RESEARCH PAPER

Delaying or delivering: identification of novel NAM-1 alleles that delay senescence to extend wheat grain fill duration

Elizabeth A. Chapman1,*†, Simon Orford1, Jacob Lage2 and Simon Griffiths1,*

1 John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, UK
2 KWS-UK, 56 Church Street, Thriplow, Hertfordshire SG8 7RE, UK

† Present address: Carlsberg Research Laboratory, J C Jacobsens Gade 4, DK-1799 Copenhagen V, Denmark.
* Correspondence: elizabeth.chapman@jic.ac.uk or simon.griffiths@jic.ac.uk

Received 13 February 2021; Editorial decision 28 July 2021; Accepted 6 August 2021

Editor: Greg Rebetzke, CSIRO Agriculture and Food, Australia

Abstract

Senescence is a complex trait under genetic and environmental control, in which resources are remobilized from vegetative tissue into grain. Delayed senescence, or ‘staygreen’ traits, can confer stress tolerance, with extended photosynthetic activity hypothetically sustaining grain filling. The genetics of senescence regulation are largely unknown, with senescence variation often correlated with phenological traits. Here, we confirm staygreen phenotypes of two Triticum aestivum cv. Paragon ethyl methane sulfonate mutants previously identified during a forward genetic screen and selected for their agronomic performance, similar phenology, and differential senescence phenotypes. Grain filling experiments confirmed a positive relationship between onset of senescence and grain fill duration, reporting an associated ~14% increase in final dry grain weight for one mutant (P<0.05). Recombinant inbred line (RIL) populations segregating for the timing of senescence were developed for trait mapping purposes and phenotyped over multiple years under field conditions. Quantification and comparison of senescence metrics aided RIL selection, facilitating exome capture-enabled bulk segregant analysis (BSA). Using BSA we mapped our two staygreen traits to two independent, dominant, loci of 4.8 and 16.7 Mb in size encompassing 56 and 142 genes, respectively. Combining association analysis with variant effect prediction, we identified single nucleotide polymorphisms encoding self-validating mutations located in NAM-1 homoeologues, which we propose as gene candidates.

Keywords: Bulk segregant analysis, exome capture, forward genetics, grain fill, mutant, NAM, remobilization, senescence, staygreen, wheat.

Introduction

Monocarpic senescence is the terminal stage in wheat development, wherein 80% of leaf nitrogen is remobilized into developing grain (Buchanan-Wollaston, 2007). Genetic regulation of senescence involves significant transcriptional reprogramming enabling timely reallocation of resources. Plants with delayed senescence are described as ‘staygreen’,
with cosmetic types resulting from impaired chlorophyll catabolism (Thomas and Ougham, 2014). Functional staygreen phenotypes are associated with enhanced or extended photosynthetic activity, conferring tolerance to heat, drought, and low nitrogen stress in multiple crops (Gregersen et al., 2013; Thomas and Ougham, 2014).

Correlations between green canopy and grain fill duration of r = 0.16–0.7 (P<0.01) (Pinto et al., 2016; de Souza Luche et al., 2017) support the potential breeding utility of staygreen traits, whereupon an extended grain filling period could increase grain size and enhance final grain yield. During grain fill, studies by Adu et al. (2011), Kitonoya et al. (2017), and Voss-Fels et al. (2019) suggesting staygreen traits have been selected over the past 50 years to sustain grain number improvement. For a Triticum durum cv. Trinkaria ethyl methane sulfonate (EMS) mutant, a 10 d delay in onset of senescence contributed to a 10–12% and 20% increase in thousand grain weight (TGW) and yield, respectively (Spano et al., 2003). Under terminal heat stress, Kumari et al. (2004) reported phenology-independent senescence QTLs located on chromosomes 2DL, 2B, and 4A, with Naruoka (2016) reported phenology-independent senescence QTLs located on chromosomes 6A and 6D. Using bulk segregant analysis (BSA) to map novel staygreen alleles underpinning delayed senescence phenotypes of two independent Triticum aestivum cv. Paragon EMS mutants. We confirm results of the initial forward screen, and the relationship between onset of senescence and grain fill duration. Following repeated in-field phenotypic assessment of segregating recombinant inbred lines (RILs), we reduced senescence from a quantitative to qualitative trait enabling construction of staygreen and non-staygreen bulks. We used mapping-by-sequencing to identify two independent loci located on chromosomes 6A and 6D. Using variant effect prediction and single marker association analysis we refined these identified regions to likely gene candidates. Here, results converged upon self-validating, dominant mutations in homoeologous copies of known senescence regulator NAM-B1 (Uauy et al., 2006b), with marker development enabling selection of identified novel diversity.

Materials and methods

Plant material

Mutagenesis and initial screen

Seven thousand seeds of Triticum aestivum cv. Paragon (spring wheat) were treated with a 1% EMS solution for 16 h to obtain a 50% lethal dose for viability, in accordance with Rakszegi et al. (2010). Three thousand five hundred M₁ seeds were sown to obtain M₂ plants, with the surviving 3461 bagged to ensure self-fertilization. Two M₂ seeds were sown per line, deriving ‘a’ and ‘b’ sibling lines (n=6922) and advanced via multiple rounds of self-fertilization to the M₃ generation. In spring 2006, ~6500 M₃ lines were grown as single ear rows in 1 m² plots at Church Farm, Norwich (52°38′N 1°10′E) (John Innes Centre, JIC). A single visual assessment of the 6500 M₃ lines identified 18 early senescing and 43 staygreen mutants. Original data are available from: www.wgin.org.uk/wgin_2003-2008/index.php?page=Resources&op=results. In 2007, forward genetic screening of the Triticum aestivum cv. Paragon EMS population was repeated under low nitrogen conditions (100 kg N ha⁻¹). Seed of the M₄₄ generation (n=6500) was sown as single rows in 1 m² plots. Phenotypic observations identified, or re-confirmed, differential senescence of ~80 lines (~1.2% in total). In 2008, 70 differentially senescing lines were included in a nitrogen use efficiency trial and grown as replicated 1 m² plots receiving 20 kg N ha⁻¹ and 240 kg N ha⁻¹ (n=3). Fifty-four lines were subject to glasshouse experimentation, with lines 555a, 862a, 1389a, 2056a, and 2514a undergoing in-depth physiological characterization by Derkx et al. (2012). This study concerns genetic mapping of 1189a and 2316b, chosen for their environmentally stable, differential, staygreen phenotypes unconfounded by heading variation, and strong agronomic performance.

RIL population development

RIL populations segregating for senescence traits were developed for trait mapping purposes. 1189a and 2316b were crossed to parental Triticum aestivum cv. Paragon, and F₁ populations developed through single seed descent (SSD): Paragon × 1189a (n=85), Paragon × 2316b (n=95). Additional RIL populations were developed segregating for senescence and heading date. 1189a and 2316b were crossed to a triple photoperiod insensitive (Ppd-1a) Triticum aestivum cv. Paragon near isogenic line (NIL), and F₃ populations developed through SSD: Ppd × 1189a...
Phenotypic characterization

Field experimentation

In-field phenotyping of F1 Paragon × staygreen RIL populations was conducted between 2016 and 2018. Experiments were performed at Church Farm, Norwich (52°38′N 1°10′E), JIC. In 2016, 36 RILs per Paragon × staygreen population were sown as unreplicated 1 m² spaced plots on 26 October 2015, alongside 36 Ppd × staygreen RILs. The selection of Ppd × staygreen RILs sown was informed by in-field phenotyping conducted in 2015, with nine RILs representing each ‘early’ or ‘late’ heading × staygreen combination selected per population. Ppd × staygreen RILs were also sown as 7 m² yield plots in Meldreth, Cambridgeshire (52°05′N 0°00′W), KWS-UK, in mid-October 2015. Plots consisted of 10 rows drilled at a rate of 275 seeds m⁻², and followed a latin-square design with cultivars Paragon, Soissons and KWS Santiago used as repeated checks.

In 2017 and 2018 experiments concerning Paragon × staygreen RILs consisted of replicated 6 m² plots and incorporated additional RILs per population (2017, n=43, three replicates; 2018, n=75, two replicates) and were sown on 26 October 2016 and 12 October 2017 (see Supplementary Fig. S1A). All experiments followed a randomized complete block design, with control plots (cv. Paragon, Soissons, 1189a, and 2316b mutant lines) randomly sown throughout. Seed used for field experiments was produced during multiplication of RILs in 2015 when the most recent source, but otherwise resulted from the preceding year.

The soil at Church Farm is described as sandy loam overlying alluvial clay, whereas the soil at the Cambridgeshire site is sandy clay loam. All experiments were rainfed but required supplemental irrigation in 2017. At Church Farm, seeds were dressed with Redigo Dieter (Bayer CropScience, Germany) and sown at a rate of 2750 seeds per 6 m² (~300 plants m⁻²), with fertilizer applied over three occasions from late February to the end of April, totalling 214–228.5 kg N ha⁻¹ and 62 kg SO₃ ha⁻¹. In Cambridgeshire nitrogen was applied when plants reached GS30 and GS32 and totalled 204 kg N ha⁻¹. For both sites, all plots received standard fungicide and herbicide treatment. Mean daily temperature and rainfall data are provided in Supplementary Fig. S2 and were obtained from weather stations at Church Farm (52°38′N 1°10′E, Norwich) and Royston (52°2′N, 0°1′W, Cambridgeshire; http://dajda.net/).

Phenotypic assessment

Phenotyping was conducted at the plot level. Ear emergence (GS55) was scored when 50% of ears had emerged halfway from the flag leaf (Zadoks, et al., 1974). Senescence was scored visually every 2–4 d after anthesis (daa) using a 0–100 scale (intervals of 5) based on Pask et al. (2012). Flag leaves were scored according to the proportion of leaf yellowing. A score of 5 represents leaf tip necrosis, whilst 100 indicates complete chlorosis or death (Pask et al., 2012). Multiple flag leaves were assessed simultaneously to give a plot score. Peduncle senescence was scored in 2015 and 2018 and assessed as the percentage of yellow peduncles (top 3–5 cm) per plot based on three to four batches of 10 tillers.

To interpret senescence dynamics, senescence scores were plotted against thermal time in day °C from ear emergence (T₀) to standardize for heading variation. For thermal time calculation, mean daily temperatures were calculated using minimum and maximum daily temperatures recorded by Church Farm weather station (location: 52°37′52.29″N, 1°10′23.57″E; Supplementary Fig. S2). Using time course senescence data corresponding to RILs and controls, senescence profiles were quantified and RILs classified into senescence types by deriving senescence metrics. Senescence metrics include mean senescence, onset, duration (from ear emergence or onset to terminal senescence) and thermal time to different leaf senescence scores. Onset and termination of senescence were considered the first time points for which senescence scores above 10 or 90, respectively, were first recorded. Calculation of time taken (in day °C) to reach specific senescence scores (TT30, TT40 …) is similar to MsDS (50% senescence) (Christopher et al., 2016), with senescence assumed to progress linearly between scoring points and time interpolated.

Grain filling experiments

To explore the relationship between senescence and grain filling traits, in 2017 and 2018 grain weight and moisture content were recorded for 1189a, 2316b, and cv. Paragon from anthesis to maturation. To standardize for developmental differences between tillers, ~50 ears per plot per genotype (2017, n=1; 2018, n=2) were tagged when 1–2 cm of peduncle became exposed. At 4–5 d intervals, starting from anthesis, five tagged ears per plot were sampled and sealed into a labelled ziplock bag to prevent moisture loss. Ears were then refrigerated whilst awaiting dissection (maximum 10 h post-sampling) and senescence of sampled plots scored. Ten grains per ear were dissected from the central region of the spike from floret positions 1 and 2 (the oldest grains; numbered according to Brinton and Uauy (2019)), placed into a single Eppendorf tube and weighed to determine fresh grain weight. With lids open, tubes were transferred to a 65 °C drying oven for grains to dry down, with oven doorajar to prevent condensation. After 48 h tubes were re-weighed to determine final dry grain weight and grain moisture content (%) calculated by dividing dry by fresh grain weight.

Exome capture and sequencing

DNA extraction

Seeds of Paragon × 1189a and Paragon × 2316b F₁ RIL populations, cv. Paragon, 1189a, and 2316b mutant parents were sown into individual cells of 96-well seed trays containing peat and sand and transferred to the glasshouse following 2–3 d of cold treatment. When plants reached the three-leaf stage, 5 cm of leaf tissue was harvested and collected into a 96-well collection tray (Qagen, Germany, 19560) and material stored at −80 °C until required. DNA was extracted using a Qiacube (Qagen) according to the QIAamp 96 DNA Qiacube HT Kit Protocol. DNA quality and quantity were analysed using a DS-11 spectrophotometer (Denoix, DE, USA), Qubit-4 fluorometer (dDNA BR assay, Thermo Fisher Scientific Q32850) and by running a DNA sample on an agarose gel (1%) to detect high molecular weight DNA.

Exome capture

Following classification of F₁ RILs into ‘staygreen’ and ‘non-staygreen’ types, DNA of selected individuals was pooled to form staygreen and non-staygreen bulks (see Fig. 3) and submitted for exome capture and sequencing. RILs for inclusion in bulks were selected based on within- and between-year concordance of senescence metrics, typically informed by a minimum of 2 years of phenotypic data. Details concerning bulk selection can be found in Supplementary Dataset S1. Provided quality control requirements specified by the sequencing provider were met, DNA of selected RILs was pooled, standardizing for DNA concentration to ensure equal RIL representation (1189a, n=17 for both bulks; 2316b, n=15 for staygreen, n=12 for non-staygreen) (see Supplementary Fig. S1B).
Quality and quantity of bulked DNA was checked by running a DNA sample on an agarose gel (1%) and final DNA concentration determined using the Quibit-4 fluorometer (d/dNA BR assay).

Exome capture was used to sequence predicted gene-encoding regions (Krasileva et al., 2017) of the four bulks and three parents (1189a, 2316b, and cv. Paragon). Library preparation, amplification, and sequencing were performed by Novogene (Hong Kong) using the SeqCapEZ probe set 1407430_Wheat_TGAC_D14_REZ_HX1 for Triticum aestivum (Roche, Nimblegen, WI, USA), described by Krasileva et al. (2017). Libraries were sequenced using the HiSeq4000 platform (Illumina, CA, USA) producing 150 bp paired end reads. Sequencing read quality was analysed using FastQC (Andrews, 2010) with low quality sequences and adapter remnants removed using 'sickle' (version 1.2; paired end (pe) mode, default parameters (-q 20 -i 15) (Joshi and Fass, 2011).

Sequence alignment
Processed reads were aligned to Triticum aestivum cv. Chinese Spring RefSeq v1.0 (IWGSC et al., 2018) using ‘BWA’ (version 0.7.17; command aln, default parameters (-n 4); sampe, default parameters (-n 10 -N 0)) (Li and Durbin, 2009). Aligned read pairs were retained and files converted from binary alignment/map (BAM) to sequence alignment/map (SAM) format and ordered, duplicates removed and read pairs indexed using ‘samtools’ (version 1.7; command view -f2, -S -h -u -b -o; sort -n), remapping 150 bp paired end reads. Sequencing read quality was analysed using 'sickle' (version 1.2; paired end (pe) mode, default parameters (-q 20 -i 15) (Joshi and Fass, 2011).

Bulk segregant analysis
To conduct BSA, multi-sample vcf files corresponding to each staygreen phenotypic classification were imported into RStudio (R Core team, 2015), R version 3.5.2 (R Core Team, 2018), using the package ‘veCR’ (Knaus and Grünwald, 2017). To identify variants enriched in staygreen versus non-staygreen bulks, single nucleotide polymorphism (SNP) indices were calculated for each position by dividing the number of reads with the alternate allele by the total read depth. Variants common to phenotypically contrasting bulks for which the SNP index >1 indicated variatal differences, occurring between cv. Paragon and Chinese Spring, and were removed. Remaining variants were filtered based on SNP index (staygreen >0.9, non-staygreen <0.1) and sequencing depth (DP>5). Mutations characteristic of EMS mutagenesis (G:A and C:T transitions) were prioritized for visualization and investigation in iGV (Integrated Genomics Viewer) (Robinson et al., 2011).

Genotyping and genetic mapping
For SNPs of interest, homoeologue-specific competitive allele specific PCR (KASP) markers were designed manually and RIL populations genotyped. When designing primers, SNPs of interest plus 150 bases of upstream and downstream flanking sequence were extracted from the Triticum aestivum cv. Chinese Spring RefSeq v1.0 (IWGSC et al., 2018) using a script supplied by Sophie Harrington. Extracted sequences were subjected to BLAST searches against RefSeq v1.0 (IWGSC et al., 2018) and Paragon assembly (Bian et al., 2017) to detect homoeologous and varietal variants. Primer assay mixes contained 46 μl dH2O, 30 μl common primer (100 μM) and 12 μl of each tailed primer (100 μM). Genotyping assays were performed in 384-well format with a 2.5 μl KASP reaction volume consisting 14–18 ng DNA, 1.25 μl PACE 2X Mastermix (3crbio, UK), 1.25 μl dH2O and 0.047 μl primer assay mix. KASP assays were performed using a hydrocycler or thermocycler, with cycling conditions as follows: 15 min at 94 °C, 10 cycles of 20 s at 94 °C, 60 s at 65–57 °C (decreasing by 0.8 °C per cycle), followed by 26–40 cycles of 20 s at 94 °C, 60 s at 57 °C. PCR cycling conditions required optimization for some markers. KASP markers developed are listed in Supplementary Table S1 (1189a) and Supplementary Table S2 (2316b). Fluorescence was measured using a Pherestar plate reader (BMG Labtech, Germany) and data analysed using KlusterCaller software (version 4.1; LGC Genomics, UK).

Genetic maps for RIL populations were constructed from KASP genotyping data using the ‘Kosambi’ mapping function, specifying a LOD score of 6 in MapDisto v2.0 (Hettfellinger et al., 2017). Loci were initially ordered using ‘Automap’ and ‘Find group’ functions and loci with missing data removed. Loci were reordered using ‘ripple order’ and selected for specific time points: and sequencing depth (DP>3). Mutations characteristic of EMS mutagenesis (G:A and C:T transitions) were prioritized for visualization and investigation in iGV (Integrated Genomics Viewer) (Robinson et al., 2011). Variants enrichment across the genome was visualized by plotting ΔSNP index (SNP indexG − SNP indexNon-st) using ggplot2 (Wickham et al., 2018a).

Data analysis
Data analysis was performed using R (version 3.5.2) (R Core Team, 2018) in RStudio (R Studio team, 2015) and data manipulated using the packages ‘data.table’ (Dowle et al., 2018) in RStudio (RStudio team, 2015) and data manipulated using the packages ‘data.table’ (Dowle et al., 2018) and ‘dplyr’ (Wickham et al., 2018b), ‘plyr’ (Wickham, 2015) and ‘tidyverse’ (Wickham et al., 2019). Senescence metrics were derived from raw senescence phenotyping data in the absence of spatial correction and means calculated per line when replicated. Analysis of senescence and grain filling profiles of individual lines or groups was conducted using linear mixed modelling using the packages ‘lme4’ (Bates et al., 2019) and ‘lmerTest’ (Kuznetsova et al., 2017). Data for each year/site were analysed separately. When conducting time course senescence or grain filling analysis two models were used, one to determine the overall difference, the other for specific time points:

\[
\text{TimeCourse} \sim \gamma \text{Lmer(Phenotype} \sim \text{Date)} \\
+ \text{Genotype} \sim (1|\text{Plot : Rep})
\]

\[
\text{TimePoint} \sim \gamma \text{Lmer(Phenotype} \sim \text{Date/Genotype} \\
+ (1|\text{Plot : Rep})
\]

When analysing differences in senescence metrics once genotyped, a complete linear mixed model was initially applied, detailed below:

\[
\text{SenescenceModelling} \sim \gamma \text{Lmer(SenMetric} \sim \text{Rep + Row} \\
+ \text{Column + Variety/Population/ (NAM.A1 + NAM.D1)} * \text{GSS5} + (1|\text{Population : Genotype})
\]

In all cases, fixed-effect terms for retention were identified through ANOVA, with non-significant terms (P>0.05) dropped in an iterative manner.
normal distribution. Tukey post hoc tests were performed using the package ‘lsmeans’ (Lenth, 2018) to determine the significance of differences between genotypic groups, or individual lines, both overall and at specific time points. Graphs were constructed using ‘ggplot2’ (Wickham et al., 2018a).

**Results**

**Identification of staygreen mutants**

Six thousand five hundred M₅:₆ generation *Triticum aestivum* cv. Paragon EMS mutant lines were phenotyped under field conditions at JIC between 2006 and 2007. Approximately 80 lines (~1.2%) displayed differential senescence phenotypes, with staygreen phenotypes of 1189a and 2316b unconfounded by heading-date variation (Fig. 1A). To confirm these results detailed phenotyping of 1189a and 2316b was conducted between 2016 and 2018, identifying staygreen phenotypes as characterized by delays in the onset of senescence. Time course leaf and peduncle senescence profiles of 1189a and 2316b differed significantly when compared with cv. Paragon (*P*<0.05) (Fig. 1B; Supplementary Fig. S3). Compared to cv. Paragon leaf senescence of 1189a and 2316b is initiated 6–10 and 3–4 d later, respectively, whilst senescence rate is unaffected (Table 1), with a similar pattern observed for peduncle senescence (Fig. 1B; Supplementary Fig. S3).

**Extended grain fill duration of staygreens**

Maintenance of green leaves has been associated with extending grain fill duration and grain weight enhancement (Wiegand and Cuellar, 1981; Gelang et al., 2000; Bogard et al., 2011). To test this, we recorded grain weight and moisture content to determine grain fill duration of 1189a, 2316b, and cv. Paragon in 2017 and 2018.

Grain moisture content declined more slowly for 1189a and 2316b when compared with cv. Paragon (*P*<0.0001) (Fig. 2A, C; Supplementary Fig. S4). Forty-two days after anthesis (daa) grain moisture content of our staygreens was 17–20% greater compared to cv. Paragon, *P*≤0.007 (Fig. 2A, C; Supplementary Fig. S4; Supplementary Table S3). Subsequently, grain moisture content of 1189a remained elevated (*P*<0.001), suggesting an extended time to reach grain maturity (Fig. 2A; Supplementary Fig. S4). Between 48 and 52 daa, differences in grain moisture content between 2316b and cv. Paragon were not significant, with time to grain maturation similar (*P*>0.2) (Fig. 2C; Supplementary Fig. S4). Relative to cv. Paragon, differences in grain moisture for 1189a and 2316b were significant at four and three time points, respectively (Supplementary Table S3), reflecting the differences in onset of senescence (Table 1; Supplementary Figs S4, S5). Extended duration of grain filling observed for our staygreens did not consistently increase final dry grain weight. Dry grain weight accumulation for 2316b matched cv. Paragon in both years (*P*>0.1) (Fig. 2D; Supplementary Fig. S4), but was greater for 1189a in 2018 (*P*<0.001) (Fig. 2B). In 2017, between 37 and 47 daa dry grain weights for 1189a and 2316b were greater relative to cv. Paragon (*P*<0.05) (Supplementary Fig. S4), but these differences did not contribute to a significant increase in final grain weight as recorded on 23 July (*P*>0.05) (Supplementary Table S3). Conversely, for 1189a the significant differences in dry grain weight recorded between 42 and 48 daa (*P*<0.01) did translate to an 11–14.4% increase in final dry grain weight (*P*<0.001) in 2018 (Fig. 2B; Supplementary Table S3).

**Identifying senescence extremes**

To genetically map our staygreen traits we adopted a complexity reduction BSA approach. Repeated in-field phenotyping of Paragon × 1189a and Paragon × 2316b RILs enabled their accurate classification into senescence types. For each experiment senescence profiles of individual RILs were quantified by deriving senescence metrics. RILs were considered ‘non-staygreen’ or ‘staygreen’ based on whether their
mean phenotypic scores were lower when compared with cv. Paragon, or respective staygreen parent. RILs for which senescence metrics fell between parental values were classified manually based on concordance of metrics able to be readily classified, alongside parental senescence curve comparison. To assess phenotypic stability of RILs inter-year comparisons were performed, with bulk selections guided by a minimum of one, but typically two, years of data for 1189a and 2316b, respectively. Therefore, despite some of the additional RILs grown in 2018 displaying senescence phenotypes towards the extreme ends, these were largely unselected due to multi-year data providing greater confidence in our selections. Details of RIL classification and bulk selections are supplied in Supplementary Dataset S1.

Senescence progression was highly dynamic between years, and when classifying and selecting RILs the discriminatory power of senescence metrics varied, with reference to multiple metrics preferred. In 2017, mean peduncle senescence scores were most discriminative, opposed to duration of leaf senescence in 2018 (Fig. 3). The metric TT70 consistently discriminated senescence variation, with large differences always visible between parental lines and segregating RILs (Fig. 5A, B). Single marker association analysis facilitated genetic map construction utilizing all available markers (Table 2, 3), Additional positions for which a ΔSNP index=1 include variants located on chromosome 4B for 1189a, and 2A, 2D, and 7D for 2316b, but none were predicted to be deleterious, and in the absence of enriched flanking SNPs (ΔSNP index>0.9) these regions were not considered to be of interest (Tables 2, 3).

1189a and 2316b staygreen traits are underpinned by mutations in NAM-1

To determine if SNPs identified as enriched by BSA are causative, variant effect prediction was conducted. For 1189a, 13 SNPs located on chromosome 6A are predicted to encode missense mutations, with nine deleterious to protein function (SIFT≤0.001) (Table 2). For 2316b, five SNPs located on chromosome 6D are predicted to encode missense mutations, three of which are deleterious (SIFT≤0.02) (Table 3). These deleterious SNPs were prioritized as gene candidates according to ΔSNP indices. Results for 1189a and 2316b converge upon NAM-A1 (Table 2) and NAM-D1 (Table 3) (ΔSNP index=1), respectively (Tables 2, 3). Additional positions for which a ΔSNP index=1 include variants located on chromosome 4B for 1189a, and 2A, 2D, and 7D for 2316b, but none were predicted to be deleterious, and in the absence of enriched flanking SNPs (ΔSNP index>0.9) these regions were not considered to be of interest (Tables 2, 3).

1189a and 2316b staygreen traits are underpinned by mutations in NAM-1

To determine if SNPs identified as enriched by BSA are causative, variant effect prediction was conducted. For 1189a, 13 SNPs located on chromosome 6A are predicted to encode missense mutations, with nine deleterious to protein function (SIFT≤0.001) (Table 2). For 2316b, five SNPs located on chromosome 6D are predicted to encode missense mutations, three of which are deleterious (SIFT≤0.02) (Table 3). These deleterious SNPs were prioritized as gene candidates according to ΔSNP indices. Results for 1189a and 2316b converge upon NAM-A1 (Table 2) and NAM-D1 (Table 3) (ΔSNP index=1), respectively (Tables 2, 3). Additional positions for which a ΔSNP index=1 include variants located on chromosome 4B for 1189a, and 2A, 2D, and 7D for 2316b, but none were predicted to be deleterious, and in the absence of enriched flanking SNPs (ΔSNP index>0.9) these regions were not considered to be of interest (Tables 2, 3).

1189a and 2316b staygreen traits are underpinned by mutations in NAM-1

To determine if SNPs identified as enriched by BSA are causative, variant effect prediction was conducted. For 1189a, 13 SNPs located on chromosome 6A are predicted to encode missense mutations, with nine deleterious to protein function (SIFT≤0.001) (Table 2). For 2316b, five SNPs located on chromosome 6D are predicted to encode missense mutations, three of which are deleterious (SIFT≤0.02) (Table 3). These deleterious SNPs were prioritized as gene candidates according to ΔSNP indices. Results for 1189a and 2316b converge upon NAM-A1 (Table 2) and NAM-D1 (Table 3) (ΔSNP index=1), homologues of known senescence regulator NAM-B1 (Uauy et al., 2006a,b).

To validate these results, we developed KASP markers for SNPs located on chromosomes 6A (1189a) and 6D (2316b) (Supplementary Tables S1, S2). Genotyping of F4 RIL populations facilitated genetic map construction utilizing all available RILs (Fig. 5A, B). Single marker association analysis reported increasing phenotypic associations with genetic proximity to NAM-A1 (−log(P)e3.5) of 1189a (Fig. 5C) and

---

**Table 1.** Senescence pairwise comparison (P-value, staygreen mutant versus cv. Paragon)

| Year | Ear emergence (GS55) (dd/mm ±SD) | Onset (dd/mm) | Leaf | Peduncle | Ear emergence (GS55) (dd/mm ±SD) | Onset (dd/mm) | Leaf | Peduncle |
|------|----------------------------------|---------------|------|----------|----------------------------------|---------------|------|----------|
| 2016 | 08/06±1.1 (+1)                   | 29/07 (+10)   | 0.11 | —        | 08/06±1.9 (+1)                   | 23/07 (+4)    | 0.0005 | —        |
| 2017 | 30/05±1.4 (+1)                   | 06/07 (+6)    | < 0.0001 | 0.0001 | 29/05±0.8 (=)                    | 04/07 (+4)    | < 0.0001 | 0.002   |
| 2018 | 01/06±0.9 (+1)                   | 07/06 (+7)    | < 0.0001 | 0.0001 | 31/05±1.2 (=)                    | 03/07 (+3)    | 0.08 | 0.048    |

Results of Tukey post hoc tests comparing overall leaf and peduncle time course senescence. Date of ear emergence (GS55) and onset of leaf senescence with days difference relative to cv. Paragon in parentheses. Peduncle senescence was unscored in 2016.
NAM-D1, $(-\log_{10}P \geq 2.35)$ of 2316b (Fig. 5D) (Holm corrected for multiple testing), with size of association reflecting subtlety of senescence phenotype (Fig. 1). Here, we mapped the 1189a interval to a 16.7 Mb region on chromosome 6A and the 2316b interval to a 4.8 Mb region on chromosome 6D, containing 142 and 56 high confidence genes, respectively (Supplementary Dataset S1; IWGSC et al., 2018).

1189a and 2316b represent novel sources of NAM-1 allelic variation

SNPs identified in NAM-1 homoeologues encode missense mutations (Tables 2, 3). Isoleucine replaces threonine at amino acid (AA) position 159 of NAM-A1 for 1189a (T159I). Glycine replaces glutamate at AA 151 of NAM-D1 for 2316b (G151E) (Fig. 6). NAC transcription factors operate as heterodimers and homodimers, which ensures stable–DNA binding (Olsen et al., 2005). The G151E and T159I AA substitutions are located within subdomain D of the NAC transcription factor domain at highly conserved positions known to be involved in DNA binding (Ooka et al., 2003; Ernst et al., 2004; Welner et al., 2012; Harrington et al., 2019c). Examination of the NAC domain crystal structure shows that variants mirror one another, with the T159I AA variant located one residue after the $\beta_4$ structure, and G151E AA variant located one residue before the $\beta_5$ structure of the antiparallel $\beta$-sheet secondary structure (Ernst et al., 2004). The substitution of positively charged threonine for hydrophobic isoleucine, or glycine for a large, positively charged glutamate may affect NAM protein function by altering protein dimerization, as demonstrated for alternative EMS-induced mutations in NAM-A1 by Harrington et al. (2019c) (Fig. 6).

Differential inheritance of 1189a and 2316b staygreen traits

RIL populations were developed by SSD in the glasshouse with segregation of senescence phenotypes unobserved in earlier generations. Phenotype by genotype plots constructed for Paragon × 1189a and Paragon × 2316b F4 RILs homozygous for alternative NAM-1 alleles form distinct groups ($P<0.0001$) (Fig. 7; Supplementary Fig. S8). Assessment of RILs heterozygous for the NAM-A1 mutation reveal the allele is dominantly
Fig. 3. Senescence variation amongst F4 RIL populations, highlighting RILs included in bulks. (A, B) Mean peduncle senescence scores for Paragon × 1189a (A) and Paragon × 2316b (B) F4 RILs grown in 2017; mean ± SD, n=3. (C, D) Duration of leaf senescence (from ear emergence) for Paragon × 1189a (C) and Paragon × 2316b (D) F4 RILs grown in 2018; mean ± SD, n=2. Coloured bars represent parents and RILs included in bulks: 1189a (orange), ‘staygreen’ (light orange, n=17), ‘non-staygreen’ (light purple, n=17); 2316b (dark blue), ‘staygreen’ (light blue, n=15), ‘non-staygreen’ (red, n=12); Paragon (black). Classification and selection of RILs guided by multiple senescence metrics with intra- and inter-year comparisons performed.
inherited, as RILs resemble those homozygous for the mutation \( (P>0.3) \), not the cv. Paragon allele \( (P<0.01) \) (Fig. 7).

Inheritance of the 2316b NAM-D1 mutation is more complex. In 2016 and 2018, senescence phenotypes of heterozygous Paragon \( \times \) 2316b F4 RILs were indistinct from RILs homozygous for either NAM-D1 allele (2016, \( P>0.7 \); 2018, \( P>0.28 \)). In 2017 the NAM-D1 mutation appears dominantly inherited, as senescence of RILs heterozygous and homozygous for the NAM-D1 mutation was similarly delayed compared with RILs homozygous for the cv. Paragon allele \( (P<0.01) \) (Fig. 7). Together, this suggests the NAM-D1 mutant allele is semi-dominant.

Validation of differential modes of inheritance for NAM-A1 and NAM-D1 mutations arose from phenotyping heterozygous Ppd \( \times \) staygreen F3 RILs grown in Cambridgeshire and Norwich. Senescence profiles of homozygous RILs contrasting for NAM-A1 alleles were significantly different \( (P<0.01) \), with heterozygotes resembling RILs homozygous for the mutation \( (P>0.66) \) (Supplementary Fig. S9). Penetration of the NAM-D1 mutant allele is environmentally dependent. Differences in senescence phenotypes of homozygous RILs contrasting for NAM-D1 alleles were significant in Cambridgeshire \( (P<0.05) \) but not in Norwich \( (P>0.5) \) (Supplementary Fig. S9). NAM-D1 heterozygotes more closely resemble RILs homozygous for the mutant allele \( (P<0.6) \) compared with the cv. Paragon allele \( (P>0.9) \), supporting semi-dominant inheritance of the NAM-D1 mutation (Supplementary Fig. S9).

**Discussion**

**Two novel wheat senescence mutants that extend grain fill duration**

Grain filling experiments confirmed the hypothesized positive relationship between staygreen traits and grain fill duration (Wiegand and Cuellar, 1981; Gelang et al., 2000). Grain fill extensions reported for lines 1189a and 2316b mirror observed delays in onset of senescence (Fig. 2; Supplementary Figs S4, S5). Differences in grain fill between staygreen lines and cv. Paragon occur towards the end of the rapid grain filling phase, which Neghliz et al. (2016) estimates to occur 39 daa for *Triticum aestivum* cv. Recital when grain moisture content reaches ~45%. Photosynthesis terminates halfway through this rapid phase, whereupon translocation of stored reserves and remobilization of fructose and sucrose occur (Takahashi et al.,...
Table 2. Bulk segregant analysis maps the 1189a allele to a chromosomal region on 6A encoding a mutation in NAM-A1

| Positiona | SNP   | ΔSNP index | Effectb | ΔAAc | SIFTc | Gened | Functionf |
|------------|-------|------------|---------|------|-------|-------|-----------|
| chr4B_17232065 | C:T   | 1.000      | Intronic | —    | —     | TraesCS4B02G024000 | Argonaute (T. urartu) |
| chr6A_46782541 | G:A   | 0.851      | Missense | R/C  | 0     | TraesCS6A02G076600 | Uncharacterized |
| chr6A_55741222 | G:A   | 0.888      | Intronic | —    | —     | TraesCS6A02G087400 | HBP-1a transcription factor (T. urartu) |
| chr6A_57668862 | G:A   | 0.876      | Missense | A/T  | 0     | TraesCS6A02G099900 | Snare region anchored in the vesicle membrane C-terminus |
| chr6A_67373891 | G:A   | 1.000      | Missense | A/T  | 0     | TraesCS6A02G099900 | ZIP zinc transporter |
| chr6A_76523438 | G:A   | 1.000      | Intronic | —    | —     | TraesCS6A02G107700 | UDP-N-acetylglucosamine–peptide N-acetylglucosaminyltransferase |
| chr6A_77099433 | G:A   | 1.000      | Missense | T/I  | 0     | TraesCS6A02G108300 | NAM-A1 (no apical meristem) |
| chr6A_80699949 | G:A   | 1.000      | Upstream | —    | —     | TraesCS6A02G111400 | Cycloartenol synthase (T. urartu) |
| chr6A_84072498 | G:A   | 1.000      | Intronic | —    | —     | TraesCS6A02G114100 | bHLH95 (T. urartu) |
| chr6A_93819244 | G:A   | 0.964      | Missense | H/Y  | 1     | TraesCS6A02G121600 | DUF1644 |
| chr6A_94086104 | G:A   | 0.933      | Missense | G/S  | 0     | TraesCS6A02G121900 | Cellulase synthase |
| chr6A_96601065 | G:A   | 0.943      | Synonymous | A   | —     | TraesCS6A02G123000 | CASP-like protein 16 (Zea mays) |
| chr6A_98048315 | G:A   | 1.000      | Missense | P/L  | 0     | TraesCS6A02G124200 | Cytochrome c oxidase subunit II (Hordeum vulgare) |
| chr6A_104309957 | G:A   | 0.892      | Intergenic | —    | —     | —    | —         |
| chr6A_116709274 | G:A   | 0.915      | Missense | A/T  | 0.02  | TraesCS6A02G141800 | E3-ubiquitin ligase (Aegilops tauschii) |
| chr6A_137292502 | G:A   | 0.923      | Intergenic | —    | —     | —    | —         |
| chr6A_142559812 | C:T   | 0.886      | Missense | S/N  | 0     | TraesCS6A02G184000 | Leucine-rich repeat receptor-like protein kinase (Zea mays) |
| chr6A_297671234 | G:A   | 0.896      | Missense | V/I  | 0.72  | TraesCS6A02G197400 | Uncharacterized |
| chr6A_371730508 | G:A   | 0.852      | Missense | A/V  | 0.51  | TraesCS6A02G245600 | U-box domain-containing protein (Aegilops tauschii) |
| chr6A_458203666 | G:A   | 0.893      | Intronic | —    | —     | TraesCS6A02G247100 | BEACH domain-containing protein (Aegilops tauschii) |
| chr6A_490764102 | G:A   | 0.875      | Missense | P/S  | 0.35  | TraesCS6A02G247300 | GRAS transcription factor (Zea mays) |
| chr6A_479267518 | G:A   | 0.823      | Missense | T/A  | 0     | TraesCS6A02G257000 | Uncharacterized |
| chr6A_504123510 | G:A   | 0.883      | Intergenic | —    | —     | —    | —         |
| chr6A_517559399 | G:A   | 0.879      | Synonymous | R   | —     | TraesCS6A02G285800 | Ethylene receptor (Aegilops tauschii) |

Bold text indicates the genetic region reporting ΔSNP index=1.

a IWGSC RefSeq v1.0 co-ordinates (IWGSC et al., 2018).
b Variant effect prediction (McLaren et al., 2016).
c IWGSC annotation v1.1 (Alaux et al., 2018).
Table 3. Bulk segregant analysis maps the 2316b allele to a chromosomal region on 6D encoding a mutation in NAM-D1.

| Position* | SNP       | ΔSNP index | Effect#   | ΔAA#     | SIFT#     | Gene*     | Function*            |
|-----------|-----------|------------|-----------|----------|-----------|-----------|-----------------------|
| chr2A_732959676 | C:T      | 1.000      | Intergenic| —        | —         | —         | —                     |
| chr2D_154726777 | C:T      | 1.000      | Missense  | A/T      | 0.38      | TraesCS2D02G042900 | NBS-LRR resistance like protein (Hordeum vulgare) |
| chr3A_8326626   | C:T      | 0.833      | Intronic  | —        | —         | TraesCS3A02G009200 | NB-ARC domain         |
| chr3B_125660977 | C:T      | 0.875      | Intergenic| —        | —         | —         | —                     |
| chr3D_588999865 | G:A      | 0.833      | Splice region, synonymous | — | — | TraesCS3D02G498000 | Protein tyrosine kinase |
| chr3D_666283121 | G:A      | 0.909      | 3' UTR    | —        | —         | TraesCS3D02G072100 | PR17c precursor (Hordeum vulgare) |
| chr4D_42296410  | C:T      | 1.000      | Intergenic| —        | —         | —         | —                     |
| chr4D_509581375 | G:A      | 0.875      | Downstream| —        | —         | TraesCS4D02G085600 | Uncharacterized        |
| chr6D_57044844  | C:T      | 1.000      | Intergenic| —        | —         | —         | —                     |
| chr6D_604873215 | C:T      | 1.000      | Missense  | G/E      | 0         | TraesCS6D02G096300 | NAM-D1 (no apical meristem) |
| chr6D_653416945 | C:T      | 1.000      | Missense  | G/S      | 0.56      | TraesCS6D02G101900 | F-box domain          |
| chr6D_720227625 | C:T      | 0.960      | Intronic  | —        | —         | TraesCS6D02G107900 | SNF2 family N-terminal domain |
| chr6D_790760455 | G:A      | 0.909      | Missense  | A/T      | 0.32      | TraesCS6D02G112400 | Diaminopimelate decarboxylase, chloroplastic (T. urartu) |
| chr6D_842226705 | G:A      | 0.929      | Downstream| —        | —         | TraesCS6D02G119000 | Tyrosine-sulfated glycopeptide receptor 1-like (Aegilops tauschii) |
| chr6D_867330525 | G:A      | 1.000      | Intronic  | —        | —         | TraesCS6D02G122300 | GDP-fucose protein O-fucosyltransferase |
| chr6D_110872402 | G:A      | 0.756      | Synonymous| —        | —         | TraesCS6D02G141300 | FGGY carbohydrate kinase domain-containing protein (Aegilops tauschii) |
| chr6D_119464714 | G:A      | 0.755      | Intergenic| —        | —         | —         | —                     |
| chr6D_139310981 | G:A      | 0.817      | Intronic  | —        | —         | TraesCS6D02G161000 | Lycopene β-cyclase (T. urartu) |
| chr6D_140102996 | G:A      | 0.833      | Intronic  | —        | —         | TraesCS6D02G161800 | Aminotransferase (Zea mays) |
| chr6D_148078666 | G:A      | 0.790      | Missense  | P/S      | 0.01      | TraesCS6D02G166300 | Glycosyltransferase (Aegilops tauschii) |
| chr6D_264681956 | G:A      | 0.815      | Missense, splice region | G/D | 0 | TraesCS6D02G192000 | Hypothetical methyltransferase |
| chr6D_292379919 | G:A      | 0.941      | Intronic  | —        | —         | TraesCS6D02G206900 | S1 RNA binding domain |
| chr6D_352357451 | C:T      | 1.000      | 5' UTR    | —        | —         | TraesCS6D02G249000 | —                     |
| chr6D_363004043 | G:A      | 1.000      | Synonymous| —        | —         | TraesCS6D02G256800 | GPI-anchored protein (Aegilops tauschii) |
| chr7D_81790837 | C:T      | 0.833      | Synonymous| —        | —         | TraesCS7D02G018200 | NBS-LRR type resistance |
| chr7D_583986610 | G:A      | 1.000      | Intergenic| —        | —         | TraesCS7D02G470700 | —                     |

Bold text indicates the genetic region reporting ΔSNP index=1.

---

* IWGSC RefSeq v1.0 co-ordinates (IWGSC et al., 2018)

# Variant effect prediction (McLaren et al., 2016)

# IWGSC annotation v1.1 (Alaux et al., 2018)
Alongside the significant differences in grain moisture content recorded (Supplementary Table S3), we propose a potential extension in the rapid grain filling phase of ~5 d for 1189a and 2316b. The additional grain fill extension observed for 1189a likely relates to differences in the final lag phase of grain fill (Takahashi et al., 2001) as unlike 2316b, grain moisture content was significantly greater compared with cv. Paragon on 23 July in both years ($P<0.001$) (Supplementary Table S3). Delayed grain maturation of 1189a may disrupt depletion of stem reserves and deposition of triticin, glutenin, and gliadin storage proteins occurring during this final phase (Takahashi et al., 2001; Triboï et al., 2003), potentially reducing grain quality and requires further investigation.

Although the pattern of grain moisture decline for our staygreens is environmentally stable, dry grain weight accumulation is under greater environmental influence (Fig. 2; Supplementary Fig. S5). In 2018, the grain fill extension reported for 1189a was associated with increasing final grain weight ($P<0.001$), but the same trend was not evident for 2316b ($P>0.05$) (Supplementary Table 1). In 2018, grain fill was curtailed by ~4–5 d for all lines compared with 2017, with temperatures exceeding the 12–22 °C range considered optimal (Dias and Lidon, 2009; Farooq et al., 2011; Supplementary Fig. S2). Therefore, the greater final grain weight of 1189a reveals an association between staygreen traits and stress tolerance, as reviewed by Gregersen et al. (2013) and Thomas and Ougham (2014).

Final grain weight and grain filling rate are significantly correlated (Dias and Lidon, 2009), with slower rates reducing remobilization efficiency (Xie et al., 2015). Grain filling rate of these staygreens may be affected, illustrated by the shallower gradients between time points when grain moisture contents are significantly different, and are potentially slowest for 1189a (Fig. 2; Supplementary Fig. S4). The later termination of photosynthesis for 1189a, and greater availability of photosynthates, may counteract the reduction in stem reserve remobilization under stress, explaining the perhaps contradictory final grain weight increase in 2018. Evidence of additional photosynthates sustaining grain fill of staygreens are the greater dry grain weights recorded for 1189a from 42 daa, and 2316b from 37 daa ($P<0.05$) (Supplementary Table S3). Grain filling experiments by Borrill et al. (2015) support this, with flag leaves of NAM-RNAi lines producing 2079 mg more glucose per plant compared with controls. For 2316b, these earlier increases did not improve final grain weight suggesting remobilization efficiency may be impaired like 1189a, with earlier termination of senescence unable to compensate. Alternatively, any final grain weight improvement associated with 2316b may be diluted due to background mutations.

Evaluating mapping by exome capture

Adopting an exome capture-enabled BSA we mapped two staygreen traits to likely causative mutations in NAM-1 homoeologues. In wheat, the use of exome capture-enabled Fig. 5. Genetic mapping of NAM-1 homoeologues as identified by association analysis. (A, B) KASP markers were developed for validation of positions enriched in staygreen bulks, F$_2$ RILs genotyped, and genetic maps constructed for regions on chromosome 6A for 1189a (A) and 6D for 2316b (B). (C, D) Single marker association tests were performed using mapped SNPs and senescence phenotypes of Paragon × 1189a (C) and Paragon × 2316b (D) F$_2$ RILs (n=75) (2018, two replicates). Marker associations increase with proximity to NAM-A1 and NAM-D1, confirming candidate likelihood. Phenotypic abbreviations: EEtoLeafSen and EEtoPedSen, time from ear emergence to terminal flag leaf and peduncle senescence (day °C); MeanLeaf and MeanPed, overall mean flag leaf and peduncle senescence score; TT40, TT50 ... TT80, time to flag leaf senescence scores of 40, 50 ... 80 (day °C). Highlighted regions indicate ΔSNP index=1 and include SNPs within NAM-A1 (chr6A_77099433) and NAM-D1 (chr6D_60487321). Markers named according to RefSeq v1.0 (IWGSC et al., 2018). Marker distances calculated in MapDisto v2.0 (Heffelfinger et al., 2017). Maps constructed using MapChart (Voorrips, 2002).
BSA has typically been limited to mapping of qualitative traits, including yellow rust resistance (\textit{TaYr6}; Gardiner \textit{et al.}, 2016), plant height (\textit{TaRhtB1}; Mo \textit{et al.}, 2018) and a dominant chlorosis phenotype (\textit{YES-1} locus; Harrington \textit{et al.}, 2019). However, in combination with the recent mapping of a novel \textit{TaMKK-3} allele conferring pre-harvest sprouting resistance (Martinez \textit{et al.}, 2020), our results illustrate BSA can be used to map quantitative traits.

Whilst parental senescence phenotypes were distinct (Table 1), variation between RILs was often limited (Fig. 3), preventing senescence being scored as a binary trait as per Harrington \textit{et al.} (2019). Instead, RILs were classified relative to parents whereupon within- and between-year comparisons of multiple senescence metrics addressed variable trait expressivity and stability. Subsequent calculation and reporting of ΔSNP index=1 indicate high accuracy of RIL selection, contributing to the success of the BSA approach. Conversely, if RILs for bulk inclusion were decided based solely on 2016 data, accuracy would have been reduced, as retrospective analysis revealed 25–30\% of RILs were classified differently upon evaluation of 2017 data.

BSA mapped 1189a and 2316b staygreen traits to 16.7 and 8.2 Mb genetic regions (Tables 2, 3), with use of additional recombinants halving this to 4.8 Mb for 2316 (Fig. 5). Conversely, for 1189a no additional recombinants were identified despite similar size of Paragon × staygreen F\textsubscript{4} RIL populations. Indeed, markers developed for mutations within the identified 6A region for 1189a were in complete linkage, located in a reportedly highly conserved haplotype block (Brinton \textit{et al.}, 2020), thereby preventing dissection of the locus in the absence of hundreds of additional RILs. More widely, the size of regions we identified compared favourably with studies applying similar methods, which range from 1.9 to 32.9 Mb (Mo \textit{et al.}, 2018; Harrington \textit{et al.}, 2019; Martinez \textit{et al.}, 2020).

\textbf{NAM-A1 and NAM-D1 as gene candidates}  
Regions identified by BSA for 1189a and 2316b encode mutations in \textit{NAM-1} homoeologues. NAM-\textit{B1} is a known senescence regulator, previously identified in \textit{Triticum turgidum} ssp. \textit{dicoccoides} during dissection of locus \textit{GPC-1} (Uauy \textit{et al.}, 2006, b). In most hexaploid wheats NAM-\textit{B1} is non-functional due to

![Fig. 6. Sequence alignment of wheat NAM-1 homoeologous proteins. Boxes denote NAC subdomains (Ooka \textit{et al.}, 2003). Bold black lettering indicates homoeologous variation, and red dotted lines exon-junctions. Asterisks and bold coloured lettering indicate known variants, including AA substitutions identified for 1189a in orange (G159E, NAM-A1), 2316b in blue (T151, NAM-D1), natural NAM-A1 variants in pink (Cormier \textit{et al.}, 2015), EMS-induced NAM-A1 missense mutations in yellow (Harrington \textit{et al.}, 2019a), EMS-induced knockout mutations in green (Avni \textit{et al.}, 2014) and red (Pearce \textit{et al.}, 2014). In \textit{Triticum aestivum} NAM-B1 is largely non-functional due to a +1 bp frameshift mutation, or complete deletion (Uauy \textit{et al.}, 2006b; Hagenblad \textit{et al.}, 2012; Alhabbar \textit{et al.}, 2018a, b). Accession numbers are, NAM-A1, TracesCS6A02G108300.1; NAM-B1, UniProtKB/Swiss-Prot: A0SPJ4.1 (\textit{Triticum turgidum} ssp. \textit{dicoccoides}); NAM-D1, TracesCS6D02G096300.1.]

| Variant: | 
| --- | 
| Subdomain A | Subdomain B |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain C | Subdomain D |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain E | Subdomain F |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain G | Subdomain H |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain I | Subdomain J |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain K | Subdomain L |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain M | Subdomain N |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain O | Subdomain P |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain Q | Subdomain R |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain S | Subdomain T |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain U | Subdomain V |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain W | Subdomain X |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |

| Variant: | 
| --- | 
| Subdomain Y | Subdomain Z |
| 101 | 110 |
| 201 | 210 |
| 301 | 310 |
| 401 | 410 |
a +1 bp frameshift-encoding insertion or its complete deletion (Uauy et al., 2006b; Hagenblad et al., 2012; Lundström et al., 2017). The role of NAM-A1 and NAM-D1 homoeologues in senescence was confirmed by Avni et al. (2014), with natural variation limited to A and B homoeologues with no D variants reported. Of the NAM-A1 alleles detected, one encodes an alanine to valine AA substitution between subdomains D and E (A171V), the other a frameshift-induced truncation mutation (Fig. 6) (Cormier et al., 2015).

SNPs within NAM-1 homoeologues of 1189a and 2316b are considered deleterious to protein function (SIFT=0) (Tables 2, 3) and are completely associated with senescence phenotypes (Fig. 5). Supporting the proposal of these mutations as causative is a reverse genetic study concerning gpc-1 (NAM-1) TILLING mutants by Avni et al. (2014) (Fig. 6). A delay in onset of senescence of ~6 d and ~3 d was reported for the Triticum aestivum cv. Express gpc-a1 W196* truncation mutant and gpc-d1 W114* knockout mutant, respectively (Avni et al., 2014), matching the phenotypes recorded for 1189a and 2316b (Table 1). Likewise, senescence of gpc-1 mutants and 1189a and 2316b progresses in parallel, terminating 6–10 d and 5 d later compared with cv. Express, respectively (Avni et al., 2014; Supplementary Fig. S3).

Within a protein context, mutations within NAM-A1 and NAM-D1 are self-validating, encoding AA substitutions within subdomain D of the NAC domain that encompasses a DNA binding region (Fig. 6) (Ooka et al., 2003; Welner et al., 2012).

Mutations in subdomain D can alter NAM protein functionality, with the affected G151 and T159 residues conserved in over 65% of NAC transcription factor encoding genes (Ooka et al., 2003; Puranik et al., 2012; Welner et al., 2012; Fan et al., 2014). In Triticum turgidum TILLING mutants, a P154L mutation in NAC subdomain D of NAM-A1 disrupted protein dimerization in the absence of a senescence phenotype (Harrington et al., 2019c), whilst a G133D mutation in subdomain D of NAM-A2, the NAM-A1 paralogue, significantly delayed peduncle senescence (Borrill et al., 2019). Similarly, NAM-1 homoeologues encoding subdomain D allelic variants in NAM-G1 of Triticum timopheevi and NAM-1 of Hordeum vulgare are associated with reducing grain protein content (P<0.05), illustrating loss of function (Jamar et al., 2010; Hu et al., 2013).

Welner et al. (2012) proposed that one NAC monomer initially sub-optimally binds DNA with the other scanning and searching for a binding site. Changes in charge or polarity introduced by G151E and T159I mutations may disrupt initial DNA-binding to prevent dimerization, with NAM-1 homoeologues affected similarly due to structural palindromicity of residues (Ernst et al., 2004; Welner et al., 2012). Performance of yeast-2-hybrid and cell death assays for mutated NAM-1 proteins would test for altered binding activity, as performed by Harrington et al. (2019c), to identify residues critical to NAM-A1 protein function. The extremity of the 1189a staygreen phenotype compared with
2316b likely reflects homoeologous dominance of NAM-A1 over NAM-D1 and not mutation type, although involvement of linked mutations within mapped intervals not captured by exome sequencing, including promoter variants, cannot be disregarded. However, the delays in onset of senescence recorded for 1189a and 2316b match those reported by Avni et al. (2014) in a study of T. aestivum cv. Express gpc-1 mutants, thereby providing independent supporting evidence.

Grain fill phenotypes of 1189a and 2316b compare favourably with NAM-1 variants

When characterizing GPC-B1 (NAM-B1) Uauy et al. (2006a) reported an association between a non-functional allele and longer grain filling period. The results of Avni et al. (2014) validate the association between variation and grain fill extension observed for 1189a (NAM-A1) and 2316b (NAM-D1) mutants. From 42 daa spike moisture content of gpc-1 mutants was greater when compared with parental controls (P<0.05) and remained so until 49 daa and 57 daa for the gpc-d1 and gpc-a1 mutant, respectively, matching the pattern of grain moisture loss recorded for 1189a and 2316b (Fig. 2; Supplementary Fig. S4). NAM-A1 variants are common in Australian wheat cultivars with variation characteristic of mid to mid-late maturity types (Alhabbar et al., 2018a), just as observed for 1189a (Fig. 2).

Contrary to the hypotheses of Wiegand and Cuellar (1981), Gelang et al. (2000), and Bogard et al. (2011) delayed senescence and grain fill extension, as associated with NAM-1 variation, may not improve final grain weight. During grain fill, dry grain weights recorded for 1189a and 2316b were greater (P<0.05) (Supplementary Table S3), contradicting the lower weights recorded for NAM-1 RNAi lines (P<0.05) (Borrill et al., 2015) and similar dry spike weights recorded for gpc-1 mutants (P>0.05) (Avni et al., 2014). Differences in final grain weight between NAM-1 RNAi, 2316b, and gpc-1 mutant lines and controls were not significant (P>0.05) (Avni et al., 2014; Borrill et al., 2015), but grain weight of 1189a was greater in 2018 (P<0.001) (Supplementary Table S3).

The influence of NAM-1 variation on final grain weight depends on genetic background and environment. Differences in TGW of isogenic lines carrying a non-functional GPC-B1 copy were inconsistent and both higher and lower (P<0.05) (Uauy et al., 2006a), and likewise for cultivars carrying NAM-A1 variants (Cormier et al., 2015; Alhabbar et al., 2018a). Borrill et al. (2015) hypothesized similarity in TGW of NAM-1 RNAi lines and controls (P=0.25) was due to inadequate starch synthase activity. Instead of contributing to grain filling activities, Borrill et al. (2015) demonstrated the additional sucrose synthesized by NAM-1 RNAi lines was retained as stem fructan within the internodes. Improved stem fructan remobilization is associated with TGW improvement (Zhang et al., 2015), with fructan providing a source of water-soluble carbohydrates for sustainment of grain filling (Fischer, 2011; Borrill et al., 2015). Greater final grain weight of 1189a in 2018 and recorded during grain filling for 2316b (P<0.05) (Supplementary Table S3) demonstrate that the novel NAM-1 alleles we identified could increase TGW. Therefore, combining these NAM-1 alleles with a mutation in gene 1-FEH-w3 regulating stem fructan remobilization (Zhang et al., 2015) could overcome problems associated with fructan retention (Borrill et al., 2015) and consistently improve TGW.

Dominance of NAM genes in regulation of wheat senescence

Genetic mapping of 1189a and 2316b converged upon homoeologous copies of known senescence regulator NAM-B1 (Uauy et al., 2006b). Similarly, a forward genetic screen of a Triticum turgidum cv. Kronos TILLING population to identify senescence mutants converged upon NAM-A1 mutations (Harrington et al., 2019). The differential onset of senescence observed for 1189a and 2316b (Table 1) reflects the reported dominance of NAM-1 homoeologues (Avni et al., 2014), with our results the first forward genetic screen identifying NAM-D1.

NAM-1 is a positive regulator of senescence, with expression up-regulated following anthesis (Uauy et al., 2006b), and is associated with transcriptional reprogramming. At 12 daa RNA-seq studies of gpc-1 (NAM-I) mutants identified ≥691 differentially expressed genes, with protein catabolism and stress response genes up-regulated, and photosynthetic and housekeeping genes down-regulated (Cantu et al., 2011; Pearce et al., 2014; Borrill et al., 2019). The role of NAM genes in senescence regulation is complicated by the NAM-1 parologue NAM-2. RNA-seq studies of tetraploid wheat gpc-1 and gpc-2 mutants identified NAM-1 as dominant over NAM-2, with expression associated with 64% of senescence-regulated genes compared with 37%, respectively (Pearce et al., 2014). Phenotypic characterization of NAM-B2 mutants confirmed its role in senescence regulation, but phenotypic differences were only significant when combined with mutations in NAM-A1 or NAM-A2 (P<0.05) (Pearce et al., 2014; Borrill et al., 2019). These findings illustrate the complexity of senescence and the problems associated with identification of genetic regulators of senescence using forward genetic techniques.

Mutations identified in NAM-1 homoeologues were penetrative and dominant (Fig. 7), with their detection relatively unconfounded by homoeologues as cv. Paragon encodes a non-functional copy of NAM-B1. Due to the apparent dominance of NAM-1, it is possible mutations in genes acting downstream result in more subtle phenotypes. Inconsistencies between leaf and peduncle phenotypes (Borrill et al., 2019; Harrington et al., 2019b, c), spatial variation or effects of background mutations may prevent identification of other contributing loci. Whilst the original screen of the Triticum aestivum cv. Paragon EMS mutant population identified lines 1189a and 2316b to be of interest, delayed and accelerated senescence phenotypes were
confirmed for additional lines. Therefore, this resource is worth further exploration to shed further light of the genetic regulation of senescence in wheat. For example, this could include sequencing of NAM-1 and NAM-2 homoeologues of staygreen mutants to identify causative mutations due to their known, and overarching, role in senescence regulation (Avni et al., 2014; Pearce et al., 2014; Borrill et al., 2019; Harrington et al., 2019c). Conversely, early senescing mutants may encode gain of function mutations affecting NAM-1 gene regulatory targets including C2C2-CO like transcription factors, RWD-RK, or GRAS genes identified during transcriptional network modelling (Borrill et al., 2019; Harrington et al., 2019a).

Conclusions

Here, we confirm the central role of NAM-1 in the genetic regulation of wheat senescence through identification of novel mutant NAM-A1 and NAM-D1 alleles following a forward genetic screen. Both mutations occurred within subdomain D of the NAC domain, highlighting the importance of this subdomain in modulating NAM-1 function. Altered senescence profiles associated with these mutations are independent of heading-date variation and contribute to a grain fill extension and potential increased grain weight, whereby introduction and selection of these novel NAM-1 alleles could enhance final grain yield.

Supplementary data

The following supplementary data are available at JXB online.
Fig. S1. Development of RIL populations segregating for senescence traits and mapping strategy.
Fig. S2. Daily rainfall and mean daily temperature data.
Fig. S3. Senescence phenotypes for lines 1189a and 2316b (2016 and 2018).
Fig. S4. Delayed senescence is associated with grain fill extension in 2017.
Fig. S5. Senescence phenotypes corresponding to grain filling experiments (2018).
Fig. S6. TT70 scores for Paragon × 1189a F4 RILs (2016 to 2018).
Fig. S7. TT70 scores for Paragon × 2316b F4 RILs (2016 to 2018).
Fig. S8. Additional phenotype×genotype plots illustrating mode of inheritance.
Fig. S9. Independent confirmation of modes of inheritance for NAM-1 mutations.
Table S1. KASP primers for 1189a.
Table S2. KASP primers for 2316b.
Table S3. Differences in grain filling parameters, pairwise-comparison (2017 and 2018).
Table S4. Exome capture coverage and identified SNPs.

Genetic mapping of staygreen traits in wheat | 7725

Dataset S1. RIL senescence classification & bulk selection; Gene lists for mapped intervals according to Chinese Spring on chromosome 6A and 6D of 1189a and 2316b, respectively.

Acknowledgements

The authors wish to thank the JIC field experimentation team, without whom field trials would not have been possible, in addition to horticultural services and glasshouse staff. Thanks go to Burkhard Steuernagel for bioinformatics assistance, members of the Uauy group (JIC) for idea exchange, Luzie Wingen for statistical support, Rajani Awal and Richard Goram for tissue collection and DNA extraction. Thank you to Clare Lister for editorial assistance. The Triticum aestivum cv. Paragon population was developed by Robert Koebner and Leodie Alibert.

Author contributions

EAC, JL, and SG conceived the study. SO screened, developed and maintained the genetic material. EAC designed and conducted the experiments with supervision from SG and JL. JL and SO provided technical assistance. EAC analysed the data and wrote the article in correspondence with SG and JL.

Conflict of interest

JL was employed by the company KWS UK Limited. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This work was funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC) grants (BB/M011216/1). EAC received a BBSRC CASE-Doctoral Training Partnership studentship with additional funding provided by KWS-UK (BB/M011216/1-1654063). The Triticum aestivum cv. Paragon EMS mutant population was developed at John Innes Centre as part of the Wheat Genetic Improvement Network (WGIN), funded by the UK Department for Environment and Rural Affairs (DEFRA; Defra Project Code: AR0709). This work was supported by the ‘NUE traits’ project joint funded by Institut National de la Recherche Agronomique (INRA) and BBSRC (IN-BB-06; BB/E527146/1).

Data availability

Raw exome capture sequencing reads for all samples have been deposited on the European Nucleotide Archive (ENA) (PRJEB40428) at https://www.ebi.ac.uk/ena. All germplasm, including original EMS mutants and associated RIL populations, are available from the Germplasm Resources
References

Adu MO, Sparkes DL, Parmar A, Yawson DO. 2011. *Stay Green* in wheat: comparative study of modern bread wheat and ancient wheat cultivars. *Journal of Agricultural & Biological Science* 6, 16–24.

Ahloowalia BS, Maluszynski M, Nichterlein K. 2004. Global impact of mutation-derived varieties. *Euphytica* 135, 187–204.

Alaix M, Rogers J, Letellier T, et al. International Wheat Genome Sequencing Consortium. 2016. Linking the International Wheat Genome Sequencing Consortium bread wheat reference genome sequence to wheat genetic and phenomic data. *Genome Biology* 19, 111.

Alhabar Z, Islam S, Yang R, et al. 2018a. Associations of NAM-A1 alleles with the onset of senescence and nitrogen use efficiency under Western Australian conditions. *Euphytica* 214, 180.

Alhabar Z, Yang R, Juhasz A, Xin H, She M, Anwar M, Sultana N, Diepeveen D, Ma W, Islam S. 2018b. NAM gene allele composition and its relation to grain-filling duration and nitrogen utilisation efficiency of Australian wheat. *PloS ONE* 13, e0205448.

Andrews S. 2010. FastQC: A quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

Arends D, Prins P, Jansen RC, Broman KW. 2010. R/qtl: high-throughput multiple QTL mapping. *Bioinformatics* 26, 2990–2992.

Avni R, Zhao 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, 313–324.

Bates D, Maechler M, Bolker B, et al. 2014. *lme4: Linear mixed-effects models using 'lme4'*. R package version 1.1-21. https://cran.r-project.org/package=lme4.

Bentley A, Turner A, Gosman N, et al. 2011. Frequency of photoperiod-insensitive *Ppd-A1a* alleles in tetraploid, hexaploid and synthetic hexaploid wheat germplasm. *Plant Breeding* 130, 10–15.

Bian X, Tyrell S, Davey RP. 2017. The Grassroots life science data infrastructure. https://grassroots.tools.

Bogard M, Jourdan M, Allard V, et al. 2011. Anthesis date mainly explained correlations between post-anthesis leaf senescence, grain yield, and grain protein concentration in a winter wheat population segregating for flowering time QTLs. *Journal of Experimental Botany* 62, 3621–3636.

Borrill P, Fahy B, Smith AM, Uauy C. 2015. Wheat grain filling is limited by grain filling capacity rather than the duration of flag leaf photosynthesis: a case study using NAM RINAI Plants. *PloS ONE* 10, e0134947.

Borrill P, Harrington SA, Simmonds J, Uauy C. 2019. Identification of transcription factors regulating senescence in wheat through gene regulatory network modelling. *Plant Physiology* 180, 1740–1755.

Brinton J, Ramirez-Gonzalez RH, Simmonds J, et al. 10 Wheat Genome Project. 2020. A haplotype-led approach to increase the precision of wheat breeding. *Communications Biology* 3, 712.

Brinton J, Uauy C. 2019. A reductionist approach to dissecting grain weight and yield in wheat. *Journal of Integrative Plant Biology* 61, 337–358.

Buchanan-Wollaston V. 2007. Senescence in plants, eLS. Chichester, UK: John Wiley & Sons, Ltd.

Camargo AV, Mott R, Gardner KA, Mackay IJ, Corke F, Doonan JH, Kim JT, Bentley AR. 2016. Determining phenological patterns associated with the onset of senescence in a wheat MAGIC mapping population. Frontiers in *Plant Science* 7, 1540.

Cantu D, Pearce SP, Distelfeld A, Christiansen MW, Uauy C, Akhunov E, Fahima T, Dubcovsky J. 2011. Effect of the down-regulation of the high Grain-Protein Content (GPC) genes on the wheat transcriptome during monocarpic senescence. *BMC Genomics* 12, 492.

Christopher JT, Christopher MJ, Borrell AK, Fletcher S, Chenu K. 2016. Stay-green traits to improve wheat adaptation in well-watered and water-limited environments. *Journal of Experimental Botany* 67, 5159–5172.

Cormier F, Throude M, Ravel C, Le Gouis J, Leveugle M, Lefarge S, Exbrayat S, Duranton N, Praud S. 2015. Detection of NAM-A1 natural variants in bread wheat reveals differences in haplotype distribution between a worldwide core collection and European elite germplasm. *Agronomy* 5, 143–151.

Danecek P, Schiefs S, Durbin R. 2014. Multi allelic calling model in bcftools (-m). https://samtools.github.io/bcftools/call-m.pdf

Derky AP, Orford S, Griffiths S, Foulkes MJ, Hawkesford MJ. 2012. Identification of differentially senescing mutants of wheat and impacts on yield, biomass and nitrogen partitioning. *Journal of Integrative Plant Biology* 54, 555–566.

de Souza Luche H, Gonzalez da Silva JA, Nornberg R, Hawerthoc MC, da Silveira Silveira SF, da Rosa Caetano V, Santos RL, Figueiredo RG, da Maia LC, de Oliveira AC. 2017. *Stay-green* character and its contribution in Brazilian wheats. *Ciência Rural* 47, doi: 10.1590/0103-8478cr20160583.

 Dias AS, Lidon FC. 2009. Evaluation of grain filling rate and duration in bread and durum wheat, under heat stress after anthesis. Journal of Agronomy and Crop Science 195, 137–147.

Dowle M, Srinivasan A, Gorecki J, et al. 2019. data.table: Extension of ‘data.frame’. R package version 1.1.2.0. https://cran.r-project.org/package=data.table.

Ernst HA, Olsen AN, 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, 297–303.

Fan K, Wang M, Miao Y, Ni M, Bibi N, Yuan S, Li F, Wang X. 2014. Molecular evolution and expansion analysis of the NAC transcription factor in *Zea mays*. *PloS ONE* 9, e111837.

Farooq M, Bramley H, Palta JA, Siddique KH. 2011. Heat stress in wheat during reproductive and grain-filling phases. Critical Reviews in Plant Sciences 30, 491–507.

Fischer RA. 2011. Wheat physiology: a review of recent developments. *Crop and Pasture Science* 62, 95–114.

Gardiner LJ, Bansept-Basler P, Olohan L, Joynson R, Brenchley R, Hall N, O’Sullivan DM, Hall A. 2016. Mapping-by-sequencing in complex polyploid genomes using genic sequence capture: a case study to map yellow rust resistance in hexaploid wheat. *The Plant Journal* 87, 403–419.

Gelang J, Pleijel H, Sild E, Danielsson H, Younis S, Sellén D. 2000. Rate and duration of grain filling in relation to flag leaf senescence and grain yield in spring wheat (*Triticum aestivum*) exposed to different concentrations of ozone. *Physiologia Plantarum* 110, 366–375.

Gregersen PL, Culetic A, Boschian L, Krupinska K. 2013. Plant senescence and crop productivity. *Plant Molecular Biology* 82, 603–622.

Hagenblad J, Asplund L, Balfourier F, Ravel C, Leino MW. 2012. *Senescence in plants, eLS*. Chichester, UK: John Wiley & Sons, Ltd.

Harrington SA, Backhaus AE, Singh A, Hassan-Pak K, Uauy C. 2019a. Validation and characterisation of a wheat GENIES network using an independent RNA-Seq dataset. *G3 Genes|Genomes|Genetics* 10, 3675–3686.

Harrington SA, Cobo N, Karafátová M, Doležel J, Borrill P, Uauy C. 2019b. Identification of a dominant chlorosis phenotype through a forward screen of the *Triticum turgidum* cv. *Kronos* TILLING population. Frontiers in *Plant Science* 10, 963.

Harrington SA, Overend LE, Cobo N, Borrill P, Uauy C. 2019c. Conserved residues in the wheat (*Triticum aestivum*) NAM-A1 NAC domain are required for protein binding and when mutated lead to delayed peduncle and flag leaf senescence. *BMC Plant Biology* 19, 407.

Heffelfinger C, Fragoso CA, Lorieux M. 2017. Constructing linkage maps in the genomics era with MapDisto 2.0. *Bioinformatics* 33, 2224–2225.

Henry IM, Nagalakshmi U, Lieberman MC, et al. 2014. Efficient genome-wide detection and cataloging of EMS-induced mutations using exome capture and next-generation sequencing. *The Plant Cell* 26, 1382–1397.


Hu XG, Wu BH, Liu DC, Wei YM, Gao SB, Zheng YL. 2013. Variation and their relationship of NAM-G1 gene and grain protein content in Triticum timopheevii Zhuk. Journal of Plant Physiology 170, 330–337.

International Wheat Genome Sequencing Consortium (IWGSC). Apels R, Eversole K, et al. 2018. Shifting the limits in wheat research and breeding using a fully annotated reference genome. Science 361, eaar7191.

Jamar C, Loffet F, Frettiger P, Ramsay L, Fauconnier ML, du Jardin P. 2010. NAM-I gene polymorphism and grain protein content in Hordeum. Journal of Plant Physiology 167, 497–501.

Joshi N, Fass J. 2011. Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.2). https://github.com/najoshi/sickle

Kitonyo OM, Sadras VO, Zhou Y, Denton MD. 2017. Evaluation of historical Australian wheat varieties reveals increased grain yield and changes in senescence patterns but limited adaptation to tillage systems. Field Crops Research 206, 65–73.

Knaus BJ, Grünwald NJ. 2017. vcfr: a package to manipulate and visualize variant call format data in R. Molecular Ecology Resources 17, 44–53.

Krasileva KV, Vasquez-Gross HA, Howell T, et al. 2017. Uncovering hidden variation in polyploid wheat. Proceedings of the National Academy of Sciences, USA 114, E913–E921.

Kumari M, Singh VP, Tripathi R, Joshi AK. 2007. Variation for staygreen, association mapping and population genetical parameter estimation. Crop Science 47, 920.

Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 2010. 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2079.

Lundström M, Leino MW, Hagenblad J. 2017. Evolutionary history of the NAM-B1 gene in wild and domesticated tetraploid wheat. BMC Genetics 18, 118.

Martinez S, Shorinola O, Conselman S, See D, Skinner DZ, Uauy C, Steber CM. 2020. Exome sequencing of bulk segregants identified a novel TaMKK3-A allele linked to the wheat ERA8 ABA-hypersensitive germination phenotype. Theoretical and Applied Genetics 133, 719–736.

McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GR, Thornam A, Flicek P, Cunningham F. 2016. The ensembl variant effect predictor. Genome Biology 17, 122.

Michelmore RW, Paran I, Kesseli RV. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proceedings of the National Academy of Sciences, USA 88, 9828–9832.

Mo Y, Howell T, Vasquez-Gross H, de Haro LA, Dubcovsky J, Pearce S. 2018. Mapping causal mutations by exome sequencing in a wheat TILLING population: a tall mutant case study. Molecular Genetics and Genomics 293, 463–477.

Narahuka Y, Sherman JD, Lanning SP, Blake NK, Martin JM, Talbert LE. 2012. Genetic analysis of green leaf duration in spring wheat. Crop Science 52, 99–109.

Neghiz H, Cochard H, Brunel N, Martre P. 2016. Ear rachis xylem occlusion and associated loss in hydraulic conductance coincide with the end of grain filling for wheat. Frontiers in Plant Science 7, 920.
Voorrips RE. 2002. MapChart: software for the graphical presentation of linkage maps and QTLs. The Journal of Heredity 93, 77–78.

Voss-Fels KP, Stahl A, Wittkop B, et al. 2019. Breeding improves wheat productivity under contrasting agrochemical input levels. Nature Plants 5, 706–714.

Wang L, Zhang W, Li Q, Zhu W, et al. 2017. AssocTests: Genetic association studies. R package version 0.0-4. https://cran.r-project.org/package=AssocTests.

Welner DH, Lindemose S, Grossmann NE, Olsen AN, 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. The Biochemical Journal 444, 395–404.

Wickham H. 2015. plyr: Tools for splitting, applying and combining data. R package version 1.8.3. http://cran.r-project.org/web/packages/plyr/.

Wickham H, Chang W, Henry L, Pedersen TL, Takahashi K, Wilke C, Woo K. 2018a. ggplot2: Create elegant data visualisations using the grammar of graphics description. R package version 3.1.0. https://cran.r-project.org/package=ggplot2.

Wickham H, François R, Henry L, Müller K. 2018b. dplyr: A grammar of data manipulation. R package version 0.7.8. https://cran.r-project.org/package=dplyr.

Wickham H, Henry L, and RStudio. 2019. tidyr: Tidy messy data. R package version 0.8.3. https://cran.r-project.org/package=tidy.

Wiegand CL, Cuellar JA. 1981. Duration of grain filling and kernel weight of wheat as affected by temperature. Crop Science 21, 95–101.

Xie Q, Mayes S, Sparkes DL. 2015. Analysis of effects in wheat of high temperature on grain filling attributes estimated from mathematical models of grain filling. Journal of Agricultural Science 141, 203–212.

Xie Q, Mayes S, Sparkes DL. 2016. Early anthesis and delayed but fast leaf senescence contribute to individual grain dry matter and water accumulation in wheat. Field Crops Research 187, 24–34.

Zadoks JC, Chang TT, Konzak CF. 1974. A decimal code for the growth stages of cereals. Weed Research 14, 415–421.

Zhang J, Xu Y, Chen W, Dell B, Vergauwen R, Biddulph B, Khan N, Luo H, Appels R, Van den Ende W. 2015. A wheat 1-FEH w3 variant underlies enzyme activity for stem WSC remobilization to grain under drought. New Phytologist 205, 293–305.

Zhao D, Derkx AP, Liu DC, Buchner P, Hawkesford MJ. 2015. Overexpression of a NAC transcription factor delays leaf senescence and increases grain nitrogen concentration in wheat. Plant Biology 17, 904–913.