Segregations for Onion Bulb Colors Reveal That Red Is Controlled by at Least Three Loci

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ABSTRACT. Onion (Allium cepa L.) bulb color is controlled by at least five major loci (I, C, G, L, and R) and seedcoat color by one locus (B). The authors developed families segregating for bulb and seedcoat colors, simple sequence repeats (SSRs), and single nucleotide polymorphisms (SNPs) in genomic amplicons of dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS). The B and C loci were linked to SSRs on chromosomes 1 and 6 respectively. For all of three families, SNPs in DFR cosegregated with the R locus conditioning red bulb color. In the family from B2246 × B11159, red bulbs versus yellow bulbs were controlled by DFR and a locus (L2) linked at 6.3 cM to ANS. The authors propose that yellow bulb onions have been independently selected numerous times and that yellow populations carry independent mutations in structural or regulatory genes controlling the production of red bulb color in onion.

Bulb color in onion is an economically important trait and is conditioned by the interaction of at least five major loci (Clarke et al., 1944; El-Shafie and Davis, 1967; Reiman, 1931). White bulbs result from a dominant allele at the I locus or recessive alleles at the C locus. Colored bulbs (chartreuse, light red, red, or yellow) require the homozygous recessive genotype at the I locus and a dominant allele at the C locus. In plants that are iiC-, a dominant allele at the G locus produces golden yellow bulbs, and the homozygous recessive genotype (iiC-gg) conditions chartreuse bulbs. Light-red bulbs are produced when the plant is iiC-G- and has dominant alleles at both the L and R loci; bulbs with deeper red colors are produced when both the L and R loci are homozygous dominant (El-Shafie and Davis, 1967). If either the L or R locus is homozygous recessive, yellow bulbs are produced. El-Shafie and Davis (1967) proposed that light-red bulb color produced when crossing two yellow onions [termed complementary light red by Jones and Peterson (1952)] results when the yellow parental plants have the genotypes iiCCGGLRrr and iiCCGGLRrr.

Onion bulbs accumulate significant amounts of flavonoids, such as quercetin derivatives in yellow and anthocyanins in red bulbs (Fossen et al., 1996; Tsushida and Suzuki, 1995). Many of the structural genes in the flavonoid biosynthetic pathway have been cloned (Holton and Cornish, 1995). Kim et al. (2004a, 2005c) correlated expression and segregation of dihydroflavonol 4-reductase (DFR) with yellow versus red bulb colors. In a confusing series of papers, variation at anthocyanidin synthase (ANS) was associated first with a new recessive locus (p) conditioning pink bulbs (Kim et al., 2004b, 2005b), and then subsequently with four alleles at the L locus [ANS-p] for recessive pink, ANS-L and ANS-L for the Brazilian and North American alleles of the complementary light-red system respectively (Kim et al., 2005a), and ANS-h1 for dark red (Kim et al., 2006). No candidate genes have been identified for the I, C, or G loci.

In this study we developed families segregating for dominant white (I-), recessive white (cc), yellow (iiC-llR-, iiC-L-rr, and iiC-Llrr), and light-red to red (iiC-L-R-) bulb colors, as well as black (B-) versus brown (bb) seeds (Davis, 1966). We scored segregations for simple sequence repeats (SSRs), bulb and seed colors, and single nucleotide polymorphisms (SNPs) in ANS and DFR to assess linkages among these important morphological and molecular markers. Our results support DFR as the R locus and reveal a new locus (L2) linked at 6.3 cM to ANS that conditions red bulb color in onion.

Materials and Methods

We obtained from N. Molenaar (formerly of Crookham Seed Co., Caldwell, ID) seed of a white onion (B11159) that produced flattened bulbs when grown in southern Wisconsin. Randomly selected bulbs from B11159 were individually
self-pollinated to produce S1 families and testcrossed to MSU7518A × MSU8155B, a yellow bulb, male-sterile hybrid. Randomly selected B11159 plants were individually crossed as the male with plants from the inbred B2246, which has yellow bulbs (iiCCl-rr or iiCClrr) and brown (bb) seeds. All self-pollinations and crosses were completed with mesh cages and house flies [Musca domestica L. (Rincon-Vitova Insectaries, Ventura, CA)] as described by Havey (1993). Hybrids were identified by white bulb color for progenies from B2246 as the maternal parent. Individual F2 plants were self-pollinated and testcrossed to the yellow bulb (iiCCl-rr or iiCClrr), black-seed (BB), male-sterile (S msms) hybrids MSU7518A × MSU8155B, B3350A × B2352B, or B1750A × B1794B. At least 50 F3 and testcross plants were scored for white or beige, red or light red, versus yellow bulb colors in field plots over at least 3 years. No effort was made to distinguish white from beige or red from light red. F3 seed was scored for black versus brown colors to assign genotypes at the B locus to F2 plants. Goodness-of-fit to expect ratios for F2 and testcross families were completed using chi-square analyses on pooled data after confirming homogeneity of ratios (Gomez and Gomez, 1984).

A second family segregating for red versus yellow bulbs was developed from an inbred population (B8667) derived from a cross of U.S. Department of Agriculture Plant Introduction (PI) 262985 with the red inbred line B5361. Random bulbs from the F1 MSM3 generation (where M is mass random pollinations among three to five bulbs) were self-pollinated and one family was segregated for yellow versus red bulbs. Random plants from this F1 MSM3 generation were again self-pollinated to produce the F1 MSM3S family and numbers of red bulbs versus yellow bulbs were scored over at least 3 years.

The third segregating family was BYG15-23 × AC43. We previously reported segregation of complementary light-red bulb color in testcrosses of F2 plants from this family with male-sterile lines (King et al., 1998). Polymorphisms in ANS and DFR have been associated with red versus yellow bulb colors in onion (Kim et al., 2004b, 2005b, c, 2006). Amplicons from ANS and DFR were generated using primers across promoter (Kim et al., 2004a, b, 2005b, 2005c, 2006) and coding regions (5′-CGTGATAATTGCAACCCAG and 5′-ACAACATCGTTCAGGATGC for ANS and 5′-ATCCGCGTCTATTTGCTGT and 5′-TCCCCACACAGTGCTTAATAA for DFR). Polymerase chain reactions (PCR) had 50 ng onion genomic DNA, 1 μM each primer, 250 μM deoxyribonucleotides (dNTPs), 5 U Taq Polymerase (Promega, Madison, WI), 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH, 9.0), and 0.1% Triton X-100 (Sigma, St. Louis, MO) in a total volume of 25 μL. Cycling conditions were 94 °C for 5 min. followed by 39 cycles of 92 °C for 1.15 min., 58.7 °C for 1 min., and 72 °C for 2 min. Amplicons were gel purified and sequenced to confirm identity with ANS or DFR. DNA from parents and progenies of the three segregating families were scored for SNPs in the ANS and DFR coding regions.

Table 1. Segregations of onion bulb colors in S1, F2, and testcross families from B2246 by B11159.

| Pedigree | Gen. | Fam. | W | R | Y | Exp. | Assigned genotypes | P value |
|----------|------|------|---|---|---|------|-------------------|---------|
| A × B11159 | TC | 4 | 348 | 19 | 0 | 2 | 1 | 0 | R | 0.005 |
| B11159 | S1 | 4 | 182 | 0 | 0 | 1 | 1 | 1 | 0.093 |
| A × (B2246 × B11159) | TC | 6 | 232 | 103 | 92 | 2 | 1 | 1 | 0.005 |
| (B2246 × B11159) | F2 | 6 | 351 | 60 | 21 | 208 | 22 | 21 | 0.093 |
| A × (B2246 × B11159) | TC | 2 | 112 | 76 | 57 | 2 | 1 | 1 | 0.005 |
| (B2246 × B11159) | F2 | 2 | 111 | 12 | 15 | 208 | 27 | 21 | 0.093 |
| A × (B2246 × B11159) | TC | 1 | 0 | 49 | 33 | 2 | 1 | 1 | 0.005 |
| (B2246 × B11159) | F2 | 1 | 6 | 10 | 4 | 16 | 27 | 21 | 0.093 |
| A × (B2246 × B11159) | TC | 2 | 122 | 0 | 81 | 1 | 0 | 0 | 0.005 |
| (B2246 × B11159) | F2 | 2 | 132 | 0 | 23 | 13 | 1 | 3 | 0.005 |
| A × (B2246 × B11159) | TC | 1 | 47 | 16 | 23 | 2 | 1 | 1 | 0.005 |
| (B2246 × B11159) | F2 | 1 | 55 | 22 | 3 | 28 | 12 | 2 | 0.005 |

* A, yellow bulb, male-sterile plant with expected genotype iiCClrr.
* Gen., generation; TC, testcross.
* Numbers of families (Fam.).
* Observed (Obs.) and expected (Exp.) ratios of white (W), red (R), and yellow (Y) bulbs. Data were pooled across families after establishing homozygosity of ratios.
* Probability (P) of goodness-of-fit from chi-square analyses.

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mapping function to assess linkages among SNPs in ANS and DFR, SSRs, and the bulb and seed color loci.

Copy numbers of ANS and DFR in the onion genome were estimated by hybridization of PCR amplicons to DNAs isolated from leaf tissue combined from at least 25 F2 progenies from the B2246 × B11159 family. Digestion of DNAs with EcoRI, EcoRV, or HindIII, gel electrophoresis, labeling and hybridization conditions, and autoradiography were described by King et al. (1998).

Results and Discussion

Segregations for bulb and seed color loci in the B2246 × B11159 family. All seed from self-pollination of random bulbs from B11159 was black (BB) and produced only white bulbs (Table 1). In crosses of B11159 plants to yellow bulb, male-sterile lines, only white bulbs were produced (Table 1), revealing that B11159 was homozygous dominant at the I locus. The testcross and F2 progenies from hybrids between B2246 by B11159 segregated for white, red, and yellow bulbs (Table 1). No chartreuse bulbs were produced.

One family (17400) from B2246 × B11159 was chosen for further study because it segregated for bulb and seed colors. Individual F2 plants were self-pollinated to produce F3 families and crossed to male-sterile plants to produce the testcross generations. Because these male-sterile lines produce only yellow bulbs in crosses with North American long-day, yellow bulb inbreds, they should be homozygous recessive at the R locus; however, they could carry dominant alleles at the L locus (El-Shafie and Davis, 1967; Jones and Peterson, 1952). Testcross segregations for bulb colors fit expected ratios for 46 of 51 families, assuming that the yellow bulb, male-sterile lines were iiCCLlrr or less commonly iiCCLrr (Table 2). F3 segregations fit expected ratios for 45 of 55 F3 families, excluding 30 families with all-white bulbs (II or cc). Segregations revealed that B2246 must have contributed

### Table 2. Segregations of onion bulb colors in F3 and testcross families from B2246 × B11159.

| Pedigree | Gen. | Assigned genotype | Fam. | W | R | Y | Obs. | Exp. | P value |
|----------|------|-------------------|------|---|---|---|------|------|---------|
| A × [(B2246 × B11159) ⊗] | TC | H— | 27 | 1721 | 0 | 0 | 1 | 0 | 0 |
| [(B2246 × B11159) ⊗] | F3 | iiCCLLRR | 27 | 883 | 0 | 0 | 1 | 0 | 0 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 3 | 112 | 131 | 0 | 1 | 1 | 0 | 0.476 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 3 | 102 | 20 | 0 | 3 | 1 | 0 | 0.090 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 2 | 60 | 36 | 33 | 2 | 1 | 1 | 0.681 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 3 | 111 | 34 | 18 | 12 | 3 | 1 | 0.025 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 10 | 378 | 219 | 191 | 2 | 1 | 1 | 0.193 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 12 | 494 | 101 | 68 | 48 | 9 | 7 | 0.622 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 1 | 30 | 15 | 0 | 2 | 1 | 1 | 0.975 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 2 | 58 | 36 | 0 | 1 | 0 | 0 | 0.769 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 4 | 180 | 28 | 1 | 13 | 3 | 0 | 0.149 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 3 | 150 | 18 | 9 | 52 | 9 | 3 | 0.327 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 1 | 35 | 0 | 32 | 0 | 1 | 0 | 1 | 0.935 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 3 | 161 | 0 | 66 | 13 | 0 | 3 | 0.000 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 3 | 38 | 27 | 0 | 1 | 1 | 0 | 0.394 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 1 | 33 | 4 | 0 | 52 | 9 | 3 | 0.317 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 3 | 115 | 62 | 49 | 2 | 1 | 1 | 0.457 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 6 | 359 | 28 | 12 | 208 | 27 | 21 | 0.000 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 3 | 103 | 13 | 83 | 2 | 1 | 1 | 0.000 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 3 | 125 | 0 | 35 | 52 | 0 | 12 | 0.557 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 9 | 288 | 0 | 272 | 1 | 0 | 1 | 0.796 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 9 | 277 | 0 | 72 | 13 | 0 | 3 | 0.667 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 1 | 33 | 18 | 16 | 2 | 1 | 1 | 0.935 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRr | 1 | 44 | 0 | 0 | 1 | 0 | 0 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 1 | 17 | 6 | 5 | 2 | 1 | 1 | 0.507 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRR | 1 | 51 | 0 | 0 | 1 | 0 | 0 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 1 | 40 | 21 | 25 | 2 | 1 | 1 | 0.673 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRR | 1 | 54 | 0 | 0 | 1 | 0 | 0 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 2 | 58 | 32 | 39 | 2 | 1 | 1 | 0.355 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRR | 3 | 97 | 1 | 0 | 1 | 0 | 0 |
| A × [(B2246 × B11159) ⊗] | TC | iicCLlRR | 4 | 0 | 143 | 129 | 0 | 9 | 7 | 0.474 |
| [(B2246 × B11159) ⊗] | F3 | iicCLlRR | 4 | 77 | 98 | 88 | 16 | 27 | 21 | 0.176 |

*A, yellow bulb, male-sterile plant with expected genotype iiCCLlrr or iicCLlrr.

*Gen., generation; TC, family from testcross of F2 plants to male-sterile (A) line.

*Numbers of families (Fam.).

*Observed (Obs.) and expected (Exp.) ratios of white (W), red (R), and yellow (Y) bulbs. Data were pooled across families after establishing homogeneity of ratios.

*Probability (P) of goodness-of-fit from chi-square analyses.
recessive alleles and B11159 dominant alleles at the L and R loci.

Amplicons were produced from the promoter regions of ANS and DFR; however, we never observed any of the polymorphisms reported by Kim et al. (2004a, b, 2005a, b, c, 2006). Amplicons produced from the coding regions of ANS and DFR were essentially identical to Genbank accessions AY585678 and AY221252 respectively, and SNPs were revealed that segregated in the B2246 × B11159 family. For ANS, one T/C SNP was identified at position 358 (Genbank accessions BV722873 and BV722872 for B2246 and B11159 respectively). One C/T SNP in DFR segregated at position 257 (Genbank accessions BV722871 and BV722870). Linkage analyses revealed that ANS and DFR were independently inherited, as expected, because Masuzaki et al. (2006) assigned these genes to chromosomes 4 and 7 respectively of onion. Red bulbs versus yellow bulbs in the B2246 × B11159 family cosegregated with DFR and a locus linked at 6.3 cM to ANS. Bulb color segregations and SNP genotypes for recombinant families are shown in Table 3. Our results demonstrate that light-red to red bulb color in the B2246 × B11159 family is conditioned by dominant alleles at two loci, consistent with the model proposed by El-Shafie and Davis (1967). One of these loci is identical or tightly linked to DFR. The second locus did not cosegregate with ANS, but showed linkage to ANS at 6.3 cM and was named L2.

**Segregations for red bulbs versus yellow bulbs from B8667B.** Segregations for red bulbs versus yellow bulbs in the B8667B family fit the expected 1:2:1 ratio (13:39:20, P = 0.394). Amplicons produced for ANS were monomorphic in this family. Amplicons from DFR were more than 98% similar to Genbank accession AY221252, and a T/C SNP in DFR was identified at position 296 (Genbank accessions BV722868 and BV722869 for red and yellow alleles respectively). Linkage analyses demonstrated that red bulbs versus yellow bulbs cosegregated with SNPs in DFR.

**Segregations for red bulbs versus yellow bulbs in testcrosses of BYG15-23 × AC43 progenies.** We previously reported that yellow F2 bulbs from the BYG15-23 × AC43 segregating family occasionally produced red progenies in testcrosses with yellow male-sterile lines (King et al., 1998). An A/T SNP was identified at position 120 of genomic amplicons of DFR (Genbank accessions BV722867 and BV722866 and AC43 and BYG15-23 respectively); no SNPs were revealed in ANS amplicons. Single nucleotide polymorphisms in DFR cosegregated with complementary light-red bulbs in testcrosses of 115 plants from BYG15-23 × AC43. We originally named a locus (Crb-1 for complementary red bulb-I) for this trait (King et al., 1998); however, this name is not appropriate because a dominant allele at DFR conditioned complementary light-red bulbs in this family. Dihydroflavonol 4-reductase mapped to chromosome 7 of onion at 11.1 cM from AOB212 (Martin et al., 2005), in agreement with Masuzaki et al. (2006), who used alien addition lines to assign DFR to chromosome 7.

**Linkage analyses for bulb and seedcoat colors in onion.** We previously described mapping of SSR markers in onion (Martin et al., 2005), of which 22 segregated in the B2246 × B11159 family and nine in the B8667B family. Linkage analyses among the bulb color loci and SSRs revealed that I was linked at 21.0 cM to ACM006 (currently unmapped in onion), B was assigned to chromosome 1, and C to chromosome 5 (Fig. 1). Single nucleotide polymorphisms in ANS and DFR were not linked to any of the SSRs.

**Genetic model for red bulb color in onion.** Crosses among populations of yellow bulb onions occasionally produce light-red progenies, for example between North American long-day storage inbreds or short-day Grano types with European Rijnburger or Brazilian populations (Jones and Peterson, 1952). These light-red hybrids complicate the production of yellow hybrids from crosses among divergent inbreds. El-Shafie and Davis (1967) proposed that complementary light-red bulbs are conditioned by dominant alleles at the R and L loci, with deeper red colors occurring when R or L loci are homozygous dominant. The L locus was proposed to be ANS (Kim et al., 2005c) and the R locus may be DFR (Kim et al., 2004b). Hybridizations revealed that ANS is single copy, and

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**Table 3. Parental genotypes and examples of segregations for onion bulb colors and single nucleotide polymorphisms at anthocyanidin synthase (ANS) and dihydroflavonol 4-reductase (DFR) for F2 and testcross families from B2246 by B11159.**

| Family     | Gen.  | SNP | Obs." | Exp." | P value=" |
|------------|-------|-----|--------|--------|-----------|
| B11159     | P1    | T   | C      | IIcLLrr| All 0 0 0 | 0 0 0 0 | 0.975 |
| B2246      | P2    | C   | T      | iiCllrr| 0 0 0 All | 0 0 1 1 | 0.897 |
| A × 19100  | TC    |     |        |        | 30 14 24 2 | 1 1 1 1 | 0.153 |
| 19100      | F3    | C   | H      | IiCllrr| 28 7 8 48 | 9 7 7 2 | 0.722 |
| A × 19124  | TC    |     |        |        | 35 23 26 2 | 1 1 1 1 | 0.975 |
| 19124      | F3    | C   | H      | IiCllrr| 36 8 5 48 | 9 7 7 2 | 0.897 |
| A × 19136  | TC    |     |        |        | 30 15 14 2 | 1 1 1 1 | 0.103 |
| 19136      | F3    | C   | H      | IiCllrr| 21 9 4 48 | 9 7 7 2 | 0.722 |
| A × 19142  | TC    |     |        |        | 33 13 17 2 | 1 1 1 1 | 0.153 |
| 19142      | F3    | C   | H      | IIcLlr | 49 4 1 208 | 27 21 2 | 0.103 |

* A, yellow bulb, male-sterile plant with expected genotype IiCllrr.
  * Gen., generation; P, parent; TC, testcross family to male-sterile (A) plant.
  * SNP, single nucleotide polymorphism.
  *Genotype assigned based on observed segregations.
  *Observed (Obs.) and expected (Exp.) ratios of white (W), red (R), and yellow (Y) bulbs.
  *P = probability of goodness-of-fit from chi-square analyses.

Dihydroflavonol 4-reductase (DFR) cosegregated with one of the two loci conditioning red bulb color. If anthocyanidin synthase (ANS) were the second locus, F2 plants homozygous for the C allele at ANS should not produce red bulbs in the F3 and testcross families.
numerous copies of DFR exist in the onion genome (Fig. 2). Our analyses support DFR as the R locus of onion; however, duplicated copies of DFR may also control the appearance of red bulbs. In the B2246 × B11159 family, red bulb color did not cosegregate with single-copy ANS, but with a second locus (L2) linked at 6.3 cM to ANS on chromosome 4. We propose that yellow bulb onions have been independently selected numerous times from white or red populations and that yellow onions carry independent mutations in structural or regulatory genes controlling the production of anthocyanins. Two of these genes are DFR and ANS, and correspond to the R and L loci, respectively. Our results demonstrate that dominant alleles at both DFR and L2, an additional locus linked at 6.3 cM to ANS on chromosome 4, also condition red bulb color in onion.

Fig. 2. (A, B) Autoradiograms from hybridizations of anthocyanidin synthase (A) and dihydroflavonol 4-reductase (B) to gel blots of EcoRV-digested DNA from four segregating progenies from the B2246 × B11159 family of onion.
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