


Genetic analysis of seed coat colour in adzuki bean (*Vigna angularis* L.)

Liwei Chu^{1,2}, Pu Zhao¹, Xueqi Huang¹, Bo Zhao¹, Yisong Li³, Kai Yang^{1*} and Ping Wan^{1*} 

¹Beijing Key Laboratory of New Technology in Agricultural Application, College of Plant Science and Technology, Beijing University of Agriculture, Beijing, 102206, China, ²State Key Laboratory of Tree Genetics and Breeding, Key Laboratory of Tree Breeding and Cultivation of the State Forestry Administration, Research Institute of Forestry, Chinese Academy of Forestry, Beijing, 100091, China and ³Key Laboratory of Urban Agriculture (North) of Ministry of Agriculture, College of Biological Science and Resources Environment, Beijing University of Agriculture, Beijing, 102206, China

Received 1 February 2020, Revised 15 February 2021; Accepted 17 February 2021 – First published online 22 March 2021

Abstract

Seed coat colour is an important quality trait, domestication trait and morphological marker, and is closely associated with flavonoid and anthocyanin metabolism pathways. The seed coat colour of adzuki bean, an important legume crop, influences the processing quality of its paste, the commodity and its nutritional quality. In this study, the genetic relationships of seed coat colour were analysed using 12 hybridized combinations of F₂ individuals and four F₃ families derived from hybridized combinations between the accessions of eight seed coat colours. The loci of the colour traits were analysed based on phenotypes and using the chi-square test. Ivory colour is recessive to red and is controlled by a single R locus. Black, black mottle on grey, black mottle on red, light brown, golden and brown are all dominant to red. The phenotypes of black mottle on red, light brown, golden and brown are all controlled by a single genetic locus. Black mottle on grey is controlled by two loci. Black is controlled with two loci, and the black locus shows dominant epistasis to another locus. A genetic model of these seed coat colours was predicted. Our results will be important for gene mapping and cloning of seed coat colour characters and for providing further insight into the regulatory network of seed coat colour.

Keywords: Chi-square test, genetic loci, qualitative trait, seed coat colour, segregation, *Vigna angularis*

Introduction

Adzuki bean is rich in protein, iron, zinc and flavonoids. It has been considered the ‘red pearl’ of beans because of its low calories and fat, high digestible protein content and abundant bioactive compounds (Kitanookada *et al.*, 2012; Kramer *et al.*, 2012).

Seed coat colour is an important domestication trait and morphological marker for genetics, as well as for evaluating offspring. Almost all wild adzuki beans (*Vigna angularis*

var. *nipponensis*) are black mottle on grey seed coat, and the vast majority of landrace and improved varieties are red. However, landraces have a diversity of colours, including red, black, ivory, black mottle on red, beige, greenish yellow, light brown, brown, green, golden (strong orange yellow), black mottle on grey and some others.

Flavonoid biosynthesis causes the differences in seed coat colour, and gives adzuki beans their diversity of colours. These flavonoids have medicinal and nutritional value and also certain antioxidant and anti-mutation functions, which are related to their health benefits (Sato *et al.*, 2005; Yang *et al.*, 2010). Seed coat colour is also an important commodity-quality and nutrition-quality trait (Jiang *et al.*, 2015).

*Corresponding authors. E-mail: yangkai8978@126.com; pingwan3@163.com

Genetic analyses of the hybridized combinations of red versus light grey and ivory have shown that ivory, red and light grey are controlled by two loci (Naruwa, 1976). Kaga *et al.* (2008) and Isemura *et al.* (2007) mapped a red seed coat quantitative trait locus (QTL) controlling brown to red and gene *sdc3.1.1* controlling red to ivory, respectively. However, seed coat colour loci have not been extensively studied. Major QTL *OLB1* and a minor QTL *OLB2* of *L**, *a* and *b** values were mapped to linkage group (LG) 1, and ivory yellow gene *IVY* and pale olive buff gene *POB* were mapped to LG 8 and LG 10, respectively (Horiuchi *et al.*, 2015). We mapped the genes of red and black seed coats to the top of chromosomes 1 and 3, respectively (Li *et al.*, 2017). The genome draft was published in 2015 (Yang *et al.*, 2015).

In this study, genetic analysis of F₂ and F₃ segregation populations derived from 12 hybridized combinations were used to build a genetic model of the relationships between red and other seed coat colours. Four F₃ families were used to confirm the model. The genetic rules of each locus were analysed and the genetic background of different seed coat colours was first predicted. This research intended to lay a foundation for further gene mapping and cloning of seed coat colour and dissecting adzuki bean seed coat colour regulatory network.

Materials and methods

Plant materials

Accessions of eight different seed coat colours were selected for crosses (Fig. 1), and 12 hybridized combinations were constructed (Table 1). Red (R) parents included GM276, GM892, GM285, GM278, JN6, AG29 and AG9. Black (B) parents were cultivar AG118 and a wild adzuki bean CWA108. Light brown (LB) parents were semi-wild adzuki bean CWA067 from China and a cultivar AG89. The brown (BR) parent was semi-wild adzuki bean JWA010 from Japan. Strong orange yellow (golden) (G), ivory and black mottle on red (RB) parents were cultivated adzuki bean GM177, AG110 and GM633, respectively. Twelve hybridized combinations were attained, and all their F₂ and four F₃ populations were planted. Then the seed coat colour traits of these populations were investigated.

All parents and the F₁, F₂ and F₃ generations were grown at the Experimental Farm of Beijing University of Agriculture (BUA) in 2012, 2013, 2014 and 2015, respectively. F₁ hybrid seeds and the F₂ of each hybridized combination, as well as one row of each parent, were planted. Four F₃ families were planted (Table 1). Each line in F₃ families was derived from an F₂ individual. Eighty seeds were planted for each line. Rows were 3 m long and 45 cm apart; 40 seeds were planted evenly in each row. The seed coat

colour traits of F₂, F₃ and F_{3:4} families were investigated in units of individual plants.

Data analyses

The seed coat colour phenotypes of F₁ and F₂ individuals were calculated, and F₃ families of each hybridized combination were analysed as the unit for the segregation of seed coat colour. The locus number controlling phenotype and the dominant and recessive genetic relationships were analysed based on the seed coat colour segregation data of the different hybridized combinations and Mendel's law. The chi-square test was conducted using SPSS17.0 software. The dominant and recessive relationship and the loci number controlling seed coat colour were determined in accordance with the *P* value of $\chi^2 < \chi^2_{P=0.05}$.

Results

Analyses of phenotypes and genetic relationships of F₁, F₂ and F₃ progenies

The seed coat colour phenotypes of F₂ and F₃ progeny from 12 hybridized combinations were obtained (Table 1). The seed coat of F_{2:3} from the combination of red versus black mottle on red, and black mottle on red versus red including the reciprocal cross (GM276 × GM633, GM633 × AG9 and AG9 × GM633) showed that black mottle on red was dominant to red. The phenotype of red versus ivory (GM892 × AG110) F_{1:2} was red, indicating that red was dominant to ivory. In the crosses between black and red (AG118 × GM285, JN6 × AG118 and JN6 × CWA108) all F_{1:2} were black, indicating that black was dominant to red. The F_{1:2} phenotype of the combinations between black mottle on grey versus red (GM167 × GM278) and black mottle on grey versus light brown (GM544 × AG89) were black mottle on grey, revealing that that colour pattern was dominant to red and light brown. Red versus brown, light brown versus red and golden versus red were brown, light brown and golden, respectively.

Segregation of seed coat colour and chi-square test in F₂ populations

The segregation ratio of F_{2:3} phenotypes from 12 hybridized combinations between seven coat colours and red was calculated, and the number of genetic loci controlling these colours was analysed using a chi-square test (online Supplementary Table S1). The segregation ratio between black mottle on red and red was close to 3:1, and it was assumed that the difference was controlled by a single locus (BR). Results of chi-square test of the progenies from the GM276 × GM633, GM633 × AG9 and AG9 × GM633

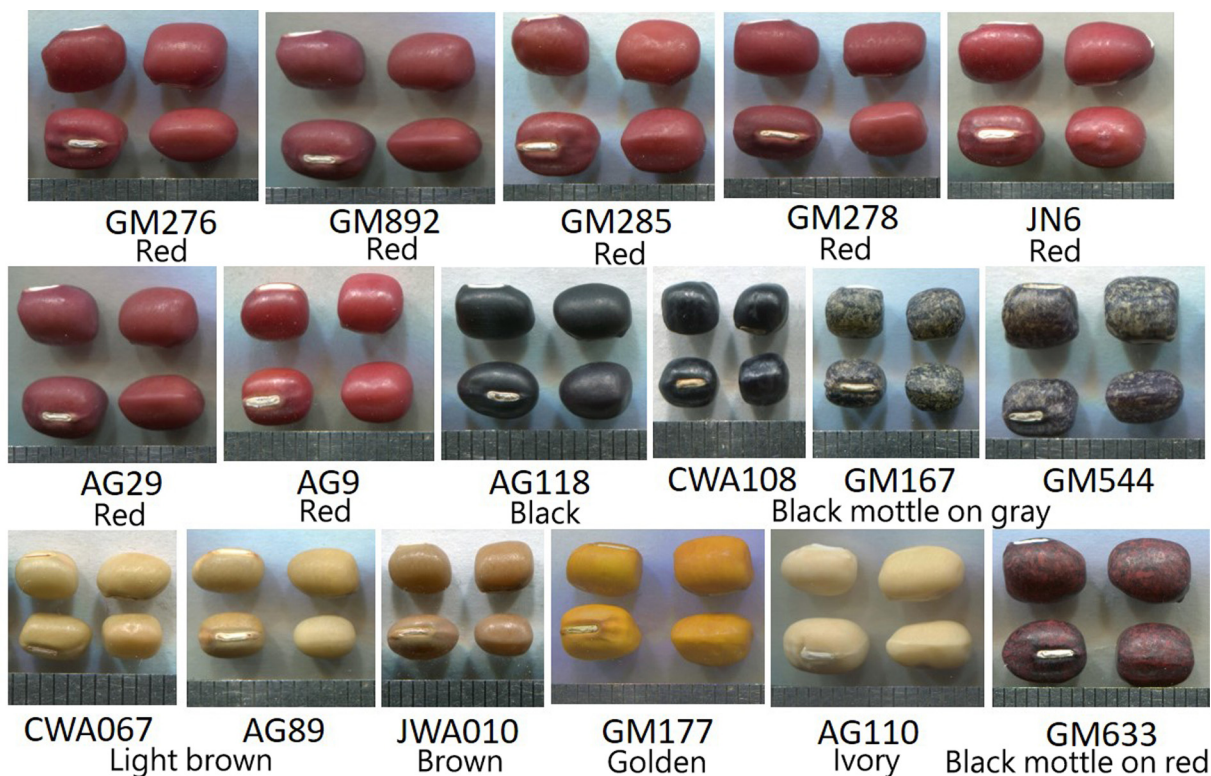


Fig. 1. Seed coat colour of parents in adzuki bean.

Table 1. Information of 12 hybridized combinations in adzuki bean

Hybridized combination	Female parent	Male parent	Number of plants in F ₂ population	Number of lines in F ₃ family
Red × Black mottle on red	GM276	GM633	281	121
Red × Black mottle on red	AG9	GM633	118	
Black mottle on red × Red	GM63	AG9	74	
Black mottle on grey × Red	GM16	GM278	174	156
Black mottle on grey × Light brown	GM544	AG89	76	
Black × Red	AG118	GM285	148	148
Red × Black	JN6	AG118	367	
Red × Black	JN6	CWA108	366	
Golden × Red	GM177	AG29	252	
Red × Brown	GM276	JWA010	303	
Red × Light brown	JN6	CWA067	249	
Red × Ivory	GM892	AG110	98	76

hybridized combinations were 0.58, 0.10 and 0.02 and less than $\chi^2_{(df=1)} P=0.05 = 3.84$, according to hypothesis that black mottle on red was dominant to red and that this was controlled by a single genetic locus was accepted within the 95% confidence interval. The hybridized combination of red versus ivory (GM892 × AG110) had a segregation ratio of 3:1 and was assumed to be controlled

by a single locus (R). The chi-square test value was 0.67 less than $\chi^2_{(df=1)} P=0.05 = 3.84$, supporting the prediction that red is dominant to ivory and is controlled by a single genetic locus. In the hybridized combination between black and red, the beige (Y) seed coat, which did not exist in the parents, was segregated out, and the segregation ratio of black:beige:red was 12:3:1. The chi-square test value

was 4.36 less than $\chi^2_{(df=2)} P=0.05 = 5.99$, suggesting that two loci regulated black and red seed coat colour, and the locus controlling the difference between black and beige showed dominant epistasis on the locus regulating the difference between beige and red. In the two hybridized combinations between red and black (JN6 × AG118 and JN6 × CWA108), the segregation ratio of black:light brown:red was 12:3:1 in $F_{2,3}$, and the locus regulating the trait between black and light brown (B) showed dominant epistasis to that regulating the trait of light brown and red (T). The chi-square test values were 4.10 and 4.32, less than $\chi^2_{(df=2)} P=0.05 = 5.99$ indicating that two loci regulated the traits between black and red seed coat and between black and light brown coat, respectively. The locus controlling black and light brown showed dominant epistasis to the locus regulating light brown and red.

In hybridized combinations between black mottle on grey and red, the individual plants with black mottle on red and light brown seed coat colour, which did not exist in their parents were segregated out. The segregation ratio of black mottle on grey:black mottle on red:light brown:red was 9:3:3:1 in $F_{2,3}$. We predicted that two genetic loci controlled black mottle on grey and red. Black mottle (RB) was dominant to non-black mottle, and light brown was dominant to red. The chi-square test values for GM167 × GM278 and GM544 × AG89 were 0.36 and 0.77, all lower than $\chi^2_{(df=3)} P=0.05 = 7.82$. It showed that two genetic loci controlled black mottle on grey and red.

In the hybridized combinations of red versus light brown, golden (strong orange yellow) versus red and red versus brown, the segregation ratios of light brown:red, golden:red and brown:red were all close to 3:1. Light brown (T), golden (G) and brown (BR) loci were predicted to be dominant to red and controlled by a single genetic locus. The chi-square values for JN6 × CWA067, GM177 × AG29 and GM276 × JWA010 were 0.30, 2.71 and 0.58, respectively, and less than $\chi^2_{(df=1)} P=0.05 = 3.84$ indicating that light brown (T), golden (strong orange yellow) (G) and brown (BR) were all dominant to red and controlled by single genetic locus.

These results show that seed coat colours in adzuki bean are generally controlled by one or two genetic loci. In addition, we found an interaction between the black mottle/non-black mottle locus (RB) and the light brown/red locus (T). The phenotype of black mottle on grey was exhibited when both loci were dominant. The locus regulating black and light brown showed dominant epistasis to the locus controlling light brown and red.

Segregation of seed coat colour and chi-square tests for F_3 families

To verify further whether the genotypes of $F_{2,3}$ seeds were controlled by one or two loci, and two loci with dominant

epistasis, the segregation of colours from four F_3 families was analysed and assessed using chi-square tests.

In genetic analyses of the hybridized combination between red and black mottle on red, it was assumed that these patterns were controlled by a single locus (RB). Based on Mendel's law, the genotype segregation ratio of corresponding family phenotypes from F_2 individuals of homozygous dominant (*RBrb*):heterozygous (*rbrb*):homozygous recessive (*RBRB*) was 1:2:1; that of black mottle on red no-segregation family:black mottle on red segregation family:red no-segregation family was 1:2:1. For GM276 × GM633, the chi-square test value was 3.13 less than $\chi^2_{(df=1)} P=0.05 = 3.84$, confirming the result that black mottle on red was dominant to red and controlled by a single locus (RB) (online Supplementary Fig. S1 and Table S2).

For red and ivory (online Supplementary Fig. S2 and Table S3), genetic analyses showed that the difference between red and ivory was controlled by a single locus (R). The genotype segregation ratio of homozygous dominant (*RR*):heterozygous (*Rr*):homozygous recessive (*rr*) was 1:2:1, based on Mendel's law. The segregation ratio of non-segregation red family:ivory segregated from red family:non-segregation ivory family was 1:2:1. The chi-square test value was 3.13 ($\chi^2_{(df=1)} P=0.05 = 3.84$), and, confirming that red was dominant to ivory and controlled by a single locus (R).

Genetic analyses of F_2 individuals indicated that the difference between red and black mottle on grey was controlled by two genetic loci, RB and T. The segregation ratio of the $F_{3,4}$ phenotype derived from the cross between GM167 and GM278 was also analysed to further confirm this result (online Supplementary Fig. S3 and Table S4). The segregation ratio of black mottle on grey:black mottle on red:light brown:red was 9:3:3:1. Based on Mendel's law, there were four genotypes in black mottle on grey progeny of F_2 individuals, and the segregation ratio of *RBrb Tt:RBrb TT:RBRB Tt:RBRB TT* was 4:2:2:1. The corresponding $F_{3,4}$ phenotype should have been black mottle on grey segregated all four seed coat colour families:black mottle on grey segregated only black mottle on red family:black mottle on grey segregated only light brown family:pure black mottle on grey family = 4:2:2:1. The chi-square test value was 1.57 ($\chi^2_{(df=3)} P=0.05 = 7.82$), two genotypes in F_2 individuals with light brown progeny were exhibited, and the segregation ratio of *tt RBrb:tt RBRB* was 2:1. The phenotype of the $F_{3,4}$ derived from the corresponding F_2 individuals was light brown family segregated red seed coat:non-segregation light brown family was 2:1. The chi-square test value was 0.07 ($\chi^2_{(df=1)} P=0.05 = 3.84$), there were two genotypes of F_3 families derived from F_2 individuals with black mottle on red, and the segregation ratio of *Tt rbrb:TT rbrb* was 2:1. The phenotypes of the corresponding F_3 families should have been black mottle on red families segregated red seed coat:homozygous black mottle on red

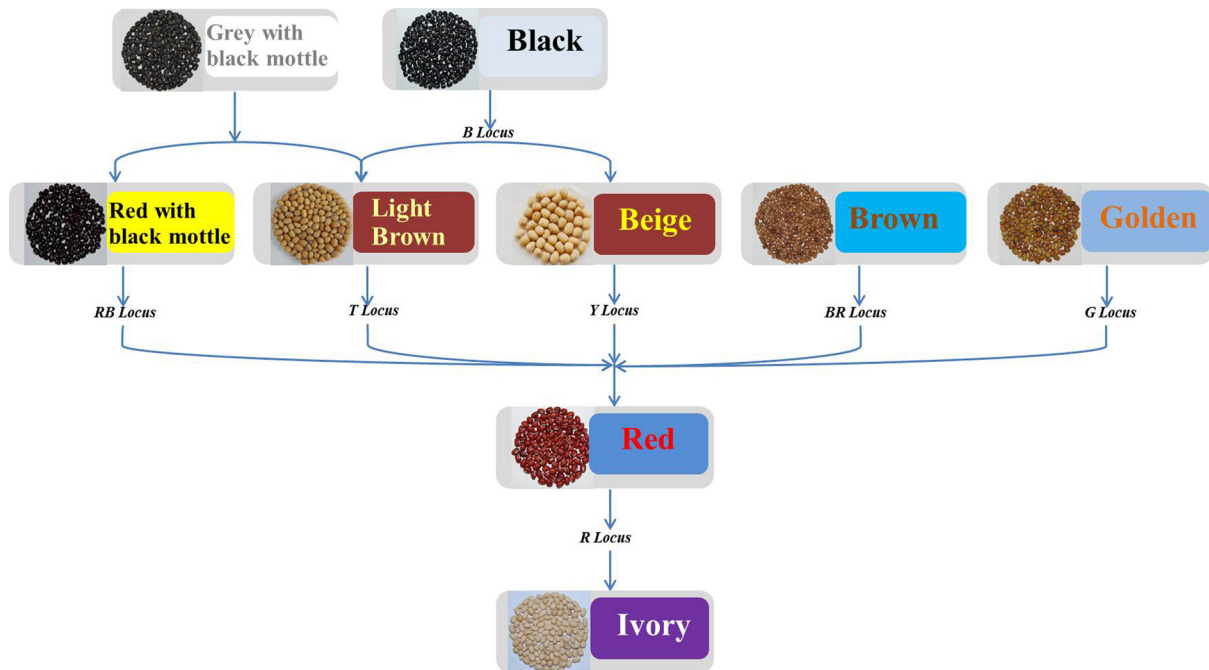


Fig. 2. Dominant and recessive relationships of seed coat colour loci in adzuki bean.

family = 2:1. The chi-square test value was 0.02 ($\chi^2_{(df=1)} P=0.05 = 3.84$), and conformed to the segregation ratio of 2:1. One genotype, *rrrb*, existed in the F_2 individuals with red progeny, and the colour of its $F_{3,4}$ were all red. The 11 families were homozygous red. According to the chi-square test, two genetic loci controlling seed coat colour between red and black mottle on grey were verified.

The segregation ratio of F_3 families between black and red derived from the hybridization between AG118 and GM285 was also calculated. The segregation ratio of black:beige:red was 12:3:1. According to Mendel's law, there were six genotypes in black progeny of F_2 individuals, and the segregation ratio of *BBYY:BBYy:BByy:BbYY:BbYy:Bbyy* was 1:2:1:2:4:2 (online Supplementary Fig. S4 and Table S5). The chi-square test results verify the prediction that seed coat colour between red and black was controlled by two loci, one locus regulating black and beige showing dominant epistasis to another locus regulating beige and red.

Discussion

The dominant and recessive genetic relationships between each pair of seed coat colours are controlled by one to two loci; a model is predicted based on these genetic analyses of 12 hybridized combinations between eight seed coat colours (Fig. 2). The vast majority of cultigens of adzuki bean have red seed coats. Genetic analyses and chi-square tests of 12 hybridized combinations between seven other types of seed coat colours and red showed that red is

recessive to other colours, except for ivory, which is the only seed coat colour in adzuki bean recessive to red. The difference between ivory and red is caused by a single R locus. In the genetic backgrounds of other loci are all recessive; the seed coat colour appears red when the R locus is dominant and the seed coat colour appears white when the R locus is recessive conversely (Fig. 2). Recessive red seed coats have been selected during the process of domestication and improvement of adzuki bean. The distinction between ivory and red is controlled by a single R locus, and red is dominant. Horiuchi *et al.* (2015) reported that ivory–yellow is recessive to red and is controlled by a single *IVY* gene. Ivory in our study may be the same as the ivory–yellow colour in Horiuchi's study (Fig. 3). In this research, the segregation ratio of its F_3 family is first used to confirm this result.

Under the genetic background of a dominant R locus, black mottle on red, brown, golden, light brown and beige showed dominance to red, respectively, and these are all regulated by a single genetic locus. The seed coat showed black mottle on red, light brown, beige, brown and golden colour, respectively, when RB, T, Y, BR and G is dominant, respectively, otherwise exhibiting red (Fig. 2). The segregation ratio between light brown and red is consistent with the result of Isemura *et al.* (2007). The result of black mottle on red being dominant to red and controlled by a single locus is consistent with Jin *et al.* (1996). The relationships between brown, golden, beige and red are first reported and the F_3 family of red versus black mottle on red is first used to confirm the result of RB locus in this study. The phenotypes of RB, T, Y, BR and



Fig. 3. Ivory yellow (Horiuchi *et al.*, 2015) and ivory (right) seed coat adzuki bean.

G on the recessive genetic background of the R locus should be further analysed under the genetic background of ivory using the hybridized combinations between each seed coat colour and ivory.

The difference between black mottle on grey and red is controlled by RB locus and T locus and the segregation ratio of its F₃ family confirmed this result. Individual functions of both two loci had been shown in other hybridized combinations. The causing factors of black mottle on grey may be RB locus controlling the black mottle interacting with T locus regulating light brown and red. When both genotype loci are dominant, the phenotype expressed mottle on grey. The BR locus is thought to affect background colour among non-red background colours.

The difference between black and red is controlled by B locus and T/Y locus. In this research and previous analyses (Li *et al.*, 2014), the black locus B is shown to display dominant epistasis on the light brown locus T and the beige locus Y, respectively. The segregation ratio of a F₃ family between black and red confirmed the dominant epistasis of B locus to Y locus. The black seed coat colour gene is mapped in soybean (*Glycine max*), and the B locus is deduced to encode a key enzyme for the synthesis of black flavonoid (Yang *et al.*, 2010). We predict that when the genotype is dominant B, black pigment will be synthesized and cover light brown and beige colour. In addition, the hybridized combination between black and red can segregate beige or light brown which don't exist in their parent. The cross of light brown and beige is also supposed to be controlled by a single locus, and the hybridized combination between light brown and beige needs be constructed for further genetic analyses.

Table 2. Genetic background of different seed coat colours in adzuki bean

Seed coat colour	Genetic background
Ivory	<i>rr rrbbrb brbr yy tt gg bb</i>
Red	<i>RR rrbbrb brbr yy tt gg bb</i>
Light brown	<i>RR rrbbrb brbr yy TT gg bb</i>
Beige	<i>RR rrbbrb brbr YY tt gg bb</i>
Golden	<i>RR rrbbrb brbr yy tt GG bb</i>
Brown	<i>RR rrbbrb BRBR yy tt gg bb</i>
Red with black mottle	<i>RR RBRB brbr yy tt gg bb</i>
Grey with black mottle	<i>RR RBRB BRBR yy tt gg bb</i>
Black	<i>RR rrbbrb brbr yy TT gg BB/RR rrbbrb brbr YY tt gg BB</i>

Seven genetic loci controlling seed coat colour in adzuki bean are explained based on different genetic backgrounds (Table 2). The genetic loci predictions indicate that the complex traits of seed coat colour can be separated into traits of different qualities controlled by a single locus. Our results have important implications for gene mapping and cloning and regulatory network dissection of seed coat colour, and provide further insight into flavonoid metabolic mechanisms in adzuki bean.

Supplementary material

The supplementary material for this article can be found at <https://doi.org/10.1017/S1479262121000101>.

Financial support

This study was funded by the Beijing Natural Science Foundation–Beijing Municipal Education Committee (grant number KZ201710020013), the National Natural Science Foundation of China (grant number 31871697) and the Open foundation of Beijing Key Laboratory of New Technology in Agricultural Application (grant number kf2017022).

Conflict of interest

The authors declare that they have no conflict of interest.

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