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).
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 FeSO₄ (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, F₁ and F₂ progenies derived from the crosses IR 36 × Acc. No. 2693 and IR 64 × Acc. No. 2693 by chemical tests.

Results and Discussion
Aleurone Layer Colouration in Parents IR 36 × Acc. No. 2693 and IR 64 × 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 F₁ to different chemical tests

| Chemical tests | IR 36 | IR 64 | Acc. No. 2693 | F₁  |
|----------------|-------|-------|---------------|-----|
| NaOH           | LY    | LY    | WR            | LWR |
| KOH            | LY    | LY    | DWR           | LWR |
| Phenol         | B     | DB    | LB/NC         | DB  |
| Modified Phenol| B     | DB    | LB/NC         | DB  |

**Aleurone Layer Colour Inheritance in F₁ Plant of IR 36 × Acc. No. 2693 and IR 64 × Acc. No. 2693**

The F₁s were derived from the crosses IR 36 × Acc. No. 2693 and IR64 × Acc. No. 2693, respectively (Table 1). The freshly harvested F₁ seeds (derived from cross IR 36 × 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 F₁ seeds derived from the cross IR 64 × 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 × Acc. No. 2693 and the similar colour pattern was recorded in case of IR 36 × Acc. No. 2693 that implies that the observed colour is dominant over light yellow.

Aleurone Layer Colour Inheritance in F₂ Progenies Derived from IR 36 × Acc. No. 2693 and IR 64 × Acc. No. 2693 with Standard Phenol and Modified Phenol (copper sulphate) Tests

Out of 484 F₂ progenies of cross IR 36 × Acc. No. 2693; 273 and 255 F₂ progenies were brown/dark brown in colour, whereas 211 and 229 F₂ progenies showed light brown/no colour with standard phenol and modified phenol tests, respectively. Further, 420 F₂ progenies derived from cross IR 64 × Acc. No. 2693 were evaluated. It is observed that 251 and 246 F₂ progenies were brown/dark brown in colour; 169 and 174 F₂ 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 F₂ progenies were segregated with complementary gene interaction with a ratio of 9:7 indicates a goodness of fit with observed ratio (Table 2). The F₂ colour segregation was consistent with the complementary gene interaction (9:7) for all F₂ progenies of both the crosses. Therefore, two major genes and their alleles
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, B₁ and D₂ e.g. B₁B₁ D₂D₂ (Fig. 4). When either B₁ (e.g. b₁b₁ D₂D₂) or D₂ (B₁B₁ d₂d₂) or both the genes (e.g. b₁b₁ d₂d₂) 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 (B₁B₁ D₂D₂) was crossed to a light brown/no reaction variety (Acc. No 2693) with the genotype b₁b₁ d₂d₂ showed dark brown colour in the F₁ (B₁b₁ D₂d₂) progeny. In the F₂ progenies, on an average nine progenies had one dominant allele of both the genes B₁ and D₂. These plants, therefore has brown/dark brown colour. Three, out of sixteen F₂ progenies, shall have dominant B₁ but had homozygous recessive genotype b₁b₁; three others shall have dominant D₂ but had homozygous recessive genotype d₂d₂, while one plant had both the genes in homozygous recessive genotype b₁b₁ d₂d₂. 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
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. F2 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

| Cross       | Chemical tests | Class       | Brown/dark brown colour | Light brown/ No reaction | Total | Ratio | χ2     | P value (at 1 degrees of freedom) |
|-------------|----------------|-------------|-------------------------|--------------------------|-------|-------|-------|----------------------------------|
| IR 36 × 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 × 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.

| Gametes | B$_1$D$_2$ | b$_1$d$_2$ | B$_1$D$_2$ | b$_1$d$_2$ |
|---------|------------|------------|------------|------------|
| B$_1$B$_1$D$_2$D$_2$ | Brown/Dark Brown | Brown/Dark Brown | Brown/Dark Brown | Brown/Dark Brown |
| B$_1$B$_1$d$_2$d$_2$ | Brown/Dark Brown | Light brown/No reaction | Brown/Dark Brown | Light brown/No reaction |
| b$_1$b$_1$D$_2$D$_2$ | Brown/Dark Brown | Light brown/No reaction | Light brown/No reaction | Light brown/No reaction |
| b$_1$b$_1$d$_2$d$_2$ | Brown/Dark Brown | Light brown/No reaction | Light brown/No reaction | Light brown/No reaction |

**Phenotypic ratio:** 09 Brown/dark brown: 07 Light brown/No reaction

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 wr₁ wr₁ reverses the dominance relationship at the other locus; hence, the genotype wr₁ wr₁ WR₂ wr₂ produces the same phenotype as the homozygous double recessive wr₁ wr₁, wr₂ wr₂ of light yellow/no reaction. Similarly, homozygous recessive condition of the other gene, wr₂ wr₂ has the same effect on the dominance relationship at the wr₁ locus. As a consequence, the genotype WR₁ wr₁ wr₂ wr₂ produces the same phenotype as the double recessive homozygote wr₁ wr₁, wr₂ wr₂ of light yellow/no reaction. Therefore, the genotypes WR₁ wr₁ wr₂ wr₂; wr₁ wr₂ WR₂ wr₂ all produce light brown/no reaction condition. Thus, the dominant genes WR₁ and WR₂ behave as if, they were recessive to their allele's wr₁ and wr₂ respectively; whenever they are present in association with the homozygous recessive state at the other locus that is with wr₂ wr₂ and wr₁ wr₁, 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 wr₁ wr₁, wr₂ wr₂, the derived F₁ (WR₁ wr₁ WR₂ wr₂) 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 WR₁ and WR₂; these plants develop wine red and dark wine red colour.

One plant has the genotype WR₁ WR₁ wr₂ wr₂, while another has the genotype wr₁ wr₁ 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 red colour.

| Cross          | Chemical tests | Class F₂ | Wine red/dark wine red | Light yellow/ No reaction | Total Ratio | χ²  | 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                    |             |     |                                 |      |

**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.
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 \(WR_1/ wr_1\) and \(WR_2 / wr_2\), 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 \(G_1\) \(g_1\) \(G_2\) \(G_2\), \(G_1\) \(g_1\) \(g_2\) \(G_2\) \(G_2\) and \(g_1\) \(g_1\) \(G_2\) \(G_2\) all produce glandless condition; hence, the \(F_2\) 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), \(urdbean\) (Chakrabarth 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; Kallilhal 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; Chakrabarth 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.

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**Diagram:**

![Diagram of duplicate gene action](image)

**Table:**

| Parents | F\(_1\) | F\(_2\) | G\(_1\) | G\(_2\) |
|---------|--------|--------|--------|--------|
| \(WR_1\) \(WR_2\) | \(WR_1\) \(WR_2\) | \(WR_1\) \(WR_2\) | \(WR_1\) \(WR_2\) | \(WR_1\) \(WR_2\) |
| \(wr_1\) \(wr_2\) | \(WR_1\) \(wr_2\) | \(WR_1\) \(wr_2\) | \(WR_1\) \(wr_2\) | \(WR_1\) \(wr_2\) |

**Fig. 6** Dominance modification of duplicate genes leading to a 11:5 phenotypic ratio in the \(F_2\) progenies for the presence of wine red / dark wine red and light yellow/no reaction colouration of aleurone layer of rice seed with NaOH and KOH tests.
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 F₂ progenies derived from crosses, IR 36 × Acc. No. 2693 and IR 64 × 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 F₂ 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 (CuSO₄), respectively. Further, the colour trait in F₂ 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), F₁ and F₂ progenies. The F₁s were derived from cross between IR 36 × Acc. No. 2693 and IR 64 × Acc. No. 2693, respectively during kharif 2014 at ICAR-Directorate of Seed Research (ICAR-DSR), Mau, Uttar Pradesh, India. Both the crosses (F₁ seeds) were raised during off-season 2014–15 at regional station, ICAR-DSR, GKVK campus, Bengaluru. Further, F₂ progenies of both the crosses were raised during kharif – 2015 at ICAR-DSR, Mau; 484 and 420 F₂ seeds of both crosses IR 36 × Acc. No. 2693 and IR 64 × 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 (CuSO₄), 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, F₁ and F₂ 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 (CuSO₄)**

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.HBO43212, New Delhi, India) solution for 24 h. Colour reaction was noted after 48 h of incubation and the parents; F₁ and F₂ 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, F₁ and F₂ 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, F₁ and F₂ 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 (χ²) Goodness of Fit Test**

Chi-square (χ²) goodness of fit test was performed to analyse phenotypic segregation between observed values (O) to the expected values (E) for F₂ 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;
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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; KVS. 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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