

Histochemical localization of storage components in caryopsis of rice (*Oryza sativa* L.)

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The pattern of distribution of major storage components of IR50 rice caryopsis was investigated and compared with several other cultivars and 17 wild species of *Oryza*. Starch occurs abundantly in the pericarp during early stages of grain filling. Starch begins to accumulate within the endosperm about 5 days after fertilization (DAF), and by 14 DAF starch in the pericarp is completely depleted, presumably transported as sucrose into the endosperm. Lipids are stored mostly in the aleurone cells. Proteins occur as aleurone grains and as discrete particles of three different sizes in the endosperm. 80% of protein occurs in the subaleurone layers. The dead endosperm cells contain remnants of nuclear material. The rice embryo is rich in lipids and proteins and accumulates smaller amounts of starch. The aleurone and embryo also store phytin granules which contain abundant calcium, potassium and iron. The above pattern of distribution of major storage reserves is remarkably alike in all cultivars and species examined. The same pattern is also observed in grains stored for 12 years.

HISTOCHEMISTRY and fluorescence microscopy are major tools in the localization of trace quantities of substances present in plant and animal tissues¹⁻⁵. Histochemical techniques and ultrastructural studies have been employed to characterize rice embryo from fertilization to maturity, and understand the deposition of storage proteins in developing caryopsis, and gene expression in transgenic rice plants⁶⁻⁸. Histochemistry of other cereal grains such as wheat and barley have been described by Fulcher⁹. We initiated histochemical studies of developing, mature and germinating rice grains in order to localize various storage components such as starch, proteins, lipids, calcium, iron and potassium, and to understand their entry into the caryopsis during grain filling in rice grain. The pattern of distribution of major storage components is described in this report.

This histochemical investigation was confined to a light microscopic analysis of free-hand sections, and wax and Spurr plastic-embedded thin sections. An indica rice, *Oryza sativa* cv IR50 was the central focus of

study. However, several other cultivars and species obtained from the International Rice Research Institute (IRRI), Philippines and local sources were examined to compare and confirm the observations made on IR50. These include: cv. Ponni, IR20, and ADT36 (from Tamil Nadu Agricultural University, Coimbatore), J13 (from J-Farm, Kelambakkam, Tamil Nadu) and *Oryza alta* Swallen, *O. australiensis* Domin, *O. barthii* A. Chev., *O. brachyantha* A. Chev & Roehr., *O. eichingeri* A. Peter, *O. glaberrima* Steud., *O. grandiglumis* Proehl., *O. granulata* Nees. et Arn., *O. latifolia* Desv., *O. longiglumis* Jansen, *O. longistaminata* A. Chev. et Roehr., *O. minuta* J. S. ex C. B. Presl, *O. nivara* Shastri, *O. officinalis* Wall. ex Watt, *O. punctata* Kotschy, *O. ridleyi* Hook. f. and *O. rufipogon* Griff. (from IRRI). Unless otherwise specified, observation and figures refer to IR50.

A number of sensitive reagents and procedures are now available for the detection of storage substances in cereal grains¹⁻¹¹. Specimens were stained with a variety of bright-field dyes and fluorochromes as described in the literature^{5,9}. Microchemical tests and selected enzyme histochemical procedures were also carried out. Specimens were examined and photographed with a Nikon Microphot-FXA research microscope. Specimens were examined in bright-field, dark-field, phase-contrast, Nomarski-DIC, polarized light and fluorescence modes.

At the time of anthesis all cells of the ovary wall contain starch. The amount of starch in the pericarp reaches maximum level about 5 DAF. Thereafter starch decreases in the pericarp as the endosperm cells begin to accumulate starch (Figure 1 a-g).

The storage reserves in mature caryopsis are partitioned into two major compartments (Figure 2 a). One is the triploid endosperm and the other is the embryo. The endosperm consists of the aleurone layer of living cells, and the dead cells of starchy endosperm. The embryo consists of living cells organized into tissues/organs such as scutellum, coleoptile, radicle, coleorhiza, ventral and lateral scales and epiblast.

The cells of the aleurone layer contain numerous lipid droplets and aleurone grains. The latter store protein and phytate in granules (Figure 2 c-f). The lipid in the aleurone cells can be easily detected with Sudan dyes and Nile Blue A. The phytin granules consist of myoinositol hexaphosphate and associated cations. Alizarin red can be used both as a bright-field reagent and a fluorochrome to detect calcium associated with phytin granules (Figure 3 b). The sodium cobaltinitrite reagent is a powerful tool for localization of K⁺ associated with phytin (Figure 3 c). In most plant tissues, K⁺ is a highly mobile ion and the staining procedure has to be stringently controlled. Rice caryopsis is one of the easiest plant materials to demonstrate the presence of K⁺. The Prussian blue technique and the Turnbull's method

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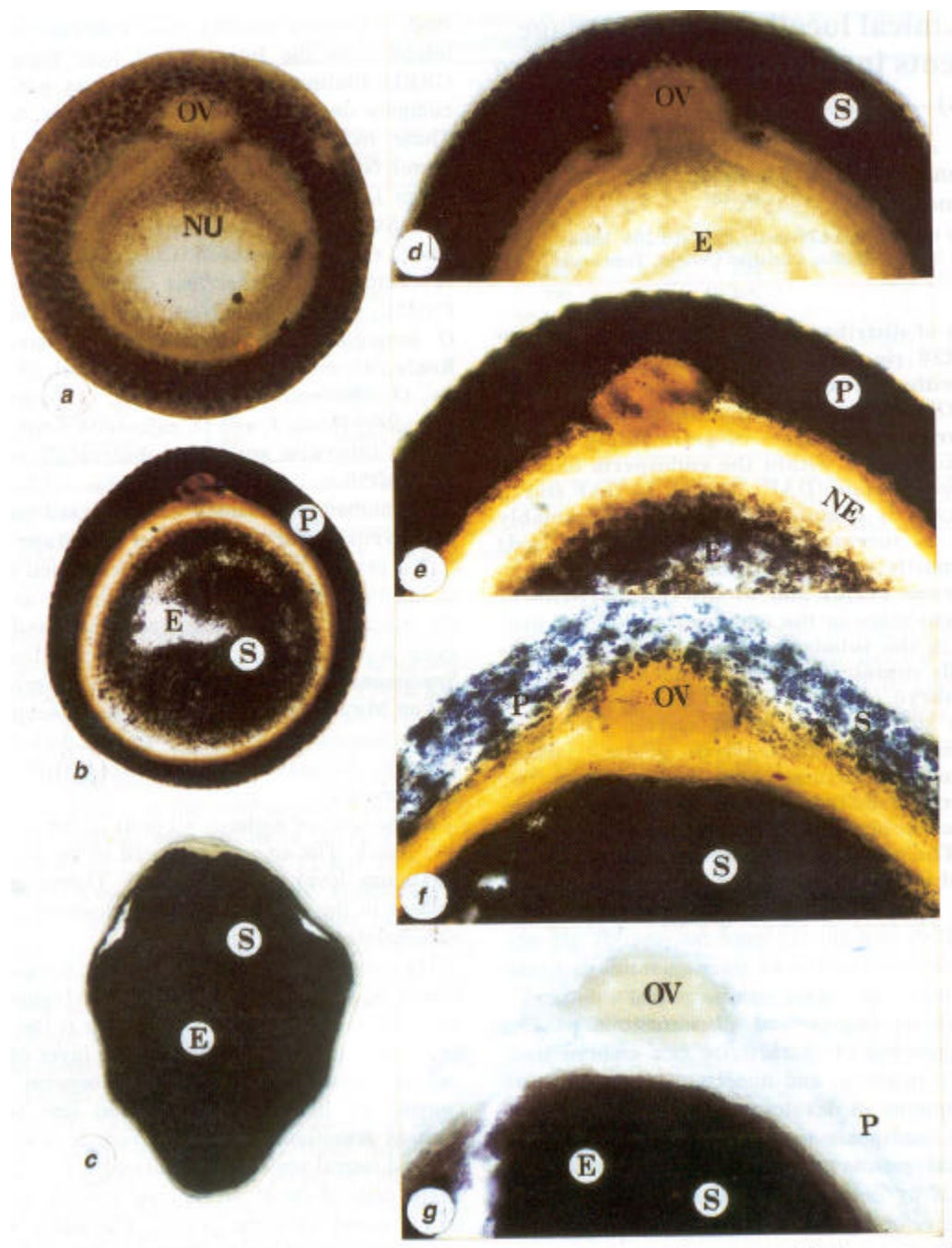


Figure 1 a-g. Transverse sections of ovary and caryopsis stained with I_2KI at various stages of development. *a*, Pre-anthesis ovary. Starch is present in the pericarp and nucellus. $\times 125$; *b*, Starch in the pericarp reaches maximum level and endosperm begins to accumulate starch, five DAF, $\times 125$; *c*, Mature caryopsis about 30 DAF. Endosperm cells are completely filled with starch, $\times 50$; *d*, Three DAF starch is present only in the pericarp, $\times 250$; *e*, Five DAF the endosperm accumulates starch. Nucellus between endosperm and pericarp is devoid of starch, $\times 250$; *f*, Starch in the pericarp begins to disappear 10 DAF, $\times 250$; *g*, Starch in the pericarp has completely disappeared by 14 DAF, $\times 250$. E, endosperm; NU, nucellus; OV, ovular vascular bundle; P, pericarp; S, starch.

reveal the presence of iron in the aleurone cells (Figure 3 *d*).

The scutellum, a major storage tissue, is similar in many ways to the aleurone tissue, although the former is diploid and the latter triploid. Scutellar cells store large amounts of protein, phytin and lipids. Acriflavin

HCl, toluidine blue O, alizarin red and other reagents reveal the presence of phytin and protein in the scutellum (Figure 3 *a, f*). Calcium and iron are also present in scutellar cells and can be revealed by staining reactions. During germination, within 12 h of imbibition, the protein bodies in the scutellum swell (Figure 3 *f*). The

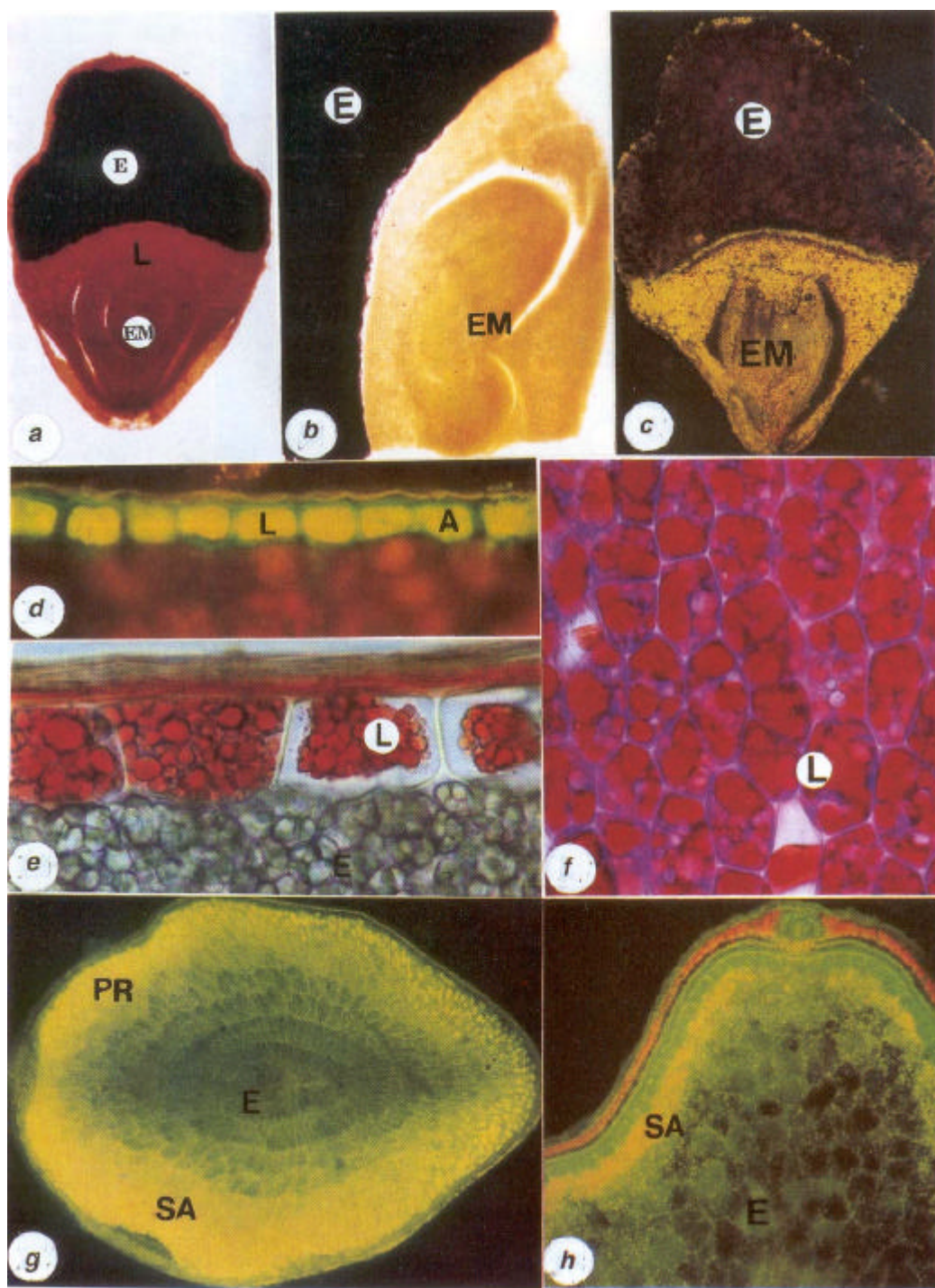


Figure 2 a-h. *a*, Simultaneous staining with I₂KI and Sudan IV reveals the presence of starch in the endosperm (E) and lipids (L) in the aleurone layer and the embryo (EM) 1 day after imbibition, $\times 50$; *b*, Free-hand longitudinal section of a caryopsis 1 day after imbibition. I₂KI reveals the presence of starch in the endosperm (E) and near absence of starch in the embryo (EM). Close observation shows that starch is beginning to accumulate in the scutellum, $\times 500$; *c*, *d*, Presence of lipids (L) in the aleurone (A) and in the embryo (EM) can be detected by staining with Nile Blue A and excited with blue light. *c*, $\times 60$; *d*, $\times 125$; *e*, Localization of lipids (L) with Sudan IV in mature grain. Lipids in the aleurone and the cuticle over the nucellar epidermis are stained red. Starch is not stained but can be easily seen, $\times 500$; *f*, Aleurone peel from caryopsis one week after germination still showing the presence of lipids (L) and protein. Simultaneously stained with Sudan IV and Coomassie brilliant blue, $\times 450$; *g*, Localization of proteins (PR) in subaleurone (SA) region with barbituric acid; Blue excitation, $\times 62$; *h*, Localization of storage reserves in wild species of rice, *Oryza punctata*. Proteins stained with barbituric acid, Blue excitation, Chlorophyll in pericarp is autofluorescing in red, $\times 125$.

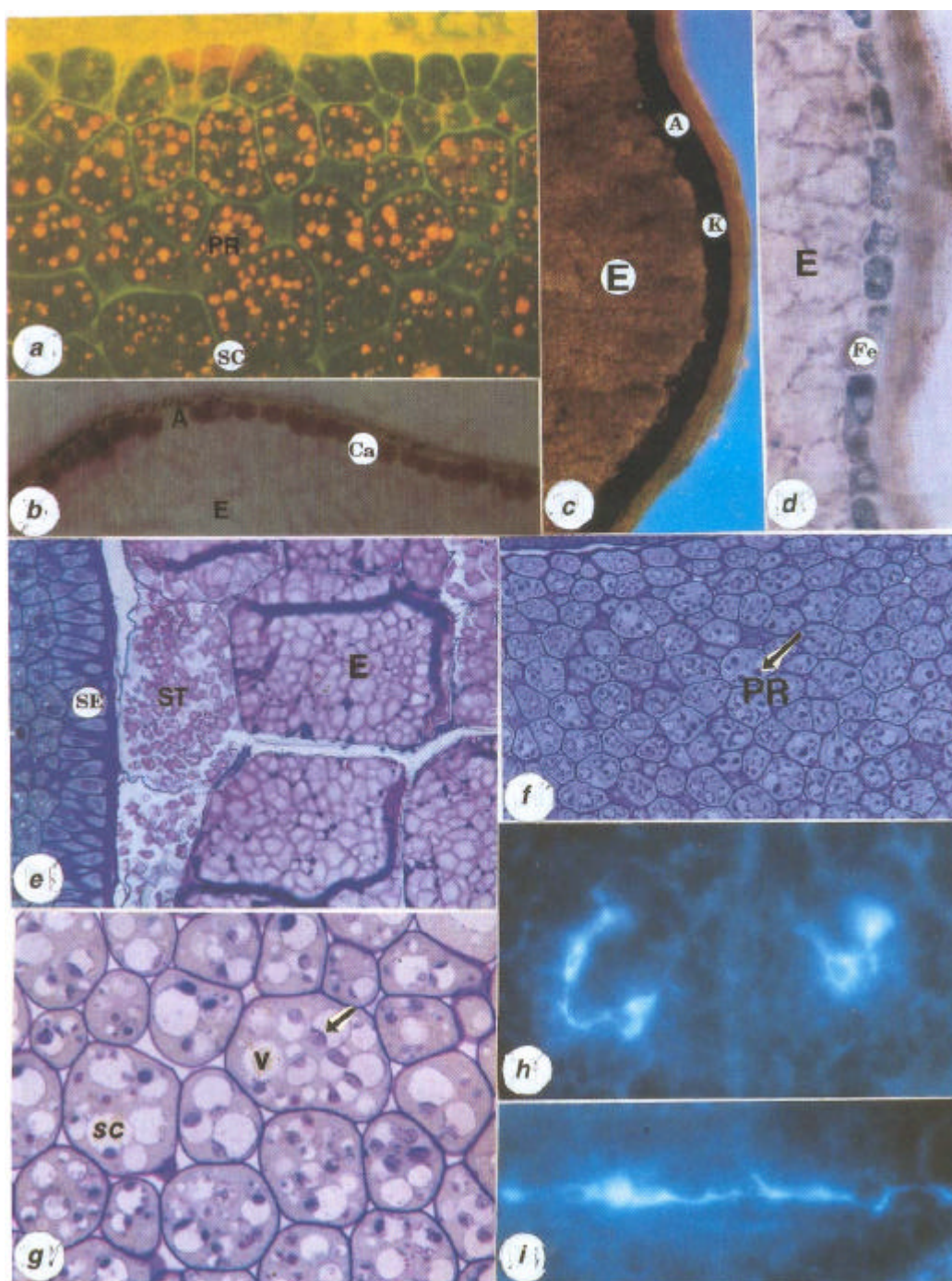


Figure 3 a-i. *a*, Thin plastic sections of scutellum (SC) from ungerminated caryopsis, stained with acriflavine HCl. Phytin granules appear yellow within the protein bodies (PB), Blue excitation, $\times 950$; *b*, Phytin stained with alizarin red in the aleurone (A) layer 3 days after imbibition. The red colour has developed due to reaction with calcium (Ca) associated with phytin granules, $\times 200$; *c*, Localization of potassium (K) in the aleurone (A). Sections of caryopsis 3 days after imbibition were incubated in sodium cobaltinitrite reagent and mounted in ammonium sulfide. Abundant potassium is present in the phytin granules in the aleurone, $\times 200$; *d*, Localization of iron (Fe) in the protein bodies of aleurone cells; Turnbull's technique, $\times 200$; *e*, Starch (ST) is being degraded close to the scutellar epithelium (SE). The thin wall of the starchy endosperm (E) can be seen, $\times 450$; *f*, Thin plastic section stained with toluidine blue O. Protein bodies (PB) swell (arrow) within the scutellum one day after imbibition, $\times 450$; *g*, Various stages of protein body vacuole formation in scutellum (SC). Lenticular bodies (arrow) are proteins getting digested at the periphery of the vacuolar (V) membrane, $\times 950$; *h*, *i*, DNA in the endosperm cells of mature caryopsis stained with DAPI and excited with UV; *i*, DNA is stretched lengthwise in the direction of elongation of the endosperm cell, both $\times 1250$.

protein bodies enlarge and turn into vacuoles as their contents are progressively digested (Figure 3g). The vacuoles then fuse together and form one large vacuole per cell. This process can be observed not only in the scutellar parenchyma cells but also in all embryonal organs. The scutellar epithelial cells contain small protein bodies. The epithelium secretes enzymes into the starchy endosperm to digest the macromolecules, and reabsorbs low molecular weight substances to be transported to the embryo (Figure 3e). Ungerminated embryo does not contain any starch (Figure 2b). During germination, within 12 h of imbibition of water, starch deposition begins in the embryo and continues for several days.

The endosperm is primarily a storehouse of starch (Figures 1c, 2a, b, e, 3e). However, each dead endosperm cell retains the remnants of a triploid nucleus. The DNA-specific fluorochrome DAPI reveals the nuclear material in the endosperm cells (Figure 3h, i). Obviously the starchy endosperm can also make a significant contribution of nitrogen bases to the germinating embryo and human nutrition. The starchy endosperm also contains proteins (Figure 2g, h). Most of the proteins occur in the subaleurone layers (Figure 2g, h). Fluorochromes specific for proteins (8-anilino-1-naphthalenesulfonic acid and dansylchloride) as well as non-specific fluorochromes such as barbituric acid, aniline blue, acridine orange and calcofluor white M2R can be used to detect proteins in the endosperm. Proteins are stored in discrete structures known as protein bodies (PB). The spherical PB I stores prolamins and the slightly larger, irregularly shaped PB II stores glutelins and globulins¹².

A survey of the wild species of rice and grains stored for more than 12 years reveals the same pattern of distribution of all storage substances, including the nuclear remains and minerals detected in IR50.

This histochemical survey of rice caryopsis provides a broad framework of reference for the understanding of time and place of deposition of storage material within the caryopsis, as well as its removal during seed germination. The emerging picture of the rice grain will help biotechnologists to sharpen their focus on spatial and temporal events for genetic manipulation to improve grain quality. Genetic manipulation altering the quality of rice lipids should focus on the aleurone as well as the embryo since these are the major tissues that store lipids. The subaleurone layers should be the major target for controlling the expression of protein genes for quality and quantity enhancement in transgenic plants. The deeper layers of the endosperm could be manipulated to promote deposition of more proteins so that the total protein content of the grain can be increased. Enhancement of carotene content may be attempted by the manipulation of cross cells and the embryonal tissue which possess plastids/proplastids. Structural and histochemical investigations will continue to complement the efforts of researchers interested in all aspects of improvement of rice.

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A simple and rapid molecular method for distinguishing between races of *Fusarium oxysporum* f.sp. *ciceris* from India

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***EcoRI* restriction pattern of the nuclear ribosomal DNA from four isolates of *Fusarium oxysporum* f.sp. *ciceris* (FOC) representing four races prevalent in India indicated that the races could be grouped into three distinct groups; races 1 and 4 representing one group and race 2 and race 3, the other two. The restriction pattern indicated presence of three *EcoRI* sites on the nuclear rDNA of this species, one each on the 5.8S and the 25S regions, conserved to all, and the other one, i.e. the variable site, on the intergenic spacer (IGS) region of the nuclear rDNA. The same was confirmed by PCR-amplification of the IGS region followed by digestion with *EcoRI* and a set of other enzymes. It is suggested that amplification of the IGS region and digestion with restriction enzymes could be used to study polymorphism in FOC, and to rapidly identify the races existing in India. We also propose that out of the four types of races described from India, races 1 and 4 are the same.**

FUSARIUM oxysporum Schl. Fr. f.sp. *ciceris* (Padwick) Mauto & Sato, which incites wilt, is one of the major

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