BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF RICE (*Oryza sativa* L.) ALEURONE PROTEIN

Thesis submitted in part fulfilment of the requirements for the degree of
MASTER OF SCIENCE IN BIOTECHNOLOGY
to.the Tamil Nadu Agricultural University,
Coimbatore – 641 003.

By

ANAND MOHAN PRASAD, B.Sc. (Ag.)
I.D.No.: 98-607-001

DEPARTMENT OF BIOTECHNOLOGY
CENTRE FOR PLANT MOLECULAR BIOLOGY
TAMIL NADU AGRICULTURAL UNIVERSITY
COIMBATORE – 641 003

2000

CERTIFICATE

This is to certify that the thesis entitled "**BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF RICE (*Oryza sativa* L.) ALEURONE PROTEIN**" submitted in part fulfilment of the requirements for the award of the degree of **Master of Science** in **Biotechnology** to the Tamil Nadu Agricultural University, Coimbatore, is a record of **bonafide** research work carried out by **Mr. ANAND MOHAN PRASAD** under my supervision and guidance and that no part of this thesis has been submitted for the award for any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular Journal or Magazine.

Place : Coimbatore – 3
Date : 28/7/2011

Kapildeo Narayan Singh
(Dr. KAPILDEO N. SINGH)
Chairman

Approved by Kapildeo Narayan Singh
Chairman : **(Dr. KAPILDEO N. SINGH)**

B Thayumanavan
Members : **(Dr. B. THAYUMANAVAN)**

R. Velazhahan
(Dr. R. VELAZHAHAN)

External Examiner [illegible]
Date : 29/12/2011

ACKNOWLEDGMENT

I wish to express sincere thanks and gratitude to my Chairman **Dr. Kapildeo N. Singh,** Assistant Professor, Centre for Plant Molecular Biology, Tamil Nadu, Agricultural University, Coimbatore for his active and valuable guidance, keen interest, constant encouragement, inspired suggestions and constructive criticism through out the course of study and preparation of the thesis.

I also wish to express my deep sense of indebtedness to the members of the advisory committee **Dr.B.Thayumanavan,** Professor and Head, Department of Biochemistry, **Dr.R.Velazhahan**, Assistant Professor, Department of Plant Pathology, for their valuable suggestions and guidance during this study period.

I am delighted to acknowledge **Dr.S.Sadasivam,** Director, Centre for Plant Molecular Biology, for his kind help and encouragement to complete the thesis.

I am also extending sincere thanks to **Dr.A.Manickam,** Professor and Head, Department of Biotechnology, **Dr.P.Balasubramanian,** Professor, Centre for Plant Molecular Biology, **Dr.P.Shanmugasundaram**, Professor, Centre for Plant Molecular Biology, for providing laboratory facilities and their suggestions during the course work.

I wish to express sincere thanks to all the teachers, who inspired, guided and encouraged to carry out research. Nevertheless, the help and advice of *Dr.R.Balasaraswathi, Dr.R.Chandra Babu, Dr.G.Gopalaswamy, Dr.P.Nagarajan, Dr.V.Udayasuriyan, Dr.S.Sukumar, Dr.S.Krishnaveni Dr.D.Sudhakar, Dr.K.Parvathi* and *Dr. R.Gnanam* deserve special mention.

My sincere thanks are due to Dr.J.A.J.Raja, Dr.Selvi Subramanian, Mr.Mahesh Babu and Mr.L.Arul for their friendly and timely help in all aspects in the completion of study.

I wish to thank my classmates Anupam, Bala, Giri, Indu, Bhoopathi, Rakhi, Sudarshan, Lenin, Savithri, Suma, Suresh, Shantanu, Phap, my Junior and Senior friends for their kind co-operation.

I also thank to Dr.T.Parimelazhagan, V.Venkateswaran and others for inspiring and keeping days in laboratory lively.

I am highly thankful to Indian Council of Agricultural Research for awarding me the Junior Research Fellowship.

I am thankful to Mr. Santha Kumar of M/s. Sowmiya Communications, TNAU Campus, Coimbatore, for meticulously typing out the thesis.

Last, but not the least, I wish to express my heartful thanks to my parents, brother, sister and relatives for their love, affection and support.

Anand Mohan Prasad

ANAND MOHAN PRASAD

Abstract

ABSTRACT

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF RICE (*Oryza sativa* L.) ALEURONE PROTEIN

By

ANAND MOHAN PRASAD

DEGREE : **Master of Science (Biotechnology)**

CHAIRMAN : **Dr. KAPILDEO N. SINGH**
Assistant Professor
Department of Biotechnology
Centre for Plant Molecular Biology
Tamil Nadu Agricultural University
Coimbatore – 641 003.

2000

Rice (*Oryza sativa* L.) is an important staple food crop for most of the population in Asian countries. It is not very rich in protein (7 to 9 %), further the protein rich aleurone layer always gets removed during milling.

The goal of the study is to translocate such proteins inside the seed in a way to protect the nutritive loss during milling. With this aim the present work was undertaken to identify and characterize the rice aleurone (surface) specific protein and its cDNA.

The rice aleurone (surface) layer protein was extracted and electrophoresed on SDS-PAGE (12 %) and compared with the marker. Several distinct size polypeptide bands were observed, among them

30 kDa and 15 kDa were unique. An antiserum was raised against purified preparation of the putative aleurone specific polypeptide (30 $\pm$ 2 kDa).

Western blot analysis of rice aleurone (surface) layer protein using anti 30 $\pm$ 2 kDa aleurone specific protein antiserum detected a 30 kDa protein as expected. In addition, a 15 kDa polypeptide was also detected due to cross contamination. Dot blot analysis of proteins isolated at various stages of growth using the above antibody proved the stage specific expression of the rice aleurone (surface) layer protein. This aleurone specific protein expression was found higher at dough and maturity stages of the plant growth.

A rice seed cDNA library was immunoscreened to isolate the rice aleurone (surface) protein specific cDNA. One putative cDNA clone was identified containing an insert of 1.2 $\pm$ 0.2 kb, which was confirmed by Southern hybridization experiment. Authenticity of this putative clone is yet to be determined.

CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

ABBREVIATIONS

µg	:	Microgram
µl	:	Microlitre
2-ME	:	2-Mercapto ethanol
AP	:	Alkaline phosphatase
APS	:	Ammonium per sulphate
AR	:	Analytical reagent
BSA	:	Bovine serum albumin
cDNA	:	Complementary DNA
CFA	:	Complete Freund's adjuvant
CNBr-	:	Cyanogen bromide
DAS	:	Days after sowing
dCTP	:	Deoxycytidine triphosphate
DEAE	:	Diethyl amino ethyl
dNTP	:	Deoxyribonucleotide triphosphate
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme Linked Immuno-sorbent Assay
HCl	:	Hydrochloric acid
HRP	:	Horse radish peroxidase
IFA	:	Incomplete Freund's adjuvant
IgG	:	Immunoglobulin G
IPTG	:	Isopropyl thiogalactose
IRRI	:	International Rice Research Institute
kbp	:	Kilobase pair
KCl	:	Potassium chloride
kDa	:	Kilodalton

LB broth	:	Luria-Bertani broth
$MgCl_2$	:	Magnesium chloride
mM	:	Milli molar
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
°C	:	Degree centigrade
PB	:	Phosphate buffer
PBS	:	Phosphate buffered saline
PBST	:	Phosphate buffered saline Tween 20
PCR	:	Polymerase chain reaction
PMSF	:	Phenylmethyl sulfonyl fluoride
PVP	:	Polyvinyl pyrollidine
RP-HPLC	:	Reverse phase - High performance liquid chromatography.
rpm	:	Rotation per minute
SDS-PAGE	:	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SSC	:	Saline sodium citrate
TBS	:	Tris buffered saline
TBST	:	Tris buffered saline Tween 20
TCA	:	Trichloro acetic acid
TE	:	Tris EDTA
TEMED	:	N, N, N', N', Tetramethyl ethylene diamine

Introduction

Chapter - I

INTRODUCTION

Rice (*Oryza sativa* L.) has one of the most nutritious protein content among the cereals (Juliano *et al.,* 1972). However, the protein content of milled or polished rice is relatively low about 7 per cent at 14 per cent moisture. Next to starch, protein is the most abundant constituent of rice endosperm. In Asian diets, milled rice provides 40 to 80 per cent of the calorie and at least 40 per cent of the protein. Brown or dehulled rice usually contains about 8 per cent protein. Its bran, which includes the aleurone layers and the germ (scutellum, plumule & radicals) contains more proteins than the starchy endosperm (Coffman and Juliano, 1979). Hence, the proportion of protein lost in milling is higher than the proportion of weight lost. In tropical places, milling is unavoidable since, the oil-rich aleurone layer turns rancid upon storage.

Rice, like other cereals is deficient in some of the essential amino acids (Johnson and Lay, 1974). The most important essential amino acid deficiency in cereals, including rice, is lysine, although threonine and tryptophan are also deficient in some species. In addition, the protein content in cereals is quite low when compared to legumes. Among the cereals, rice has the lowest amount of total protein i.e. 7.5 per cent (Johnson and Lay, 1974). Thus rice presents two major nutritional problems imbalance of essential amino acids and low protein content, particularly in the milled grain. (Coffman and Juliano, 1979).

For decades, plant breeders have put considerable effort in developing rice varieties that are more productive and high nutritious than the one we

now have but the improvement have been modest. Since 1966, the International Rice Research Institute (IRRI) has been involved in improving the protein quality of rice. Among the 10,500 cultivars that were screened, no high lysine variety was identified (Khush and Juliano, 1984). High protein lines have also been sought, but none of them has commercial value. Similarly, no high protein rice variety has been released by any of the national programmes (Coffman and Juliano, 1979; Monyo *et al.,* 1979; Ratho *et al.,* 1984; Kihupi, 1984 and Kvassay - Sajo, 1984).

Beachell *et al.* (1972) suggested that protein content in rice variety IR8 could be increased from 7.5 per cent to 9.4 per cent without a reduction in yield. However, the task is problematic. High grain protein content in many crops tends to be accompanied with depressed grain yield as well as with an undesirable shift in amino acid balance, particularly in the cereals (Johnson and Lay, 1974; Kvassay - Sajo, 1984; Shewrg *et al.,* 1981). When high protein lines of rice were analyzed for amino acid composition, lysine and tryptophan were decreased as protein content increased (Johnson and Lay, 1974).

Thus, all classical plant breeding approaches have been rather ineffective in improving the nutritional properties of rice. With the advancement of recombinant DNA technology there is hope to bypass the dead lock encountered in breeding programmes especially in this direction. There are two principal approaches of applying recombinant DNA technologies to solve the nutritional deficiencies inherent in rice. First, one could isolate the storage protein genes, modify them *in vitro* either by replacing existing codon with one encoding more nutritionally desirable amino acids or by inserting a DNA fragment enriched in codons for amino

acids of interest. A second approach is to enhance or modify the gene expression of storage proteins in order to increase synthesis of proteins having desirable amino acid composition (Shewrg *et al.*, 1981 and Barton and Brill, 1983).

Since storage proteins are synthesized only in endosperm cells their expression and regulation are strictly controlled during grain development (Yamagata *et al.*, 1982). Using recombinant DNA technology, it should be possible to understand the gene expression of storage proteins and biosynthesis of these polypeptides in the cells. This provides us indispensable knowledge for manipulating genes of interest in order to improve the nutritional quality of proteins. Recently, Ye *et al.* (2000) have introduced the complete β-carotene (pro vitamin A) biosynthesis pathway into rice endosperm by genetic engineering. This finding has revolutionized the biotechnology field and opened a new avenue for research.

All the earlier works were done on the proteins present on the endosperm. There are very few reports regarding the work on aleurone specific protein. The aleurone layer is present on the surface of the rice seed, which gets removed during polishing and milling processes. This loss can be stopped or reduced by isolating the gene for the rice aleurone specific protein and making it to express inside the seed using an endosperm specific promoter. Resulting transgenic plant will express this protein only inside the seed and there will not be any loss during polishing and milling processes. With this major goal, the present study was undertaken in rice (*Oryza sativa* L.) variety Nootripathu, a land race with the following objectives.

1. Isolation of rice surface protein from the aleurone layer of the seed and its analysis on SDS- PAGE.
2. Raising antiserum for the aleurone specific protein.
3. Western blot analysis of the rice seed aleurone protein with the raised antibody.
4. Dot blot analysis of rice seed aleurone protein from different stages of growth.
5. Immunoscreening of a rice seed cDNA library for identifying cDNA clones for aleurone specific proteins.
6. Characterization of cDNA clones.

Review of Literature

Chapter - II

REVIEW OF LITERATURE

Rice (*Oryza sativa* L.) is an important staple food crop for most of the population in Asian countries. In India, it occupies an area of 42.7 million hectares with an annual production of 84.7 million tones of grains (Venkataramani, 2000). Rice is the main source of protein and energy in human diets in Asia as well as in parts of Africa and Latin America. It is depended upon not only as a source of calories but also as a source of nitrogen and essential amino acids (Kartoprawiro and Mugiono, 1984). Next to starch, protein is the most abundant constituent of rice endosperm. In Asian diets, milled rice provides 40-80 per cent of the calories and at least 40 per cent of the protein.

2.1. Structure and localization of rice protein

Endosperm protein exists as discrete particles located between the compound starch granules (Rosario *et al.,* 1968 and Harris and Juliano, 1977). The particles are called protein bodies. They are smaller and more numerous in peripheral cells and near the cell walls. Rice protein bodies generally have a lamellar structure. A crystalline type of protein body was reported to be present in the sub-aleurone layer of milled rice (Harris and Juliano, 1977).

Storage proteins are synthesized in Rough Endoplasmic Reticulum (RER) translocated through membranes into the lumen area of rough endoplasmic reticulum and finally deposited in protein bodies *via* Golgi apparatus (Tanaka *et al.,* 1980; Oparka and Harris, 1982; Yamagata *et al.,* 1982; Krishnan *et al.,* 1986 and Yamagata and Tanaka, 1986). Glutelin is

found exclusively in the protein body-II (Yamagata *et al.,* 1982 and Tanaka *et al.,* 1980) which is referred as the crystalline type of protein body (Oparka and Harris, 1982).

Krishnan and White (1997) studied the immuno cytochemical localization of globulin and glutelin in developing rice embryo. The electron microscope immuno cytochemistry revealed that proteins similar to α-globulins and glutelins were deposited within the vacuoles. As in endosperm tissue, the glutelins and the α-globulins were deposited within the same protein bodies. However, the embryo protein bodies were morphologically distinct from endosperm protein bodies. This study demonstrated the occurrence and localization of protein similar to endosperm glutelins and globulins within the protein bodies of rice embryos.

Muench *et al.* (1998) investigated the association of prolamine polysomes of the endoplasmic reticulum that delimit the prolamine protein bodies. They suggested that translation complexes are anchored to the protein body surface through a second binding site in addition to the well characterized ribosome binding site of the ER localized protein translocation complex. Earlier studies already demonstrated that the mRNA encoding the prolamin and glutelin storage proteins are localized in morphologically distinct membranes of the endoplasmic reticulum (ER) complex in developing rice endosperm cells.

2.2. Protein classification based on solubility

Proteins were classified on the basis of solubility by Osborne (1907).

Albumins : soluble in water and dilute buffers at neutral pH

globulins : soluble in salt solutions but insoluble in water

glutelins : soluble in dilute acid or alkali solution

prolamins : soluble in aqueous alcohols (70-90 per cent)

2.3. Composition of different parts of rice grain.

Hinton (1948) reported the percentage composition of different parts of rice grain such as germ, bran and endosperm's shown in Table 1.

Table 1. Percentage composition of different parts of the rice grain.

Rice grain	Water (%)	Protein (%)	Fat (%)	Carbohydrate (%)	Minerals (%)	Fibre (%)
Germ	9	32	18	33	6.0	2.0
Bran	9	18	10	48	6.0	9.0
Endosperm	11	7	1	79	0.5	0.8

Hinton (1948).

The bran accounts for 5-9 per cent of rough rice weight in most milling processes (Houston, 1972). However, this portion of the rice grain comprises, 17-30 per cent of the total proteins in the whole grain, 95-100 per cent of the oil, 60-70 per cent of the vitamins, and 70-75 per cent of the minerals including a high percentage of calcium and iron. Juliano (1972) reported the percentage composition of different constituents in whole rice, polished rice and bran, shown in table 2.

Table 2. Percentage composition of different constituents in whole rice, polished rice and bran

Rice	Water (%)	Protein (%)	Fat (%)	Carbohydrate (%)	Mineral (%)
Whole rice	13.10	7.85	0.88	76.50	1.00
Polished	12.55	7.90	0.52	77.80	0.35
Bran	12.90	11.10	7.85	62.10	4.55

Juliano (1972)

2.4. Composition of rice protein

The major fraction of milled rice protein is glutelin (alkali-soluble protein) (at least 80 %) (Cagampang *et al.*, 1966). The other fractions, including prolamin (alcohol-soluble protein) are present in small amounts. Albumin (water-soluble protein) and globulin (salt soluble protein) are concentrated in the bran, whereas prolamin is evenly distributed in the grain (Cagampang *et al.*, 1966). The composition of these rice protein in percentage is as follows. : 5 per cent albumin, 10 per cent globulin, 5 per cent prolamin (Oryzin) and 80 per cent glutelin (Oryzenin) (Houston et al., 1968). Glutelin is the major protein which is present in highest percentage in rice compared to other proteins fractions.

2.5. Amino acid composition of rice seed protein

Rice protein includes various amino acids. Seed storage proteins are rich in arginine, glutamine, glutamate and asparagine. Cagampang *et al.* (1976) has reported the amino acid composition of IR8 brown rice. Following data shows the amino acid composition of IR8 brown rice and its fractions

Table 3. Amino acid composition of brown rice IR8 and its fractions (g/16.8 g N).

Property	Brown rice	Milled rice	Rice pericarp	Rice embryo	Rice aleurone layer
Alanine	5.8	5.8	6.7	6.6	6.2
Arginine	9.0	8.5	7.8	9.6	8.7
Aspartic acid	9.0	9.0	11.2	9.1	9.4
Cystine	2.4	2.6	2.9	2.0	2.7
Glutamic acid	16.8	18.3	12.4	15.1	13.9
Glycine	4.8	4.7	6.1	6.0	5.5

Property	Brown rice	Milled rice	Rice pericarp	Rice embryo	Rice aleurone layer
Histidine	2.6	2.7	2.9	3.8	3.1
Isoleucine	4.5	4.7	4.5	3.8	4.3
Leucine	8.3	8.5	8.2	6.8	7.8
Lysine	4.4	4.0	5.7	6.8	5.1
Methionine	2.6	2.6	1.7	1.9	1.8
Phenylalanine	5.3	5.5	5.3	4.2	5.0
Proline	4.9	5.3	5.8	4.5	4.9
Serine	5.8	5.9	5.8	5.3	5.7
Threonine	3.9	3.9	4.6	4.5	4.0
Tryptophan	1.2	1.3	1.0	1.4	1.3
Tyrosine	4.0	3.8	3.1	3.3	3.6
Valine	6.6	6.8	6.9	6.3	6.3
Protein (% N X 5.95)	7.8	7.2	16.0	25.3	15.8

Cagampang *et al.* (1976)

Compared with other cereal proteins, rice protein has an excellent nutritional value due to its high content of lysine (about 4 per cent). The high lysine content of rice is due to the low prolamin and high glutelin content. Lysine is the first limiting essential amino acid of cereal proteins. Among the protein fractions, albumin has the highest lysine content, followed by glutelin, globulin and prolamin (Tecson *et al.*, 1971). Milled rice protein generally has a lower lysine content than brown rice protein because the protein in the pericarp, embryo and aleurone layers are richer in lysine due to their higher content of albumin (Cagampang *et al.*, 1976 and Baldi *et al.*, 1976).

There is little prospect of improving the lysine content of rice because of the relatively low prolamin content. But it should be possible to improve the protein content, since, differences in protein content exist among varieties grown under similar cultural management. (Juliano *et al.,* 1964; De Datta *et al.,* 1972 and Juliano *et al.,* 1972). It appears reasonable to expect that protein content of brown rice can be increased 20 to 25 per cent (Beachell *et al.,* 1972). This corresponds to an improvement in milled rice protein by the percentage points from 7 to 9 per cent and to an increase in protein intake in the Asian diet of from 10 to 20 per cent (Beachell *et al.,* 1972). The nutritional utility of this increase in protein content has been demonstrated in co-operative feeding trials in rats and man (Coffman and Juliano, 1979).

2.6. Nutrient content of rice

Nutrient content and its distribution in a low-protein (7.5 %) rice IR32 and a high protein (10.8 %) rice (IR480-5-9) were studied by analyzing successive abrasive milling fractions of brown rice (Resurreccion *et al.,* 1979). It was reported that non starch constituents decreased from the surface to the center of the grain in both rices, except that the highest protein fraction in high-protein rice was the sub aleurone layer (Houston *et al.,* 1964; Normand *et al.,* 1966; Barber, 1972 and Kennedy and Schelstraete, 1974). Starch and amylose contents of starch increased progressively from the surface to the center of the grain and were lower in high-protein grain.

The outer layers of milled rice are richer in nonstarch constituents including B vitamin than the core of the endosperm. Although, brown rices contained similar B vitamin contents (as that of milled rice), high protein milled rice had higher contents of thiamin, riboflavin, and phytin P than

low-protein rice (Resurreccion *et al.,* 1979). Juliano (1972) has estimated the vitamin content of rice bran (Table 4).

Table 4. Vitamin content of rice bran

Vitamin	Content (µg/g, dry basis)
Vitamin A (Carotenes)	4.2
Thiamin	10.1 to 27.9
Riboflavin	1.7 - 3.4
Niacin (nicotinic acid)	236 - 590
Pyridoxine	10.3 - 32.1
Pantothenic acid	27.7 - 71.3
Biotin	0.16 - 0.60
Inositol	4627 - 9270
Choline	1279 - 1700
p-Amino benzoic acid	0.75
Folic acid	0.50 - 1.46
Vitamin B_{12}	0.005
Vitamin E (Tocopherols)	149.2

Juliano (1972)

Recently, "Golden rice", a rice with accumulated carotenoids were obtained by genetic engineering (Ye *et al.*, 2000). In order to accomplish this, cDNAs from *Narcissus pseudonarcissus* coding for the carotenogenic enzymes phytoene-synthase and lycopene-cyclase, both placed under the control of the rice glutelin (Gt 1) promoter, as well as a bacterial (*Erwinia*) gene, coding for phytoene-desaturase under 35 S promoter control were used to transform rice (TP 309).

Albumin content of the protein was higher in the sub-aleurone layer than in the inner endosperm (Houston *et al.,* 1968). Low protein rice had lower total ash content than the high-protein rice. Glutelin accounted for the 87 to 93 per cent of milled rice protein. Protein and protein bodies of the sub-aluerone layer and inner endosperm have similar amino grams and electrophoretic pattern.(Resurreccion *et al.,* 1979).

In the high-protein rice the proportion of globulin in the protein of sub-aleurone was higher than that in the inner endosperm. Crystalline protein bodies and the small spherical protein bodies were reported to be present only in the sub-aleurone layer (Bechtel and Pomeranz, 1978 and Jones and Rost, 1989). Breeding efforts for improved protein content in rice began as early as 1950 (Johnston *et al.,* 1974). An increased protein content has been found to be associated with a higher proportion of brown rice protein residing in the endosperm (milled rice) and improved grain resistance to abrasive milling (Juliano *et al.,* 1973).

Biological tests in co-operating laboratories with weaning rats and humans verified that the protein quality of milled rice was predictable from amino acid scores based on lysine (Bressani *et al.,* 1971; Clark *et al.,* 1971; Eggum and Juliano, 1973; Hegsted and Juliano, 1974; Roxas *et al.,* 1975 and Murata *et al.,* 1978). The content of utilizable protein of milled rice in diets adequate in calories was affected more by the protein content than by the protein quality. Replacing average protein rice with an equal weight of high-protein rice improved the nitrogen retention in humans in studies where adults were fed a rice diet and preschool children were fed rice based diets (Clark *et al.,* 1971).

2 7. Rice proteins and their genes

2.7.1. Rice glutelin

Rice seeds contain four classical protein fractions albumin, globulin, prolamin and glutelin. Glutelin accounts for atleast 80 per cent of the total protein of milled rice (Houston and Mohammad, 1970 and Villareal and Juliano, 1978). Rice glutelin consists of two major set of polypeptides with approximate molecular weight of 37 kDa and 22 kDa (Juliano and Boulter, 1976: Yamagata *et al.*, 1982; Luthe, 1983 and Sarker *et al.*, 1986). Electrofocussing showed that each of the two sets of polypeptide is composed of several bands with the isoelectric points from 6.5 to 7.5 and from 9.4 to 10.3 in the 37 kDa and 22 kDa respectively (Pan, 1983; Robert *et al.*, 1985 and Wen and Luthe, 1985).

According to Zhao *et al.* (1983) the 37 kDa and 22 kDa polypeptide are linked by one or more disulfide bonds. Based on labelling experiments carried out both *in vivo* and *in vitro*, it was suggested that the disulfide linked pairs are split from 57 kDa precursor (Yamagata *et al.*, 1982 and Luthe, 1983). This was confirmed by Krishnan and Okita (1986) who presented immunological evidence that the 37 kDa and 22 kDa polypeptides originate from a common precursor.

The glutelin precursor is fairly stable *in vivo*, since it can be isolated from cells (Krishnan and Okita, 1986). This indicates that the proteolytic cleavage of the 57 kDa precursor into the 37 kDa and 22 kDa polypeptides occurs post translationally and not co-translationally (Yamagata *et al.*, 1982 and Krishnan *et al.*, 1986).

Lycett *et al.* (1984) reported the electrophoretic pattern of rice glutelin, which was found very similar to that of oat globulin, the major oat seed storage protein. It was also similar to legumin like protein found in soybean and pea (Derbyshire *et al.*, 1976 and Shotwell and Larkins, 1989). The legumin like protein have α and β (6) sub units joined by disulfide bond and have a molecular weight of about 360 kDa. The α and β subunits have molecular weight of 20-40 kDa and 20 kDa respectively (Derbyshire *et al.*, 1976). The α and β peptides are formed after translation (Shotwell and Larkins, 1989). The rice glutelin is similar to legume-like proteins because of its similarity in apparent molecular weight and the facts that it was synthesized as precursor, which was post translationally cleaved to form the α and β subunits (Yamagata *et al.*, 1982 and Luthe, 1983).

2.7.1.1. Glutelin a Homolog of 11S globulin protein

Zhao *et al.* (1983) reported that a partial amino acid sequence of the purified 22 kDa glutelin polypeptide is similar to the basic polypeptide of pea legumin. This was the first solid evidence to prove rice glutelin protein to be a legumin type protein. Leguminous proteins including soybean glycinin pea legumin and oat globulin form a group of storage proteins classified as 11S globulin (Derbyshire *et al.*, 1976; Croy *et al.*, 1980; Matlashewski, *et al.*, 1982 and Lycett *et al.*, 1984).

Rice glutelin like 11S globulin is synthesized as a high molecular weight precursor that comprises both an acidic (around 37 kDa) and a basic (around 22 kDa) polypeptide (Wen and Luthe, 1985). The amino acid composition of the basic polypeptide is similar to that of all species of 11S globulin, while the acidic polypeptide is similar to that of oats, a closely

related species, and coconut, a monocotyledonous plant. (Wen and Luthe, 1985).

Inspite of those similarities the solubility of rice glutelin is quite different from that of 11S globulins. The marked difference in solubility between rice glutelin and 11S globulin may be associated with the structure of C-terminal region of the 37 kDa polypeptide of the glutelin protein (Wen and Luthe, 1985 and Higuchi and Fukazawa, 1987).

2.7.1.2. cDNA clones coding for rice glutelin protein

cDNA clones encoding glutelin protein have been reported (Takaiwa, *et al.*, 1986 and Higuchi and Fukazawa, 1987). The inferred amino acid sequence showed that the 57 kDa precursor contains a signal peptide at the N-terminal end of the sequence. The signal peptide is removed during co-translational processing (Takaiwa *et al.*, 1987) after which the polypeptide is inserted into the lumen of rough endoplasmic reticulum. Higuchi and Fukazawa (1987) have suggested that the signal sequence with mostly hydrophobic residues is a segment of 24 amino acids upon cleavage. The molecular weight of this sequence is close to that of the signal peptide estimated by polyacrylamide gel electrophoresis (PAGE) when the *in vitro* translational product of glutelin polypeptide is compared to a putative precursor (Krishnan and Okita, 1986). However, a different cleavage site for the signal peptide was proposed resulting in a fragment of 37 amino acids (Takaiwa *et al.*, 1986).

Glutelin cDNA library clones were isolated by antibody screening of a λgt 11 rice seed library. The nucleotide sequence of clone pRG206 reported by

Higuchi and Fukazawa (1987) is very similar to the sequence of cDNA clone PREE61 (Takaiwa *et al.,* 1986). They differ at 0.9 per cent of the positions in their coding region and that produces 7 amino acid substitution. The most striking observation is that both nucleotide sequence in the 3' non coding region are exactly identical. The putative polyadenylation signals observed in the 3' noncoding region of both cDNA clones were also 100 per cent identical.

2.7.1.3. Rice glutelin gene

A rice glutelin gene representing a second subfamily of glutelin gene has been reported (Takaiwa *et al.,* 1987). The genomic glutelin gene contains three introns. The first two introns are in the 37 kDa coding region while the last one is in the 22 kDa coding region (Takaiwa *et al.,* 1987). They are found at exactly the same positions as in Leg A, a legumin gene. (Matlashewski *et al.,* 1982). At the 5' flanking region of the rice glutelin gene a 19 bp sequence around 60 bp upstream from the transcription start site, this sequence may be specific to the genes grouped in 11S globulin type gene and may play an important role in specific developmental expression (Takaiwa *et al.,* 1987). Based on this sequence analysis, it was confirmed that rice glutelin and 11S globulin genes originate from a common ancestral gene.

2.7.2. Rice Globulin

Among the salt - soluble proteins, globulin constitutes about 10 per cent and albumin about 5 per cent of milled rice protein. Very less work has been done on these proteins of milled rice. The major globulins were studied in the rice endosperm of well-milled (12 per cent bran-polish removal) IR 480-5-9 rice with 11 per cent protein (Morita and Yoshida, 1968). Salt soluble proteins were extracted from defatted milled - rice flour with 5 per cent NaCl and the globulin was precipitated either by dialysis against water

or by adding ammonium sulphate to 30 per cent saturation. Higher recoveries were obtained by dialysis than by ammonium sulphate precipitation (4 per cent of total protein Vs 2.4 per cent). In sodium dodecyl sulphate-PAGE (SDS-PAGE) analysis two major slow band and three minor faster migrating bands were found. The slower of the two bands was the main globulin. On the other hand the albumin had faster migrating bands than globulin (Morita and Yoshida, 1968 and Morita and Horikoshi, 1972).

Shorrosh *et al.* (1992) constructed λ gt-11 cDNA library, from poly (A)+ RNA isolated from immature rice seed endosperm and identified a clone for rice seed storage protein called globulin or the 19 kDa globulin. They sequenced a positive clone encoding for a 21 kDa globulin.

2.7.3. Prolamin

It is an alcohol soluble storage protein that is high in proline (Tecson *et al.*, 1971). Rice is unique among the cereals in having only about 3 per cent prolamin (Tecson *et al.*, 1971). Prolamin has the poorest amino acid balance among the four Osborne solubility fractions of rice protein. Tecson *et al.* (1971) reported that there is one major protein band for the prolamin of the milled rice of molecular weight 17 kDa. Linear gradient elution chromatography in a diethyl amino ethyl cellulose (DEAE cellulose) column of prolamin (0.1 M Tris-HCl buffer pH 8.0 from 0 to 1 M NaCl) resulted in only one protein peak which eluted at 0.42 N NaCl and had the same electrophoretic properties as the starting protein.

2.7.3.1. cDNA clones coding for prolamin

Masumura *et al.* (1989) constructed cDNA library from developing rice endosperm mRNAs and isolated clones which code for the rice 10 kDa

prolamin. Structural analysis of the entire cDNA of this prolamin revealed that the signal sequence composed of 24 amino acids which had quite a high homology to that of major prolamins of maize (Masumura *et al.*, 1989).

Shyur *et al.* (1992) sequenced a full-length cDNA (PS18) encoding the 16 kDa rice prolamin composed of 158 amino acid. Analysis of N- terminal amino acid sequence of a major rice prolamin indicated that an 18 amino acid signal peptide was removed from 16 kDa precursor prolamins to form the 14 kDa prolamin during seed development.

Nakase *et al.* (1996) characterized the expression mechanism of the rice seed storage protein genes (16 kDa albumin, 13 kDa prolamin and type II glutelin). Their coordinate expression suggested that the transcriptional regulatory machinery is shared among the glutelin, prolamin and albumin-genes. They isolated two novel genomic genes for prolamins and obtained the promoter region of the glutelin gene by PCR. They identified that the 5' - flanking regions of three rice seed storage protein genes contain some similar conserved sequences. Further they identified that these three genes share transcription factors.

2.8. Developmental regulation of seed storage proteins

Changes in the properties of crude prolamin were studied in dehulled developing grains of IR 26 and IR 480-5-9 rices. Analytical PAGE of both the rices showed only one protein band in the sample about 4 days after flowering. Subsequent samples (7 DAF) showed a faint, slightly slower moving protein band in addition to the major prolamin band. Amino acid analysis of prolamin from developing grains of both rice 4, 7, 10, 14 and

21 days after flowering showed progressive decrease in lysine and aspartic acid (IRRI Annual Report 1976). Appearance of the slower electrophoretic band coincided with a drastic drop in lysine, aspartic acid, alanine, valine, and methionine and increase in histidine, arginine and leucine in both rices.

Kim *et al.* (1993) assessed the expression of the genes that encode for both glutelin and prolamin proteins during seed development, the transcription activities and mRNA levels of several gene classes that comprise these storage protein multigene family. They reported that the expression of these gene classes for each multigene family was differentially regulated at both transcriptional and post transcriptional levels. They also suggested that in addition to regulation at the transcriptional and post-transcriptional levels, endoplasmic reticulum membrane - associated translational control is also involved in the expression of rice seed storage protein multigene families.

Liu *et al.* (1995) analyzed the partial nucleotide sequence of randomly isolated clones from an endosperm cDNA library. They identified that 21 per cent of cDNA clones were found to code for rice seed storage protein gene. Consequently, 37 independently genes were identified. They compared the accumulation of mRNA during rice seed development. Genes associated with seed storage protein for example glutelin and prolamin was expressed more at 10 and 20 days of growth. Prolamin also showed the same pattern of expression, but, the expression at maturity (three months of plant) stage was found very low.

Till date all classical plant breeding approaches have been rather ineffective in improving the nutritional properties of rice. With recombinant DNA technologies, there is hope to bypass the deadlock encountered in breeding programme in this aspect. All the earlier works were done on the protein present on the endosperm. There are very less report regarding the work on aleurone specific protein. But aleurone layers are found to be very rich in protein and its loss in milling has to be compensated by expressing those genes in endosperm. To accomplish this, the aleurone specific proteins and their genes has to be identified.

Materials and Methods

Chapter - III

MATERIALS AND METHODS

3.1. Materials

3.1.1. Rice (*Oryza sativa* L.)

A land race of rice variety Nootripathu was used for the experiment. It is a local cultivar, which has got coloured aleurone (rice surface) layer. The main idea behind selecting this variety was to differentiate easily between aleurone (surface) layer and the seed without aleurone layer during the process of peeling. The top aleurone (rice surface) layer was separated from the rice seed using a sharp blade and it was used for the experiment. The structure of the rice grain and the aleurone layer was shown in figure 1.

3.1.2. Growing of Nootripathu plants

Rice (*Oryza sativa* L.) seeds, variety Nootripathu, were obtained from the Paddy Breeding Station, Tamil Nadu Agricultural University Coimbatore. Seeds were rinsed in water and allowed to imbibe overnight. Next day seeds were planted in earthen pots. The leaf, materials were collected at different stages of growth and stored at -70°C.

3.2. Methods

3.2.1. Protein extraction

Proteins were extracted from whole seeds, aleurone (rice surface) layer and white seed (endosperm only) i.e., without aleurone (rice surface) layer. The protein concentration was measured by the dye binding method of Bradford (1976). All protein extracts were electrophoresed on SDS-PAGE (12 %) (Laemmli, 1970).

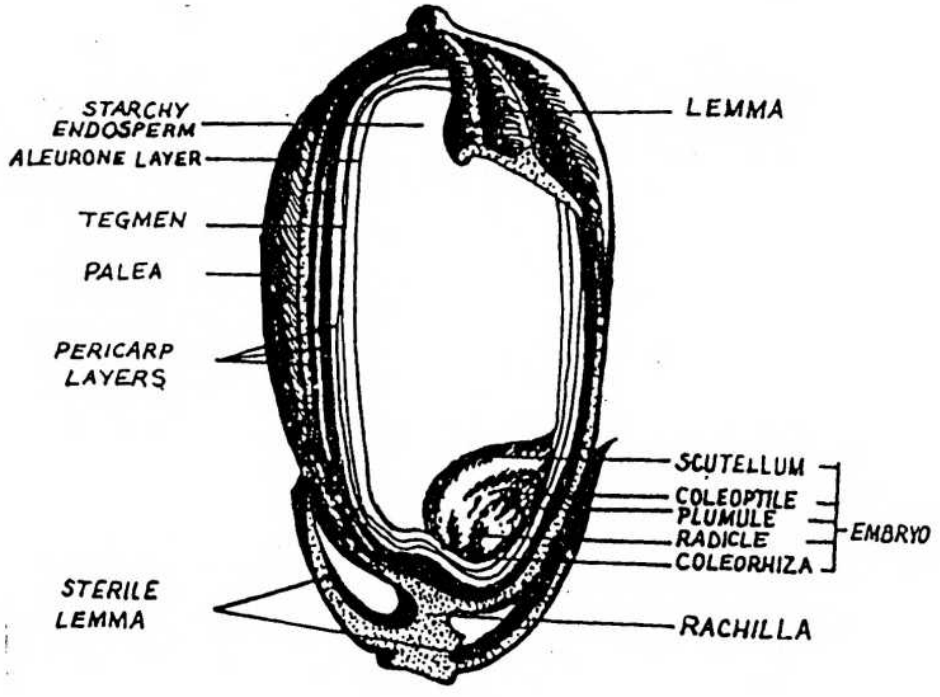


Figure 1. Structure of a rice grain (Singh, 1983)

3.2.1.1 Protein extraction from whole seed

Whole seed protein was extracted by the method as described by Nakase *et al.* (1996).

The whole seed of the rice variety Nootripathu was soaked in seed protein extraction buffer (Tris- HCl, 10 mM, pH 7.4; NaCl 50 mM; PMSF (Phenylmethylsulfonyl fluoride) 1 mM; 2-Mercaptoethanol (2-ME) 10 mM) for 30 minutes. It was ground to fine powder in a pestle and mortar and it was transferred to 30 ml centrifuge tubes. Centrifugation was carried out at 10,000 x g at 4°C for 10 minutes. The supernatant containing the protein alone was dialyzed against polyethylene glycol 2000 for 1 hour to concentrate the sample.

3.2.1.2. Protein extraction from aleurone (rice surface) layer of seed

The top aleurone (rice surface) layer was peeled off from the seed using a sharp blade. Protein was extracted in seed protein extraction buffer (pH 7.4) mentioned in 3.2.1.1. by grinding in a pestle and mortar. Crude extract was centrifuged at 10,000 x g at 4°C for 10 minutes. The supernatant containing the protein alone was dialyzed against polyethylene glycol 2000 for concentrating the sample.

3.2.1.3. Protein extraction from white seed (endosperm only)

The complete removal of top layer was done by rubbing the seed with emory paper (sand paper). The protein was extracted from white seed (endosperm only) by following the method described in 3.2.1.1 or 3.2.1.2.

3.2.1.4. Leaf protein extraction

Leaf protein at different stages of growth were extracted in phosphate buffer (Phosphate buffer 0.1 M, pH 7.2; PMSF 50 mM; 2-Mercaptoethanol 10 mM) as described by Velazhahan and Vidhyasekran (1999).

The leaf samples of different stages of growth were cut into small bits. It was ground in 2 ml phosphate buffer (pH 7.2) in a pestle and mortar. Crude extract was centrifuged at 10,000 x g for 20 minutes. The supernatant containing protein was stored at -20°C.

3.2.2. Protein precipitation

3.2.2.1. Trichloroacetic acid (TCA) precipitation

It was done essentially as described by Harlow and Lane (1988)

Protocol

Protein sample (500 µl) was taken in a eppendorf tube and 500 µl of cold TCA (10 %) was added and mixed properly. It was kept at 4°C for one hour for precipitation. Then it was centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was discarded and precipitate was washed with 500 µl of ethanol (70 %) at 10,000 x g for 10 minutes. Supernatant was discarded and the pellet was dissolved in 100 µl of seed protein extraction buffer. This protocol was followed for all the three protein samples *viz.* whole seed, aleurone (rice surface) layer and white seed (endosperm only).

3.2.2.2. Ammonium sulphate precipitation.

Protocol

The extracted protein samples were subjected to ammonium sulphate precipitation. Ammonium sulphate was added slowly to the crude protein extract with constant stirring to bring to 75 per cent saturation. Then the contents were centrifuged at 9000 x g for 30 minutes at 4°C. The pellets were collected and redissolved in 5 ml of extraction buffer. The solution was then transferred to pretreated (as per manufacturer's instruction) dialysis bags

and dialyzed against distilled water with three change for 24 hours. The dialysates were centrifuged and the supernatant were pooled and lyophilized.

3.2.3. Electrophoresis of proteins

The protein extracts from aleurone (rice surface) layer, whole seed and white seed (endosperm only) were separated on SDS-PAGE (12 %) to analyze the quality of protein extracts, characterization prior to western blot analysis. The SDS-PAGE separation of proteins was done in a discontinuous buffer system essentially as described by Laemmli (1970).

Materials

1. Slab gel electrophoresis apparatus and power supply unit (A Hoefer SE 600 slab gel unit and a PS 250 power supply unit was used).
2. Electrode Buffer : Tris-HCl 25 mM, pH 8.3 ; Glycine, 192 mM; SDS (0.1 %) .
3. Acrylamide stock (30 %)

Acrylamide	29.2 gm
Bis acrylamide	0.8 gm
Distilled water to make up volume	100 ml

4. 4 X Resolving Gel buffer : Tris -HCl, 1.5 M, pH 8.8
5. 8 X stacking gel buffer : Tris- HCl, 1.0 M, pH 6.8
6. SDS (10 %)
7. Ammonium persulphate (APS) (10 %)
8. N, N, N', N'-Tetramethyl ethylenediamine (TEMED)

9.	2X sample buffer :	
	Tris-HCl	25 mM pH 6.8
	Glycerol	20 %
	SDS	4 %
	2-ME	10%
	Bromophenol blue	0.025%
10.	Staining solution:	
	Coomassie brilliant blue R-250	0.025 %
	Methanol	40 %
	Acetic acid	7 %
11.	Destaining solution:	
	Methanol	40 %
	Acetic acid	10 %

Protocol

A. Preparation of separating gel

The gel unit was assembled as the manufacturer's instruction using the 1-mm spacer set. In a side arm flask 20 ml 12 per cent gel solution was mixed (Monomer 8 ml, separating gel buffer 5 ml, distilled water 6.6 ml, SDS (10 %) 0.2 ml and APS (10 %) 0.2 ml). The solution was degassed for few minutes, and 12 µl TEMED was mixed by gently swirling the flask. The solution was poured in between the gel plates upto a level about 4 cm from the top. The gel solution was overlaid with water and left to polymerize for several hours. The water was removed and the assembly was inverted on a filter paper and allowed to dry.

B. Preparation of stacking gel

Stacking gel mixture	
Monomer	1.1 ml
Stacking gel buffer	1.0 ml
SDS (10 %)	0.08 ml
Initiator (APS) (10 %)	0.08 ml
Distilled Water to make up the volume	8.0 ml

The mixture was degassed for few minutes and 10 μl of TEMED was mixed by gentle swirling. The plate was rinsed with 1ml of the gel mixture and filled with the stacking gel solution. Appropriate comb was inserted in place and gel was allowed to polymerize for about half an hour.

C. Preparation of sample

Protein sample was mixed with equal volume of 2X sample buffer in microfuge tubes. The tubes were placed in a boiling water bath for 2 minutes.

D. Loading and Running the gel

The comb was removed and the wells were flushed with electrode buffer to remove any unpolymerized acrylic acid. The upper buffer chamber was placed according to manufacturer's instruction. The gel assembly was placed in the lower buffer chamber with the heat exchanger in place. Both the buffer chambers were filled with tank buffer. With a loading tip protein samples (100 μg) were under laid in each well. The lid was connected to the power supply and the heat exchanger to a circulating water bath set at

20°C. Power supply was turned on and adjusted for 20 mA constant current for each 1mm gel. When the dye reached the bottom of the gel the power supply was disconnected and the assembly was removed.

E. Staining and destaining of gels

The gel setup was disassembled and the gel was removed carefully and transferred to a tray containing about 100 ml of coomassie brilliant blue R 250 staining solution. It was allowed to shake gently overnight on a rocking platform. Next day the gel was removed and rinsed briefly in distilled water and placed in destaining solution. The gel was allowed to destain on a rocking platform with several changes of destaining solution.

3.2.4. RP-HPLC (Reverse phase High Performance Liquid Chromatography)

Materials

1. HPLC model RP 4a equipped with a variable wave length UV detector. (Wave length set at 280 nm) and a microprocessor.
2. Hypersil BDS, C18, 5 µl column (250 x 4.6 mm) (Varian)
3. Mobile phase : Methanol (HPLC graded) mixed with water

Protocol

1. The extracted protein samples were subjected to ammonium sulphate precipitation followed by filtration through 0.45 µm membrane filter (Durapore). The filtered protein sample (50 µg) was injected into the sample introduction system and the flow rate was maintained at 1 ml per minute.

2. The proteins were eluted and fractions corresponding to the peaks were collected separately, dialyzed against distilled water and lyophilized.
4. The peak showing unique rice aleurone (surface) protein was collected through repeated injections, dialyzed against distilled water, lyophilized and stored at -20°C.

3.2.5. Production of rice aleurone protein specific antiserum

Antiserum was raised in rabbits (New Zealand white) against aleurone (rice surface) layer protein. Rabbit was procured from Pasteur Institute of India, Coonoor, Tamil Nadu.

3.2.5.1. Raising antiserum

Immunization of rabbits, collection and purification of antiserum and all other immunological assays were done following the protocols described by Harlow and Lane (1988).

Protocol

1. The extracted protein samples were separated on SDS-PAGE (12 %) and stained with coomassie brilliant blue R 250 followed by destained. The 30 $\pm$ 2 kDa band was cut with a sharp blade and passed through a 2 ml syringe with seed protein extraction buffer pH 7.4 and kept for overnight at 4°C. Next day it was centrifuged at 10,000 x g for 10 minutes supernatant was collected dialyzed against polyethylene glycol. Alternatively it was lyophilized. This protein sample was used as antigen. This protein sample (150 µg) was dissolved in 500 µl of

phosphate buffer saline (10 mM sodium potassium phosphate, pH 7.2 containing 15 mM NaCl) and used as antigen.

2. Complete Freund's Adjuvant (CFA) (500 µl) was added to the protein solution of 500 µl and mixed thoroughly by passing through a 22 gauge needle repeatedly until a white emulsion was formed.
3. A small test bleed (1 ml) was taken from the marginal ear vein of the test animal to prepare pre-immune serum.
4. The protein adjuvant emulsion was injected in the hind thigh of the rabbit intra-muscularly with a syringe fitted with 18-gauge needle.
5. Another test bleed was collected from the marginal ear vein two weeks after the first injection to prepare post immune serum.
6. Three weeks after the first injection, the rabbit was given a booster with 300 µg of protein extract in Incomplete Freund's Adjuvant (IFA). Protein extracts (300 µg) was diluted in 500 µl PBS and emulsified in 500 µg IFA. The emulsion was injected in the hind thigh intramuscularly.
7. Ten days after the first boost approximately 10 ml of blood was collected from the marginal ear vein and immune serum was prepared as described below.
8. The animal was given booster dose regularly at monthly intervals and bled routinely from the marginal ear vein ten days after each boost.

3.2.5.2. Isolation of Antiserum

The blood collected, was incubated at 37°C for 1 hour. The blood clot was separated from the tube by ringing with a sterile needle, and stored at 4°C overnight for the clot to contract. Next day clear straw colored serum were collected by centrifugation at 5,000 x g for 20 minutes at 4°C. The sera was stored with sodium azide (0.02 %) at -20°C in aliquots.

3.2.5.3. Affinity purification of rice seed aleurone (surface) antisera

The immune serum was made "rice seed aleurone specific" by pre-adsorption with proteins from rice white seed (endosperm only) on CNBr-activated sepharose 4B column (Affinity chromatography, principle and methods, pharmacia fine chemicals, Uppsala, Sweden).

Protocol

1. The seed aleurone protein extract was desalted by extensive dialysis against distilled water to remove all Tris salt.
2. One gram of freeze-dried CNBr-activated sepharose 4B (Sigma) was swollen for 30 minutes on a scintered glass funnel (porocity 63) with 200 ml of 1 mM HCl to get about 3 ml of swollen matrix.
3. About 150 mg of protein extract was suspended in 7 ml of coupling buffer (Sodium-carbonate-bicarbonate, 0.1 M, pH 8.3; NaCl, 0.5 M)
4. The protein suspension was mixed with the pre-swollen matrix in end-over-end motion at 4°C overnight. The matrix was washed with coupling buffer followed by acetate buffer (sodium-acetate, 0.1 M, pH 4.0; NaCl, 0.5 M).
5. The remaining active groups on the matrix were blocked by incubating with glycine (0.2 M, pH 8.0).
6. The excess adsorbed protein and the glycine was washed away by repeated washing with coupling buffer and acetate buffer alternately.
7. The end of a 10 ml glass syringe was blocked with sterile glass wool to make a home made column.
8. The protein sepharose conjugate was packed in the column and the column was equilibrated with PBS.

9. Crude antiserum (2 ml) was loaded into the column and allowed to pass through the column slowly.
10. After the antiserum, PBS was allowed to flow slowly on to the column.
11. The eluate was again eluted twice through the column with PBS, so that all the antibody species common to white seed (endosperm only) were bound and the final eluate was free of antibodies common to white seed (endosperm only).
12. The purified antisera was tested for its titre and specificity by direct ELISA against aleurone protein and also on western blots.

3.2.5.4. Enzyme linked immuno-sorbent assay (ELISA)

The titre of the serum isolated from the different bleeds was assayed by antigen excess antibody capture ELISA technique essentially as described by Engvall and Perlmann (1971) and Engvall and Perlmann (1972) in microtitre plates. The crude protein extract from aleurone (rice surface) layer was used in excess as antigen, and alkaline phosphatase (AP) conjugated monoclonal anti-rabbit IgG from Sigma chemical USA was used as secondary antibody.

Protocol

1. The antigen was diluted to 50 μg/ml in PBS.
2. Antigen solution (50 μl) was loaded into each well of microtitre plate.
3. The plates were incubated in a humid atmosphere to prevent evaporation for 2 hours.
4. The antigen solution was removed and the plates were washed thrice with excess PBS for 5 minute each time.

5. All the wells were filled to the brim with blocking solution (1 % gelatin in PBS with 0.02 % NaN_3) and incubated for further two hours in humid atmosphere to block all protein binding sites.
6. At the end of two hours the blocking solution was removed and plates were again washed thrice with PBS for two minutes each.
7. The test-immune sera (pre immune serum and the immune serum preparations after the second boosts) was serially diluted in fresh blocking solutions.
8. Each dilution (50 µl) was loaded in the respective wells in two replications and the plate was again incubated for two hours in a humid atmosphere.
9. The plate was washed thrice with PBS and once with PBST (Phosphate buffered saline tween) (PBS with 0.02% Tween) for two minutes each.
10. Alkaline phosphatase conjugated anti-rabbit IgG (50 µl) (from sigma chemical Co., USA) at 1:5000 dilution was pipetted into each well and allowed to incubate for two hours.
11. The plate was washed thrice with PBST and once with AP buffer (Tris-HCl, 100 mM, pH 9.5; NaCl, 150 mM ; $MgCl_2$ 5 mM)
12. 50 µl of substrate was prepared by dissolving 1 mg of Paranitro phenyl phosphate (Pnpp), 1ml of substrate buffer (Diethanolamine, 10 mM; $MgCl_2$, 5 mM, pH 9.5) was loaded in each well and the plate was incubated for 15 minute, and the reaction was stopped by adding 50 µl of 0.1N NaOH.
13. The plate was read at 405 nm in ELISA reader (BIORAD).

3.2.6. Western blot analysis

3.2.6.1. Electrophoretic separation of proteins

The extracts from seed, aleurone (rice surface) layer and white seed (endosperm only) were electrophoresed on SDS-PAGE (12 %) as described previously (3.2.3.).

3.2.6.2. Electrophoretic transfer of proteins

The polypeptide bands from SDS-PAGE were transferred to nitrocellulose membrane electrophoretically essentially by the method of Towbin *et al.* (1979) in a Hoefer, T.E.42 (Transphor unit).

Materials

1. Electro transfer unit (Hoefer TE42) and power supply unit (Hoefer PS250)
2. Nitrocellulose membrane (Millipore, USA)
3. Towbin buffer :

   Tris-HCl 50 mM

   Glycine 190 mM

   Methanol 20 %

Protocol

1. The scotch bright pads and thick pads supplied with the unit were allowed to imbibe in the transfer buffer in a big tray, so that all air bubbles were removed.
2. A piece of nitrocellulose membrane was cut equal to the gel size and gently floated on the Towbin buffer and allowed to wet slowly for five minutes.

3. In the large tray, on one lid of the transfer cassette the gel was laid on a thick pad on a scotch bright.
4. A corner of the nitrocellulose was marked with a soft pencil to know the orientation and laid on the gel carefully avoiding entrapment of any air bubble.
5. The other pair of pad and scotch bright was laid on the gel carefully and the cassette was closed.
6. The cassette was placed on the transfer tank filled with buffer and with the heat exchanger in place.
7. The unit was connected to the power supply taking note that the membrane was towards the anode.
8. Proteins were transferred at 20 V (constant voltage) over night or at 100 V constant for two hours at 4°C.

3.2.6.3. Visualization of proteins by Ponceau S staining

Materials

1. Ponceau S concentrate (10 X) : Ponceau S (2 %) (Sigma) in trichloroacetic acid (30 %) and sulphosalicylic acid (30 %)
2. Tris buffered saline (TBS) : pH 8.0

   Tris-HCl 10 mM

   NaCl 150 mM

Protocol

The blot was washed briefly with TBS to remove any traces of polyacrylamide etc., and placed on a plastic tray slightly larger than the blot. Ponceau S (1 ml) solution was diluted to 1 X with TBS and slowly poured on the blot. The blot was allowed to absorb the dye for about

30 seconds and then the dye was removed by washing with TBS briefly. Purple bands appeared as soon as background started clearing. The standard marker bands were marked with a soft pencil quickly before the bands get vanished.

3.2.6.4. Immunostaining

Materials

1. Tris Buffered Saline (TBS):

   Tris-HCl 10 mM, pH 8.0

   NaCl 150 mM

2. TBST : TBS with Tween 20 (0.02 %).
3. Blocking solution : gelatin (1 %) in TBS.
4. Primary antibody : 1:50 dilution of aleurone specific antiserum in fresh TBS.
5. Secondary antibody : 1:5000 dilution of HRP (Horse Radish Peroxidase) conjugated anti-rabbit IgG in TBS.
6. HRP substrate : H_2O_2 (immunolocalization solution 10X) commercially available (Genei).

Protocol

1. **The blots were washed in a plastic tray with TBST.**
2. Blots were incubated separately in blocking solution for two hours with slight shaking. From this point onwards the blots were kept constantly wet and shaking till the end of the process.
3. The blots were washed briefly with TBST and primary antibody was added, just enough to cover the blot and incubated for two hours on a slow shaking platform.

4. The primary antibody solution was removed (stored for later use) and the blot was washed thrice with TBST for 5 minute each with slow shaking (60 rpm).
5. After the final wash, the blot was removed and incubated in secondary antibody for two hours.
6. The secondary antibody was removed and stored for later use and excess antibody from the blot was removed by washing thrice with TBST for 5 minutes each.
7. The H_2O_2 substrate, available in 10 X was made to 1 X and the membrane was transferred to 1 X substrate immediately.
8. Reactive areas turned magenta colour within 1 to 5 minutes. The blot was washed in water to stop the colour development.

3.2.7. Dot blot analysis

Protocol

A nitrocellulose membrane dipped in TBS buffer was fixed in the dot blot apparatus. The protein samples (30 μg) at different stages were loaded in different slots. The membrane was kept in gelatin (1.5 %) solution (in TBST) for 2 hours. Rest of the protocol followed was same as that of western blot discussed earlier (3.2.6.4.).

3.2.8. cDNA library screening

cDNA library was made previously in laboratory from Nootripathu immature seed. The library was made in vector pBlue script and were screened for identification of putative clones. The protocol was followed as described by Perbal (1988).

3.2.8.1. Making master plate

Protocol

1. LB-ampicillin plates were prepared by pouring out LB agar media (Trypton (1 %); yeast extract 0.5 (%); NaCl (1.0 %), pH 7.2) with 50 ppm ampicillin, in Petri plates.
2. *E.coli* strain (with pBlucescript vector containing gene of interest) was plated out (40 µl) in LB ampicillin plates and incubated overnight at 37°C to get single colonies.
3. Well-isolated colonies were collected and inoculated in another plate, numbered and incubated at 37°C overnight.
4. Like wise 1200 colonies were plated on LB ampicillin plate.

3.2.8.2. Immunoscreening protocol

A. Plating of library for immunoscreening

The library was plated out in 150 mm plates using LB-agar. The plates were allowed to incubate at 37°C for 4-5 hours till the colonies appeared.

B. Induction of fusion proteins expression and colony lifting

Materials

1. IPTG (Isopropyl thio galactose) 10 mM.
2. Nitrocellulose membrane (sterilized).
3. TBS buffer : Tris-HCl, 10 mM; NaCl, 150 mM; pH 8.0
4. TBST : TBS with Tween 20 (0.02 %).
5. Needle and forceps.
6. LB medium : Trypton 1 gm, NaCl 1 gm, yeast extracts, 0.5 gm, Agar 1.9 gm/100 ml.

Protocol

1. The nitrocellulose membrane discs were presoaked in 10 mM IPTG solution and allowed to dry on a pad of tissue paper in a laminar flow hood for 1-2 hours.
2. Once the colonies attained regular size, the plate was transferred to laminar flow chamber and an IPTG presoaked nitrocellulose membrane was carefully laid on the plate avoiding any air bubble. Plate was marked in 3 places asymmetrically.
3. The plate was transferred to a 37°C incubator and allowed to incubate right side up for 3 to 4 hours. The membrane was removed carefully and placed in a container containing TBS buffer to remove any agar sticking to the membrane.
4. After incubation, filter was kept in blocking solution (1 % gelatin in TBS) for 2 hours, and the plate was wrapped with parafilm immediately and stored in refrigerator.

C. Immuno adsorption and immunostaining

1. The membrane was washed thrice with TBST for 5 minutes each and finally washed with TBS.
2. Primary antibody was added (100 µl in 10 ml TBS)
3. It was incubated overnight at slow shaking at 60 rpm.
4. The filter was washed with TBST, thrice 5 minutes each and finally washed with TBS.
5. Goat anti-rabbit IgG HRP conjugate was added (10 µl in 25 ml TBS) and the filter was incubated for 3 hours at 37°C with slow shaking (60 rpm).

6. The filter was washed with TBST thrice, 5 minutes each and finally washed with TBS.

D. Detection assay

Materials

1. Solution I : 45 mg 4 chloro - 1 Naphthol in 15 ml methanol (AR)
2. Solution II : 100 ml of TBS + 80 ml of H_2O_2
3. Solution III : Mix Solution I and II before use, 10 ml for one filter.

Protocol

1. Solution III was prepared just before use.
2. Solution III (10 ml) was added to each plate.
3. After reaction with HRP conjugated secondary antibody, the positive colonies appeared as magenta rings.

E. Picking of positive clones

1. The membrane showing positive signal was matched with the plates, the position of the positive clone was clearly marked with the marker pen.
2. The marked colony was picked with a toothpick and inoculated in 5 ml LB ampicillin (50 ppm) broth.
3. It was incubated for 12 hours at 37°C with 150 rpm shaking.
4. The culture was plated on LB ampicillin by following the spread plate method, and incubated at 37°C for 12 hours.
5. Grown colonies were collected and arranged in another plate for second immunoscreening.

6. The second immunoscreening was done by following the same method as in the first immunoscreening.
7. The positive clones of second immunoscreening was picked and grown in LB ampicillin (50 ppm) broth.
8. Again this was plated and third immunoscreening was carried out.
9. Positive clones were selected and glycerol stocks were maintained in freezer.

3.2.9. Plasmid isolation

The plasmid DNA from the putative clone was isolated using miniprep method for subsequent analysis. It was essentially done as described by Birnboim and Doly (1979).

Materials

1. Solution I : Glucose, 50 mM, EDTA 10 mM, Tris-Cl, 25 mM pH 8.0
2. Solution II : NaOH 0.2 N; SDS (1 %).
3. Solution III : Sodium acetate, 3M , pH 4.6.
4. DNAse free RNase A: 20 μg per ml.
5. Chloroform : Isoamyl alcohol (24:1).
6. Ethanol (95 % and 70 %).
7. Isopropanol.
8. TE buffer: Tris, 10 mM pH 8.0; EDTA 1 mM.

Protocol

1. The selected colony was inoculated in 5ml LB ampicillin broth and incubated at 37°C, with 150 rpm shaking.
2. The culture was taken in eppendorf tube and kept as glycerol stock.

3. Cells were harvested by centrifugation at 5000 x g for 5 minutes at 4°C.
4. Supernatant was discarded pellet was resuspended in 100 µl of ice cold solution I, mixed properly and kept in crushed ice for 5 minutes
5. Freshly prepared solution II (200 µl) was added, mixed gently, kept in room temperature for five minutes.
6. Ice cold solution III (150 µl) was added mixed gently and kept in ice for 5 minutes.
7. Then it was centrifuged for 10 minutes and supernatant was transferred carefully to a fresh tube and 500 µl of cold iso-propanol was added and left for 30 minutes at room temperature.
8. It was again centrifuged for 5 minutes at 5000 x g. Supernatant was discarded and pellet was dissolved in 100 µl of TE buffer.
9. NaCl (5 µl) and of chloroform : 1soamyl alcohol (24:1) (100 µl) was added, centrifuged, and supernatant was taken in a new tube.
10. Ethanol (95 %) (300 µl) was added and kept overnight for precipitation at -20°C /freezer.
11. It was centrifuged at 12000 x g for 3 minutes and pellet was washed with ethanol (70 %) (200 µl).
12. TE (50 µl) (pH 8.0) containing DNAase free pancreatic RNase (20 µg/ml) was added and centrifuged for 5 minutes at 5000 x g. Supernatant was collected.
13. Further steps followed as described in steps 9, 10, 11.
14. Pellet was dried at room temperature for 30 minutes and dissolved in TE (40 µl) buffer.
15. Agarose gel (1.2 %) was casted and 5 µl of plasmid preparation with 3 µl loading dye was loaded for checking the plasmid profile.

3.2.10. Agarose gel electrophoresis

The isolated plasmids were checked on submarine agarose gel electrophoresis. It was done essentially as described by Sharp *et al.* (1973).

Protocol

The gel template was cleaned and both the ends were closed with cellophane tapes. 1.2 per cent agarose was prepared in 1 X TBE (10 X TBE buffer : Tris-HCl, 0.8 M; Boric acid, 0.002 M; EDTA, 0.02 M) and cooled to 50°C. 2 µl of Ethidium bromide (10 mg/ml) was added to the melted agarose. TBE was added to a final concentration of 1 X. The gel solution was poured into the template and appropriate sized comb was set in place. The gel was allowed to solidify for about 30 minutes. When the gel was ready, the cellophane tapes and the comb were removed. The gel along with the template was placed in the platform of the submarine gel apparatus "well" side towards the cathode. 1 X TBE was poured in both the buffer chamber so that the gel was submerged under about 1 mm buffer. Plasmid samples were mixed with 0.1 volume of 10 X sample buffer (10 X sample buffer : Ficoll, 50 % ; EDTA, 1 mM ; Xylene cyanole FF 0.4 %, Bromophenol blue, 0.4 %) and loaded in the well with the help of a micropipette. The apparatus was connected to the power supply unit and power was applied at 5 V/cm. Plasmid bands where visualized by UV transillumination.

3.2.11. Polymerase Chain Reaction (PCR) amplification of cDNA clones

The putative cDNA clones were amplified by polymerase chain reaction using M13 primers (forward and reverse).

Protocol

1. In 250 µl sterile microfuge tubes, the PCR cocktail was prepared.

Plasmid DNA (100 ηg)	1.0 µl
Sterile H_2O	14.5µl
Taq DNA Polymerase	0.5 µl
10 x Taq buffer	2.0 µl
2 mM dNTP mixture	1.0 µl
M13 forward primer (5 ηg)	0.5 µl
M 13 reverse primer (5 ηg)	0.5 µl
Total	20.0 µl

2. The reaction mixture was covered by 20 µl mineral oil and placed in the thermocycler.
3. Amplification of DNA was achieved by the programme as shown in figure 2.
4. The PCR products were analysed on 1.2 per cent agarose gels.

3.2.12. Restriction digestion of Plasmid DNA

Protocol

1. The reagents required and plasmid DNA were taken out of the freezer and thawed on ice.
2. In sterile tubes, the reaction was mixed as follows

Plasmid DNA (0.5 µg/µl)	4.0 µl
10X restriction buffer	3.0 µl
Restriction enzyme (4 units)	2.0 µl
Water (sterile)	21.0 µl
Total volume	30.0 µl

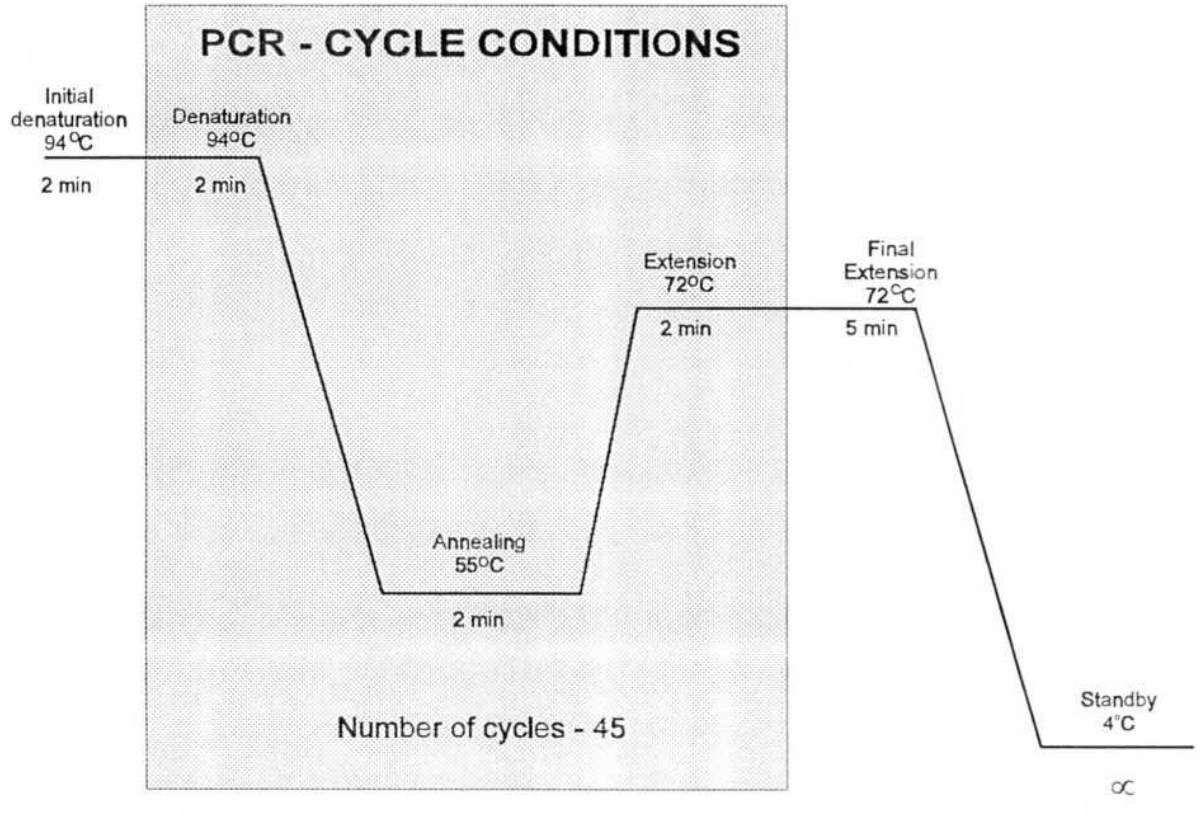


Figure 2. The PCR condition for DNA amplification

3. The reaction components were mixed gently by tapping the bottom of the microfuge tube and again gathered at the bottom of the tube by gentle spinning.
4. The tubes were sealed with parafilm and incubated at 37°C overnight.
5. Next day the sample was checked in agarose gel (1.5 %) electrophoresis (3.2.10).

3.2.13. Southern transfer

It was essentially done as described by Southern (1975).

Protocol

1. The restricted fragments run on a gel were denatured by soaking in several volume of 0.4 N NaOH for 20 minutes at room temperature with constant stirring.
2. A platform was made in the 0.4 N NaOH filled dish with a glass plate and covered with a Whatman 3 MM paper touching the bottom of the dish on both the sides.
3. The gel was placed on top of the Whatman 3 MM filter paper and a prewetted positive nylon membrane (Hybond from Amersham) cut exactly to the size was placed on top carefully avoiding air bubbles.
4. Two pieces of wet Whatman 3 MM paper, cut to the exact size of the gel was placed on top of the nylon membrane.
5. A stack of paper towel slightly smaller than 3 MM paper were placed carefully on top and covered with a glass plate and weighed down with a 1 kilogram weight.

6. The transfer of DNA was allowed to proceed for 16 hours by capillary action.
7. Next day the set up was dismantled and the nylon membrane was carefully removed marking the position of the wells on the gel by a pencil. The membrane was washed in 2 X SSC (Sodium citrate 0.3 M; Sodium chloride 0.15 M) for few minutes to remove any agarose bit sticking to it.
8. It was wrapped in saran wrap and kept in 4°C.

3.2.14.1. Preparation of radio active probe

It was essentially done as described by Feinberg and Vogelstein (1983) and Feinberg and Vogelstein (1984). Molecules to be used as probe for hybridisation of southern blots were radio labelled by the random primer labelling method of using (α^{32}P) dCTP.

Protocol

The PCR amplified product (as described in 3.2.11) was eluted from agarose gel. The DNA band (1.2 ± 0.2 kb fragment) was cut and passed through 2 ml syringe with phenol and kept at 4°C overnight. Next day it was centrifuged. Supernatant was mixed with ethanol (95 %) and 3 M Sodium acetate for DNA precipitation. Finally the DNA pellet was washed with ethanol (70 %) and dissolved in of TE buffer (20 µl) (pH 8.0).

1. The template DNA (25ηg) was dissolved in sterile water at 5 µl/ml and denatured by boiling for 3 minutes and immediately chilling on ice.
2. The labelling reaction was mixed in the following order.

Labeling 5 X buffer (Tris-Cl, 250mM, pH 8.0, $MgCl_2$, 25 mM; DTT 10 mM, HEPES, 1mM, pH 6.6).	10 µl
dNTP mix (dATP, dTTP, dGTP, 20 mM each)	2 µl
Random primer (2.5 ng/µl)	1 µl
DNA template (denatured)(25 ηg)	5 µl
Acylated BSA (10 mg/ml)	2 µl
(α^{32}P) dCTP	5 µl
Klenow enzyme	5 units
sterile water to make up the volume	50 µl

3. It was incubated at 37°C for 60 minutes. Reaction was terminated by boiling for 3 minutes and snap chilling in ice.

3.2.14.2. Hybridization of Southern filters

The blot was hybridized to labelled DNA probes for recognition of specific DNA fragments of interest (Sambrook *et al.*, 1989).

Materials

1. Denhardt's solution (50 X) Ficoll, 1%; PVP (polyvinyl pyrollidine), 1%; BSA (Bovine Serum Albumin) 1 %
2. Prehybridisation buffer
   SSC, 6 X; SDS (0.5 %); Denhardt's solution (5 X); Denatured salmon sperm DNA, 100 µg/ml and EDTA 10 mM.
3. Hybridization solution: labelled denatured probe DNA in pre-hybridization buffer.
4. Washing buffer : 2 X SSC, SDS (0.5 %); 1 X SSC, SDS (0.5 %); 5 X SSC, SDS (0.5 %)

Protocol

1. The blot was floated on 2 X SSC and immersed for two minutes and transferred to hybridization bottles.
2. Prewarmed (65°C) prehybridization solution (10 ml) was poured in the bottle and the bottle was transferred to the hybridization chamber
3. The blots were allowed to incubate for 4 hours in the pre-hybridization solution at 65°C.
4. The pre-hybridization solution was removed and the probe was added to 10 ml of fresh hybridization solution into the tube taking care not to pour the solution directly on the blot.
5. The blots were hybridized for 16 hours at 65°C.
6. The bottle was removed from the chamber and blots were removed carefully and incubated in washing buffer at room temperature for 15 minutes.
7. The blots were given high stringency washes.
8. Blots were covered by saran wrap and autoradiographed using kodak X AR2 film with intensifying screen.
9. The X-ray film was developed by using developer and fixer solution.

Results

Chapter - IV

RESULTS

4.1. Extraction and estimation of proteins from rice

Proteins were extracted from whole rice seed, aleurone (rice surface) layer and white seed (endosperm only) by using seed protein extraction buffer (pH 7.4). In addition, proteins were isolated from leaf samples of different stages (20 days after sowing, tillering stage and boot leaf stage) by using phosphate buffer (pH 7.0) and panicle tissue of different stages (flowering, milky, dough and maturity) by using seed protein extraction buffer (pH 7.4). These proteins were subjected to TCA (10 %) precipitation and the pellets were dissolved in their respective extraction buffers.

Proteins from different samples were measured by the dye binding method of Bradford (1976). The concentration of protein was expressed in mg/g of tissue. The protein concentration of aleurone (rice surface) layer, whole seed and white seed (endosperm only) were 100 mg/g, 80 mg/g and 30 mg/g of tissue respectively.

4.2. SDS-PAGE analysis

Proteins extracted from whole rice seed, aleurone (rice surface) layer and white seed (endosperm only) were resolved by SDS-PAGE (12%). The bands were detected after staining with coomassie brilliant blue R 250 followed by destaining.

Protein profile of all the three-protein samples were shown in plate (1, 2 and 3). The whole seed proteins showed bands of molecular weight

Plate 1. SDS-PAGE analysis of rice aleurone (surface), whole seed and white seed (endosperm only) proteins.

Proteins were extracted from whole seed, aleurone (rice surface) layer and the white seed (endosperm only) in seed protein extraction buffer pH 7.4. The crude supernatant was loaded (100 μg) on SDS-PAGE (12 %)and stained with coomassie brilliant blue R 250. M represents the protein marker in kDa. The arrow (→) indicates 30 kDa and (→♣) indicates 15 kDa polypeptide band respectively. Numbers in the right side refer to the apparent molecular weight in kilodalton of the major polypeptides in the protein marker. Lane 1-3: Rice aleurone (surface) layer protein ; Lane 4-6: Rice whole seed protein. Lane 7-9: White seed (endosperm only) protein.

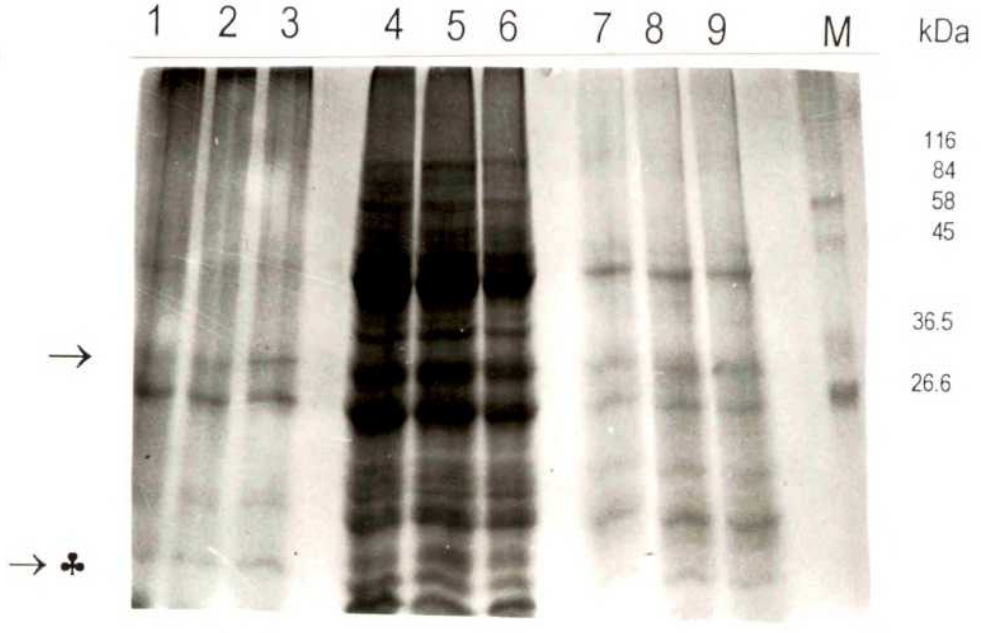


Plate.1

Plate 2. SDS-PAGE analysis of rice aleurone (surface) layer and white seed (endosperm only) proteins.

Proteins were extracted from the aleurone (rice surface) layer and white seed (endosperm only) in seed protein extraction buffer pH 7.4. The crude supernatant was loaded (100 μg) in SDS-PAGE (12 %) and stained with Coomassie brilliant blue R 250. M denotes the protein marker in kDa. The arrow (→) indicates 15 kDa polypeptide band. Numbers in the right side refer to the apparent molecular weight in kilodaltons of major polypeptide in the protein marker. Lane 1-2: Rice aleurone (surface) layer protein. Lane 3-4: Commercial rice white seed (endosperm only) protein

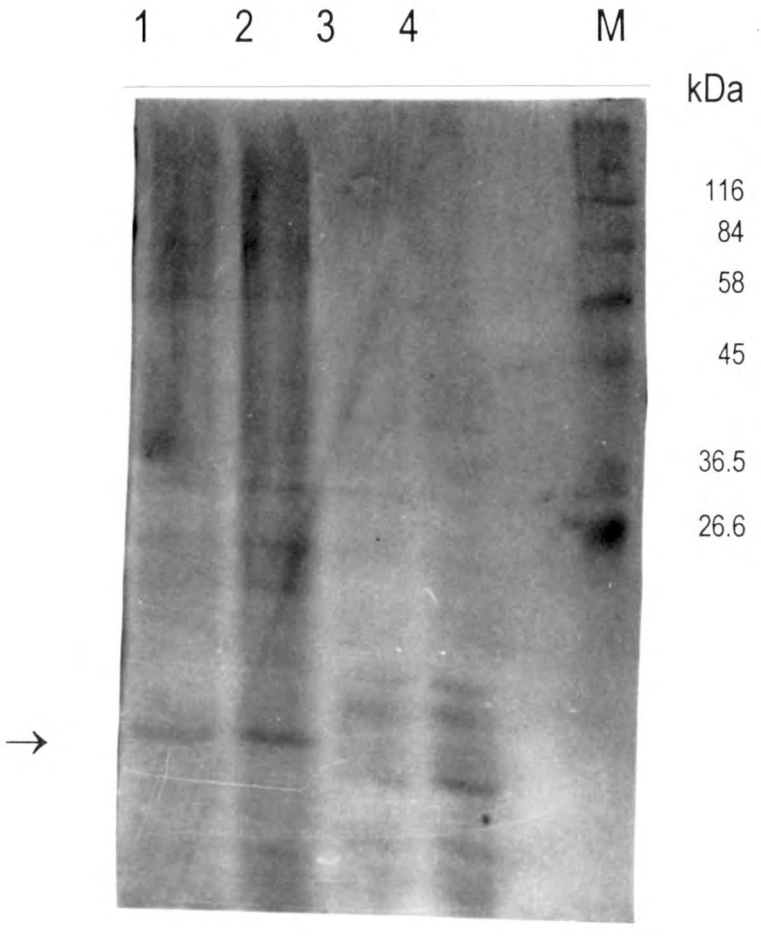


Plate.2

Plate 3. SDS-PAGE analysis of rice aleurone (surface) layer whole seed and white seed (endosperm only) proteins.

Proteins were extracted from whole seed, aleurone layer and white seed by using seed protein extraction buffer, pH 7.4. The crude supernatant was loaded (100 μg) on SDS-PAGE (12 %) and stained with Coomassie brilliant blue R 250. Arrow (→) indicates the 30 kDa polypeptide present in rice seed. M_1 represents the high molecular weight marker, M_2 represents the low molecular weight marker, numbers in the margin refer to the apparent molecular weight in kilodalton of the major polypeptides. Lane 1: Whole seed protein. Lane 2: Rice aleurone (surface) layer protein. Lane 3 : white seed (endosperm only) protein.

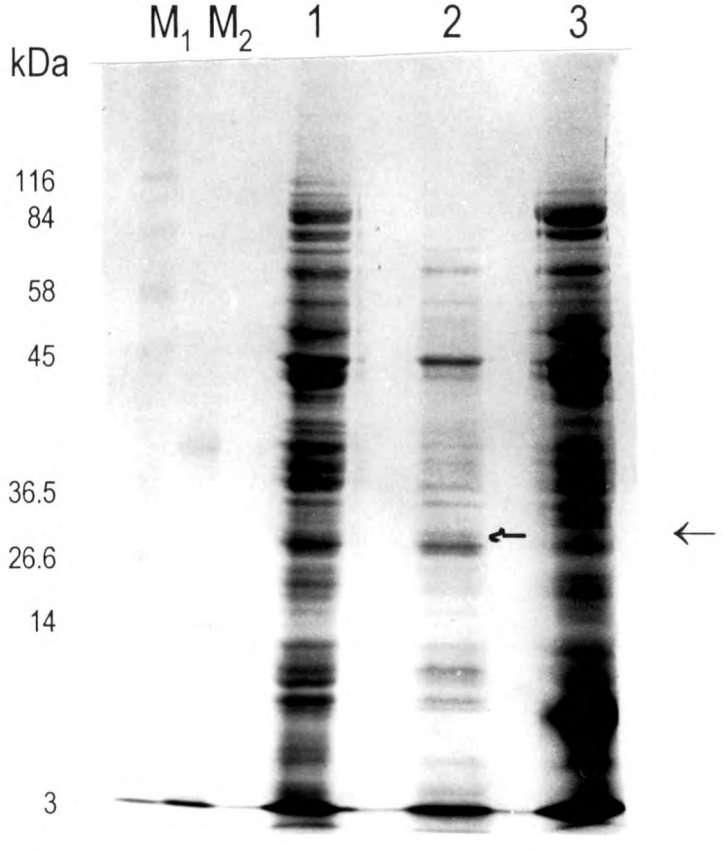


Plate.3

ranging from 3 kDa to 110 kDa (Plate 1 and 3). The lane in which rice aleurone (surface) proteins was loaded showed distinct bands of molecular weight 43 kDa, 30 kDa, 26 kDa, 19 kDa and 15 kDa (Plate 1). Among them the 30 kDa (Plate 1 and 3) and 15 kDa (Plate 2) bands were prominent and unique to aleurone layers. Lanes corresponding to white seed (endosperm only) protein samples also showed several bands of molecular weight ranging from 10 kDa to 84 kDa (Plate 1 and 3), among them a 22 kDa band was found, which was absent in aleurone layer specific protein sample (Plate 1).

The above result indicated that there were two main distinct bands present in the aleurone (rice surface) layer protein specifically, but absent in white seed (only endosperm). They were of 30 kDa and 15 kDa in size. Like wise, white seed protein banding pattern indicated the presence of an unique 22 kDa polypeptide.

4.3. Analysis of rice protein by RP-HPLC

The extracted protein samples were subjected to ammonium sulphate precipitation (20-75 %). The precipitate was dissolved in seed protein extraction buffer, dialyzed against distilled water and lyophilized. The sample was dissolved in distilled water and filtered through 0.45 μm membrane filter (Durapore). Approximately 50 μg of protein was loaded to a BDS C18 column (Varian). The column was run at the flow rate of 1 ml/minute and the eluted samples were collected at different time interval. The HPLC profile displayed various peaks for different samples. Each ammonium sulphate precipitated protein sample produced a different profile based on the absorbance at 280 nm (Figure 3).

Figure 3. Elution profile of rice seed protein by RP-HPLC.

RP-HPLC elution profile of rice aleurone (surface) layer protein (---), whole seed protein (-·-), and white seed (endosperm only) (—) protein using a BDS C-18 column (Varion). Proteins were extracted in seed protein extraction buffer pH 7.4 (3.2.1.1.) The flow rate was maintained at 1ml/minute. Arrow (↓) indicates the unique peak present in rice aleurone (surface) protein

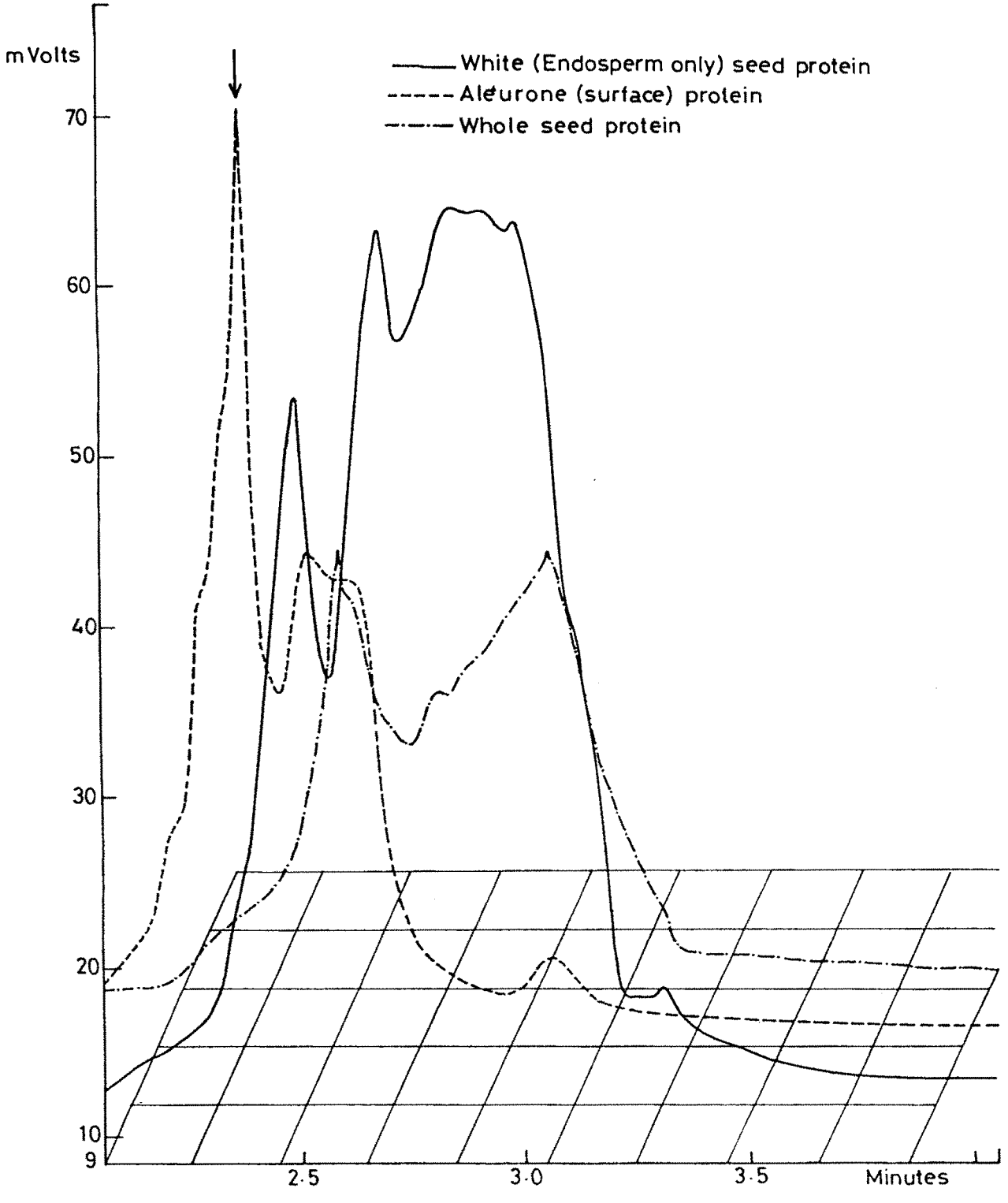


Figure 3. Elution profile of rice seed proteins by RP-HPLC

HPLC profile of whole seed protein sample indicated three major peaks, one narrow sharp, and two broad peaks. Similarly in aleurone (rice surface) layer protein samples three major peaks were found, one peak was sharp and other two were broad peaks whereas white seed (endosperm only) protein sample showed two sharp and one broad peak.

The above results indicated that the aleurone layer protein sample has a unique polypeptide. Most of the peaks of the whole seed protein and white seed (only endosperm) sample were found to be similar. Among the three protein samples, aleurone protein was distinctly separated from others by a sharp major peak which was absent in white (endosperm only) but not clearly found in whole seed protein sample.

4.4. Raising antiserum

Rice seed aleurone (surface) specific proteins were extracted by the method (3.2.1.1.) described by Nakase *et al.* (1996). Antiserum was raised in New Zealand white rabbit against the aleurone (rice surface) layer specific proteins (30 $\pm$ 2 kDa). The titre of the antiserum and its reactivity with seed aleurone (surface) proteins were estimated by antigen excess antibody capture Enzyme Linked Immuno-Sorbent Assay (ELISA) in microtitre plate.

The antiserum collected after the first booster injection showed reaction against both aleurone (rice surface) layer protein as well as white seed (endosperm only) proteins. The intensity of the reaction was however considerably higher against aleurone (rice surface) layer specific proteins. Significant strong reactivity was observed at 1:500 dilution as determined by ELISA test using antiserum collected after second booster dose (data not

shown). The intensity of the reaction decreased, as the antiserum was further diluted. The antiserum collected after further boosts also reacted similarly against both white seed (endosperm only) and aleurone specific proteins. To increase the specificity of anti- 30 ± 2 kDa aleurone protein antiserum, the initial antiserum was passed through cyanogen bromide activated sepharose 4B column with white seed (endosperm only) proteins as ligands. The purified antiserum was tested for its titre and specificity against aleurone (rice surface) specific proteins. Relatively strong reactivity was observed at 1:100 dilution (1:1000 effective dilution) after purification.

4.5. Western blot analysis

Western blotting analysis was performed with proteins extracted from rice seed aleurone (surface) layer, and white seed (endosperm only). The anti-30 ± 2 kDa aleurone protein antiserum was used to detect the aleurone specific polypeptides.

The crude protein extracts from aleurone (rice surface) layer and white seed (endosperm only) were electrophoresed on SDS-PAGE (12 %) and transblotted on nitrocellulose membrane as described in 3.2.6.2 to test the aleurone specificity of the antiserum. The nitrocellulose membrane (blot) was incubated with the purified antiserum at 1:50 dilution. The antigen antibody complexes on the blot were recognized by using horse radish peroxidase conjugated anti rabbit IgG as described in 3.2.6.4.

The results of the western blot analysis of protein samples from aleurone (rice surface) layer and white seed (endosperm only) were shown in plate 4. The lane in which aleurone (rice surface) layer protein sample was

Plate 4. Western blot analysis of rice aleurone (surface) protein and white seed (endosperm only) protein with anti- 30 ± 2 kDa aleurone (surface) protein antiserum.

Proteins were extracted from aleurone (surface) layer and white seed (endosperm only) were separated on SDS-PAGE (12 %) and transblotted on nitrocellulose membrane. It was probed with aleurone specific antiserum. Bands were detected by anti rabbit IgG conjugated horse radish peroxidase.

a) Lane 1: Rice aleurone (surface) protein. Lane 2: white seed (endosperm only) protein. Arrow (→) indicates the 30 kDa protein. M represents protein marker in kDa. Numbers in the middle refer to the apparent molecular weight in kilodalton of the major polypeptide.

b) Lane 1: Rice aleurone (surface) protein. Lane 2: white seed (endosperm only) protein. Arrow (→) indicates the 30 kDa and (→♣) indicates the 15 kDa protein.

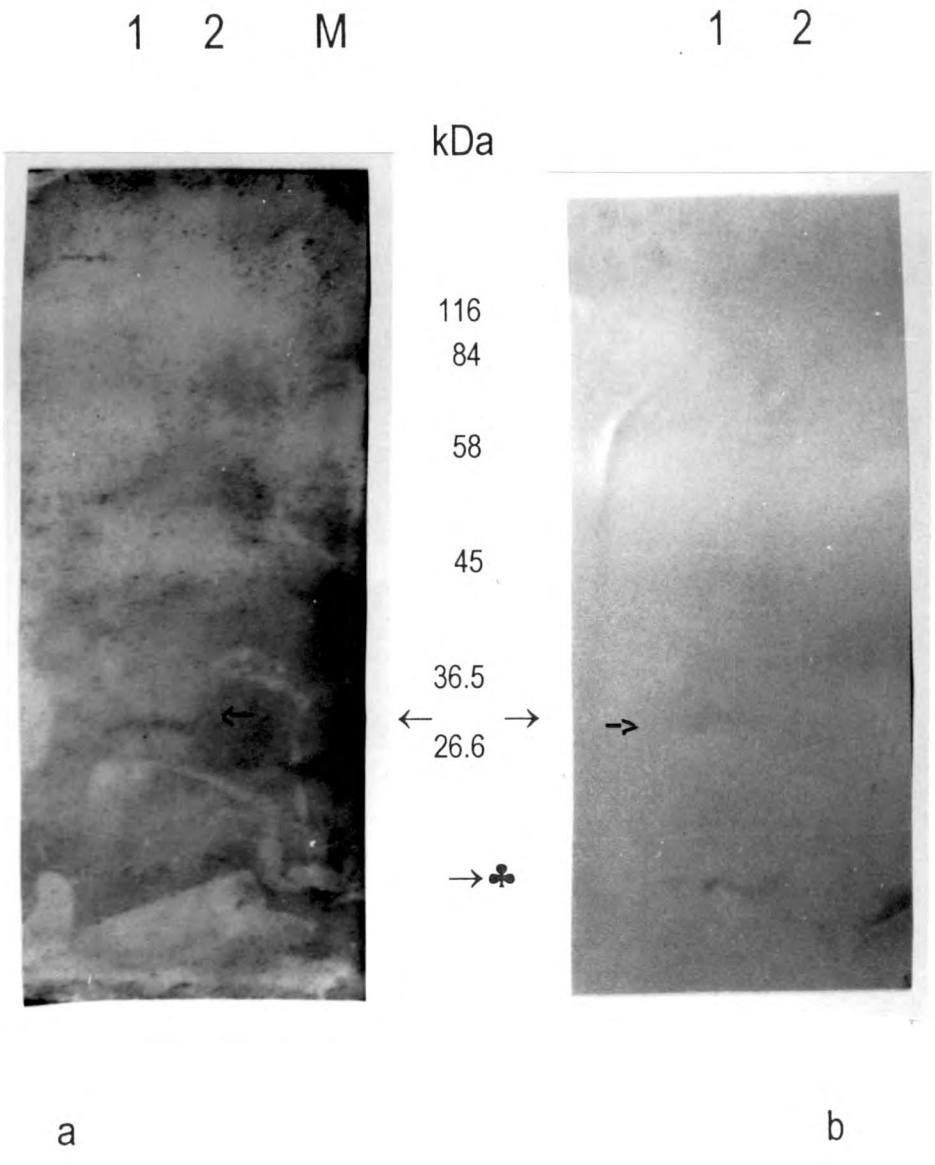


Plate. 4a & 4b

loaded showed signals at 30 kDa (Plate 4 a & b). The anti- 30 ± 2 kDa aleurone protein antiserum not only detected the expected 30 kDa proteins but also a 15 kDa proteins (Plate 4b). However, the 15 kDa band was not always recognized by the anti- 30 ± 2 kDa aleurone protein antiserum.

4.6. Dot blot analysis

Dot blot analysis was performed to study the pattern of expression of rice aleurone protein (rice surface) in different stages of crop growth. Proteins were extracted from seven different stages (20 days after sowing, tillering, boot leaf, flowering, milky, dough and maturity stages). Panicle tissues were selected in flowering, milky dough and maturity stages. Equal quantity of protein (30 μg) was loaded on each slot on the nitrocellulose membrane. The samples were probed with anti- 30 ± 2 kDa aleurone protein antiserum and positive signals were recognized by using HRP conjugated IgG.

The results are shown in plate 5. The boot leaf stage proteins (Plate 5a & 5b) showed very faint signals, where as the protein of flowering, milky, dough and maturity stages showed intense signal. It was observed that the signal intensity gradually increased from boot leaf stage to maturity stages, stating an increase in aleurone (rice surface) layer specific protein during flowering and subsequent stages. The aleurone (rice surface) protein expression was low during 20 days of growth and tillering stages (Plate 5b). The expression of aleurone (rice surface) protein increases gradually as the crop attains maturity (Plate 5 a & b).

4.7. Immunoscreening of cDNA library

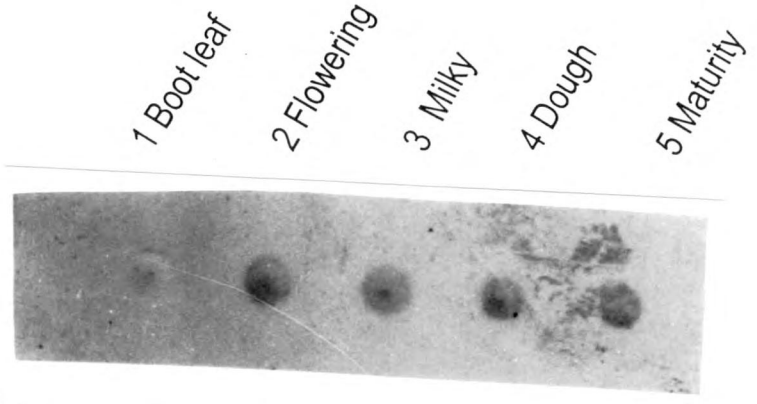
An attempt was made to isolate the cDNA clones coding for the aleurone (rice surface) layer specific proteins by screening a rice cDNA

Plate 5. Dot blot analysis of proteins isolated from samples at different stages of crop growth.

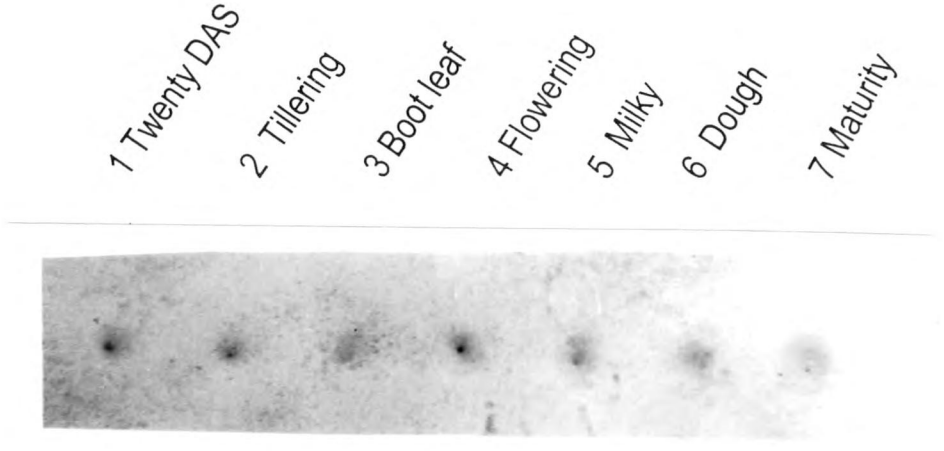
Proteins were extracted from different stages such as 20 days after sowing, tillering, boot leaf (phosphate buffer pH 7.0), flowering, milky, dough and maturity stage (seed protein extraction buffer pH 7.4). Protein samples were quantified by Bradford method and equal quantity (30 μg) of proteins were loaded in each slot. The membrane was probed with rice aleurone specific antiserum. Signals were detected by anti-rabbit Ig G conjugated horse radish peroxidase.

a) Lane 1-5: Boot leaf, flowering, milky, dough and maturity stages respectively.

b) Lane 1- 7: 20 days after sowing (20 DAS), tillering, boot leaf, flowering, milky dough and maturity respectively.



(a)



(b)

Plate. 5a & 5b

Plate 6. Immunostained putative cDNA clones on nitrocellulose filter (first immunoscreening).

IPTG presoaked nitrocellulose membrane containing proteins expressed by the colonies were probed with anti- 30 $\pm$ 2 kDa aleurone (surface) protein antiserum. One positive signal was detected by anti rabbit IgG conjugated horse radish peroxidase using hydrogen peroxide as substrate. Arrow (➔) indicates the putative cDNA clone hybridized to the antibody after expressing in terms of protein.

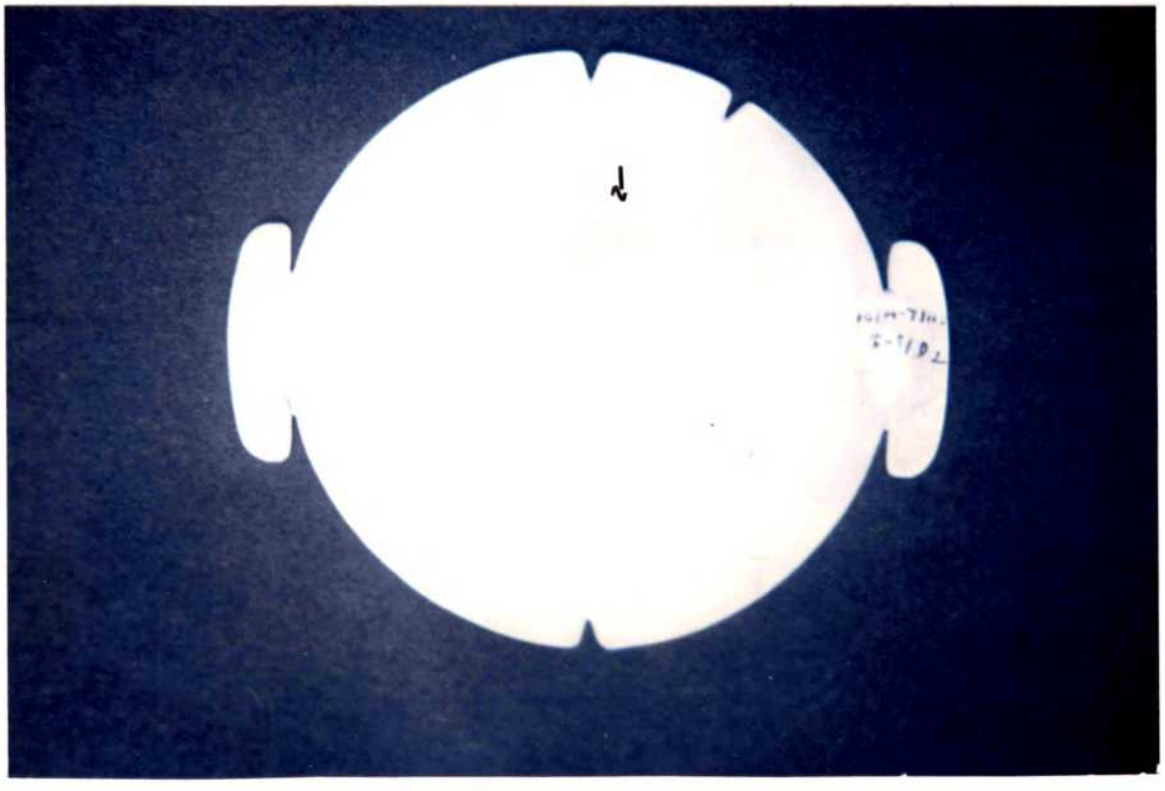


Plate. 6

Plate 7. Immunostained putative cDNA clones on nitrocellulose filter (second immunoscreening).

IPTG presoaked nitrocellulose membrane containing proteins expressed by the colonies were probed with anti-30 $\pm$ 2 kDa aleurone (surface) protein antiserum. Signals were detected by anti rabbit Ig G conjugated horse radish peroxidase using hydrogen peroxide as substrate. Membrane showing the putative cDNA clones hybridized to the antibody after expressing in terms of protein. Most of the clones were positive at this stage as shown in the plate.

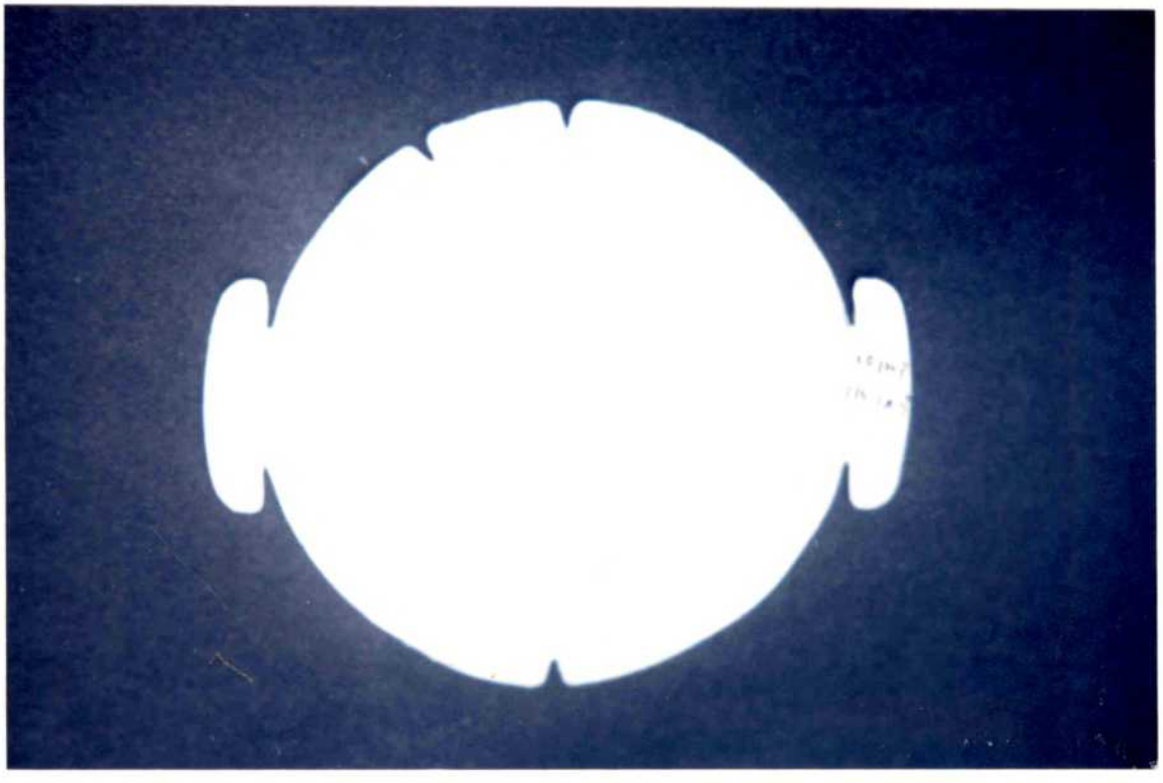


Plate. 7

Plate 8. Immunostained putative cDNA clones on nitrocellulose filter (third immunoscreening).

IPTG presoaked nitrocellulose membrane containing proteins expressed by the colonies were probed with anti-30 $\pm$ 2 kDa aleurone (surface) protein antiserum. Signals were detected by anti rabbit Ig G conjugated Horse radish peroxidase using hydrogen peroxide as substrate. Membrane showing the putative cDNA clones hybridized to the antibody after expressing in terms of protein. All the clones were positive as shown in the plate.

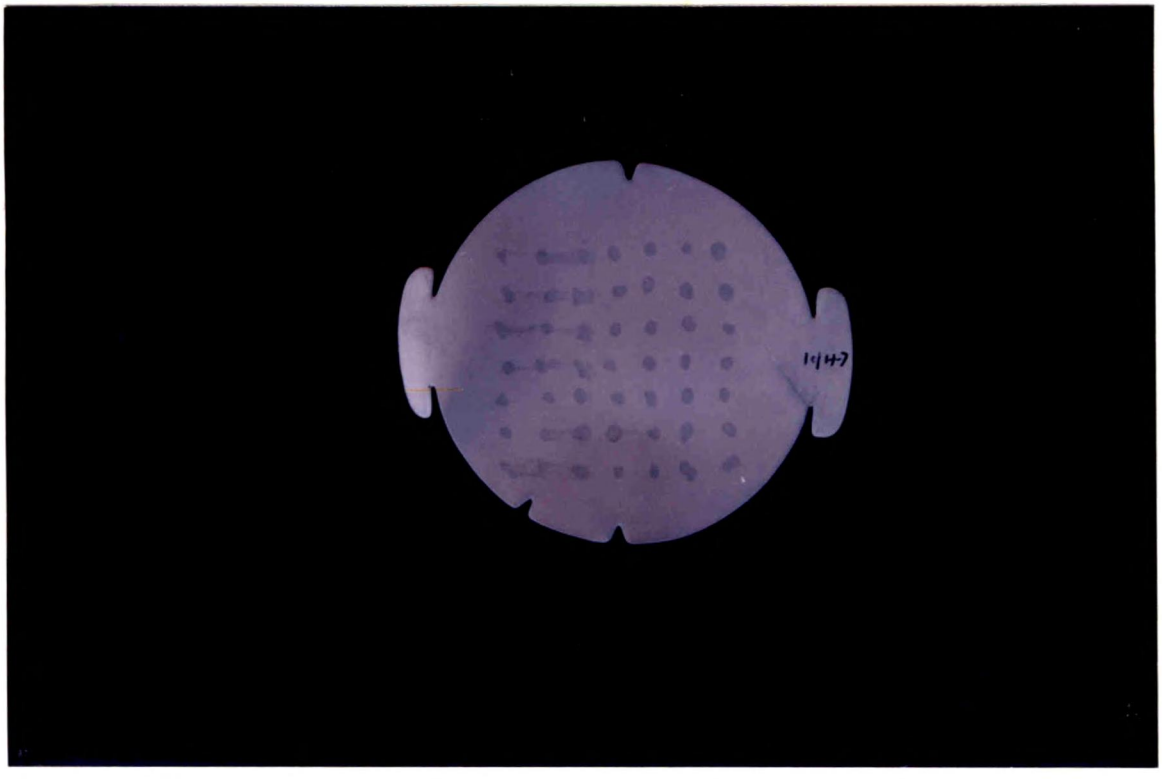


Plate.8

cDNA LIBRARY SCREENING
FLOW CHART

FIRST SCREENING

Plate Numbers

1	2	3	4	5	6	7	8	9	10	11	12

No. of positive clone 1/1200

Positive clone selected 10 /H-7

SECOND SCREENING

No. of positive clone 22/250

Positive clone selected 10/H-7/100

THIRD SCREENING

No. of positive clones **ALL POSITIVE**

10/H-7/100 (1...49)
(Homogenized)

Positive clone selected 10/H-7/100 (A, B, C, D, E & F)

Figure 4. Flow diagram of immunoscreening strategy of the rice seed specific cDNA library using rice aleurone specific antiserum. Rice seed cDNA library was screened and putative cDNA clones **(10/H-7/100)** were identified after third screening.

library made from rice immature seed of variety Nootripathu. The rice cDNA library was immunoscreened with the purified anti- 30 ± 2 kDa aleurone protein antiserum (3.2.8) to identify putative cDNA clones.

The initial library titre was calculated by plating the library in LB ampicillin (50 ppm) agar plates at different dilutions. One fourth of the cDNA library (around 1200 clones) was screened immunologically using the purified anti- 30 ± 2 kDa aleurone protein antiserum. The flow chart shown in figure 4 exhibits the screening strategy.

The primary screening of 1200 colonies, identified only one positively reacting colony (plate 6). In the secondary screening a total of 22 colonies were found to be positive in a population of 250 colonies (Plate 7). In the third screening, all the colonies were found to be positive, suggesting that all clones were similar and containing the same insert (Plate 8). Among these colonies six colonies were selected, randomly, for further analysis.

4.8. Isolation of plasmid DNA from the selected clones

The plasmid DNA isolated from the positive putative clones were electrophoresed on agarose gel (1.2 %) prestained with ethidium bromide. The bands were visualized by UV illumination. The plasmid profile of the 6 putative clones separated on a agarose gel (1.2 %) was shown in plate 9. Plasmids were found intact in all the lanes and showed various forms of a typical plasmid (supercoiled, covalently closed circular and open circular). The isolated plasmids were used for restriction digestion and PCR amplification (for preparing probes). They were further used for Southern hybridization reaction.

Plate 9. Plasmid DNA profile of the six putative cDNA clones.

Plasmid DNA was isolated from six positive clones by alkali lysis method (Birnboim and Doly, 1979). Isolated plasmids were separated on a agarose gel (1.2 %), which was prestained with ethidium bromide. DNA bands were visualized under UV illumination. All three forms of plasmid DNA (Supercoiled, covalently closed circular and open circular) were present. Lane 1-6: 10/H-7/100 (A, B, C, D, E & F) clones.

1 2 3 4 5 6

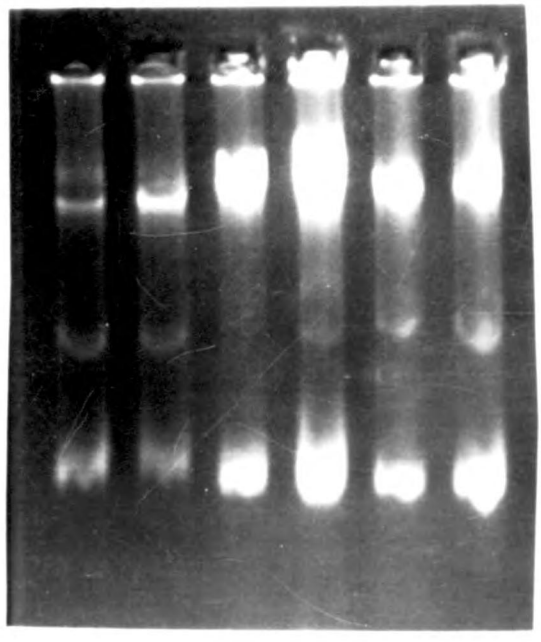


Plate. 9

4.9. PCR amplification of cDNA clones

The putative cDNA clones were amplified by polymerase chain reaction using M13 primers (forward and reverse) so that only the insert could be amplified. PCR products (10 µl) were analyzed on agarose gel (1.2 %) prestained with ethidium bromide. DNA bands were visualized by UV illumination.

The amplified product profile was shown in plate 10. Among the six cDNA clones used only, four clones showed the amplification of 1.2 ± 0.2 kb band. These 1.2 ±0.2 kb fragments were eluted from the agarose gel and used for probe preparation in Southern hybridization experiment.

In order to make a probe, one of the PCR amplified product was eluted from agarose gel. For eluting the (1.2 ± 0.2 kb) fragment the DNA band was cut and passed through a 2 ml syringe with phenol and kept at -80°C for over night. Supernatant was collected after centrifugation and mixed with ethanol (95 %) and 3 M sodium acetate for DNA precipitation. Finally the DNA pellet was washed with ethanol (70 %) and dissolved in 20 µl of TE buffer. The recovery of DNA was estimated to be 60-80 per cent.

4.10. Restriction digestion and Southern hybridization of restricted cDNA clones against PCR amplified product

The cDNA clones, which showed amplification in PCR, were taken up for characterization by Southern analysis. The clones were digested with the restriction enzyme *Eco*R I to release the inserted fragment (Plate 11a). Analysis of the restriction product on agarose gel (1.5 %) showed the release

Plate 10. PCR amplification of insert of the putative cDNA clone.

PCR amplified products of putative rice cDNA clones using M13 primer (forward and reverse) were electrophoresed on a agarose gel (1.2 %) prestained with ethidium bromide. Gel was illuminated under UV. 1.2 $\pm$ 0.2 kbp bands were observed in four lanes. Lane 1-6: 10/H-7/100 (A, B, C, D, E & F) clones, M represents DNA marker (1 kb ladder). Arrow ($\rightarrow$) indicates the 1.2 $\pm$ 0.2 kbp band.

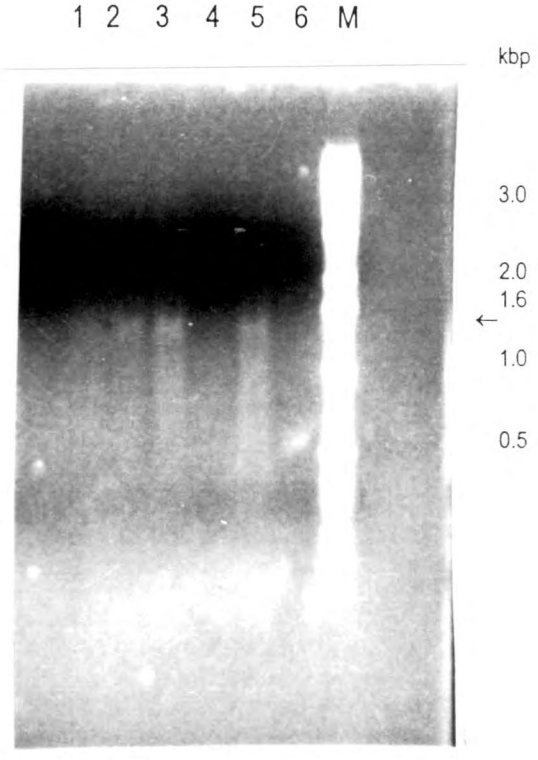


Plate. 10

Plate 11. Restriction digestion and Southern hybridization of putative cDNA clones.

11a) Putative cDNA clones were digested with restriction enzyme *Eco*R I electrophoresed in agarose gel (1.5 %).

11b) Southern blot hybridization of putative cDNA clones, digested with *Eco*R I, were electrophoresed on agarose gel (1.5 %) and blotted on the nylon membrane. The blots were probed with radiolabelled 1.2 $\pm$ 0.2 kb fragment and washed under high stringency condition. The *Eco*R I digest showed the signal of insert 1.2 $\pm$ 0.2 kb and vector 2.9 kb.

Lane 1-4: 10/H-7/100/ A, B, C and E and M represents the DNA marker (λ Hind III digest). Arrow ($\rightarrow$) indicates the 1.2 $\pm$ 0.2 kb band and ($\clubsuit$) indicates the 2.9 kb band.

Plate. 11a &11b

of the expected fragment. The DNA bands were transferred to Hybond positive nylon membrane for Southern hybridization (3.2.13).

The blot was probed with PCR amplified 1.2 ± 0.2 kb fragment radiolabelled by random primer labelling method using (α^{32}P) dCTP (Feinberg and Vogelstein, 1983). Hybridization was carried out using standard protocol (Sambrook *et al.*, 1989) and the blot was subjected to autoradiography (Plate 11b). The bands detected were of the same size as found in restriction analysis indicating that all the clones were identical.

Discussion

Chapter - V

DISCUSSION

Plants are critical components of the dietary food chain since they provide almost all essential minerals and organic nutrients to humans. Therefore, the nutritional health and well being of humans are dependent on plant foods. Estimates are that 40 per cent of the world's population do not receive adequate and balanced nutrients to meet their basic dietary requirements. Around 840 million people have insufficient intake of protein and calories. Hence, it is imperative to improve the status of plant food by understanding the genes involved in the nutritional upliftment.

Rice (*Oryza sativa* L) has one of the most nutritious protein contents among the cereals (Juliano, 1972). The protein content of milled and polished rice is relatively low, about 7 per cent at 14 per cent moisture. Rice is the main source of protein and energy in human diets in Asia as well as in parts of Africa and Latin America. In Asian diets, milled rice provides 40 to 80 per cent of the calorie and at least 40 per cent of the protein. Its bran, which includes the aleurone (rice surface) layer and the germ, contains more proteins (17-30 per cent) than the starchy endosperm.

The bran accounts for 5-9 per cent of rough rice weight in most milling processes. However, this portion of the rice grain comprises, 17-30 per cent of the total proteins in the whole grain (Houston, 1972). The aleurone (rice surface) layer present on the surface of the rice seed, gets removed during polishing and milling. This loss can be stopped or reduced by any of the following approaches. 1) Identifying genes specific to aleurone (rice surface)

layer protein and allowing it to express in the endosperm using an endosperm specific promoter. 2) Finding out the signal peptides responsible for the tissue specific expression of the protein present in the aleurone (rice surface) layer and manipulating them to express in the endosperm, a targeting expression approach. Such transgenic plants will express this protein throughout the endosperm and there will not be any loss during polishing and milling processes.

In this work, first approach was chosen and efforts were made to identify the aleurone (rice surface) layer specific proteins present in the rice seed. Further the cDNA clones coding for the related protein was identified after raising antibodies for the purified aleurone (rice surface) layer protein.

5.1. Protein characterization

A red rice variety Nootripathu was used in the experiment for easy differentiation of the aleurone (rice surface) layer (red) and endosperm (white). Total proteins were extracted from whole rice seed, aleurone (rice surface) layer, and white seed (endosperm only). The extracted crude sample was subjected to TCA (10 %) precipitation with an aim to precipitate only proteins and discard any other materials like carbohydrate, which are often carried along with protein during grinding.

SDS-PAGE analysis was performed as described by Laemmli (1970) to identify and characterize the proteins present in rice whole seed, aleurone (rice surface) layer specific and white seed (Plate 1, 2 and 3). Yamagata *et al.* (1982) has successfully used SDS-PAGE system for the characterization of rice protein. They have identified two major set of polypeptides (37 kDa and 22 kDa) as rice glutelins. Kim and Okita (1988 a) have reported the presence

of 51 kDa and 21-22 kDa glutelin proteins as well as contaminating 14 kDa prolamin in SDS-PAGE fractionation.

In the present study, a complex protein profile was observed both in rice whole seed and white seed (endosperm only) (Plate 3). Numerous polypeptides ranging from 3 kDa to 110 kDa were observed at varying intensity. Similar results were also reported for milled rice glutelin in several rice commercial varieties (IRRI annual report 1976). However, in the present study the aleurone (rice surface) layer showed limited number of bands, among them the 30 kDa and 15 kDa proteins were unique (Plates 1, 2 and 3). Similarly in barley a 12.5 kDa aleurone (rice surface) layer specific protein was identified which was not present in starchy endosperm and embroys (Jakobsen *et al.*, 1989).

Resurreccion *et al.* (1979) reported that sub-aleurone layer contains higher albumin content than in the inner endosperm. Presence of 33 kDa and 53 kDa along with 13-16 kDa low molecular weight proteins were reported in both the albumin and globulin fractions of rice (Yamagata *et al.*,1982). When pericarp and milled glutelin proteins were compared in IR 8, milled grain had 38 kDa, 25 kDa and 16 kDa proteins, among them pericarp showed just the 16 kDa protein. (IRRI Annual report 1976).

Aleurone (rice surface) layer specific 30 kDa and 15 kDa proteins were identified in this study, may not be fractions like albumin, globulin, glutelin. Since purified fractions of these proteins were reported to give a complex SDS-PAGE profile (Tanaka *et al.*, 1980 and Yamagata *et al.*, 1982).

In order to make a comparison and clearly distinguish seed aleurone (rice surface) layer specific proteins from white seed (endosperm only) RP- HPLC was carried out. The protein samples were loaded in C-18 column, (a reverse phase column). RP-HPLC separates protein on the basis of hydrophobic interactions among protein, matrix and mobile phase (Bietz, 1986 and Gooding, 1986). It elutes the protein on the basis of increase in hydrophobicity. RP-HPLC resolved the ammonium sulphate precipitated protein into numerous peaks. One major peak was found specifically in rice aleurone (surface) layer protein sample (Figure 3). The white seed protein showed three peaks, two sharp and one broad. The retention times of these peaks were different than that of aleurone (rice surface) layer protein peak. Hence it can be interpreted that the rice aleurone (rice surface) layer has got some unique proteins which was not found in white (endosperm only) seed. Feng *et al.* (1991) reported the use of C-18 column in RP- HPLC for separating wheat flour proteinaceous fractions (by ammonium sulphate precipitation) through RP-HPLC. More than 10 peaks in each fraction were resolved by RP-HPLC with the mixture of acetonitrile and H_2O as the mobile phase.

Further to study the translational control of the protein, developmental regulations and to isolate the corresponding gene, antibody was raised against the 30 $\pm$ 2 kDa aleurone (rice surface) layer specific protein in New Zealand white rabbit. In the ELISA test, though the antiserum was raised against the 30 $\pm$ 2 kDa aleurone layer protein, it also showed cross-reaction against white seed proteins. This could be attributed to the fact that aleurone proteins used for raising antibody, always contained some contaminant white seed proteins, as extrication of pure aleurone layer is practically impossible by conventional means. The cross-reacting antibodies present in the antiserum

intended to be against the 30 ± 2 kDa aleurone proteins, were depleted by passing through a column of $CNBr^-$ activated sepharose 4B with white seed proteins as ligands. Krishnan *et al.* (1992); Krishnan and Pueppke, (1993) and Krishnan and white, (1995) have used this strategy to make the antiserum α-globulin specific.

However, the overall titre of the enriched antibodies remained moderate since, very weak signal was detected in western blotting. The failure of the animal, a herbivore, to produce a high titre antibody against the 30 ± 2 kDa protein may be ascribed to the possible presence of the antigen and / or like proteins in its usual diet.

Western blot analysis was performed to detect the aleurone (rice surface) specific proteins present in the surface of the rice seeds. The result (Plate 4a) showed that the anti- 30 ± 2 kDa aleurone protein antiserum has recognized only one band, which was 30 kDa and no other band was detected in the lane where white (endosperm only) seed protein sample was loaded. However, occasionally we also observed a 15 kDa polypeptide band along with 30 kDa (Plate 4b). The detection of 15 kDa signal may either be due to the presence of cross reacting 15 kDa protein or the existence of a 15 kDa cleaved product of 30 kDa. Alternatively, the 15 kDa would also be the heteromer of 30 kDa, of the same native protein. However, this is yet to be confirmed.

Earlier, cross-reaction of a 51 kDa antibody of rice storage protein to 34-37 kDa and 21-22 kDa polypeptides were reported by Yamagata *et al.*

(1982); Luthe (1983) and Krishnan and Okita (1986). They have indicated that the 34-37 kDa and 21-22 kDa proteins were post-translationally proteolytic products of 51 kDa proteins.

To study the pattern of expression of the rice aleurone (surface) protein in different stages of crop (rice) growth, proteins were extracted from each of the seven different critical stages (20 days after sowing, tillering, boot leaf, flowering, milky, dough and maturity stages) of rice. Dot blot analysis of these proteins showed clear increase in intensity of the signal at developmental stages (Plate 5). A typical developmentally regulated pattern was observed. The result clearly indicated that the aleurone (rice surface) layer specific protein is present at all the stages of crop growth and the gene responsible for this protein expresses more at dough and maturity stages of the crop growth.

It was observed that the aleurone (rice surface) layer specific protein was expressed even in vegetative stage. However, in this stage the level of expression is low as less intense signal was detected in dot blot (Plate 5a & 5b). At the reproductive stage the expression of aleurone (rice surface) layer protein was found to be higher. The gene responsible for the expression of aleurone (rice surface) layer specific protein might be up regulated resulting in high expression of the specific proteins (Plate 5). This also throws light at translational control of the protein and this control seems to be developmental stage dependent. In addition tissue specific regulation is also possible. Since, we have used only the panicle tissues. The possible tissue specific expression of this protein is to be studied by analyzing proteins extracted from various tissues at different stages of reproductive phase.

5.2. Identification of aleurone specific cDNA clones.

Antibodies raised against rice aleurone (surface) 30 ± 2 kDa proteins were used in immunoscreening a rice seed cDNA library to identify the putative cDNA clones coding for the aleurone (rice surface) layer specific proteins as described in 3.2.8. In the first screening only one clone was identified as positive. In subsequent screenings, the number of positive putative clones increased. Thus an expected pattern of the clone enrichment was observed with an increase in number of screening. Only one type of cDNA clones were identified during immunoscreening as shown in the flow chart for immunoscreening strategy (Figure 4). Various clones of second and third screening are virtually derivatives or originated from a particular positive clone of first screening therefore it shows that the clones code for the same gene / protein.

Rice globulin cDNA was successfully isolated by immunoscreening a λgt11 library using affinity purified antibodies against rice storage protein called α globulin or 19 kDa globulin (Shorrosh *et al.*, 1992)

In addition there are other reports for identification of clones from seed library using antibody raised against glutelin and prolamin (Kim and Okita 1988 b). However it is yet to be confirmed by them whether isolated clones resemble to any of the known genes as sequence analysis has not been performed.

Further, to characterize the isolated cDNA clone, plasmid DNA was isolated from six positive putative clones. PCR amplification was carried out using M13 primers (forward and reverse) to find out whether the plasmids

have insert. The isolated recombinant plasmids were subjected to restriction digestion analysis to determine the size of the insert of the cDNA. As the cDNA library was made at *Eco*R I site to insert the random clones. *Eco*R I restriction enzyme was used to release the insert. *Eco*R I restriction of the plasmid DNAs isolated from the positive clone released a fragment of 1.2 ± 0.2 kb.

To check whether the 1.2 ± 0.2 kb fragments released from all the clones were the same, cross hybridization experiment was done. Southern blot experiment was performed with one of the clone used as probe to hybridize with others. The probe was synthesized by radiolabelling the amplified cDNA insert of one of the clones. Antoradiograph showed cross-reaction for all the bands of 1.2 ± 0.2 kb and vector 2.9 kb. It is natural, because probe was prepared from the amplified product, which had multicloning site, and this multicloning site is a part of the vector (pblue script). Because of that 2.9 kb vector band was also detected in autoradiography.

Theoretically, 1.2 ± 0.2 kb cDNA approximately codes for 50-52 kDa protein. Hence a 800 bp cDNA is sufficient to encode a 30 kDa polypeptide. However, the western blot and southern hybridization experiments indicated that the 1.2 ± 0.2 kb cDNA corresponds to the 30 kDa polypeptide.

Nevertheless, Shyur *et al.* (1992) have isolated a cDNA of 629 bp coding for a 16 kDa rice prolamin protein. Similarly, Shorrosh *et al.* (1992) have also reported the identification of a 750 bp cDNA coding for the 19 kDa globulin in rice. These reports suggest that the size of the cDNA can vary according to the

length of the 5' untranslated leader and 3' untranslated tail. Accordingly, in this study it is possible that 1.2 ± 0.2 kb cDNA could code for a rather small (30 kDa) polypeptide.

Alternatively, the size of the native protein could also influence the size of the cDNA, i.e., if the size of the native protein is larger, naturally the size of the cDNA encoding that protein will also be large. Krishnan and Okita (1986) have reported earlier that post translational proteolytic products could cross react in western blotting. Similarly, in this study there is a possibility that 30 kDa protein may be a product of post-translational proteolytic cleavage and native protein might be a larger one, hence a larger cDNA (1.2 ± 0.2 kb) was detected by the anti- 30 ± 2 kDa aleurone protein antiserum. In addition as mentioned earlier, if 30 kDa and 15 kDa are subunits of one protein it is always possible that the 1.2 ± 0.2 kb cDNA could code for the 45 kDa (30 and 15 kDa) heteromer.

The HPLC data (Figure 3) indirectly suggests that rice aleurone (surface) layer contain some novel specific polypeptide as one sharp peak eluted in the beginning and did not coincide to white (endosperm only) seed protein. However, since it is in native form, it is just a speculation as polypeptide and its size need to be confirmed by SDS-PAGE analysis. N-terminal sequencing of both 15 kDa and 30 kDa proteins and further sequencing of the isolated cDNA clone and later their comparison will solve this ambiguity.

5.3. Conclusion

Aleurone layer, which accounts for 5-9 per cent of rough rice weight, contains 17-30 per cent of the total proteins present in the whole grains. In addition, 95-100 per cent of the oil, 60-70 per cent of vitamins an 70-75 per cent of minerals and a high per cent of calcium and iron is present in this nutritive layer. Unfortunately, the polished rice, which we consume, does not contain this layer. This loss can be compensated by several approaches. One of them is to identify the aleurone-specific protein genes and use them in transgenic studies in order to express it in the endosperm.

In the present study, protein specific to aleurone layer and a corresponding putative cDNA specific to aleurone proteins were identified. The sequence analysis of the cDNA would give more information regarding aleurone-specific proteins and its coding genes. After confirmation, the clone can directly be used in transformation experiments with an endosperm-specific promoter to express its product in rice endosperm. This type of biotechnological approach is more reliable because in the traditional plant breeding programmes, the chance of achieving tissue specific expression (endosperm-specific, in this case) is very meagre.

Summary

Chapter – VI

SUMMARY

The present study was aimed at biochemical and molecular characterization of rice aleurone (surface) specific protein in order to isolate the corresponding genes. The salient findings of the study are summarized below.

1. Isolation of rice surface protein from the aleurone layer of the seed variety Nootripathu as well as from white seed (endosperm only) were done.
2. SDS-PAGE analysis of the isolated protein showed different protein bands ranging from 3 kDa to 110 kDa. Among them a 30 kDa and 15 kDa was found to be aleurone layer specific and 22 kDa as an endosperm specific.
3. RP-HPLC analysis revealed the presence of an unique protein in aleurone (rice surface) layer of the rice seed, confirming the extra protein lying in aleurone layer.
4. Reaction against 30 kDa and 15 kDa was detected on western blot analysis using the raised antibody with aleurone surface proteins.
5. Dot blot analysis revealed the developmentally regulated expression of the rice aleurone (surface) layer protein in different stages of crop growth. Expression of the gene in terms of protein was more at dough and maturity stages than the vegetative stage.
6. A rice seed cDNA library was immunoscreened and one putative clone was identified by using the 'aleurone-specific' antiserum.

PCR amplification was carried out to select the cDNA clones containing insert.

7. The insert size of the putative cDNA clones were found to be 1.2 ± 0.2 kb as shown by restriction digestion analysis.
8. The various cDNA putative clones were cross checked by Southern hybridization and were found to be identical.

As a whole, an aleurone specific protein in Nootripathu rice seed was identified and characterized employing immunological and molecular biological techniques. The putative cDNA clone of 1.2 ± 0.2 kb has been isolated for the identified aleurone protein (30 kDa). Further experiments are needed to confirm the cDNA clone for the corresponding protein.

References

REFERENCES

Baldi, G., G. Fossati and G.C. Fantone. 1976. By-products of rice II. Protein fractions and amino acid composition. **Riso, 25**: 347-356.

Barber, S. 1972. Milled Rice and changes during aging. In : **Rice Chemistry and Technology.** D.F. Houston (Ed.) Amer. Assoc. Cereal Chem. Incorp., St. Paul, Minn., pp : 215-263.

Barton, K.A. and W.J. Brill. 1983. Prospects in plant genetic engineering. **Science, 219**: 671-676.

Beachell, H.M., G.S. Khush and B.O. Juliano. 1972. Breeding for high protein content in rice. **Rice breeding, Int. Rice Res. Inst.**, Los Banos, pp : 419-428.

Bechtel, D.B. and Y. Pomeranz. 1978. Ultrastructure of the mature ungerminated rice (*Oryza sativa*) caryopsis. The germ. **Am. J. Bot., 65**: 75-85.

Bietz, J.A. 1986. High performance liquid chromatography of cereal proteins. **Adv. Cereal Sci. Technol., 8**:105-107.

Birnboim, H.C. and J. Doly. 1979. A rapid alkali extraction procedure for screening recombinant plasmid DNA. **Nucleic Acids Res., 6**: 1513-1524.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.,** 72: 248-254.

Bressani, R., L.G. Elias and B.O. Juliano. 1971. Evaluation of the protein quality of milled rice differing in protein content. **J. Agric. Food Chem., 19**: 1028-1034.

Cagampang, G.B., A.A. Pedron and B.O. Juliano. 1976.Changes in salt soluble proteins of rice during grain development. **Phytochem., 15**: 1425-1430.

Cagampang, G.B., L.J. Cruz, S.G. Espiritu, R.G. Santiago and B.O. Juliano. 1966. Studies on the extraction and composition of rice protein. **Cereal Chem., 43**: 145-155.

Clark, H.E., J.M. Howe and C.J. Lee. 1971. Nitrogen retention of adult human subjects fed a high protein rice. **Am. J. Chem. Nutr., 24**: 324-328.

Coffman, W.R. and B.O. Juliano. 1979. Seed protein improvement in rice. In: **Seed Protein Improvement in Cereals and Grain Legumes** (Proc. Symp. Neuherberg, 1978), IAEA, Vienna, Vol. II, pp : 261-276,

Croy, R.R.D., J.A. Gatehouse, I.M. Evans and D. Boulter 1980. Characterization of the storage protein subunits synthesized in *vitro* by polyribosome and RNA from developing pea (*Pisum sativum* L.) Legumin. **Planta, 148**: 49-56.

De Datta, S.K., W.N. Obcemea and R.K. Jana. 1972. Protein content of rice as affected by nitrogen fertilizer and some triazines and substituted ureas. **Agron. J., 64**: 785-788.

Derbyshire, E., D.J. Wright and D. Boulter. 1976. Legumin and vicilin. Storage proteins of legume seeds. **Phytochem., 15**: 3-24.

Eggum, B.O. and B.O Juliano. 1973. Nitogen balance in rats fed rices differing in protein content, **J. Sci. Food Agric., 24**: 921-927

Engvall, E. and P. Perlmann. 1971. Enzyme-linked immunosorbent assay (ELISA): quantitative assay of immunoglobulin G. **Immunochem., 8**: 871-879.

Engvall, E. and P. Perlmann. 1972. Enzyme-linked immuno-sorbent assay, ELISA III quantification of specific antibodies by enzyme labelled anti-immunoglobulin in antigen-coated tubes. **J. Immunol., 109**: 129-135.

Feinberg, A.P. and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonucleases fragments to high specific activity. **Anal. Biochem., 132**: 6-13.

Feinberg, A.P. and B. Vogelstein. 1984. Addendum to A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. **Anal. Biochem., 137**: 266-267.

Feng, G., M. Chen, K.J. Kramer and G.R. Reeck. 1991. Reverse Phase high performance liquid chromatography separation of wheat proteinaceous inhibitors of insect and mammalian α amylase. **Cereal Chem., 68(1)**: 95-99.

Gooding, K.M. 1986. High performance liquid chromatography of proteins. In : **A current look at the state of the technique biochromatography,** 1: 34-38.

Harlow, E. and D. Lane. 1988. **Antibodies - A laboratory manual**, Cold spring, Harbor Laboratory, New York, 678-679.

Harris, N. and B.O. Juliano. 1977. Ultrastructure of endosperm protein bodies in developing rice grains differing in protein content. **Ann. Bot., 41(171)**: 1-5.

Hegsted, D.M. and B.O. Juliano. 1974. Difficulties in assessing the nutritional quality of rice protein. **J. Nutr., 104**: 772-781.

Higuchi, W. and C. Fukazawa. 1987. A rice glutelin and a soybean glycinin have evolved from a common ancestral gene. **Gene(Amst.), 55**: 245-254.

Hinton, J.J.C. 1948. The distribution of vitamin B_1 in the rice grain. **Brit. J. Nutr., 2**: 237-241

Houston, D.F. 1972. Rice bran and polish In : **Rice Chemistry and Technology**, D.F.Houston. (Ed.) Amer. Ass. Cereal Chem., St. Paul, Minn.

Houston, D.F. and A. Mohammad. 1970. Purification and partial characterization of a major globulin from endosperm. **Cereal Chem., 47**: 5-12.

Houston, D.F., A. Mohammad, T. Wasserman and E.B. Kester. 1964. High protein rice flours. **Cereal Chem., 41**: 514-523.

Houston, D.F., T. Iwasaki, A. Mohammad and L. Chen. 1968. Radial distribution of protein by solubility classes in the milled rice kernel. **J. Agric. Food Chem., 16**: 720-724.

IRRI - International Rice Research Institute. 1976. Protein content, Genetic evaluation and utilization programme. Annual Report for 1976. Los Banos, Philippines, pp : 63-70.

Jakobsen, K., S.S. Klemsdal, R.B. Aalen, M. Bosnes, D. Alexander and O.A.Olsen. 1989. Barley aleurone cell development : molecular cloning of aleurone-specific cDNAs from immature grains. **Plant Mol. Biol., 12** (3): 285-293.

Johnson, V.A. and C.L. Lay. 1974. Genetic improvement of plant protein. **J. Agric. Food Chem., 22**(4): 558-566.

Johnston, T.H., W.J. Wiser, B.D.Webb and C.R. Adair. 1974. Breeding for increased protein content in brown rice -progress after two decades'. (Abstr.) Proc. 15th Rice Tech. Working Group, USDA, Washington. D.C., pp : 28.

Jones, T.J. and T.L. Rost. 1989. Histo chemistry and ultrastructure of rice (*Oryzae sativa*) zygotic embryogenesis. **Am. J. Bot., 76**: 504-520.

Juliano, B.O. 1972. The rice caryopsis and its composition. In : **Rice chemistry and technology.** D.F Houston. (Ed.). Amer. Assoc. Cereal Chem., St. Paul, Minn., pp : 16-74

Juliano, B.O. and D. Boulter, 1976. Extraction and composition of rice endosperm glutelin. **Phytochem., 15**: 1601-1606.

Juliano, B.O., A.A. Antonio and B.V. Esmama. 1973. Effect of protein content on distribution and properties of rice protein. **J. Sci. Food Agric., 24**: 295-306.

Juliano, B.O., C.M. Ferez and K.A. Gomez. 1972. Variability in protein content of rice. **Phil. J. Biol., 1**: 74-81.

Juliano, B.O., E.L. Albano and G.B. Cagampang. 1964. Variability in protein content ,amylose content and alkali digestibility of rice varieties in Asia. **Phil. Agric., 48**: 234-239.

Kartoprawiro, M.I. and K. Mugiono. 1984. Possible variability in rice protein caused by environmental conditions. In: **Cereal Grain Protein Improvement** (Proc. Symp., Vienna, 1982), IAEA, Vienna, pp : 71-80.

Kennedy, B.M. and M. Schelstraete. 1974. Chemical physical and nutritional properties of high protein flours and residual kernel from the over-milling of uncoated milled rice, 1. Milling procedure and protein , fat, ash, amylase and starch content. **Cereal Chem., 51**: 435-448.

Khush, G.S. and B.O. Juliano. 1984. Rice varietal improvement for protein content at the International Rice Research Institute In : **Cereal Grain Protein Improvment** (Proc. Symp., Vienna, 1982). IAEA, Vienna, pp :199-202.

Kihupi, A.N. 1984. Utilization of induced mutants for rice improvement in Tanzania. In : **Cereal Grain Protein Improvement** (Proc. Symp. Vienna, 1982), IAEA, Vienna, pp : 81-86.

Kim, W.T. and T.W. Okita. 1988 (a). Structure and expression and heterogeneity of the rice seed prolamines. **Plant Physiol., 88**: 649-655.

Kim, W.T. and T.W. Okita. 1988 (b). Nucleotide and primary sequence of a major rice prolamines. **FEBS Lett., 231**: 308-310.

Kim, W.T., X. Li and T.W. Okita. 1993. Expression of storage protein multigene families in developing rice endosperm. **Plant Cell Physiol., 34**(4): 595-603.

Krishnan, H.B. and S.G. Pueppke. 1993. Nucleotide sequence of an abundant rice seed globulin: Homology with the high molecular weight glutelins of wheat, rye and triticale. **Biochem. Biophys. Res. Commun., 193**: 460-466.

Krishnan, H.B. and J.A. White. 1997. Protein body formation and immunocytochemical localization of globulins and glutelins in developing rice (*Oryza sativa* L.) embryos. **Crop Sci., 37**: 932-939.

Krishnan, H.B. and J.A.White. 1995. Morphometric analysis of rice seed protein bodies: Implication for a significant contribution of prolamine to the total protein content of rice endosperm. **Plant Physiol., 109**: 1491-1495.

Krishnan, H.B. and T.W. Okita. 1986. Structural relationship among the rice glutelin polypeptides. **Plant Physiol., 81**: 748-753.

Krishnan, H.B., J.A. White and S.G. Pueppke. 1992. Characterization and localization of rice (*Oryza sativa* L.) seed globulins. **Plant Sci., 81**: 1-11.

Krishnan, H.B., V.R. Franceschi and T.W. Okita. 1986. Immunochemical studies on the role of the Golgi complex in protein body formation in rice seeds. **Planta, 169**: 471-480.

Kvassay-Sajo, Z. 1984. Mutation breeding for high protein content of rice grain. In : **Cereal Grain Protein. Improvement** (Proc. Symp. Vienna, 1982), IAEA, Vienna, pp : 87-90.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. **Nature, 227**: 680-685.

Liu, Q., C. Hara, M. Umeda, Y. Zhao, T.W. Okita and H. Uchimiya. 1995. Analysis for randomly isolated cDNAs from developing endosperm of rice (*Oryza sativa* L.): evaluation of expressed sequence tags and expression levels of mRNAs. **Plant Mol. Biol., 29**: 685-689.

Luthe, D.S. 1983. Storage protein accumulation in developing rice seeds. **Plant Sci. Lett., 32**: 147-158.

Lycett, G.W., R.R.D. Croy, A.H. Shirsat and D. Boulter. 1984. The complete nucleotide sequence of legumin gene from pea (*Pisum sativum* L.) **Nucleic Acids Res., 12**: 4493-4506.

Masumura, T., D. Shibata, T. Hibino, T. Kato, K. Kawabe , G. Takeba, K. Tanaka and S. Fujii. 1989. cDNA cloning of an mRNA encoding a sulfur rich 10 KDa prolamin polypeptide in rice seeds. **Plant Mol. Biol., 12**: 123-130.

Matlashewski, G.J., K. Adeli, I. Altosaar and B.J.Miflin. 1982. *In vitro* synthesis of globulin. **FEBS Lett., 145**: 208-212.

Monyo, S.H., T. Sugiyama and A.N. Kihupi. 1979. Potentially high-yielding and high-protein rice in induced mutation breeding. In: **Seed Protein improvement in Cereals and Grain Legumes** (Proc. Symp. Neuherberg, 1978), Vol. II, IAEA, Vienna, pp : 293-300.

Morita, Y. and C. Yoshida. 1968. Studies on globulin of rice embryo. Part I. Isolation and purification of γ globulin from rice embryo. **Agric. Biol. Chem., 32**: 664-670.

Morita, Y. and M. Horikoshi. 1972. Studies on γ globulin of rice embryo. Part VI. Molecular dimension and chemical composition of globulin. **Agric. Biol. Chem., 36**: 651-657.

Muench, D.G., S.J. Coughlan and T.W. Okita. 1998. Evidence for a cytoskeleton- associated binding site involved in prolamin mRNA localization to the protein bodies in rice endosperm tissue. **Plant Physiol., 116**: 559-569.

Murata, K., T. Kitagawa and B.O. Juliano. 1978. Protein quality of a high protein milled rice in rats. **Agric. Biol. Chem., 42**: 565-570.

Nakase, M., T. Yamada, T. Kiro, J. Yamaguchi, N. Aoki, R. Nakamura, T. Matsuda and T. Adachi. 1996. The same nuclear proteins bind to the 5'-flanking regions of genes for the rice seed storage protein: 16 kDa albumin, 13 kDa prolamin and type II glutelin, **Plant Mol. Biol., 32**: 621-630.

Normand, F.L., D.M. Soignet, J.T, Hogan and H.J. Deobald. 1966. Content of certain nutrients and amino acids patterns in high protein rice flour. **Rice J., 69** (9): 13-18.

Oparka, K.J. and N. Harris. 1982. Rice protein body formation: all types are initiated by dilation of endoplasmic reticulum. **Planta, 154**: 184-188.

Osborne, T.B. 1907. The proteins of the wheat kernel. Carnegie Inst. Washington, Washington, D.C. Pub.No. 84.

Pan, S. 1983. Electrophoretic studies of rice proteins and characterization of rice endosperm α globulin. M.S. Thesis (Kansas State University).

Perbal, B. 1988. In : **A practical guide to molecular cloning.** Wiley-inter science publication, John Wiley & Sons, pp : 752-776.

Ratho, S.N., B.B. Nanda, K.K. Jena and R.N. Misra. 1984. Studies on the protein content in rice. In: **Cereal Grain Protein Improvement** (Proc. Symp. Vienna, 1982, IAEA, Vienna, pp : 191-198.

Resurreccion, A.P., B.O. Juliano and Y. Tanaka. 1979. Nutrient content and distribution in milling fraction, of rice grain. **J. Sci. Food Agric., 30**: 475-481.

Robert, L.S., C. Nozzolillo and I. Altosaar. 1985. Homology between rice glutelin and oat 12 S globulin. **Biochem. Biophys. Acta., 829**: 19-26.

Rosario, A.R., V.P. DelBriones, A.J. Vidtal and B.O Juliano. 1968. Composition and endosperm structure of developing and mature rice kernel. **Cereal Chem., 45(3)**: 225-235.

Roxas, B.V., C.L.L. Intengan and B.O. Juliano. 1975. Effect of protein content of milled rice on nitrogen retention of Filipino children fed a rich-fish diet. **Nutr. Rep. Int., 11**: 393-398.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. In : **Molecular cloning - A laboratory manual.** 2nd Ed. Cold spring Harbor Laboratory press, Cold Spring Harbor, N Y.

Sarker, S.C., M. Ogawa, M. Takahashi. and Asada. 1986. The processing of a 57-kDa precursor peptide subunits of rice glutelin. **Plant Cell Physiol., 27**: 1579-1586.

Sharp, P.A., B. Sugden and J. Sambrook. 1973. Detection of two restriction endonucleases activities in *Haemophilus para-influenzae* using analytical agarose. **Biochem., 12**: 3055-3057.

Shewrg, P.R., B.J. Miflin, B.G. Forde and S.W.J. Bright. 1981. Conventional and novel approaches to the improvement of the nutritional quality of cereal and legume seeds. **Sci. Prog. Oxf., 67**: 575-600.

Shorrosh, B.S., L. Wen, K. Zen, J. Huang, J. Pan, M.A. Hermodson, K.Tanaka, S. Muthukrishnan and P.R. Gerald 1992. A novel cereal storage protein : Molecular genetics of the 19 KDa globulin of rice. **Plant Mol. Biol., 18**: 151-159.

Shotwell, M.A. and B.A. Larkins.1989. The molecular biology and biochemistry of seed storage proteins. A. Marcus (Ed.). In : **The biochemistry of plants,** A comprehensive treatis. Academic Press, San Diego, **15**: 297-345.

Shyur. L., T. Win and C. Chen. 1992. cDNA cloning and gene expression of the major prolamin of rice. **Plant Mol. Bio., 20**: 323-326.

Singh, C. 1993. In : **Modern techniques of raising field crops**. Oxford and IBH Publishing Co. Pvt. Ltd., Ninth Printing, pp : 3-30.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. **J. Mol. Biol., 98**: 503-517.

Takaiwa, F., H. Ebinuma, S. Kikuchi and K. Oono. 1987. Nucleotide sequence of a rice glutelin gene. **FEBS Lett., 221(1)**: 43-47.

Takaiwa, F., S. Kikuchi and K. Oono. 1986. The structure of rice storage protein glutelin precursor deduced from cDNA. **FEBS Lett., 206**(1): 33-35.

Tanaka, K., T. Sugimoto, M. Ogawa and Z. Kasai. 1980. Isolation and characterization of two types protein bodies in the rice endosperm. **J. Agri. Biol. Chem., 44**(7): 1633-1639.

Tecson, E.M.S., B.V. Esmama, L.P. Lontok and B.O. Juliano. 1971. Studies on the extraction and composition of rice endosperm : glutelin and prolamin, **Cereal Chem., 48**: 186-191.

Towbin, H., T. Staehelin.and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose sheets: procedure and some application. **Proc. Natl. Acad. Sci., USA., 76**: 4350-4354.

Velazhahan, R. and P. Vidhyasekran. 1999. An elicitor of the rice sheath blight pathogen *Rhioctonia solani* exhibits dual function: Elicitation and suppression of tissue. Necrotization in rice. **Acta Phytopathologica et Entomologica Hungarica, 34(3-4)**: 1-6.

Venkataramani, G. 2000. Scaling new heights. In : **Survey of Indian Agriculture, 2000.** The Hindu Publication, pp : 5-7.

Villareal, R.M. and B.O. Juliano. 1978. Properties of glutelin from mature and developing rice grain. **Phytochem., 17**: 177-182.

Wen, T.N. and D.S. Luthe. 1985. Biochemical characterization of rice glutein. **Plant Physiol., 78**: 172-177.

Yamagata, H. and K. Tanaka. 1986. The site for synthesis and accumulation of rice storage protein. **Plant Cell Physiol., 271**: 135-145.

Yamagata, H., T. Sugimoto, K. Tanaka and Z. Kasai. 1982. Biosynthesis of storage proteins in developing rice seeds. **Plant Physiol., 70**: 1094-1100.

Ye, X., S. Al-Babili, A. Kloti, J. Zhang, P. Lucca, P. Beyer and I. Potrykus. 2000. Engineering the provitamin A (beta-carotene) biosynthesis pathway into (carotenoid-free) rice endosperm. **Science, 287**(5451): 303-305.

Zhao, W.M., J.A. Gatehouse and D. Boulter. 1983. The purification and partial amino acid sequences of a polypeptide glutelin fraction of rice grain homology to pea legumin. **FFBS Lett., 162**(1): 96-101.