MOLECULAR ANALYSIS OF WHEAT - *Ae. kotschyi* DERIVATIVES WITH HIGH GRAIN IRON AND ZINC CONTENT

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

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in

BIOTECHNOLOGY

by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **MOLECULAR ANALYSIS OF WHEAT - *Ae. kotschyi* DERIVATIVES WITH HIGH GRAIN IRON AND ZINC CONTENT** in fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 2004 to December 2008 under the supervision of Dr. H. S. Dhaliwal and Dr. G. S. Randhawa, Department of Biotechnology Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(NIDHI RAWAT)

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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ABSTRACT

Over two billion people of the world suffer from micronutrient deficiency also known as 'hidden hunger'. Among the various approaches to overcome micronutrient deficiency, biofortification is the most sustainable, cheapest and long lasting solution. Combination of conventional and molecular breeding methods is the most desirable approach for biofortification of wheat having diverse germplasm sources. Grains of 80 accessions of nine species of wild *Triticum* and *Aegilops* along with 15 semi-dwarf cultivars of wheat and durum grown over two years at Indian Institute of Technology, Roorkee, were analyzed for grain iron and zinc contents. The wheat and durum cultivars had very low content and little variability for both of these micronutrients. The related non-progenitor wild species with S, U and M genomes showed upto 2-3 fold higher grain iron and zinc content. There were highly significant differences for iron and zinc contents among various cultivars and wild relatives over both the years with very high broad sense heritability. There was a significantly high positive correlation between flag leaf iron with grain iron content (r=0.82) and flag leaf zinc with grain zinc content (r=0.92) of the selected donors suggesting that the leaf analysis could be used for early selection and enrichment of segregants with high iron and zinc content for effective breeding for yield and other traits. Chinese Spring with Ph^{I} gene from *Aegilops speltoides* was used to transfer useful variability from *Aegilops* to elite cultivars for inducing homoeologous chromosome pairing between *Aegilops* and wheat genomes.

A majority of the interspecific hybrids had higher leaf iron and zinc content than their wheat parents and equivalent or higher content than their *Aegilops* parents, strongly supporting a proof of the concept that the parental *Aegilops* donors possess superior genetic systems for efficient uptake and translocation of the micronutrients

which could ultimately be utilized for wheat grain biofortification. Meiotic metaphase chromosome analysis of the F_1 hybrids ($ABDUS^{l}$) showed expected chromosome number of 35 and very little but variable homoeologous chromosome pairing. Partially fertile to sterile BC_1 derivatives with variable chromosomes of *Aegilops* species and nearly 75% of the expected wheat background had also higher leaf iron and zinc content confirming the transfer of required variability and ultimate expression of the efficient superior genetic systems of the donor parents for the micronutrient content in wheat grains. BC_2F_1 and BC_1F_2 progenies were cytologically and morphologically nearer to wheat cultivars. Selection among these progenies was done on the basis of grain iron and zinc content. Subsequently BC_2F_2 and BC_1F_3 progenies were analysed for grain micronutrient content. The recovery of fertile derivatives with seeds as bold as that of the wheat cultivars and micronutrient content as high as that of the wild donors gives unequivocal proof of the concept that *Aegilops kotschyi* possess efficient genetic system for uptake and translocation of the micronutrients which could be effectively used for biofortification of wheat cultivars. Thirteen derivatives were finally selected for detailed analyses using morphological markers, chromosome pairing, HMW- Glutenin subunit profile, GISH and anchored wheat SSR markers. Group 1, 2 and 7 chromosomes of *Ae. kotschyi* were found to be present in the selected derivatives carrying genes for high grain iron and zinc.

Synthetic amphiploids between *Triticum aestivum* (AABBDD) landrace Chinese Spring (*Ph*^{l}) and cultivar WL711 with different accessions of *Aegilops kotschyi* ($UUS^{l}S^{l}$) were developed through colchicine treatment of sterile F_1 hybrids. The F_1 hybrids and amphiploid plants were intermediate between the parents for plant morphology and spike characteristics. The amphiploids ($AABBDDUUS^{l}S^{l}$), however, had variable frequency of univalents at meiotic metaphase-I. The SDS-PAGE of

HMW glutenin subunits of amphiploids along with the parents showed the presence and expression of all the parental genomes in the amphiploids. The amphiploids with bolder seeds had higher grain and grain ash iron and zinc content than the wheat parents and comparable to those of their *Ae. kotschyi* parents suggesting that *Ae. kotschyi* possesses superior genetic system for the micronutrient uptake and translocation than the wheat cultivars. The variable chromosome number in PMCs of different tillers, spikelets and florets in some of the amphiploids suggests somatic chromosome elimination in the amphiploids. The amphiploids can be used for transfer of high iron and zinc content and development of alien addition and substitution lines in wheat.

Lowering the content of anti-nutritional factor, phytic acid, in wheat may increase the bioavailable content of micronutrients iron and zinc. A set of 76 EMS-induced mutants of *T. monococcum* obtained from P.A.U. Ludhiana were screened for low phytic acid content. On the basis of initial screening two mutants having high inorganic phosphate content were selected as putative low phytic acid (*lpa*) mutants. Phytic acid content in the two putative mutants *viz.*, MM225 and MM169 was reduced by 57 % and 46 % respectively over the wild type. The decrease in phytic acid content of the mutants was paralleled by increase in total Fe, Zn and P. Available iron content increased by 57 % and 19 % in *lpa* mutants MM225 and MM169, respectively over wild type *T. monococcum*. Scanning Electron Microscope-Energy Dispersive X-Ray (SEM-EDX) mapping of grains of wild type *T. monococcum* showed compactly arranged phytic acid granules whereas phosphorus was more loosely packed in the aleurone of *lpa* mutant MM225. Higher Fe and Zn content in the endosperm of MM225 than that of *T. monococcum* wild type was also visible in SEM-EDX maps. Thus the novel *T. monococcum lpa* mutants- MM225 and MM169

had lower phytic acid, higher micronutrient content and increased bioavailability. Thorough understanding of increase in phosphate and micronutrients in *lpa* mutants will be of tremendous help in biofortification with enhanced bioavailability.

Effect of germination of wheat grains on phytic acid content, mineral elements P, K, Mg, Ca, Fe and Zn was studied using SEM-EDX analysis along with other constituents. The minerals showed peripheral distribution within the wheat grain, being sequestered mainly in the aleurone layer of the grains. Phytic acid represented by the phosphorus rich granules of the aleurone layer of grains was reduced by 81 % after 120 hours of germination. The SEM-EDX profile of the minerals also showed their reduction in the aleurone layer with the progress of germination. SEM images revealed significant degradation of starch granules after 72 hours of germination. SDS-PAGE of the seed storage proteins indicated that the protein profile remained unaffected till 96 hours of germination. HMW glutenin proteins remained intact even after the fifth day of germination, whereas LMW glutenin proteins were preferentially degraded. Analysis of protein content on dry weight basis of the partially germinated seeds indicated a progressive increase over control. To reduce phytic acid content and enhance iron and zinc bioavailability to humans and monogastric animals, wheat seeds can be partially germinated upto 72 hours without any significant deterioration of processing, nutritional characteristics and palatability.

The precise transfer of *Ae. kotschyi* genes for high grain Fe and Zn content and their marker assisted pyramiding in elite wheat cultivars can nearly double the micronutrient content over the existing levels. The combination of biofortified wheat with low phytic acid mutants and improvised processing will be absolutely essential to enhance bioavailability of micronutrients to alleviate the hidden hunger.

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Rawat

Date: 30/Dec/08

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ABBREVIATIONS USED

Abbreviation	Extended form
%	Percentage
AAS	Atomic Absorption Spectrometer
AAS	Atomic Absorption Spectrophotometer
BC	Backcross
BC_1	First back cross generation
BC_2	Second back cross generation
bp	Base pairs
CGIAR	Consultative group of International Agricultural Research
CIMMYT	Centro Internacional de Mejoramiento de Maíz y Trigo
CTAB	Cetyl-trimethyl ammonium bromide
DArT	Diversity Array Technology
DMA	2-deoxymugineic acid
DMSO	Dimethyl sulphoxide
dNTPs	Nucleotide Triphosphates
EDTA	Ethylenediaminetetraaceticacid
EDTA	Ethylene di-amine tetra acetic acid
EMS	Ethane methyl sulphonate
epi-HDMA	epihydroxy-2hydroxy mugineic acid
epi-HMA)	3-epi-hydroxymugineic acid
EST	Expressed sequence tag
F_1	First Filial Generation
FAO	Food and Agricultural organisation

FAO	Food and Agriculture Organization
Fig.	Figure
FISH	Fluoresence *in situ* hybridization
GISH	Genomic *in situ* hybridisation
HMW-GS	High Molecular Weight glutenin subunit
HPLC	High Performance Liquid Chromatography
ICPMS	Inductively Coupled Plasma Mass Spectrometer
IRT	Iron regulatory transporter protein
IZiNCG	International Zinc Nutrition Consultative Group
MA	Mugineic acid
mg/kg	Milligram per kilogram
MTP	Metal tolerance proteins
NRAMP	Natural Resistance Associated Macrophage Proteins
NRAMP	Natural Resistance Associated Macrophage Proteins
IRT	Iron regulatory protein
PCR	Polymerase Chain Reaction
PMCs	Pollen Mother Cells
ppm	Parts per million
QTL	Quantitaive trait loci
RDA	Recommended dietary allowance
RAPD	Random amplified polymorhic DNA
RFLP	Restriction Fragment Length Polymorphism
SDS-PAGE	Sodium dodecyl sulphate- Poly acrylamide gel electrophoresis
SEM-EDX	Scanning Electron Microscopy – Energy Dispersive X Ray analysis
SSRs	Simple Sequence Repeats

TAE	Tris Acetate
TE	Tris EDTA
TEMED	Tetramethylene diamine
VIT1	Vacuolar Iron Transporter
WHO	World Health Organisation
WHO	World Health Organization
YS	Yellow Stripe
ZIP	Zinc regulated- Iron regulated transporter proteins
µg	Micrograms

Chapter I

Introduction

1. INTRODUCTION

More than half of the world population suffers from iron and zinc deficiency, also known as 'hidden hunger' because of externally not so visible symptoms associated with it. The high frequency of micronutrient deficiency has substantial health and economic costs. Marginal intake of micronutrients contribute to increased morbidity and mortality rates, diminished livelihoods, and adverse effects on learning ability, development and growth in infants and children (Caballero, 2002; Gitlin, 2006). By any measure, micronutrient malnutrition is currently of alarming proportions particularly in developing nations (WHO, 2002).

The intervention approaches being adopted to overcome micronutrient deficiency include dietary diversification, supplementation, fortification and biofortification (Brinch-Pederson *et al.*, 2007). Dietary diversification is an efficient strategy of fighting hidden hunger, but the diets rich in micronutrients are too costly for most of the affected poor people. Supplementation involves therapeutic administration of iron and zinc as syrups or pills while fortification entails adding minerals to food items. These approaches require recurring expenses and careful implementation each time. Biofortification refers to increasing the bioavailable mineral contents of the edible plant parts such as grains of cereals, tubers of sweet potato, cassava etc. This is the most sustainable and permanent approach for solving the hidden hunger problem of the poor countries as only one time expenditure on production of biofortified crops is required (Welch and Graham, 2004). Crops can be biofortified either by genetic engineering or by molecular and traditional breeding systems. The complex mechanisms of mineral uptake, transport and sequestration in the crop plants are poorly understood till date. Presently the most feasible strategy to enhance iron and zinc contents in crop plants is the plant breeding based approach.

With the mapping of QTLs in many plants for high iron and zinc, molecular breeding is the method of choice of HarvestPlus initiative of CGIAR.

Wheat is the staple food of one third of the world population. It accounts for 50 % of the calorie intake in Asian countries, which are the worst sufferers of hidden hunger. Biofortifying wheat is, therefore, a promising solution of this problem. The popular cultivars of wheat have very little iron and zinc in their grains (Cakmak *et al.*, 2000). Wild germplasm is a rich source of useful variability for grain iron and zinc content (Rawat *et al.*, 2008; Chhuneja *et al.*, 2006) and can be effectively utilized for enhancing the micronutrient status of the elite cultivars. Wide hybridization with wild relatives having high iron and zinc content followed by selection of the desirable trait can be a feasible approach. Many genes for pest and disease resistance, abiotic stresses and quality traits have been transferred to wheat from wild relatives. Among various approaches of alien introgression in wheat from non-progenitor genomes, the induction homoeologous pairing with *Ph*^{I} gene transferred from *Ae. speltoides* (Chen *et al.*, 1994) has been found to be very effective and feasible (Aghaee-Sarbarzeh *et al.*, 2002). For selection of precise transfer of useful variability among introgressive derivatives, approaches like GISH, FISH, C-Banding, HMW glutenin profiles, molecular markers etc. have been used frequently. The useful variability from related germplasm can be stored immortally as amphiploids which may be used to develop addition and substitution lines to study more than one required trait anytime (Jiang *et al.*, 1994).

Simply enhancing the micronutrient content of the plants would not solve the iron and zinc deficiency problem. The frequently present inhibitors of micronutrient absorption like phytic acid make the job more tedious. Therefore, to enhance the bioavailability of micronutrients in food, antinutritional factor phytic acid has to be

minimized. Bioprocessing methods such as soaking, fermentation, roasting, milling and polishing reduce phytic acid levels by the action of phytate degrading phytase produced endogenously in seeds or added exogenously from microorganisms like yeast (Frontela *et al.*, 2008). It will be useful to characterize strictly the optimum period of bioprocessing treatments to maximize the benefits of these methods.

In order to increase the nutritive value of crops, it would be highly appropriate to reduce the levels of phytic acid in the plants themselves. Thus low phytic acid (*lpa*) mutants are one of the most sought after plant mutants (Raboy, 2001; Bouis, 2000). The *lpa* mutants have been identified and characterized in a number of plants including maize, rice, barley, wheat and soybean. In fact experiments on yield trials are currently going on at various places to analyse the possibility of using the *lpa* mutants in these crops with higher percentage of bioavailable nutrients (Bregitzer and Raboy, 2006; Gutteieri *et al.*, 2006). It will be useful to identify some more *lpa* mutants and to study their effect on iron and zinc content in the seeds and actual change in bioavailability of minerals if any without any yield penalty.

Keeping in view the above facts, the present investigation was carried with the following broad objectives:

- Identification of potential donors for high iron and zinc concentration in grains of wild *Triticum* and *Aegilops* species by rigorous analyses with AAS and ICPMS.
- The use of a selected donor for transfer of high iron and zinc content into elite wheat cultivars through interspecific hybridization.
- Molecular characterization of the introgressive derivatives on the basis of morphology, HMW-glutenin subunit proteins, Genomic *in situ* hybridization

(GISH) and microsatellite markers for locating alien chromosomes with QTL/genes controlling higher iron and zinc in the derivatives.

- Development of amphiploids with high grain iron and zinc to combine the useful variability forever with the wheat genome.
- Screening and characterization of some low phytic acid mutants in a set of *T. monococcum* mutants and to study the iron zinc content and enhancement of bioavailability of minerals in them.
- Studying the effects of germination as a bioprocessing method to lower the phytic acid content of wheat grains.

Chapter II

Review of Literature

2. REVIEW OF LITERATURE

2.1 Micronutrient Deficiency

More than half of the world's population suffers from iron and zinc deficiency (Brinch-Pederson *et al.*, 2007; Welch and Graham, 2004). According to the World Health Organization (WHO) iron and zinc malnutrition accounts for 1.5% and 1.4% of the total annual deaths respectively (WHO, 2004). Even mild deficiencies of these micronutrients have serious implications. Thirty nine percent of children younger than 5 years, 48% of children between 5 and 14 years, 42% of all women, and 52% of pregnant women in developing countries are anaemic (Zimmerman & Hurrel, 2007). Zinc deficiency alone is estimated to affect more than 25% of the world population (Maret and Sandtead, 2006). In fact Zn deficiency ranks as the fifth most serious health risk factor in developing countries (immediately behind inhalation of cigarette smoke indoors) and is equally important as iron deficiency (WHO, 2002). Marginal intake of iron and zinc results in increased morbidity and mortality rates, diminished livelihoods, adverse effects on learning ability, development, growth and long-term neurocognitive and psychomotor impairment in infants and children (Gitlin, 2006; Caballero, 2002). The worst affected parts of the world are the developing countries of the southern part of the globe (Mason and Garcia, 1993) where 59 % of the daily calorie intake is comprised of starch rich cereal, root tubers, banana and plaintain diet (FAO, 2004). The non-diversified diets of the people of these regions meet only the carbohydrate and protein requirements of the body but lack the micronutrients. Moreover the cereal based diets is further depleted of the little mineral content it has, during processing steps such as polishing, milling etc. leaving the cereal based diets lag far behind the recommended dietary allowance (RDA). WHO has declared RDA for iron as 10 mg for men and 15 mg for women in the age group of 25-50 years. For

zinc the RDA in the same age group is 15 mg and 12 mg for men and women respectively (FAO/WHO, 2000). Dietary inhibitors such as phytic acid, polyphenols, food fibers, tannins and lignins also reduce bioavailability of the minerals resulting in micronutrient malnutrition (Zimmerman and Hurrel, 2007; Welch, 2002).

2.2 Role of iron and zinc in humans and plants

Mineral nutrition is an important aspect of growth and development in animals and plants. Among the micronutrients, iron is important because of its physico-chemical properties. It participates in most of the basic redox reactions required in both the production and consumption of oxygen besides being involved as cofactor in numerous vital enzymatic reactions (Kim and Guerinot, 2007). Metal-containing proteins of the photosynthetic machinery are particularly abundant in plants, with a predominance of iron, for instance in the reaction centres of photosystems I and II (Krämer *et al.*, 2007).

Krämer *et al.* (2007) report the largest number of metallo-proteins to be functionally associated with zinc (1272), followed by copper (108) and iron (106). Taking only enzyme proteins into account, zinc is a cofactor in more than 300 enzymes that help in maintaining structural integrity of proteins and regulate gene expression in both plants and animals. The biological function of zinc can be catalytic, structural or regulatory. In humans more than 85% of total body zinc is found in skeletal muscles and bones (King & Keen, 1999). As such iron and zinc are indispensable for existence of plants and animals.

2.3 Strategies for alleviating micronutrient malnutrition

Various strategies like supplementation, fortification, dietary diversification and biofortification have been suggested to alleviate micronutrient deficiency (Zimmerman and Hurrel, 2007). Provision of iron and zinc in higher doses without

food is referred to as supplementation, whereas fortification involves adding minerals in available forms to food stuffs. Supplementation and fortification have met with several difficulties in solving the problem (Allen, 2008). The uptake of micronutrients is dependent upon the food matrix as well as on the presence of compounds that may promote or inhibit the uptake. Cook (2005) reported that food reduces medicinal iron (given as supplementation) absorption by two thirds. Moreover, micronutrients added externally are often lost during processing and cooking of the food (Binch-Pederson, 2007). Besides, approaches other than biofortification need recurring costs and demand careful implementation at each level every time. It is this multiplier aspect of biofortification across time and distance that makes it so cost-effective (Welch and Graham, 2004; Subbulakshmi and Naik, 1999; Yip, 1997). Dietary modification and dietary diversification, although is sustainable approach, change of dietary practices and preferences is difficult and foods that provide highly bioavailable iron and zinc are expensive (Zimmerman and Hurrel, 2007).

The word 'biofortification' refers to increasing the bioavailable micronutrient content of food crops through genetic selection via plant breeding (Welch and Graham, 2004). Biofortified crops are the most promising intervention tools for overcoming micronutrient deficiencies (Lonnerdal, 2003; Zimmerman and Hurrel, 2002). Moreover, increased storage of minerals in seeds of staple food crops increases crop productivity in micronutrient poor soils (Welch, 2002). Adequate nutrition is as important to disease resistance and stress tolerance in plants as it is in humans. Roots of plant genotypes that are efficient in mobilizing surrounding external minerals not only are more disease resistant, but are better able to penetrate deficient subsoils and so make use of the moisture and minerals contained in subsoils. This reduces the need for fertilizers and irrigation (Brinch-Pederson *et al.*, 2007).

Work is being done worldwide to biofortify crops with micronutrients- iron, zinc and vitamin A (Pfeiffer and McClafferty, 2007; Lucca *et al.*, 2006; www.HarvestPlus.org). Conventional breeding, molecular breeding and genetic engineering techniques have been considered to be the most feasible and cost effective approaches for biofortification of cereals with high iron and zinc content (Hirschi, 2008; Nestel *et al.*, 2006; Lonnerdal, 2003; Bouis, 1999; DellaPena, 1999). Attempts to enhance the micronutrient content of crops using transgenic approach are being made, but the complex mechanisms of mineral uptake, transport and deposition in grains are yet not very thoroughly understood. For instance transformation of rice with soybean *ferritin* led to higher expression of ferritin in seeds and leaves but this increase did not parallel iron levels in seeds (Qu *et al.*, 2005). Moreover variability for traits needed in biofortification programmes can be explored in germplasm collections. In case the desired trait is unavailable within the species or when the crop is not amenable to conventional and molecular breeding approaches, transgenes from various sources can be explored (Mayer *et al.*, 2008).

2.4 Biofortification of wheat

Wheat is currently the primary staple food for almost one-third of the world's population (FAO, 2004). With an annual global production of 619 million tonnes from 213 million hectares of land, it stands second only to rice in global production (Feuillet *et al.*, 2007). Wheat provides nearly 20% (one fifth) of total food calories and protein in human nutrition (Gupta *et al.*, 2008). However the popular bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L. ssp. *durum* (Desf.)) cultivars grown worldwide have low micronutrient content (Rawat *et al.*, 2008; Chhuneja, 2006; Cakmak *et al.*, 2000; Monasterio and Graham, 2000). Therefore biofortification of wheat is the thrust area of HarvestPlus initiave of CGIAR.

2.5 Origin of wheat

The word 'wheat' includes several related grain crops *viz.*, hexaploid bread wheat- *Triticum aestivum*, tetraploid pasta wheat- *T. turgidum* and diploid einkorn wheat- *T. monococcum*. Hexaploid bread wheat accounts for 90% of world wheat production today (http://faostat.fao.org/). All these wheats share a common basic set of seven chromosomes. The species of *Triticum* and related *Aegilops* species with their genomic constitution have been listed in Table 2.1. Einkorn wheat was the first

Table 2.1 Species of genus *Triticum* and *Aegilops* and their genomic constitution based on chromosome pairing and DNA analysis

Species	Genomic constitution
Triticum aestivum L. (Bread wheat)	ABD
Triticum turgidum L. (Pasta wheat)	AB
Triticum monococcum (Einkorn wheat)	A^m
Triticum zhukovskyi Menabde & Ericz.	A^tA^mG
Triticum timopheevii (Zhuk.) Zhuk. (cultivated form)	A^tG
Triticum urartu ex Gamdilyan (wild form)	A^u
Aegilops speltoides Tausch	S
Aegilops longissima Schweinf. & Muschl.	S^l
Aegilops searsii Feldman & Kislev ex Hammer	S^s
Aegilops sharonensis Eig	S^{sh}
Aegilops bicornis (Forssk.) Jaub. & Spach	S^b
Aegilops tauschii Coss. var. *tauschii*, var. *strangulata*	D
Aegilops uniaristata Vis.	N
Aegilops comosa Sm. in Sibth. & Sm. var. *heldreichii*	M
Aegilops caudata L.	C
Aegilops umbellulata Zhuk.	U
Aegilops mutica Boiss.	T
Aegilops cylindrica Host	D^cC^c
Aegilops ventricosa Tausch	D^VN^V
Aegilops crassa Boiss.	$D^{c1}M^c(D^{c1}X^C)$
Aegilops juvenalis (Thell.) Eig	DMU ($D^cX^cU^j$)
Aegilops vavilovii (Zhuk.) Chennav.	DMS ($D^cX^cS^v$)
Aegilops triuncialis L.	UC^t
Aegilops columnaris Zhuk.	UM (UX^{CO})
Aegilops neglecta Req. ex Bertol. (syn. *Ae. triaristata)*	UM (UX^n)
var. *recta* (Zhuk.) Hammer	UMN (UX^tN)
Aegilops geniculata Roth (syn. *Ae. ovata)*	UM (UM^0)
Aegilops biuncialis Vis.	UM (UM^0)
Aegilops kotschyi Boiss.	US (US^l)
Aegilops peregrina (Hack. in J. Fraser) Maire & Weiller (syn. *Ae. variabilis*)	US (US^l)

Source: Gill and Friebe, 2002, FAO Document repository

wheat to be cultivated in the Fertile Crescent (Fig. 2.1), which is regarded to be its place of origin some 10,000 years ago (Dubcovsky and Dvörak, 2007). Several related species of genus *Aegilops* having similar basic set of seven chromosomes also evolved in this region.

Triticum urartu (AA) is considered as the A genome donor of hexaploid wheat *Triticum aestivum* (AABBDD) and *Ae. tauschii* (DD) contributed the D genome (Faris *et al.*, 2002; Dvörak, 1993; McFadden and Sears, 1946; Kihara, 1944). *Ae. speltoides* (SS) or a closely related species is regarded to have donated the B genome (Faris *et al.*, 2002; Riley *et al.*, 1958). However the origin of the B genome is still debated. A schematic representation of origin of wheat is given in Fig. 2.2.

2.6 Utilization of wild germplasm of wheat

The tribe Triticeae contains more than 500 species of 26 genera (Feuillet *et al.*, 2007). This vast germplasm has been divided into primary, secondary and tertiary gene pools with respect to utilization for improvement of cultivated wheat (Jiang *et al.*, 1994). Primary gene pool consists of landraces, traditional varieties and wild species with a common genome that hybridize directly with wheat like tetraploid *T. turgidum* (AABB) and diploid *T. urartu* (AA) and *T. tauschii* (DD). Secondary gene pool contains species that share at least one genome in common with wheat, as for instance, *T. timopheevi* (AAGG) and *Ae. cylindrica* (CCDD). Tertiary gene pool is comprised of distantly related diploid and polyploid species without any common genome. In their hybrids with wheat, homoeologous recombination is not possible due to the pairing inhibition activity of *Ph1* located on chromosome 5BS of wheat. Special techniques have to be employed here like irradiation, 5B nullisomic condition or *Ph1* deletions, the use of Ph^{I} (inhibitor of *Ph1*) and use of gametocidal genes (Hossain *et*

Fig. 2.1 The dark green shaded area on the map shows the region of the near east (Israel,Jordan, Turkey, Syria, Iran, Iraq) known as the Fertile Crescent where cultivation of wheat first began about 10,000 years ago. Source: Feuillet *et al.*, 2007

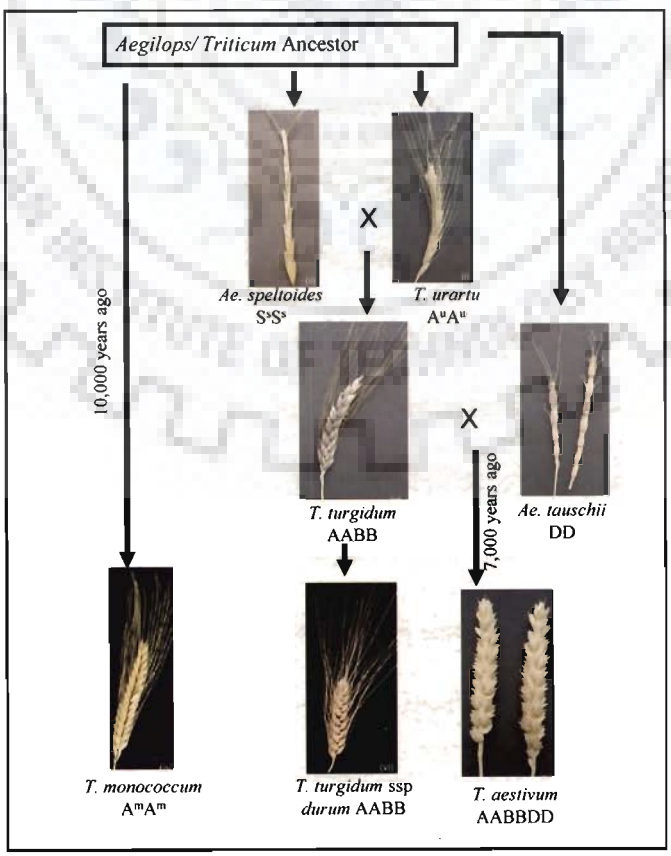


Fig. 2.2 Origin of polyploid wheat

al., 2004; Masoudi-Nejad *et al.*, 2002; Fedak, 1999; Vega and Feldman, 1998; Dubcovsky *et al.*, 1995; Chen *et al.*, 1994; Sears, 1977).

The wild germplasm of wheat has been a source of unlimited useful variability for crop improvement. A number of genes for resistance against various wheat diseases have been introgresed into wheat from related progenitor and non progenitor species (McIntosh *et al.*, 2005; Marais *et al.*, 2005; Friebe *et al.*, 1996) and commercially exploited. A few examples from the long list of alien transfers include *Lr9* from *Ae. umbellulata* (Sears, 1956), *Yr8* from *Ae. camosa* (Riley *et al.*, 1968), wheat streak mosaic resistance from *Agropyron elongatum* (Sebesta *et al.*, 1972), *Pm13* from *Ae. longissima* (Ceoloni *et al.*, 1988), *Lr35* and *Sr39* from *Ae. speltoides* (Kerber and Dyck, 1990), *H21* and *H25* (Hessian Fly resistance) from rye (Friebe, 1990), *Pm29* from *Ae. geniculata* (Stoilova and Spetsov, 2006), *Lr57* and *Yr40* from *Ae. geniculata* (Kuraparthy *et al.*, 2007a), *Lr58* from *Ae. truncialis* (Kuraparthy *et al.*, 2007 b), *Pm19* and *Pm35* from *Ae. tauschii* (Miranda *et al.*, 2007). Not only for pest and disease resistance, but genes also for yield and quality improvement have been transferred from wild species to cultivars (Hajjar and Hodgkin, 2007).

For the identification of useful variability for wheat biofortification major emphasis has been on the screening of progenitor species including diploid wheat, *T. monococcum*, *T. dicoccoides, T. dicoccon, T. tauschii,* etc. (White and Broadley, 2005; Cakmak *et al.*, 2004; Monasterio and Graham, 2000). Scientists at CIMMYT, Mexico in collaboration with HarvestPlus have used synthetic hexaploid wheat from crosses between *T. dicoccoides* and *T. tauschii* with high iron and zinc contents in breeding programmes and have developed wheat lines with higher level of these micronutrients which are being tested in farmers' fields in India, Pakistan and other countries (Calderini and Monasterio, 2003b). However the level of enhancement of

iron and zinc through the breeding programme using wheat synthetics has remained low because of the limited variability for iron and zinc in the wild progenitor species. Greater variability for micronutrients exists among the secondary and tertiary gene pools (Rawat *et al.*, 2008; Chhuneja *et al.*, 2006) that lies little explored so far.

It will be highly appropriate to identify diverse sources for high iron and zinc content among related species and genera for introgression and pyramiding of the desired variability to achieve high level of iron and zinc content in wheat. Approaches used to transfer useful variability from alien germplasm either involve ionising radiation of the hybrids or manipulation of the genetic control of homoeologous chromosome pairing. Sears (1956) transferred *Lr9* from *Ae. umbellulata* using irradiation technique. Among various approaches of induced homoeologus pairing mediated introgression in wheat from non progenitor genomes, the use of *Ph*^{I} gene transferred from *Ae. speltoides* (Chen *et al.*, 1994) has been found to be very effective and feasible (Aghaee-Sarbarzeh *et al.*, 2002).

2.7 Study of Alien Introgression

Various cytological and molecular techniques have been used to analyse the alien chromosome introgressed during chromatin transfers from wild germplasm to wheat.

2.7.1 HMW-Glutenin Subunits

The genes controlling High Molecular Weight (HMW) subunits of glutenin proteins are located on long arm of group 1 homoeologous chromosomes of wheat (Payne, 1987; Payne *et al.*, 1980). In bread wheat these loci have been named *Glu-A1*, *Glu-B1* and *Glu-D1*. Each locus includes two genes linked together encoding two different types of HMW-GS, x- and y-type subunits (Shewry *et al.*, 1992; Payne,

1987). The x-type subunits have generally lower electrophoretic mobility in SDS-PAGE and higher molecular weight than the y-type subunits. Electrophoresis studies show appreciable polymorphism in the number and mobility of HMW-GS coded by loci of different genomes. As such the HMW glutenin subunits have been used to monitor group 1 alien addition/ substitution to wheat (Dou *et al.*, 2006; Koebner and Shepherd, 1985). Koebner and Shepherd (1987) used HMW-GS to study allosyndetic recombination between a chromosome of *Aegilops umbellulata* and wheat chromosomes.

2.7.2 Chromosome C-Banding and *in situ* Hybridization

Standard C-banding karyotypes of many wild relatives have been developed and used for monitoring alien introgressions (Friebe, 1995a; 1995 b). Friebe *et al.* (1999) used C-banding to develop and identify complete set of wheat-*Ae. geniculata* addition lines.

GISH (Genomic *in situ* hybridization) involves labelling total genomic DNA and using it as a probe to identify alien chromosomes in a wheat background by in situ hybridization (Heslop-Harrison *et al.*, 1992; Le *et al.*, 1989). GISH is of potentially wide application in plant breeding programmes involving alien translocations (Mukai and Gill, 1991; Heslop-Harrison *et al.*, 1992). This technique has identified the parental origin of each chromosome in hybrids of *Hordeum chilense* and *H. vulgare* and in the hybrid *H. vulgare* X *H. bulbosum* L. (Schwarzacher *et al.*, 1992; Leitch *et al.*, 1990), as well as alien chromosomes and chromosome segments from *S. cereale* and *H. vulgare* in hexaploid wheat cultivars (Mukai and Gill, 1991) and triticale (Le and Armstrong, 1991).

FISH (Fluoresence *in situ* hybridization) and isozymes have been used to characterize addition lines of *Thinopyrum bessarabicum* (William and Mujeeb-Kazi,

1995) and addition lines of *Lophopyrum elongatum* showing resistance to *Cephalosporium gramineum* (Cai *et al.*, 1996). Partial amphiploids derived from crosses of wheat with *Thinopyrum intermedium* and *L. elongatum* with resistance to barley yellow dwarf virus (Zhang *et al.*, 1996) have been identified using FISH. Badaeva *et al.* (2004) used both GISH and C-banding to study genome differentiation in *Aegilops* and evolution of the U-genome species. BACs have also been utilized as probes for the so-called BAC-FISH which helped not only to discriminate between the three sub-genomes, but also in the identification of intergenomic translocations, molecular cytogenetic markers, and individual chromosomes (Zhang *et al.*, 2002).

2.7.3 Molecular markers

Molecular markers have been a tool of choice for identification of alien chromatin in addition and substitution lines (Schneider *et al.*, 2008; Ma *et al.*, 1994). RAPD (randomly amplified polymorphic DNA) markers have been used for characterization of addition lines in wheat of *Hordeum vulgare* (Devos and Gale, 1993), *Thinopyrum bessarabicum* (King *et al.*, 1993), *Hordeum chilense* (Hernandez *et al.*, 1995), *Aegilops searsii* (Diaz-Salazar and Orellana, 1995), *Dasypyrum villosum* (Qi *et al.*, 1996) and *Aegilops markgrafii* (Peil *et al.*, 1998). RFLP (restriction fragment length polymorphism) markers have been used to identify addition lines from *Th. intermedium* (Francki *et al.*, 1997) and addition and substitution lines of *Leymus racemosus* (Qi *et al.*, 1997). Autrique *et al.* (1995) tagged *Lr9*, *Lr19*, *Lr24*, *Lr32*, and *Sr25* using a large number of RFLP probes. Kuraparthy *et al.* (2007a and 2007b) used RFLP markers in combination with GISH to identify cryptic translocations in wheat from *Ae. geniculata* and *Ae. triuncialis* conferring new leaf rust and stripe rust resistance genes. With the advent of PCR based markers systems such as SSR, AFLP the molecular maps became denser and identification of alien

translocations became more promising (Somers, 2004; Roder *et al.*, 1998). Peil *et al.* (1998) used microsatellite markers to identify addition lines of *Ae. markgrafii*. With the increase in number of expressed sequence tag (EST) databases, EST-based microsatellite markers also known as genic microsatellite markers are being used to assay functional diversity in introgressive populations (Varshney *et al.*, 2005). These SSRs are useful because they represent transcribed genes and a putative function can often be deduced by homology search. Mullan *et al.* (2005) used EST-derived SSR markers from defined regions of the wheat genome to identify *Lophopyrum elongatum* specific loci in introgression lines. In a translocation line (T6BS-6SS) of *Ae. speltoides* in wheat, Song *et al.* (2007) using EST-SSR markers characterized the location of *Pm12* in the short arm of 6S conferring effective resistance to powdery mildew. Dou *et al.* (2006) used HMW-GS, GISH and SSR markers to study molecular cytogenetics of hexaploid lines spontaneously appearing in octoploid *Triticale*.

DArT (Diversity Array Technology) is the latest technique based on microarray to analyse DNA polymorphisms. The technology was originally developed for rice, a diploid crop with a small genome of 430 Mbp (Jaccoud *et al.*, 2001) and was subsequently applied to a range of other crops (the list currently includes 19 plant species and three fungal plant pathogens). Akbari *et al.* (2006) have recently developed a DArT molecular map for wheat with 339 markers across all the 21 chromosomes. DArT generates whole-genome fingerprints by scoring the presence versus absence of DNA fragments in genomic representations generated from samples of genomic DNA in microarray platform. The DArT technology is very promising especially for related wild germplasm as it does not require their prior DNA sequence information.

2.7.4 Radiation hybrid mapping:

Hybrid sterility and lack of recombination between wheat and alien chromosomes are the major barriers in alien gene transfer in wheat. Radiation hybrid mapping is a recent approach which does not rely on meiotic recombination and can be used in generating high resolution Radiation hybrid maps of wheat (Michalak *et al.*, 2008). Kalavacharla *et al.* (2006) generated a radiation hybrid map of 1D chromosome of wheat with the resolution of about 200kb/break. Hossain *et al.* (2004) were able to locate an alien scs^{ae} gene of *Ae. longissima* (Species Cytoplasm-Specific) in wheat by using radiation hybrid mapping approach.

A very high resolution physical map of wheat chromosome 3B has been recently generated using radiation hybrid mapping along with other approaches of mapping (Paux *et al.*, 2008).

2.8 Iron and zinc acquisition in plants

Although abundant in the earth's crust, iron is present in the soil almost exclusively in its oxidized form [Fe (III)], which has a very low solubility in water, affected by both pH and oxygen. Plants require approximately 10^{-8} M iron, but in calcareous soils, total soluble iron is below 10^{-10} M. Without active mechanisms for extracting iron from the soil, most plants would, therefore, exhibit iron-deficiency symptoms, such as leaf interveinal chlorosis (Kim and Geurinot, 2007). Similarly very little free Zn^{2+} ions occur in soils. Therefore specific uptake strategies are required for absorbing them from soil (Haydon and Cobbett, 2007; Palmgren *et al.*, 2008).

2.8.1 Metal uptake from the soil: from soil to roots

Plants have evolved two mechanisms for taking up metal ions from soil. Dicotyledonous plants adopt reduction based Strategy-I whereas grasses adopt

chelation based strategy-II for uptake of metal ions under deficiency conditions (Kim and Geurinot, 2007; Romheld and Marschner, 1986).

The strategy-I plants secrete protons into the rhizosphere for lowering the soil pH, due to which metal ions in the soil are converted into soluble forms and are readily taken up by the plants. As an example, Fe^{3+} is 1000 times more soluble when reduced to Fe^{2+} (Olsen, 1981). Plasma membrane H^+-ATPases generate the required membrane potential (Palmgren *et al.*, 2008). FRO2, a ferric chelate reductase is required for Fe uptake from Fe deficient soils. *Arabidopsis frd1* mutant (Ferric-chelate reductase defective 1) has no inducible root Fe(III) chelate reductase activity and develops severe chlorosis under Fe deficiency (Yi and Geurinot, 1996). Fe^{2+} is transported into the root by metal transporters of the ZIP (Zinc regulated- Iron regulated transporter Proteins) family. Iron regulated transporter 1 and 2 (IRT1 and IRT2) are representatives of this family and are located in the plasma membrane of epidermal of roots. IRT1 can transport many divalent metals (Fe, Zn, Mn and Cd) and as such the *Arabidopsis irt1* mutants exhibit severe chlorosis and impaired growth (Vert *et al.*, 2002; Henriques *et al.*, 2002). Rice has the ability to transport Fe^{2+} via OsIRT1 in addition to its strategy II uptake (Ishimaru *et al.*, 2006).

Strategy II plants such as wheat, rice, maize, barley secrete low molecular weight compounds known as the mugineic acid (MA) family of phytosiderophores (PS) under low Fe conditions. The MA family comprises derivatives of nicotinamine (NA) of mugineic acid (MA), 2-deoxymugineic acid (DMA), 3-epi-hydroxymugineic acid (epi-HMA) and 3-epihydroxy-2hydroxy mugineic acid (epi-HDMA). Each species produces its own sets of MAs and regulates their secretion in response to the metal deficiency wheat, rice, maize secrete only MA, whereas barley secretes MA, HMA and epi-HMA. After metal-ion chelation, the metal-PS complex is taken up by

the YS1 (Yellow-Stripe 1) transporters located in the plasma membrane of root cells (Roberts *et al.*, 2004). YS1 was the first transporter of a metal ion-ligand identified in plants (Curie *et al.*, 2001). The *ys1* maize mutants were defective in uptake of Fe-PS leading to interveinal necrosis (Curie *et al.*, 2001).

2.8.2 From soil to grain

2.8.2.1 Xylem loading and long distance transport

Once inside the root cells the metal ions undergo symplastic diffusion between interconnected root cells towards the stele (Fig. 2.3). Movement across the xylem parenchyma to the vessels is brought about by HMA2 (Heavy Metal Transporting ATPase2) and HMA4, which pump metal ions into the root vascular system. HMA2 and HMA4 are specific transporters of Zn and Cd but not of Fe or Mn, for which YSL2 and AtIREG1 are suggested to be responsible (Kim and Geurinot, 2007; Colangelo and Geurinot, 2006). Thus *hma2hma4* double mutants suffer from inadequate Zn supply to the shoot, resulting in stunted growth and chlorosis (Hussain *et al.*, 2004). Long distance transport through the xylem sap where pH is around 5.5-6 involves chelation of metal ions with mobile low molecular weight ligands. For example Fe is present as Fe (III)-citrate complexes in the xylem for transport to aerial parts over long distances (Hell and Stephan, 2003).

2.8.2.2 Xylem Unloading and transport to fruits

From xylem vessels, micronutrients undergo active transport to the leaf mesophyll tissue using metal uptake transporters of the parenchyma cells. Their further movement within the leaf cells is symplastic. The transport to the developing

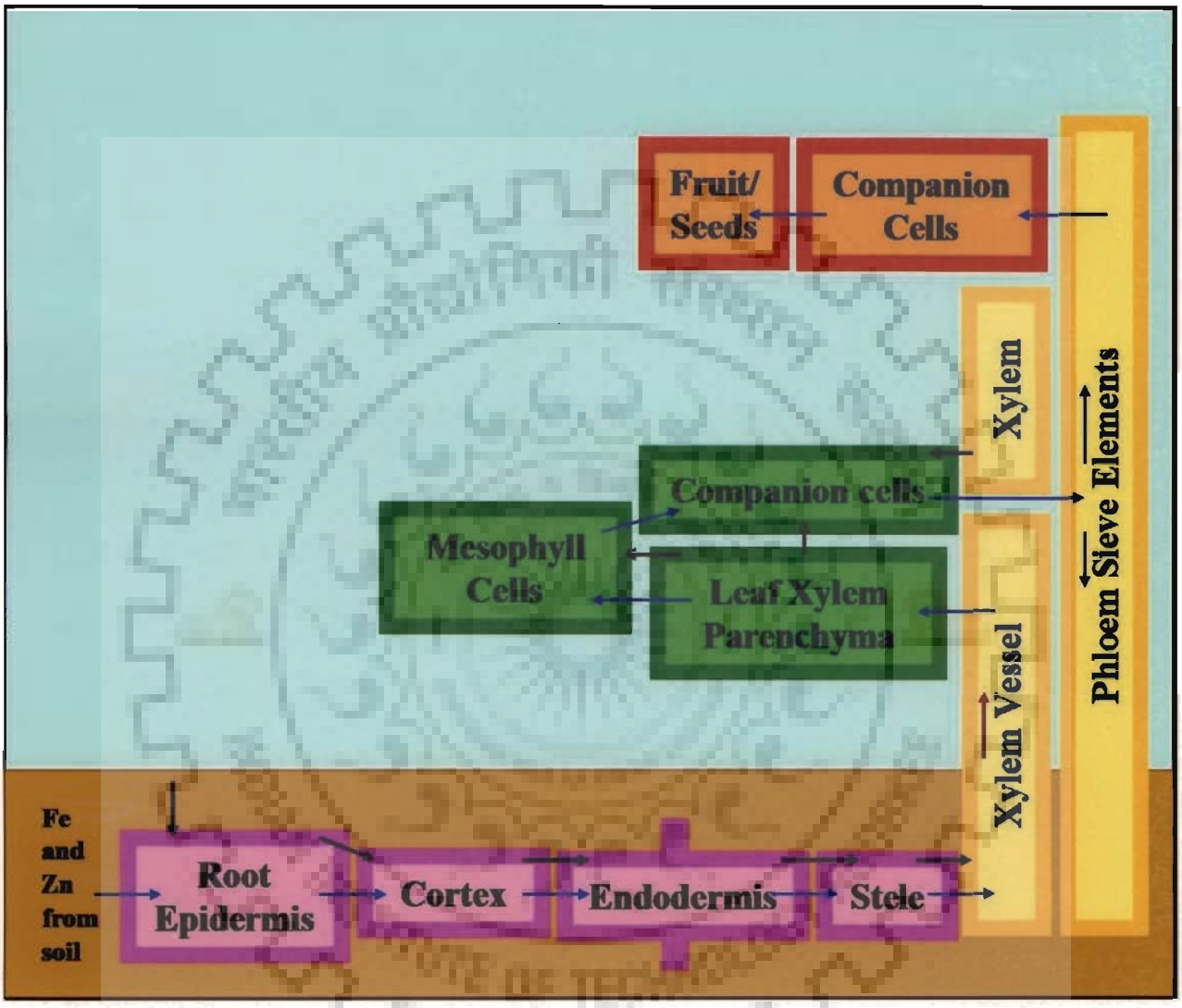


Fig. 2.3 Fe and Zn transport from soil to the grains. Blue arrows show the symplastic and black arrows show apoplastic movement of minerals.

grains takes place either by direct upake from the soil, or from remobilization of stored minerals in the senescing leaves (Uauy *et al.*, 2006). YSL transporters have been suggested to be involved in this transport (Waters and Grusak, 2008).

2.8.2.3 Loading of metals into the seeds and partitioning therein

The embryo and the endosperm of the seed are symplastically isolated from the mother plant and therefore transporter systems are required for metal loading (Patrick and Offler, 2001). Not much is known till date about the nature of transporters regulating phloem unloading. A few studies have been reported explaining the dyanamics of iron and zinc deposition during grain filling. During wheat grain development iron and zinc accumulate in the glumes and testa, but in the mature grain they predominantly remain confined to the aleurone cells and the embryo (Cakmak, 2008; Ozturk *et al.*, 2006).

2.8.2.4 Intracellular metal transport

Metal ion homeostasis is maintained precisely within the cells as both deficiency and hyperaccumulation are hazardous. Fig. 2.4 shows various metal transporters involved in metal homeostasis in plant cells. Pea mutants *brz* and *dgl* show necrotic or degenerative leaves (Fe toxicity) due to Fe overaccumulation (Grusak and Pezeshgi, 1996; Kneen *et al.*, 1990). Vacuoles provide site for accumulating excess minerals and releasing them into the cytoplasm when external supply is suboptimal. Kim *et al.* (2006) reported VIT1 (Vacuolar Iron Transporter) localised in the vacuolar membrane as an Fe^{2+} transporter that functions in vacuolar iron storage in *Arabidopsis*. The *vit1* mutants grow poorly in Fe-limiting soils, highlighting the critical role played by vacuoles in mineral homeostasis in the cells. Members of the Cation Diffusion Facilitator (CDF) family also called the metal tolerance proteins (MTP) control hyperaccumulation of metals particularly Zn, Cd

Table 2.2 Tissue expression, cellular localizations, factors affecting expression and known substrates of metal transporter proteins. The number of family members known in *Arabidopsis* is given in parentheses. Source: Colangelo and Geurinot, 2006.

	Tissue expression	Cellular localization	Inducing conditions	Proposed/ known substrates	Reference(s)
(A) Metal efflux proteins					
P1B-ATPase (8)					
AtHMA2/HMA4	Vasculature of root and shoot, anther	Plasma membrane		Zn, Cd	Eren *et al.*, 2004; Mills *et al.*, 2005
AtHMA5	Root, flower		+Cu	Cu	Andres-Colas *et al.*, 2006
AtHMA6(PAA1)	Root, shoot	Plastid envelope		Cu	Abdel-Ghany *et al.*, 2005
AtHMA8 (PAA2)	Shoot	Thylakoid membrane		Cu	Abdel-Ghany *et al.*, 2005
AtHMA1	Root, shoot	Chloroplast envelope		Cu	Seigneurin-Berny *et al.*, 2005
CDF (12)					
AtMTP1	Root, shoot, flower	Vacuolar Membrane		Zn	Kobae *et al.*, 2004
AhMTP1	Root	Vacuolar Membrane	+Zn	Zn	Drager *et al.*, 2004
TgMTP1		Plasma membrane		Zn	Kim *et al.*, 2004
(B) Metal Uptake Proteins					
YSL (8)					
ZmYS1	Root, shoot		-Fe	Fe^{3+}-PS, Fe^{3+}, Fe-, Ni-, Cu-NA,	Roberts *et al.*, 2004
AtYSL1	Silique, leaf (xylem parenchyma), flower		+Fe	Fe-NA	Le Jean *et al.*, 2005
AtYSL2	Root (endoderm pericycle), shoot	Plasma membrane	+Fe, downregulated by –Zn		Di Donato *et al.*, 2004
OsYSL2	Leaf (phloem), root, seed	Plasma membrane	-Fe	Fe-, Mn-NA	Koike *et al.*, 2004
NRAMP (6)					
AtNRAMP3/4	Root, shoot, seed	Vacuolar Membrane		Fe	Lanquar *et al.*, 2005
TjNRAMP4		Plasma membrane		Ni	Mizuno *et al.*, 2005
ZIP (16)					
OsZIP4	Root, shoot (phloem meristem)		-Zn	Zn	Ishimaru *et al.*, 2005
MtZIP1	Root, leaf		-Zn	Zn	Lopez-Millan *et al.*, 2004
MtZIP3	Root, leaf		Downregulated by -Mn, -Fe	Fe	Lopez-Millan *et al.*, 2004
MtZIP4	Root leaf		-Zn	Mn	Lopez-Millan *et al.*, 2004
MtZIP5	Leaf		-Zn, -Mn	Zn, Fe	Lopez-Millan *et al.*, 2004
MtZIP6	Root, leaf			Zn, Fe	Lopez-Millan *et al.*, 2004
MtZIP7	Leaf			Mn	Lopez-Millan *et al.*, 2004
TjZNT1				Ni, Cd, Mn, Zn	Mizuno *et al.*, 2005
COPT (5)					
AtCOPT1	Root, pollen, embryo, stomata, trichome		Downregulated by Cu	Cu	Sancenon *et al.*, 2004

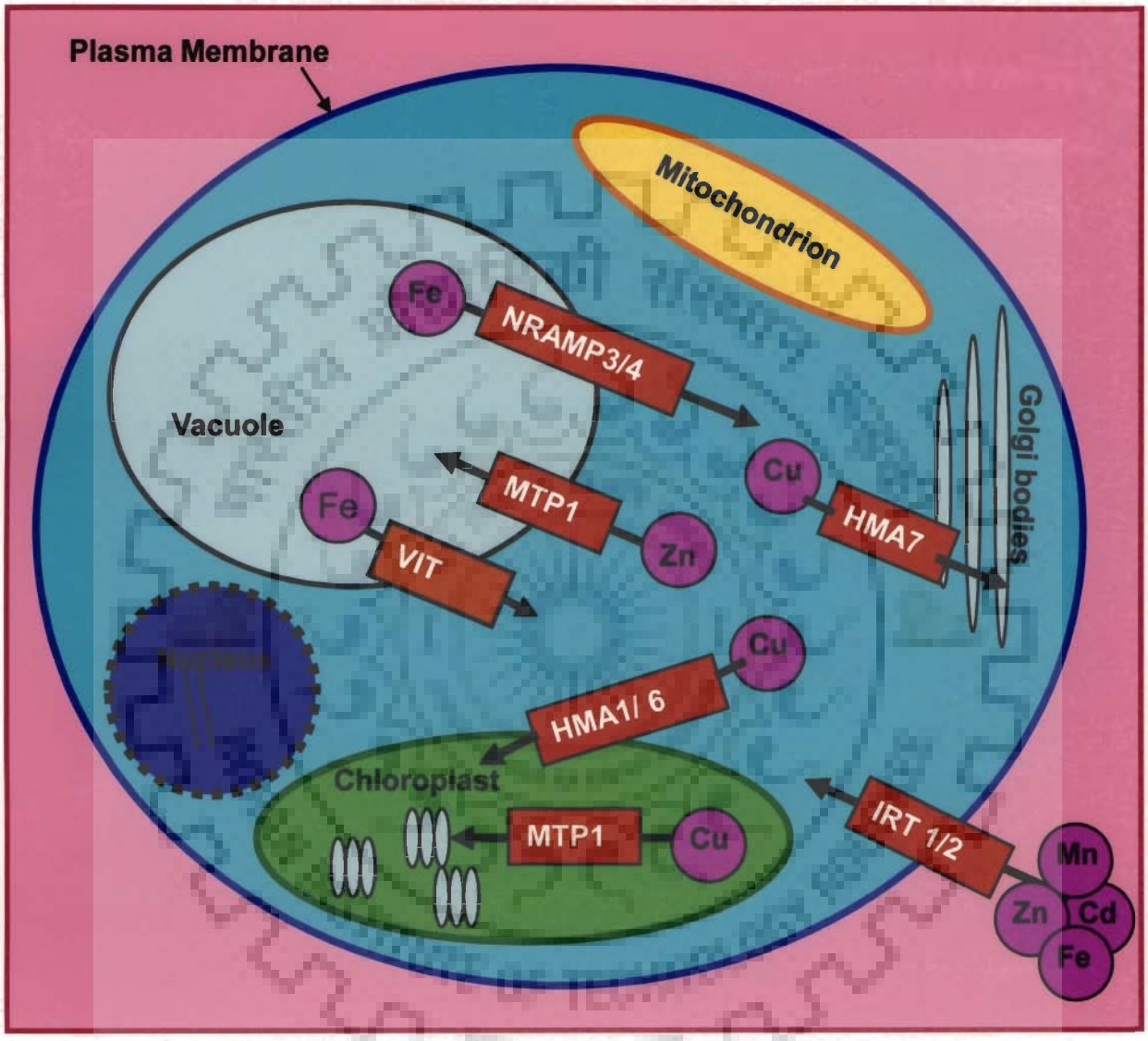


Fig. 2.4 Intracellular metal transporter proteins involved with uptake and efflux of metal ions. Source: Colangelo and Geurinot, 2006.

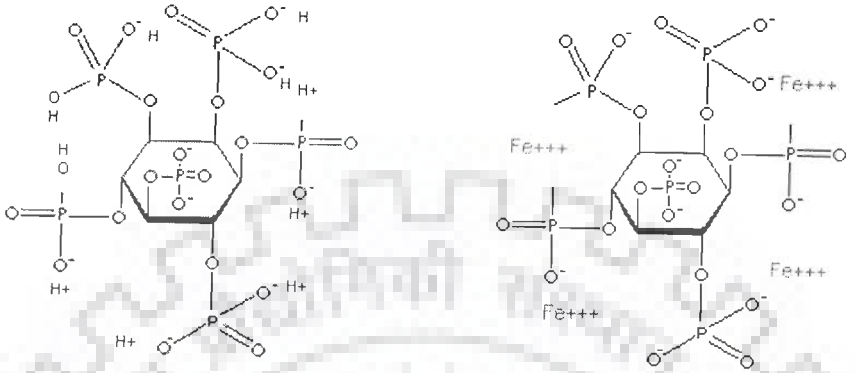
and Co in the cytoplasm by facilitating their movement into the vacuoles when present in excess. NRAMP (Natural Resistance Associated Macrophage Proteins) family transporters AtNRAMP3 and AtNRAMP4 facilitate mobilization of vacuolar iron for seed germination on low iron (Lanquar *et al.*, 2005). *Arabidopsis* double mutants *nramp3 nramp4* is arrested under low Fe nutrition and fully rescued by Fe supply, but single knockout mutants *nramp3-1* and *nramp4-1*do not show change in phenotype, suggesting a functional redundancy. The NRAMPs are highly conserved family of integral membrane proteins involved in metal ion transport not only in plants but also in bacteria, fungi, plants and animals (Hall and Williams, 2003). A list of metal efflux transporters (that decrease metal ions in the cytoplasm) and metal uptake transporter proteins (that increase metal ion concentration in the cytoplasm) are given with their known localization in *Arabidopsis* in Table 2.2.

Thus plants have delicately balanced systems for metal homeostasis which has to be taken care of while exploiting them for mineral accumulation.

2.9 Bioavailability of micronutrients

Phytic acid (*myo*-inositol-(1,2,3,4,5,6)-hexakisphosphate, Fig. 2.5a) accounts for about 1% of the seed weight (Lott *et al.*, 2000; Cosgrove, 1996) and stores 50-80% of total seed phosphorus. Having dense negative charges as phytate ion (Fig. 2.5b), it has strong tendency to bind metal cations in seeds forming stable salts with metals (Brinch-Pederson, 2002). Monogastric animals (like humans, poultry, pigs and fish) unlike ruminants are unable to utilize phytic acid due to the absence of microbial flora in their gut capable of degrading phytic acid. Thus presence of phytic acid further aggravates micronutrient deficiency in human diet and animal feeds acting as a strong antinutrient. Phytic acid is considered to be the single most important anti-nutritional factor in food (Bouis, 2000). Plant based diets with high phytate content, tannins,

fibers and polyphenols have low bioavailability of minerals (Welch, 1984). The antinutritional problem of phytic acid is so severe that Zimmerman and Hurrel (2007)



(a) (b)

Fig. 2.5 Structure of (a) Phytic acid and (b) Fe(III) salt of phytic acid

recommend daily intakes for micronutrients based on the type of diet one takes (Table 2.3) the values of which are upto 4 folds higher than WHO recommended RDI values (WHO, 2000).

Table 2.3 Recommended daily intakes of iron and zinc estimated by dietary iron bioavailability in age group of 19-50 years (Table based on Zimmerman and Hurrel, 2007 and IZiNCG)

Type of diet	Bioavailability	Daily intake			
		Iron		Zinc	
		Women	Men	Women	Men
Diet rich in viamin C and animal protein	15 %	19.6	9.1	8.0	14.0
Diet rich in viamin C but poor in animal protein	10%	29.4	13.7	12.0	21.0
Diet poor in vitamin C and animal protein (vegetable diet)	5%	58.8	27.4	24.0	42.0

Due to the stability of phytate complex, phosphorus fertilizers have to be added externally, inspite of excess presence of phytate-P to maintain fertility of the soil (Brinch- Pederson *et al.*, 2002). Stable phytate complexes are carried away to

water sources where they cause excessive growth of phytoplanktons (eutrophication) and increase the biological oxygen demand (BOD), depriving the aquatic animal life of the oxygen supply. Because of its fundamental importance to human diet, poultry feed, agriculture and environmental problems phytic acid has been studied in detail (Vats *et al.*, 2009; Raboy, 2001; Pederson *et al.*, 2000; Mallin, 2000). Various approaches to reduce phytic acid in food and feed have been studied.

2.9.1 Bioprocessing methods

Bioprocessing methods such as germination, fermentation, dehulling, roasting etc. have been used unknowingly by man for several centuries to reduce phytic acid of food. These processes are known to reduce phytic acid content of food due to phytase activity (Frontela *et al.*, 2008; Brinch-Pederson *et al.*, 2002; Khetarpaul and Chauhan, 1989). Endogenous phytases are produced in the germinating seeds, thus significantly reducing the phytic acid content and simultaneously enhancing the bioavailability of minerals like phosphorus, iron, zinc, calcium, potassium, magnesium etc. (Oloyo, 2004; De Ruiz and Bressani, 1990). Lintschinger *et al.* (1997) reported increase in bioavailability of various trace elements during germination of wheat, buckwheat and quinoa. Al-Numair *et al.* (2009) found HCL-extractability, a measure of increase in bioavailability, of minerals to increase in sprouted beans. Milling and polishing reduce phytic acid by mechanically eliminating phytate rich aleurone layer from grains. Fermentation, on the other hand utilizes phytase produced by yeasts to break down phytic acid in dough.

2.9.2 Phytase addition:

External phytase addition to poultry and pig feed for increased phosphorus availability is practised in livestock units (Boiling *et al.*, 2000). Wyss *et al.* (1999) studied *in vitro* phosphate liberation kinetics using feed suspensions supplemented

with *Aspergillus fumigatus* and *A. niger* phytase (Natuphos). Phytases from many microbes have been studied and are commercially available (Verwoerd *et al.*, 1995) and search for new sources of thermotolerant phytases with broad subsrate specificity is going on (Vats, 2009; Vats and Banerjee, 2005; Wyss *et al.*, 1998).

2.9.3 Transgenics with phytase:

To reduce phytase production costs, new prokaryotic and eukaryotic expression systems were explored. Phytase genes from various *Aspergillus* species have been expressed in transgenic tobacco seeds and leaves (Ullah *et al.*, 1999; Verwoerd *et al.*, 1995; Reddy *et al.*, 1982), transformed soybean cell suspension cultures (Li *et al.*, 1997), transgenic soybean and alfalfa (Ullah, 2002) and transgenic wheat, rice and canola seeds (Brinch-Pederson *et al.*, 2000; Zhang *et al*, 2000; Hong *et al.*, 2004; Lucca *et al.*, 2001). The plant produced phytases have a lower molecular mass than the fungal enzymes due to difference in glycosylation (Li *et al.*, 1997). Poultry-feeding studies demonstrated that the plant-produced phytase can substitute for the enzyme produced from microbial fermentation (Zhang *et al.*, 2000). Chen *et al.* (2008) developed transgenic maize by overexpressing *A. niger phy*A2 gene using maize embryo specific globulin-1 promoter. This transgenic maize produced 2,200 units of phytase per kg seed, a 50 fold increase compared to non-transgenic maize seeds. These phytase expressing transgenics can be used on commercial scales to improve P bioavailability and solve environmental problems associated with commercial phytase production.

2.9.4 Low Phytic Acid mutants:

Plants have a biochemical pathway by which $InsP_6$ is synthesized in a stepwise manner, each step catalysed by enzymes (Pederson *et al.*, 2002; Loewus and Murthy, 2000; Stephens and Irvine, 1990). Lesion in any of the genes coding the enzymes

impairs phytic acid synthesis, generating low phytic acid (*lpa*) mutants. For instance mutation in MIPS (myo-inositol-3-P_1 synthase), the enzyme catalysing glucose-6-P conversion to Ins(3)P_1, results in lower phytic acid and reduced seed raffinosaccharides (Hitz *et al.*, 2002). Low phytic acid mutants have been reported in a number of plants like maize (Raboy *et al.*, 2000), rice (Larson *et al.*, 2000), soybean (Wilcox *et al.*, 2000), barley (Rasmussen and Hatzack, 1998) and wheat (Guttieri *et al.*, 2004). These mutants have higher free phosphorus, mineral cations and are thus more nutritious as food and feed. Besides they also provide opportunity for better understanding of synthetic pathways of Inositol phosphates in developing grains. Recently Zhao *et al.* (2008) developed CAPs markers after identification of the locus of two *lpa* mutants of rice.

2.9.5 RNAi for silencing MIPS (Myoinositol synthase)

The gene controlling MIPS is under the control of *RINO1* gene expressed in developing rice seeds specifically in aleurone and embryo. Recently Kuwano *et al.* (2008) used antisense *RINO1* under *Ole18* promoter. *Ole18* codes an oleosin Ole18, an 18kDa protein expressed in the aleurone and embryo of developing seeds. The transgenic rice had 68% lower phytic acid levels than the wild type and had normal seed weight, germination and plant growth. The concomitant increase in inorganic phosphate levels was higher than the currently available rice *lpa* mutants.

Though Phytic acid is infamous for its antinutrient action, new studies reveal its positive roles as an antioxidant and anti-cancerous compound (Harland and Morris, 1995). Pros and cons have to be analysed carefully by feed trials and cell-cultures.

Chapter III

Materials and Methods

3. MATERIALS AND METHODS

3.1 Plant Materials

3.1.1 Plant material for development of introgressive derivatives

The experimental material comprising eighty accessions of nine related *Aegilops* and wild *Triticum* species from different geographical regions was obtained from the wheat germplasm collection maintained at the Punjab Agricultural University, Ludhiana, India. The related wild species, wheat and durum cultivars were grown at the experimental fields of the Indian Institute of Technology Roorkee, Roorkee for two consecutive seasons of 2004-05 and 2005-06 as unreplicated single row of two meter length with plant to plant distance of 10 cm and row to row spacing of 30 cm with recommended fertilizers and irrigation as that of wheat. Grains, spikelets and spikes were harvested and threshed from cultivars and wild accessions at physiological maturity. Due to frequent shattering of spikes in various wild species, collection of mature spikelets and spikes had to be done repeatedly at different intervals over two-three weeks. Due to tough glumes and hard threshing in wild species the grains had to be taken out manually.

F_1 hybrids

For transfer of useful variability for higher concentration of iron and zinc from selected wild donors, interspecific crosses were made using wheat and durum cultivars as the maternal parent. A bread wheat line Chinese Spring with *Ph*^{I} transferred from *Ae. speltoides* obtained from Dr. B.S. Gill of Kansas State University, Kansas was used for making crosses for induced homoeologus pairing whereas interspecific crosses were also made with wheat and durum cultivars without *Ph*^{I} gene.

Backcross derivatives

In the following season of 2005-06 the hybrid F_1 CS(*Ph*^{l})/*Ae. kotschyi* 396 was backcrossed with elite cultivars. The BC_1 plants were either crossed with recurrent parent next year or allowed to self depending upon the fertility of the plants. The BC_2 and BC_1F_2 seeds were analyzed for their micronutrient content and the selected progenies were sown next year. Finally BC_1F_3 and BC_1F_4 seeds were put to rigorous chemical analysis to select a few derivatives with exceptionally higher micronutrient content than the control wheat cultivar WL711. Fig. 3.1 shows schematic presentation of the development of *Aegilops kotschyi*. These selected derivatives were characterized on the basis of morphology, cytology, HMW-glutenin subunit profiles, microsatellite markers and finally Genomic *in situ* Hybridization (GISH).

Period	Generation	Remarks
Nov, 2004-April, 2005	CS(*Ph*^{l}) ♀ X *Ae. kotschyi* ♂	
Nov, 2005-April, 2006	F_1 X Wheat cultivar	F_1 hybrids sterile
Nov, 2006-April, 2007	BC_1 X Wheat cultivar	Partially fertile plants allowed to self
Nov, 2007-April, 2008	BC_1F_2 BC_2F_1	Sufficient selfed seed set
	selfed	High Fe and Zn derivatives sown
	BC_2F_2 and BC_1F_3 seeds	Selected derivatives analysed

Fig. 3.1 A schematic presentation of the development of introgressive derivatives

3.1.2 Synthetic wheat-*Aegilops kotshyi* amphiploids

Seven F_1 hybrids were produced using CS(*Ph*^{1}) or WL711 as the female parent and six *Ae. kotschyi* accessions (acc. no. 396, 395, 393, 3774, 391 and 3790) as the male parent. In the following year, the F_1 seeds were sterilized with 1% sodium hypochlorite for five minutes, washed thrice with distilled water and germinated on two layers of sterilized moist filter paper in Petri plates. The chromosomes of the F_1 hybrids were doubled by treating coleoptiles of germinating seeds with 0.25% of colchicine (in 5 % DMSO solution) for 5 hours. The colchicine treated seedlings were transplanted in the field.

During flowering the spikes with dehiscing viable pollen grains and seed set, evidently due to chromosome doubling were identified and tagged. Seeds (C_0 generation of amphiploids) from the doubled sectors of the tagged spikes were harvested carefully before shattering of spikes. The C_1 generation of these amphiploids was grown in the field during 2006-2007. Collection of mature spikelets and spikes of the F_1 hybrids and synthetic amphiploids had to be done repeatedly at different intervals over two-three weeks because of frequent shattering of spikes. Due to tough glumes and hard threshing in the amphiploids and wild donors, the grains were threshed manually. Mean number of seeds per spike was determined for each amphiploid by taking average of seeds of 10 spikes.

3.1.3 Plant material for characterization of low phytic acid mutants

A set of 76 EMS-induced mutants of *Triticum monococcum* isolated at Punjab Agricultural University, Ludhiana and maintained at I.I.T. Roorkee was initially screened for low seed phytic acid content using the method of Rasmussen and Hatzack (1998). Thereafter two putative *lpa* mutants were selected for further characterization.

3.1.4 Seed material for study of effects of germination on phytic acid

Seeds of 10 Indian bread (*Triticum aestivum* L.) and 5 durum (*T. durum* Desf.) wheat cultivars were analysed for their phytic acid content. Detailed study of effects of germination on nutrient mobilization was done in *T. aestivum* L. 'WL711'.

3.2 Chemical Analyses

3.2.1 Grain analysis:

For chemical analysis whole grain samples from cultivated and wild accessions were washed with N/10 HCl (Merck), dried till constant weight (0.5g) and dried in hot air oven at 80°C for 4 hours. Grain samples were digested in a mixture of two parts of concentrated nitric acid (Merck) and one part perchloric acid (Merck) as per the standard procedure described by Zarcinas *et al.* (1987). Digestion was continued till white residue was obtained. Required volume was made after the completion of digestion process and digests were analyzed by Atomic Absorption Spectrophotometer (AAS); (GBC- Avanta Garde M). A minimum of five replications of chemical analysis was made in each of cultivars and wild accessions. Grain iron and zinc status of selected donors and selected derivatives were also reconfirmed by Inductively Coupled Plasma Mass Spectrometer (ICPMS) (Perkin Elmer).

3.2.2 Flag leaf analysis

Flag leaves from selected potential donors, recipient parents and their F_1 hybrids were collected at the pre-anthesis stage, washed thoroughly with N/10 HCl, dried at 80° C for 8 hrs in oven prior to digestion. Dried leaf samples were then digested as a minimum of five replications using diacid mixture of nitric acid and perchloric acid (Zarcinas *et al.*, 1987). Iron and zinc concentrations in the digests were analyzed by AAS.

3.2.3 Grain ash analysis

Grains (1 g) were ashed in muffle furnace at 500° C for 10 hours. The ash was carefully collected and weighed. Further the ash samples were processed like grains for iron and zinc analyses.

3.3 Cytological Studies

For meiotic analysis spikes of interspecific F_1 plants were fixed in Cornoy's solution (6 ethanol: 3 chloroform: 1 acetic acid) for 24 hours and transferred to 70% ethanol. Anthers at various stages of meiotic division-I were squashed in 2% acetocarmine and the pollen mother cells (PMCs) were scored for chromosomal pairing in all the crosses. Photographs were taken with a digital camera (Canon PC1049, No. 6934108049). Pollen stainability was measured by staining the pollen grains after squashing the anthers in Iodine-Potassium Iodide solution (I-KI).

3.4 Protein analysis

HMW gluentenin subunit proteins were analysed using method described by Smith and Payne (1984) with some modifications.

3.4.1 HMW glutenin subunit extraction reagents and procedure

Extraction buffer: SDS (sodium dodecyl sulphate, Merck) 2.0g, ß- mercaptoethanol (HiMedia) 5ml; Total volume made upto 100ml with distilled water after setting pH to 6.8

Dye: Bromophenol Blue (Merck) 0.5g, Glycerol 50ml (SRL), Distilled water 50ml

3.4.2 Extraction procedure:

Single seed was crushed and weighed (Say its weight was X mg). Extraction buffer (13.2 x X) was added to it in an eppendorf tube, vortexed for 1.5 minutes and

then incubated in water bath at 80°C for 18 minutes. 1.2 x X dye was added to it and then it was centrifuged at 4000rpm for 10 minutes. Supernatant was retained.

3.4.3 HMW glutenin SDS PAGE reagents

Acrylamide 40%: Acrylamide (SRL) 100g; total volume was made upto 250ml with distilled water.

Bis-acrylamide 2%: Bisacrylamide (SRL) 2.0g; total volume was made upto 100ml with distilled water.

Stain : Commassie Brilliant Blue R-250 (SRL) 2.0g; 100% Methanol 800ml (SRL); 100% Trichloro Acetic acid (SRL) 200ml; Total volume was made upto 2000ml with distilled water.

1.5M Running gel buffer: Tris 18.17g, SDS 0.4g; Total volume was made upto 100ml with distilled water after setting pH to 8.8.

0.5M Stacking gel buffer (pH 6.8):

Tris 6.06g, SDS 0.4g; Total volume was made upto 100ml with distilled water after setting pH to 6.8.

10X Tank buffer (pH 8.3): Tris 30.3g, Glycine 142.0g, SDS 10.0g; Total volume was made upto 1000ml with distilled water after setting pH to 8.3.

Ammonium per sulphate (APS, SRL) 0.06g in 600µl distilled water.

Tetramethylene diamine (TEMED, HiMedia)

Butan-2-ol (SRL)

Running gel (10%)

Acyrlamide: 5.0ml, Bis-acrylamide: 1.3ml, Running gel buffer: 5.0ml, Distilled water: 8.7ml, APS: 250µl, TEMED: 50µl

Stacking Gel (5ml)

Acrylamide: 0.55ml, Bisacrylamide: 0.30ml, Stacking gel buffer: 1.25ml, Distilled water: 2.90ml, APS: 55.55µl, TEMED: 20µl

3.4.4 HMW glutenin SDS PAGE procedure:

The 10% running gel was poured in the preset gel casting unit (Atto, Japan), overlaid with butanol and allowed to polymerize for 40 minutes. Butanol was drained off and thoroughly washed with distilled water. Stacking gel was poured over the running gel and comb was inserted. It was left for 15 minutes for polymerizing. Comb was pulled and gel was washed by pushing distilled water with mild pressure. Chilled 1X tank buffer was poured in the assembly, gel was inserted into it and samples (10 µl) were loaded in the wells.

3.5 Genomic *In situ* hybridization

Genomic *in situ* hybridization was done in order to finally visualize the alien introgression in the selected derivatives using the method described by Dou *et al.* (2006). Seeds were germinated at room temperature. Root tips were collected at a length of 0.5–2 cm, pretreated in ice-water for 24h, and fixed in 99% ethanol–glacial acetic acid (3:1). Slides were prepared by squashing in 45% acetic acid. Genomic DNA of *Aegilops longissima* and *Ae. umbelullata* were used as probes in GISH. Clones pAs1 (Rayburn and Gill, 1986) and a synthesized 30-base length $(AAG)_{10}$ repetitive oligomer were used as probes in FISH. pAs1 is regarded to be a D-genome-specific clone. The pattern of FISH with this clone permits identification of the D-genome chromosomes, though there are other weak hybridization signals on some B and A-genome chromosomes (Pedersen and Langridge, 1997). The pattern of AAG-

satellites together with pAs1 can identify the entire chromosome complement of bread wheat by two-color FISH (Pedersen and Langridge, 1997).

3.6 Isolation and purification of genomic DNA from leaf tissues

DNA was extracted from young leaves of the parents and selected BC_2F_2 and BC_1F_4 plants using CTAB method described by Murray and Thompson (1980).

3.6.1 DNA Extraction buffer:

200mM Tris (pH 8.0), 20mM ethylene diamine tetra-acetic acid (pH 8.0)

140mM NaCl, 2% CTAB (Cetyl-trimethyl ammonium bromide)

0.01% β mercaptoethanol). All chemicals used were of HiMedia (Molecular biology grade).

3.6.2 DNA isolation and purification reagents:

TE buffer (10 mM Tris (pH 8.0) 1mM EDTA (pH 8.0)), RNAse solution (10mg/ml), Phenol: Chloroform: Isoamyl alcohol (25:24:1), Isoproponal, Absolute Ethanol, 70% ethanol

3.6.3 DNA isolation procedure

About 5-7g of young, healthy and disease free leaves from each plant were collected and kept in the plastic bags on ice. Leaves were frozen in liquid nitrogen and crushed to fine powder using autoclaved and pre-chilled mortar and pestle. The powder was transferred to 50 ml Oakridge tubes containing pre-warmed (65°C) DNA extraction buffer (15ml for approximately 3g of leaves). It was gently mixed and incubated in 65°C water bath for 1 hour, mixing briefly every 15 minutes. Equal volumes of phenyl: chloroform: isoamyl alcohol (25:24:1) solution was added to the samples followed by gentle mixing for 15 minutes to ensure emulsification of phases. The samples were centrifuged at 10,000rpm for 20 minutes at 25°C. Supernatants were transferred to the falcon tubes with the help of micropipettes. Equal volume of

ice cold propon-2-ol was added and left overnight at 4°C for complete precipitation of DNA. DNA was spooled out using large bore pipette tips into the 1.5ml microcentrifuge tubes. It was centrifuged at 8000 rpm to get a pellet of DNA. Supernatant was discarded and pellet was washed with 400µl 70% ethanol. It was centrifuged at 8000rpm for 5 minutes. Ethanol was drained out, pellets were air dried and resuspended in 500µl TE buffer. Subsequently RNAse treatment at final concentration of 100µg/mL was done at 37°C for 1 hour. The DNA was re-extracted with fresh chloroform: isoamyl alcohol followed by reprecipitation with ethanol and pelleting by centrifugation (8000 rpm, 4°C). Pellet was collected, air dried (37°C) for few hours and dissolved in appropriate volume of 1X TE. For DNA quantification, spectrophotometric readings of the DNA samples were taken at wavelengths 260nm and 280nm. Ratio of OD260/OD280 was checked to be around 1.8 as a measure of DNA purity. At wavelength 260 nm, the concentrations of DNA (OD260x 50x dilution factor) were determined and subsequently samples were diluted to 50ng/µl concentration. Electrophoresis (Sambrook, 2001) was carried out finally for the qualitative and quantitative analysis in 0.8% agarose gel with 0.5µg/ml ethidium bromide (10mg/ml) in 1X TAE.

3.7 Application of microsatellite markers

Wheat microsatellite markers (401 in number) representing all the 21 chromosomes of wheat covering both chromosomal arms were selected from publications of Roder *et al.* (1998), Pestsova *et al.* (2000) and Somers *et al.* (2004). A list of the markers used has been given in Annexure-I. Parental polymorphism between wheat cultivars and *Ae. kotschyi* accession 396 was checked. PCR was carried out according to Roder *et al.* (1998) with some modifications. The primers were synthesized from Hysel India (Pvt.) Ltd. Distal transferable polymorphic

markers of each chromosome arm were applied in the finally selected derivatives to identify the introgressed chromosome. Finally the introgression was characterized by applying additional polymorphic markers of whole introgressed chromosome(s) to the selected derivatives.

3.7.1 Composition of reaction mix:

PCR Buffer (10X) - 2µl, dNTP mix (1mM each dATP, dCTP, dGTP and dTTP) - 4µl Primer f (5mM) - 1µl, Primer r (5mM) - 1µl, Taq polymerase - 1 unit, $MgCl_2$ (25mM) - 1.2 µl, DNA (50ng/ µL) - 2µl : Total volume-20µl

3.7.2 PCR conditions:

The PCR was carried on Eppendorf Thermocycler with following conditions: Initial denaturation at 94°C for 4 min; 35 cycles of - denaturation at 94°C for 1 min and annealing at 50-68°C depending upon the primer T_a for 1 min; extension at 72°C for 1 min; Final extension at 72°C for 7 min

3.7.3 Resolution of the amplified SSR product:

4µl of 6X gel loading dye (New England Biolabs) was added to the 20µl PCR product. The PCR products were loaded on 3% high resolution agarose (Amresco) having 0.5µg/ml ethidium bromide (10mg/ml) and prepared with 1X TAE buffer. The gels were visualized and photographed using BioRad gel documentation system.

3.8 Low phytic acid characterization

3.8.1 Reagents for qualitative screening

Extraction reagent: 0.4M HCl

Chen's Reagent (Molybdate reagent): For 50 ml

6N H_2SO_4	-	10ml
Ammonium molybdate (2.5%)	-	10ml

Ascorbic acid (10%) - 10ml

Milli-Q water (with specific resistance 18.2mΩcm or higher) - 20ml

3.8.2 Procedure for Qualitative screening:

Initial screening of the *T. monococcum* mutants was done by colorimetric method used by Rasmussen and Hatzack (1998). The experiment was carried out in two replications in microtitre plates. Two adjacent seeds from the middle of a spike of the plants were crushed in each microtitre plate. Extraction of phytic acid was done in 200µl 0.4 M HCl and kept for 12 hours at 4°C. Tapping was done briefly and then 10µl of the extract was taken in another microtitre plate to which 90µl of Milli-Q water was added. To this 100µl of freshly prepared Chen's reagent was added. Inorganic phosphate standards were prepared from KH_2PO_4 with P*i* equal to 0.15, 0.50, 1.00, 1.5, 2.0 and 2.5 (all values in µg/ml) and 100µl of each was mixed with 100µl Chen's reagent. Scoring was done visually. In case of high free phosphate content a dark-blue coloured phospho-molybdate complex formed in 1-2 hours.

3.8.3 Reagents for HPLC of putative *lpa* mutants:

The putative mutants selected on the basis of colorimetric screening were analysed for their phytic acid content using the method described by Dost and Tokul (2006). All chemicals were of A.R. grade from Merck:

For Fe-SCN complex (100 ml).

Ferric chloride hexahydrate - 100 µg/ml - 25 ml

Ammonium thiocyanate - 500 µg/ml -25 ml

Conc. HNO_3 -0.2 ml

Milli-Q water -to make volume 100 ml

Extraction reagent

HCl (0.5M) -100 ml

Standards:

Stock standard - 1mg/ml phytic acid

Calibration standards of 10, 25, 50, 75 and 100µg/ml were prepared from the stock using Milli-Q water for dilution.

HPLC reagents:

Mobile phase – 30% Acetonitrile in 0.1 M HNO_3

Instrument:

HPLC instrument - Agilent 1100 Series, 2006 Make

Column – Novapack C-18 HPLC column

3.8.4 Sample Preparation:

T. monococcum wild type and two mutants MM225 and MM169 were used for HPLC analysis in two replicates. All samples were finely powdered in Agate mortar pestle. Each homogenised sample was carefully weighed (0.2 g) and transferred to 20 ml beaker with 10 ml extraction reagent. Shaking was done for 1 hour at room temperature, there after the contents were transferred to 10 ml centrifuge tube, followed by centrifugation for 15 min at 4000rpm. The supernatants were stored at 4 °C till the addition of Fe-SCN complex.

Prior to analysis, 900 µl of Milli Q- water was added to 100 µl of the extracted samples, followed by 2 ml of freshly prepared Fe-SCN complex. The samples were syringe filtered using Millipore membrane filter. One ml of each standard was mixed with 2 ml of Fe-SCN complex before analysis.

3.8.5 Estimation:

The flow rate of mobile phase was set at 1ml/min and was left to run for half an hour for baseline development. Samples and standards (20 µl) were injected into the instrument using Hamilton syringe and allowed to run for 5 minutes each. The data

was collected using Agilent-software. Since the method of detection is based on the formation of iron(III)-thiocyanate complex, a reduction in peak area would give increase in phytic acid in the sample.

3.9 In vitro digestion and Iron availability

3.9.1Chemicals

All the chemicals were purchased from Sigma, St Louis, MO, USA, unless otherwise specified. Sodium-bi-carbonate, Sodium Acetate, Trichloroacetic acid and Hydroxylamine hydrochloride were purchased from Sisco Research Laboratories Pvt. Ltd., India.

3.9.2 Method

The method used was that described by Bergqvis *et al.* (2006), with slight modification. Briefly, 1gm of cooked, homogenized and lyophilized wheat samples were taken in triplicate and suspended in 8 to 10 ml of Milli Q water. The pH of each suspension was adjusted to pH 2 with 5 mol/l HCl, and 0·3ml of freshly made and chelex treated pepsin (P7000; Sigma, St Louis, MO, USA) solution (0·16 g (1130 U/mg protein) per ml 0·1 mol/l HCl) was added. The mixture was incubated for simulated gastric digestion, on an incubator shaker at 120 rpm at 37°C for 2 hrs. The pH of the gastric digest was brought to 6 by the dropwise addition of 1 mol/l NaHCO3. A volume of 1·7ml of chelex treated pancreatin–bile mixture (0·12 g bile extract and 0·02 g Pancreatin (4 X USP activity, in 5ml 0·1 mol/l NaHCO3) was mixed with each sample and incubated for another 2 hrs on an incubator shaker at 120 rpm at 37°C. The pepsin and bile-pancreatin solutions used for the assay were treated with Chelex-100 resin from Sigma, St Louis, MO, USA to eliminate the contaminant iron from Pepsin, Bile extract and Pancreatin as described by Glahn et al (1998). An assay blank was also maintained for simulated digestion without any wheat sample. The incubation times followed were according to Miller *et al.* (1981). The digests

were placed on ice to stop pancreatic activity. Volumes of all samples were brought to 18 ml and then centrifuged at 5000 g for 30 min at 4°C.

The soluble iron in the supernatants was quantified according to Miller et al. (1981), briefly the protein precipitant solution (10% Trichloroacetic acid, 10% Hydroxylamine hydrochloride and 10% Conc. HCL) was added to the supernatant at a ratio of 1:2, protein precipitant/supernatant, and thoroughly mixed. The mixture was heated in a boiling water bath for 20 mins and centrifuged. An aliquot of the clear supernatant was transferred to a clean test tube and the chromogen solution (25mg Bathophenanthroline sulphonate from Sigma, St Louis, MO, USA dissolved in 100 ml of 2M Sodium Acetate) was added with thorough mixing at a ratio of 2:1, supernatant/chromogen. To obtain a standard curve, 0·05–1.00 µg/ml Iron (Iron Standard for AAS 1mg/ml from Sigma, St Louis, MO, USA), was mixed with bathophenanthrolinesulfonate. The formation of Fe^{2+} –bathophenathrolinesulfonate was measured spectrophotometrically at 535 nm after 10 min against a reagent blank. The level of Fe^{2+} was quantified based on the standard curve.

3.10 Study of effects of germination on phytic acid and mineral content in wheat grains

3.10.1 Sample preparation

Sufficient quantity of WL711 grains were sterilized with 1% sodium hypochlorite, thoroughly washed with distilled water, presoaked for 6 hrs and germinated in plastic trays on two layers of sterilized moistened blotting paper for 24-144 hrs. Regular watering was done with Millipore- ultra pure water. The control, germinating seeds and seedlings after different intervals of germination were oven dried at 50°C till constant weight and moisture. Replicated samples were used for estimation of phytic acid, carbohydrates, proteins and lipid contents. Fresh samples were processed

for Scanning Electron Microscopy- Energy Dispersive X ray studies (SEM-EDX). Each analysis was carried out in three replications.

3.10.2 SEM-EDX analysis of minerals

Electron dispersive elemental microanalysis of germinating grains was done using SEM-EDX model FEI Quanta 200F. This instrument is sensitive enough to detect 0.01 to 0.1% of elements. Phosphorus, potassium, magnesium, calcium, iron and zinc were analysed for comparative distribution within aleurone and endosperm regions of wheat grains. Their subsequent analysis was also done in the aleurone layer of grains after varying duration of germination. Sample preparation was done according to the method of Feeney *et al.* (2003). Transverse sections cut through the centre of grains were fixed using a solution of 2.5% glutaraldehyde in 25mM PIPES buffer (pH 6.9). Vaccuum infiltration of fixation solution was done for half an hour. Thereafter 4 hours of incubation in fixative was done with continuous rotation at atmospheric pressure, followed by washing in 25mM PIPES buffer for 15 minutes thrice. Subsequently dehydration in an increasing ethanol: water series (30, 50, 70, 80, 90 and 100) by 30 minutes of rotation in each solution was done. Fixed dehydrated samples were placed on double sided carbon tape adhered on aluminium stubs. To make samples conducting, gold coating was done for 60 seconds at 30 mA current for depositing a 5-7 nm thick layer using BAL-TEC SCD 005 model sputter coater. Silver enhancement technology was used to increase the size of colloidal gold particle (Scopsi *et al.*, 1986).

3.10.3 Phytic Acid Estimation

Method of Villanova and de Lope (1982) was used for extraction and estimation of phytic acid content in germinating wheat seeds. This method is based on formation of Iron (III)-Phytate complex and utilizes sulphosalicylic acid as an indicator of the end point of titration.

4. RESULTS

Results of this study are presented in the following sections

1. Development of introgressive derivatives of *Aegilops kotschyi* acc. no. 396 with high grain iron and zinc and their cytological and molecular characterization.
2. Development of wheat- *Ae. kotschyi* synthetic amphiploids with high grain iron and zinc content.
3. Identification and characterization of two low phytic acid mutants of *T. monococcum*.
4. Partial germination as a bioprocessing method to reduce phytic acid content of wheat grains.

Chapter IV

Results

4.1 Development of wheat- *Ae. kotshyi* acc. no. 396 introgression lines and their cytological and molecular characterization

4.1.1 Grain iron and zinc content and selection of donor parent

The range and mean of grain iron and zinc content in the cultivars of bread wheat and durum and accessions of various genomes of wild *Aegilops* and *Triticum* species of wheat, grown over two years are given in Table 4.1. All the 15 wheat and durum cultivars recommended for commercial cultivation in northern India, possess low level of grain iron and zinc content with very limited variability, thus emphasizing the necessity of their biofortification for high iron and zinc content.

Among the *Aegilops* species, *Ae. longissima* (S^l) and *Ae. kotschyi* (US) had on an average high grain iron and zinc content suggesting that the S genome possesses useful variability for effective uptake, translocation and deposition of the micronutrients in the grains. Some other D genome *Aegilops* species like *Ae. cylindrica* (CD) and *Ae. ventricosa* (DN) and non-progenitor genomes such as *Ae. ovata* (UM) also had high iron and zinc content. The wild diploid and tetraploid *Triticum* species viz., *T. dicoccoides*, *T. araraticum*, and *T. boeoticum* had however lower mean and limited variability for iron and zinc content as compared to the *Aegilops* species.

Comparison of iron and zinc content among some representative cultivars and accessions of wild *Triticum* and *Aegilops* species shows that the mean grain iron content is consistently higher than the mean zinc content with the exception of *T. dicoccoides* where the zinc content was higher than that of iron (Table 4.2). Most of the accessions with high iron also had higher zinc content. For unequivocal identification of potential donors among related wild species of wheat the grain iron and zinc content were analyzed from the germplasm grown over two seasons at the Indian Institute of Technology Roorkee, Roorkee. A minimum of five sets of digestion and estimation using AAS were done (Table

4.2). *Ae. kotschyi* and *Ae. longissima* show two to three folds higher iron and zinc content as compared to a very popular and high yielding semi-dwarf wheat variety WL 711. The accessions for high iron or zinc content were also analyzed for other micronutrients (copper, manganese etc.) and they were found to have their higher content (data not shown) further confirming similar mechanisms for their uptake, translocation and deposition in the grains. *T. dicoccoides* accessions 4640 and 4641 with bold seeds also had nearly three fold higher grain zinc content indicating that the higher iron and zinc content in the wild relatives with smaller seed is due to their superior genetic system rather than higher number of seeds per unit weight.

Table 4.1 Range and mean of grain iron and zinc content of bread and durum wheat cultivars and wild *Triticum* and *Aegilops* species

S.No.	*Species*	*Number of accessions*	*Genome*	*Iron mg/kg*		*Zinc mg/kg*	
				Range	*Mean*	*Range*	*Mean*
1	*T. aestivum*	13	ABD	21.26- 30.59	27.69	14.88 - 19.33	22.15
2	*T. durum*	2	AB	21.91 – 25.60	23.58	13.68- 19.60	18.79
3	*T. boeoticum*	19	A^m	23.88 – 40.50	30.91	22.12 - 39.06	29.27
4	*T. dicoccoides*	17	AB	27.67 – 42.67	32.98	22.50 – 66.51	35.33*
5	*T. arraraticum*	6	AG	23.10 – 59.06	29.85	19.27 – 30.54	23.52
6	*Ae. longissima*	5	S^l	59.12 – 81.59	73.24*	24.99 – 50.52	41.66*
7	*Ae. kotschyi*	14	US	22.89 – 90.96	67.46*	22.29 – 58.61	49.27*
8	*Ae. peregrina*	10	US	34.37 – 82.32	52.85*	33.13 – 49.49	39.54*
9	*Ae. cylindrica*	3	CD	52.21- 93.27	66.76*	32.38 – 52.18	38.51*
10	*Ae. ventricosa*	3	DN	55.41 – 93.52	65.75*	24.01 – 39.08	33.81*
11	*Ae. ovata*	3	UM	52.25 – 81.97	69.95*	31.93- 40.81	37.70*

*Significant at 5 % level of probability

Table 4.2 Grain iron and zinc content of bread and drum wheat cultivars and selected accessions of *Aegilops* and wild *Triticum* species over 2 years

Species	Variety/Accession	Year 2004-05		Year 2005-06	
		Iron	Zinc	Iron	Zinc
		Mean ±S.D	Mean ±S.D	Mean ±S.D.	Mean ±S.D
Triticum.aestivum	WL 711	22.01^{a} ± 1.65	19.28^{bc}±1.67	26.09 ^{b} ±2.24	18.15^{ab}±0.99
Triticum.aestivum	PBW343	25.39^{b} ± 1.44	18.25^{bc}±1.16	30.59^{cd}±2.023	19.33^{b}±1.73
Triticum.aestivum	UP2338	27.07^{b} ± 3.98	16.64^{b}±1.09	28.77^{c}±2.65	16.32^{a}±0.67
Triticum aestivum	Chinese spring (*Ph*$^{-}$)	21.86^{a} ± 3.25	14.88^{a}±1.18	23.45^{a}±0.50	16.91^{ab}±1.53
Triticum.aestivum	UP2382	23.08^{ab} ± 2.65	16.75^{b}±0.96	22.76^{a}±1.25	15.19^{a}±1.46
Triticum.aestivum	UP 2565	21.26^{a} ± 2.90	16.02^{ab}±1.22	25.89^{b}±1.72	16.94^{ab}±1.49
Triticum durum	PDW274	21.94^{a} ± 2.93	13.68^{a}± 0.90	23.62^{a} ± 2.72	16.31^{a}±1.31
Triticum durum	PDW233	21.91^{a} ± 1.27	19.60^{c}±0.62	25.60^{b} ±0.76	15.85^{a}±1.88
T.boeoticum	4873	37.61^{ef} ±2.12	27.97^{ef}± 3.18	40.5^{g}±2.87	29.27^{ef}±2.68
T. boeoticum	4874	23.88^{ab} ± 2.59	22.12^{cd}±1.58	26.43^{b} ±2.20	24.27^{de}±2.27
T. dicoccoides	4630	38.03^{ef}± 2.51	35.74^{hi}±2.46	42.67^{h}±3.07	32.88^{fg}±2.98
T. dicoccoides	4640	34.37^{de}± 2.02	52.12^{op}±2.20	39.50^{gh}±3.69	52.05^{m}±0.81
T. dicoccoides	4641	37.87^{ef}± 2.18	65.62st±2.98	40.09^{g}±5.06	66.51^{qr}±2.60
T. dicoccoides	4772	27.67^{bc}± 0.67	43.93^{lm}±2.23	33.25^{ef}±3.11	48.95^{k}±2.06
T.arraraticum	4770	58.59^{ik}± 2.09	27.32^{ef}±1.62	59.06^{mn}±5.60	30.54^{f}±4.04
Ae.longissima	3507	75.00^{p}± 2.39	50.52^{no}±2.16	81.59^{u}±2.61	49.95^{kl}±4.06
Ae.longissima	3506	59.12^{ik}± 1.94	35.95^{hi}±2.31	69.66^{q}±3.25	35.15^{gh}±3.19
Ae.longissima	28	65.06^{ml}± 3.12	43.08^{l}±1.62	69.96^{q}±5.28	38.48^{hi}±2.49
Ae.longissima	3819	57.66^{jk}± 3.05	24.99^{de}±2.76	67.21^{pq}±4.01	27.69^{e}±2.51
Ae.longissima	3770	78.60^{q}± 5.08	43.14^{l}±1.83	76.52st±2.48	39.99^{i}±3.86
Ae. kotschyi	3774	82.42^{r} ± 3.83	36.61^{hi}±2.12	82.92^{uv}±6.25	45.82^{k}±2.60
Ae. kotschyi	3790	73.01^{no} ± 2.67	36.96^{i}±2.93	76.08st±1.79	46.37^{kl}±3.91
Ae. kotschyi	3573	82.28^{r} ± 3.27	50.43^{mn}±3.01	90.96^{wy}±4.75	52.77^{m}±2.43
Ae. kotschyi	387	69.97^{mn} ± 6.57	45.17^{kl}±2.01	73.46^{sr}±4.85	49.93^{kl} ±1.48
Ae. kotschyi	388	67.53^{mn} ± 3.90	54.57^{p}±2.79	68.18^{q}±5.48	50.43^{lm}±3.21
Ae. kotschyi	389	22.89^{ab} ± 1.27	48.08^{l}±2.18	30.45^{cd}±2.19	51.70^{m}±2.18
Ae. kotschyi	390	70.48^{mn} ± 3.74	22.29^{cd}±2.38	69.06^{q}±3.39	24.44d±3.05
Ae. kotschyi	393	66.47^{lm} ± 3.95	23.11^{d}±2.70	66.43^{p}±6.15	26.91^{de}±2.09
Ae. kotschyi	394	61.16^{k}± 5.55	25.75^{e}±3.02	74.45^{s}±2.97	51.45^{lm}±3.37
Ae. kotschyi	396	78.49^{p}± 3.23	48.76^{m}±2.08	75.49^{s}±2.60	53.38^{m}±3.43
Ae. kotschyi	400804	78.42^{p}± 2.23	44.23^{kl}±1.50	82.51^{v}±2.92	40.62^{j}±3.34
Ae. kotschyi	401021	37.43^{e}± 4.59	52.41^{n}±1.64	40.22^{g}±4.90	58.61^{o}±3.10
Ae. peregrina	13772	61.28^{l}± 1.43	41.71^{jk}±1.26	68.76^{r}±1.39	40.19^{i}±2.47
Ae. peregrina	3477	56.21^{j}± 1.83	38.92^{ij}±2.61	62.19^{o}±2.45	40.75^{i}±2.34
Ae. peregrina	3519	49.43^{ih}± 1.79	38.00^{ij}±1.59	54.24^{l}±2.45	38.47^{ij}±0.98
Ae. peregrina	3791	78.14^{q}± 1.37	33.13^{h}±1.47	82.32^{uv}±4.60	35.75^{gh}±1.99
Ae. peregrina	1155-1-1	46.35^{gh}± 4.19	42.57^{jk}±1.55	51.77^{l}±3.64	45.23^{k}±0.91
Ae. peregrina	1155-2-2	34.37^{e}± 1.84	35.04^{hi}±2.46	41.72^{gh}±3.18	37.66^{h}±1.11
Ae. peregrina	1155-2-4	37.74^{e} ± 0.89	33.80^{gh}±3.67	42.69^{gh}±3.55	35.65^{gh}±2.76
Ae. peregrina	1155-4-1	40.66^{f}± 2.10	38.35^{ij}±1.68	47.14^{ij}± 2.67	38.54^{hi}±2.35
Ae. peregrina	1155-2-8	35.21^{ed}± 1.60	35.54^{hi}±1.71	41.93^{gh}± 3.34	38.57^{hi}±2.30
Ae. peregrina	1155-5-3	55.97^{j}± 2.51	49.49^{m}±2.36	56.57^{lm}± 3.98	47.94^{kl}±2.45
Ae.cylindrica	3472	84.85^{rq}± 2.34	32.38^{gh}±2.61	93.27^{yz}± 2.67	35.74^{gh}±2.95
Ae.cylindrica	3511	78.8^{p}± 1.92	52.18^{n}±1.36	82.85^{uv}± 3.12	51.78^{lm}±2.22
Ae.cylindrica	3705	52.21^{l} ± 2.60	46.77^{l}±1.75	53.61^{kl}± 2.74	47.83^{kl}±1.83
Ae.ventricosa	401027	92.23st ± 4.32	37.97^{i}±1.91	93.52^{yz}±3.18	39.08^{hi}±1.66
Ae.ventricosa	401447	60.89^{k}± 1.63	25.06^{de}±2.22	67.97^{pq}±1.70	24.01^{cd}±1.02
Ae.ventricosa	3520	55.41^{j}± 2.75	37.10^{i}±1.90	55.88^{l}±2.24	38.70^{hi}±2.71
Ae.ovata	3800	79.29^{p}± 3.93	31.93^{g}±2.23	81.97^{u}±4.72	34.93g^{h}±1.36
Ae.ovata	3548	52.64^{l}± 4.35	40.17^{j}±2.18	52.25^{k}±3.78	40.43^{i}±1.07
Ae.ovata	3565	76.10^{op}± 2.30	40.81^{j}±1.48	72.58^{r}±4.86	37.58^{h}±1.39

Note: Similar lower case alphabet letters as superscripts within each column indicate non significant differences in the means of different accessions for micronutrient contents.

In the analysis of variance for iron and zinc content over replicated chemical analysis, highly significant differences were found among wild accessions and cultivars (Table 4.3).

Table 4.3 Analysis of variance for grain iron and zinc content

Source of variation	D.F.	MSS			
		Grain Iron		Grain Zinc	
		Year 1	Year 2	Year 1	Year 2
Replication	2	25.33	6.48	2.63	16.31
Accession	50	1382.10**	1413.13**	373.16**	381.57**
Error	100	8.13	11.29	4.70	5.28

**Significant at 1% level of probability

4.2 Correlation between leaf and grain iron and zinc content

To confirm whether the accessions with high iron and zinc content in the grains also had higher content of these micronutrients in leaves, iron and zinc content in the flag leaves of some of the selected *Aegilops* donors were analyzed at the pre-anthesis stage. Significant positive correlations were found between leaf and grain iron ($r = +0.82$) and zinc content ($r = +0.92$) for 17 accessions of these species analyzed (Fig. 4.1). This indicates that all the selected accessions with high iron and zinc content in grains also had higher iron and zinc content in their flag leaves.

4.3 The F_1 hybrids

4.3.1 Morphology of the F_1 hybrids

The wheat x *Ae. kotschyi* F_1 hybrids were morphologically intermediate between wheat and *Ae. kotschyi* parents. All the F_1 hybrids were completely self sterile and had spelta heads with brittle rachis above the basal spikelet. The hybrids with CS (Ph^I) had awnless lemma and glumes whereas those with WL711 had one glume awn and one

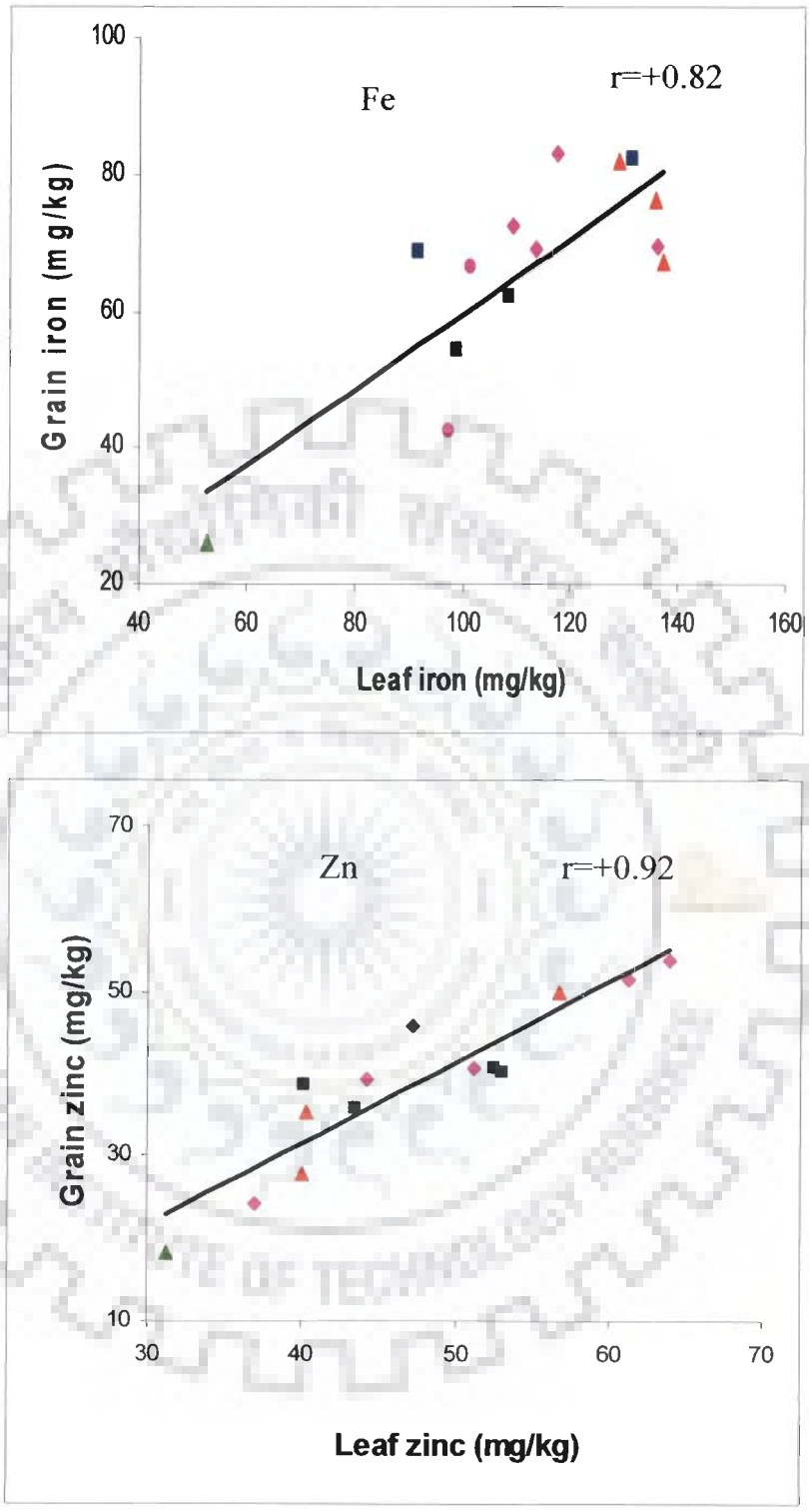


Fig. 4.1 Correlation between leaf and grain micronutrients (▲WL711(NN), ♦ *Ae. kotschyi*, ■ *Ae. peregrina* and ▲ *Ae. longissima*)

lemma awn. Chinese Spring has been known to possess awn inhibitor genes on chromosomes 4A and 6B (Sourdille *et al.*, 2002). The rachis of F_1 hybrids disarticulated only above the basal spikelets like that of *Ae. kotschyi*.

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4.3.2 Cytology

The details of fertility and chromosome pairing of seven F_1 hybrids between *T. aestivum* [WL711 or CS (*Ph*^{l})] and six accessions of *Ae. kotschyi* are given in Table 4.2. There was very limited intergenomic pairing in the F_1 hybrids (Table 4.4 and Fig. 4.2) with very high frequency of univalents (25.69-32.74), low frequency of rod bivalents (1.0- 4.17), and an occasional trivalent (0.09-0.32). One of the F_1 hybrids, CS (*Ph*^{l})/ *Ae. kotschyi*

Table 4.4 Mean and range (within parentheses) of induced homoeologous pairing configuration of F_1 hybrids between wheat (Chinese Spring (*Ph*^{l}), WL711) and different accessions of *Ae. kotschyi*

Cross	2n	Number of PMCs studied	Mean ± S.E. (Range)	Mean ± S.E. (Range)	Mean ± S.E. (Range)	Pollen Stainability %
			Univalent (I)	Bivalent (II)	Trivalent (III)	
CS (Ph^l) X *Ae. kotschyi* 3790	35	100	29.40 ± 0.42 (23-35)	2.86 ± 0.28 (0-6)	0.16 ± 0.02 (0-1)	18.65
CS (*Ph*^{l}) X *Ae. kotschyi* 3573	35	100	29.38 ± 0.29 (21-35)	2.52 ± 0.14 (0-7)	0.20 ± 0.04 (0-1)	19.22
CS (*Ph*^{l}) X *Ae .kotschyi* 396	35	100	25.69 ± 0.61 (13-35)	4.17 ± 0.24 (0-11)	0.32 ± 0.06 (0-3)	23.51
CS (*Ph*^{l}) X *Ae. kotschyi* 390	35	100	31.88 ± 1.68 (24-35)	2.19 ± 0.23 (0-4)	0.15 ± 0.07 (0-1)	16.98
CS (*Ph*^{l}) X *Ae. kotschyi* 393	35	100	31.63 ± 0.56 (26-35)	1.5 ± 0.23 (0-3)	0.13 ± 0.07 (0-1)	17.56
CS (*Ph*^{l}) X *Ae. kotschyi* 395	35	100	32.74 ± 0.39 (26-35)	1.0 ± 0.16 (0-3)	0.09 ± .05 (0-1)	19.72
CS (*Ph*^{l}) X *Ae. kotschyi* 3774	35	100	30.35 ± 1.7 (29-35)	2.04 ± 1.2 (0-4)	0.19 ± 0.6 (0-1)	18.48
WL 711 X *Ae. kotschyi* 391	35	100	29.82 ± 0.7 (21-27)	2..35 ± 3.2 (0-3)	0.18 ± 1.3 0-2	17.62
WL 711 X *Ae. kotschyi* 393	35	100	30.21 ± 6.0 (18-35)	2.14 ± 2.6 (0-6)	0.17 ± 0.7 (0-1)	21.93
WL 711 X *Ae. kotschyi* 3790	35	100	29.16 ± 6.5 (16-35)	2.63 ± 2.8 (0-4)	0.19 ± 0.4 (0-2)	21.09

396 showed higher chromosome pairing (25.69 Is, 4.17 IIs and 0.32 IIIs) as compared with other hybrids (Table 4.4). This may be attributed to induced homoeologous pairing due to Ph^I in CS which is epistatic to *Ph1* gene on the long arm of chromosome 5B (Aghaee-Sarbarzeh *et al.*, 2002; Chen *et al.*, 1994; Jiang *et al.*, 1994; Riley and Chapman 1958). However, in the other three hybrids of CS (Ph^I) with different *Ae. kotschyi* accessions comparatively less homoeologous chromosome pairing was observed (Table 4.4). The F_1 WL711/ *Ae. kotschyi* 393 without (Ph^I) also had relatively higher frequency of bivalents (up to 6 II). All the F_1 plants showed very low pollen stainability (17.6 % to 23.5 %), no anther dehiscence and no seed set (Table 4.4).

4.3.3 Flag Leaf analysis

The iron and zinc content of flag leaves of sterile F_1 hybrids was also analyzed and compared with their wheat and *Aegilops* parents for each of the hybrids (Fig. 4.3). The flag leaves of all the F_1 hybrids had higher iron content than their wheat and some of the *Aegilops* parents, whereas only three hybrids had lower zinc content than either of the parents. This suggests that all the selected wild donors with high grain iron and zinc content possess superior genetic systems for efficient uptake and better transport of high iron and zinc content to leaves which could ultimately be deposited in grains.

Iron content in flag leaves of about half of the hybrids exceeded the level of flag leaves in the *Aegilops* parents suggesting a synergistic interaction between wheat and *Aegilops.* In all hybrids, bread wheat lines Chinese Spring (Ph^I) or WL711 were used as the female parents. Higher levels of iron and zinc in majority of the F_1 hybrids suggest that the superior genetic system of *Aegilops* is partially due to dominant gene(s) and is capable of expression in association or in background of cultivated wheat, and hence could be transferred and exploited.

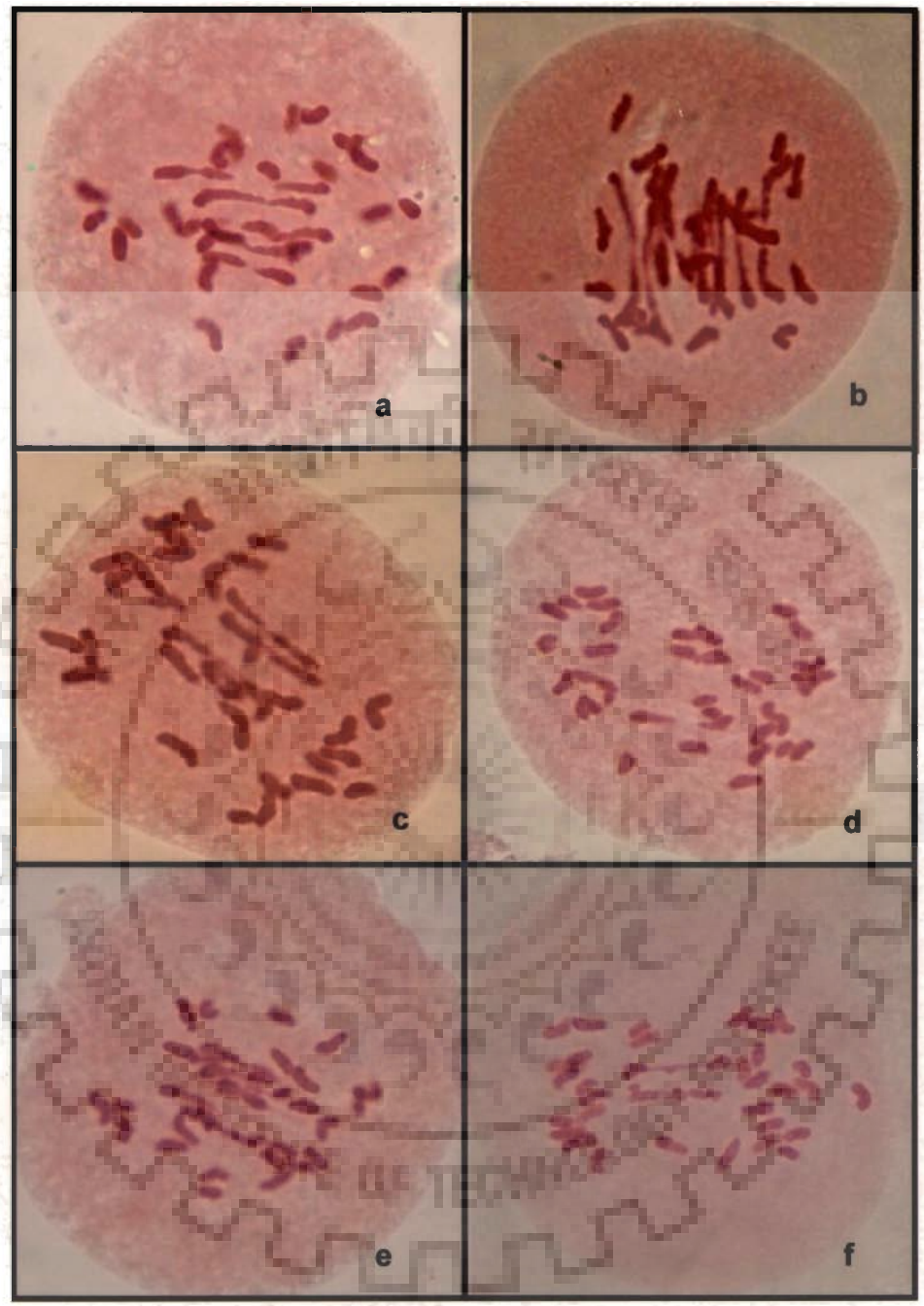
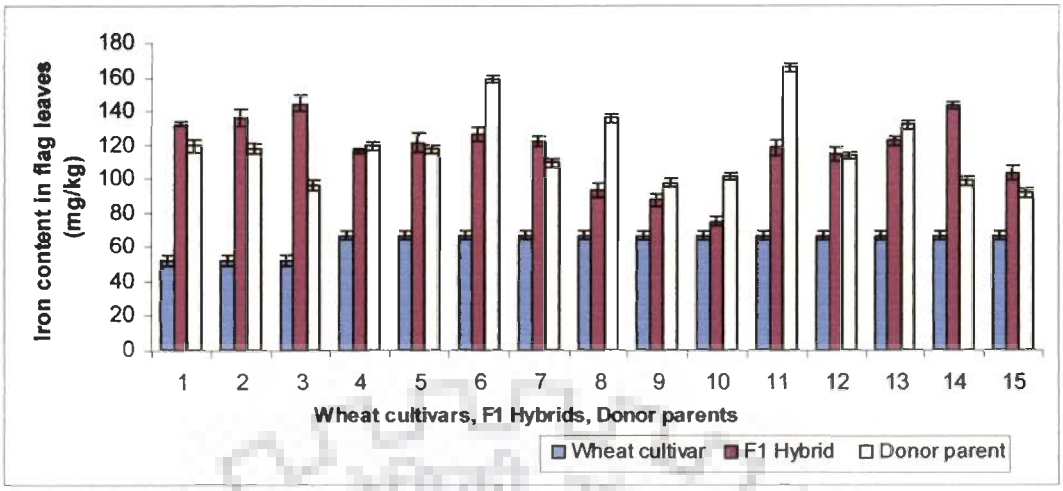


Fig. 4.2 Chromosome pairing at metaphase-I of wheat/ *Ae. kotschyi* F_1 hybrids, (a) F_1 CS(*Ph*^{l})/ *Ae. kotschyi* 393 (6 II + 23 I), (b) CS(*Ph*^{l})/ *Ae. kotschyi* 396 (1III+7II +18 I), (c) F_1 CS (*Ph*^{l})/*Ae. kotschyi* 393 (5II+25I), (d) F_1 WL711/ *Ae. kotschyi* 3774 (1 II + 32 I), (e) F_1 WL711/ *Ae. kotschyi* 391 (1 III + 3 II + 27 I) and (f) F_1 WL711/ *Ae. kotschyi* 3790 (2 II + 31 I)



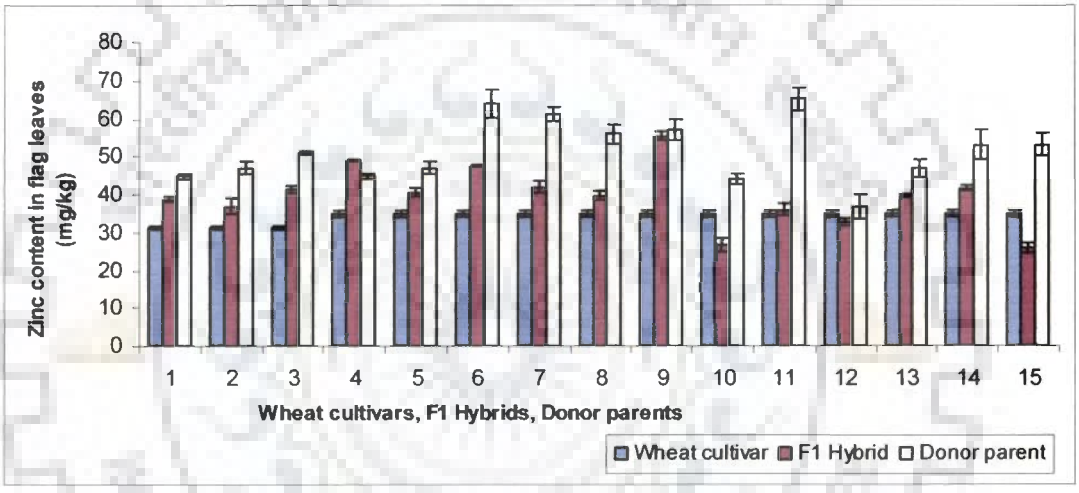


Fig. 4.3 Iron and zinc content in flag leaves of wheat cultivars, their F_1 hybrids and *Aegilops* parents; crosses 1-3 have WL711 as recipient parent with donor being (1) *Ae. kotschyi* 3573, (2) *Ae. kotschyi* 3774, (3) *Ae. kotschyi* 400804; crosses 4-15 have Chinese Spring (*Ph*^{I}) as recipient parent, donors being (4) *Ae. kotschyi* 3573, (5) *Ae. kotschyi* 3774, (6) *Ae. kotschyi* 396, (7) *Ae. kotschyi* 394, (8) *Ae. kotschyi* 3790, (9) *Ae. kotschyi* 395, (10) *Ae. kotschyi* 393, (11) *Ae. kotschyi* 388, (12) *Ae. kotschyi* 390, (13) *Ae. peregrina* 3791, (14) *Ae. peregrina* 3519 and (15) *Ae. peregrina* 13772.

An analysis of Table 4.2 shows that *Ae. kotschyi* 396 has very high grain iron and zinc concentrations. The hybrid F_1CS(*Ph*^{l})/*Ae. kotschyi* 396 had iron and zinc concentrations in the flag leaves comparable to the wild parent (Fig. 4.3). Moreover this hybrid showed highest pairing at metaphase-I in pollen mother cells (Fig. 4.2), indicating the highest possibility of recombination between the homoeologous chromosomes among all the F_1 hybrids. Therefore F_1CS(*Ph*^{l})/*Ae. kotschyi* 396 was used to generate further backcross derivatives.

4.1.4 BC_1 Derivatives

4.1.3.1 Morphology

The sterile F_1 CS(*Ph*^{l})/*Ae. kotschyi* 396 plants were extensively backcrossed with the recurrent or other wheat parent to get BC_1 seeds. The male sterile F_1 hybrids on backcrossing as female parent with wheat cultivars gave a very low backcross seed set (0.53%) suggesting that the hybrids had only partial female fertility. Table 4.5 shows the morphology, chromosome number and fertility status of the BC_1 plants. The growth habit of the plants varied from erect like the recurrent wheat parent to spreading like the *Aegilops* parent or intermediate between the two. Tiller number, plant height, awning and waxiness also varied among the different BC_1 plants.

4.3.2 Cytology

Chromosome number varied from 2n = 31 to 54. Pollen stainability was low in general and the plants with a little higher pollen stainability (26 % or above) were partially fertile and set a few seeds. The chromosome number and pairing of some of the BC_1 plants has been shown in Fig. 4.4. The pairing percentage between the chromosomes was somewhat higher than that in the F_1 hybrids, though many univalents were still visible, indicating increasing stability of the plants.

Table 4.5 Morphological characteristics, chromosome number and fertility of the parents and BC_1 plants

Pedigree	No. of tillers per plant	Plant Height (cm)	Waxiness	Awnness	Chr. No.	Pollen stainability (%)	Female Fertility
CS (*Ph*^{1})/ *Ae. kotschyi* 396// PBW 343-1	43	60	Waxy	Awned	31	7.4	Sterile
CS (*Ph*^{1})/ *Ae. kotschyi* 396// PBW 343-2	29	40	Nonwaxy	Awnless	33	8.3	Sterile
CS (*Ph*^{1})/ *Ae. kotschyi* 396// PBW 343-3	112	80	Nonwaxy	Awnless	43	27.4	Partially fertile
CS (*Ph*^{1})/ *Ae. kotschyi* 396//PDW274-1	45	70	Nonwaxy	Awnless	31	7.4	Sterile
CS (*Ph*^{1})/ *Ae. kotschyi* 396//PDW274-2	33	60	Nonwaxy	Awned	32	10.5	Sterile
CS (*Ph*^{1})/ *Ae. kotschyi* 396//PDW274-4	9	50	Nonwaxy	Awned	36	9.2	Sterile
CS (*Ph*^{1})/ *Ae. kotschyi* 396//PDW274-5	63	50	Nonwaxy	Awned	32	11.2	Sterile
CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2382-1	16	90	Nonwaxy	Awnless	54	26.2	Partially fertile
CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2382-2	46	35	Waxy	Awnless	54	8.7	Sterile
CS (*Ph*^{1})/ *Ae. kotschyi* 396// UP2425-1	30	75	Nonwaxy	Awned	44	17.6	Sterile
CS (*Ph*^{1})/ *Ae. kotschyi* 396// UP2425-2	7	35	Nonwaxy	Awned	44	9.1	Sterile
PBW343	14.8	93	Waxy	Awned	42	85.4	Fertile
CS (*Ph*^{1})	18.3	100	Nonwaxy	Awnless	42	80.9	Fertile
Ae. kotschyi 396	275.5	35	Nonwaxy	Awned	28	78.6	Fertile

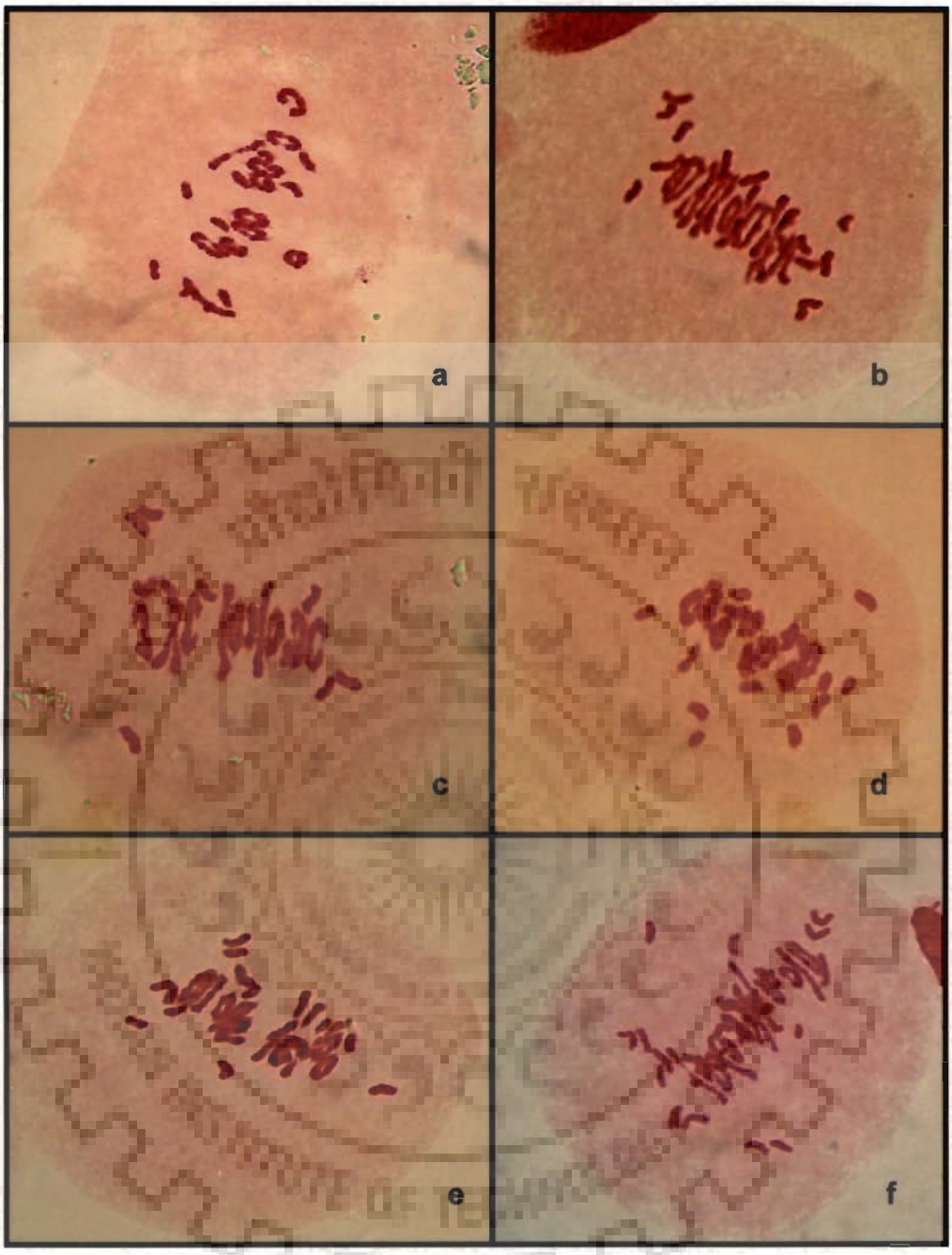


Fig 4.4 Chromosome pairing at metaphase-I of some BC_1 plants (a) BC_1 CS(*Ph*^{I})/ *Ae. kotschyi* 396//PBW343 Pl-1 (31 Chr, 12II+7I), (b) BC_1CS(*Ph*^{I})/ *Ae. kotschyi* 396//PBW343 Pl-3 (43 Chr, 17II+9I), (c) BC_1 CS (*Ph*^{I})/*Ae. kotschyi* 396//PDW274 Pl-1 (31 Chr, 13II+5I), (d) BC_1 CS (*Ph*^{I})/*Ae. kotschyi* 396//PDW274 Pl- 2 (32 Chr, 10II+12I), (e) BC_1 CS (*Ph*^{I})/*Ae. kotschyi* 396//UP2425 Pl-1 (44 Chr, 17II+10I) and (f) BC_1 CS (*Ph*^{I})/*Ae. kotschyi* 396//UP2382 Pl-1 (54 Chr, 20II+14I)

4.3.3 Micronutrient analysis

Iron and zinc content in the flag leaves of some BC_1 plants is given in Table 4.6. Most of the BC_1 plants with variable number of chromosomes from *Aegilops* parents had higher iron content. Some of the BC_1 plants in certain crosses had even higher iron content in their leaves than those of the wild donors, indicating the synergistic interaction between parents and the possibility of obtaining transgressive segregants for high iron. Comparatively lower level of zinc in the flag leaves among the BC_1 progenies may be due to epistatic effect of increasing wheat background on the uptake and translocation of zinc to the leaves. Some BC_1 plants were self fertile giving sufficient BC_1F_2 seeds while others with partial fertility were further backcrossed with recurrent wheat cultivars to get BC_2F_1 seeds.

Table 4.6 Iron and zinc content in flag leaves of some partially fertile and sterile BC_1 plants

S.No.	BC_1 Plants	Iron (mg/kg)	Zinc (mg/kg)
1	CS (*Ph*^{I})/ *Ae. kotschyi* 396// PBW 343-1	183.6	32.6
2	CS (*Ph*^{I})/ *Ae. kotschyi* 396// PBW 343-2	141.7	28.3
3	CS (*Ph*^{I})/ *Ae. kotschyi* 396// PBW 343-3	129.8	26.5
4	CS (*Ph*^{I})/ *Ae. kotschyi* 396//PDW274-1	110.6	30.3
5	CS (*Ph*^{I})/ *Ae. kotschyi* 396//PDW274-2	95.3	31.0
6	CS (*Ph*^{I})/ *Ae. kotschyi* 396// UP2425-1	131.2	32.
7	CS (*Ph*^{I})/ *Ae. kotschyi* 396// UP2425-2	132.0	32.5
8	CS (*Ph*^{I})/ *Ae. kotschyi* 396//UP2382-1	79.0	36.5
9	*T. aestivum* lr. CS (*Ph*^{I})	66.7	35.0
10	*T. aestivum* cv. WL711	56.5	29.3
11	*Ae. kotschyi* acc. 396	158.6	63.9

4.4 BC_2F_1 and BC_1F_2 Derivatives

4.4.1 Morphology

Table 4.7 shows the morphology of some $BC_2F_1$and BC_1F_2 plants. The plants resembled recurrent parent in tiller number, plant height, head type etc. whereas seed colour was like *Aegilops* parent in many derivatives. The BC_1F_2 and

Table 4.7 Morphological characteristics, chrosomome number and grain iron and zinc content of some BC_2F_1 and BC_1F_2 plants

I.D. No.	Pedigree	No. of tillers	Plant Height (cm)	Head type and waxiness	Chr. No.	Seed colour and shape	Grain iron (mg/kg)	Grain zinc (mg/kg)
BC_2F_1 46-1	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711-1	8	98	Square, Non-waxy	40	Red, plump, medium sized	46.4	41.0
$BC_2F_1$46-15	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711-15	28	95	Square, non-waxy	44	Red, plump, medium sized	44.8	19.4
BC_2F_1 48-2	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-2	24	85	Square , waxy	44	Red, shrivelled. slender	24.3	19.8
$BC_2F_1$48-7	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-7	8	90	Square , waxy	44	Red, shrivelled, medium sized	30.7	26.3
BC_2F_1 48-12	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-12	9	105	Square, waxy	42	Amber, bold	31.0	20.6
$BC_2F_1$48-25	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-25	15	105	Square, waxy	42	Red, shrivelled, slender	36.2	19.8
$BC_2F_1$48-27	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-27	19	75	Square, nonwaxy	44	Red, shrivelled, slender	36.4	43.4
$BC_2F_1$48-35	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-35	8	70	Square, waxy	44	Red, shrivelled, slender	33.4	22.8
BC_2F_1 48-37	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-37	24	115	Square, waxy	42	Red, angular seeds, slender	40.1	23.5
BC_2F_1 48-43	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-43	9	70	Square, waxy	44	Red, shrivelled, slender	26.4	20.4
BC_2F_1 48-41	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-41	42	110	Square, nonwaxy	42	Red, slender, plump seeds	52.4	29.2
$BC_2F_1$48-42	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-42	31	65	Square, nonwaxy	42	Red, slighty shrivelled seeds	57.2	27.2

I.D. No.	Pedigree	No. of tillers	Plant Height (cm)	Awnness and waxiness	Chr. No.	Seed colour and shape	Grain iron (mg/kg)	Grain zinc (mg/kg)
BC_2F_1 48-44	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-44	25	125	Square, nonwaxy	42	Amber, bold, slender	57.3	48.4
BC_2F_1 48-45	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-45	15	110	Square, nonwaxy	42	Amber, bold, slender	45.3	38.1
$BC_2F_1$48-65	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-65	31	112	Square, waxy	42	Amber, bold, slender	33.3	28.4
$BC_2F_1$49-1	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1	52	85	Square, nonwaxy	46	Red, slighty shrivelled seeds	58.3	39.2
$BC_2F_1$49-2	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///UP2425-2	29	115	Square, nonwaxy	46	Red, bold, slender seeds	36.8	31.5
$BC_2F_1$49-3	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///UP2425-3	49	108	Square, nonwaxy	42	Red, slighty shrivelled seeds	46.3	35.2
$BC_2F_1$53-1	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PDW274-2///PBW373-1	19	112	Square, nonwaxy	40	Red, round shrivelled seeds	51.4	44.1
BC_2F_1 54-1	CS (*Ph*^{l})/ *Ae. kotschyi* 396//UP425-1///PBW373-1	13	95	Square, waxy	40	Red, round shrivelled seeds	32.2	20.2
BC_2F_1 54-3	CS (*Ph*^{l})/ *Ae. kotschyi* 396//UP2425-2///PBW373-3	18	93	Square, waxy	40	Red, plump medium sized seeds	33.7	23.4
BC_2F_1 54-4	CS (*Ph*^{l})/ *Ae. kotschyi* 396//UP2425-2///PBW373-4	7	96	Square, waxy	40	Red, slender medium sized seeds	51.4	44.1
BC_1F_2 74-1	CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3-1	92	65	Square, waxy	44	Red, slender plump seeds	24.7	21.8
$BC_1F_2$75-1	CS (*Ph*^{l})/ *Ae. kotschyi* 396//UP2382-1-1	35	105	Square, nonwaxy	46	Red, plump medium sized seeds	61.5	39.7

BC_2F_1plants having high tillering, good biomass and fair seed set were analysed for their grain iron and zinc content (Table 4.7), whereas the plants which were still sterile or partially fertile with low seed set were discarded. Seeds of these plants were as bold as or even bolder than their wheat parents and had iron and zinc concentrations in the range of 24.3 to 61.5 mg/kg and 19.4 to 48.4 mg/kg respectively (Table 4.7).

4.4.2 Cytology

The chromosome number varied from 2n = 40 to 46 (Table 4.7). The highest number of chromosomes (2n = 46) were observed in $BC_2F_1$49-1, BC_2 49-2 and BC_1F_2 75-1. Cytological analysis of the fertile high iron and zinc BC_1F_2 and BC_2F_1plants showed the presence of many bivalents and some univalents (Fig. 4.5) indicating towards increasing stability of the plants. The number of bivalents ranged from 15-20, while univalents varied from 4-10.

4.4.3 Grain ash analysis

Grain ash analysis of some selected BC_1F_2 and BC_2F_1plants along with their parents was carried out using atomic absorption spectrophotometer (AAS) to examine the actual increase in iron and zinc concentrations (Table 4.8). The seeds of *Ae. kotschyi* 396 were richer in inorganic content than wheat as is visible in its higher ash percentage (2.07 %) than WL711 (1.58 %) and Chinese Spring (1.65 %). Among the three derivatives, $BC_2F_1$49-1 had highest grain ash percentage (2.05 %). The grain ash of *Ae. kotschyi* had higher percentage of iron and zinc in it than that of wheat. Ash of the derivatives was also richer in iron and zinc content than control.

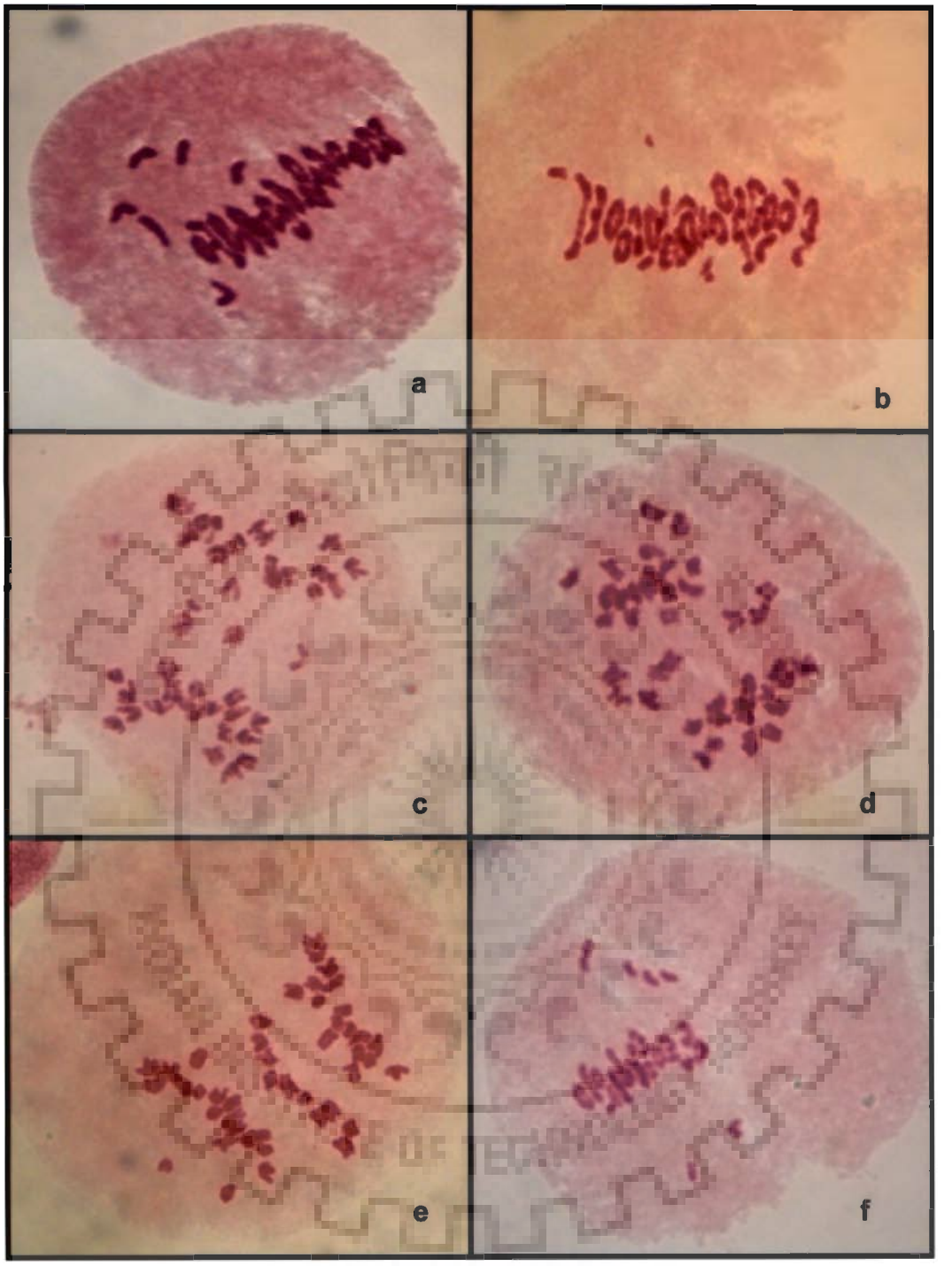


Fig 4.5 Chromosome pairing at metaphase-I of some BC2F1 plants (a) BC2F1CS(*PhI*)/ *Ae. kotschyi* 396//PBW343-3///WL711 Pl-1 (40 Chr, 18II+4I), (b) BC2F1CS (*PhI*)/ *Ae. kotschyi* 396//PBW343-3///WL711 Pl-15 (44 Chr, 20II+4I), (c) BC2F1CS (*PhI*)/ *Ae. kotschyi* 396//PBW343-3///PBW373 Pl-41 (42 Chr, 19II+4I), (d) BC2F1CS (*PhI*)/ *Ae. kotschyi* 396//PDW274-2///PBW373 Pl-1 (40 Chr, 15II+10I), (e) BC2F1CS (*PhI*)/ *Ae. kotschyi* 396//PBW343-3///UP2425 Pl-1 (46 Chr, 18II+10I) and (f) BC2F1CS (*PhI*)/ *Ae. kotschyi* 396//UP2425 Pl-3 (42 Chr, 17II+8I)

Table 4.8 Grain ash content in wheat cultivars, *Ae. kotschyi* 396 and their BC_1F_2 and BC_2F_1 derivatives and their grain ash iron and zinc content

I.D. No.	Grain material	Ash %	Fe (µg/g) of ash	% Change in ash Fe over WL711	Zn (µg/g) of ash	% Change in ash Zn over WL711
Control	WL711	1.58	1,607	-	1,342	0
-	CS(*Ph*^{l})	1.65	1,702	5.9	1,181	-11.9
-	*Ae. kotschyi* 396	2.07	3,058	90.3	2,197	63.7
BC_2F_1 46-1	BC_2CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711-1	2.02	2,755	71.4	2,248	67.5
BC_2F_2 49-1	BC_2CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1	2.05	2,864	78.2	2,116	57.7
BC_1F_2 75-1	BC_1F_2 CS (*Ph*^{l})/ *Ae. kotschyi* 396//UP2382-1-1	1.98	2,691	67.5	1,990	48.3

About 100-150 seeds of eight BC_2F_1 and BC_1F_2 progenies with significantly high micronutrient content were sown in 2007-08.

4.5 BC_2F_2 and BC_1F_3 derivatives

4.5.1 Morphology

The morphology of these BC_2F_2 and BC_1F_3 plants was recorded at regular intervals and harvesting was done individual plant-wise. Morphological characteristics of some of the BC_2F_2 and BC_1F_3 plants are given in Table 4.9. Waxiness of wheat plants is known to be controlled by genes present on homoeologous group 2 chromosomes (McIntosh, 1983; Levy and Feldman, 1989). As such plants with waxy leaf sheaths indicated introgression of group 2 chromosomes morphologically. Variations in general plant morphology among the different families were distinct; however within the families greater uniformity was visible. For instance BC_2F_2 46-1 and BC_2F_2 53-1 progenies had all amber grains, whereas BC_2F_2 49-1 and BC_1F_3 75-1 showed red coloured grains. Rachis was non-brittle in most of the

Table 4.9 Morphological characteristics of some representative plants of BC_2F_2 and BC_1F_3 derivatives. The selected derivatives are in bold.

ID. No.	Pedigree	No. of tillers/ plant	Plant Height (cm)	Days to Flowering	Waxiness	Head type	Rachis	Grain color	No of seeds per spike	Harvest Index (%)
BC_2F_2 46-1-11	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -1-11	15	107	102	Waxy	Awned, square	NB	Amber	21	24.1
BC_2F_2 46-1-15	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -1-15**	**33**	**107**	**106**	**Nonwaxy**	**Awned, square**	**B**	**Amber**	**23**	**27.5**
BC_2F_2 46-1-27	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -1-27**	**11**	**100**	**108**	**Waxy**	**Awned square**	**NB**	**Amber**	**20**	**21.1**
BC_2F_2 46-1-45	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -1-45	9	85	108	Waxy	Awned square	NB	Amber	18	26.7
BC_2F_2 46-15-16	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -15-1	24	100	105	Waxy	Awnless, square	NB	Amber	30	34.3
BC_2F_2 46-15-37	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -15-37**	**14**	**75**	**108**	**Waxy**	**Awnless, square**	**B**	**Red**	**22**	**29.2**
BC_2F_2 46-15-48	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -15-48	15	88	106	Waxy	Awnless, square	NB	Red	36	38.0
BC_2F_2 46-15-105	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -15-105	19	100	105	Waxy	Awned, square	NB	Amber	38	41.8
BC_2F_2 48-41-3	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-41-3	5	70	101	Nonwaxy	Awned, square	NB	Red	23	16.7
BC_2F_2 48-41-6	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-41-6**	**12**	**81**	**110**	**Waxy**	**Awned, square**	**NB**	**Amber**	**24**	**17.2**
BC_2F_2 48-41-49	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-41-49	10	57	112	Nonwaxy	Awned, square	NB	Red	22	18.8
BC_2F_2 48-42-16	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-42-16**	**11**	**77**	**106**	**Nonwaxy**	**Awned, square**	**NB**	**Red**	**21**	**35.9**
BC_2F_2 48-42-61	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-42-61	8	66	110	Nonwaxy	Awnless square	NB	Red	18	45.0
BC_2F_2 48-42-82	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-42-82	13	80	108	Nonwaxy	Awnless, square	NB	Amber	18	22.0
BC_2F_2 48-44-3	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-44-3	10	103	105	Nonwaxy	Awnless, square	NB	Red	20	30.0
BC_2F_2 48-44-4	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-44-4	8	65	108	Nonwaxy	Awned, square	NB	Red	19	25.0
BC_2F_2 48-44-23	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-44-23**	**11**	**85**	**108**	**Nonwaxy**	**Awned, square**	**NB**	**Amber**	**21**	**16.7**
BC_2F_2 49-1-9	**CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-9**	**19**	**90**	**102**	**Nonwaxy**	**Awned, square**	**NB**	**Red**	**18**	**33.3**
BC_2F_2 49-1-11	**CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-11**	**38**	**85**	**105**	**Nonwaxy**	**Awned, square**	**NB**	**Red**	**22**	**22.8**

ID. No.	Pedigree	No. of tillers/ plant	Plant Height (cm)	Days to Flowering	Waxiness	Head type	Rachis	Grain color	No of seeds per spike	Harvest Index (%)
BC_2F_2 49-1-18	CS (*Ph*^{1})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-18	17	97	105	Nonwaxy	Awned, square	NB	Red	22	28.6
BC_2F_2 49-1-22	CS (*Ph*^{1})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-22	23	85	108	Waxy	Awned, square	NB	Red	17	8.0
BC_2F_2 49-1-29	CS (*Ph*^{1})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-29	26	82	106	Nonwaxy	Awned, square	NB	Red	20	20.0
BC_2F_2 49-1-45	CS (*Ph*^{1})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-45	33	70	110	Nonwaxy	Awned, square	NB	Red	18	29.3
BC_2F_2 49-1-73	**CS (*Ph*^{1})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-73**	**14**	**88**	**110**	**Nonwaxy**	**Awned, square**	**NB**	**Red**	**21**	**23.9**
BC_2F_2 49-1-104	CS (*Ph*^{1})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-104	19	104	110	Waxy	Awned, square	NB	Red	21	25.0
BC_2F_2 53-1-18	CS (*Ph*^{1})/ *Ae. kotschyi* 396//PDW274-2///PBW373-1-18	11	123	108	Waxy	Awnless, square	NB	Amber	26	25.5
BC_2F_2 53-1-20	CS (*Ph*^{1})/ *Ae. kotschyi* 396//PDW274-2///PBW373-1-20	13	124	108	Waxy	Awnless, square	NB	Amber	23	27.0
BC_2F_2 53-1-23	CS (*Ph*^{1})/ *Ae. kotschyi* 396//PDW274-2///PBW373-1-23	25	130	108	Waxy	Awnless, square	NB	Amber	23	26.0
BC_2F_2 53-1-25	CS (*Ph*^{1})/ *Ae. kotschyi* 396//PDW274-2///PBW373-1-25	12	115	115	Waxy	Awnless, square	NB	Amber	24	25.6
BC_1F_3 75-1-4	**CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2382-1-1-4**	**27**	**115**	**124**	**Nonwaxy**	**Awnless, square**	**B**	**Red**	**26**	**32.3**
BC_1F_3 75-1-6	CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2382-1-1-6	23	105	124	Nonwaxy	Awnless, square	B	Red	24	35.4
BC_1F_3 75-1-39	**CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2382-1-1-39**	**16**	**85**	**115**	**Waxy**	**Awnless, square**	**B**	**Amber**	**17**	**13.3**
BC_1F_3 75-1-62	CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2382-1-1-62	14	105	112	Waxy	Awnless, square	B	Red	23	36.0
BC_1F_3 75-1-76	CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2382-1-1-76	22	115	112	Nonwaxy	Awnless, square	NB	Red	21	31.1
BC_1F_3 75-1-105	CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2382-1-1-105	18	97	112	Waxy	Awnless, square	B	Red	23	27.5
BC_2F_2 54-3-1	CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2425-2///PBW373-3-1	10	100	108	Waxy	Awned, square	NB	Amber	24	36.4
BC_2F_2 54-3-11	CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2425-2///PBW373-3-11	14	105	115	Waxy	Awned, square	NB	Amber	24	35.7
BC_2F_2 54-3-12	**CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2425-2///PBW373-3-12**	**10**	**105**	**115**	**Waxy**	**Awned, square**	**NB**	**Amber**	**21**	**36.4**
BC_2F_2 54-3-15	**CS (*Ph*^{1})/ *Ae. kotschyi* 396//UP2425-2///PBW373-3-15**	**9**	**112**	**120**	**Waxy**	**Awned, square**	**NB**	**Amber**	**24**	**15.0**

derivatives, but a few plants showed shattering at maturity on account of having brittle rachis like the *Aegilops* parents. Havest index varied from 13.3 % to 41.8 %. Fig. 4.6 illustrates plants of two BC_2F_2 families showing recovered wheat background as against *Ae. kotschyi* parent.

4.5.2 Grain iron and zinc

Grain iron and zinc concentrations of at least 10 healthy plants from each BC_2F_2 and BC_1F_3 family having good harvest index in recovered wheat background were analysed for their grain micronutrient content thrice in two replications using AAS. Finally the plants with high iron and zinc concentrations were analyzed once again using Inductively Coupled Plasma Mass Spectrometer (ICPMS) for the precise estimation of the micronutrients. Table 4.10 gives the grain iron and zinc content of some of the BC_2F_2 and BC_1F_3 derivatives. The derivatives showed variation for grain iron and zinc content among different families and within the families, although the variation within families was limited. BC_2F_2 49-1 Pl-73 with grain iron and zinc content of 58.3 mg/kg and 61.6 mg/kg showed highest increase for grain iron (155.6 %) and zinc (184.6 %) over WL711. Plants having more than 60 % and 70 % increase over WL711 for grain iron and zinc, respectively were selected as high iron and zinc plants for cytological and molecular characterization. All plants of BC_2F_2 53-1 progeny were discarded owing to their low grain iron as well as zinc content. Plant BC_2F_2 49-1 Pl-22 was discarded inspite of high iron and zinc content because of its low harvest index which might be contributing to its high micronutrient content due to concentration effect. The selected derivatives were as fertile as the wheat parent (Fig. 4.6) and had seeds as bold as or even bolder than the cultivar seeds (Fig. 4.7). Thus increase in micronutrient content of these plants was genuine and not due to concentration effect.

Table 4.10 Grain iron zinc content in some BC_2F_2 and BC_1F_3 derivatives. The plants selected for molecular analysis are coloured

ID. No.	Pedigree	Grain iron content (mg/kg)				% Change	Grain zinc content (mg/kg)				% Change
		I	II	III	Mean ± SE	over WL711	I	II	III	Mean ± SE	over WL711
Control	WL 711	21.5	25.0	22.0	22.8 ± 1.1	-	21.7	20.1	23.1	21.6 ± 0.9	-
BC_2F_2 46-1-11	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -1-11	22.0	25.1	22.5	23.2 ± 1.0	1.6	34.3	28.0	29.9	30.7 ± 1.9	42.0
BC_2F_2 46-1-15	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -1-15**	**38.4**	**35.2**	**37.1**	**36.9 ± 0.9**	**61.7**	**34.9**	**27.0**	**36.9**	**32.9 ± 3.0**	**52.1**
BC_2F_2 46-1-27	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -1-27**	**41.2**	**37.0**	**38.3**	**38.8 ± 1.2**	**70.1**	**36.7**	**35.0**	**36.0**	**35.9 ± 0.5**	**65.8**
BC_2F_2 46-1-45	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -1-45	22.0	26.5	24.3	24.3 ± 1.3	6.3	35.0	30.3	32.2	32.5 ± 1.3	50.2
BC_2F_2 46-15-16	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -15-1	25.3	23.1	21.4	23.3 ± 1.2	2.0	28.1	24.7	23.6	25.5 ± 1.4	17.7
BC_2F_2 46-15-37	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -15-37**	**38.5**	**36.3**	**39.1**	**38.0 ± 0.8**	**66.5**	**25.4**	**17.9**	**23.7**	**22.3 ± 2.3**	**3.1**
BC_2F_2 46-15-48	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -15-48	22.7	25.5	22.0	23.4 ± 1.1	2.6	21.8	29.6	22.8	24.7 ± 2.5	14.2
BC_2F_2 46-15-105	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///WL711 -15-105	23.2	24.8	21.6	23.2 ± 0.9	1.6	25.2	25.6	21.9	24.2 ± 1.2	12.0
BC_2F_2 48-41-3	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-41-3	27.9	28.3	26.3	27.5 ± 0.6	20.5	24.4	25.2	22.7	24.1 ± 0.8	11.3
BC_2F_2 48-41-6	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-41-6**	**49.1**	**51.5**	**46.5**	**49.0 ± 1.5**	**114.9**	**41.1**	**42.4**	**41.7**	**41.7 ± 0.4**	92.8
BC_2F_2 48-41-49	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-41-49	38.0	39.3	35.9	37.7 ± 1.0	65.3	42.8	40.5	38.3	40.6 ± 1.3	87.4
BC_2F_2 48-42-16	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-42-16**	**40.3**	**36.5**	**38.5**	**38.4 ± 1.1**	**68.4**	**25.6**	**34.8**	**32.7**	**31.0 ± 2.8**	**43.5**
BC_2F_2 48-42-61	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-42-61	39.0	39.6	36.3	38.3 ± 1.0	67.9	34.4	35.9	33.2	34.5 ± 0.8	59.3
BC_2F_2 48-42-82	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-42-82	34.9	36.2	32.5	34.5 ± 1.1	51.3	30.1	35.0	33.5	32.9 ± 1.5	51.9
BC_2F_2 48-44-3	CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-44-3	28.7	30.1	26.5	28.5 ± 1.0	24.7	28.1	25.7	23.1	25.6 ± 1.4	18.4
BC_2F_2 48-44-23	**CS(*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///PBW373-44-23**	**49.2**	**51.5**	**46.9**	**49.2 ± 1.3**	**115.7**	**33.2**	**29.6**	**35.5**	**32.8 ± 1.7**	**51.4**
BC_2F_2 49-1-9	**CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-9**	**54.9**	**56.8**	**52.3**	**54.7 ± 1.3**	**139.6**	**42.7**	**39.4**	**44.5**	**42.2 ± 1.5**	**95.0**
BC_2F_2 49-1-11	**CS (*Ph*^{l})/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-11**	**53.7**	**51.3**	**55.7**	**53.5 ± 1.3**	**134.6**	**51.0**	**51.7**	**50.3**	**51.0 ± 0.4**	**135.8**

ID. No.	Pedigree	Grain iron content (mg/kg)				% Change	Grain zinc content (mg/kg)				% Change
		I	II	III	Mean±SE	over WL711	I	II	III	Mean±SE	over WL711
BC_2F_2 49-1-18	CS (*Ph^l*)/ *Ae. kotschyi*396//PBW343-3///UP2425-1-18	37.3	38.5	35.3	37.0 ± 0.9	62.3	47.7	45.4	49.9	47.6 ± 1.3	120.1
BC_2F_2 49-1-22	CS (*Ph^l*)/ *Ae. kotschyi*396//PBW343-3///UP2425-1-22	53.0	55.8	52.0	53.6 ± 1.1	134.9	62.2	64.7	62.5	63.1 ± 0.8	191.8
BC_2F_2 49-1-29	CS (*Ph^l*)/ *Ae. kotschyi*396//PBW343-3///UP2425-1-29	41.3	40.1	41.7	41.0 ± 0.5	79.7	52.1	51.2	50.5	51.3 ± 0.5	137.0
BC_2F_2 49-1-45	CS (*Ph^l*)/ *Ae. kotschyi*396//PBW343-3///UP2425-1-45	46.4	48.3	45.4	46.7 ± 0.9	104.7	46.4	42.5	44.5	44.5 ± 1.1	105.5
BC_2F_2 49-1-73	**CS (*Ph^l*)/ *Ae. kotschyi*396//PBW343-3///UP2425-1-73**	**58.1**	**59.2**	**57.8**	**58.3 ± 0.4**	**155.6**	**61.3**	**59.8**	**63.7**	**61.6 ± 1.1**	**184.6**
BC_2F_2 49-1-104	CS (*Ph^l*)/ *Ae. kotschyi*396//PBW343-3///UP2425-1-104	55.2	54.3	56.2	55.2 ± 0.5	142.1	59.2	56.6	54.7	56.8 ± 1.3	162.5
BC_2F_2 53-1-18	CS (*Ph^l*)/ *Ae. kotschyi*396//PDW274-2///PBW373-1-18	34.6	36.5	32.7	34.6 ± 1.1	51.8	28.7	33.8	34.7	32.4 ± 1.9	49.5
BC_2F_2 53-1-20	CS (*Ph^l*)/ *Ae. kotschyi*396//PDW274-2///PBW373-1-20	25.3	26.7	23.5	25.2 ± 0.9	10.3	24.5	23.8	23.7	24.0 ± 0.3	10.9
BC_2F_2 53-1-23	CS (*Ph^l*)/ *Ae. kotschyi*396//PDW274-2///PBW373-1-23	25.9	23.1	27.6	25.5 ± 1.3	11.9	22.5	23.7	27.6	24.6 ± 1.5	13.6
BC_2F_2 53-1-25	CS (*Ph^l*)/ *Ae. kotschyi*396//PDW274-2///PBW373-1-25	33.7	35.6	32.2	33.8 ± 1.0	48.1	32.6	35.6	31.9	33.4 ± 1.2	54.1
BC_2F_2 53-1-26	CS (*Ph^l*)/ *Ae. kotschyi*396//PDW274-2///PBW373-1-26	24.9	26.8	22.3	24.7 ± 1.3	8.1	22.3	19.7	24.3	22.1 ± 1.3	2.1
BC_1F_3 75-1-4	**CS (*Ph^l*)/ *Ae. kotschyi*396//UP2382-1-1-4**	**48.1**	**40.2**	**46.2**	**44.8 ± 2.4**	**96.5**	**66.7**	**60.4**	**64.5**	**63.9 ± 1.9**	**195.1**
BC_1F_3 75-1-6	CS (*Ph^l*)/ *Ae. kotschyi*396//UP2382-1-1-6	26.0	24.6	22.5	24.3 ± 1.0	6.7	65.2	50.9	53.8	56.6 ± 4.4	161.6
BC_1F_3 75-1-39	**CS (*Ph^l*)/ *Ae. kotschyi*396//UP2382-1-1-39**	**39.2**	**35.9**	**42.5**	**39.2 ± 1.9**	**71.8**	**54.6**	**47.6**	**52.3**	**51.5 ± 2.1**	**137.8**
BC_1F_3 75-1-62	CS (*Ph^l*)/ *Ae. kotschyi*396//UP2382-1-1-62	24.7	28.4	30.4	27.8 ± 1.7	21.9	47.7	51.2	45.7	48.2 ± 1.6	122.6
BC_1F_3 75-1-76	CS (*Ph^l*)/ *Ae. kotschyi*396//UP2382-1-1-76	32.6	31.7	31.9	32.1 ± 0.3	40.5	60.4	65.2	59.3	61.6 ± 1.8	184.8
BC_1F_3 75-1-105	CS (*Ph^l*)/ *Ae. kotschyi*396//UP2382-1-1-105	33.9	32.8	30.9	32.5 ± 0.9	42.4	57.6	59.1	56.8	57.8 ± 0.7	167.2
BC_2F_2 54-3-1	CS (*Ph^l*)/ *Ae. kotschyi*396//UP2425-2///PBW373-3-1	17.4	19.6	16.3	17.8 ± 1.0	-22.2	31.7	27.6	25.7	28.3 ± 1.8	30.8
BC_2F_2 54-3-11	CS (*Ph^l*)/ *Ae. kotschyi*396//UP2425-2///PBW373-3-11	21.0	23.1	22.5	22.2 ± 0.6	-2.7	38.1	35.6	33.6	35.7 ± 1.3	65.2
BC_2F_2 54-3-12	**CS (*Ph^l*)/ *Ae. kotschyi*396//UP2425-2///PBW373-3-12**	**18.1**	**22.3**	**19.7**	**20.1 ± 1.2**	**-12.1**	**38.9**	**37.8**	**37.6**	**38.1 ± 0.4**	**76.0**
BC_2F_2 54-3-15	**CS (*Ph^l*)/ *Ae. kotschyi*396//UP2425-2///PBW373-3-15**	**19.4**	**22.8**	**20.6**	**20.9 ±1.0**	**-8.3**	**49.2**	**50.5**	**47.8**	**49.2 ± 0.8**	**127.2**

Fig. 4.6 Morphology of two BC2F2 progenies a- CS(*PhI*), BC2F2 46-1: Pl-16, Pl-18, Pl-20, Pl-37, Pl-47, Pl-48, Pl-54, Pl-60, WL711 and *Ae. kotschyi* 396 (on top); b- CS(*PhI*), BC2F2 48-42: Pl-16, Pl-17, Pl-20, Pl-24, Pl-49, Pl-64, Pl-76, Pl-82 and WL711.

Fig. 4.7 Seeds of selected BC_2F_2 and BC_1F_3 derivatives. 1- CS(*Ph*^{l}), 2- WL711, 3- BC_2F_2 46-1 Pl-15, 4- BC_2F_2 48-42 Pl-16, 5- BC_2F_2 49-1 Pl-11, 6- BC_2F_2 54-3 Pl-15,7- BC_1F_3 75-1 Pl-4 and 8-*Ae. kotschyi* 396

Generally plants do not have uptake mechanism for aluminium, therefore, presence of aluminum in the digested samples would indicate contamination during sampling or digestion. Therefore, aluminium contamination in the digested samples was checked through ICPMS study. Aluminium was found undetectable in all the samples, proving the reliability of the analyses free from any contamination.

Three other elements in addition to iron and zinc *viz.*, manganese, copper and calcium were also analysed in the digested seed samples using ICPMS and were found to be higher in most of the samples than in control WL 711 (Table 4.11).

Table 4.11 Grain mineral content in selected BC_2F_2 and BC_1F_3 derivatives using ICPMS

ID. No.	Fe (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Cu (mg/kg)	Ca (mg/kg)
WL 711	22.2	18.2	19.5	4.6	40.6
BC_2F_2 46-1-15	42.1	36.3	43.6	6.0	43.6
BC_2F_2 46-1-27	41.8	35.6	25.8	4.0	43.4
BC_2F_2 46-15-37	45.0	23.8	27.0	15.8	82.5
BC_2F_2 48-41-6	50.8	36.0	45.8	4.9	80.5
BC_2F_2 48-42-16	42.6	40.1	42.6	5.4	48.1
BC_2F_2 48-44-23	45.8	34.2	38.5	6.9	60.4
BC_2F_2 49-1-9	56.2	23.3	44.1	7.2	55.9
BC_2F_2 49-1-11	55.6	38.7	54.2	6.5	36.8
BC_2F_2 49-1-73	56.8	38.4	47.4	5.6	46.2
BC_1F_3 75-1-4	50.8	38.0	27.6	8.6	69.7
BC_1F_3 75-1-39	36.5	22.8	35.6	4.6	50.1
BC_2F_2 54-3-12	19.8	30.1	38.5	6.9	60.4
BC_2F_2 54-3-15	23.0	35.4	35.1	5.5	44.2

4.5.3 Grain ash analysis

Ash analysis of grains of some of the high iron and zinc containing plants was done to examine the actual iron and zinc percentages in the inorganic constituents of the grains. Table 4.12 provides the ash analysis data of six high grain iron and zinc BC_2F_2 and BC_1F_3

derivatives. Like their *Aegilops* parent, the seeds of these plants had higher ash content than control. Moreover their iron and zinc percentages of ash were also higher than WL711 indicating the transfer of the trait of interest from the alien genome of *Ae. kotschyi* 396 to them.

Table 4.12 Grain ash percentage and ash iron and zinc content in some selected BC_2F_2 and BC_1F_3 derivatives

I.D. No.	Grain material	Ash %	Fe (µg/g) of ash	% Change in ash Fe over WL711	Zn (µg/g) of ash	% Change in ash Zn over WL711
Control	WL711	1.58	1,607	-	1,342	-
-	CS(*Ph*^{l})	1.65	1,702	5.9	1,181	-12.0
-	*Ae. kotschyi* 396	2.07	3,058	90.3	2,197	63.7
BC_2F_2 46-1-15	CS (*Ph*^{l})/*Ae. kotschyi* 396//PBW343-3///WL711-1-15	1.86	2,563	59.5	1,858	38.5
$BC_2F_2$46-15-37	CS (*Ph*^{l}) *Ae. kotschyi* 396//PBW343-3///WL711-15-37	1.89	2,681	66.8	1,893	41.1
BC_2F_2 48-41-6	CS(*Ph*^{l})/*Ae. kotschyi* 396//PBW343-3///PBW373-41-6	1.92	2,752	71.3	1,986	48.0
BC_2F_2 48-42-16	CS(*Ph*^{l})/*Ae. kotschyi* 396//PBW343-3///PBW373-42-16	1.80	2,639	64.2	1,884	40.4
BC_2F_2 49-1-9	CS (*Ph*^{l})/*Ae. kotschyi* 396//PBW343-3///UP2425-1-9	2.04	2,826	75.9	1,932	44.0
BC_2F_2 49-1-11	CS (*Ph*^{l})/*Ae. kotschyi* 396//PBW343-3///UP2425-1-11	2.03	2,819	75.4	1,968	46.6
BC_2F_2 54-3-15	CS (*Ph*^{l})/*Ae. kotschyi* 396//UP2425-2///PBW373-3-15	1.96	2,674	66.4	1,929	43.7
BC_1F_3 75-1-4	CS (*Ph*^{l})/*Ae. kotschyi* 396//UP2382-1-1-4	2.02	2,798	74.1	1,951	45.4

Thirteen BC_2F_2 and BC_1F_3 derivatives having high grain iron and zinc concentrations were selected for characterization based on cytology, HMW-Glutenin subunit profile, molecular markers and GISH.

4.5. Cytology

The chromosome number and pairing behaviour at metaphase-I of PMCs in the selected high grain iron and zinc BC_2F_2 and BC_1F_3 plants is given in Table 4.13. Many plants had 42 chromosomes, and none had any trivalents showing the increased stability over the previous generation. However there were still some univalents particularly in high

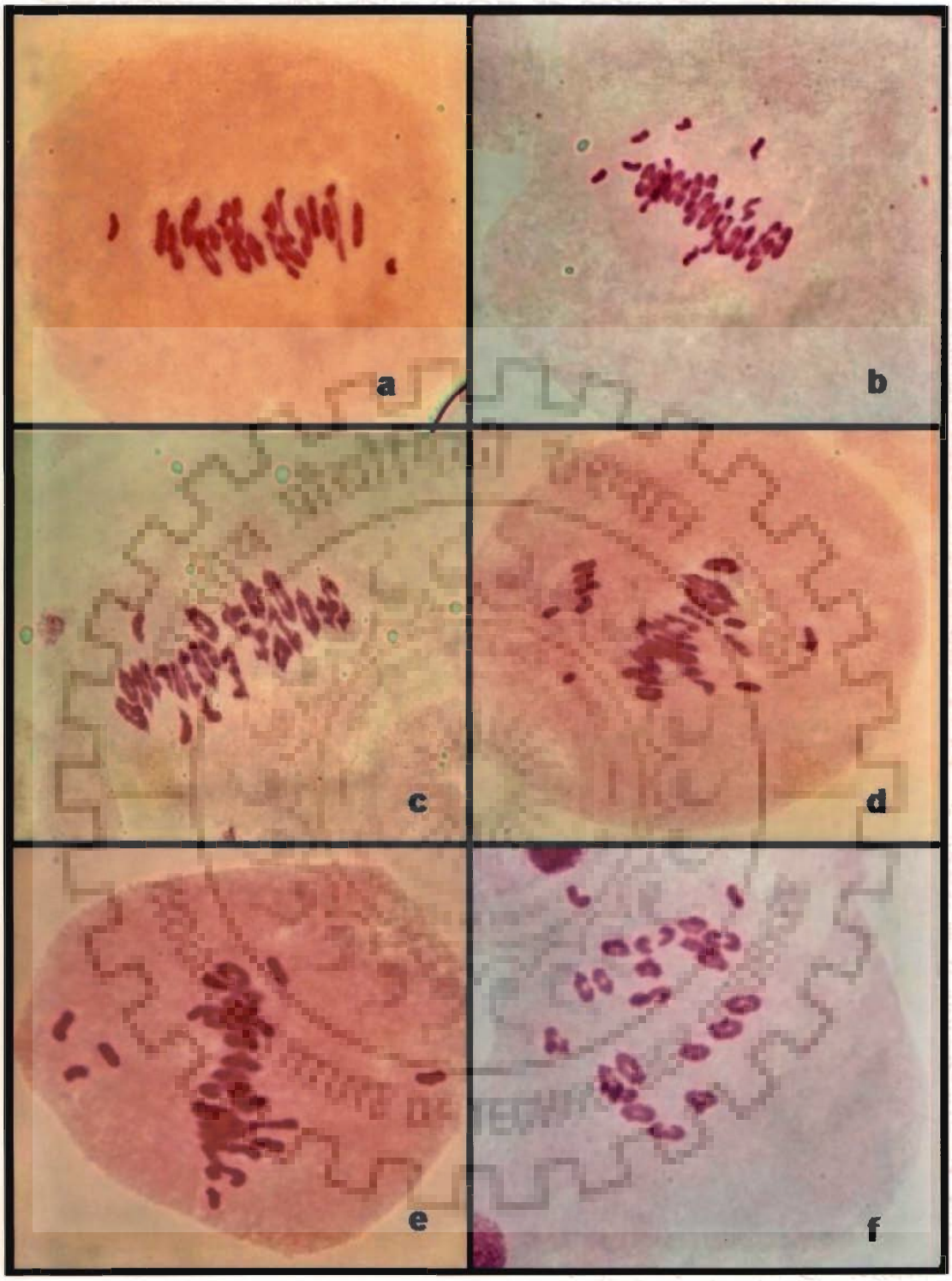


Fig. 4.8 Chromosome pairing at metaphase-I of PMCs of some selected BC_2F_2 and BC_1F_3 derivatives (a) BC_2F_2 46-1-15 (40 Chr, 1IV+17II+2I), (b) BC_2F_2 46-15-37 (43 Chr, 19 II + 5 I), (c) BC_2F_2 48-42-16 (42 Chr, 21 II) (d) BC_2F_2 49-1-73 (43 Chr, 17II+9I), (e) BC_1F_3 75-1-4 (42 Chr, 18II+6I) and (f) BC_2F_2 54-3-15 (42 Chr, 20II+2I)

Table 4.13 Chromosome number and mean and range (within parenthesis) of chromosome pairing at metaphase-I of selected BC_2F_2 and BC_1F_3 derivatives

Derivative I.D. No.	2n	PMCs	Mean ± S.E. Univalent (I)	Mean ± S.E. Bivalent (II)
BC_2F_2 46-1-15	40	25	1.28 ± 0.26	19.36 ± 0.34
BC_2F_2 46-1-27	40	25	1.52 ± 0.31	19.24 ± 0.29
$BC_2F_2$46-15-37	43	25	3.56 ± 0.33	19.72 ± 0.25
BC_2F_2 48-41-6	42	25	2.53 ± 0.29	19.74 ± 0.37
BC_2F_2 48-42-16	42	25	1.16 ± 0.21	20.42 ± 0.34
BC_2F_2 48-44-23	42	25	1.23 ± 0.15	20.37 ± 0.17
BC_2F_2 49-1-9	43	25	5.36 ± 0.29	18.87 ± 0.35
BC_2F_2 49-1-11	42	25	5.42 ± 0.37	18.29 ± 0.44
BC_2F_2 49-1-73	43	25	6.37 ± 0.42	18.32 ± 0.51
BC_1F_3 75-1-4	42	25	4.45 ± 0.29	18.76 ± 0.37
BC_1F_3 75-1-39	44	25	6.19 ± 0.34	18.91 ± 0.39
BC_2F_2 54-3-12	42	25	0.86 ± 0.18	20.57 ± 0.21
BC_2F_2 54-3-15	42	25	0.94 ± 0.19	20.53 ± 0.23

iron and zinc plants BC_2F_2 49-1 and BC_1F_3 75-1 progenies. Fig. 4.8 shows chromosome pairing behavior in some representative plants of the selected BC_2F_2 and BC_1F_3 families. Most of the plants had 42 chromosomes. BC_2F_2 46-1 Pl-15 and 27 had 40 chromosomes, whereas BC_1F_3 75-1 Pl-39 had maximum number (44) of chromosomes. BC_2F_2 48-42-16, BC_2F_2 54-3-12 and BC_2F_2 54-3-15 had 21 bivalents while BC_2F_2 49-1-73 had highest number of univalents.

4.6.1 High molecular weight glutenin subunit (HMW-GS) profile

High Molecular Weight Glutenin Subunit (HMW-GS) profile of nine seeds of some of the selected BC_2F_2 and BC_1F_3 derivatives are shown in Fig. 4.9 and Fig. 4.10. The 1U/1S HMW glutenin subunits of *Ae. kotschyi* were distinctly heavier than the wheat HMW subunits controlled by *Glu A1*, *Glu B1* and *Glu D1* loci. So the HMW-GS profile can be used to monitor group 1 alien introgression in the selected derivatives. 1U/1S

HMW-glutenin subunits were present in all the 9 seeds analysed in BC_2F_2 49-1-73 and BC_1F_3 75-1-4 in addition to the wheat HMWglutenin subunits suggesting that these plants were homozygous for 1U/1S chromosome of *Ae. kotschyi* 396. In BC_2F_2 48-44-23, BC_2F_2 49-1-9, BC_1F_3 75-1-39 1U/1S HMW-GS bands of *Ae. kotschyi* were present in 5 or 6 seeds out of the total 9 single seeds indicating that these derivatives may be segregating for the addition 1U/1S glutenin subunit (Table 4.14 and Fig. 4.9 and Fig. 4.10. The *Ae. kotschyi* 1U/1S HMW-GS bands wherever present in the derivatives, were in addition to the 1A, 1B and 1D HMW-GS bands of wheat in all the cases.

Table 4.14 Number of seeds of selected derivatives showing additional 1U/1S HMW-Gluenin Subunits of the progenies analysed.

Derivative I.D. No.	Number of individual seeds analysed	Number of seeds with 1U/1S HMW-GS addition
BC_2F_2 46-1-15	9	0
BC_2F_2 46-1-27	9	0
$BC_2F_2$46-15-37	9	0
BC_2F_2 48-41-6	9	0
BC_2F_2 48-42-16	9	0
BC_2F_2 48-44-23	9	6
BC_2F_2 49-1-9	9	6
BC_2F_2 49-1-11	9	5
BC_2F_2 49-1-73	9	9
BC_1F_3 75-1-4	9	9
BC_1F_3 75-1-39	9	6
BC_2F_2 54-3-12	9	0
BC_2F_2 54-3-15	9	0

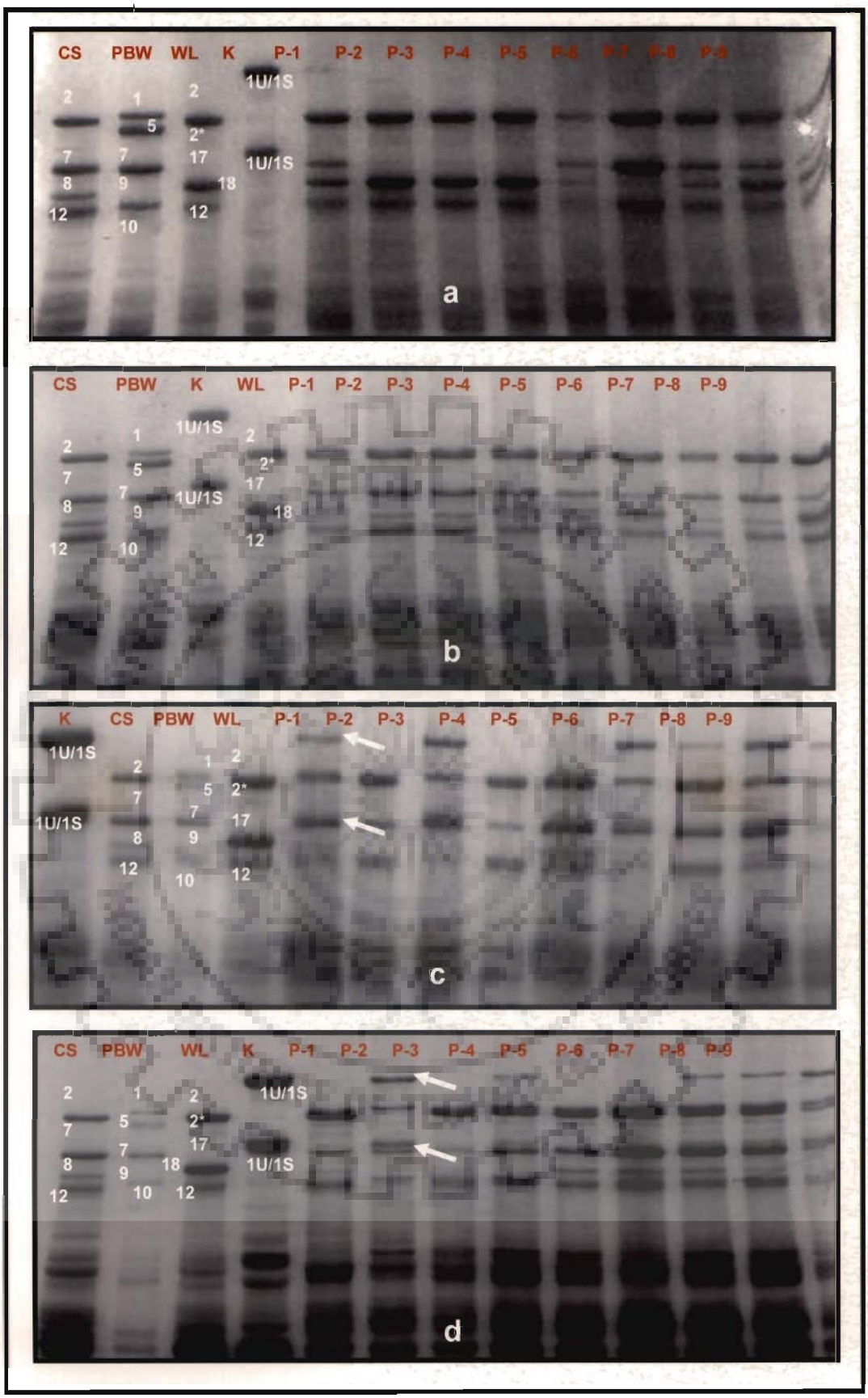


Fig. 4.9 High Molecular Weight- Glutenin Subunit profile of some selected BC_2F_2 and BC_1F_3 derivatives a- BC_2F_2 46-1-15, b- BC_2F_2 48-42-16, c- BC_2F_2 49-1-9 and d- BC_2F_2 49-1-11

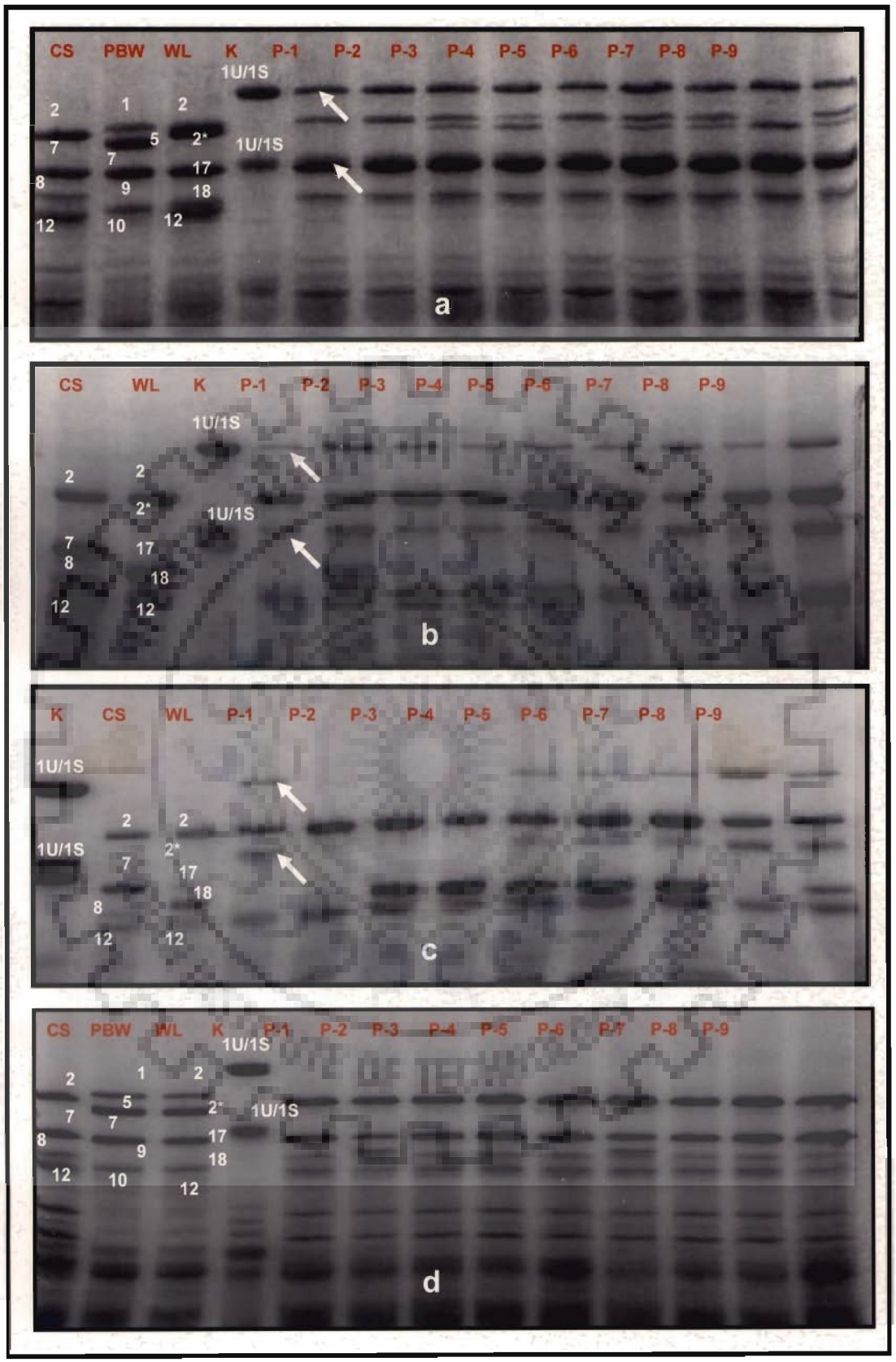


Fig. 4.10 High Molecular Weight- Glutenin Subunit profile of some selected BC_2F_2 and BC_1F_3 derivatives a- BC_2F_2 49-1-73, b- BC_1F_3 75-1 Pl-4, c- BC_1F_3 75-1-39 and BC_2F_2 54-3- Pl-15

4.1. Genomic *in situ* hybridization (GISH)

GISH of the selected derivatives was carried out to characterize alien introgressions conferring high grain iron and zinc content. Fig. 4.11 shows GISH of some of the selected derivatives. BC_2F_2 46-1-15 showed introgression of two chromosomes of *Ae. kotschyi*, visible in the GISH photographs as two pink coloured metacentric S genome chromosomes. Two different cells of BC_2F_2 46-15-37 were analyzed. One cell showed a pink (S genome) chromosome and other showed one green (U genome) chromosome. Derivative BC_1F_3 75-1-4 showed three chromosomes of *Ae. kotschyi*. A Robertsonian translocation was also seen in BC_1F_3 75-1-4.

4.1.6 Molecular Marker Analysis

Microsatellite markers were applied to the selected high grain iron and zinc derivatives to monitor the alien chromosome/ chromosome arm, containing high grain micronutrients. Out of total 401 markers applied on the parents, 63.6 % were transferable to *Ae. koschyi* while 46.3 % of the transferable markers showed polymorphism between recipient wheat cultivars and the *Ae. kotschyi* donor (Table 4.15). Highest transferability was shown by B genome markers (70.5 %) while A and D genome markers showed 63.6 % and 61.4 % transferability to *Ae. kotschyi.* The highest transferability of B genome markers indicates closeness of the B genome of wheat to the S genome. Fig. 4.12 shows wheat SSR markers having polymorphism between *Ae. kotschyi* 396 and wheat (CS(*Ph*^{I}) and PBW343).

Firstly anchored wheat SSR markers at distal positions of each of the the 42 chromosome arms transferable to *Ae. kotschyi* and showing distinct polymorphism between wheat and the *Aegilops* parent were applied on the selected derivatives to examine the alien chromosome/chromosome arm introgressed. The details of alien chromosome introgression

Table: 4.15 Transferability and polymorphism of wheat SSR markers in *Ae. kotschyi*

Homoeologous genome	Chromosome	Markers Tested	Markers Transferable	Markers Polymorphic	Transferable (%)	Polymorphism in transferable markers (%)	Genome wise transferability of markers
A	1A	23	15	7	65.2	46.7	63.6
	2A	28	18	9	64.3	50.0	
	3A	20	14	6	70.0	42.9	
	4A	12	7	4	58.3	57.1	
	5A	12	7	4	58.3	57.1	
	6A	16	10	5	62.5	50.0	
	7A	24	16	6	66.7	37.5	
B	1B	24	17	8	70.8	47.1	70.5
	2B	27	20	9	74.1	45.0	
	3B	18	12	3	66.7	25.0	
	4B	14	10	4	71.4	40.0	
	5B	14	9	4	64.3	44.4	
	6B	15	11	4	73.3	36.4	
	7B	26	19	7	73.1	36.8	
D	1D	23	14	7	60.9	50.0	61.4
	2D	26	18	7	69.2	38.9	
	3D	15	9	5	60.0	55.6	
	4D	15	9	5	60.0	55.6	
	5D	14	8	4	57.1	50.0	
	6D	16	10	4	62.5	40.0	
	7D	25	15	6	60.0	40.0	
Total		401	255	118	63.6	46.3	

in all the selected derivatives on the basis of microsatellite markers are given in Table 4.16. The table shows that only group 2 and group 7 chromosomes of *Ae. kotschyi* were introgressed in the derivatives having high grain iron and zinc concentrations. Plants 46-1-15, 48-42-16, 48-44-23, 49-1-9, 49-1-11, 54-3-15, 49-1-73, 75-1-4, 75-1-39 and 54-3-12 showed addition of a group 2 chromosome of *Ae. kotschyi,* whereas 46-1-15, 48-42-16, 49-1-9, 49-1-11, 49-1-73, 75-1-4, 75-1-39 and 54-3-15 showed the addition of group 7 chromosome (7U/7S) of *Ae. kotschyi.* Thus the genes for high grain iron and zinc may be present on *Ae. kotschyi* group 2 or group 7 chromosomes.

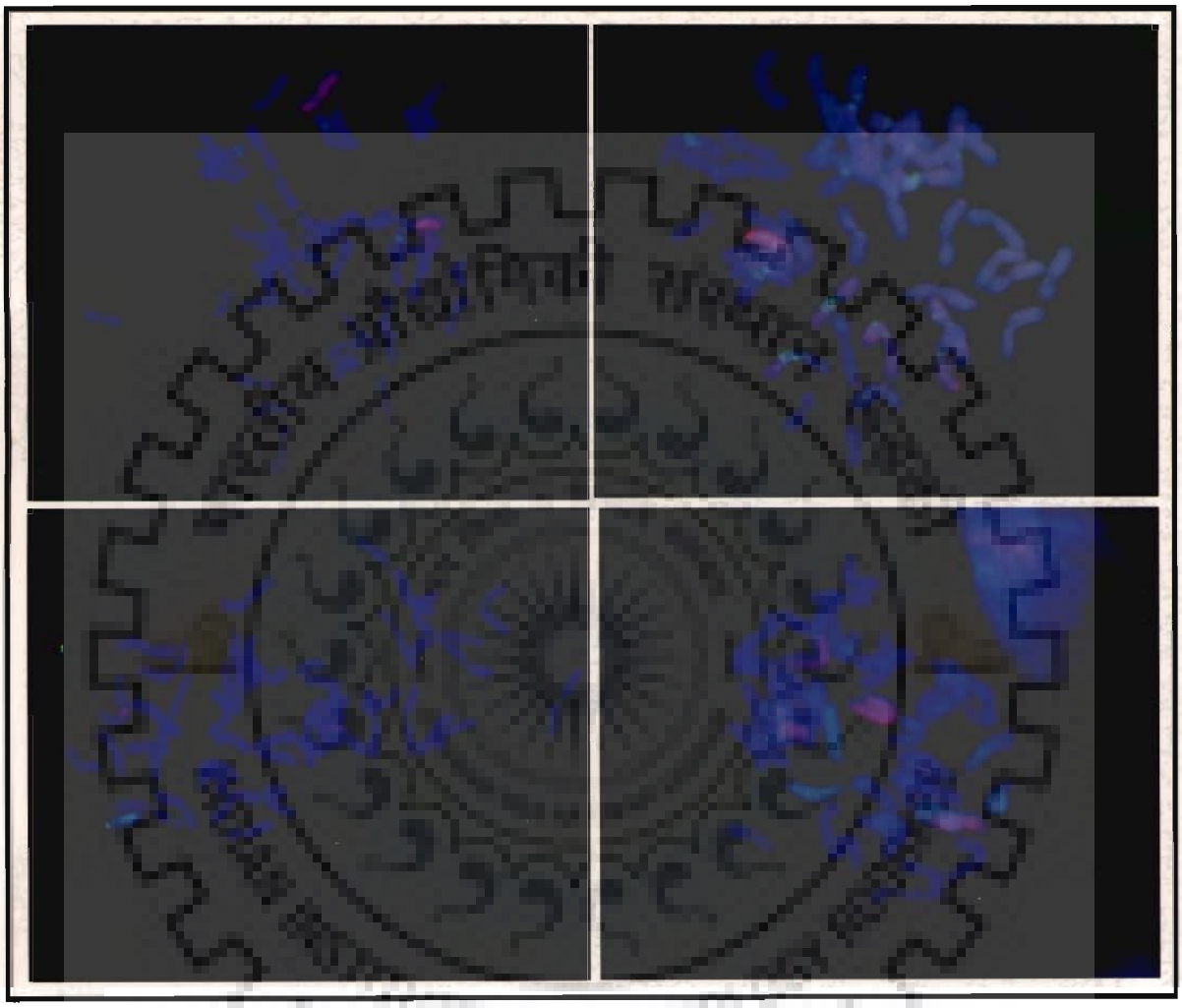


Fig. 4.11 Genomic *in situ* Hybridization of some of the selected derivatives showing introgressed alien chromosomes

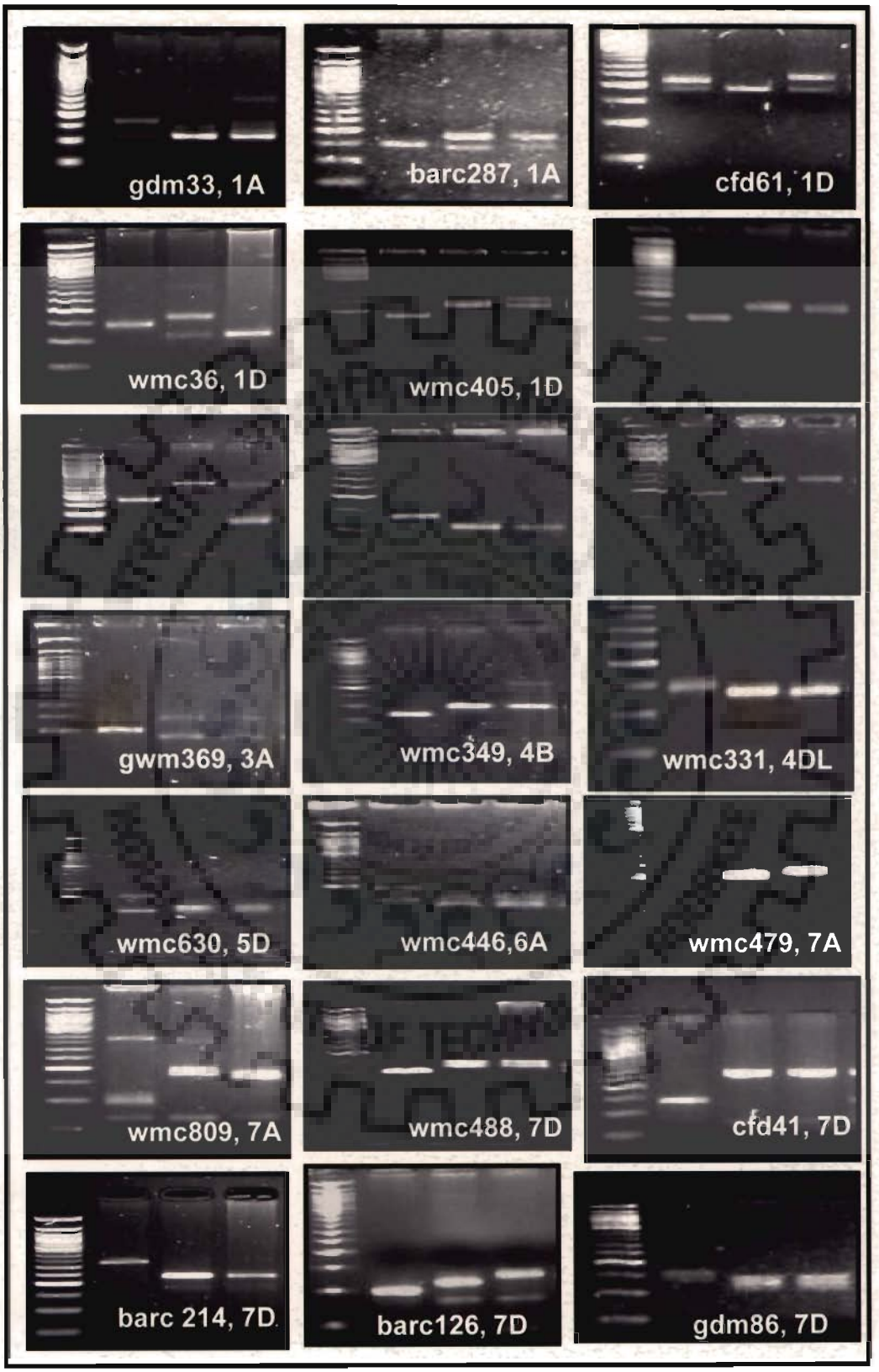


Fig. 4.12 Wheat SSR markers showing polymorphism between *Ae. kotschyi* and wheat. In all gels Lane 1-*Ae. kotschyi* 396, 2- CS(*PhI*) and 3-PBW343.

Table 4.16 Details of alien chromosome introgression in the selected BC_2F_2 and BC_1F_3 derivatives with high grain iron and zinc content.

Chromosome specific SSR markers →

I.D. No.	1AS	1AL	1BS	1BL	1DS	1DL	2AS	2AL	2BS	2BL	2DS	2DL	3AS	3AL	3BS	3BL	3DS	3DL	4AS	4AL	4BS
→ *SSR Markers used*	*gdm33*	*barc287gwm403*		*Wmc500*	*cfd 61*	*wmc405*	*Cfd36*	*wmc63*	*barc318*	*wmc474*	*cfd56*	*gdm148*	*barc12*	*wmc169*	*barc75*	*gwm340*	*cfd79*	*wmc552*	*barc206*	*wmc468*	*barc10*
Ae. Kot.	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
CS(*PhI*)	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
PBW343	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
46-1-15	W	W	W	W	W	W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W
46-1-27	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
46-15-37	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
48-41-6	W	W	W	W	W	K+W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
48-42-16	W	W	W	W	W	W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W
48-44-23	W	W	W	W	W	W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W
49-1-9	W	W	W	W	W	W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W
49-1-11	W	W	W	W	W	K+W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W
49-1-73	W	W	W	K+W	W	K+W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W
75-1-4	W	W	W	W	W	W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W
75-1-39	W	W	W	W	W	W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W
54-3-12	W	W	W	W	W	K+W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W
54-3-15	W	W	W	W	W	K+W	W	W	W	W	K+W	K+W	W	W	W	W	W	W	W	W	W

Continued…

I.D. No.	4BL	4DS	4DL	5AS	5AL	5BS	5BL	5DS	5DL	6AS	6AL	6BS	6BL	6DS	6DL	7AS	7AL	7BS	7BL	7DS	7DL
→ *SSR Markers used*	*wmc349*	*barc225*	*wmc331*	*barc180*	*wmc415*	*cfd5*	*wmc386*	*gwm190*	*wmc630*	*wmc182*	*wmc446*	*gwm613*	*wmc486*	*cfd49*	*gdm98*	*gwm350*	*wmc809*	*barc65*	*wmc396*	*cfd41*	*wmc488*
Ae. Kot.	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
CS(*PhI*)	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
PBW343	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
46-1-15	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	K+W
46-1-27	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
46-15-37	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
48-41-6	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
48-42-16	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	K+W
48-44-23	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
49-1-9	K+W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	K+W	W	W	W	K+W
49-1-11	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	K+W
49-1-73	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	K+W	W
75-1-4	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	K+W	W	W	W	K+W
75-1-39	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	K+W	W	W	K+W	K+W
54-3-12	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
54-3-15	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	K+W	W

It is interesting to note that HMW-Glutenin subunit profile of derivatives showed the presence of group 1U/1S chromosome in BC_2F_2 49-1-9, BC_2F_2 49-1-11, BC_2F_2 48-44-23, BC_2F_2 49-1-73, BC_1F_3 75-1-4 and BC_1F_3 75-1-39 progenies but molecular markers of 1A, 1B or 1D could not monitor introgression in BC_2F_2 49-1-9, BC_2F_2 48-44-23, BC_1F_3 75-1-4, and BC_1F_3 75-1-39. This may be attributed to the highly reorganized chromosome 1U (Badaeva *et al.*, 2004). May be the glutenin subunits of HMW corresponded to 1U alien chromosome while remained undetected by the SSR markers. BC_2F_2 54-3-12 and 54-3-15 showed introgression with wmc405 of 1DL, but did not have HMW-GS of *Ae. kotschyi*, this may be because of the presence of this marker on chromosome 7D as well (Xue, *et al.*, 2007).

Additional molecular markers of group 2 and group 7 chromosomes were applied to the derivatives to monitor the alien chromosome/chromosome arm introgression (Fig. 4.13 and Fig. 4.14). Addition of group 2 alien chromsome was evident in 10 out of 13 derivatives. Group 7 was also present in 46-1-15, 48-41-6, 48-42-16, 49-1-9, 49-1-11, 49-1-73, 75-1-4, 75-1-39, 54-3-12 and 54-3-15 derivative. Molecular markers did not show any alien introgression in derivatives BC_2F_2 46-1-27, BC_2F_2 46-15-27 and BC_2F_2 46-15-37. This may be because of fine transfers of *Ae. kotschyi* group 2 or group 7 chromosome not linked to the molecular markers used and as such remained undetected using molecular markers.

The alien chromosomes introgressed in the selected high grain iron and zinc derivatives on the bases of HMW-GS profile and SSR markers and percent increase in iron and zinc content over WL711 are summarized in Table 4.17. The derivatives with simultaneous introgression of *Ae. kotschyi* group 1, 2 and 7 had highest grain iron and zinc concentrations. It may be noted here that plants with group 2 or 7 only had 60-70 % increase in grain iron and zinc content, whereas the plants with group 1 had more

Table 4.17 Chromosome introgression on the bases of HMW-GS profile and SSR markers and increase of high grain iron and zinc content in selected derivatives.

Derivative I.D. No.	Group of Introgressed alien chromosome(s)	% Increase in Fe over WL711	% Increase in Zn over WL711
46-1-15	2, 7	61.7	52.1
46-1-27	-	70.1	65.8
46-15-37	-	66.5	3.1
48-41-6	7	114.9	92.8
48-42-16	2,7	68.4	43.5
48-44-23	1,2	115.7	51.4
49-1-9	1,2,7	139.6	95.0
49-1-11	1,2,7	134.6	135.8
49-1-73	1,2	155.6	184.6
75-1-4	1, 2, 7	96.5	195.1
75-1-39	1,2,7	71.8	137.8
54-3-12	2,7	-12.1	76.0
54-3-15	2,7	-8.3	127.2

than double the micronutrient content than the control. Thus genes on group 1 chromosomes of *Ae. kotschyi* may be presumed to have greatest effect on increasing grain micronutrient content of the derivative.

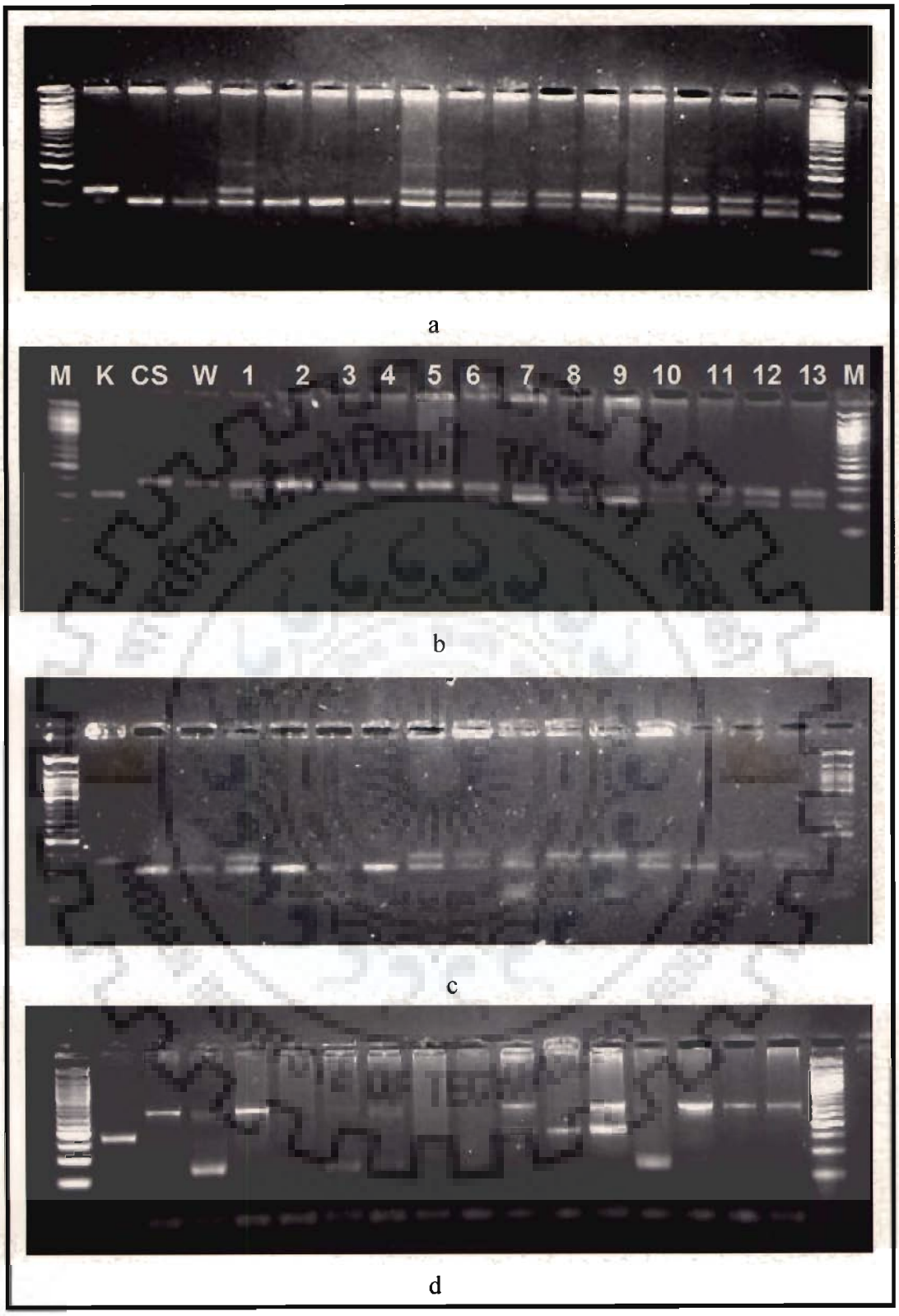


Fig. 4.13 Analysis of introgression of group 2 chromosome of *Ae. kotschyi* with wheat SSR markers a- cfd56, b- gwm539, c-gdm148 and d- barc11 in selected BC_2F_2 and BC_1F_3 derivatives: Ladder (50bp),, CS(*PhI*), PBW343, 1: 46-1-15, 2: 46-1-27,3: 346-15-37,4: 48-41-6, 5: 48-42-16, 6: 48-4-23, 7: 49-1-9, 8: 49-1-11, 9: 49-1-73, 10: 75-1-4, 11: 75-1-39, 12: 54-3-12 and 13: 54-3-15.

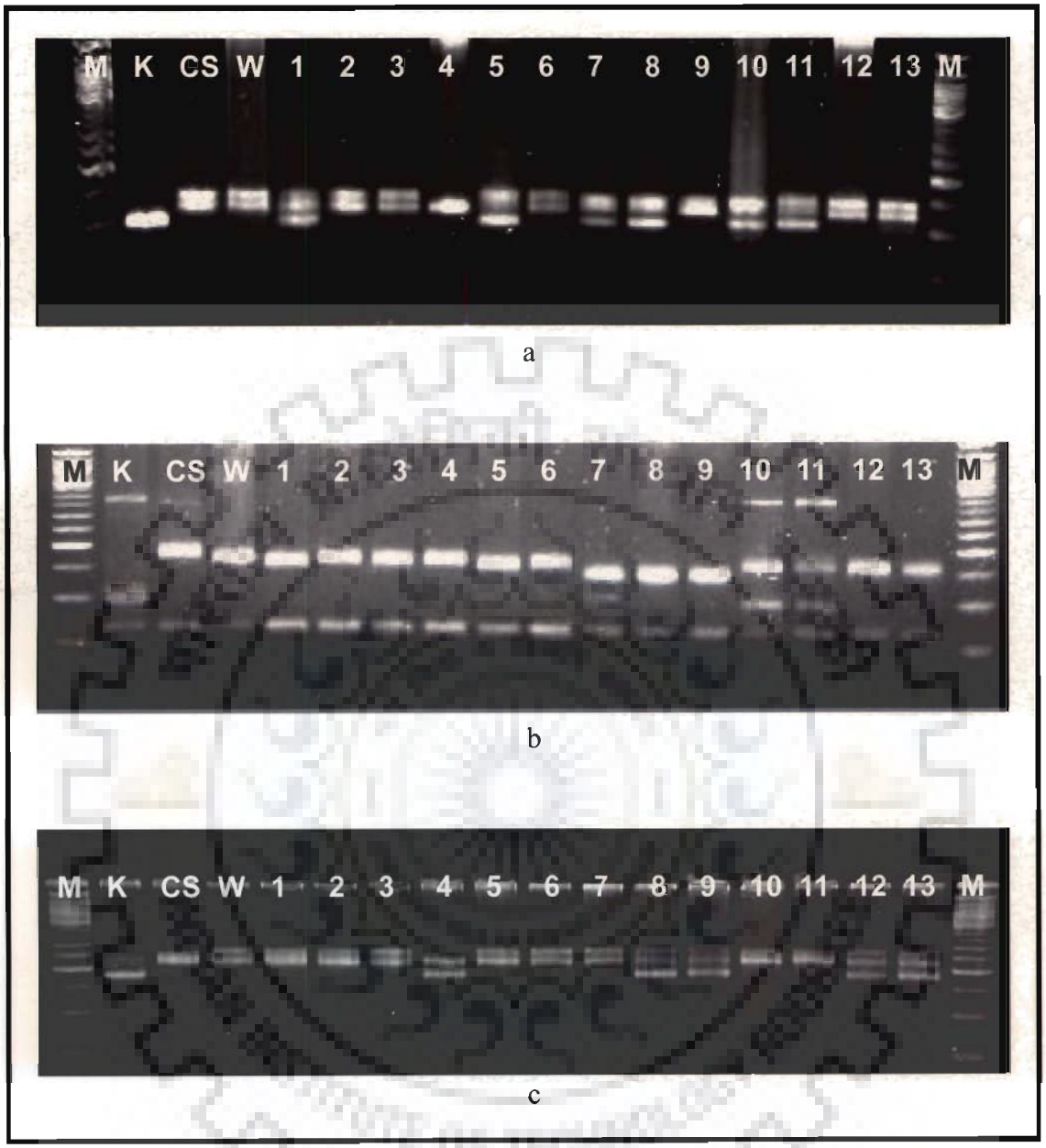


Fig. 4.14 Analysis of introgression of group 7 chromosome of *Ae. kotschyi* with wheat SSR markers a- wmc488, b-wmc809 and c- wmc405 in selected BC_2F_2 and BC_1F_3 derivatives: Ladder (50bp),, CS(*PhI*), PBW343, 1: 46-1-15, 2: 46-1-27,3: 346-15-37,4: 48-41-6, 5: 48-42-16, 6: 48-4-23, 7: 49-1-9, 8: 49-1-11, 9: 49-1-73, 10: 75-1-4, 11: 75-1-39, 12: 54-3-12 and 13: 54-3-15.

Section-2 Synthesis and characterization of wheat- *Ae. kotschyi* amphiploids with high grain iron and zinc concentrations

4.2.1 Colchicine treated C_0 generation

The colchicine treated F_1 hybrids were exactly like the untreated F_1 hybrids except having some doubled sectors or spikes with dehiscing anthers which were readily distinguished from non-dehiscent sterile anthers in other spikes. These spikes/sectors with dehiscing anthers had high seed set where as there was no seed set on the otherwise sterile F_1 plants without chromosome doubling. The seeds thus obtained were identified as the potential synthetic amphiploids (C_0 generation) for further studies.

4.2.2 Morphology and fertility of the synthetic amphiploids

Comparative morphology of the synthetic amphiploids with the parents showed their intermediate growth habit, tiller number and plant height (Table 4.18) like the F_1 hybrids. The amphiploids displayed some of the characteristics of the *Ae. kotschyi* parent such as spelta head, brittle rachis and red seed colour and others of the wheat parents such as 1000 grain weight. The number of spikelets per spike exceeded that of both the parents. Most of the spike characteristics like number and length of awns of glumes and lemmas were again intermediate to both the parents (Fig. 4.15). *Ae. kotschyi* accessions had 5-7 glumed awns against none and single awn in CS(*Ph*^{I}) and WL711, respectively. The glumes of amphiploids with WL711 had single awn while those with CS (*Ph*^{I}) were awnless and had a tooth only. The long lemma awn of WL711 was replaced by small awns in the amphiploids whereas *Ae. kotschyi* had two lemma awns (Fig. 4.15). Pollen stainability and seed set in the amphiploids varied within the season.

Table 4.18 Morphological characteristics of *T. aestivum*, *Ae. kotschyi*, their F_1 hybrids and amphiploids

Plant material*	Average No. of tillers per plant	Average plant height (cm)	Ear shape	Spikelets per spike	Glume awn	Lemma awn	Rachis	1000 grain weight (g)	Average seed set per spike
Parents									
T. aestivum cv. WL 711	14.6	92.8	Square	13.4	0	1 long	Tough	32.4	30
T. aestivum lr. CS(Ph^l)	18.3	100.3	Square	14.6	0	0	Tough	30.2	28
Ae. kotschyi acc. 396 (14267)	275.5	35.7	Spelta	7.2	5-7	2 small	Brittle	10.7	12
Ae. kotschyi acc.395 (14266)	280.9	40.1	Spelta	5.1	5-7	2 small	Brittle	8.3	10
Ae. kotschyi acc.393 (14264)	270.6	35.9	Spelta	6.5	5-7	2 small	Brittle	8.7	10
Ae. kotschyi acc.3774	285.5	38.2	Spelta	7.2	5-7	2 small	Brittle	12.9	11
Ae. kotschyi acc.3790	273.4	36.8	Spelta	6.1	5-7	2 small	Brittle	10.8	12
Ae. kotschyi acc.391 (14262)	287.6	43.6	Spelta	7.4	5-7	2 small	Brittle	11.6	10
F_1 hybrids									
F_1 CS(Ph^l)/ *Ae. kotschyi* 396	110.5	90.1	Spelta	14.5	0	0	Brittle	N.A.**	-
F_1 CS(Ph^l)/ *Ae. kotschyi* 395	105.7	95.5	Spelta	13.8	0	0	Brittle	N.A.	-
F_1 CS(Ph^l)/ *Ae. kotschyi* 393	100.6	95.8	Spelta	15.3	0	0	Brittle	N.A.	-
F_1 CS(Ph^l)/ *Ae. kotschyi* 3774	111.7	90.2	Spelta	13.6	0	0	Brittle	N.A.	-
F_1 WL711/ *Ae. kotschyi* 3790	104.1	100.4	Spelta	16.6	1	1 medium	Brittle	N.A.	-
F_1 WL711/ *Ae. kotschyi* 391	113.0	95.6	Spelta	18.2	1	1 medium	Brittle	N.A.	-
F_1 WL711/ *Ae. kotschyi* 393	103.8	100.3	Spelta	18.4	1	1 medium	Brittle	N.A.	-
Amphiploids									
Amphi. CS(Ph^l)-*Ae. kotschyi* 396	60.5	80.1	Spelta	19.3	0	0	Brittle	34.93	5.6
Amphi CS(Ph^l)-*Ae. kotschyi* 395	68.6	82.8	Spelta	18.5	0	0	Brittle	29.93	15.3
Amphi CS(Ph^l)-*Ae. kotschyi* 393	65.3	75.5	Spelta	17.2	0	0	Brittle	30.58	14.2
Amphi CS(Ph^l)-*Ae. kotschyi* 3774	70.2	79.1	Spelta	15.7	0	0	Brittle	36.98	17.5
Amphi WL711-*Ae. kotschyi* 3790	59.9	75.2	Spelta	13.9	1	1 medium	Brittle	35.39	4.1
Amphi WL711-*Ae. kotschyi* 391	58.7	70.8	Spelta	11.8	1	1 medium	Brittle	28.54	15.9
Amphi. WL711-*Ae. kotschyi* 393	61.8	76.4	Spelta	17.1	1	1 medium	Brittle	27.49	8.3

* The accession numbers of the PAU accsssion register are given in parentheses

** N.A. data not available due to sterility of F_1 hybrids

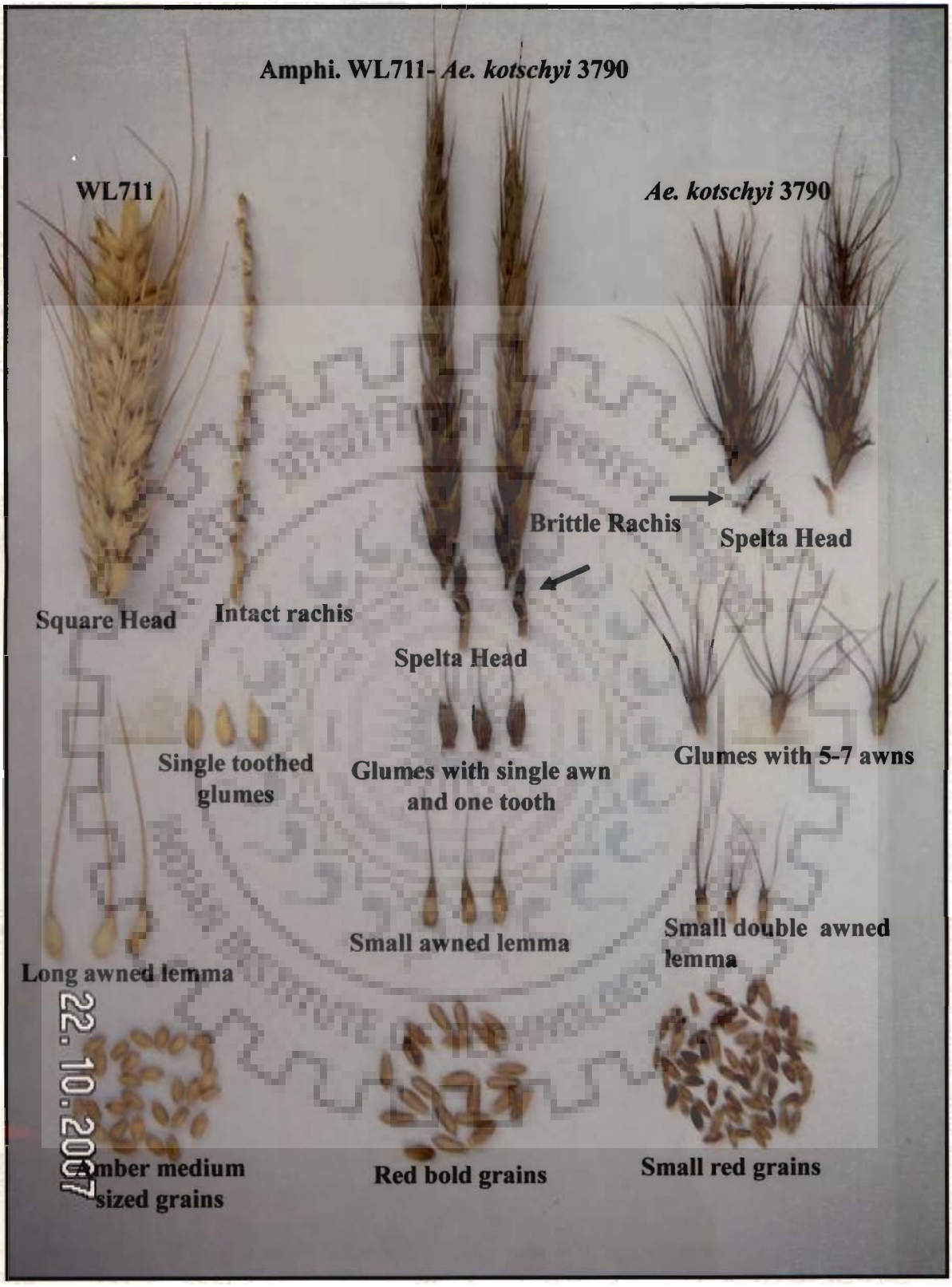


Fig 4.15. Spike and grain characteristics of amphiploid WL711-*Ae. kotschyi* 3790 (center) along with parents WL711 (left) and *Ae. kotschyi* 3790 (right).

The early flowering spikes had non-dehiscent anthers, low pollen stainability and less seed set whereas the late flowering tillers had dehiscing anthers, higher pollen stainability and good seed set. Pollen stainability varied from 62.6 to 81.8 percent in different amphiploids of CS (Ph^I)-*Ae. kotschyi* accessions, while in amphiploids of WL711-*Ae. kotschyi* accessions it ranged from 57.4 to 79.0 percent (Table 4.19). Variation in the seed set was observed for different combinations of bread wheat lines and *Ae. kotschyi* accessions. Maximum seed set was observed in the amphiploid CS(Ph^I)-*Ae. kotschyi* 3774 (17.5 seeds/ spike) and least in WL 711- *Ae. kotschyi* 391 (4.1 seeds/ spike). Seeds of the amphiploids were bold, long, red and had 1000 grain weight comparable to those of the wheat parents (Table 4.18, Fig. 4.15).

4.2.3 Cytology of the synthetic amphiploids

Chromosome number in the amphiploids was highly variable (Table 4.19, Fig. 4.16) ranging from 35-70 chromosomes in CS (Ph^I)-*Ae. kotschyi* 396, 39-69 in CS (Ph^I)-*Ae. kotschyi* 395 and 42-70 in CS (Ph^I)-*Ae. kotschyi* 393 and 37-70 in CS (Ph^I)- *Ae. kotschyi*

Table 4.19 Chromosome number, meiotic pairing and seed set in *T. aestivum/ Ae. kotschyi* synthetic amphiploids (C_1)

Amphiploid	No. of PMCs studied	Chromo some number (Range)	Mean ± S.D. (Range) Univalent (I)	Mean ± S.D. (Range) Bivalent (II)	Mean ± S.D. (Range) Trivalent (III)	Pollen Stain-ability %
Amphi. CS (Ph^I)-*Ae .kotschyi* 396	25	35 - 70	8.63 ± 2.03 (2-21)	24.25 ± 2.91 (2-34)		62.6
Amphi. CS (Ph^I)-*Ae .kotschyi* 395	25	39 - 69	2.60 ± 0.81 (1-5)	32.80 ± 0.58 (31-34)	-	76.6
Amphi. CS (Ph^I)- *Ae .kotschyi* 393	25	42 - 70	12.40 ± 0.75 (10-14)	25.80 ± 1.86 (21-29)	-	72.5
Amphi. CS (Ph^I)-*Ae .kotschyi* 3774	25	37 - 70	2.92 ± 0.71 (1-10)	32.80 ± 0.58 (27-34)	0.27 ± 0.14 (0-1)	81.8
Amphi. WL 711-*Ae .kotschyi* 391	25	39 - 67	11.00 ± 2.81 (2-18)	20.43 ± 3.76 (12-32)	-	57.4
Amphi. WL 711-*Ae .kotschyi* 393	25	47-68	8.51 ± 0.97 (2-15)	23.17 ± 2.14 (16-32)	-	79.0
Amphi. WL 711-*Ae .kotschyi* 3790	25	57-68	3.56 ± 1.78 (4-12)	27.75 ± 0.97 (24-30)	-	70.5

3774. There was only a small proportion of PMCs in all the amphiploids with the expected double chromosome number (70) of the F_1 hybrids (35). Comparatively higher number of bivalents and lower number of univalents in the amphiploids CS (*Ph*^{l})-*Ae. kotschyi* 395 (32.8II, 2.6I), CS(*Ph*^{l})-*Ae. kotschyi* 3774 (32.8 II, 2.92 I) and WL 711-*Ae. kotschyi* 393 (23.2 II, 8.5I) might have resulted in higher seed set in these amphiploids (Table 4.18), whereas irregular meiotic behaviour of F_1CS (*Ph*^{l})-*Ae. kotschyi* 396 and F_1WL 711-*Ae. kotschyi* 391 with very wide range of chromosome number, higher frequency of univalents and lower bivalent frequency was associated with low seed set percentage (4 seeds per spike).

To get a better insight into the stage of chromosome elimination in amphiploids, chromosome number and pairing in different PMCs from different tillers and different spikelets of a plant of amphiploid CS (*Ph*^{l})-*Ae. kotschyi* 3774 were studied. The number of chromosomes showed variation between different tillers, different spikelets and even within a floret (Fig. 4.17, Fig. 4.18). This indicates that the chromosome elimination occurring due to imbalanced gamete formation also occurs in the somatic tissue of amphiploids during germination and continued during tillering, floret initiation and PMCs differentiation.

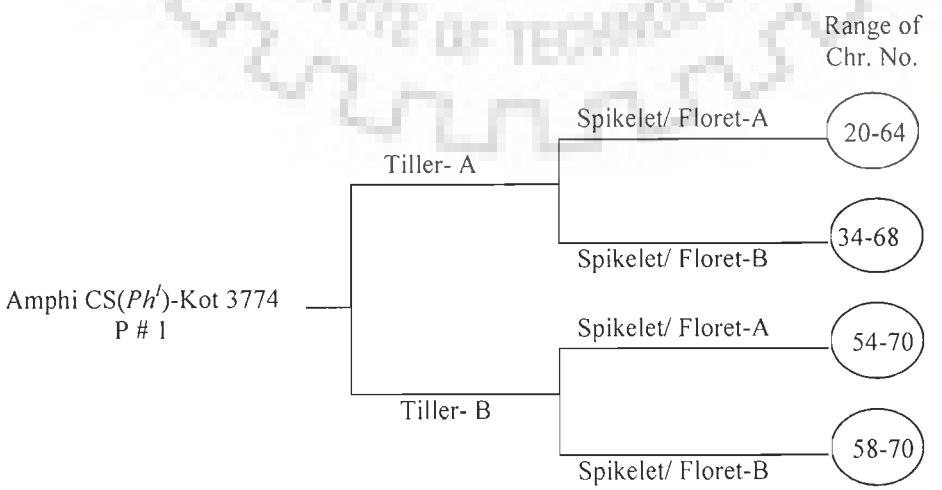


Fig. 4.17 Range of chromosome numbers in anthers of different florets on two tillers of a plant of amphiploid CS(*Ph*^{l})-*Ae. kotschyi* 3774

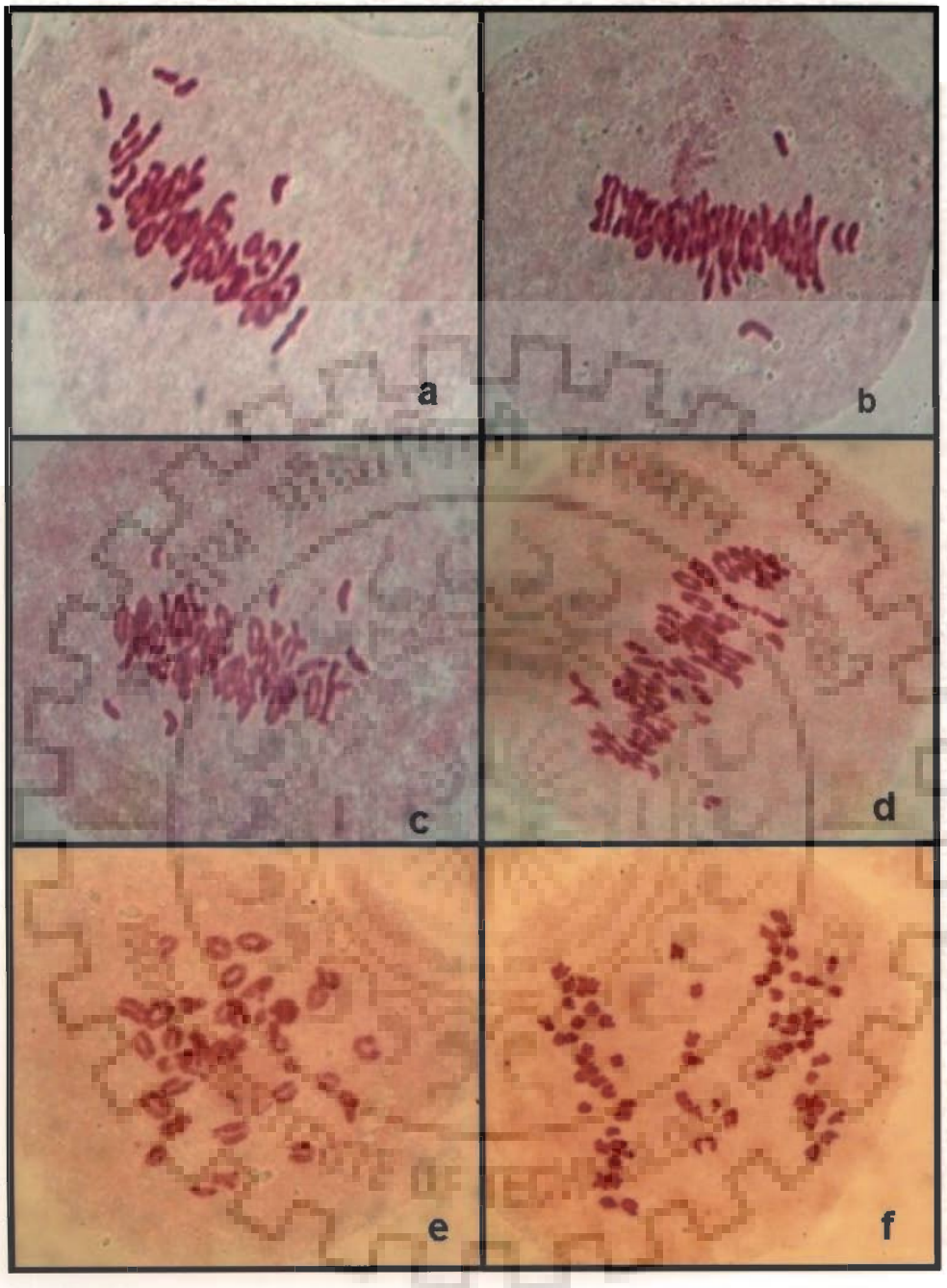


Fig 4.16 Chromosome pairing in wheat- *Ae. kotschyi* amphiploids (a) Amphi. CS(*PhI*)-*Ae. kotschyi* 3774 (Chr- 64, 1 III+ 28 II+ 5 I) (b) Amphi. CS(*PhI*)-*Ae. kotschyi* 393 (Chr-68, 32 II + 4 I) (c) Amphi. CS(*PhI*)-*Ae. kotschyi* 393 (chr-69, 31 II + 7 I) (d) Amphi. WL711-*Ae. kotschyi* 393 (Chr- 69, 32 II + 5 I), (e) Amphi. WL711-*Ae. kotschyi* 3790 (Chr-64, 30 II + 4 I) and (f) Amphi. WL711-*Ae. kotschyi* 391 (Chr- 67, 29 II + 8 I)

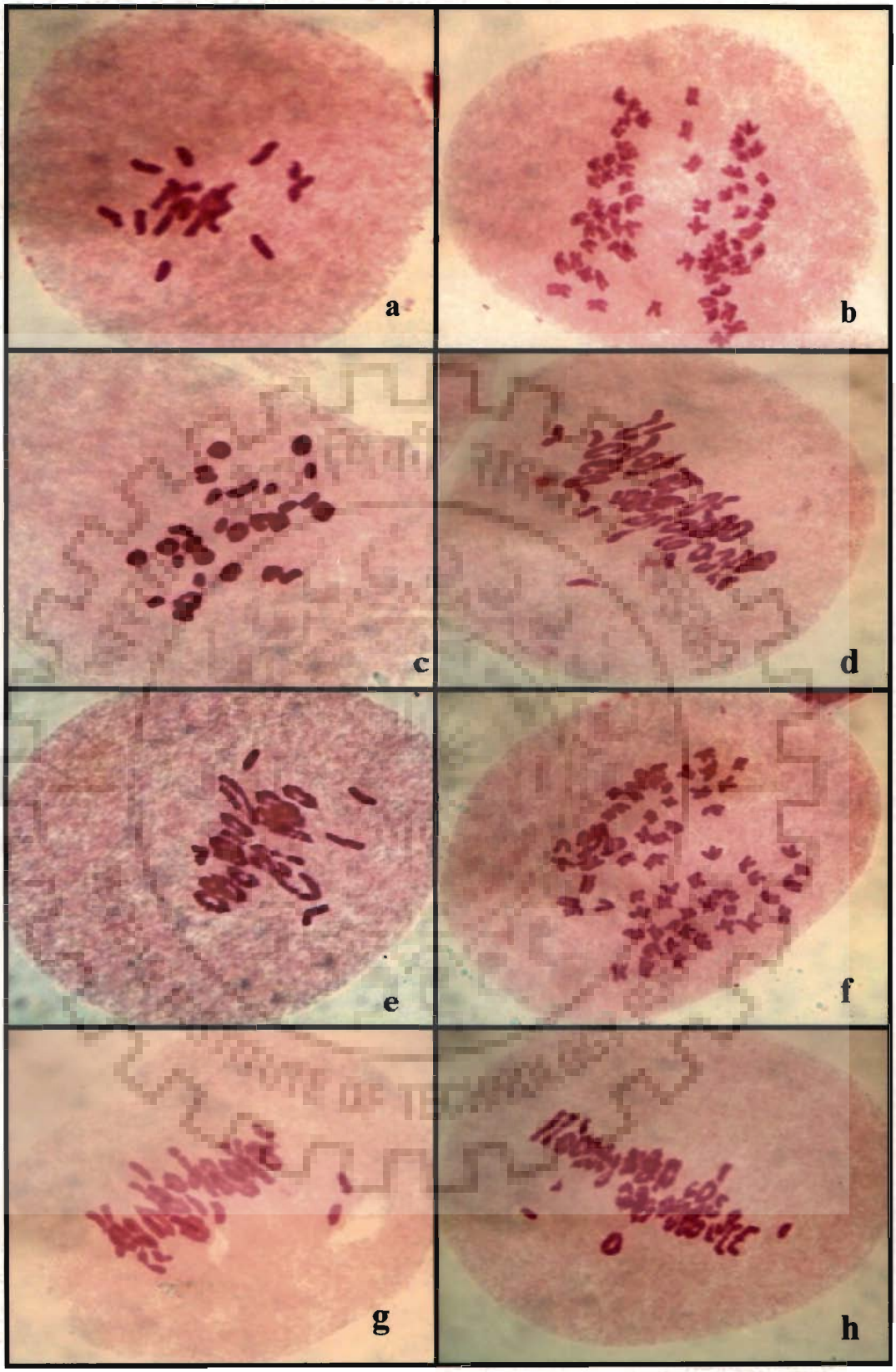


Fig 4.18 Chromosome pairing in wheat- *Ae. kotschyi* amphiploids (a) Amphi. CS(*PhI*)-*Ae. kotschyi* 3774 (Chr- 64, 1 III+ 28 II+ 5 I) (b) Amphi. CS(*PhI*)-*Ae. kotschyi* 393 (Chr-68, 32 II + 4 I) (c) Amphi. CS(*PhI*)-*Ae. kotschyi* 393 (chr-69, 31 II + 7 I) (d) Amphi. WL711-*Ae. kotschyi* 393 (Chr- 69, 32 II + 5 I), (e) Amphi. WL711-*Ae. kotschyi* 3790 (Chr-64, 30 II + 4 I) and (f) Amphi. WL711-*Ae. kotschyi* 391 (Chr- 67, 29 II + 8 I)

4.2.4 HMW glutenin subunit profiles of amphiploids

The SDS-PAGE profiles of the HMW glutenin subunits of CS(*Ph*^{I}), *Ae. kotschyi* accessions and the CS(*Ph*^{I})-*Ae. kotschyi* amphiploids are given in Fig. 4.18. *Triticum aestivum* cultivars PBW343, Kalyan Sona and landrace CS and CS(*Ph*^{I}) were taken as the control. CS and CS (*Ph*^{I}) had similar subunit pattern for *Glu 1B* controlled 7+8 subunits and *Glu 1D* controlled 2+12 subunits of HMW glutenins. All the accessions of *Ae. kotschyi* (UUSS) expressed 3-5 novel subunits of high molecular weight glutenin subunits. Two of the slowest migrating *x* subunits had lower electrophoretic mobility than the Glu D1 subunit 5 while the faster migrating two *y* subunits were slower than the subunit 7. HMW glutenin subunits of both the wheat and *Ae. kotschyi* parents were present in all the amphiploids confirming the presence and expression of both the parental genomes (Fig. 4.19). Similar additive profile of HMW glutenin subunits was observed in the three amphiploids of WL 711-*Ae. kotschyi.*

4.2.5 Grain iron and zinc content of amphiploids

Table 4.20 shows grain iron and zinc content of the amphiploids along with both the parents viz., WL711, CS (*Ph*^{I}) and *Ae. kotschyi* accessions. The micronutrient content of wild parents was 2-3 fold higher as compared to wheat parents. *Ae. kotschyi* accessions 3774 had highest grain iron (70.8 mg/kg) and zinc content (35.7mg/kg), respectively. The micronutrient contents of wheat parents were quite low, iron being 22.8 mg/kg in WL 711 and 30.2 mg/kg in CS (*Ph*^{I}) and zinc 16.6 mg/kg and 18.3 mg/kg in WL711 and CS (*Ph*^{I}), respectively. The micronutrient contents of amphiploids were comparable to those of *Ae. kotschyi*. Amphiploid CS (*Ph*^{I})-*Ae. kotschyi* 395 (64.2 mg/kg iron and 29.5 mg/kg zinc) had lower grain micronutrient than the wild parent *Ae. kotschyi* 395 (63.2 mg/kg iron and 31.5 mg/kg zinc), on the other hand WL711-*Ae.*

Table 4.20 Whole grain iron and zinc, ash and ash iron and zinc concentrations of amphiploids and their parents

Plant material	Whole grain			Grain ash			
	Iron (mg/kg) ± S.D.	Zinc (mg/kg) ± S.D.	Grain ash %	Fe (µg/g) of ash	% Change in ash Fe content over WL711	Zn (µg/g) of ash	% Change in ash Zn content over WL711
Triticum aestivum cv. WL711	22.8 ± 0.8	16.6 ± 1.3	1.69	1667	-	1341	-
Chinese Spring (*Ph*^{I})	30.2 ± 0.5	18.3 ± 0.9	1.70	1867	12.00	1568	16.93
Ae. kotschyi 391	63.1 ± 1.1	35.2 ± 0.5	2.11	3089	85.30	2178	62.42
Ae. kotschyi 393	58.4 ± 0.9	29.8 ± 0.8	1.98	3119	87.10	2099	56.52
Ae. kotschyi 395	63.2 ± 1.0	31.5 ± 1.5	2.05	3151	89.02	2195	63.68
Ae. kotschyi 396	65.6 ± 1.4	32.3 ± 1.2	2.07	3272	96.28	2373	76.96
Ae. kotschyi 3774	70.8 ± 0.7	35.7 ± 0.7	2.02	3170	90.16	2261	68.61
Ae. kotschyi 3790	67.5 ± 0.6	30.8 ± 2.1	2.21	3176	90.52	2265	68.90
Amphi. CS(*Ph*^{I})-*Ae. kotschyi* 393	65.0 ± 1.3	36.5 ± 0.6	1.89	2687	61.19	1998	48.99
Amphi. CS(*Ph*^{I})-*Ae. kotschyi* 395	64.2 ± 1.8	29.5 ± 1.5	1.83	2712	62.69	2118	57.94
Amphi. CS(*Ph*^{I})-*Ae. kotschyi* 396	62.9 ± 1.5	43.2 ± 1.2	1.72	2697	61.79	2013	50.11
Amphi. CS(*Ph*^{I})-*Ae. kotschyi* 3774	59.2 ± 0.5	28.8 ± 0.6	1.88	3082	84.88	2188	63.16
Amphi. WL711-*Ae. kotschyi* 391	61.8 ± 1.7	33.1 ± 0.8	1.92	2705	62.27	2059	53.54
Amphi. WL711-*Ae. kotschyi* 393	64.6 ± 2.0	30.7 ± 1.3	1.86	2609	56.51	2016	50.34
Amphi. WL711-*Ae. kotschyi* 3790	65.2 ± 2.2	33.1 ± 2.0	1.76	2798	67.85	2148	60.18

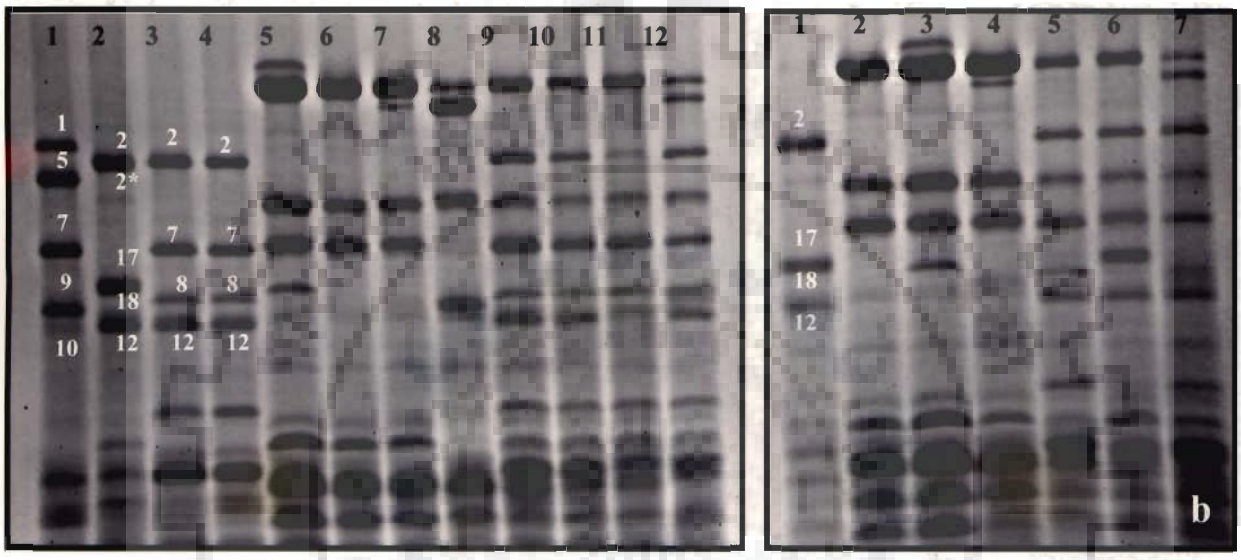


Fig. 4.19 HMW glutenin subunit profile of *T. aestivum* cultivars, *Ae. kotschyi* accessions and the amphiploids of CS(*PhI*) and WL711 with *Ae. kotschyi* accessions. a. Lane 1- PBW343, 2- Kalyan Sona, 3- Chinese Spring, 4- Chinese Spring(*PhI*), 5- *Ae. kotschyi* 393, 6- *Ae. kotschyi* 395, 7- *Ae. kotschyi* 396, 8- *Ae. kotschyi* 3774, 9- Amphi. CS(*PhI*)-*Ae. kotschyi* 393, 10- Amphi. CS(*PhI*)-*Ae. kotschyi* 395, 11- Amphi. CS(*PhI*)-*Ae. kotschyi* 396 and 12- Amphi. CS(*PhI*)-*Ae. kotschyi* 3774. b. Lane 1- WL711, 2- *Ae. kotschyi* 391, 3- *Ae. kotschyi* 393, 4- *Ae. kotschyi* 3790, 5- Amphi. WL711- *Ae. kotschyi* 391, 6- Amphi. WL711-*Ae. kotschyi* 393 and 7- Amphi. WL711-*Ae. kotschyi* 3790

3790 showed higher levels (65.2 mg/kg iron and 33.1 mg/kg zinc) of these than their wild parent *Ae. kotschyi.*

4.2.6 Ash content and ash iron and zinc content of amphiploids

The grain ash content in *Ae. kotschyi* was nearly 20% higher than that of the wheat cultivars indicating higher inorganic nutrients whereas for the wheat *Ae. kotschyi* amphiploids it was intermediate between the parental species (Table 4.20). The grain iron and zinc content in *Ae. kotschyi* amphiploids was more than double than that of the wheat cultivars where as the iron content in the grain ash was 61-85% and zinc was 50-63% higher than that of control WL711.

4.3 Low phytic acid mutants

4.3.1 Morphology of the mutants

The low phytic acid (*lpa*) mutant plants were morphologically similar to the *T. monococcum* wild type plants in colour, tillering, height and fertility. The seeds of *lpa* mutant MM225, however, were strikingly round as against the normal seeds of MM169 (Fig. 4.20).

4.3.2 Qualitative screening:

On the basis of qualitative screening two samples with deepest blue colour i.e. MM225 and MM169 out of a total 76 were isolated as putative low phytic acid mutants (Fig. 4.21). The deepest blue colour depicted highest inorganic phosphate (low phytate) content in these samples.

4.3.3 HPLC based quantification of phytic acid:

The seeds of selected putative mutants were analyzed by HPLC for phytic acid content. Fig. 4.22 shows the HPLC peaks of phytic acid standard of 75 µg/ml concentration. The retention time (RT) of phytic acid was found to be 1.8 minutes. The equation of calibration curve calculated was $y = -261.75x + 55865$. Since the method of phytic acid quantification is based on iron-thiocyanate complex measurement, increasing phytic acid causes a decrease in the peak area. Slope of the calibration curve prepared was therefore negative. The HPLC peaks of phytic acid (RT 1.8 min) in the samples are shown in Fig. 4.22. The smaller peaks in the graphs at lower RT (1.7 min) represent lower phosphates of inositol. Based on the equation of calibration curve and using peak area as a function of concentration of phytic acid standard solutions, phytic acid contents in the samples were calculated (Table 4.21). Mutant MM225 had 17.1 mg/g phytic acid content

Fig 4.20 Spikes and seeds of *T. monococcum* wild type and *lpa* mutants MM225 and MM169

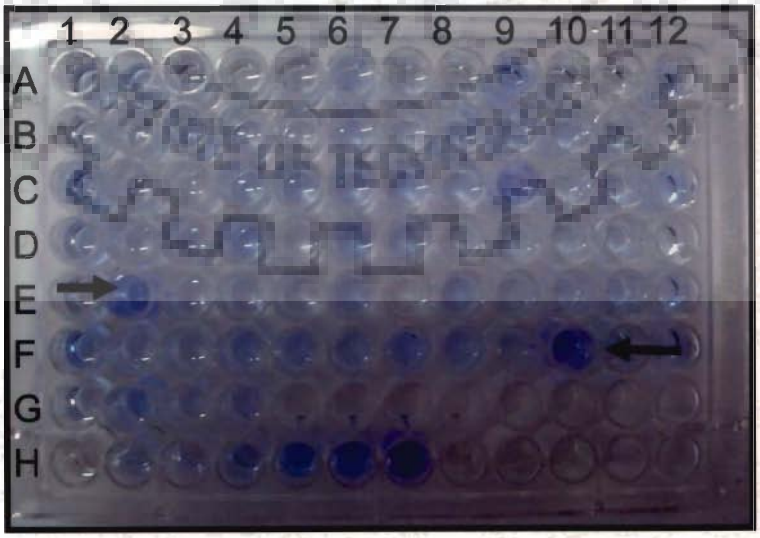


Fig. 4.21 Detection of higher inorganic levels of P*i* in *T. monococcum* mutant grains in a microtitre plate. H2-H7 show P*i* standards, H1- *T. monococcum* wild type. Arrows point to the two selected mutants MM169 (E-2) and MM225 (F-10)

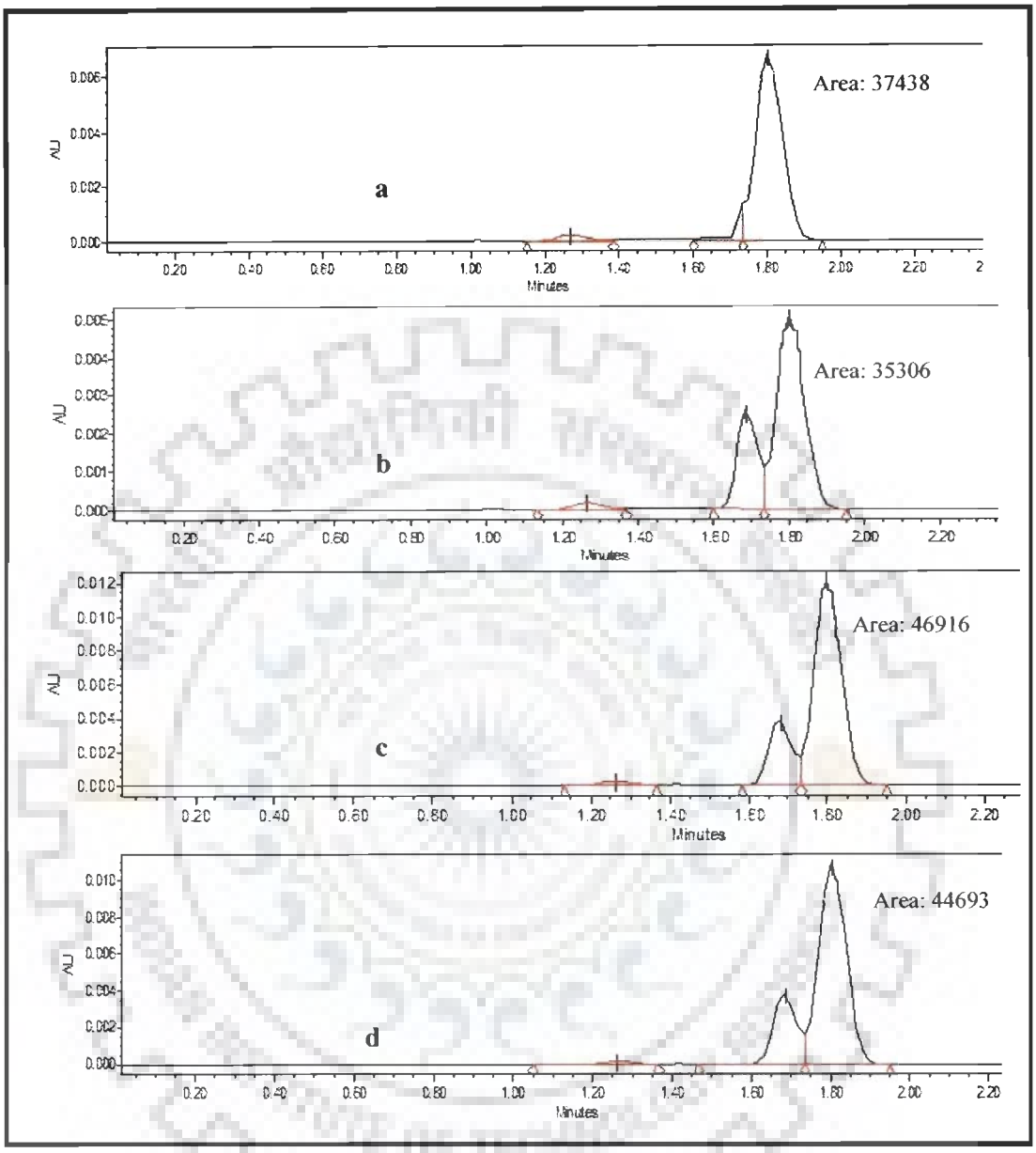


Fig. 4.21 HPLC graphs for phytic acid estimation in *T. monococcum* wild type and low phytic acid; a- Phytic acid standard (75µg/ml), b- *T. monococcum* wild type c-*lpa* mutant MM225 and d-*lpa* mutant MM169.

which was lower by 56.6 % than the wild type *T. monococcum.* Mutant MM169 was close behind with a phytic acid content of 21.3 mg/g which was reduced by 46.2 % than the wild type.

4.3.4 Mineral concentrations of *lpa* mutants

Seed iron, zinc and phosphorus concentrations measured using ICPMS are given in Table 4.21. The mineral content of seeds of mutants was higher than that of the *T. monococcum* wild type. MM225 had 40.4 mg/kg iron and 48.4 mg/kg zinc which were 49 % and 53 % higher than the wild type. MM169 had 41.2 mg/kg iron

Table 4.21 Phytic acid, iron, zinc and phsphorus concentrations of *T. monococcum* wild type and *lpa* mutants

Sample	Phytic Acid		Fe		Zn		P	
	Content (mg/g)	% Change over w.t.	Content (mg/kg)	% Change over w.t.	Content (mg/kg)	% Change over w.t.	Content (mg/kg)	% Change over w.t.
T. monococcum w.t.	39.5	-	27.1	-	31.7	-	3004.3	-
MM225	17.1	-56.6	40.4	48.8	48.4	52.7	7568.0	151.9
MM169	21.3	-46.2	41.2	51.9	32.5	2.5	5527.8	84

was 52 % higher than wild type, but it did not show significant increase in seed zinc content. Total phosphorus contents of seeds of the mutants MM225 (7568 mg/kg) and MM169 (5527 mg/kg) were also higher than the wild type *T. monococcum* seeds (3004 mg/kg).

4.3.5 Micronutrient availability of *lpa* mutants

To study the actual effect of decrease in phytic acid content of the mutants over *T. monococcum* wild type, availability of iron was measured. Availability

percentage of iron in *T. mocococcum* wild type was 2.3 %, whereas the mutants MM225 and MM169 had 3.7 % and 2.8 % bioavailable iron respectively. Thus mutant MM225 had 57.2 % higher availability and MM169 had 19.0 % higher availability than the wild type. Correlation factor was calculated between phytic acid content and percent available iron in wild type *T. monococcum* and mutants. The value of correlation coefficient was r = – 0.86. This highlights the fact that phytic acid is the chief antinutritional factor affecting bioavailability of iron in the seeds.

4.3.6 SEM-EDX analysis of the *lpa* mutants

Scanning Electron Microscopy- Energy Dispersive X-ray (SEM-EDX) analysis was done to study the distribution pattern of minerals in the seeds of *T. monococcum* wild type and *lpa* mutants. Comparative SEM-EDX maps of wild type *T. monococcum* and mutant MM225 for phosphorus (P), potassium (K), iron (Fe) and zinc (Zn) is given in Fig. 4.23 In *T. monococcum* wild type P and K were conspicuously concentrated in the aleurone layer whereas in mutant MM225, P and K were distributed more densely in the endosperm than being strictly restricted to the aleurone layer. Potassium distribution also followed similar distribution pattern. Fe and Zn were also more in MM225 as compared to the wild type. Thus mapping clearly shows the low phytic acid content of the characterized *lpa* mutants.

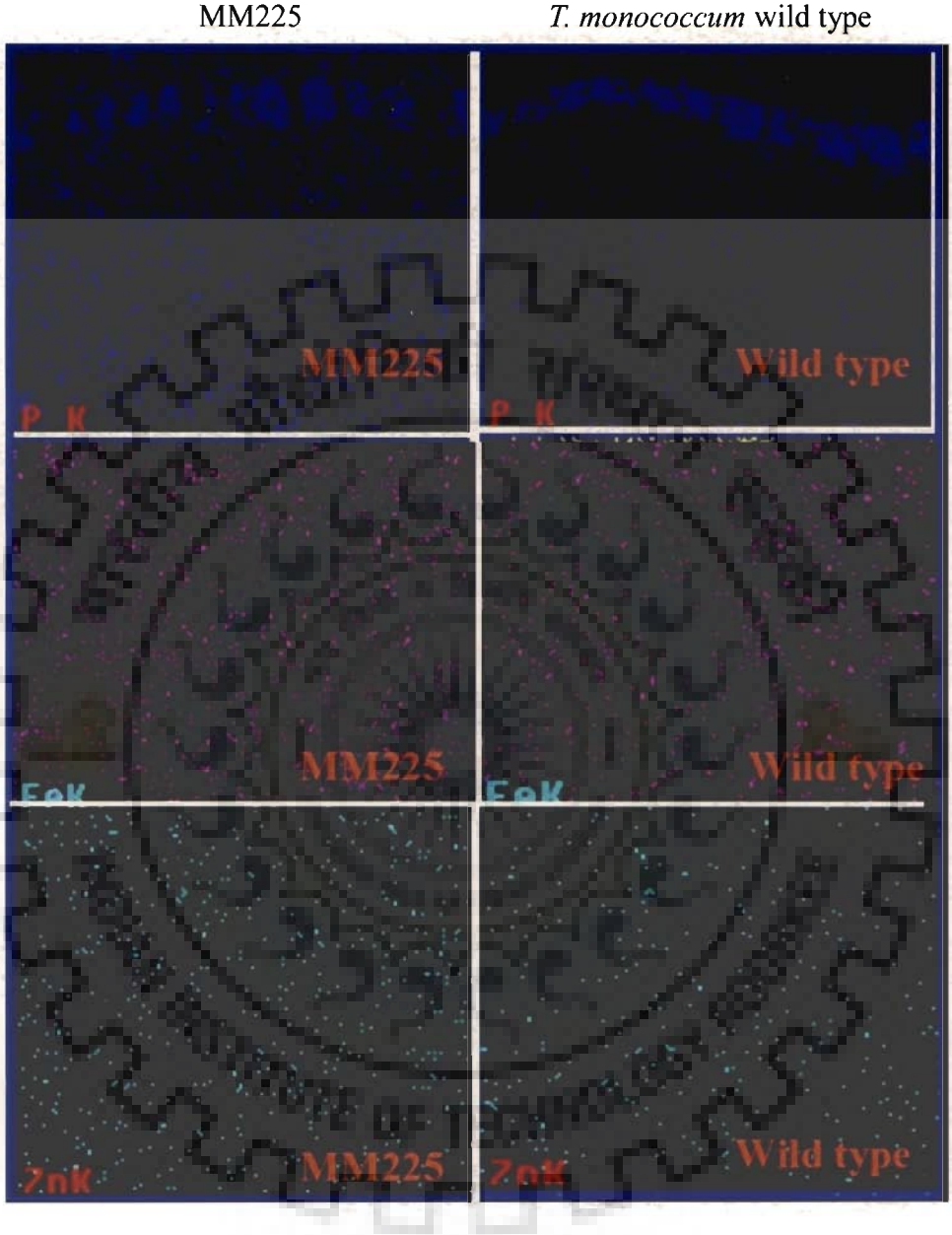


Fig. 4.23 SEM-EDX mapping of T.S. of seed of *T. monococcum lpa* mutant MM225 as compared to the wild type seed

4.4 Distribution and bioavailability of micronutrients in germinating wheat grains

4.4.1 SEM-EDX localization of Phosphorus

The phytic acid content of 10 bread and 5 durum wheat cultivar grains was found to vary from 0.989 to 1.122 %. SEM-EDX of the grains showed upto 94 % of the total grain phosphorus to be deposited in the aleurone layer (Fig. 4.24).

SEM-EDX for minerals potassium, calcium, magnesium, iron and zinc showed similar distribution pattern within the aleurone and the endosperm regions as that of phytic acid (Fig. 4.24 c, d). The concentrations of elements are expressed as weight% by the instrument. Potassium was 2.21% of weight in aleurone while only 0.20% in endosperm. Calcium was 0.09% in endosperm against 1.13% in the aleurone. Similarly magnesium constituted 3.31% by weight of aleurone and only 0.04% of that of endosperm. Iron distribution also followed the same trend, although the gradation from aleurone towards the centre of endosperm was not that abrupt, being 1.15% in the aleurone, 0.75% in the endosperm region just after the aleurone and 0.5% in the centre of the endosperm. Zinc was detected in traces (0.27% in aleurone and 0.12% in endosperm). Phytic acid chelates these metal cations and as such confines them to the aleurone layer. Fig. 4.24 shows coloured mapping images of the minerals studied. C and O can be seen as the principal constituents of the grain; P, K, and Mg are confined to the aleurone layer of the seed, whereas Ca, Fe and Zn are present in traces in the grain.

Phytic acid content after different intervals of germination of wheat grains and ungerminated control is given in Table 4.22. There was consistent reduction in phytic acid content up to fifth day of germination, from 1.056 % in control to 0.264 % after 120 hours. The phytic acid content again started rising on the sixth day to 0.48 %. Percentage reduction from 18.1% in 24 hours to 80.6% after 120 hours of germination was observed. SEM-EDX values of phophorus content in the aleurone also decreased from 5.93% in ungerminated seeds to 1.98% of grain weight on the fifth day of germination (Fig. 4.25), indicating clearly the degradation of phytic acid by endogenous phytases during germination for the mobilization of phosphorus.

Table 4.22 Phytic acid content of grains of bread wheat variety WL711 after different hours of germination

S.No.	Hours of germination	Phytic Acid (g/100g) ± S.D.	Reduction in Phytic Acid %
1	Control	1.06 ± 0.06	-
2	24	0.92 ± 0.11	18.1
3	48	0.79 ± 0.10	30.6
4	72	0.53 ± 0.09	55.6
5	96	0.40 ± 0.05	68.1
6	120	0.26 ± 0.05	80.6
7	144	0.48 ± 0.02	54.5

Consistent reduction in minerals potassium, calcium, magnesium, iron and zinc content in aleurone layer of grains on various days of germination was observed using SEM-EDX (Fig. 4.25). Potassium content showed a reduction of 81.9% from 2.21%

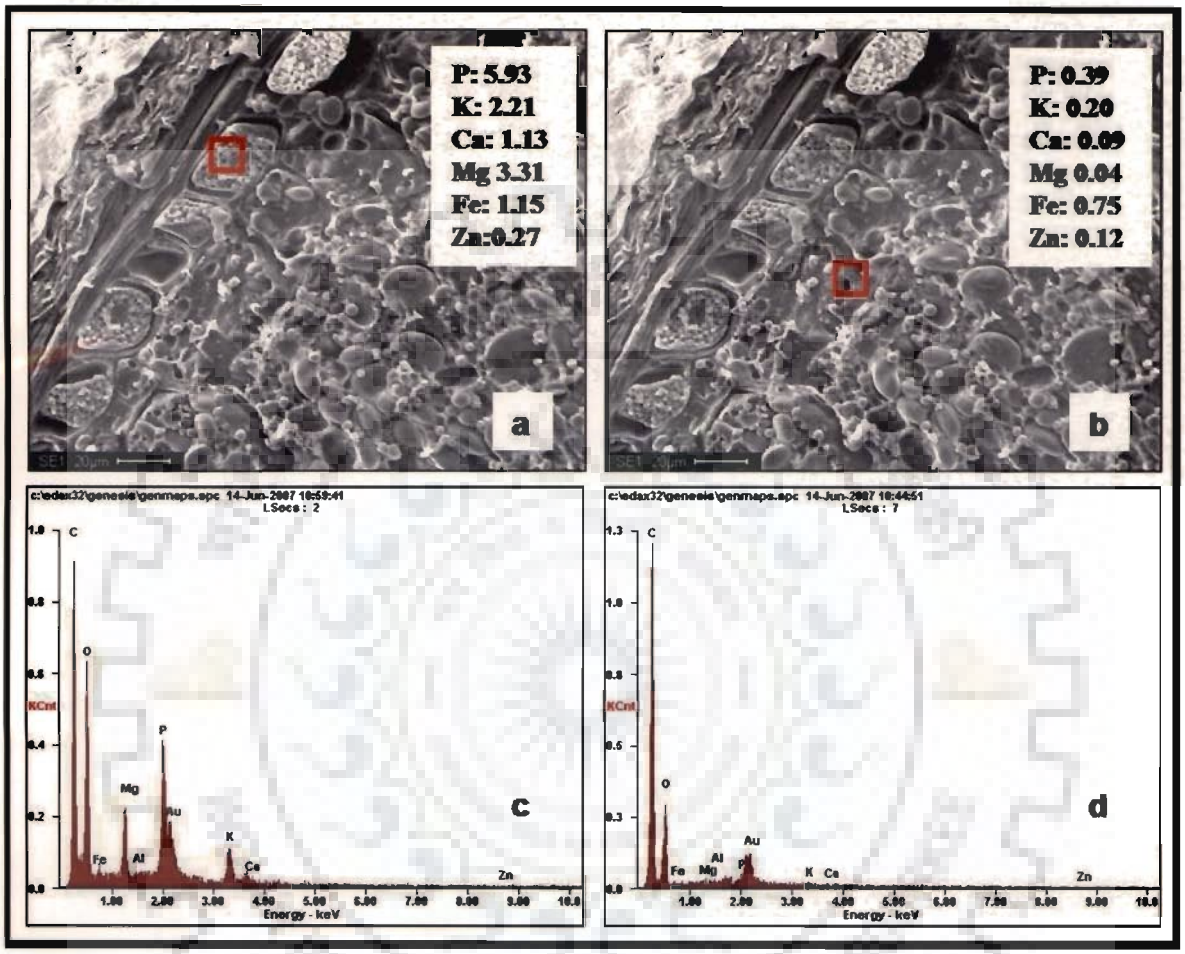


Fig. 4.24 Distribution of minerals in selected areas (□) in Scanning Electron Micrographs (a) aleurone and (b) endosperm and EDX profiles of (c) aleurone and (d) endosperm of dry wheat grains

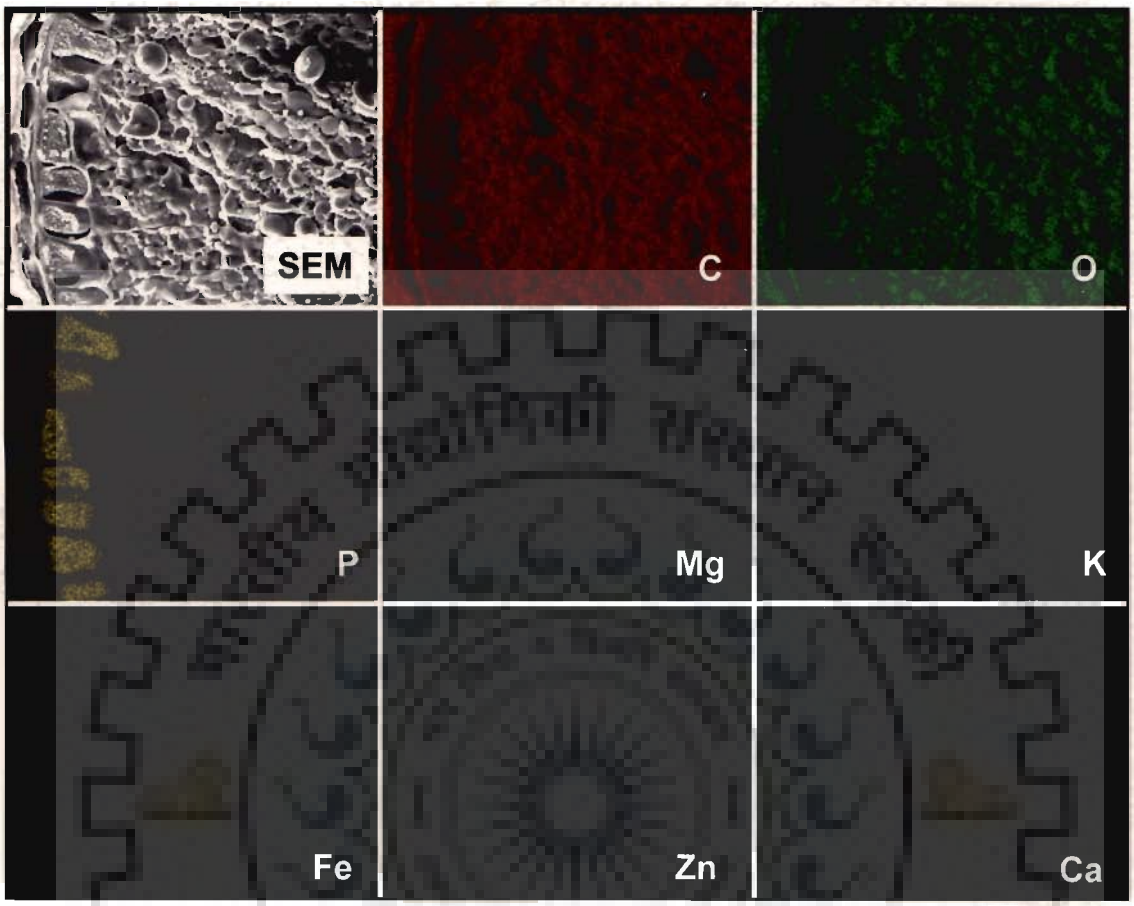


Fig. 4.25 Colour display images of minerals along T.S. of a wheat grain showing C and O as the chief constituents; P, Mg and K being preferentially confined to aleurone cells and Fe, Zn and Ca present in traces in the grain.

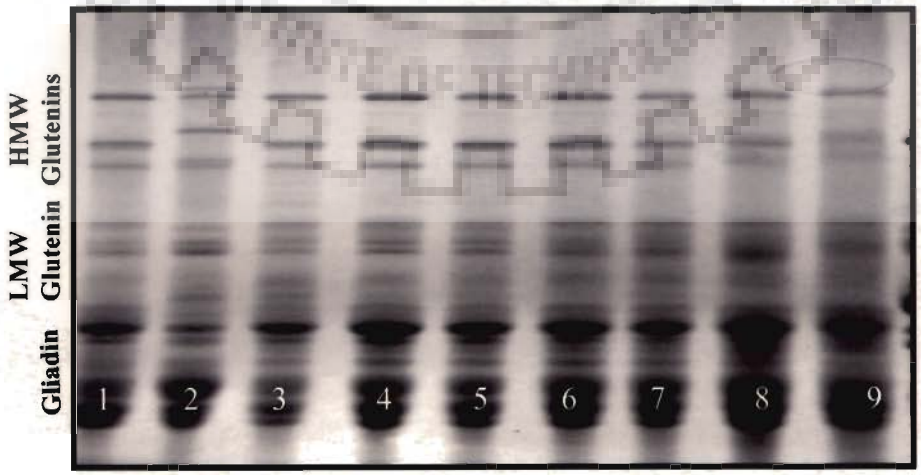


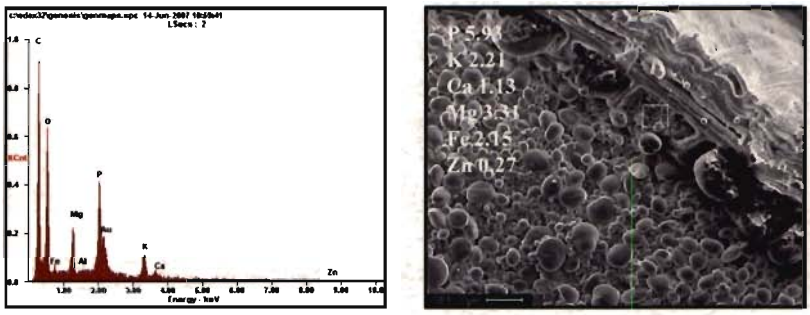
Fig. 4.26 SDS PAGE of grain proteins in germinating wheat grains. Lanes 1-3 bread wheat cultivars Sonalika, Chinese Spring, WL711, respectively as ungerminated controls. Lanes 4-9 samples after germination for 24, 48, 72, 96, 120 and 144 hours.

(w/w) in control to 0.40% on fifth day of germination. Calcium content was reduced to one-sixth of the control upon 120 hours (0.18%) from initial content of 1.13%. Similarly magnesium, which was 3.31% in ungerminated grains, was reduced by 86.71 % to to 0.44% on fifth day of germination. Iron content was reduced by one and a half time on fifth day, from 2.15% in control to 1.47%. A reduction of 74.07% in zinc content from 0.27% in control to 0.07% on fifth day was found. This highly correlated decline of minerals and phytic acid in the aleurone due to the action of endogenous phytases in the grains further confirms the chelation of the minerals by phytic acid.

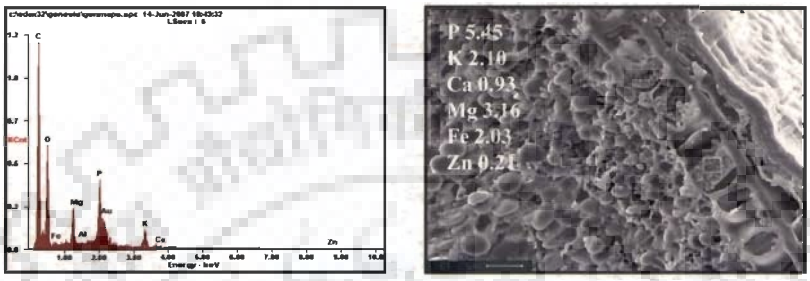
The SDS-PAGE of storage proteins indicated that the composition of high molecular weight glutenin was fairly maintained intact by 96 hours of germination with progressive degradation subsequently while the low molecular weight glutenins and gliadins were rapidly degraded (Fig. 4.26).

Scanning Electron Micrographs showed progressive loosening and dissolution of starch granules as germination progressed (Fig. 4.27). The changes became remarkably visible after 72 hours of germination, where matrix started showing interstitial spaces due to dissolution of starch granules. After 120 hours the granules were completely reduced to an amorphous mass.

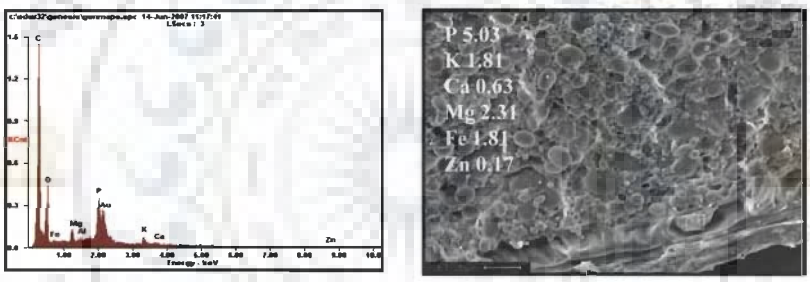
Control



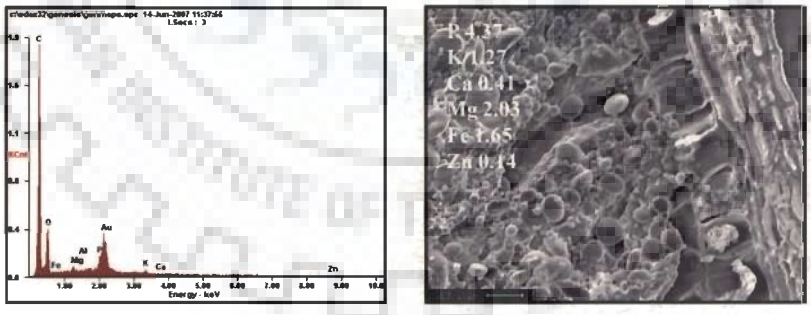
24 hours



48 hours



72 hours



120 hours

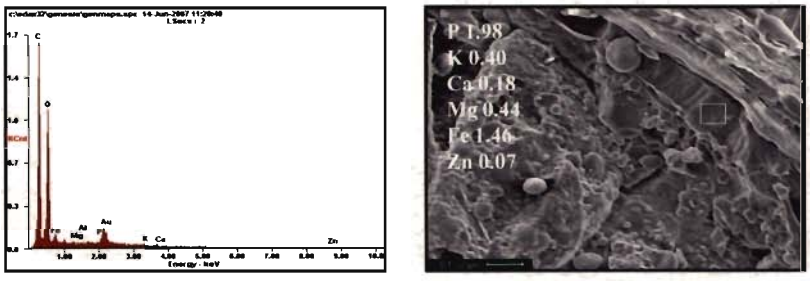


Fig. 4.27 SEM-EDX graphs and Scanning Electron Micrographs showing progressive mobilization of minerals from aleurone layer and structural changes in germinating wheat grains.

Chapter V

Discussion

5. DISCUSSION

5.1 Development and molecular analysis of wheat-*Aegilops kotschyi* derivatives with high grain iron and zinc content

The low mean and range of grain iron and zinc concentrations in elite wheat and durum cultivars in this study strongly emphasize the need of screening and identification of useful variability among related wild species of wheat for an effective biofortification programme (Monasterio and Graham, 2000). Cakmak *et al.* (2000) also reported lower iron and zinc content among *T. durum* and *T. aestivum* cultivars as compared to wild and primitive *Triticum* species. CIMMYT and HarvestPlus have already used *T. tauschii* and *T. dicoccoides* synthetics in wheat breeding programme for biofortification of wheat for iron and zinc content (Calderini and Monasterio, 2003a, b).

Ae. longissima (S), *Ae. kotschyi* (US), and *Ae. peregrina* (US) all having S genome gave consistently higher levels and range of both iron and zinc concentrations. Exhaustive screening of a number of accessions of diploid and tetraploid wild *Triticum* and *Aegilops* species has shown that the non-progenitor S and U genomes may be the useful sources for transferring genes controlling high iron and zinc content to wheat. The S genome being closely related to the B genome of polyploid wheat (Faris *et al.*, 2002; Dvorak and Zhang, 1990; Daud and Gustaffson, 1996) can be effectively used for transferring useful variability for high iron and zinc content into wheat. *T. boeoticum* and *T. monococcum* with A^m genome most closely related to that of A genome of polyploid wheat, *T. tauschii* with D genome and *T. dicoccoides* having AB genome have already been reported to have high grain iron and zinc content (Monasterio and Graham, 2000 and Cakmak *et al.*, 2000). The useful variability from S genome can be transferred to wheat through induced homoeologous

chromosome pairing using *Ph*^{I} (Chen *et al.*, 1994; Aghaee-Sarbarzeh et *al.*, 2002). In addition to the S genome, some other non-progenitor genomes (U, M) also controlling high iron and zinc concentrations can be exploited for biofortification using *Ph*^{I} mediated induced homeologus chromosome pairing.

Among the screened wild relatives in general and selected donors in particular, accessions with high grain iron content were also found to have high grain zinc content, which strongly suggests similar mechanism of uptake, translocation and deposition of the two micronutrients Welch and Graham (2004) also reported high correlation between grain iron and grain zinc concentrations in wheat cultivars and related species. Phytosiderophores like mugineic acids are known to facilitate uptake of iron, zinc and other micronutrients (Takagi *et al.*, 1998; Marschner and Romheld, 1994). Some of the accessions of *Aegilops* species with high grain and leaf iron and zinc also possess high level of phytosiderophores (our unpublished results).

Analysis of variance for iron and zinc content in wild accessions and wheat cultivars revealed highly significant variation among them and high heritability indicating suitability of the set of donor parents for biofortification of wheat cultivars.

Leaves of wild species have higher micronutrient content than cultivars. *T. aestivum* c.v. WL711 had 52.16 mg/kg iron and 31.72 mg/kg zinc. Garnett and Graham (2005) also reported high iron content of leaves of wheat cultivar 'Warigal' at anthesis to be about 55 mg/kg. Significantly high positive correlation between grain and flag leaf content of both iron and zinc in the selected donors strongly suggests the presence of a genetic system(s) in the donors for more efficient uptake and transport of the micronutrients as compared to that of wheat and durum cultivars. This high positive correlation between flag leaf and grain iron and zinc suggests the possibility of using flag or other leaves for the early selection of plants with potentially high iron

and zinc in the segregating generations of inter-specific crosses rather than waiting till harvest for grain analysis. This could facilitate selection of high yielding and disease resistant plants among those with high iron and zinc concentrations in leaves similar to that of marker assisted selection. Garnett and Graham (2005) reported nearly 77%, 62% and 42 % remobilization of wheat shoot iron, copper and zinc, respectively in wheat into grains during anthesis to maturity under controlled experimental conditions. This supports our observations of higher content of leaf and grain iron than their zinc content. It appears that remobilization of zinc follows the same pattern as that of iron but in lower proportion than the latter (Garnett and Graham, 2005). The ancestral wild allele of transcription factor NAM-B1 responsible for accelerated senescence during grain filling period of wheat (Uauy *et al.*, 2006) can be used for high level of translocation of iron and zinc from the biofortified leaves to grains.

Higher iron and zinc concentrations in flag leaves of most of the interspecific hybrids than their parents provides an excellent 'proof of the concept' that the screened and selected *Aegilops* accessions have the requisite superior genetic system(s) for higher uptake and transport of micronutrients which expresses in the wheat background. Higher leaf iron content in most of the BC_1 plants with approximately 75% of the wheat complement further confirms the effectiveness of their superior genetic system.

Chemical analysis of the selfed seeds of fertile derivatives in advanced backcross generations was done for further confirmation of the proof of concept. The iron and zinc content of grains of fertile BC_2 and BC_1F_2 plants showed variation ranging from that of the wheat parent to the wild donors. The variation could be attributed to the presence of one or more chromosomes of the wild donors controlling the efficient uptake and translocation of the micronutrients. As the grain size of the

fertile derivatives was almost similar or even greater than that of the wheat parent, the higher iron and zinc concentrations found in their seeds is not due to biomass dilution, unlike synthetic hexaploids where Calderini and Monasterio (2000) found lower grain yield to be a major contributing factor to their higher micronutrient contents. The recovery of fertile derivatives with seeds as bold as that of the wheat cultivars and micronutrient content as high as that of the wild donors gives unequivocal proof of the concept that *Aegilops kotschyi* possess efficient genetic system for uptake and translocation of the micronutrients which could be effectively used for biofortification of wheat cultivars.

Waxiness of leaf sheaths is controlled by genes on homoeologous group 2 chromosomes (Levy and Feldman, 1989; McIntosh, 1983). Many derivatives had non-waxy leaf sheaths indicating presence of alien group 2 chromosomes. HMW glutenin subunits have been used to monitor group 1 chromosomes in alien introgression in wheat (Dou *et al.*, 2006; Koebner and Shepherd, 1985). Some plants with very high grain iron and zinc content had additional HMW glutenin subunits of *Ae. kotschyi* 396 showing that group 1 is associated with very high grain iron and zinc content in them.

Anchored wheat SSR markers showed 63.6 % transferability and high polymorphism to *Aegilops kotschyi* indicating their utility to study introgression of *Ae. kotschyi* in addition, substitution and translocation lines (Dou *et al.*, 2006). B genome markers had the highest transferability to *Ae. kotschyi* among markers of all the three genomes. This indicates the closeness of the B and S genomes and some 'S' genome species to be the donor of B genome of wheat. Adonina *et al.* (2005) also reported highest transferability of B genome wheat microsatellite markers to species of Sitopsis section.

On the basis of HMW glutenin subunit profiles and GISH of high grain iron and zinc derivatives, chromosome 1U/1S was found to be present in the plants with very high micronutrient content. However SSR markers of group 1A, 1B and 1D could not detect alien group 1 present in some plants. This may be due to the introgression of 1U chromosome, which is highly reorganised and has little synteny with homoeologous group 1 chromosomes (Badaeva *et al.*, 2004). The presence of group 2 and 7 alien chromosomes in the high grain iron and zinc derivatives was confirmed using SSR molecular markers. QTLs for grain iron and zinc content have been mapped previously to homoeologous chromosomes 2 and 7 (Tiwari *et al.*, in press). Shi *et al.* (2008) also detected QTLs for grain Zn content on chromosome 7A. Stangoulis *et al.* (2007) using a doubled haploid (DH) population in rice mapped three QTLs for grain Fe accumulation on rice chromosomes 2S, 8L and 12L and two QTL for grain Zn on chromosomes 1L and 12L. The region where grain Zn QTL was mapped on rice chromosome 1 is orthologous to wheat chromosome 7. Chromosomes 1, 2 and 7 of *Ae. kotschyi* may be thus concluded to have genes for high grain and zinc content in selected derivatives.

5.2 Development of wheat- *Ae. kotschyi* synthetic amphiploids with high grain iron and zinc content

The wheat/*Ae. kotschyi* F_1 hybrids as well as the amphiploids were morphologically intermediate between the wheat and *Ae. kotschyi* parents for plant height, growth habit, tiller numbers per plant etc. However other characters like ear shape, glume awns, hard threshing and brittle rachis were more like their *Ae. kotschyi* parents. The intermediate morphology of the F_1 hybrids and their synthetic amphiploids have been reported in several studies (Sears, 1954, Martin and Laguna,

1982; Sharma *et al.*, 1987; Oliver *et al.*, 2005). The genes controlling brittle rachis (*Br*), tenacious glumes (*Tg*) of *Ae. kotschyi* appear to be epistatic over the *Q* locus in controlling square head, tough rachis and free threshing in *T. aestivum* (Li and Gill, 2006 and Endo and Gill, 1996) as the amphiploids resembled their *Ae. kotschyi* parents.

All the F_1 hybrids ($ABUS^l$) had the expected thirty five chromosomes indicating complete parental chromosome complement and chromosome stability. Low to high intergenomic homoeologous chromosome pairing was observed in different F_1 hybrids. High choromosome pairing observed in F_1 CS (*Ph*^{l})/ *Ae. kotschyi* 396 is probably due to the presence of *Ph1* inhibitor gene *Ph*^{l} transferred from *Ae. speltoides* which is known to induce considerable amount of wheat – alien pairing even in a single dose (Chen *et al.*, 1994). The CS (*Ph*^{l}) stock with us seems to be heterogeneous as some other F_1 hybrids with CS (*Ph*^{l}) had limited pairing. Intermediate homoelogous pairing in hybrids with cultivar WL 711 may also be explained due to some pairing promoters in *Aegilops* species which are known to suppress or enhance pairing in Triticeae (Riley and Chapman, 1958; Sears, 1976; Jauhar, 2007). Mello-Sampayo (1973) also observed the interaction of pairing promoters which inactivate *Ph1* or *Ph1*-like genes in wheat/ *Ae. speltoides* and wheat/ *Ae. longissima* hybrids.

The F_1 hybrids had too low pollen stainability to permit anther dehiscence and hence had no selfed seed set. The low to medium chromosome pairing permitted some of the paired chromosomes to undergo reduction division and move to anaphase poles before the large number of unpaired univalents aligned on the metaphase-I plate and divide. Only those paired chromosomes with intact sister chromatids would divide equationally in the second meiotic metaphase while the univalent chromatids already

separated in metaphase-I are expected to move randomly resulting in tetrads with unbalanced chromosome number and micronuclei. However, no fertile first division restitution nucleus was observed as reported for *T. durum/ Ae. tauschii* crosses (Jauhar, 2007 and Matsouka and Nasuda, 2004;). Medium to highly fertile synthetic amphiploids ($AABBDDUUS^{l}S^{l}$) with nearly expected chromosome number (2n=10x=70) were obtained indicating the effectiveness of colchicine treatment for doubling the chromosome number of the F_1 hybrids. Variable chromosome number, pollen fertility, seed set and HMW glutenin subunit profiles of individual seeds of amphiploids indicated chromosomal instability among them.

The varied chromosome numbers in different plants, different tillers from same plant, and different florets of same spike and even in different PMCs from same anther suggested that the chromosome elimination occurs only in the somatic tissues of the amphiploids. Once initiated in the somatic tissues it would continue through germinal tissues and meiosis due to variable chromosome number, pairing and univalent frequencies. Competition among pollen grains and gametes with different chromosome number would lead to the elimination of the gametes with aneuploidy and chromosomal rearrangements and hence the amphiploids are expected to stabilize as complete or partial segmental amphiploids as observed in wheat-*Thinopyrum* amphiploids (Cauderon *et al.,* 1973; Sun, 1981; Yang *et al.,* 2006). Somatic chromosome elimination leading to variable chromosome number in newly synthesized amphiploids has been extensively reported in the interspecific F_1 hybrids up to several generations of synthetic amphiploids in tetraploid to octaploid amphiploids (Schulz-Schaeffer and McNeal, 1977; Ozkan, 2001). Ozkan *et al.* (2001) named this phenomenon as rapid genome evolution and divided it into two levels viz., the genomic level taking place in the F_1, C_1, C_2 and C_3 generations at which genome-

specific sequences (GSS) are eliminated and the other at chromosomal level involving elimination of chromosome specific sequences (CSS). Feldman *et al.* (1997) found that some low copy CSS and GSS in a newly synthesized wheat although present in all the three diploid parents of the amphiploids were specifically eliminated at the ploidy level without any change in chromosome number as observed in the wheat-*Ae. kotschyi* amphiploids. Liu (1998) called this as "programmed" pattern of evolution. All the amphiploids developed in the present study showed nearly additive parental electrophoretic pattern of HMW glutenin subunits. Preferential elimination of either whole or a part of 1D chromosome was observed in one of the amphiploids as indicated by missing 1D specific HMW glutenin subunits. Preferential loss of whole or a part of 1D chromosome that controls HMW glutenin loci was also observed in *Agropyron intermedium* chromosome addition lines in 'Vilmorin 27' background and $1S^{v}$ addition lines of *Ae. peregrina* with chromosome by Garg *et al.* (2007). FISH analysis of these addition lines actually revealed shorter 1D chromosome or loss of a pair of 1D chromosome. The D genome of *T. aestivum* and polyploid *Aegilops* species, *Ae. cylindrica* and *Ae. ventricosa* has been found to show complete homology with the D genome of *Ae. tauschii* showing no or little change or elimination during evolution (Huang *et al.*, 2002; Boyko *et al.*, 1999; McGuire and Dvorak, 1982). The reason of preferential elimination of 1D in certain aneuploids and amphiploids is however not known.

It would be worthwhile to monitor the behavior of certain chromosomes or genomes in the subsequent generations of wheat-*Ae. kotschyi* amphiploids. This suggests that the higher micronutrient content of Ae. kotschyi is partially due to concentration effect because of their smaller seeds. Higher ash and ash micronutrient content in amphiploids with seeds bolder than or as bold as wheat cultivars further

suggests that *Ae. kotschyi* have genetically superior micronutrient uptake translocation or seed sequestration system which can be commercially exploited in elite wheat cultivars Most of the amphiploids having bolder grains than the wheat parents, and nearly as high grain iron and zinc content as that of the *Ae. kotschyi* parent strongly suggested that the higher micronutrient content of *Ae. kotschyi* as reported earlier (Chhuneja *et al.,* 2006) is due to its superior genetic system(s) for uptake, translocation and/or sequestration in grain. *Ae. kotschyi* is thus a potential source of useful variability for wheat biofortification for high grain iron and zinc in addition to other progenitor species reported earlier (Chhuneja, 2006; White and Broadley 2005; Calderini and Monasterio, 2003 a,b). The work to transfer and dissect useful variability of *Ae. kotschyi* through recurrent backcrossing and development of alien addition and substitution lines in wheat background is in progress.

5.3 Low phytic acid mutants

The results of the above investigation clearly identify *T. monococcum* (einkorn wheat) mutants MM225 and MM169 as low phytic acid mutants (*lpa*). Low phytic acid mutants have been characterized in several other crops like maize, barley, bread wheat, soybean and rice. A comparison of reduction in phytic acid content in the *T. monococcum lpa* mutants hereby identified with the available *lpa* mutants of other plants has been given in Table 5.1 The reduction in phytic acid content in the diploid *T. monococcum lpa mutants* (57 % in MM225 and 46 % in MM169) is higher than the hexaploid wheat *lpa* mutants (37%) (Guttieri *et al.,* 2004) and comparable to the reduction in other plants. This is because the diploid wheat *T. monococcum* mutants fully exhibit the impact of mutation while hexaploid wheat has buffering for mutation effect (Guttieri, 2004).

Table 5.1 Reduction in phytic acid content of *lpa* mutants of *T. monococcum* mutants MM225 and MM169 as compared to *lpa* mutants in other crops

Plant	Mutant name	Percentage decrease in P.A.	Reference
T. monococcum	MM225	57 %	-
"	MM169	46 %	-
Wheat	*lpa-1*	37%	Guttieri *et al.*, 2004
Barley	*lpa-1-1, lpa2-1*	50%, 40%	Rasmussen and Hatzack, 1998
Maize	*lpa-1-1, lpa2-1*	55%, 66 %	Raboy *et al.*, 2000
Rice	*Os-lpa*-XS110-1, *Os-lpa*-XS110-2	46 %, 23 %	Larson *et al.*, 2000
Soyabean	HIP	50%	Wilcox *et al.*, 2000

Interestingly total phosphorus, iron and zinc showed an increase over the wild type in the identified *lpa* mutants. Very high correlation coefficient (r = -0.86) between percent decrease in phytic acid content and percent increase in availability of iron in the *lpa* mutants over the wild type *T. monococcum* shows the importance of low phytic acid seeds in mineral nutrition and as solution to alleviate bioavailability bottlenecks.

SEM-EDX mapping showed visual manifestation of the ICPMS data. P and K were distributed more freely in the endosperm as well as in the aleurone in the mutant MM225, as against the wild type which had P and K dense aleurone. The Fe and Zn SEM-EDX maps were denser for the mutant MM225 than the wild type *T. monococcum*. Thus the maps support the ICPMS results of higher iron and zinc content in the mutants.

Thus *T. monococcum* mutant MM225 with low phytic acid had high bioavailability of minerals. Whether higher micronutrient conten in the *lpa* muant MM225 is pleiotropic to the phytic acid content or another independent mutation needs to be investigated.

5.4 Partial grain germination lowers phytic acid

Phytic acid is the chief storage form of phosphorus. In general there is about 1-2% of phytic acid in cereal seeds on weight basis (Qazi, 2003). Wang *et al.* (1992) found the phytate content of cereals and oil seeds to vary in rice (0.89%), soybeans (1.4%), peanut meal (1.7%) and sesame (5%). In the SEM-EDX profiles the aleurone layer is visible as the major accumulation site of phosphorus. Lott *et al.* (2004) studied phytic acid phosphorus in low phytic acid and normal rice grain using EDX and found the aleurone to be the site of occurrence of phytic acid-phosphorus. About 90% of phytic acid in rice, barley and wheat has been reported to be present in the aleurone layer (Shi *et al.*, 2003). Heard *et al.* (2001) actually showed the phytate granules to be embedded in protein rich globoid structures in the aleurone cells of wheat grains using Secondary Ion Mass Spectrometer. Joyce *et al.* (2005) reported similar results of STEM-EDX analyses of wild type and low phytic acid wheat. SEM-EDX for minerals K, Ca Mg, Fe and Zn showed localization pattern similar to P. Heard *et al.*, (2001) also reported presence of Na^+, K^+, Mg^+ and Ca^+ in the phytate granules of aleurone cells. Ren *et al.*, (2006) found similar results in rice where, in addition to phytic acid, mineral elements Fe, Zn, Ca and Mg were highly concentrated in bran (including pericarp, seed coat, embryo and aleurone), in contrast to the starchy endosperm. Formation and deposition of phytate complexes of these elements as globoids in bran has been attributed for this co-enrichment (Ren *et al.*, 2006).

Reduction in phytic acid content of wheat grains upon germination has been known since long (Mihailovic *et al.*, 1965). Oloyo (2004) also reported a reduction in phytate content in seeds of *Cajanus cajan* upon germination reaching the minimum level on the fifth day. This decline in phytic acid is due to the enzymatic breakdown of phytin by endogenous phytases produced in the seeds during germination

(Sangronis and Machado, 2007). The phytic acid content was found to increase slightly from sixth day onwards. Walker (1974) while working on *Phaseolus vulgaris* found similar results and attributed this increase in phytic acid after 6 days of germination due to decrease in phytase activity and synthesis of phytic acid thereafter.

SEM-EDX images show minerals iron, zinc, potassium, magnesium, calcium and phosphorus to reduce with germination in the aleurone layer. Liu and Pomeranz (1976) using X ray microanalysis also found a large decrease in the concentration of most minerals in the aleurone layer during malting in barley. Breakdown of phytates not only makes inorganic phosphorus available to the growing embryo, but also meets its mineral requirements by the release of cations complexed in them (Sung *et al.*, 2005). Sangronis and Machado (2007) similarly found reduction in iron and magnesium in cotyledons of *Cajanus cajan* and *Phaseolus* upon germination and attributed this decrease to the transfer of these minerals from their storehouses to the sink i.e. from cotyledons to the developing embryo.

The SDS-PAGE of storage proteins indicated that the composition of high molecular weight glutenin was fairly maintained intact by 96 hours of germination with progressive degradation subsequently while the low molecular weight glutenins and gliadins were rapidly degraded. Torrent *et al.* (1989) while working with *Zea mays,* also reported enzymatic breakdown of storage proteins by endopeptidases and exopeptidases responsible for this trend.

Scanning Electron Micrographs showed progressive loosening and dissolution of starch granules as germination progressed. The changes became remarkably visible after 72 hours of germination, where matrix started showing interstitial spaces due to dissolution of starch granules. After 120 hours the granules were completely reduced to

an amorphous mass. De Ruiz and Bressani (1990) also found that damaged starch content of germinating amaranth grains increased upon germination and on 72 hours of germination, this damaged starch content became double of that in the ungerminated samples. Thus these micrographs present visual manifestation of amylolytic activities in grains during germination.

The precise transfer of *Ae. kotschyi* genes for high grain Fe and Zn content and their marker assisted pyramiding in elite wheat cultivars can nearly double the micronutrient content over the existing levels. The combination of biofortified wheat with low phytic acid mutants and improvised processing will be absolutely essential to enhance bioavailability of micronutrients to alleviate the hidden hunger.

Chapter VI

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6. REFERENCES

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Annexure

Annexure-I

List of wheat SSR markers used

	Primer	Forward Sequence[5'-3']	Reverse Sequence[5'-3']	Tm
Chr1A				
1	gdm33	GGCTCAATTCAACCGTTCTT	TACGTTCTGGTGGCTGCTC	56
2	barc119	CACCCGATGATGAAAAT	GATGGCACAAGAAATGAT	48
3	barc263	GGAAGCGCGTCAGCACTAGGCAAC	GGCTTCTAGGTGCTGCGGCTTTTGTC	70
4	gwm136	GACAGCACCTTGCCCTTTG	CATCGGCAACATGCTCATC	59
5	gwm11	GGATAGTCAGACAATTCTTGTG	GTGAATTGTGTCTTGTATGCTTCC	58
6	cfd16	GGATCCAAGGGAATCCAAAT	TCCTTCGGTTCCCATATCAC	56
7	wmc336	GTCTTACCCCGCGATCTGC	GCGGCCTGAGCTTCTTGAG	62
8	barc148	GCGCAACCACAATGTATGCT	GGGGTGTTTTCCTATTTCTT	54
9	barc162	GCGTTTAAAGACAAGGTGGTAGGTATT	GCGTGTCCCATCATGCATAGA	61
10	barc120	CCCCCTCTCTTCCTCAT	ATATAGCTCCCCCATTTCCT	55
11	barc213	GCGTAGATTCTCGGTTTGTTGGCTTGC	CCGTCCCTCCTTCCTGGTCT	64
12	barc83	AAGCAAGGAACGAGCAAGAGCAGTAG	TGGATTTACGACGACGATGAAGATGA	73
13	gwm164	ACATTTCTCCCCCATCGTC	TTGTAAACAAATCGCATGCG	54
14	gwm135	TGTCAACATCGTTTTGAAAAGG	ACACTGTCAACCTGGCAATG	57
15	barc240	AGAGGACGCTGAGAACTTTAGAGAA	GCGATCTTTGTAATGCATGGTGAAC	64
16	gwm357	TATGGTCAAAGTTGGACCTCG	AGGCTGCAGCTCTTCTTCAG	59
17	wmc716	CATTTATGTGCACGCCGAAG	CCATAAGCATCGTCACCCTG	58
18	wmc312	TGTGCCCGCTGGTGCGAAG	CCGACGCAGGTGAGCGAAG	64
19	barc158	TGTGTGGGAAGAAACTGAGTCATC	AGGAATACCAAAAGAAGCAAACCAAC	63
20	gwm99	AAGATGGACGTATGCATCACA	GCCATATTTGATGACGCATA	54
21	barc17	GCGCAACATATTCAGCTCAACA	TCCACATCTCGTCCCTCATAGTTTG	60
22	barc287	CGGATGGGTTACTTACTTAGGATG	CGCAACTCCATTTCAGAATCATT	59
23	wmc611	GGTTCGCTTTCAAGGTCCACTC	CGGGACACTAGTGCTCGATTCT	66
Chr1B				
24	gwm33	GGAGTCACACTTGTTTGTGCA	CACTGCACACCTAACTACCTGC	60
25	wmc134	CCAAGCTGTCTGACTGCCATAG	AGTATAGACCTCTGGCTCACGG	64
26	gwm403	CGACATTGGCTTCGGTG	ATAAAACAGTGCGGTCCAGG	52
27	gwm268	AGGGGATATGTTGTCACTCCA	TTATGTGATTGCGTACGTACCC	59
28	wmc719	TTGTGGGAATCTACATCAGAAGG	AACAGCCACGCTCTATCTTCAGT	61
29	wmc367	CTGACGTTGATGGGCCACTATT	GTGGTGGAAGAGGAAGGAGAGG	62
30	gwm259	AGGGAAAAGACATCTTTTTTTTC	CGACCGACTTCGGGTTC	56
31	gwm140	ATGGAGATATTTGGCCTACAAC	CTTGACTTCAAGGCGTGACA	60
32	wmc419	GTTTCGGATAAAACCGGAGTGC	ACTACTTGTGGGTTATCACCAGCC	62
33	wmc269	GCACCTTCTAACCTTCCCCAGC	CCCTAATCCAGGACTCCCTCAG	66
34	barc137	GGCCCATTTCCCACTTTCCA	CCAGCCCCTCTACACATTTT	58
35	barc187	GTGGTATTTCAGGTGGAGTTGTTTTA	CGGAGGAGCAGTAAGGAAGG	63
36	barc61	TGCATACATTGATTCATAACTCTCT	TCTTCGAGCGTTATGATTGAT	55
37	barc188	CGTGAGATCATGTTATCAGGACAAG	GCGTTGAAAGGTGTTAGTGGGATGG	64
38	barc81	GCGCTAGTGACCAAGTTGTTATATGA	GCGGTTCGGAAAGTGCTATTCTACAGT AA	65
39	barc80	GCGAATTAGCATCTGCATCTGTTTGAG	CGGTCAACCAACTACTGCACAAC	65
40	wmc500	ATAGCATGTTGGAACAGAGCAC	CTTAGATGCAACTCTATGCGGT	60
41	wmc619	TTCCCTTTCCCCTCTTTCCG	TACAATCGCCACGAGCACCT	60
42	wmc626	AGCCCATAAACATCCAACACGG	AGGTGGGCTTGGTTACGCTCTC	62
Chr1D				
43	cfd61	ATTCAAATGCAACGCAAACA	GTTAGCCAAGGACCCCTTTC	52

44	gwm106	CTGTTCTTGCGTGGCATTAA	AATAAGGACACAATTGGGATGG	62
45	gwm232	ATCTCAACGGCAAGCCG	CTGATGCAAGCAATCCACC	52
46	gdm111	CACTCACCCCAAACCAAAGT	GATGCAATCGGGTCGTTAGT	58
47	wmc216	ACGTATCCAGACACTGTGGTAA	TAATGGTGGATCCATGATAGCC	60
48	cfd19	TACGCAGGTTTGCTGCTTCT	GGAGTTCACAAGCATGGGTT	58
49	wmc36	TTCTCTTTTCCTTTCGCACTCC	CATCAGTTGTGGGGTTTCTTCA	60
50	wmc93	ACAACTTGCTGCAAAGTTGACG	CCAACTGAGCTGAGCAACGAAT	60
51	cfd63	TCCTGAGGATGTTGAGGACC	GAGAGAGGCGAAACATGGAC	60
52	wmc813	TGTTGGATGCGTGCGAC	CCTCTCCCGGACTCCTGC	52
53	barc66	CGCGATCGATCTCCCGGTTTGCT	GGGAAGAGGACCAAGGCCACTA	66
54	wmc609	CATCCAGCCCATGTAGACGC	AACGGTGCCCATCATCTCCC	63
55	wmc222	AAAGGTGCGTTCATAGAAAATTAGA	AGAGGTGTTTGAGACTAATTTGGTA	59
56	wmc339	CCGCTCGCCTTCTTCCAG	TCCGGAACATGCCGATAC	52
57	wmc590	CGCACGAAGCTATCTGATACCA	GGAAAACCTAACCCTAGCCACC	62
58	barc152	CTTCCTAAAATCGGGCAACCGCTTGTTG	GCGTAATGATGGGAGTGGCTATAGGGC AGTT	70
59	barc229	GGCCGCTGGGGATTGCTATGAT	TCGGGATAAGGCAGACCACAT	61
60	barc99	CGCATTCTTTCGCATTCTCTGTCATA	CGCATACTGTGTCGTGTTCCTGGTTTAG A	65
61	barc169	CCGCGAACCATACAAAGGAAAC	GCTATAGAGGCGCCTTGGAGTACC	62
62	barc271	CGCACCTAATATCGTAAAACAATGTA	CGCTTTCCCAGAATATTATTTGTATTGT	62
63	barc346	ACCGCCTCAGCCTTATTCCTTG	TGGGCTCGGGTTGGTCTCT	62
64	gwm458	AATGGCAATTGGAAGACATAGC	TTCGCAATGTTGATTTGGC	52
65	wmc405	GTGCGGAAAGAGACGAGGTT	TATGTCCACGTTGGCAGAGG	60
Chr2A				
66	cfd36	GCAAAGTGTAGCCGAGGAAG	TTAGAGTTTTGCAGCGCCTT	56
67	gwm512	AGCCACCATCAGCAAAAATT	GAACATGAGCAGTTTGGCAC	54
68	barc1138	GCGATGTCATGCTCACCAATGTGT	GCGTGCTCCACTCAGAGACTATCATAA A	65
69	wmc382	CATGAATGGAGGCACTGAAACA	CCTTCCGGTCGACGCAAC	60
70	gwm359	CTAATTGCAACAGGTCATGGG	TACTTGTGTTCTGGGACAATGG	59
71	wmc602	TACTCCGCTTTGATATCCGTCC	GTTTGTTGTTGCCATCACATTC	58
72	wmc453	ACTTGTGTCCATAACCGACCTT	ATCTTTTGAGGTTACAACCCGA	58
73	barc124	TGCACCCCTTCCAAATCT	TGCGAGTCGTGTGGTTGT	54
74	wmc382	CATGAATGGAGGCACTGAAACA	CCTTCCGGTCGACGCAAC	60
75	gwm515	AACACAATGGCAAATGCAGA	CCTTCCTAGTAAGTGTGCCTCA	54
76	gwm473	TCATACGGGTATGGTTGGAC	CACCCCCTTGTTGGTCAC	58
77	wmc261	GATGTGCATGTGAATCTCAAAAGTA	AAAGAGGGTCACAGAATAACCTAAA	61
78	gwm47	TTGCTACCATGCATGACCAT	TTCACCTCGATTGAGGTCCT	56
79	wmc109	AATTCGGGAAGAGTCTCAGGGG	TTCGAAGGGCTCAAGGGATACG	64
80	barc231	GCGATCAATAACCGTGCCACCA	GCACTTGCGATGTCACTAAAATG	61
81	barc309	GCGAAAGCCCTAAAGTTACAA	AAGCCGCAGAGAAGGTCAGC	57
82	cfd168	CTTCGCAAATCGAGGATGAT	TTCACGCCCAGTATTAAGGC	56
83	barc353	GAAGTTCCCAAAATGCCTCTGTC	GCGGATCGAAGACCTAAGAAAAG	63
84	wmc181	TCCTTGACCCCTTGCACTAACT	ATGGTTGGGAGCACTAGCTTGG	62
85	barc279	GCGTTTTTTACCTAAAGAAAAGGTGATTG	CGCAACACACATTCCATTCCATTTCAC	65
86	gwm356	AGCGTTCTTGGGAATTAGAGA	CCAATCAGCCTGCAACAAC	57
87	barc76	ATTCGTTGCTGCCACTTGCTG	GCGCGACACGGAGTAAGGACACC	61
88	wmc658	CTCATCGTCCTCCTCCACTTTG	GCCATCCGTTGACTTGAGGTTA	62
89	gwm311	TCACGTGGAAGACGCTCC	CTACGTGCACCACCATTTTG	58
90	gwm425	GAGCCCACAAGCTGGCA	TCGTTCTCCCAAGGCTTG	56
91	wmc407	GGTAATTCTAGGCTGACATATGCTC	CATATTTCCAAATCCCCAACTC	58
92	wmc177	AGGGCTCTCTTTAATTCTTGCT	GGTCTATCGTAATCCACCTGTA	58
93	wmc63	GTGCTCTGGAAACCTTCTACGA	CAGTAGTTTAGCCTTGGTGTGA	60

Chr2B				
94	wmc764	CCTCGAACCTGAAGCTCTGA	TTCGCAAGGACTCCGTAACA	58
95	barc318	CGACTAACAATTTTTCATTT	TGATTTCGCTAACAAGGAG	48
96	barc200	GCGATATGATTTGGAGCTGATTG	GCGATGACGTTAGATGCGGAATTGT	61
97	barc349	CGAATAGCCGCTGCACAAG	TATGCATGCCTTTCTTTACAAT	55
98	barc13	GCAGGAACAACCACGCCATCTTAC	GCGTCGCAATTTGAAGAAAATCATC	63
99	wmc154	ATGCTCGTCAGTGTCATGTTTG	AAACGGAACCTACCTCACTCTT	60
100	barc128	GCGGGTAGCATTTATGTTGA	CAAACCAGGCAAGAGTCTGA	56
101	gwm429	TTGTACATTAAGTTCCCATTA	TTTAAGGACCTACATGACAC	52
102	barc101	GCTCCTCTCACGATCACGCAAAG	GCGAGTCGATCACACTATGAGCCAATG	66
103	gwm148	GTGAGGCAGCAAGAGAGAAA	CAAAGCTTGACTCAGACCAAA	57
104	barc183	CCCGGGACCACCAGTAAGT	GGATGGGGAATTGGAGATACAGAG	62
105	wmc272	TCAGGCCATGTATTATGCAGTA	ACGACCAGGATAGCCAATTCAA	58
106	wmc265	GTGGATAACATCATGGTCAAC	TACTTCGCACTAGATGAGCCT	57
107	gwm129	TCAGTGGGCAAGCTACACAG	AAAACTTAGTAGCCGCGT	52
108	cfd73	GATAGATCAATGTGGGCCGT	AACTGTTCTGCCATCTGAGC	58
109	gwm501	GGCTATCTCTGGCGCTAAAA	TCCACAAACAAGTAGCGCC	57
110	wmc332	CATTTACAAAGCGCATGAAGCC	GAAAACTTTGGGAACAAGAGCA	58
111	wmc149	ACAGACTTGGTTGGTGCCGAGC	ATGGGCGGGGGTGTAGAGTTTG	66
112	wmc317	TGCTAGCAATGCTCCGGGTAAC	TCACGAAACCTTTTCCTCCTCC	62
113	gwm382	GTCAGATAACGCCGTCCAAT	CTACGTGCACCACCATTTTG	58
114	gwm526	CAATAGTTCTGTGAGAGCTGCG	CCAACCCAAATACACATTCTCA	58
115	gwm374	ATAGTGTGTTGCATGCTGTGTG	TCTAATTAGCGTTGGCTGCC	58
116	gwm410	GCTTGAGACCGGCACAGT	CGAGACCTTGAGGGTCTAGA	58
117	wmc474	ATGCTATTAAACTAGCATGTGTCG	AGTGGAAACATCATTCCTGGTA	58
118	wmc445	AGAATAGGTTCTTGGGCCAGTC	GAGATGATCTCCTCCATCAGCA	62
119	wmc356	GCCGTTGCCCAATGTAGAAG	CCAGAGAAACTCGCCGTGTC	60
120	wmc592	GGTGGCATGAACTTTCACCTGT	TGTGTGGTGCCCATTAGGTAGA	62
Chr2D				
121	cfd56	TTGCATAATTACTTGCCCTCC	CTGGTCCAACTTCCATCCAT	57
122	barc297	GCGTAGGAGAGATGCCCCAAAGGTT	GCGTGCGGACTCGTGAATCATTACA	69
123	wmc25	TCTGGCCAGGATCAATATTACT	TAAGATACATAGATCCAACACC	58
124	gwm261	CTCCCTGTACGCCTAAGGC	CTCGCGCTACTAGCCATTG	59
125	barc168	GCGATGCATATGAGATAAGGAACAAATG	GCGGCTCTAAGGCGGTTTCAAAT	65
126	wmc470	ACTTGCAACTGGGGACTCTC	TCCCCAATTGCATATTGACC	56
127	barc228	CCCTCCTCTCTTTAGCCATCC	GCACGTACTATTCGCCTTCACTTA	63
128	barc145	GCAGCCTCGAATCACA	GGGGTGTTGAAGATGA	48
129	barc11	GCGATGCGTGTAAAGTCTGAAGATGA	GCGTCCATGGAGCTCTGTTTTATCTGA	66
130	gwm249	CAAATGGATCGAGAAAGGGA	CTGCCATTTTTCTGGATCTACC	56
131	wmc601	ACAGAGGCATATGCAAAGGAGG	CTTGTCTCTTTATCGAGGGTGG	62
132	barc219	GCGATCCCACAATGCATGACAACTTC	GGACGTCCGATCGAATTGGTTT	62
133	barc159	CGCAATTTATTATCGGTTTTAGGAA	CGCCCGATAGTTTTTCTAATTTCTGA	62
134	wmc41	TCCCTCTTCCAAGCGCGGATAG	GGAGGAAGATCTCCCGGAGCAG	66
135	gwm608	ACATTGTGTGTGCGGCC	GATCCCTCTCCGCTAGAAGC	55
136	gwm349	GGCTTCCAGAAAACAACAGG	ATCGGTGCGTACCATCCTAC	58
137	wmc175	GCTCAGTCAAACCGCTACTTCT	CACTACTCCAATCTATCGCCGT	62
138	wmc817	TGACGGGGATGATGATAACG	CGGTGAGATGAGAAAGGAAAAC	58
139	gwm301	GAGGAGTAAGACACATGCCC	GTGGCTGGAGATTCAGGTTC	60
140	gdm5	CTAGCCAGAAGGTTACTTTG	CAACATTAACATTAACGCAC	52
141	gwm455	ATTCGGTTCGCTAGCTACCA	ACGGAGAGCAACCTGCC	57
142	gwm484	ACATCGCTCTTCACAAACCC	AGTTCCGGTCATGGCTAGG	58
143	gwm102	TCTCCCATCCAACGCCTC	TGTTGGTGGCTTGACTATTG	58
144	gwm539	CTGCTCTAAGATTCATGCAACC	GAGGCTTGTGCCCTCTGTAG	60
145	gdm148	GATTTGACCGTCTGAGGTCG	AACTAGTTCTGTGGCAAGCT	56

146	gwm296	AATTCAACCTACCAATCTCTG	GCCTAATAAACTGAAAACGAG	55
Chr3A				
147	wmc11	TTGTGATCCTGGTTGTGTTGTGA	CACCCAGCCGTTATATATGTTGA	61
148	barc310	GGGCGGCGCATGTGCACCTA	GCGTGGAAGCGACTAAATCAACT	63
149	barc12	CGACAGAGTGATCACCCAAATATAA	CATCGGTCTAATTGTCAATGTA	57
150	gwm369	CTGCAGGCCATGATGATG	ACCGTGGGTGTTGTGAGC	56
151	barc179	GCGTCGTCATAATTGCCTTTCACTTG	GCGAGCCCATATTGCCTTGTCTTCT	66
152	barc45	CCCAGATGCAATGAAACCACAAT	GCGTAGAACTGAAGCGTAAAATTA	60
153	wmc505	AGGGGAGGAAAACCTTGTAATC	ACGACCTACGTGGTAGTTCTTG	60
154	barc67	GCGGCATTTACATTTCAGATAGA	TGTGCCTGATTGTAGTAACGTATGTA	59
155	barc19	GCGACCCGAGTAGCCTGAA	GGTGGACCATTAGACGCTTACTTG	62
156	barc25	GCGGTGCATCAAGGACGACAT	GCGTAGTTCATCCATCCGTAAT	60
157	wmc428	TTAATCCTAGCCGTCCCTTTTT	CGACCTTCGTTGGTTATTTGTG	58
158	barc314	CTGTGGAAACCAATAAAAACAA	GTGCGCGAATAACTACAAGAAA	55
159	gwm494	ATTGAACAGGAAGACATCAGGG	TTCCTGGAGCTGTCTGGC	58
160	wmc96	TAGCAGCCATGCTTAGCATCAA	GTTTCAGTCTTTCACGAACACG	60
161	wmc173	TGCAGTTGCGGATCCTTGA	TAACCAAGCAGCACGTATT	53
162	wmc153	ATGAGGACTCGAAGCTTGGC	CTGAGCTTTTGCGCGTTGAG	60
163	cfa2076	CGAAAAACCATGATCGACAG	ACCTGTCCAGCTAGCCTCCA	56
164	gwm666	GCACCCACATCTTCGACC	TGCTGCTGGTCTCTGTGC	58
165	gwm480	TGCTGCTACTTGTACAGAGGAC	CCGAATTGTCCGCCATAG	56
166	barc284	GCGTCAGAAATGCAAGAAAAATAGG	GCGGAAGAAAAGGACGAAGACAAG	63
167	wmc289	CATATGCATGCTATGCTGGCTA	AGCCTTTCAAATCCATCCACTG	60
168	gwm155	CAATCATTTCCCCCTCCC	AATCATTGGAAATCCATATGCC	56
169	wmc83	TGGAGGAAACACAATGGATGCC	GAGTATCGCCGACGAAAGGGAA	62
170	wmc169	TACCCGAATCTGGAAAATCAAT	TGGAAGCTTGCTAACTTTGGAG	57
Chr3B				
171	barc75	AGGGTTACAGTTTGCTCTTTTAC	CCCGACGACCTATCTATACTTCTCTA	59
172	gwm533	AAGGCGAATCAAACGGAATA	GTTGCTTTAGGGGAAAAGCC	54
173	barc133	AGCGCTCGAAAAGTCAG	GGCAGGTCCAACTCCAG	50
174	wmc597	AACACACCTTGCTTCTCTGGGA	GACTAGGGTTTCGGTTGTTGGC	62
175	cfd28	TGCATCTTATTACTGGAGGCATT	CGCATGCCCTTATACCAACT	58
176	barc102	GGAGAGGACCTGCTAAAATCGAAGACA	GCGTTTACGGATCAGTGTTGGAGA	65
177	wmc679	TAGGGGACAGGAGGGAGGG	CGGATCCAGACCAGGAAGGT	63
178	barc218	GGTGAGGAGATGGCCCAAAGTAAC	GGGGGTGTGAGGAGAACGTATCAACTCT	67
179	wmc625	CACAGACCTCAACCTCTTCTT	AGTACTGTTCACAGCAGACGA	59
180	gwm77	ACAAAGGTAAGCAGCACCTG	ACCCTCTTGCCCGTGTTG	58
181	barc164	TGCAAACTAATCACCAGCGTAA	CGCTTTCTAAAACTGTTCGGGATTTCTAA	58
182	cfa2134	TTTACGGGGACAGTATTCGG	AAGACACTCGATGCGGAGAG	58
183	barc84	CGCATAACCGTTGGGAAGACATCTG	GGTGCAACTAGAACGTACTTCCAGTC	67
184	barc77	GCGTATTCTCCCTCGTTTCCAAGTCTG	GTGGGAATTTCTTGGGAGTCTGTA	64
185	gwm108	CGACAATGGGGTCTTAGCAT	TGCACACTTAAATTACATCCGC	58
186	wmc687	AGGACGCCTGAATCCGAG	GGGAGCGTAGGAGGACTAACA	58
187	wmc206	TTGTGCTCGTGAATTGCATACC	GCCAAAATGGCAGCTTCTCTTA	60
188	gwm114	ACAAACAGAAAATCAAAACCCG	ATCCATCGCCATTGGAGTG	57
189	gwm547	GTTGTCCCTATGAGAAGGAACG	TTCTGCTGCTGTTTTCATTTAC	57
190	wmc632	GTTTGATTGGTCGTTCCTGGTC	AACAGCGAATGGAGGGCTTTAG	62
191	gwm493	TTCCCATAACTAAAACCGCG	GGAACATCATTTCTGGACTTTG	56
192	gwm566	TCTGTCTACCCATGGGATTTG	CTGGCTTCGAGGTAAGCAAC	59
193	wmc231	CATGGCGAGGAGCTCGGTGGTC	GTGGAGCACAGGCGGAGCAAGG	70
194	gwm340	GCAATCTTTTTTCTGACCACG	ACGAGGCAAGAACACACATG	57

195	wmc307	GTTTGAAGACCAAGCTCCTCCT	ACCATAACCTCTCAAGAACCCA	60
196	wmc471	GGCAATAATAGTGCAAGGAATG	GCCGATAATGGGCAATATAAGT	58
Chr3D				
197	cfd35	GGGATGACACATAACGGACA	ATCAGCGGCGCTATAGTACG	58
198	cfd141	CGTAAAGATCCGAGAGGGTG	TCCGAGGTGCTACCTACCAG	60
199	barc321	TGCACTTCCCACAACACATC	TTGCCACGTAGGTGATTTATGA	58
200	cfd79	TCTGGTTCTTGGGAGGAAGA	CATCCAACAATTTGCCCAT	53
201	gwm52	CTATGAGGCGGAGGTTGAAG	TGCGGTGCTCTTCCATTT	54
202	barc6	TTCGGTCGTTGAGGTGACCAATTATG	GACAAAGGATTAGCCCAAAGTAAGAG	65
203	barc135	ATCGCCATCTCCTCTACCA	GCGAACCCATGTGCTAAGT	57
204	wmc631	TTGCTCGCCCACCTTCTACC	GGAAACCATGCGCTTCACAC	60
205	cfd211	AGAAGACTGCACGCAAGGAT	TGCACTAAAGCATCTTCGTGTT	58
206	barc42	GCGACTCCTACTGTTGATAGTTC	GCGTTCTTTTATTACTCATTTTGCAT	60
207	gdm72	TGGTTTTCTCGAGCATTCAA	TGCAACGATGAAGACCAGAA	54
208	barc71	GCGCTTGTTCCTCACCTGCTCATA	GCGTATATTCTCTCGTCTTCTTGTTGGTT	67
209	barc270	GCGCATTGTGACAGGTGAAC	GGAGGGAGTACTTGGTTATTAGGGT	60
210	barc323	GCGAATCTGATGTGGCATGTTAGTT	GGCATATTTCCTTCACAGTTTT	57
211	gwm456	TCTGAACATTACACAACCCTGA	TGCTCTCTCTGAACCTGAAGC	58
212	gwm383	ACGCCAGTTGATCCGTAAAC	GACATCAATAACCGTGGATGG	58
213	gwm314	AGGAGCTCCTCTGTGCCAC	TTCGGGACTCTCTTCCCTG	59
214	gwm341	TTCAGTGGTAGCGGTCGAG	CCGACATCTCATGGATCCAC	59
215	gwm497	GTAGTGAAGACAAGGGCATT	CCGAAAGTTGGGTGATATAC	56
216	wmc552	ACTAAGGAGTGTGAGGGCTGTG	CTCTCGCGCTATAAAAGAAGGA	60
217	wmc533	AATTGGATCGGCAGTTGGAG	AGCAAGCAGAGCATTGCGTT	58
Chr4A				
218	barc206	GCTTTGCCAGGTGAGCACTCT	TGGCCGGGTATTTGAGTTGGAGTTT	63
219	barc138	CTCGATTCGCCGTCAG	GTGGGGGAAGAAGAAACC	53
220	barc106	GCCCTCAAATAATTACGCCAATCCCTATG	GCGTCAAGATCAGAAGGCATCCTATTATTG	69
221	barc170	CGCTTGACTTTGAATGGCTGAACA	CGCCCACTTTTTACCTAATCCTTTTGAA	64
222	gwm637	AAAGAGGTCTGCCGCTAACA	TATACGGTTTTGTGAGGGGG	58
223	wmc707	GCTAGCTGACACTTTTCCTTTG	TCAGTTTCCCACTCACTTCTTT	58
224	barc343	GGCCTAATTACAAGTCCAAAAG	GCTCAAAGTAAAGTTCACGAATAT	58
225	wmc718	GGTCGGTGTTGATGCACTTG	TCGGGGTGTCTTAGTCCTGG	60
226	wmc698	GTGAAGGGAGAGCTAGCAA	ACAGTTGGCCCAGCTAGTA	57
227	barc70	GCGAAAAACGATGCGACTCAAAG	GCGCCATATAATTCAGACCCACAAAA	63
228	gwm160	TTCAATTCAGTCTTGGCTTGG	CTGCAGGAAAAAAAAGTACACCC	57
229	barc78	CTCCCCGGTCAAGTTTAATCTCT	GCGACATGGGAATTTCAGAAGTGCCTAA	63
230	wmc219	TGCTAGTTTGTCATCCGGGCGA	CAATCCCGTTCTACAAGTCCA	59
231	barc52	GCGCCATCCATCAACCGTCATCGTCATA	GCGAGGAAGGCGGCCACCAGAATGA	72
232	barc315	CATCCAGGCGGGCGCACGAGA	CAAGCCTCCGTGCACACCGTAT	66
233	barc184	TTCGGTGATATCTTTTCCCCTTGA	CCGAGTTGACTGTGTGGGCTTGCTG	62
234	barc153	CGCGCCTTGCTTTATTAGTATTAGTATT	GCGGCATGCACATATAATTCTCATTGACT	64
235	gwm397	TGTCATGGATTATTTGGTCGG	CTGCACTCTCGGTATACCAGC	57
236	wmc513	TGAATTGAATCTGGTTGCGG	TGGCAATTCACAGGCACATA	56
237	wmc468	AGCTGGGTTAATAACAGAGGAT	CACATAACTGTCCACTCCTTTC	58
238	wmc283	CGTTGGCTGGGTTATATCATCT	GACCCGCGTGTAAGTGATAGGA	60
239	wmc313	GCAGTCTAATTATCTGCTGGCG	GGGTCCTTGTCTACTCATGTCT	62
Chr4B				
240	barc193	GCGCATCCATATTTTTCCAGCAAGCACTT	GCGTTCTTGTTTGTGGTTTCTATTTTTCT	69

241	barc10	GCGTGCCACTGTAACCTTTAGAAGA	GCGAGTTGGAATTATTTGAATTAAACAAG	63
242	wmc47	GAAACAGGGTTAACCATGCCAA	ATGGTGCTGCCAACAACATACA	60
243	barc292	GCGTGTGAGTCAATCCGTGCTTTAT	GCGTTGGTTTTAAGAGGTGCCTGAA	66
244	barc163	GCGTGTTTTAAGGTATTTTCCATTTTCT	GCGCATCCTGTTCCTCCATTCATA	63
245	cfd22	GGTTGCAAACCGTCTTGTTT	AGTCGAGTTGCGACCAAAGT	56
246	barc60	CATGCTCACAAAACCCACAAGACT	CTCGAAAGGCGGCACCACTA	63
247	wmc546	CGGCTAAAATCGTACACTACACA	CTCACTTGCACGATTTCCCTAT	60
248	wmc710	GTAAGAAGGCAGCACGTATGAA	TAAGCATTCCCAATCACTCTCA	58
249	wmc617	CCACTAGGAAGAAGGGGAAACT	ATCTGGATTACTGGCCAACTGT	60
250	wmc42	GCCCTTGGTCCTGGGGTGAGCC	GCCTCATCCAGAGAGCCTGCGG	70
251	gwm149	CATTGTTTTCTGCCTCTAGCC	CTAGCATCGAACCTGAACAAG	59
252	gwm375	ATTGGCGACTCTAGCATATACG	GGGATGTCTGTTCCATCTTAGC	60
253	gwm6	CGTATCACCTCCTAGCTAAACTAG	AGCCTTATCATGACCCTACCTT	60
254	wmc125	ATACCACCATGCATGTGGAAGT	ACCGCTTGTCATTTCCTTCTGT	60
255	wmc349	ACACACACTCGATCGCAC	GCAGTTGATCATCAAAACACA	55
Chr4D				
256	wmc285	TGTGGTTGTATTTGCGGTATGG	TTGTGGTGCTGAGTTAGCTTGT	60
257	barc225	CGCAATAATTCAGTACTACTTCCCCGCAATA	CGAAGGATTTGCATGGTACTGTGGGTGAT	70
258	gwm213	TGCCTGGCTCGTTCTATCTC	CTAGCTTAGCACTGTCGCCC	60
259	barc308	GCGATCTTGCGTGTGCGTAGGA	GCGTGGGATGCAAGTGAACAAT	62
260	barc288	GGGTTTTGCTTGGTTGACA	CGGGACGATTTTATTTAGGAGT	55
261	barc98	CCGTCCTATTCGCAAACCAGATT	GCGGATATGTTCTCTAACTCAAGCAATG	63
262	cfd39	CCACAGCTACATCATCTTTCCTT	CAAAGTTTGAACAGCAGCCA	56
263	cfd84	GTTGCCTCGGTGTCGTTTAT	TCCTCGAGGTCCAAAACATC	58
264	wmc622	CAGGAAGAAGAGCTCCGAGAAA	CTTGCTAACCCGCGCC	56
265	barc48	GCGAGCTGCAGAGGTCCATC	GCGTTAGTCTTCTTGGTCAATCAC	64
266	wmc825	GCTAGCTGCTGGTTCCACTTG	TGTCCACTCCACTCCAGCATTAC	63
267	gwm624	TTGATATTAAATCTCTCTATGTG	AATTTTATTTGAGCTATGCG	50
268	gwm609	GCGACATGACCATTTTGTTG	GATATTAAATCTCTCTATGTGTG	56
269	wmc51	TTATCTTGGTGTCTCATGTCAG	TCGCAAGATCATCAGAACAGTA	58
270	wmc48	GAGGGTTCTGAAATGTTTTGCC	ACGTGCTAGGGAGGTATCTTGC	60
271	wmc52	TCCAATCAATCAGGGAGGAGTA	GAACGCATCAAGGCATGAAGTA	60
272	wmc89	ATGTCCACGTGCTAGGGAGGTA	TTGCCTCCCAAGACGAAATAAC	60
273	wmc331	CCTGTTGCATACTTGACCTTTTT	GGAGTTCAATCTTTCATCACCAT	59
Chr5A				
274	barc122	CCCGTGTATATCCAGGAGTG	CAGCCCTTGTGATGTGATG	56
275	barc316	GCGTCCCACCTGTCATTAACTGTC	GCGGGCCCACTCCTGTTAGATTA	70
276	barc180	GCGATGCTTGTTTGTTACTTCTC	GCGATGGAACTTCTTTTTGCTCTA	61
277	barc186	GGAGTGTCGAGATGATGTGGAAAC	CGCAGACGTCAGCAGCTCGAGAGG	65
278	gwm443	GGGTCTTCATCCGGAACTCT	CCATGATTTATAAATTCCACC	54
279	barc360	GCGATGGCAAAAACTGTGACC	GCGCTCCAGCAGATACATAAGATAAC	61
280	barc40	GCCGCCTACCACAGAGTTGCAGCT	GCGGCATTGACAAGACCATAGC	64
281	cfa2104	CCTGGCAGAGAAAGTGAAGG	AGTCGCCGTTGTATAGTGCC	60
282	barc141	GGCCCATGGATAATTTTTGAAATG	CAATTCGGCCAAAGAAGAAGTCA	60
283	barc330	GCACTAAGCGCTCTTTATTTAC	CCTGCATCTGGTATGGAGA	57
284	gwm293	TACTGGTTCACATTGGTGCG	TCGCCATCACTCGTTCAAG	57
285	barc56	GCGGGAATTTACGGGAAGTCAAGAA	GCGAGTGGTTCAAATTTATGTCTGT	63
286	gwm186	GCAGAGCCTGGTTCAAAAAG	CGCCTCTAGCGAGAGCTATG	58
287	wmc492	AGGATCAGAATAGTGCTACCC	ATCCCGTGATCAGAATAGTGT	57
288	gwm156	CCAACCGTGCTATTAGTCATTC	CAATGCAGGCCCTCCTAAC	59
289	barc230	CCCCTCCTCCTTCTCCCTCCTCCTA	GGCTCATGCGGGCGTGTTTGG	67

290	barc319	GCAGAGCTACGGCAATGT	GCGTAAGTCCCGGAAGTAACAGAA	56
291	barc151	TGAGGAAAATGTCTCTATAGCATCC	CGCATAAACACCTTCGCTCTTCCACTC	63
292	cfa2155	TTTGTTACAACCCAGGGGG	TTGTGTGGCGAAAGAAACAG	56
293	barc232	CGCATCCAACCATCCCCACCCAACA	CGCAGTAGATCCACCACCCCGCCAGA	71
294	cfa2185	TTCTTCAGTTGTTTTGGGGG	TTTGGTCGACAAGCAAATCA	54
295	wmc110	GCAGATGAGTTGAGTTGGATTG	GTACTTGGAAACTGTGTTTGGG	60
296	gwm126	CACACGCTCCACCATGAC	GTTGAGTTGATGCGGGAGG	58
297	wmc577	CTGTCCGACTCCCCAGATG	CCCTGTCAGAGGCTGGTTG	62
298	gwm595	GCATAGCATCGCATATGCAT	GCCACGCTTGGACAAGATAT	56
299	wmc727	CATAATCAGGACAGCCGCAC	TAGTGGCCTGATGTATCTAGTTGG	60
300	gwm291	CATCCCTACGCCACTCTGC	AATGGTATCTATTCCGACCCG	59
301	gwm154	TCACAGAGAGAGAGGGAGGG	ATGTGTACATGTTGCCTGCA	56
302	wmc415	AATTCGATACCTCTCACTCACG	TCAACTGCTACAACCTAGACCC	60
303	wmc497	CCCGTGGTTTTCTTTCCTTCT	AACGACAGGGATGAAAAGCAA	58
Chr5B				
304	cfd5	TGCCCTGTCCACAGTGAAG	TTGCCAGTTCCAAGGAGAAT	56
305	wmc773	GAGGCTTGCATGTGCTTGA	GCCAACTGCAACCGGTACTCT	56
306	barc32	GCGTGAATCCGGAAACCCAATCTGTG	TGGAGAACCTTCGCATTGTGTCATTA	65
307	barc216	TGACGACCCAATCCATAGACA	GGTGATTATTCGTGAGTTCCCTGTG	59
308	barc340	GCAACCAAGGCAGCGTAAATG	GCGTGTAGCCGTCCATAAGCATCAT	61
309	barc4	GCGTGTTTGTGTCTGCGTTCTA	CACCACACATGCCACCTTCTTT	62
310	barc89	GGGCGCGGCACCAGCACTACC	CTCCGAGGCCACCGAAGACAAGATG	71
311	wmc728	GCAGGCTCTGCATCTTCTTG	CGCAGAGCTGAGCTGAAATC	60
312	cfa2121	TAAATGGCCATCAAGCAATG	GCTTGTGAACTAATGCCTCCC	54
313	gdm146	ATCCTGACGGCCACCAC	CAAAGCCTGCGATACATCAA	56
314	gwm66	CCAAAGACTGCCATCTTTCA	CATGACTAGCTAGGGTGTGACA	56
315	gwm274	AACTTGCAAAACTGTTCTGA	TATTTGAAGCGGTTTGATTT	50
316	barc140	CGCCAACACCTACCATT	TTCTCCGCACTCACAAAC	52
317	cfd2	GGTTGCAGTTTCCACCTTGT	CATCTATTGCCAAAATCGCA	54
318	barc156	CGCATCGAGGTCTTCCCCGCTGTCCAA	CGCACCCACACATGTATCTGAGTTTCCTA	70
319	barc142	CCGGTGAGAGGACTAAAA	GGCCTGTCAATTATGAGC	54
320	barc69	AGGCGGCGGTCGTGGAACA	GCGTACCGAGAAGTGATCAAGAACAT	64
321	gwm408	TCGATTTATTTGGGCCACTG	GTATAATTCGTTCACAGCACGC	56
322	wmc118	AGAATTAGCCCTTGAGTTGGTC	CTCCCATCGCTAAAGATGGTAT	60
323	wmc640	AATTTATCTCGATCATGTGAGC	TGAGTAGTTCCCTTAGGACCTT	57
324	wmc783	AGGTTGGAGATGCAGGTGGG	TCTTCCTTCTCCTGCCGCTA	60
325	wmc258	GCGATGTCAGATATCCGAAAGG	ACCAGGACACCAGAACAGCAAT	62
326	wmc503	GCAATAGTTCCCGCAAGAAAAG	ATCAACTACCTCCAGATCCCGT	60
327	gwm234	GAGTCCTGATGTGAAGCTGTTG	CTCATTGGGGTGTGTACGTG	60
328	gwm499	ACTTGTATGCTCCATTGATTGG	GGGGAGTGGAAACTGCATAA	58
329	wmc386	ATCACTGAAACGAAATGAGCGG	TGGTTGGCGGTTTTTCTCTACA	60
330	wmc363	TCTGTAACGCATAATAGAATAGCCC	ATGATTGCGTTATCTTCATATTTGG	64
Chr5D				
331	barc130	CGGCTAGTAGTTGGAGTGTTGG	ACCGCCTCTAGTTATTGCTCTC	66
332	gwm190	GTGCTTGCTGAGCTATGAGTC	GTGCCACGTGGTACCTTTG	58
333	barc205	GCGACAGTTGTAGCGGCAGTAGC	GAGCGTAGTAGAAGCAGAAGGAG	70
334	cfd81	TATCCCCAATCCCCTCTTTC	GTCAATTGTGGCTTGTCCCT	60
335	barc143	TTGTGCCAAATCAAGAACAT	GGTTGGGCTAGGATGAAAAT	54
336	barc44	CCCTACAAAATACGAACATGAAGTCAG	GGGTCCTACTCAGATAGTGACAGTCAAC	65
337	gdm136	CTCATCCGGTGAGTGCATC	CCCGCATGTCTACATGAGAA	58
338	cfd7	AGCTACCAGCCTAGCAGCAG	TCAGACACGTCTCCTGACAAA	59

339	gwm174	GGGTTCCTATCTGGTAAATCCC	GACACACATGTTCCTGCCAC	60
340	wmc215	CATGCATGGTTGCAAGCAAAAG	CATCCCGGTGCAACATCTGAAA	60
341	barc286	GCGAAGAAAACATTAGACCAAAA	GCGATATGTTTCCCGACAACTA	58
342	gwm654	TGCTGATGTTGTAAGAAGGC	TGCGTCAGATATGCCTACCT	56
343	barc347	GCGCACCTCTCCTCACCTTCT	GCGAACATGGAAATGAAAACTATCT	61
344	cfd86	TTAATGAGCGTCAGTACTCCC	GCAACCATGTTTAAGCCGAT	56
345	barc320	CGTCTTCATCAAATCCGAACTG	AAAATCTATGCGCAGGAGAAAC	58
346	wmc161	ACCTTCTTTGGGATGGAAGTAA	GTACTGAACCACTTGTAACGCA	58
347	gwm469	CAACTCAGTGCTCACACAACG	CGATAACCACTCATCCACACC	61
348	barc93	GCCGGACGGATTTAGGTGGAGGAGA	CGCAACCTCACCATCACCGCCTCATC	71
349	wmc443	CCTCCTCTGTTTTCCCTCTGTT	CACACTCTGTGCTTCTGTTTGC	62
350	barc322	GAGAACATGAACGTGATTTACC	CGCAAACTTGTGTATCCTTATC	58
351	barc110	CCCGAACAATGGCTTTGGTGTCGTAAT	CATGGTGACGGCAAGTGTGAGGT	66
352	barc177	GCGATCCTGTTGTTGAGCGTTTGCATAA	TCCCGTTTTCCCGTGTGTTAGTCTA	66
353	barc144	GCGTTTTAGGTGGACGACATAGATAGA	GCGCCACGGGCATTTCTCATAC	66
354	gwm182	TGATGTAGTGAGCCCATAGGC	TTGCACACAGCCAAATAAGG	56
355	gdm63	GCCCCCTATTCCATAGGAAT	CCTTTTGATGGTGCATAGGA	56
356	wmc97	GTCCATATATGCAAGGAGTC	GTACTCTATCGCAAAACACA	54
357	wmc630	ATAATGCACGGTAGGACTGAGG	CATACTGAGACAATTTGGGGGT	60
Chr6A				
358	gwm459	ATGGAGTGGTCACACTTTGAA	AGCTTCTCTGACCAACTTCTCG	57
359	gwm334	AATTTCAAAAAGGAGAGAGA	AACATGTGTTTTTAGCTATC	50
360	barc23	GCGTGAAATAGTGCAAGCCAGAGAT	GCGCTAACACCTCGGCAAGACAA	66
361	wmc182	GTATCTCACGAGCATAACACAA	GAAAGTGTATGGATCATTAGGC	58
362	barc37	CAGCGCTCCCCGACTCAGATCCTT	GCGCCATGTTTCTTTTATTACTCACTTT	64
363	wmc672	GGAGGAGCAAGCTAGGCAA	TTTATAGAGGGAGGGGAGGCAG	59
364	barc3	TTCCCTGTGTCTTTCTAATTTTTTTT	GCGAACTCCCGAACATTTTTAT	58
365	gwm570	TCGCCTTTTACAGTCGGC	ATGGGTAGCTGAGAGCCAAA	56
366	wmc179	CATGGTGGCCATGAGTGGAGGT	CATGATCTTGCGTGTGCGTAGG	64
367	barc195	CCCACATGTCATTGGCTGTTTAA	GCCCGGCCCAGAACGATTTAAATG	61
368	barc113	GCGCACAACAACGGACACTTAACAATT	GGGACTCATTTAGCTTCTACTCGCCATTA	67
369	barc204	CGCAGAAGAAAAACCTCGCAGAAAAACC	CGCAGTGTATCCAAATGGGCAAGC	67
370	gwm169	ACCACTGCAGAGAACACATACG	GTGCTCTGCTCTAAGTGTGGG	62
371	wmc417	GTTCTTTTAGTTGCGACTGAGG	CGATGTATGCCGTATGAATGTT	58
372	wmc580	AAGGCGCACAACACAATGAC	GGTCTTTTGTGCAGTGAACTGAAG	58
373	gwm617	GATCTTGGCGCTGAGAGAGA	CTCCGATGGATTACTCGCAC	60
374	wmc621	GACGTAGGGCGGCGGATA	TGCGCCGTGTTTAATTGCTC	58
375	wmc254	AGTAATCTGGTCCTCTCTTCTTCT	AGGTAATCTCCGAGTGCACTTCAT	62
376	wmc59	TCATTCGTTGCAGATACACCAC	TCAATGCCCTTGTTTCTGACCT	60
377	wmc446	CCAGCTAGTACTCTATATCTACATC	TATTTGAACAAGAGTTATGTGG	55
378	wmc256	CCAAATCTTCGAACAAGAACCC	ACCGATCGATGGTGTATACTGA	60
379	wmc201	CATGCTCTTTCACTTGGGTTCG	GCGCTTGCAGGAATTCAACACT	62
Chr6B				
380	gwm613	CCGACCCGACCTACTTCTCT	TTGCCGTCGTAGACTGG	52
381	wmc487	CAAATTTGGCCACCATTTTACA	CGGTTCAATCCTTGGATTTACA	58
382	wmc104	TCTCCCTCATTAGAGTTGTCCA	ATGCAAGTTTAGAGCAACACCA	58
383	gwm518	AATCACAACAAGGCGTGACA	CAGGGTGGTGCATGCAT	55
384	barc198	CGCTGAAAAGAAGTGCCGCATTATGA	CGCTGCCTTTTCTGGATTGCTTGTCA	66
385	barc24	CGCCTCTTATGGACCAGCCTAT	GCGGTGAGCCATCGGGTTACAAAG	64
386	barc178	GCGTATTAGCAAAACAGAAGTGAG	GCGACTAGTACGAACACCACAAAA	62
387	barc134	CCGTGCTGCAAATGAACAC	AGTTGCCGGTTCCCATTGTCA	57
388	gwm219	GATGAGCGACACCTAGCCTC	GGGGTCCGAGTCCACAAC	61

389	gwm132	TACCAAATCGAAACACATCAGG	CATATCAAGGTCTCCTTCCCC	58
390	wmc105	AATGTCATGCGTGTAGTAGCCA	AAGCGCACTTAACAGAAGAGGG	60
391	wmc486	CCGGTAGTGGGATGCATTTT	ATGCATGCTGAATCCGGTAA	56
392	gwm133	ATCTAAACAAGACGGCGGTG	ATCTGTGACAACCGGTGAGA	58
Chr6D				
393	cfd49	TGAGTTCTTCTGGTGAGGCA	GAATCGGTTCACAAGGGAAA	56
394	cfd135	GGATCTCGGGGATGTCCT	TAAGCACCTTCTTCATGGGG	56
395	barc173	GGGGATCCTTCAACAATAACA	GCGAGATGGCATTTTTAAATAAAGAGAC	57
396	cfd13	CCACTAACCAAGCTGCCATT	TTTTTGGCATTGATCTGCTG	54
397	wmc749	GGGTACAGGAGGATCTGACAGG	TCTCGTCTCCGTCTAGGTTCG	63
398	cfd132	CAAATGCTAATCCCCGCC	TGTAAACAAGGTCGCAGGTG	56
399	barc54	GCGAACAGGAGGACAGAGGGCACGAGAG	GCGCTTTCCCACGTTCCATGTTTCT	67
400	cfd287	TCAAGAAGATGCGTTCATGC	GGGAGCTTTCCCTAGTGCTT	56
401	wmc469	AGGTGGCTGCCAACG	CAATTTTATCAGATGCCCGA	52
402	wmc786	GGGTCACCAACCCGCTC	CGTGGGTGCAATTCTCAGG	59
403	barc1121	GCGAGCAAACTGATCCCAAAAAG	TATCGGTGAGTACGCCAAAAACA	61
404	barc175	GCGTAACAGAAGCGGAGAAAGC	GCGAATCATTTAGTGTTAGGTGGCAGTG	64
405	barc96	AAGCCTTGTTGTTCCGTATTATT	GCGGTTTATATTTTGTGGTTGAGCATTTT	58
406	gdm132	ACCGCTCGGAGAAAATCC	AGGGGGGCAGAGGTAGG	56
407	gdm98	CCATCCATGAAATGGCG	GCCCTTCACTAGCCTTCATG	50
Chr7A				
408	wmc158	AACTGGCATCATGTTTTGTAGG	AATGTAGTCAAAAGAGGTGGTG	60
409	gwm350	ACCTCATCCACATGTTCTACG	GCATGGATAGGACGCCC	54
410	gwm471	CGGCCCTATCATGGCTG	GCTTGCAAGTTCCATTTTGC	56
411	wmc479	GACCTAAGCCCAGTGTCATCAG	AGACTCTTGGCTTTGGATACGG	66
412	wmc168	AACACAAAAGATCCAACGACAC	CAGTATAGAAGGATTTTGAGAG	58
413	gwm60	TGTCCTACACGGACCACGT	GCATTGACAGATGCACACG	58
414	cfa2049	TAATTTGATTGGGTCGGAGC	CGTGTCGATGGTCTCCTTG	56
415	barc127	TGCATGCACTGTCCTTTGTATT	AAGATGCGGGCTGTTTTCTA	56
416	cfa2028	TGGGTATGAAAGGCTGAAGG	ATCGCGACTATTCAACGCTT	56
417	barc64	GCG GAG TCT GCA ATT AGT ATA GGT AT	GCA TCC ACC TCC GCA GTC AGT	65
418	wmc826	GAGGTAGATGACCACGCCG	CACGATCCCCCAAGCAC	57
419	barc174	TGGCATTTTTCTAGCACCAATACAT	GCGAACTGGACCAGCCTTCTATCTGTTC	61
420	barc108	GCGGGTCGTTTCCTGGAAATTCATCTAA	GCGAAATGATTGGCGTTACACCTGTTG	68
421	barc121	ACTGATCAGCAATGTCAACTGAA	CCGGTGTCTTTCCTAACGCTATG	59
422	barc29	GCACGCAGGAGCACCACCACGAC	GCGAGAGTAAGCAGCACCGAGGCACGAC	72
423	gwm282	TTGGCCGTGTAAGGCAG	TCTCATTCACACACAACACTAGC	52
424	wmc633	ACACCAGCGGGGATATTTGTTAC	GTGCACAAGACATGAGGTGGATT	63
425	wmc525	GTTTGACGTGTTTGCTGCTTAC	CTACGGATAATGATTGCTGGCT	60
426	cfa2040	TCAAATGATTTCAGGTAACCACTA	TTCCTGATCCCACCAAACAT	56
427	wmc809	CAGGTCGTAGTTGGTACCCTGAA	TGAACACGGCTGGATGTGA	57
428	barc275	GCG TTT GGT CAG AAT AGG GAA GAT	GCG TAT GTT CGT GTT AGT GTT GGT TAT GC	64
429	gwm130	AGCTCTGCTTCACGAGGAAG	CTCCTCTTTATATCGCGTCCC	60
430	wmc9	AACTAGTCAAATAGTCGTGTCCG	GTCAAGTCATCTGACTTAACCCG	61
431	gwm332	AGCCAGCAAGTCACCAAAAC	AGTGCTGGAAAGAGTAGTGAAGC	56
432	wmc139	TGTAACTGAGGGCCATGAAT	CATCGACTCACAACTAGGGT	56
433	wmc603	ACAAACGGTGACAATGCAAGGA	CGCCTCTCTCGTAAGCCTCAAC	62

Chr7B				
434	gwm569	GGAAACTTATTGATTGAAAT	TCAATTTTGACAGAAGAATT	48
435	barc65	CCCATGGCCAAGTATAATAT	GCGAAAAGTCCATAGTCCATAGTCTC	54
436	barc72	CGTCCTCCCCCTCTCAATCTACTCTC	CGTCCCTCCATCGTCTCATCA	63
437	barc176	GCGAAAGCCATCAAACACTATCCAACT	GGTAACTAAGCACGTCACAAGCATAAA	65
438	barc278	GCATGCACTACGCTCAGAATAAAC	TAAAAGGCCCGTCAACATACAAGTA	63
439	gwm68	AGGCCAGAATCTGGGAATG	CTCCCTAGATGGGAGAAGGG	56
440	barc85	GCGAACGCTGCCCGGAGGAATCA	GCGTCGCAGATGAGATGGTGGAGCAAT	70
441	wmc476	TACCAACCACACCTGCGAGT	CTAGATGAACCTTCGTGCGG	60
442	gwm333	GCCCGGTCATGTAAAACG	TTTCAGTTTGCGTTAAGCTTTG	54
443	cfa2106	GCTGCTAAGTGCTCATGGTG	TGAAACAGGGGAATCAGAGG	58
444	wmc540	CGGGGTCCTAACTACGGTGA	CCTGTAATGGAGGACGGCTG	63
445	wmc517	ATCCTGACGTTACACGCACC	ACCTGGAACACCACGACAAA	58
446	wmc792	GGATGCAGTAGCAGTCAGGGA	CTCCATCGCTAGGCAGGG	61
447	barc20	GCGATCCACACTTTGCCTCTTTTACA	GCGATGTCGGTTTTCAGCCTTTT	63
448	wmc557	GGTGCTTGTTCATACGGGCT	AGGTCCTCGATCCGCTCAT	59
449	barc123	GGCCGAATTGAAAAAGCC	CCTGCCGTGTGCCGACTA	52
450	gwm146	CCAAAAAAACTGCCTGCATG	CTCTGGCATTGCTCCTTGG	56
451	gwm344	CAAGGAAATAGGCGGTAACT	ATTTGAGTCTGAAGTTTGCA	52
452	wmc398	GGAGATTGACCGAGTGGAT	CGTGAGAGCGGTTCTTTG	56
453	wmc273	AGTTATGTATTCTCTCGAGCCTG	GGTAACCACTAGAGTATGTCCTT	61
454	wmc323	ACATGATTGTGGAGGATGAGGG	TCAAGAGGCAGACATGTGTTCG	62
455	wmc396	TGCACTGTTTTACCTTCACGGA	CAAAGCAAGAACCAGAGCCACT	60
456	wmc10	GATCCGTTCTGAGGTGAGTT	GGCAGCACCCTCTATTGTCT	58
457	wmc526	TCCCATTGGTTCACAAACTCG	GATGGTATCGCATTCATCGGT	59
458	wmc70	GGGGAGCACCCTCTATTGTCTA	TAATGCTCCCAGGAGAGAGTCG	64
Chr7D				
459	wmc646	GGAGTAAATGGAGACGGGGAC	GCCAGTGTGATGCATGTGAC	60
460	barc154	GTAATTCCGGTTCCACTTGACATT	GGATGGGCAGCTTCAAGGTATGTT	62
461	barc352	CCCTTTCTCGCTCGCCTATCCC	CTGTTTCGCCCAATCTCGGTGTG	66
462	wmc450	GCAGGACAGGAGGTGAAGAAG	AGGCGTTGCTGATGACACTAC	61
463	barc126	CCATTGAAACCGGATTTGAGTCG	CGTTCCATCCGAAATCAGCAC	61
464	cfd41	TAAAGTCTCAGGCGACCCAC	AGTGATAGACGGATGGCACC	60
465	barc214	CGCTTTCGGGACAGTGAAGGTGTAT	CGGTACGCGCGAGGAGGAAGAAGG	67
466	gdm88	TCCCACCTTTTTGCTGTAGA	AAGGACAAATCCCTGCATGA	56
467	wmc606	CCGATGAACAGACTCGACAAGG	GGCTTCGGCCAGTAGTACAGGA	64
468	barc26	GCGCTGGGTAAAAAGTGAAATTC	TGCAAGTGGAGGGGGAGGCGAGAG	61
469	barc87	GCTCACCGGGCATTGGGATCA	GCGATGACGAGATAAAGGTGGAGAAC	65
470	barc172	GCGAAATGTGATGGGGTTTATCTA	GCGATTTGATTTAACTTTAGCAGTGAG	62
471	barc105	CAGGAAGAAAAGGAAAGCATGCGACAA	GCGGTGTGGCAATAATTACTTTTT	60
472	barc111	GCGGTCACCAGTAGTTCAACA	GCGTATCCCATTGCTCTTCTTCACTAAC	61
473	wmc488	AAAGCACAACCAGTTATGCCAC	GAACCATAGTCACATATCACGAGG	60
474	gwm121	TCCTCTACAAACAAACACAC	CTCGCAACTAGAGGTGTATG	54
475	barc235	GCGCTCACCCTCCTACACTTCCTA	GCGCAAGTCTGTCAAAGCCTAA	62
476	cfd25	CATCGCTCATGCTAAGGTCA	CGTGTCTGTTAGCTGGGTGG	58
477	wmc824	CCGATGAACTTAAAAGTACCACCTG	CATGGATTGACACGATTGGC	58
478	barc53	GCGTCGTTCCTTTGCTTGTACCAGTA	GCGCGTCCTTCCAATGCAGAGTAGA	68
479	cfd69	AAATACCTTGAATTGTGAGCTGC	TCTGTTCATCCCCAAAGTCC	58
480	wmc14	ACCCGTCACCGGTTTATGGATG	TCCACTTCAAGATGGAGGGCAG	64
481	cfd175	TGTCGGGGACACTCTCTCTT	ACCAATGGGATGCTTCTTTG	56
482	gdm86	GGTCACCCTCTCCCATCC	GGCGCTCCATTCAATCTG	54
483	gwm295	GTGAAGCAGACCCACAACAC	GACGGCTGCGACGTAGAG	60

Publications

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1. **Rawat, N.**, Tiwari, V.K., Singh, N., Randhawa, G.S., Singh, K., Chhuneja, P. and Dhaliwal, H.S. 2008. Evaluation and utilization of *Aegilops* and wild *Triticum* species for enhancing iron and zinc content in wheat. *Genet. Res. Cr. Evol.* DOI: 10.1007/s10722-008-9344-8.

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9. **Rawat, N.**, Tiwari, V.K., Singh, N., Kumar, M., Randhawa, G.S., and Dhaliwal, H.S. Phytate analysis in wheat germplasm and their products, in International Conference on Plant Genetics and Biotechnology. Raipur, India (November, 2005).

10. **Rawat, N.**, Tiwari, V.K., Singh, N., Randhawa, G.S., Singh, K., Chhuneja, P., Dhaliwal, H.S. Development and characterization of wheat-*Aegilops kotschyi* amphiploids with high grain iron and zinc. 2008. Plant Genet. Resour. (Communicated).

11. **Rawat, N.**, Tiwari, V.K., Neelam, K., Randhawa, G.S., Singh, A.M., and Dhaliwal, H.S. 2008. Scanning Electron Microscope – Energy Dispersive X ray analysis of phytic acid and minerals and nutritional changes in germinating wheat grains. (Communicated).

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15. **Rawat, N.**, Tiwari, V.K., Singh, N., Randhawa, G.S., Singh, K. and Dhaliwal, H.S. 2008. Identification and characterization of two novel low phytic acid mutants of *Triticum monococcum.* (Communicated).

16. **Rawat, N.**, Tiwari, V.K., Singh, N., Randhawa, G.S., Singh, K. and Dhaliwal, H.S. Molecular and *in situ* hybridization based characterization of introgressive derivatives of *Ae. kotschyi* with high grain iron and zinc content. (Manuscript under preparation)

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