

Identification of transcription factors regulating senescence in wheat through gene regulatory network modelling

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1 Short title: Transcription factors regulating wheat senescence

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6 Article title:

7 **Identification of transcription factors regulating senescence in wheat**
8 **through gene regulatory network modelling**

9

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15 One sentence summary: Integrating gene regulatory network modelling during a gene expression
16 timecourse with publicly available genomic datasets identifies transcription factors regulating
17 senescence in wheat.

18

19

20

21 Footnotes:

22 **Author contributions**

23 PB and CU conceived, designed and coordinated the study. PB harvested tissue for the timecourse and
24 collected the associated chlorophyll and grain moisture content phenotypic data. PB carried out the
25 RNA extraction, analysed the RNA-Seq data, and built the gene regulatory network model. PB
26 identified mutations in *NAM-A2* and *NAM-B2* for crossing and designed KASP markers. JS carried out
27 crossing of *NAM-A2* and *NAM-B2* mutant lines. JS and PB carried out KASP genotyping. PB and SH
28 carried out phenotyping of the *NAM2* mutant lines. PB wrote the manuscript. CU, SH, and JS edited
29 the manuscript.

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38 **Abstract**

39 Senescence is a tightly regulated developmental programme coordinated by transcription factors.
40 Identifying these transcription factors in crops will provide opportunities to tailor the senescence
41 process to different environmental conditions and regulate the balance between yield and grain
42 nutrient content. Here, we use ten time points of gene expression data along with gene network
43 modelling to identify transcription factors regulating senescence in polyploid wheat (*Triticum*
44 *aestivum* L.). We observe two main phases of transcriptional changes during senescence: early
45 downregulation of housekeeping functions and metabolic processes followed by upregulation of
46 transport and hormone related genes. These two phases are largely conserved with *Arabidopsis*
47 (*Arabidopsis thaliana*), although the individual genes underlying these changes are often not
48 orthologous. We have identified transcription factor families associated with these early and later
49 waves of differential expression. Using gene regulatory network modelling, we identified candidate
50 transcription factors that may control senescence. Using independent, publicly available datasets, we
51 found that the most highly ranked candidate genes in the network were enriched for senescence-
52 related functions compared to all genes in the network. We validated the function of one of these
53 candidate transcription factors in senescence using wheat chemically-induced mutants. This study lays
54 the groundwork to understand the transcription factors that regulate senescence in polyploid wheat
55 and exemplifies the integration of time-series data with publicly available expression atlases and
56 networks to identify candidate regulatory genes.

57 **Introduction**

58 Grain yield and nutrient content in cereal crops is determined by the accumulation of carbon, nitrogen,
59 and other nutrients in the grain towards the end of a plant's life. The availability of these nutrients is
60 strongly influenced by the process of senescence, a regulated developmental programme to
61 remobilise nutrients from the vegetative tissues to the developing grain. Both the onset and rate of
62 senescence influence grain yield and nutrient content. A delay in senescence may be associated with
63 increased yield due to an extended period of photosynthesis (Thomas and Howarth, 2000; Gregersen
64 et al., 2013), although this is not always the case (Borrill et al., 2015a). Delayed senescence may also
65 be associated with a decrease in grain nutrient content due to reduced nutrient remobilisation from
66 green tissues (Distelfeld et al., 2014). Senescence is often associated with the visual loss of chlorophyll,
67 however the initiation of senescence through signalling cascades, and early stages such as degradation
68 of protein and RNA, are not visible (Buchanan-Wollaston et al., 2003; Fischer, 2012). Through these
69 initial stages, and later during visual senescence, a programme of tightly-regulated changes occurs in
70 gene expression (Buchanan-Wollaston et al., 2003; Fischer, 2012). Despite its importance, we know

71 relatively little about the molecular control of senescence in crops such as wheat (Distelfeld et al.,
72 2014).

73 This lack of knowledge is partly due to the difficulty of identifying genes regulating quantitative traits
74 in the large wheat genome (IWGSC et al., 2018) as well as the subtle effects of individual gene copies
75 (homoeologs) within the polyploid context (Borrill et al., 2015b). These challenges mean that
76 conventional genetic mapping approaches often take many years to identify causal genes. To date two
77 genes have been identified to regulate senescence in wheat. The *NAM-B1* NAC transcription factor
78 was identified to underlie a quantitative trait locus (QTL) for grain protein content and senescence
79 (Uauy et al., 2006). A second NAC transcription factor, *NAC-S*, was found to have a strong correlation
80 between its expression level and leaf nitrogen concentration in tandem with a role in regulating
81 senescence (Zhao et al., 2015). However, to realise the potential to manipulate the rate and onset of
82 senescence in wheat it will be necessary to gain a more comprehensive understanding of the network
83 of transcription factors regulating this process. Identifying these transcription factors may enable the
84 development of wheat varieties with a senescence profile tailored to maximise nutrient remobilisation
85 whilst maintaining yield and providing adaption to local growing conditions.

86 The first step towards manipulating senescence at the molecular level is to understand the genes
87 which are involved in the process, and the transcription factors which orchestrate gene expression
88 changes during senescence. Over 50% of micro- and macro-nutrients remobilised to the developing
89 grain originate from the uppermost (flag) leaf of the senescing wheat plant (Garnett and Graham,
90 2005; Kichey et al., 2007), making it a key tissue in which to understand the senescence process.
91 Previous attempts have been made to characterise transcriptional changes in wheat flag leaves,
92 however these studies have been either carried out with microarrays which were limited to a small
93 set of 9,000 genes (Gregersen and Holm, 2007) or had a limited number of samples and time points
94 (Pearce et al., 2014; Zhang et al., 2018). Decreases in the cost of RNA-Seq now mean that these
95 constraints can be overcome through genome-wide expression studies across multiple time points.
96 The recent publication of the wheat genome sequence with over 100,000 high confidence gene
97 models (IWGSC et al., 2018) and accompanying functional annotations, enhances the ease and
98 accuracy with which RNA-Seq data can be analysed in wheat. Systems biology approaches can start to
99 make sense of the vast quantities of data produced and identify the regulatory pathways controlling
100 quantitative traits (Kumar et al., 2015).

101 Our aim in this study was to identify the molecular pathways involved in senescence in wheat and to
102 determine candidate transcription factors controlling these processes in the flag leaf. We sequenced
103 a ten time point expression timecourse of wheat senescence in the flag leaf from 3 days post anthesis

104 until 26 days post anthesis which corresponded to the first signs of visual senescence. We identified
105 the temporal progression of the senescence process at the molecular level and used gene regulatory
106 network modelling to predict transcription factors which coordinate this developmental process. We
107 confirmed the role of one of these candidate genes, *TraesCS2A02G201800* (*NAM-A2*), in wheat itself.

108 **Results**

109 **Growth and physiological measurements**

110 To understand the transcriptional control of the initiation of senescence we harvested an early
111 timecourse of senescence at 3, 7, 10, 13, 15, 17, 19, 21, 23, and 26 days after anthesis (DAA) (Fig. 1A).
112 SPAD chlorophyll meter readings in the flag leaf were maintained at a similar level from 3 to 21 DAA,
113 with a significant decrease from 23 DAA (Supplemental Fig. S1). Percentage moisture of the grains
114 decreased from 80.0% at 3 DAA to 54.7% at 26 DAA which corresponds to the soft dough stage (GS85
115 (Zadoks et al., 1974)) (Supplemental Fig. S2), indicating that the time period sampled included the
116 majority of the grain filling period.

117 **Strong transcriptional changes occur during flag leaf senescence**

118 RNA was extracted from the flag leaf blade with three replicates for each of the ten time points and
119 sequenced. The RNA-Seq data was aligned to the RefSeqv1.1 transcriptome annotation (IWGSC et al.,
120 2018) using kallisto (Bray et al., 2016). On average, each sample had 38.7 M reads, of which 30.9 M
121 mapped (78.9%) (Supplemental Table S1). We found that 52,905 high confidence genes were
122 expressed at > 0.5 transcripts per million (TPM) in at least one time point during flag leaf senescence,
123 which corresponds to 49.0% of high confidence genes. To identify genes differentially expressed
124 during the timecourse, we used two programmes specifically designed for timecourse data:
125 ImpulseDE2 (Fischer et al., 2018) and gradient tool (Breeze et al., 2011). In total 9,533 genes were
126 identified as differentially expressed by both programmes, giving a high confidence set of differentially
127 expressed genes (DEGs). In addition, gradient tool identified the time points at which the genes
128 became differentially expressed which we used to determine the temporal changes in gene expression
129 associated with senescence (Supplemental Table S2).

130 To define the biological roles of these 9,533 genes, we grouped them according to the first time point
131 at which they were up or downregulated. For example, a gene first upregulated at 10 DAA was in
132 group "U10" (up 10 DAA), whereas a gene first downregulated at this time point was assigned to group
133 "D10" (down 10 DAA). Fewer than 4% of genes were both up and down regulated during the
134 timecourse and these were excluded from further analysis of global expression patterns, resulting in
135 17 expression patterns (Supplemental Table S2). In total, approximately twice as many genes were
136 upregulated during this senescence timecourse than downregulated (5,343 compared to 2,715). This
137 indicates that senescence is actively regulated through transcriptional upregulation rather than a
138 general downregulation of biological processes.

139 We found that the patterns of up and downregulation were not equally spaced throughout the
140 timecourse. During the early stages of senescence, the majority of DEGs were downregulated
141 (825/1035 DEGs at 3 DAA), and these continued to be downregulated throughout the timecourse (Fig.
142 1B). At the later stages of senescence relatively few genes started to be downregulated (e.g. 50 genes
143 at 19 DAA). Instead the number of genes which started to be upregulated increased from 210 genes
144 at 3 DAA to 1,324 genes at 13 DAA. After this peak of upregulation at 13 DAA, fewer genes started to
145 be upregulated, although there were still over 500 genes upregulated at each of 15, 17, and 19 DAA.
146 Genes that were upregulated even at early stages of senescence tended to continue to increase in
147 expression throughout the timecourse. At the latest stages of the timecourse, when chlorophyll loss
148 was visible (23 and 26 DAA), very few genes started to be differentially expressed; no genes started to
149 be downregulated at either time point or upregulated at 26 DAA, whilst only 31 genes started to be
150 upregulated at 23 DAA (too few to be visible in Fig. 1B). The major shift from downregulation at the
151 early stages of senescence to upregulation at the later stages was also observed in Arabidopsis (Breeze
152 et al., 2011), and strikingly this occurs at a very similar period during the senescence process in both
153 species, prior to the visual loss of chlorophyll (29 to 33 DAS in Arabidopsis and 13 to 21 DAA in wheat,
154 Supplemental Fig S3).

155 We found that this temporal divide between downregulation at the early stages of senescence and
156 upregulation at the later stages was also reflected in different GO term enrichments in these groups
157 of DEGs (Fig. 1C; Supplemental Table S3). The large numbers of genes which started to be
158 downregulated at 3 and 7 DAA were enriched for GO terms relating to housekeeping functions (e.g.
159 translation, photosynthesis, and rRNA processing) as well as for central metabolic processes such as
160 amino acid biosynthesis and starch biosynthesis. These are very similar to the results found in
161 Arabidopsis where during senescence downregulated genes were significantly enriched for functions
162 in photosynthesis and carbohydrate and amino acid metabolism (Breeze et al., 2011). Alongside these
163 housekeeping functions, downregulated genes were enriched for defence responses and hormone
164 biosynthesis and signalling, indicating a reduction in the transcriptional responses to stimuli. This
165 differs from results in Arabidopsis where these GO terms were not enriched in downregulated genes.
166 Later in the timecourse, from 10 to 13 DAA, groups of genes started to be upregulated which were
167 involved in vesicle mediated transport and the proteasome, indicating a remobilisation of components
168 from the existing proteins. This is supported by the upregulation from 13 DAA of genes involved in
169 phosphate and protein transport. GO terms related to transport function were also enriched amongst
170 the upregulated genes in Arabidopsis, but this was principally before anthesis, a time point we did not
171 test (Breeze et al., 2011). From 15 DAA to 21 DAA waves of genes enriched for responses to cytokinin,
172 ABA and ethylene were upregulated, indicating a temporal hierarchy of hormone responses during

173 senescence. This hierarchy appears to be conserved between wheat and Arabidopsis with both species
174 showing earlier upregulation of genes enriched for ABA and JA signalling and responses (17 days after
175 anthesis in wheat, 23 days after sowing in Arabidopsis) and later upregulation of genes enriched for
176 ethylene signalling and responses (21 days and 33 days, respectively). However, the time separating
177 these different hormone responsive genes is longer in Arabidopsis (Supplemental Fig. S3).

178 Conservation of senescence-related genes between wheat, rice and Arabidopsis

179 Given the high degree of conservation of enriched GO terms between up and downregulated genes in
180 wheat and Arabidopsis, we compared the expression profiles of previously identified senescence-
181 associated genes from Arabidopsis with their orthologs in wheat. We first explored ten genes from
182 Arabidopsis which have been proposed as a basic set of genes to assess the progress of senescence
183 (Bresson et al., 2017). We found that their expression profiles between Bresson et al. (2017) and
184 Breeze et al. (2011) were largely consistent, although *CAT2* was described as a down-regulated gene
185 in Bresson et al. (2017), whereas its expression was upregulated during senescence in Breeze et al.
186 (2011) (Fig. 2, Supplemental Table S4), and *WRKY53* and *CAT3* were not differentially expressed in
187 Breeze et al. (2011). We were able to identify wheat orthologs for seven of these ten genes, and four
188 of these were differentially expressed during wheat senescence in a similar direction to that observed
189 in Arabidopsis (Fig. 2). However, the wheat orthologs of two well-known senescence associated genes
190 in Arabidopsis (*SAG12* and *ANAC092/ORE1*) were not differentially expressed during our wheat
191 senescence timecourse, consistent with independent wheat RNA-Seq datasets (Fig. 2, Supplemental
192 Table S4).

193 Since *ANAC092/ORE1*, part of a robust system controlling senescence in Arabidopsis (Kim et al., 2009),
194 was not differentially expressed in wheat we investigated whether other NAC transcription factors
195 (TFs) known to regulate senescence in Arabidopsis may be contributing to senescence in wheat
196 instead. We focussed on nine additional NAC TFs that regulate senescence in Arabidopsis (Fig. 2,
197 Supplemental Table S4). For five of these nine NACs, we were able to identify wheat orthologs.
198 *AtNAP/ANAC029* was identified as a key regulator of senescence in Arabidopsis (Guo and Gan, 2006)
199 and the ortholog in wheat displays a very similar increase in expression during senescence in our
200 timecourse and in an independent dataset (Fig. 2, Supplemental Table S4), suggesting it too may play
201 a role in regulating senescence in wheat. *ANAC082* and *ANAC090* form part of a NAC regulatory
202 module that governs the shift from positive to negative regulation amongst NACs at a pre-senescence
203 stage in Arabidopsis (Kim et al., 2018). However, *ANAC082* and *ANAC090* were not differentially
204 expressed in Arabidopsis, and the majority of their wheat orthologs were also not differentially
205 expressed during senescence (the third member of the regulatory module *ANAC017* did not have a
206 wheat ortholog). In contrast two other NACs known to regulate senescence in Arabidopsis, namely

207 *ANAC059/ORS1* (Balazadeh et al., 2011), a paralog of *ANAC092/ORE1*, and *ANAC042/JUB1* (Wu et al.,
208 2012), were upregulated during the earlier part of the timecourse in Arabidopsis, but no change in
209 expression was observed in the wheat orthologs. Together, these results show that although the broad
210 scale biological processes governing senescence are similar between Arabidopsis and wheat, many of
211 the individual genes influencing senescence are not conserved between the two species.

212 The lack of conservation between orthologous genes in wheat and Arabidopsis may be explained by
213 their evolutionary separation ~200 million years ago when dicots and monocots diverged (Bowers et
214 al., 2003; Wolfe et al., 1989). To put this finding in context, we examined the conservation of
215 senescence-related genes in rice, a monocot species which is more closely related to wheat (50 million
216 years since divergence (Charles et al., 2009)). Leng et al. (2017) identified 32 leaf senescence-related
217 genes in rice, of which we identified 26 to have orthologs in wheat (Supplemental Table S5). We
218 examined the expression of these rice genes during a timecourse of rice senescence from 4 to 28 days
219 after heading (Lee et al., 2017). The majority of these genes with wheat orthologs (17 out of 26) had
220 a conserved pattern of expression between wheat and rice (Supplemental Table S6). Five were
221 upregulated in both species including *SGR* which regulates chlorophyll degradation in rice (Park et al.,
222 2007), *OsNAC106* which inhibits leaf senescence (Sakuraba et al., 2015), and *OsNAP/PS1* which fine-
223 tunes ABA biosynthesis and leaf senescence in rice (Liang et al., 2014). One gene (*LTS1/OsNaPRT1*)
224 which plays a role in the NAD salvage pathway in rice (Wu et al., 2016) was downregulated in both
225 species whilst eleven genes were not differentially expressed during leaf senescence in either species.
226 Nine genes showed non-conserved patterns of expression between wheat and rice, including two
227 genes affecting jasmonate content (*OsPME1* and *OstSD2*) (Fang et al., 2016) that showed opposite
228 expression patterns between rice (upregulated) and one wheat homoeolog (downregulated; the other
229 homoeologs were not differentially expressed). Interestingly, of the seven senescence-related TFs
230 examined from rice, four showed conserved expression patterns between rice and wheat. We were
231 not able to identify wheat orthologs for the remaining three rice senescence-related TFs, suggesting
232 that the transcriptional control of senescence is not completely conserved between wheat and rice,
233 as previously demonstrated for *NAM-B1* (Distelfeld et al., 2012).

234 **Transcription factors regulating senescence**

235 We next examined TF expression patterns to understand how these highly ordered and coordinated
236 transcriptional changes are regulated in wheat. We found that 2,210 TFs were expressed (> 0.5 TPM)
237 during the timecourse but only 341 TFs (15.4%) were differentially expressed. A small number of TFs
238 (18 TFs; 5.2%) were both up and downregulated during the timecourse (Supplemental Table S2),
239 including *NAM-A1*, a known regulator of senescence in wheat. It is possible these TFs are bifunctional,
240 acting to both activate and repress senescence-related pathways, similar to the positive and negative

241 regulation exerted by *ANAC083* on other senescence associated NACs in Arabidopsis (Kim et al., 2018).
242 We calculated the percentage of differentially expressed TF per TF family across time (Fig. 3). In
243 general, each TF family tended to either be upregulated or downregulated as a whole (Fig. 3), although
244 there are exceptions such as the C2C2_CO-like and MADS_II family which showed upregulation and
245 downregulation of different family members during the timecourse.

246 While we observed a temporal gradient of TF families starting to be up and downregulated throughout
247 the timecourse, we defined an initial (3 to 7 DAA) and later wave (13-19 DAA) when many TF families
248 were up or downregulated. TF families that were upregulated in the initial wave from 3 to 7 DAA
249 include the RWP-RK, pseudo ARR-B, and CCAAT_HAP2 (NF-YA) families (Fig. 3A). A distinct set of TF
250 families were upregulated in the later wave from 13 to 19 DAA including CAMTA, GRAS and MADS_II.
251 After these waves of upregulation were initiated, the same families tended to continue to be
252 upregulated throughout the rest of the timecourse. Compared to all genes, the RWP-RK, CCAAT_HAP2
253 (NF-YA), and NAC families were significantly enriched ($p_{adj} < 0.01$, Fisher test; Fig. 3A) for upregulated
254 genes at early (RWP-RK and CCAAT_HAP2 (NF-YA)) and late (NAC) time points. In all three families,
255 over 30% of the expressed genes were upregulated during senescence corresponding to 61 NAC TFs
256 (32.4% of expressed NAC TFs) and eight RWP-RK and seven CCAAT_HAP2 (NF-YA) TFs (33.3% and
257 38.9% of expressed genes per family, respectively). The finding that NAC and CCAAT_HAP2 (NF-YA)
258 TFs are enriched for upregulated genes in senescing tissues is consistent with results in Arabidopsis
259 but, as discussed above, the exact gene family members involved in the senescence process may be
260 different between the two species. The RWP-RK family was not identified to be enriched for
261 upregulation in Arabidopsis, although this may be due to the RWP-RK genes being included within the
262 NLP family in the Arabidopsis analysis (Breeze et al., 2011).

263 In parallel with certain TF families being upregulated, another group of TF families were
264 downregulated during the senescence timecourse. The initial wave of downregulation largely
265 occurred at 7 DAA and included the AS2/LOB, bHLH_TCP, and MADS_I families. The later wave of
266 downregulation, initiated from 17 to 19 DAA, included the C2C2 GATA, GARP G2-like, and MADS_II
267 families. Similar to upregulation of TFs, the downregulation tended to continue throughout the rest
268 of the timecourse, indicating a gradual change in TF expression levels. None of the TF families were
269 significantly enriched for downregulated genes compared to all genes. These results differ from
270 Arabidopsis, where several families (C2C2-CO-like and TCP) were significantly enriched for
271 downregulated genes (Breeze et al., 2011).

272 These two waves of TF differential expression are analogous to the two waves of differential
273 expression observed for all gene classes (Fig. 1). This is consistent with TF roles as activators and

274 repressors of gene expression. These results suggest that specific TF families initiate temporally
275 distinct changes in gene expression, broadly classed into an initial (3 to 7 DAA) and later (13 to 19 DAA)
276 response.

277 Understand regulation using network modelling

278 Our results indicate that there are two main temporal waves of expression during senescence (from 3
279 to 7 DAA and from 13 to 19 DAA) that may be regulated by the associated up and downregulation of
280 particular TF families. However, to understand the interactions between TFs and predict which ones
281 may be key regulators (hub genes) driving this transcriptional programme, we constructed a gene
282 regulatory network. We used Causal Structure Inference (Penfold and Wild, 2011), which produces a
283 directional network of TF interactions. We used the 341 TFs that were differentially expressed during
284 the timecourse to build the network.

285 To interpret the network it is necessary to determine the 'edge weight threshold' at which to include
286 edges. Since our aim was to identify the most important TFs within the network to test as candidate
287 genes for the regulation of senescence, we decided to compare the network across different edge
288 weight thresholds. We hypothesised that by identifying TFs which were important across multiple
289 thresholds, we would be more likely to identify robust candidate genes. We found that from an edge
290 weight threshold of 0.01 to 0.3, the number of edges decreased from 12,832 to 61 (Table 1). *NAM-A1*,
291 a known regulator of senescence in wheat, was only present in the network at the lower thresholds
292 of 0.01, 0.05, and 0.1. We therefore decided to focus on the networks that included *NAM-A1*, as it is
293 likely that the more stringent thresholds (0.2 and 0.3) would also have excluded other TFs relevant to
294 the senescence process. The other TF which had previously been identified to regulate senescence in
295 wheat (*NAC-S*) was not detected as differentially expressed during our timecourse so it was not used
296 to construct the network or determine appropriate thresholds.

297 We determined the importance of a gene within the network using two measures: 'degree' which is
298 the number of direct connections to other genes, and 'betweenness centrality' which is a measure of
299 the number of shortest paths which pass through that gene and represents a measure of how essential
300 the gene is to the flow of information around the network. We calculated percentage rankings of
301 genes in each of the three edge weight thresholds that included *NAM-A1* (0.01, 0.05, and 0.1)
302 according to their degree and betweenness centrality to allow comparison across networks with
303 different numbers of genes (Supplemental Table S7).

304 Differentiating between top ranked candidate genes using complementary datasets

305 We hypothesised that TFs which were ranked highly in at least one threshold for degree and one
306 threshold for betweenness centrality would represent good candidate genes for further investigation.

307 To explore this, we selected the TFs which were ranked in the top 5%, 10%, 20%, and 30% in at least
308 one threshold for degree and one threshold for betweenness centrality (Table 2; Supplemental Table
309 S7). We evaluated these different top ranked candidate TFs using two additional datasets: 1)
310 expression data from an independent experiment with 70 tissues/time points in the spring wheat
311 cultivar Azhurnaya which included senescing leaves (Ramirez-Gonzalez et al., 2018) and 2) a GENIE3
312 network of predicted TF – target relationships from 850 independent expression samples (Ramirez-
313 Gonzalez et al., 2018).

314 Across all four thresholds, a higher percentage of TFs were upregulated in senescing tissues in
315 Azhurnaya (27.6% to 36.1%) than in the network as a whole (22.0%, Table 2). Similarly, a higher
316 percentage of these top ranked TFs shared predicted target genes with *NAM-A1* in the GENIE3
317 network than in our network as a whole (ranging from 30.4% to 45.1%, compared to 20.1%, Table 2).
318 The highest enrichments (36.1% for senescence expression and 45.1% for *NAM-A1* shared targets)
319 were both observed when we selected the top 10% of TFs in the network ($p < 0.06$ and $p < 0.001$,
320 Fisher's exact test). We also examined whether the predicted target genes from the GENIE3 network
321 were enriched for senescence-related GO terms. In this case only the TFs in the top 20% and top 30%
322 had more targets with senescence-related GO terms (8.0% and 8.9%, respectively) than TFs in the
323 whole network (4.9%). We also noticed that *NAM-A1* did not have target genes in the GENIE3 network
324 enriched for senescence GO terms, suggesting that this GO enrichment data may miss some relevant
325 candidate genes. Nevertheless, together these complementary data sources show that ranking the
326 genes within a CSI network by degree and betweenness centrality can be an effective strategy to
327 narrow down a candidate gene list.

328 The top 10% of TFs in the network were most likely to be upregulated during senescence in Azhurnaya
329 and to share predicted downstream targets with *NAM-A1*, therefore we considered these to represent
330 good candidate genes for further investigation. More stringent thresholds (top 5% of TFs) failed to
331 increase the enrichment of senescence-related TFs, and indeed failed to include *NAM-A1* which is a
332 known regulator of senescence. This highlights that the threshold chosen must be integrated with
333 biological knowledge to ensure it remains biologically relevant.

334 Amongst the 36 top ranked genes (top 10% threshold), we found that three TF families were enriched
335 compared to all 341 TFs in the network: GRAS, HSF, and RWP-RK ($\chi^2 < 0.001$, 0.01, and 0.05
336 respectively). Interestingly the RWP-RK family was also significantly enriched for upregulation during
337 senescence (Fig. 3A), in addition to being enriched amongst top ranked genes in the network.

338 Using the information from the other datasets mentioned above (Azhurnaya expression and GENIE3
339 network) we found that 13 out of the 36 top ranked genes from the network were expressed over

340 two-fold higher in senescing tissues than in other tissues across the Azhurnaya developmental
341 experiment (Fig. 4A). This independent dataset suggests that these 13 genes may play a specific role
342 in senescing tissues and we hypothesise that they would be less likely to induce pleiotropic effects
343 when their expression is altered in mutant or transgenic lines. We examined the function of the
344 orthologs of all 36 top ranked candidate genes in Arabidopsis and rice (Supplemental Table S7), but
345 only four of them had reported leaf senescence phenotypes in Arabidopsis, and none had senescence
346 phenotypes in rice. This supports the low conservation of ortholog function across species. Using the
347 independent GENIE3 TF - target network, we tested whether the 36 top ranked candidate genes had
348 any shared target genes with *NAM-A1*, which might indicate they act together in the same senescence-
349 related pathway. We found that 14 genes had one or more shared target genes with *NAM-A1* (Fig.
350 4B), and several of these were also upregulated in senescing Azhurnaya tissues (Fig. 4A). Even an
351 overlap of one target gene is significantly more than the expected zero overlap between *NAM-A1* and
352 a random TF (Sign test, $p < 0.001$). We found that only three of the candidate genes were direct targets
353 of *NAM-A1* in the GENIE3 network, and these included *NAM-D1*, the D-genome homoeolog of *NAM-*
354 *A1*, and *NAM-A2* and *NAM-D2*, paralogs of *NAM-A1* located on different chromosomes.

355 Validation of candidate gene *NAM-A2*

356 Using the additional information sources above, we selected *NAM-A2* (*TraesCS2A02G201800*) for
357 phenotypic characterisation in wheat because it was amongst our 36 top ranked candidate genes, was
358 upregulated in senescing leaves, and shared many downstream target genes with *NAM-A1*.
359 Furthermore, the *NAM-A2* homoeologs *NAM-B2* (*TraesCS2B02G228900*) and *NAM-D2*
360 (*TraesCS2D02G214100*) were also amongst the top 36 candidate genes. *NAM-A2* is a paralog of *NAM-*
361 *A1* which regulates senescence and nutrient remobilisation (Avni et al., 2014; Harrington et al., 2019;
362 Uauy et al., 2006). The homoeolog of *NAM-A2*, *NAM-B2*, was previously found to cause a slight delay
363 in senescence (Pearce et al., 2014), but *NAM-A2* has not been previously characterised so was a strong
364 candidate TF that might regulate senescence.

365 To test the predictions of our model, we identified TILLING mutations in *NAM-A2* and *NAM-B2* in a
366 tetraploid Kronos background (Uauy et al., 2009; Krasileva et al., 2017). Due to the potential
367 redundancy between homoeologs in wheat (Borrill et al., 2015b), we decided to generate double
368 *NAM-A2/NAM-B2* mutants through crossing. We identified a mutation leading to a premature stop
369 codon in *NAM-B2* (R170*; between subdomains D and E of the NAC domain (Kikuchi et al., 2000)),
370 which is predicted to abolish protein function by creating a truncated protein lacking part of the NAC
371 DNA-binding domain. For *NAM-A2*, we could not identify any mutations which would cause
372 truncations, so instead we selected three missense mutations that were in highly conserved domains
373 and were thus expected to play important roles in protein function (Fig. 5A), as has recently been

374 shown for *NAM-A1* (Harrington et al., 2019). These were located in the A, C, and D NAC subdomain
375 and were predicted to be highly deleterious according to SIFT (Ng and Henikoff, 2003) and PSSM scores
376 for the NAM family (pfam02365) (Marchler-Bauer et al., 2017). We crossed each of the *NAM-A2*
377 missense mutants to the *NAM-B2* truncation mutant to create segregating populations from which
378 wild type, single and double mutants were phenotyped in the F₃ generation.

379 Across the three populations with different missense mutations in *NAM-A2*, and a common truncation
380 mutation in *NAM-B2*, there was a significant delay of 4.9 days in flag leaf senescence in the double
381 mutant compared to wild type (padj <0.01, ANOVA post-hoc Tukey HSD; Fig. 5B-C). There were no
382 significant differences between the single mutants and wild type in flag leaf senescence. Peduncle
383 senescence was significantly delayed by 7.4 days in the double mutant compared to wild type (padj
384 <0.001, ANOVA post-hoc Tukey HSD; Fig. 5D). In addition, the single A mutant was significantly later
385 in peduncle senescence than wild type (3.9 days, padj <0.001, ANOVA post-hoc Tukey HSD). The single
386 B mutant was not significantly different from wild type suggesting that the A genome homoeolog has
387 a stronger effect on senescence than the B genome homoeolog. Since the comparison is between
388 different types of mutations (missense compared to a truncation mutation) interpretation of the
389 relative magnitudes is difficult, although the truncation mutation in the B genome would have been
390 expected to produce at least an equivalent effect to the missense mutation in the A genome. These
391 effects were largely consistent across the three different missense mutations, although the mutation
392 in subdomain C (G111R) had the largest effect when combined into a double mutant compared to wild
393 type (Supplemental Fig. S4).

394

395

396 Discussion

397 In this work, we have characterised the transcriptional processes associated with senescence in the
398 wheat flag leaf. We found that specific TF families are associated with these changes in transcription
399 and have used gene regulatory network modelling, alongside additional complementary information,
400 to identify candidate genes controlling this process. We confirmed that one of these genes, *NAM-A2*,
401 plays a role in senescence in wheat.

402 Time-resolved transcriptional control of senescence in wheat

403 We found that although 52,905 genes were expressed in senescing flag leaves, only 9,533 genes were
404 differentially expressed during this time period. Sampling ten time points allowed us to observe that
405 these 9,533 DEGs were largely divided into two temporal waves of transcriptional changes which may
406 not have been captured using a less time-resolved set of data. Frequent sampling has also proven
407 informative in other time-dependent processes in wheat, such as pathogen infection (Dobon et al.,
408 2016), and represents a powerful approach to understand the co-ordination and regulation of gene
409 expression changes throughout development and environmental responses (Bar-Joseph et al., 2012;
410 Lavarenne et al., 2018).

411 We found that during the first wave of transcriptional changes, the majority of DEGs were
412 downregulated, and these groups were enriched for GO terms related to translation, photosynthesis
413 and amino acid biosynthesis. During the second wave, genes started to be upregulated with
414 enrichment for GO terms related to vesicle mediated transport, protein transport, and phosphate
415 transport. The chronology of biological processes is well conserved with Arabidopsis. For example,
416 early downregulation of chlorophyll-related genes is observed in both Arabidopsis (Breeze et al., 2011)
417 and wheat, whilst transport processes are upregulated later during senescence. The temporal order
418 of senescence-related processes is also broadly conserved in maize although only three time points
419 were sampled making fine-grain comparisons difficult (Zhang et al., 2014). In rice, a longer timecourse
420 of flag leaf senescence has been studied (Lee et al., 2017), where the authors mainly focused on
421 comparing flag leaf and second leaf senescence; nevertheless broadly similar processes were observed
422 in both senescing rice leaves and the wheat flag leaf.

423 To test whether the conservation of biological processes during senescence between plant species is
424 controlled by orthologous genes, we examined the expression profiles of wheat orthologs of
425 Arabidopsis and rice senescence-related genes. We found that the expression profiles of wheat and
426 rice genes were conserved more frequently than those of Arabidopsis and wheat genes, although even
427 between the monocots (i.e. rice and wheat), only 65% of orthologs had similar expression profiles.

428 This result suggests that individual genes' functions may not be well conserved between species,
429 although similar gene families are involved. These results are consistent with results from experiments
430 studying flowering time, in which similar gene families were co-opted to regulate flowering time in
431 both monocots and dicots, although the individual genes' functions and their interactions are re-
432 arranged (Li and Dubcovsky, 2008).

433 The importance of TFs in tightly coordinating transcriptional changes during senescence is well known
434 from other plant species (Podzimska-Sroka et al., 2015; Woo et al., 2016). We found that particular TF
435 families were up and downregulated in two distinct waves, an initial and later response, following the
436 pattern for all DEGs. We found that three TF families were enriched for upregulated genes during
437 senescence at early (CCAAT_HAP2 and RWP-RK) and late (NAC) stages. Members of the NAC family
438 have been characterised to play a role in regulating senescence in both wheat (Uauy et al., 2006; Zhao
439 et al., 2015) and other plant species (Podzimska-Sroka et al., 2015). The CCAAT_HAP2 (NF-YA) family
440 is less well characterised in this process, but one member has been shown to delay nitrate-induced
441 senescence in Arabidopsis (Leyva-González et al., 2012) and the family was found to be enriched for
442 genes upregulated during Arabidopsis senescence (Breeze et al., 2011) suggesting a potential
443 conserved role. The RWP-RK family is known in Arabidopsis to control nitrogen responses (Chardin et
444 al., 2014), and in cereals nitrogen remobilisation is closely connected with senescence, highlighting
445 the potential for further investigations into this family in the future. Surprisingly, the WRKY TF family,
446 which has been reported to play important roles in senescence in several other species such as
447 Arabidopsis (Breeze et al., 2011; Woo et al., 2013), cotton (Lin et al., 2015), and soybean (Brown and
448 Hudson, 2017), was not enriched for upregulation during senescence in wheat. It is possible that
449 relatively few members of the WRKY family function in regulating senescence in wheat or that the
450 function of WRKY TFs has diverged between wheat and other plant species. This potential for
451 divergence in the regulation of senescence between species is supported by experiments
452 characterising the rice ortholog of *NAM-B1*. Whilst the *NAM-B1* TF in wheat regulates monocarpic
453 senescence (Uauy et al., 2006), the ortholog in rice (*Os07g37920*) regulates anther dehiscence and
454 does not affect monocarpic senescence (Distelfeld et al., 2012).

455 Identifying candidate genes in networks

456 One of the aims of this study was to identify TFs which regulate the process of senescence. The
457 rationale behind this approach was that TFs control other genes and therefore may have a strong and
458 readily detectable effect on the process of senescence. Secondly, in crops, TFs have been frequently
459 selected under quantitative trait loci for important traits such as flowering time (*PPD1*, *VRN1*) (Yan et
460 al., 2003; Beales et al., 2007) and cold tolerance (*CBF*) (Knox et al., 2008) due to their strong phenotypic

461 effects. Thus, identified candidate TFs regulating senescence might also prove to be useful breeding
462 targets.

463 Through examining the expression patterns of TFs in detail, we identified TF families which were
464 enriched for upregulation during senescence, however this analysis cannot provide information about
465 which of the individual TFs within the family might be more important in regulating the senescence
466 process. To address this question, we used Causal Structure Inference (Penfold and Wild, 2011) to
467 identify interactions between TFs. Our hypothesis was that central transcriptional regulators of
468 senescence would regulate other TFs to create a regulatory cascade to influence the thousands of
469 genes differentially expressed during senescence. We found that the most highly ranked candidate
470 genes from the network were enriched for senescence-related functions compared to all genes within
471 the network. We therefore propose that the ranking of candidate genes based on degree and
472 betweenness centrality can be a practical strategy to narrow down long lists of candidate genes from
473 network analyses.

474 Amongst the 36 top ranked TFs in the network, three TF families were enriched: GRAS, HSF, and RWP-
475 RK. Members of the GRAS family play diverse roles in plant development, and in particular the DELLA
476 subfamily has been reported to play a role in senescence in Arabidopsis (Chen et al., 2014). HSF TFs
477 are associated with stress responses, and although no members have been associated with
478 developmental senescence, stress-responsive genes are also closely associated with environmentally-
479 induced senescence, and common regulation has been observed in Arabidopsis (Woo et al., 2013).
480 The RWP-RK family is of interest because it is also significantly enriched for upregulation during
481 senescence, in addition to being enriched amongst top ranked genes in the network. This adds further
482 weight to the hypothesis that the RWP-RK TFs may play a role in senescence, in addition to their known
483 role in nitrogen responses (Chardin et al., 2014). The roles of these identified TFs can now be directly
484 tested in wheat to determine whether they regulate senescence using gene editing and TILLING
485 (Borrill et al., 2019).

486 To further delimit this list of candidate genes, we used information from independent datasets
487 (developmental timecourse of expression and GENIE3 TF-target network) to prioritise candidate
488 genes. The approach to combine additional data sets was also applied in Arabidopsis where a Y1H
489 screen was used in conjunction with Causal Structure Inference to help to identify regulatory
490 interactions in senescence and pathogen infection (Hickman et al., 2013). Another approach that can
491 be used to narrow down candidate genes is to examine how the network is perturbed in TF mutants.
492 This approach was used in Arabidopsis to identify three NAC TFs which regulate senescence (Kim et

493 al., 2018) and could now be applied in wheat using the TILLING mutant resource (Krasileva et al., 2017),
494 for example starting with the mutants generated in this study.

495 To test the predicted function of these candidate genes in regulating wheat senescence, we focused
496 on *NAM-A2*, which is a paralog of the known *NAM-B1* gene (Uauy et al., 2006). We found significant
497 delays in flag leaf and peduncle senescence in *NAM-A2/NAM-B2* double mutants, indicating that the
498 genes predicted by the network play roles in senescence. The peduncle senescence phenotype
499 indicates that this approach can identify genes that regulate senescence across different tissues, not
500 only in the flag leaf, and may reflect that monocarpic senescence in wheat is a developmental process
501 regulated across the whole plant (Harrington et al., 2019). Ongoing work is currently characterising
502 the additional candidate genes through the development of wheat double mutants for phenotypic
503 characterisation.

504 **Future directions**

505 This study has uncovered candidate TFs that may regulate senescence in wheat and has confirmed the
506 role of one of these genes in regulating senescence. It will be of great interest to determine whether
507 these genes only control senescence or also affect nutrient remobilisation and hence influence final
508 grain nutrient content. In addition to deepening our understanding of the molecular regulation of
509 senescence, this study lays the groundwork to use this network-enabled approach to identify TFs
510 regulating a range of different biological processes which happen across a timecourse. This approach
511 is not only applicable to developmental processes but could equally be applied to abiotic and biotic
512 stresses, as has been carried out in other plant species (Hickman et al., 2013). This approach could
513 also be applied to identify candidate genes for traits in species without genome sequences, although
514 a transcriptome would need to be assembled from the RNA-Seq data. The advent of genome-editing
515 means that the prediction of gene function could readily be tested in any transformable species.

516

517 **Conclusion**

518 The availability of a fully sequenced reference genome for wheat, alongside functional genomic
519 resources such as the TILLING population, have brought wheat biology into the genomics era and have
520 made possible studies which even a few years ago would have been unthinkable. Here we have used
521 these new resources to characterise the transcriptional processes occurring during wheat senescence.
522 We found that specific TF families are associated with this process in wheat, some of which have been
523 reported in other species, but others present new links between TF families and the process of
524 senescence. Although these associations do not prove causality, the hypotheses generated can now

525 be tested experimentally in wheat using TILLING or gene editing. Gene network modelling, when used
526 in conjunction with complementary datasets, is a powerful approach that can accelerate the discovery
527 of genes regulating biological processes in both model and crop species.

528

529 **Methods**

530 **Plant growth for RNA-Seq timecourse**

531 We pre-germinated seeds of hexaploid wheat cv. Bobwhite on moist filter paper for 48 h at 4°C
532 followed by 48 h in the dark at room temperature (~20°C). These pre-germinated seeds were sown in
533 P40 trays in 85% fine peat with 15% horticultural grit. Plants were potted on at 2–3 leaf stage to 1L
534 square pots with 1 plant per pot in Petersfield Cereal Mix (Petersfield, Leicester, UK). Plants were
535 grown in 16 h light at 20°C, with 8 h dark at 15°C. The main tiller was tagged at anthesis, and the
536 anthesis date was recorded.

537 *Phenotyping for RNA-Seq timecourse*

538 We measured the chlorophyll content of flag leaves across the timecourse from 3 to 26 days after
539 anthesis (DAA) using a SPAD-502 chlorophyll meter (Konica Minolta). The time points used were 3, 7,
540 10, 13, 15, 17, 19, 21, 23, and 26 DAA. We measured the flag leaf from the main tiller (tagged at
541 anthesis) for five separate plants for each time point, taking measurements at eight different locations
542 distributed along the length of each flag leaf. Three of these measured leaves were subsequently
543 harvested for RNA extraction.

544 We measured the grain moisture content across the timecourse from 3 to 26 days after anthesis, using
545 the same time points as for chlorophyll measurements. We harvested eight grains from central
546 spikelets (florete positions 1 and 2) within the primary spike of five separate plants at each time point,
547 these grains were weighed, and then dried at 65°C for 72 hours before re-weighing. The difference in
548 weight was used to calculate the percentage grain moisture content.

549 **Tissue harvest, RNA extraction and sequencing**

550 *Harvesting*

551 The flag leaf from the main tiller was harvested at 3, 7, 10, 13, 15, 17, 19, 21, 23, and 26 DAA from
552 three separate plants (three biological replicates). We harvested the middle 3 cm of the flag leaf
553 lengthways to have a region of the leaf which was synchronised in its developmental stage. We flash
554 froze the samples in liquid nitrogen, then stored them at -80°C prior to processing. In total we
555 harvested 30 samples.

556 *RNA extraction*

557 We ground the samples to a fine powder in mortar and pestles which had been pre-chilled with liquid
558 nitrogen. We extracted RNA using Trizol (ThermoFisher) according to the manufacturer's instructions,
559 using 100 mg ground flag leaf per 1 ml Trizol. We removed genomic DNA contamination using DNaseI
560 (Qiagen) according to the manufacturer's instructions and cleaned up the samples using the RNeasy
561 Mini Kit (Qiagen) according to the manufacturer's instructions.

562 *Library preparation*

563 The quality of the RNA was checked using a Tecan plate reader with the Quant-iT™ RNA Assay Kit (Life
564 technologies/Invitrogen Q-33140) and also the Quant-iT™ DNA Assay Kit, high sensitivity (Life
565 technologies/Invitrogen Q-33120). Finally the quality of the RNA was established using the
566 PerkinElmer GX with a high sensitivity chip and High Sensitivity DNA reagents (PerkinElmer 5067-
567 4626). 30 Illumina TruSeq RNA libraries were constructed on the PerkinElmer Sciclone using the
568 TruSeq RNA protocol v2 (Illumina 15026495 Rev.F). After adaptor ligation, the libraries were size
569 selected using Beckman Coulter XP beads (Beckman Coulter A63880). This removed the majority of
570 un-ligated adapters, as well as any adapters that may have ligated to one another. The PCR was
571 performed with a primer cocktail that annealed to the ends of the adapter to enrich DNA fragments
572 that had adaptor molecules on both ends. The insert size of the libraries was verified by running an
573 aliquot of the DNA library on a PerkinElmer GX using the High Sensitivity DNA chip and reagents
574 (PerkinElmer CLS760672) and the concentration was determined by using the Tecan plate reader.

575 *Sequencing*

576 The TruSeq RNA libraries were normalised and equimolar pooled into one final pool using elution
577 buffer (Qiagen). The library pool was diluted to 2 nM with NaOH and 5 µL transferred into 995 µL HT1
578 (Illumina) to give a final concentration of 10 pM. 120 µL of the diluted library pool was then
579 transferred into a 200 µL strip tube, spiked with 1% v/v PhiX Control v3 and placed on ice before
580 loading onto the Illumina cBot. The flow cell was clustered using HiSeq PE Cluster Kit v3, utilising the
581 Illumina PE_Amp_Lin_Block_Hyb_V8.0 method on the Illumina cBot. Following the clustering
582 procedure, the flow cell was loaded onto the Illumina HiSeq 2000/2500 instrument following the
583 manufacturer's instructions. The sequencing chemistry used was HiSeq SBS Kit v3 with HiSeq Control
584 Software 2.2.58 and RTA 1.18.64. Reads (100 bp, paired end) in bcl format were demultiplexed based
585 on the 6bp Illumina index by CASAVA 1.8, allowing for a one base-pair mismatch per library, and
586 converted to FASTQ format by bcl2fastq.

587 RNA-Seq data analysis

588 *Mapping*

589 We pseudoaligned the samples using kallisto v0.44.0 with default parameters to the RefSeqv1.0
590 annotation v1.1 (IWGSC et al., 2018). Transcripts per million (TPM) and counts for all samples were
591 merged into a single dataframe using tximport v1.0.3 (Soneson et al., 2016). Scripts for data analysis
592 are available from <https://github.com/Borrill-Lab/WheatFlagLeafSenescence>.

593 *Differential expression analysis*

594 We filtered for high confidence genes which were expressed on average > 0.5 TPM in at least one time
595 point; this excluded low expressed genes and low confidence gene models from further analysis,
596 consistent with previous analyses in wheat (Ramirez-Gonzalez et al., 2018). In total 52,905 genes met
597 this condition. We used the count expression level of these genes for differential expression analysis
598 using the R package ImpulseDE2 v1.4.0 (Fischer et al., 2018), all counts were rounded to the nearest
599 integer before they were analysed with ImpulseDE2. In parallel we used the TPM expression level of
600 these 52,905 genes for differential expression analysis using Gradient Tool v1.0 (Breeze et al., 2011)
601 with the normalisation enabled on Cyverse (<https://de.cyverse.org/de/>) (Merchant et al., 2016). To
602 select a high confidence set of DEGs we only retained genes which were differentially expressed padj
603 < 0.001 from ImpulseDE2 and which were differentially expressed according to Gradient Tool with a
604 z-score of $> |2|$. We grouped the 9,533 high confidence DEGs according to the first time point at which
605 they were up or downregulated, according to Gradient Tool. The Gradient Tool uses Gaussian process
606 regression to identify whether gene expression is increasing or decreasing at each time point (Breeze
607 et al., 2011). For example, a gene first upregulated at 10 DAA was in group "U10" (up 10 DAA), whereas
608 a gene first downregulated at this time point was assigned to group "D10" (down 10 DAA). Genes
609 which were both up and downregulated during the timecourse ($< 4\%$ of all DEGs) were grouped
610 according to the time point of first differential expression with the opposite change also indicated. For
611 example a gene upregulated at 10 DAA and then downregulated at 15 DAA was grouped as U10D (the
612 second time point of differential expression was not recorded in the grouping). These groupings are
613 available in (Supplemental Table S2). The minority of genes with both up and downregulation ($< 4\%$
614 of all DEGs) were excluded from GO term enrichment analysis.

615 *GO term enrichment*

616 We obtained GO terms from the RefSeqv1.0 annotation and transferred them from the annotation
617 v1.0 to v1.1. We only transferred GO terms for genes which shared $> 99\%$ identity across $> 90\%$ of the
618 sequence (105,182 genes; 97.5% of all HC genes annotated in v1.1). GO term enrichment was carried

619 out for each group of DEGs (groups defined according to the first time point at which genes were
620 upregulated or downregulated, see above) using Goseq v1.24.0.

621 *Ortholog identification*

622 We identified the rice and Arabidopsis orthologs of the wheat genes using *EnsemblPlants* ortholog
623 information downloaded via BioMart (Kersey et al., 2018). Functional annotation for Supplemental
624 Table S7 was obtained from funricegenes (Li et al., 2017), RAP-DB (Sakai et al., 2013), Araport (Cheng
625 et al., 2017), and literature searches.

626 *Arabidopsis and rice leaf senescence gene expression*

627 Arabidopsis gene expression data during a timecourse of leaf senescence was obtained from Breeze
628 et al. (2011). Gene expression patterns had already been assigned using Gradient Tool v1.0 by Breeze
629 et al. (2011). Rice gene expression data for a timecourse of flag leaf senescence were obtained from
630 Lee et al. (2017). Gene expression data was presented in this publication as the log₂ normalised read
631 count for each time point compared to expression level at the initial time point (4 days after heading).
632 The gradient tool had not been used on this dataset therefore we assigned a general trend in
633 expression (increasing or decreasing according to the log₂ fold change) for time points up to 28 days
634 after heading when the first loss of chlorophyll was observed (comparable to the end of our wheat
635 senescence timecourse) (Supplemental Table S6).

636 *TF annotation*

637 Genes which were annotated as TFs were obtained from
638 [https://opendata.earlham.ac.uk/wheat/under_license/toronto/Ramirez-Gonzalez_et_al_2018-06025-
639 Transcriptome-Landscape/data/data_tables/](https://opendata.earlham.ac.uk/wheat/under_license/toronto/Ramirez-Gonzalez_et_al_2018-06025-Transcriptome-Landscape/data/data_tables/) (Ramirez-Gonzalez et al., 2018).

640 *Gene regulatory network construction*

641 We selected the 341 TFs which were amongst the 9,533 DEGs. We used the TPM gene expression
642 values as input to Causal Structure Inference (CSI) v1.0 (Penfold and Wild, 2011) which was run
643 through Cyverse (<https://de.cyverse.org/de/>) (Merchant et al., 2016). The parameters used with CSI
644 were the defaults (parental set depth =2, gaussian process prior = 10;0.1, weight truncation = 1.0E-5,
645 data normalisation = standardise (zero mean, unit variance), weight sampling = FALSE). The output
646 marginal file was converted to Cytoscape format using hCSI_MarginalThreshold v1.0 in Cyverse with
647 a probability threshold of 0.01. We used this file for directed network analysis in Cytoscape v3.6.1
648 (Shannon et al., 2003) which produced network statistics. We used Cytoscape to filter the network for
649 degree and betweenness centrality at 0.01, 0.05, 0.1, 0.2, and 0.3.

650 *GENIE3 data*

651 We identified the targets of TF using a TF-target network which was previously published (Ramirez-
652 Gonzalez et al., 2018). Only connections amongst the top one million links were considered in this
653 analysis. The network had been produced by a random forest approach (GENIE3) (Huynh-Thu et al.,
654 2010) using 850 RNA-Seq samples.

655 *Visualisation*

656 Graphs were made in R using the packages ggplot2 (Wickham, 2016), NMF (aheatmap function)
657 (Gaujoux and Seoighe, 2010), and pheatmap (Kolde, 2013).

658 **Candidate gene validation**

659 *Phenotyping of NAM-2 mutants*

660 We selected mutant lines from the Kronos TILLING population (K0282, K0427, K3240) (Krasileva et al.,
661 2017) with missense mutations (G111R, G133D, P40S, respectively) in *NAM-A2*
662 (*TraesCS2A02G201800*). These *NAM-A2* mutant lines were crossed with a line containing a mutation
663 inducing a premature stop codon in *NAM-B2* (*TraesCS2B02G228900*) (K4452; R170*). For each of the
664 three crosses, heterozygous F₁ seeds (AaBb) were self-pollinated to produce an F₂ population. We
665 selected double homozygous mutant (aabb), single homozygous mutant (aaBB or AAbb), and double
666 homozygous wild type plants (AABB) in the F₂ using KASP markers (Supplemental Table S8) as
667 described in Ramirez-Gonzalez et al. (2015). Seeds from two individuals of each genotype in the F₂
668 population were grown in greenhouse conditions for phenotyping from Jan 2018 – May 2018 in
669 Norwich with 16 h supplemental lighting and a daytime temperature of 18°C, and a night-time
670 temperature of 12°C. For each genotype we tagged the main tiller at anthesis and recorded the
671 anthesis date for 16-20 individual plants. We scored flag leaf senescence as the date when the flag
672 leaf of the main tiller had lost chlorophyll from 25% of the flag leaf blade. We scored peduncle
673 senescence as the date when the top 3 cm of the peduncle lost all green colour and turned straw-
674 yellow.

675 *Statistical analyses*

676 Statistical analyses were carried out using the base R package. Statistical tests used and the number
677 of samples are indicated in the appropriate figure legends.

678 *Accession numbers*

679 RNA-Seq raw reads have been deposited in the SRA accession PRJNA497810. The original TILLING
680 mutant lines can be ordered from JIC Germplasm Resources Unit (<https://www.seedstor.ac.uk/>).

681 *NAM-A2* is *TraesCS2A02G201800* in the RefSeqv1.1 gene annotation available from EnsemblPlants
 682 (https://plants.ensembl.org/Triticum_aestivum/Info/Index).

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689

690 Tables

691 **Table 1.** Comparing CSI network at different thresholds for edge weight.

Edge weight threshold	# TFs	# edges	<i>NAM-A1</i> included in network
0.01	341	12,832	Yes
0.05	295	843	Yes
0.1	190	277	Yes
0.2	99	109	No
0.3	64	61	No

692

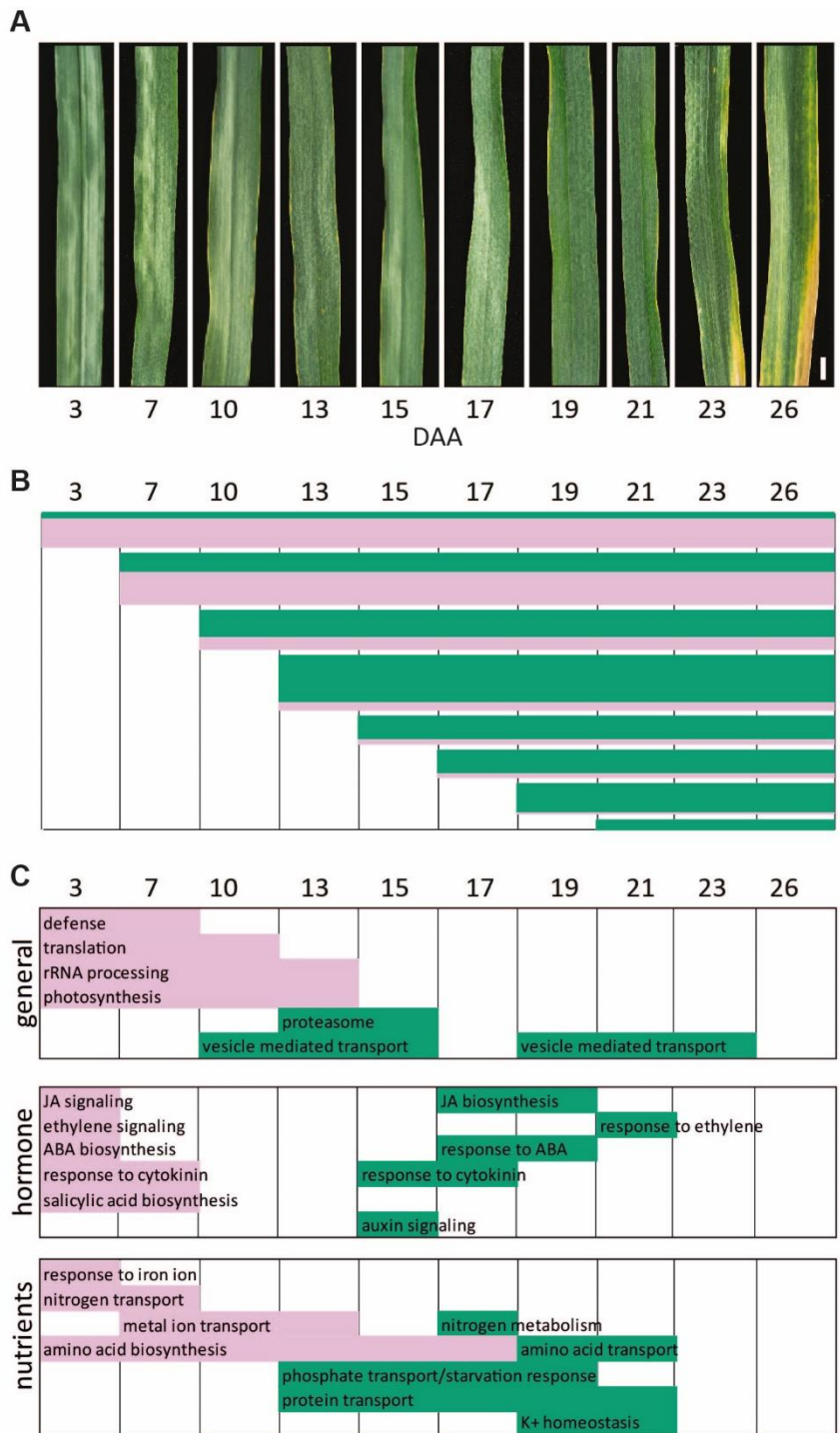
693 **Table 2.** Evaluation of candidate TFs selected from the CSI network using independent data sources.
 694 We selected the TFs which were ranked in the top 5%, 10%, 20%, and 30% in at least one threshold
 695 for degree and one threshold for betweenness centrality as potential candidate genes for further
 696 investigation. Independent data sources were used to determine whether these top candidate genes
 697 were enriched for TFs which 1) were upregulated in independent gene expression data (Azhurnaya
 698 developmental timecourse), 2) had target genes predicted in a GENIE3 network which were shared
 699 with *NAM-A1*, and 3) had target genes predicted in a GENIE3 network which were enriched for
 700 senescence GO terms.

TF group	Number of TFs	<i>NAM-A1</i> included	Percentage of TFs upregulated 2x in Azhurnaya	Percentage of TFs with GENIE3 targets	Percentage of TFs with GENIE3 targets enriched for
----------	---------------	------------------------	---	---------------------------------------	--

				shared with <i>NAM-A1</i>	senescence GO terms
Entire network	341	Yes	22.0	20.1	4.9
Top 30%	140	Yes	28.6	30.4	8.9
Top 20%	105	Yes	27.6	34.0	8.0
Top 10%	36	Yes	36.1	45.1	3.2
Top 5%	15	No	33.3	33.3	0

701

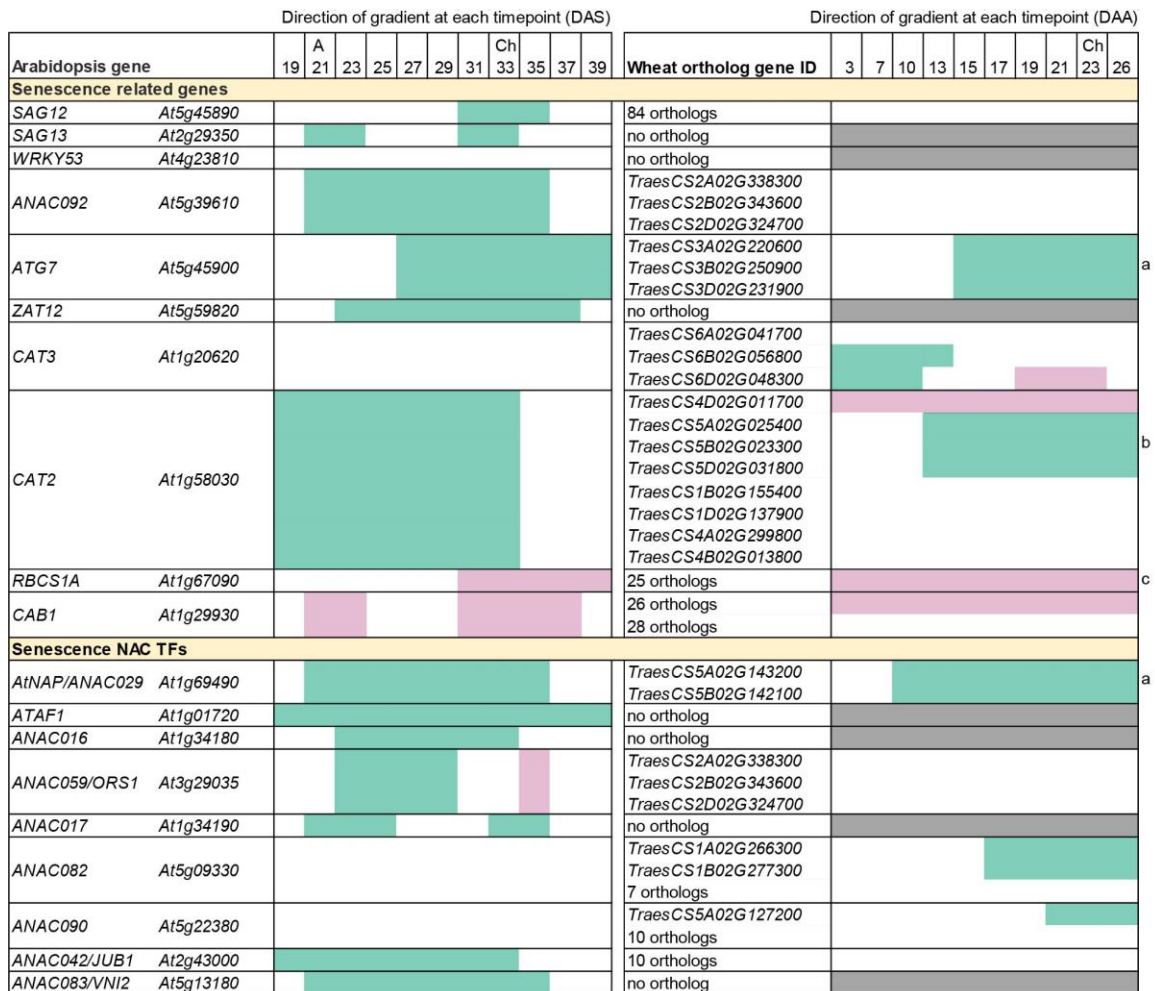
702 **Figures**



703

704 **Figure 1. Transcriptional re-programming during flag leaf senescence.** A) Timecourse of flag leaf
 705 senescence from 3 to 26 days after anthesis (DAA), scale bar represents 1 cm. B) Diagram showing
 706 representative patterns for genes which are consistently upregulated (green) or consistently
 707 downregulated (pink) during senescence (96.2% of DEGs). Genes were grouped according to the first
 708 time of up or downregulation. The majority of genes in each pattern continued to be up or
 709 downregulated across the whole timecourse. Bar heights represent the number of genes in each

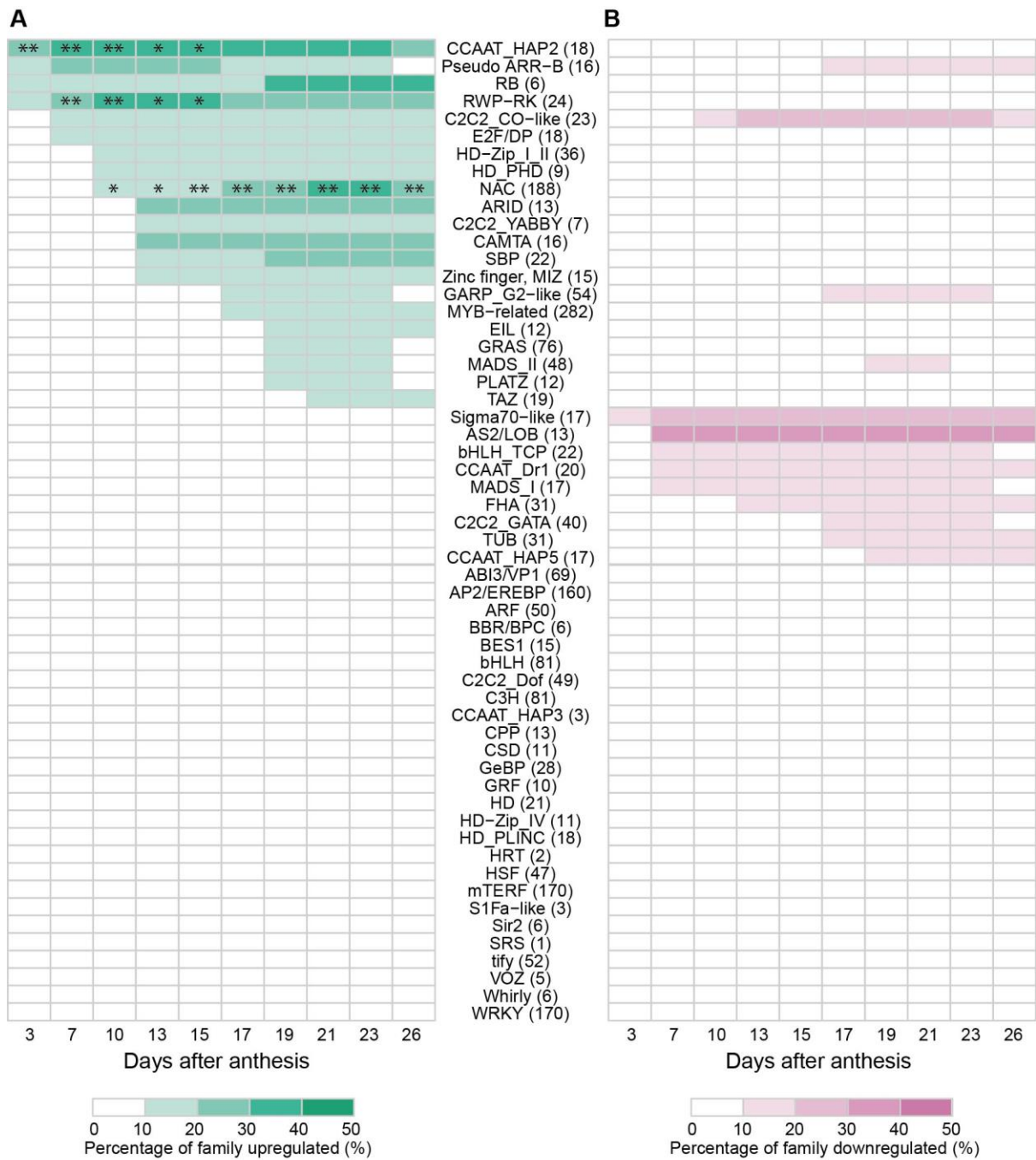
710 expression pattern. The x axis represents time after anthesis, the axis is represented uniformly
 711 although time points are not evenly spaced. C) GO term enrichments are shown related to general,
 712 hormone and nutrient related processes. Filled rectangles represent that genes starting to be
 713 differentially expressed at that time point are enriched for that specific GO terms. Green rectangles
 714 represent upregulated genes, pink rectangles represent downregulated genes.



715 Figure 2. Expression profiles of Arabidopsis senescence-related genes and NAC transcription factors (left) and their
 716 wheat orthologs (right). The significant gradient changes are indicated over the course of an 11 time point timecourse
 717 in Arabidopsis (4th rosette leaf from 19 to 39 days after sowing (DAS), Breeze et al., 2011) and over a 10 time point
 718 timecourse in wheat (flag leaf from 3 to 26 days after anthesis (DAA), this study, see Figure S3). The 21 DAS time
 719 point in the Arabidopsis data corresponds to anthesis (A), equivalent to 0 DAA for the wheat data. Likewise, the first
 visible loss of chlorophyll (Ch) occurs at 33 DAS and 23 DAA, respectively (see Figure S3). The direction of the
 gradient at each time point is highlighted as either upregulated (green) or downregulated (pink). Cells in white
 indicate there was no change at this time point. Grey cells indicate that no wheat ortholog was identified. One wheat
 ortholog is represented per row, except where five or more wheat orthologs with the same pattern of expression were
 identified, for clarity these are represented within a single row. a Representative pattern shown, full details in Table
 S4. b Representative pattern shown for chromosome 5 homoeologs, full details in Table S4. c 23 out of 25 orthologs
 were DE, all with this pattern.

715
 716 **Figure 2. Expression profiles of Arabidopsis senescence-related genes and NAC transcription factors**
 717 **(left) and their wheat orthologs (right).** The significant gradient changes are indicated over the course
 718 of an 11 time point timecourse in Arabidopsis (4th rosette leaf from 19 to 39 days after sowing (DAS),
 719 Breeze et al., 2011) and over a 10 time point timecourse in wheat (flag leaf from 3 to 26 days after

720 anthesis (DAA), this study, see Figure S3). The 21 DAS time point in the Arabidopsis data corresponds
721 to anthesis (A), equivalent to 0 DAA for the wheat data. Likewise, the first visible loss of chlorophyll
722 (Ch) occurs at 33 DAS and 23 DAA, respectively (see Figure S3). The direction of the gradient at each
723 time point is highlighted as either upregulated (green) or downregulated (pink). Cells in white indicate
724 there was no change at this time point. Grey cells indicate that no wheat ortholog was identified. One
725 wheat ortholog is represented per row, except where five or more wheat orthologs with the same
726 pattern of expression were identified, for clarity these are represented within a single row. ^a
727 Representative pattern shown, full details in Supplemental Table S4. ^b Representative pattern shown
728 for chromosome 5 homoeologs, full details in Supplemental Table S4. ^c 23 out of 25 orthologs were
729 DE, all with this pattern.



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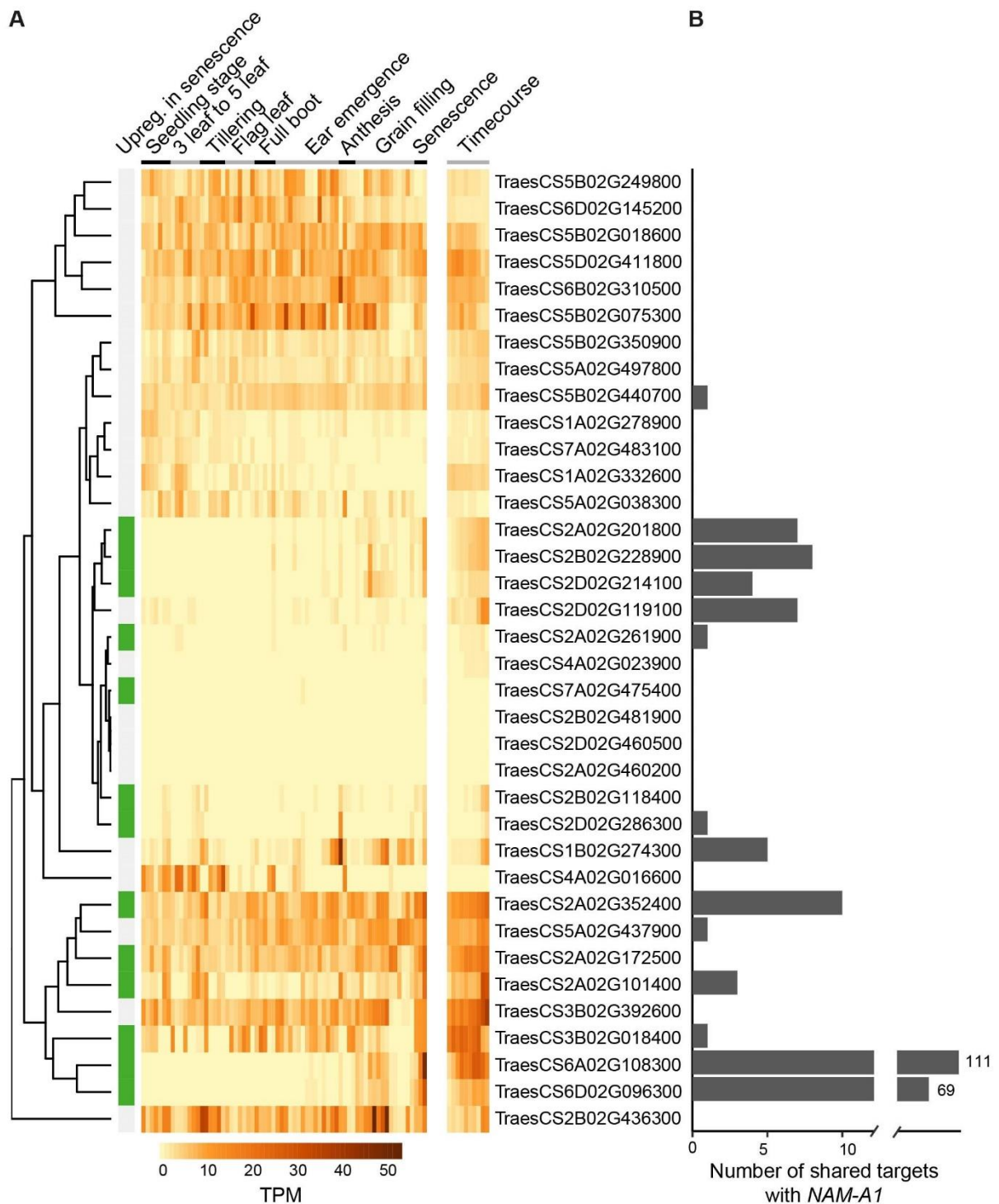
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Figure 3. Percentage of expressed genes which were differentially expressed per transcription factor family at each time point. Upregulated (A) and downregulated (B) genes are shown. The total number of genes expressed in each family is shown in brackets after the family name. Time points during which specific transcription factor families which were significantly enriched for upregulation are indicated with asterisks (* = $p < 0.05$, ** = $p < 0.01$). No families were significantly enriched for downregulation.



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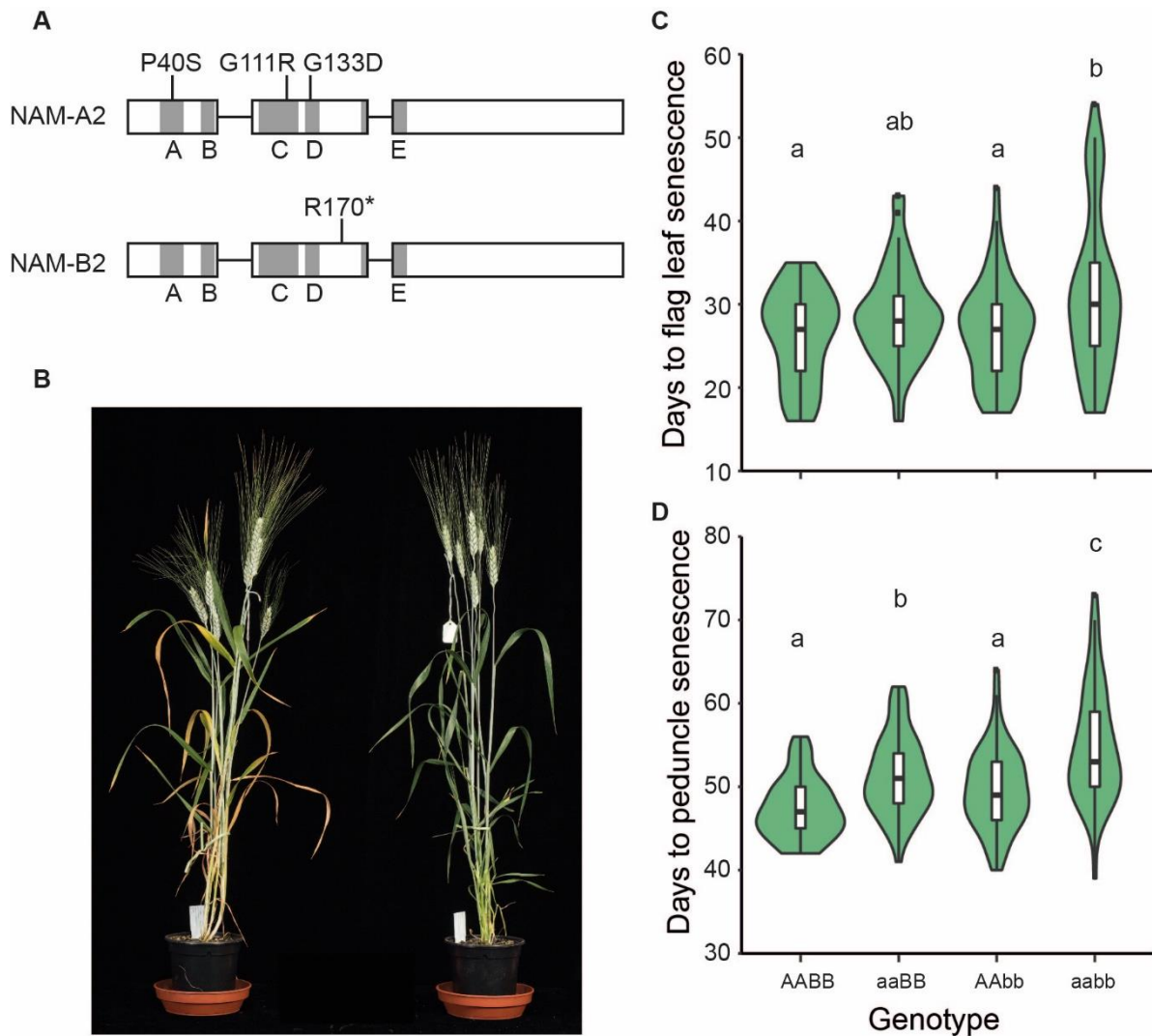
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Figure 4. Additional information for 36 top ranked candidate genes. A) Expression from an independent RNA-Seq experiment using Azhurnaya spring wheat (left part of heatmap) and expression in the senescence timecourse (right part of heatmap, “Timecourse”). Each of the 36 genes is represented in one row, and rows are sorted according to the similarity of the expression patterns (dendrogram to left). Expression level is measured in transcripts per million (TPM). Genes which were over two-fold upregulated in senescence compared to other tissues/time points in Azhurnaya are highlighted by green boxes in the left-hand column (“Upreg. in senescence”). B) The number of targets

744 each transcription factor shares with *NAM-A1*, predicted by the independent GENIE3 network. *NAM-*
 745 *A1* (*TraesCS6A02G108300*) has 111 targets and its homoeolog *NAM-D1* (*TraesCS6D02G096300*) has
 746 69 shared targets, shown with broken axis.



747
 748 **Figure 5. Mutants in *NAM-A2* and *NAM-B2*.** A) Selected missense mutations in *NAM-A2* and stop
 749 mutation in *NAM-B2*. Grey regions are the NAC subdomains A-E. Subdomain E spans the end of exon
 750 2 and the start of exon 3. B) Wild type sister line (left) and *NAM-A2 NAM-B2* double homozygous
 751 (*aabb*) mutant (right), 37 days after anthesis. C) Days from heading to flag leaf senescence and D) days
 752 from heading to peduncle senescence in wild type, single and double mutants across all three
 753 populations (different missense mutation in *NAM-A2*, common truncation mutation in *NAM-B2*).
 754 Letters indicate significant differences $p < 0.05$, with ANOVA post-hoc Tukey HSD, $n = 53-61$ individual
 755 plants per genotype. The populations are shown separately in Supplemental Figure S4.

756

757 **Supplemental Data**

758 The following supplemental materials are available.

759 **Supplemental Figure S1.** SPAD chlorophyll meter readings for flag leaves across the timecourse.

760 **Supplemental Figure S2.** Grain moisture content across the timecourse.

761 **Supplemental Figure S3.** Comparison of senescence progress in wheat and Arabidopsis leaves.

762 **Supplemental Figure S4.** Senescence phenotypes of individual missense mutations in *NAM-A2*.

763

764 **Supplemental Table S1.** Total number of reads and pseudoaligned reads per sample.

765 **Supplemental Table S2.** The 9,533 genes differentially expressed during senescence.

766 **Supplemental Table S3.** GO terms enriched per grouped expression pattern.

767 **Supplemental Table S4.** Wheat orthologs of Arabidopsis senescence-related genes.

768 **Supplemental Table S5.** Wheat orthologs of senescence-related rice genes.

769 **Supplemental Table S6.** Expression patterns of wheat orthologs and senescence-related rice genes.

770 **Supplemental Table S7.** The 341 transcription factors in the CSI network with additional information.

771 **Supplemental Table S8.** Primers used for KASP genotyping of *NAM-2* mutants.

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