

1 **A high soluble-fibre allele in wheat encodes a defective cell wall peroxidase responsible for  
2 dimerization of ferulate moieties on arabinoxylan**

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12

13 **Abstract**

14 Increasing dietary fibre (DF) intake is an important target to improve health and an attractive  
15 strategy for this is to increase the fibre content of staple foods, particularly white bread which is the  
16 staple food in many countries. DF in wheat white flour is derived principally from the endosperm cell  
17 wall polysaccharide arabinoxylan (AX) and the water-extractable form of this (WE-AX) accounts for  
18 the majority of soluble dietary fibre (SDF), which is believed to confer particular health benefits. We  
19 previously identified QTLs for soluble dietary fibre (SDF) on 1B and 6B chromosomes in wheat in  
20 biparental populations. Here we show that the 6B high SDF allele encodes a peroxidase protein  
21 (PER1-v) with a single missense compared to the more common low SDF form (PER1). Wheat lines  
22 with the natural PER1-v allele and with an induced knock-out mutation in PER1 showed similar  
23 characteristics of reduced dimerization of ferulate associated with water-extractable WE-AX.  
24 Decreased ferulate dimerization is associated with decreased cross-linking of the WE-AX chains and  
25 increased solubility of AX. Transiently expressed PER1\_RFP fusion driven by native promoter in  
26 wheat endosperm was shown to localise to cell walls whereas PER1-v\_RFP did not; we therefore  
27 propose that PER1-v lacks capacity to dimerise AX ferulate *in vivo* due to mis-localisation. PER1 is  
28 the first peroxidase reported to be responsible for oxidative coupling of ferulate on AX, a key process  
29 in all grass cell walls. Understanding its role and the effect of variants on AX properties offers a route  
30 to control the properties of wheat DF in the human diet.

31

32 Background

33 Bread wheat is the most widely grown food crop globally and this single species accounts for ~20%  
34 of human food calories [[www.fao.org/faostat](http://www.fao.org/faostat)]. Dietary fibre (DF) is an essential component of the  
35 human diet and the intake of cereal fibre is associated with a reduced risk of a range of chronic  
36 diseases (Gill et al., 2021). The mechanisms of action of fibre are complex and still incompletely  
37 understood. However, it has been suggested that the balance of three properties, solubility, viscosity  
38 and fermentability in the colon, are important in determining the behaviour of fibre in the GI tract  
39 (Gill et al., 2021). Hence, soluble and insoluble forms of fibre share some health benefits but also  
40 differ in others.

41 Wheat is a major source of DF in the western diet, but most products consumed are made from  
42 white flour which has a lower content of DF than whole grain (about 4% dry wt. compared with over  
43 10% dry wt.). Increasing the fibre content of white flour is therefore an attractive strategy to deliver  
44 health benefits to consumers. The major DF component in white flour is the cell wall polysaccharide  
45 arabinoxylan (AX), accounting for about half of the total DF with water-extractable (WE)-AX  
46 accounting for most SDF and nearly all of the associated viscosity (Freeman et al., 2016). The  
47 amounts of both total AX and WE-AX in white flour vary between wheat genotypes, from about 1.35  
48 to 2.75% dry wt. and 20% and 50% of the total, respectively (Gebruers et al., 2008). Several studies  
49 have found QTLs and Marker-Trait Associations (MTAs) for total and WE-AX in wheat (Charmet et al.,  
50 2009; Nguyen et al., 2011; Quraishi et al., 2011; Yang et al., 2014; Marcotuli et al., 2015; Yang et al.,  
51 2016; Zhan et al., 2019; Lovegrove et al., 2020; Ibba et al., 2021) but no causal genes have been  
52 experimentally demonstrated.

53 Possible candidate genes include those involved in synthesis of AX. AX is a chain of  $\beta$ -1,4-linked  
54 xylopyraonsyl units decorated with  $\alpha$ -1,3- and  $\alpha$ -1,2-linked arabinofuranosyl units; some of  $\alpha$ -1,3-  
55 linked arabinofuranosyl are themselves 5-O- substituted with ester-linked feruloyl residues. These  
56 ferulate moieties are key for functionality because they allow for radical oxidative coupling to form  
57 ferulate dimers that can cross-link chains of AX (Burr and Fry, 2009; Pellny et al., 2020). This cross-  
58 linking is believed to bind AX more tightly into the endosperm cell wall (Saulnier et al., 2007) so  
59 decreasing the extent of cross-linking would be expected to increase the amount of WE-AX. Cross-  
60 linking of ferulate moieties on AX has significance beyond wheat grain as it is a key process in all grass  
61 cell walls, determining primary wall extensibility and biomass recalcitrance to digestion and is likely a  
62 capability that contributed to evolutionary success of the grasses (Chandrakanth et al., 2023). It has  
63 been shown that dimerization of ferulate on AX in grass cell walls requires the action of an

64 apoplastic peroxidase (Burr and Fry, 2009) but the peroxidase gene family is large (Passardi et al.,  
65 2004), and the identity of these peroxidases has not been established for any grass cell wall.

66 Previous studies have identified a QTL for SDF on chromosome 6B in a Yumai34 x Valoris wheat  
67 population (Charmet et al., 2009; Lovegrove et al., 2020). Here we show that the causal gene is  
68 *TraesCS6B02G042500* encoding a peroxidase responsible for cross-linking ferulate moieties on AX,  
69 decreasing water extractability. The high SDF Valoris allele contains a single missense SNP that  
70 appears to prevent localisation of the encoded protein to the cell wall thus disrupting function,  
71 resulting in less cross-linking and more WE-AX.

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73

74 Results

75 *RNAseq from endosperm of lines from Yumai34 x Valoris population identifies PER1 as candidate*  
76 *causal gene*

77 We previously mapped significant QTLs for relative viscosity of grain extracts (a property conferred  
78 by WE-AX) on chromosomes 1B and 6B and a more minor QTL on 1A in the Yumai34 x Valoris  
79 population of doubled-haploid lines (DHLs) (Lovegrove et al., 2020). To identify candidate genes for  
80 the 1B and 6B QTLs we analysed RNA-seq from pools of 4 DHLs with contrasting genotypes at these  
81 two QTLs (but all with Yumai34 allele at 1A).

82 Transcripts for enzymes of AX synthesis typically peak around 12-18 days post anthesis (dpa) (Pellny  
83 et al., 2012), so we isolated RNA from endosperm at 17 dpa. The 95% confidence intervals  
84 [19,946,488 – 28,116,185] for the 6B QTL encompass 323 protein-coding genes annotated in wheat  
85 reference genome IWGSC Refseq 1.1; of these 27 were expressed (average FPKM>0.5) and 16 were  
86 differentially expressed (FDR<0.05) and/or had splicing differences or coding polymorphisms. From  
87 these 16, we identified neighbouring genes TraesCS6B02G042500 and TraesCS6B02G042600  
88 annotated as peroxidases as top candidates due to their expression pattern (high in endosperm  
89 during grain fill, not expressed in non-grain tissue) and proximity to the QTL peak. The two genes are  
90 result of tandem duplication (sharing 85% CDS identity) and each has 4 exons. RNA-seq mapped to  
91 these two genes (Figure 1A) shows that all abundant transcripts are made up of 4 exons but can be  
92 from either gene or from TraesCS6B02G042500 exons 1 and 2 combined with TraesCS6B02G042600  
93 exons 3 and 4. Therefore these two separate gene models actually represent splice variants, albeit  
94 ones sharing no exons. We named the transcripts and encoded proteins PER1 (identical to  
95 TraesCS6B02G042500.1) PER2 (identical to TraesCS6B02G04600.1) and PER1&2 for the hybrid  
96 comprised of PER1 exons 1,2 and PER2 exons 3,4. DHLs with the Valoris allele at this 6B locus  
97 showed highly significant ( $P<0.001$ ) differences in expression and splicing with marked decreases in  
98 PER1&2 and increases in PER2 expression (Fig. 1B); the Yumai34 sequence is identical to Chinese  
99 Spring reference whereas Valoris PER1 has 3 synonymous SNPs and one missense SNP in exon 1 of  
100 PER1. The missense results in a Ser51Phe change in the protein encoded by the Valoris allele which  
101 we call PER1-v. Both PER1 and PER2 have homeologues on the A and D subgenomes but PER1A is  
102 not expressed, and the other forms are less expressed than PER1 and PER2 (Fig. 1B). We  
103 hypothesised that, despite the likely redundancy between these forms, the missense and/or lower  
104 expression of PER1-v in Valoris might be the cause of the higher SDF.

105

106 *DHLs with Valoris allele at 6B have lower dimerization of ferulate in endosperm WE-AX*

107 The identification of PER1 as candidate causal gene suggested that the mode of action for the 6B  
108 QTL could be via the dimerization of ferulate on AX, with lower dimerization associated with the  
109 Valoris PER1-v allele. We have previously determined dimerization of wheat endosperm AX ferulate  
110 in total and WE endosperm fractions and increases were seen in both when genes responsible for  
111 synthesis of the AX xylan backbone were downregulated (Freeman et al., 2017; Pellny et al., 2020).  
112 Here, we observed significantly lower ferulate dimerization on WE-AX in DHLs with the Valoris  
113 genotype (6B:H) to those with the Yumai34 genotype (6B:L) in the 6B QTL region (Table 1). Out of  
114 the five ferulate dimer (diFA) peaks detected, the ratios to FA monomer were all decreased in 6B:H  
115 DHLs and the sum of all dimers expressed as % dimerization was significantly decreased (F. prob =  
116 0.014) by ~30%. The effects of 1B:H were more complex, decreasing some dimers significantly but  
117 having no significant effect on overall dimerization. There were also significant effects of 1B and 6B  
118 genotypes and interaction of these on FA monomer amount per unit dwt endosperm (Table 1).

119

120

121

122 Table 1 Amounts of ferulate monomer (FA) and dimers (diFA) in WE fractions from endosperm of  
123 DHLs grouped according to genotype at 1B and 6B QTLs. Results from a 2-way ANOVA of 1B x 6B  
124 genotype showing means for each group (n=3) and F probability for effects.

diFA / FA (w/w)								
		Dimerisation						
means	Dimerisation [tot diFA / (tot diFA + FA)]					diF8-0-4	FA ( g / g dwt)	
		diF8-8AT	diF8-8	diF8-5	diF5-5	& diF8- 5BF		
	1B:L 6B:L	22%	0.065	0.024	0.046	0.08	0.061	6.3
	1B:H 6B:L	19%	0.038	0.072	0.032	0.048	0.049	12.9
	1B:L 6B:H	15%	0.032	0.017	0.023	0.058	0.05	10.5
	1B:H 6B:H	14%	0.032	0.015	0.027	0.046	0.04	13.4
F prob.								
	1B	0.319	0.11	0.405	0.665	0.004	0.024	<.001
	6B	0.014	0.026	0.251	0.266	0.072	0.041	0.013
	interaction	0.794	0.09	0.362	0.477	0.125	0.755	0.035

125

126

127

128 Table 2 Amounts of ferulate monomer (FA) and dimers (diFA) in endosperm of Cadenza BC2F2 lines  
129 segregating for KO mutation in PER1. Results from a 1-way ANOVA of mutation showing means for  
130 each group (n=7) and F probability for effect.

131

diFA / FA (w/w)									
		Dimerisation							
means	Dimerisation [tot diFA / (tot diFA + FA)]					diF8- 0-4	FA ( g / g dwt)		
		diF8-8AT	diF8-8	diF8-5	diF5-5	diF8- 5BF			
	mutant	15.5%	0.018	0.036	0.049	0.027	0.052	0.053	88.8
	null	17.0%	0.024	0.04	0.05	0.035	0.056	0.061	79.3
F prob.									
	mutation	0.037	0.019	0.227	0.830	0.030	0.346	0.046	0.194

132

133

134

135

136 *Wheat lines with an induced mutation in PER1 have lower dimerization of ferulate in endosperm*

137 Using the mutagenised, exome-sequenced population of wheat var. Cadenza (Krasileva et al., 2017),  
138 we identified a line carrying a premature stop codon in exon 2 of PER1. We twice backcrossed lines  
139 to Cadenza wild-type and allowed lines to self-fertilise for 2 further generations (BC2F2 lines) and  
140 identified segregants that were homozygous for the mutation and those that were homozygous for  
141 the wild-type allele. We then compared the ferulate dimerization in endosperm tissues from these;  
142 BC2F2 lines carrying the mutation had significantly (F prob. = 0.037) lower ferulate dimerization in  
143 endosperm (Table 2) supporting the putative role of PER1.

144

145 *PER1\_RFP fusion protein transiently expressed in wheat endosperm localises partially to cell wall but*  
146 *PER1-v\_RFP does not*

147 To investigate whether the missense SNP could explain the effect of PER1-v allele (as opposed to the  
148 expression difference; Fig. 1B) we made constructs with and without the missense SNP to encode  
149 PER1 and PER1-v, both fused at C-terminal to RFP reporter and driven by the endogenous promoter.  
150 We transiently transformed wheat endosperm tissue with plasmids containing these constructs. For  
151 both constructs, the RFP signal was mostly in the cytoplasm, possibly as a result of high expression  
152 overloading the capacity of the cell to normally process the protein which is predicted to require  
153 signal peptide cleavage and N-glycosylation. However in plasmolysed cells expressing PER1\_RFP we  
154 also observed RFP signal associated with the cell wall whereas in plasmolysed cells expressing PER1-  
155 v\_RFP we never observed cell wall RFP signal (examples shown in Figure 2, the complete set of  
156 images with clearly plasmolysed cells are in Supplemental Figures S1-S4). In total, we observed cell  
157 wall RFP signal in 41 out of 43 plasmolysed cells with PER1\_RFP whereas there were none in 40  
158 plasmolysed cells expressing PER1-v\_RFP). Since we would expect most or all AX ferulate  
159 dimerization to occur in the cell wall (Burr and Fry, 2009), the failure of PER1-v to localise to the cell  
160 wall means it is likely defective in function.

161

162 *Occurrence of missense SNP in wheat varieties*

163 The effect of the single missense SNP on PER1 localisation strongly suggests that this is the cause of  
164 the 6B QTL for SDF. If this is the case, this SNP which is chr6B 26076727 C->T on IWGSC 1.0 reference  
165 (Axiom marker AX-94816599) represents a perfect marker for the trait. This SNP was also one of 5  
166 closely neighbouring SNPs on 6B found by GWAS as associated with equal significance to high

167 relative viscosity of aqueous extracts in the WHEALBI panel (Lovegrove et al., 2020). The increasing  
168 allele found in Valoris is not rare, being present in 150 out of 426 panel members ([www.whealbi.eu](http://www.whealbi.eu)).  
169 We further investigated allele frequency in another panel (an elite high fibre panel, comprised of  
170 advanced commercial breeding lines of UK, French and German winter wheats) using a KASP marker  
171 designed to the SNP. In this panel, we found the Valoris allele in 57 lines, 287 giving reference allele  
172 and 27 scored as het.

173

174 Discussion

175 Soluble dietary fibre may confer health benefits via several mechanisms including fermentation to  
176 release short-chain fatty acids in the colon, and by increasing viscosity which may reduce the rate of  
177 glucose release from starch in the duodenum (Gill et al., 2021). In the case of wheat SDF, nearly all of  
178 the viscosity of aqueous extracts of white flour is due to WE-AX (Freeman et al., 2016). AX is a major  
179 component of all grass cell walls, but is normally not extractable in water, so wheat endosperm WE-  
180 AX is unusual in this respect. Extractability of AX in water is determined by several structural factors  
181 of which cross-linking via ferulate dimerization may be the most important (Saulnier et al., 2007).  
182 The AX in the starchy endosperm, and particularly the WE-AX fraction, has less ferulate [AX ~0.5%;  
183 WE-AX ~0.2% (Freeman et al., 2017)] , and therefore potential for cross-linking, than AX from other  
184 tissues in wheat and in grasses generally, which varies greatly but can be up to 20% (Hatfield et al.,  
185 2017).

186 It has been shown that oxidative coupling of ferulate that results in dimers and higher oligomers  
187 bound to AX in grass cell walls occurs in the apoplast and is mediated by peroxidases (Burr and Fry,  
188 2009) but these have not been identified. Here we provide strong evidence that the dimerization of  
189 ferulate on wheat endosperm AX is mediated by the PER1 protein, since both a natural allele and an  
190 induced KO mutant result in lower dimerization (Tables 1 and 2). The natural allele has a missense  
191 SNP that prevents localisation of PER1 to the cell wall (Fig. 2), the likely site for AX dimerization. We  
192 therefore attribute the 6B QTL effect on SDF to this SNP resulting in a defective PER1 that lowers the  
193 capacity for AX ferulate dimerization, resulting in a higher proportion of AX being extractable in  
194 water. This conclusion is clear despite the changes in expression of PER1, PER1&2 and PER2  
195 associated with the Valoris 6B genotype (Fig. 1) which could be due to nearby *cis* factors rather the  
196 missense SNP itself.

197 PER1 is a member of a large gene family of class III haem peroxidases that are specific to green  
198 plants. These peroxidases apparently all catalyse the same reaction *in vitro*, generating radical  
199 oxygen species from H<sub>2</sub>O<sub>2</sub> and it has been hypothesised that *in vivo* specificity is achieved by  
200 localisation close to the intended substrate (Francoz et al., 2015). This is supported by observation of  
201 precise localisation of peroxidases associated with lignification in *Arabidopsis*, (Hoffmann et al.,  
202 2020) and has been demonstrated for a peroxidase that binds to a particular cell wall microdomain  
203 on the polysaccharide homogalacturonan in order to function in mucilage extrusion (Francoz et al.,  
204 2019) and for a lignin peroxidase that localises to the Casparyan strip via binding to another protein  
205 (Lee et al., 2013). It is therefore probable that a large family of peroxidase genes is required for the  
206 multiplicity of peroxidase binding sites that confer specificity of function. This is consistent with our

207 result showing that PER1 is present in the cell wall whilst PER1-v is not and this difference is  
208 associated with decreased dimerization of AX ferulate. The single amino acid difference between the  
209 natural and mutant peroxidases is at a serine residue in PER1 which is predicted by AlphaFold to be  
210 involved in H-bonding in an  $\alpha$ -helix on the exterior of the protein (Figure S5). This is changed in  
211 PER1-v to the aromatic amino acid phenylalanine which would prevent H-bonding and disrupt the  $\alpha$ -  
212 helix. PER1 and PER2 are in a grass-specific subclade of Group VI of the class III peroxidase gene  
213 family as defined in (Passardi et al., 2004) (Figure S6). This is consistent with a specific role in cross-  
214 linking of AX ferulate, which is confined to grasses and other commelinid monocots (Chandrakanth  
215 et al., 2023). PER1 and PER2 are only expressed in grain out of the major wheat tissues (Figure S5)  
216 suggesting they may have specific properties related to the unusual structure of grain AX.  
217 Our identification of PER1 as the cause of the high SDF trait from the 6B QTL will allow the specific  
218 tailoring of the solubility of wheat grain AX to increase benefits for human health. It also represents  
219 an important step forward in our broader understanding of grass cell walls.

220

221 Methods

222 Plant Growth

223 We grew the Yumai34 x Valoris population in the field in 2018 and in the glasshouse in 2022 in  
224 randomised designs at Rothamsted Research, UK. In both experiments, we selected DHLs as having  
225 the same genotype for all Axiom genotyping markers on 35K SNP platform within the 1A, 1B, 6B  
226 QTLs and assigned these to 4 pools of contrasting parental genotype at 1B and 6B but all with  
227 Yumai34 genotype at 1A. Three biological reps of these pools from different blocks were used from  
228 2018 field experiment for transcriptome analysis and from 2022 glasshouse experiment for ferulate  
229 determination.

230 KASP marker for Valoris missense SNP

231 We designed KASP marker to Valoris chr6B 26076727 SNP and used this to confirm SNP genotype of  
232 the selected lines in Yumai34 x Valoris population and in elite high fibre panel. Primers and PCR  
233 conditions are given in Table S1.

234 RNA-seq analysis

235 Total RNA was isolated from developing endosperm of 20 selected DHLs at 17 days post anthesis as  
236 previously described (Pellny et al., 2012; Pellny et al., 2020) to give 3 biological replicates of each  
237 pool. Library preparation and mRNA sequencing was done by Novogene (HK, China). We mapped  
238 reads to IWGSC refseq 1.1 genome using HISAT2 (Kim et al., 2015), visualised alignments using  
239 Integrative Genomics Viewer (Robinson et al., 2011) and estimated transcript abundance and  
240 differentially expressed genes using DESeq2 (Love et al., 2014). Transcript abundances for  
241 TraesCS6B02G042500.1 and TraesCS6B02G042600.1 gene models in reference were reallocated to  
242 PER1, PER1&2 and PER2 based on manually determined ratios of counts of read pairs that could be  
243 clearly assigned to these.

244

245 Ferulate Monomer and Dimer Analysis

246 We analysed the ferulate monomer and dimer content of endosperm fraction from mature grain by  
247 HPLC analysis as previously described (Freeman et al., 2017) using pure ferulate dimer standards  
248 kindly supplied by Professor John Ralph (Lu et al., 2012).

249

250 PER1 Knock-Out Mutant

251 From the database of mutations in the exome sequenced collection of mutagenised wheat cv  
252 Cadenza available at Ensembl Plants, we identified line Cadenza1644 carrying a G->A mutation at  
253 26,076,941 on chr6B causing a premature stop codon Trp94STOP in exon 2 of PER1. We backcrossed  
254 this line twice to Cadenza, allowed to self-fertilise over two further generations and identified BC2F2  
255 homozygotes for both mutation and wild-type allele using crossing and genotyping procedures  
256 previously described (Pellny et al., 2020); subgenome specific primers for genotyping are given in  
257 Table S1. We grew these lines in a randomised design in the glasshouse with 7 replicate pots per  
258 line.

259

260 Transient expression of PER1\_RFP and PER1-v\_RFP in immature wheat endosperm  
261 We designed a version of PER1 CDS (TraesCS6B02G042500.1) that contained additional cloning sites  
262 immediately before the natural Ncol site present over the ATG codon and an Hpal site at the C'  
263 terminal end of the gene that involved the addition of an extra serine residue to the protein. Nine  
264 synonymous changes were also engineered into the coding sequence to facilitate cloning while  
265 retaining the natural PER1 protein sequence. We designed PER1-v from this with a single bp change  
266 corresponding to the Valoris allele missense SNP giving rise to the Ser51Phe peptide sequence  
267 change. From alignment analysis of the 5' upstream sequences of PER1 and the Chinese Spring, A,  
268 and D homeologues and other wheat relatives, we selected 2135 bp of the PER1 sequence as likely  
269 to contain a complete promoter including the 5' transcribed untranslated sequences. Bases  
270 immediately upstream of the ATG codon were modified to contain the same restriction sites as  
271 engineered into the PER1 and PER1-v genes sequences for cloning purposes and to remove the out  
272 of frame ATG codon present 5bp upstream of the PER1 start codon. The designed PER1, PER1-v and  
273 PER1 promoter sequences were synthesised by Genscript (Oxford, UK) and transferred using  
274 standard cloning techniques to pRRes208, a biolistic vector backbone. Two plasmids were generated  
275 with the PER1 promoter driving either PER1 [pRRes208.618] or PER1-v [pRRes208.617]. In each  
276 construct the PER1 sequences were fused in frame upstream of a six amino acid linker sequence and  
277 the fluorescence marker gene Tag-RFP\_T. Immature wheat endosperm sections were isolated from  
278 7-10 dpa wheat seeds cv Cadenza, as described in (Jones et al., 2008). The tissues were bombarded  
279 on the same day as isolation using 0.6µm gold particles (Bio-Rad Laboratories Ltd, UK) coated with  
280 the constructs pRRes208.617, pRRes208.618 or pRRes.380, a plasmid containing Tag-RFP under the  
281 control of the constitutive rice actin promoter which acted as a positive control. Bombardment was  
282 carried out using the PDS-1000/He particle gun with 650psi rupture pressure and 29" Hg vacuum;

283 full details of bombardment parameters can be found in (Sparks and Doherty, 2020). Plates were  
284 incubated in the dark at 22°C for 1 day prior to bioimaging.

285 Imaging of wheat endosperm transiently expressing PER1\_RFP and PER1-v\_RFP  
286 Transformed, immature wheat endosperm sections were mounted on glass slides with coverslips for  
287 imaging with a Zeiss LSM 780 confocal microscope (Carl Zeiss Ltd. Cambourne, Cambridge, UK). The  
288 endosperm tissue was mounted in distilled water for initial imaging and the water replaced under  
289 the coverslip with 2M NaCl by capillary action to induce plasmolysis. Cells were monitored for up to  
290 10 mins to observe plasmolysis and imaged with the 40x objective using 405nm excitation, 428nm –  
291 490nm emission for cell wall auto-fluorescence, and 561nm excitation, 580nm – 700nm emission for  
292 Tag-RFP.

293 Figure captions

294 Figure 1. RNAseq analysis of PER transcripts from 17 dpa endosperm of DHLs in Yumai34 x Valoris  
295 mapping population. **A.** Read coverage for 6B chromosome region containing TraesCS6B02G042500  
296 and TraesCS6B02G042600 genes from 12 samples of DHLs selected to have Yumai34 (Y34) or Valoris  
297 alleles (VAL) at 1B and 6B QTLs with 3 reps of each combination. The arrow indicates the missense  
298 SNP in Valoris. Analysis of paired reads showed nearly all transcripts were one of the 3 forms  
299 depicted PER1, PER1&2 or PER2. These inferred transcripts are shown as blocks and lines where  
300 blocks represent coding exons and the missense SNP in Valoris allele is marked in red. **B.** Transcript  
301 abundance of the splice variants PER1, PER1&2 or PER2 and their homeologues on sub-genomes A  
302 and D. PER1A exons are not expressed. Bar heights and error bars are estimated means and 5% LSD  
303 for 6B alleles of each transcript from a 2-way ANOVA of 1B and 6B allele effects. No 1B effects were  
304 significant ( $P>0.05$ ) so results shown group DHLs differing in 1B alleles (n=6).

305

306 Figure 2. Example images of sections of plasmolysed wheat endosperm cells transiently expressing  
307 PER1\_RFP and PER1-v\_RFP proteins. Pairs of images show RFP fluorescence signal alone in top panel  
308 and combined with autofluorescence (+AF) in lower panel to show cell walls. Plasmolysis caused  
309 complete (**A, B**) or partial (**C, D**) separation of cell contents from cell wall allowing identification of  
310 RFP signal from cell wall indicated by arrows. Cell wall localisation of RFP signal is present in cells  
311 expressing PER1\_RFP (**A, C**) but not those expressing PER1-v\_RFP (**B, D**).

312

313 Supplementary material

314 Figures S1. Images from endosperm grain sections transiently expressing PER1\_RFP and PER1-v\_RFP  
315 where clear cell plasmolysis was observed. Pairs of images show RFP fluorescence signal alone in top  
316 panel and combined with autofluorescence (+AF) in lower panel to show cell walls. Plasmolysis  
317 separation of cell contents from cell wall allowing identification of RFP signal from cell wall indicated  
318 by arrows. Cell wall localisation of RFP signal is present in cells expressing PER1\_RFP (**A, B, E, F, I, J**)  
319 but not those expressing PER1-v\_RFP (**C, D, G, H, K, L**).

320 Figures S2. Further images from endosperm grain sections transiently expressing PER1\_RFP and  
321 PER1-v\_RFP where clear cell plasmolysis was observed. Details as for Fig. S1, except for image pairs  
322 (**D, G, H, K, L**) where image in upper panel is pre-plasmolysis and after plasmolysis in lower panel.

323 Figures S3. Further images from endosperm grain sections transiently expressing PER1\_RFP and  
324 PER1-v\_RFP where clear cell plasmolysis was observed. Details as for Fig. S1, except for image pairs  
325 (**C-L**) where image in upper panel is pre-plasmolysis and after plasmolysis in lower panel. Image pair  
326 **F** is the single exception where we could not see clear cell wall localisation of RFP signal in cells  
327 expressing PER1\_RFP.

328 Figures S4. Further images from endosperm grain sections transiently expressing PER1\_RFP and  
329 PER1-v\_RFP where clear cell plasmolysis was observed. Pairs of images show pre-plasmolysis image  
330 in upper panel and after plasmolysis in lower panel. Plasmolysis causes separation of cell contents  
331 from cell wall allowing identification of RFP signal from cell wall indicated by arrows. Cell wall  
332 localisation of RFP signal is present in cells expressing PER1\_RFP (**A**) but not those expressing PER1-  
333 v\_RFP (**B, C, D**).

334 Figure S5. AlphaFold predicted structure of PER1 (TraesCS6B02G04600.1) protein with Ser51 residue  
335 that is changed in PER1-v highlighted. Predicted N-glycosylation Asn, haem-binding and signal  
336 peptide cleavage sites are indicated.

337 Figure S6. Phylogeny of subclade within class III peroxidase family containing PER1 and PER2 genes.  
338 For phylogenetic analyses of protein sequences, we selected the rice ortholog of PER genes  
339 (OsPRX91) and its three closest paralogs (OsPrx90, OsPrx92, OsPrx93) which are all neighbouring  
340 genes on rice chromosome 6. We then selected all orthologs of these from wheat, *Arabidopsis*,  
341 *Ananas comosus*, *Glycine max*, *Hordeum vulgare*, *Populus trichocarpa* and *Sorghum bicolor* as  
342 defined Ensembl Plants release 56 (Yates et al., 2021) and aligned with MUSCLE (Edgar, 2004) and  
343 generated phylogenetic tree with Phymol (Guindon et al., 2010) as previously described (Pellny et al.,

344 2012). Wheat expression data was taken from wheat-expression.com (Borrill et al., 2016) averaged  
345 across 34 studies encompassing many genotypes and environments.

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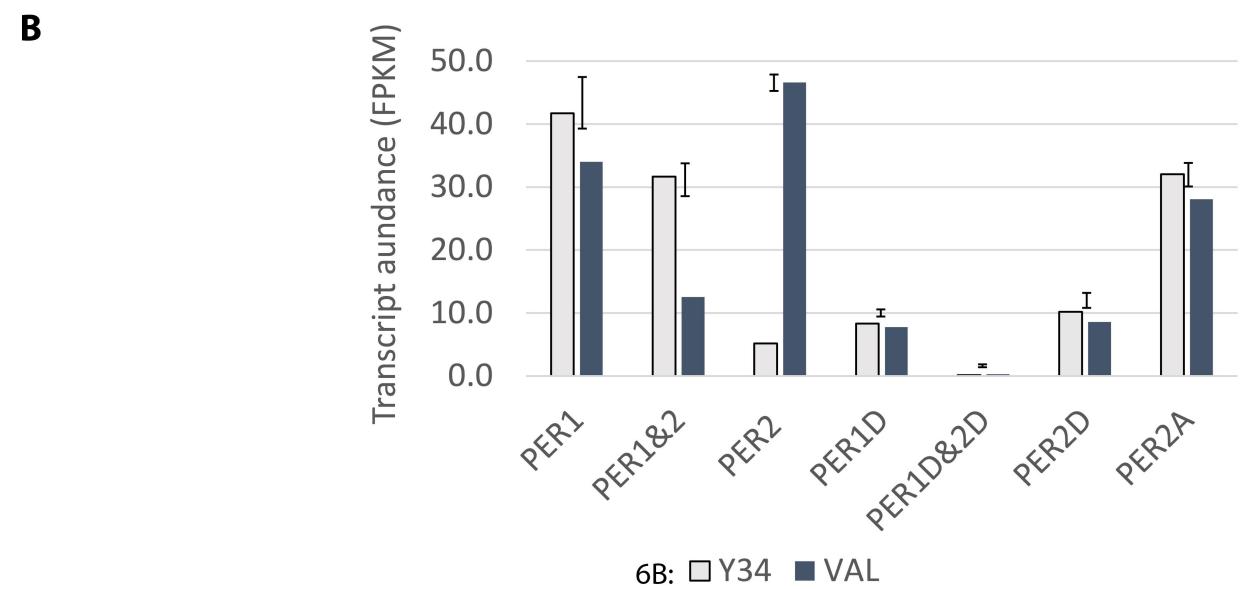
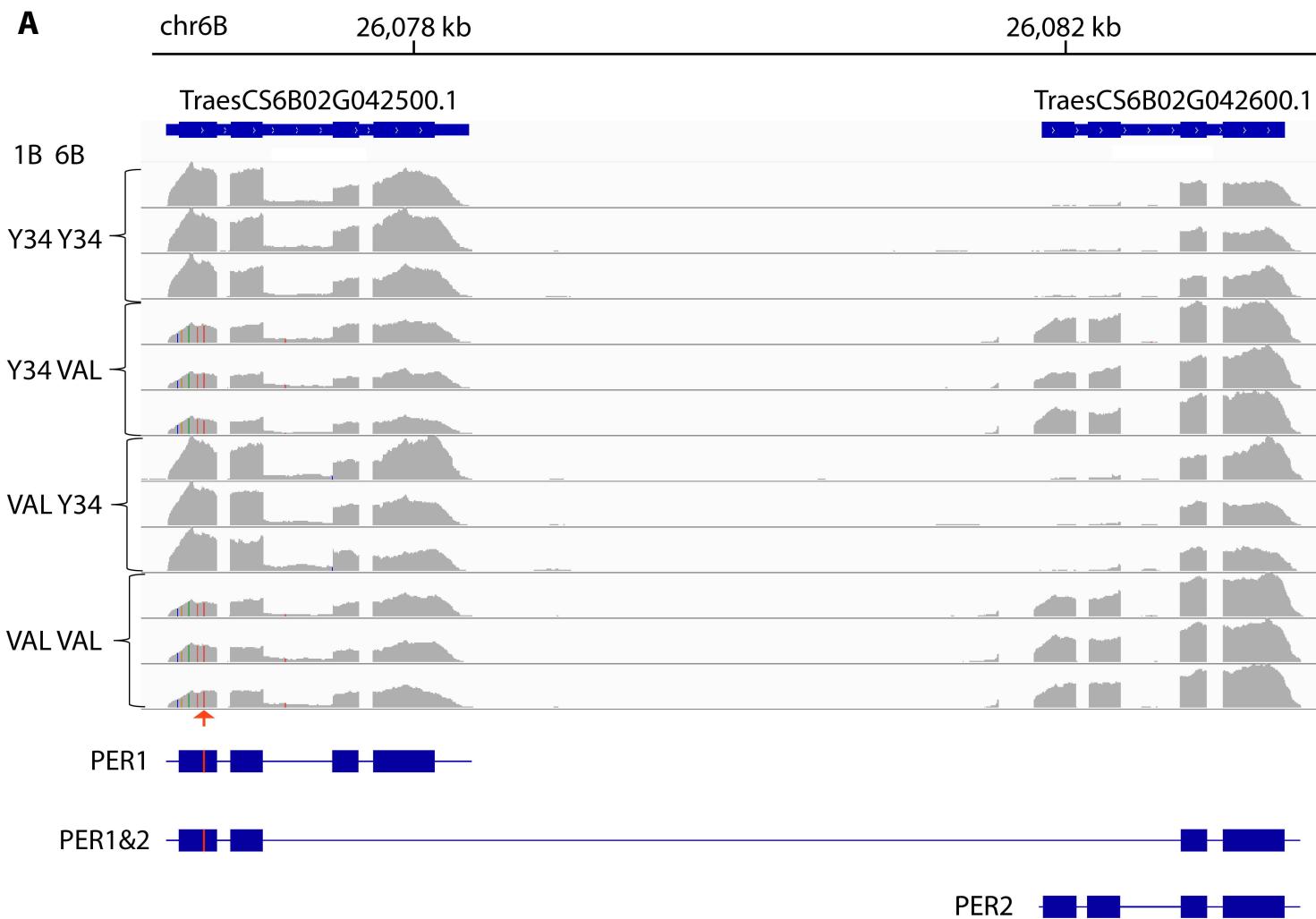
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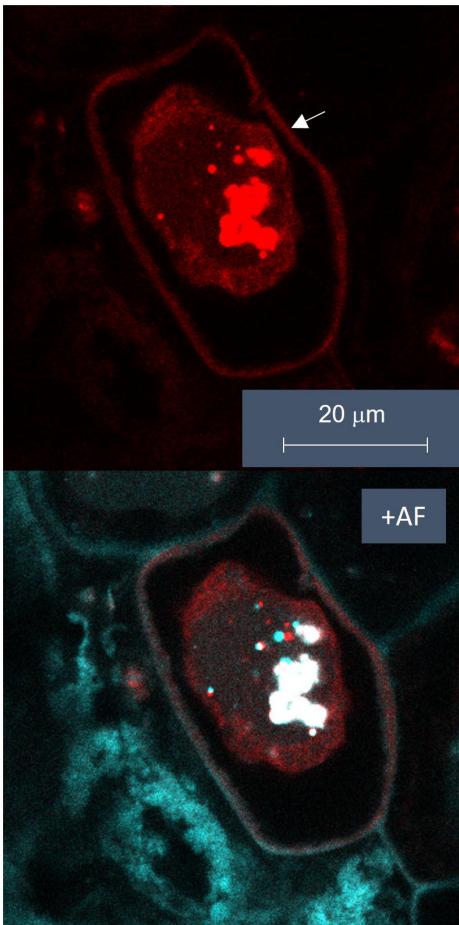
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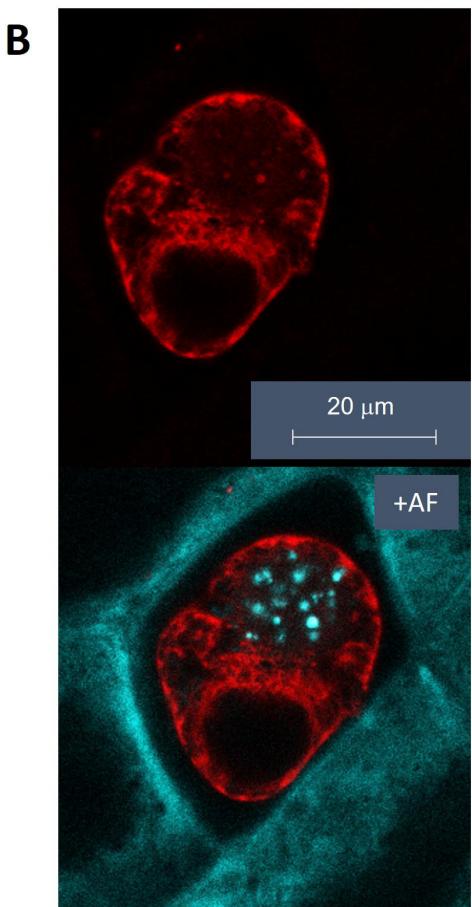
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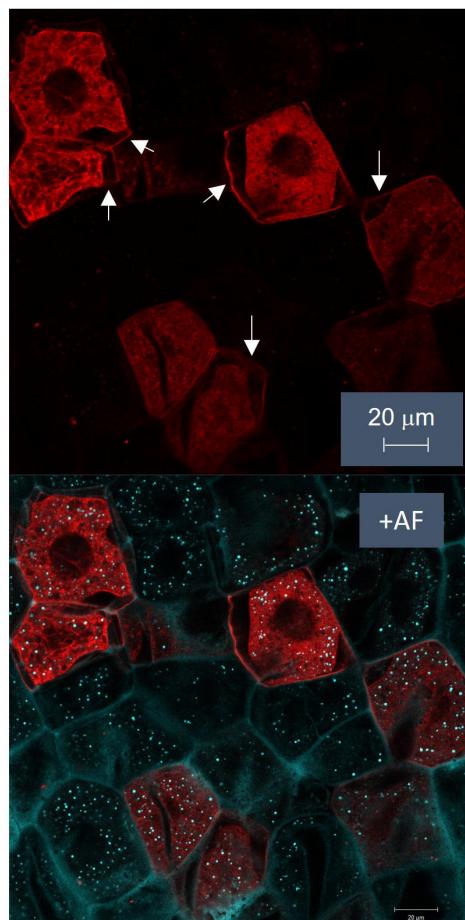
PER1\_RFP



PER1-v\_RFP



C



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