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Marker-assisted development of bread wheat near-isogenic lines carrying various combinations of purple pericarp (*Pp*) alleles

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Abstract The commercial interest in pigmented wheat grain flows from an understanding that they are nutritionally superior to white kernels. The pigment of purple coloured bread and durum wheat grains results from the accumulation of anthocyanins in the pericarp; its genetic basis is the action of *Pp-1* and *Pp3* genes. Here, the development of a set of bread wheat near isogenic lines (NILs) carrying various combinations of *Pp* alleles is described, along with a demonstration of their utility for the genetic dissection of the purple pericarp trait. A marker-assisted backcrossing strategy was based on the use of microsatellite markers linked to *Pp3* (chromosome 2A), *Pp-A1* (7A) and *Pp-D1* (7D). *Pp-A1* is a newly uncovered gene of weak effect. A qRT-PCR-based analysis of the anthocyanin synthesis structural genes [*Chi* (chalcone-flavanone isomerase) and *F3h* (flavanone 3-hydroxylase)] transcript abundance in the pericarp of the NILs suggested that the *Pp* genes up-regulate their transcription in contrasting ways. These NILs represent a resource for studying the effect of grain pigmentation on other wheat traits and end products.

Keywords *Triticum* · Microsatellites · Genotyping · Transcription regulation · Anthocyanin synthesis

Introduction

Descriptions of grain colour in wheat are generally qualitative in nature: white, red, blue or purple. Red pigmentation is associated with the deposition of proanthocyanidin in the testa, whereas both blue and purple pigmentation flows from the accumulation of anthocyanin in respectively the aleurone and the pericarp (reviewed by Zeven 1991; Khlestkina 2013). As the consumption of anthocyanins is associated with a number of health benefits (Lila 2004), wheat grains enriched for these compounds are an attractive product.

The mode of inheritance of the purple pericarp has been known for many years: in some situations it appears to be monogenic (Sharman 1958; Dobrovolskaya et al. 2006), while in others it is digenic (Bolton 1970; Piech and Evans 1979; Arbuzova et al. 1998; Dobrovolskaya et al. 2006; Khlestkina et al. 2010a). According to Dobrovolskaya et al. (2006), the *Pp3* locus maps to a marker-defined region of chromosome 2A of bread wheat, and the corresponding region of

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tetraploid (durum) wheat chromosome arm 2AL also harbours a probable *Pp3* orthologue (Khlestkina et al. 2010a). Durum wheat also houses *Pp-B1* (complementary to *Pp3*), mapping to chromosome arm 7BS (Khlestkina et al. 2010a), while its D-genomic homoeologue in bread wheat (*Pp-D1*) lies on chromosome arm 7DS (Tereshchenko et al. 2012a). Based on an analysis of introgression lines, Tereshchenko et al. (2012b) have shown that a functional allele of at least one purple pericarp gene is retained in both *Aegilops speltoides* and *Triticum timopheevii*, although in the latter species, the purple grain trait has to date never been described (Tereshchenko et al. 2012b).

Analysis of the transcription of various structural genes encoding enzymes active in anthocyanin synthesis in near-isogenic lines (NILs) varying for their *Pp* gene content has suggested that *Pp-D1* and *Pp3* are both transcriptional regulators (Tereshchenko et al. 2013). However, the *Pp-1* and *Pp3* genes have been not sequenced yet. The goal of the current study was to develop a set of NILs carrying various combinations of *Pp* alleles as a tool for genetically dissecting the purple pericarp trait, including analysis of the transcription of the key anthocyanin biosynthesis structural genes *Chi* (encoding chalcone-flavanone isomerase and cloned in wheat by Shoeva et al. 2014) and *F3h* (flavanone 3-hydroxylase; Khlestkina et al. 2008, 2013) in the pericarp of newly developed NILs.

To accelerate development of NILs microsatellite markers were utilized for genotyping of plant material obtained in crosses. Microsatellite (or SSR—simple sequence repeats) markers are widely used for marker-assistant selection in wheat (Reviewed by Leonova 2013), since they are abundant, convenient, reliable and characterized by precise positions in wheat genome (Röder et al. 1998; Ganal and Röder 2007).

Materials and methods

Plant material and phenotyping

A description of the parental materials is given in Table 1. All these lines were developed in the genetic background of spring bread wheat 'Saratovskaya 29' (i:S29*Pp-A1pp-D1pp3*). The *pp-A1* donor was spring bread wheat 'Janetzkis Probat', the *Pp3* and the *Pp-D1* donors were spring wheats 'Purple' (k-46990) and 'Purple Feed' (k-49426). Derivatives carrying both *Pp3* and *Pp-D1* were generated by Arbuzova et al. (1998). The line i:*S29pp-A1pp-D1pp3* was selected from a set of doubled haploid lines described by Khlestkina et al. (2010b). The crossing scheme and the marker-assisted selection interventions are illustrated in Fig. 1. Since the same scheme was used to produce lines from both i:S29*Pp-A1Pp-D1Pp3*^{PF} and i:S29*Pp-A1Pp-D1Pp3*^{P}, only the one used for the first line has been illustrated. To evaluate anthocyanin pigmentation in the coleoptile (which was exploited as an additional marker for the selection of *Pp-A1* and *Pp-D1*), F_2 populations bred from each cross were evaluated according to Khlestkina et al. (2011). Pericarp pigmentation was scored in developing seeds within 55th–75th day after sowing. Pericarp samples for RNA extraction were collected from immature grains. Three biological replicates (from a bulk of 3–5 plants) were collected for each entry line marked in Table 1 with asterisks. The plants were grown using resources of ICG Greenhouse Core Facilities (Novosibirsk, Russia) under 12 h of light per day at 20–25 °C.

DNA extraction and microsatellite analysis

DNA was extracted from leaf material harvested from each segregant and the parental lines using a procedure described by Plaschke et al. (1995). A set of informative microsatellites chosen from the GWM series (Röder et al. 1998; Ganal and Röder 2007) was assembled for marker assisted selection purposes (Fig. 1). The PCR-conditions were as described in Röder et al. (1998). Amplicons were separated either through 5 % ACTGene agarose gels (ACTGene, Inc., Piscataway, NJ, USA) or by capillary electrophoresis using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). In the latter case, GeneScan v2.1 0 software was used to determine fragment sizes. The capillary electrophoresis was performed using resources of SB RAS Genomics Core Facilities (Novosibirsk, Russia, http://www.niboch.nsc.ru/doku.php/corefacility).

RNA extraction, reverse transcription and qRT-PCR

RNA was extracted from the pericarp of immature grains using a Plant RNA MiniPrep^{TM} kit (Zymo

Table 1 The set of NILs carrying various combinations of *Pp* alleles

Designation	Other name	*Pp-A1* (7A)	*Pp-D1* (7D)	*Pp3* (2A)	Pericarp **	Coleoptile color (dominant gene(s))	References
*i:S29*Pp-A1Pp-D1Pp3*^{PF}	i:S29*Pp1Pp2*	Dominant	Dominant	Dominant	Dark purple	Dark red (*Rc-A1* + *Rc-D1*)	Arbuzova et al. (1998); Tereshchenko et al. (2012a); current study***
***i:S29*Pp-A1Pp-D1pp3*^{PF}**	**–**	**Dominant**	**Dominant**	**Recessive**	**Uncolored**	**Dark red (*Rc-A1* + *Rc-D1*)**	**Current study**
***i:S29*Pp-A1pp-D1Pp3*^{PF}**	**–**	**Dominant**	**Recessive**	**Dominant**	**Light purple**	**Light red (*Rc-A1*)**	**Current study**
i:S29*pp-A1pp-D1Pp3*^{PF}	**–**	**Recessive**	**Recessive**	**Dominant**	**Uncolored**	**Uncolored**	**Current study**
*i:S29*Pp-A1pp-D1pp3*	Saratovskaya 29	Dominant	Recessive	Recessive	Uncolored	Light red (*Rc-A1*)	*Pp-A1* was identified in the current study
*i:S29*pp-A1pp-D1pp3*	Line 140	Recessive	Recessive	Recessive	Uncolored	Uncolored	Khlestkina et al. (2010b); current study***
*i:S29*Pp-A1Pp-D1Pp3*^{P}	i:S29*Pp1Pp3*	Dominant	Dominant	Dominant	Dark purple	Dark red (*Rc-A1* + *Rc-D1*)	Arbuzova et al. (1998); Tereshchenko et al. (2012a); current study***
***i:S29*Pp-A1Pp-D1pp3*^{P}**	**–**	**Dominant**	**Dominant**	**Recessive**	**Uncolored**	**Dark red (*Rc-A1* + *Rc-D1*)**	**Current study**
***i:S29*Pp-A1pp-D1Pp3*^{P}**	**–**	**Dominant**	**Recessive**	**Dominant**	**Light purple**	**Light red (*Rc-A1*)**	**Current study**
i:S29*pp-A1pp-D1Pp3*^{P}	**–**	**Recessive**	**Recessive**	**Dominant**	**Uncolored**	**Uncolored**	**Current study**
i:S29*Ra*	–	Dominant	Dominant	Recessive	Uncolored	Dark red (*Rc-A1* + *Rc-D1*)	Arbuzova et al. (1998); Khlestkina et al. (2014); current study****

The lines obtained by marker-assisted backcrossing in the current study are *bold*, other lines were used as parents in crosses (see Fig. 1)

* The NILs used for the anthocyanin biosynthesis genes transcription analysis

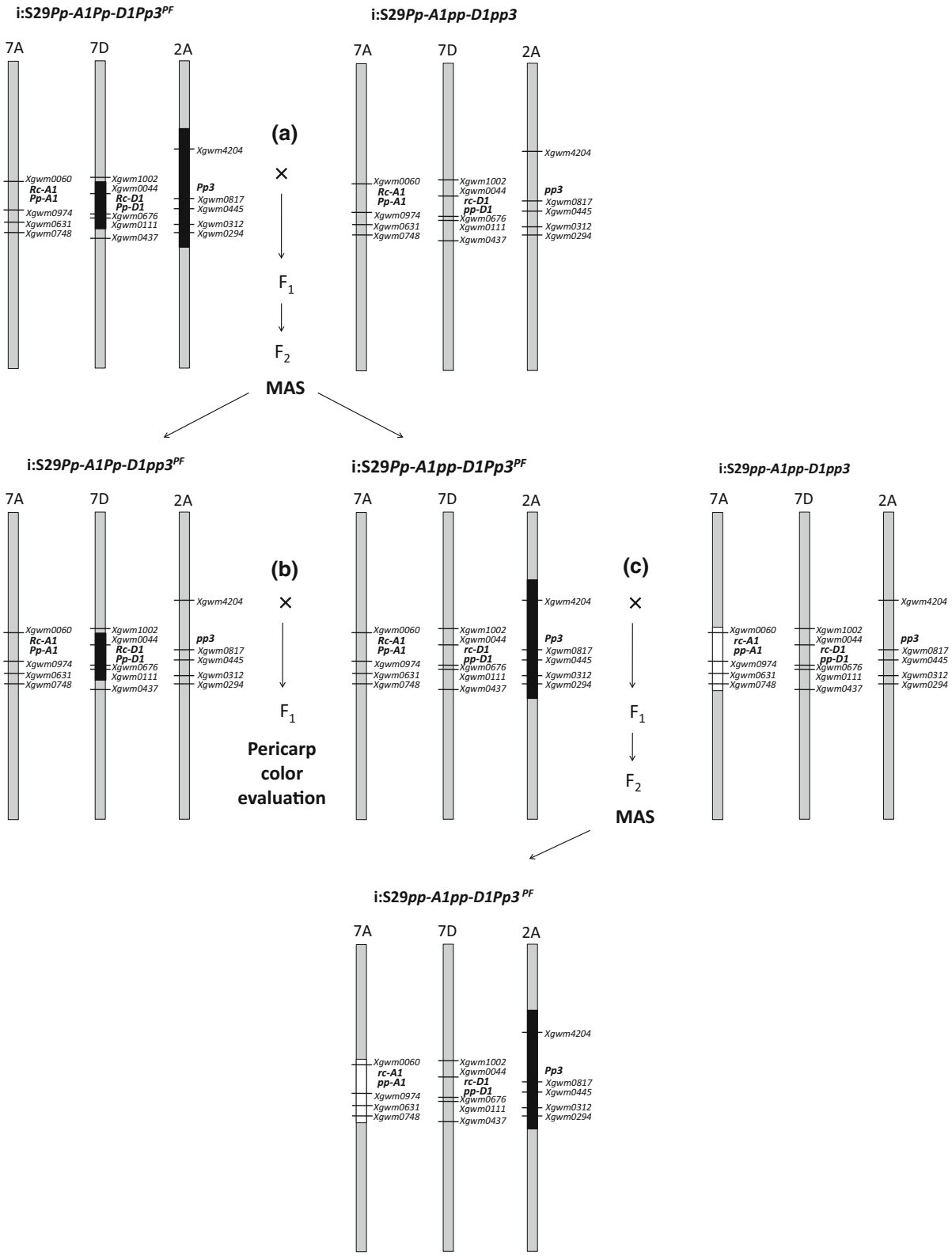
** The patterns of developing seeds with dark purple, light purple and uncolored pericarp are shown at Fig S1

*** Allelic composition at the *Pp-A1* locus was determined in the current study

**** Allelic composition at the *Pp-D1* locus was determined in the current study

^{P} The source of the *Pp3* and *Pp-D1* dominant alleles is Purple

^{PF} The source of the *Pp3* and *Pp-D1* dominant alleles is Purple Feed


i:S29Pp-A1Pp-D1Pp3PF
i:S29Pp-A1pp-D1pp3
7A
7D
2A
Xgwm0060
Rc-A1
Pp-A1
Xgwm0974
Xgwm0631
Xgwm0748
Xgwm1002
Xgwm0044
Rc-D1
Pp-D1
Xgwm0676
Xgwm0111
Xgwm0437
Xgwm4204
Pp3
Xgwm0817
Xgwm0445
Xgwm0312
Xgwm0294
(a)
×
F1
F2
MAS
rc-A1
pp-A1
rc-D1
pp-D1
pp3
i:S29Pp-A1Pp-D1pp3PF
i:S29Pp-A1pp-D1Pp3PF
i:S29pp-A1pp-D1pp3
(b)
(c)
Pericarp color evaluation
i:S29pp-A1pp-D1Pp3 PF

◀**Fig. 1** Crossing scheme and marker interventions used to obtain and validate NILs carrying various combination of *Pp* alleles. Chromosomal segments inherited from Purple Feed is marked in *black*, from Janetzkis Probat in *white* and from Saratovskaya 29 in *grey*. *MAS* marker-assisted selection. **a**–**c** Crosses designations

Research Corporation, Irvine, CA, USA), then treated with DNAse. Each entry was represented by three biological replicates. A 0.7 µg aliquot of RNA was used to prepare single-stranded cDNA by reverse transcription, based on a RevertAid^{TM} kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and a $(dT)_{15}$ primer. The subsequent qRT-PCR was based on a SYNTOL SYBR Green I kit (Syntol, Moscow, Russia). *Chi* and *F3h* transcript abundance was assessed using the respective primer pairs 5-CTCGC CGCCAAGTGGG/5-TTCTCGAACTCGCCGGTG AC and 5-AAGGGCGGCTTCATCGTCTC/5-CCCTCCAGTCCTGCACCGC. The primers were designed using OLIGO software (Offerman and Rychlik 2003) based on multiple alignment of the *Chi* (Khlestkina and Shoeva 2014; Shoeva et al. 2014) and *F3h* (Khlestkina et al. 2008, 2013) sequences. MULTALIN software (Corpet 1988) was used to obtain multiple sequence alignments. The reference sequence used was *Ubc* (ubiquitin), assayed using primers suggested by Himi et al. (2005). Three technical replicates of each reaction were run. The significance of differences in transcript abundance between entries was tested using the Mann–Whitney *U*-test, with a $p = 0.05$ significance level.

Results

Marker-assisted development of NILs

Each of i:S29*Pp-A1Pp-D1Pp3*^{PF} and i:S29*Pp-A1Pp-D1Pp3*^{P} was crossed with i:S29*Pp-A1pp-D1pp3* (Fig. 1a); pericarps of the F_1 hybrid plants were dark purple in both cases. F_2 progeny expressing a dark red coleoptile (carriers of *Rc-D1* on chromosome 7D) were genotyped using microsatellites flanking *Pp-D1* and *Pp3* (Fig. 1) to select homozygous *Pp-A1Pp-A1Pp-D1Pp-D1pp3pp3* plants, while those with light red coleoptiles (genotype *Rc-A1Rc-A1rc-D1rc-D1*) were used to select homozygous *Pp-A1Pp-A1pp-D1pp-D1Pp3Pp3* plants. F_2 segregants homozygous for the Purple Feed and Purple chromosome 7D microsatellite alleles and homozygous for the Saratovskaya 29 chromosome 2A microsatellite alleles were designated i:S29*Pp-A1Pp-D1pp3*^{PF} and i:S29*Pp-A1Pp-D1pp3*^{P}, respectively. F_2 segregants homozygous for the Purple Feed and Purple chromosome 2A microsatellite alleles and homozygous for the Saratovskaya 29 chromosome 7D microsatellite alleles were designated i:S29*Pp-A1pp-D1Pp3*^{PF} and i:S29*Pp-A1pp-D1Pp3*^{P}, respectively. Both i:S29*Pp-A1Pp-D1pp3*^{PF} and i:S29*Pp-A1Pp-D1pp3*^{P} exhibited strongly pigmented coleoptiles and an uncolored pericarp, while i:S29*Pp-A1pp-D1Pp3*^{PF} and i:S29*Pp-A1pp-D1Pp3*^{P} plants produced weakly pigmented coleoptiles (due to the presence of *Rc-A1*) and a light purple pericarp.

Each of i:S29*Pp-A1pp-D1Pp3*^{PF} and i:S29*Pp-A1pp-D1Pp3*^{P} was crossed with i:S29*pp-A1pp-D1pp3* (Fig. 1c); pericarps of the F_1 hybrid plants were light purple in both cases. F_2 segregants from each cross were selected on the basis of an uncolored coleoptile (genotype *rc-A1/rc-A1*), and were then genotyped using microsatellite markers flanking *Pp-A1* and *Pp3* (Fig. 1). Segregants homozygous for Janetzkis Probat chromosome 7A microsatellite alleles and Purple Feed or Purple chromosome 2A microsatellite alleles were designated i:S29*pp-A1pp-D1Pp3*^{PF} and i:S29*pp-A1pp-D1Pp3*^{P}, respectively. These lines produced uncolored coleoptiles and an uncolored pericarp.

Test crosses

The crosses i:S29*Pp-A1Pp-D1pp3*^{PF}/i:S29*Pp-A1pp-D1Pp3*^{PF} and i:S29*Pp-A1Pp-D1pp3*^{P}/i:S29*Pp-A1pp-D1Pp3*^{P} were made to verify the presence of the dominant alleles at the *Pp-D1* and *Pp3* loci in the lines, which had been selected either for *Pp-D1* or *Pp3* exclusively using markers (Fig. 1b). The purple pericarp trait was restored in the F_1 plants in each cross. This confirmed the presence of the dominant allele *Pp-D1* in i:S29*Pp-A1Pp-D1pp3*$^{PF/P}$ and *Pp3* allele in i:S29*Pp-A1pp-D1Pp3*$^{PF/P}$.

Lines i:S29*Pp-A1Pp-D1pp3*$^{PF/P}$ and i:S29*Pp-A1pp-D1Pp3*$^{PF/P}$ are suitable as testers to reveal the presence of the dominant allele at, respectively, *Pp3* and *Pp-D1*. When i:S29*Pp-A1pp-D1Pp3*^{PF} was

crossed with i:S29*Ra* (Table 1), the F_1 plants bore grains exhibiting a purple pericarp, meaning that i:S29*Ra* carries the *Pp-1* allele.

Transcription of *Chi* and *F3h* in the pericarp of the NILs

The qRT-PCR-based evaluation of transcription of *Chi* and *F3h* in the pericarp of the NILs is summarized in Fig. 2. *Chi* transcript was detectable even in plants lacking dominant alleles in the *Pp-1* and *Pp3* loci. The presence of *Pp-A1* increased the transcript abundance by five fold, and a further increase (1.5–2 fold) was induced by the additional presence of either *Pp-D1* or *Pp3*. In plants carrying both *Pp-D1* and *Pp3,* the abundance of *Chi* transcript was increased eight fold compared to i:S29*Pp-A1pp-D1pp3* and 40 fold compared to i:S29*pp-A1pp-D1pp3* (Fig. 2). The level of *F3h* transcription was 2,000 fold higher in i:S29*Pp-A1Pp-D1Pp3* than in i:S29*pp-A1pp-D1pp3*. Other combinations of the *Pp* alleles had no effect on the abundance of *F3h* transcript (Fig. 2).

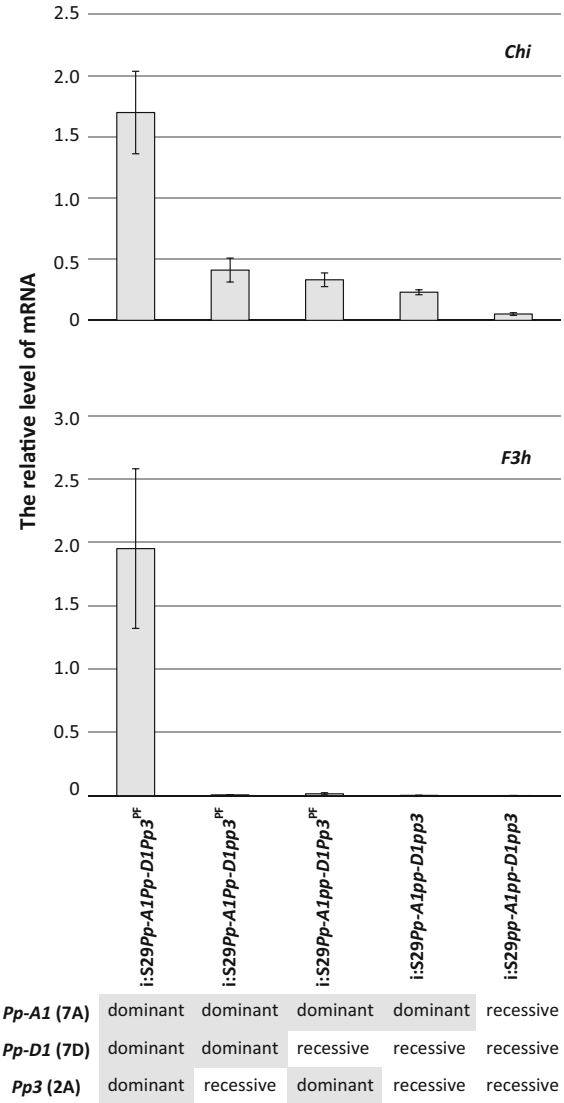


Fig. 2 *Chi* and *F3h* transcription in the pericarp of NILs carrying various combinations of *Pp* alleles. The *lines* illustrated are those which inherited the dominant allele at *Pp3* and *Pp-D1* from Purple Feed. Similar results were obtained for those which had inherited the dominant alleles from Purple. A statistical analysis is given in Tables S1 and S2

Discussion

The use of molecular markers can accelerate the selection process, lead to a greater accuracy of selection, reduce the acreage occupied by breeding material, and save labour and material resources (Moose and Mumm 2008; Leonova 2013; Khlestkina 2014a). Their use has halved the time needed to split i:S29*Pp-A1Pp-D1Pp3* into the two homozygous lines i:S29*Pp-A1pp-D1Pp3* and i:S29*Pp-A1Pp-D1pp3*, since the process took just three growing seasons, rather than the six which would have been required relying only on phenotypic selection. The volume of plant material needed (and hence the planting area required) was reduced by some 70 fold.

The NILs harbouring *Pp-D1* and *pp3* produced an uncolored pericarp, while those with *pp-D1* and *Pp3* had a light purple pericarp. The incomplete inhibition of anthocyanin production in the pericarp of the latter plants may be due to the continuing presence of the putative *Pp-A1* gene lying within the cluster of anthocyanin synthesis regulatory genes present on cv. Saratovskaya 29 chromosome 7A (Khlestkina et al. 2010b). The suggestion is that the effect of *Pp-A1* is much weaker than that of its homoeologue *Pp-D1*, in the same way that the effect of cv. Saratovskaya 29 *Rc-A1* is less than that of *Rc-D1*, a gene which is quite widely distributed (Khlestkina et al. 2002, 2009, 2014). The introgression of the critical part of chromosome 7A from a non-pigmented cultivar such as cv. Janetzkis Probat into the lines i:S29*Pp-A1pp-D1Pp3*^{PF} and i:S29*Pp-A1pp-D1Pp3*^{P} having light purple pericarp resulted in an uncolored pericarp (in the lines i:S29*pp-A1pp-D1Pp3*^{PF} and i:S29*pp-A1pp-D1Pp3*^{P}; Table 1),

thereby confirming the location of *Pp-A1* in the genetic interval defined by *Xgwm0060* and *Xgwm0974* (Fig. 1). This location fits well those of *Pp-D1* on chromosome 7D (Tereshchenko et al. 2012a) and *Pp-B1* on chromosome 7B (Khlestkina et al. 2010a).

The utility of the NILs as testers for the presence of *Pp* genes has been successfully demonstrated. When i:S29*Pp-A1pp-D1Pp3*^{PF} was crossed with i:S29*Ra* (a line which expresses intense anthocyanin pigmentation of its coleoptile, auricles, leaf blades and leaf sheaths, but develops a non-pigmented pericarp), the resulting F_1 hybrids bore grains exhibiting a dark purple pericarp, implying that i:S29*Ra* harbours a dominant allele at a *Pp-1* gene. This gene is likely *Pp-D1*, a member of a complex of pigmentation genes (*Rc-D1*, *Pc-D1*, *Pls-D1*, *Plb-D1* and *Ra-D1*) present on chromosome 7D (Khlestkina et al. 2014).

A previous analysis has concluded that the *Pp* genes act as regulators of anthocyanin synthesis in the pericarp (Tereshchenko et al. 2013). Here, the qRT-PCR method was exploited to analyze transcript abundances of *Chi* and *F3h*, the key structural genes with respect to anthocyanin synthesis, in the pericarp of the NILs carrying the various combinations of *Pp* alleles. The outcome of the analysis confirmed that the *Pp* genes indeed acted to up-regulate these anthocyanin synthesis genes. However, the conversion of chalcone to flavanone (catalyzed by CHI) did not require the presence of both *Pp-1* and *Pp3*; one of these genes (even the weak *Pp-A1*) was sufficient to allow *Chi* transcription activation (Fig. 2). In contrast, the F3H-enabled conversion of naringenin to dihydroflavonol relied on the presence of both *Pp-D1* and *Pp3*, since the presence of just one of these without the other was ineffective (Fig. 2). *Pp-1* and *Pp3* have been not sequenced, but some evidence (based on comparative mapping) points to their belonging to, respectively, the *myb* and *myc* families of transcription factors (reviewed by Khlestkina 2013). Unlike the synthesis of anthocyanin in the pericarp, that of anthocyanin in the coleoptile (and some other organs) requires the presence of a single dominant *myb*-like transcription factor (Himi et al. 2005; Khlestkina et al. 2008; Khlestkina 2013; Tereshchenko et al. 2013). The set of the lines developed here is suitable for clarifying the mechanisms underpinning the regulation of tissue-specific (and species-specific; Shoeva and Khlestkina 2014) anthocyanin synthesis in wheat.

The NILs developed here may aid in elucidating the physiological role of anthocyanins in the wheat pericarp. NILs represent a powerful means of establishing gene function, since they allow contrasts between a set of closely related genotypes which differ from each other largely only in and around a known target gene (reviewed by Khlestkina 2014b). Furthermore, the NILs developed here may have breeding value as donors of particular *Pp* alleles. The commercial interest in pigmented wheat grain flows from an understanding that they are nutritionally superior to white kernels. A combination of functional regulatory genes underlying both purple and blue grained materials may be particularly attractive. Syed Jaafar et al. (2013) have demonstrated that anthocyanin content can be boosted by stacking the purple pericarp and blue aleurone trait in a number of genetic backgrounds. The anthocyanin composition of pigmented grains (Abdel-Aal et al. 2006; Ficco et al. 2014) and the contribution of anthocyanin to the grain's antioxidant potential (Abdel-Aal et al. 2008) are well studied. In combination with an understanding of the genetic basis of the trait and associated mapping data (Dobrovolskaya et al. 2006; Khlestkina et al. 2010a; Arbuzova et al. 2012; Tereshchenko et al. 2012a, 2013; current study), there is now a strong basis for using marker-assisted selection to increase the antioxidant content of the wheat grain.

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