Lv et al., 2020). In this study, these genes will be referred to as *NAC5-A1* (*TraesCS4A02G219700*), *NAC5-B1* (*TraesCS4B02G098200*) and *NAC5-D1* (*TraesCS4D02G094400*) (Table S4), in accordance with guidelines on wheat gene nomenclature based on homology (Boden et al., 2023). *NAC5-A1* shows 97.3% and 98.5% identity with homoeologs *NAC5-B1* and *NAC5-D1* respectively, and 81.9% identity with *OsNAC5* at the peptide level (Figure S3a). *NAC5-1* was the only wheat gene triad to cluster with *OsNAC5* based on peptide alignment of related wheat and rice NAC transcription factors, confirming that *NAC5-1* and *OsNAC5* have 1:1 orthology (Figure S3b). To assess the suitability of *NAC5-1* as a candidate senescence regulator, the expression of *NAC5-1* in a time-course of flag leaf senescence was extracted from an RNA-seq dataset in hexaploid wheat (Borrill et al., 2019). *NAC5-A1*, *NAC5-B1*, and *NAC5-D1* increase in expression from 3 days after anthesis (DAA) to 26DAA, with *NAC5-D1* also showing a peak in expression at 15DAA (Figure S3c).

3.2 | Missense mutations in *NAC5-1* are associated with a delay in leaf senescence

In rice, *OsNAC5* expression is positively correlated with earlier senescence. Therefore, to test the hypothesis that loss-of-function of *NAC5-1* will delay senescence, lines with mutations in all copies of *NAC5-1* were developed by crossing together lines from the tetraploid wheat cv. Kronos TILLING population (Krasileva et al., 2017). Tetraploid wheat was selected to accelerate the crossing program as the tetraploid wheat genome has two subgenomes, A and B, hence contains only two homoeologs of *NAC5-1*, *NAC5-A1* and *NAC5-B1*. Missense mutations in the highly conserved NAC domain responsible for both DNA binding and dimerization (Ernst et al., 2004; Welner et al., 2012) were identified in both *NAC5-A1* and *NAC5-B1* (Table 1; Figure 1a). Line K2546, with a missense mutation in subdomain iii of the NAC domain of *NAC5-A1*, was crossed with line K2036 and independently with line K3328, which both had missense mutations in subdomain iv of the NAC domain of *NAC5-B1* (Figure S1). Two backcrosses were carried out with wild-type Kronos to reduce the background mutation load. In preliminary trials at the BC_1 generation, delayed leaf senescence was observed in *NAC5-1* double mutant lines compared to controls (Figure 1b).

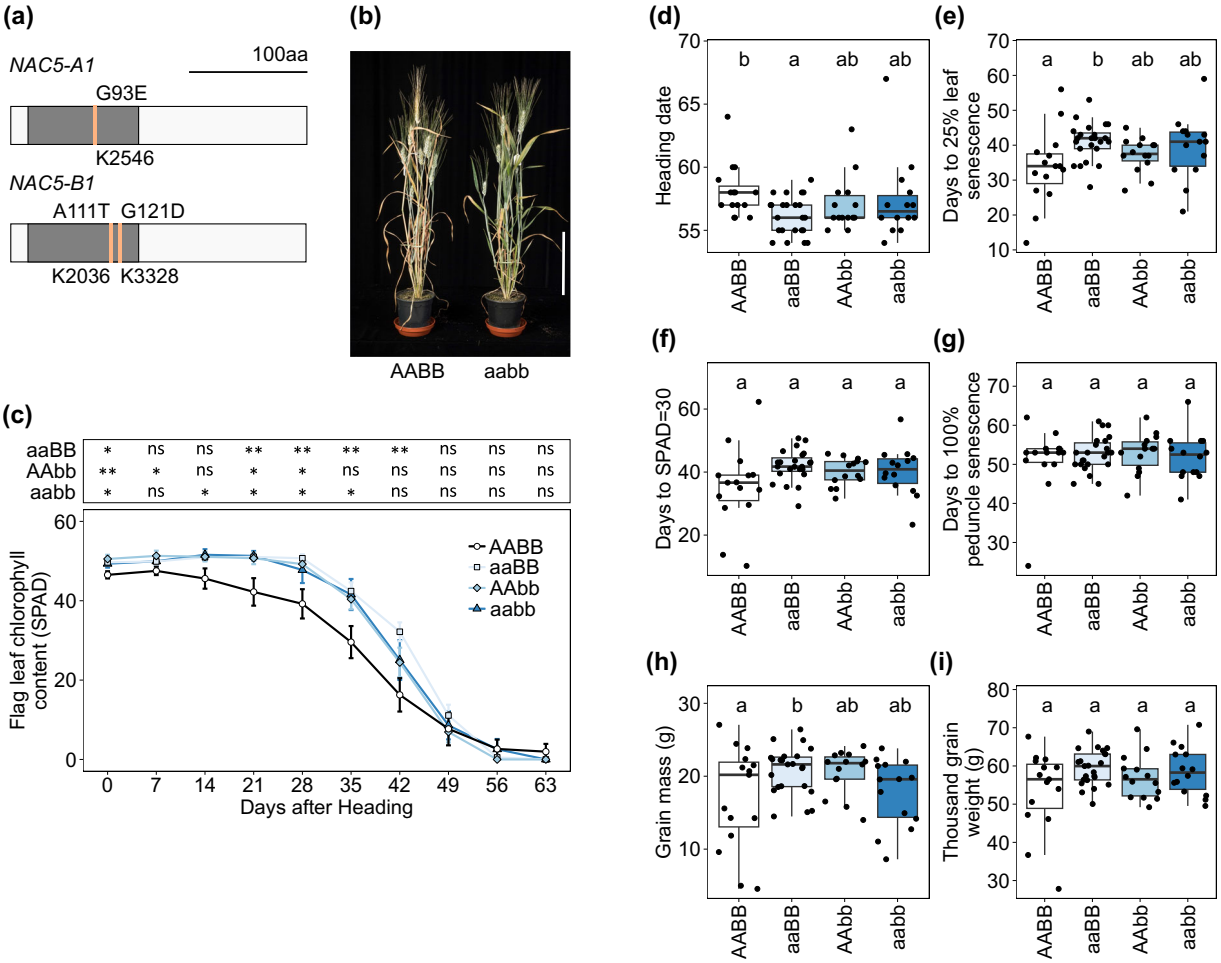

FIGURE 1 *NAC5-1* TILLING lines retain higher chlorophyll content than controls. (a) Peptide sequences of *NAC5-1*, annotated with selected TILLING mutations in Kronos. Pale lines mark mutations, white boxes show peptide sequence, gray boxes show conserved NAC domain. (b) Image at 50 days after anthesis of wild type (AABB) and double (aabb) *NAC5-1* BC_1 TILLING mutant plants from cross K2546*K2036. Scale bar shows 20 cm. (c-i) *NAC5-1* BC_2 TILLING lines, combined data from crosses K2546*K2036 and K2546*K3328 (n = 14 to 24). (c) Flag leaf chlorophyll content (SPAD) in days after heading (DAH) +/− 1 day was compared between wild type (AABB), single (aaBB/AAbb), and double (aabb) *NAC5-1* mutants. Average SPAD value at each timepoint was compared against wild type by Wilcoxon test, * = p < .05; ** = p < .01. (d) Heading date (days after sowing), (e) 25% flag leaf yellowing (DAH), (f) flag leaf SPAD value of 30 (DAH), (g) 100% peduncle yellowing (DAH), (h) grain mass (g), (i) thousand grain weight (g). (d-i) ANOVA with post-hoc Tukey test, formula ~ row + block + genotype, letters show significance groups at p < .05.

To test whether these mutations in *NAC5-A1* and *NAC5-B1* delay senescence, a time-course of flag leaf senescence was measured in TILLING lines at the BC_2F_3 generation in the glasshouse. Results show combined data from the two independent crosses, sharing the same mutation in *NAC5-A1* and differing in the mutation in *NAC5-B1*, as both showed similar trends. Wild-type segregants derived from the same crosses were used as controls. A SPAD spectrometer was used as a non-destructive indicator of flag leaf chlorophyll contents, expressed in SPAD units. On average, *NAC5-1* double mutant lines retained significantly higher flag leaf chlorophyll contents relative to controls from 14 to 35 days after heading (DAH) (Figure 1c). Similarly, *NAC5-A1* single mutants retained higher chlorophyll contents from 21 to 42 DAH, and *NAC5-B1* mutants from 21 to 28 DAH (Figure 1c). Interestingly, all three *NAC5-1* TILLING lines also showed slightly higher chlorophyll contents at the heading (Figure 1c). The heading date was earlier in *NAC5-A1* single mutants than controls but did not differ in *NAC5-B1* mutants and double mutants (Figure 1d). The onset of flag leaf senescence was assessed by two methods: a visual score of 25% leaf yellowing and a calculation of the point at which the SPAD value passed 30. *NAC5-1* single and double mutant lines trended toward delayed onset of leaf senescence by both metrics, although this was only significant for days to 25% leaf yellowing in *NAC5-A1* single mutant lines (Figure 1e,f). The time from heading to complete peduncle senescence did not differ between lines (Figure 1g).

To explore whether mutation in *NAC5-1* affects the trade-off between grain mass and protein content, grain traits were also measured in this experiment. Grain mass was significantly higher in

NAC5-A1 single mutants compared to controls but did not differ in *NAC5-B1* mutants or double mutants (Figure 1h). This increase in grain mass may derive from a combination of factors, as neither thousand-grain weight, tiller number nor grain number per tiller differed between lines (Figure 1i; Figure S4a,b). *NAC5-1* double mutants showed increased grain length (Figure S4c). There were no significant differences in grain width, grain area, grain protein content, or plant height (Figure S4d–g).

3.3 | Development of transgenic lines to overexpress *NAC5-A1*

To further assess the effect of *NAC5-1* on senescence timing we expressed *NAC5-A1* from a constitutive rice Actin promoter with a 5' FLAG tag in hexaploid spring wheat cv. Fielder, selected for its high transformation efficiency (Figure S2). Two wheat transformation experiments were carried out with binary construct pMSH30 and transformation efficiencies of 21.8 and 68.4% were achieved (percentage of inoculated embryos that regenerate a transformed plant).

To assess the expression of *NAC5-1* in transgenic lines, three sets of qPCR primers were designed to amplify the transgenic construct, the homeolog *NAC5-A1* (including both endogenous and construct-derived transcripts), or all three homoeologs of *NAC5-1* (Table S1). Initially, each independently transformed line was assessed by a pooled leaf sample from 12 individuals at the T_1 generation. As expected, the construct-specific primers amplified in all transgenic lines but did not amplify in non-transformed controls (Figure S5a). Based on pooled samples, line 5.4 showed the highest transcript level of *NAC5-A1* among single-copy transformants, while line 8.2 showed the highest transcript level of *NAC5-A1* among multi-copy transformants (Figure S5b). Therefore, lines 5.4 and 8.2 were selected. A copy number assay of plants from line 5.4 in the T_1 generation identified individuals homozygous for the transgenic construct, heterozygous, and wild-type segregants (Figure S5c).

To account for variability in *NAC5-A1* expression between individual plants, transcript levels were assessed from leaf samples of each plant in the selected lines. Again, the construct was expressed in the majority of plants from transgenic lines but was not amplified in non-transformed controls (Figure S5d). Over half of the individuals of multi-copy line 8.2 showed a higher transcript level of *NAC5-A1* and of all homoeologs of *NAC5-1* compared to the non-transformed control (Figure 2a,b). On average, homozygous and heterozygous plants of single copy line 5.4 did not show overexpression of *NAC5-A1* or of all homoeologs of *NAC5-1* relative to the non-transformed control (Figure 2a,b). Nevertheless, some individuals within single copy line 5.4 expressed *NAC5-A1* more highly than all non-transformed control individuals (Figure 2a).

If *NAC5-1* regulates senescence, it follows that overexpression of *NAC5-1* would lead to earlier leaf senescence. To test this hypothesis, T_1 plants were selected to advance to the T_2 generation for phenotyping according to the following criteria:

1. For overexpression of *NAC5-A1*, from multi-copy line 8.2 the plant with the highest transcript level of *NAC5-A1* was selected (Figure 2a).
2. For slight overexpression of *NAC5-A1*, from single-copy line 5.4, a homozygous plant in T_1 with a higher transcript level of *NAC5-A1* than non-transformed controls was selected (Figure 2a).
3. As negative control, from single-copy line 5.4 a wild-type segregant control with no amplification of the construct was selected (Figure 2a).

3.4 | Overexpression of *NAC5-1* leads to slightly earlier leaf senescence

NAC5-1 overexpression line 5.4 showed reduced flag leaf chlorophyll content at 28 DAH compared to the matched wild-type segregant control, and no difference at other timepoints (Figure 2c). *NAC5-1* overexpression line 8.2 showed a significantly lower average SPAD value from 21 to 35 DAH than the wild-type control (Figure 2c). *NAC5-1* overexpression lines did not differ from the control in heading date (Figure 2d). Both overexpression lines showed significantly earlier onset of flag leaf senescence scored as days to a SPAD value of 30, and line 5.4 also when scored as day of 25% flag leaf yellowing (Figure 2e,f) The timing of peduncle senescence did not differ between lines (Figure 2g). There were no significant differences in grain mass, thousand-grain weight, tiller number, grain length, grain width, or grain area, although line 8.2 had fewer grains per tiller than the control (Figure 2h,i; Figure S6a–e).

3.5 | Putative downstream targets of *NAC5-1* include senescence-related genes

To investigate the potential direct downstream target genes of the transcription factor *NAC5-1*, DAP-seq was carried out on *NAC5-A1* and *NAC5-B1* (Gene IDs in Table S4). *NAC5-D1* was omitted because the peptide sequence of the DNA-binding domain is identical between *NAC5-D1* and *NAC5-A1* (Figure S3a). All homoeologs of transcription factors *NAM-1* and *NAM-2* (Gene IDs in Table S4), previously shown to be associated with senescence timing, were also tested with DAP-seq.

For each transcription factor homoeolog, the number of reads obtained ranged from 55.7 M to 94.6 M raw read pairs and 14.7 M to 24.3 M filtered read pairs (Table 2). After filtering, a total of 277 peaks were called across all eight transcription factors, including 39 for *NAC5-A1* and six for *NAC5-B1* (Table 2). *NAM-B1* had both the highest number of input reads and highest number of peaks called (96) indicating that variability in number of peaks called is partly explained by variability in input read depth (Table 2).

To assess the quality of the DAP-seq peak data, a motif analysis was carried out on samples with more than 30 peaks. For *NAM-A1*, *NAM-B1*, *NAM-A2*, and *NAM-D2*, at least two of the de novo motifs identified correlated with motifs assigned to NAC family transcription

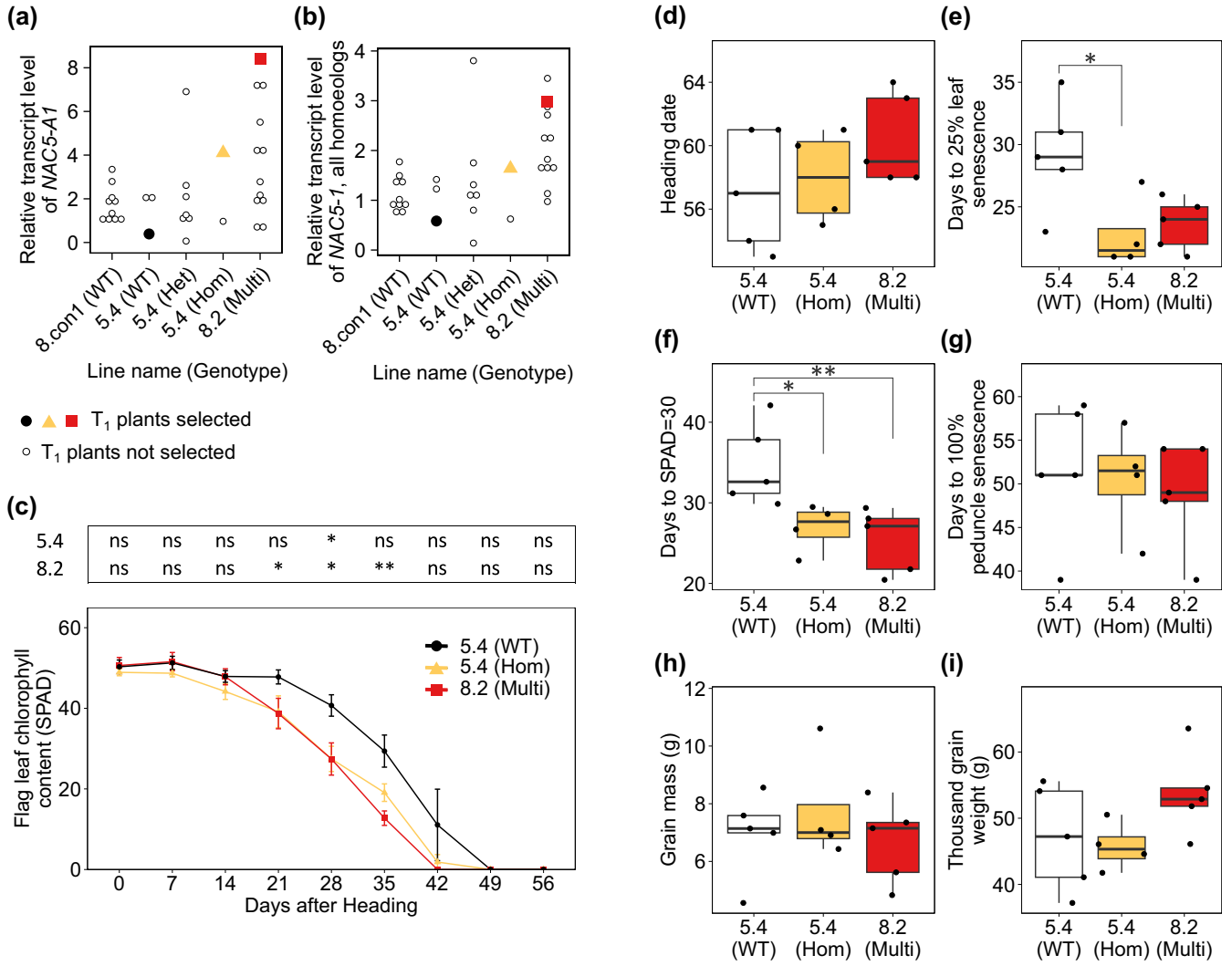

FIGURE 2 Transgenic lines expressing *NAC5-A1* show an earlier onset of flag leaf senescence. (a, b) Relative transcript level of individual 3-week-old T_1 leaf samples was analyzed using the Pfaffl method with primer efficiencies and actin as reference gene, average of three technical replicates normalized against average of single copy plants. Non-transformed control (8.con1), single-copy line (5.4) categorized by construct genotype, and multi-copy line (8.2) are shown. Highlighted points mark T_1 plants for which T_2 progeny were used for phenotyping. (a) Relative transcript level of *NAC5-A1*; (b) relative transcript level of *NAC5-A1*, *NAC5-B1* and *NAC5-D1* combined. (c-i) Phenotypes of T_2 plants. (c) Flag leaf chlorophyll content (SPAD) in days after heading (DAH) +/− 1 day. (d) Heading date (days after sowing), (e) 25% flag leaf yellowing (DAH); (f) flag leaf SPAD value of 30 (DAH); (g) 100% peduncle yellowing (DAH); (h) grain mass (g); (i) thousand grain weight (g). (c-i) Pairwise comparisons by Wilcoxon test for single-copy transgenic line 5.4 (n = 4) and multi-copy transgenic line 8.2 (n = 5) against wild-type segregant control from line 5.4 (n = 5), ns = not significant; * = p < .05; ** = p < .01.

factors in the Jaspar database (Table 2). In addition, the most significant motif for *NAM-B1* and *NAM-A2* showed an E-value less than 1×10^{-5} (Table 2). However, for *NAC5-A1* DAP-seq data, and for control data (peaks shuffled to randomized genome locations), none of the motifs identified correlated with NAC transcription factor motifs (Table 2). This indicates that *NAM-A1*, *NAM-B1*, *NAM-A2*, and *NAM-D2* DAP-seq peak datasets are enriched for transcription factor binding sites, while the *NAC5-A1* dataset may not be.

The closest gene to each DAP-seq peak was identified (Dataset S1). The closest genes to peaks in *NAM-A1* and *NAM-B1* shared two genes in common, a higher overlap than would be expected if gene sets were independent (jaccard index .016, q < .01) (Dataset S2). Similarly, 11 out of 15 pairwise overlaps between the closest genes to peaks in *NAM-A1*, *NAM-B1*, *NAM-D1*, *NAM-A2*, *NAM-B2*, and *NAM-D2* were significant (Dataset S2). There was no overlap in the closest genes to peaks between *NAC5-A1* and *NAC5-B1*, although an Alpha/beta gliadin gene was shared between *NAC5-A1* and *NAM-B2*, and an uncharacterized gene was shared between *NAC5-B1*, *NAM-A1*, and *NAM-D1* (Dataset S3).

These gene sets were also compared to independent datasets for candidate target genes of *NAC5-1*, *NAM-1*, *NAM-2*, and *OsNAC5*. Candidate target genes were obtained from the top 1 million connections of a GENIE3 gene network in wheat (Ramírez-González et al., 2018). Predicted target genes from this gene network showed significant

TABLE 2 Summary of DAP-seq peaks called for *NAC5-1*, *NAM-1*, and *NAM-2*. Peaks were called using two or three pooled technical replicates and filtered for q-value < .01 and a greenscreen was applied. Randomized control peak data was generated by randomizing the location of *NAM-A1* peaks with bedtools shuffle. Motif analysis was run for genes with > 30 peaks.

Gene	Technical replicates	Total raw read pairs	Total read pairs input for peak calling	Raw peaks	Filtered peaks	Motifs called (out of 15)	Lowest motif E-value	Motifs correlated with a NAC motif	Predicted target genes
NAC5-A1	2	76,184,457	19,945,930	78	39	9	0.042	0	39
NAC5-B1	2	63,572,448	15,941,210	19	6	NA	NA	NA	6
NAM-A1	3	92,107,807	22,677,438	59	35	8	0.0069	2	34
NAM-B1	3	94,588,293	24,265,952	149	96	14	4.90×10^{-6}	6	93
NAM-D1	2	66,774,874	17,158,800	15	6	NA	NA	NA	6
NAM-A2	2	57,646,546	14,796,529	44	31	12	1.30×10^{-6}	5	30
NAM-B2	2	57,907,246	14,973,598	72	49	11	0.011	5	48
NAM-D2	2	55,724,124	14,698,721	22	15	NA	NA	NA	14
Randomized control	3	NA	NA	59	35	8	0.15	0	NA
pIX-HALO control	3	90,048,334	23,115,763	NA	NA	NA	NA	NA	NA

pairwise overlaps between homoeologs within each triad for *NAC5-1*, *NAM-1* and *NAM-2*, and between *NAM-1* and *NAM-2* (Dataset S2). However, while a few genes were common between the gene network and DAP-seq gene sets, none of the pairwise overlaps were significant (Dataset S2). Comparison was also made with genes differentially expressed in RNA-seq of *NAC5-A1* overexpression lines compared to wild-type in watered conditions and identified by DAP-seq in wheat (Mao et al., 2022). Finally, wheat orthologs of candidate target genes of *OsNAC5* in rice based on ChIP-seq and RNA-seq of *OsNAC5* overexpression lines were added (Chung et al., 2018). The genes upregulated in *NAC5-A1* overexpression lines showed a significant pairwise overlap with gene-network predicted targets of *NAC5-A1* and with orthologs of ChIP-seq predicted targets of *OsNAC5* (Dataset S2). Overall, 67 genes were associated with *NAC5-1* according to more than one gene set (Figure 3, Dataset S3). Of these, 37 were identified as interacting with two or three of the homoeologs of *NAC5-1* within the GENIE3 gene network, while 30 were identified by independent methods in two or more studies (Figure 3, Dataset S4). These 67 genes come from a range of different families including cytochrome P450 genes which have been associated with chlorophyll catabolism (Christ et al., 2013) and chloroplast function (Cui et al., 2021), transcription factors in the MYB, bHLH and NAC families which may be part of the senescence regulatory cascade, peptidases which have been associated with protein catabolism in senescence (Roberts et al., 2012) and peroxidases which are known to be upregulated during senescence (Bhattacharjee, 2005).

4 | DISCUSSION

4.1 | Summary of key findings

We found that mutation of *NAC5-1* was associated with a delay in the onset of leaf senescence, while overexpression of *NAC5-1* was associated with earlier leaf senescence, supporting the hypothesis that *NAC5-1* positively regulates the timing of leaf senescence. Thousand-grain weight did not differ in *NAC5-1* mutant lines or overexpression lines, and grain protein content did not differ in *NAC5-1* mutant lines, providing no evidence for the hypothesis that by promoting senescence, *NAC5-1* decreases yield and increases protein content. The DAP-seq data generated in this study provided few peaks, and the *NAC5-A1* dataset did not identify a known NAC transcription factor motif, indicating that these data are of limited value. However, a comparison with other datasets identified putative downstream target genes of *NAC5-1* which may be involved in senescence.

4.2 | *NAC5-1* has a conserved role to promote senescence

The retention of higher flag leaf chlorophyll content in *NAC5-1* single and double mutant TILLING lines and more rapid loss of chlorophyll content in *NAC5-1* overexpression lines suggest a role for *NAC5-1* as a positive regulator of the onset of leaf senescence. The chlorophyll retention in *NAC5-1* TILLING lines occurred around 21–35 DAH, coinciding with the upregulation of *NAC5-1* transcripts in wild-type plants at 23-26DAA (Borrill et al., 2019). This is consistent with its ortholog in rice, *OsNAC5*, which was shown to be upregulated in leaf senescence (Sperotto et al., 2009). Grain mass did not differ significantly in *NAC5-1* double mutant lines. However, *NAC5-A1* single mutants showed slightly increased grain mass and *NAC5-1* double mutants showed increased grain length. These results are consistent with rice T-DNA insertion lines with enhanced *OsNAC5* expression, which showed decreased grain length, grain number, and kernel weight (Wairich et al., 2023), however, overexpression of *OsNAC5* in a separate study resulted in increased grain mass (Jeong et al., 2013). To ascertain the effects of *NAC5-1* on yield components and grain yields larger-scale field experiments will be required. Missense

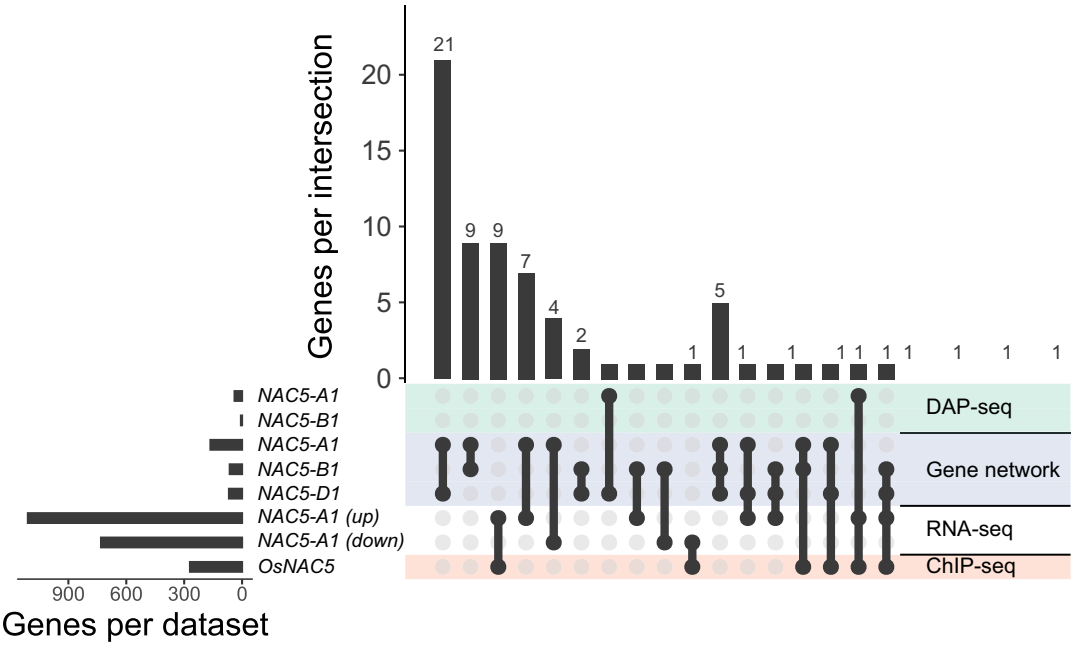

FIGURE 3 Candidate target genes of *NAC5-1* which overlap in two or more datasets. Left panel shows total number of genes per dataset. Right panel shows the intersection of two or more datasets with black dots, and above, the number of genes in this intersection. DAP-seq: genes closest to peaks in DAP-seq data of *NAC5-A1* and *NAC5-B1*, from this study. Gene network: candidate target genes of *NAC5-A1*, *NAC5-B1*, and *NAC5-D1* based on a GENIE3 gene network (Ramírez-González et al., 2018). RNA-seq: genes both significantly upregulated (up) or downregulated (down) in RNA-seq of young leaves of *NAC5-A1* overexpression lines in wheat and identified in a DAP-seq experiment (Mao et al., 2022). ChIP-seq: wheat orthologs of rice genes identified from both a ChIP-seq experiment of *OsNAC5*, and differential expression in RNA-seq of roots of *OsNAC5* overexpression lines (Chung et al., 2018).

mutations in *NAC5-1* did not affect grain protein content in these experiments, contrasting with rice, where increased *OsNAC5* expression correlated with increased grain protein content (Sharma et al., 2019). Further work is needed to assess the grain protein content of *NAC5-1* mutant lines in larger-scale trials in the field, which may result in different findings from our experiments in greenhouse conditions, as has been shown for photosynthetic traits in wheat (Sales et al., 2022). Previously, it was shown that *NAC5-A1* shares a role in promoting drought tolerance with *OsNAC5* (Mao et al., 2022; Takasaki et al., 2010). Our experiments provide evidence that wheat *NAC5-A1* may share further conserved roles with its rice ortholog *OsNAC5*, as it is associated with earlier leaf senescence and decreased grain number and length.

To clarify the extent of the effect of *NAC5-A1* on senescence and effects on grain phenotypes it would be valuable to generate complete null mutants, for example using CRISPR-Cas9. Null mutants may show stronger phenotypic effects than the missense mutants characterized here. Similarly, our transgenic lines showed only a moderate increase in expression and future work using a stronger overexpression promoter could reveal stronger or additional effects. Moreover, our study measuring flag leaf chlorophyll content and peduncle yellowing only captures two aspects of the complex senescence process and additional work will be needed to understand any effects of *NAC5-1* at the biochemical, transcriptomic, cellular, and whole plant scales (Borrill et al., 2019; Davies & Gan, 2012; Lim et al., 2007).

4.3 | Integrating multiple datasets identified putative downstream targets of *NAC5-1*

This study aimed to identify direct downstream targets of *NAC5-1* using DAP-seq. The 17Gb genome of wheat is significantly larger than the 100 Mb genome of *Arabidopsis thaliana*, on which the DAP-seq technique was initially validated (O'Malley et al., 2016). Despite combining technical replicates, it is likely that in this experiment there was insufficient read depth for effective peak calling in wheat. A peak q-value filter of .01 was analyzed because the more stringent q-value filter of 1×10^{-10}, used in other studies (Klasfeld et al., 2022), returned very few peaks. Future DAP-seq experiments on polyploid wheat or other species with large genomes should increase the number of reads and maximize mapped read depth by increasing DNA library fragment size (to increase the probability of uniquely mapping a read to a subgenome).

Nevertheless, DAP-seq works more efficiently for some transcription factors or transcription factor families than others: previously only 529 of 1,812 (29%) *Arabidopsis* transcription factors and 45 of 189 (24%) wheat transcription factors yielded high-quality datasets with de novo motifs (O'Malley et al., 2016; Zhang et al., 2022). This variability is not random, as out of transcription factors that did not work on the first attempt, only 10% were recovered on a second attempt (O'Malley et al., 2016). Specifically, DAP-seq of *NAC5-A1* did not identify a known NAC transcription factor motif either in this

dataset or in the A-genome donor of wheat (*Triticum urartu* Thumanjan ex Gandilyan), implying that *NAC5-1* may be one of the transcription factors less compatible with DAP-seq (Zhang et al., 2021). As codon usage can impact protein folding, it is possible that some alteration to transcription factor protein folding affected DAP-seq results (Liu, 2020). Further research is needed to optimize DAP-seq to work efficiently with a wider proportion of transcription factors. This would unlock the potential of this technique for research focussed on specific transcription factors, or on developing a detailed regulatory network for a specific trait, such as senescence timing.

Although the DAP-seq for *NAC5-1* was not very informative, adding independently published datasets revealed 67 genes that are putative target genes of *NAC5-1* based on two or more gene sets. Some of these putative target genes have potential associations with senescence. Among these, several target genes have associations with nitrogen remobilization including two serine peptidases that were identified as putative targets of *NAC5-1* from the gene network approach and from downregulation in *NAC5-A1* overexpression lines. One of these serine peptidases was also identified as a target of *NAM-A1* by DAP-seq in this study, suggesting a shared pathway. Serine peptidases are upregulated in senescing wheat flag leaves (Gregersen & Holm, 2007), and protein catabolism by peptidases in the flag leaf is a necessary first step in nitrogen remobilization. These genes may provide a direct mechanism by which *NAC5* and *NAM-1* promote nitrogen remobilization. *NAC5-1* may also regulate the release of nitrogen from chlorophyll (Christ et al., 2013) through cytochrome P450 genes, of which six were identified as putative targets of two *NAC5-1* homoeologs in the gene network. Remobilized nitrogen may be stored in the form of gliadin in the seeds (Cauvain, 2012), consistent with our identification of an alpha/beta gliadin gene as a putative target of *NAC5-A1* and *NAM-B2* based on DAP-seq in this study and as a target of *NAC5-D1* in the gene network.

Other genes associated with senescence were also identified as putative targets. For example "Senescence regulator S40", related to *AtS40-3*, which is associated with earlier leaf senescence in *Arabidopsis*, was downregulated in *NAC5-A1* overexpression lines and a target of *NAC5-A1* in the gene network (Fischer-Kilbienski et al., 2010). Two closely related heavy metal-associated genes were also identified from the gene network, which is interesting given the role of *OsNAC5* in metal ion remobilization (Wairich et al., 2023). Three homoeologs of a wheat peroxidase gene were upregulated in *NAC5-1* overexpression lines, and orthologous to an *OsNAC5* ChIP-seq target. Peroxidases contribute to the catabolism of lipids during leaf senescence (Bhattacharjee, 2005). Finally, six of the 67 putative *NAC5-1* targets are transcription factor genes. Investigating these transcription factor gene interactions will aid in building the regulatory network of wheat senescence. In summary, some of the putative target genes of *NAC5-1* identified from the DAP-seq from this study together with independent datasets may be connected with senescence and nitrogen remobilization. Biochemical validation of these putative downstream targets, for example using electrophoretic mobility shift assays, chromatin immunoprecipitation, or luciferase assays would be valuable future work to clarify the mechanism of senescence regulation by *NAC5-1*.

4.4 | Conclusion

In conclusion, these results indicate that NAC transcription factor *NAC5-1* is a positive regulator of leaf senescence in wheat. Results of one out of two experiments suggest that *NAC5-1* is associated with decreased grain length and grain number. Putative downstream targets of *NAC5-1* feature gene families which have roles in senescence and nitrogen remobilization, but further research is needed to explore whether *NAC5-1* affects yield or grain protein content in the field. Transcription factors regulating senescence could be targeted to develop wheats with a range of earlier and later flag leaf senescence, to balance yield and protein content.

AUTHOR CONTRIBUTIONS

CE and PB conceived and designed the research with contributions from SLM, EW, and JC. CE, SLM, CS, and PB performed the research. CS generated and validated the transgenic wheat lines, PB carried out the crossing of TILLING mutants and CE carried out genotyping and phenotypic experiments for TILLING and transgenic lines. CE carried out the DAP-seq with contributions from SLM. CE analyzed data including phenotypic and genomic data. CE and PB wrote the paper, and all authors contributed comments to the manuscript. All authors have read and approved the manuscript.

ACKNOWLEDGMENTS

The authors thank Dr Ruth Bryant for valuable suggestions during this project and Matthew Hope for assistance with binary construct preparation. We thank the John Innes Centre and University of Birmingham Horticultural Services teams for their support in glasshouse experiments.

CONFLICT OF INTEREST STATEMENT

The Authors did not report any conflict of interest.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

ORCID

Sophie Louise Mogg https://orcid.org/0000-0003-3025-5525
Emma Wallington https://orcid.org/0000-0003-3715-7901
Juliet Coates https://orcid.org/0000-0002-2381-0298
Philippa Borrill https://orcid.org/0000-0002-7623-8256

REFERENCES

AHDB. (2023). *AHDB recommended lists for cereals and oilseeds 2023/24*. Retrieved 28/08/2023, from https://ahdb.org.uk/knowledge-library/recommended-lists-for-cereals-and-oilseeds-rl

Alexaki, A., Kames, J., Holcomb, D. D., Athey, J., Santana-Quintero, L. V., Lam, P. V. N., Hamasaki-Katagiri, N., Osipova, E., Simonyan, V., Bar, H., Komar, A. A., & Kimchi-Sarfaty, C. (2019). Codon and codon-pair usage tables (CoCoPUTs): Facilitating genetic variation analyses and recombinant gene design. *Journal of Molecular Biology*, *431*(13), 2434–2441. https://doi.org/10.1016/j.jmb.2019.04.021

Appels, R., Eversole, K., Feuillet, C., Keller, B., Rogers, J., Stein, N., Pozniak, C. J., Choulet, F., Distelfeld, A., Poland, J., Ronen, G., Sharpe, A. G., Pozniak, C., Barad, O., Baruch, K., Keeble-Gagnere, G., Mascher, M., Ben-Zvi, G., Josselin, A. A., … IWGSC. (2018). Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science*, *361*(6403), eaar7191. https://doi.org/10.1126/science.aar7191

Avni, R., Zhao, R. R., Pearce, S., Jun, Y., Uauy, C., Tabbita, F., Fahima, T., Slade, A., Dubcovsky, J., & Distelfeld, A. (2014). Functional characterization of *GPC-1* genes in hexaploid wheat. *Planta*, *239*(2), 313–324. https://doi.org/10.1007/s00425-013-1977-y

Bartlett, A., O'Malley, R. C., Huang, S.-S. C., Galli, M., Nery, J. R., Gallavotti, A., & Ecker, J. R. (2017). Mapping genome-wide transcription-factor binding sites using DAP-seq. *Nature Protocols*, *12*(8), 1659–1672. https://doi.org/10.1038/nprot.2017.055

Bates, R., Craze, M., & Wallington, E. J. (2017). Agrobacterium-mediated transformation of oilseed rape (*Brassica napus*). *Current Protocols in Plant Biology*, *2*(4), 287–298. https://doi.org/10.1002/cppb.20060

Bhattacharjee, S. (2005). Reactive oxygen species and oxidative burst: Roles in stress, senescence and signal transduction in plants. *Current Science*, *89*(7), 1113–1121. ://WOS:000232711500024

Biomatters. (2022). *Geneious version 2022.2*. https://www.geneious.com

Boden, S. A., McIntosh, R. A., Uauy, C., Krattinger, S. G., Dubcovsky, J., Rogers, W. J., Xia, X. C., Badaeva, E. D., Bentley, A. R., Brown-Guedira, G., Caccamo, M., Cattivelli, L., Chhuneja, P., Cockram, J., Contreras-Moreira, B., Dreisigacker, S., Edwards, D., González, F. G., Guzmán, C., … the Wheat Initiative. (2023). Updated guidelines for gene nomenclature in wheat. *Theoretical and Applied Genetics*, *136*(4), 72. https://doi.org/10.1007/s00122-023-04253-w

Bogard, M., Allard, V., Brancourt-Hulmel, M., Heumez, E., Machet, J. M., Jeuffroy, M. H., Gate, P., Martre, P., & Le Gouis, J. (2010). Deviation from the grain protein concentration-grain yield negative relationship is highly correlated to post-anthesis N uptake in winter wheat. *Journal of Experimental Botany*, *61*(15), 4303–4312. https://doi.org/10.1093/jxb/erq238

Borrill, P., Harrington, S. A., Simmonds, J., & Uauy, C. (2019). Identification of transcription factors regulating senescence in wheat through gene regulatory network modelling. *Plant Physiology*, *180*(3), 1740–1755. https://doi.org/10.1104/pp.19.00380

Borrill, P., Harrington, S. A., & Uauy, C. (2017). Genome-wide sequence and expression analysis of the NAC transcription factor family in polyploid wheat. *G3: Genes, Genomes, Genetics*, *7*(9), 3019–3029. https://doi.org/10.1534/g3.117.043679

Borrill, P., Ramirez-Gonzalez, R., & Uauy, C. (2016). expVIP: A customizable RNA-seq data analysis and visualization platform. *Plant Physiology*, *170*(4), 2172–2186. https://doi.org/10.1104/pp.15.01667

Buchanan-Wollaston, V. (1997). The molecular biology of leaf senescence. *Journal of Experimental Botany*, *48*(307), 181–199. https://doi.org/10.1093/jxb/48.2.181

Cauvain, S. P. (2012). *Breadmaking : Improving quality/edited by Stanley P. Cauvain* (2nd ed.). Woodhead Pub.

Chapman, E. A., Orford, S., Lage, J., & Griffiths, S. (2021). Capturing and selecting senescence variation in wheat. *Frontiers in Plant Science*, *12*(17), 638738. https://doi.org/10.3389/fpls.2021.638738

Christ, B., Süssenbacher, I., Moser, S., Bichsel, N., Egert, A., Müller, T., Kräutler, B., & Hörtensteiner, S. (2013). Cytochrome P450 CYP89A9 is involved in the formation of major chlorophyll catabolites during leaf senescence in *Arabidopsis*. *The Plant Cell*, *25*(5), 1868–1880. https://doi.org/10.1105/tpc.113.112151

Christiansen, M. W., Matthewman, C., Podzimska-Sroka, D., O'Shea, C., Lindemose, S., Mollegaard, N. E., Holme, I. B., Hebelstrup, K., Skriver, K., & Gregersen, P. L. (2016). Barley plants over-expressing the NAC transcription factor gene *HvNAC005* show stunting and delay in development combined with early senescence. *Journal of Experimental Botany*, *67*(17), 5259–5273. https://doi.org/10.1093/jxb/erw286

Chung, P. J., Jung, H., Choi, Y. D., & Kim, J. K. (2018). Genome-wide analyses of direct target genes of four rice NAC-domain transcription factors involved in drought tolerance. *BMC Genomics*, *19*, 40. https://doi.org/10.1186/s12864-017-4367-1

Chung, N. C., Miasojedow, B., Startek, M., & Gambin, A. (2019). Jaccard/-Tanimoto similarity test and estimation methods for biological presence-absence data. *BMC Bioinformatics*, *20*(Suppl 15), 644. https://doi.org/10.1186/s12859-019-3118-5

Clarke, T. F. I. V., & Clark, P. L. (2008). Rare codons cluster. *PLoS ONE*, *3*(10), e3412. https://doi.org/10.1371/journal.pone.0003412

Clavijo, B. J., Venturini, L., Schudoma, C., Accinelli, G. G., Kaithakottil, G., Wright, J., Borrill, P., Kettleborough, G., Heavens, D., Chapman, H., Lipscombe, J., Barker, T., Lu, F.-H., McKenzie, N., Raats, D., Ramirez-Gonzalez, R. H., Coince, A., Peel, N., Percival-Alwyn, L., … Clark, M. D. (2017). An improved assembly and annotation of the allohexaploid wheat genome identifies complete families of agronomic genes and provides genomic evidence for chromosomal translocations. *Genome Research*, *27*(5), 885–896. https://doi.org/10.1101/gr.217117.116

Conway, J. R., Lex, A., & Gehlenborg, N. (2017). UpSetR: An R package for the visualization of intersecting sets and their properties. *Bioinformatics*, *33*(18), 2938–2940. https://doi.org/10.1093/bioinformatics/btx364

Cui, Y., Peng, Y., Zhang, Q., Xia, S., Ruan, B., Xu, Q., Yu, X., Zhou, T., Liu, H., Zeng, D., Zhang, G., Gao, Z., Hu, J., Zhu, L., Shen, L., Guo, L., Qian, Q., & Ren, D. (2021). Disruption of *EARLY LESION LEAF 1*, encoding a cytochrome P450 monooxygenase, induces ROS accumulation and cell death in rice. *The Plant Journal*, *105*(4), 942–956. https://doi.org/10.1111/tpj.15079

Daniel, E., Onwukwe, G. U., Wierenga, R. K., Quaggin, S. E., Vainio, S. J., & Krause, M. (2015). ATGme: Open-source web application for rare codon identification and custom DNA sequence optimization. *BMC Bioinformatics*, *16*(1), 303. https://doi.org/10.1186/s12859-015-0743-5

Davies, P. J., & Gan, S. (2012). Towards an integrated view of monocarpic plant senescence. *Russian Journal of Plant Physiology*, *59*(4), 467–478. https://doi.org/10.1134/s102144371204005x

Distelfeld, A., Pearce, S. P., Avni, R., Scherer, B., Uauy, C., Piston, F., Slade, A., Zhao, R. R., & Dubcovsky, J. (2012). Divergent functions of orthologous NAC transcription factors in wheat and rice. *Plant Molecular Biology*, *78*(4–5), 515–524. https://doi.org/10.1007/s11103-012-9881-6

Ernst, H. A., Olsen, A. N., Skriver, K., Larsen, S., & Lo Leggio, L. (2004). Structure of the conserved domain of *ANAC*, a member of the NAC family of transcription factors. *EMBO Reports*, *5*(3), 297–303. https://doi.org/10.1038/sj.embor.7400093

FAO. (2020). *FAOSTAT* http://www.fao.org/faostat/en/

Fischer-Kilbienski, I., Miao, Y., Roitsch, T., Zschiesche, W., Humbeck, K., & Krupinska, K. (2010). Nuclear targeted *AtS40* modulates senescence associated gene expression in *Arabidopsis thaliana* during natural development and in darkness. *Plant Molecular Biology*, *73*(4), 379–390. https://doi.org/10.1007/s11103-010-9618-3

Fradgley, N. S., Gardner, K., Kerton, M., Swarbreck, S. M., & Bentley, A. R. (2022). Trade-offs in the genetic control of functional and nutritional quality traits in UK winter wheat. *Heredity*, *128*(6), 420–433. https://doi.org/10.1038/s41437-022-00503-7

Gregersen, P. L., & Holm, P. B. (2007). Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal*, *5*(1), 192–206. https://doi.org/10.1111/j.1467-7652.2006.00232.x

Gregersen, P. L., Holm, P. B., & Krupinska, K. (2008). Leaf senescence and nutrient remobilisation in barley and wheat. *Plant Biology*, *10*, 37–49. https://doi.org/10.1111/j.1438-8677.2008.00114.x

Harrington, S. A. (2019). *Understanding the molecular and genetic mechanisms regulating senescence in wheat*. (Publication Number uea: 74201) [Doctoral, University of East Anglia]. https://ueaeprints.uea.ac.uk/id/eprint/74201/.

Ishida, Y., Tsunashima, M., Hiei, Y., & Komari, T. (2015). Wheat (*Triticum aestivum* L.) transformation using immature embryos. *Agrobacterium Protocols*, *1*, 189–198. https://doi.org/10.1007/978-1-4939-1695-5_15

Jeong, J. S., Kim, Y. S., Redillas, M., Jang, G., Jung, H., Bang, S. W., Choi, Y. D., Ha, S. H., Reuzeau, C., & Kim, J. K. (2013). *OsNAC5* overexpression enlarges root diameter in rice plants leading to enhanced drought tolerance and increased grain yield in the field. *Plant Biotechnology Journal*, *11*(1), 101–114. https://doi.org/10.1111/pbi.12011

Jukanti, A. K., & Fischer, A. M. (2008). A high-grain protein content locus on barley (*Hordeum vulgare*) chromosome 6 is associated with increased flag leaf proteolysis and nitrogen remobilization. *Physiologia Plantarum*, *132*(4), 426–439. https://doi.org/10.1111/j.1399-3054.2007.01044.x

Kawahara, Y., de la Bastide, M., Hamilton, J. P., Kanamori, H., McCombie, W. R., Ouyang, S., Schwartz, D. C., Tanaka, T., Wu, J., Zhou, S., Childs, K. L., Davidson, R. M., Lin, H., Quesada-Ocampo, L., Vaillancourt, B., Sakai, H., Lee, S. S., Kim, J., Numa, H., … Matsumoto, T. (2013). Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. *Rice*, *6*(1), 4. https://doi.org/10.1186/1939-8433-6-4

Kichey, T., Hirel, B., Heumez, E., Dubois, F., & Le Gouis, J. (2007). In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers. *Field Crops Research*, *102*(1), 22–32. https://doi.org/10.1016/j.fcr.2007.01.002

Kikuchi, K., Ueguchi-Tanaka, M., Yoshida, K. T., Nagato, Y., Matsusoka, M., & Hirano, H. Y. (2000). Molecular analysis of the NAC gene family in rice. *Molecular & General Genetics*, *262*(6), 1047–1051. https://doi.org/10.1007/pl00008647

Kinsella, R. J., Kähäri, A., Haider, S., Zamora, J., Proctor, G., Spudich, G., Almeida-King, J., Staines, D., Derwent, P., Kerhornou, A., Kersey, P., & Flicek, P. (2011). Ensembl BioMarts: A hub for data retrieval across taxonomic space. *Database*, *2011*, bar030. https://doi.org/10.1093/database/bar030

Klasfeld, S., Roulé, T., & Wagner, D. (2022). Greenscreen: A simple method to remove artifactual signals and enrich for true peaks in genomic datasets including ChIP-seq data. *The Plant Cell*, *34*, 4795–4815. https://doi.org/10.1093/plcell/koac282

Krasileva, K. V., Vasquez-Gross, H. A., Howell, T., Bailey, P., Paraiso, F., Clissold, L., Simmonds, J., Ramirez-Gonzalez, R. H., Wang, X., Borrill, P., Fosker, C., Ayling, S., Phillips, A. L., Uauy, C., & Dubcovsky, J. (2017). Uncovering hidden variation in polyploid wheat. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(6), E913–E921. https://doi.org/10.1073/pnas.1619268114

Lim, P. O., Kim, H. J., & Nam, H. G. (2007). Leaf senescence. *Annual Review of Plant Biology*, *58*, 115–136. https://doi.org/10.1146/annurev.arplant.57.032905.105316

Liu, Y. (2020). A code within the genetic code: Codon usage regulates co-translational protein folding. *Cell Communication and Signaling: CCS*, *18*(1), 145. https://doi.org/10.1186/s12964-020-00642-6

Lv, S. K., Guo, H., Zhang, M., Wang, Q. H., Zhang, H., & Ji, W. Q. (2020). Large-scale cloning and comparative analysis of TaNAC genes in response to stripe rust and powdery mildew in wheat (*Triticum aestivum* L.) [article]. *Genes*, *11*(9), 1073. https://doi.org/10.3390/genes11091073

Madeira, F., Pearce, M., Tivey, A. R. N., Basutkar, P., Lee, J., Edbali, O., Madhusoodanan, N., Kolesnikov, A., & Lopez, R. (2022). Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Research*, *50*(W1), W276–W279. https://doi.org/10.1093/nar/gkac240

Maeoka, R. E., Sadras, V. O., Ciampitti, I. A., Diaz, D. R., Fritz, A. K., & Lollato, R. P. (2020). Changes in the phenotype of winter wheat varieties released between 1920 and 2016 in response to in-furrow fertilizer: Biomass allocation, yield, and grain protein concentration. *Frontiers in Plant Science*, *10*, 1786. https://doi.org/10.3389/fpls.2019.01786

Mao, H., Li, S., Chen, B., Jian, C., Mei, F., Zhang, Y., Li, F., Chen, N., Li, T., Du, L., Ding, L., Wang, Z., Cheng, X., Wang, X., & Kang, Z. (2022). Variation in cis-regulation of a NAC transcription factor contributes to drought tolerance in wheat. *Molecular Plant*, *15*(2), 276–292. https://doi.org/10.1016/j.molp.2021.11.007

McElroy, D., Zhang, W., Cao, J., & Wu, R. (1990). Isolation of an efficient actin promoter for use in rice transformation. *The Plant Cell*, *2*(2), 163–171.

Milner, M. J., Howells, R. M., Craze, M., Bowden, S., Graham, N., & Wallington, E. J. (2018). A PSTOL-like gene, *TaPSTOL*, controls a number of agronomically important traits in wheat. *BMC Plant Biology*, *18*(1), 115. https://doi.org/10.1186/s12870-018-1331-4

Murozuka, E., Massange-Sanchez, J. A., Nielsen, K., Gregersen, P. L., & Braumann, I. (2018). Genome wide characterization of barley NAC transcription factors enables the identification of grain-specific transcription factors exclusive for the Poaceae family of monocotyledonous plants. *PLoS ONE*, *13*(12), e0209769. https://doi.org/10.1371/journal.pone.0209769

Nakashima, K., Takasaki, H., Mizoi, J., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2012). NAC transcription factors in plant abiotic stress responses. *Biochimica et Biophysica Acta-Gene Regulatory Mechanisms*, *1819*(2), 97–103. https://doi.org/10.1016/j.bbagrm.2011.10.005

O'Malley, R. C., Huang, S.-s. C., Song, L., Lewsey, M. G., Bartlett, A., Nery, J. R., Galli, M., Gallavotti, A., & Ecker, J. R. (2016). Cistrome and epicistrome features shape the regulatory DNA landscape. *Cell*, *165*(5), 1280–1292. https://doi.org/10.1016/j.cell.2016.04.038

Pearce, S., Tabbita, F., Cantu, D., Buffalo, V., Avni, R., Vazquez-Gross, H., Zhao, R. R., Conley, C. J., Distelfeld, A., & Dubcovksy, J. (2014). Regulation of Zn and Fe transporters by the *GPC1* gene during early wheat monocarpic senescence. *BMC Plant Biology*, *14*, 368. https://doi.org/10.1186/s12870-014-0368-2

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), e45. https://doi.org/10.1093/nar/29.9.e45

Ramírez-González, R. H., Borrill, P., Lang, D., Harrington, S. A., Brinton, J., Venturini, L., Davey, M., Jacobs, J., van Ex, F., Pasha, A., Khedikar, Y., Robinson, S. J., Cory, A. T., Florio, T., Concia, L., Juery, C., Schoonbeek, H., Steuernagel, B., Xiang, D., … Uauy, C. (2018). The transcriptional landscape of polyploid wheat. *Science*, *361*(6403), eaar6089. https://doi.org/10.1126/science.aar6089

Ramirez-Gonzalez, R. H., Segovia, V., Bird, N., Fenwick, P., Holdgate, S., Berry, S., Jack, P., Caccamo, M., & Uauy, C. (2015b). RNA-Seq bulked segregant analysis enables the identification of high-resolution genetic markers for breeding in hexaploid wheat. *Plant Biotechnology Journal*, *13*(5), 613–624. https://doi.org/10.1111/pbi.12281

Ramirez-Gonzalez, R. H., Uauy, C., & Caccamo, M. (2015a). PolyMarker: A fast polyploid primer design pipeline. *Bioinformatics*, *31*(12), 2038–2039. https://doi.org/10.1093/bioinformatics/btv069

Ricachenevsky, F. K., Menguer, P. K., & Sperotto, R. A. (2013). kNACking on heaven's door: How important are NAC transcription factors for leaf senescence and Fe/Zn remobilization to seeds? *Frontiers in Plant Science*, *4*, 226. https://doi.org/10.3389/fpls.2013.00226

Risacher, T., Craze, M., Bowden, S., Paul, W., Barsby, T. (2009). Highly Efficient Agrobacterium-Mediated Transformation of Wheat Via In Planta Inoculation. In: Jones, H., Shewry, P. (eds) *Transgenic Wheat, Barley and Oats. Methods in Molecular Biology™*, vol 478 (pp.

115–124). Humana Press. https://doi.org/10.1007/978-1-59745-379-0_7

Roberts, I. N., Caputo, C., Criado, M. V., & Funk, C. (2012). Senescence-associated proteases in plants. *Physiologia Plantarum*, *145*(1), 130–139. https://doi.org/10.1111/j.1399-3054.2012.01574.x

Sakai, H., Lee, S. S., Tanaka, T., Numa, H., Kim, J., Kawahara, Y., Wakimoto, H., Yang, C.-C., Iwamoto, M., Abe, T., Yamada, Y., Muto, A., Inokuchi, H., Ikemura, T., Matsumoto, T., Sasaki, T., & Itoh, T. (2013). Rice annotation project database (RAP-DB): An integrative and interactive database for rice genomics. *Plant & Cell Physiology*, *54*(2), e6. https://doi.org/10.1093/pcp/pcs183

Sales, C. R. G., Molero, G., Evans, J. R., Taylor, S. H., Joynson, R., Furbank, R. T., Hall, A., & Carmo-Silva, E. (2022). Phenotypic variation in photosynthetic traits in wheat grown under field versus glasshouse conditions. *Journal of Experimental Botany*, *73*(10), 3221–3237. https://doi.org/10.1093/jxb/erac096

Schippers, J. H. M. (2015). Transcriptional networks in leaf senescence. *Current Opinion in Plant Biology*, *27*, 77–83. https://doi.org/10.1016/j.pbi.2015.06.018

Sharma, G., Upadyay, A. K., Biradar, H., Sonia, & Hittalmani, S. (2019). OsNAC-like transcription factor involved in regulating seed-storage protein content at different stages of grain filling in rice under aerobic conditions. *Journal of Genetics*, *98*(1), Article 18. https://doi.org/10.1007/s12041-019-1066-5

Simmonds, N. W. (1995). The relation between yield and protein in cereal grain. *Journal of the Science of Food and Agriculture*, *67*(3), 309–315. https://doi.org/10.1002/jsfa.2740670306

Song, S. Y., Chen, Y., Chen, J., Dai, X. Y., & Zhang, W. H. (2011). Physiological mechanisms underlying *OsNAC5*-dependent tolerance of rice plants to abiotic stress. *Planta*, *234*(2), 331–345. https://doi.org/10.1007/s00425-011-1403-2

Sperotto, R., Ricachenevsky, F., Duarte, G., Boff, T., Lopes, K., Sperb, E., Grusak, M., & Fett, J. (2009). Identification of up-regulated genes in flag leaves during rice grain filling and characterization of *OsNAC5*, a new ABA-dependent transcription factor. *An International Journal of Plant Biology*, *230*(5), 985–1002. https://doi.org/10.1007/s00425-009-1000-9

Takasaki, H., Maruyama, K., Kidokoro, S., Ito, Y., Fujita, Y., Shinozaki, K., Yamaguchi-Shinozaki, K., & Nakashima, K. (2010). The abiotic stress-responsive NAC-type transcription factor *OsNAC5* regulates stress-inducible genes and stress tolerance in rice. *Molecular Genetics and Genomics*, *284*(3), 173–183. https://doi.org/10.1007/s00438-010-0557-0

Tenea, G. N., Peres Bota, A., Cordeiro Raposo, F., & Maquet, A. (2011). Reference genes for gene expression studies in wheat flag leaves grown under different farming conditions. *BMC Research Notes*, *4*, 373. https://doi.org/10.1186/1756-0500-4-373

Thomas, H., & Ougham, H. (2014). The stay-green trait. *Journal of Experimental Botany*, *65*(14), 3889–3900. https://doi.org/10.1093/jxb/eru037

Uauy, C., Brevis, J. C., & Dubcovsky, J. (2006). The high grain protein content gene *Gpc-B1* accelerates senescence and has pleiotropic effects on protein content in wheat. *Journal of Experimental Botany*, *57*(11), 2785–2794. https://doi.org/10.1093/jxb/erl047

Uauy, C., Distelfeld, A., Fahima, T., Blechl, A., & Dubcovsky, J. (2006). A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science*, *314*(5803), 1298–1301. https://doi.org/10.1126/science.1133649

Uddling, J., Gelang-Alfredsson, J., Piikki, K., & Pleijel, H. (2007). Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. *Photosynthesis Research*, *91*, 37–46. https://doi.org/10.1007/s11120-006-9077-5

Vranic, M., Perochon, A., Benbow, H., & Doohan, F. M. (2022). Comprehensive analysis of pathogen-responsive wheat NAC transcription factors: New candidates for crop improvement. *G3: Genes, Genomes, Genetics*, *12*(11), jkac247. https://doi.org/10.1093/g3journal/jkac247

Wairich, A., Vitali, A., Adamski, J. M., Lopes, K. L., Duarte, G. L., Ponte, L. R., Costa, H. K., Menguer, P. K., dos Santos, R. P., Fett, J. P., Sperotto, R. A., & Ricachenevsky, F. K. (2023). Enhanced expression of *OsNAC5* leads to up-regulation of *OsNAC6* and changes rice (*Oryza sativa* L.) ionome. *Genetics and Molecular Biology*, *46*(1), e20220190. https://doi.org/10.1590/1678-4685-gmb-2022-0190

Welner, D. H., Lindemose, S., Grossmann, J. G., Mollegaard, N. E., Olsen, A. N., Helgstrand, C., Skriver, K., & Lo Leggio, L. (2012). DNA binding by the plant-specific NAC transcription factors in crystal and solution: A firm link to WRKY and GCM transcription factors. *Biochemical Journal*, *444*, 395–404. https://doi.org/10.1042/Bj20111742

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer-Verlag. https://doi.org/10.1007/978-3-319-24277-4

Yang, X. L., Lu, Y. L., Ding, Y., Yin, X. F., Raza, S., & Tong, Y. A. (2017). Optimising nitrogen fertilisation: A key to improving nitrogen-use efficiency and minimising nitrate leaching losses in an intensive wheat/maize rotation (2008-2014). *Field Crops Research*, *206*, 1–10. https://doi.org/10.1016/j.fcr.2017.02.016

Yates, A. D., Allen, J., Amode, R. M., Azov, A. G., Barba, M., Becerra, A., Bhai, J., Campbell, L. I., Carbajo Martinez, M., Chakiachvili, M., Chougule, K., Christensen, M., Contreras-Moreira, B., Cuzick, A., Da Rin Fioretto, L., Davis, P., Silva, D., Nishadi, H., Diamantakis, S., … Flicek, P. (2022). Ensembl genomes 2022: An expanding genome resource for non-vertebrates. *Nucleic Acids Research*, *50*(D1), D996–D1003. https://doi.org/10.1093/nar/gkab1007

Zhang, Y., Li, Z., Liu, J., Zhang, Y., Ye, L., Peng, Y., Wang, H., Diao, H., Ma, Y., Wang, M., Xie, Y., Tang, T., Zhuang, Y., Teng, W., Tong, Y., Zhang, W., Lang, Z., Xue, Y., & Zhang, Y. (2022). Transposable elements orchestrate subgenome-convergent and -divergent transcription in common wheat. *Nature Communications*, *13*(1), 6940. https://doi.org/10.1038/s41467-022-34290-w

Zhang, Y. Y., Li, Z. J., Zhang, Y. E., Lin, K. D., Peng, Y., Ye, L. H., Zhuang, Y. L., Wang, M. Y., Xie, Y. L., Guo, J. Y., Teng, W., Tong, Y. P., Zhang, W. L., Xue, Y. B. A., Lang, Z. B., & Zhang, Y. J. (2021). Evolutionary rewiring of the wheat transcriptional regulatory network by lineage-specific transposable elements. *Genome Research*, *31*(12), 2276–2289. https://doi.org/10.1101/gr.275658.121

Zhao, D., Derkx, A. P., Liu, D. C., Buchner, P., & Hawkesford, M. J. (2015). Overexpression of a NAC transcription factor delays leaf senescence and increases grain nitrogen concentration in wheat. *Plant Biology*, *17*(4), 904–913. https://doi.org/10.1111/plb.12296

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Evans, C., Mogg, S. L., Soraru, C., Wallington, E., Coates, J., & Borrill, P. (2024). Wheat NAC transcription factor *NAC5-1* is a positive regulator of senescence. *Plant Direct*, *8*(7), e620. https://doi.org/10.1002/pld3.620