

Identification of a Candidate Gene Responsible for the *G* Locus Determining Chartreuse Bulb Color in Onion (*Allium Cepa* L.) Using Bulked Segregant RNA-Seq

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Abstract

To identify a causal gene for the *G* locus determining chartreuse bulb color in onion (*Allium cepa* L.), bulked segregant RNA-Seq (BSR-Seq) was performed using yellow and chartreuse individuals of a segregating population. Through customized single nucleotide polymorphism (SNP) and differentially expressed gene (DEG) screening processes, 163 and 143 contigs were selected, respectively. One contig encoding a laccase-like protein was commonly identified from SNP and DEG screening. This contig contained four highly conserved copper-binding domains known to be signature sequences of laccases. This gene was designated *AcLAC12* since it showed high homology with *Arabidopsis AtLAC12*. A 4-bp deletion creating a premature stop codon was identified in exon 5 of the chartreuse allele. Another mutant allele in which an intact LTR-retrotransposon was transposed in exon 5 was identified from other chartreuse breeding lines. Genotypes of molecular markers tagging *AcLAC12* were perfectly matched with bulb color phenotypes in segregating populations and diverse breeding lines. All chartreuse breeding lines contained inactive alleles of *DFR-A* gene determining red bulb color, indicating that chartreuse color appeared when both *DFR-A* and *AcLAC12* genes were inactivated. Linkage maps showed that *AcLAC12* was positioned at the end of chromosome 7. Transcription levels of structural genes encoding enzymes in anthocyanin biosynthesis pathway were generally reduced in chartreuse bulk compared with yellow bulk. Concentrations of total quercetins were also reduced in chartreuse onion. However, significant amounts of quercetins were detected in chartreuse onion, implying that *AcLAC12* might be involved in modification of quercetin derivatives in onion.

Key Message

A gene encoding a laccase responsible for chartreuse onion bulb color was identified. Markers tagging this gene showed perfect linkage with bulb colors among diverse germplasm.

Introduction

Onion (*Allium cepa* L.) was the second most important vegetable crop in the world following tomato in terms of value of products in 2019. Worldwide production quantity and harvested area of dry onion have steadily increased during the last decade (<http://faostat.fao.org>). Despite its economical importance, genetic and genomic studies of onion are relatively far behind than those of tomato and other major vegetable crops. Unfavorable features of onion such as huge genome (~16 Gb), severe inbreeding depression, and biennial growth habit might be responsible for the paucity of its genomic information (Brewster 2008).

Bulb color is one of major traits in onion breeding. Diverse bulb colors such as white, yellow, red, pink, gold, and chartreuse have been reported (El-Shafie and Davis 1967; Kim et al. 2004b; Khandagale and Gawande 2019). Flavonoid compounds are responsible for diverse bulb colors (Slimestad et al. 2007; Cao et al. 2010). Flavonoids consisting of more than 6,000 derivatives are among the largest plant secondary metabolites (Zakaryan et al. 2017). It has been reported that quercetin and anthocyanin are

main flavonoids determining yellow and red bulb colors, respectively (Slimestad et al. 2007; Cao et al. 2010). Flavonoid plays diverse roles in plants such as UV protectant and pigments (Dixon and Pasinetti 2010; Fini et al. 2011; Chin et al. 2018; Scarano et al. 2018; Zaynab et al. 2018). In addition, numerous studies have reported health benefits of flavonoid compounds, such as anti-inflammatory and anticancer activities (Amawi et al. 2017; Farhadi et al. 2018; Fernández-Rojas and Gutiérrez-Venegas 2018; Kopustinskiene et al. 2020).

Flavonoid biosynthesis pathway (Fig. 1) has been extensively studied in model plants such as *Arabidopsis*, petunia, and maize (Holton and Cornish 1995; Yamazaki et al. 2003; Passeri et al. 2016; Xu et al. 2017). Many mutants exhibiting different color phenotypes have been used to identify structural genes encoding enzymes involved in pathway and regulatory genes controlling expression of structural genes. A 'MBW complex' consisting of MYB, bHLH, and WD40 transcription factors is known to be a major regulator of flavonoid biosynthesis pathway (Ramsay and Glover 2005; Hichri et al. 2011; Petroni and Tonelli 2011; Jaakola 2013). Most structural and regulatory genes in onions have been isolated (Kim et al. 2005c; Baek et al. 2017; Jo and Kim 2020).

Five major loci (*C*, *I*, *G*, *L*, *R*) are known to determine onion bulb color (Clarke et al. 1944; El-Shafie and Davis 1967). The *C* locus, called a basic color factor, is responsible for a white bulb color. When the genotype of *C* locus is homozygous recessive, white color appears regardless of other loci. A gene coding for a bHLH transcription factor has been revealed to be the causal gene for the *C* locus (Jo and Kim 2020). The *I* locus, a color inhibiting factor, is also involved in the white bulb color. Bulb color becomes white regardless of other loci if the genotype of *I* locus is homozygous dominant. When its genotype is heterozygous, creamy or reddish white bulb color will appear depending on genotypes of *R* and *L* loci (El-Shafie and Davis 1967; Seo et al. 2020).

The *R* and *L* loci are complementarily involved in the production of red bulb color. Red color appears when dominant alleles (at least one) of both *R* and *L* loci are present (El-Shafie and Davis 1967). Genes encoding dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) are causal genes for the *R* and *L* loci, respectively (Kim et al. 2005a, b). In the case of the DFR-coding gene, at least three paralogs (*DFR-A*, *DFR-B* and *DFR-C*) are present in the onion genome, although only *DFR-A* gene corresponding to the *R* locus is active (Kim et al. 2005b). Twelve inactive *DFR-A* alleles containing deletion, substitution, and insertion mutations have been identified from diverse onion germplasm (Song et al. 2014; Kim et al. 2015). In the case of the *ANS* gene, three inactive and one leaky alleles have been reported (Kim et al. 2004a, 2005a, 2016).

The *G* locus determining the chartreuse bulb color was first reported by El-Shafie and Davis (1967). Since then, few studies have been performed on the chartreuse bulb color. Recently, Havey (2020) has reported that the *G* locus is positioned at the tip of chromosome 7. In the present study, a candidate gene responsible for the *G* locus was identified using bulked segregant RNA-seq (BSR-Seq) analysis. Molecular markers developed based on critical mutations in the causal gene were used to demonstrate perfect

linkage disequilibrium between the causal gene and the *G* locus. An underlying model showing a role of the causal gene in appearance of a chartreuse bulb color is also presented in this study.

Materials And Methods

Plant materials

A segregating population (JNU21C) was produced by crossing a male-sterile yellow breeding line (JNUA12) with a male-fertile yellow breeding line (JNUA25). Bulb color phenotypes of JNU21C were investigated after harvesting mature bulbs. This population was used to perform BSR-Seq. Another segregating population was produced from self-pollination of JNUA25 and used to construct a linkage map flanking the *G* locus. An F₂ population originating from the cross between chartreuse SG21 and red UG19 was used to develop a molecular marker tagging the *G* locus. Seventeen chartreuse breeding lines bred by two institutes were used to analyze linkage relationship between the candidate gene and the *G* locus (Supplementary Table 1). A total of 96 diverse accessions were used to analyze distribution of mutant alleles of the causal gene (Supplementary Table 2). Total genomic DNAs of these accessions isolated from a previous study (Song et al. 2014) were used.

Identification of candidate genes responsible for the *G* locus using BSR-Seq

BSR-Seq is a combined analysis of bulked segregant analysis (BSA) and RNA-Seq (Liu et al. 2012). To perform BSR-Seq, total RNAs were extracted from two pooled samples of leaf sheath tissues sprouted from 10 yellow and 10 chartreuse onion bulbs, respectively. An RNA extraction kit (RNeasy Plant Mini Kit, QIAGEN, Valencia, CA, USA) was used for RNA extraction following the manufacturer's instructions. RNA-Seq analysis was carried out by a specialized company (Phyzen Genomics Institute, Seoul, Republic of Korea). Transcriptome sequences were produced using a HiSeq X Ten platform (Illumina, Hayward, CA, USA). Trimmed reads were mapped to a reference transcriptome (Fujito et al. 2021). More detailed procedures of RNA-Seq analysis were described in our previous studies (Baek et al. 2017; Seo et al. 2020).

PCR amplification and high resolution melting (HRM) analysis

Total genomic DNAs were extracted from seedlings or bulbs of onions using a cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). To obtain full-length genomic DNA sequences of a candidate gene, PCR was performed in a 25-μL reaction mixture containing 0.05 μg DNA template, 2.5 μL 10× PCR buffer, 0.2 μL forward primer (10 μM), 0.2 μL reverse primer (10 μM), 0.2 μL dNTPs (10 mM each), and 0.25 μL polymerase mix (Advantage 2 Polymerase Mix; Takara Bio, Shiga, Japan). PCR amplification conditions consisted of an initial denaturation step at 95°C for 4 min; 10 cycles at 95°C for 30 s, 65°C (0.8°C decrements in each cycle) for 30 s, and 72°C for 1 min; 35 cycles at 95°C for 30 s, 57°C

for 30 s, and 72°C for 1 min; and a final 10 min extension step at 72°C. PCR products were visualized on 1.5% agarose gels after ethidium bromide staining. PCR products were purified using a QIAquick PCR Purification kit (QIAGEN) and sequenced by a specialized company (Macrogen, Seoul, Republic of Korea).

To obtain full-length sequences of a variant of the candidate gene, long PCR amplification was performed using a TaKaRa LA PCR™ Kit Ver. 2.1 (Takara Bio) following the manufacturer's instructions. Sequences of putative promoter regions of the candidate gene were obtained by PCR amplification using scaffold sequence information retrieved from the Onion Genome Sequencing Project (www.oniongenome.wur.nl). For analysis of HRM markers, SYTO®9 green fluorescent nucleic acid stain (Thermo Fisher Scientific, Waltham, MA, USA) and a LightCycler® 96 system (Roche Molecular Systems, Pleasanton, CA, USA) were used. Detail procedures were described in a previous study (Kim and Kim 2019). Primer sequences of molecular markers used in this study are shown in Supplementary Table 3. Regarding an InDel marker, PCR products of the marker were visualized on 1.5% agarose gels after ethidium bromide staining.

Real-time RT-PCR amplification

Total RNAs were extracted from leaf sheath tissues of yellow and chartreuse individuals of the JNU21C population using an RNeasy Plant Mini Kit (QIAGEN). cDNAs were synthesized using a cDNA synthesis kit (SuperScript™ III first-strand synthesis system for RT-PCR, Invitrogen, Carlsbad, CA, USA). Real-time RT-PCRs were carried out using SYBR® Green Realtime PCR Master Mix (Toyobo Co. Ltd, Osaka, Japan) and a LightCycler®96 system (Roche Molecular Systems) according to each manufacturer's instructions with four technical replicates. Onion tubulin sequence (Unigene24335) was retrieved from a reference transcriptome (Fujito et al. 2021) and used as an internal control.

Construction of linkage maps and phylogenetic tree

A total of 96 individuals of a segregating population produced by self-pollination of JNUA25 were used for linkage map construction. The linkage map was constructed using JoinMap 4.1 (Van Ooijen and Voorrips 2001). Linkage maps constructed by other studies (Havey 2020; Cho et al. 2021) were drawn using MapChart 2.3 (Voorrips 2002). Deduced amino acid sequences of genes coding for laccases were aligned using BioEdit software (Hall 1999). Gaps in the alignment were removed using Gblocks software (Castresana 2000). A phylogenetic tree was constructed with MEGA version X (Kumar et al. 2018) using a neighbor-joining method. Node support of the phylogenetic tree was assessed by 1,000 bootstrap replicates.

Measurements of concentrations of quercetin derivatives

Concentrations of four quercetin derivatives in yellow and chartreuse onions were measured using HPLC analysis. Detail procedures were described in previous studies (Lee et al. 2016; Seo et al. 2020). Concentrations of four quercetin derivatives of red and white onions previously measured by Seo et al. (2020) were adopted for comparison in this study.

Results

Identification of a candidate gene responsible for chartreuse bulb color in onion using BSR-Seq analysis

Bulb colors of onions showing typical chartreuse and yellow colors segregated in a population (JNU21C) originating from a cross between male-sterile and male-fertile single plants (Fig. 2). The segregation ratio of bulb colors (591 yellow and 179 chartreuse) was fitted into a single-gene inheritance pattern ($\chi^2 = 1.26$, $P = 0.26$). Since bulb color of both parental plants was yellow, genotype of the *G* locus must be heterozygous (*Gg*) for both parental plants. To identify a causal gene for the *G* locus controlling the chartreuse bulb color, BSR-Seq analysis was carried out using yellow and chartreuse bulked RNAs of the JNU21C population.

RNA-Seq of chartreuse and yellow bulked RNAs produced a total of 10.9 Gb and 10.5 Gb raw reads, respectively (Supplementary Table 4). Approximately 360,000 single nucleotide polymorphisms (SNPs) or insertion/deletions (InDels) were identified after mapping of trimmed reads to a reference transcriptome (Fujito et al. 2021). Through a customized process for screening polymorphisms (Table 1), a total of 163 contigs were selected as candidate genes (Supplementary Table 5). In addition, differentially expressed genes (DEGs) between yellow and chartreuse bulked RNAs were screened. A total of 143 contigs showing more than three-fold decreased transcription in the chartreuse bulk and more than 3 FPKM values in the yellow bulk were selected (Supplementary Table 6).

Table 1
A customized stepwise process for selecting contigs linked to the *G* locus.

Step	Criterion	Number of SNPs/InDels or contigs
1	Number of SNPs or InDels identified by software	360,065
2	Number of homozygous SNPs or InDels in the chartreuse bulk	108,790
3	Number of SNPs or InDels of which yellow allele frequencies are more than 0.4	29,709
4	Number of SNPs or InDels of which read counts are more than 5	12,225
5	Number of contigs containing SNPs or InDels	3,641
6 ^a	Number of contigs after removal of contigs positioned at other than chromosome 7	2,375
7	Number of contigs containing SNPs or InDels confirmed by visual investigation using Integrative Genomics Viewer (IGV) software (Robinson et al. 2011)	163

^aChromosomal positions of contigs identified by Fujito et al. (2021) were used.

SNP and DEG screening commonly identified three contigs (CL835, Unigene35885, and Unigene39335). Lengths of two contigs, Unigene35885 and Unigene39335, were 292 bp and 171 bp, respectively. In these two contigs, there was no apparent open reading frame (ORF) or annotated function. In contrast, CL835 had a length of 1,934 base pairs, containing an ORF of 1,701-bp in length. Alignment of mapped reads clearly showed that all chartreuse reads contained a 4-bp deletion. However, reads containing a wild type or the 4-bp deletion were mixed in the yellow bulk (Supplementary Fig. 1). Both heterozygous (*Gg*) and homozygous dominant (*GG*) individuals were included in the yellow bulk because bulb color phenotypes of these two genotypes were indistinguishable.

Full-length genomic DNA sequences including approximately 2.5 kb putative promoter regions of CL835 were obtained from yellow and chartreuse individuals (Fig. 3A). Nucleotide sequences of yellow and chartreuse alleles were deposited in GenBank with accession numbers of OK33673 and OK336704, respectively. Although two small InDels (1-bp and 2-bp) were identified in their putative promoter regions, additional polymorphism except for the 4-bp deletion was not identified between the two alleles. The 4-bp deletion positioned in the exon5 resulted in a frame shift and created a premature stop codon 11-bp downstream of the deletion (Fig. 3A). These results suggest that CL835 is a strong candidate gene for the *G* locus in onion.

Development of molecular markers linked to the *G* locus and linkage relationship between the candidate gene and *G* locus

Nucleotide sequences of CL835 showed high homologies with those of genes encoding laccase enzymes. Laccase is a member of the multicopper oxidase superfamily. It has signature sequences of four highly conserved copper-binding domains (Kumar et al. 2003; McCaig et al. 2005). Alignment of deduced amino acid sequences of CL835 and other known laccase enzymes showed that four copper-binding domains (L1-L4) were also conserved in CL835 (Fig. 4A), indicating that CL835 encoded a laccase. In previous studies (McCaig et al. 2005; Pourcel et al. 2005), plant laccases were clearly classified into six groups based on phylogenetic relationships. The phylogenetic tree showed that CL835 belonged to group 3 with a close relationship with *Arabidopsis* LAC12 (Fig. 4B). Therefore, CL835 was designated *AcLAC12*.

To confirm co-segregation between the mutation in *AcLAC12* and bulb color phenotypes in segregating populations, an HRM marker was developed by designing a primer pair (LAC12-F1 and LAC12-R1) flanking the 4-bp deletion. Peak patterns of HRM clearly distinguished three genotypes of the *G* locus (Fig. 3B). This molecular marker was designated LAC12-DEL. Marker genotypes of LAC12-DEL were perfectly matched with bulb color phenotypes of the JNU21C population. The chromosomal position of *G* locus is known to be located at the tip of chromosome 7 (Havey 2020). To analyze linkage relationship between *AcLAC12* and *G* locus, 12 HRM markers were developed based on linked contigs selected in this study or reported by other studies (Havey 2020; Cho et al. 2021; Fujito et al. 2021). Homologous contigs

among three linkage maps were all collinear. The *G* locus and *AcLAC12* were positioned together at the end of chromosome 7 (Fig. 5; Supplementary Table 7). Interestingly, the *G* locus was linked to the *R* locus with a distance of approximately 30 cM (Fig. 5).

To obtain more evidences supporting that *AcLAC12* was the causal gene for the *G* locus, 17 chartreuse breeding lines and 96 diverse yellow and red accessions were analyzed using the LAC12-DEL marker. While LAC12-DEL genotypes of eight chartreuse breeding lines were homozygous recessive as expected, marker genotypes of the other nine chartreuse lines were homozygous dominant. However, sequencing of *AcLAC12* in these nine breeding lines revealed that an intact 6,449-bp long terminal repeat (LTR)-retrotransposon was transposed in exon 5 (Fig. 3A), indicating that this mutant allele was also inactive. The wild type and two mutant *AcLAC12* alleles containing a 4-bp deletion and an LTR-retrotransposon insertion were designated as *AcLAC12^{G1}*, *AcLAC12^{DEL}*, and *AcLAC12^{LTR}*, respectively. Nucleotide sequences of *AcLAC12^{LTR}* were deposited in GenBank with an accession number of OK336705. A PCR-based marker designated LAC12-LTR was developed to identify the *AcLAC12^{LTR}* allele (Fig. 3C). Using two LAC12 markers, 96 red and yellow accessions were analyzed. However, mutant *AcLAC12* alleles were not identified (Supplementary Table 2). Perfect linkage disequilibrium between bulb color phenotypes and marker genotypes among diverse accessions indicated that *AcLAC12* was probably the causal gene for the *G* locus.

Analysis of DEGs showing reduced transcription in chartreuse bulked RNA

Overall transcription levels of the majority of 56,161 contigs were similar between yellow and chartreuse bulked RNAs with a correlation coefficient of 0.94 (Supplementary Fig. 2). Among DEGs, *AcLAC12* showed significantly reduced transcription level in chartreuse bulked RNA (Fig. 6A). Real-time RT-PCR results confirmed that transcripts of *AcLAC12* were reduced in chartreuse individuals of the JNU21C population (Fig. 6B). Regarding structural genes encoding enzymes involved in flavonoid biosynthesis pathway (Fig. 1), transcription levels of genes involved in anthocyanin biosynthesis pathway from *CHS-A* to *UFGT2* were generally reduced in chartreuse bulked RNA (Supplementary Fig. 3). Similarly, concentrations of total quercetin in chartreuse bulb were also lower than those in yellow and red bulbs (Fig. 7).

In the case of *DFR-A* gene, few transcripts were detected in both yellow and chartreuse bulked RNAs. Sequencing of *DFR-A* of the JNU21C population revealed that both yellow and chartreuse onions contained an inactive *DFR-A^{2AT}* allele having a premature stop codon (Song et al. 2014). Further sequence analysis of *DFR-A* alleles of 17 chartreuse breeding lines showed that all chartreuse breeding lines contained inactive *DFR-A* alleles as homozygous genotypes (Supplementary Table 1). These results imply that chartreuse bulb color might appear when both *AcLAC12* and *DFR-A* genes are inactive at the same time.

Discussion

Identification of a candidate gene responsible for the G locus in onion

A gene encoding a laccase enzyme was proposed as a strong candidate gene for the *G* locus controlling the chartreuse bulb color in this study. Since *Agrobacterium*-mediated genetic transformation is hardly applicable in onion, a complementation test using genetic transformation could not be performed to prove that *AcLAC12* was responsible for the chartreuse bulb color. However, several evidences presented in this study supported a role of *AcLAC12* in the appearance of the chartreuse bulb color. First, a perfect linkage between LAC12 markers and *G* locus in segregating populations and diverse accessions was demonstrated in this study. Perfect linkage disequilibrium among diverse breeding lines implies that no recombinant has been produced during breeding periods of such accessions and that both *AcLAC12* and *G* loci might be identical to each other.

Second, chartreuse alleles of *AcLAC12* harbored critical mutations leading to complete inactivation of gene products. The 4-bp deletion in exon5 was a frame-shift mutation that created a premature stop codon before two highly conserved copper-binding domains, L3 and L4 (Fig. 3A). Significantly reduced transcripts of *AcLAC12* in chartreuse onions were probably caused by a mechanism of nonsense-mediated RNA decay (NMD). Since 2.5 kb putative promoter regions showed no significant polymorphic sequence between yellow and chartreuse alleles, reduced transcripts might be resulted from degradation of mRNA by the NMD mechanism. NMD is conserved among eukaryotes. It plays an essential role in maintaining the quality of transcriptome by eliminating mutant transcripts containing premature stop codons (Nickless et al. 2017).

Another novel mutant *AcLAC12* allele harboring an LTR-retrotransposon was identified from nine chartreuse breeding lines. Since the 6,449-bp LTR-retrotransposon was transposed at the upstream sequence of a 4-bp deletion in exon 5 (Fig. 3A), this mutant allele must be inactive as well. Nucleotide sequences of 954-bp LTRs were identical to each other. A 3,891-bp intact ORF encoded a polyprotein containing three major domains (GAG, INT, and RT) of LTR-retrotransposons. These features imply that this LTR-retrotransposon might be active and recently transposed into the *AcLAC12* gene. Organization of integrase (INT) and reverse transcriptase (RT) domains indicated that this element belonged to *Copia* superfamily (Wicker et al. 2007). Interestingly, an unusually long 9-bp target site duplication (TSD) was identified in this LTR-retrotransposon. In general, the length of TSD ranges from four to six base pairs (Wicker et al. 2007).

Underlying relationship between inactivation of *AcLAC12* and appearance of chartreuse bulb color in onion

Laccase, a member of the multicopper oxidase superfamily, is widely distributed in all kingdoms of life ranging from bacteria to mammals. Although laccase was first discovered from Japanese lacquer tree almost 138 years ago, its precise biochemical and physiological roles in plants remain mostly unresolved. Their wide taxonomic distribution and broad substrate specificity might be obstacles to resolve precise roles of laccases (Turlapati et al. 2011; Janusz et al. 2020). Plant laccases are known to

be involved in diverse processes such as lignification, defense responses, wound healing, iron metabolism, and polymerization of phenolic compounds (Janusz et al. 2020).

Seventeen genes coding for laccases were found in *Arabidopsis* genome. However, physiological functions of only two genes (*AtLAC3* and *AtLAC15*) have been elucidated (Pourcel et al. 2005; Zhuang et al. 2020). Regarding mutants of *AtLAC12* showing the highest homology with *AcLAC12*, no apparent mutant phenotype was observed (Cai et al. 2006). In the case of *AtLAC3*, this gene is known to be involved in directing Caspary strip formation in *Arabidopsis* (Zhuang et al. 2020). Caspary strip is a ring-like water-impermeable lignin polymer positioned in the endodermis of root (Barbosa et al. 2019). Meanwhile, *Arabidopsis AtLAC15* gene was revealed to be the causal gene for *transparent testa10* (*tt10*) mutant showing delayed browning of seed coats. The *AtLAC15* gene has been shown to be involved in oxidative polymerization of flavonoids (Pourcel et al. 2005). Likewise, *AcLAC12* might be involved in the modification of flavonoid derivatives in onions since laccases have a broad spectrum of substrate specificity (Fig. 1).

The *G* locus determining chartreuse bulb color in onion was first proposed by El-Shafie and Davis (1967). They hypothesized that the *G* locus might be involved in sequential pigment synthesis pathway. It acted after the *C* locus and before the *R* (*DFR-A*) locus based on the fact that chartreuse color was dominant over recessive white and recessive to yellow. However, since quercetin derivatives were detected in chartreuse onions (Fig. 7), genes coding for enzymes from chalcone synthase (CHS) to flavonol synthase (FLS) should be active in chartreuse onions. Therefore, the *G* locus is likely to be involved in later steps than FLS in the flavonoid biosynthesis pathway (Fig. 1).

If we assume that chartreuse color would appear only when both *R* and *G* loci are homozygous recessive, inheritance patterns of chartreuse color could be clearly explained (Fig. 1). Indeed, all chartreuse breeding lines analyzed in this study contained four different kinds of inactive *DFR-A* alleles (Supplementary Table 1). The *AcLAC12* gene might be involved in the modification of quercetin derivatives. Since *Arabidopsis AtLAC15* catalyzes oxidative polymerization of flavonoids, *AcLAC12* might also catalyze polymerization of quercetin derivatives (Fig. 1), although polymers of flavonoid compounds in onions have not been reported yet. Involvement of a laccase enzyme in the production of onion flavonoids will provide an important clue to resolve precise compounds responsible for chartreuse and yellow bulb colors. Furthermore, chartreuse onion will be a precious material to elucidate the role of laccase enzyme in plant pigmentation.

Application of molecular markers linked to the *G* locus in onion breeding programs

Compared with white, yellow, and red bulb colors, chartreuse bulb color has been rarely used to produce commercial cultivars. The complex inheritance of the *G* locus might have partly contributed to the rare usage of chartreuse bulb color. Linkage between the *G* and *R* loci might also complicate inheritance. We showed that the *R* (*DFR-A*) locus was linked to the *G* locus in the same chromosome (Fig. 4). Information about the chromosomal location of the *G* locus and molecular markers linked to the *G* locus will be useful for developing commercial chartreuse cultivars in onion breeding programs. In particular, if *AcLAC12* is

the genuine causal gene for the *G* locus, LAC12 markers would be functional markers developed based on critical mutations (Andersen and Lübbestedt 2003). Therefore, prediction of *AcLAC12* genotypes could be accurate without any errors.

Declarations

Conflict of interest

The authors have no conflicts of interest relevant to this study to disclose.

Ethical standards

All experiments performed in this study were in compliance with current laws of the Republic of Korea.

Authors' contributions

SeongChan Jeon performed experiments and drafted the manuscript. JiWon Han and Cheol-Woo Kim produced segregating populations. Ju-Gyeong Kim and Jae-Hak Moon performed measurements of concentrations of quercetin derivatives. Sunggil Kim organized and coordinated this research project and edited the final manuscript.

Data availability

Nucleotide sequences of *AcLAC12* alleles are accessible at NCBI Database under the accession numbers from OK33673 to OK336705.

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Figures

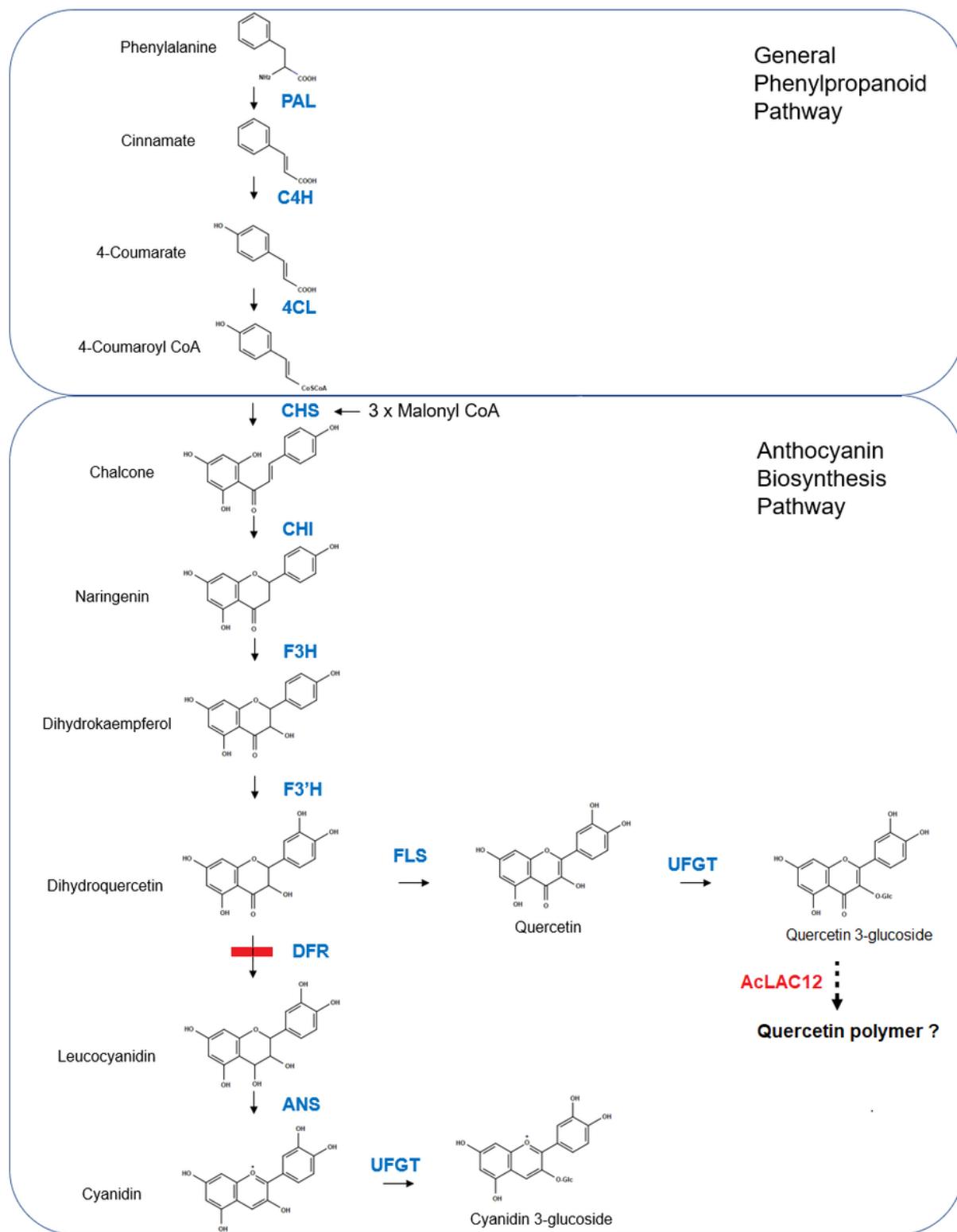


Figure 1

Flavonoid biosynthesis pathway and a proposed model showing an underlying mechanism of appearance of chartreuse bulb color in onion. Red bar indicates inactivation of the DFR enzyme. Dotted arrow implies that two or more steps of modifications can be involved in this step. PAL: phenylalanine ammonia lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumaroyl-coenzyme A ligase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; DFR:

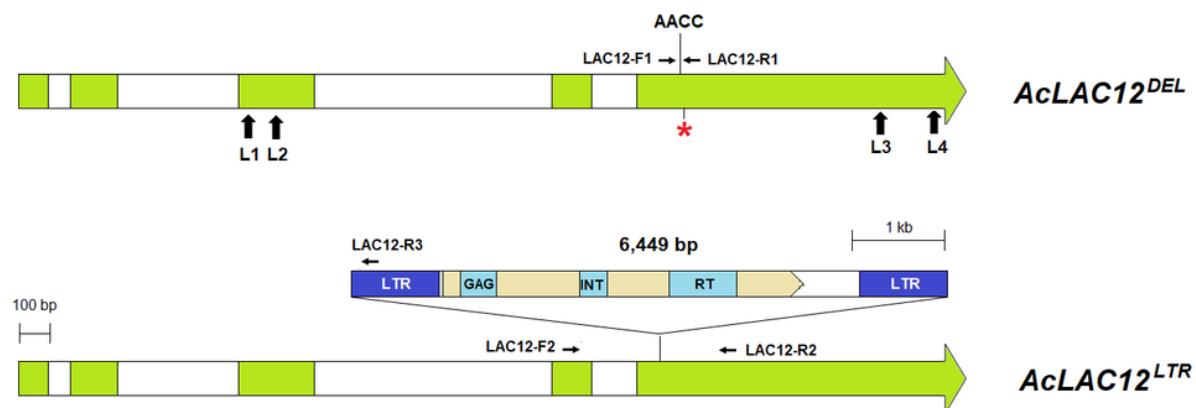
dihydroflavonol 4-reductase; ANS: anthocyanidin synthase; FLS: flavonol synthase; UFGT: UDP glucose-flavonoid 3-O-glucosyl transferase.



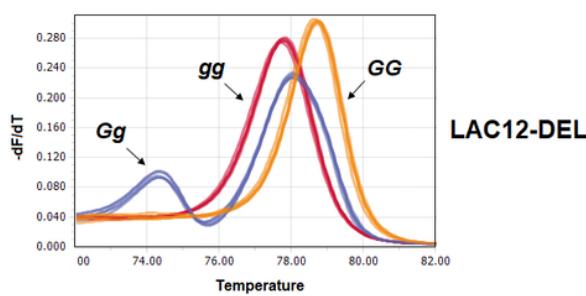
Figure 2

Onion bulbs showing chartreuse and yellow bulb color phenotypes.

A



B



C

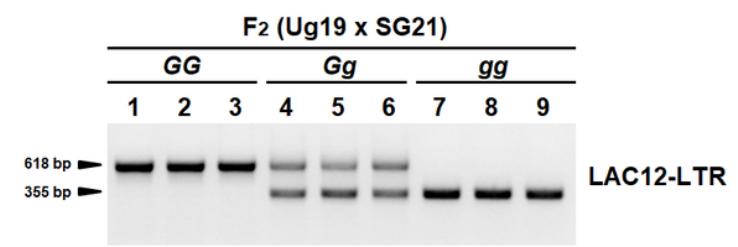
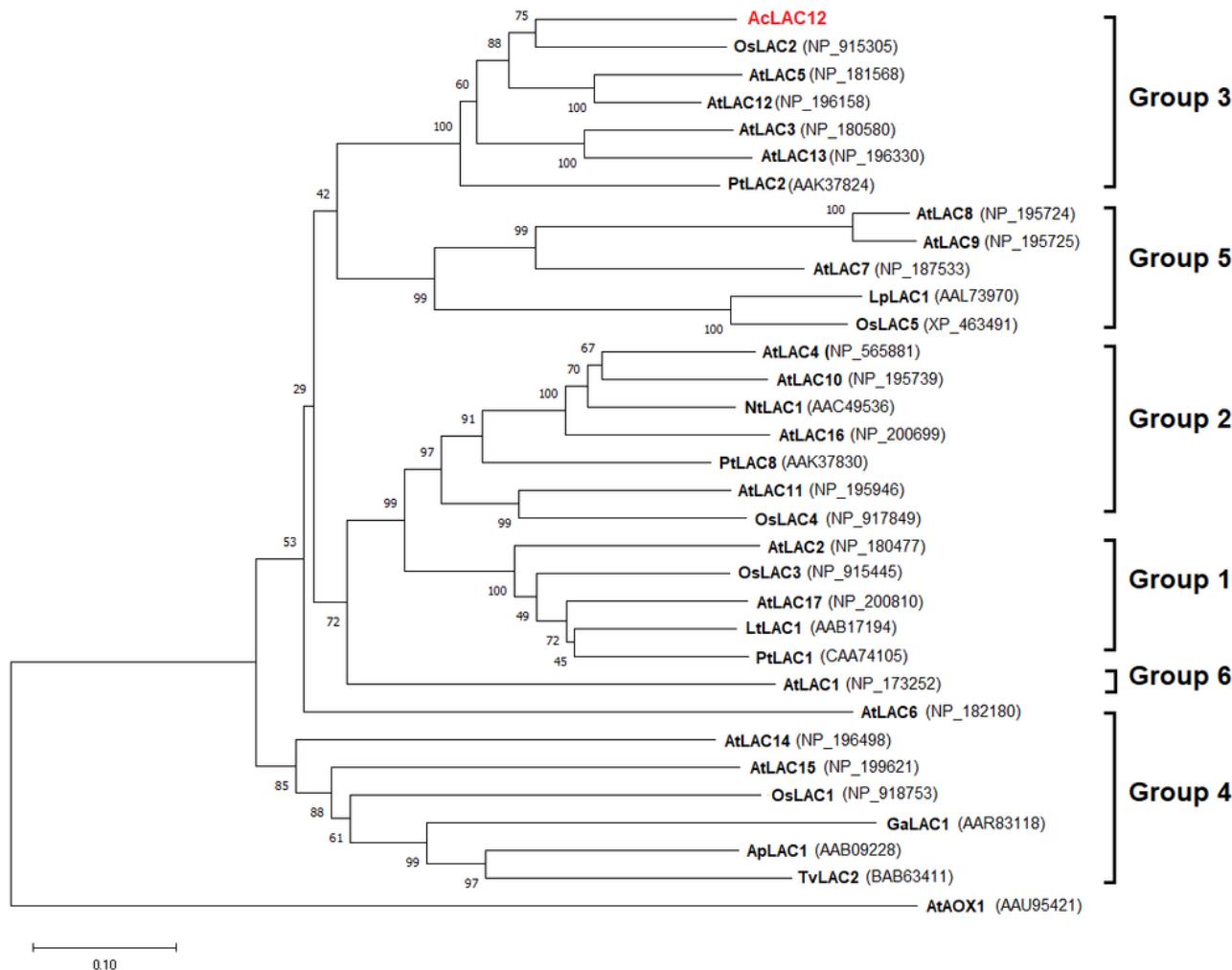


Figure 3

Development of molecular markers for genotyping of AcLAC12. A. Gene organizations of AcLAC12, a candidate gene for the G locus. Arrow-shaped box indicates 5'-to-3' orientation. Green and empty boxes indicate exons and introns, respectively. 'AACC' on a vertical line indicates the position of a 4-bp deletion. Vertical arrows indicate positions of highly conserved copper-binding motifs. A red asterisk shows the position of a premature stop codon. The position of LTR-retrotransposon insertion is shown on an inverted triangle. The LTR-retrotransposon is drawn with a more reduced scale. An arrow-shaped box in the LTR-retrotransposon indicates an ORF encoding a polyprotein and the 5'-to-3' direction. LTR: long terminal repeat; GAG: Capsid protein; INT: Integrase; RT: Reverse transcriptase. Horizontal arrows indicate primer binding positions of molecular markers. B. Normalized melting peak patterns of the LAC12-DEL marker. C. Band patterns of PCR products of the LAC12-LTR marker.

A

	L1	L2	L3	L4
AcLAC12	HMGVRQMRCTWADGPEYITQCPI 105	GTLFWH[AH] 129	HPIHL[HGY 475	GAWFMHC[LDVHTWGL 541
AtLAC1	HWHGLRQYRTGWADGPA[YITQCPI 107	GTLW[HAH 131	HPIHV[HGH 489	GVWF[HCHLEHTSWGL 555
ApLAC1	HWHGVKMPRN[PNSDGP[EYVTQCPI 104	GTLW[HAH 128	HPMHL[HGF 470	GVWF[HCHFERHTTWGM 536
GaLAC1	HWHGVKQPRNPNSDGP[EYVTQCPI 106	GTLW[HAH 130	HPIHL[HGS 471	GVWF[HCHLERHSSWGM 537
LpLAC1	HWHGIMQLRTPWADGPSMVTQCPI 118	GTLW[HAH 142	HPIHL[HGF 472	GYWF[HCHLDAHVPML 537
LtLAC1	HWHGVRQLRGWADGPA[YVTQCPI 115	GTLFWH[AH 139	HPIHL[HGF 493	GVWF[HCHLEHTSWGL 559
NtLAC1	HWHGIRQLRTGWADGPA[YITQCPI 103	GTLFWH[AH 127	HPIHL[HGF 465	GVWF[HCHLEHTTWGL 531
OsLAC1	HWHGVDQPRNPNSDGP[EFTQCPI 110	GTLW[HAH 134	HPMHL[HGF 507	GVWF[HCHFDRHVVWGM 573
PtLAC1	HWHGVRQLTTGWADGPA[YVTQCPI 114	GTLW[HAH 138	HPIHL[HGF 488	GVWF[HCHLDVHTSWGL 554
****:	****:****:****:	****:	****:	****:****:****:

B**Figure 4**

Phylogenetic analysis of AcLAC12 with other laccases isolated from other plant species. A. Alignment of deduced amino acid sequences of four highly conserved copper-binding domains. Amino acids involved in copper binding are enclosed with red boxes. B. Phylogenetic tree of AcLAC12 and other laccases isolated from other plant species. AtAOX1 is included as an outgroup. GenBank accession numbers are shown in parentheses.

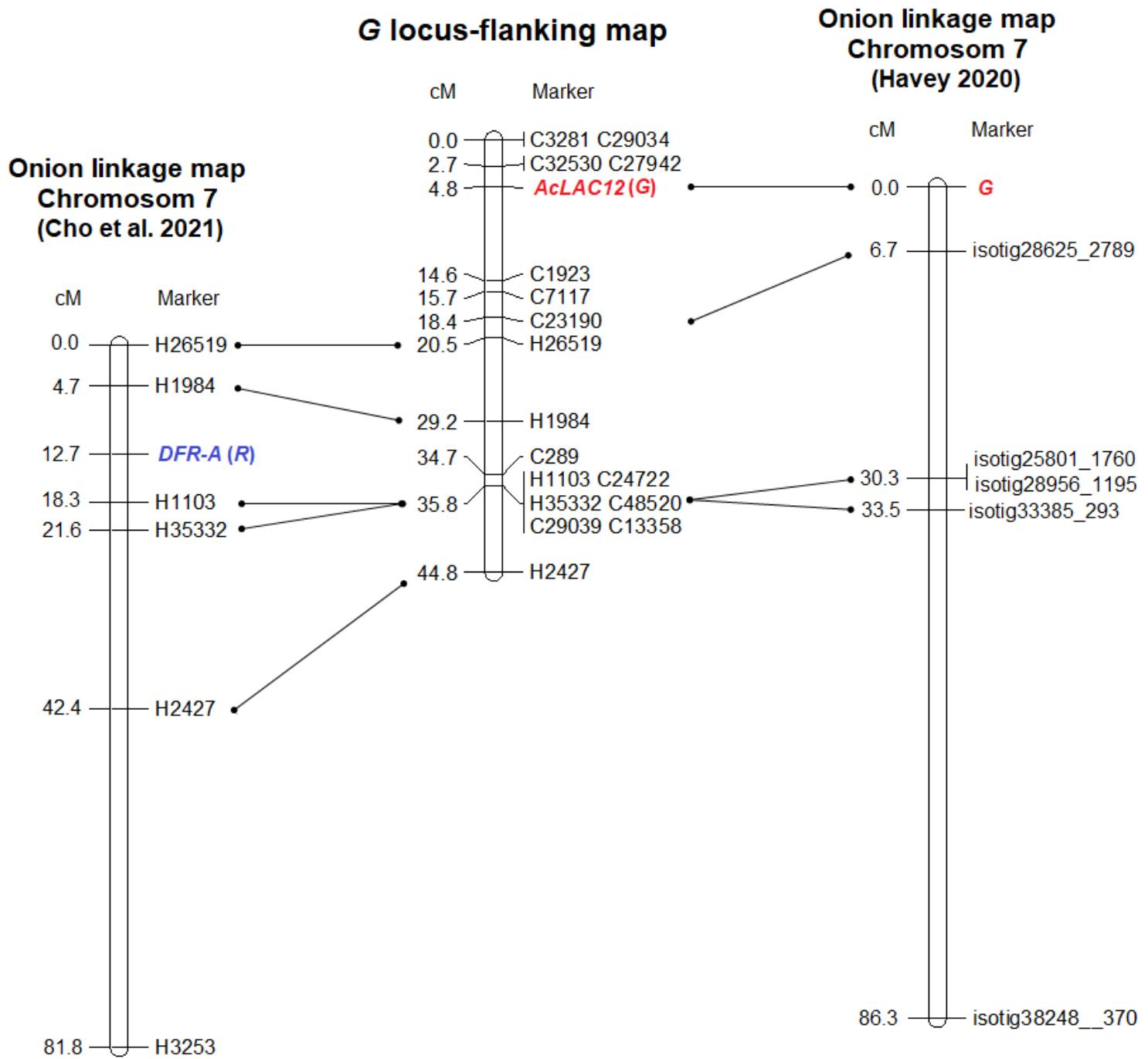


Figure 5

Linkage maps flanking the G locus controlling chartreuse bulb color in onion. (Left) A partial linkage map of chromosome 7 constructed by Cho et al. (2021); (Center) An G locus-flanking linkage map constructed in this study; (Right) A partial linkage map of chromosome 7 constructed by Havey (2020). Markers designed based on homologous contigs are connected with horizontal lines. Detail information of homologous contigs is shown in Supplementary Table 7. Markers positioned at both ends of chromosome 7 in two linkage maps (Havey 2020; Cho et al. 2021) are included to show relative positions of the G locus in chromosome 7.

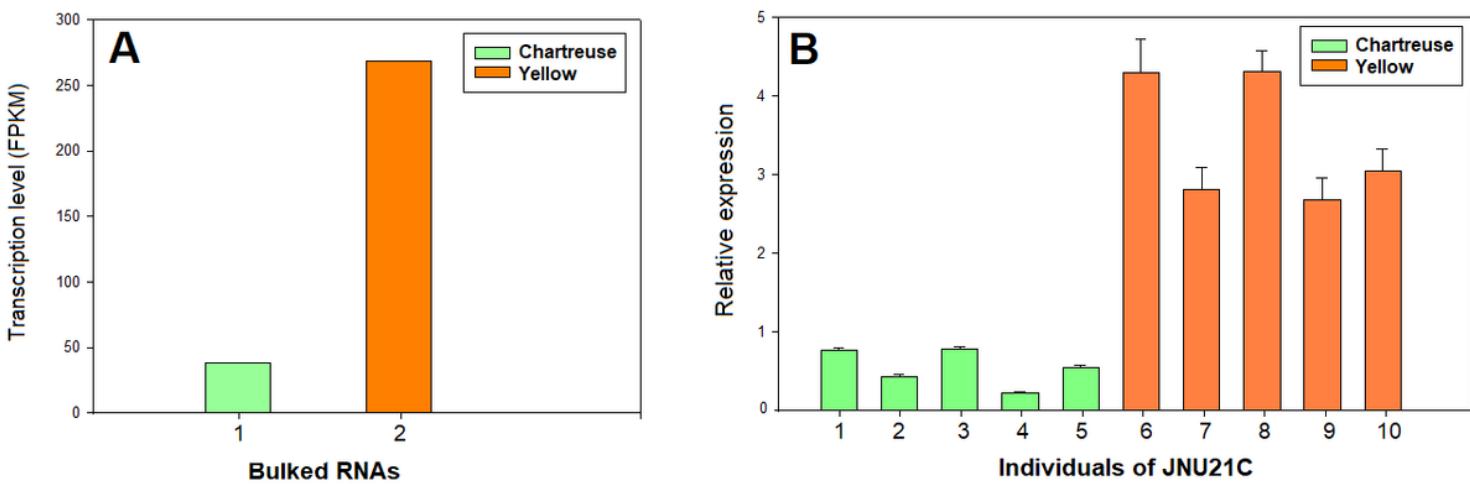


Figure 6

Differential expression of AcLAC12 between chartreuse and yellow onions. A. Transcription levels estimated by RNA-Seq. B. Relative transcription levels of AcLAC12 in chartreuse and yellow individuals of the JNU21C population estimated by real-time RT-PCR.

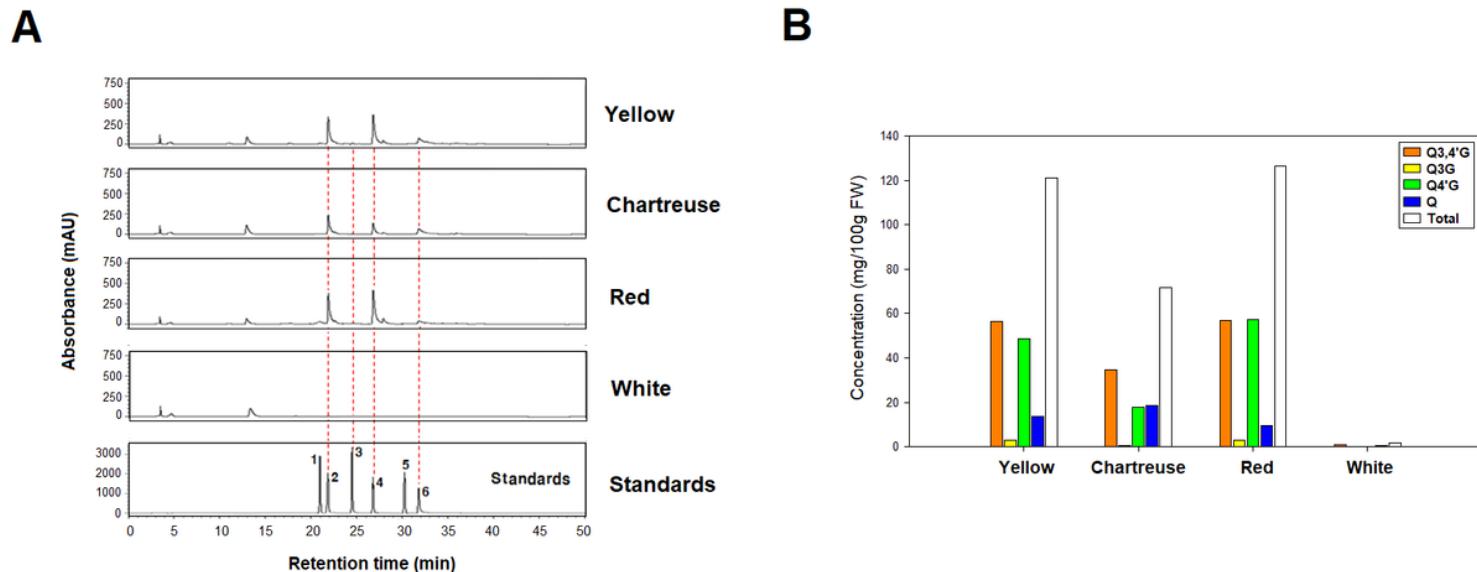


Figure 7

Concentrations of quercetin derivatives in yellow and chartreuse onions. A. HPLC peak patterns of yellow, chartreuse, red, and white onions. HPLC analyses of red and white onions performed in a previous study (Seo et al. 2020) were included for comparison with the chartreuse onion. Standard 1: Quercetin 3,7-diglucopyranoside; Standard 2: Quercetin 3,4'-diglucopyranoside; Standard 3: Quercetin 3-glucopyranoside; Standard 4: Quercetin 4'-glucopyranoside; Standard 5: Isohamnetin 3-glucopyranoside; and Standard 6: Quercetin aglycon. B. Estimated concentrations of four quercetin derivatives in yellow, chartreuse, red, and white onions.

Supplementary Files

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- [SupplementaryFig.1.ReadmappingofCL835.tif](#)
- [SupplementaryFig.2.Scatterplotofexpressions.tif](#)
- [SupplementaryFig.3.Expressionofstructuralgenes.tif](#)
- [SupplementaryTable1.GenotypesofDFRA.xlsx](#)
- [SupplementaryTable2.Listofbreedinglines.xlsx](#)
- [SupplementaryTable3.Primersequenceusedinthisstudy.xlsx](#)
- [SupplementaryTable4.SummaryofRNASeq.docx](#)
- [SupplementaryTable5.ListofcontigscontainingSNPs.xlsx](#)
- [SupplementaryTable6.ListofDEGs.xlsx](#)
- [SupplementaryTable7.Mappedhomologouscontigs.xlsx](#)