INTROGRESSION OF GENES FOR HIGH GRAIN Fe AND Zn OF GROUP 2 CHROMOSOME OF *Aegilops* INTO WHEAT

Ph.D. THESIS

by

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INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE - 247 667 (INDIA)
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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **INTROGRESSION OF GENES FOR HIGH GRAIN Fe AND Zn OF GROUP 2 CHROMOSOME OF *Aegilops* INTO WHEAT** in partial fulfillment 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 January, 2009 to October, 2013 under the supervision of Dr. H.S. Dhaliwal, (Retired) Professor, Department of Biotechnology and Dr. R. Prasad, Professor, 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.

(SHAILENDER KUMAR VERMA)

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

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Dated: October, 2013

The Ph.D. Viva-Voce Examination of **Mr. SHAILENDER KUMAR VERMA**, Research Scholar, has been held on.. .

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ABSTRACT

In the developing world, more than 3 billion peoples are affected by iron and zinc deficiency. Deficiency of Fe leads to impaired physical growth, anaemia, mental retardation, weak learning capacity and ability to do physical work. While Zn pays important role in normal growth and development, maintenance of body tissues, cognitive ability, vision, immune system and cofactor for more than 300 enzymes. Micronutrient deficiency can be alleviated by diet diversification, supplementation, fortification and biofortification. However biofortification is the most sustainable and effective approach for improving nutritional quality of crop plants. There are several approaches for biofortification of crops including agronomic fortification conventional and molecular breeding and genetic engineering.

Wheat is the primary staple food for majority of global population and accounts for major proportion for daily dietary calorie intake. Acid digestion of seeds of parents and derivatives were done for mineral micronutrient analysis through atomic absorption spectrophotometer (AAS) and inductively coupled plasma mass (ICPMS). Most of the wheat cultivars are low in mineral micronutrient content. Several *Aegilops species* had 2-3 folds higher Fe and Zn content in comparison to elite wheat cultivars and can be utilized for biofortification of wheat. Several addition and substitution lines of 2U or 2S chromosome of *Ae. kotschyi* with high grain Fe and Zn content have been developed. The linkage drag with low yield and harvest index is the major bottleneck for their exploitation. Precise transfer of useful variability from wheat- *Ae. kotschyi* addition lines into elite wheat cultivars can easily be achieved by induced homoeologous pairing by seed and pollen irradiation, *ph1b* deletion and 5B deficiency.

A total of 189 anchored wheat SSR microsatellite molecular markers specific for group 2 chromosomes were used for analysis of their transferability and polymorphism between genomic DNA of three bread wheat cultivars and six accessions of *Aegilops species.* A total of 143 markers (75.66%) mapped on group 2 chromosomes of wheat were transferable among all the selected *Aegilops species*. Polymorphism varied from 37-77% for 2A, 2B and 2D chromosome markers. Among the polymorphic transferable markers, there were 27 markers for chromosome 2A, 22 markers for chromosome 2B and 40 markers for chromosome 2D were polymorphic in all the selected *Aegilops species*. The high transferability of D genome specific marker indicates greater similarity of D genomes with S and U genome than those to the A and B genomes. A tentative consensus map of A, B, D,U and S genomes was prepared by BioMercator V3.0 These polymorphic markers were highly informative, reliable and useful for molecular characterization of the introgressed derivatives.

All the five selected wheat-*Aegilops* derivatives with introgression of group 2 chromosomes from *Aegilops species* showed consistently high grain Fe and Zn content. Most of the derivatives (except 77-50-8-1) had non-waxy leaf sheaths, a character controlled by group 2 chromosome indicating the introgression of group 2 chromosome of *Ae. kotschyi*. Wheat-*Aegilops* derivatives 49-1-73 had also shown introgression of 1U/1S, a confirmed through HMW- glutenin subunit profiling of seeds. 46-1-15-15 with 22 bivalents had brittle rachis and red seed colour indicating disomic addition of chromosome 3^k in addition to $2S^k$. All the derivatives showed ~70-90% higher grain Fe and Zn indicating that the introgressed chromosomes were responsible for high grain Fe and Zn. These derivatives were further used for induced homoeologous pairing through *ph1b* mutants, mono 5B approach or irradiation induced transfers

Seeds of wheat-*Aegilops* addition/substitution lines for group 2 chromosomes were irradiated at 40 krad of gamma radiation. In the introgressed derivatives through seed irradiation (SRH_3, WL711/77-36-2⊗), the maximum concentration of grain iron was found in SRH_3-62-1 (43.34±1.02 mg/kg) and zinc content 60 ±1.08 mg/kg in SRH_3-115-1. GISH analysis of A-108 and A-115 showed several translocations of $2S^k$ in wheat genome. A-108 was waxy plant indicating translocation of long arm of $2S^k$.

For pollen irradiation, spikes of wheat-*Aegilops* addition/substitution lines with introgression of group 2 chromosomes were irradiated at 2 krad of gamma radiation and crossed with elite wheat cultivar PBW343. Among the pollen irradiated (PRH_2, PBW343/49-1-73⊗) plants the maximum concentration of grain iron (64.47±3.17 mg/kg) and zinc (88.42±3.04 mg/kg) was found in B-45-1.HMW glutenin subunit profiling of all the pollen irradiated hybrids were showed introgression of 1U/1S except PRH_1-14, PRH_1-48, PRH_1-52, PRH_1-97 and PRH_1-1 progenies. GISH analysis of B-52 and B-56 showed several translocations of U and S chromosome. However absence of chromosome 1 introgression confirms the presence of only 2S and 2U in B-52. Waxy leaf sheaths of B-52 plants also indicates presence of long arm of $2S^k$, as the gene for waxiness trait has been mapped on the short arm of group 2 chromosomes.

As an alternative strategy for gene transfer, 2U/S wheat-*Aegilops* derivatives were crossed with *ph1bph1b* mutant and the F_1 plants were further backcrossed with *ph1b* mutant. The BC_1F_1 plants homozygous for *ph1bph1b* were screened through *Ph1* locus specific markers psr574 and psr2120. The shrivelled seeds and leaf yellowing seemed to be associated with the presence of *ph1b* mutant. Group 2 chromosome specific SSR markers gwm265, barc349, barc11, gwm539 and gwm71 were used for monitoring introgression of group 2 chromosome

into the BC_1F_1 plants homozygous for *ph1b* mutant. The BC_1F_2 plants were screened for introgression of fragment(s) of group 2 chromosome and grain iron and zinc content. The BC_1F_3 seeds were mostly shrivelled and plants had yellow leaves. Only a few plants (PH-34, PH-110, PH-199, PH-208, PH-301 and PH-305) having harvest index equivalent to that of PBW343 and 40-65% increase in grain iron and zinc content were selected for further propagation and analysis. There was no translocation was detected through GISH analysis, indicating transfer of small fragment beyond the GISH resolution.

The mono 5B plants of *T. aestivum* cv. Pavon were cytologically identified and crossed with *Aegilops species* as the male parent and the F_1 plants were analyzed by molecular markers, psr574 and psr128, for the absence of chromosome 5B. These plants were further confirmed for homeologous pairing by cytological analysis. The F_1 plants without 5B having 34 chromosomes showed high chromosome pairing up to 2V+4III+2II+1I, while the plants with 5B having 35 chromosomes, had highly reduced homeologous pairing, 6II+23I. The plants with 34 chromosomes without 5B were selected and backcrossed extensively with wheat cultivar PBW343 for the transfer of useful variability of *Aegilop*s for micronutrient biofortification.. Fe and Zn content of mono5B BC_1F_2 and BC_2F_1 plants ranged from 43-84 mg/kg and 53-96 mg/kg, respectively. The chromosome number of BC_1F_2 and BC_2F_1 plants ranged from 43-60 with several univalents and multivalent formation. Fertile derivatives will be screened for high grain Fe and Zn content and backcrossed with recurrent wheat cultivar for maximum background recovery

All the selected wheat-*Aegilops* derivatives with translocation of 2S/2U had better system for uptake, transport and translocation. However the overall nutrient content of these derivatives were less than the donor *Aegilops species*. The biofortification of wheat for grain Fe and Zn content could be achieved up to 40-60%, without any linkage drag. Tagging, localization and pyramiding of the introgressed genes/ QTLs for grain micronutrient content in wheat could be achieved through biofortification of wheat for grain Fe and Zn content.

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-Psalm 28:7; Holy Bible

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Date: **SHAILENDER KUMAR VERMA**

ABBREVIATIONS USED

Abbreviation	Extended from
%	Percentage
⊗	Bulk
µ	Micron
µg	Microgram
♀	Female
♂	Male
AAS	Atomic Absorption Spectrometer
AFLP	Amplified Fragment Length Polymorphism
BAC	Bacterial Artificial Chromosome
BC	Backcross
BC_1	First backcross generation
BC_2	Second backcross generation
bp	Base Pair
CAPS	Cleaved amplified polymorphic sequence
cDNA	Complimentary DNA
CGIAR	Consultative Group of International Agricultural Research
CIMMYT	Centro Internacional de Mejoramiento de Maíz y Trigo
cM	CentiMorgan
cR	CentiRay
CS	Chinese Spring
CTAB	Cetyl Trimethyl Ammonium Bromide
Cu	Copper
cv.	Cultivated Variety
DArT	Diversity Array Technology
DMA	2-Deoxy mugineic acid
DNase	Deoxyribonuclease
dNTPs	Deoxy Nucleotide Triphosphates
EDTA	Ethylene Diamine Tetraacetic Acid
epi-HDMA	epihydroxy-2hydroxy mugineic acid
epi-HMA	3-epi-hydroxymugineic acid

EST	Expressed Sequence Tag
EtBr	Ethidium Bromide
F_1	First Filial Generation
F_2	Second Filial Generation
F_3	Third Filial Generation
FAO	Food and Agriculture Organisation
Fe	Iron
Fig.	Figure
FISH	Fluorescent *in situ* Hybridization
g	Gram
GGT	Graphical Genotyping
GISH	Genome *in situ* Hybridization
GPC	Grain Protein Content
HMA2	Heavy Metal Transporter Atpase
HMW-GS	High Molecular Weight Glutenin Subunit
ICP-MS	Inductively Coupled Plasma Mass Spectrometer
IRT	Iron Regulatory Transporters Proteins
Kb	Kilobase pairs (10^3 bp)
KCl	Potassium Chloride
kg	Kelogram
L	Litre
M	Molarity
MA	Mugineic acid
MAS	Marker Assisted Selection
Mb	Megabase pairs (10^6 bp)
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimole
mm	Millimetre
mRNA	Messenger Ribonucleic Acid
MTP	Metal tolerance proteins

NAAT	Nicotianamine Amino Transferase
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NAS	Nicotianamine Amino Synthase
ng	Nanogram
NRAMP	Natural Resistance Associated Macrophage Protein
ºC	ºCentigrade
PAGE	Poly-Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
Ph 1	Pairing homoeologous 1
ph 1b	*Ph 1* deletion
Ph^I	Pairing homoeologous inhibitor
PMCs	Pollen mother cells
ppm	Parts Per Million
PRH	Pollen irradiated hybrids
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RDA	Recommended dietary allowance
RFLP	Restriction Fragment Length Polymorphism
RIL	Recombinant inbred line
RNase	Ribonuclease
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacryamide Gel Electrophoresis
sec	Second
SEM-EDX	Scanning Electron Microscopy-Energy Dispersive X Ray analysis
SNPs	Single Nucleotide Polymorphism
SRH	Seed Radiation Hybrid
SSC	Sodium Citrate
SSCP	Single Strand Conformation Polymorphism
SSR	Simple Sequence Repeats
STMS	Sequence Tagged Microsatellite Site
STS	Sequence Tagged Sight
TAE	Tris Acetate
TBE	Tris Borate
TE	Tris EDTA

TEMED	Tetramethylene diamine
T_m	Melting Temperature
U	Units
v/v	Volume/Volume
VIT1	Vaculor Iron Transporter
w/v	Weight/Volume
WHO	World Health Organisation
YS	Yellow Stripe
YSL	Yellow Strip Like
ZIP	Zinc regulated- Iron regulated transporter Proteins
Zn	Zinc
γ radiation	Gamma radiation

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1. Introduction

Iron and zinc deficiency also known as 'Hidden hunger' affects more than half of the world's population. The high frequencies of micronutrient deficiency have several health and economic impacts. Marginal intake of iron contributes to increased morbidity and mortality, mental retardation, diminished livelihood, week stamina and adverse effect on learning capacity. Iron deficiency is the major risk factor for more than 841000 deaths and 35057000 disability adjusted life year lost (Caballero, 2002; Stoltzfus, 2003; Gitlin, 2006). Zinc deficiency is also a major risk factor affecting more than two billion people. Zinc plays necessary role in several enzyme systems, proper growth, development and maintenance of body tissue, immune system, vision and reproductive functions. Micronutrient malnutrition is currently at alarming stage especially in developing nations and requires immediate steps to alleviate it (WHO, 2002).

There are several approaches to overcome micronutrient deficiency including diet diversification, micronutrient supplementation, fortification and bio-fortification (Brinch-Pederson *et al.,* 2007). Diet diversification is very efficient strategy to cure hidden hunger but most of the nutrient rich diets are costly and un-affordable to poor people. Supplementation strategy requires therapeutic administration of iron and zinc rich syrup or pills. Fortification mainly relies on addition of minerals in food items. All the above three approaches require careful administration and recurring expenses. The best and sustainable approach alleviate micronutrient deficiency is biofortification as it requires only one time investment on development and production of biofortified crop (Welch and Graham, 2004). Crops can be either agronomically biofortified by application of extra micronutrient on plant rhizosphere, by genetic engineering or by conventional and molecular breeding methods. The mechanisms of mineral uptake, transport and sequestration and are very complex and are poorly understood till date. Biofortification through conventional and molecular breeding techniques is the most sustainable, targeted, cost effective and best approach for improving nutritional traits of staple crops. Bio-fortification of cereal crops depends on efficient uptake, translocation and sequestration of mineral micronutrients into edible part of the plant. Dicotyledonous plants rely on reduction based strategy for mineral micronutrient uptake where Fe^{3+} is reduced to Fe^{2+} by excretion of proton cloud in the rhizosphere (Olsen, 1981). While on the other hand the graminaceous plants on chelation based strategy where the phytosiderophores (Mugineic acids) helps in the absorption and uptake of mineral micronutrients (Marschner *et al*., 1986; Ueno *et al*., 2009).

Consultative Group of International Agricultural Research (CGIAR) and HarvestPlus Challenge had initiated biofortification of crop program with special emphasis on three critical micronutrient namely Fe, Zn and Vitamin-A. Mapping of QTLs for several nutritional traits in many plants, whole genome sequences, high density molecular maps and SSR markers can be method of choice for biofortification of crops through conventional and molecular breeding techniques.

Wheat is the staple food for more than one third of the world population. Wheat provides 50% of calorie intake of Asian peoples, which are worst affected by micronutrient deficiency. Most of the elite wheat cultivars are poor in grain micronutrient content. Several wild progenitor and non-progenitor species of wheat have been used for development of alien addition, translocation and substitution lines for transfer of several useful traits into elite wheat cultivars (Raupp *et al*., 1995; Friebe *et al*., 2000; Qi *et al*., 2007). The related wild *Triticum* and *Aegilops species* have useful variability for high grain iron and zinc content (Cakmak *et al*., 2000; Chhuneja *et al*., 2006; Rawat *et al*., 2009). The QTL for grain Fe was mapped on short arm of chromosome 2A (Tiwari *et al*., 2009). Wheat-*Aegilops* derivatives with introgression of group 2 and 7 chromosome from *Aegilops* into wheat enhance grain iron, zinc and protein content (Rawat *et al*., 2011; Tiwari *et al*., 2010).

Among various approaches of precise transfer of useful variability from non-progenitor species and derivatives into wheat through induction of homoeologous pairing with Ph^I, absence of chromosome 5B and *ph1b* deletion mutant are very effective (Sears 1977; Chen *et al*., 1994; Aghaee-Sarbarzeh *et al*., 2002). Several disease resistance genes including *Sr39, Lr9* and *Pm21* were transferred into wheat through induced homoeologous pairing approaches (Sears *et al*., 1956, Yu *et al*., 2010; You *et al*., 2012).

Radiation hybrid (RH) approach relying on radiation induced chromosome breakage and analysis of loss and retention of alien chromosome fragments through molecular markers. It is an alternative approach for gene transfer, tagging and mapping. Pollen and seed radiation method is very useful for precise gene transfer and suitable method for stable introgression of alien chromosome fragment into elite wheat cultivars (Snape *et al*., 1983; Hossain *et al*., 2004; Chen *et al*., 2012).

High density genetic microsatellite map of wheat (Somers *et al.*, 2004), modern cytological techniques like GISH and FISH and efficient analytical techniques like inductively coupled plasma mass spectrophotometer (ICPMS) can be used for precise transfer and tagging of genes

responsible for grain mineral micronutrient content. For precise transfer and introgression of genes/ chromosomal fragments, wheat-*Aegilops* derivatives (Tiwari *et al*., 2010; Rawat *et al*., 2010) with 2U/2S were used in induced homoeologous pairing approach and irradiation mediated transfer of genes. Pedigree of several wheat-*Aegilops* derivatives which were further backcrossed with *ph1bph1b* and elite wheat cultivars for induced homoeologous pairing and irradiation mediated gene transfer in Table 1.1.

Keeping in view the above facts, the present study aims at the transfer of genes responsible for high grain micronutrient from *Aegilops species* and their derivatives into elite wheat cultivars with following objectives-

- Identification of group 2 *Aegilops* chromosome introgression lines with high grain Fe and Zn through molecular and cytological techniques.
- Induced homeologous pairing between group 2 *Aegilops* and wheat chromosomes for precise transfer of useful variability.
- Radiation induced transfer of genes for high grain Fe and Zn content.
- Biochemical analysis of introgressed derivatives with high grain micronutrients.
- Molecular mapping and tagging of introgressed *Aegilops* genes for high grain iron and zinc content.

2. Review of Literature

2.1. Micronutrient malnutrition in humans

Micronutrient deficiency is serious public health concern which affects more than three billion people worldwide leading to malnutrition syndromes (Welch and Graham, 2004; Bouis, 2007). Micronutrient deficiencies particularly iron and zinc in diet is known as “hidden hunger” (Poletti *et al.*, 2004; White and Broadley, 2005, Palmgren, 2008). Dietary deficiency of micronutrient including iron, zinc, selenium, calcium, iodine and vitamin A has serious health implications especially in developing countries (Demment *et al.*, 2003; Holtz and Brown, 2004; Bhaskaram, 2008; Ramakrishnan *et al.*, 2009).

According to reports of “Hunger Portal” of Food and Agricultural Organization of the United Nations (FAO) in the year 2010-2012, total world population is estimated to be 6,953.3 million with 868 million peoples undernourished (13% prevalence of under nourishment) while total population of India was 1,241.5 million with 217 million peoples under nourished (18% prevalence of under nourishment) (http://www.fao.org/hunger/en/). The under nourishment in 2010-12 by region and in developing countries has been shown in Fig. 2.1

More than 30% of world population has been severely affected by iron deficiency mainly affecting children (47.4%), pregnant women (41.8%) and non pregnant women (30.2%) (FAO/WHO, 2001; Mclean *et al*, 2008). Iron deficiency during pregnancy causes pregnancy complications, maternal death, birth defects, low birth weight (LBW) (Pathak *et al*. 2004; Stoltzfus, 2004; Monasterio *et al*, 2007). Clinical or subclinical micronutrient deficiency may affect growth, cognition and reproductive performance (Sesadri, 2001).

In India, 70-80% children, 70% pregnant women and 24% adult men were found to be anemic due to iron deficiency (WHO, 2007). Low intake of dietary iron results in higher morbidity and mortality rates, prenatal birth defects, impairment in cognitive skills and physical strength and adverse affects on neuropsychological functions (Stein, 2005; Barbin *et al.*, 2001).

Zinc deficiency causes serious health problems including poor physical growth, immune system, learning capacity, reproductive ability and adverse effect on mother and child during pregnancy. According to World Health Organization (WHO) reports zinc deficiency accounts 11th in world and 5th in developing countries as the major risk factor causing disease burden in humans (Cakmak, 2008). Zinc deficiency is major cause for more than 8,00,000 child death

worldwide (Micronutrient Initiative, 2006). Micronutrient deficiency has prolonged effect on entire human life cycle as shown in Fig. 2.3.

The non-diversified diet of developing countries contains mainly starch rich cereals, roots tubers, banana and plantain food for calorie requirements and lag far behind the recommended dietary allowance (RDA) in terms of micronutrients (FAO, 2004). WHO has declared RDA for men, iron as 10 mg and zinc as 15 mg and for women, iron as 15 mg and zinc as 12 mg in the age group of 25-50 years (FAO/WHO, 2000).

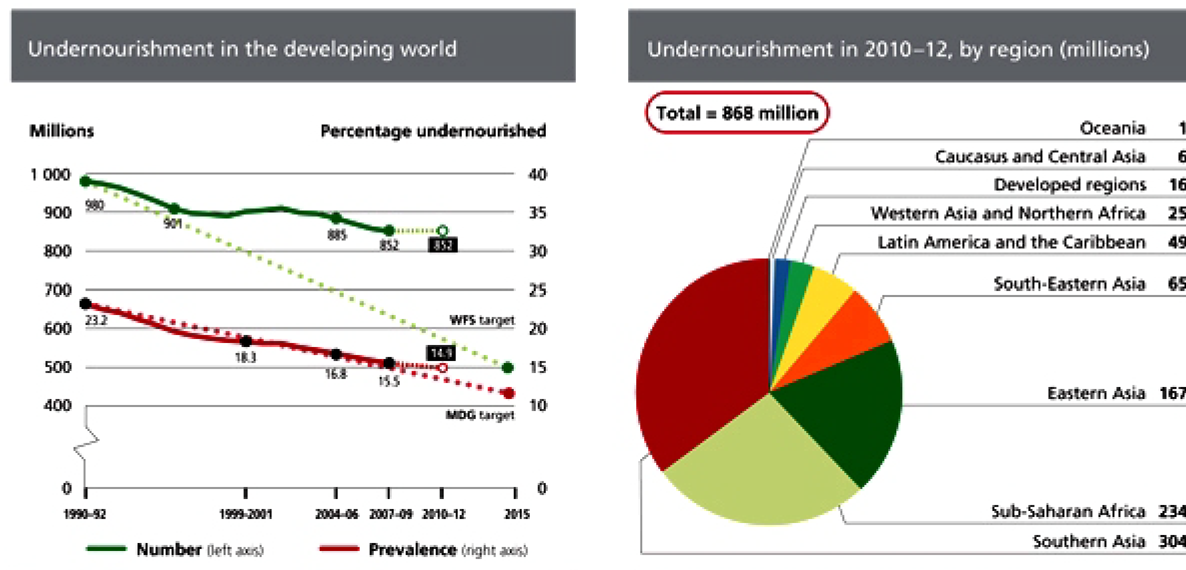


Fig. 2.1 Trait and region wise undernourishment in the developing world in 2010-12, (millions); Source: FAO: Hunger portal

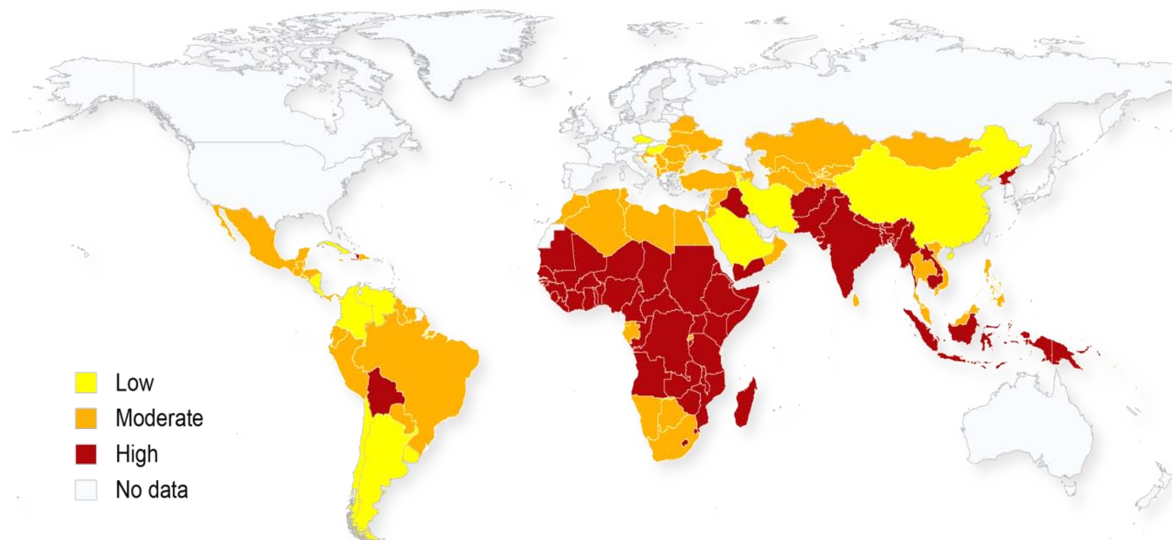


Fig. 2.2 Effect of micronutrient malnutrition across globe (FAO. 2012)

2.1.1. Role of iron and zinc in plants

Iron is very essential micronutrient and is involved in various plant metabolic reactions including most of the redox processes of electron transport chain, photosynthesis (photosystem I and II) and respiration reactions, chlorophyll synthesis, nitrogen fixation (Garrido *et al.*, 2006; Kim and Guerinot, 2007). Although iron is the fourth most abundant mineral in earth crust but constitutes only 0.005% of plant mass (Graham and Welch, 2002; Meng *et al.*, 2005). Most of the iron is present in the form of oxides, hydroxides, phosphates and other complex forms in plants.

Zinc is very important mineral for plant and is involved as co-factor in nearly 300 enzymes and play very crucial role in the structure of many proteins and gene regulatory elements (Hershfinkel, 2006; Krämer and Clemens, 2006). About one third of the world's cereal growing area is iron deficient with high soil pH and half has zinc deficient soil (Mori, 1999; Cakmak, 2002). Inefficient micronutrient mineral uptake due to calcareous or salt stressed alkaline soil results in severe loss in yield, plant growth and nutritional quality of grains (Brown, 1961; Mori, 1990, Cakmak, 2008). In these mineral deficient soils with abiotic stress conditions, plants show high susceptibility to environmental stress including drought stress, pathogenic infections, stunted growth and leaf necrosis. Grain micronutrient depends on amount of mineral uptake from soil to roots during developmental stages, their remobilization and distribution in grains from the vegetative tissues via phloem. The mobility of each mineral element differs significantly from each other in the phloem tissues. It has been found than Zn shows good mobility, Fe has intermediate mobility and Copper (Cu) and Manganese (Mn) have lower mobility in the phloem tissues (Kochian, 1991; Pearson and Rengel, 1994). It has been found that in wheat and rice only 4 % to 5% of shoot iron is being translocated into grain at maturity level (Hocking, 1994; Marr *et al.*, 1995). It is a necessity for further detailed and focused studies of the genes, proteins and metabolites involved in metal uptake, transport and translocation in plant. Higher efficiency for uptake, translocation and storage are high priority areas of research for biofortification of cereal crops.

2.1.2. Role of iron and zinc in humans

Iron is very essential mineral required in several vital functions in all living organisms including several metabolic processes, electron transport, oxygen transport and DNA synthesis (Lieu *et al.*, 2001). Fe plays significant role in hematopoiesis (Red blood cell production), hemoglobin formation and conversion of blood sugar to energy, regulation of metabolic energy, immune system, normal brain development, muscle development and proper growth

and development of body. With respect to absorption mechanism, dietary iron is classified a two types of iron: heme iron and non-heme iron. Primary source of heme iron is haemoglobin and myoglobin from fish, poultry and meat products whereas cereals, legumes, fruits and vegetables are primary source of non-heme iron (Hallberg, 1981). There are three mechanisms to control iron balance and regulation of iron absorption. First mechanism is the continuous re-utilization of Fe from catabolised erythrocytes. Second mechanism is through iron storage protein ferritin to store and release iron especially in excessive iron demand conditions, menstrual cycle and pregnancy. Third mechanism involves regulation of iron absorption in intestinal tract by establishing equilibrium between absorption and requirement (Hallberg *et al*., 1998).

Zinc is present in all the tissues and cells of human body. Skeletal muscle constitutes 60% of total body mass with Zn concentration of 100 – 200 mg g-1 (approximately 30%) (Hambidge, 1987). Zn is an essential component of several enzymes (>300), play significant role in synthesis and degradation of carbohydrates, lipids, nucleic acid and proteins. Zinc also plays significant role in maintenance of cell and organ integrity by stabilizing molecular structure of cellular components and membranes. Zinc plays central role in several immune processes (Shakar and Prasad, 1998). The clinical features of zinc deficiency in humans are the stunted growth, poor bone maturation and fertility, skin lesions, alopecia, diarhoea, impaired appetite, defect in immune system and wound healing (Hambidge, 1987). Zinc metabolism and absorption are concentration dependent and occurs in small intestine (Sandström, 1997).

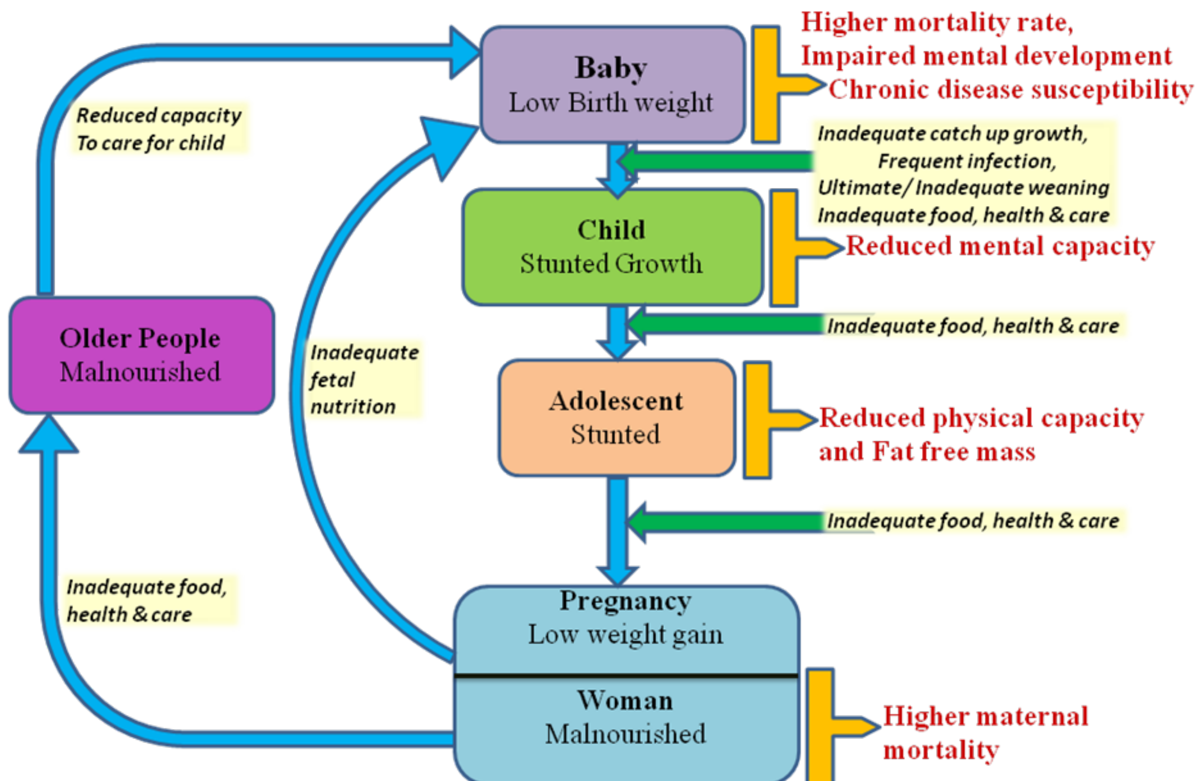


Fig. 2.3 Effect of micronutrient malnutrition in human life cycle.

2.1.3. Reasons for micronutrient deficiency and bioavailability of micronutrients

In the developing countries most of the dietary calories come from non-diversified carbohydrate rich food including rice, wheat, potato, maize and banana while fruits vegetables, milk and dairy products accounts negligible proportion (FAO, 2004). Most of the staple food crops have very low micronutrient content. Furthermore most of the micronutrients are present in the aleurone layer of the grain and various processing methods like milling, polishing etc. damage and remove the outermost micronutrient rich layer of grain resulting in nutrient poor diet. This nutrient poor diet is not sufficient and meet the recommended dietary allowance (RDA).

According to IRRI report (2006), it was found that polished rice contains only 2 mg/kg of Fe and 12 mg/kg of Zn whereas minimum RDA for Fe and Zn is 10-15 mg and 12-15 mg, respectively. Hence to fulfil RDA for better nutrition, cereal grain should contain around 40-60 mg/kg of Fe and Zn (Cakmak *et al.,* 2000).

In addition to nutrients content, there are several anti-nutrient factors present in the grains. These anti-nutrient factors are the major constrains for bioavailability of mineral micronutrients for proper human growth and development. The anti-nutrient factors such as

phytic acid, polyphenols, fibers, tannins, hemagglutinins reduce proper absorption of mineral micronutrients. Phytic acid chelates metal cations (iron, zinc, calcium and so on) and makes them unavailable by inhibiting there absorption as there is no phytase activity in human digestive track (Zimmerman and Hurrel, 2007; Campion *et al*., 2009). It was also reported that finger millet (having dark seed coat) have high tannin content which forms complexes with proteins and enzyme inhibitors leading to poor digestion of proteins in body (Antony and Chandra, 1998). There are certain organic acids, amino acids, heme-proteins, β-carotene and chemical compounds that can increase Fe and Zn bioavailability (Graham *et al.,* 2001). Bioavailability of micronutrients also depends on ionic forms of minerals such as ferric ions in ferritin proteins have relatively high bioavailability.

Majority of the agricultural land is Fe and Zn deficit (Mori, 1999; Cakmak, 2002). Zinc deficient soil covers major area in India, South East Asia, China and Australia (Alloway, 2007). Soil quality and Fe and Zn content have significant role in mineral micronutrient uptake and concentration in plants. This ultimately results in severe yield loss, stunted plant growth, poor grain quality and poor nutrition content of grains (Brown, 1961; Mori, 1990; Cakmak, 2008)

2.2. Wheat: An agriculturally important crop

Wheat (*Triticum spp.*) is the one of the major staple food crop of the world in terms of food source and cultivated area. Wheat alone contributes ~28% of world's edible dry matter and up to 60 % of daily calorie intake in several developing nations (Welch and Graham 2004; Distelfield *et al*., 2007). As wheat is staple food for over 35 % of the global population (Peng *et al*., 2004), its biofortification will help in combating the threat of hidden hunger.

2.2.1. Evolution of wheat

The hexaploid wheat, *Triticum aestivum* (2n(6x)=42) has three different genomes designated as A, B and D. Two separate natural amphiploidization events were occurred during the evolution of allohexploid wheat.

Diploid einkorn types of wheat are the oldest while the hexaploid wheat (*T. aestivum*) is recently evolved and latest step in the evolution of the wheat complex. Approximately 0.5 million years ago (MYA), after spontaneous chromosome doubling due to crossing of two wild diploid species, a wild tetraploid species *T. turgidum ssp dicoccoides* (BBAA) got created. *Triticum urartu* (AA) is considered as A genome donor of wheat (Dvörak *et al*., 1993)

while *Aegilops speltoides* (SS) is considered as the potential donor of B genome to the common wheat (Maestra & Naranjo, 1998). Tetraploid species (wild emmer wheat) *Triticum turgidum* ssp. *dicoccoides* (genomes BBAA) was evolved after the crossing of A and B genome donor.

These two diploids crossed and produced the wild tetraploid species, also known as wild emmer wheat. Bread wheat (*Triticum aestivum* L.) arose 8000- 10,000 years ago (Feldman, 1995) from the spontaneous hybridization of the tetraploid wheat *T. turgidum* L. (2n= 4×= 28, BBAA, genomes), with diploid goat grass *Triticum tauschii* Coss. (2n= 2×= 14, DD genomes) (Kihara, 1944; McFadden and Sears, 1946; Huang *et al.,* 2002; Jauhar, 2007).

2.2.2. Wheat taxonomy and its gene pool

Wild *Triticum*, *Aegilops* and related species are very important sources of numerous genes of agronomic importance including resistance against biotic and abiotic stresses. The genus *Aegilops* comprises 11 diploids, 10 tetraploids and 2 hexaploid species (Table 2.1)**.** Out of these species various diploid and polyploid species were used to develop alien introgression lines (Feuillet *et al*., 2007; Schneider *et al.*, 2008).

The primary gene pool of wheat includes hexaploid landraces, cultivated tetraploids, wild *T. dicoccoides* and diploid donors of the A and D genomes to tetraploid and hexaploid wheats. Transfers of genes from these two genomes occur as a consequence of direct hybridization and homologous recombination with conventional breeding approaches. The secondary gene pool includes polyploid *Triticum* and *Aegilops species,* which share one genome with any of the three genomes of wheat. Gene transfers occur as a consequence of direct crosses, breeding protocols, homologous exchange between the related genome or through use of special manipulation strategies among the homoeologous genomes. Embryo rescue is a complementary aid for obtaining hybrids. Diploid and polyploid species with non-progenitor genomes constitute the tertiary gene pool; hence, gene transfers require special techniques that assist homoeologous exchanges (Kazi and Rajaram, 2002).

Table 2.1 Genomic constitution of *Triticum* and *Aegilops* species

Species	Genomic constitution
Triticum aestivum L. (common or bread wheat)	ABD
Triticum turgidum L. (pollard wheat) ssp. *carthlicum* (Nevski)	AB
Triticum zhukovskyi Menabde & Ericz.	A^tA^mG
Triticum timopheevii (Zhuk.) Zhuk. (cultivated form) Subspecies: *armeniacum* (Jakubz.) van Slageren (wild form)	A^tG
Triticum monococcum L. Subspecies: *aegilopoides* (Link) Thell.	A^m
Triticum urartu Tumanian ex Gandilyan	A
Aegilops speltoides Tausch	S
Aegilops longissima Schweinf. & Muschl.	S^1
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 caudate 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.	$D^{c1}M^c(D^{c1}X^C)$
var. *glumiaristata*	$D^{c1}D^{c2}M^c(D^{c1}D^{c2}X^c)$
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^1)
Aegilops peregrina (Hack. in J. Fraser) Maire & Weiller (syn. *Ae. variabilis)*	US (US^1)

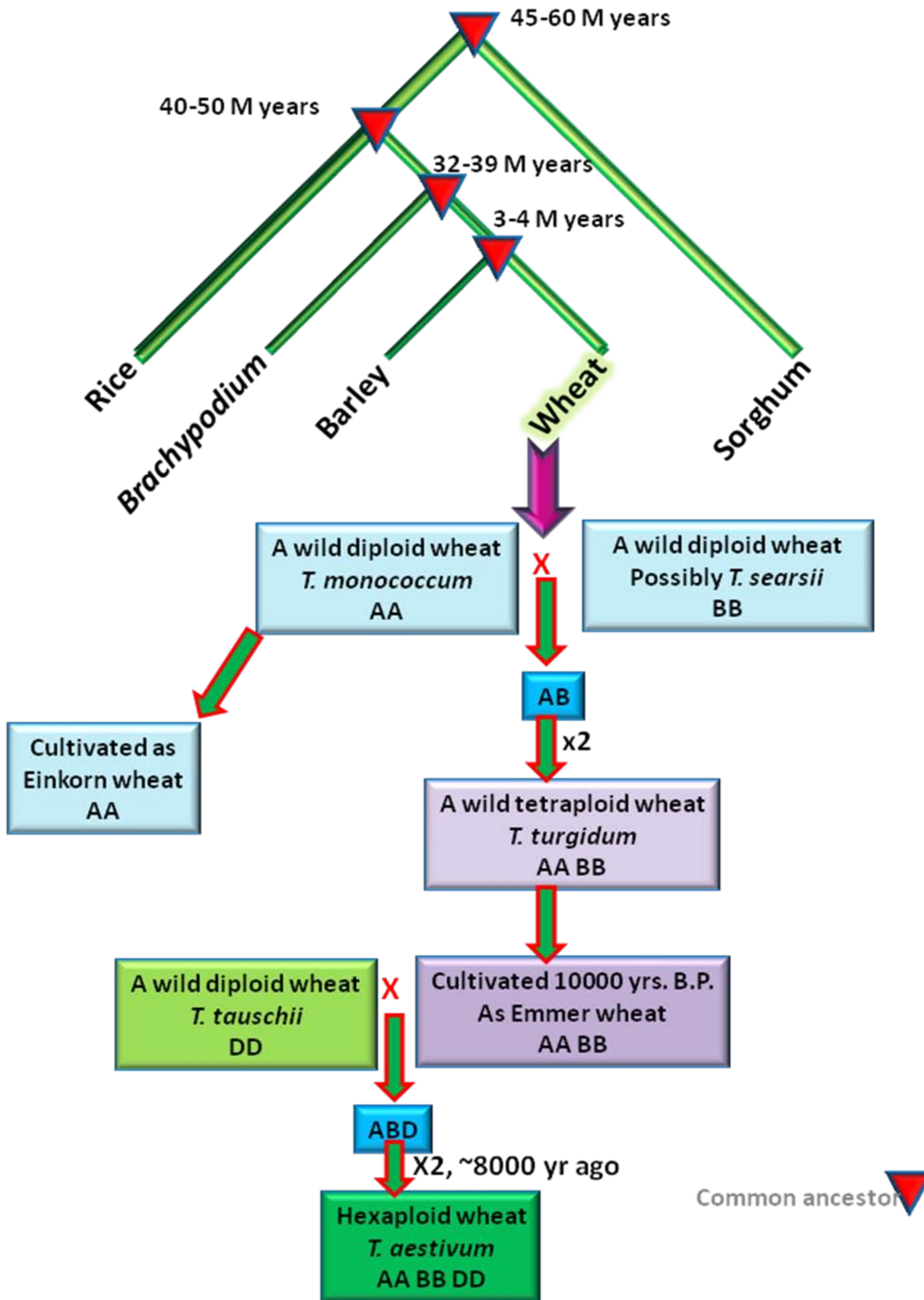


Fig. 2.4 Timeline for wheat genome evolution. Modified from Bristol wheat genomics site (http://www.wheatbp.net/WheatBP/Documents/DOC_Evolution.php).

2.3. Wheat Genome

Wheat is an agriculturally important crop with very large genome size and very high proportion of repetitive sequences. Presence of extensive and numerous cytogenetic stocks is the prime asset for research in the area of wheat genomics. However, polyploid nature of the genome and large number of repetitive sequences are making, wheat as a very complex crop to study. At genetics level, wheat has three subgenomes (A, B and D). Each genome consists of seven chromosome making homoeologous groups. Due to the presence of homoeologous pairing controlling genes *Ph1*, wheat behaves like diploid possesses very high genome buffering capacity for most of the chromosomal changes and aneuploid states due to presence of 3 sets of genes in three genomes (Gupta *et al*., 2008). Molecular tools, cytogenetic stocks, genetic, physical maps and genome sequencing makes further easier and accurate to analyze the genomes. Determination of functional domains in the whole genome is one of the most challenging tasks in this post genomic era. Functional genomics analysis of wheat through serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), Affymetrix GeneChip wheat genome array, RT-PCR, RNAi, Targeting Induced Local Lesions in Genome (TILLING) and RNA-Seq were has been taken up extensively. International wheat genome sequencing consortium (IWGSC) works on flow sorting and sequencing of individual wheat chromosome. Due to this international collaboration, chromosome 3B, 4A and group 7 chromosomes has been sequenced and other chromosomes are in pipeline (Paux *et al*. 2008; Hernandez *et al*., 2012). Wheat genome showed dominancy of transposable elements (TEs; ~80%). While most of the small non-coding RNAs (sRNA) originated from TEs specifically miniature inverted repeats transposable element (MITEs). It was also observed that there were 3 fold chances of transposable element mutation in comparison to introns and untranslated genic regions (Cantu *et al*., 2010). There was an integrated database were generated to access and analyze wheat genome through web interface (www.wheatgenome.info).

2.3.1. Genome of *Triticum urartu*

A genome is the basic genome of bread wheat and other polyploid wheats. *Triticum urartu* is the progenitor species of A genome. *Triticum urartu* works as a diploid reference species to analyze the durum and bread wheat genomes. The genome size of *Triticum urartu* was estimated to be approximately 4.94 Gb. About 66.88% of *Triticum urartu* genome consists of repetitive elements including long terminal repeats (LTR) retrotransposons (49.07%), DNA transposons (9.7%) and unclassified elements (8.04%).There are about 34879 protein coding families and 34,800 genes predicted in *Triticum urartu* with average gene size of 3,207 bp.

Average gene size of *Triticum urartu* was similar to that of *Brachipodium* but larger in comparison to rice, maize and sorghum (IRGSP, 2005; Schnable *et al*., 2009; Paterson *et al*., 2009). NB-ARC domain (R genes) of protein, NBS-LRR proteins and serine-threonine/ protein kinase domains are longer in *Triticum urartu* has comparison to other grass genomes. Although *Triticum urartu* have average gene size similar to *Brachypodium* and only 1.37 fold numbers of genes, but its genome size is 18 fold longer than *Brachypodium* genome due to abundance of intergenic regions in the A genome (Ling *et al*., 2013). About 739534 insertion site based polymorphism (ISBP) markers and1,66,309 simple sequence repeats (SSRs) may further help in several marker assisted selection, QTL analysis and plant breeding approaches to study, analyze and transfer elite traits into commercial wheat cultivars.

2.3.2. Genome of *Triticum tauschii*

About 8000 years ago due to spontaneous hybridization of *Aegilops tauschii* (2n=14, DD) with *Triticum turgidum* (2n=28, AA BB) a rise to hexaploid bread wheat. Through whole genome sequencing of *Aegilops tauschii* by shotgun strategy, about 90 fold deep short read libraries with 83.4% assembled scaffolds showed that about 65.9% of genome consists of transposable elements. That assembly estimates 4.34 Gb genome of *Aegilops tauschii* genome consisting of 34,498 high confidence protein coding loci, 2,505 transfer RNA, 358 ribosomal RNA, 78 small nucleolar RNA genes and 35 small nuclear RNA genes (Jia *et al*., 2013). It was also found that about 8,443 gene groups were common in 5 major grass species including *Aegilops*, barley, *Barchypodium*, rice and sorghum. While only 234 gene groups were found in Pooideae (*Aegilops, Barchypodium* and barley) and 587 were found in Triticeae (*Aegilops* and barley). In comparison to other grass genomes, *Aegilops tauschii* showed abundance of NBS-LRR genes (Nucleotide-binding site leucine rich repeats; 1,219 genes), M-Type MADS box gene (58 genes), Cytochrome P450 family (485 genes) and cold acclimation related genes (216 genes) including CCAAT binding transcription factor (CBF), Late embryogenesis abundant protein (LEA), DREB2 transcription factor and COR proteins. Recently a total of 159 novel microRNAs were indentified including miR2118, miR399 and miR2275 family which may help to withstand *Aegilops* into low nutrient soil and enhanced disease resistance (Jia *et al*., 2013). However two color genome mapping showed 2.1 Mb *de novo* genome assemblies with very high percentage of repetitive sequence and complexity in D genome of *Aegilops tauschii* (Hastie *et al*., 2013). A high density 4 Gb physical map of *Aegilops tauschii* suggested that genetic map length of chromosome 2D (235.1 cM) were longest while of 6D (172.2 cM) was the lowest (Luo *et al*., 2013). The whole genome sequence of *Aegilops tauschii* in addition to

4 Gb physical map of the D genome can help in identification of elite genes for agriculturally important traits to alleviate the problem of global food security.

2.3.3. Genome of *Triticum aestivum*

About 94,000 and 96,000 genes were assigned on 17 Gb paired genome sequence of bread wheat through 454 pyrosequencing technique. Dynamic nature of hexaploid wheat genome may be due to significant loss of gene families in the due course of evolution, domestication and polyploidization. Approximately 28,000, 38,000 and 36,000 genes were estimated on A, B and D genome of wheat. Loss of 10,000 and 16,000 genes in hexaploid wheat in comparison to 3 diploid progenitor species shows erosion of genetic diversity. Hydrogen ion trans-membrane transporters were very high in *Aegilops tauschii* in comparison to bread wheat. It was also observed that different subunits of ATPase and proton gradient related genes may further be helpful in Na ion exclusion and accumulation of minerals in other *Aegilops species* (Wang *et al*., 2011). In the whole genome sequence analysis of bread wheat, 66% of gene assemblies were assigned onto A (28.3%), B (29.2%) and D (33.8%) genomes. It was also estimated that through GO slim molecular function analysis of A, B and D genomes that there were no biased gene loss at the level of functional characterization. Gene loss in hexaploid wheat was very less in comparison to maize and *Brassica rapa*. Most of the transcriptional factors were retained in all the three genomes (Brenchley *et al*., 2013). The identification of 132000 SNPs in all the three subgenome (A,B and D) can be very useful in QTL analysis, association studies, gene tagging and identification of several agronomically important traits in wheat.

2.4. Group 2 Chromosomes of wheat

Group 2 chromosomes contain many genes controlling several important agronomic traits. Through whole genome shotgun sequencing of D genome donor *Aegilops tauschii*, it was identified that chromosome 2D had highest genetic map length and physical map length among all the 7 chromosomes of D genome (Luo *et al*., 2013). Previously it was observed that 2D had highest EST density in comparison to chromosome 2B and 2A. 2600 loci bin map of homoeologous group 2 chromosome was showed that 29.6% ESTs was mapped on chromosome 2A, 36.9% was mapped on chromosome 2B while 33.5% was mapped on chromosome 2D. Long arms of all the group 2 chromosomes showed higher EST density as compared to the short arm. However, 2,600 loci chromosome bin map of homoeologous group 2 chromosomes showed that gene density was high in distal ends while lower at the proximal

ends. Recombination frequency was also higher at least 10 times at distal ends in comparison to proximal ends in group 2 chromosomes of wheat (Delanay *et al*., 1995). Wheat group chromosome 2 shows very good synteny with chromosomes 4 and 7 of rice and location of two specific ESTs showed small pericentric inversion on chromosome 2B of bread wheat. Several structural rearrangements in gene order and content of chromosome signifies that, there was no perfect conservation among all the three group 2 homoeologous chromosomes (Coonley *et al*., 2004). Although there was conservedness among group 2 homoeologous chromosomes of wheat, barley and rye genomes, but the distal ends of chromosome 2RS and 2BS were showed major inter-chromosomal translocations. However inter-chromosomal translocation was also observed between 2BS and 6AS & 6DS of bread wheat (Devos *et al*., 1993; Weng and Lazar 2002). Chromosome 2DS also showed existence of some pairing promoter gene (Naranjo *et al*., 1994). List of several agronomical important genes, QTLs and orthologs sets at group 2 chromosomes were discussed further and given in Table 2.2.

Table: 2.2 Genes on group 2 chromosome for various traits

Gene	Trait	Reference
	Chromosome 2AS	
Bh	Branched spike	Klindworth *et al*., 1997
Hf	Resistance to hessian fly	Tan *et al*., 2013
Lr17, Lr11	Reaction to *Puccinia recondite*	Dyck and Kerber, 1977
Sog	Soft glume	Taenxler *et al*., 2002
	Chromosome 2AL	
Br	Brittle rachis	Peleg *et al*., 2011
Nax-1	Sodium exclusion	Lindsay *et al*., 2004
Pm4a, Pm4b	Reaction to *Erysiphe graminis*	McIntosh and Arts, 1996; The *et al*., 1999
Ppo-A1	Polyphenol oxidase	He *et al*., 2007
Sr21	Reaction to *Puccinia graminis tritici*	The *et al*., 1999
Yr1	Reaction to *Puccinia striiformis*	McIntosh and Arts, 1996
	Chromosome 2BS	
D2	Grass-clump dwarfness	Gfellar and Whiteside, 1961

Dfq1	Difenzoquat insensitivity	Leckie *et al*., 1988
Hst2a-B1	Histone proteins	Masua *et al*., 1993
Lr13, Lr16, Lr23	Reaction to *Puccinia recondite*	Singh and McIntosh, 1986; McIntosh and Luig, 1973; Nelson *et al*., 1997
Ne2	Hybrid necrosis	Gfellar and Whiteside, 1961
Ppd2	Response to photoperiod	Leckie *et al*., 1988
Sr9b,Sr9d,Sr9e, Sr9g, Sr19,Sr28, Sr36, Sr40	Reaction to *Puccinia graminis*	Singh and McIntosh, 1986; McIntosh and Luig, 1973; Scrath and Law, 1984; William and Maan, 1973; McIntosh 1978; Dyck, 1992.
wptms1, wptms2	Photoperiod and/or temperature sensitive male sterility	Guo *et al*., 2006
	Chromosome 2BL	
D2	Grass-clump dwarfness	McIntosh and Baker, 1969
Dfq1	Difenzoquat insensitivity	Leckie *et al*., 1988
Sr9a, Sr9b, Sr9g, Sr16, Sr19, Sr20, Sr28	Reaction to *Puccinia graminis*	Sears and Loegering, 1968; McIntosh and Baker, 1969; Leckie *et al*., 1988; McIntosh 1978; Anderson *et al*., 1971
Yr5, Yr7	Reaction to *Puccinia striiformis*	Leckie *et al*., 1988; Johnson and Dyck, 1984
	Chromosome 2DS	
C	Club spike shape	Luig and McIntosh 1978
D1	Grass-clump dwarfness	McIntosh and Baker 1968
Lr2a, Lr15, Lr22, Lr22b	Reaction to *Puccinia recondite*	Luig and McIntosh 1978; McIntosh and Baker 1968; Rowland and Kerber1974, Dyck 1979.
Ra	Red auricles	McIntosh and Baker 1968
Sr6	Reaction to *Puccinia graminis*	McIntosh and Baker 1968
Tg	Tenacious glumes	Dyck, 1979
W2I, Iw2	Galucousness/waxiness,glossiness	Dyck, 1979, Liu *et al*., 2007
	Chromosome 2DL	

C	Club spike shape	Rao, 1972
D1, D4	Grass-clump dwarfness	Worland and Law, 1986
LMF, Area_leaf3, Cell	Rate and duration of leaf elongation, cell production rate and cell length	Steege *et al*., 2005
Ppd1	Response to photoperiod	Worland and Law, 1986
Rht8	Reduced height	Worland and Law, 1986
Su	Insensitivity to chlortoluron	Worland and Law, 1986
YmIb	Reaction to wheat yellow mosaic virus	Nishio *et al*., 2010
Yr16	Reaction to *Puccinia striiformis*	Worland and Law, 1986

Table: 2.3 Chromosomal locations of wheat genes on group 2 chromosome that were known to be members of orthologous sets of Triticeae genes.

Gene	Trait
	Chromosome 2AS
Est-A6	Esterase-A6
	Chromosome 2AL
Est-A7	Esterase-A7
F3h-A1, F3h-B1, F3h-B2	Flavone-3-hydroxylase-A1, B1 and B2
Hyd-A1	Carotenoid beta-hydroxylase (non-heme di iron type)-A1
Isa-A1	Bifunctional α-amylase/subtilisin inhibitor-A1
Sod-A1	Superoxide dismutase –A1
Zds-A1	Zeta carotene desaturase-A1
	Chromosome 2BS
Est-B6	Esterase-B6
F3h-B1	Flavone-3-hydroxylase -B1
Per-B2	Peroxidase-B2
Tsc-2	Reaction to tan spot toxin (chlorosis)
	Chromosome 2BL
Est-B7	Esterase-B7

Hyd-B1	Carotenoid beta-hydroxylase (non-heme di iron type)-B1
Isa-B1	Bifunctional α-amylase/subtilisin inhibitor-B1
Sod-B1	Superoxide dismutase-B1
Stb-9	Reaction to *Mycosphaerella graminicola*
Zds-B1	Zeta carotene desaturase-B1
	Chromosome 2DS
Est-D6	Esterase-D6
Per-D2, Per-D5	Peroxidase 2 and 5
	Chromosome 2DL
Acph-D2	Acid phosphatase–D2
Est-D7	Esterase-D7
F3h-D1	Flavone-3-hydroxylase -D1
Hyd-D1	Carotenoid beta-hydroxylase (non-heme di iron type)-D1
Isa-D1	Bifunctional α-amylase/subtilisin inhibitor-D1
Sod-D1	Superoxide dismutase-D1
Zds-D1	Zeta carotene desaturase-D1

*Source: http://wheat.pw.usda.gov/ggpages/wgc/98/SumTabl2.htm & Wheat genetic resources database: http://www.shigen.nig.ac.jp/wheat/komugi/genes/symbolClassList.jsp

Table: 2.4 List of selected QTLs mapped on homoeologous group 2 chromosomes of bread wheat

Trait	Gene/QTL	Chromosome	Reference
		Disease resistance	
Reaction to *Fusarium graminearum*	*Fhs*	2AL	Anderson *et al*., 2001; Waldron *et al*., 1999
	Fhs	2B	Gervais *et al*., 2003
	Fhs	2D	Shen *et al*., 2003
Reaction to head scab	*Fhs*	2AL	Waldron *et al*., 1999
	Fhs	2D	Somars *et al*., 2003
Reaction to	*Cre*	2AS	Singh *et al*., 2010

Heterodera avenae			
Reaction to powdery mildew	*Pm*	2BS	Borner *et al*., 2002
Reaction to *Puccinia recondite*	*Lr*	2B	Xu *et al*., 2005
Reaction to *Puccinia striiformis*	*Yr*	2B	Ramburan *et al*., 2004
Reaction to *Stagonopora* glume blotch	*Sng*	2DL	Uphaus *et al*., 2007
Reaction to wheat spindle streak mosaic virus	*Wss*	2DL	Khan *et al*., 2000
	Quality and quality related traits		
Endosperm color	*Psy-1*	2A	Pozniak *et al*., 2007
Quality traits	*Ppd-1, Ha, Gli-D2*	2DS	Nelson *et al*., 2006
Grain dry matter	*Gli, Glu-1B*	2B	Charmet *et al*., 2005
Grain protein content	*Pro*	2A	Groos *et al*., 2003
	Gpc	2BL, 2DL	Prasad *et al*.,2003
	Agronomic traits		
Days to heading	*Eet*	2B, 2DS	Gervais *et al*., 2003
	Hd	2DS	Narasimhamoorthy *et al*., 2006
Grain yield	*Yld*	2BS	McCartney *et al*., 2005
	Yld	2DS	Narasimhamoorthy *et al*., 2006
Kernel weight	*Gw1*	2B	Groos *et al*., 2003
	Gwe	2DL	Borner *et al*., 2002
Plant height	*Ht*	2D	Heidari *et al*., 2012
	Growth related traits		
Coleoptile growth	*Rht1, Rht2*	2B	Rebetzke *et al*., 2007
Ear emergence	*Eet*	2DS	Borner *et al*., 2002

Kernel shattering	*Ks*	2B	Zhang and Mergoum, 2007
Lodging resistance	*Ld*	2A	Keller *et al*., 1999
Stem strength and related traits	*Pd, Cwt*	2D	Hai *et al*., 2005
Viridescent phenotype	*Vir*	2B	Simmonds *et al*., 2005
	Biochemical related		
Grain iron	*Fe*	2A	Tiwari *et al*., 2009
Polyphenol oxidase	*PPO*	2A	He *et al*., 2007
Response to tissue culture	*Gpp*	2AL, 2BL	Torp *et al*., 2001

2.5. Radiation hybrid approach

Radiation hybrid approach is very efficient approach for transfer, localization, tagging and mapping of genes in living organisms. This technique relies on radiation induced breakage and analysis of marker loss and retention in radiation hybrid population. Recombination event is not homogenous in the whole chromosome and genetic mapping relies mainly on recombination and crossing over events, hence genetic/linkage mapping does not give accurate physical position and location of genes on chromosomes. In wheat genes and markers are being allocated on chromosomes and chromosomal arms with the help of aneuploid and deletion stocks (Endo 1990; Qi and Gill, 2001). Radiation hybrid mapping is recombination independent method for physical mapping of genome (Goss and Harris 1975, 1977; Cox *et al*., 1990). Co-retention frequency between molecular marker and chromosomal fragments is the main basis for physical mapping through radiation hybrid approach. Due to advancenment of this method in physical mapping of genome, it has been applied on several species (Table: 2.5).

The scale for construction of radiation hybrid maps is centiRay (cR). It is not a constant value but depends on dose of radiation to break one DNA into several fragments. For example if distance between 2 genes is 1 centiRay then there may be at least one percent chances having break between them. For example the distance between two SSR markers was $1cR_{2000}$=1%, then chances that the two SSR markers will break apart at the dose of 2,000 rads in chromosome (Strachan and Read, 1997). One can easily calculate and convert the centiRay

(cR) to centiMorgan (cM) to megabase (Mb) to kilobase (Kb) distance, in chromosomes. For example the total genetic distance of chromosome 2S was estimated to be 120 cM and cR_{2000} value for the 2S genome is 6264.5 cR, hence 1 cR corresponds to 120/6264.5 cM i.e. 0.019 cM.

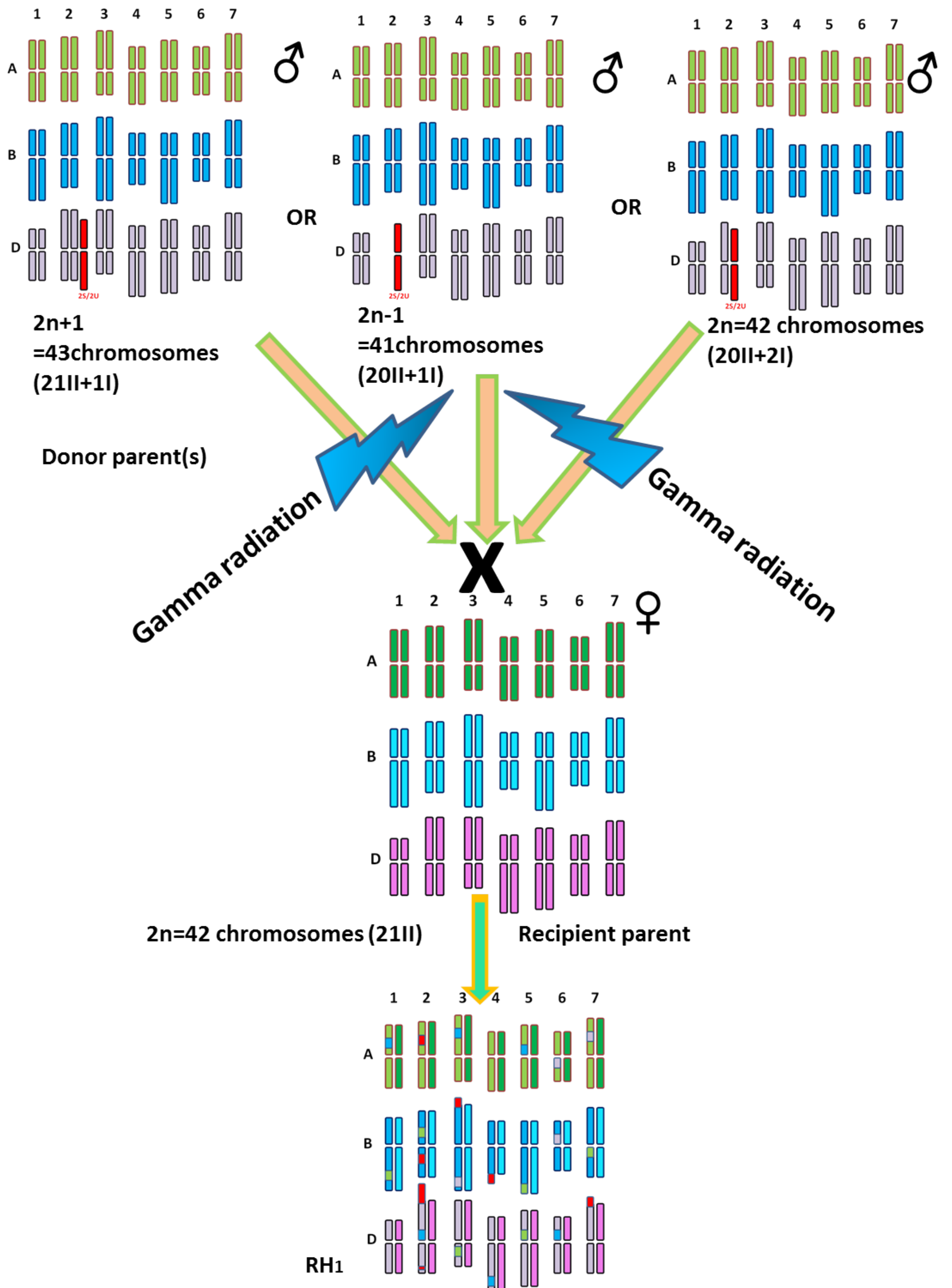


Fig.2.5 Schematic representation of strategy for irradiation induced gene transfer

Due to presence of rich robust molecular markers, efficient genotyping technologies, dense and integrated genetic and physical maps, large insert libraries, BAC contigs and sequencing technologies, radiation hybrid mapping can play key role in mapping, genome annotation, localization and identification of gene for various plant breeding and crop improvement programs. There are several agriculturally important crop plants where this technology has been used (Table: 2.5).

Table: 2.5 Radiation hybrid mapping in crops

Species/ lines	Radiation dose (krad)	Important features	Reference
RH mapping in maize			
Oat-Maize addition line	30-35	Allohexaploid oat line carrying single chromosome 9 of maize was irradiated. Self pollinated plants showed variable degree of rearrangement for chromosome 9 of maize. It was estimated that due to 1-10 radiation induced breaks, the population of 100 radiation hybrids can permit mapping of 191 Mb maize chromosome with resolution of 0.6 Mb with average retention frequency of 75% to 85%	(Riera-Lizarazu *et al*., 2000
Oat-Maize addition lines	20-50	There were 50 disomic addition lines for individual chromosomes of maize ranging from 1-9 while short arm of chromosome10. To further analyse maize genome, irradiated monosomic addition lines showed low and medium map resolution of maize genome.	Kynast *et al*., 2004
Oat-Maize radiation hybrids	30-35	In self fertilization of radiation hybrids, all the simple as well as complex re-arrangements were maintained on chromosome 9 of maize in subsequent generations.	Vales *et al.* (2004)

		RH mapping in barley	
Tobacco-barley protoplast fusion	5	The RH panel was developed by fusion of radiated protoplast of transgenic barley containing bar gene as selectable marker and gene of bialaphos resistance, with protoplast of tobacco plant. For proper genome coverage 35 barley specific molecular markers were tested in those lines. Mean marker retention frequency observed was ~26%.	Wardrop *et al.*, 2002
		RH mapping in cotton	
Cotton	5	Whole genome radiation hybrid panel was generated by using diploid cell lines. Radiated pollen of *Gossypium hirsutum* fused with egg of *G. barbadense.* 102 SSR markers were mapped on different chromosomes. Marker retention frequency varied from 77% to 100%.	Gao *et al.* (2004)
		RH mapping in wheat	
Durum wheat	35	Alloplasmic durum wheat line, which contain nucleus from durum and cytoplasm from *Triticum logissimum* carries scs^{ae} gene on long arm of chromosome 1D. 39 molecular marker on 87 individuals revealed 88 radiation induced chromosome breakages	Hossain *et al.*, 2004
Hexaploid-tetraploid wheat line	35	RH map of chromosome 1D was made in the background of durum wheat (AABB). With the help of 378 D genome specific molecular markers on 87 lines have detected 2312 chromosomal breaks. A total map distance ranged ~3341 cR for 5 linkage groups.	Kalvacharla *et al.*, 2006
Hexaploid-tetraploid wheat line	35	Mapping of all D genome of bread wheat by making quasi-pentaploid by crossing hexaploid and tetraploid wheat. Marker retention varied from 94.4% to 98.6% for all	Riera-Lizarazu *et al.*, 2010

		the D genome chromosomes	
Bread wheat	35	3B-RH panel was generated by crossing durum wheat with aneuploid "Langdon" 3D (3B). With the help of 541 markers, a high density RH map of 1871.9 cR map of chromosome 3B was generated.	Kumar *et al.* 2012
Hexaploid wheat (Chinese Spring)	2	RH mapping of germplasm with the help of endosperm (pollen radiation hybrid). RH map of seven D genome chromosome were constructed with the help of 737 D genome specific markers.	Tiwari *et al.* 2012
Hexaploid Wheat-*Bupleurum species*	3, 5 and 7	Somatic hybridization between hexaploid wheat and *Bupleurum scorzonerifolium* was performed. The comprehensive map distance calculated was 2103 cR with mean marker retention frequency of was 15.5% and map resolution of ~501.6 Kb/break.	Zhou *et al.*, 2012
Aegilops tauschii	35-40	Synthetic hexaploid (AABBDD) wheat with D-genome of *Aegilops tauschii* was irradiated and crossed with durum wheat, to obtain pentaploid RH_1 generation (AABBD)	Kumar *et al.* 2012
Bread wheat	35	A population of 188 radiation hybrids was utilized to fine map the location of scs^{ae} gene in chromosome 1D. 57 markers were used to make a map of 205.2 cR	Michalak *et al.*, 2013

Other than mapping of genome, radiation hybrid approach is equally useful for transfer of alien chromosome fragment of agronomically important genes from distinctly related species in elite cultivars. Snape *et al.*, 1983 suggested this as a efficient method for differential gene transfer in wheat through pollen radiation hybrid approach. However Hossain *et al.*, 2004 had transferred and mapped scs^{ae} gene in chromosome 1D of wheat through seed radiation hybrid approach. Small alien segments also precisely and stably transfer from *Haynaldia villosa* into wheat through pollen radiation (Chen *et al.*, 2008). Pollen as well as seed radiation techniques

to generate radiation hybrids for precise transfer, tagging and localization of genes can be very useful and efficient tool for breeding by design.

Table: 2.6.Radiation induced transfer of traits in wheat

Trait/Gene	Line/Plant	Salient features	References
Resistance to leaf rust (*Lr19*)	Wheat-*Agropyron elongatum* translocation line	Chromosome $7el_1$ of *Agropyron* translocated on chromosome 7D of wheat	Dvořák and Knott, 1977
Resistance to hessian fly	Wheat-rye translocation line	Translocation of chromosomal arm 6RL of rye onto wheat chromosome 6B, 4B and 4A	Friebe *et al.* 1991
Resistance to hessian fly	Wheat-rye translocation line	57% of rye 6RL translocated on long arms of 6B and 4B	Mukai *et al.* 1993
Resistance to leaf rust	Wheat-*Agropyron intermedium* translocation line	Chromosome 7A of *Agropyron* translocated on chromosome 2A, 5A, 1D and 3D of wheat	Friebe *et al.* 1993
Resistance to wheat- scab	Wheat-*Leymus racemosus* ditelosomic substitution lines	Substitution of telocentric chromosome 7Lr for chromosome 7A of wheat	Wang and Chen, 2008
Resistance to wheat- scab	Wheat-*Leymus racemosus* translocation lines	Chromosome 5Lr translocated onto chromosome 7DL of wheat	Wang *et al.* 2010
Powdery mildew resistance (*Pm21*)	Wheat-*Haynaldia villosa* translocation line	6VS chromosomal segment was translocated onto 6AL of wheat and small fragment inserted in 4B while 1A showed terminal translocation of minute fragment of 6VS	Chen *et al.*, 2012

2.5.1. Prospects of genome mapping and gene tagging by radiation hybrid approach.

Radiation hybrid mapping is a statistical approach, hence best map constructed based on data obtained from radiation hybrid panel may or may not be considered as the actual position of sequence or marker. Optimum radiation dose for appropriate chromosome breakage, recovery, population size and robust genotyping platform are the main requirements for construction of accurate radiation hybrid map. Variety of molecular markers, genomic sequences and BAC contigs, further increases the efficacy of the technique. Recently several new technologies like individual chromosome sorting and isolation and fusion of microprotoplast, further increase the scope of this technique (Kubala´kova´ *et al*., 2005; Saito and Nakano, 2002). Radiation hybrid mapping gives better results as compared to recombination based genetic mapping as it requires ~80% less population to achieve similar results. Comparative mapping to study evolutionary relationship of two different genomes can be done by radiation hybrid mapping (Chowdhary *et al*., 1996; Solinas-Toldo *et al*., 1995; Womack and Moll, 1986). Several genes of wheat have been ordered by using bin maps and radiation hybrid maps (Kalvacharla *et al*., 2006). Recently radiation hybrid maps were also used to assemble and annotate whole genome shot gun sequences. This technique not only helps in analysis of sequence organization and genome structure analysis but can also be used for transfer of useful variability in agriculturally important crops (Kalvacharla *et al*., 2009).

2.6. Crop improvement through homoeologous recombination

Wild relatives and related species of crop plants have been used as the important genetic resource for transfer of useful variability. However, due to evolutionary divergence between crop plants and their wild relatives, highly differentiated homoeologous genomes were established with very limited genetic recombination. Unpaired wild chromosomal fragments transferred as a single inherited unit due to suppressed genetic recombination in polyploid crops, these entire chromosomal segments in wheat background often lead to undesirable phenomenon of linkage drag (Qi *et al*., 2007). Genetic manipulation through chromosome engineering is an approach to overcome linkage drag by reducing the size of alien chromosomal segment in the recipient plant genome.

2.6.1. Induced homoeologous pairing through *ph1b* approach

Ph1 gene in wheat allows homologous chromosomal pairing but prevents homoeologous pairing. Griffiths *et al*., 2006 had localized *Ph1* to 2.5 megabase interstitial region on chromosome 5B with subtelomeric heterochromatin in clusters of *cdc2* related genes. There

are several ways to manipulate the *Ph1* gene in wheat for induced homoeologous pairing. One approach is to eliminate the *Ph1* gene either through nullisomy for 5B or by using deletion mutants of *Ph1* locus such as *ph1b* and *ph1c* (Sears, 1977; Giorgi, 1983). The use of Ph^{I} genes transferred from *T. speltoides* (syn *Ae. speltoides*) to *T. aestivum* through induced homoeologous pairing was another option (Chen *et al.,* 1994). The *ph* mutants were developed by pollen irradiation through X-rays and pollinating them on mono 5B plants (Sears, 1977). The *Ph1* gene was fine mapped on wheat long arm of chromosome 5B relative to the breakpoints of various deletion and mutant lines (Gill and Gill, 1991; Gill *et al.,* 1993). Two mutant lines for the *Ph1* gene, *ph1b* and *ph1c* were generated in hexaploid wheat cultivar Chinese Spring (Sears, 1977), and in tetraploid wheat cultivar Cappelli (Jampates and DvoÍák, 1986), respectively. The *Ph1* locus was flanked by the breakpoints of two deletions (5BL-1 and *ph1c*) and marked by a DNA probe (XksuS1). The *ph1b* deletion is linked to Xpsr128, Xpsr2120 and Xpsr574 markers. The deletion size in *ph1c* was about 0.89 µm and was smaller than that in *ph1b*, which is 1.05 µm in length (Gill *et al.,* 1993). However meiotic pairing promoted by *ph1b* mutation may not affect all types of pairing combinations but mainly allow distant homoeologous partners (Benavente *et al*., 1998). The *Ph1* gene has been localized to a much smaller region within the gene rich regions (*Ph1* gene region). Rice chromosome 7 and 9 showed synteny with *Ph1* region of wheat (Sidhu *et al.,* 2008). Several alien addition, substitution, translocation, deletion, ditelosomics, monosomic and nullisomic lines have been used for alien introgression using *ph1b* mutants and further used for plant breeding and genetics. Homoeologous recombination was induced and detected between wheat chromosome 2B and goat grass 2S through *ph1b* deletion mutants (Niu *et al*., 2011). Non-homologous pairing was also observed in *Thinopyrum bessarabicum* chromosome in the presence of *ph1c* mutation (King *et al*., 1993). Translocation line with stem rust resistance gene *Sr39* conferring resistance to seven rust races was developed in wheat (Yu *et al*., 2010).

2.6.2. Induced homoeologous pairing through mono5B approach

Ph1 gene suppresses homoeologous pairing in wheat and to locate on the long arm of chromosome 5B (5BL) (Holm, 1988). Deficiency in chromosome 5B allows homologous and homoeologous recombination between wheat chromosome and those of related species and useful tool to introduce alien chromosome for useful variability in wheat. The study based on hybrids between *Ae. peregrina* and *T. aestivum* cv. Chinese Spring (CS) substitution lines showed that chromosome 5B in hybrids was replaced by either 5B of *T. turgidum* or 5G of *Triticum timopheevii* ssp. *timopheevii.*(Ozkan and Feldman, 2001). About 37% homoeologous

pairing was observed in wheat-rye crosses and 50% in case of wheat-*Ae. columnaris* crosses (Lacadena, 1966) due to deficiency of chromosome 5B. Disomic-5D nullisomic-5B [5D (5B)-substitution-disomic] line of durum wheat (*Triticum turgidum* L.) was developed using durum lines showing deficiency of *Ph* gene on chromosome 5B (Joppa and Williams, 1988). Exploiting this strategies T1BL.1RS spontaneous translocation and induced T1AL.1RS lines were used for alien introgression (Rajaram *et al*., 1983; Islam-Faridi and Mujeeb-Kazi 1995; Villareal *et al*., 1996)

Homoeologous recombination was observed in triploids of *Festuca arundinacea* var. glaucescens (GGGG′) and tetraploid *Lolium multiflorum* (*LmLmLmLm*) hybrids (Morgan *et al.,* 2001). Phenomenal cytogenetic flexibility due to manipulation in *Ph* region offers remarkable opportunities for alien gene transfer through induced homoeologous pairing.

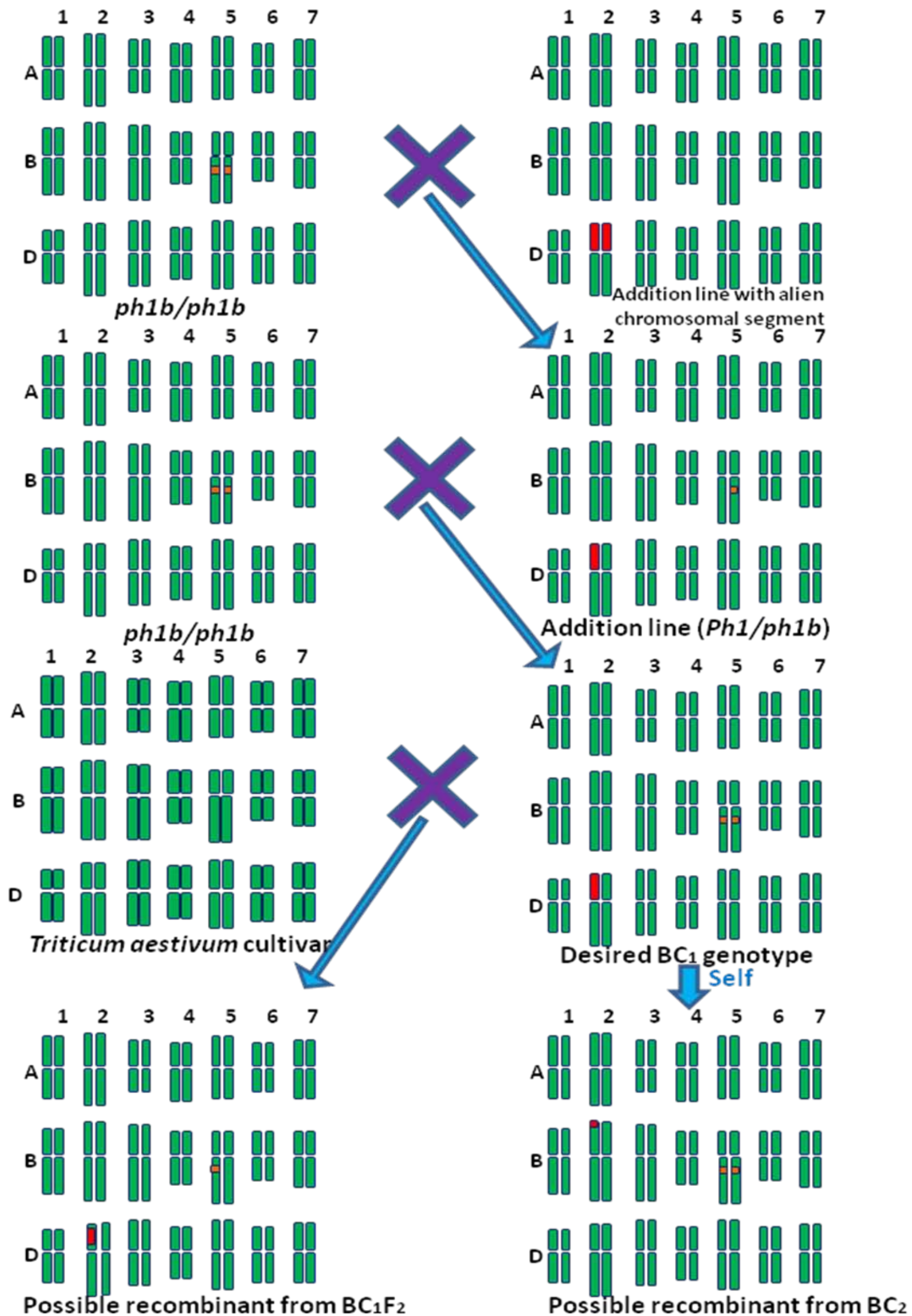


Fig. 2.6 Strategy to produce wheat-alien recombinant chromosome using induced homoeologous pairing, Orange colour represents *ph1b*, red colour represents as *Aegilops* chromosome segment

2.7. Wheat improvement through alien introgression

Aegilops germplasm has been extensively utilized for the wheat improvement and development of various addition, substitution and translocation lines for different chromosomes for transfer of useful variability (Schineder *et al.*, 2008). Several genes for resistance against various diseases has 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. Sears (1956) transferred leaf rust resistance gene *Lr9* from *Ae. umbellulata* to wheat using irradiation. Since then various workers have utilized wild wheat germplasm for wheat improvement. Some of the examples are *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). Genes for yield, grain micronutrinet and quality improvement are also transferred from wild species to cultivars (Hajjar and Hodgkin, 2007; Rawat *et al*., 2010; Tiwari *et al*., 2010).

2.7.1. Characterization of alien introgression through molecular markers

Molecular markers are the most powerful and robust diagnostic tools to detect DNA polymorphism at the level of specific loci and whole genome level. Molecular markers act as signpost on the chromosome and are usually associated with some gene. The markers which were tightly linked with gene of interest can be used in marker assisted selection and several breeding programs. Molecular markers were also used in gene pyramiding, gene tagging and localization of desirable traits in plants. Molecular markers were also exploited for dissecting polygenic traits (QTLs) and hence help in understanding of inheritance of gene and other important traits through MAS and conventional molecular breeding techniques. In addition to that molecular markers were also investigated for comparative genomics, genetic fingerprinting and linkage mapping. Previously DNA based markers were developed either through genomic libraries (RFLPs and microsatellites) or from randomly amplified genomic DNA (RAPDs) or both (AFLP). However, recently due to the availability of whole genome sequence and cDNA sequences (ESTs) in the public databases marker development has become more direct, cost effective and accurate. Most of the molecular markers are divided into PCR based and non-PCR based marker. RFLP (restriction fragment length

polymorphism) are non PCR based markers used for genetic studies of plants. RAPDs (random amplified polymorphic DNA), AFLPs (amplified fragment length polymorphism), STSs (sequence tagged sites), SNPs (single nucleotide polymorphisms), and microsatellites are all PCR-based markers. RAPD (Williams *et al.,* 1990) are short (10mer) random oligonucleotides as primers to amplify genomic DNA sequences. RAPDs were show lack of reproducibility and are considered as dominant marker. DNA fragments (80-500 bp) obtained from endonuclease restriction, followed by ligation of oligonucleotide adapters to the fragments and selective amplification by polymerase chain reaction (PCR). AFLPs (Vos *et al*., 1995) are widely used in plants for various genetic analyses including genetic mapping. The best feature of AFLP technique is a higher degree of polymorphism and reproducibility. Many genetic diversity studies in wheat and related species have been conducted using AFLPs (Heun *et al*., 1997). Simple sequence repeats (SSRs) or microsatellites markers consist of di-, tri-, or tetranucleotide repeats and DNA sequences flanking the repeats are used as priming sites in PCR reactions. SSR markers were extensively used to develop genetic maps of wheat (Röder *et al*., 1998; Somers *et al.,* 2004). STS (sequence tagged sites) markers were usually designed from known sequence in the genomic region of interest. SNP (Single nucleotide polymorphism) markers were based on single base differences within a given segment of DNA between any two individuals. Wheat have highest number of SNPs among most of the crop plants (Brenchley *et al*., 2012)

Diversity Arrays Technology (DArT), markers are sequence-independent, high-throughput, able to discover hundreds of markers in a single experiment (Kilian *et al*., 2008). DArT have been successfully developed for rice, barley, wheat and cassava and exploited in various plant breeding programs. A DArT marker is a segment of genomic DNA, the presence of which is polymorphic in a defined genomic representation. DArT markers are biallelic and behave in a dominant or co-dominant manner (Hurtado *et al.,* 2008).

2.7.2. Characterization of alien introgression through modern cytological techniques

C-banding was considered as a very good technique for monitoring alien introgression in wheat (Friebe, 1995a; 1995 b). A complete set of wheat-*Ae. geniculata* addition lines was identified and characterized through C-banding technique. Chromosome number and pairing at metaphase-I from meiotic associations were analyzed to study the pairing affinities between wheat and *Aegilops.*

GISH and FISH are also considered as very effective to analyze the alien introgression in wheat background. GISH (Genomic *in situ* hybridization) involves labeling of total genomic DNA and using it as a probe to identify alien chromosomes in a wheat background (Le *et al*., 1989; Heslop-Harrison *et al*., 1992). GISH is of potentially wide application in plant breeding programs involving alien translocations (Mukai and Gill, 1991; Heslop-Harrison *et al*., 1992). GISH was also used to identify the parental origin of each chromosome in hybrids of *Hordeum chilense* X *H. vulgare*, *H. vulgare* X *H. bulbosum* L. and *S. cereale* X *H. vulgare* (Leitch *et al.,* 1990; Mukai and Gill, 1991; Schwarzacher *et al.*, 1992) as well as alien introgression in triticale (Le and Armstrong, 1991).

FISH (Fluoresence *in situ* hybridization) and isozymes were used to characterize addition lines of *Lophopyrum elongatum* and *Thinopyrum bessarabicum* showing resistance to *Cephalosporium gramineum* (William and Mujeeb-Kazi, 1995; 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) and wheat-*Aegilops* amphiploid with resistance to powdery mildew were identified using FISH (Kumari *et al*., 2011). Badaeva *et al*. (2004) used both GISH and C-banding to study genome differentiation in *Aegilops* and evolution of the U-genome in wheat.

2.8. Biofortification of wheat and related species

Biofortification stands for increasing the bioavailability and content of micronutrient in food crops through plant breeding (Welch and Graham, 2004). The ways of meeting this objective, are screening for high grain Fe and Zn containing varieties, and genetic engineering of plants with grain micronutrient uptake, transport and sequestration genes. These approaches were combined with modern plant breeding techniques to develop superior varieties (Welch, 2005). The biofortified crops can reach the poor in rural area, have one time investment and sustainable in nature. Additionally several biofortified crops can withstand mineral deficient soils, arid regions and have added benefits of resistance to several diseases and environmental stress (Welch, 2005; Monasterio *et al*., 2007). Genetic engineering and conventional and molecular techniques were considered as the most effective and cost effective approach for biofortification of cereals with high grain micronutrinets content (Bouis *et al*., 1999; Lonnerdal, 2003). Masuda *et al*., (2012) obtained iron biofortification of rice through introduction of multiple alleles involved in iron nutrition namely, Ferritin, iron(II) nicotianaamine transporter (NAS-YSL). QTLs for high grain Fe and Zn content was reported on chromosome 2A and 7A of diploid wheat (Peleg *et al*., 2009; Tiwari *et al*., 2009). Group 1,

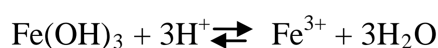
2 and 7 chromosomes of *Aegilops kotschyi* and *Aegilops longissima* have genes for high grain Fe and Zn content (Rawat *et al*., 2011; Tiwari *et al*., 2011; Kumari *et al*., 2012). Chromosome 2H and 5H of *Hordeum vulgare* (Lonergan *et al*., 2009), L1, L4 and L7 of *Agropyron intermedium* (Schlegel *et al*., 1998), V2 and V7 of *Hynaldia villosa* (Schlegel *et al*., 1998) and 1R, 2R and 7R of *Secale cerale* (Cakmak *et al*., 1997; Schlegel *et al*., 1997) were reported to have genes controlling grain Fe and Zn. Tiwari *et al*. (2011) had transferred 2S and 7U chromosomal fragments from *Aegilops kotschyi* into wheat showing 2-3 fold higher grain Fe and Zn content in derivatives seeds.

2.9. Metal homeostasis in plants

Metallic micronutrient play very important role in plant life cycle. Other than that many minerals act as micronutrient to human beings. Oxidized form of iron (Fe^{3+}) is very abundant in earth crust. Among most of the metal micronutrients, Fe, Zn and Cu plays very significant role in plant growth and physiological functioning. Metal hyperaccumulation in association with metal hypertolerance acts as important phenomenon for most of the phytoremediation, phytomining or biofortification strategies (Krämer, 2010). The Fe^{3+} shows very less solubility in water and its absorbance in plants is affected by slight changes in pH and oxygen content of soil. In calcareous soils or Fe deficient soil, plants show extensive iron deficiency symptoms like inter-veinal leaf chlorosis, and stunted growth (Kim and Geurinot, 2007). Similarly specific uptake strategies have been developed by plants for Zn uptake in mineral deficient soil (Haydon and Cobbett, 2007; Palmgren *et al.*, 2008). In plant, Fe uses its redox properties while Zn has ability to work as Lewis acid.

2.9.1. Mechanism of Fe and Zn uptake in plants

Soil is the primary source of all the metals and micronutrients for plants. Efficient uptake of metals and micronutrients is very essential for plants for their normal life processes. Despite abundance of these metals in soil, Fe^{3+} and Zn^{2+} are not readily available to the plants. The absorption of these metals is further decreased in calcareous and alkaline soils. To overcome this inaccessibility of some metal ions, all the non-graminaceous plants reliy on reduction based methodology (Strategy-I) and graminaceous plants relies on chelation based methodology (Strategy-II). Metal limiting conditions of alkaline soil are overcome by the use of ATPase activity and synthesis of protons in root rhizosphere and decrease in pH of soil. Increase in proton concentration in soil can further generates free metal ions (Palmgren *et al*., 2007).



$$Fe(OH)_3 + 3H^+ \rightleftarrows Fe^{3+} + 3H_2O$$

There was 1000 fold increase in Fe solubility by just a unit drop in soil pH (Guerinot and Yi, 1994). Members of H^+ ATPase family are up regulated in Fe deficiency conditions and can help in proton extrusion, soil acidification, increase solubility of Zn, Fe and Cu, enhanced cation exchange and uptake (Palmgren *et al*., 2001).

All non-graminaceous plants follow reduction based strategy for metal uptake. Chelated transition metals are less accessible for uptake by plants. However, many plants show affinity for specific oxidation state of metal and rely on reduction based strategy. For example Fe^{3+} state of iron is reduced to Fe^{2+} state by ferric chelate reductase (FRO2), so that iron can be taking up by their respective promoters, IRT1 and COPT1 at plasma membrane of plant roots (Robinson *et al*., 1999; Connolly *et al*., 2003; Puig *et al*., 2007).

The graminaeous plants relies on chelation based strategy for metal uptake. According to this mechanism of metal uptake, plants secrete certain chelators known as phytosiderophores, in rhizosphere. These phytosiderophores are synthesized from methionine and are also referred as mugineic acids (MAs). MAs bind with Fe^{3+} for transport and uptake in plants and play central role in mobilization of Fe and Zn. *Oryza sativa, Triticum aestivum* and *Zea mays* secrete only 2 – deoxymugineic acid (DMA) while barley secretes 3-hydroxymugineic acid (HMA), 3-epi-hydroxymugineic acid (epi-HMA), DMA and epihydroxy-2hydroxy mugineic acid (epi-HDMA) (Singh *et al*., 1993).

Metal ions diffuse into some cells of root apoplast where they are actively transported to symplastic space to inner cell layers of root through plasmadesmata and subsequently and loaded into plant vascular system (Kim and Guerinot, 2007). Other than acquisition of metals from soil to plant through reduction based or chelation based strategy, there are several transporters involved in metal uptake from soil into plants. IRT1 was reported have high affinity Fe transporter belongs to ZIP family of metal transporter and is localized at plasma membrane of root epidermis. FRO2 and IRT1 are associated with Fe accumulation and show up-regulation in Fe deficient conditions (Palmer and Guerinot, 2009). It was also reported that Fe^{2+} can be taken up and accumulated in rice due to nicotianamine (NA) as an important component (Cheng *et al*., 2007). NA acts as a precursor for production of phytosiderophores in plants. In addition to Fe uptake, IRT1 was also reported for other divalent metal uptake (including Zn) in plants. It was previously identified that *ZmYS1* (yellow stripe 1) and

OsYSL15 (yellow stripe like) help in Fe-MA uptake in graminaceous plants (Curie *et al*., 2001; Inoue *et al*., 2009)

2.9.2. Transport of Fe and Zn in between tissues

The most critical and important stage for Fe and Zn transport in plant their initial uptake of Fe and Zn from root epidermis to vascular tissue. Although most of the metals were transported from root epidermis to transpiration stream of xylem tissues through symplastic pathways, some tissues such as developing tissues and seeds, which do not have transpiration stream or fully differentiated xylem tissues mainly rely on phloem tissues (Curie *et al*., 2009). Hence proper loading and unloading of Fe and Zn from vasculature is most essential parts for metal transport in plants.

2.9.3. Transport of Fe and Zn from root to shoot

The complete mechanism and transporter related to Fe and Zn uploading in xylem tissues are not yet fully known. However, Fe most likely transported in chelated forms with citrate and NA. Ferric reductase defective (FRD3) is a citrate transporter, which transports Fe^{3+}-citrate complex. FRD3 related with efflux of iron into xylem and its long distance transport in plant. While heavy metal ATPase (HMA2 and HMA4) required for transport of Zn from root to vascular system and helps in shoot Zn hyper-accumulation. NA and several organic acids act as ligands for Zn transport in shoot (Hussain *et al*., 2004; Curie *et al*., 2009).

2.9.4. Transport of Fe and Zn from shoot to seed

YSL group of transporters which belongs to oligopeptide transporter (OPT) family of transporter are required for loading and unloading of phloem Fe^{2+} and Zn^{2+} ions, chelated with NA into seeds. It was identified that *YSL1* and *YSL3* were required for Fe, Zn and Cu complexes with NA can be helpful in remobilization from leaf tissue and metal loading into seed (Curie *et al*., 2009). OPT3 showed significant role in transporting and loading of Fe in seed while no role in Zn and Cu loading (Stacey *et al*., 2008).

2.9.5. Intracellular transport of Fe and Zn in plants

After transportation of Fe and Zn ions in proper tissue, distribution of metal ion into sub-cellular compartment is an essential task. About 90% of plant Fe stored in chloroplast and required for electron transport chain, Fe-S cluster, as cofactor for superoxide dismutase (SODs) and heme (Alscher *et al*., 2002; Kim and Guerinot, 2007). It was also reported that FSD2 and FSD3 (chloroplastic Fe-SODs) were required to protect chloroplast from reactive

oxygen species (ROS) damage. Under limiting conditions of metal, plant induces Fe-SOD and Zn-SOD (Cohu and Pilon, 2007). Despite of the importance of metal in chloroplast, transporters responsible for the Fe and Zn uptake in chloroplast not have been identified. However, permease PIC1 at inner chloroplast showed close resemblance with several Fe and Cu uptake mutants in yeast and could be the possible transporter of Fe in chloroplast. HMA1 showed higher ATPase activity in the presence of Cu and Zn (Moreno *et al*., 2008).

In mitochondria, Fe and Cu are transported and required for electron transport chain and Fe-S cluster synthesis. An ABC transporter ortholog, STA1/AtATM3 help to export Fe-S cluster in mitochondria (Kushnir *et al*., 2001). But ATM1 and ATM2 were also identified in mitochondrion membrane, but showed no significance in Fe-S cluster export in plants (Maxfield *et al*., 2004). ZIP transporters localized at mitochondrial plasma membrane and can play important role in transport of Zn inside mitochondria.

Vacuoles are very important cell organelle in terms of Fe and Zn storage and play significant role as metal reservoir in early stages seed germination and development. VIT1 is a metal transporter in vacuole and is required for proper distribution of Fe and Zn in seed. It was reported that NRAMP3 and NRAMP4 up regulated in Fe deficient condition and play significant role in Fe remobilization. Metal tolerance protein (MTP) also referred to as cation diffusion facilitator (CDF) have important role in Zn transport. MTP1 and MTP3 are localized at vacuolar membrane and helps in Zn loading. Through proteomic analysis of rice, it was identified that ZIP2 and COPT5 may be the candidates for transporting metals in vacuole.

Regulation of Fe in plants due to fluctuating environmental conditions is governed by several important genes. FER gene and FIT (FER like iron deficiency induced transcription factor) play critical role in regulation of Fe. Co-expression of FIT with basic helix-loop-helix (bHLH) gene induces IRT1 and FRO2 which can enhance shoot Fe content. Interaction of FIT with ETHYLENE INSENSITIVE 3 (EIN3) and ETHYLENE INSENSITIVE 3-LIKE1 (EIL1) indicates tight linkage between ethylene pathway and Fe deficiency response. While in case of graminaceous plants, IDS2 genes lead to, cis-acting iron deficiency responsive element1 (IDE1 and IDE2). *IRO* gene showed very complex pathways, but was recognized as important class of genes for Fe transport in Fe deficient conditions. List of several important genes related with Fe and Zn transport in plants has been given in Table 2.7.

Table: 2.7. Genes related to Fe and Zn content in plants

Gene name	Feature	Representative gene	Reference
	Fe and Zn uptake: Strategy I		
FRO	Ferric chelate reductase	*AtFRO2*	Robinson *et al*., 1999
HA	Proton efflux transporter	*CsHA1*	Santi *et al*., 2005
IRT	Ferrous ion transporter	*AtIRT1*	Eide *et al*., 1996
PEZ	Phenolics efflux transporter	-	
	Fe and Zn uptake: Strategy II		
APRT	Adenine phosphoribosyltransferase	*HvAPT1*	Itai *et al*., 2000
DEP	Methylthioribose-1-phosphate dehydrtase-enolase-phosphatase	*OsDEP*	Kobayashi *et al*., 2005
DMAS	Deoxymugineic acid synthase	*OsDMAS1*	Bashir *et al*., 2006
FDH	Formate dehydrogenase	*HvFdh*	Suzuki *et al*., 1998
IDI1/ARD	Acireductone dioxygenase	*HvIDI1*	Yamaguchi *et al*., 2000
IDI2/MTI	Methylthioribose-1-phosphate isomerise	*HvIDI2*	Suzuki *et al*., 2006
IDI4/AAT	Putative aminotransferase catalyses the synthesis of methionine	*OsIDI4*	Kobayashi *et al*., 2005
IDS2	Putative epihydroxymugineic acid synthase	*HvIDS2*	Okumura *et al*., 1994
IDS3	Mugineic acid synthase	*HvIDS3*	Nakanishi *et al*., 2000
MTK	Methylthioribose kinase	*OsMTK1*	Kobayashi *et al*., 2005
MTN	Methylthioadenosine/S-adenosyl homocysteine nucleosidase	*OsMTN*	Kobayashi *et al*., 2005
NAAT	NA aminotransferase	*HvNAAT-A*	Takahashi *et al*., 1999
NAS	NA synthase	*HvNAS1*	Higuchi *et al*., 1999
SAMS/MAT	S-adenosyl-L-methionine synthetase	*OsSAMS2*	Kobayashi *et al*., 2005
TOM1	MAs efflux transporter	*OsTOM1*	Nozoye *et al*., 2011

YS1/YSL	Fe(III)-MAs transporter	*ZmYS1*	Curie *et al.*, 2001
	Fe and Zn translocation		
ENA	NA efflux transporter	*OsENA1*	Nozoye *et al.*, 2011
FPN/IREG	Putative Fe efflux transporter	*AtFPN1/IREG1*	Morrissey *et al.*, 2009
FRD3/FRDL	Citrate efflux transporter	*AtFRD3*	Rogers and Guerinot, 2002
FRO	Ferric-chelate reductase for translocation of Fe	-	-
IRT and NRAMP	Ferrous ion transporter for translocation of Fe	-	-
PEZ	Phenolics efflux transporter	*OsPEZ1*	Ishimaru *et al.*, 2011
TOM	MAs efflux transporter	*OsTOM1*	Nozoye *et al.*, 2011
YS1/YSL	Fe(III)-MAs/Fe(II)-NA transporter	*OsYSL2*	Koike *et al.*, 2004
ZIF	Zinc induced facilitator	*AtZIF*	Sinclair and Kramer, 2012
ZIPS	ZRT-IRT like proteins	-	Sinclair and Kramer, 2012
ZRT	Zinc regulated transporter	*OsZRT*	Sinclair and Kramer, 2012
	Fe storage		
Ferritin	Storage and sequestration of Fe	*AtFer1*	Petit *et al.*, 2001
	Fe and Zn compartmentalization		
FPN/IREG	Vacuolar Fe transporter	*AtFPN2/IREG2*	Morrissey *et al.*, 2009
FRO	Ferric chelate reductase for chloroplast Fe transport	*AtFRO7*	Jeong *et al.*, 2008
HMA	Heavy metal ATPase	*AtHMA*	Sinclair and Kramer, 2012
MIT	Mitochondrial Fe transporter	*OsMIT*	Bashir *et al.*, 2006
NRAMP	Fe transporter into cytosol from vacuole	*AtNRAMP3*	Lanquar *et al.*, 2005
PIC1	Fe transporter at chloroplast	*AtPIC1*	Duy *et al.*, 2007
VIT1	Vacuolar Fe transporter	*AtVIT1*	Kim *et al.*, 2006

	Gene regulation: Strategy I		
BTS	Putative transcriptional or posttranscriptional regulator	*AtBTS*	Long *et al*., 2010
EIN3, EIL1	Ethylene signalling regulator	*AtEIN3*	Lingam *et al*., 2011
FER/FIT	Positive transcriptional regulator for Fe transport	*SlFER*	Ling *et al*., 2002
HLH	Positive transcriptional regulator for Fe transport	*AtbHLH38*	Wang *et al*., 2007
PYE	Negative transcriptional regulator for Fe transport	*AtPYE*	Long *et al*., 2010
TIC	Circadian clock regulator	*AtTIC*	Duc *et al*., 2009
	Gene regulation: Strategy II		
IDEF1	Positive transcriptional regulator for Fe transport	*OsIDEF1*	Kobayashi *et al*., 2007
IDEF2	Positive transcriptional regulator for Fe transport	*OsIDEF2*	Ogo *et al*., 2008
IRO2	Positive transcriptional regulator for Fe transport	*OsIRO2*	Ogo *et al*., 2006
IRO3	Transcriptional regulator for Fe transport (putatively negative)	*OsIRO3*	Zheng *et al*., 2010

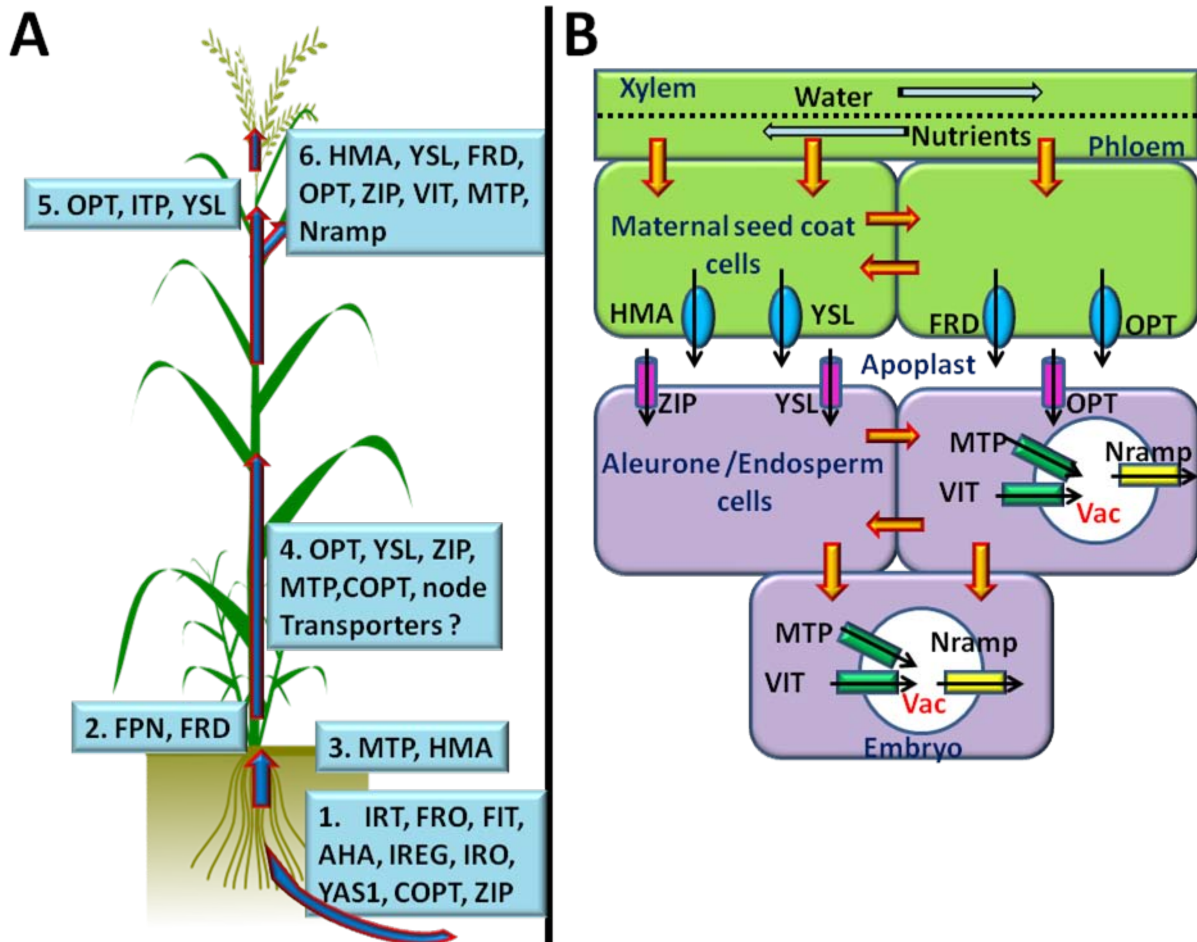


Fig. 2.7 Model for localization of Fe and Zn related genes in plants. (A) Model of wheat plant showing the following translocation steps to the seed: 1, uptake from the rhizospere; 2, xylem loading; 3, root-to-shoot transfer; 4, distribution to the leaves or seed-covering tissues; 5, phloem loading for movement to seed; 6, loading into the seed. (B) Detail of seed loading. Gene families potentially involved in seed mineral micronutrient transport are pictured in hypothetical or known localizations (Modified from Waters *et al*., 2011)

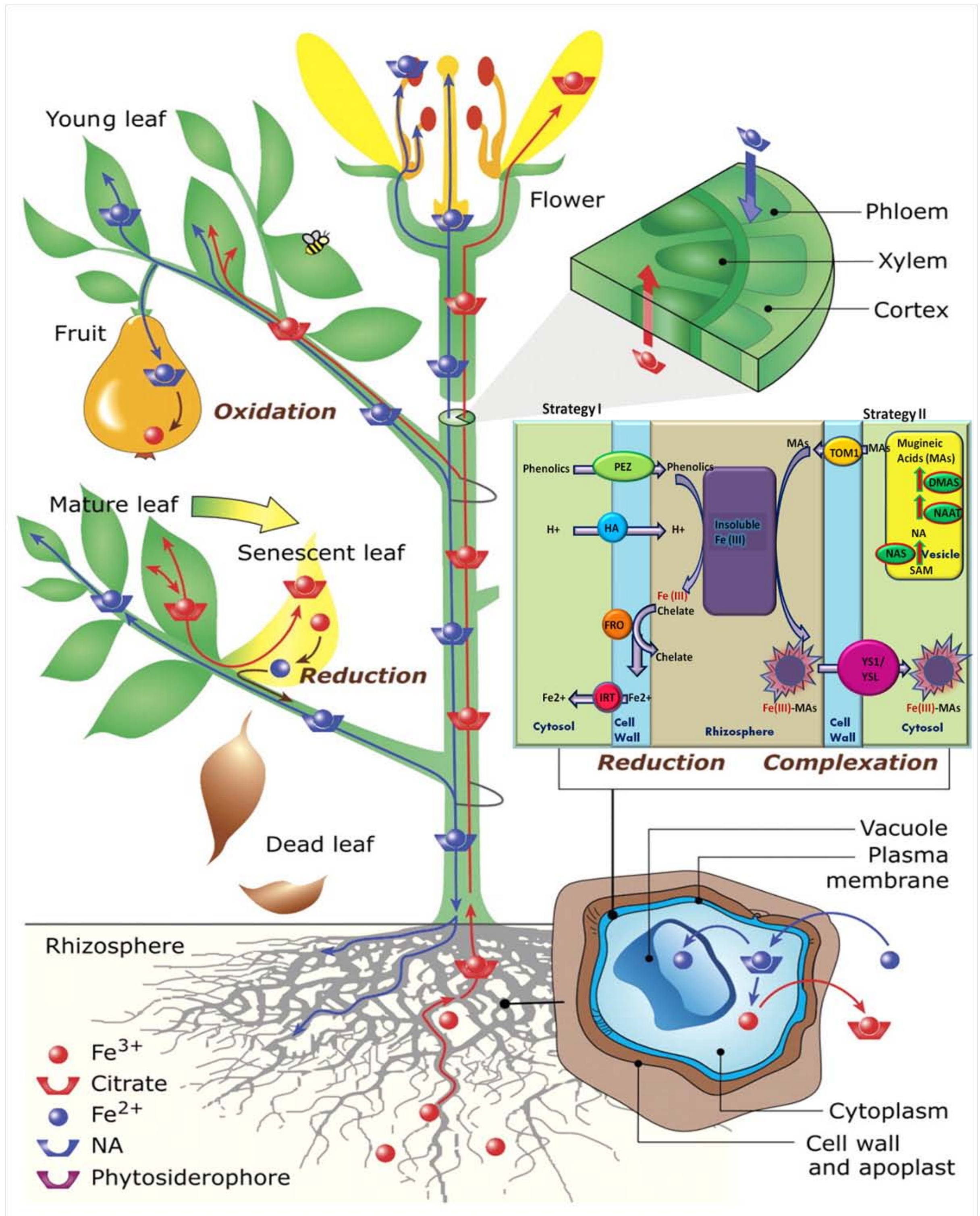


Fig.2.8 Schematic representation of the long-distance circulation of iron chelates within a flowering plant. Iron-citrate circulating in xylem vessels and iron-nicotianamine circulating in phloem vessels are represented as blue and pink arrows, respectively. (For full forms of abbreviations used, see list of abbreviation table) (Modified from Briat *et al.*, 2007 and Kobayashi and Nishizawa, 2012)

3. Materials and Methods

The present study is mainly focused on the transfer of genes responsible for high grain iron and zinc from *Aegilops* into elite wheat cultivars. For the above mentioned broad objective, several strategies have been followed including development of radiation hybrids, facilitation of homoeologous recombination through *ph1b* deletion and mono 5B stocks. The use, development of plant material and methods used in the present study are as follows

3.1. Plant Materials

3.1.1. Basic plant material, parents and introgression lines

The experimental material used for this study mainly comprises hexaploid wheat cultivars and related *Aegilops species* and *Ae. kotschyi* group 2 introgression lines. In the first stage of the dissertation work for polymorphic survey of group 2 chromosome specific SSR markers, nine wheat cultivars and *Aegilops species* were used. It included three elite wheat cultivars namely, *T. aestivum* cv. WL711, *T. aestivum* cv.PBW 343, *T. aestivum* cv. Chinese Spring and six wild related non-progenitor species including *Aegilops kotschyi* acc. 3790, 396 and.3573, *Ae. peregrina* acc. 13772 and 3519, *Ae. longissima* acc. 28. The six related non-progenitor species of wheat were used as the parent for biofortification of wheat for high grain iron and zinc. All the material was obtained from "Wheat germplasm Collection" maintained at the Punjab Agricultural University (PAU), Ludhiana, Punjab, India.

The wheat-*Aegilops* derivatives which were used for precise gene transfer through radiation hybrid approach, induced homoeologous pairing through *ph1b* and mono 5B were developed by Prof. H.S. Dhaliwal at IIT Roorkee namely 77-33-2 $\otimes$ (Cs(Ph^{I})/ *Ae. kotschyi* 3790//UP2338-2-33-2 Bulk), 77-36-2 $\otimes$ (Cs(Ph^{I})/ *Ae. kotschyi* 3790//UP2338 (77)-36-6 Bulk), 49-1-73 $\otimes$ (Cs(Ph^{I})/ *Ae. kotschyi* 396//PBW343-3///UP2425(49)-1-73 Bulk), 77-50-8-8-1(Cs(Ph^{I})/ *Ae. kotschyi* 3790//UP233(77)-50-8-8-1), 46-1-15-15 (Cs(Ph^{I})/ *Ae. kotschyi* 396//PBW343-3///UP2425(46)-1-15-15). All the plant material was grown in the experimental fields of Indian Institute of Technology (IIT), Roorkee, Uttarakhand, India from 2009-10, 2010-11 and 2011-12, Punjab Agricultural University Ludhiana for the season 2011-12 and at the Eternal University (EU), Himachal Pradesh in the season 2011-12 and 2012-13. The offseason nursery of the experimental material was grown at DWR field station at Keylong, Himachal Pradesh for the year 2010-11 and PAU field station at Keylong, Himachal Pradesh in the year 2011-12.

All the plant material was grown using the recommended agronomical filed conditions in a single row of 1.5 m length. With plant to plant distance of 15 cm and row to row spacing of 30 cm and recommended fertilizers and irrigation schedule as that for wheat. Grains and spikes were harvested after physiological maturity. Collection of mature spikelet and spikes has done at different intervals over 7-21 days due to frequent shattering of brittle spikes of wild species and derivatives. Harvesting and threshing of all the material was done manually.

Table: 3.1. Name, genome and micronutrient content of elite wheat cultivars, related non progenitor *Aegilops species* and wheat-*Aegilops* derivatives.

S. No.	Plant Name	Genome	Micronutrient content (Fe and Zn)
1	*Triticum aestivum* cv.WL711	AABBDD	Low
2	*Triticum aestivum cv.* PBW343	AABBDD	Low
3	*Triticum aestivum* cv.Chinese spring	AABBDD	Low
4	*Aegilops kotschyi* acc.*396*	UUSS	High
5	*Aegilops kotschyi* acc.*3790*	UUSS	High
6	*Aegilops kotschyi* acc.*3573*	UUSS	High
7	*Aegilops peregrina* acc.*3519*	UUSS	High
8	*Aegilops peregrina* acc.*13772*	UUSS	High
9	*Aegilops longissima* acc.*28*	SS	High
10	77-33-2 ⊗	AABBDD/U/S	High
11	77-36-2 ⊗	AABBDD/U/S	High
12	77-50-8-8-1	AABBDD/U/S	High
13	46-1-15-15	AABBDD/U/S	High
14	49-1-73 ⊗	AABBDD/U/S	High

3.1.2. Development of radiation hybrids

For precise transfer of genes for high iron and zinc content from the selected substitution and addition lines 77-33-2 ⊗, 77-36-2 ⊗ and 49-1-73 ⊗ to bread wheat through radiation hybrids. For irradiation mediated gene transfer two procedures seed radiation and pollen radiation were used.

3.1.2.1. Seed radiation hybrid approach

In the the year 2009 a wheat culivar was used for optimization of radiation dose. *Triticum aestivum* cv. Chinese Spring seeds were given variable gamma radiation doses in gamma chamber 5000 (BRIT, DAE Cesium-dose rate 01.79 KGy) from 25 krad to 55 krad. All the seeds exposed to defferential radiation dose (200 seeds per radiation dose) and control were sown in pots and examined carefully in the green house condition favourable for wheat germination and growth. Percentage seed germination and plant height was measured upto 30

days from the date of sowing. Based on seed germination condition and growth, 40 krad was selected as the dose of choice for the seed radaition induced gene transfer.

For precise transfer of genes for useful varaibility from selected wheat-*Aegilops* substitution and addition lines to bread wheat. Crosses of plants were made from irradiated seeds using *Triticum aestivum* cv WL711 as the female parent and seed radiated substitution/ addition line as male parent. The scheme for use seed radiation hybrid is shown in Fig. 3.1.

Period	**Generation**		**Remarks**
	♀		♂
Nov, 2009-April, 2010	**WL711**	**X**	**77-33-2 ⊗ and 77-36-2 ⊗** (Seed irradiation)
		↓	
Nov, 2010-April, 2011		**SRH$_1$**	Radiation Hybrid (Introgression analysis through molecular markers and seed micronutrient content)
		↓	
Nov, 2011-April, 2012		**SRH$_2$**	SRH$_2$ -Radiation Hybrid (Generation advancement, Introgression analysis through molecular markers , seed micronutrient content and GISH)
		↓	
Nov, 2012-May, 2013		**SRH$_3$**	SRH$_3$ -Radiation Hybrid (Generation advancement, Introgression analysis through molecular markers and seed micronutrient content)

Fig. 3.1 A schematic representation of the development of seed radiation hybrids

3.1.2.2. Pollen radiation hybrid (PRH) approach

Pollen radiation approach is another promising approach for radiation induced gene transfer of useful variability (Fig. 3.2). *Triticum aesivum* cv PBW343 was used for dose optimization procedure. Variable gamma radiation i.e. 1krad to 5 krad was applied to the dehiscing spikes of wheat, a day before the use of irradiation pollen for crosses. Gamma radiation exposed spikes were used as the male parent and crossed with *Triticum aesivum* cv PBW343 and the seed was recorded. The gamma radiation dose of 2 krad was the most appropriate for pollen radiation.

Selected wheat-*Aegilops* derivatives including 77-33-2-5 ⊗ (Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338(77)-33-2-5 Bulk)and 49-1-73-10 ⊗ (Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-

3///UP2425(49)-1-73-10 Bulk) was used for pollen radiation hybrid approach. Crosses were made between wheat cultivar *Triticum aestivum* cv. PBW343 having *GPC* and *Lr24* gene as female parent and 2 krad gamma irradiated substitution and addition line as male/donor parent. For proper synchronization of use of irradiated pollen for pollination, spikes were detached (expected to dehiscence next day) exposed to gamma radiation at 2 krad in the evening and kept the peduncles immersed in water. The recipient plants were emasculated two days before the dehiscence of pollen so that on the day of pollination, ovary looked feathery and receptive. Seed set was checked on regular intervals. The fully matured crossed seeds was harvested, dried and stored at 4 ºC temperature till the next sowing. In the next sowing season (2011-12) the seeds were germinated in petriplates. 4-5 days old seedlings were carefully transplanted in field and extensive care was taken until they reached a self sustaining healthy growing stage. The F_1 plants were observed regularly to analyse the effect of radiation dose.

Period	Generation		Remarks
	♀		♂
Nov., 2010-April, 2011	**PBW343** (*GPC+Lr24*)	**X** ↓	**77-33-2-5 ⊗ and 49-1-73-10 ⊗** (Spike irradiation)
Nov., 2011-April, 2012		**RH F_1** ↓	Radiation Hybrid (Introgression analysis through molecular markers, GISH and analysis of seed micronutrient content)
Nov., 2012-May, 2013		**RH F_2**	PRH_2 -Radiation Hybrid (Generation advancement, Introgression analysis through molecular markers and analysis of seed micronutrient content)

Fig. 3.2 A schematic representation of the development of pollen radiation hybrids

3.1.3. Development of *ph1b* hybrids

As an alternate strategy for precise and stable gene transfer of useful variability, wheat-*Aegilops* 2U/2S derivatives were crossed with *ph1bph1b* deletion mutant (*Ph1* gene is deleted) and the F_1 plants were further backcrossed with *ph1b* mutant. As per scheme given in Fig. 3.3. Homozygous *ph1b* leads to homoeologous pairing for alien introgression. Plants homozygous for *ph1bph1b* were screened through *Ph1* locus specific markers psr574 and psr2120. The selected wheat-*Aegilops* derivatives for this approach were 77-50-8-1-1⊗, 49-1-73-10-3⊗, 77-36-6⊗, 77-33-2⊗ and 46-1-15-15-2⊗. The BC_1F_1 plants homozygous for *ph1bph1b*

were further screened for retention of 2S/2U chromosomes. Selfed BC_1F_2 plants were screened for introgression studies and micronutrient analysis. The BC_1F_3 seeds were mostly shrivelled and the plants had yellow leaves. Leaf yellowing and shrivelled seeds seemed to be associated with the presence of *ph1b* mutant. Morphological and molecular data was recorded on regular intervals and the seeds were harvested at physiological maturity.

Period	**Generation** ♀		**Remarks** ♂
Nov., 2009-April, 2010	***ph1bph1b***	**X**	**77-50-8-1-1⊗, 49-1-73-10-3⊗, 77-36-6⊗, 77-33-2⊗ and 46-1-15-15-2⊗.** (Wheat-*Aegilops* derivatives)
		↓	
May, 2010-Sept., 2010 (Keylong offseason nursery)	**F_1**	**X**	***ph1bph1b*** (Backcrossing of plants homozygous for *ph1bph1b*)
		↓	
Nov., 2010-April, 2011		**BC_1F_1**	Selection (*Ph1/ph1b* homozygous, 2S/2U introgression and high grain Fe and Zn)
		↓	
Nov., 2011-April, 2012		**BC_1F_2**	Generation advancement and selection (2S/2U introgression, yield and high grain Fe and Zn)
		↓	
Nov., 2012-April, 2013		**BC_1F_3**	Generation advancement and selection (2S/2U introgression, high grain Fe and Zn and yield)

Fig. 3.3 A schematic representation of the development of *ph1b* hybrids

3.1.4. Development of interspecific hybrid with mono 5B stock.

Crosses were made between wheat line Pavon monosomic for 5B (Mono5B) with *Aegilops species* as male parent as the male parent as per the scheme given in Fig.3.4.

The F_1 plants were completely sterile and had 34 or 35 chromosomes. Plants with 34 chromosomes showed high pairing and were screened for further crossing through *Ph1* specific molecular markers and cytology. The F_1 plants without 5B were crossed with PBW343 (*GPC1 + Lr24*)

The BC_1F_1 plants were again backcrossed with *Triticum aestivum* cv.PBW343 to get BC_2F_1 seeds. These BC_2F_1 plants were screened for pollen viability and cytology to study fertility, chromosome number and pairing.

Period	Generation		Remarks
	♀		♂
Nov, 2009-April, 2010	**Mono5B**	**X**	***Aegilops species***
		↓	
Nov, 2010-April, 2011	**F_1** (with 34 chr.)	**X**	**PBW343(*Lr24,GPC*)** (F1 plants were sterile and had 34 or 35 chromosomes)
		↓	
Nov, 2011-April, 2012	**BC_1F_1**	**X**	**PBW343(*Lr24,GPC*)** (BC_1F_1 plants were further screened through cytology)
		↓	
Nov, 2012-May, 2013	**BC_2F_1**	**X**	**PBW343(*Lr24,GPC*)** (BC_2F_1 plants were further screened through cytology)

Fig. 3.4 Schematic presentation of the development of introgressive derivatives through Mono 5B approach

3.2. Chemical Analyses

3.2.1. Analysis of Fe and Zn of grain:

To estimate total grain iron and zinc content whole grain samples of elite wheat cultivars, non-progenitor *Aegilops species* and wheat-*Aegilops* derivatives were thoroughly washed with N/10 hydrochloric acid (Merck) for 2 min. than washed with distilled water, dried in hot air oven at 70˚C for 5 hours till constant weight of grain samples (0.5 g) were digested on hot plate at 150˚C or in microwave digester (Anton Par) in a 5 ml of di-acid mixture i.e. 2 parts of concentrated nitric acid (Merck) and 1 part percholoric acid (Merck) as per standard protocol described by Zarcinas *et al*. (1987). Hydrogen peroxide (Merck) was added at regular intervals 3-5 times (for complete oxidation) until clear residue have been obtained. After the completion of digestion process, required volume has been made by using double distilled de-ionised water (Milli-Q) and were analysed on Atomic Absorption Spectrophotometer (AAS); (GBC-Avanta and Agilent Technologies). A minimum of 6 replications were made in each sample to get uniform results. The nutrition content were again reconfirmed by Inductively Coupled

Plasma Mass Spectrometer (ICPMS) (Perkin Elmer).The Fe and Zn standards (Merck) purchased for this study being of high purity and ICPMS grade

3.2.2. Analysis of Fe and Zn of flag leaf

Flag leaves of selected donors, recipient donors, interspecific derivatives were collected at pre-anthesis stage, washed thoroughly in N/10 HCl, dried at 70˚C for 12 h. in oven prior to digestion. Dried leaves were weighed and digested in minimum of 6 replications using diacaid mixture (2 parts nitric acid and 1 part percholoric acid) as per standard protocol given by Zarcinas *et al*., 1987. Iron and zinc content of digested samples were analysed through AAS.

3.3. Analysis of mineral distribution through SEM-EDX

Electron dispersive elemental microanalysis of grains was done using SEM-EDX model FEI Quanta 200F. Phosphorus, potassium, magnesium, calcium, iron and zinc were analysed for comparative distribution within aleurone layer of wheat grains. Sample preparation was done according to the method of Feeney *et al*., (2003). Transverse sections were cut through the centre of the grains and fixed using a solution of 2.5% glutaraldehyde in 25 mM PIPES buffer (pH 6.9). Vacuum infiltration of fixation solution was done for half an hour. Thereafter 4 h. of incubation in fixative was done with continuous rotation at atmospheric pressure, followed by washing in 25 mM PIPES buffer for 15 min. 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 sec. 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). The differential mineral distribution and percentage mineral content was observed by energy dispersive X-ray spectroscopy connected to SEM.

3.4. Cytological Studies

To study the meiotic chromosome number and behaviour, spikes were fixed in Carnoy's fixative (6 ethanol: 3 chloroform: 1 acetic acid) for one day and after 24 h. transferred to 70% ethanol solution. Anthers at various stages of meiosis- I were squashed in 2% aceto-carmine (Sigma) solution and the pollen mother cells (PMCs) were scored for analysis of chromosome number and pairing in compound microscope (Zeiss). Photographs were taken with the help of digital camera (Canon PC1049, No. 6934108049).

Pollen viability test to analyse male fertility was measured by staining dehiscing pollen grain after squashing the anthers in Iodine-Potassium iodide solution (I-KI) (HiMedia). Pollen grains with dark stain (blue) were scored as viable pollens and with light or no stain were scored as dead pollen.

3.5. Genomic *in situ* hybridization

Genomic *in situ* hybridization was used to analyse and visualize alien introgression in selected radiation hybrids using the method described by Dou *et al.,* (2006). For this study selected seeds were germinated at room temperature and root tips were collected at the length of 0.5-2.5 cm. , pre-treated in ice-water for 24 h. and fixed in 99% ethanol-glacial acitic acid (3:1) solution. Slides were prepared by squashing fixed root tips in 45% acetic acid.Genomic DNA of *Aegilops umbelullata* (U genome) and *Ae. longissima* (S genome) were used as probes to analyse alien introgression.

3.6. Protein analysis

Isolation and analysis of HMW glutenin subunit proteins were done by using method described by Smith and Payne, (1984) with slight modifications.

3.6.1. HMW glutenin subunit extraction reagents and procedure

Protein extraction buffer: It consists of SDS (sodium dodecyl sulphate, Sigma Aldrich) 2 g, ß- mercaptoethanol (HiMedia) 5 ml; Total volume made up to 100 ml with distilled water (pH-6.8)

Protein loading Dye: Bromophenol Blue (Merck) 0.5 g, Glycerol 50 ml (Sigma), double distilled water 50ml

3.6.2. HMW glutenin subunit extraction procedure:

Tem mg of seeds were crushed and weighed. 132 µl of extraction buffer was added to it in an eppendorf tube, vortexed for 1.5 min. and then incubated in water bath for 18 minutes at 80°C. 12 µl dye was added to it and then it was centrifuged at 5000 rpm for 10 min. Supernatant was retained.

3.6.3. HMW glutenin SDS PAGE reagents

Acrylamide 40%: Acrylamide (Sigma) 200 g; total volume was made up to 500 ml with distilled water.

Bis-acrylamide 2%: Bisacrylamide (Sigma) 4 g; total volume was made up to 200 ml with distilled water.

Stain : Commassie Brilliant Blue R-250 (HiMedia) 4 g; 100% Methanol 1600 ml (SRL); 100% Trichloro Acetic acid (SRL) 400ml; Total volume was made up to 4000 ml with distilled water.

1.5M Running gel buffer (pH-8.8)**:**

Tris 18.17 g, SDS 0.4 g; Total volume was made up to 100 ml with distilled water.

0.5M Stacking gel buffer (pH 6.8):

Tris 6.06 g, SDS 0.4 g; Total volume was made up to 100 ml with distilled water.

10X Tank buffer (pH 8.3):

Tris 30.3 g, Glycine 142.0 g, SDS 10.0 g; Total volume was made up to 1000 ml with distilled water after setting pH to 8.3.

Ammonium per sulphate (APS, SRL) 1 g in 1000 µl distilled water.

Tetramethylene diamine (TEMED, HiMedia)

Butan-2-ol (SRL)

Running gel (10%)

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

Stacking Gel (5 ml)
Distilled water- 2.90 ml, Acrylamide- 0.55 ml, Bisacrylamide- 0.30 ml, Stacking gel buffer- 1.25 ml, APS- 55.55 µl, TEMED- 20 µl

3.6.4. HMW glutenin SDS PAGE procedure

The 10% running gel was poured in the gel casting unit (Atto, Japan), apply 1ml butanol overlaid the gel solution and allowed to polymerize for 40 min. Butanol was drained off and thoroughly washed with distilled water. After 40 min. stacking gel was poured over the running gel and comb was inserted. After 15 min. gel was solidified and comb was pulled out

and gel wells were washed with distilled water. Chilled 1X tank buffer was poured in the assembly and samples (10 µl) were loaded in the wells.

3.7. Isolation and purification of genomic DNA from leaf tissues

DNA was extracted from young leaves of experimental plant samples using CTAB method described by Murray and Thompson (1980) with some modifications.

3.7.1. Composition of DNA Extraction buffer:

S.No.	Name of the reagent	Composition
1	Tris-Cl (pH 8.0)	200 mM
2	EDTA (pH 8.0)	20 mM
3	NaCl	140 mM
4	CTAB	2%
5	β mercaptoethanol	0.01%

All chemicals used were of molecular biology grade (HiMedia).

3.7.2. DNA isolation and purification reagents:

S.No.	Name of the reagent	Composition
1	TE Buffer (Tris-Cl) (pH 8.0)	10 mM
2	EDTA (pH 8.0)	1 mM
3	RNase solution	10 mg/ml
4	Chloroform: Isoamyl alcohol	24:1
5	Ethanol	70%
6	Absolute Ethanol and Isoproponal	

3.7.3. DNA isolation procedure

About 5-10g of young, healthy and disease free leaves from each plant were collected and kept on ice in the plastic bags. One or two leaves were crushed to fine powder in liquid nirogen using autoclaved and pre-chilled mortar and pestle. About ~3 g powder was transferred to 2 ml centrifuge tubes containing pre-warmed (65°C) 900 µl DNA extraction buffer. Leaf powder and extraction buffer was gently mixed and incubated in 65°C water bath for 60 min., mixing

briefly every 10 min. After incubation in water bath, equal volumes of chloroform: isoamyl alcohol (24:1) solution was added to the samples followed by gentle mixing for 20 min. on gel rocker to ensure emulsification of phases. The samples were centrifuged at 10,000 rpm for 20 min. at room temperature. The supernatant was transferred to the fresh and sterile centrifuge tubes with the help of micropipettes. Equal volume of chilled iso- propanol was added and left for 12 h. at 4°C for complete precipitation of DNA. The DNA forms cotton like precipitate and floats on the top. DNA was precipitated and fished out by centrifuging at 5000 rpm for 5 min. Supernatant was discarded and pellet was washed twice with 400 µl of 70% ethanol by centrifuging it at 5000 rpm for 5 min. Ethanol was rinsed out, pellets were air dried and resuspended in 100 µl TE buffer. Subsequently RNase treatment with final concentration of 100 µg/mL was done at room temperature for 1 hour. The DNA was re-extracted with fresh chloroform: isoamyl alcohol followed by precipitating it with ethanol and pelleting by centrifugation at 8000 rpm, 4°C. Pellet was collected, air dried at room temperature for 4-5 h. in clean place and dissolved in appropriate volume of 1X TE. DNA quantification was done by taking spectrophotometric readings of the DNA samples at wavelengths 260 nm and 280 nm. Ratio of OD 260/280 to be around 1.8 was considered as measure of DNA purity. The concentration of DNA was determined at wavelength 260 nm, (OD260 X 50 X dilution factor) and subsequently samples were diluted to 50ng/µl concentration. Qualitative and quantitative analysis of DNA sample was carried out by analyzing it on electrophoresis in 0.8% agarose gel with 0.5 µg/ml ethidium bromide (10mg/ml) in 1X TAE (Sambrook, 2001) .

3.8. Application of microsatellite markers

Wheat microsatellite SSR markers mapped on chromosomes 2A, 2B and 2D of wheat covering both chromosomal arms were selected from Röder *et al*. (1998), Pestsova *et al*. (2000) and Somers *et al*. (2004). List of all markers used in this study has been given in Annexure-I. Parental polymorphism of group 2 chromosome specific markers was checked on wheat cultivars and *Ae. species*. PCR was carried out according to Roder *et al*. (1998) with some modifications.

3.8.1. Composition of PCR reaction mix (20 µl)

S.No.	Name of the reagent	Composition
1	DNA(50 ng/ µL)	2 µl
2	PCR Buffer (10X)	2 µl
3	dNTP mix (1 mM each)	4 µl
4	Primer Forward(5 mM)	1 µl
5	Primer Reverse(5 mM)	1 µl
6	Taq polymerase	1 unit
7	$MgCl_2$ (25 mM)	1.2 µl
8	Water	7.8 µl

dNTP mix and Taq polymerase were purchased from New England Biolabs and PCR buffer and $MgCl_2$ were purchased from BioChem, The primers were synthesized from Metabion Hysel India (Pvt.) Ltd. Transferable polymorphic markers were applied in the finally selected derivatives and interspecific hybrids to identify the introgressed chromosome fragment .

3.8.2. PCR conditions

The PCR was carried on Eppendorf Thermocycler Gradient 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_m for 1 min.; extension at 72°C for 1 min.; Final extension at 72°C for 10 min

3.8.3. Resolution of the amplified SSR product:

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

3.9. Polyacrylamide gel electrophoresis for DNA

3.9.1. Preparation of 40% Acrylamide-bis acrylamide solution

38 g of Acrylamide and 2 g of bis acrylamide were weighed and dissolved in 50ml of double distilled water. The volume was made up to 100 ml in deionized double distilled water.

3.9.2. Preparation of 10X TBE

The composition of TBE buffer is as follows

Composition of 10X TBE buffer

S.No.	Name of the reagent	Composition
1	Tris	10.9 g
2	Boric acid	5.56 g
3	EDTA	0.98 g
4	Distilled water	100 ml

3.9.3. Procedure of DNA PAGE

The composition of PAGE gel used was as follows.

Composition of 8% PAGE gel

S.No.	Name of the reagent	Composition for 150 ml
1	40% acrylamide-bis acrylamide solution	30 ml
2	10X TBE	7.5 ml
3	Ammonium persulphate	0.105 g
4	TEMED	125 µL
5	Double distilled water	92.38 ml

Ammonium persulphate is light sensitive and always recommended to fresh prepared. Ammonium persulphate and TEMED were added just before pouring. All the constituents as mentioned in Tabel 4.2 were mixed thoroughly and pour in the gel assembly as soon as possible. After pouring, the comb was fixed in the gel solution and allowed to solidify for about 1 h.

3.9.4. Silver staining

For silver staining, gel was carefully placed in staining tray. The PAGE gel must be gone through three solutions i.e. Fixative solution for 5 min., staining solution for 5 min. and developing solution for 2 min., when band becomes clear then the gel is washed with distilled water and re-suspended in fixative solution to picture the gel and increase the depth and clarity of bands. The composition of all the solutions were used as follows

Name of the reagent	Composition
Composition of fixative solution	
Methanol	20 ml
Glacial acetic acid	1 ml
Distilled water	179 ml
Composition of staining solution	
Methanol	20 ml
Glacial acetic acid	1 ml
AgNO3	0.2 g
Distilled water	179 ml
Composition of developing solution	
NaOH	5.1 g
Formaldehyde	600 µL
Distilled water	199.4 ml

3.10. Software analysis for genotyping and mapping

The entire transferable and polymorphic marker data was carefully recorded for group 2 chromosomes and a consensus map were constructed using BioMercator V3.1 through user's manual instructions. Interactive diagram of transferable and polymorphic SSR marker among A, B, D, U and S genomes were constructed by MapChart software package. Polymorphic SSR markers were applied on all the derivatives and interspecific hybrids. All the marker data of addition and substitution lines were recorded and arranged by linkage group by showing data on several linkage groups at once through graphical genotyping software GGT 2.0 software. This software was further used for marker trait association analysis.

4. Results

Results of the studies are presented in the following sections-

1. Transferability and polymorphic survey of group 2 chromosome specific wheat SSR markers in *Aegilops species.*
2. Identification and biochemical analysis of group 2 *Aegilops* chromosome introgression lines with high grain Fe and Zn.
3. Radiation induced transfer of genes for high grain Fe and Zn content.
4. Induced homoeologous pairing between group 2, *Aegilops* and wheat chromosomes for precise transfer of useful variability through *ph1b* mutants.
5. Induced homoeologous pairing between group 2, *Aegilops* and wheat chromosomes for precise transfer of useful variability through *Triticum aestivum* cv. Pavon Mono 5B.

4.1. Transferability and polymorphic survey of group 2 chromosome specific wheat SSR markers on *Aegilops species.*

In the present study, SSR markers of bread wheat from group 2 chromosomes were studied and analyzed among three bread wheat cultivars *Triticum aestivum* L. cvs. WL711, PBW343, Chinese Spring and three related wild species, namely *Aegilops kotschyi* accession 396, 3790, 3573, *Ae. peregrina* acc. 3519, 13772 and *Ae. longissima* acc. 28. These polymorphic markers were analyzed for addition or substitution of group 2 chromosomes from *Aegilops species* into wheat.

4.1.1. Analysis of transferable SSR marker in wheat and *Aegilops species*

In the present study a total of 189 markers of group 2 chromosomes showing amplification in bread wheat were utilized for transferability analysis. A total of 143 markers (75.66%) of group 2 chromosomes were transferable among all the selected genotypes. A total of 41 markers (67.21%) for chromosome 2A showed transferability with high amplification percentage of marker of 2AS (81.48%) than 2AL (55.88 %). Similarly among 50 (73.52 %) transferable markers of chromosome 2B amplification percentage of markers of 2BS was 83.33% whereas 2BL had 65.78% transferability. Among 52 transferable (86.66%) markers of chromosome 2D, the marker for both the arms on 2DS and 2DL had highest transferability of 90% and 85% respectively. Considering total number of transferable markers among A,B and D genomes, the chromosome 2D genome markers showed highest percentage of transferable markers as compared to those of A and B genomes.

4.1.2. Analysis of polymorphism among transferable SSR markers in wheat and *Aegilops species.*

All the SSR markers which were transferable to wheat and the wild relatives were either showing monomorphic or polymorphic fragment amplification profile. Screening of amplified polymorphic fragments was based on strong signals showing difference in size or number of fragments in the selected genotypes. Polymorphism varied from 37-77% for the group 2 chromosome markers. Among the transferable markers, there were 27 markers of chromosome 2A (77.27% of 2AS and 52.63% of 2AL), 22 markers of chromosome 2B (40% of 2BS and 48% of 2BL) and 40 markers of chromosome 2D (94.44% of 2DS and 67.64% of 2DL) polymorphic among all the selected genotypes. Among all the tested and amplified markers, a total of 44.26 % of chromosome 2A, 32.35% of chromosome 2B and 66.66 % of chromosome 2D were polymorphic. The markers mapped on chromosome 2 of D genome showed higher

percentage of polymorphism among the transferable markers indicating that the D genome was less modified during evolution among Triticeae species. The overall amplification percentage of transferable and polymorphic marker has been shown in Table 4.1.

Table-4.1. Amplification of group 2 chromosome specific SSR markers on bread wheat and *Aegilops sp.*

Chromosome	Chromosomal arm	Total Number of SSR Markers	Transferable markers to *Aegilops species* (%)	Polymorphic markers among transferable (%)
2A	Short arm	27	22 (81.48%)	17 (77.27%)
	Long arm	34	19 (55.88%)	10 (52.63%)
2B	Short arm	30	25 (83.33%)	10 (40.00%)
	Long arm	38	25 (65.78%)	12 (48%)
2D	Short arm	20	18 (90.00%)	17 (94.44%)
	Long arm	40	34 (85.00%)	23 (67.64%)

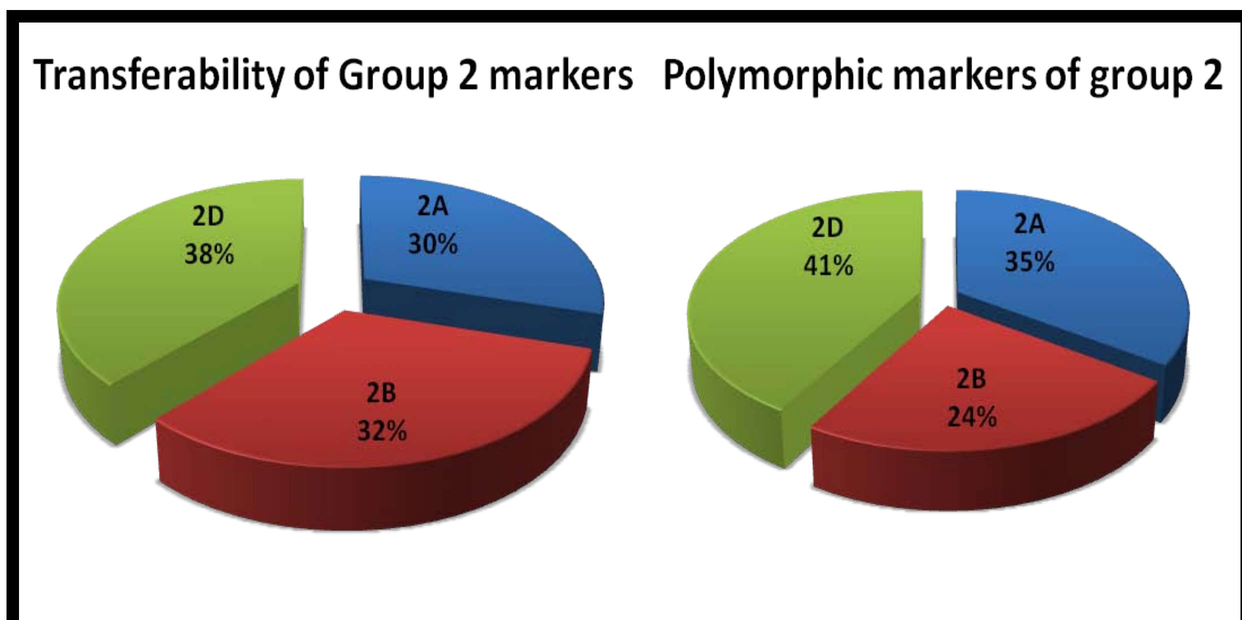


Fig. 4.1 Total percentage of transferable and polymorphic markers of group 2 chromosomes specific SSR markers in bread wheat and related non-progenitor species

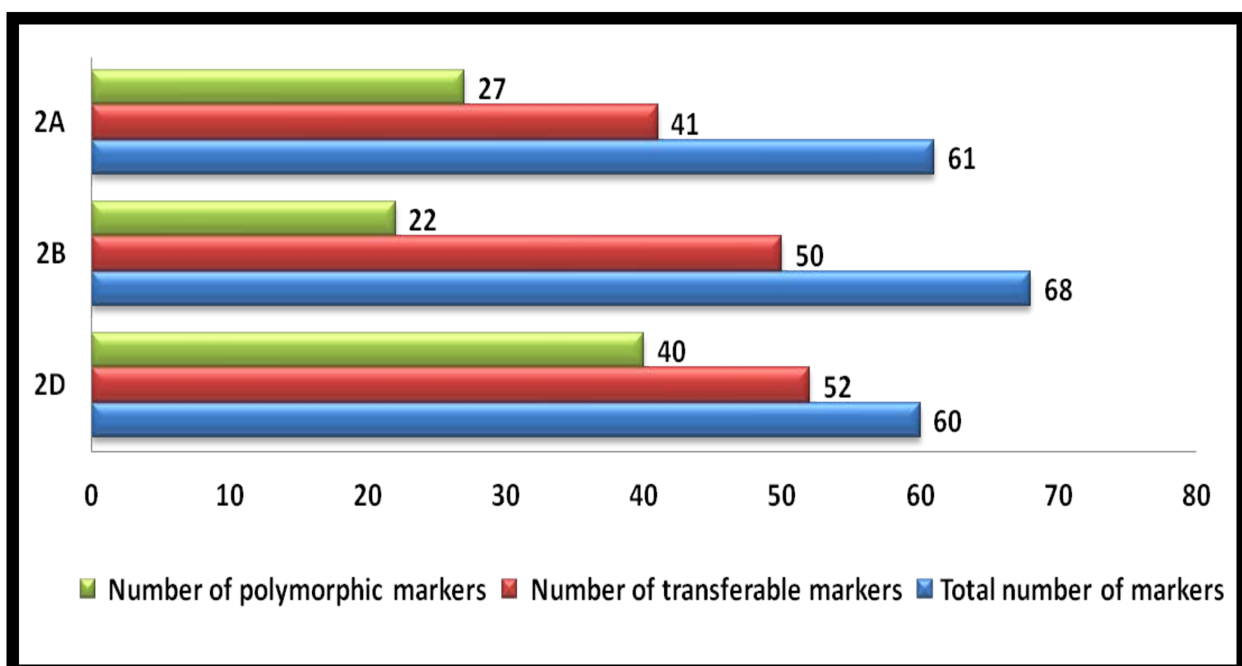


Fig. 4.2 Total number of transferable and polymorphic markers out of total number of markers tested on wheat and non-progenitor *Aegilops species* for group 2 chromosome

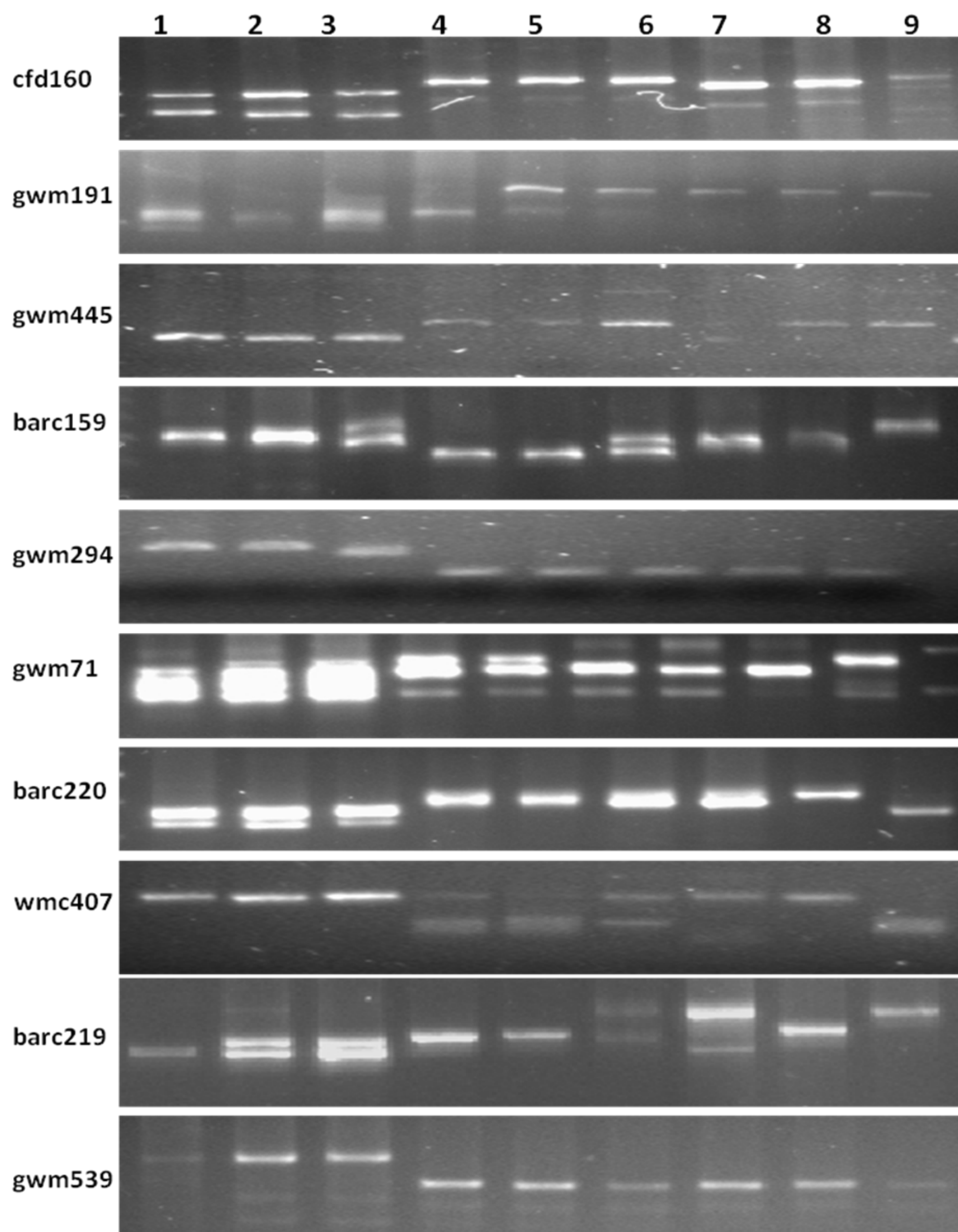


Fig. 4.3 Polymorphic survey of anchored SSR markers of group 2 chromosomes of bread wheat cultivars and *Aegilops species*. Lanes 1: *Triticum aestivum* cv. WL711, 2: *T. aestivum* cv. PBW343, 3: *T. aestivum* cv. Chinese spring, 4: *Aegilops kotschyi acc. 396*, 5: *Ae. kotschyi acc.3790,* 6: *Ae. kotschyi acc.3573,* 7: *Aegilops peregrina acc. 3519,* 8: *Ae. peregrina acc. 13772* and 9: *Aegilops longissima-28*

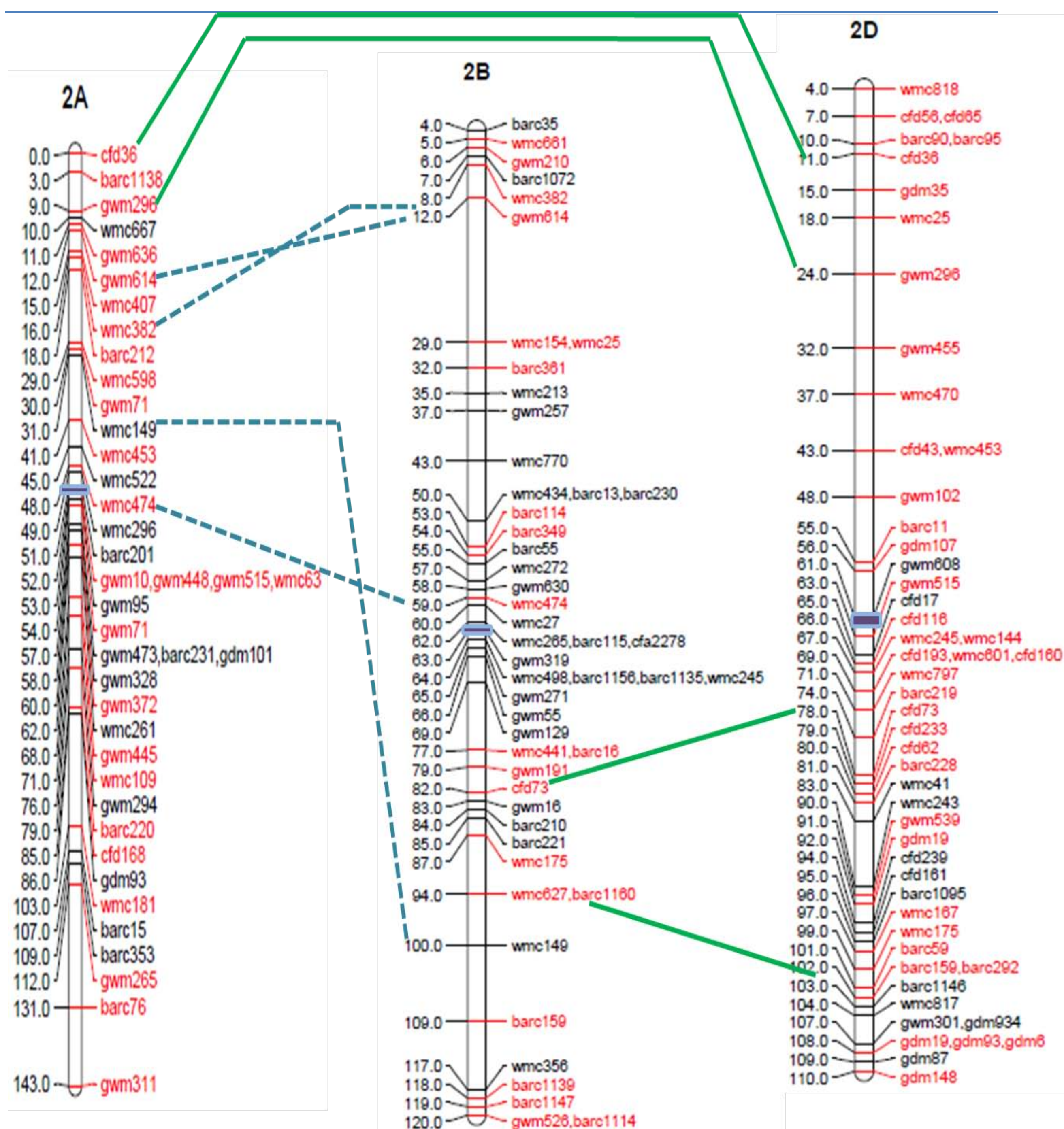


Fig. 4.4 Genetic map drawn through MapChart, showing location of transferable and polymorphic markers in bread wheat and *Aegilops species* in wheat linkage map of chromosome 2A, 2B and 2D (adapted from Somers *et al*., 2004). Black: monomorphic, red: polymorphic, blue dotted lines: inversion and green lines: colinerarity in two different chromosomes

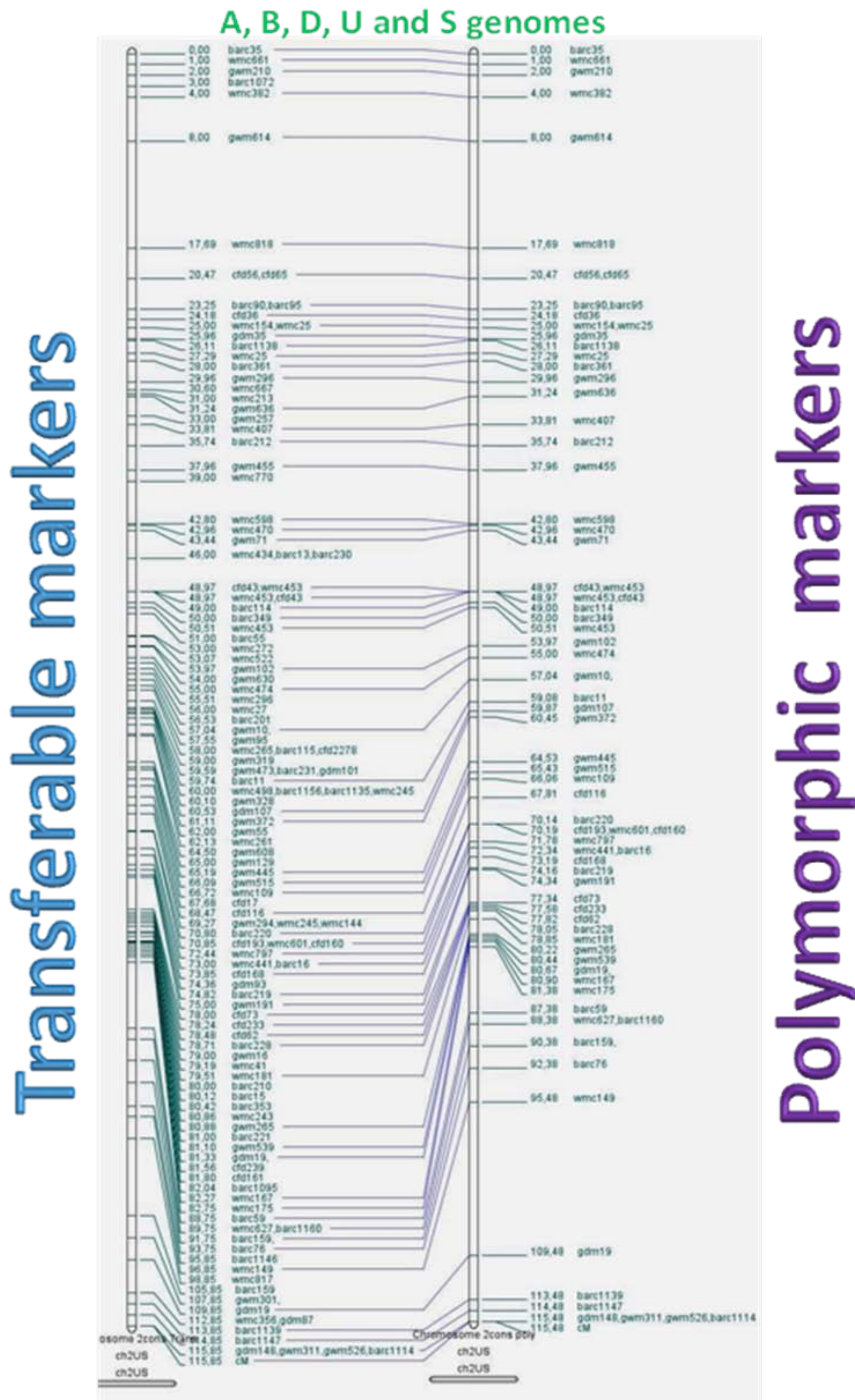


Fig.4.5 Consensus maps of transferable and polymorphic SSR markers among A, B, D, U and S genomes

All the transferable and polymorphic markers have been placed in the genetic map by assuming the distance and location of markers similar to the mentioned in high density microsatellite map developed by Somers *et al*., 2004. The location and distance of some markers, which were not included in the publication, have been taken from the related genetic maps presented at GraineGenes 2.0. Based on some shared markers among chromosomes 2A, 2B, 2D, 2U and 2S, a consensus map has been constructed using transferable and polymorphic markers. This map may give overall idea about the relative arrangement of the SSR markers in 2U/2S chromosomes based on the assumption of evolutionary conservation of colinerarity between A, B, D, U and S genomes (Fig. 4.5). The consensus map was further used for analysis of relative position of alien introgression in wheat-*Aegilops* derivatives.

The selected transferable markers have been applied on all the derivatives and induced transfers to analyse the introgression of alien chromosome fragment into wheat.

4.2. Identification and biochemical analysis of group 2 *Aegilops* chromosome introgression lines with high grain Fe and Zn.

Wheat and several *Aegilops species* were analyzed for grain micronutrient content through the biochemical and analytical techniques. The *Aegilops species* showed 2-3 fold high grain Fe and Zn content in comparison to elite wheat cultivars (Table 4.3). These were also analyzed for mineral distribution pattern in seeds. The mineral distribution pattern was very different in *Aegilops* and wheat seeds with higher percentage distribution of grain Fe and Zn in aleuronic layer of *Aegilops* in comparison to wheat indicating that *Aegilops* have superior mechanism for mineral uptake, transport and sequestration in comparison to wheat (Fig. 4.6 and 4.7)

Several wheat-*Aegilops* derivatives with high grain Fe and Zn content were developed by Tiwari *et al*., (2010), Rawat *et al*., (2011) and Neelam *et al*., (2011). Grain micronutrient content, harvest index, molecular marker analysis and cytological analysis of all the derivatives were performed for 5 consecutive seasons i.e. from 2009-10 to 2012-13. Based on the above analyses, best derivatives were forwarded to subsequent generations. These derivatives were also used for precise transfer of grain micronutrient genes through radiation hybrid approach and induced homoeologous pairing through *ph1b* and Mono 5B condition.

In the season 2009-10, segregating progenies of wheat-*Aegilops* derivatives were screened for grain micronutrients, morphological traits, and molecular marker analysis. The derivatives showed wide range of grain Fe and Zn content. It was observed that plants with high grain Fe and Zn had lower yield and harvest index.

4.2.1. Morphological characteristics and micronutrient content of selected wheat-*Aegilops* derivatives

In all the consecutive 5 years only high grain micronutrient plants with high harvest index were selected for further generation advancement. Mainly 5 wheat-*Aegilops* derivatives with introgression of 2U/2S were selected due to their high micronutrient content and grain yield. Progenies of 46-1-15-15, 49-1-73-8, 77-33-2-5, 77-36-6-15 and 77-50-8-1 were further selected for field trials and considered as important source for gene transfer through various molecular breeding techniques. Among all the selected derivatives 46-1-15-15, 49-1-73-8, 77-36-6-15 and 77-33-2-5 progenies were segregating for non-waxy leaves but all the 77-50-8-1 plants were waxy, indicating the absence of 2S chromosome. After extensive screening of wheat-*Aegilops* derivatives, in the year 2010, some plants were selected for precise gene transfer through radiation hybrid approach and homoeologous pairing. The selected derivatives showed variable number of chromosomes ranging from 41 to 44. In the year 2010, no plant screened was showing exactly 21 bivalents. The plants were highly segregating but after several generation advancements and selfing events derivatives in the season 2012-13 were more stable, except 77-50-8-1, with 42 chromosomes (21II) and regular pairing. The initially selected wheat-*Aegilops* derivatives data has been given in Annexure-2.

Cytological analysis of the selected derivatives has been shown in Fig. 4.10.Morphological characteristics of selected derivatives are given in Fig. 4.8 and Fig. 4.9.

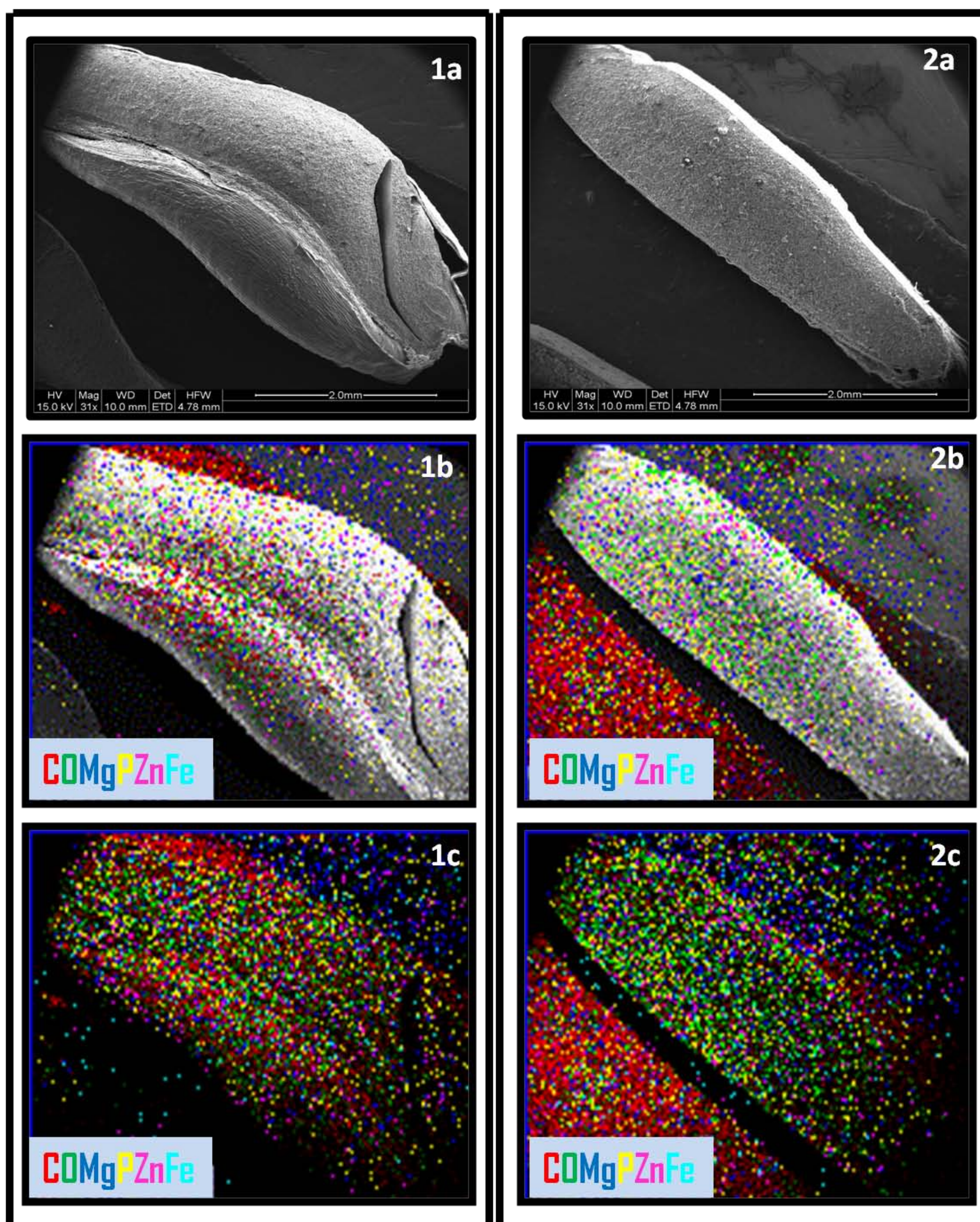
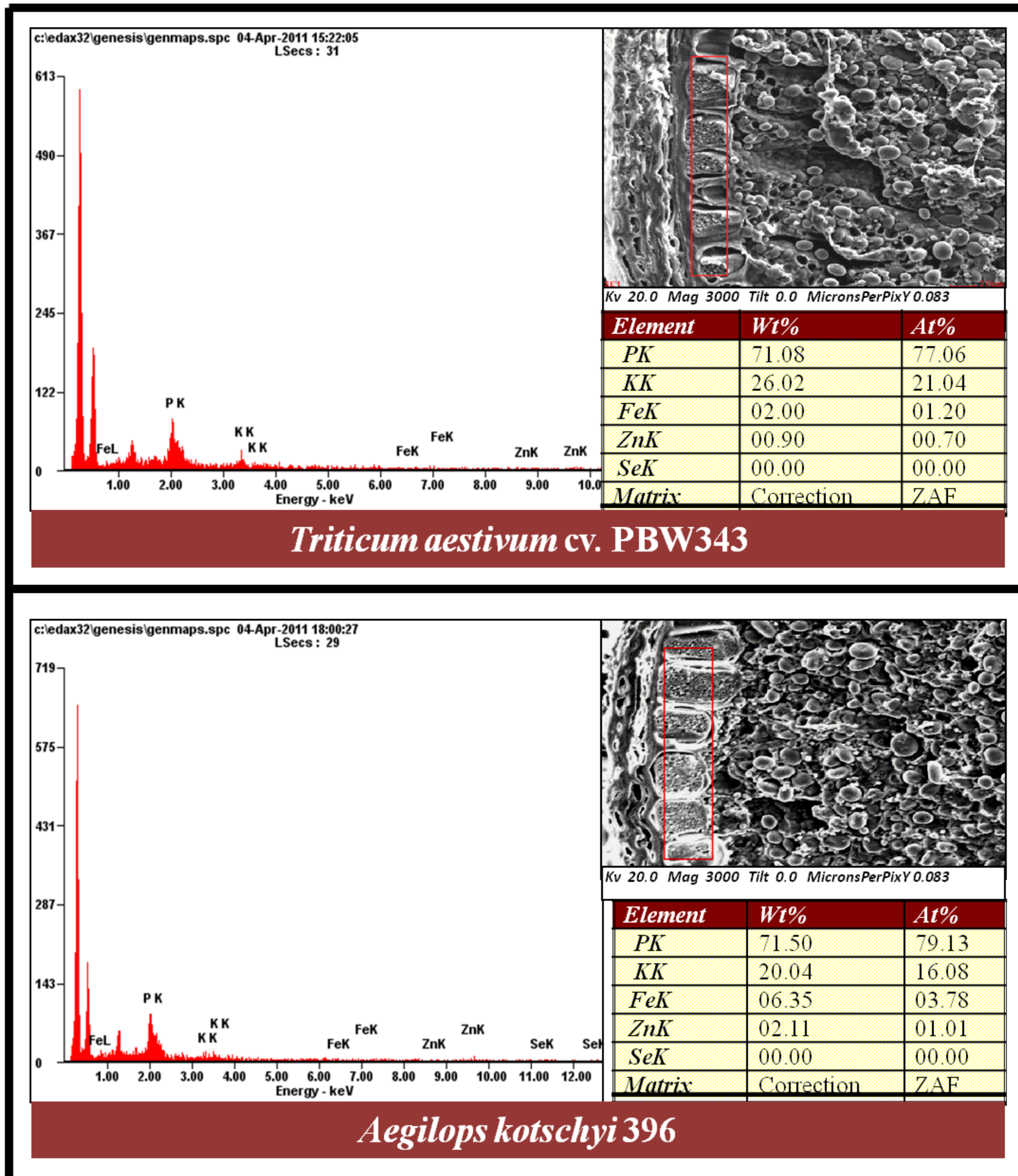


Fig. 4.6 Colour display images for distribution pattern of C, O, Mg, P, Zn and Fe along section of wheat, PBW343 (1a, 1b, 1c) and *Aegilops kotschyi* 396 (2a, 2b, 2c) by SEM-EDX. The red colour background was the tape where grain sample was embedded for SEM analysis



Triticum aestivum cv. PBW343

Element	Wt%	At%
PK	71.08	77.06
KK	26.02	21.04
FeK	02.00	01.20
ZnK	00.90	00.70
SeK	00.00	00.00
Matrix	Correction	ZAF

Aegilops kotschyi 396

Element	Wt%	At%
PK	71.50	79.13
KK	20.04	16.08
FeK	06.35	03.78
ZnK	02.11	01.01
SeK	00.00	00.00
Matrix	Correction	ZAF

Fig. 4.7 Comparative analysis of distribution of minerals in selected areas of seed of bread wheat and *Aegilops kotschyi* 396, the selected area under red rectangles is the aleurone layer, and EDX profile for mineral content in the selected area of aleurone layer is given in the tables

Fig.4.8 Morphological characteristics of selected derivatives and their respective parent (season 2009-10) at IIT Roorkee, *Triticum aestivum* cv. WL711, *T. aestivum* cv. PBW343, *Aegilops kotschyi* acc. 3790, and 77-36-6-13, 77-33-2-5, 49-1-73-8 and 46-1-15-15 are the wheat-*Aegilops* derivatives

Fig.4.9 Field overview of selected wheat-*Aegilops* derivatives at the Eternal University, Baru-Sahib, Himachal Pradesh in 2012-13

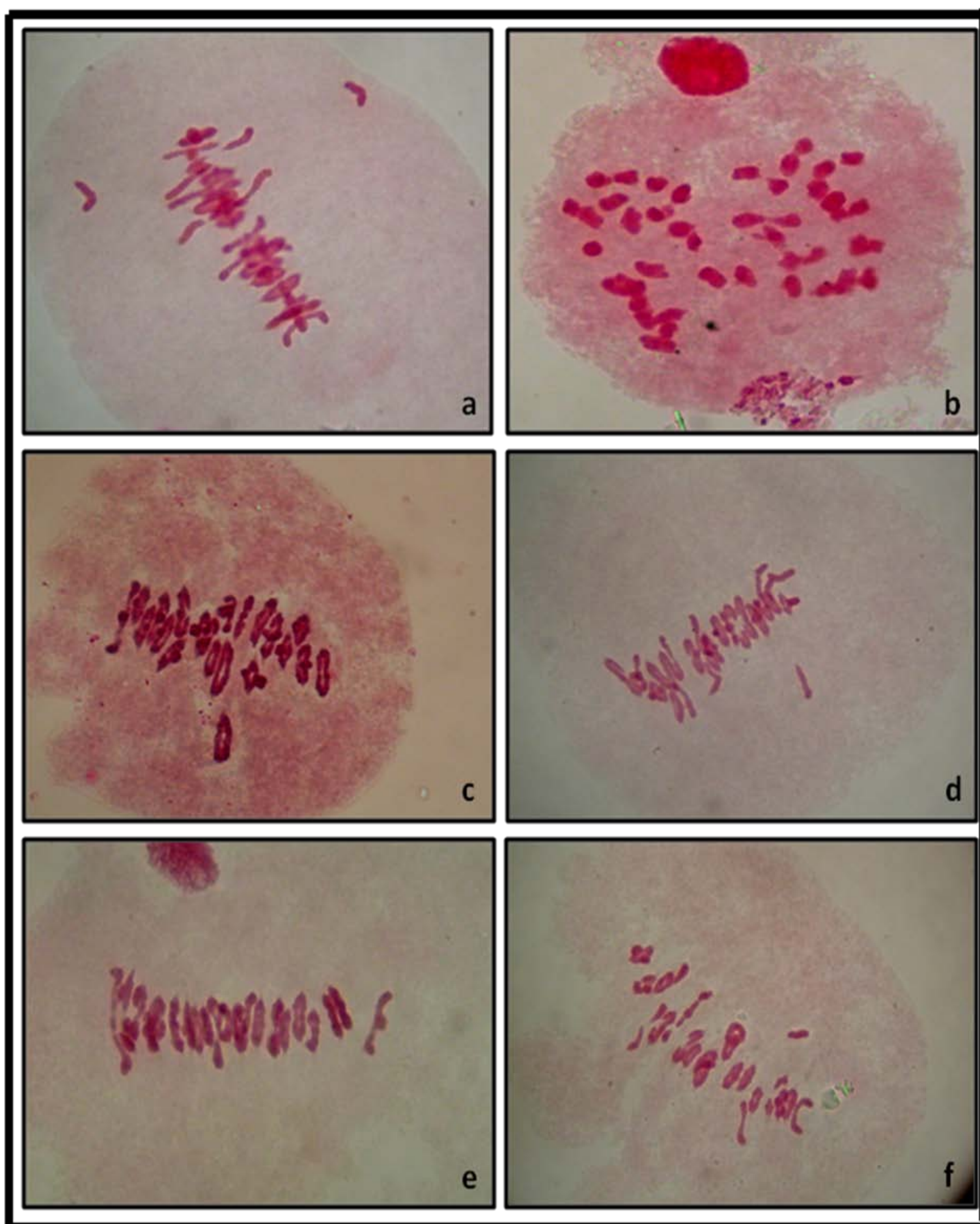


Fig.4.10 Cytological analysis of PMC at meiosis-I of selected wheat-*Aegilops* derivatives (2011-12) a) 77-50-8-1 (44; 21II + 2I), b): 77-50-8-1 (44; 22II), c): 49-1-73-8 (42; 21II); d): 77-36-6-15 (42; 21II), e) 46-1-15-15 (42; 21II), f: 77-33-2-5 (42; 20II +2I)

Table: 4.2 Introgression of group 2 chromosomes in selected wheat-*Aegilops* derivatives using transferable and polymorphic SSR markers in wheat cultivars and *Ae. kotschyi* parents

SSR markers	**WL711**	***Ae. kotschyi* 396**	**77-33-2-5**	**77-36-6-15**	**77-50-8-1**	**49-1-73-8**	**46-1-15-15**
2A-gwm71	W	K	K+W	K+W	W	K+W	K+W
2A-gwm265	W	K	K+W	K+W	W	K+W	K+W
2B-barc349	W	K	K+W	K+W	W	K+W	K+W
2B-wmc25	W	K	K+W	W	W	W	W
2D-cfd56	W	K	W	W	W	K+W	W
2D-barc90	W	K	W	W	W	K+W	W
2D-wmc470	W	K	K+W	K+W	W	K+W	K+W
2D-cfd43	W	K	K+W	K+W	W	W	W
2D-gwm539	W	K	K+W	K+W	K+W	K+W	W
2D-barc11	W	K	W	W	W	K+W	W
2D-barc219	W	K	W	W	W	K+W	W
2D-barc159	W	K	W	W	W	K+W	W
2D-barc1139	W	K	K+W	K+W	W	W	W
2D-gdm148	W	K	K+W	K+W	K+W	K+W	W

4.2.2. SSR marker analysis on wheat-*Aegilops* derivatives.

Various group 2 chromosome specific transferable and polymorphic SSR markers were used for analysis of wheat-*Aegilops* derivatives. Some markers showed addition of *Aegilops* alien chromosomes in the derivatives (Table 4.2). These derivatives were analyzed using SSRs for up to 5 generations i.e. 2009-2013. The 2 SSR markers mapped on 2A chromosome i.e. gwm71 and gwm265 showed addition of alien chromosome of group 2 four out of five derivatives. Only 2 SSR marker anchored on chromosome 2B showed addition/substitution of alien chromosome in some derivatives while 10 SSR markers anchored on 2D chromosome indicated addition of alien introgression in one or more derivatives. Among all the selected SSRs, marker barc11, cfd56, gwm539 and gdm148 were the most robust one showing clear polymorphism. All the selected derivatives have high micronutrient, good yield and introgression of alien chromosome confirmed by at least one or more SSR markers (Table 4.3).

Table 4.3: Grain micronutrient and morphological characteristics of parents and selected wheat-*Aegilops* derivatives (season 2012-13)

Plant	**Chromosome number**	**No. of Tillers per plant**	**Height (cm)**	**Waxiness of leaves**	**Grain Fe (mg/kg)**	**Grain Zn (mg/kg)**	**1000 Kernel weight (gm)**	**Harvest index**
Triticum aestivum cv.WL711	42	17	94	Waxy	24.70±0.78	22.14±1.30	39.7	41.98
Triticum aestivum cv. PBW343	42	15	91	Waxy	30.30±0.76	25.29±0.41	42.3	42.24
Triticum aestivum cv.Chinese spring	42	19	99	Waxy	27.59±0.25	23.91±0.51	36.1	39.19
Aegilops kotschyi acc.*396*	28	214	37	Non-waxy	72.20±2.00	77.56±1.35	11.9	NA
Aegilops kotschyi acc.*3790*	28	208	35	Non-waxy	70.88±3.06	77.98±0.59	11.6	NA
Aegilops kotschyi acc.*3573*	28	221	38	Non-waxy	80.19±1.94	79.18±1.20	12.1	NA
Aegilops peregrina acc.*3519*	28	198	37	Non-waxy	48.53±1.55	57.64±0.43	12.9	NA
Aegilops peregrina acc.*13772*	28	207	36	Non-waxy	65.23±1.01	69.60±1.67	12.4	NA
Aegilops longissima acc.*28*	12	172	81	Non-waxy	68.48±1.49	72.37±0.56	9.4	NA
46-1-15-15-3-1-1-2	44 (21II+2I)	24	92	Non-waxy	43.37±0.07	48.03±0.70	41.2	38.97
49-1-73-8-5-2-4 ⊗	42 (21 II)	26	86	Non-waxy	45.02±1.69	58.14±1.97	38.1	34.86
77-33-2-5-9-5-2 ⊗	42 (21 II)	21	95	Non-Waxy	36.07±4.83	50.25±4.71	37.4	36.71
77-36-6-15-1-8-2 ⊗	42 (21 II)	19	97	Non-waxy	41.19±1.40	50.49±4.24	40.2	35.68
77-50-8-1 ⊗	42 (21 II)	22	94	Waxy	42.02±0.91	37.94±0.40	39.2	37.41

NA: Not available

4.3. Radiation induced transfer of genes for high grain Fe and Zn content

For precise and stable transfer of genes responsible for high grain Fe and Zn content from wheat-*Aegilops* derivatives into elite wheat cultivars, mainly two strategies were followed: Seed irradiation and pollen irradiation

4.3.1. Transfer of high grain Fe and Zn genes through seed irradiation

4.3.1.1. Optimization of effective radiation dose

Seeds of *Triticum aestivum* cv. Chinese Spring were irradiated at dose ranging from 25 krad to 55 krad. Percentage seed germination and plant height were taken as criteria for evaluation of survival and plant growth due to radiation. Radiation dose >40 krad, results in dramatic effects on seed germination and growth. Highest seed germination (82%) was observed at 25 krad while only 61% seed germinated at 55 krad. Shoot length was recorded up to 25 days. In comparison to control, 25 krad radiation dose showed minimal effect and 55 krad showed maximum effect on plants growth. Shoot length was also significantly reduced at 55 krad. Presence of univalent, iso-chromosome and multivalents in plants from irradiated seeds indicated the effectiveness of radiation at 40 krad for chromosomal breakage and transfer. On the basis of this data, 40 krad was chosen as the effective radiation dose to irradiate seeds for radiation induced transfer of genes for high grain Fe and Zn from wheat-*Aegilops* derivatives into elite wheat cultivar *Triticum aestivum* cv. WL711.

4.3.1.2. Development of seed radiation hybrid (SRH) population.

Based on the grain micronutrient data two derivatives were selected for seed radiation at 40 krad i.e. 77-33-2⊗ (Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338(77)-2-33-2⊗) and 77-36-6⊗ (Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338(77)-2-36-6⊗). Irradiated seeds were sown in the field and the plants were analyzed for pollen stainability and cytology. Plants from the irradiated seeds of derivatives were taken and crossed with *T. aestivum* cv. WL711. From derivative 77-33-2⊗, only 90 seeds were obtained while 135 seeds from 77-36-6⊗. In the next season all the F_1 seeds were sown in the field where 90 plants from 77-33-2⊗ and only 43 plants of 77-36-6⊗ survived up to full seed maturity which were further characterized for radiation induced transfers through molecular, cytological and biochemical techniques.

4.3.1.3. Morphological and molecular analysis of seed radiation hybrids

The entire radiation hybrid F_1 (SRH_1) populations obtained were further analyzed for several morphological traits. Non-waxy trait considered as the morphological marker for introgression

of 2S/2U was recorded in all the generations. Only the healthy plants, showing better yield, good harvest index and bold seeds were selected. General morphology of selected SRH_2 population has been shown in Fig. 4.13. All the group 2 chromosome specific polymorphic SSR markers were used to analyze the derivatives. Those markers, showing introgression in the derivatives were further applied on radiation hybrid populations. Only 4 markers showed introgression in radiation hybrids at SRH_2 generation i.e. gwm265, barc1139, cfd43 and gdm148. These markers confirmed the presence of 2U/2S specific alien introgression from *Aegilops* into wheat. The comprehensive morphological and molecular data of selected SRH_2 population is present in Table 4.4.

4.3.1.4. Micronutrient analysis of seed radiation hybrids

Grain Fe and Zn were analyzed up to 3 generations. Due to the segregation in radiation hybrids, significant variation in molecular and biochemical data was observed. SRH_1 showed very high segregation while SRH_3 showed least segregation. In SRH_2 generation, plant A-150 showed highest grain Fe and Zn content i.e. 102.66±1.94 mg/kg and 121.83±0.91mg/kg respectively. While in SRH_3 generation again the A-115 plant showed highest grain Fe and Zn content but variation in content level in comparison to the previous season i.e. Fe content was 44.86±1.07 and Zn content was 60.39±1.08. In the year 2011-12 ICPMS was used for micronutrient analysis while in the year 2012-13, AAS was used for analysis. Grain Fe and Zn readings and harvest index of selected plants of SRH_2 and SRH_3 has been presented in table 4.5.

4.3.1.5. Genomic in situ hybridization study of radiation hybrids

GISH study of selected plants was carried out to characterize alien introgressions conferring high grain iron and zinc. Fig. 4.14 shows GISH of some selected SRH_2 plants. Previously it was identified that 77-36-6 ⊗ 77-33-2⊗ had 2 pairs of $2S^K$ chromosomes while the A-108 and A-115 of SRH_2 population showed several Robertsonian translocations identified through two major signals and 4 minor GISH signals (Fig. 4.14).

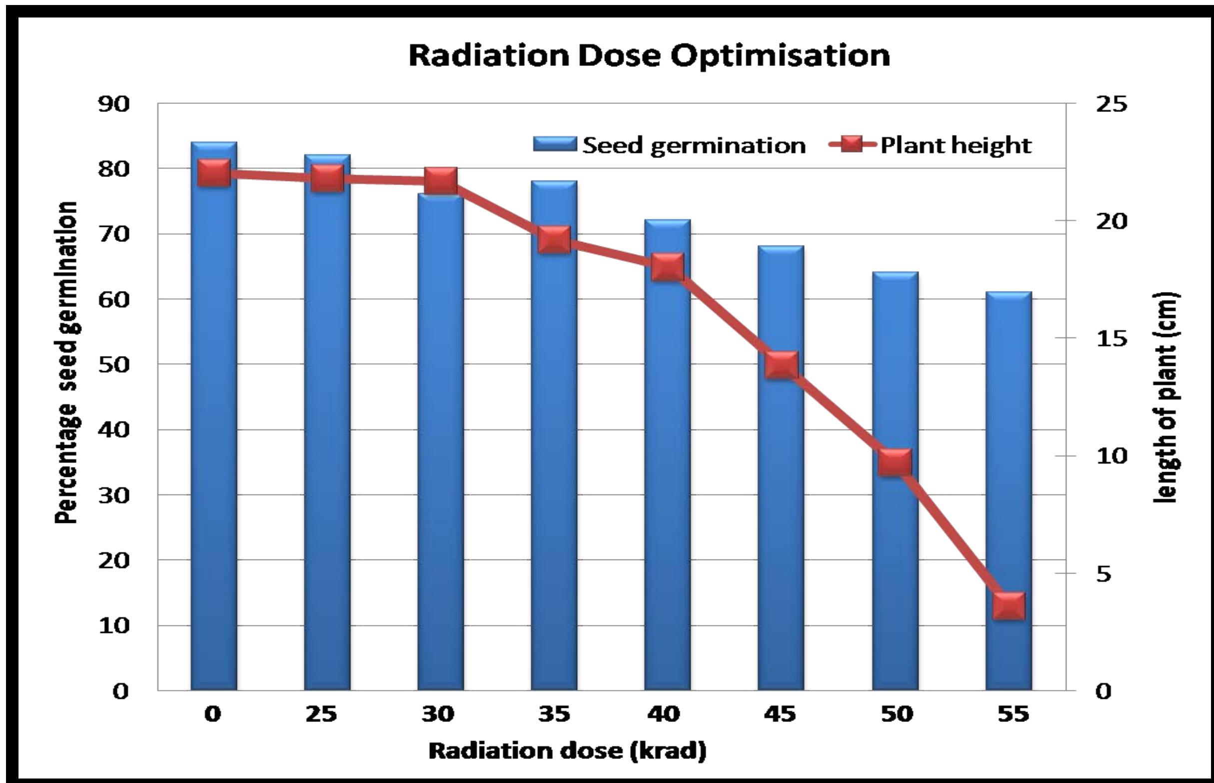


Fig. 4.11 Optimization of effective gamma radiation dose for seed radiation hybrids

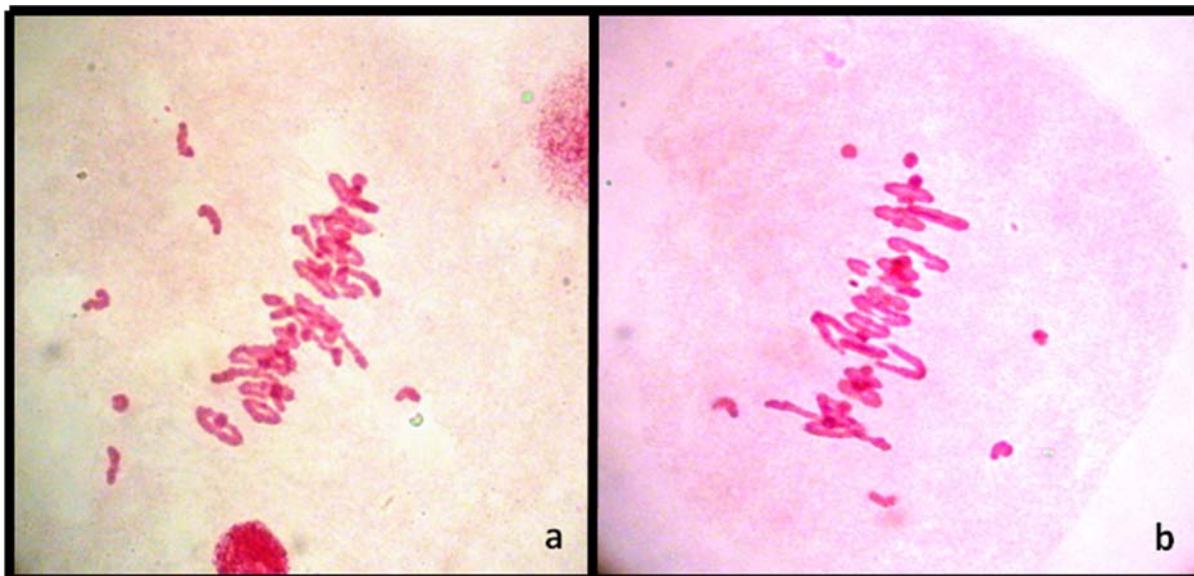


Fig. 4.12 Effect of radiation dose on chromosome pairing behaviour of wheat-*Aegilops* derivatives. a) 77-33-2⊗ b) 77-36-6⊗

Fig. 4.13 Morphology of derivatives and the selected SRH_2 seed radiation hybrids (see table4.5 for micro nutrient percentage and table 4.4 for pedigree)

Table 4.4: Morphological and molecular data of SRH_3 generation of seed radiation hybrids and parents

Plant details		SSR markers				General morphology			
Parents/ Derivatives	Pedigree	gwm 265	barc 1139	cfd 43	gdm 148	Tiller Number	Height (cm)	Appearance	Waxiness
WL711	*Triticum aestivum* cv. WL711	+	+	+	+	18	98	Healthy	Waxy
77-33-2⊗	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-33-2⊗	+	+	+	+	17	97	Weak	Non-Waxy
77-36-6⊗	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-36-6⊗	-	+	+	+	20	112	Weak	Non-Waxy
A-57	SRH_3 WL711/77-33-2⊗-57-3	+	+	+	+	2	100	Weak	Non-Waxy
A-62	SRH_3 WL711/77-33-2⊗-62-4	+	+	+	-	3	86	Weak	Non-Waxy
A-73	SRH_3 WL711/77-33-2⊗-73-8	-	-	-	+	14	103	Healthy	Non-Waxy
A-74	SRH_3 WL711/77-33-2⊗-74-9	-	-	-	+	6	32	Weak	Waxy
A-75	SRH_3 WL711/77-33-2⊗-75-9	-	-	-	-	10	97	Healthy	Waxy
A-86	SRH_3 WL711/77-33-2⊗-86-3	-	+	-	-	10	102	Healthy	Waxy
A-89	SRH_3 WL711/77-33-2⊗-89-5	-	-	-	-	10	97	Healthy	Waxy
A-94	SRH_3 WL711/77-36-2⊗-94-9	-	+	+	-	23	112	Healthy	Waxy
A-96	SRH_3 WL711/77-36-2⊗-96-5	-	+	+	-	13	110	Healthy	Waxy
A-108	SRH_3 WL711/77-36-2⊗-108-1	-	+	-	+	11	99	Healthy	Waxy
A-109	SRH_3 WL711/77-36-2⊗-109-1	-	-	+	+	18	110	Healthy	Waxy
A-111	SRH_3 WL711/77-36-2⊗-111-3	-	-	-	+	11	110	Healthy	Non-Waxy
A-112	SRH_3 WL711/77-36-2⊗-112-4	-	-	-	+	13	110	Healthy	Non-Waxy
A-115	SRH_3 WL711/77-36-2⊗-115-6	-	-	-	+	7	105	Healthy	Non-Waxy
A-117	SRH_3 WL711/77-36-2⊗-117-6	-	-	-	+	5	98	Healthy	Waxy
A-119	SRH_3 WL711/77-36-2⊗-119-6	-	+	+	+	20	112	Healthy	Non-Waxy
A-120	SRH_3WL711/77-36-2⊗-120-7	-	-	-	-	5	103	Healthy	Waxy
A-123	SRH_3 WL711/77-36-2⊗-123-2	-	-	-	+	8	105	Healthy	Waxy
A-124	SRH_3 WL711/77-36-2⊗-124-3	-	-	-	+	19	115	Healthy	Waxy
A-133	SRH_3 WL711/77-36-2⊗-133-6	-	-	-	-	8	100	Weak	Waxy

"+" means addition of 2U/2S and "-" means absence of alien chromosome fragment

Table: 4.5 Grain Fe and Zn content of seed radiation hybrids SRH_3 and parents only two consecutive seasons (2011-13)

Season 2011-12 (ICPMS)-IITR				Session 2012-13 (AAS)-EU,H.P.			
Parents/ derivatives, SRH_2	Iron concentration (mg/kg) Mean ± S.D	Zinc concentration (mg/kg) Mean ± S.D	Harvest Index (%)	Parents/ derivatives, SRH_3	Iron concentration (mg/kg) Mean ± S.D	Zinc concentration (mg/kg) Mean ± S.D	Harvest Index (%)
WL711	35.92±1.25	47.27±2.08	38.27	WL711	23.30±2.74	23.38±3.35	38.91
77-33-2$\otimes$	80.28±2.89	56.23±1.84	28.94	77-33-2$\otimes$	47.79±2.19	34.68±3.11	33.57
77-36-6$\otimes$	95.75±3.04	98.24±3.24	29.67	77-36-6$\otimes$	41.50±1.38	44.77±1.04	31.24
A-57	67.92±2.89	55.34±2.03	22.91	A-57-1	41.33±1.07	42.66±0.72	30.24
A-62	60.10±1.05	45.00±1.86	26.19	A-62-1	43.84±1.06	45.32±0.79	29.74
A-73	71.78±2.78	41.68±1.94	31.77	A-73-1	33.50±0.84	39.71±0.66	41.26
A-74	84.76±3.08	58.29±2.04	12.49	A-74-1	35.18±0.88	39.71±0.66	38.91
A-75	50.63±2.14	40.61±3.07	36.99	A-75-1	31.11±0.80	38.47±0.56	42.20
A-86	40.81±3.75	27.55±1.08	41.36	A-86-2	31.04±0.80	32.55±0.53	33.54
A-89	31.95±2.14	28.00±2.07	37.27	A-89-1	36.65±0.91	41.28±0.70	33.54
A-94	63.00±2.41	66.91±2.47	33.67	A-94-1	39.30±0.96	46.38±0.80	22.79
A-96	61.28±0.98	78.94±1.94	21.69	A-96-1	42.30±1.02	42.56±0.72	24.04
A-108	84.41±1.27	105.48±1.24	36.16	A-108-2	43.34±1.04	50.52±0.88	39.83
A-109	101.00±1.89	99.94±1.45	33.04	A-109-2	41.90±1.02	37.88±0.63	29.15
A-111	89.79±1.57	98.28±1.97	12.50	A-111-1	37.55±0.93	36.22±0.60	26.22
A-112	78.81±2.35	69.32±0.76	34.58	A-112-3	41.15±1.00	51.90±0.91	27.89
A-115	102.66±1.94	121.83±0.91	45.51	A-115-1	44.86±1.07	60.39±1.08	26.47
A-117	76.94±1.75	71.14±2.24	45.51	A-117-2	31.15±0.79	55.67±0.97	18.61
A-119	82.18±0.86	82.18±1.75	33.61	A-119-2	37.90±0.93	47.33±0.81	33.60
A-120	69.51±2.14	66.91±2.67	23.24	A-120-2	32.36±0.82	36.31±0.60	35.77
A-123	82.45±1.48	87.92±1.95	30.49	A-123-1	22.24±0.62	30.63±0.49	35.43
A-124	68.96±1.11	60.00±2.07	53.24	A-124-2	34.04±0.86	39.22±0.66	30.04
A-133	76.54±1.02	69.00±1.06	9.08	A-133-2	31.99±0.82	48.93±0.85	34.73

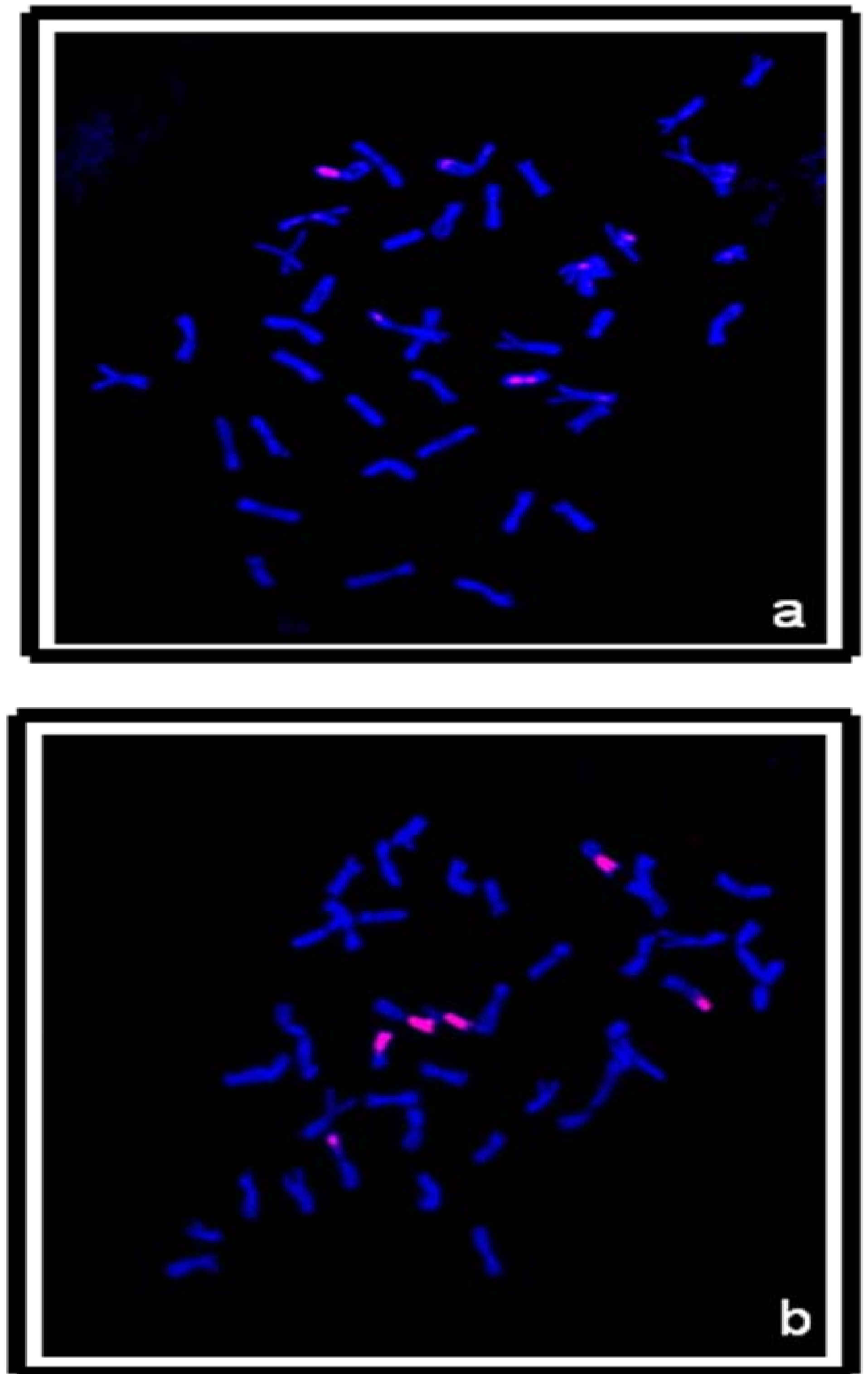


Fig. 4.14 Genomic in situ Hybridization of selected seed radiation hybrid SRH_2 showing introgression of 2S (pink) alien chromosome fragments a) A-108 b) A-115

As shown in Fig. 4.13. some of the selected plants were showing very good plant morphology, good yield and harvest index, with enhanced level of grain micronutrients. GISH analysis also reconfirmed that the single $2S^k$ chromosome was fragmented into small segments and stably introgressed into several other chromosomes. The selected plants will be further analyzed for stability, fertility and micronutrient content

4.3.2. Transfer of high grain Fe and Zn controlling chromosomal fragment(s) through pollen irradiation

4.3.2.1. Optimization of effective radiation dose for pollen irradiation

A day before dehiscence, spikes of *Triticum aestivum* cv. PBW343 were collected and subjected to variable radiation doses ranging from 1 krad to 5 krad. On the following day radiated spikes were further used as pollen source for crossing with *Triticum aestivum* cv. PBW343 as female parent. Percent seed set were recorded on each radiation dose. At 1 krad about 79.03% seed set was observed, while only 2.68% seed set was observed at 5 krad of radiation dose. Radiation dose >2 krad had dramatic effect on seed set and crossability within the same species. Considering seed set as a criterion for radiation induced translocation, 2 krad was chosen as the effective radiation dose for pollen irradiation induced transfer of grain Fe and Zn containing chromosomal fragment(s). Effect of several radiation doses on percent seed set has been shown in Fig.4.15.

4.3.2.2. Development of pollen radiation hybrid population

Wheat-*Aegilops* derivatives were analyzed for grain micronutrient content and addition/substitution of alien chromosome fragment(s). Two derivatives were selected for pollen irradiation i.e. 49-1-73-10⊗ and 77-33-2-5⊗. These derivatives were grown in the field and before dehiscence of pollen; spikes were detached from plant and irradiated at 2 krad at cobalt 60 gamma chamber. These irradiated spikes were taken as the donor parent and crossed with female parent i.e. *T. aestivum* cv. PBW343. After crossing with the irradiated pollen of derivatives, a total of 399 seeds were obtained. Only 91 from 77-33-2-5⊗ while 308 seeds from 49-1-73-10⊗ obtained. These seeds showed very poor viability and only 99 seeds of 49-1-73-10⊗, while only 51 seeds of 77-33-2-5⊗ germinated in the field. The fertility of these plants was even worst, only 4 plants from 77-33-2-5⊗ irradiation were fertile while 16 plants from 49-1-73-10⊗. These pollen radiation hybrids (PRH) were analyzed for grain Fe and Zn, harvest index, yield, introgression of alien group 2 chromosome. Only plants with good health, harvest index and desired traits were selected for next generation.

4.3.2.3. Morphological characteristics and micronutrient analysis of pollen irradiation hybrids (PRHs) of derivatives 49-1-73-10⊗ and 77-33-2-5⊗.

All the germinated pollen radiation hybrid plants (PRH_1) were transferred in the field and morphological traits were carefully recorded. Non-waxy character was chosen as a morphological identification for the presence of alien group 2 chromosomes. Both the population was segregating for non-waxy character. Among all PRH_1 plants, only B-20 and B-52 were waxy plants while all other were non-waxy. As shown in Fig 4.18. HMW Glutenin subunit profile of several pollen hybrids including B2, B8, B10, B-16, B-19, B20, B-27, B-44, B-45, B-56 and B-58 showed introgression of 1U/1S. Be not so having chromosome several plants including B-2, B-10, B-44, B-59 and C-1were discarded.

Grain Fe and Zn content of all the selected pollen irradiation hybrids (PRH_1) were carefully estimated in triplicate. Grain Fe and Zn data of PRH_1 generation was recorded using ICPMS. In PRH_1 B-44 showed highest grain Fe content i.e., 54.03 mg/kg while B-8 gave 58.79 mg/kg Zn. B-56 was healthy plant, with high harvest index i.e. 56.24 with grain Fe and Zn content of 43.71 mg/kg and 49.83 mg/kg respectively. Among all the F_1 plants, B-19 showed lowest Fe content of 36.82 mg/kg while with total Zn content of 48.71 mg/kg.

There was significant difference in the total Fe and Zn content of PRH_2 plants observed. Among all the PRH_2 plants, plant B-45-1 line showed highest grain Fe and Zn content i.e. 64.47 mg/kg and 88.42 mg/kg respectively, with harvest index of 28.52. While the plant B-16-1 gave lowest grain Fe and the plant B-8-1 showed lowest Zn content i.e., 38.85 mg/kg and 43.71 mg/kg respectively. Grain Fe and Zn content and harvest index of all the selected plants is given in table. 4.7.

4.3.2.4. GISH of selected pollen radiation hybrids

GISH analysis of selected pollen radiation hybrids was done. Wheat-*Aegilops* derivative 49-1-73 was known to have two *Ae. kotschyi* chromosomes.. During GISH analysis of several pollen radiation hybrids it was found that plant B-52 and B-56 had stable introgression of U^k and S^k chromosome. Through GISH analysis it was further observed that U and S chromosomes of wheat-*Aegilops* derivatives were fragmented and stably introgressed in the wheat genome showing Robertsonian translocation (Fig. 4.19)

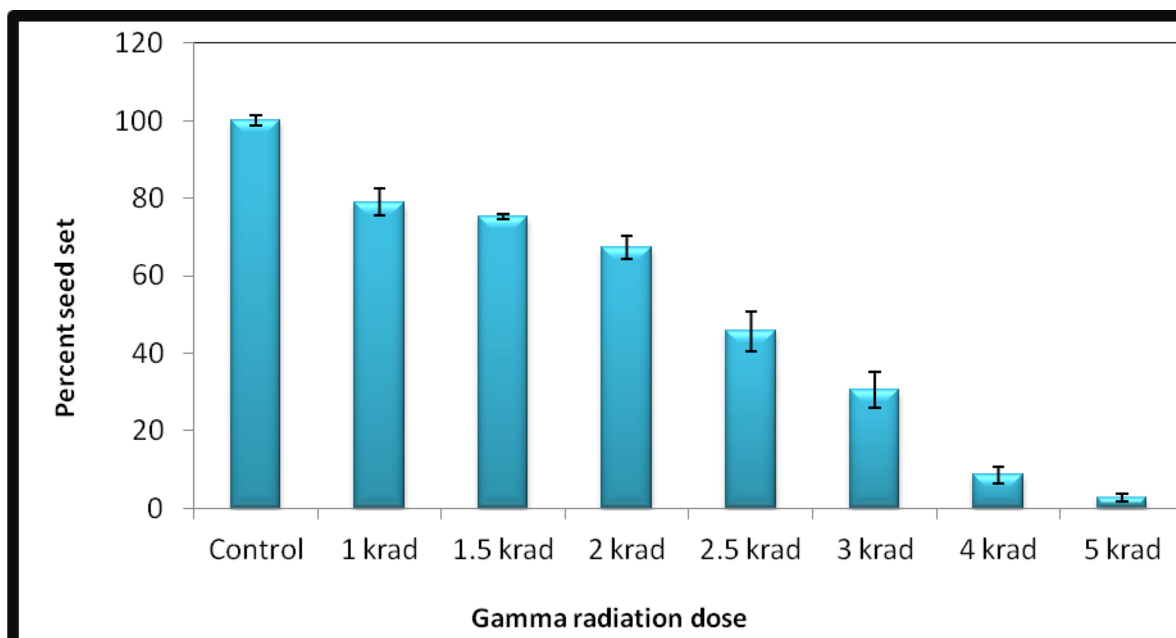


Fig 4.15 Optimization of pollen irradiation dose in *T. aestivum* cv. PBW343

Fig 4.16 Morphological characteristics of pollen radiation hybrids (PRH_1)

Fig. 4.17 Grain morphology of selected pollen irradiation hybrid (PRH_1) with their parent (see the table 4.8 for pedigree

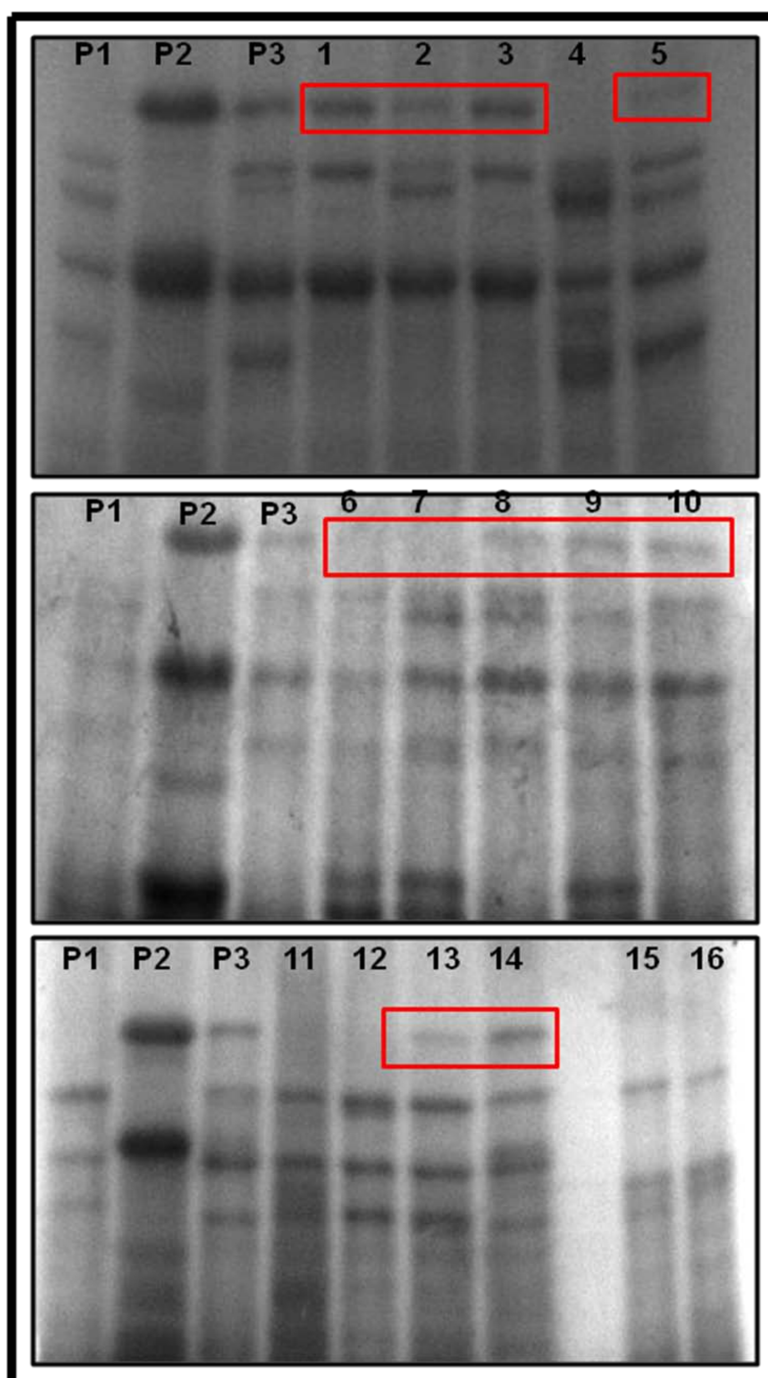


Fig. 4.18 HMW Glutenin subunit profile of some selected pollen irradiation hybrids and their parents. P1: *Triticum aestivum* cv. PBW343, P2: *Aegilops kotschyi* acc. 396, P3: 49-1-73⊗ , Lane 1-16 are B-2, B-8, B-10, B-14, B-16, B-19, B-20, B-27, B-44, B-45, B-48, B-52, B-56, B-58, B-97 and C-1 respectively

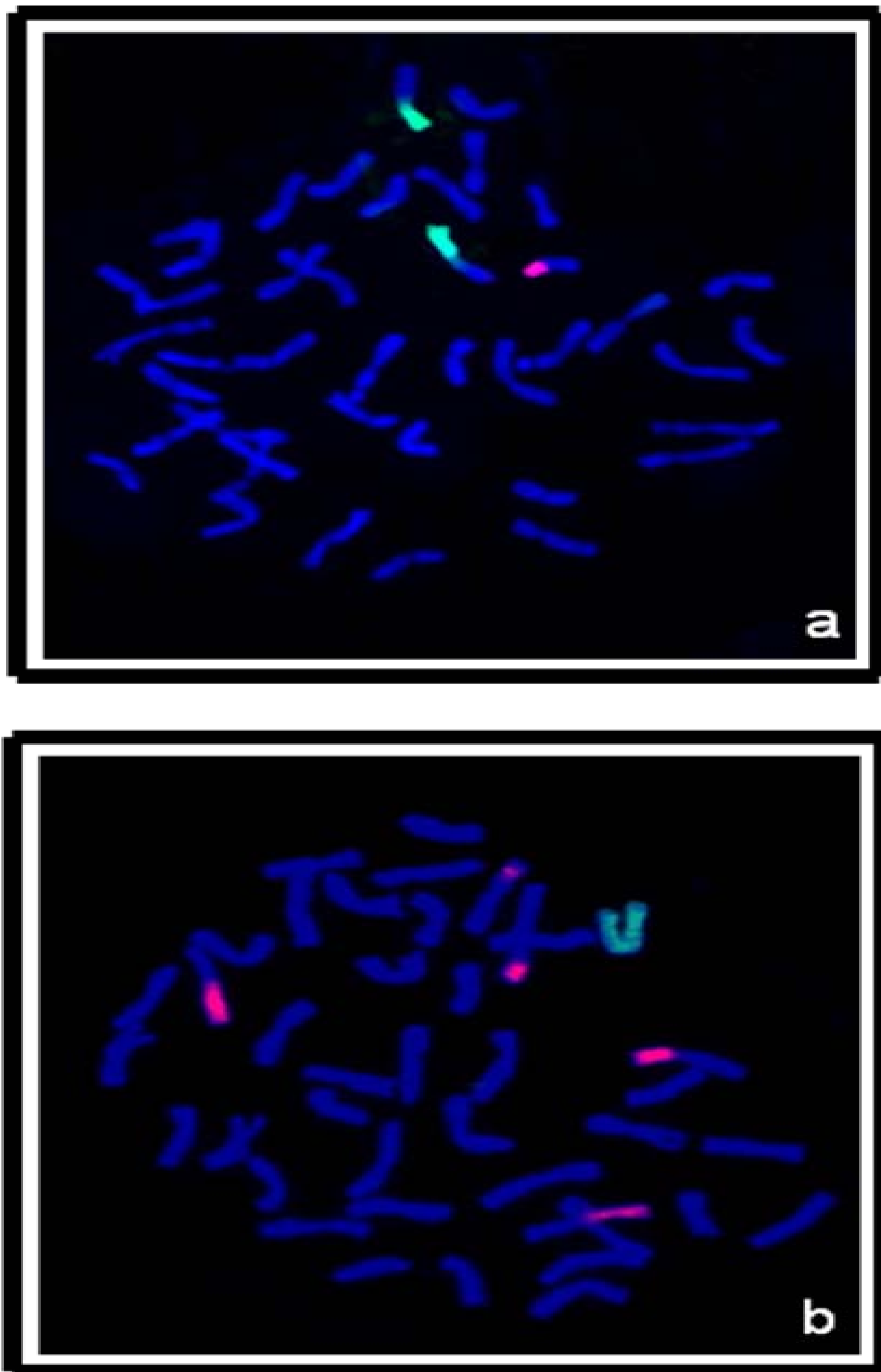


Fig. 4.19 Genomic *in-situ* hybridization (GISH) of selected pollen irradiation hybrids showing introgression of alien chromosome fragments S (greenish blue) and U (pinkish red) a) B-52 b) B-56

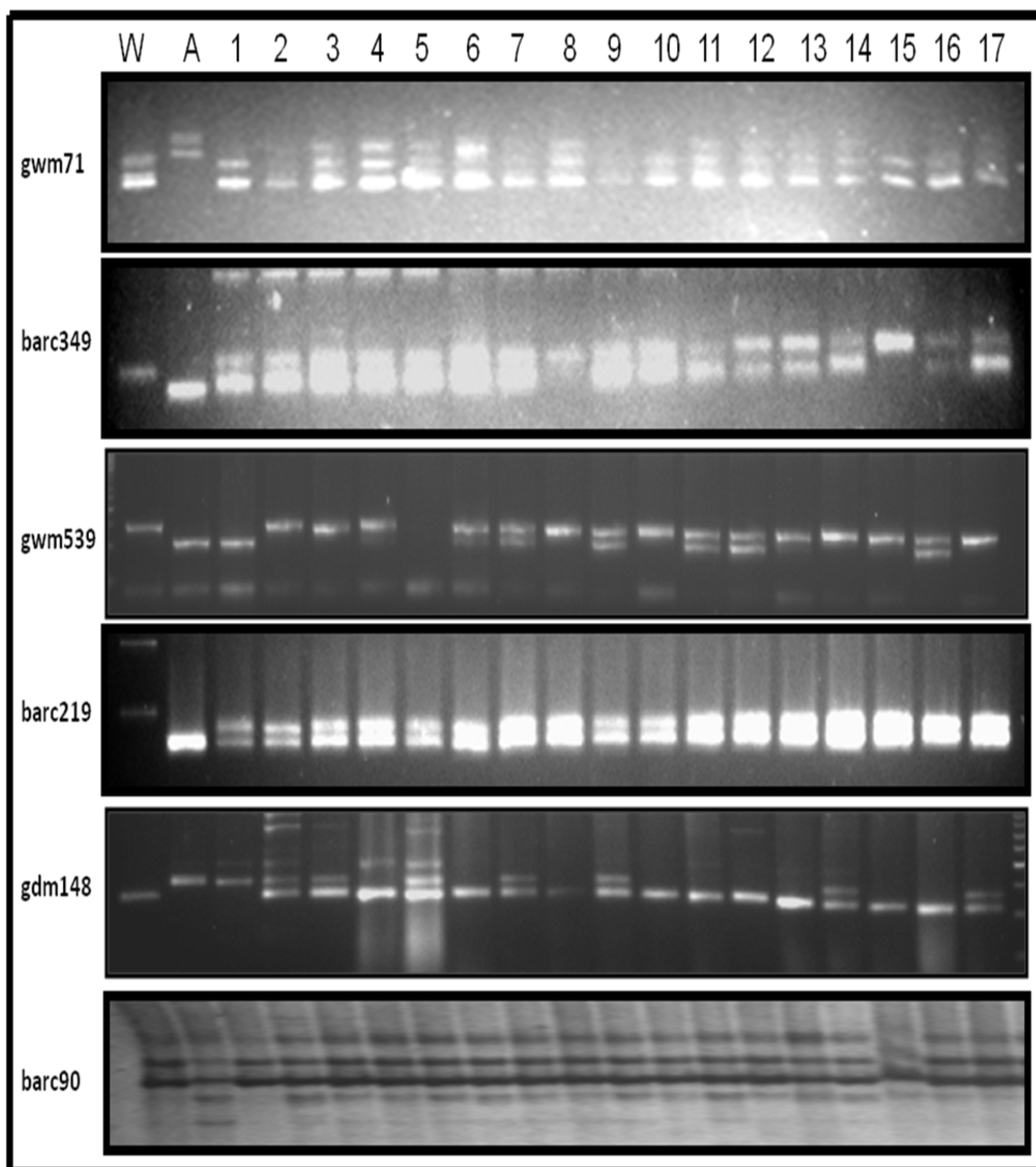


Fig. 4.20 Molecular marker analysis of introgression of 2U/2S in pollen radiation hybrids, W= *Triticum aestivum* cv. PBW343, A= *Aegilops kotschyi* acc. 396 and PRH_1 plants B2-C1

SSR Marker / Plant ID	cfd56	barc90	wmc470	gwm71	barc349	gwm319	barc11	barc219	gwm539	barc159	gdm148
WL711	-	-	-	-	-	-	-	-	-	-	-
49-1-73 (X)	+	+	+	+	+	+	+	+	+	+	+
B-2	-	-	+	-	+	-	-	+	+	-	+
B-8	-	+	+	-	+	-	+	+	-	+	+
B-10	-	+	+	+	+	-	+	+	-	+	+
B-14	-	+	+	+	+	-	-	+	+	+	-
B-16	-	+	+	+	+	-	-	+		+	+
B-19	-	+	+	+	+	-	+	+	+	+	-
B-20	+	+	+	-	+	-	+	+	+	+	+
B-27	+	+	-	+	-	-	-	+	-	+	-
B-44	-	+	+	+	+	-	+	+	+	+	+
B-45	+	+	+	-	+	-	+	+	-	+	-
B-48	+	+	+	+	+	-	+	+	+	-	-
B-52	-	+	+	+	+	+	+	+	+	+	-
B-56	-	+	+	+	+	+	+	+	-	+	-
B-58	-	+	+	+	+	-	+	+	-	+	+
B-59	-	+	-	-	-	-	+	+	-	-	-
B-97	+	+	+	-	+	-	+	+	+	+	-
C-1	+	+	+	-	+	+	+	+	-	-	+

Fig. 4.21 Pollen iradiation hybrid panel of selected pollen radiation hybrids, + indicates presence of *Aegilops kotschyi* 396 band while – indicates its absence

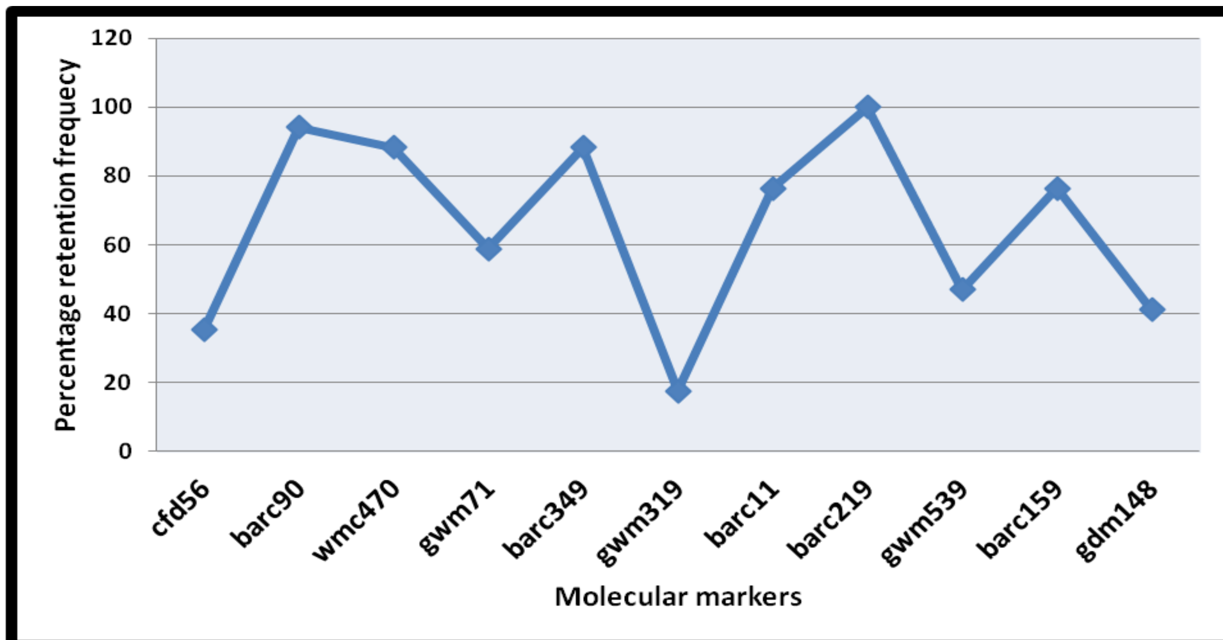


Fig. 4.22 Retention frequency of selected molecular markers in F_1 radiation hybrid population

Table: 4.6 Morphological characteristics of selected PRH_1 pollen radiation hybrids of 49-1-73⊗ and 77-33-2⊗

Parents/ Derivatives	Pedigree	No. of tillers per plant	Height (cm)	Waxi Ness	General morphology	TGW (gm)
WL711	*Triticum aestivum* cv WL711	19.00	95.00	Waxy	Healthy	39.2
49-1-73 ⊗	Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-73 ⊗	18.00	88.00	Non-Waxy	Healthy	34.2
B-2	PRH_2 PBW343/49-1-73-10⊗ -2	22.00	76.00	Non-Waxy	Weak	51.50
B-8	PRH_2 PBW343/49-1-73-10⊗ -8	39.00	95.00	Non-Waxy	Healthy	40.50
B-10	PRH_2 PBW343/49-1-73-10⊗ -10	50.00	87.00	Non-Waxy	Healthy	35.00
B-14	PRH_2 PBW343/49-1-73-10⊗ -14	20.00	79.00	Non-Waxy	Healthy	45.50
B-16	PRH_2 PBW343/49-1-73-10⊗ -16	32.00	79.00	Non-Waxy	Healthy	49.00
B-19	PRH_2 PBW343/49-1-73-10⊗ -19	6.00	69.00	Non-Waxy	Healthy	49.00
B-20	PRH_2 PBW343/49-1-73-10⊗ -20	40.00	83.00	Waxy	Healthy	36.00
B-27	PRH_2 PBW343/49-1-73-10⊗ -27	24.00	65.00	Non-Waxy	Healthy	62.50
B-44	PRH_2 PBW343/49-1-73-10⊗ -44	39.00	76.00	Non-Waxy	Healthy	49.00
B-45	PRH_2 PBW343/49-1-73-10⊗ -45	12.00	69.00	Non-Waxy	Healthy	49.00
B-48	PRH_2 PBW343/49-1-73-10⊗ -48	5.00	54.00	Non-Waxy	Weak	62.50
B-52	PRH_2 PBW343/49-1-73-10⊗ -52	50.00	96.00	Waxy	Healthy	62.50
B-56	PRH_2 PBW343/49-1-73-10⊗ -56	34.00	95.00	Non-Waxy	Healthy	61.00
B-58	PRH_2 PBW343/49-1-73-10⊗ -58	22.00	69.00	Non-Waxy	Healthy	44.50
B-59	PRH_2 PBW343/49-1-73-10⊗ -59	20.00	73.00	Non-Waxy	Healthy	39.00
B-97	PRH_2 PBW343/49-1-73-10⊗ -97	38.00	81.00	Non-Waxy	Healthy	44.50
C-1	PRH_2 PBW343/77-33-2-5 ⊗ -1	30.00	95.00	Non-Waxy	Healthy	39.00

Table: 4.7 Grain Fe and Zn content of selected pollen radiation hybrid PRH_1 (2011-12) and PRH_2 (2012-13)

Parents/ derivatives	Iron concentration (mg/kg) Mean ± S.D	Zinc concentration (mg/kg) Mean ± S.D	Harvest Index (%)	Parents/ derivatives	Iron concentration (mg/kg) Mean ± S.D	Zinc concentration (mg/kg) Mean ± S.D	Harvest Index (%)
2011-12 (ICPMS)				2012-13 (AAS)			
WL711	36.42±1.11	46.36±0.74	39.87	WL711	24.70±0.78	22.14±1.30	41.98
49-1-73 ⊗	55.59±0.62	60.40±0.81	24.62	49-1-73 ⊗	25.02±1.69	38.14±1.97	34.86
B-2	49.72±1.31	58.66±1.07	10.45	B-2	NA	NA	NA
B-8	42.14±0.52	58.79±1.17	21.47	B-8-1	39.73±1.08	43.71±1.71	30.14
B-10	40.47±0.98	49.70±0.69	17.89	B-10	NA	NA	NA
B-14	38.18±0.70	58.68±1.11	24.00	B-14-2	52.72±2.74	75.46±2.07	23.67
B-16	47.58±1.38	50.81±0.53	51.24	B-16-1	38.85±1.47	51.37±1.72	31.60
B-19	36.82±0.98	54.96±1.17	24.68	B-19-1	45.75±2.94	86.59±0.74	12.66
B-20	38.79±0.86	51.30±1.01	29.68	B-20-1	45.42±1.07	61.67±2.34	26.86
B-27	36.90±0.35	56.01±1.52	31.87	B-27-1	47.47±2.01	79.42±1.97	28.91
B-44	54.03±0.68	50.84±1.13	30.14	B-44	NA	NA	NA
B-45	49.12±0.46	48.95±0.95	32.48	B-45-1	64.47±3.17	88.42±3.04	28.52
B-48	42.47±0.45	49.38±0.97	11.20	B-48-1	42.74±0.89	63.80±2.18	30.96
B-52	46.74±0.67	48.71±1.01	50.87	B-52-1	40.98±0.71	53.14±1.97	38.86
B-56	43.71±1.21	49.83±1.05	56.24	B-56-3	45.12±1.07	51.62±1.03	30.62
B-58	46.31±2.33	57.71±1.36	42.98	B-58-2	60.19±0.61	84.26±1.54	24.73
B-59	38.40±1.36	54.32±1.85	21.14	B-59	NA	NA	NA
B-97	36.87±0.28	52.57±1.16	18.91	B-97-1	41.75±0.72	45.80±2.18	26.94
C-1	40.30±0.87	56.09±1.47	12.12	C-1	NA	NA	NA

*NA means data not available

4.3.2.5. Molecular marker analysis of radiation induced segments for transfer of group 2 chromosome.

All the transferable and polymorphic markers were applied on both the parents, 49-1-73-10⊗ and 77-33-2-5⊗. Only those markers, which showed addition or substitution of 2U/2S were further applied on the whole population. Introgression of individual marker in pollen radiation hybrid population has been described in Fig. 4.22. Among all the molecular markers gwm319 showed least retention frequency while marker barc219 showed the highest retention frequency. Some of the pollen radiation hybrids (PRHs) were very healthy, have high grain Fe and Zn, bold seeds and good harvest index.

4.4. Induced homoeologous pairing between group 2, *Aegilops* and wheat chromosomes for precise transfer of useful variability through *ph1b* deletion.

In the year 2009, several wheat-*Aegilops* interspecific derivatives were analyzed for introgression of group 2 chromosomes and grain micronutrient content. Five derivatives were shortlisted on the basis of biochemical, molecular and cytological analysis, showing uniform addition and substitution of 2U/2S chromosomes which were further used for *ph1b* induced homoeologous recombination for transfer of useful variability from *Aegilops* into wheat. In the year 2010 crosses between *ph1b* deletion and the derivatives were made in 2010. A total of 180 seeds were obtained after crossing with different derivatives. Name of derivative and number of seeds obtained after F_1 cross has been given in Table: 4.8

Table: 4.8 Wheat-*Aegilops* derivatives and *ph1b* crosses

S.No.	Cross	Number of F_1
1	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425(46)-1-15-15-2	52
2	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425(49)-1-73-10-3	30
3	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425(49)-1-73-12-3	15
4	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425(49)-1-73-12-7	6
5	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 396//UP2425-2///PBW373(77)-3-12-11-1	3
6	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 396//UP2425-2///PBW373(77)-3-12-12-8	4
7	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338(70)-2-36-6-15-1	22
8	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338(70)-2-36-6-15-3	3
9	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338(70)-2-36-6-15-9	3
10	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338(70)-2-50-8-1-1	41
11	*ph1b* X Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338(70)-2-50-8-13-1	1

The F_1 seeds grown in the field and the plants were selfed and further backcrossed with *ph1bph1b* deletion. Plants homozygous for *ph1b* were isolated using *Ph1b* linked dominant marker psr574 and psr2120 while the anchored group 2 specific SSR marker gwm539 and gdm148 were used for detection of alien introgression. As shown in Fig. 4.23 *ph1b* deletion mutants were also showing leaf yellowing and shriveled seeds. In the present study it was demonstrated that some of the F_1 plants of *ph1b* x derivatives showed yellow leaves, shriveled seeds and absence of *Ph1* specific dominant marker indicating *ph1b* is closely associated with leaf yellowing and hence shriveled seeds. Leaf yellowing and shriveled seeds can be used as

morphological markers for selection of homozygous *ph1b* plants. Similarly, non-waxy plants among crosses were considered as the phenotypic marker for introgression of group 2S alien chromosome. The screening of homozygous *ph1b* plants among the F_2 population has been shown in Fig. 4.24. Some of the F_2 plants were screened for group 2 chromosomal fragment containing grain iron and zinc. Some of the F_2 plants, which were showing high grain Fe and Zn, introgression of group 2 alien chromosomes and absence of *Ph1* specific marker were selected and backcrossed. The backcrossed plants were further screened for grain micronutrient analysis, molecular marker introgression and homozygous *ph1b* up to BC_1F_3 generations. From the population of several hundred plants only 13 plants were selected for further generation advancements. High grain micronutrient, high yield and harvest index, bold seeds, high fertility, and introgression of group 2 molecular markers were the main factors for selection of plants. Forty eight SSR markers anchored on group 2 chromosomes were used among *ph1b* induced recombinant wheat-*Aegilops* derivatives. Only 4 SSR markers showed introgression in the population, gwm71, barc349 of short arms and gwm265 and gwm539 of long arms. Graphical representation of introgression of group 2 alien chromosome fragment has been demonstrated in the consensus map of 2US among BC_1F_2 population. Fe and Zn content of all the BC_1F_2 plants was also analyzed and given in Annexure-3. Based on grain micronutrient and molecular data, a marker-trait association graph was plotted through the help of Graphical genotyping 2.0. In the association plot it was confirmed that group 2 chromosome anchored marker gwm539 and gwm265 showed tight association with grain high Fe content and weak association for grain Zn content. Some of the healthy and fertile BC_1F_2 plants with high grain micronutrient and introgression of group 2 chromosome specific SSR markers were selected for generation advancement. Comparative plant morphology of selected plants is given in Fig 4.25 and Table 4.9. The 13 selected BC_1F_3 plants had very high harvest index ranging 26.13 to 37.19 while for grain Fe ranged from 35.80 mg/kg to 54.14 mg/kg grain Zn was 50.89 mg/kg to 97.01 mg/kg.

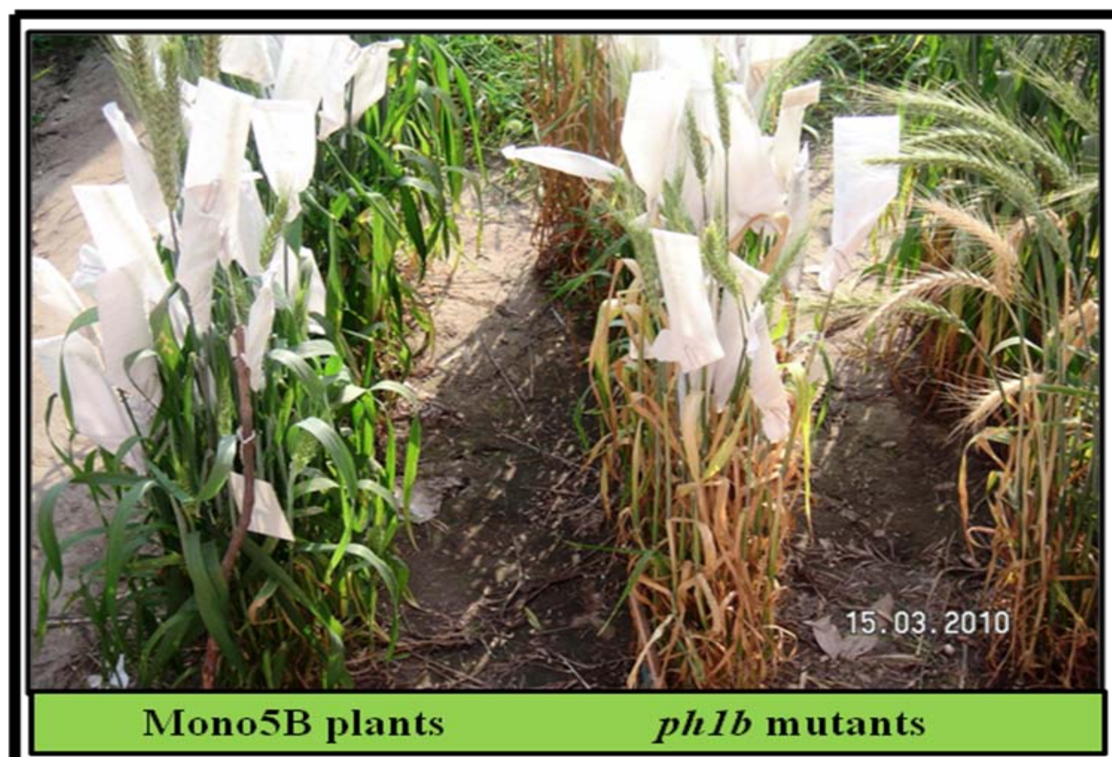


Fig. 4.23 Crosses of selected wheat-*Aegilops* derivatives with *ph1b* mutants. *ph1b* mutants lacking *Ph1* gene showing extensive leaf yellowing.

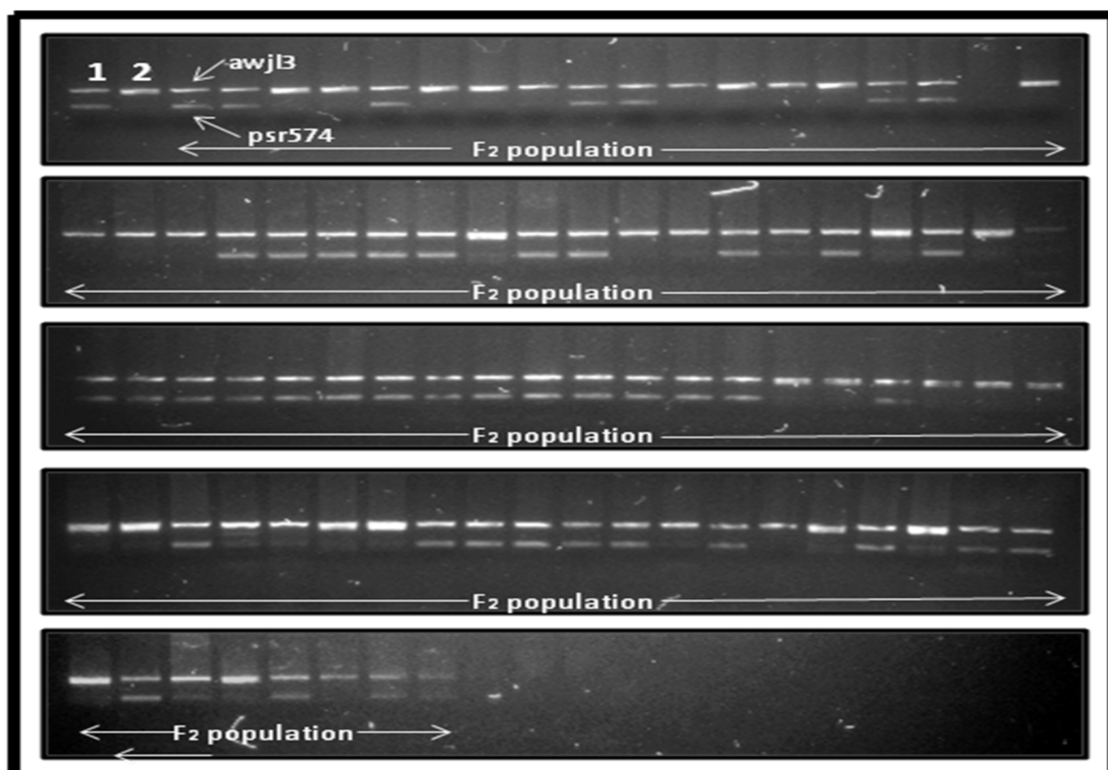


Fig. 4.24 Deletion of homozygous *ph1b* in F_2 generation, 1= *T.aestivum* cv. PBW343; 2=*ph1b* mutant, awjl3=House keeping marker and psr574= *ph1b* specific dominant marker

Fig. 4.25 Morphological characteristics of selected BC_1F_2 *ph1b* induced wheat-*Aegilops* recombinant derivatives

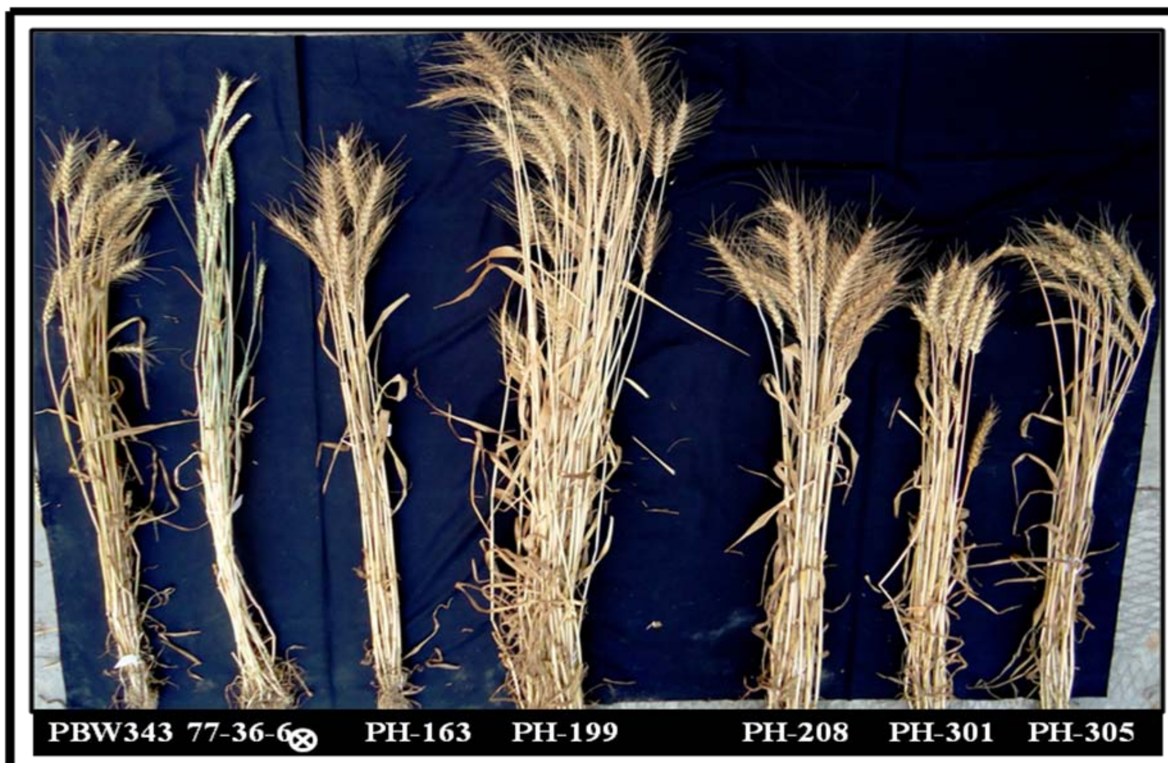


Fig. 4.26 Morphological characteristics of selected F_3, *ph1b* induced wheat-*Aegilops* recombinant derivatives

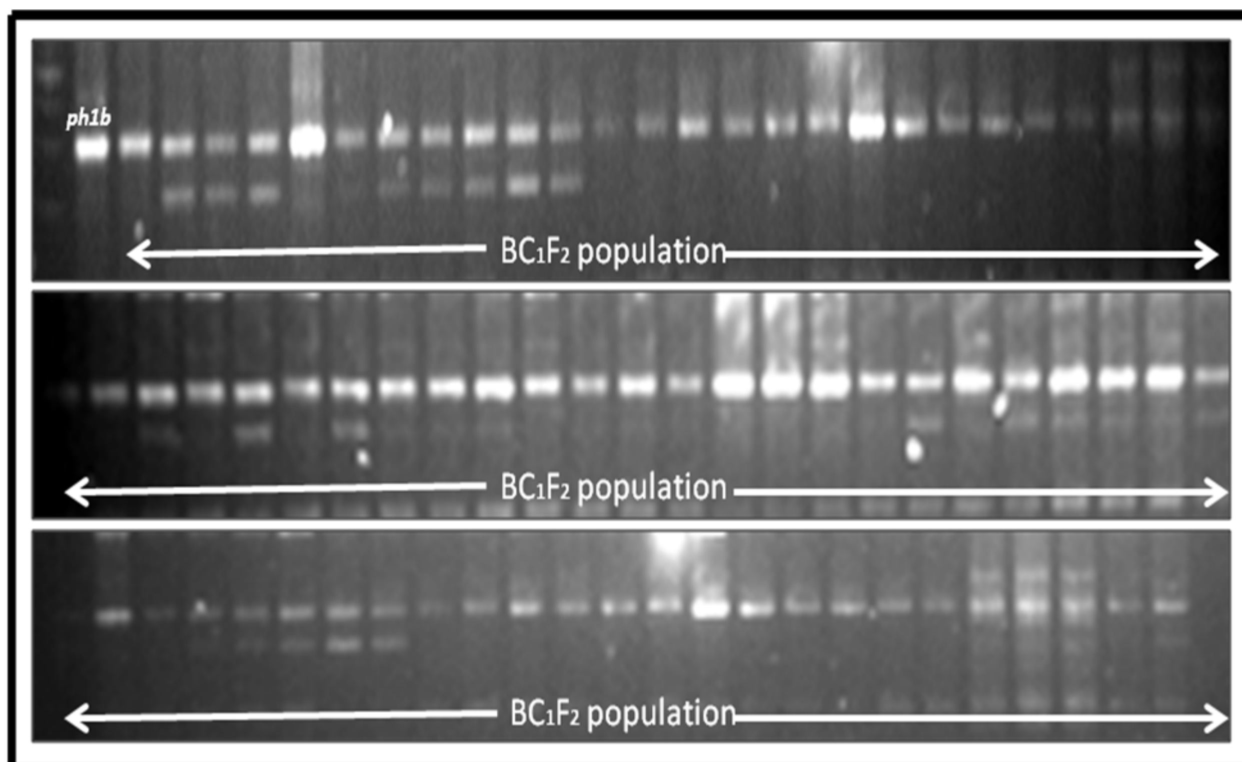


Fig. 4.27 Analysis of *Ph1* specific primer psr574 in BC_1F_2 plants. awjl3 marker has been used as the confirmatory marker for PCR amplification. Absence of *Ph1* amplicon confirms *ph1bph1b* deletion

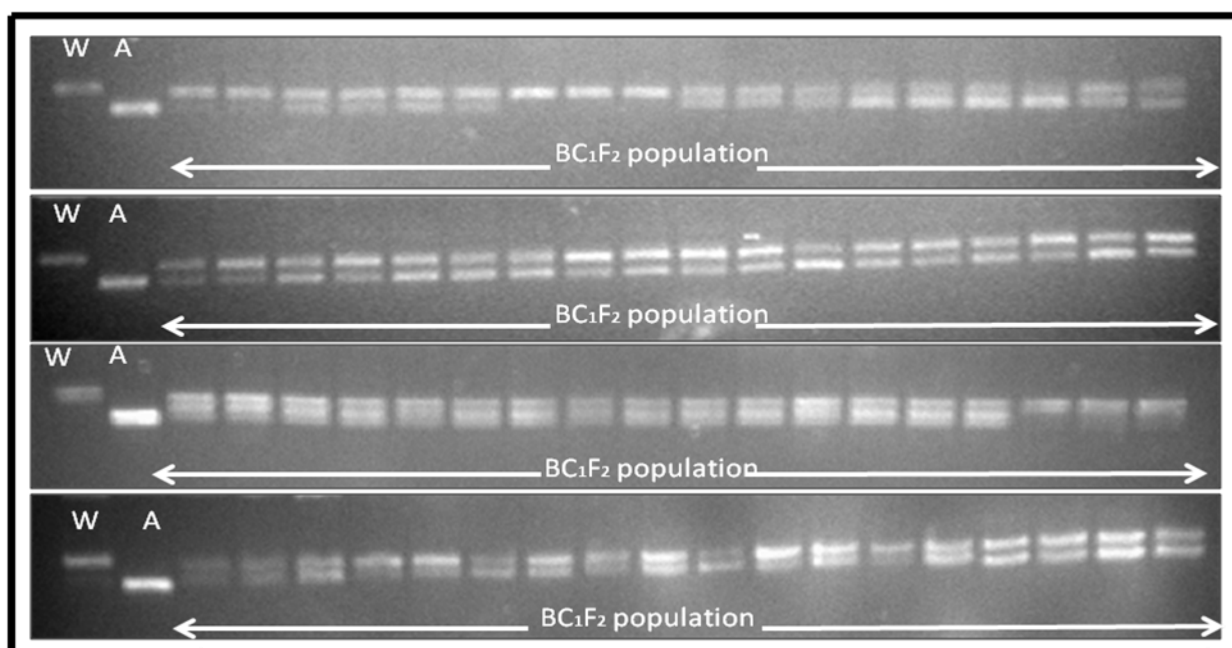


Fig. 4.28 Amplification profile of chromosome group 2 polymorphic marker barc349; W=*Triticum aestivum* cv. WL711, A=*Aegilops kotschyi* 396 and selected BC_1F_2 plants

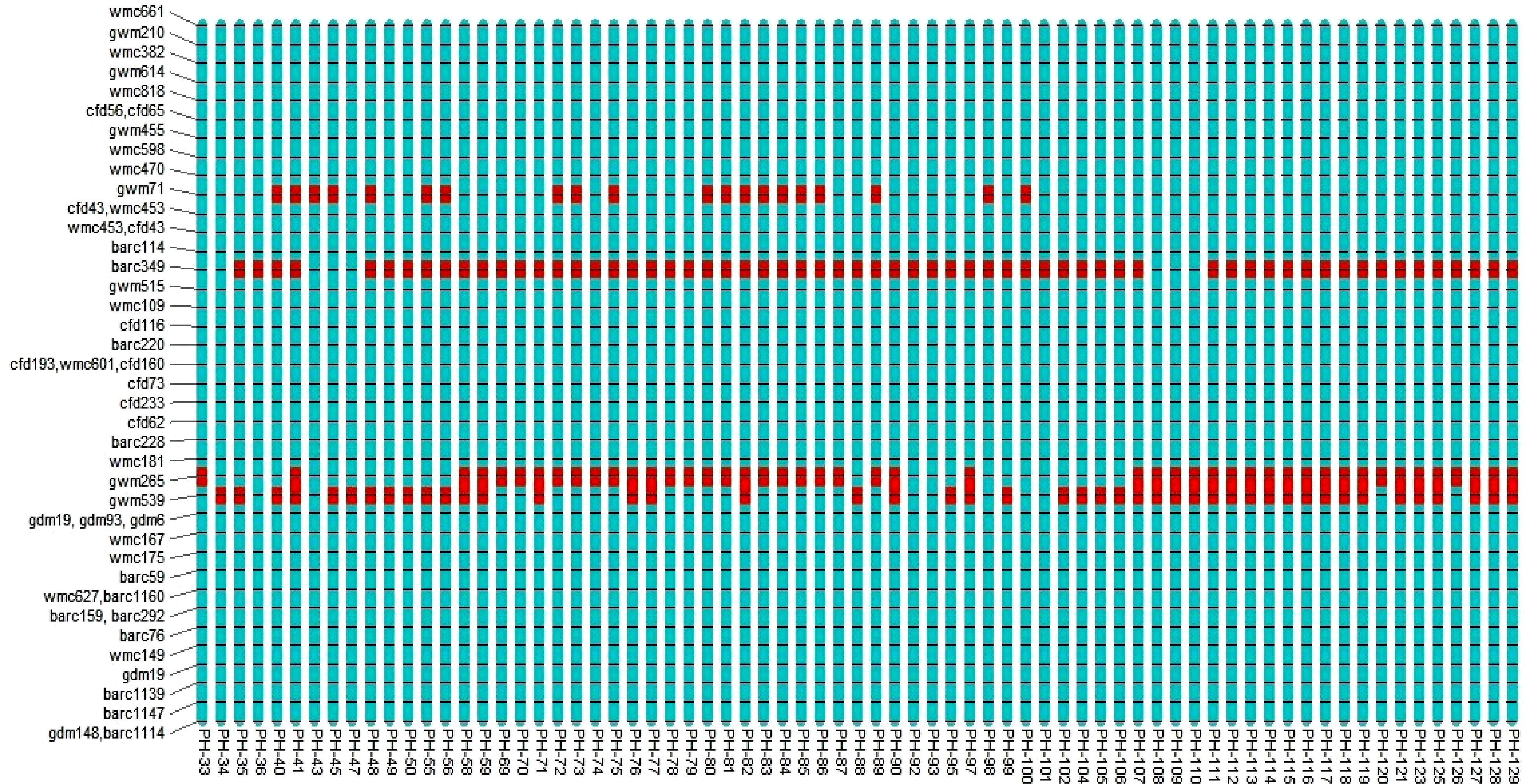


Fig. 4.29 Graphical genotypes of selected BC_1F_2 plants for alien introgression. Red colored region indicates presence of *Aegilops* chromosome segment while the blue colored region indicates wheat chromosome fragment

Table: 4.9. Morphological characteristics and grain micronutrient contents of selected BC_1F_3 recombinant plants

Plant Name	Pedigree	Tiller Number per plant	Waxyness of leaves	Fe (mg/kg)	Zn (mg/kg)	TGW (gm)	Harvest index
ph1b mutant	-	20	Waxy	28.20±06.89	33.21±1.24	31	34.51
PH-34-1	BC_1F_3 *ph1b*/46-1-15-15-2 //*ph1b*-34-1	19	Non-waxy	38.82±1.29	50.89±1.77	24	31.28
PH-48-1	BC_1F_3 *ph1b*/46-1-15-15-2 //*ph1b*-48-1	21	Non-waxy	35.80±2.45	53.37±0.24	28	30.24
PH-104-1	BC_1F_3 *ph1b*/49-1-73-10-3//*ph1b*-24-104-1	15	Non-waxy	47.40±1.47	51.91±0.89	29	29.14
PH-110-2	BC_1F_3 *ph1b*/49-1-73-10-3//*ph1b*-24-110-2	14	Waxy	42.46±2.03	55.00±0.45	30	35.13
PH-110-3	BC_1F_3 *ph1b*/49-1-73-10-3//*ph1b*-24-110-3	16	Non-waxy	42.55±1.27	55.06±1.24	33	26.13
PH-163-1	F_4 *ph1b*/77-36-6- ⊗-4-163-1	18	Waxy	32.18±2.01	61.58±2.21	31	28.91
PH-163-2	F_4 *ph1b*/77-36-6 ⊗-4-163-2	17	Waxy	42.58±0.54	55.02±2.15	27	29.14
PH-199-3	F_4 *ph1b*/77-33-2 ⊗-1-199-3	19	Waxy	54.14±0.44	97.01±0.89	29	35.17
PH-208-2	F_4 *ph1b*/77-33-2 ⊗-1-208-2	18	Waxy	40.98±1.40	54.79±0.64	29	37.19
PH-208-3	F_4 *ph1b*/77-33-2 ⊗-1-208-3	17	Waxy	40.20±1.02	59.48±0.55	32	32.84
PH-301-2	F_4 *ph1b*/46-1-15-15-2 ⊗-10-301-2	14	Waxy	43.10±0.57	65.18±1.04	31	33.97
PH-305-1	F_4 *ph1b*/46-1-15-15-2 ⊗-10-305-1	16	Waxy	36.30±0.91	70.30±0.91	30	34.18
PH-305-2	F_4 *ph1b*/46-1-15-15-2 ⊗-10-305-2	15	Waxy	41.09±0.87	62.03±0.83	29	31.94

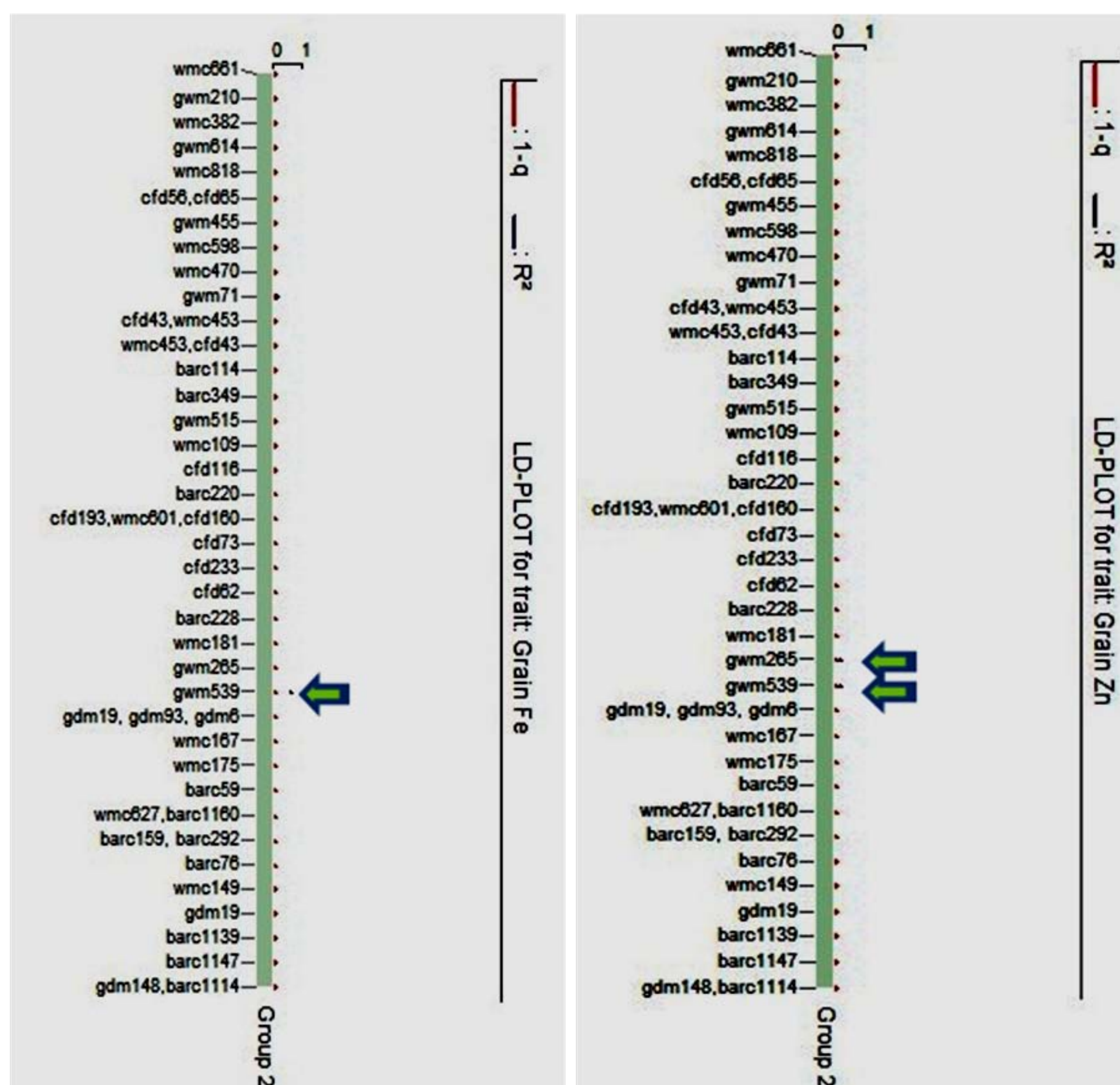


Fig. 4.30 Marker-trait association plot for grain Fe and Zn on consensus group 2U/2S chromosome

In the present study it was confirmed that genes responsible for grain micronutrient content have been precisely transferred from wheat-*Aegilops* derivatives into elite wheat cultivar *Triticum aestivum* cv. PBW343. All the plants had 42 chromosomes with normal pairing behavior of 21 bivalent. No positive signal was obtained in the selected plants for alien introgression through GISH due to transfer of small fragments beyond the resolution limit of GISH. All the selected plants were healthy, with high grain Fe and Zn content and introgression of group 2 specific SSR markers.

4.5. Induced homoeologous pairing in wheat-*Aegilops* crosses in the absence of 5B chromosome.

Triticum aestivum cv. Pavon mono 5B (41 chromosomes; 2n-1) was used for precise transfer of useful variability for grain Fe and Zn genes from *Aegilops species* through homoeologous recombination. Pavon mono 5B plants were crossed with several accessions of *Aegilops species* and a total of 190 seeds were obtained (Table 4.12). Out of 190 seeds, 131 germinated seeds were grown in the field.

Table: 4.10 Total viable F_1 seeds in crosses between *Triticum aestivum* cv. Pavon Mono 5B with *Aegilops species*

S.No.	Cross	Total seeds	Seeds germinated
1	mono 5B X *Ae. longissima* 28	8	4
2	mono 5B X *Ae. peregrina13772*	14	11
3	mono 5B X *Ae. kotschyi 3790*	110	76
4	mono 5B X *Ae. kotschyi 396*	45	31
5	mono 5B X *Ae. kotschyi 3573*	13	9

The F_1 hybrids had either 34 or 35 chromosomes. Plants with 34 chromosomes number gave high homoeologous pairing while the plants with 35 chromosomes number had more univalents due to presence of 5B. The F_1 plants with 34 chromosomes were also screened by 5B specific marker psr2120. The absence of chromosome 5B results in absence of psr2120 specific band. To confirm proper PCR amplification, multiplex PCR of a housekeeping marker awjl3 and psr2120 was done. Simultaneously the pairing behavior of some of the selected F_1 hybrids has been shown in Fig. 4.31 and PCR analysis for the presence or absence of chromosome 5B specific marker psr2120 has been shown in Fig.4.32 The plants without 5B had 34 chromosomes with high pairing were backcrossed with *Triticum aestivum* cv. PBW343 (*Lr24+GPC*). Only 48 seeds were obtained out of which 15 seeds germinated. The BC_1F_1 plants were further backcrossed with *Triticum aestivum* cv. PBW343 (*Lr24+GPC*). Only 28 BC_2F_1 seeds were obtained. In the next season all the 28 seeds were sown in the field and only 5 plants matured. These 5 plants were either self seeded or some spikes were backcrossed with *Triticum aestivum* cv. PBW343 (*Lr24+GPC*). There were 43 BC_3F_1 and 22 BC_2F_2 seeds have been obtained.

All the BC_1F_2 and BC_2F_1 plants had variable number of chromosomes and pairing behavior. Among the BC_2F_1 plants, the chromosome number varied from 41 to 60, while in the BC_1F_2 plants it was around 56 (Table 4.13 and Fig. 4.33). Some plants such as MB11 and MB16 showed at least one quadrivalent while plant MB37 and MB39 had 12 univalents. MB13 and MB27 had 42 chromosomes with 19II +4I. Similarly the pollen stainability also showed variation among all the BC_1F_2 and BC_2F_1 plants. *Triticum aestivum* cv. Pavon mono 5B had 91.28% pollen stainability. MB13 had highest pollen stainability of 90.32% while plant MB16 had only 48.19% stainable pollen viable. As shown in Fig. 4.34 pollen stainability have significant correlation with chromosome number above 40.

Flag leaf and grain Fe and Zn content of all the BC_1F_2 and BC_2F_1 plants was analyzed through AAS (Table.4.14). Highest flag leaf, Fe and Zn content was observed in plant MB39 while the lowest in plant MB31 and MB17 had lowest flag leaf Zn. Grain Fe and Zn content of all the BC_1F_2 and BC_2F_1 plants had considerable variation. Highest grain Fe and Zn was observed in plant MB37. Both the wheat cultivars had lowest flag leaf iron, low flag leaf zinc and lowest grain iron and zinc than the *Ae. kotschyi* 3790 and various backcross derivatives of wheat X *Ae. kotschyi* crosses indicating a positive correlation behind flag leaf and grain micronutrients. But the high grain iron and zinc in most of the derivatives could also be attributed to concentration effect as there was lower seed set in backcross derivatives due to high frequency of univalents

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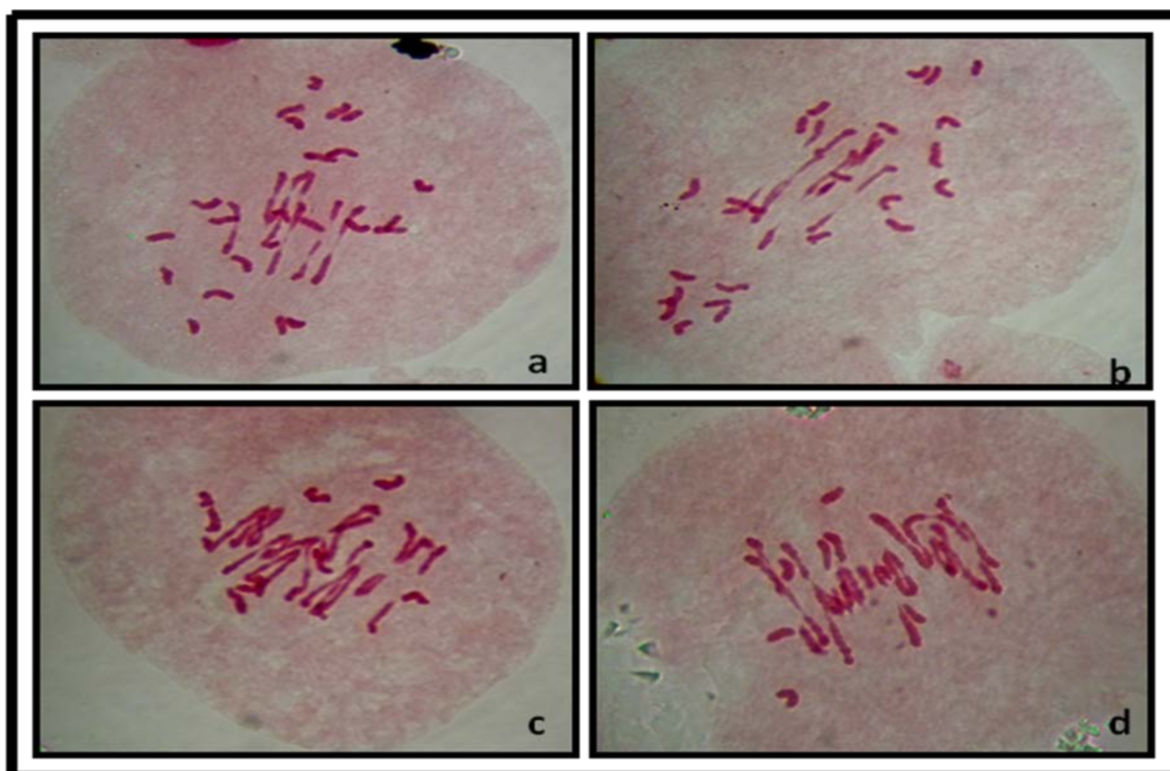

Fig. 4.31 Chromosome number and pairing at metaphase 1 of *Triticum aestivum* cv. Pavon mono 5B with and without chromosome 5B, F_1 hybrid (2n=35) with chromosome 5B: a (4 II + 1III + 24 I), b (4 II + 1III + 23 I); F_1 hybrid (2n=34) without chromosome 5B: c (2V + 4III + 2II + 8I), d (1V + 2III + 9II + 5I)

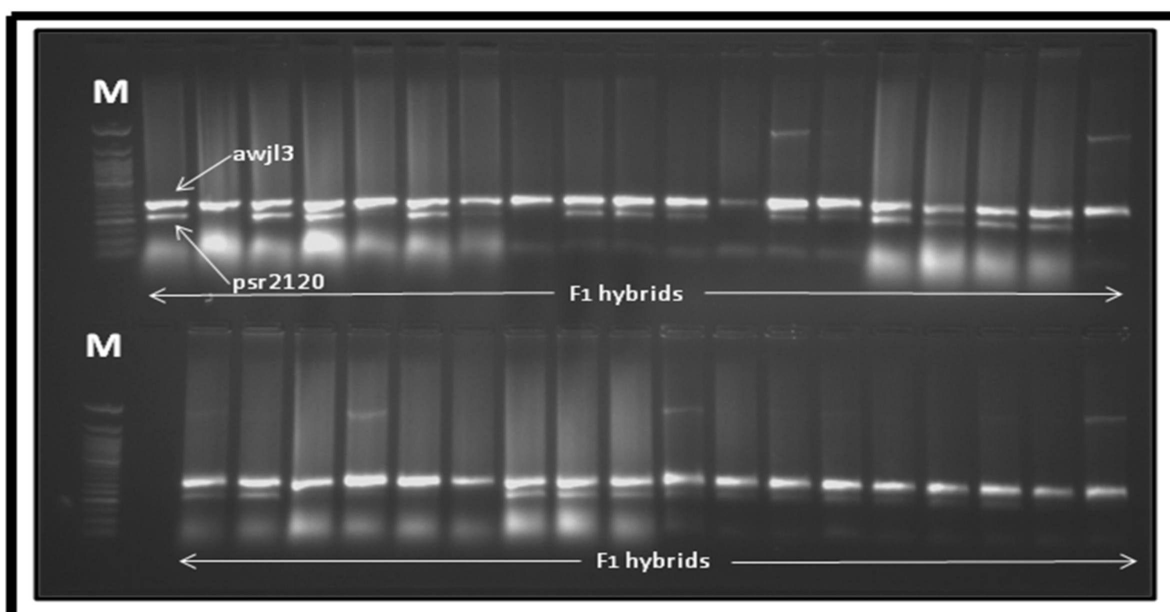

Fig. 4.32 Identification of mono5B x *Aegilops* F_1 hybrids without chromosome 5B by *Ph1* specific marker psr2120, anchored on chromosome 5B in selected F_1 hybrids; M= 50bp marker ladder, 1=*Triticum aestivum* cv. PBW343, 2= *Triticum aestivum* cv. Pavon mono 5B

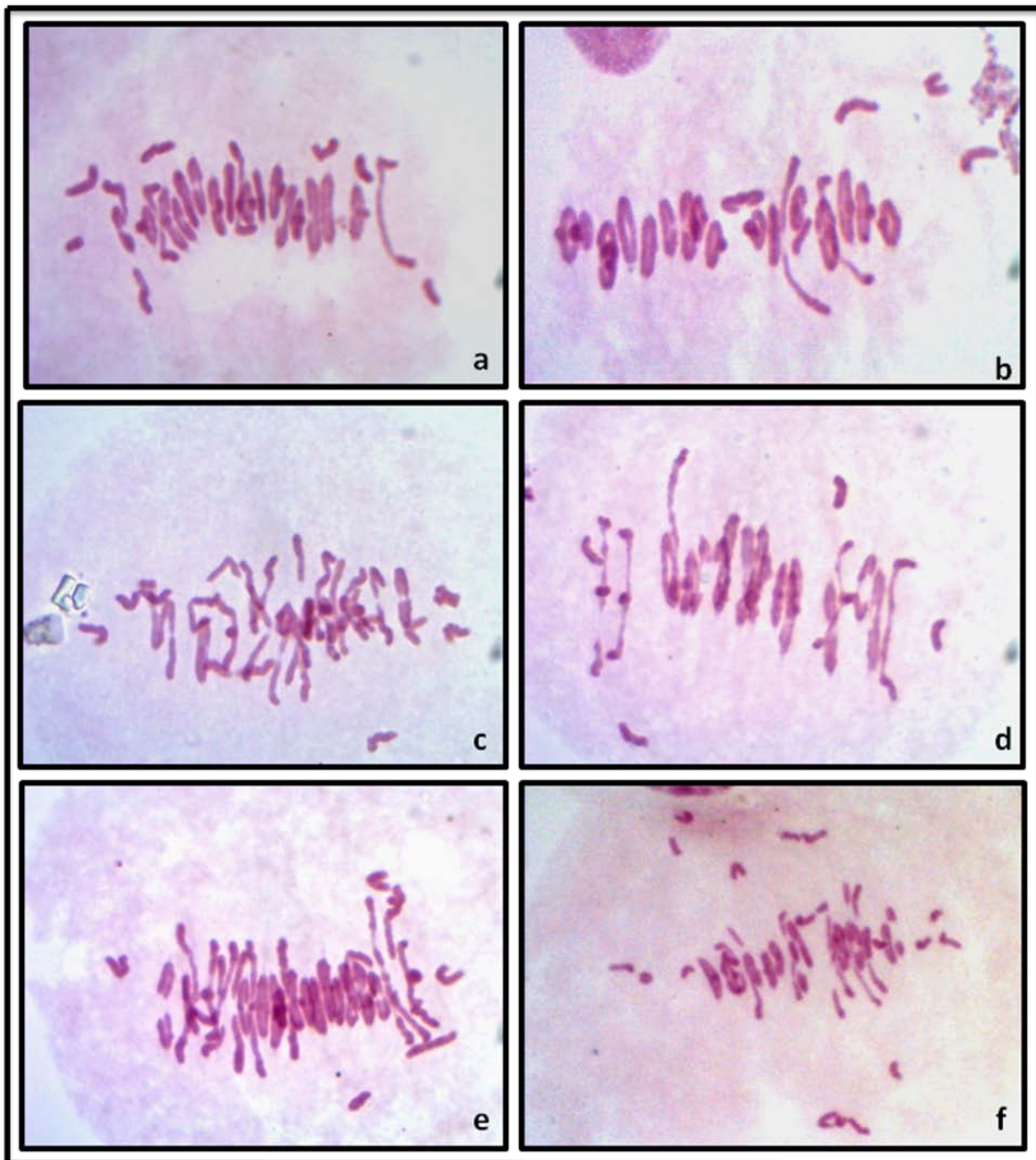

Fig. 4.33 Chromosome pairing at metaphase I of some selected BC_2F_1 and BC_1F_2 wheat-*Aegilops* derivatives, (a)MB7; 46 (20II+6I), (b)MB13; 42 (19II+4I), (c)MB16; 60 (1V+22II+11I), (d)MB27; 42 (19II+4I), (e)MB46; 56 (25II+6I) and (f)MB50; 56(21II+11I)

Table: 4.11 Pollen viability, chromosome number and pairing behaviour of BC_1F_2 (*Triticum aestivum* cv. Pavon mono 5B/*Ae. kotschyi* 3790// PBW343-3///PBW343) and BC_2F_1 (*Triticum aestivum* cv. Pavon mono 5B/*Ae. kotschyi* 3790// PBW343-3) *Aegilops*- *Triticum aestivum* cv. Pavon mono 5B interspecific hybrids

Plant ID	Pollen stainability (%)	Chromosome Number	Chromosome pairing
Mono 5B	91.28	41	20II+1I
BC_2F_1 MB 2	81.48	48	23II+2I
BC_2F_1 MB 3	80.81	48	22II+4I
BC_2F_1 MB 4	85.21	43	19II+5I, 18II+7I
BC_2F_1 MB 6	83.14	44	20II+4I
BC_2F_1 MB 7	82.15	46	20II+6I
BC_2F_1 MB 11	60.14	54	1IV+1III+20II+7I
BC_2F_1 MB 12	81.15	46	20II+6I, 21II+4I
BC_2F_1 MB 13	90.32	42	19II+4I
BC_2F_1 MB 15	59.04	54	1III+20II+11I
BC_2F_1 MB 16	48.19	60	28II+2I, 1V+22II+11I
BC_2F_1 MB 17	61.14	-	-
BC_2F_1 MB 19	83.08	45	19II+7I
BC_2F_1 MB 20	74.15	-	-
BC_2F_1 MB 21	83.81	44	20II+4I
BC_2F_1 MB 26	47.94	60	26II+8I
BC_2F_1 MB 27	89.14	42	19II+4I
BC_2F_1 MB 29	82.54	46	21II+4I
BC_2F_1 MB 31	80.20	48	20II+8I, 21II+6I
BC_2F_1 MB 32	83.47	44	21II+2I
BC_2F_1 MB 33	78.34	49	20II+9I
BC_2F_1 MB 34	74.91	-	-
BC_2F_1 MB 35	61.89	57	23II+11I
BC_2F_1 MB 37	76.97	50	19II+12I
BC_2F_1 MB 39	59.57	54	21II+12I
BC_1F_2 MB 46	54.92	56	25II+6I
BC_1F_2 MB 50	53.67	56	21II+11I
BC_2F_1 MB 67	64.38	52	23II+6I

Table: 4.12 Plant flag leaf and grain iron and zinc content of selected *Aegilops*- *Triticum aestivum* cv. Pavon Mono 5B interspecific hybrids

Plant ID	Plant Flag leaf		Plant grain	
	Fe(mg/kg)	Zn(mg/kg)	Fe(mg/kg)	Zn(mg/kg)
Aegilops kotschyi 3790	896.25±1.2	44.56±1.8	50.00±1.8	63.36±1.9
T. aestivum cv.PBW343	112.15±2.4	32.16±2.1	33.05±1.5	24.96±1.6
T. aestivum cv. Pavon	148.37±0.8	26.68±0.9	29.39±1.7	32.34±1.9
MB 2	450.00±1.4	38.33±1.6	43.55±1.7	60.17±0.2
MB 3	516.60±2.1	38.43±2.8	47.63±1.3	64.84±0.5
MB 4	396.20±0.9	59.05±1.7	44.12±1.4	68.58±0.2
MB 6	283.40±0.6	33.38±0.7	56.83±1.3	74.03±0.6
MB 7	215.00±0.4	30.28±0.9	52.26±0.8	72.08±0.5
MB 11	591.70±2.8	44.60±2.4	57.62±1.7	53.30±0.7
MB 12	262.08±1.5	35.61±1.9	43.47±2.0	53.83±0.0
MB 13	257.10±0.7	46.38±0.4	55.40±2.4	70.48±1.0
MB 15	660.50±3.0	32.70±2.9	55.23±0.2	63.84±0.4
MB 16	274.46±1.4	49.08±1.5	57.41±2.7	70.17±1.1
MB 17	261.50±1.8	20.50±1.2	70.88±1.6	93.38±2.9
MB 19	238.00±0.7	43.40±0.4	60.99±0.4	75.90±0.4
MB 20	248.90±1.6	43.56±1.4	61.47±1.3	80.11±0.2
MB 21	215.00±1.4	38.69±1.9	63.85±1.4	79.43±0.3
MB 26	271.80±2.7	43.43±2.2	65.15±1.7	81.89±0.2
MB 27	260.50±2.4	32.43±2.0	65.02±0.6	80.47±0.3
MB 29	1001.20±3.4	50.62±3.9	68.59±0.8	84.42±0.0
MB 31	195.05±2.4	33.82±2.8	65.57±1.6	82.73±0.0
MB 32	NA	NA	62.21±0.5	67.55±0.9
MB 33	202.30±1.7	37.59±1.2	71.50±1.5	87.91±0.0
MB 34	237.00±0.5	39.62±0.9	67.50±0.4	79.82±0.5
MB 35	414.50±2.7	34.00±2.4	59.21±2.7	55.67±0.6
MB 37	208.50±0.6	27.94±0.9	84.81±0.6	96.29±0.0
MB 39	1660.00±3.9	61.50±3.1	71.45±1.4	69.43±0.2
MB 46	449.50±2.1	21.82±2.6	70.73±0.2	58.48±0.4
MB 50	229.00±2.0	60.80±1.7	67.59±1.2	57.10±0.9
MB 67	474.75±1.7	32.30±1.5	66.60±2.5	58.54±0.7

NA= Data not available

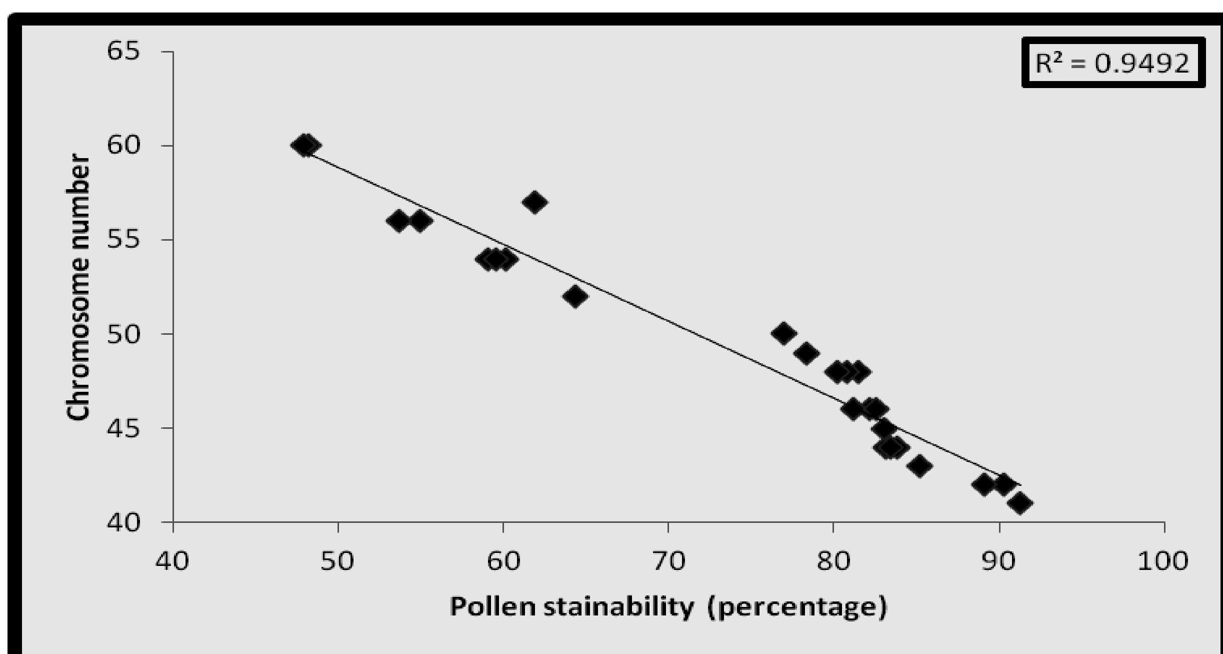

Fig. 4.34 Correlation between number of chromosomes and pollen stainability in BC_1F_2 and BC_2F_1 plants of mono 5B wheat-*Aegilops* interspecific hybrids

Although, in MB plants there was positive correlation has been observed between flag leaf Fe and Zn with grain Fe and Zn content, but in the given set of data coefficient of determination between flag leaf and grain iron and zinc, R^2= 0.0728 and 0.1422 are very low. In stable wheat cultivar and wild relatives, the R^2 value for flag leaf Fe and grain Fe; flag leaf Zn and grain Zn were 0.8216 and 0.9777, respectively. The poor association between flag leaf mineral micronutrient content and grain micronutrient content, observed in MB plants as compared to elite wheat cultivars and wild wheat relatives may be attributed the concentration effect. Few of the *Aegilops- Triticum aestivum* cv. Pavon mono 5B interspecific backcross derivatives were fertile, had high grain Fe and Zn content and bold seeds. Some of the plants are expected to have recombined wheat chromosomes during F1 hybrid without 5B with desirable variability from *Ae. kotschyi*. Such derivatives with stable chromosome number and pairing, high micronutrient without any linkage drag will be selected using wheat molecular marker and GISH etc.

5. Discussion

More than half of the world population is suffering from Fe and Zn micronutrient malnutrition (Stoltzfus, 2003; Poletti *et al*., 2004; Welch and Graham, 2004; Bouis, 2007; Pfeiffer *et al*., 2007). Many elite wheat cultivars have very low Fe and Zn content which are largely distributed in embryos and peripheral tissues of bran (Welch and Graham, 1999). Most of the cultivated crops with very low Fe and Zn content cannot fulfil the daily dietary nutrition requirements. The related non-progenitor *Aegilops species* with S and U genomes showed 2-3 fold high grain Fe and Zn content (Rawat *et al*., 2009) than the wheat and durum cultivars. The derivatives of *Aegilops kotschyi* 3790 and 396 showed high grain Fe and Zn had low harvest index, reduced yield due to linkage drag (Tiwari *et al*., 2010; Rawat *et al*., 2010; Kumari *et al*., 2011). Keeping these points in view, the present study was undertaken to reduce the linkage drag in substitution and addition lines of 2S and 2U chromosomes of *Ae. kotschyi* 396 and 3790 by precise transfer of genes/ chromosomal fragments responsible for high grain Fe and Zn content into wheat.

Among the wheat group 2 chromosome specific SSR markers the D genome specific markers showed highest transferability to the donor *Aegilops species* followed by B genome and A genome. Similarly the D genome specific markers also showed higher polymorphism with *Aegilops species* in comparison to B and A genome, respectively. This implies that *Ae. tauschii* (D genome donor) is more closely related to the S and U genome of *Ae. kotschyi* in as compared to their similarity with the B genome. The least transferability of A genome specific markers suggests that *Triticum urartu* (A genome donor) is distantly related to *Ae. kotschyi* genomes. Among all the transferable markers, 27 markers of 2A, 22 markers of 2B and 40 markers of 2D were polymorphic on all the A, B, D, U and S genome. However, several markers were non transferable on *Ae. longissima* ($S^lS^{l)}$, the donor S genome and *Ae. kotschyi* (Gu *et al*., 2004). The tentative consensus map on the basis of shared transferable markers was prepared by BioMercator V3.0 (Arcade *et al*., 2004). Consensus map of A, B and D genome of wheat were made by JoinMap (Somars *et al*., 2004). The D genome of wheat is more similar to S and U genomes in comparison to B and A genomes (Golovnina *et al*., 2007) The B genome of wheat is more closely related to the S genome of *Aegilops species* as compared to the A genome of *Triticum species* (Dvorak and Zhang, 1990; Daud and Gustaffson, 1996; Faris *et al.,* 2002).

Grain micronutrient analysis of elite wheat cultivars and *Aegilops species* showed 2-3 fold higher grain micronutrient. All the wheat-*Aegilops* derivatives screened for U/S introgression through group 1 to 7 SSR markers and showed only group 2 and 7 introgression (Tiwari, 2008) while 49-1-73 showed group 1U/1S introgression confirmed through HMW glutenin subunit profiling (Rawat; 2008). Among all the five selected wheat-*Aegilops* derivatives, four had non-waxy leaf sheaths and spikes (except 77-50-8-1, having waxy leaves) indicating that they had introgression of 2S chromosome of *Ae kotschyi*. The derivative 77-50-8-1 had waxy plant suggesting that it might have 2U introgression. All of the wheat-*Aegilops* derivatives (except 46-1-15-15) had 42 chromosomes with 21 bivalents confirming that they had 2S/2U disomic substitution. The introgression of group 2 *Ae. kotschyi* chromosome into derivatives was screened on the basis of non-waxy phenotypic trait is based on the fact that group 2 chromosome of wheat have been reported to carry the gene for non-waxy plants (Levy and Feldman, 1989; McIntosh, 1983). All the *Ae. longissima* accessions, the donor of S genome to *Ae. kotschyi* have non-waxy plants further confirming the $2S^k$ chromosome in non-waxy wheat-*Aegilops* derivatives. Most of the *Ae. longissima* derivatives with 2S introgression had non-waxy plants (Kumari *et al*., 2012). The derivative 77-50-8-1 with waxy plants had 2U substitution as its previous generations had both 2S and 2U introgression (Tiwari, 2008). The wheat-*Aegilops* derivative, 49-1-73 had high molecular weight (HMW) glutenin subunits specific to *Ae kotschyi* donor indicating that it had introgression of 1U/1S of *Ae. kotschyi* 396 (Rawat, 2008), as HMW glutenins are controlled by group 1 chromosome of Triticeae. This derivative also had U genome chromosome in addition to 1 and 2 chromosomes. The derivative 46-1-15-15 with 44 chromosomes (22II) had brittle rachis and red seed color; the trait controlled by group 3 chromosomes of Triticeae and hence could have disomic addition of chromosome 3^k (Watanbe and Ikebata, 2000; Groos *et al*., 2002; Kumar *et al*., 2009). All the five derivatives had additional *Ae. kotschyi* specific alleles of 2D SSR markers gwm539 and gdm148 indicating that they had substitution of group 2 *Ae. kotschyi* chromosome for A/B homoeologous chromosomes. All the five derivatives gave ~70-90% higher grain iron and zinc content indicating that *Ae. kotschyi* posses efficient genetic system for micronutrient uptake, translocation and sequestration in grain. Most of the derivatives with high micronutrient content had shows lower yield and harvest index than the recipient wheat cultivars indicating that the high micronutrient content could be particular due to concentration effect (Calderini and Monesterio 2003). Through SEM-EDX analysis of seeds it was confirmed that most of the grain micronutrients are concentrated in the aleurone layer suggesting that smaller and shriveled seeds with more aleurone are would have higher seed

micronutrient content than the bold ones. However, higher percentage of Fe and Zn in aleurone layer of *Aegilops kotschyi* 396 in comparison to wheat cultivar PBW343 again supports the superior genetic system for mineral acquisition and deposition in *Aegilops species.* Most of the derivatives showed consistently high but variable amount of Fe and Zn at different locations over different years indicating the superior genes/QTL for micronutrient uptake, translocation and storage on 2S and 2U chromosome of *Aegilops kotschyi*. QTL and genes for high grain Fe and Zn were reported on chromosome 2A of diploid wheat (Tiwari *et al*., 2009), 2H and 5H of *Hordeum vulgare* (Lonergan *et al*., 2009), V2 and V7 of *Hynaldia villosa* (Schlegel *et al*. 1998), L1, L4 and L7 of *Agropyron intermedium* (Schlegel *et al*. 1998) and 1R, 2R and 7R of *Secale cerale* (Cakmak *et al*., 1997; Schlegel *et al*., 1997). The 2S and 2U addition and substitution lines had higher grain Fe and Zn content (Rawat *et al*., 2010; Tiwari *et al*., 2010). Because of the associated linkage drag in derivatives, none of the addition line or substitution lines of wheat with complete chromosome of chromosomal arm could be used for cultivars, except 1RS:1BL translocation lines of *Secale cereale* (Friebe *et al.* 1996b; Conner *et al.,* 1998; Dhaliwal *et al.* 2002; Yan *et al.,* 2005).

In the study, two approaches for precise transfer of useful variability from alien substitution through irradiation induced transfer and induced homoeologous pairing were found effective. Seed irradiation hybrids (SRH) induced precise transfer of 2S substitution line 77-33-2⊗ and 77-36-6⊗ were found effective. SRH_1 plants of Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338(77)-33-2⊗ and Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338 (77)-36-6⊗ with high grain micronutrients, yield and harvest index were selected. Molecular marker data and GISH analysis of some of the SRH_1-108 and 115 confirmed the transfer of 2S chromosome fragments. Molecular marker data also indicated that the SSR markers gwm265, barc1139, cfd43 and gdm148 tentatively mapped on the long arm of consensus group 2 chromosome map were associated with grain Fe and Zn content, indicating that long arm of 2S may have the genes for grain Fe and Zn content uptake, transport or sequestration. Wheat-*Aegilops* derivative 77-36-2 had 2S chromosome from *Ae. kotschyi* 3790 (Tiwari *et al*., 2010) while A-108 (SRH_3 WL711/77-36-2⊗-108) and A-115 (SRH_3 WL711/77-36-2⊗-115) showed several translocations of 2S, indicating the effectiveness of seed irradiation induced homoeologous transfer of alien chromosome fragment containing genes for seed micronutrient content. The genes for non-waxy plants was reported on the distal region of chromosome 2DS (Liu *et al*., 2007). A-108 had waxy leaf sheaths suggesting that the long arm of $2S^k$ was translocated and have genes for high grain Fe and Zn. A-115 had non-waxy leaf sheaths indicating the presence of both long arm and short arm of $2S^k$. Grain micronutrient content of SRH_3-108 (Fe: 43.34 mg/kg; Zn: 50.52 mg/kg) and

SRH_3-115 (Fe:44.86 mg/kg; Zn:60.39 mg/kg) and introgression of U/S genome indicates transfer of useful variability from *Aegilops* into wheat. Gamma rays (10-40 krad) were used for mutagenesis in wheat for reduced plant height and grain quality improvement (Singh and Balyan, 2009). Similarly Sears (1956) first time induced translocation of *Lr9* gene in wheat-Alien chromosome by X-rays. Several genes had been transferred into wheat through radiation hybrid approach including *Lr9* from *Agropyron elongatum* (Dvorak and Knott, 1977), resistance to hessian fly from rye(Mukai *et al*., 1993), resistance to leaf rust from *A. intermedium* (Friebe *et al*., 1993), resistance to wheat scab from *Leymus racemosus* (Wang *et al*., 2010) and powdery mildew resistance from *Hynaldia villosa* (Chen *et al*., 2012) confirmed by C-banding and GISH.

Pollen irradiation hybrids (PRH) of U and S substitution derivative 49-1-73⊗ at 2 krad were equally effective for precise transfer of genes/ chromosomal fragments as that of seed irradiation hybrids. Plants PRH_1-52 and 56 of Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425(49)-1-73 ⊗. /PBW343 (*GPC1+ Lr24*) had high grain Fe and Zn content with good yield and harvest index. The Fe and Zn content of PRH_2-52 was 40.98 mg/kg and 53.14 mg/kg while for PRH_2-56 was 45.12 mg/kg and 51.62 mg/kg respectively. The plant PRH_1-52 showed translocation of S and U chromosome while plants PRH_1-56 showed translocation of only U while intact S chromosome. There was no introgression of chromosome 1U/1S in PRH_1-52 indicating presence of only group 2 chromosome. PRH_1-52 plant had waxy leaf sheaths indicating the translocation of long arm of 2S/2U while PRH_1-58 had non-waxy plants indicating the presence of whole arm of 2S/2U (Liu *et al*., 2007). Pollen irradiation transfer of gene is more precise method and then seed irradiation. Higher radiation dose causes higher damage and subsequently smaller fragments can be transfer. It was also identified that translocation lines through irradiation induced transfer showed higher grain Fe and Zn content indicating that U and S genome had genes for Fe and Zn uptake/sequestration, which might be orthologous to the genes on S chromosome. Snape *et al*., 1973 had concluded that pollen irradiation is very useful approach for differential gene transfer. ^{60}Co-γ irradiation was used for transfer of good agronomic traits, such as grain yield per spike, powdery mildew resistance and stress tolerance in wheat- *A. cristatum* disomic addition lines (Song *et al*., 2013).

Previously it was reported that, wheat *ph1b* deletion promoting meiotic pairing among homoeologous chromosomes was used to induce recombination between chromosome 2B of wheat and 2S of goat grass, which induced and detect the homoeologous recombinants with small 2S chromosomal fragment of *Aegilops species* (Niu *et al*., 2011).

Extensive leaf yellowing was screened *ph1bph1b* homozygous plants which can be used as phenotypic marker for initial screening of homozygous *ph1b* lines. However molecular marker analyses through *Ph1* specific markers (psr2120, psr574) were considered as the final confirmatory test for screening of homozygous *ph1b* lines. Some plants were selfed while some plants were backcrossed with *ph1b* mutant plants. Among the BC_1F_3 plants from *ph1bph1b* homozygous and 2S/2U introgressed plants BC_1F_2-34, 48 and 104 with high micronutrient content, high yield and harvest index had no GISH signal showing any translocation of alien chromosomal fragment. Similarly F_4 plants homozygous for *ph1b* showed very high grain Fe and Zn content, introgression of group 2 chromosomes and high yield and harvest index. BC_1F_3 plants and F_4 plants showed grain Fe content in between 35-54 mg/kg while Zn content ranged 50-97 mg/kg. 40-60% increase in grain Fe and Zn content further confirmed the transfer of high grain Fe and Zn genes from *Aegilops* into wheat. Only plants with high grain mineral micronutrient, 2U/2S introgression, good harvest index and homozygous for *ph1b* were further subjected to generation advancement. A positive correlation was observed between grain Fe and Zn content among their advanced progenies of *ph1b* induced homoeologous pairing in wheat-*Aegilops* derivatives. Marker trait association plot of grain Fe and Zn content also showed association with marker gwm539 and gwm265 on the long arm of tentative 2U/2S consensus map, indicating the presence of common mechanism/ gene for grain Fe and Zn uptake, transport and/or storage in the 2U/2S wheat-*Aegilops* derivatives. Positive correlation was also observed for Zn with Fe, P, S and Na and of Cu with Fe, K, Mg, Ni and P in bread wheat (Caballero, 2002). Yu *et al*., 2010 had developed wheat translocation lines conferring resistance to stem rust (*Sr39*) using *ph1b* deletion. Naranjo and Fernández-Rueda (1996) also found homoeologous recombination and pairing in wheat and rye interspecific hybrids using *ph1b* mutation.

Wheat-*Aegilops* derivatives with high grain Fe and Zn were developed by using Chinese Spring with Ph^I as the female parent to induce homoeologous pairing (Chen *et al*., 1994; Rawat *et al.,* 2010; Tiwari *et al.,* 2010; Kumari *et al.,* 2011). None of the derivatives showed stable transfer of alien chromosome fragment indicating that either Ph^I gene was ineffective for inducing homoeologous recombination or the Ph^I stock was not stable. Therefore, it was decided to initiate fresh crosses to transfer gene(s) responsible for high grain micronutrient from *Aegilops species* using mono 5B wheat (*T. aestivum* cv. Pavon) for induced homoeologous pairing. It was previously observed that homozygous *ph1b* plants facilitates pairing among wheat and *Aegilops* homoeologous chromosomes at meiotic metaphase I (Benavente *et al*., 1998)

Hence to facilitate homoeologous recombination, plants of Pavon monosomics for 5B were crossed with superior *Aegilops* donors. Higher chromosomal pairing and multivalent formation confirmed the effectiveness of mono 5B. The BC_1F_2 and BC_2F_1 plants of Pavon mono 5B/ *Ae. kotschyi* 3790//PBW343 (*GPC+Lr24*)///PBW343 (*GPC+Lr24*) had high grain Fe and Zn content. The chromosome number of these plants varied from 41-60 with 1 to 12 univalents. These interspecific hybrids expected to have higher rate of recombination among homoeologous chromosomes. The recombination frequency can be further analysed by molecular markers and GISH/ FISH. Deficiency of 5B chromosome enhanced pairing and recombination in between *T. aestivum* Chinese Spring and *Aegilops columnaris* interspecific hybrids (Lacadens *et al.*, 1967). While in absence of 5BL, both homologous and homoeologous chromosomes formed multivalent with enhanced pairing due to absence of *Ph1* gene (Hobolth, 1981).

The wheat-*Aegilops* derivatives showed poor yield, harvest index and grain quality. The negative correlation of Fe and Zn content in seeds may be due to concentration effect. The negative correlation between seed size and grain micronutrient content may be due to more aleurone area per unit mass in the shrivelled seeds in comparison to the bold and healthy seeds. After extensive screening and analysis of all the plants with high grain Fe and Zn content and chromosomal translocations indicated that they had better genetic system for Fe and Zn uptake, transport and sequestration in seeds. The biofortification of wheat with 40-60% enhanced grain Fe and Zn content could be achieved in some of the precise transfers without any linkage drag. Pyramiding of these introgressed fragments/ genes from different sources through conventional and molecular breeding techniques can be done to achieve enhanced biofortification of these micronutrients in elite wheat cultivars.

6. References

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Appendix - I

List of wheat SSR markers and gene specific markers used

S.No	Primer	Forward sequence [5'-3']	Reverse sequence [5'-3']	Tm
CHROMOSOME- 2A				
1	cfd36	GCAAAGTGTAGCCGAGGAAG	TTAGAGTTTTGCAGCGCCTT	56
2	gwm512	AGCCACCATCAGCAAAAATT	GAACATGAGCAGTTTGGCAC	54
3	barc1138	GCGATGTCATGCTCACCAATGTGT	GCGTGCTCCACTCAGAGACTATCATAAA	65
4	wmc382	CATGAATGGAGGCACTGAAACA	CCTTCCGGTCGACGCAAC	60
5	gwm359	CTAATTGCAACAGGTCATGGG	TACTTGTGTTCTGGGACAATGG	59
6	wmc602	TACTCCGCTTTGATATCCGTCC	GTTTGTTGTTGCCATCACATTC	58
7	wmc453	ACTTGTGTCCATAACCGACCTT	ATCTTTTGAGGTTACAACCCGA	58
8	barc124	TGCACCCCTTCCAAATCT	TGCGAGTCGTGTGGTTGT	54
9	wmc382	CATGAATGGAGGCACTGAAACA	CCTTCCGGTCGACGCAAC	60
10	gwm515	AACACAATGGCAAATGCAGA	CCTTCCTAGTAAGTGTGCCTCA	54
11	gwm473	TCATACGGGTATGGTTGGAC	CACCCCCTTGTTGGTCAC	58
12	wmc261	GATGTGCATGTGAATCTCAAAAGTA	AAAGAGGGTCACAGAATAACCTAAA	61
13	gwm47	TTGCTACCATGCATGACCAT	TTCACCTCGATTGAGGTCCT	56
14	wmc109	AATTCGGGAAGAGTCTCAGGGG	TTCGAAGGGCTCAAGGGATACG	64
15	barc231	GCGATCAATAACCGTGCCACCA	GCACTTGCGATGTCACTAAAATG	61
16	barc309	GCGAAAGCCCTAAAGTTACAA	AAGCCGCAGAGAAGGTCAGC	57
17	cfd168	CTTCGCAAATCGAGGATGAT	TTCACGCCCAGTATTAAGGC	56
18	barc353	GAAGTTCCCAAAATGCCTCTGTC	GCGGATCGAAGACCTAAGAAAAG	63
19	wmc181	TCCTTGACCCCTTGCACTAACT	ATGGTTGGGAGCACTAGCTTGG	62
20	barc279	GCGTTTTTTACCTAAAGAAAAGGTGATTG	CGCAACACACATTCCATTCCATTTCAC	65
21	gwm356	AGCGTTCTTGGGAATTAGAGA	CCAATCAGCCTGCAACAAC	57
22	barc76	ATTCGTTGCTGCCACTTGCTG	GCGCGACACGGAGTAAGGACACC	61
23	wmc658	CTCATCGTCCTCCTCCACTTTG	GCCATCCGTTGACTTGAGGTTA	62
24	gwm311	TCACGTGGAAGACGCTCC	CTACGTGCACCACCATTTTG	58
25	gwm425	GAGCCCACAAGCTGGCA	TCGTTCTCCCAAGGCTTG	56
26	wmc407	GGTAATTCTAGGCTGACATATGCTC	CATATTTCCAAATCCCCAACTC	58
27	wmc177	AGGGCTCTCTTTAATTCTTGCT	GGTCTATCGTAATCCACCTGTA	58
28	wmc63	GTGCTCTGGAAACCTTCTACGA	CAGTAGTTTAGCCTTGGTGTGA	60
29	wmc667	GAGGAGAGGAAAAGGCAGGCTA	AACTCTTGCGTGTCTCAAACCG	61
30	barc1136	CGAGTTTTGCACAGGACAACCAATA	ATGCCAGTTTCTTTCTAGACATCTC	50
31	gwm636	CGGTAGTTTTTAGCAAAGAG	CCTTACAGTTCTTGGCAGAA	50
32	gwm614	GATCACATGCATGCGTCATG	TTTTACCGTTCCGGCCTT	60
33	wmc407	GGTAATTCTAGGCTGACATATGCTC	CATATTTCCAAATCCCCAACTC	61
34	barc212	GGCAACTGGAGTGATATAAATACCG	CAGGAAGGGAGGAGAACAGAGG	52
35	wmc598	TCGAGGAGTCAACATGGGCTG	ACGGTCGCTAGGGAGGGGAG	61
36	gwm71.1	GGCAGAGCAGCGAGACTC	CAAGTGGAGCATTAGGTACACG	60
37	wmc522	AAAAATCTCACGAGTCGGGC	CCCGAGCAGGAGCTACAAAT	61
38	wmc296	GAATCTCATCTTCCCTTGCCAC	ATGGAGGGGTATAAAGACAGCG	61
39	gwm122	GGGTGGGAGAAAGGAGATG	AAACCATCCTCCATCCTGG	60
40	gwm339	AATTTTCTTCCTCACTTATT	AAACGAACAACCACTCAATC	50
41	gwm10	CGCACCATCTGTATCATTCTG	TGGTCGTACCAAAGTATACGG	57
42	gwm448	AAACCATATTGGGAGGAAAGG	CACATGGCATCACATTTGTG	60
43	gwm275	AATTTTCTTCCTCACTTATTCT	AACAAAAAAATTAGGGCC	50
44	gwm95	GATCAAACACACACCCCTCC	AATGCAAAGTGAAAAACCCG	60
45	gwm558	GGGATTGCATATGAGACAACG	TGCCATGGTTGTAGTAGCCA	55
46	gdm101	GTCTCCATGACAAGGAGGGA	TGAAACCTCAAAGGGAAAGA	55
47	gwm328	GCAATCCACGAGAAGAGAGG	CACAAACTCTTGACATGTGCG	55
48	wmc455	GCGTCATTTCCTCAAACACATC	AGAAGGAGAAGTGCCTCACCAA	61
49	gwm372	AATAGAGCCCTGGGACTGGG	GAAGGACGACATTCCACCTG	60
50	gwm445	TTTGTTGGGGGTTAGGATTAG	CCTTAACACTTGCTGGTAGTGA	55
51	gwm312	ATCGCATGATGCACGTAGAG	ACATGCATGCCTACCTAATGG	60
52	gwm294	GGA TTG GAG TTA AGA GAG AAC CG	GCA GAG TGA TCA ATG CCA GA	55
53	barc201	GCGTTACTAGCCCAGGATTTACATT	GCGTCGATGTGAGTAGCGGGAAAC	52

54	barc28	CTCCCCGGCTAGTGACCACA	GCGGCATCTTTCATTAACGAGCTAGT	55
55	barc15	ATGCAAAGGCCGGGGTTATC	CACCTCTAGCCTACGCCAACATT	52
S.No	Primer	Forward sequence [5'-3']	Reverse sequence [5'-3']	Tm
56	barc220	CCTCTGCCATAAACATCACCTCTC	GGCCTCAACATCATGTGAAAGA	55
57	gwm265	TGT TGC GGA TGG TCA CTA TT	GAG TAC ACA TTT GGC CTC TGC	55
CHROMOSOME- 2B				
58	wmc764	CCTCGAACCTGAAGCTCTGA	TTCGCAAGGACTCCGTAACA	61
59	barc318	CGACTAACAATTTTTCATTT	TGATTTCGCTAACAAGGAG	50
60	barc200	GCGATATGATTTGGAGCTGATTG	GCGATGACGTTAGATGCGGAATTGT	52
61	barc349	CGAATAGCCGCTGCACAAG	TATGCATGCCTTTCTTTACAAT	52
62	barc13	GCAGGAACAACCACGCCATCTTAC	GCGTCGCAATTTGAAGAAAATCATC	52
63	wmc154	ATGCTCGTCAGTGTCATGTTTG	AAACGGAACCTACCTCACTCTT	61
64	barc128	GCGGGTAGCATTTATGTTGA	CAAACCAGGCAAGAGTCTGA	52
65	gwm429	TTGTACATTAAGTTCCCATTA	TTTAAGGACCTACATGACAC	50
66	barc101	GCTCCTCTCACGATCACGCAAAG	GCGAGTCGATCACACTATGAGCCAATG	52
67	gwm148	GTGAGGCAGCAAGAGAGAAA	CAAAGCTTGACTCAGACCAAA	57
68	barc183	CCCGGGACCACCAGTAAGT	GGATGGGGAATTGGAGATACAGAG	58
69	wmc272	TCAGGCCATGTATTATGCAGTA	ACGACCAGGATAGCCAATTCAA	61
70	wmc265	GTGGATAACATCATGGTCAAC	TACTTCGCACTAGATGAGCCT	51
71	gwm129	TCAGTGGGCAAGCTACACAG	AAAACTTAGTAGCCGCGT	50
72	cfd73	GATAGATCAATGTGGGCCGT	AACTGTTCTGCCATCTGAGC	60
73	gwm501	GGCTATCTCTGGCGCTAAAA	TCCACAAACAAGTAGCGCC	60
74	wmc332	CATTTACAAAGCGCATGAAGCC	GAAAACTTTGGGAACAAGAGCA	61
75	wmc149	ACAGACTTGGTTGGTGCCGAGC	ATGGGCGGGGGTGTAGAGTTTG	61
76	wmc317	TGCTAGCAATGCTCCGGGTAAC	TCACGAAACCTTTTCCTCCTCC	61
77	gwm382	GTCAGATAACGCCGTCCAAT	CTACGTGCACCACCATTTTG	60
78	gwm526	CAATAGTTCTGTGAGAGCTGCG	CCAACCCAAATACACATTCTCA	55
79	gwm374	ATAGTGTGTTGCATGCTGTGTG	TCTAATTAGCGTTGGCTGCC	60
80	gwm410	GCTTGAGACCGGCACAGT	CGAGACCTTGAGGGTCTAGA	55
81	wmc474	ATGCTATTAAACTAGCATGTGTCG	AGTGGAAACATCATTCCTGGTA	55
82	wmc445	AGAATAGGTTCTTGGGCCAGTC	GAGATGATCTCCTCCATCAGCA	55
83	wmc356	GCCGTTGCCCAATGTAGAAG	CCAGAGAAACTCGCCGTGTC	55
84	wmc592	GGTGGCATGAACTTTCACCTGT	TGTGTGGTGCCCATTAGGTAGA	61
85	barc35	GCGGTGTGCATGCTTGTCGTGTAGGAGT	GCGTAGTGTAGTATGTGGCCCGATTATT	55
86	wmc661	CCACCATGGTGCTAATAGTGTC	AGCTCGTAACGTAATGCAACTG	61
87	gwm210	TGCATCAAGAATAGTGTGGAAG	TGAGAGGAAGGCTCACACCT	60
88	barc361	TGCAGGCTAGATTGGGCGACGAT	GTGGGGCCTAGGCAGTGGACG	60
89	barc91	TTCCCATAACGCCGATAGTA	GCGTTTAATATTAGCTTCAAGATCAT	50
90	barc160	GGTTGTTCCTAGGAAATTTCTATAATAACTG	GCGACTTTCATGATCAAGATGGCATC	50
91	wmc213	ATTTTCTCAAACACACCCCG	TAGCAGATGTTGACAATGGA	51
92	gwm257	AGAGTGCATGGTGGGACG	CCAAGACGATGCTGAAGTCA	60
93	wmc257	GGCTACACATGCATACCTC	CGTAGTGGGTGAATTTCGGA	51
94	wmc770	TGTCAGACTTCCTTTGATCCCC	AAGACCATGTGACGTCCAGC	61
95	wmc434	GGAGCCTGATTAGGCTGGAC	AGCCAAACAGCCAACAGAGT	61
96	barc7	GCGAAGTACCACAAATTTGAAGGA	CGCCATCTTACCCTATTTGATAACTA	50
97	barc55	GCGGTCAACACACTCCACTCCTCTCTC	CGCTGCTCCCATTGCTCGCCGTTA	55
98	gwm630	GTGCCTGTGCCATCGTC	CGAAAGTAACAGCGCAGTGA	60
99	barc18	CGCTTCCCATAACGCCGATAGTAA	CGCCCGCATCATGAGCAATTCTATCC	52
100	wmc344	ATTTCAGTCTAATTAGCGTTGG	AACAAAGAACATAATTAACCCC	51
101	wmc27	AATAGAAACAGGTCACCATCCG	TAGAGCTGGAGTAGGGCCAAAG	61
102	barc167	AAAGGCCCATCAACATGCAAGTACC	CGCAGTATTCTTAGTCCCTCAT	50
103	cfa2278	GCCTCTGCAAGTCTTTACCG	AAGTCGGCCATCTTCTTCCT	60
104	gwm319	GGTTGCTGTACAAGTGTTCACG	CGGGTGCTGTGTGTAATGAC	55
105	wmc477	CGTCGAAAACCGTACACTCTCC	GCGAAACAGAATAGCCCTGATG	61
106	wmc498	CGATGAAGAGAGCCATCAAAA	TGACATTCCGGTAGGTCAGTT	61
107	gwm271	CAAGATCGTGGAGCCAGC	AGCTGCTAGCTTTTGGGACA	60
108	gwm55	GCATCTGGTACACTAGCTGCC	TCATGGATGCATCACATCCT	60
109	gwm388	CTACAATTCGAAGGAGAGGGG	CACCGCGTCAACTACTTAAGC	60
110	wmc441	TCCAGTAGAGCACCTTTCATT	ATCACGAAGATAAACAAACGG	51

111	cfd70	GTCGGCATAGTCGCACATAC	ACTATGCCAAGGGGAGTGTG	60
112	gwm120	GATCCACCTTCCTCTCTCTC	GATTATACTGGTGCCGAAAC	60
S.No	Primer	Forward sequence [5’-3’]	Reverse sequence [5’-3’]	Tm
113	gwm191	AGACTGTTGTTTGCGGGC	TAGCACGACAGTTGTATGCATG	60
114	barc230	CCCCTCCTCCTTCTCCCTCCTCCTA	GGCTCATGCGGGCGTGTTTGG	58
115	gwm16	GCTTGGACTAGCTAGAGTATCATAC	CAATCTTCAATTCTGTCGCACGG	50
116	wmc627	GATCCGAGAAGGGCAATGGTAG	AGCAACAGCAGCGTACCATAAA	61
117	wmc361	AATGAAGATGCAAATCGACGGC	ATTCTCGCACTGAAAACAGGGG	61
118	gdm114	AGATAGCACGGGTGCGG	CACAAACGACCCCTCTGG	60
119	barc16	GCGTTGCGAGATCTTATGGGTTT	GCGCGTTTTTCGAATACCTTGT	52
120	barc1024	CGTGATGGCTGATTTGTCTTC	GCCCCTTCAAACCTCTCCAATCATC	52
121	barc1072	CGGCGGGACCCAGTTGACCAGT	GAGCCCTTGCCCCCAGAATG	52
122	barc1114	GCGGGATAAAGCACGAAAAATAAT	GCGTGCCGCTAGAGTTTAGTCAA	50
123	barc1160	CGTCCCAGCCCCAGAACATTTTT	CAGCGTGATGTCAGCATGATGTCAT	52
124	barc114	GGGTATCACGCATTGTAAAATCCGAAA	GCTGACTTCCGCAAATGCCATTCGCTGAT	50
125	barc115	GCGTCTTCAAAATCTATATCTTCACAATG	CCCATCACATTCACCTAGTTCAGA	50
126	barc210	TGAAGCAAAACCGCAATGGGATAGG	GGAGCCGAAGAGCAGGAAGGTG	52
127	barc1135	GCATCCGCAGAAAGAGAAGCAAAT	GCGTATCCCATTGCTCTTCTTCACTAA	50
128	barc221	GAAGCGACGAGTGTAGGACGGTAGC	CGGCCAGGACGGTCAGTTCT	55
129	barc1156	CCAAATAGCGCTTACCGGTTCGAAATA	CCTCTCCTCCCTCATCCGTTAG	50
130	barc1139	GCGAAAGCGTCAAGGTATTTGTGC	GCGTTCCAGCCCTGATTATCTAAGAG	50
131	barc1064	GCGATAAGAGATGGCAAAGGAAAGG	CAGCTTATTAAACAACGCCCGTCAA	50
132	barc1147	CAGGTCAGTTCCCCGATCCGA	TACGTCCGCGGAGGAGTTGACC	60
CHROMOSOME-2D				
133	cfd56	TTGCATAATTACTTGCCCTCC	CTGGTCCAACTTCCATCCAT	57
134	barc297	GCGTAGGAGAGATGCCCCAAAGGTT	GCGTGCGGACTCGTGAATCATTACA	69
135	wmc25	TCTGGCCAGGATCAATATTACT	TAAGATACATAGATCCAACACC	58
136	gwm261	CTCCCTGTACGCCTAAGGC	CTCGCGCTACTAGCCATTG	59
137	barc168	GCGATGCATATGAGATAAGGAACAAATG	GCGGCTCTAAGGCGGTTTCAAAT	65
138	wmc470	ACTTGCAACTGGGGACTCTC	TCCCCAATTGCATATTGACC	56
139	barc228	CCCTCCTCTCTTTAGCCATCC	GCACGTACTATTCGCCTTCACTTA	63
140	barc145	GCAGCCTCGAATCACA	GGGGTGTTGAAGATGA	48
141	barc11	GCGATGCGTGTAAAGTCTGAAGATGA	GCGTCCATGGAGCTCTGTTTTATCTGA	66
142	gwm249	CAAATGGATCGAGAAAGGGA	CTGCCATTTTTCTGGATCTACC	56
143	wmc601	ACAGAGGCATATGCAAAGGAGG	CTTGTCTCTTTATCGAGGGTGG	62
144	barc219	GCGATCCCACAATGCATGACAACTTC	GGACGTCCGATCGAATTGGTTT	62
145	barc159	CGCAATTTATTATCGGTTTTAGGAA	CGCCCGATAGTTTTTCTAATTTCTGA	62
146	wmc41	TCCCTCTTCCAAGCGCGGATAG	GGAGGAAGATCTCCCGGAGCAG	66
147	gwm608	ACATTGTGTGTGCGGCC	GATCCCTCTCCGCTAGAAGC	55
148	gwm349	GGCTTCCAGAAAACAACAGG	ATCGGTGCGTACCATCCTAC	58
149	wmc175	GCTCAGTCAAACCGCTACTTCT	CACTACTCCAATCTATCGCCGT	62
150	wmc817	TGACGGGGATGATGATAACG	CGGTGAGATGAGAAAGGAAAAC	58
151	gwm301	GAGGAGTAAGACACATGCCC	GTGGCTGGAGATTCAGGTTC	60
152	gdm5	CTAGCCAGAAGGTTACTTTG	CAACATTAACATTAACGCAC	52
153	gwm455	ATTCGGTTCGCTAGCTACCA	ACGGAGAGCAACCTGCC	57
154	gwm484	ACATCGCTCTTCACAAACCC	AGTTCCGGTCATGGCTAGG	58
155	gwm102	TCTCCCATCCAACGCCTC	TGTTGGTGGCTTGACTATTG	58
156	gwm539	CTGCTCTAAGATTCATGCAACC	GAGGCTTGTGCCCTCTGTAG	60
157	gdm148	GATTTGACCGTCTGAGGTCG	AACTAGTTCTGTGGCAAGCT	56
158	gwm296	AATTCAACCTACCAATCTCTG	GCCTAATAAACTGAAAACGAG	55
159	wmc818	TGAAGGGTGCGTGTGGTC	GCGTCGATTTTAATTTGATGATGG	61
160	cfd65	AGACGATGAGAAGGAAGCCA	CCTCCCTTGTTTTTGGGATT	60
161	cfd51	GGAGGCTTCTCTATGGGAGG	TGCATCTTATCCTGTGCAGC	60
162	barc90	GCGCTTGGGTTGCTTCGAGGAGGACA	CGCAATCCTCTTCCCCGTGGCATAG	62
163	wmc111	ATTGATGTGTACGATGTGCCTG	CATGTCAATGTCATGATGAAGC	61
164	gdm6	GATCAATCAAGCATGTGTGTGT	GGATGCCATGCCAAAGTATT	55
165	wmc112	TGAGTTGTGGGGTCTTGTTTGG	TGAAGGAGGGCACATATCGTTG	61
166	Wmc25	TCTGGCCAGGATCAATATTACT	TAAGATACATAGATCCAACACC	51
167	Cfd56	TTGCATAATTACTTGCCCTCC	CTGGTCCAACTTCCATCCAT	60

168	cfd43	AACAAAAGTCGGTGCAGTCC	CCAAAAACATGGTTAAAGGGG	60
169	wmc18	CTGGGGCTTGGATCACGTCATT	AGCCATGGACATGGTGTCCTTC	61
S.No	Primer	Forward sequence [5’-3’]	Reverse sequence [5’-3’]	Tm
170	gwm30	ATCTTAGCATAGAAGGGAGTGGG	TTCTGCACCCTGGGTGAT	60
171	cfd17	AGCACAGAAGGGGTTAGGGT	AGCTGCGGTGTGAGCTAAAT	60
172	gwm358	AAACAGCGGATTTCATCGAG	TCCGCTGTTGTTCTGATCTC	55
173	cfd116	TTTGCCCATTACAACAAGCA	CAAGCAGCACCTCATGACAG	60
174	wmc245	GCTCAGATCATCCACCAACTTC	AGATGCTCTGGGAGAGTCCTTA	61
175	wmc144	GGACACCAATCCAACATGAACA	AAGGATAGTTGGGTGGTGCTGA	61
176	cfd193	GCTGCCGCTACTGTCTGTC	GGCACACTCACACACCACAC	60
177	cfd160	CCACTACTGCGGCTAGGTCT	CTTTTCCGTGTCTCCCTAGC	60
178	wmc797	CGAAACCCTAGATGAAGC	ACACAACCACAGGTGAGTTGTTCT	51
179	gwm157	GTCGTCGCGGTAAGCTTG	GAGTGAACACACGAGGCTTG	60
180	cfd233	GAATTTTTGGTGGCCTGTGT	ATCACTGCACCGACTTTTGG	60
181	cfd62	CAAGAGCTGACCAATGTGGA	ACGGCGGTGAGATGAG	60
182	wmc243	CGTCATTTCCTCAAACACACCT	ACCGGCAGATGTTGACAATAGT	61
183	cfd239	CTCTCGTTCTCTCCAGGCTC	GAGAGGAGAGCTTGCCATTG	57
184	cfd161	GTAAGGCATCTTCGCGTCTC	CCATGATAGATTTGGACGGG	60
185	wmc167	AGTGGTAATGAGGTGAAAGAAG	TCGGTCGTATATGCATGTAAAG	51
186	gwm320	CGAGATACTATGGAAGGTGAGG	ATCTTTGCAAGGATTGCCC	55
187	barc59	GCGTTGGCTAATCATCGTTCCTTC	AGCACCCTACCCAGCGTCAGTCAAT	55
188	barc292	GCGTGTGAGTCAATCCGTGCTTTAT	GCGTTGGTTTTAAGAGGTGCCTGAA	52
189	gdm19	GCGTTCGAGTGACTTCCAAT	ATTGACAGCAGATGGCAGTG	55
190	gdm35	CCTGCTCTGCCCTAGATACG	ATGTGAATGTGATGCATGCA	55
191	gdm87	AATAATGTGGCAGACAGTCTTGG	CCAAGCCCCAATCTCTCTCT	60
192	gdm93	AAAAGCTGCTGGAGCATACA	GGAGCATGGCTACATCCTTC	55
193	gwm539	CTGCTCTAAGATTCATGCAACC	GAGGCTTGTGCCCTCTGTAG	60
194	gdm107	AGCAACAAACGCGAGAGC	TGACACCCGGTTGTTGG	55
195	barc11	GCGATGCGTGTAAAGTCTGAAGATGA	GCGTCCATGGAGCTCTGTTTTATCTGA	52
196	gdm29	CTAGTTGTGCTAGGCGCTCC	CTGGCTGCTCCCTCCTC	60
197	gdm77	GACACACAATAGCCAAAGCA	TGATGTCGGCACTATTTTGG	55
198	barc95	GGGGTGTGGTTGTTTGTAAGG	TGCGAATTCTATATACGATCTTGAGC	52
199	barc1146	GGCGGTTGCAGGTGGTGGGTGGT	GCTCTCCTCTGCTTTTGACCCTCTA	55
200	barc1095	ATAGGGCGGGAAAGATTGTA	CCTTCCCCTCTCGTCCAT	52
201	barc1143	CACTCCGAGTTATCACTTGATTGTCA	ATAGGTGGCGTTGGTGGGTAATA	50
202	gdm148	GATTTGACCGTCTGAGGTCG	AACTAGTTCTGTGGCAAGCT	60
***Ph1* specific markers**				
203	PSR128	ATCGCTCCTCTGCTTGCTTC	GACCGCCTGAAACCTCCC	57
204	PSR574	AGCGTATATTCACGCGCTCC	CGTAAGAACTCCCCAGGGTTTG	57
205	PSR2120	TTAACGCCAGGGCATACT C	CTGCAGGAGGCGCTGGA	57
206	AWJL3	TGGCACCCTCAATGTAGAC	GCTTGCCCATTTCACAAC	57

List of Selected Derivatives: 2009-10 (First Priority)

Progeny	Pedigree	Chromosome Introgressed	Fe (mg/kg)	Fe % increase over *T.aestivum* cv. PBW343	Zn (mg/kg)	Zn % increase over *T.aestivum* cv. PBW343	Harvest Index	Waxiness	Chromosome Number (Pairing behaviour)
77-36-6-13-1	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-36-6-13-1	2S	51.06	74.73	45.1	35.34	31.15	Non-Waxy	43 (21II+1I)
77-36-6-13-8	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-36-6-13-8	2S	32.24	10.32	48.9	46.95	32.93	Non-Waxy	43(21II+1I &20II+1III)
77-36-6-15-1	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-36-6-15-1	2S	44.77	61.16	30.6	14.48	38.24	Non-Waxy	43(20II+1III)
77-33-2-16-1	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-33-2-16-1	2S	96.8	124.78	81.5	45.72	18.43	Non-Waxy	43 (20II+1III)
77-33-2-25-2	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-33-2-25-2	2S & 7S	18.19	-39.9	39	13.7	34.62	Non-Waxy	42 (20II+2I)
77-50-8-1-2	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-50-8-1-2	2S	59.36	126.46	60.4	81.78		Non-Waxy	NA
49-1-11-10-6	Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-11-10-6	2S&7S	63.2	146.7	48.6	119.91	42.47	Non-Waxy	NA
49-1-11-28-2	Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-11-28-2	2S	39.9	55.7	33.75	52.71	37.25	Non-Waxy	NA
49-1-73-5-7	Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-73-5-7	7S & 2S	41.25	57.36	55.5	67.17	44.23	Non-Waxy	NA
49-1-73-8-5	Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-73-8-5	2S	44.1	58.73	45	68.66	44.12	Non-Waxy	43 (18II+2III+1I & 21II+1I)

NA: Data not available

List of Selected Derivatives 2009-10(Second Priority)

Progeny	**Pedigree**	**Chromosome Introgressed**	**Fe (mg/kg)**	**Fe % increase over *T.aestivum* cv. PBW343**	**Zn (mg/kg)**	**Zn % increase over *T.aestivum* cv. PBW343**	**Harvest Index**	**Waxiness**	**Chromosome Number (Pairing behaviour)**
77-33-2-5-8	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-33-2-5-8		44.61	60.57	31.4	17.48	34.25	Waxy	41(20II+1I)
77-33-2-25-4	Cs(Ph^I)/ *Ae. kotschyi* 3790//UP2338-2-33-2-25-4	2S & 7S	46.6	53.98	43.55	26.97	20.17	Non-Waxy	42 (20II+2I)
49-1-73-8-1	Cs(Ph^I)/ *Ae. kotschyi* 396//PBW343-3///UP2425-1-73-8-1	2S	43.01	54.82	45.4	70.04	43.28	Non-Waxy	43 (21II+1I)
54-3-12-11-2	Cs(Ph^I)/ *Ae. kotschyi* 396//UP2425-2///PBW373-3-12-11-2	2S & 7S	39.2	34.69	53.1	72.96	29.23	Non-Waxy	43 (21II+1I)
54-3-12-12-3	Cs(Ph^I)/ *Ae. kotschyi* 396//UP2425-2///PBW373-3-12-12-3	2S& 7S	68.43	161.06	55.2	66.27	18.60	Non-Waxy	43 (21II+1I)

Grain Fe and Zn of selected BC_1F_2 and F_3 plants, (*ph1b* X wheat-*Aegilops* derivatives) (years 2011-12)

Plant Name	Pedigree	Mean Fe (mg/kg) ± SD	Mean Zn (mg/kg) ± SD	Harvest Index
PH-33	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-33	25.82 ± 3.34	30.90 ± 1.80	15.75
PH-34	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-34	78.04 ± 1.47	53.02 ± 0.80	19.16
PH-35	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-35	68.48 ± 0.63	52.27 ± 1.12	42.16
PH-36	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-36	42.03 ± 0.79	39.33 ± 0.94	18.62
PH-40	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-40	60.62 ± 1.04	51.64 ± 1.13	13.41
PH-41	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-41	56.47 ± 1.34	52.31 ± 0.67	13.41
PH-43	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-43	42.87 ± 0.62	32.18 ± 2.09	18.20
PH-45	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-45	36.67 ± 1.32	34.27 ± 0.72	11.50
PH-47	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-47	66.02 ± 0.64	42.44 ± 0.05	16.29
PH-48	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-48	76.13 ± 0.27	40.45 ± 1.36	18.20
PH-49	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-49	61.82 ± 1.51	36.27 ± 0.37	13.83
PH-50	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-50	57.39 ± 0.97	43.01 ± 0.53	13.83
PH-55	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-55	42.15 ± 1.75	51.51 ± 4.31	10.54
PH-56	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-56	67.40 ± 0.95	44.11 ± 0.67	17.67
PH-58	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-58	35.38 ± 1.70	34.77 ± 0.99	24.91
PH-59	BC_1F_2 *ph1b*/46-1-15-15-2//*ph1b*-1-59	58.48 ± 0.58	47.53 ± 1.20	16.71
PH-69	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-69	40.29 ± 0.87	29.37 ± 1.17	31.62
PH-70	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-70	42.44 ± 0.76	26.21 ± 0.56	19.16
PH-71	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-71	55.42 ± 1.06	25.58 ± 0.75	10.54
PH-72	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-72	28.76 ± 1.44	25.73 ± 1.04	20.12
PH-73	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-73	35.66 ± 1.35	29.02 ± 1.04	18.20
PH-74	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-74	29.54 ± 0.59	26.85 ± 0.24	15.33
PH-75	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-75	37.53 ± 0.57	23.53 ± 1.15	41.20
PH-76	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-76	38.31 ± 1.20	27.08 ± 0.78	28.74
PH-77	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-77	69.25 ± 1.26	29.57 ± 0.54	29.70
PH-78	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-78	44.04 ± 1.85	28.54 ± 0.89	17.25
PH-79	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-79	23.94 ± 2.63	20.88 ± 1.95	15.33
PH-80	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-80	28.28 ± 1.33	32.76 ± 0.59	27.79
PH-81	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-81	25.24 ± 2.01	24.65 ± 0.74	13.41
PH-82	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-82	51.59 ± 1.02	34.29 ± 0.74	10.54
PH-83	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-83	47.05 ± 0.75	34.81 ± 0.72	10.54
PH-84	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-84	36.01 ± 0.83	31.39 ± 0.72	23.00
PH-85	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-85	41.16 ± 0.75	38.84 ± 0.87	17.25
PH-86	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-86	36.82 ± 1.00	33.68 ± 1.13	15.33
PH-87	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-87	39.75 ± 0.95	40.67 ± 0.53	17.67
PH-88	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-88	71.31 ± 0.61	47.26 ± 0.80	16.71
PH-89	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-89	46.84 ± 0.38	42.88 ± 0.45	11.50
PH-90	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-90	79.60 ± 0.62	77.89 ± 1.44	22.04
PH-92	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-92	24.68 ± 1.16	24.64 ± 1.01	10.54

PH-93	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-93	41.93 ± 0.94	38.93 ± 0.56	17.67
PH-95	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-95	45.95 ± 0.37	29.83 ± 0.68	17.25
PH-97	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-97	61.91 ± 0.87	21.70 ± 3.23	14.37
PH-98	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-98	49.02 ± 0.57	39.53 ± 0.06	18.62
PH-99	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-99	59.83 ± 0.95	50.55 ± 1.50	10.54
Plant Name	**Pedigree**	**Mean Fe (mg/kg) ± SD**	**Mean Zn (mg/kg) ± SD**	**Harvest Index**
PH-100	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-100	23.70 ± 0.56	24.49 ± 1.24	11.50
PH-101	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-101	28.24 ± 1.27	24.94 ± 0.88	21.97
PH-102	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-102	81.77 ± 0.90	54.98 ± 0.63	47.91
PH-104	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-104	82.59 ± 1.20	43.17 ± 1.60	12.46
PH-105	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-105	69.46 ± 0.91	36.77 ± 1.00	25.87
PH-106	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-106	59.39 ± 0.93	40.85 ± 0.45	23.95
PH-107	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-107	67.41 ± 0.77	36.34 ± 1.03	11.50
PH-108	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-108	89.45 ± 1.44	54.43 ± 0.60	39.28
PH-109	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-109	65.54 ± 1.21	36.16 ± 0.55	17.25
PH-110	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-110	73.47 ± 1.37	43.08 ± 0.89	15.33
PH-111	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-111	58.57 ± 1.26	44.95 ± 0.57	11.50
PH-112	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-112	76.15 ± 1.02	50.86 ± 0.47	16.29
PH-113	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-113	68.40 ± 0.69	35.58 ± 0.57	20.12
PH-114	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-114	53.16 ± 1.77	29.64 ± 0.47	12.46
PH-115	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-115	61.42 ± 1.06	34.99 ± 0.66	26.83
PH-116	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-116	61.62 ± 1.41	29.79 ± 0.75	23.95
PH-117	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-117	26.50 ± 2.64	26.86 ± 0.95	22.04
PH-118	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-118	61.44 ± 1.37	26.70 ± 0.82	16.29
PH-119	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-119	29.23 ± 1.27	25.16 ± 0.39	10.54
PH-120	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-120	31.18 ± 1.36	27.55 ± 0.53	45.99
PH-121	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-121	63.09 ± 2.68	25.88 ± 0.50	19.16
PH-123	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-123	75.55 ± 0.69	31.02 ± 0.72	22.04
PH-125	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-125	61.45 ± 0.63	26.33 ± 0.62	38.33
PH-126	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-126	22.90 ± 2.40	23.76 ± 0.87	27.79
PH-127	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-127	35.98 ± 1.83	25.51 ± 0.98	24.91
PH-128	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-128	62.55 ± 1.23	29.40 ± 1.18	10.54
PH-129	BC_1F_2 *ph1b*/49-1-73-10-3//*ph1b*-24-129	77.48 ± 1.13	27.44 ± 0.61	10.54
PH-130	F_3 *ph1b*/77-36-6 ⊗-4-130	61.27 ± 1.05	30.69 ± 0.20	21.08
PH-131	F_3 *ph1b*/77-36-6 ⊗-4-131	42.11 ± 0.30	31.35 ± 0.18	44.07
PH-132	F_3 *ph1b*/77-36-6 ⊗-4-132	76.05 ± 1.84	39.84 ± 0.42	35.45
PH-133	F_3 *ph1b*/77-36-6 ⊗-4-133	48.06 ± 1.62	31.41 ± 1.22	14.37
PH-134	F_3 *ph1b*/77-36-6 ⊗-4-134	52.39 ± 1.14	36.67 ± 1.44	19.58
PH-136	F_3 *ph1b*/77-36-6 ⊗-4-136	60.47 ± 0.83	28.43 ± 1.07	18.20
PH-137	F_3 *ph1b*/77-36-6 ⊗-25-137	31.90 ± 0.87	23.82± 1.66	18.20
PH-138	F_3 *ph1b*/77-36-6 ⊗-25-138	30.61 ± 0.68	27.24 ± 0.44	19.58
PH-139	F_3 *ph1b*/77-36-6 ⊗-25-139	45.73 ± 1.09	37.57 ± 0.50	15.33
PH-140	F_3 *ph1b*/77-36-6 ⊗-25-140	68.34 ± 1.62	39.26 ± 0.57	15.33
PH-141	F_3 *ph1b*/77-36-6 ⊗-25-141	62.12 ± 0.80	33.12 ± 0.56	40.24
PH-142	F_3 *ph1b*/77-36-6 ⊗-25-142	67.67 ± 1.15	31.87 ± 0.64	23.95

PH-143	F_3 *ph1b*/77-36-6 ⊗-25-143	48.06 ± 1.57	26.45 ± 1.00	22.04
PH-144	F_3 *ph1b*/77-36-6 ⊗-25-144	63.56 ± 0.70	40.05 ± 0.55	30.00
PH-145	F_3 *ph1b*/77-36-6 ⊗-25-145	63.65 ± 0.74	32.61 ± 0.87	32.58
PH-146	F_3 *ph1b*/77-36-6 ⊗-25-146	68.34 ± 0.82	33.22 ± 0.64	12.46
PH-147	F_3 *ph1b*/77-36-6 ⊗-25-147	52.60 ± 0.73	30.61 ± 0.60	24.91
PH-148	F_3 *ph1b*/77-36-6 ⊗-25-148	62.14 ± 1.62	28.15 ± 0.67	21.08
PH-149	F_3 *ph1b*/77-36-6 ⊗-25-149	62.85 ± 1.02	35.31 ± 1.01	16.29
PH-150	F_3 *ph1b*/77-36-6 ⊗-25-150	51.99 ± 1.68	28.01 ± 0.79	20.96

Plant Name	**Pedigree**	**Mean Fe (mg/kg) ± SD**	**Mean Zn (mg/kg) ± SD**	**Harvest Index**
PH-151	F_3 *ph1b*/77-36-6 ⊗-25-151	56.58 ± 1.13	33.48 ± 1.19	11.50
PH-152	F_3 *ph1b*/77-36-6 ⊗-25-152	76.78 ± 0.89	37.55 ± 0.55	14.37
PH-153	F_3 *ph1b*/77-36-6 ⊗-25-153	70.25 ± 0.99	38.84 ± 0.27	23.00
PH-154	F_3 *ph1b*/77-36-6 ⊗-25-154	30.46 ± 0.96	28.31 ±1.05	26.83
PH-155	F_3 *ph1b*/77-36-6 ⊗-25-155	70.93 ± 1.45	30.93 ± 0.96	17.25
PH-156	F_3 *ph1b*/77-36-6 ⊗-25-156	70.00 ± 1.70	32.13 ± 0.53	22.04
PH-157	F_3 *ph1b*/77-36-6 ⊗-25-157	38.83 ± 0.70	29.11 ± 0.57	31.62
PH-158	F_3 *ph1b*/77-36-6 ⊗-25-158	63.71 ± 1.35	35.27 ± 0.87	30.66
PH-159	F_3 *ph1b*/77-36-6 ⊗-25-159	73.43 ± 0.66	34.33 ± 0.90	15.33
PH-160	F_3 *ph1b*/77-36-6 ⊗-25-160	35.86 ± 0.90	24.46 ± 0.92	17.25
PH-161	F_3 *ph1b*/77-36-6 ⊗-25-161	74.71 ± 0.51	43.33 ± 0.35	21.08
PH-162	F_3 *ph1b*/77-36-6 ⊗-25-162	54.75 ± 1.02	36.96 ± 0.59	13.41
PH-163	F_3 *ph1b*/77-36-6 ⊗-25-163	68.63 ± 2.01	40.72 ± 0.51	10.54
PH-165	F_3 *ph1b*/77-36-6 ⊗-25-165	52.99 ± 0.94	33.24 ± 0.64	15.33
PH-166	F_3 *ph1b*/77-36-6 ⊗-25-166	74.47 ± 0.70	29.89 ± 0.54	21.08
PH-167	F_3 *ph1b*/77-36-6 ⊗-25-167	75.14 ± 0.56	37.06 ± 0.58	20.12
PH-169	F_3 *ph1b*/77-36-6 ⊗-42-169	79.28 ± 0.84	47.21 ± 0.43	16.71
PH-170	F_3 *ph1b*/77-36-6 ⊗-42-170	82.07 ± 1.72	42.29 ± 0.68	18.20
PH-171	F_3 *ph1b*/77-36-6 ⊗-42-171	70.83 ± 1.30	36.96 ± 0.86	19.16
PH-172	F_3 *ph1b*/77-36-6 ⊗-42-172	78.21 ± 1.50	36.95 ± 0.60	23.00
PH-173	F_3 *ph1b*/77-36-6 ⊗-42-173	67.96 ± 1.46	33.01 ± 0.44	16.29
PH-174	F_3 *ph1b*/77-36-6 ⊗-42-174	86.55 ± 2.00	44.01 ± 0.68	14.37
PH-175	F_3 *ph1b*/77-36-6 ⊗-42-175	29.17 ± 1.56	29.72 ± 0.66	15.33
PH-177	F_3 *ph1b*/77-36-6 ⊗-42-177	73.45 ± 0.63	33.50 ± 0.59	36.41
PH-178	F_3 *ph1b*/77-36-6 ⊗-42-178	70.91 ± 0.73	37.69 ± 0.50	11.50
PH-179	F_3 *ph1b*/77-36-6 ⊗-42-179	54.73 ± 0.99	31.65 ± 1.39	14.37
PH-180	F_3 *ph1b*/77-36-6 ⊗-42-180	69.10 ± 1.00	36.83 ± 1.14	20.12
PH-181	F_3 *ph1b*/77-36-6 ⊗-27-181	71.90 ± 1.62	36.73 ± 0.71	19.58
PH-182	F_3 *ph1b*/77-36-6 ⊗-27-182	76.07 ± 0.98	42.59 ± 0.51	17.25
PH-183	F_3 *ph1b*/77-36-6 ⊗-27-183	68.52 ± 1.48	36.47 ± 0.86	21.08
PH-184	F_3 *ph1b*/77-36-6 ⊗-27-184	78.00 ± 1.75	41.03 ± 0.78	22.04
PH-185	F_3 *ph1b*/77-36-6 ⊗-27-185	46.54 ± 1.22	33.79 ± 0.32	19.16
PH-186	F_3 *ph1b*/77-36-6 ⊗-27-186	41.58 ± 0.91	34.13 ± 0.63	19.58
PH-187	F_3 *ph1b*/77-36-6 ⊗-27-187	47.63 ± 1.79	34.07 ± 0.63	27.79
PH-188	F_3 *ph1b*/77-36-6 ⊗-27-188	72.27 ± 1.70	34.94 ± 0.63	33.53
PH-189	F_3 *ph1b*/77-36-6 ⊗-27-189	69.50 ± 1.42	34.98 ± 0.78	17.25

PH-190	F_3 *ph1b*/77-36-6 ⊗-27-190	71.17 ± 0.65	32.54 ± 1.38	20.12
PH-195	F_3 *ph1b*/77-33-2 ⊗-1-195	53.31 ± 1.30	28.75 ± 0.90	21.08
PH-196	F_3 *ph1b*/77-33-2 ⊗-1-196	51.64 ± 1.19	34.38 ± 0.80	21.08
PH-197	F_3 *ph1b*/77-33-2 ⊗-1-197	67.00 ± 0.41	36.56 ± 0.80	20.12
PH-199	F_3 *ph1b*/77-33-2 ⊗-1-199	80.92 ± 1.63	47.03 ± 0.43	11.50
PH-200	F_3 *ph1b*/77-33-2 ⊗-1-200	57.30 ± 1.89	35.55 ± 0.60	29.70
PH-201	F_3 *ph1b*/77-33-2 ⊗-1-201	38.71 ± 1.17	36.78 ± 1.06	14.37
PH-202	F_3 *ph1b*/77-33-2 ⊗-1-202	62.53 ± 1.33	35.26 ± 0.65	23.00
PH-203	F_3 *ph1b*/77-33-2 ⊗-1-203	51.58 ± 0.86	33.73 ± 0.80	25.87
PH-204	F_3 *ph1b*/77-33-2 ⊗-1-204	50.19 ± 1.93	33.66 ± 1.00	27.79
PH-205	F_3 *ph1b*/77-33-2 ⊗-1-205	79.13 ± 0.95	40.98 ± 0.26	60.36

Plant Name	**Pedigree**	**Mean Fe (mg/kg) ± SD**	**Mean Zn (mg/kg) ± SD**	**Harvest Index**
PH-206	F_3 *ph1b*/77-33-2 ⊗-1-206	77.32 ± 0.89	37.56 ± 0.86	31.62
PH-207	F_3 *ph1b*/77-33-2 ⊗-1-207	71.99 ± 0.65	31.32 ± 1.18	11.50
PH-208	F_3 *ph1b*/77-33-2 ⊗-1-208	80.17 ± 0.65	42.93 ± 0.66	11.50
PH-209	F_3*ph1b*/46-1-15-15-2 ⊗-10-209	62.72 ± 1.10	45.98 ± 0.84	16.29
PH-210	F_3*ph1b*/46-1-15-15-2 ⊗-10-210	65.60 ± 1.19	47.30 ± 0.94	08.62
PH-211	F_3*ph1b*/46-1-15-15-2 ⊗-10-211	55.48 ± 0.89	50.60 ± 0.66	15.33
PH-212	F_3*ph1b*/46-1-15-15-2 ⊗-10-212	58.21 ± 0.40	45.13 ± 0.45	16.71
PH-213	F_3*ph1b*/46-1-15-15-2 ⊗-10-213	60.30 ± 1.01	38.61 ± 1.20	27.67
PH-214	F_3*ph1b*/46-1-15-15-2 ⊗-10-214	64.26 ± 0.34	46.95 ± 6.14	15.33
PH-215	F_3*ph1b*/46-1-15-15-2 ⊗-10-215	52.85 ± 0.77	37.41 ± 0.55	20.12
PH-216	F_3*ph1b*/46-1-15-15-2 ⊗-10-216	76.37 ± 0.85	39.04 ± 1.28	27.79
PH-217	F_3*ph1b*/46-1-15-15-2 ⊗-10-217	62.48 ± 1.31	40.84 ± 1.59	22.04
PH-218	F_3*ph1b*/46-1-15-15-2 ⊗-10-218	61.79 ± 0.44	43.20 ± 2.24	23.95
PH-219	F_3*ph1b*/46-1-15-15-2 ⊗-10-219	66.21 ± 1.18	52.60 ± 0.67	18.62
PH-220	F_3*ph1b*/46-1-15-15-2 ⊗-10-220	60.17 ± 1.18	47.80 ± 1.01	11.50
PH-221	F_3*ph1b*/46-1-15-15-2 ⊗-10-221	91.02 ± 1.18	55.62 ± 0.46	15.75
PH-222	F_3*ph1b*/46-1-15-15-2 ⊗-10-222	95.20 ± 1.79	60.41 ± 0.53	14.79
PH-223	F_3*ph1b*/46-1-15-15-2 ⊗-10-223	55.87 ± 1.03	40.84 ± 1.25	10.54
PH-224	F_3*ph1b*/46-1-15-15-2 ⊗-10-224	53.33 ± 1.15	32.28 ± 1.11	16.71
PH-225	F_3*ph1b*/46-1-15-15-2 ⊗-10-225	62.08 ± 0.68	34.06 ± 0.44	42.16
PH-226	F_3*ph1b*/46-1-15-15-2 ⊗-10-226	67.77 ± 1.03	32.77 ± 0.62	10.54
PH-227	F_3*ph1b*/46-1-15-15-2 ⊗-10-227	66.12 ± 0.62	42.58 ± 0.91	19.16
PH-228	F_3*ph1b*/46-1-15-15-2 ⊗-10-228	49.73 ± 1.32	44.29 ± 1.05	37.37
PH-229	F_3*ph1b*/46-1-15-15-2 ⊗-10-229	74.44 ± 1.21	42.74 ± 0.78	11.50
PH-230	F_3*ph1b*/46-1-15-15-2 ⊗-10-230	61.44 ± 1.29	38.11 ± 0.73	23.00
PH-231	F_3*ph1b*/46-1-15-15-2 ⊗-10-231	34.94 ± 0.62	30.45 ± 0.88	26.71
PH-232	F_3*ph1b*/46-1-15-15-2 ⊗-10-232	54.62 ± 0.46	42.59 ± 0.43	14.37
PH-233	F_3*ph1b*/46-1-15-15-2 ⊗-10-233	57.82 ± 0.94	41.66 ± 0.90	11.50
PH-234	F_3*ph1b*/46-1-15-15-2 ⊗-10-234	41.33 ± 1.26	37.49 ± 0.68	32.58
PH-235	F_3*ph1b*/46-1-15-15-2 ⊗-10-235	63.40 ± 1.19	42.50 ± 1.41	16.77
PH-236	F_3*ph1b*/46-1-15-15-2 ⊗-10-236	62.14 ± 0.35	49.15 ± 1.04	18.20
PH-237	F_3*ph1b*/46-1-15-15-2 ⊗-10-237	66.53 ± 0.43	40.95 ± 1.84	18.20
PH-238	F_3*ph1b*/46-1-15-15-2 ⊗-10-238	74.13 ± 0.48	44.40 ± 1.31	16.29

Plant Name	Pedigree	Mean Fe (mg/kg) ± SD	Mean Zn (mg/kg) ± SD	Harvest Index
PH-239	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-239	46.97 ± 0.80	32.16 ± 2.27	20.12
PH-240	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-240	57.72 ± 1.31	46.17 ± 1.50	17.67
PH-241	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-241	69.24 ± 1.01	35.62 ± 1.33	14.37
PH-242	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-242	48.67 ± 0.56	30.32 ± 1.48	18.62
PH-243	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-243	46.65 ± 0.85	36.66 ± 1.01	19.16
PH-244	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-244	55.12 ± 0.22	42.06 ± 1.01	19.58
PH-245	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-245	59.40 ± 1.10	41.53 ± 1.18	15.75
PH-246	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-246	46.26 ± 1.05	30.77 ± 1.03	10.54
PH-247	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-247	59.62 ± 1.19	52.52 ± 1.60	24.00
PH-248	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-248	73.45 ± 1.22	42.80 ± 1.09	32.58
PH-249	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-249	78.50 ± 0.73	39.85 ± 0.95	13.41
PH-250	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-250	52.35 ± 0.88	37.75 ± 1.22	19.58
PH-251	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-251	51.67 ± 0.91	35.37 ± 1.30	11.50
PH-252	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-252	50.27 ± 0.84	41.14 ± 1.62	20.87
PH-253	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-253	66.58 ± 1.19	46.83 ± 1.01	30.66
PH-254	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-254	65.40 ± 0.93	46.92 ± 0.90	16.29
PH-255	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-255	76.74 ± 0.64	47.64 ± 2.33	07.67
PH-256	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-256	76.90 ± 1.46	51.59 ± 0.96	09.58
PH-257	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-257	76.65 ± 2.23	48.05 ± 1.71	14.37
PH-258	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-258	42.12 ± 1.60	42.79 ± 1.72	26.83
PH-259	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-259	48.10 ± 1.59	37.43 ± 0.99	36.41
PH-260	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-260	68.31 ± 0.68	60.07 ± 1.68	20.12
PH-261	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-261	28.22 ± 2.71	45.99 ± 1.68	38.33
PH-262	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-262	29.69 ± 1.51	35.73 ± 1.88	27.79
PH-263	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-263	67.07 ± 0.79	41.02 ± 1.35	23.00
PH-264	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-264	63.80 ± 0.54	47.45 ± 1.27	10.54
PH-265	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-265	51.53 ± 1.19	43.23 ± 0.57	19.58
PH-266	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-266	79.05 ± 1.53	47.15 ± 0.53	20.12
PH-267	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-267	38.70 ± 0.64	32.25 ± 2.17	29.58
PH-268	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-268	58.24 ± 1.12	35.85 ± 1.01	31.62
PH-269	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-269	80.26 ± 1.51	54.75 ± 2.43	23.00
PH-270	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-270	57.30 ± 0.53	42.82 ± 1.11	26.71
PH-271	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-271	60.63 ± 1.27	44.70 ± 0.19	22.04
PH-272	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-272	92.77 ± 1.57	60.13 ± 0.93	09.58
PH-273	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-273	66.32 ± 1.03	46.69 ± 0.77	80.48
PH-274	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-274	90.86 ± 1.96	51.62 ± 0.94	15.33
PH-275	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-275	49.23 ± 0.90	41.45 ± 1.00	11.50
PH-276	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-276	24.45 ± 1.92	25.56 ± 1.35	38.62
PH-277	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-277	70.10 ± 1.13	46.70 ± 0.77	18.20
PH-278	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-278	68.75 ± 1.80	48.24 ± 0.71	19.58
PH-279	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-279	79.88 ± 1.54	44.71 ± 1.11	22.04
PH-280	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-280	67.55 ± 0.96	38.82 ± 1.11	28.74
PH-281	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-281	39.95 ± 2.57	27.63 ± 1.41	10.54
PH-283	F$_3$*ph1b*/46-1-15-15-2 ⊗-10-283	77.45 ± 1.12	49.89 ± 0.81	19.16

PH-284	F_3*ph1b*/46-1-15-15-2 ⊗-10-284	65.28 ± 0.91	45.28 ± 0.81	20.12
PH-285	F_3*ph1b*/46-1-15-15-2 ⊗-10-285	94.61 ± 1.25	47.27 ± 0.81	32.58
PH-286	F_3*ph1b*/46-1-15-15-2 ⊗-10-286	83.50 ± 0.77	46.55 ± 0.92	15.33
PH-287	F_3*ph1b*/46-1-15-15-2 ⊗-10-287	79.21 ± 0.84	49.88 ± 0.69	33.53
PH-288	F_3*ph1b*/46-1-15-15-2 ⊗-10-288	49.71 ± 1.08	35.18 ± 1.00	23.83
PH-289	F_3*ph1b*/46-1-15-15-2 ⊗-10-289	43.57 ± 0.70	29.71 ± 0.82	17.67
PH-290	F_3*ph1b*/46-1-15-15-2 ⊗-10-290	31.08 ± 1.43	29.66 ± 0.84	30.66
PH-291	F_3*ph1b*/46-1-15-15-2 ⊗-10-291	35.29 ± 1.43	27.20 ± 1.83	11.50
PH-292	F_3*ph1b*/46-1-15-15-2 ⊗-10-292	68.93 ± 1.52	47.46 ± 1.00	18.62
PH-293	F_3*ph1b*/46-1-15-15-2 ⊗-10-293	36.61 ± 0.71	31.44 ± 0.70	23.00
PH-295	F_3*ph1b*/46-1-15-15-2 ⊗-10-295	36.43 ± 2.17	24.65 ± 1.43	13.41
PH-297	F_3*ph1b*/46-1-15-15-2 ⊗-10-297	83.78 ± 1.38	47.93 ± 1.00	35.45
PH-298	F_3*ph1b*/46-1-15-15-2 ⊗-10-298	58.88 ± 0.93	37.74 ± 1.50	36.41
PH-299	F_3*ph1b*/46-1-15-15-2 ⊗-10-299	75.31 ± 0.59	44.76 ± 1.04	08.62
PH-300	F_3*ph1b*/46-1-15-15-2 ⊗-10-300	76.07 ± 1.62	49.45 ± 1.38	13.41
PH-301	F_3*ph1b*/46-1-15-15-2 ⊗-10-301	66.81 ± 0.90	48.20 ± 0.66	29.70
PH-302	F_3*ph1b*/46-1-15-15-2 ⊗-10-302	60.56 ± 0.65	54.49 ± 1.23	36.41
Plant Name	**Pedigree**	**Mean Fe (mg/kg) ± SD**	**Mean Zn (mg/kg) ± SD**	**Harvest Index**
PH-303	F_3*ph1b*/46-1-15-15-2 ⊗-10-303	78.47 ± 1.01	51.10 ± 0.67	18.20
PH-304	F_3*ph1b*/46-1-15-15-2 ⊗-10-304	56.43 ± 0.86	43.64 ± 1.24	17.25
PH-305	F_3 *ph1b*/77-36-6 ⊗-34-305	82.33 ± 1.45	43.60 ± 1.10	28.74
PH-306	F_3 *ph1b*/77-36-6 ⊗-34-306	56.09 ± 0.70	31.84 ± 1.52	19.58
PH-307	F_3 *ph1b*/77-36-6 ⊗-34-307	59.12 ± 1.21	30.60 ± 2.35	18.20
PH-308	F_3 *ph1b*/77-36-6 ⊗-34-308	62.43 ± 0.57	36.44 ± 1.85	12.46
PH-309	F_3 *ph1b*/77-36-6 ⊗-34-309	33.09 ± 1.29	26.35 ± 2.58	23.95
PH-311	F_3 *ph1b*/77-36-6 ⊗-34-311	61.59 ± 1.21	43.42 ± 0.68	11.50
PH-312	F_3 *ph1b*/77-36-6 ⊗-34-312	63.86 ± 1.03	35.90 ± 0.83	15.33
PH-313	F_3 *ph1b*/77-36-6 ⊗-34-313	67.47 ± 0.61	39.97 ± 0.94	10.54
PH-314	F_3 *ph1b*/77-36-6 ⊗-34-314	59.66 ± 0.75	35.92 ± 0.85	19.16
PH-315	F_3 *ph1b*/77-36-6 ⊗-34-315	61.90 ± 1.56	38.90 ± 0.30	21.08
PH-316	F_3 *ph1b*/77-36-6 ⊗-34-316	38.55 ± 0.85	37.81 ± 0.65	19.16
PH-317	F_3 *ph1b*/77-36-6 ⊗-34-317	37.24 ± 1.57	32.83 ± 2.40	20.12
PH-318	F_3 *ph1b*/77-36-6 ⊗-34-318	71.56 ± 1.16	43.59 ± 0.80	32.58
PH-319	F_3 *ph1b*/77-36-6 ⊗-34-319	37.01 ± 0.86	39.25 ± 0.97	11.50
PH-320	F_3 *ph1b*/77-36-6 ⊗-34-320	71.57 ± 1.28	41.50 ± 1.39	21.50
PH-321	F_3 *ph1b*/77-36-6 ⊗-34-321	59.18 ± 1.06	33.23 ± 0.33	11.80
PH-322	F_3 *ph1b*/77-36-6 ⊗-34-322	57.67 ± 1.29	43.32 ± 0.74	11.97
PH-323	F_3 *ph1b*/77-36-6 ⊗-34-323	49.62 ± 1.30	39.97 ± 0.24	19.16
PH-324	F_3 *ph1b*/77-36-6 ⊗-34-324	49.35 ± 0.98	41.90 ± 2.16	21.00
PH-325	F_3 *ph1b*/77-36-6 ⊗-34-325	36.51 ± 0.87	26.33 ± 1.14	46.95
PH-326	F_3 *ph1b*/77-36-6 ⊗-34-326	61.39 ± 0.92	70.22 ± 2.05	34.49
PH-327	F_3 *ph1b*/77-36-6 ⊗-34-327	58.31 ± 1.70	36.45 ± 0.98	12.46
PH-328	F_3 *ph1b*/77-36-6 ⊗-34-328	49.33 ± 1.64	42.15 ± 0.41	28.74
PH-329	F_3 *ph1b*/77-36-6 ⊗-34-329	59.23 ± 1.53	40.54 ± 0.66	24.91
PH-331	F_3 *ph1b*/77-36-6 ⊗-34-331	43.36 ± 1.11	30.39 ± 1.17	12.46