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Delineation of Inheritance Pattern of Aleurone Layer Colour Through Chemical Tests in Rice

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Abstract

Background: Rice aleurone layer develops different colours with various chemical tests that may help to develop some rapid tests for identification/grouping of rice varieties. Understanding the colour inheritance pattern could enable to develop chemical clues that may help for genetic purity analysis along with grow-out-test.

Results: In this study, inheritance pattern of aleurone layer colour was studied in parents, F₁ and F₂ progenies derived from the crosses IR 36 × Acc. No. 2693 and IR 64 × Acc. No. 2693. The parent IR 36 showed light yellow (NaOH/KOH) and brown (phenol/modified phenol test) colour; whereas, Acc. No. 2693 revealed wine red/dark wine red (NaOH/KOH) and light brown colour/no reaction (phenol/modified phenol test). In contrary, another parent IR 64 exhibited light yellow (KOH/NaOH) and dark brown (phenol, modified phenol) colour. Both the F₁ showed an intermediate light wine red colour (NaOH/KOH) and dark brown (phenol and modified phenol) colour, which is dominant over their one of the parents. The colour pattern with standard phenol/modified phenol, NaOH and KOH tests in F₂ progenies of both the crosses showed 9:7 (complementary gene interaction) and 11:5 ratios (reciprocal dominance modification of recessive alleles), respectively.

Conclusions: Our findings clearly elucidate the colour inheritance pattern in rice that may facilitate to develop rapid chemical tests to identify/ group the varieties for genetic purity analysis.

Keywords: Aleurone layer, Alleles, Complementary gene action, Duplicate gene action, Rice

Background

Chemical tests in rice, so far have been extrapolated for identification/characterization of cultivars on the basis of colour pattern in aleurone layer that might developed through enzyme mediated reactions. Aleurone layer is a living entity, which constitute outermost layer of endosperm, specialised in de novo synthesis of reserve mobilizing enzyme complex during seed germination process (Kumar et al. 2015). In addition, the aleurone layer is also involved in the synthesis of oxidase enzymes such as laccase, tyrosinase, polyphenol oxidase, monophenol oxidase and horse-radish peroxidase, which catalyzes to form a colour reaction (Cabaj et al. 2010; Fernandes

et al. 2005). Among these oxidases, polyphenol oxidase (PPO) is one of the enzyme that is involved in oxidation of phenol colour reaction through formation of brown coloured melanin pigment (Steffens et al. 1994; Kumar et al. 2016). Polyphenol oxidases avail molecular oxygen, which undergoes hydroxylation and dehydrogenation of phenolic compounds to form reactive *o*-quinones. These *o*-quinones alkylate nucleophilic groups and self-polymerize to form melanin polymers (Fuerst et al. 2014). Role of oxidases is reported to be multifaceted, wherein they are involved in potential seed defense pathways and located in aleurone layer as indicated by their increased levels in the aleuronic fraction (Fraignier et al. 2000; Kumar et al. 2017a; Sinha et al. 2016). Proteomic analysis of aleurone layer in wheat suggested the presence of oxidases along with proteins involved in metabolism (Jerkovic et al. 2010; Kumar et al. 2017b).

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In rice, genetic studies on inheritance of colour formation in aleurone layer have been limited. However, biochemical aspects of various enzymes secreted through aleurone layer especially PPO and other reserve mobilizing enzymes have been well studied. Many workers (Joshi and Banerjee 1970; Joshi and Banerjee 1969; Jimenez and Dubcovsky 1999) studied the colour formation of wheat polyploids that had developed colour when reacted with phenol and tyrosine substrates mediated by PPO. Further, reactions of whole-wheat seeds with phenol (Joshi et al. 1969; Maguire et al. 1975) and catechol (Milner and Gould 1951) have been used for cultivar purity testing. Miczynski (1938) reported the presence of one or two genes in bread wheat, which controlled the phenol colour reaction. Moreover, various chemical tests were used so far in identification and differentiation of crop varieties based on the colour differences generated in the aleurone layer when reacted with different chemical tests. Besides, genetic background of the varieties also plays an important role in the identification of varieties. The colour formation by enzyme system has been reported in pearl millet (Varier et al. 1995), foxtail millet (Pallares et al. 2004), sorghum (Thangavel et al. 2005), rice (Mor et al. 2006; Dileepkumar et al., 2015; Chandu et al. 2017) and wheat (Joshi et al. 2007), respectively. Chemical tests such as FeSO_4 (Pallares et al. 2004), KOH (Mor et al. 2006), phenol and modified phenol tests (Joshi et al. 2007; Banerjee and Chandra 1977) etc. were studied for the development of seed keys. The purpose of the present study is to determine the inheritance pattern and segregation of colour formation trait in aleurone layer of rice using parents, F_1 and F_2 progenies derived from the crosses IR 36 \times Acc. No. 2693 and IR 64 \times Acc. No. 2693 by chemical tests.

Results and Discussion

Aleurone Layer Colouration in Parents IR 36 \times Acc. No. 2693 and IR 64 \times Acc. No. 2693

Studies on colour formation of aleurone layer revealed that IR 36 showed light yellow with NaOH and KOH tests, whereas, phenol and modified phenol tests recorded brown colour. The Acc. No. 2693 recorded wine red / dark wine red colour with NaOH, KOH and light brown colour/ no reaction colour with phenol and modified phenol tests, respectively. Similarly, IR 64 recorded light yellow colour with NaOH and KOH tests; while phenol and modified phenol tests were recorded dark brown colour as shown in Table 1. Colour formation with phenol test is depicted in Fig. 1, where the genotypes were grouped based on the biochemical tests and were in congruence with other studies (Thangavel et al. 2005; Nethra et al. 2007; Vijayalakshmi and Vijay 2009; Singh et al. 2011; Anitalakshmi et al. 2014; Kumar et al. 2015).

Table 1 Aleurone layer color reaction of parents and F_1 to different chemical tests

Chemical tests	IR 36	IR 64	Acc. No. 2693	F_1
NaOH	LY	LY	WR	LWR
KOH	LY	LY	DWR	LWR
Phenol	B	DB	LB/NC	DB
Modified Phenol	B	DB	LB/NC	DB

LY Light Yellow, WR Wine Red, DWR Dark Wine Red, LWR light wine red, LB Light Brown, B Brown, DB Dark Brown, NC No reaction

Aleurone Layer Colour Inheritance in F_1 Plant of IR 36 \times Acc. No. 2693 and IR 64 \times Acc. No. 2693

The F_1 s were derived from the crosses IR 36 \times Acc. No. 2693 and IR64 \times Acc. No. 2693, respectively (Table 1). The freshly harvested F_1 seeds (derived from cross IR 36 \times Acc. No. 2693, as depicted in Fig. 2) showed light wine red colour with NaOH and KOH tests; while brown colour was recorded with phenol and modified phenol tests, respectively.

Further, the freshly harvested F_1 seeds derived from the cross IR 64 \times Acc. No. 2693 (Fig. 3) showed light wine red colour with NaOH and KOH tests, respectively. In contrary, reaction with phenol and modified phenol tests showed dark brown colour, respectively. NaOH and KOH tests showed intermediate colour segregation i.e. light wine red colour compared with parents. In the same way, in case of phenol and modified phenol tests showed dark brown colour from cross IR 64 \times Acc. No. 2693 and the similar colour pattern was recorded in case of IR 36 \times Acc. No. 2693 that implies that the observed colour is dominant over light yellow.

Aleurone Layer Colour Inheritance in F_2 Progenies Derived from IR 36 \times Acc. No. 2693 and IR 64 \times Acc. No. 2693 with Standard Phenol and Modified Phenol (copper sulphate) Tests

Out of 484 F_2 progenies of cross IR 36 \times Acc. No. 2693; 273 and 255 F_2 progenies were brown/dark brown in colour, whereas 211 and 229 F_2 progenies showed light brown/no colour with standard phenol and modified phenol tests, respectively. Further, 420 F_2 progenies derived from cross IR 64 \times Acc. No. 2693 were evaluated. It is observed that 251 and 246 F_2 progenies were brown/dark brown in colour; 169 and 174 F_2 progenies showed light brown/no reaction with standard phenol and modified phenol tests, respectively. Therefore, the investigation revealed that the colour trait in the aleurone layer of F_2 progenies were segregated with complementary gene interaction with a ratio of 9:7 indicates a goodness of fit with observed ratio (Table 2). The F_2 colour segregation was consistent with the complementary gene interaction (9:7) for all F_2 progenies of both the crosses. Therefore, two major genes and their alleles

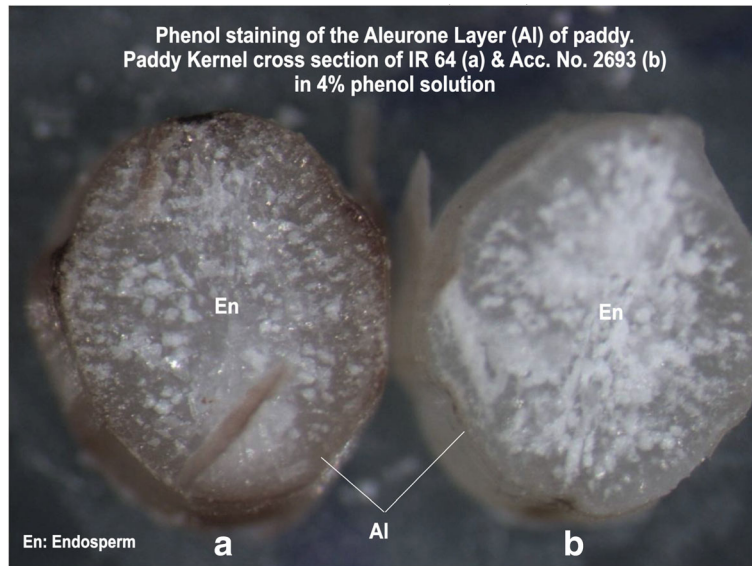


Fig. 1 Cross section of rice kernel derived from IR 64 (coloured) and Acc. No. 2693 after subjection to phenol test. Phenol staining of the Aleurone Layer (AL) of paddy. Paddy Kernel cross section of IR 64 (a) & Acc. No. 2693 (b) in 4% phenol solution

with complementary gene action controls the colour formation in aleurone layer.

The development of colour in aleurone layer (brown/dark brown) with standard phenol and modified phenol tests (copper sulphate) in rice requires the presence of two dominant genes, B_1 and D_2 e.g. $B_1B_1 D_2D_2$ (Fig. 4). When either B_1 (e.g. $b_1b_1 D_2D_2$) or D_2 ($B_1B_1 d_2d_2$) or both the genes (e.g. $b_1b_1 d_2d_2$) are present in homozygous recessive condition, brown/ dark brown colour cannot be produced; as a result, light brown / no reactions were obtained. The brown/dark brown colour variety (IR 36 and IR 64) of rice ($B_1B_1 D_2D_2$) was crossed to a light brown/no reaction variety (Acc. No 2693) with

the genotype $b_1b_1 d_2d_2$ showed dark brown colour in the F_1 ($B_1b_1 D_2d_2$) progeny. In the F_2 progenies, on an average nine progenies had one dominant allele of both the genes B_1 and D_2 . These plants, therefore has brown/dark brown colour. Three, out of sixteen F_2 progenies, shall have dominant B_1 but had homozygous recessive genotype b_1b_1 ; three others shall have dominant D_2 but had homozygous recessive genotype d_2d_2 , while one plant had both the genes in homozygous recessive genotype $b_1b_1 d_2d_2$. All these (seven progenies) had light brown/no reaction. In this type of gene interaction, the production of one of the two phenotypes of colour trait requires the presence of dominant alleles of both the

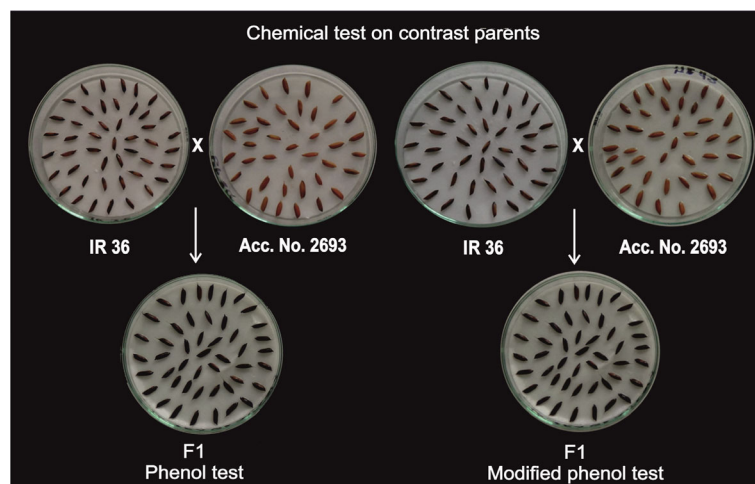


Fig. 2 Colour formation in aleurone layer of parents IR 36 (brown colour), Acc. No. 2693 (light brown/no reaction) and their F_1 progeny (brown colour) with phenol and modified phenol tests

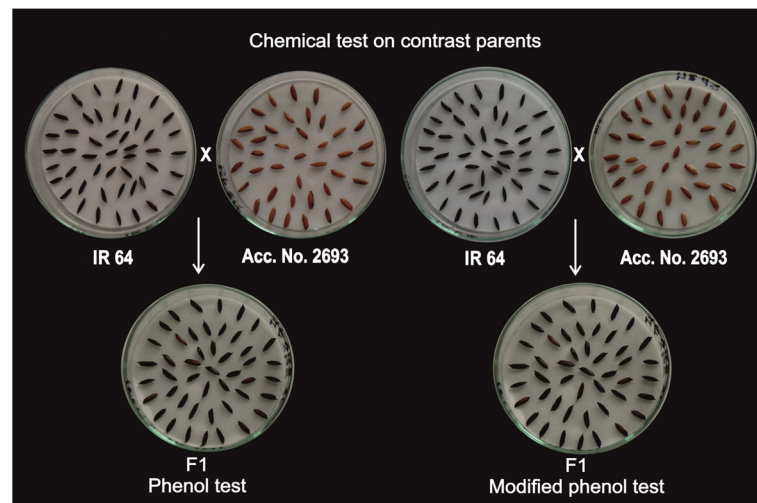


Fig. 3 Colour formation in aleurone layer of parents IR 64 (dark brown colour), Acc. No. 2693 (light brown/No reaction) and their F_1 progeny (dark brown colour) with phenol and modified phenol tests

genes controlling the concerned trait. When any one of the two or both the genes are present in the homozygous recessive state, the contrasting phenotype is produced, which leads to various modifications of the typical dihybrid, trihybrid etc. F_2 ratio.

The Mechanism of Colour Formation in Aleurone Layer

Phenol test, which is an index of polyphenol oxidase activity, has been reported to be associated with intra-varietal diversity that has been used in ascertaining varietal purity. Colour formation in aleurone layer occurs by phenol oxidation in two reactions (Fig. 5). In the first reaction, the aromatic ring of phenol can be hydroxylated to form catechols or quinols, respectively. In the second reaction, the quinols or catechols undergo oxidation to form quinones (Takahashi, 1984). Two major genes and their allelic interactions control this reaction, which is localized in seed aleurone layer in rice. The ability of genotype to form colour depends on the tyrosinase activity, which is

located at aleuronic layer (Masuthi et al. 2015). The extent of colour intensity among germplasms varied because of differences in enzyme activity, temperature, light, aeration and genetic background, respectively (Sivasubramanian and Ramakrishnan 1974). On the other hand, the germplasms with no colour might have resulted because of inability to hydroxylate the aromatic ring of phenol either due to shortage of electron donor or hydroxylating enzyme (Takahashi and Hamza 1983).

The results of phenol test are usually distinct and easily interpreted. Walls (1965), reported that the phenol colour reaction depends on the quality and quantity of oxidases present in the seeds, whereas Takahashi and Hamza (1983), reported that monophenol oxidase was extremely localized in aleurone layer of grains even though it is present in all other plant parts of a variety. Presence of enzyme system has been utilized to distinguish the crop varieties in rice and tomato (Pieper 1922; Joshi and Banerjee 1970; Mansing 2010; Vijayalakshmi

Table 2 Aleurone layer color segregation in F_2 progenies of the crosses IR 36 \times Acc.No. 2693 and IR 64 \times Acc. No. 2693 for standard phenol and modified phenol with copper sulphate tests

Cross	Chemical tests	Class	Brown/dark brown colour	Light brown/ No reaction	Total	Ratio	χ^2	<i>P</i> value (at 1 degrees of freedom)
IR 36 \times Acc. No. 2693	Phenol test	Observed	273.00	211.00	484	9:7	0.005	0.945
		Expected	272.25	211.75				
	Modified phenol test	Observed	255.00	229.00	484	9:7	2.498	0.114
		Expected	272.25	211.75				
IR 64 \times Acc. No. 2693	Phenol test	Observed	251.00	169.00	420	9:7	2.104	0.147
		Expected	236.25	183.75				
	Modified phenol test	Observed	246.00	174.00	420	9:7	0.920	0.338
		Expected	236.25	183.75				

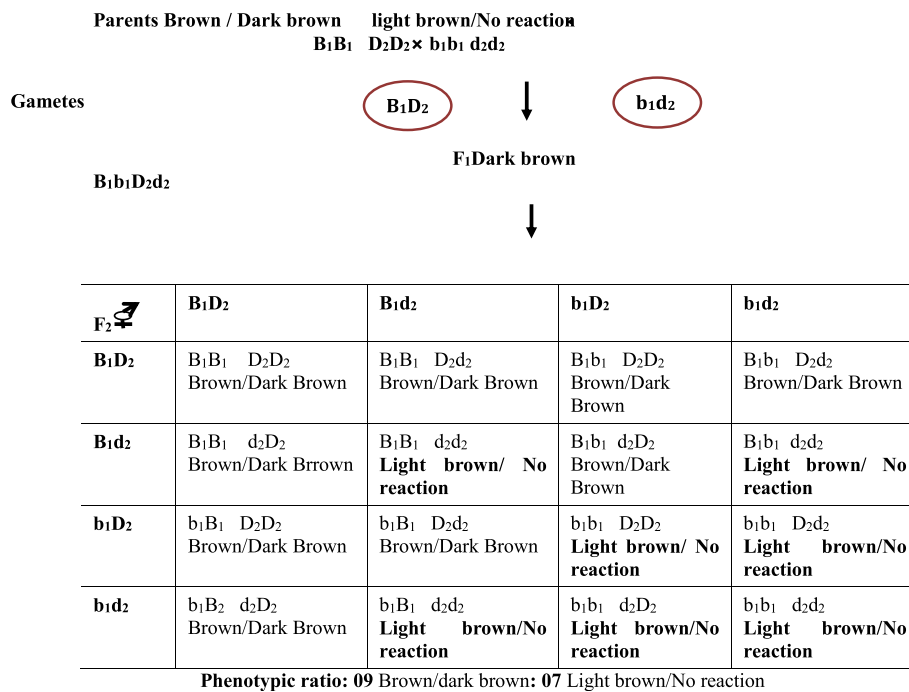


Fig. 4 Complementary gene interaction in the development of aleurone layer colour through standard phenol and modified phenol tests in rice seed giving rise to the phenotypic ratio of 9:7 (brown/dark brown: light brown/no reaction) in F_2 progenies

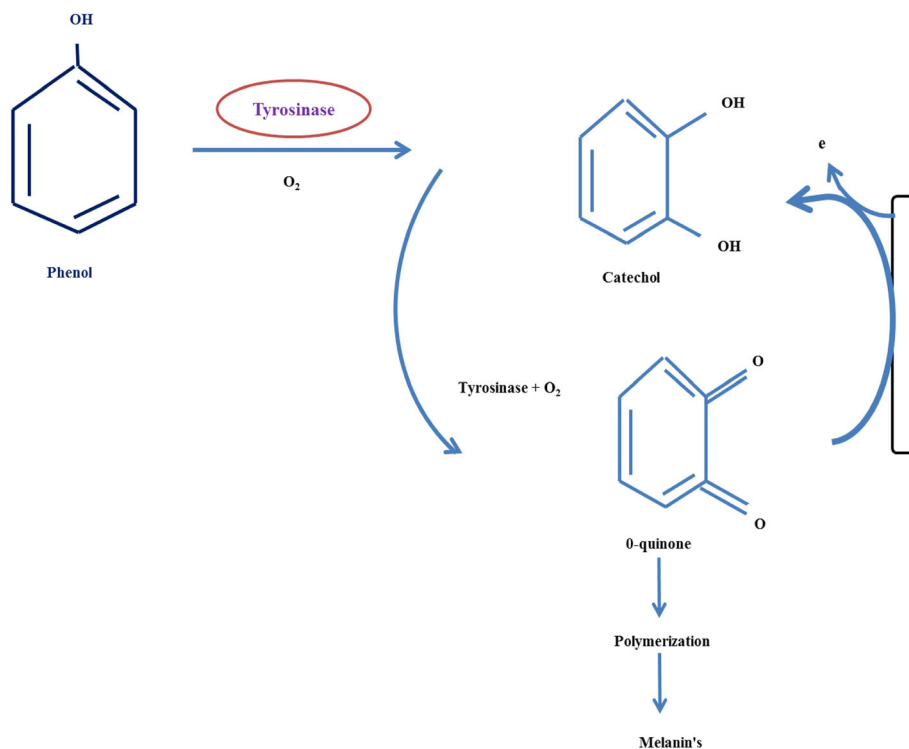


Fig. 5 Mechanism of melanin colour formation in seed aleurone layer using enzyme system upon reaction with phenol test

and Vijay 2009; Anitalakshmi et al. 2014; Sripunitha and Sivasubramaniam 2014; Vishwanath et al. 2013).

Qian et al. (2000) reported major QTL (qPH-4a) that is responsible for phenol colour has been located on chromosome 4, which explained the total phenotypic variation as high as 94.6%. Hence, this QTL is controlled by major gene. Moreover, two minor QTLs (qPH-1 and qPH-4b) located on chromosome 1 and 4 have account of total phenotypic variation of 14.9 and 29.5%, respectively. Hence, minor genes that code for these QTLs, which control phenol colour have showed positive additive effects. Phenol showed a bimodal distribution in the double haploid population and a major gene detected was close to the *Ph* gene located on chromosome 4 (Lin et al. 1994).

Aleurone Layer Colour Inheritance in F₂ Population Derived from IR 36 × Acc. No. 2693 and IR 64 × Acc. No. 2693 with Sodium Hydroxide (NaOH) and Potassium Hydroxide (KOH) Tests

The 484 F₂ progenies of the cross IR 36 × Acc. No. 2693 were evaluated. Among them, 319 and 335 F₂ progenies were wine red/dark wine red colour, whereas 165 and 149 F₂ progenies were light yellow coloured with NaOH and KOH tests, respectively. Further, 420 F₂ progenies derived from the cross IR 64 × Acc. No. 2693 were evaluated, of which 281 and 290 F₂ progenies were wine red/dark wine red colour and 139 and 130 F₂ progenies were light yellow coloured with NaOH and KOH tests, respectively.

The segregation of aleurone layer colour in 11:5 ratio for wine red/dark wine red and light yellow in F₂ progenies of both the crosses showed that the colour trait is governed by two genes, where a dominance of one gene is modified by homozygous recessive condition of another gene. The two genes that interact to produce a single character may also reciprocally modify the dominance relationship between the alleles at the other locus. Thus, the typical 15:1 ratio for duplicate gene action is modified as 11:5 due to the reciprocal dominance

modification of recessive alleles. This indicates a goodness of fit with expected ratio of 11:5 for the reciprocal dominance of duplicate genes as given in Table 3.

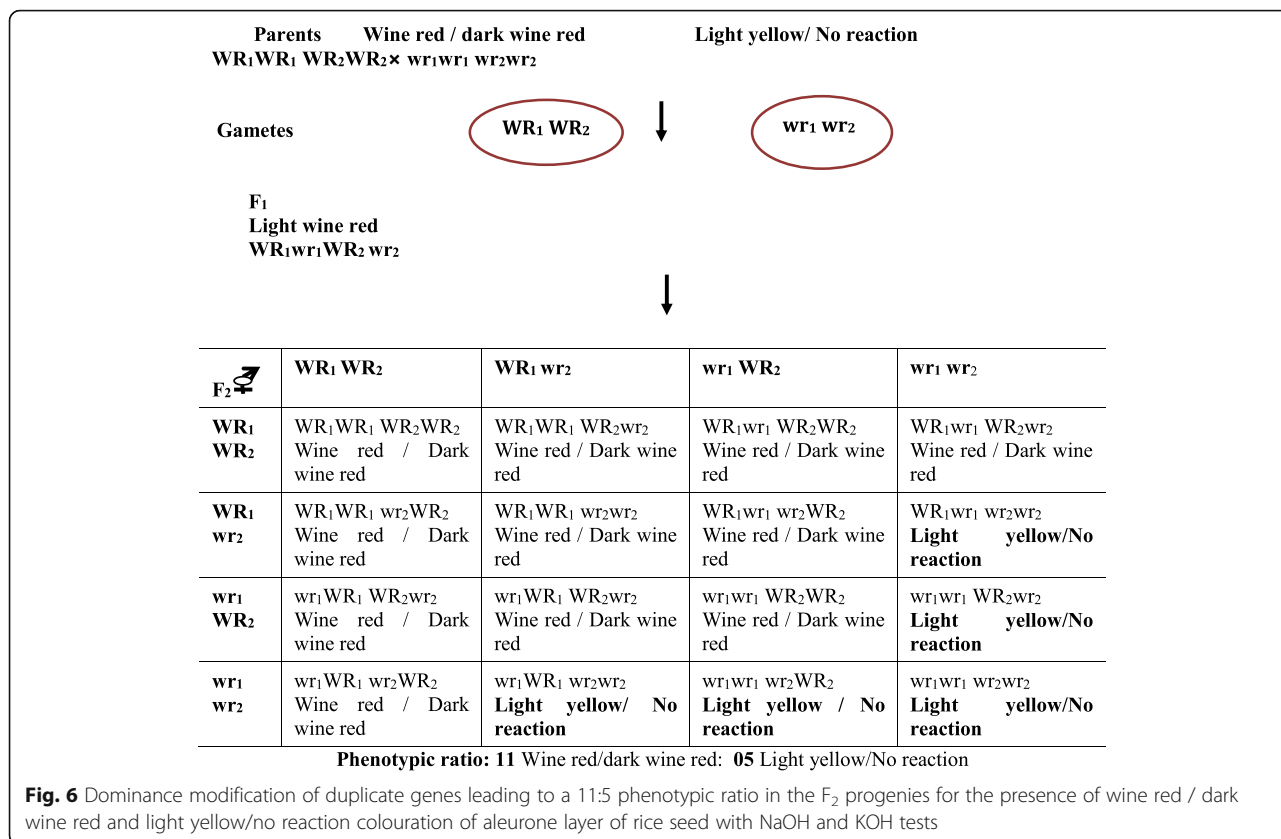
Two genes, WR₁ and WR₂ showing duplicate interaction governs the development of aleurone layer colour (wine red / dark wine red/light) with NaOH and KOH in rice plants. However, the recessive homozygous condition of one gene, say *wr*₁ *wr*₁ reverses the dominance relationship at the other locus; hence, the genotype *wr*₁*wr*₁ WR₂ *wr*₂ produces the same phenotype as the homozygous double recessive *wr*₁ *wr*₁, *wr*₂ *wr*₂ of light yellow/no reaction. Similarly, homozygous recessive condition of the other gene, *wr*₂ *wr*₂ has the same effect on the dominance relationship at the *wr*₁ locus. As a consequence, the genotype WR₁ *wr*₁ *wr*₂ *wr*₂ produces the same phenotype as the double recessive homozygote *wr*₁ *wr*₁ *wr*₂ *wr*₂ of light yellow/no reaction. Therefore, the genotypes WR₁ *wr*₁ *wr*₂ *wr*₂; *wr*₁ *wr*₁ WR₂ *wr*₂ and *wr*₁ *wr*₁ *wr*₁ *wr*₂ all produce light brown/no reaction condition. Thus, the dominant genes WR₁ and WR₂ behave as if, they were recessive to their allele's *wr*₁ and *wr*₂, respectively; whenever they are present in association with the homozygous recessive state at the other locus that is with *wr*₂ *wr*₂ and *wr*₁ *wr*₁, respectively as depicted in Fig. 6.

The wine red/ dark wine red colour variety (Acc. No. 2693) of rice (WR₁WR₁ WR₂ WR₂) was crossed with a light yellow/no reaction variety (IR 36 and IR 64) with the genotype *wr*₁*wr*₁, *wr*₂*wr*₂, the derived F₁ (WR₁ *wr*₁ WR₂ *wr*₂) has produced light wine red colour (intermediate) even in the presence of dominant alleles of both the genes. In the F₂ generation, on an average nine plants out of 16, have at least one dominant allele of both the genes WR₁ and WR₂; these plants develop wine red and dark wine red colour.

One plant has the genotype WR₁ WR₁ *wr*₂ *wr*₂, while another has the genotype *wr*₁ *wr*₁ WR₂ WR₂. These two plants also develop wine red and dark wine red colour; since, they have either WR₁ / WR₂ in the homozygous state, which is able to produce wine red and dark wine

Table 3 Aleurone layer color segregation in F₂ progenies of the crosses IR 36 × Acc.No. 2693 and IR 64 × Acc. No. 2693 for NaOH and KOH tests

Cross	Chemical tests	Class	Wine red/dark wine red	Light yellow/ No reaction	Total	Ratio	χ ²	P value(at 1 degrees of freedom)
IR 36 × Acc. No. 2693	NaOH	Observed	319.00	165.00	484	11:5	1.818	0.178
		Expected	332.75	151.25				
	KOH	Observed	335.00	149.00	484	11:5	0.048	0.825
		Expected	332.75	151.25				
IR 64 × Acc. No. 2693	NaOH	Observed	281.00	139.00	420	11:5	0.665	0.415
		Expected	288.75	131.25				
	KOH	Observed	290.00	130.00	420	11:5	0.017	0.895
		Expected	288.75	131.25				



red colour. Two plants out of 16 are heterozygous for WR_2 and homozygous for wr_1 and vice versa. These four plants do not develop wine red and dark wine red colour; since, the homozygous recessive state of wr_2 and wr_1 , reverses the dominance relationship between WR_1/wr_1 , WR_2/wr_2 , respectively. The remaining one plant is also light yellow/no reaction because it is homozygous recessive for both the genes $wr_1 wr_1 wr_2 wr_2$. Thus, the typical dihybrid 15:1 ratio for duplicate gene action is modified as 11:5 due to the reciprocal dominance modification of wr_1 and wr_2 .

The present findings were reported in case of cotton (Fuchs et al. 1972). Two genes, G_1 and G_2 showing duplicate gene interaction governs the presence of pigment glands on cotton plants. However, the recessive homozygous condition of one gene, say $g_1 g_1$, reverses the dominance relationship at the other locus so that genotype $g_1 g_1 G_2 g_2$ produces the same phenotype as the homozygous double recessive $g_1 g_1 g_2 g_2$. Similarly, homozygous recessive condition of the other gene, $g_2 g_2$ has the same effect on the dominance relationship at the g_1 locus. As a consequence, the genotype $G_1 g_1 g_2 g_2$ produces the same phenotype as the double recessive homozygote $g_1 g_1 g_2 g_2$ produces glandless plants. Therefore, the genotypes $G_1 g_1 g_2 g_2$, $g_1 g_1 G_2 g_2$ and $g_1 g_1 g_2 g_2$ all produce glandless condition; hence, the F₂

phenotypic ratio 11:5 was observed. Similar classification was noticed in the present study with NaOH, which is in congruence with the report in wheat (Mansing 2010), rice (Vanangamudi et al. 1988; Sripunitha and Sivasubramaniam 2014), *urdbean* (Chakrabarthy and Agrawal 1990); cotton (Ponnuswamy et al. 2003; Reddy et al. 2008), safflower (Biradar Patil et al. 2006). In addition, the same findings corroborate with the crops such as sesame (Suhasini 2006) soybean (Chavan 2010), sunflower (Sathisha et al. 2012; Kallihal et al. 2013) and tomato (Qian et al. 2000), respectively. The reasons for various colour attributed when reacted with sodium hydroxide and potassium hydroxide might be due to inherent chemical difference, stability of genetic characters and secondary metabolites present in the seeds (Masuthi et al. 2015; Vanderburg and Vanzwol 1991; Chakrabarthy and Agrawal 1990).

Therefore, these studies are particularly useful, where non-availability of distinct stable morphological markers for identification of increased varieties. These chemical tests along with other parameters like 1000 seed weight, seed size, response to GA_3 , 2,4-D and soluble proteins acts as a descriptors for identification of the rice varieties. Further, these tests could help to develop a rapid varietal identification that may help the breeders and seed inspectors to monitor the quality seed production

(Nethra et al. 2007). Thus, chemical tests are one of the important characters that help in easy identification of varieties for genetic purity.

Conclusions

Based on the response of biochemical tests with 904 F_2 progenies derived from crosses, IR 36 \times Acc. No. 2693 and IR 64 \times Acc. No. 2693 were utilised for delineation of inheritance pattern of aleurone layer colour in rice. In this investigation, it was found that the colour trait in aleurone layer of the F_2 progenies derived from both the crosses were segregated with complementary gene interaction of 9:7 ratio (brown/dark brown: light brown/no reaction), indicating a goodness of fit with observed ratio for standard phenol and modified phenol tests (CuSO_4), respectively. Further, the colour trait in F_2 progenies of both the crosses with NaOH and KOH tests were observed to segregate in 11:5 ratio (wine red/dark wine red: light yellow/ no reaction), wherein typical 15:1 ratio for duplicate gene action is modified as 11:5 due to the reciprocal dominance modification of recessive alleles. Therefore, it is deduced that the colour trait in aleurone layer was found to be controlled by two major genes and their allelic interactions. These findings could be utilised for easy identification of varieties in rice breeding programme, gene expression analysis, cloning and tagging of gene and also to develop the seed keys to precisely define cultivars that would serve an alternative for Grow-out-test.

Methods

Plant Materials

The present work was carried out using IR 36, IR 64 and Acc. No. 2693 (as parents), F_1 and F_2 progenies. The F_1 s were derived from cross between IR 36 \times Acc. No. 2693 and IR 64 \times Acc. No. 2693, respectively during *kharif* 2014 at ICAR-Directorate of Seed Research (ICAR-DSR), Mau, Uttar Pradesh, India. Both the crosses (F_1 seeds) were raised during off-season 2014–15 at regional station, ICAR-DSR, GKVK campus, Bengaluru. Further, F_2 progenies of both the crosses were raised during *kharif* – 2015 at ICAR-DSR, Mau; 484 and 420 F_2 seeds of both crosses IR 36 \times Acc. No. 2693 and IR 64 \times Acc. No. 2693 were used for present investigation to delineate the inheritance pattern of colour trait in aleurone layer using chemical tests, respectively.

Chemical Tests

To know the segregation pattern of colour trait in aleuronic layer, study has been performed using different chemical tests such as standard phenol, modified phenol (CuSO_4), potassium hydroxide and sodium hydroxide tests. These chemical tests are insensitive to environment and serves not only as basis for grouping of

varieties, but also used for genotype identification (Naga Padma et al. 1996) with consistent results.

Phenol Test

For phenol test, seeds were pre-soaked in distilled water for 24 h. Thereafter, they were transferred on two layers of Whatman No.1 filter paper saturated with 4 % phenol solution (Merck, Cat. No. AL8AF58565, Merck Specialities Private Ltd. Mumbai, India). The Petri-dishes were covered and incubated at 25 ± 1 °C and the change in colour of aleurone layer in response to phenol reaction was evaluated after 24 h. The parents, F_1 and F_2 progenies were categorized into five categories as no reaction, light brown, brown, dark brown and black colour (Jaiswal and Agrawal 1995).

Modified Phenol Test-A (CuSO_4)

Modified phenol test was conducted, which is alike to standard phenol test except that seeds were pre-soaked in 0.5% (w/v) copper sulphate (Helix Bio-Science, Cat. No.HBC043212, New Delhi, India) solution for 24 h. Colour reaction was noted after 48 h of incubation and the parents; F_1 and F_2 progenies were categorized into five categories as no reaction, light brown, brown, dark brown and black colour (Jaiswal and Agrawal 1995).

Sodium Hydroxide (NaOH) Test

Parents, F_1 and F_2 seeds were subjected to sodium hydroxide test where, seeds were pre-soaked in 5 % sodium hydroxide solution (Merck, Cat. No. MJ8D580230, Merck Specialities Private Ltd. Mumbai, India) and kept at room temperature for one hour and change in colour of the solution was observed. Chakrabarty et al. 1989, categorized the reaction into light yellow and wine red based on the intensity of change in colour solution.

Potassium Hydroxide (KOH) Test

Seeds of parents, F_1 and F_2 progenies were pre-soaked in 5 % potassium hydroxide solution (Helix Bio-Science, Cat. No. A3641, New Delhi, India) and kept at room temperature for 4 h and a change in colour of the solution was observed. Based on the intensity of reaction, the populations were categorized into various groups viz., light yellow, dark yellow, light wine red, dark wine red (Vanangamudi et al. 1988).

Chi-square (χ^2) Goodness of Fit Test

Chi-square (χ^2) goodness of fit test was performed to analyse phenotypic segregation between observed values (O) to the expected values (E) for F_2 population data, using $\chi^2 = \sum(O - E)^2 / E$.

Abbreviations

Acc. No.: Accession number; B: Brown; b₁b₁ d₂d₂: Light brown/no reaction; B₁B₁: Brown; D₂D₂: Dark brown; DB: Dark brown; DWR: Dark wine red; ICAR: Indian Council of Agricultural Research; KOH: Potassium hydroxide;

LB: Light brown; LWR: Light wine red; LY: Light yellow; NaOH: Sodium hydroxide; NR: No reaction; WR: Wine red; wr₁ wr₁ wr₂ wr₂: Light yellow/ no reaction; WR₁ WR₁: Wine red; WR₂ WR₂: Dark wine red

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Availability of Data and Materials

The datasets for supporting the conclusions of this article have been provided in the research article.

Authors' Contributions

Conceived, Designed & Performed the Experiments: CS. Formal analysis: CS; KBN. Contributing reagents: KVS; KUB; GS. Writing-original draft: CS; KVS. Writing-review & editing: CS; GP; SPJK. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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