

Isolation of candidate genes for the barley *Ant1* and wheat *Rc* genes controlling anthocyanin pigmentation in different vegetative tissues

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Abstract MYB transcription factors exist in a large copy number and control various plant phenotypes. We cloned R2R3 MYB-type transcription factors that determine the coloration of basal sheaths in barley and wheat coleoptiles. These genes are highly homologous to maize *Cl* and rice *OsCl*, regulators for anthocyanin biosynthesis, but they control seed pigmentation in maize and rice. On the basis of high homology, barley and wheat counterparts are designated *HvCl* and *TaCl*, respectively. *HvCl* gene is located on the short arm of chromosome 7H, and *TaCl* genes are located on the short arms of chromosomes 7A, 7B, and 7D (*TaCl-A1*, *B1*, and *D1*, respectively). *HvCl* is a strong candidate for *Ant1* because of (1) complete cosegregation of anthocyanin pigmentation phenotype of the basal sheath with the *HvCl* genotype in genetic mapping, and (2) complete deletion of the *HvCl* gene in two anthocyanin-decreased allelic mutants (*ant1.1* and *ant1.2*) that were induced by irradiation. In contrast, colorless coleoptile wheat lines had lesions in all three genomes consisting of a single-nucleotide substitution or a 1-bp deletion

of *TaCl-A1*, a 1.7-kb insertion of *TaCl-B1*, and a 2.0-kb insertion of *TaCl-D1*. At least one normal *TaCl* gene appears to be sufficient to produce anthocyanin pigments in wheat coleoptiles. Previous crossing experiments localized *Rc* (red coleoptile) genes to homoeologous group 7 chromosomes and deduced *Rc* genotypes of several wheat lines. Their *TaCl* gene sequence variation coincided with deduced *Rc* genotypes; therefore, the present molecular genetic study demonstrates that *TaCl* is a strong candidate for *Rc* in wheat.

Keywords Anthocyanin biosynthesis · Barley · R2R3 MYB · Wheat

Introduction

Plant pigmentations that are stably expressed in specific growth stages and organs have been used as classical genetic markers because they are easily scored with the naked eye. Particularly, anthocyanin pigmentation has been utilized as a reliable genetic marker in grasses. In wheat (*Triticum aestivum* L. $2n = 6x = 42$, genomes AABBDD), many genes have been reported for anthocyanin pigmentation, such as *Pan* (Purple anther), *Pc* (Purple culm), *Pg* (Purple glume), *Plb* (Purple leaf blade), *Pp* (Purple pericarp), *R* (Red grain), *Ra* (Red auricle), and *Rc* (Red coleoptile) (McIntosh et al. 2010). However, among the genes noted above, only the *R* gene has been molecularly identified to encode an R2R3 MYB transcription factor that regulates proanthocyanidin synthesis in grains (Himi et al. 2011; Himi and Noda 2005).

Anthocyanin and proanthocyanidin are synthesized via a common flavonoid biosynthetic pathway. In this pathway, anthocyanin and proanthocyanidin precursors are generated

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by enzymes encoded by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), or flavonoid 3'/5' hydroxylase (F3'/5'H), dihydroflavonol 4-reductase (DFR), and then the pathway branches into anthocyanin- or proanthocyanidin-specific synthesis (Winkel-Shirley 2001). These enzymatic genes are grouped into early flavonoid biosynthetic genes (EBG), including CHS, CHI, and F3H, and late flavonoid biosynthetic genes (LBG), including DFR and further downstream catalytic enzymes, for anthocyanin and proanthocyanidin production. These enzymatic genes are controlled by specific regulatory genes. In *Arabidopsis* (*Arabidopsis thaliana*), EBGs are transcriptionally activated by R2R3 MYB proteins (MYB11, MYB12, and MYB111); however, several LBGs are activated by the MYB-bHLH-WD40 (MBW) ternary complex (Li 2014). Anthocyanin synthesis of *Arabidopsis* is controlled by the MBW ternary complex of (1) one R2R3 MYB protein from PRODUCTION OF ANTHOCYANIN PIGMENTS (PAP1), PAP2, MYB113, or MYB114, (2) one bHLH protein from TRANSPARENT TESTA8 (TT8), GLABROUS3 (GL3), or ENHANCER OF GLABRA3 (EGL3), and (3) TRANSPARENT TESTA GLABROUS1 (TTG1) of WD40. In the case of maize (*Zea mays*), both EBGs and LBGs seemed to be regulated by an MBW complex consisting of C1/P1 of MYB, R/B1/SN1/HOPI1 of bHLH, and PAC1 of WD40 for anthocyanin production (Petroni and Tonelli 2011).

In barley (*Hordeum vulgare* L. $2n = 2x = 14$, HH), a number of anthocyanin/proanthocyanidin-decreased (*ant*) mutants, with both spontaneous and artificial origins, were isolated. Allelism tests grouped *ant* mutants into 30 genetically independent loci (*Ant1* to *Ant30*), but the molecular identities of only four loci have been clarified: *Ant2* (*HvbHLH1*) (Cockram et al. 2010), *Ant18* (*dihydroflavonol 4-reductase*; *DFR*) (Kristiansen and Rohde 1991), *Ant28* (*Hvmyb10*) (Himi et al. 2012), and *Ant30* (*chalcone isomerase*; *CHI*) (Druka et al. 2003). *Ant2* regulates the anthocyanin pigmentation of the auricle, awn, and lemma, but it is not involved in grain proanthocyanidin pigmentation. In contrast, *Ant28* specifically regulates proanthocyanidin synthesis in grain.

In rice (*Oryza sativa*), the allelic series at the *C* locus controls the degree of anthocyanin pigmentation in the apiculus. The rice *C* gene encodes an R2R3 MYB-type transcription factor that is an ortholog of maize *CI* (Saitoh et al. 2004). Additionally, two *R* genes were identified in rice, designated *Ra* and *Rb*; the active *Ra* gene showed similarity to the maize *R* (*Lc*) gene (Hu et al. 1996).

Wheat *Rc* genes remain molecularly unidentified, but their recessive (colorless coleoptiles) lines did not express *F3H*, one of the enzymatic genes. This observation suggests that *Rc* is a regulatory gene, such as MYB or bHLH (Khlestkina et al. 2008). Wheat *Rc* genes mapped the short arms of chromosomes 7A (*Rc-A1*), 7B (*Rc-B1*), and 7D

(*Rc-D1*) (Khlestkina et al. 2002). Triple recessive genotypes (gene symbol *Rc-A1a/Rc-B1a/Rc-D1a*) exhibit colorless coleoptiles, while those with at least one dominant genes (*Rc-A1b*, *Rc-B1b*, or *Rc-D1b*) show anthocyanin pigmentation in the coleoptiles (Khlestkina et al. 2010). Interestingly, barley *ant1* mutants lack anthocyanin pigmentation in the basal leaf sheath that appears to have a similar phenotype to that of wheat *Rc* triple recessives with colorless coleoptiles (Jende-Strid and Lundqvist 1978).

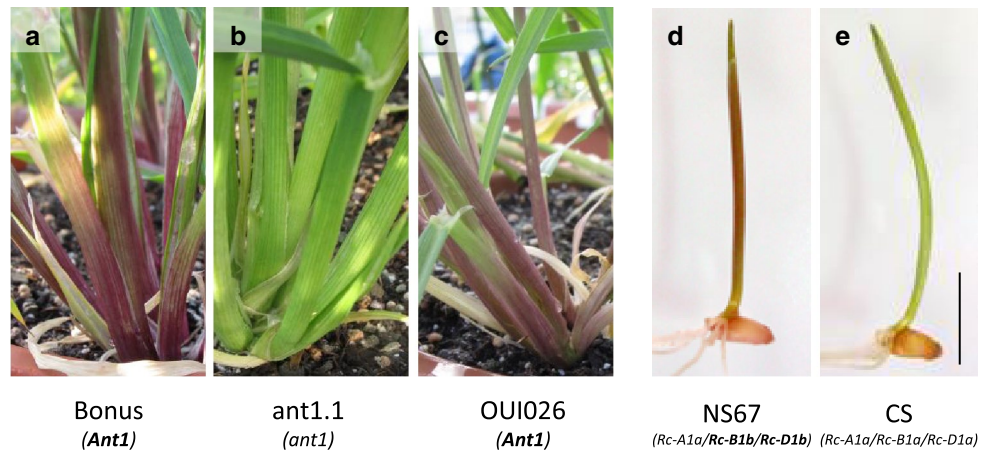
In this study, we cloned new members of the R2R3 MYB genes that control the anthocyanin biosynthesis pathway in wheat and barley. These MYB genes were designated *TaCI-A1*, *TaCI-B1*, and *TaCI-D1* derived from the A, B, and D genomes of hexaploid wheat, respectively, and their barley ortholog was named *HvCI*. This is because they showed high similarity to the previously reported maize *CI* and rice *OsCI*. *TaCI* genes and the *HvCI* gene mapped on the short arms of wheat homoeologous group 7 and barley 7H, which are syntenic to the rice chromosome 6 region that harbors the *OsCI* gene. Genomic sequencing analysis revealed that a single-nucleotide substitution or a 1-bp deletion was found in the *TaCI-A1* gene of recessive *Rc-A1a* lines, a 1.7-kb insertion was found in the *TaCI-B1* gene of recessive *Rc-B1a* lines, and a 2.0-kb insertion was found in the *TaCI-D1* gene of recessive *Rc-D1a* lines. Substitutions or insertions are predicted to cause amino acid changes or frame-shift, and the resultant protein may be dysfunctional as regulators to activate the flavonoid biosynthetic genes. Additionally, it was found that two independent barley *ant1* mutants deleted at least a 7-kb 7HS genomic region, including the entire *HvCI* gene. On the basis of multiple mutant analyses, both in wheat and barley, we conclude that wheat *Rc* genes and barley *Ant1* loci may encode new members of the R2R3 MYB transcription factor that show a high homology to the maize *CI* gene. The unique aspect of wheat *Rc* and barley *Ant1* genes is their vegetative tissue-specific regulation of anthocyanin pigmentation, in contrast to the grain color regulation of maize *CI* and rice *OsCI*.

Materials and methods

Materials

Wild-type barley (*Hordeum vulgare* L., cv. Bonus) and its anthocyaninless *ant1* mutants (*ant1.1*, *ant1.2*, *ant1.4*, and *ant1.56*) were supplied by Dr. Udda Lundqvist (NordGen) for use in this study (Supplementary Table S1). As compared to the wild type, *ant1.1* does not show red pigmentation in leaf sheath (Fig. 1a, b). *ant1.2* shows a similar colorless phenotype, but *ant1.4* and *ant1.56* show a red-colored phenotype that disagrees with the original mutant description (Supplementary Fig. S1a). The latter two dubious mutants were also included in the initial molecular analysis.

Fig. 1 Leaf sheath bases of barley (**a–c**), and coleoptiles of wheat (**d, e**). Bonus and OUI026, which have dominant *Ant1* loci, show red (**a** and **c**); whereas *ant1.1*, which lacks *Ant1*, does not show the red color (**b**). NS67 genotyped *Rc-A1a/Rc-B1b/Rc-D1b* accumulates anthocyanin in a coleoptile (**d**), but CS (*Rc-A1a/Rc-B1a/Rc-D1a*) does not accumulate red pigment in a coleoptile (**e**). *Ant1*, *Rc-B1b*, and *Rc-D1b* written in *bold* represent dominant alleles (color figure online)



Four wheat cultivars with deduced Red coleoptile (*Rc*) genotypes in a previous study (Khlestkina et al. 2011) were used: Chinese Spring (CS; *Rc-A1a/Rc-B1a/Rc-D1a*), Hope (*Rc-A1b/Rc-B1b/Rc-D1a*), Novosibirskaya67 (NS67; *Rc-A1a/Rc-B1a/Rc-D1b*), and Mironovskaya808 (M808; *Rc-A1a/Rc-B1a/Rc-D1b*) (Supplementary Table S1). Since CS is a triple recessive *Rc* genotype, its coleoptile does not have red pigmentation as compared to NS67 (Fig. 1d, e). Moreover, 6 additional lines/cultivars with unknown *Rc* genotypes were included: 8019R1, Douson1, Cornell595-W, Ardito, AUS1408, and OW104. All these show red pigmentation in the coleoptile.

Wheat cv. CS and barley cv. Betzes chromosome disomic addition lines harboring barley chromosomes 2H, 3H, 4H, 5H, 6H, and 7H as well as ditelosomic addition lines for the short arms of Betzes chromosome 1HS, 7HS, and 7HL (Islam et al. 1981) listed in Supplementary Table S1 were also used. Nulli-tetrasomic lines of Chinese Spring for homoeologous group 7 and CS deletion lines for the short arms of 7A, 7B, and 7D (Endo and Gill 1996) provided by the National BioResource Project were also used.

Analysis of anthocyanin content

Relative anthocyanin levels were determined according to Neff and Chory (1998), with some modification. Auricles of flag leaves and 3 cm of the upper part of the awns of Bonus and *ant1.1* were collected at the heading stage. Frozen tissues were ground with liquid N₂ and incubated for 16 h in 300 μL of methanol acidified with 1 % HCl at 4 °C. After the addition of 200 μL of distilled water, anthocyanins were separated from chlorophylls with 500 μL of chloroform. Total anthocyanins were determined by measuring the OD535 and OD657 of the aqueous phase using a spectrophotometer (Ubest, Japan Spectroscopic Co.). By subtracting the OD657 from the OD530, the relative amount of anthocyanin was calculated. Anthocyanin

extraction was performed in triplicate with different tillers. A *t* test was used for statistical analyses.

DNA extraction and PCR amplification

DNA was isolated from 0.1 g of leaves with DNeasy Plant Mini Kit (Qiagen) according to instructions from the supplier. Primers used for this study are listed in Supplementary Table S2.

Barley cDNA (accession number AK359777) that showed high similarity to *OsCI* (AB111869; Os06g0205100) on rice chromosome 6 was used to design the primers (HvC1-LP1, 2, 3, and HvC1-RP1, 2, 3) (Fig. 2a). The cDNA sequence showed identification with Morex_contig_137164 (IPK Blast Server; <http://webblast.ipk-gatersleben.de/barley/>), and about 4-kb upper and 1.5-kb lower sequences from the identification area in the contig were used to design the pairs of primers shown in Fig. 2a (137164-LP1 and RP1 and 137164-LP2 and RP2, respectively). Also, pairs of primers (48315-LP1/RP1 and 1562627-LP1/RP1) were designed based on the sequences of Morex_contig_48315 and Morex_contig_1562627, which correspond to Os6t0204800 and Os6t0205600, respectively (Fig. 2a).

A partial sequence of *TaCI* was obtained from the amplification with a pair of HvC1-LP1 and -RP2. The obtained sequence was compared to Cereals DB (http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB/search_reads.php), and high similarity between contig559744 and contig383159 derived from CS was found. Then, primers for *TaCI* were designed based on the obtained sequences.

The PCR mixture (10 μL) included 5 ng of genomic DNA, 1 × Ex Taq buffer (TaKaRa), 0.2 mM dNTP, 0.5 μM primers, 10 % glycerol, and 0.25 U of ExTaq (TaKaRa). A total of 35 PCR cycles were performed, with each cycle involving 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, except for some pairs of primers, for which the annealing temperatures used are mentioned later. PCR products were checked by electrophoresis and then cloned into

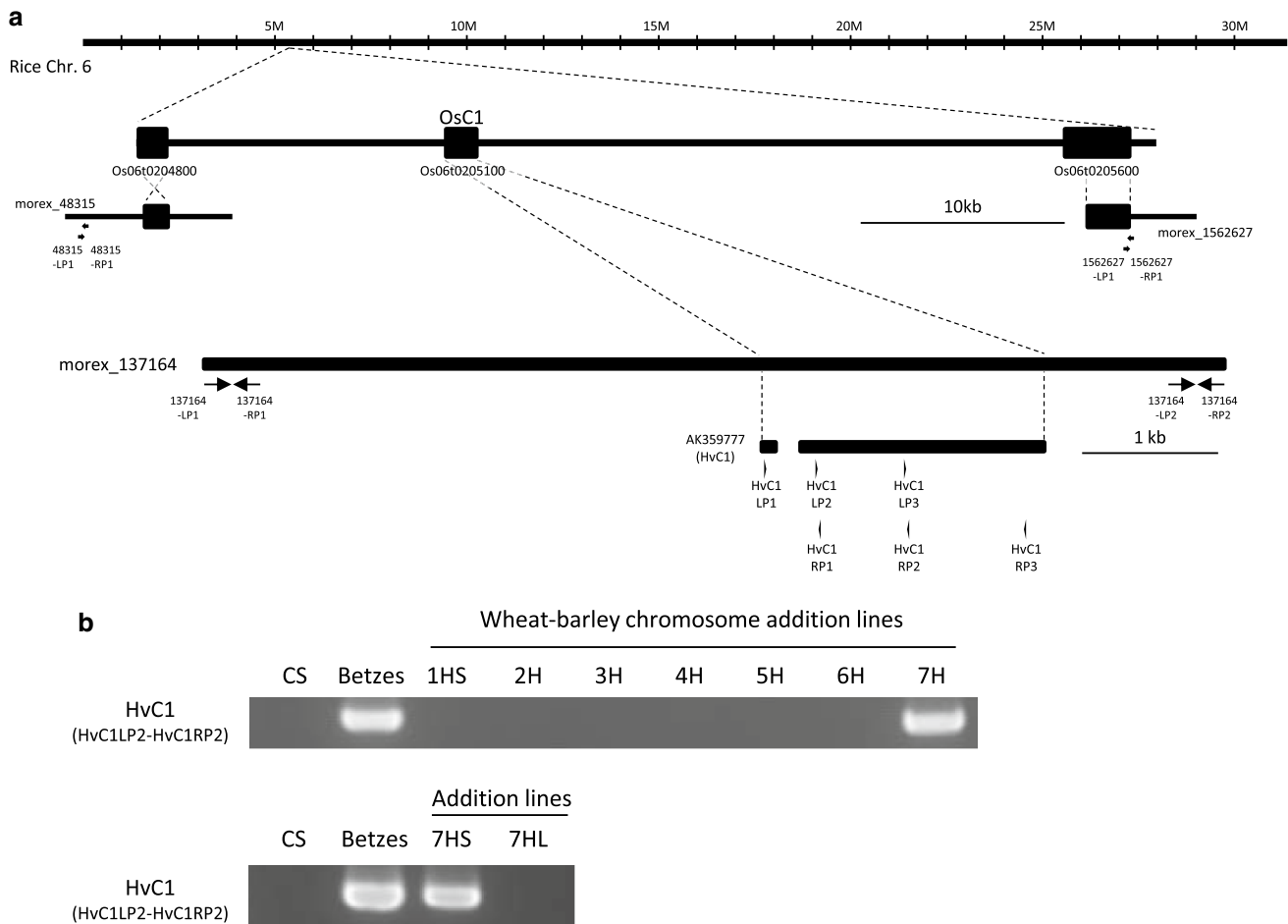


Fig. 2 **a** Chromosomal localization of rice *CI* (*OsCI*) and the relation between *OsCI* and *HvCI*. *OsCI* (syn.Os06t0205100) is located on the rice chromosome 6 pseudomolecule positioned from 5315163 to 5316640 (+strand). The *OsCI* shows high homology to a barley contig, morex_contig_137164, and a cDNA, AK359777. Two neighbor genes of *OsCI*, Os06t0204800 and Os06t0205600, show high homology to barley contigs, morex_contig_48315 and morex_con-

tig_1562627, respectively. *Arrows* represent primers based on these contigs (48315-LP1 and 48315-RP1, 1562627-LP1 and 1562627-RP1, 137164-LP1 and 137164-RP1, and 137164-LP2 and 137164-RP2). *Black arrowheads* represent primers for *HvCI*. **b** PCR amplification of *HvCI* using wheat cv. CS, barley cv. Betzes, and wheat-barley chromosome addition lines

pGEM-T Easy Vector (Promega) and sequenced with ABI 3100 sequencer. Obtained sequences were analyzed using GENETYX ver. 10.0.3 (GENETYX CORPORATION).

The 5' regions of *TaCI* were amplified by inverse PCR. The genomic DNA (2.5 µg) of NS67 was digested with 25 U of one of the following enzymes: *ClaI* or *PmlI*. The digested DNA was self-ligated using T4-ligase (Promega) and used as a template for subsequent PCRs, with TaC1-LP4 and TaC1-RP2 for the first PCR and TaC1-LP5 and TaC1-RP5 for the nested PCR.

TaCI genotyping

In recessive *TaCI-A1* lines such as CS, single-nucleotide substitutions were found in the 3' terminal region. A dCAPS (Derived Cleaved Amplified Polymorphic Sequences) primer,

TaC1-LP15, was designed using the dCAPS Finder ver. 2.0 (<http://helix.wustl.edu/dcaps/dcaps.html>). *TaCI-A1* fragments were first amplified with TaC1-LP11 and TaC1-RP13 with 35 cycles of 30 s at 94 °C, 30 s at 64 °C, and 1 min at 72 °C, and then nested PCR was performed with TaC1-LP15 and TaC1-RP4 with 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. Ten µL of the PCR product was incubated with/without 10 U of *ApaI* and separated in 3 % agarose gel.

Two types of *TaCI-B1* were separately amplified with pairs of primers: TaC1-LP10 and TaC1-RP8, and TaC1-LP11 and TaC1-RP8. Also, two types of *TaCI-D1* were amplified with combinations of TaC1-LP7 and TaC1-RP11, and TaC1-LP3 and TaC1-RP11.

TaCI-D1 genotyping was performed with 96 F₂ plants of NS67 and CS crossing, using the primers for *TaCI-D1* mentioned above.

Mapping of *HvCl* and *TaCl*

A total of 96 F₂ plants derived from a cross between OUI026 and ant1.1 were used for the molecular mapping of *ant1* in relation to *HvCl* and other markers. OUI026 shows pigmentation in the leaf sheath base (Fig. 1c). The presence or absence of purple pigmentation at the leaf sheath base was individually scored at the tillering stage. Recombination values were calculated as reported in Taketa et al. (2011).

To map the *HvCl* gene, HvC1-LP1 and HvC1-RP1 primers were used. Other molecular markers used for mapping listed in Supplementary Table S2 were from public sources (Li et al. 2003). The map was compared to previously reported maps by Varshney et al. (2007), Rostoks et al. (2005), and BinMap (<http://barleygenomics.wsu.edu/>).

For chromosomal localization of *TaCl-A1*, *-B1*, and *-D1* of CS, the amplification specificity from each homoeologous group 7 chromosome was confirmed with nulli-tetrasomic lines. Cytogenetic mapping was also performed with the aid of partial deletion lines of the short arm of homoeologous group 7 chromosomes of CS. The primer pairs for 7A, 7B, and 7D were TaC1-LP3 and TaC1-RP4, TaC1-LP10 and TaC1-RP8, and TaC1-LP7 and TaC1-RP11, respectively. The chromosomal location of *TaCl-A1* was compared to maps by Khlestkina et al. (2002) and Somers et al. (2004).

Results

Isolation of *HvCl*

Rice *Cl* (*OsCl*), a homolog of maize *Cl*, was previously isolated and is located on the short arm of chromosome 6 (Saitoh et al. 2004) (Fig. 2a). A predicted amino acid sequence of AK359777 derived from a cDNA library from a germinated shoot of barley cv. Haruna Nijo showed a high homology (59 % identity) to *OsCl*. It showed a high identity (94 %) to a contig (morex_contig_137164) derived from barley cv. Morex. From the comparison of the genomic and cDNA sequences, this gene consists of two exons and one intron and is predicted to encode a 253-amino acid protein. We designated this gene *HvCl* as a homolog of maize *Cl* and *OsCl*.

PCR primers specific for *HvCl* were designed (Fig. 2a) and used for chromosome localization with wheat–barley chromosome addition lines. As shown in Fig. 2b, an amplified fragment was observed in barley cv. Betzes but not in wheat cv. CS. An amplified fragment of the same size was observed in 7H and the 7HS addition lines. From this result, *HvCl* is located on the short arm of chromosome 7H, which was reported to be syntenic to rice chromosome 6.

In barley, a number of anthocyanin-/proanthocyanidin-reduced or -deficient mutants were discovered and classified from *Ant1* to *Ant30*. It was reported that *ant1* reduces the anthocyanin content in the stem, and *Ant1* is located on chromosome 7H (Jende-Strid 1984). Four alleles of *ant1* mutants (*ant1.1*, *ant1.2*, *ant1.4*, and *ant1.56*) have been isolated. Both *ant1.1* and *ant1.2* showed anthocyaninless leaf sheaths. However, *ant1.4* showed pink pigmentation, and *ant1.56* showed dark red pigmentation in the leaf sheath at a level similar to that of the original cv. Bonus (Fig. 1a and Supplementary Fig. S1a). Red pigment is visible in the auricles of Bonus and *ant1.1*, but the *ant1.1* awn appeared anthocyaninless (Supplementary Fig. S1b–e). Quantification of the red pigments in the auricle and awn showed that *ant1.1* accumulated reduced amounts of anthocyanin as compared with Bonus in both organs; the *ant1.1* awn accumulated a significantly lower amount of red pigment (Supplementary Fig. S1f).

PCR amplification of *HvCl* was attempted using three pairs of primers. Bonus, *ant1.4*, and *ant1.56* showed amplification in all primer pairs, whereas no amplification was observed with *ant1.1* or *ant1.2* (Fig. 3a). Thus, positive or negative PCR amplification corresponded to the presence or absence of anthocyanin pigmentation in the leaf sheath. In *ant1.1* and *ant1.2*, no amplification was also found with primers designed from the regions of 4-kb above the 5' terminus of *HvCl* and 1.3-kb below the 3' terminus of *HvCl* in morex_contig_137164 (Figs. 2a, 3b). Os06t0204800 and Os06t0205600, which show similarities to barley contig (morex_contig_48315 and morex_contig_1562627, respectively), are located 14-kb above and 29-kb below Os06t0205100 (*OsCl*) (Fig. 2a). Primers designed for each farther distant barley contig amplified the expected size of the fragments in *ant1.1* and *ant1.2* as their wild type (Fig. 3b). Therefore, at least a 7-kb length of DNA, including the entire *HvCl* gene, seemed to be deleted in *ant1.1* and *ant1.2*; both are irradiation-induced mutants (Franckowiak and Lundqvist 2012).

Isolation of *TaCl*

In wheat, a gene for controlling the coleoptile color has been reported and designated the *Rc* gene. *Rc* genes have been shown to be located on the short arm of chromosomes 7A, 7B, and 7D (Khlestkina et al. 2002). In this study, CS (colorless coleoptile; *Rc-A1a/Rc-B1a/Rc-D1a*), NS67 (red coleoptile; *Rc-A1a/Rc-B1a/Rc-D1b*), and Hope (red coleoptile; *Rc-A1b/Rc-B1b/Rc-D1a*) were used. A partial sequence was obtained from amplification with a primer pair, HvC1-LP1 and -RP2, using an NS67 DNA template. This sequence showed 83 % identity to *HvCl* (deduced amino acid showed 90 % identity), and the position of an intron is identical. Thus, we designated *TaCl* as being

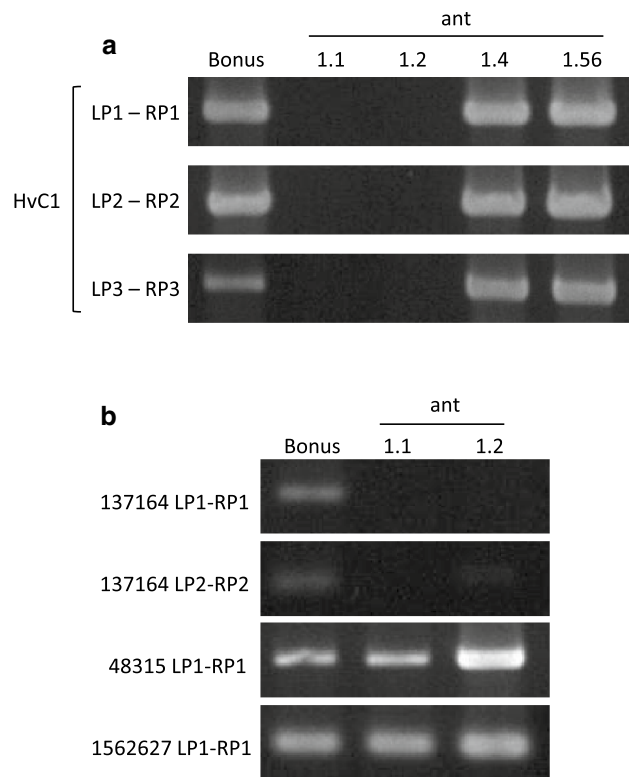


Fig. 3 Amplification of the *HvCl* gene and the neighbor genes of *HvCl*. **a** PCR amplification of *HvCl* genes of three sets of primers with Bonus and its mutants, ant1.1, 2, 4, and 56. **b** PCR amplification of the neighbor genes of *HvCl*. 137164-LP1 and -RP1 amplified 4-kb above and 137164-LP2 and -RP2 amplified 1.3-kb below the region from *HvCl*. 48315-LP1 and -RP1, and 156267-LP1 and -RP1 were designed based on the barley contigs, morex_contig_48315 and morex_contig_156267, which showed high similarity to Os06t0204800 and Os06t0205600, located on 14-kb above and 29-kb below Os06t0205100 (*OsCl*). Positions of the primers are depicted in Fig. 2a

homologous to *HvCl*, maize *Cl*, and *OsCl*. Unique primers for *TaCl* were designed, and the primer pair, TaCl-LP3 and -RP4, recognized only wheat chromosome 7A because an amplified fragment was absent in the Nulli7A-tetra7B and Nulli7A-tetra 7D lines but present in other group 7 nulli-tetrasomic lines that retained 7A (Fig. 4a). Therefore, the amplified sequence was named *TaCl-AI*. PCR using the TaCl-LP3 and -RP4 primer pair was performed with partial chromosome deletion lines for 7AS. *TaCl-AI* was amplified in 7AS-1, 9, 12, and 2, but not in 7AS-11, 5, 8, 10, 13, 3, 4, or 6 (Fig. 4b). This result showed that *TaCl-AI* is located between fraction lengths 0.66 and 0.73.

The *TaCl* sequence was compared to Cereals DB (http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB/search_reads.php) and showed high similarity to contig 559744 and contig 383159 derived from CS. Contig 559744 has a partial sequence of *TaCl* that starts from 10-bp above the second exon. An inverse PCR product of

NS67 was identical to contig 559744; therefore, primers were designed based on these sequences. A pair of primers (TaCl-LP10 and -RP8) based on the inverse PCR product and contig 559744 recognized only *TaCl* on 7B, designated as *TaCl-BI* (Fig. 4a). Also, a pair of primers (TaCl-LP7 and -RP11) based on contig 383159 amplified only *TaCl* on 7D, designated as *TaCl-DI* (Fig. 4a). *TaCl-BI* did not amplify in partial chromosome deletion lines 7BS-1, -2, and -3. Similarly, *TaCl-DI* did not amplify in any of the five deletion lines tested, 7DS-1, -2, -3, -4, or -5. From these results, *TaCl-BI* and *D1* appeared to be located on the distal region of chromosomes 7B and 7D, respectively (Supplementary Fig. S2a, b).

In *TaCl-AI* of CS (recessive *Rc-AIa*), one nucleotide deletion was found that causes a frame-shift that disrupts the amino acid sequence from position 239 to the C-terminal (Figs. 5, 6a, and Supplementary Fig. S3).

Interestingly, another recessive *Rc-AIa* line, Mironovskaya808 (M808), had the same 1-bp deletion at the identical position. On the other hand, NS67 (recessive *Rc-AIa*) has a normal C-terminal region, but a single-nucleotide substitution that changes an amino acid residue from N (Asn) to D (Asp) in R3 MYB repeats (Figs. 5, 6a, and Supplementary Fig. S3). As compared to the *TaCl-BI* of Hope (dominant *Rc-BIb*), that of CS (recessive *Rc-BIa*) is longer because of a 1.7-kb insertion sequence in the first exon (Fig. 6a and Supplementary Fig. S3). Transcription of the sequence that is 1.7 kb longer may not have a functional MYB factor. As does *TaCl-BI*, *TaCl-DI* of CS (recessive *Rc-DIa*) has a 2.0-kb insertion in the second exon (Fig. 6a and Supplementary Fig. S3).

The *TaCl* genotype coincides with the *Rc* genotype

A dCAPS primer, TaCl-LP15, was designed to detect the 1-bp deletion and the *TaCl-AI* genotyping (Fig. 6b). PCR products of CS and M808 were not digested after *ApaI* treatment because their *TaCl-AI* lacked 1-bp, and the recognition sites of *ApaI* were not produced (Fig. 6c). On the other hand, PCR products of Hope and NS67 were digested (Fig. 6c). Undigested upper fragments might be derived from the homoeologous *TaCl*. This dCAPS marker was also used with other red coleoptile lines which were not genotyped. 8019R1 and Douson1 showed same digested pattern to Hope and NS67 whereas Cornell595-W, Ardito, AUS1408 and OW104 showed same undigested pattern to CS and M808 (Supplementary Fig. S4a). This result indicated that the *Rc-AI* genotype of Cornell595-W, Ardito, AUS1408 and OW104 is recessive *Rc-AIa*. Although NS67 is also an *Rc-AIa* line, the amplified fragment was digested, since the mutation site of NS67 was different (Fig. 6a, Supplementary Fig. S3). Then, other dCAPS marker, TaCl-RP15, was designed to detect 1-bp substitution of NS67 (Supplementary

Fig. 4 Chromosomal locations of *TaCl* genes. **a** Three sets of primers recognized as *TaCl-A1*, *B1*, and *D1* on chromosomes 7A, 7B, and 7D, respectively. **b** Amplification of *TaCl-A1* in CS and partial deletion lines of 7AS. Fraction length (FL) shows the relative remaining length of the short arm

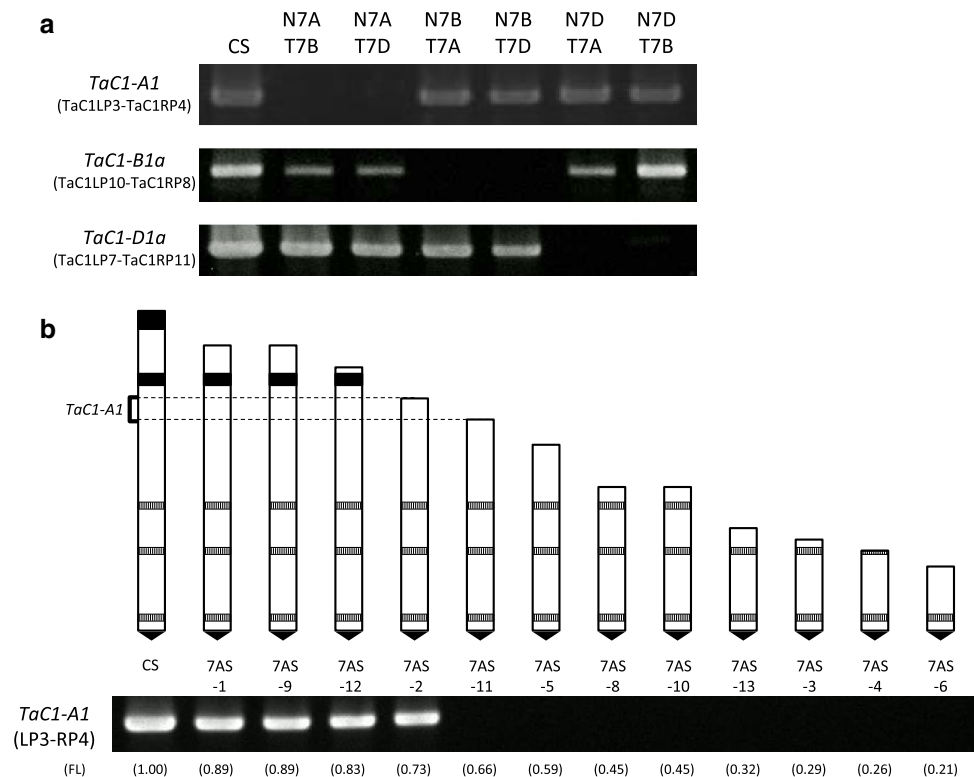


Fig. S4b). After treatment with *EcoRV*, PCR fragments of NS67 and Douson1 were digested (Supplementary Fig. S4c). Therefore, Douson1 has same allele of *TaCl-A1* of NS67, and is diagnosed as *Rc-A1a*. However, this NS67-type allele of *TaCl-A1* seems to be rare because other colorless coleoptile lines belong to the CS type with a 1-bp deletion in the second exon (data not shown). 8019R1 showed same pattern to Hope with the two dCAPS markers; 8019R1 has dominant *Rc-A1b* allele. Then, a primer, TaC1-LP10, was designed based on the 1.7-kb insertion sequence of *TaCl-B1*. Amplified fragments with this primer and TaC1-RP8 were observed only in recessive *Rc-B1a* lines. In contrast, a primer for the normal sequence of *TaCl-B1* (TaC1-LP11) recognized a dominant *Rc-B1b* line (Hope) (Fig. 6d and Supplementary Fig. S4d). Similarly, TaC1-LP7, designed based on the 2.0-kb insertion sequence of *TaCl-D1*, amplified fragments in recessive *Rc-D1a* lines, whereas a primer of the normal sequence of *TaCl-D1* (TaC1-LP3) was amplified in dominant *Rc-D1b* lines (NS67 and M808) (Fig. 6e and Supplementary Fig. S4e). From these results, normal and mutated sequences of *TaCl-A1*, *B1*, and *D1* were clarified, and the pattern of the *TaCl* genotype coincides with the *Rc* genotype. Additionally, *Rc* genotypes of other red coleoptile lines were estimated as follows: 8019R1 (*Rc-A1b/Rc-B1a/Rc-D1a*), Douson1 (*Rc-A1a/Rc-B1a/Rc-D1b*), Cornell595-W (*Rc-A1a/Rc-B1a/Rc-D1b*), Ardito (*Rc-A1a/Rc-B1a/Rc-D1b*), AUS1408 (*Rc-A1a/Rc-B1a/Rc-D1b*), and OW104 (*Rc-A1a/Rc-B1a/Rc-D1b*).

Mapping of the *HvCl* gene

To map the *HvCl* gene, an *HvCl*-LP1 and -RP1 primer pair was used. No amplification was observed in *ant1.1*, whereas the expected fragment was amplified from the wild-type mapping parent, OUI026. This is probably due to the irradiation-induced origin of *ant1.1*, deleting the entire *Ant1* gene. Thus, molecular mapping for *HvCl* was conducted as a dominant marker.

Mapping of the *HvCl* gene was carried out along with the basal leaf sheath color segregating in the F_2 population consisting of 96 individuals derived from an OUI026 and *ant1.1* cross. The *ant1* basal leaf sheath color and the *HvCl* gene cosegregated completely and mapped to the same position (Fig. 7a). *Ant1* and the *HvCl* genes were mapped on chromosome 7HS by comparison with other barley maps (Rostoks et al. 2005; Varshney et al. 2007) (BinMap, <http://barleygenomics.wsu.edu/>).

Chromosomal location of the *TaCl* gene

By cytogenetic mapping using CS deletion lines for 7AS, *TaCl-A1* was localized in a rather distal region between breaking points 0.66 and 0.73 (Fig. 4b). Previous bin mapping studies localized many wheat EST clones to physical regions delimited by series of partial chromosome deletion lines (<http://wheat.pw.usda.gov/wEST/binmaps/>). Between 7AS-5 and -1 (fraction length 0.59–0.89), 13 ESTs may

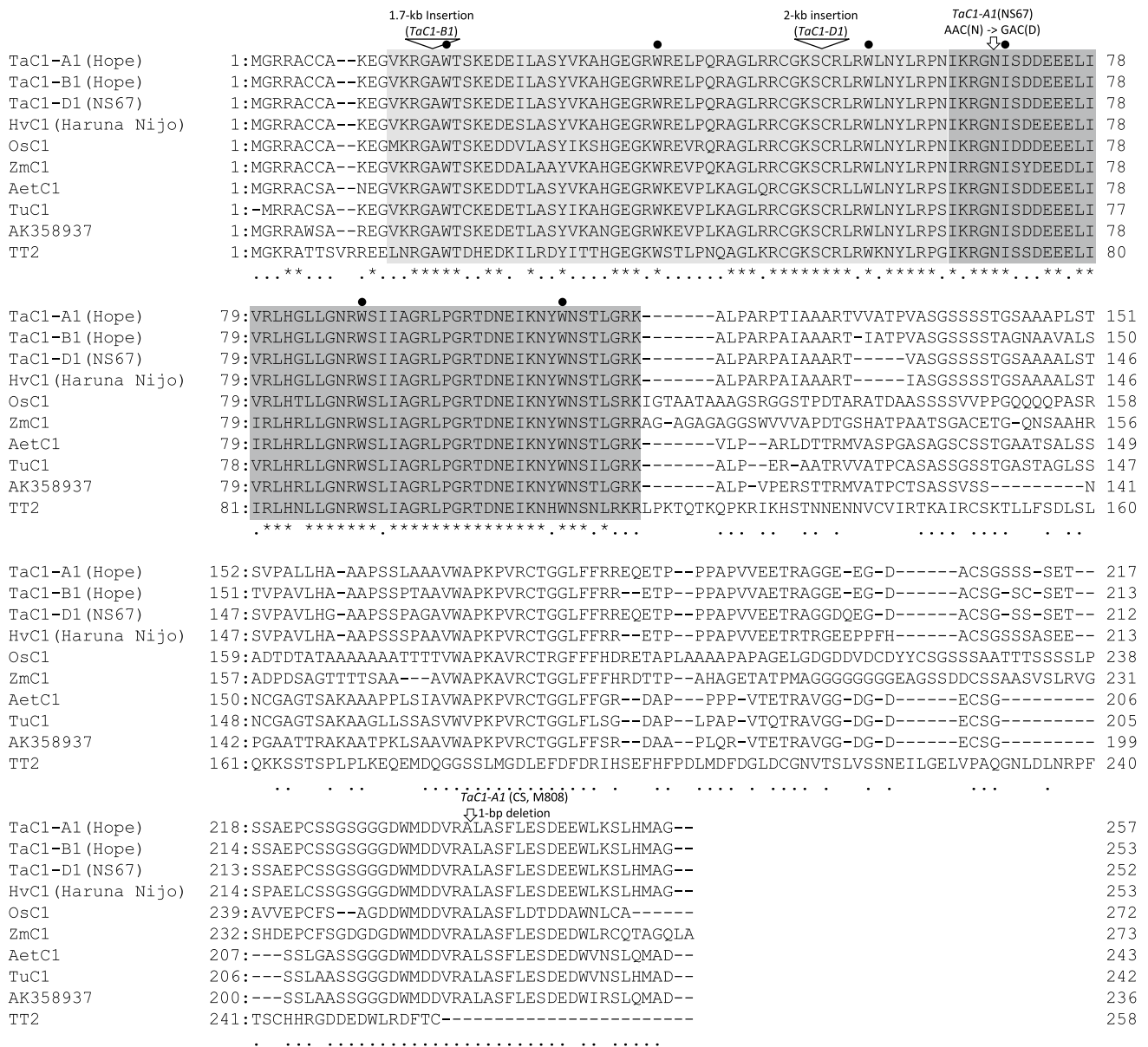


Fig. 5 Alignment of deduced amino acid sequences of *C1* genes of wheat (TaC1-A1 of Hope, TaC1-B1 of Hope, and TaC1-D1 of NS67; accession numbers AB983540, AB983541, and AB983542), barley (HvC1; BAJ90986), maize (ZmC1; NP_001147547), *Aegilops tauschii* (AetC1; EMT07248), *Triticum urartu* (TuC1; EMS50544). Deduced amino acid sequences of an HvC1-like gene (AK358937) and the seed color regulator of Arabidopsis (AtTT2; CAC40021) are

also aligned. Regions shaded *light gray* and *dark gray* represent R2 and R3 MYB repeats, respectively. Conserved W (Trp) and I (Ile) in the R2R3 MYB repeats are denoted with *black circles*. *White arrows* indicate the positions of single-nucleotide substitution in NS67 and 1-bp deletion in other *Rc-A1a* lines of the *TaC1-A1* gene. *White arrowheads* represent the insertion of *TaC1-B1* and *-D1* in *Rc-B1a* and *Rc-D1a*, respectively

correspond to rice chromosome 6 (Fig. 7b). This region contains BE493812, which corresponds to Os06t0138200, located at 2.0 Mb, and BE590476, which corresponds to Os06t0211200, located at 5.7 Mb on a rice chromosome 6 pseudomolecule. *OsC1* (syn. Os06t0205100) is located between these two rice genes separated by 3.7 Mb, and *TaC1-A1* is located within the region. Rough genetic mapping using SSR markers localized *Rc-A1* between

Xgwm913 and Xgwm60 on the short arm of chromosome 7A (Khlestkina et al. 2002). Markers on chromosome 7A reported by Somers et al. (2004) were used for PCR with partial chromosome deletion lines and showed that Xgwm471 is located on a region between 0.83 and 0.89; Xgwm60 is located on a region between 0.73 and 0.83, and Xgwm260 is located on a region between 0.32 and 0.45 (Fig. 7b). These results suggest that *TaC1-A1*

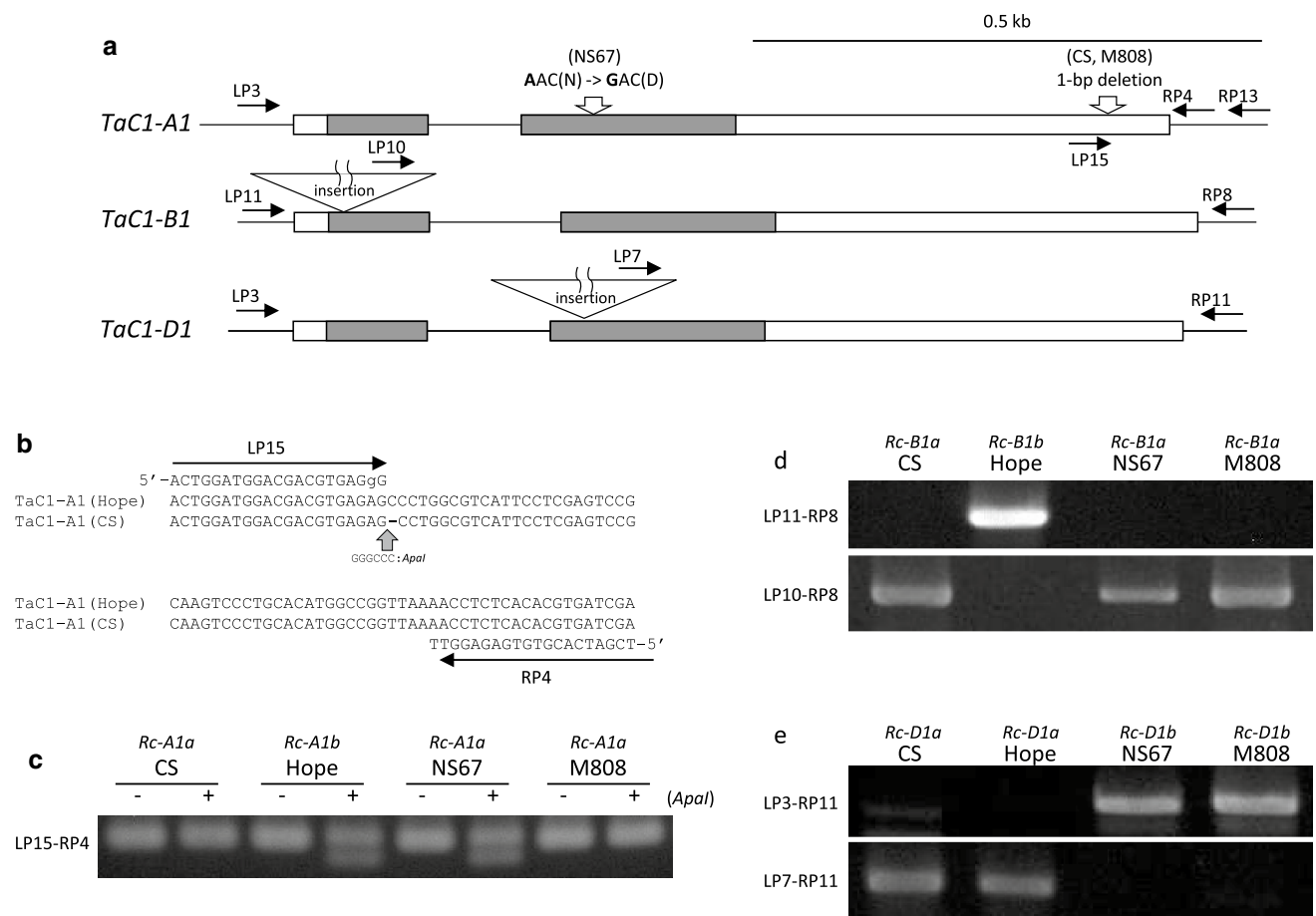


Fig. 6 *TaC1* genotyping. **a** Schematic diagrams of *TaC1-A1*, *B1*, and *D1* genes. Boxes represent exons, and lines represent introns and untranslated regions. Gray-boxed region encodes R2R3 MYB repeats. Positions and directions of primers are shown by arrows. White arrows on *TaC1-A1* indicate the positions of a single-nucleotide substitution in NS67 and a 1-bp deletion in CS and M808. **b** Partial alignments of *TaC1-A1* sequences of Hope (*Rc-A1b*) and CS (*Rc-A1a*). Primers for PCR are shown above (TaC1-LP15) and below (TaC1-RP4) with black arrows in alignment. The TaC1-LP15 primer generates a recognition site of *Apa*I (GGGCC) with a mismatch base printed in lower case and shown by a gray arrow. **c** PCR

products of *TaC1-A1* with TaC1-LP15 and TaC1-RP4 from CS (*Rc-A1a*), Hope (*Rc-A1b*), NS67 (*Rc-A1a*), and M808 (*Rc-A1a*) with (+) or without (–) *Apa*I treatment. **d** PCR product of *TaC1-B1* with the pairs of TaC1-LP11 and TaC1-RP8 from Hope (*Rc-B1b*) (above), and PCR product of *TaC1-B1* with the pairs of TaC1-LP10 and TaC1-RP8 from CS (*Rc-B1a*), NS67 (*Rc-B1a*), and M808 (*Rc-B1a*) (below). **e** PCR products of *TaC1-D1* with pairs of TaC1-LP3 and TaC1-RP11 from NS67 (*Rc-D1b*) and M808 (*Rc-D1b*), respectively (above), and PCR products of *TaC1-D1* with pairs of TaC1-LP7 and TaC1-RP11 from CS (*Rc-D1a*) and Hope (*Rc-D1a*), respectively (below)

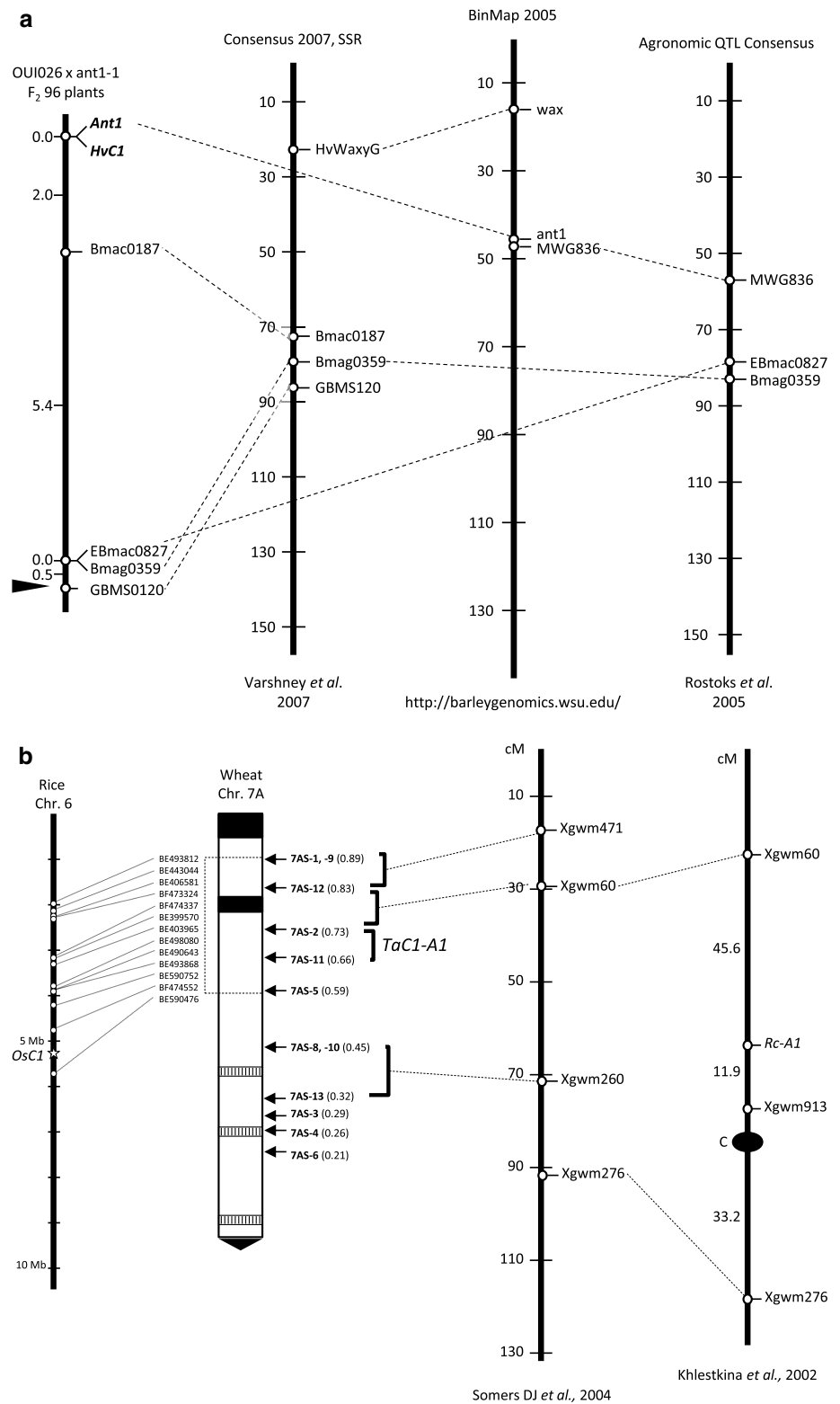
and *Rc* occupy a similar chromosome map location. Taken together, it is suggested that barley *Ant1* and wheat *Rc* are strong candidates for R2R3-type MYB *HvC1* and *TaC1* genes, respectively, which are orthologous to maize *C1* and rice *OsC1*.

Discussion

In this paper, four R2R3 MYB-type transcription factors of barley and wheat were isolated, i.e., *Hordeum vulgare* *HvC1*, *Triticum aestivum* *TaC1-A1*, *B1*, and *D1*. These were highly homologous to maize *C1* and rice *OsC1* genes and are located on barley chromosome 7HS and wheat

chromosomes 7AS, 7BS, and 7DS, which are syntenic to rice chromosome 6S. In maize, *C1* and *Pl* determine the tissue-specific synthesis of anthocyanin, i.e., *C1* in the aleurone layer of the kernel and *Pl* in the vegetative and floral tissues (Cone et al. 1993). The amino acid sequences of *C1* and *Pl* are 96 % identical and are located on chromosomes 9S and 6L, respectively. Since it has been considered that these chromosome arms have duplicated segments, *Pl* is considered to be a duplicate of *C1* (Cone et al. 1993). The *OsC1* gene in rice is also responsible for anthocyanin pigmentation in the tip of husk that covers the caryopsis. A duplicated *OsC1* gene was, however, not discovered in the Rice Annotation Project Database (RAP-DB, <http://rapdb.dna.affrc.go.jp/>). The expression of *OsC1* was detected in

Fig. 7 a Location of *HvCl* as compared with other genetic maps of barley chromosome 7H. *Left to right* partial linkage map of barley chromosome 7H in the F_2 population of OUI026 and ant1.1 (the locations of the *ant1* trait and *HvCl* are shown in **bold**); the microsatellite consensus map (Varshney et al. 2007); BinMap from Washington State University (<http://barleygenomics.wsu.edu/>); and agronomic QTL map (Rostoks et al. 2005). A **black triangle** indicates the centromere. Numbers at the *left side* of the *left* map show the distance in centiMorgans, and numbers on the *left side* of the three maps on the *right* show the distance in centiMorgans from the *top* of each chromosome. **b** Comparative mapping of wheat *TaCl-A1* and rice *OsCl*. *Left to right* part of rice chromosome 6 pseudomolecule from the Rice Annotation Project Database (RAP-DB, <http://rapdb.dna.affrc.go.jp/>); BIN map of wheat chromosome 7AS from the wheat genetics resource center, Kansas University (<http://www.k-state.edu/wgrc/Germplasm/Deletions/delindex.html>); and the microsatellite consensus maps by Somers et al. (2004) and Khlestkina et al. (2002). *OsCl* is located from 5315163 to 5316640 on the short arm of chromosome 6, indicated by a **star**. **White circles** show genes that correspond to ESTs on chromosome 7A of wheat, as reported by Sorrells et al. (2003). Chromosomal breaking positions of partial *deletion lines* are shown with **arrows**, and numbers in parentheses indicate fraction lengths. **c** centromere



the shoot, seedling of the four-leaf stage, anther, and pistil, but not in seed tissues such as immature seeds 5 and 10 days after pollination or embryo and endosperm of 25 days after pollination in RNA-Seq FPKM Expression

Values (Ouyang et al. 2007). These results showed that *OsCl* controls only plant color synthesis like maize *Pl*, and *OsCl* has no function to regulate seed color. In barley and wheat, *HvCl* and *TaCl*s also seem to act as regulators

for plant pigmentation, but they are likely to be active in vegetative tissue and inactive in grain. Between barley and wheat, pigmented tissues differ; thus, the leaf sheath and culm are pigmented in barley, but the coleoptile is pigmented in wheat. Therefore, *HvCI* does not control the pigmentation of the coleoptiles of barley, but *TaCI* controls the coleoptile color. Every *CI* controls anthocyanin pigmentation, but the tissue specificity of the pigmentation varies among plant species. Comparative analyses of *CI*-orthologs may clarify their functional diversity.

Two full-length cDNA clones of barley, AK359777 and AK358937, are highly similar to maize *CI*, while AK359777 is a synonym of *HvCI*, which is located on chromosome 7HS. The other AK358937 of morex_contig_317820 is located on chromosome 4HL. We previously isolated a *CI*-like gene of wheat, *Tamyb9* (AB252148), which showed 72 % identity to AK358937 (data not shown). Although the draft sequences of the wheat D-genome progenitor *Aegilops tauschii* and the wheat A-genome progenitor *Triticum urartu* (Ling et al. 2013) include genes annotated as *CI*, they are more similar to AK358937 than *HvCI* (AK359777) (Fig. 5). Furthermore, the deduced amino acid sequence of the *CI*-like gene of *Triticum urartu* is ~98 % identical to wheat *Tamyb9* (data not shown). Interestingly, Li et al. (1999) found 6 loci which were hybridized to maize *CI* probe on chromosome 4B, 4D, 5A, 7A, 7B, and 7D. Moreover, Devos et al. (1995) reported that the translocation of chromosomes 4AL and 5AL was occurred at the diploid level and it is present in hexaploid wheat. These results consist with the results of barley, i.e., *HvCI* is located on chromosome 7H and *HvCI*-like gene is located on chromosome 4HL. And it is presumed that *CI*-like gene might be located on chromosome 5AL of *Triticum urartu* and 4DL of *Aegilops tauschii*. As mentioned, maize *CI* and *Pl* are considered duplicated genes, but rice *OsCI* seems to be present as a single gene. These results suggest that the duplication of the *CI* gene occurred in barley and wheat, but not in rice. Analysis of duplicated *CI* genes in cultivated and wild barley and wheat may reveal their different roles in controlling pigmentation and other biological processes.

In *Arabidopsis*, anthocyanin synthesis is regulated by the MYB-bHLH-WD40 (MBW) complex composed of TRANSPARENT TESTA GLABROUS1 (TTG1; also termed WD40), one R2R3 MYB protein from PRODUCTION OF ANTHOCYANIN PIGMENTS (PAP1), PAP2, MYB113, or MYB114, as well as one bHLH protein from TRANSPARENT TESTA8 (TT8), GLABROUS3 (GL3), or ENHANCER OF GLABRA3 (EGL3) (Li 2014). In barley, the *Ant2* locus was identified as a regulator of anthocyanin biosynthesis in plant tissues such as the awn, the auricle, and the lemma (Cockram et al. 2010). *Ant2* encodes *HvbHLH1*, which shows 35 % identity to *TT8*. This study

also showed that the deletion of *Ant1* reduced the anthocyanin content in the awn and the auricle (Supplementary Fig. S1b–f). This result suggests that MYB (*Ant1*) and bHLH (*Ant2*) may produce a complex to control anthocyanin production in barley. Although barley WD40 has not been isolated, findings from other model plants suggest that barley also is likely to form an MBW complex to control anthocyanin synthesis. Barley anthocyaninless (*Ant*) loci have been classified as *Ant1* to *Ant30*, but many loci are not yet determined. It is possible that the WD40 gene is the one of the loci which acts as a regulator of anthocyanin synthesis with *Ant1* and *Ant2* in barley. Similarly in wheat, the *Rc* gene of MYB may also act with unknown bHLH and WD40 as a regulator of anthocyanin synthesis.

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Conflict of interest The authors declare no conflict of interest.

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