MOLECULAR CHARACTERIZATION AND PROTEOMICS OF PREHARVEST SPROUTING TOLERANCE IN WHEAT

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **MOLECULAR CHARACTERIZATION AND PROTEOMICS OF PREHARVEST SPROUTING TOLERANCE IN WHEAT** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2007 to July 2011 under the supervision of Dr. R. Prasad, Associate Professor and Dr. G. S. Randhawa, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

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

(NAINCY GIRDHARWAL)

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

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Abstract

ABSTRACT

Wheat (*Triticum aestivum* L.) is a staple food crop for India and the world. Around the world wheat is cultivated in EU-25, China, India, America, Russia, Australia, Canada, Pakistan, Turkey and Argentina. Pre harvest sprouting (PHS) is a worldwide problem and is greater in white kernelled wheat than red kernelled wheat. PHS is a condition when physiologically mature grain sprouts while still in the ear under moist and wet conditions. In damaged food grains α-amylase activity is upregulated which damages wheat grains. Hence, seed viability is lost and milled flour has inferior baking properties. Therefore, sprouted wheat looses market value and is rendered unfit for human consumption. Taking above problems into account the development of PHS tolerant lines is a long term solution to PHS as these cultivars will be able to tolerate damaging effects of rain.

To address the problem of PHS the present study was undertaken using near isogenic lines (NILs) derived from a cross between amber and PHS susceptible cultivar PBW343 and red and PHS tolerant cultivar SPR8198. The NILs were developed at PAU Ludhiana. The two NILs used in the study were PHST9 (amber coloured and PHS susceptible) and PHST0 (red coloured and PHS tolerant). Using PHS test it was determined that NIL PHST0 did not germinate under moist and wet conditions till ten days. Sprouting tolerance in wheat is lost between two months after harvesting. Dormancy is dissipated by after ripening in dry storage at room temperature in a similar manner for the red and white lines. We preserved PHS tolerance after harvesting by storing seeds of NIL PHST0 at -86°C. It was interesting to observe that the (preharvest sprouting tolerance) PHST in NIL PHST0 was preserved for around two years. The preserved seeds of PHST0 were dormant and did not germinate till ten days after imbibition. To the best of my knowledge this is the first report of prolonged maintenance of PHST under deep freezer storage conditions. It is important to mention here, that the plant material stored at -86°C was used for phytohormone analysis in our study.

The effect of exogenous application of growth regulators, abscisic acid (ABA) and gibberellic acid (GA) on PBW343 and two NILs was tested at different phytohormone concentrations. This test with growth regulators demonstrated that the susceptible parent cultivar PBW343 and NIL PHST9 were ABA insensitive while tolerant NIL PHST0 was ABA sensitive. Taking seed morphology into account seed anatomy was studied for the three experimental lines using scanning electron microscopy (SEM) which demonstrated that in PHST0 three layers i.e. epidermis, aleurone layer and intermediate layer between epidermis and aleurone were thicker than PBW343 and PHST9. These differences in the thickness of the three layers might add to seed dormancy. Moreover thousand grain weight of PHST0 was also greater than PBW343 and PHST9.

Other than the above mentioned genes and transcription factors quantitative trait loci (QTL) studies have also been employed to localize agronomically important genes like PHS on wheat chromosomes. QTLs involved in PHS tolerance or seed dormancy have been mapped on 20 out of 21 chromosomes in wheat. The QTLs other than those on 3AL and 4AL are minor QTLs affecting PHS. QTL *QPhs.ccsu-3A.1*, on chromosome 3AL is the major QTL explaining 78% phenotypic variation. This major QTL was flanked by SSR markers *Xwmc153* and *Xgwm155*. In addition to 3AL QTL flanking markers we also used seven SSR markers lying between Xwmc153 and Xgwm155 on wheat genetic map. Similarly dormancy in white grained wheat has been linked to chromosome 4AL as indicated by QTL studies. The 4AL QTL is flanked by *Xbarc170*, *Xgwm269* and *Xgwm397*. The marker zxq118 has been mapped between the flanking markers reported for 4AL. In present study we tried to validate 3AL and 4AL QTL flanking markers. The lines used for marker validation included ten selected IIT Roorkee (IITR) landraces in addition to PBW343, NILs PHST0 and PHST9. In our study we could not validate chromosome 3AL QTL flanking markers wmc153 and gwm155 but chromosome 4AL QTL flanking markers *Xgwm269* and *Xzxq118* were validated in our plant material. In our

study gwm269, differentiated between amber tolerant and amber susceptible lines and can therefore be used for marker assisted breeding.

The main aim of the present study was proteome analysis to identify a broad spectrum of proteins that are expressed in wheat during PHS. For proteome study two dimensional electrophoresis (2-DE) was coupled with mass spectrometric (MS) tool, LC-ESI-MS/MS and the information generated was utilized for protein identification. 2-DE was done for endosperm, mature embryo and immature embryo proteins. For proteome analysis seed material was collected and stored at -80°C at two developmental stages i.e stage87 (hard dough) and stage95 (dry caryopses). From endosperm LC-ESI-MS/MS was done for three proteins and from embryo LC-ESI-MS/MS was done for four proteins. The endosperm proteins designated as PH-ENDO-1, PH-ENDO-2 and PH-ENDO-3 were identified as LMW proteins for spot 1 and 2 while protein disulfide isomerise (PDI) for spot3. PDI is responsible for protein folding and on consultation of literature it was found to have an alternate function as well. The enzyme is also responsible for production of hydrogen peroxide via oxalate oxidase during stress/ pathogen attack. This is important in our context as the protein spot for PDI was identified in tolerant NIL PHST0. Therefore PDI was further used for transcriptomic studies using gene specific primers.

For mature embryo LC-ESI-MS/MS was done for four protein spots which were designated as PH-EMB-3, PH-EMB-5, PH-EMB-6 and PH-EMB-7. PH-EMB-3 was identified as an unknown protein from gymnosperm, PH-EMB-5 was identified as serine carboxypeptidase, PH-EMB-6 was identified as unknown protein Os01g0749000 from *Oryza sativa* and PH-EMB-7 was identified as avenin like precursor from *T. aestivum*.

The results of LC-ESI-MS/MS for the two protein spots i.e PH-EMB-3 and PH-EMB-6 were further analyzed using blastp. The peptide for PH-EMB-3 blasted with *Oryza sativa* sequences and the sequences showing 100% coverage were selected for further study. Hence, nine *Oryza* sequences were selected for the study. The mRNA sequences of these nine selected protein

sequences were further blasted with wheat mapped ESTs on graingenes and three mRNA sequences blasted on group 3 chromosomes of wheat. The second protein spot i.e PH-EMB-6 from immature embryo proteins 2-DE was analyzed using Blastp. Two peptides were identified for PH-EMB-6 i.e. Os01g0749000 and Os10g0125700 were unknown proteins. Os01g0749000 belongs to DUF1264 superfamily and is an embryo specific protein. Upon Blastp of Os01g0749000 a wide range of hypothetical proteins from *oryza* were identified, while for Os10g0125700, which is blast resistance protein in *oryza*, blastp identified *RGA-1*(repressor of gibberellic acid-1) like protein from *T.aestivum* along with hypothetical proteins from *oryza* and sorghum. For the same spot DNA binding / transcription factor from *Arabidopsis thaliana* was identified with a low probability which was identified as dehydration response element binding protein (DREB). Therefore for same protein spot taken from 2-DE gel of PHST0, two physiologically important proteins i.e. RGA-1 and DREB were identified. Here it can be said that the above study has been helpful as with the help of NIL PHST0 we could identify two important genes.

For transcriptome study gene specific primers were designed for myb10, dihydro flavonoid-4-reductase (DFR) and PDI (protein disulfide isomerase-enzyme responsible for protein folding). The plant material used for the analysis included parent PBW343 and NILs PHST9 and PHST0. Differences were observed among the three lines for PDI and myb10 but in case of DFR expression was observed only in PHST0. The DFR transcription factor is present on chromosome3A which is consistent with the observation of major PHS gene on chromosome 3AL as indicated by QTL study. These results suggest that PHS/ dormancy gene might be present on chromosome 3A.

Chromosome bin maps of wheat are available for all the 21 chromosomes. With the help of chromosome bin mapping genes can be assigned to specific bins based on expressed sequence tags (ESTs). We also employed chromosome bin mapping for chromosome 3AL for markers wmc153, gwm155, zxq118 and gwm269 to identify the chromosome bin carrying the

PHS/dormancy gene. The same was also done for proteins. The results from both markers and proteins were same as in both the case two bins 3AL3-0.42-0.78 and 3AL5-0.78-1.00 were identified. In our study, bin mapping was done with an objective to identify either of the two above mentioned bins, but we could not narrow down on either bin as both had almost equal number of ESTs. Hence we would propose that the gene for PHST might be present in either of the bins, 3AL3-0.42-0.78 or 3AL5-0.78-1.00, as indicated both by markers and proteins. This bin identification also raises the possibility of the gene being present in distal region of chromosome 3AL.

Therefore, with above study it can also be said that dormancy in wheat might exist both on chromosome 3AL and 4AL and extensive studies are required at the proteome level for identification of the dormancy/PHST gene.

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NAINCY GIRDHARWAL

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Centigrade
µg	Micrgram
µM	Micro Molar
2-DE	Two-Dimensional Gel Electrophoresis
ABA	Abscisic Acid
ABA8'OH1	ABA 8'hydroxylase
ABI	ABA-Insensitive
AGL	AGAMOUS-LIKE
APS	Ammonium per sulfate
BAC	Bacterial Artificial Chromosome
bp	Basepair
BR	Brassinosteroid
BRI	Brassinosteroid Insensitive
BRS	Brassinosteroid Supressor
BSA	Bovine Serum Albumin
CBB	Coommassie Brilliant Blue
CBF	CRT binding factor
cDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl) dimethlammonio]-1-propane sulfonate
CHI	Chalcone Isomerase
CHS	Chalcone Synthase
cM	Centi-Morgan
CTAB	Cetyl-trimethyl ammonium bromide
DAP	Days After Pollination
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
DDT	Di thio trietol
DEPC	Diethylpyrocarbonate
DFR	Dihydro-flavonol-4-reductase
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribo Nucleic Acid
DNS	Dinitrosalicylic Acid
dNTP	Deoxyribonucleotide triphosphate
DOG1	Delay of Germination 1
DPA	Dihydro Phaseic Acid
DREB	Dehydration Responsive Element Binding Protein
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
EBR	24-epibrassinolide
ECD	Electron Capture Dissociation
EDTA	Ethylenediaminetetra acetic acid
EKO	ent-Kaurene 19-oxidase
ESI	Electrospray ionization
EST	Expressed Sequence Tags

ETD	Electron Transfer Dissociation
F3H	Favanone 3-hydroxylase
FAA	Formaldehyde-acetic acid-alcohol
FAB	Fast Atom Bombardment
FCI	Food Corporation of India
FRY1	Inositol polyphosphate 1-phosphatase
g	Gram
GA20ox	GA 20-oxidase
GA2ox	GA 2-oxidase
GA_3	Gibberellic acid
GA3ox	GA3-hydroxylase
GA7ox	GA12-aldehyde 7-oxidase
GAI	GA-Insensitive
GER	Germin
GFP	Green Fluorescence Protein
H_2O_2	Hydrogen peroxide
ha	Hectare
HCl	Hydrochloric Acid
HCOOH	Formic acid
HMW glutenin subunit	High molecular Weight Glutenin Subunits
HPLC	High Performance Liquid Chromatography
hsp	Heat-Shock Protein
IEF	Isoelectric Focusing
IPG	Immobilized pH gradient
IRGSP	International Rice Genome Sequencing Project
kD	KiloDalton
kg	Kilogram
KS	ent-Kaurene synthase
l	Liter
LC	Liquid Chromatography
LEA	Late Embryogenesis Abundant
LiCl	Lithium chloride
LMW- glutenin subunit	Low Molecular Weight Glutenin Subunit
m	Meter
M	Molar
MALDI	Matrix-Assisted Laser Desorption/Ionization
mg	Milligram
ml	Milliliter
mM	MilliMolar
MS	Mass Spectrometry
NaN_3	Sodium azide
NCBI	National Centre for Biotechnology Information
NCED	9-*cis*-epoxycarotenoid dioxygenase
NDB	Nucleic Acids Database
ng	Nanogram
NIL	Near Isogenic Lines
nm	Nanometer
nM	NanoMolar

PA	Phaseic Acid
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PDF	Portable document format
PDI	Protein Disulfide Isomerase
PHOR	Photoperiod Responsive
PHS	Preharvest Sprouting
PHSS	Preharvest Sprouting Susceptible
PHST	Preharvest Sprouting Tolerant
PMF	Peptide Mass Fingerprinting
ppm	parts per million
PSD	Post-Source Decay
PVDF	Polyvinylidine fluoride
QTL	Quantitative Trait Loci
R gene	Red Colour Gene
Rc gene	Gene for Coleoptiles Colour
RFLP	Restriction Fragment Length Polymorphism
RGA	Repressor of Gibberallic Acid1-3
RGL1	RGA-like 1
RT-PCR	Reverse Transcriptase PCR
SCP	Serine Carboxypeptiase
SDS	Sodium do-decyl sulphate
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
SSP	Sample Spot Protein
SSR	Simple Sequence Repeats
TCGA	The Centre for Genomic Application
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethlenediamine
TFA	Trifluoroacetic acid
TGS	Tris-glycine-SDS
TIGR	The Institute for Genomic Research
TOF	Time of flight
URL	Uniform resource locator
Vp1	Viviparous-1
α	Alpha

Chapter I
Introduction

Wheat (*Triticum aestivum* L.) is the staple food for one third of the world's population. In India only spring wheat is grown, which is raised in winter. Total area under wheat cultivation in India is 266.92 lakh ha and crop production of wheat in India is approximately 721.40 lakh tonnes per year (Food Corporation of India, 2010). Around the world wheat cultivation is estimated to be 215.26 million ha with 584.76 million tonnes of production per annum. PHS in cereal crops such as wheat leads to major crop losses every year. In north India alone approximately 35.5 million tonnes of wheat had rotten because of unprecedented rainfall in year 2010-11 according to FCI.

PHS is one of the major causes associated with wheat productivity losses in India and the world. The phenomenon of germination of physiologically mature cereal grains in the ear or panicle, usually under wet conditions before harvest, is termed as PHS. PHS occurs in cereal crops such as wheat, barley, maize, and rice in many parts of the world. PHS results in lower yields due to decreased test weight and limits end-use applications of wheat due to deleterious grain quality. The extent of PHS depends on the duration and severity of moist conditions, the stage of ripening of the grain, and the inherent level of dormancy of the variety. Sprouting begins as the kernels absorb moisture and swell, which activates a number of enzymes that breakdown starch, proteins, and other constituents during respiration and growth. α-amylase enzyme activity is closely associated with PHS, as it causes breakdown of starch, and so is often used as a measure of sprouting damage. PHS tolerance is a complex trait and therefore genes, transcription factors and phytohormones are associated with this phenomenon. Tolerance to PHS depends on various factors like water uptake, drying rate of the grain, grain dormancy, velocity of after ripening and the mobilization of nutrients to support germination. These factors are controlled by a number of genes that strongly interact with environmental conditions.

Seed dormancy has often been associated with grain colour in wheat, with red seed coloured wheat being PHS tolerant (PHST). Red seed colour occurs due to polyphenol pigment

phlobaphene which is synthesized via flavonoid biosynthetic pathway (Miyamato and Everson, 1958). *R* genes responsible for red grain colour are involved in activation of early flavonoid biosynthesis genes. All the major genes responsible for PHST like R genes, dihydro-flavonol-4-reductase (DFR) genes, myb10 transcription factor are present on homeologous group 3 chromosomes of wheat. Dormancy QTLs have been mapped on 20 chromosomes of wheat but chromosome 3AL carries the major QTL which explains 78% phenotypic variation (Kulwal *et al.*, 2005). *T. aestivum* viviparous 1 (*taVp1*) gene, an orthologue of *Vp1*, has also been located in a region 30 cM from *R* locus on chromosome 3AL. In addition the major QTL, QPhs.ccsu-3A.1, of PHST has also been mapped on chromosome 3AL of wheat (Kulwal *et al.*, 2005). Major dormancy QTLs associated with white wheat has been mapped on chromosome 4AL in wheat (Mares *et al.*, 2005). QTL studies have shown that gene(s) for ABA sensitivity are located in the middle region of chromosome arm 4AL (Noda *et al.*, 2002). Therefore, most of the studies conducted to unravel the phenomenon of dormancy in wheat have largely relied upon studies of ABA biosynthetic genes using mutants in *Arabidopsis*, viviparous-1 (Vp1) mutants in maize, delay of germination 1 (*DOG1*) mutants in wheat (Rikiishi and Maekawa, 2010) and QTL studies. Hence, in hexaploid bread wheat (*Triticum aestivum*) both mutant (Kawakami *et al.*, 1997) and QTL-mapping approaches (Noda *et al.*, 2002) have shown that ABA sensitivity is important for the acquisition of dormancy.

Abscisic acid (ABA) is the major hormone involved in induction and maintenance of dormancy but the mechanism of its action has not been fully understood (Wang *et al.*, 1994). The presence of ABA is required for the maintenance of dormancy following imbibition and analysis of *Arabidopsis* mutants defective in ABA catabolism have indicated that the removal of active ABA by ABA 8'-hydroxylase (CYP707A1, CYP707A2) is required for the loss of dormancy (Kushiro *et al.*, 2004, Okamoto *et al.*, 2006). 8'-hydroxy ABA is spontaneously isomerized to form phaseic acid (PA) which is reduced to dihydro phaseic acid (DPA). The catabolites 8'-hydroxy ABA, PA and 7'-hydroxy ABA all have some hormonal activity and

might play roles in mediating ABA-like effects (Hill *et al.*, 1995). Members of *AtNCED* (9-*cis*-epoxycarotenoid dioxygenase) family (*NCED6* and *NCED9*) have been identified to be essential for ABA synthesis in both the embryo and endosperm during dormancy induction. Expression of *AtNCED6* and *AtNCED9* was observed in the endosperm and embryo respectively. This demonstrates that ABA, synthesized both in endosperm and embryo participates in the hormonal balance that controls seed dormancy and germination (Lefebvre *et al.*, 2006).

Transcriptomic studies have also been done using gene specific primers for either flavonoid synthesis genes (Himi *et al.*, 2005a) or myb10 transcription factor (Himi and Noda, 2005b). Himi and Noda (2005b) identified three Myb-type transcription factors (*Tamyb10-A1* on 3A, *Tamyb10-B1* on 3B and *Tamyb10-D1*on 3D), located in the same region as the *R* loci (red colour gene loci), expressing predominantly in developing grains. Single R gene is able to up regulate the four genes in the flavonoid biosynthesis pathway, suggesting that the R gene might be a transcription factor. In the promoter region of DFR, three myb binding elements are found which might play an important role in upregulation of DFR. This further suggests that flavonoid genes control grain colour through activation of flavonoid biosynthetic genes. Though R genes are considered important in dormancy but these are not absolutely essential and dormancy might be controlled by genes tightly linked to R gene.

Proteomic studies have been conducted in wheat to compare and identify proteins using MS techniques. Such studies have been done for analysis of endosperm proteins (Vensel *et al.*, 2005), wheat germ proteins (Mak *et al.*, 2006), metabolic proteins of durum wheat (Laino *et al.*, 2010), proteome analysis of peripheral layer during grain development (Tasleem-Tahir *et al.*, 2011), comparison of biotic stress related proteins (Kamal *et al.*, 2010), qualitative and quantitative evolution of proteins was analysed during early stages of grain development (Naduad *et al.*, 2010) etc. Other than the studies mentioned above, little has been done to study the proteome differences between the PHS tolerant and susceptible varieties in wheat.

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

- To study near isogenic lines (NILs) of bread wheat and IITR landraces for preharvest sprouting (PHS)
- To validate QTLs on chromosome 3AL and 4AL for PHS tolerance in NILs and IITR landraces using linked SSR markers
- To study comparative proteome of NIL PHST0 (PHS tolerant) and PBW343 (PHS susceptible)

Chapter II
Review of Literature

CHAPTER-2

2.1 Preharvest sprouting

The phenomenon of germination of physiologically mature cereal grains in ear or panicle, under wet conditions shortly before harvest is termed as PHS (Groos *et al.*, 2002). PHS occurs in cereal crops like wheat, barley, maize and rice in most parts of the world. PHS in wheat results in loss of grain yield. The main effects of PHS are low yield due to harvest losses and a reduction in the end product quality. Tolerance to sprouting damage is affected by water uptake and drying rate of the ear, grain dormancy and the mobilisation of storage reserves to support germination. These processes are regulated by a number of key tolerance genes that interact strongly with environmental conditions. A major component of the observed genetic variation in sprouting of grains within the ear is the degree of embryo dormancy at grain harvest. Embryo dormancy can be influenced by environmental conditions during grain development and the stage of grain maturity. Therefore, environmental conditions both before and after seed maturity influence the rate of PHS. In general, more dormant grains are produced under cool growing conditions whereas high temperature during the later stages of grain development breaks embryo dormancy allowing grains to germinate in the ear if rain occurs around harvest time. Other factors that contribute to increased tolerance to PHS are reduced levels of α-amylase activity in the grain, presence of inhibitors of germination, reduced water absorption by the grains and altered responses to hormones. Tolerance to PHS is a highly desirable trait sought by plant breeders as PHS tolerance and grain dormancy are complex traits affected by different environmental factors (Trethowan *et al.*, 1996; Johansson, 2002) and controlled by several genes and QTL (Bailey *et al.*, 1999; Mares *et al.*, 2005; Yang *et al.*, 2007a). PHS has a high economic cost for both growers and the end-users. As the seed germinates starch and protein are degraded, reducing the quality of seed and commercial demand. Flour from the degraded grains will produce products that are porous, sticky, off-color and generally of poor quality. If the produce has over four percent damaged kernels then it is unacceptable for human

consumption and severely sprouted grains are often used for animal feed, reducing the price by 20% to 50%. Considerable damage occurs due to PHS in wheat every year. In 2010-11 in India 35.5 million tonnes of wheat was damaged amounting to losses of 60,000 crores. The wheat crop grown in the north eastern and far-eastern states of India (West Bengal, Assam, and other eastern hill states) has also been reported to be prone to PHS losses due to pre-monsoon rains and high humidity around maturity. The development of PHS tolerant wheat cultivars has been difficult through conventional breeding as PHS tolerance is a quantitative trait influenced by many genes. As PHS in wheat poses a major problem of reducing grain yield and quality, PHS tolerant wheat varieties need to be developed.

2.1.1 Measurement of PHS

Preharvest sprouting can be quantified using a falling number determination. The Hagberg falling number indicates the level of α-amylase activity in a wheat flour/water suspension which has been rapidly heated to gelatenize the starch (Hagberg, 1960, 1961). Falling-number tests are conducted by creating a slurry of flour and water in a test tube. The time required for a plunger to fall through the slurry is measured. Sprouted grain has lower falling number because the active α-amylase enzymes degrade starch, which liquefies the gelatinous suspension. Hence, low falling number is associated with poor end-use quality. Loss in functional baking quality due to PHS may include reduction in test weight, low milling yield, low absorption, reduced dough strength and loaf volume and poor crumb structure (Derera, 1989). Sound, starchy grains produce thick slurry (and a bigger falling number). Sprout-damaged grain will result in a flour and water mixture that is thinner. In general, falling-number values less than 250 seconds to 300 seconds indicate flours that are of poor quality for milling and baking purposes. Wheat that does not meet the minimum falling-number standard is unsuitable for milling. Since flour quality wheat generally receives a significant premium over feed wheat, low falling-number wheat represents a loss to the producer.

Other methods of measuring PHS tolerance include determination of α-amylase activity and use of germination test. α-amylase causes breakdown of starch in the endosperm which is necessary for seed germination. Hence, measurement of the enzyme activity can help in the determination of PHS tolerance in susceptible and dormant seeds. For determination of α-amylase activity either Iodine or dinitrosalicylic acid (DNS) method can be used. In DNS method, amount of reducing sugars is measured and glucoamylase activity is measured at 540nm (Miller *et al.,* 1959). Iodine method is more specific and is based on colour development that results from iodine binding to starch and the enzyme activity is measured at 620nm. Dextrinizing activit is measured as $D(D_0\text{-}D)\div D_0 \times 100\div 10$, where D is the absorbance of the enzyme sample and D_0 is the absorbance of the amylose control without the addition of enzyme (Fuwa, 1954).

The germination test has been reported to be more reliable for PHS tolerance measurement then falling number and α-amylase activity measurement. The reason being that seed dormancy provides tolerance to PHS while α-amylase activity can occur without causing PHS also (Singh *et al.,* 2008). In addition to above mentioned tests, standard laboratory germination test includes immersion of freshly harvested spikes in water for 4-6 hours, followed by transfer of spikes to moist 7.5-cm-thick sand bed covered with a double layer of moist jute bags. The spikes are sprinkled with water every 3–4 h to prevent drying and subsequent data recording (Kulwal *et al.*, 2005).

2.1.2 Pre harvest sprouting and seed colour

White/amber kernel wheat cultivars are more susceptible to PHS than the red kernel wheat (Lawson *et al.*, 1997). Seed dormancy has been associated with the grain colour in cultivated wheat, with red seed coloured varieties more resistant to PHS than white coloured ones. It is proposed that the association is either the pleiotropic effect of the red colour genes (R1 to R3) on dormancy or the dormancy genes are tightly linked to the R loci. So far, it has not been possible to use the dormancy genes in the R gene regions to improve white grain-coloured

wheat varieties for resistance to PHS. R genes play a role in dormancy but it is not clear whether R gene itself or some genes linked to R locus affect dormancy. R genes have been mapped on the distal regions of long arm of group 3 chromosomes. Therefore, R gene is not solely responsible for PHS tolerance but might affect the sensitivity of embryo to ABA (Himi *et al.,* 2002). A study to elucidate the same was conducted by Flintham (2000) and it was concluded that R gene on chromosome 3 enhances level of grain dormancy. In addition R genes have also been suggested to accumulate germination inhibitors in seed coat like catechin and catechin-tannins (Miyamato and Everson, 1958). Stoy and Sundin (1976) reported that catechins and catechin-tannin precursors also inhibit embryo germination. Wheat bran has been reported to contain water soluble extract equivalent to 1-10μM ABA which inhibits embryo germination (Himi *et al.*, 2002). In another study white grained and red grained dormant wheat were compared and it was reported that under wet and humid conditions the red seed coat is essential for the maintenance of a suitable level of seed dormancy but under dryer conditions white wheat dormancy is adequate. It was also suggested that the effect of seed coat colour on seed dormancy is variable according to environment during seed development (Torada and Amano, 2002).

2.2 Dormancy and role of ABA

Grain dormancy is an important mechanism that enables the grain to remain quiescent until the conditions become favourable for germination. Seed dormancy and germination are regulated by a wide range of hormones. Several studies have shown that ethylene, gibberellic acid and brassinosteroids promote the germination of dormant seeds. The plant hormone abscisic acid (ABA) is ubiquitous for many crucial aspects of plant growth and development (Hetherington *et al.*, 1991). Physiological studies on seed dormancy in cereals has shown that the phytohormone ABA plays a great role in embryo dormancy to PHS (Koornneef *et al.*, 1984, McCarty, 1995, Kawakami *et al.*, 1997). ABA, mutant screens in *Arabidopsis* have relied on the inhibitory effect of exogenously applied ABA on seed germination and

seedling growth. Using these screens approximately ten ABI (ABA-insensitive) loci, several of which have not yet been described in detail among higher plants, where it participates in the control of many crucial aspects of plant growth and development (Koornneef *et al.*, 1984, Finkelstein *et al.*, 1994, Finkelstein and Somerville, 1990, Nambara *et al.*, 1992). The list of ABA biosynthetic mutants has been given in Table 2.1. It is well known that the plant hormone, abscisic acid (ABA), is involved in the regulation of embryo dormancy. In wheat, no increase in ABA content is associated with the induction of dormancy but there is evidence that changes in ABA responsiveness are related to the maintenance of dormancy (Kawakami *et al.*, 1997).

It has been reported that in general wheat embryos maintain 3-4μM ABA level 40 days after pollination (DAP), which is sufficient to maintain dormancy (Walker-Simmons, 1987). However, the ABA level decrease upon imbibitions. The endogenous ABA level in non-dormant embryos in wheat and barley seeds dropped to 0.45μM and 0.1μM upon imbibitions (Ried and Walker-Simmons, 1990 and Wang *et al.* (1994). The same has also been observed in Kitakei (wheat) embryos in which endogenous ABA levels dropped to 0.1μM from 5μM within 24 hours of imbibitions (Kawakami *et al.*, 1997). It was proposed that dormant embryos had the ability to resynthesize ABA during imbibitions (Ried and Walker-Simmons, 1990 and Wang *et al.*, 1995). In another study, six wheat ABA-responsive mutants (W*arm*) i.e Warm1-6 have been isolated in Chinese Spring background. Embryos of the W*arm1* and W*arm4* (Wheat ABA-responsive mutant) lines were reported to show strong increase in embryo sensitivity to ABA inhibition of germination. This sensitivity was associated with their tendency to after-ripen more slowly than wild-type Chinese Spring (Schramm *et al.*, 2010). In barley, after-ripened grains show lower expression of ABA biosynthetic gene *HvNCED1* and increased expression of the ABA catabolic gene *HvABA8'OH1* (ABA 8'hydroxylase) (Millar *et al.*, 2006). If this mechanism is conserved in wheat, then ABA 8'hydroxylase activity in after-

Table 2.1: A list of mutants defective in ABA synthesis in plants

Species	Mutation/ accession no.	ABA Phenotype	locus	Allele/Ortholog	References
Arabidopsis thaliana	aba1	ABA-deficient	*los 6*	Zeanthin epoxidase	Koornneef *et al.* (1982), Xiong *et al.* (2002)
	aba2	ABA-deficient	*gin1* *sis4* *sis5*	Xanthoxinoxidase short chain dehydrogenase/ reductase	Leon-kloosterziel *et al.* (1996), Laby *et al.* (2000), Rook *et al* .(2001)
	aba3	ABA- deficient	*frs1* *los1*	Aldehyde oxidase Molybdenum Cofactor sulfurase	Leon-kloosterziel *et al.* (1996), Llorente *et al.* (2000), Rook *et al.* (2001), Xiong *et al.* (2001)
	abi 1-1 AT4G26080	ABA resistant (Pleiotropic)		Protein Phosphatase 2C	Koornneef *et al.* (1984), Meyer *et al.* (1994), Moes *et al.* (2008)
	abi 2-1 AT5G57050			-	Kormneef *et al.* (1984)
	abi 3 AT3G24650	ABA resistant ABA resistant ABA resistant	Cereal *Vp1*	B3 domain transcription factor	Kormneef *et al.* (1984), Giraudat *et al.* (1992)
	abi 4 AT2G40220		*gin 6,* *isi 3,* *san5,* *sis 5,* *sun 6*	APTELA2 domain transcription factor	Finkelstein (1994), Finkelstein *et al.* (1998), Dekkers *et al.* (2008)
	abi 5 At2G36270		*At DPBF1* *gia 1*	bZIP domain transcription factor	Finkelstein (1994), Finkelstein and Lynch (2000), Garcia *et al.* (2008)
	Myb 44 AT5G67300	Drought/Salt sensitivity ABA induced		Myb transcription factor	Huang *et al.* (2007), Jung *et al.* (2008)
	Hos 10 ATIG35515	Deregu;ated reporter expression	*MYB 8*	R2R3-MYB transcription factor	Zhu *et al.* (2005)
	Ost 1 AT4G33950	Open stomata	*SNRK2-6* *Vicia faba AAPK*	Calcium dependent ABA activated protein kinase	Li and Assmann 1996, Mustilli *et al.* (2002)
	Ib 5 AT2G04550	Indole-3-Butyric Acid responses, ABA insensitive		Tyr/Ser/Thr protein phosphatase	Strader *et al.* (2008)
Triticum aestivum	ABA 27	ABA hypersensitive		-	Kobayashi *et al.* (2008)
Hordeum vulgare	Cool	ABA insensitivity in guard cells		-	Raskin and Ladymann (1988)
Zea mays	Vp I	Viviparous seeds	ABA insensitive	AtAB13/B4 domain transcription factor	Robertson (1955), McCarty *et al.* (1991)
	Vp 14	Viviparous, ABA deficient	*AtNCEB3* *AT3G14440*	9 cis-neo xanthin epoxycarotenoid dioxygenase	Neill *et al.* (1986), Schwartz *et al.* (1997)
	Vp 8	Viviparous, altered anthocyanin and aleurone cell differentiation	*AtAMP1* *AT3G54720*	Peptidase	Suzuki *et al.* (2008a)
	Rea	ABA resistant germination occasional vivipary red embryos		-	Sturaro *et al.* (1996)
Oryza sativa	Phs	Pre-harvest sprouting ABA deficient		Phytoene desaturase, Zea carotene desaturase, carotenoid isomerase, lycopene beta cyclise	Fang *et al.* (2008)

(Source: Pareek *et al.*, 2010)

ripened wheat may rapidly degrade applied ABA leading to apparent insensitivity to exogenous ABA. Alternatively, after-ripening may also alter ABA sensitivity directly through changes in signalling gene expression (Schramm *et al.,* 2010).

In a study conducted, optically active forms of abscisic acid and their oxygenated metabolites were tested for their activity and reversal of gibberellic acid induced α-amylase activity in barley aleurone layers (Jacobsen *et al.*, 2002). The induction of gene expression in barley aleurone protoplasts transformed with a chimeric construct containing the promoter region of an albumin storage protein gene was also studied. The two experimental systems gave different results and it was found that promotion of albumin storage protein gene response had a stricter stereochemical requirement for elicitation of an ABA response than inhibition of α-amylase gene expression. In addition it was also demonstrated that ABA showed highest activity followed by 7'-hydroxyABA while phaseic acid was the least active (Hill *et al.*, 1995). Racemic 8'-hydroxy-2',3'-dihydroABA, an analog of 8'-hydroxyABA, was inactive, whereas racemic 2',3'- dihydroABA was as effective as ABA. These differences in response of the same tissue to the ABA enantiomers leads to the conclusion that there exists more than one type of ABA receptor and/or multiple signal transduction pathways in barley aleurone tissue (Hill *et al.,* 1995). The structures of the various intermediates studied by Hill *et al.* (1995) have been shown in Fig 2.1. Studies on a series of viviparous mutations of maize have indicated that the phytohormone abscisic acid (ABA) and transcription factor *VP1* are involved in seed maturation and prevention of premature germination (Robichaud *et al.*, 1980; McCarty *et al.*, 1989). Seed development is characterized by progressive differentiation of organs and tissues resulting in developmental gradients. The whole process is prone to metabolic control and distinct metabolite profiles specify the differentiation state. Early embryo growth is mainly

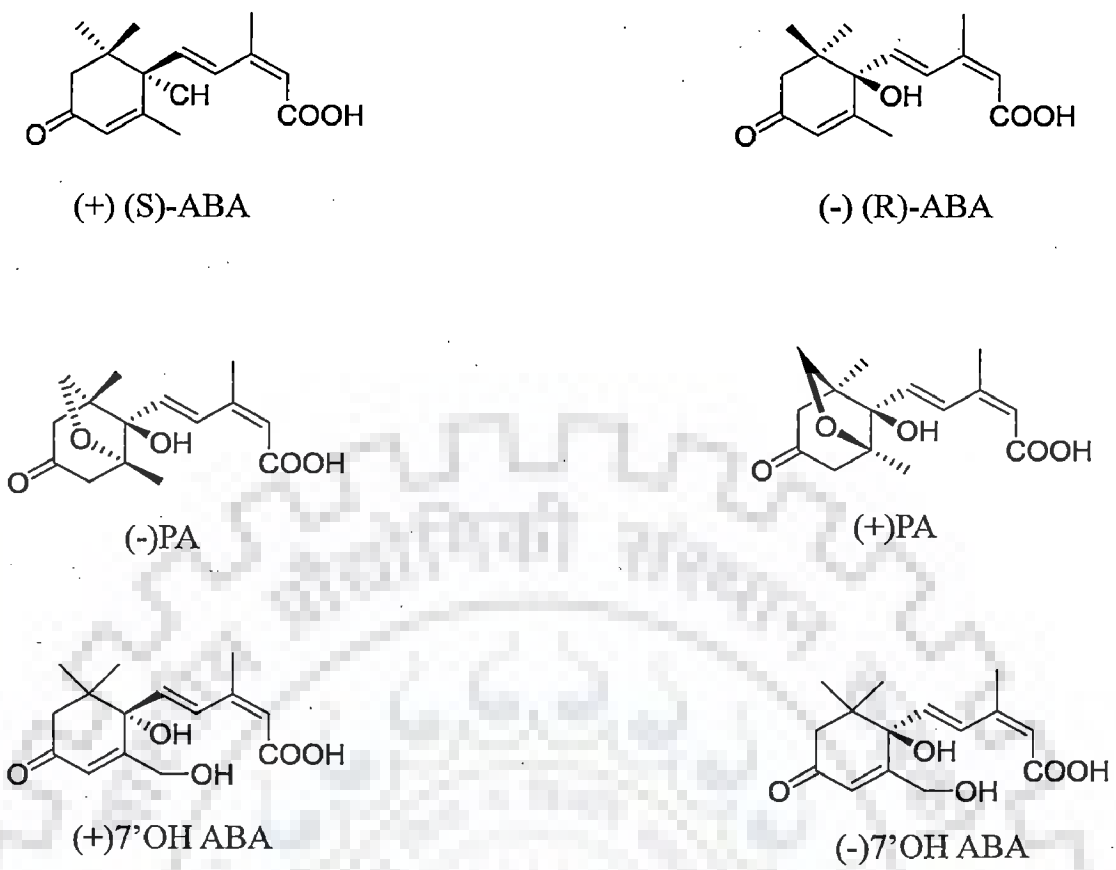


Fig 2.1: Structures of various intermediates of ABA (adapted from Hill *et al.*, 1995)

maternally controlled and the transition into maturation implies a switch to filial control. A signaling network involving sugars, ABA, and SnRK1 protein kinases governs maturation. Processes of maturation are activated by changing oxygen/energy levels and/or a changing nutrient state, which trigger responses at the level of transcription and protein phosphorylation. This way seed metabolism becomes adapted to altering conditions (Weber *et al.*, 2005).The level of ABA in any particular tissue in a plant is determined by the rate of biosynthesis and catabolism of the hormone. Therefore, identifying all the genes involved in the metabolism is essential for a complete understanding of how this hormone directs plant growth and development. To date, almost all the biosynthetic genes have been identified through the isolation of auxotrophic mutants. On the other hand, among several ABA catabolic pathways, current genomic approaches have revealed that *Arabidopsis* cytochrome P450 CYP707A gene encodes ABA 8′-hydroxylases, which catalyzes the first committed step in the predominant

ABA catabolic pathway (Kushiro *et al.,* 2004). Identification of ABA metabolic genes has revealed that multiple metabolic steps are differentially regulated to fine-tune the ABA level at both transcriptional and post-transcriptional levels. Furthermore, recent studies have given new insights into the regulation and site of ABA metabolism in relation to its physiological roles (Nambara and Marion-Poll, 2005). Mutant studies indicate that the CYP707A1 is important for reducing ABA levels during mid-maturation while CYP707A2 is responsible for the regulation of ABA levels from late-maturation to germination. Therefore each CYP707A gene plays a distinct role during seed development and post germination growth. Although ABA decreases to relatively low levels in dry dormant seeds owing to catabolism by specific ABA 8'-hydroxylases encoded by the cytochrome P450 CYP707A family, dormancy is maintained by renewed accumulation of ABA following imbibition of dormant seeds. Expression and genetic analysis of specific genes encoding key enzymes in ABA metabolism have identified members of the *AtNCED* (9-*cis*-epoxycarotenoid dioxygenase) family (*NCED6* and *NCED9*) that are essential for ABA synthesis in both the embryo and endosperm during dormancy induction. Expression of *AtNCED6* was observed in the endosperm during seed development and that of *AtNCED9* was observed in both embryo and endosperm at mid development. This demonstrates that ABA synthesized in both the endosperm and the embryo participates in the hormonal balance that controls seed dormancy and germination (Lefebvre *et al*., 2006). Thus, seed ABA levels and dormancy are controlled by the combined action of differentially expressed enzymes involved in several steps of both synthesis and catabolism. Dormancy release by after-ripening and stratification presumably causes a switch to ABA catabolism, resulting in a decrease in ABA content in the embryo and a corresponding increase in inactive ABA metabolites such as phaseic acid (PA) and dihydrophaseic acid (DPA) (Fig 2.2). In imbibing after-ripened barley grains, ABA is rapidly converted to PA in the embryo and does not appear to be released into the incubation medium or into the endosperm (Jacobsen *et al.,* 2002).

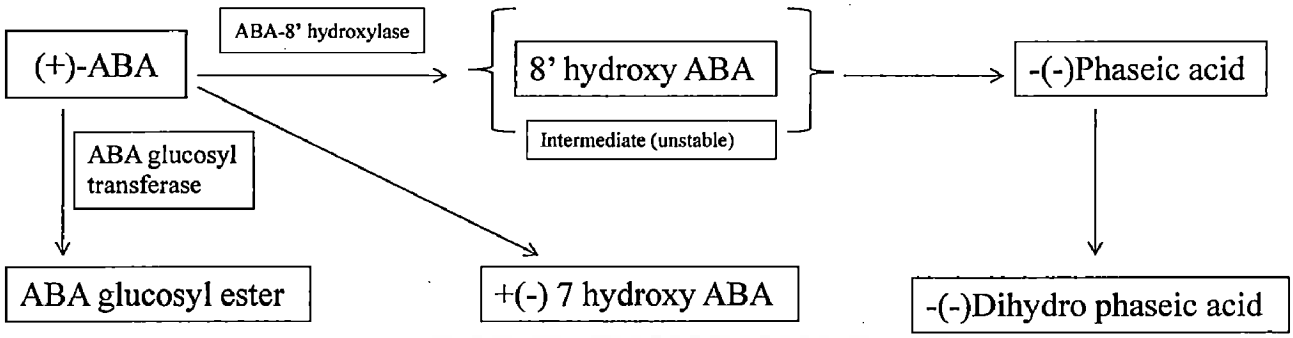


Fig 2.2: ABA catabolic pathway in plants

Similarly, increases in PA and DPA, have been observed during the first 24 h of imbibition of after-ripened *Arabidopsis* seeds (Kushiro *et al.*, 2004). Fig 2.2 shows that how ABA is catabolized into inactive forms either by oxidation or conjugation. The oxidative pathway ABA catabolism takes place by hydroxylation at C-8' to produce 8'-hydroxy ABA which is then spontaneously isomerized to form phaseic acid (PA). Further PA is reduced to dihydro phaseic acid (DPA). The catabolites 8'-hydroxy ABA, PA and 7'-hydroxy ABA all have some hormonal activity and might play roles in mediating ABA-like effects but not much is known in the context (Cutler and Krochko, 1999).

In wheat seedling roots, 8'-hydroxyABA and (+)-PA both induce group 3 *LEA* (late embryogenesis abundant 3), while (-)-7'-hydroxyABA had significant (10 to 40% of effective ABA activity) inhibititory activity on wheat embryo germination suggesting that ABA metabolites can maintain and prolong *LEA* gene expression (Walker-Simmons *et al.*,1997). In contrast to activities of above mentioned intermediates DPA does not exhibit any ABA like activities. A balance exists between ABA catabolism and biosynthesis and during dehydration when ABA levels increase PA levels also increase (Zeevaart, 1980; Pierce and Raschke, 1981). ABA 8'-hydroxylase has been reported to be cytochrome P-450 in *Arabidopsis*. *CYP707A* family, *CYP707A1–CYP707A4*, encode ABA 8'-hydroxylase. Okamoto et al (2006) demonstrated that each CYP707A gene plays a distinct role during seed development and post germination growth. Further expression studies have shown that *CYP707A2* is

responsible for the decrease in ABA level during seed imbibitions (Kushiro *et al.*, 2004) suggesting important role for *CYP707A* family in ABA regulation.

QRT-PCR studies demonstrated that expression of *CYP707A* and *NCED3* genes decrease upon dehydration but upon rehydration *CYP707A* expression increased while that of *NCED3* decreased (Kushiro *et al.*, 2004). The expression pattern of these genes upon rehydration suggests that ABA catabolism and biosynthesis are regulated to control ABA level during water stress.

2.3 Release of germination

Dormancy release can be mediated by gibberellins, brassinosteroids, ethylene, reactive oxygen species and nitrogen-containing compounds such as nitrate and nitric oxide (NO). Gibberellins are a family of 136 tetracyclic diterpenes, a small subset of which are active as plant hormones and known to stimulate seed germination in a wide range of plant species. Gibberellins stimulate germination by inducing hydrolytic enzymes that weaken the barrier tissues such as the endosperm or seed coat, inducing mobilization of seed storage reserves and stimulating expansion of the embryo (Bewley and Black, 1994). GAs are not involved in the control of dormancy but act to promote germination after the ABA-mediated inhibition of germination has been overcome (Bewley, 1997). More precisely Page-Degivry, (1997) proposed that ABA is the primary hormone involved at any step during dormancy maintenance and release, and that GAs are present at sufficient levels to promote germination as soon as ABA synthesis is inhibited. GAs are known to promote germination, and counteract the inhibitory effects of ABA, frequently in combination with cytokinins (Bewley and Black, 1982, 1994). GA is important for two distinct functions, firstly to overcome the mechanical restraint conferred by tissues that surround the embryo, such as aleurone and testa and secondly to increase the growth potential of the embryo, as indicated by the reduced growth rate of GA-deficient embryos (Groot and Karssen, 1987). Fig 2.3 shows the movement of enzymes during course of seed germination. Several GA-inducible genes related to cell wall loosening have been

identified in tomato seeds, including those encoding endo-β-mannanase (Nonogaki *et al.*, 2000), xyloglucan endotransglycosylase/hydrolase (Chen *et al.*, 2002), expansin (Chen and Bradford, 2000; Chen *et al.*, 2001) and β-1,3-glucanase and chitinase (Wu *et al.*, 2001), some of which are expressed specifically in the micropylar endosperm cap around the radicle.

2.4 Genes in GA response

A number of genes involved in the GA signaling pathway have been identified. These genes include *GAI* (GA-insensitive), *RGA* (Repressor of ga1-3), *RGL1* (RGA-like 1), *RGL2*, *AGL20* (AGAMOUS-LIKE 20), *SUPERMAN*, *SPY* (SPINDLEY) and *PICKLED* in *Arabidopsis*, *GAMYB*, *PKABA1* and *SLN1* (Slender 1) in barley, *SLR1* (Slender Rice 1) in rice, *PHOR1* (PHOTOPERIOD RESPONSIVE 1) in potato, *KNOX* in tobacco, *rht* (reduced height) in wheat

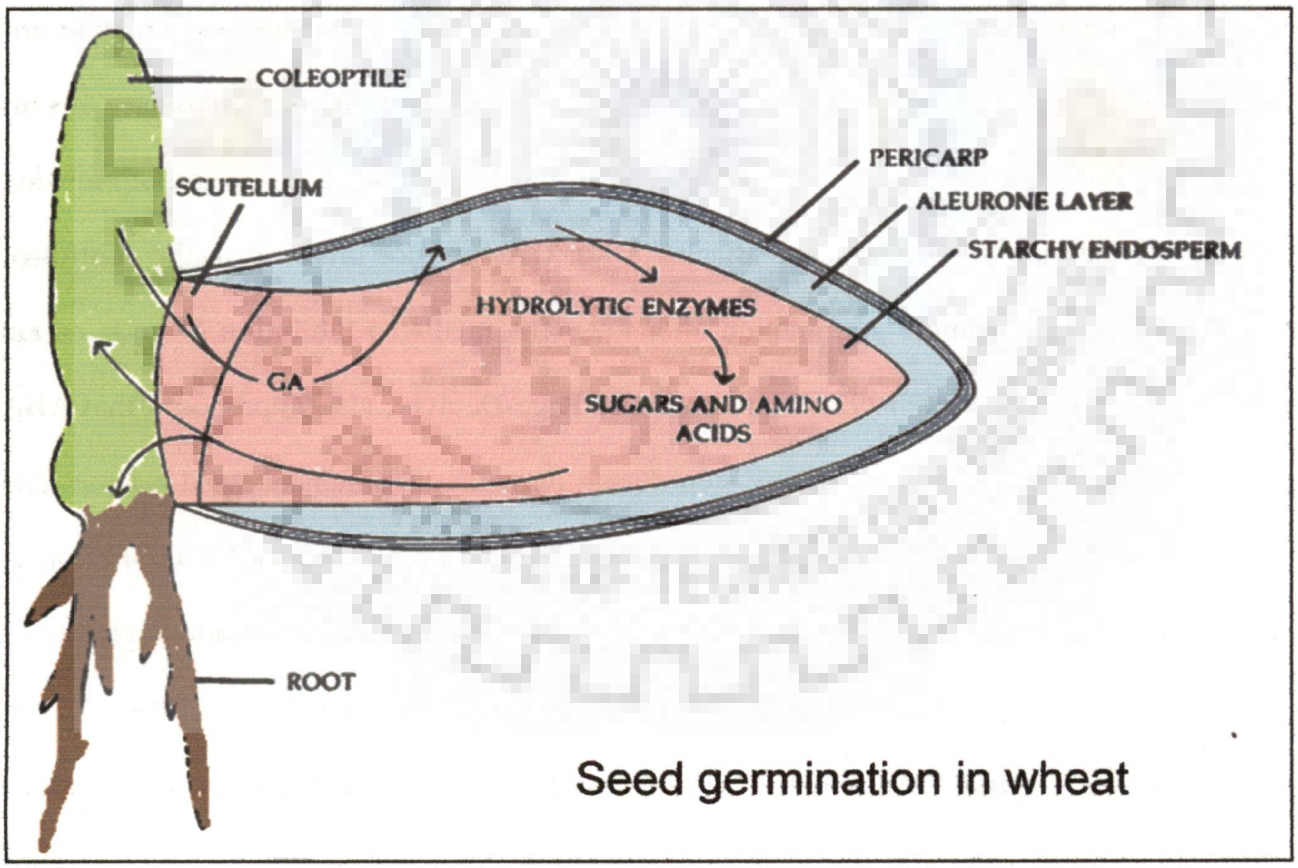


Fig 2.3: GA induced seed germination in wheat

(Borner *et al.*, 1996), *d8* (dwarf 8) in maize (Phinney, 1956) and *VvGAI1* (*Vitis Vinifera* GA insensitive 1) in grapevine (Peng and Harberd, 2002; Boss and Thomas, 2002). *RGA-LIKE1* (RGL1) and *RGL2*, members of the DELLA subfamily of GRAS proteins and SPINDLY act as negative regulators of GA-dependent seed germination (Jacobsen and Olszewski, 1993; Jacobsen *et al.*, 1996; Lee *et al.*, 2002; Wen and Chang, 2002).

RGA and *GAI* are negative regulators of GA signals transduction in *Arabidopsis* and have redundant functions similar functions with *RGA* being more dominant (Dill and Sun, 2001). By contrast, *SLEEPY1* (*SLY1*) is proposed to be a positive regulator in the GA response pathway (Steber *et al.*, 1998). *RGA* (Repressor of ga1-3) and *SPINDLY* (SPY) are repressors of GA signalling in *Arabidopsis*. GA signalling controls GA biosynthesis through feedback mechanisms (Bethke and Jones, 1998; Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). GA signal represses the GA signalling pathway by degrading repressor protein RGA as indicated by (green fluorescence protein) GFP-RGA fusion protein studies. In the study levels of GFP-RGA fusion protein and RGA were both reduced by exogenous application of GA. Further *GA4* (*AtGA3ox1)* expression is reduced by *rga* mutation suggesting a feedback inhibition of *GA4* expression (Silverstone *et al.,* 2001). *FRY1* (inositol polyphosphate 1-phosphatase) functions as negative regulator of ABA in *Arabidopsis* (Xiong *et al.,* 2001). In addition microarray studies have shown that *ABI3*, *ABI5*, and *FRY1* partially mediate the down regulation of (abscisic acid responsive element) ABRE-containing genes by *GA4* and that many GA downregulated genes contained ABRE in their promoter region (Ogawa *et al.,* 2003).

2.4.1 Mechanism of GA action

In germinating cereal grains, GA-induced α-amylase gene expression in aleurone cells has been a useful model system to study the GA response pathway (Olszewski *et al.,* 2002). It has been postulated that GA-down regulated genes contain ABRE in their putative promoter regions (Ogawa *et al.*, 2003). There are at least three possible mechanisms by which GA downregulates ABA upregulated genes: (i) GA reduces ABA levels by affecting ABA biosynthesis, (ii) GA

negatively regulates the ABA response pathway and (iii) GA and ABA signals are targeted independently to distinct cis-regulatory sequences of a single gene. It has been demonstrated that some genes that encode GA 20-oxidases and GA 3-oxidases are negatively regulated by GA activity through feedback inhibition while GA upregulates genes that encode GA-deactivating GA 2-oxidases by a positive feed forward loop (Olszewski *et al.,* 2002). It has also been reported that the synthesis of active GAs, rather than their deactivation, plays an important regulatory role in determining active GA levels during *Arabidopsis* seed germination (Ogawa *et al.,* 2003). After germination, bioactive GAs promote stem elongation, leaf expansion, and root growth (Davies, 1995; Yaxley *et al.*, 2001). Table 2.2 summarizes GA biosynthetic enzymes whose genes have been cloned.

The biosynthesis of GA in higher plants can be divided into three stages: (i) biosynthesis of ent-kaurene in proplastids, (ii) conversion of ent-kaurene to GA12 via microsomal cytochrome P450 monooxygenase and (iii) formation of C20 - and C19 -GAs in the cytoplasm. In the first stage, geranylgeranyl diphosphate, which serves as a common precursor for diterpenes (e.g.,

Table2.2: GA biosynthetic enzymes genes for which have been cloned

Enzyme	**Gene name**	**Arabidopsis locus**	**First cloned from**
ent-Copalyl diphosphate synthase	*CPS*	GA1	*Arabidopsis*
ent-Kaurene synthase	*KS*	GA2	*Cucurbita maxima* (Pumpkin)
ent-Kaurene 19-oxidase	*EKO*	GA3 Arabidopsis	*Arabidopsis*
GA12-aldehyde 7-oxidase	*GA7ox*		*Cucurbita maxima* (Pumpkin)
GA 20-oxidase	GA20ox	GA5	Pumpkin
GA3-hydroxylase	*GA3ox*	GA4	*Arabidopsis*
GA 2-oxidase	*GA2ox*		*Phaseolus coccineus* (Runner bean)

(Source: Hedden and Phillips, 2000)

GAs and the phytol chain of chlorophyll) and tetraterpenes (carotenoids), is synthesized by either a mevalonate-dependent or a non mevalonate pathway (Lange, 1998; Hedden and Phillips, 2000). Geranylgeranyl diphosphate is then converted to ent-kaurene in a two-step cyclization reaction and catalyzed by ent-Copalyl diphosphate synthase (CPS) and ent-Kaurene synthase (KS), with ent-Copalyl diphosphate as the intermediate. In plants ent-Kaurene–synthesizing activities are present mainly in rapidly developing tissues, such as the shoot tips, expanding leaves and petioles near elongating internodes and developing seed (Choi *et al.*, 1995; Aach *et al.*, 1997).

2.5 Dormancy and transcription factors

Red pigment of wheat grain has been reported to be polyphenol compounds, phlobaphene or proanthocyanidin, which are synthesized through the flavonoid biosynthesis pathway (Grotewold *et al.*, 1994). The genes in flavonoid biosynthesis pathway are chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), and dihydro-flavonol-4-reductase (DFR). The expression of these genes in the white-grained wheat lines is reduced or completely supressed, compared with that in the red-grained wheat (Himi *et al.*, 2005b). A single *R* gene is able to up regulate the four genes in the flavonoid biosynthesis pathway, suggesting that the R gene is a transcription factor. In addition to above mentioned genes Myb-type transcription factors (*Tamyb10-A1* on 3A, *Tamyb10-B1* on 3B and *Tamyb10-D1* on 3D) are also located in the same region as the *R* loci and are expressed in developing grains (Himi and Noda, 2005a). Further Himi *et al.* (2005b) reported that pigmentation of grain and coleoptiles is controlled by *R* and *Rc* genes respectively. The expression of flavonoid biosynthetic genes CHS, CHI, F3H and DFR was also found to be highly upregulated in red grained lines. The function of the *R* gene on grain dormancy has been suggested to accumulate germination inhibitors since a water-soluble precursor of the red pigment, catechin, inhibits grain germination (Miyamoto and Everson, 1958; Stoy and Sundin, 1976). Warner *et al.* (2000)

using sodium azide induced mutants in a red (*R1R1r2r2r3r3*) Chinese spring genotype showed that a functional R1 allele is not absolutely required for dormancy in wheat but it does enhance its expression in caryopses with dormant (sensitive) embryos. Himi *et al.* (2002) have also shown that *R* gene is not responsible for dormancy which may be controlled by genes tightly linked to *R* gene. Several genes for ABA sensitivity such as *ABI3*, *ABI5* and *Vp1*, which are expressed in seeds, have been isolated in *Arabidopsis* and maize (McCarty, 1995; Giraudat, 1995; Finkelstein and Lynch, 2000). Putative linkage between genes and transcription factors has been shown in Fig 2.4. Maize *Vp1* has also been reported to be involved in flavonoid synthesis and grain maturation besides the sensitivity to ABA (McCarty, 1995). In maize most viviparous mutants are blocked in biosynthesis of the carotenoid precursors for *de novo* ABA synthesis and so these mutants produce albino or pale green, non-viable seedlings. *taVp1*gene, an orthologue of *Vp1*, has been located in a region 30 cM from *R* locus, that controls wheat grain colour and is located in the distal region of the long arm of wheat chromosome

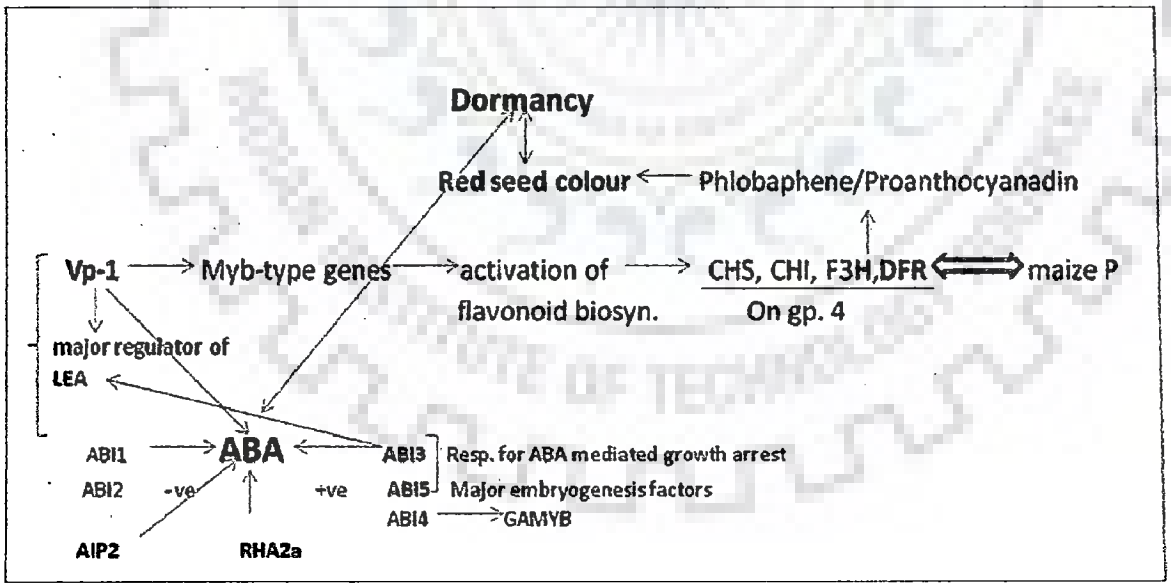


Fig 2.4: Putative linkage between various genes and transcription factors

3 (Bailey *et al.*, 1999). *Viviparous-1* (*Vp-1*) homologues play important roles in seed maturation, dormancy and dessication (McCarty *et al.*, 1989, 1991; Giraudat *et al.*, 1992; Bailey *et al.*, 1999).

The *Vp-1* gene is a major regulator of late embryo development in maize and inactivation of this locus leads to disruption of embryo maturation, resulting in the promotion of germination of embryos while still attached to the cob (vivipary) (McCarty *et al.*, 1991). The viviparous and germination mutants of maize and *Arabidopsis thaliana* help in our understanding of dormancy and germination pathways in seed development. This mechanism includes (a) developmental control of abscisic acid and gibberellin hormone synthesis and perception, (b) integration of maturation and anthocyanin pathways in the maize seed, (c) functions of the *VP1* and *ABI3* factors in abscisic acid-regulated gene expression, and (d) intrinsic developmental genes and transcription factors that couple seed maturation to the program of embryo morphogenesis. The scarcity of mutants that affect timing or tissue specificity of hormone synthesis in the seed is an important constraint to progress in understanding the role of hormonal signals. The interactions among the abscisic acid-insensitive *abi1*, *abi2*, *abi3*, *abi4*, and *abi5* mutants of *A. thaliana* indicate presence of multiple pathways of abscisic acid signal transduction in the seed. The maize *Vp1* and *A. thaliana Abi3* genes are functional homologs that mediate a seed-specific abscisic acid response necessary for maturation. Laethauwer *et al.* (2009) used quantitative RT-PCR to detect the expression of *Vp1* genes in both wheat and triticale and concluded that the expression level of *Vp1* gene at 50 days post anthesis are not useful to select for PHS tolerance indicating that *Vp1* genes are synthesized at a later stage in seed development. Functional analysis indicates that *VP1* is a transcriptional activator of the Em and C1 promoters in maize, although its specific role in abscisic acid signal transduction remains poorly understood. Yang *et al.* (2007) demonstrated using semi-quantitative RT-PCR that there was no difference in spliced transcripts of *Vp-1A*, and *Vp-1D* between PHS tolerant and PHS susceptible cultivars. They also demonstrated after genomic sequence comparison that among *Vp-1Ba*, *Vp-1Bb* and

Vp-1Bc only one transcript is correctly spliced and can encode full length Vp-1 protein. Difference in *Vp-1B* alleles was observed after ABA treatment suggesting that a deletion or insertion in third intron can affect *Vp-1B* gene and its ABA sensitivity. Furthermore, it was also observed that as genotypes with different levels of tolerance to PHS show differing responses on ABA exposure and similarly differences in transcript levels of *Vp-1Ba*, *Vp-1Bb* and *Vp-1Bc* is also observed after ABA treatment (Yang *et al.*, 2007a). Keeping above said in view a *Vp1* STS marker, Vp1B3 was developed and validated among a set of Chinese wheat germplasm. Vp1B3 amplifies either 845 or 569-bp fragment from the tolerant cultivars and 652-bp from the susceptible cultivar suggesting that there is a strong association between the polymorphic fragments of Vp1B3 and PHS tolerance. Therefore, Vp1B3 could be used as an efficient and reliable co-dominant marker in the evaluation of wheat germplasm for PHS tolerance and marker-assisted breeding for PHS tolerant cultivars (Yang *et al.*, 2007b). Another mutant *TaDOG1* (delay of germination1) has also been reported to participate in the regulation of wheat seed dormancy. The *DOG1* gene has been identified as a QTL for the regulation of seed dormancy in *Arabidopsis*. This novel wheat (*Triticum aestivum* L.) mutant with reduced seed dormancy, RSD32, was isolated from NaN_3-treated population of a dormant cultivar, Norin61 (Rikiishi and Maekawa, 2010) in wheat.

2.6 Mapping of QTLs for PHS tolerance

QTL mapping is a powerful strategy to identify dormancy QTLs independent of the R genes and ultimately to use the QTL alleles in breeding white grain-coloured varieties for resistance to preharvest sprouting. In wheat dormancy is associated with red colour (R) genes located on the distal region of the long arm of wheat group 3 chromosome. A number of genes and QTLs involved in PHS tolerance or seed dormancy have been found and mapped in wheat. PHST is a quantitative trait controlled by many quantitative trait loci (QTL)/genes, and a number of QTL controlling this trait have been identified (Anderson *et al.*, 1993; Zanetti *et al.*, 2000; Mares *et*

al., 2001; Kato *et al.*, 2001; Flintham *et al.*, 2002; Groos *et al.*, 2002; Osa *et al.*, 2003; Kulwal *et al.*, 2004). In bread wheat, 20 chromosomes with the solitary exception of chromosome 1D are known to carry QTLs/genes for PHST/dormancy. QTLs mapped for PHS across populations in different studies has been summarized in Table 2.3. Anderson *et al.*, (1993) and Sorrells and Anderson (1996) reported that several RFLP markers for resistance to preharvest sprouting were located on chromosomes of groups 1, 2, 3, 4, 5, and 6 using quantitative trait loci (QTL) analysis of wheat. These results suggest that many genes are involved in grain dormancy. However, chromosomes 3A, 3B, 3D and 4A have been considered to be more important than other chromosomes for the study of genetics of PHST/dormancy (Flintham and Gale, 1996; Bailey *et al.*, 1999; Zanetti *et al.*, 2000; Warner *et al.*, 2000; Kato *et al,* .2001; Flintham *et al.*, 2002; Groos *et al.*, 2002; Himi *et al.*, 2002; Osa *et al.*, 2003; Kulwal *et al.*, 2004; Mori *et al.*, 2005; Chen *et al.,* 2008). Group 3 chromosomes have received attention as these carry R genes for seed coat colour and *taVp1* genes that are orthologous to maize *Vp1*gene that encode a dormancy-related transcription factor. Several recent studies demonstrated that the major QTL on 4AL is present in both white and red wheat cultivars (Chen *et al.*, 2008; Lohwasser *et al.*, 2005; Mares *et al.,* 2005; Torada *et al.*, 2005). A major PHS gene on chromosome 4AL was identified as controlling dormancy (Flintham *et al.,* 2002). The PHS gene exerts effect on embryo while R gene expresses in maternal testa tissue.

2.7 Comparative genomics

Comparative genome analyses have shown the existence of conserved gene orders (colinearity) in the genomes of different plant and mammal species. The first comparative genetic maps were produced in the late 1980s (Bonierbale *et al.*, 1988 and Chao *et al.,* 1989). Orthologous genes that have conserved functions produce similar phenotypes across species. For example, *GAI, Rht-1* and *D8* orthologous dwarfing genes reduce plant height in *Arabidopsis*, wheat and maize, respectively (Peng *et al.*, 1999). Restriction fragment length polymorphism (RFLP)

maps for each of the three genomes in hexaploid (bread) wheat were constructed for each of the three diploid ancestors of hexaploid wheat - *Triticum urartu*, *Aegilops squarrosa* and *Triticum speltoides* were found to be remarkably similar (Chao *et al., 1989*). In plants, this order is best documented in the grass family, where colinearity has been maintained over evolutionary periods as long as 60 million years (Devos and Gale, 1997; Gale and Devos, 1998). Among plants *Arabidopsis* (Kaul *et al.* 2002), rice (Matsumoto *et al.*, 2005), maize (Schnable *et al.*, 2009), *Sorghum* (Paterson *et al.*, 2009), *Brassica* (Wang *et al.*, 2011) have been sequenced. Progress in rice genomics has made it possible to undertake detailed structural and functional comparisons of genes involved in various biological processes among rice and other plant species. Comparative mapping has been done using 48 DNA probes, including six with known functions, on the group 2 chromosomes of wheat, rye and barley. Therefore, a genetic map was constructed comprising of 114 loci in wheat and 34 loci in rye. It was found that the gene orders in cereal genomes of barley, wheat and rice is highly conserved except for the distal ends of chromosome arms 2BS and 2RS (Devos *et al.*, 1993). In another study comparative genetic maps of chromosomes $4A^{m}$ and $5A^{m}$ of *Triticum monococcum* and chromosome groups 4, 5 and 7 of *T.aestivum* were constructed (Devos *et al.*, 1995). Comparative genomics approaches have been used to identify genes controlling seed dormancy in rice, wheat and barley. In the study it has been reported that barley dormancy QTL on long arm of chromosome 5H showed synteny with terminal region of long arm of chromosome 3 of rice. The dormancy gene in rice was annotated as *GA20-oxidase* and the same was found to control dormancy in barley on chromosome 5HL. This chromosomal region also shared synteny with chromosome 4AL in wheat. Therefore, chromosome 4AL of wheat was found syntenic to both chromosome 3 and 11 in rice and the dormancy QTLs have been reported on both rice chromosomes (Li *et al.*, 2004). The most popular applications of comparative genomics since the completion of rice genomic sequence has been the generation of new markers to tag traits in other species and to identify the candidate genes for these traits. In another study using rice as a reference genome with 41,046 gene models, 4,454 maize orthologs (defining 30 syntenic blocks), 6,147 *Sorghum*

Table 2.3: Wheat PHS QTLs mapped in various studies across different populations

S.No.	Chromosome	QTL loci	Markers	References
1.	1AS 1AS ? 2S 2L 2L 5DL 6BL 4AL? 3BL		XcnI.BCD1434 XcnI.CDO431 XcnI.CDO795 XcnI.CDO64 Xcnl.WG996a XcnI.BCD120a Xcnl.BCD450 Xcnl.BCD1426 XcnI.CDO545 XcnI.CDO482	Anderson *et al.*, 1993
2.	6BS 7DL		Xwmc104 Xmst101	Roy *et al.*, 1999
3.	2AL 3AS 3BL 4DL 5AS 5AL 6DL 7BL		Xplp ap-Xglk684α Xpsr304-Xpsr598 Xglk80-Xpsr1054 Xpsr1101a-Xpsr160b Xglk163a-Xpsr945a Xpsr1194-Xpsr918b Xpsr167a-Xmwg684b Xpsr350-Xpwir232b	Zanetti *et al.*, 2000
4.	2AL, 2DL, 4AL			Mares *et al.*, 2001
5.	4A 4B 4D	QPhs.ocs-4A.1 QPhs.ocs-4B.2 QPhs.ocs-4D.2	Xcdo795-Xpsr115 Xbcd1431.1- Xbcd1431.2 Xbcd1431.1- Xbcd1431.2	Kato *et al.*, 2001
6.	3A 3B 3D 5A		Xfbb293 Xgwm403 Xbcd131 Xbcd1871	Groos *et al.*,2002
7.	7BL 3B		Xpsr547-Xpsr680b	Mrva & Mares, 2002
8.	3A	QPhs.ocs.3A.1 QPhs.ocs.3A.2		Osa *et al.*, 2003
9.	4A		Xbarc 170-Xgwm 269-Xgwm 397	Mares *et al.*, 2005
10.	4AL		Xhbe03 and Xhbe11	Torada *et al.*, 2005
11.	3A	QPhs.ocs.3A.1	Xbarc310-Xbcd907	Mori *et al.*, 2005
12.	3AL	QPhs.ccsu-3A.1	Xwmc153-Xgwm155	Kulwal *et al.*,2005
13.	4A		Xbarc170-Xzxq118-Xwg622 Xgwm269-Xzxq118-Xbarc170	Zhang *et al.*, 2008
14.	3A 2B	QPhs.pseru-3AS QPhs.pseru-2B.1	Xbarc12-Xbarc57-Xbarc321 Xdup398-Xbarc54	Liu *et al.*, 2008
15.	3A	QPhs.pseru-3AS	(XpACT-mCAC144-XpCGA-mACAG172)-Xbarc57-(XpGCGT-mCTGA160-XpGCGA-nAGG242)	Liu and Bai, 2010

orthologs (12 syntenic blocks), 827 wheat orthologs (13 syntenic blocks), and 309 barley orthologs (13 syntenic blocks) were identified. In total, 11,737 orthologous pairs and 68 synteny blocks that covered 99%, 82%, 99%, 91%, and 84% of the rice, maize, sorghum, wheat, and barley genomes, respectively, were observed, suggesting rapid changes in plant genome architecture (Salse *et al.,* 2008). In another study by Singh *et al.* (2004) rice-wheat synteny was studied using gene-by-gene comparison of the sequence homology of 2,932 predicted rice genes from the long arm of chromosome 11 with wheat ESTs and their location on wheat chromosome bins. The largest number of genes, about one-third, were mapped to the homoeologous group 4 chromosomes of wheat, while the remaining genes were located on wheat chromosomes of different groups with significantly higher numbers for groups 3 and 5. Therefore, the location of bin-mapped wheat contigs to all seven homoeologous groups can possibly be due to movement of genes (transpositions) or chromosome segments (translocations) within rice or the hexaploid wheat genomes. The results of this study demonstrate that there is definite conservation of gene sequences and the ancestral chromosomal identity between rice and wheat but there is no conservation of the gene order (Singh *et al.*, 2004).

2.8 Chromosome bin mapping in wheat

Bin maps have been prepared in wheat using the vast EST database in wheat which are valuable for gene discovery and genome analysis. The bin maps of EST loci and chromosome specific BAC libraries (Šafaàř *et al.*, 2004) can be used for preparation of BAC contig physical map of wheat. BAC physical map has been prepared for chromosome 3B of wheat assembling 1036 contigs anchored with 1443 molecular markers (Paux *et al.,* 2008). Similar BAC physical maps have also been prepared for wheat chromosome 3DS (Bartos *et al.,* 2010). For chromosome 7D the BAC library comprised of the short arm (7DS) consisting of 49,152 clones with 113 kb insert size representing 12.1 arm equivalents while the long arm (7DL) consisted

of 50,304 clones of 116 kb providing 14.9 fold arm coverage. The 7DS library was screened with markers for the *DnCI2401* (Russian wheat aphid resistance) gene and 7DL was screened with a probe linked to greenbug resistance gene *Gb3* and can be considered as a first step towards positional cloning of the genes. (Šimková *et al.,* 2011). A high-density deletion bin maps of wheat chromosomes 5A, 5B, and 5D including 2338 loci mapped with 1052 EST probes and 217 previously mapped loci (total 2555 loci) has been constructed. The information generated was combined to construct a consensus chromosome bin map of group 5 including 24 bins (Linkiewicz *et al.*, 2004). 7104 expressed sequence tag (EST) unigenes by Southern hybridization and chromosome bin map using a set of wheat aneuploids and deletion stocks was prepared for wheat. In the study more loci mapped to B genome (5774) than to A (5173) or D (5146) genomes (Qi *et al.,* 2004). Chromosome bin-mapping of expressed sequence tags (ESTs) representing genes onto the seven homoeologous chromosome groups of wheat was prepared by Lazo *et al.* (2004) and a wheat EST collection comprising of 113,220 ESTs was also developed. In another study 996 chromosome bin-mapped expressed sequence tags (ESTs) accounting for 2266 restriction fragments (loci) on the homoeologous group 3 chromosomes of hexaploid wheat (*Triticum aestivum* L.) were mapped. Of these loci, 634, 884, and 748 were mapped on chromosomes 3A, 3B, and 3D, respectively (Munkvold *et al.*, 2004). Based on wheat-rice comparison, group 3 chromosomes of wheat are homologous to rice chromosome1 (Devos *et al.*, 1992; Ahn *et al.*, 1993; Kurata *et al.*, 1994; Van Deynze *et al.*, 1995). A high-density EST chromosome bin map of wheat homoeologous group 2 chromosomes was constructed. A total of 2600 loci were generated from 1110 ESTs using Southern hybridization onto wheat aneuploid chromosome and deletion stocks and 552 ESTs mapped to more than one group 2 chromosome (Conley *et al.,* 2004). For chromosome 1 a total of 944 expressed sequence tags (ESTs) were used which generated 2212 EST loci. The EST loci distribution among chromosomes 1A, 1B, and 1D were 660, 826, and 726, respectively (Peng *et al.*, 2004).

2.9 Bioinformatics in cereals

The term bioinformatics was coined by Hogeweg and Hesper (1978) for the study of informatic processes in biotic systems. Bioinformatics can be defined as the application of statistics and computer science to the field of molecular biology. Bioinformatic applications include sequence alignment, gene identification, genome assembly, protein structure alignment, protein structure prediction, prediction of gene expression and protein-protein interactions genome annotations, comparative genomics and genome-wide association studies. Using bioinformatics various biological data like nucleotide and amino acid sequences, protein domains and protein structures can be analyzed. The list of databases available for data mining has been shown in Table 2.4. The International Rice Genome Sequencing Project (IRGSP), a consortium of publicly funded laboratories, was established in 1997 to obtain a high quality, map-based sequence of the rice genome using the cultivar Nipponbare of *Oryza sativa* ssp. japonica. It was comprised of ten members including Japan, United States of America, China, Taiwan, Korea, India, Thailand, France, Brazil, and United Kingdom. The IRGSP website (http://rgp.dna.affrc.go.jp/IRGSP/) can be used for information regarding rice. Another website the TIGR (The Institute for Genomic Research) database http://www.tigr.org/tdb/e2k1/osa1/) has also been annotated (Yuan *et al.*, 2002). On TIGR rice genome has been annotated for gene content, identified motifs/domains within the predicted genes, constructed a rice repeat database, identified related sequences in other plant species, and syntenic sequences between rice and maize. Analyses include anchoring publicly available rice bacterial artificial chromosome and P1 artificial chromosome clones (BAC, PAC) to the genetic map, annotation of BAC/PAC sequences, classification of domains and motifs within proteins predicted in the genome, construction of a rice repeat database have been performed by Yuan *et al.* (2002). In wheat on the other hand as the whole genome sequence is not available expressed sequenced tags (ESTs) and comparative maps are used for gene discovery. The wheat EST information is present on Graingenes

(http://wheat.pw.usda.gov/GG2/index.shtml). The site also provides bin map viewer in wheat as shown with synteny to rice chromosome and barley bin map is also available. NCBI (http://www.ncbi.nlm.nih.gov/dbEST) also provides EST sequence information while another site Gramene (http://www.gramene.org/) provides with the similar information.

2.10 Proteomics

The term proteome is used for total protein content expressed by a genome in a cell, tissue or organism under a defined set of conditions (Wasinger *et al.*, 1995 and Wilkins *et al.*, 1996) and the study of proteome is called proteomics. The schematic diagram of 2-DE SDS PAGE has been shown in Fig 2.5. Proteomic approach is informative as the cell responds to internal and external effects by changing the level and activity of its proteins. Proteins are made from basic building blocks called amino acids which are joined together by peptide bonds. Each protein is

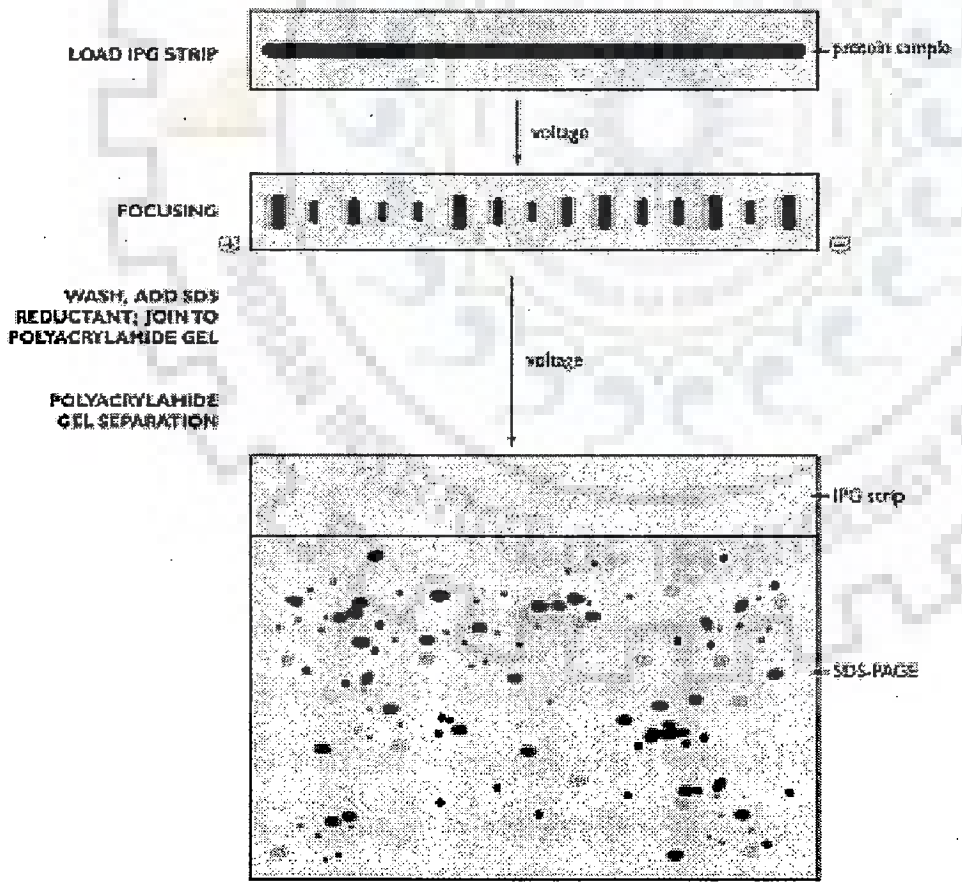


Fig 2.5: Schematic representation of 2-DE SDS-PAGE (Source: Liebler, 2002)

Table 2.4: List of URLs for the databases available for bioinformatic studies

Sl No.	Database	URL
1.	**Protein sequence (primary)**	
	Swiss-Prot PIR-International	www.expasy.ch/sprot/sprot-top.html www.mips.biochem.mpg.de/proj/protseqdb
2.	**Protein sequence (composite)**	
	OWL NRDB	www.bioinf.man.ac.uk/dbbrowser/OWL www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein
3.	**Protein sequence (secondary)**	
	PROSITE PRINTS Pfam	www.expasy.ch/prosite www.bioinf.man.ac.uk/dbbrowser/PRINTS/PRINTS.html www.sanger.ac.uk/Pfam/
4.	**Macromolecular structures**	
	Protein Data Bank (PDB) Nucleic Acids Database (NDB) HIV Protease Database ReLiBase PDBsum CATH SCOP FSSP	www.rcsb.org/pdb ndbserver.rutgers.edu/ www.ncifcrf.gov/CRYS/HIVdb/NEW_DATABASE www2.ebi.ac.uk:8081/home.html www.biochem.ucl.ac.uk/bsm/pdbsum www.biochem.ucl.ac.uk/bsm/cath scop.mrc-lmb.cam.ac.uk/scop www2.embl-ebi.ac.uk/dali/fssp
5	**Nucleotide sequences**	
	GenBank EMBL DDBJ	www.ncbi.nlm.nih.gov/Genbank www.ebi.ac.uk/embl www.ddbj.nig.ac.jp
6	**Genome sequences**	
	Entrez genomes GeneCensus COGs	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome bioinfo.mbb.yale.edu/genome www.ncbi.nlm.nih.gov/COG
7.	**Integrated databases**	
	InterPro Sequence retrieval system (SRS) Entrez	www.ebi.ac.uk/interpro www.expasy.ch/srs5 www.ncbi.nlm.nih.gov/Entrez

built from different arrangements of twenty natural amino acids. Proteins are the expressed part of a genome so it is very important to identify proteins individually. According to the central dogma of molecular biology the flow of genetic information takes place from DNA to mRNA and on to the synthesis of polypeptides. There are mainly three types of proteomics which are classified as expression, structural, and functional proteomics. Expression proteomics aims to identify the protein expression level of biological systems in terms of its response to any physical and biochemical stresses. It deals with change in entire proteome in a cell, tissue, or organism at a certain time. Structural proteomics identifies all the proteins within the cell or any organelle with their location. Its major role is mapping out the structure of protein in three dimensional space using X-ray crystallography and NMR spectroscopy methods. Functional proteomics deals with the identification of functions, activities, and interactions of all the proteins in a proteome.

In the past ten years, advancements in proteomic techniques such as two-dimensional gel electrophoresis, mass spectrometry and mass spectral data mining using bioinformatic tools have made proteomics a method of choice for researchers. Moreover the ever increasing protein databases such as NCBI protein database, ExPASy, Swiss-Prot etc. have made protein mining easy.

2.10.1 Plant proteomics

Plant proteomics is the study of total proteins in a plant at any stage of development. Systematic analysis of the function of genes can be done at either oligonucleotide or protein level, but proteins essentially offer advantage of being closest to functions as they form part of the expressed part of the genome. Proteomic employs mass spectrometry, electrophoresis and chromatography for the detection, identification and characterization of proteins. Commonly used proteomic techniques include cell fractionation protein separation by electrophoresis,

chromatography, electroblotting etc. In a study by Sivaprakash *et al.* (2006) the expression of soyabean ferritin under the control of rice endosperm-specific glutelin promoter (GluB-1) was studied using histochemical staining after immunoblotting the extracted protein to localize ferritin protein in both non transgenic and transgenic rice in the study.

But in addition to the above mentioned techniques, protein identification techniques have received a boost with advent of mass spectrometry. Therefore, out of most protein based approaches used to identify gene function, mass spectrometry has lately been the method of choice as mass spectrometry identifies proteins with very high sensitivity and medium to high throughput depending on mass spectrometric method used. Genome annotation is in progress and with the genome sequencing of *Arabidopsis* and rice it has become easy to annotate proteins. As identification of proteins requires knowledge of all the coding genes of an organism, gene annotation and functional characterization benefit greatly from the findings of proteome analysis. In addition large-scale EST sequencing projects from various crop plants have facilitated the use of tools that aid in the determination of gene functions. The technique of proteome analysis with two-dimensional PAGE helps to monitor changes that occur in the protein expression of tissues and organisms and/or expression that occurs under abiotic and biotic stresses. The classical method of gene expression studies in terms of proteomics is two-dimensional gel electrophoresis (2DE), which is often combined with subsequent protein identification by mass spectrometry (MS). This approach allows the generation of information on expressed proteins in a cell or tissue of interest. Proteome analysis using 2-D gel electrophoresis of an *Arabidopsis* mutant with disturbed cell division indicated an elevated protein expression of stress-responsive proteins (Lee *et al.,* 2008). In global gene expression profiling studies during abiotic stress treatment, stress-inducible genes were identified enabling the elucidation of stress response mechanisms (Vij and Tyagi, 2007). A total of 10,589 protein spots were identified in rice out of which 252 proteins were electroblotted onto PVDF (polyvinyl difluoride) membrane after which N-terminal sequencing was done. Internal amino

acid sequences of 633 proteins were further determined using a protein sequencer or mass spectrometry after enzyme digestion of the proteins. Out of some of the proteins determined, calreticulin and a gibberellin-binding protein, ribulose-1,5-bisphosphate carboxylase/oxygenase activase were identified in rice and have functions in the signal transduction pathway (Komatsu *et al.*, 2003). According to a review 549 proteins have been resolved using 2D-PAGE in rice nucleus. Among these 257 proteins have been systematically analyzed by Edman sequencing and mass spectrometry and 190 proteins have been identified following database searching (http://gene64.dna.affrc.go.jp/RPD/main.html). The identified proteins have been sorted into different functional categories and most of the proteins were found to be involved in signaling and gene regulations thus, reflecting the role of nucleus in gene expression and regulation (Khan and Komatsu, 2004). PEG stimulated drought stress responsive proteins were studied using 2-DE and twelve proteins were identified using mass spectrometry. The proteins identified were involved in redox metabolism, photosynthesis, cytoskeleton stability, defense, protein metabolism and signal transduction. The corresponding gene expression of identified proteins were studied using real time PCR and it was concluded that the differentially displayed proteins might play a role in redox metabolism, photosynthesis, protein degradation, cytoskeleton organization and programmed cell death during drought in rice (Xiong *et al.*, 2010). To understand the mechanism of dehydration response, four week old rice seedlings were subjected to dehydration and changes in extracellular matrix proteome were examined using 2-DE. This study revealed 192 proteins that changed their intensities by more than 2.5-fold during dehydration. The proteomic analysis led to the identification of about 100 differentially regulated proteins presumably involved in carbohydrate metabolism, cell defence and rescue, cell wall modification, cell signaling and molecular chaperones and upon comparison with database an evolutionary divergence in the dehydration response was revealed (Pandey *et al.*, 2010). To understand the molecular mechanisms of dehydration-responsive adaptation in plants nuclear proteome of rice was studied by Choudhary *et al.* (2009). The

study identified 109 differentially regulated proteins using LC-ESI-MS/MS with varying functions like transcriptional regulation and chromatin remodeling, signaling and gene regulation, cell defense and rescue, and protein degradation. Of the total 109 proteins identified 14% were annotated as hypothetical proteins which were further studied and conserved domains for seven proteins were identified. In addition dehydration responsive nuclear proteome of rice was also compared with chickpea, *Arabidopsis* and *Medicago* (Choudhary *et al.*, 2009). In another study changes in 2-DE spot patterns during barley seed development and germination were studied and proteins like thioredoxin, cold regulated protein cor14b, α amylase/trypsin inhibitors were identified using MS. Two isoforms of thioredoxin h, HvTrxh1 and HvTrxh2 were cloned (Finnie *et al.*, 2004). Salinity tolerance during germination was studied by comparing proteomic profiles of mature barley grains and tolerant lines were found to express a higher level of 6-phosphogluconate dehydrogenase and glucose/ribitol dehydrogenase (Glc/RibDH). QTL studies using different salt concentrations led to the identification of two of heat-shock protein (hsp) 70 and Glc/RibDH which co-localized with the identified QTL on chromosome 5H (Witzel *et al.*, 2010). Proteomic analysis of developing rachis (cob) from maize was studied using two proteomic approaches i.e. 2-DE and 2-D LC. A total of 967 proteins were identified and functional classification of identified proteins revealed that proteins involved in cellular metabolisms, response to stimulus and transport, were the most abundant (Pechanova *et al.*, 2010). In another study abiotic stress tolerance was studied in genetically engineered rice by Sahoo *et al.* (2007).

2.10.2 Wheat proteomics

Wheat is an important cereal crop with a large genome size of the order of 16,000Mb i.e almost forty times the size of rice genome (420 Mb). This large genome size and functional redundancy of genes on all three homeologues (A, B and D genome) is posing difficulties in the whole genome sequencing of wheat. Proteomics offers a new approach to identify a broad spectrum of genes that are expressed in wheat during a wide range of abiotic and biotic

stresses. The 2-DE database of wheat seed proteins is available for public access at http://www.proteome.ir. Vensel *et al.* (2005) compared endosperm proteins at 10dpa (days post anthesis), and 36 dpa of wheat grain development. MALDI-TOF MS was used to identify albumins and globulins of wheat endosperm. It was therefore, concluded that at 10dpa predominant proteins were metabolism related and at 35 dpa stress and storage proteins were present (Vensel *et al.,* 2005). Proteomic study was conducted by Mak *et al.* (2006) on wheat germ and 612 proteins were subjected to peptide mass fingerprinting (PMF). Using Swiss-Prot and TrEMBL databases 347 proteins were identified and grouped categorically into energy, cell growth, transcription, protein synthesis, transporters, cell structure, signal transduction, disease, secondary metabolism and storage proteins (Mak *et al.,* 2006). The proteome analyses of diploid, tetraploid and hexaploid wheats were studied using 2-DE and peptides were identified using ESI-Q TOF-MS. With the study it was demonstrated that the expression of proteins in hexaploid wheat is a product of the interaction of the diploid genomes (AA and BB) in hexaploid nuclear constitutions. It was also concluded with this study that the non storage proteins are present on the D genome (Islam *et al.,* 2003). In another study differences in the redox status of protein Cys thiols upon wheat seed dormancy were analysed using thiol-specific fluorescent labelling. Proteins with reversible oxidoreductive changes were characterized and 193 reactive Cys were found in 79 unique proteins responding differentially in dormant, non-dormant, abscisic or gibberellic acid treated seed protein extracts. It was demonstrated that during after-ripening, accumulated ROS target different redox-sensitive pathways by altering protein function through oxidative modifications, which might displace the phytohormonal balance. This displays antioxidant defence mechanisms are active for seed protection during dormancy stage (Bykova *et al.,* 2010). In another study different methods of protein extraction from wheat endosperm were compared followed by 2-DE (Hurkman and Tanaka, 2007). It was concluded that accumulation of stress proteins and storage proteins are the predominant activities at above mentioned stages (Hurkman and Tanaka, 2007). In a recent study two

hundred and seventeen protein spots were detected by using two-dimensional electrophoresis. After tryptic digestion MALDI-TOF/MS analysis was performed and database searching of some of the identified 185 differentially expressed proteins which were involved in biotic stress related functions as disease associate with pathogens (Kamal *et al.,* 2010). In another study Laino *et al.* (2010) performed comparative proteome analysis of metabolic proteins from seeds of durum wheat and demonstrated that heat stress alters durum seed proteome by 1.2 to 2.2 fold. The study identified 132 differentially expressed polypeptides, 47 of which were identified by MALDI-TOF and MALDI-TOF-TOF MS and most of the heat-induced polypeptides were allergenic to humans (Laino *et al.,* 2010). Proteome of the peripheral layer of wheat grain during development was done followed by MALDI-TOF MS. In study two hundred and seventeen proteins were identified using data mining tools. Proteins identified were grouped into five categories and four distinct phases of development. Therefore, this study helped to understand the processes taking place in peripheral layers during seed development (Tasleem-Tahir *et al.,* 2011). In another study the qualitative and quantitative evolution of proteins was analysed during early stages of grain development and changes in proteome was observed between 125°C (grain development in thermal time) and 195°C day. It was observed that at 195°C day storage proteins and globulins were present in addition to proteins responsible for degradation and proteasome subunits, while DNA repair enzymes and cellular metabolism enzymes were present at 125°C. This study helped to identify the early processes in wheat seed development (Nadaud *et al.,* 2010).

2.11 2D gel analysis softwares

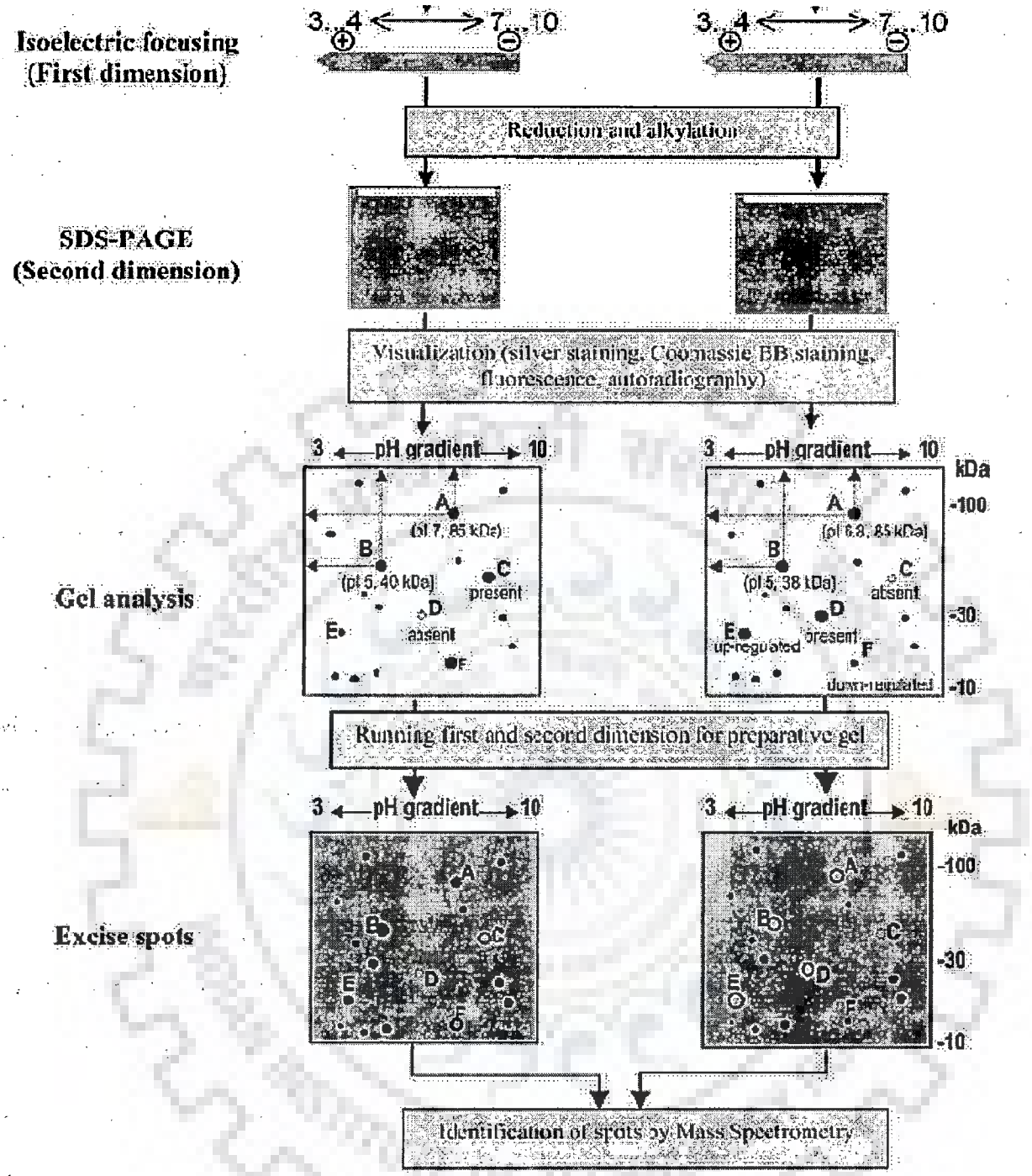
In proteomic studies bioinformatics helps in the analysis of proteome of an organism, 2 dimensional studies, mass spectrometry and prediction of 2D and 3D structures. Comparative analysis of 2-DE gels can be done by softwares like PDQuest, Delta2D, ImageMaster Progenesis Samespots, Melanie-II and REDFIN. These 2DE gel analysis softwares have auto matching algorithms which facilitate spot detection, matching and editing functions.

2.11.1 PDQuest 8.0 2D analysis software

PDQuest is a gel analysis software by BIORAD (UK) limited. Bio-Rad PDQuest 2D is fully integrated with Bio-Rad's ProteomeWorks system for proteome discovery research, offering solutions to bottlenecks in sample tracking, data assimilation, and protein identification and characterization. Other than gel analysis PDQuest also controls the spot cutter excision robot and also tracks samples and data through mass spectrometric protein identification. It is a user friendly software and is fully automated. The software is designed for automated spot matching algorithms which use mathematical algorithms like Gaussian model for higher accuracy in spot quantitation. Another feature included filtering based on spot quantity and quality and import of SSP (sample spot protein) numbers after conversion to PDF files which can be viewed in windows. The features include sharing and matching gel images in multiple experiments, import and export of images and data as excel compatible files. Gels can be improved and templates can be set designed for image analysis in which parameters like faint spot, small spot, largest spot cluster and hiding of vertical and horizontal streaks can be done and settings can be stored. All the images for which analysis has to be done can be visualized in a tile format and cross hair can be placed in them. When image analysis has to be done then master gel can be selected followed by loading of template file.

2.12 Mass spectrometry

Mass Spectrometry (MS) can be described as the study of gas-phase ions. The main goal of mass spectrometric experiments is to characterize the structure of protein/ peptide molecules. MS has become a valuable tool in the field of biochemistry with the development of fast atom bombardment (FAB) in 1981 (Barber *et al.*, 1981). Electrospray ionization (ESI) was introduced by Fenn and co-workers (Fenn *et al.*, 1989) and matrix-assisted laser desorption/ionization (MALDI) was introduced by Karas and Hillenkamp (1988). MS is helpful in the identification and characterization of large biomolecules such as proteins and peptides.



(Source: Salekdeh, *et al.* 2002)

Fig 2.6: A schematic representation of comparative proteomics using 2-DE followed by MS

The most attractive properties of MS with these new ionization methods are high informational content of MS data, high sensitivity (low attomole levels), rapidity, versatility, and accuracy. The diagram in Fig 2.6 shows a flow chart for the analysis of proteomes by MS. Proteins are separated by 1-DE or 2-DE, after which they are visualized and selected for identification. The protein spots or bands are excised and after removal of SDS, stain, and salts, the proteins are digested by a site-specific protease usually trypsin. Peptides are extracted and a small fraction is used to determine the mono-isotopic peptide ion masses by MALDI-time-of-flight (TOF) MS. The experimental peptide ion masses (Mr expt.) are then searched against peptide mass predicted (Mr calc) from protein databases. If no positive identification can be achieved, (partial) sequences of the remaining peptides are determined by MALDI-TOF post-source decay (PSD)/MS or ESI-MS/MS. The sequence tags (partial sequence, together with the parent- and fragment ion masses) are used to search against EST or genomic data. Fig 2.6 illustrates the steps from 2-DE to mass spectrometry and protein identification as adapted from Salekdeh *et al.* (2002) with minor modifications.

2.12.1 An overview of the instrumentation

Mass spectrometers have mainly three essential parts, namely the ionization source, the mass analyzer, and the detector. The first part is the ionization source (e.g. ESI, MALDI) which produces ions from liquid and solid the sample. The second part is the mass analyzer (e.g. quadrupole, time-of-flight (TOF)) which resolves ions based on their mass-to-charge. The third and last part is the detector (photomultiplier, microchannel plate, electron multiplier) which detects the ions resolved by the mass analyzer. In short, the basic process associated with a mass spectrometer is generation of gas-phase ions derived from an analyte and the measurement of those ions according to their mass-to-charge ratio. Each of these three parts of mass spectrometer is housed inside a vacuum-pump system. Fig 2.7 shows schematic diagram of a typical MS.

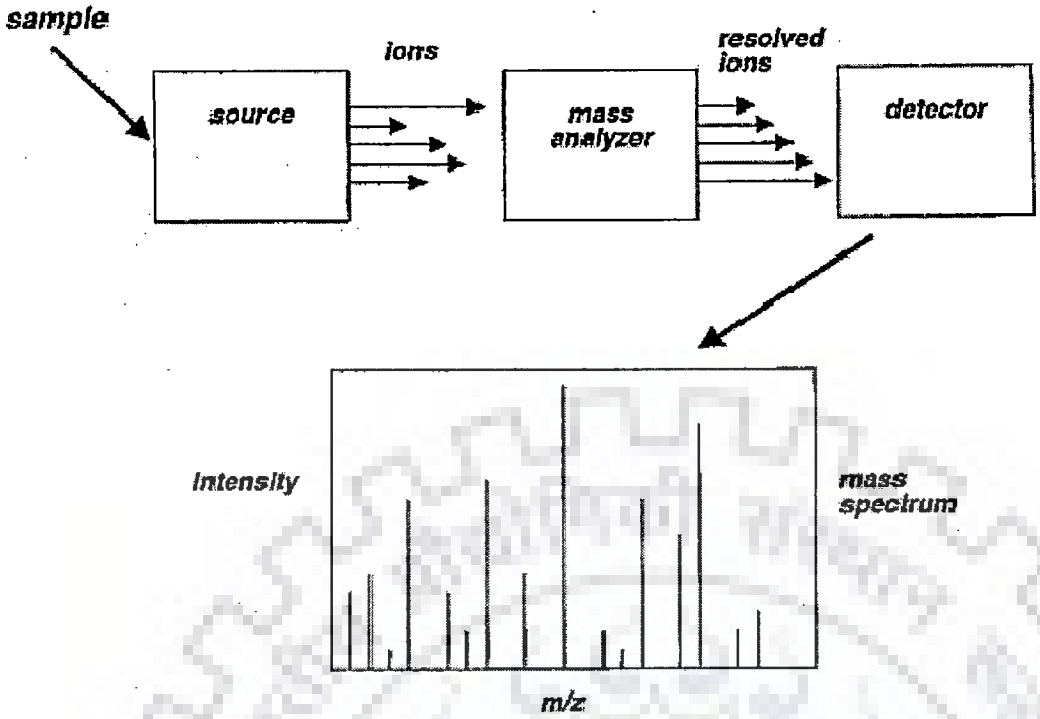


Fig 2.7: Schematic representation of mass spectrometer (Source: Liebler,2002)

2.12.2 Nano-LC-MS-MS

Vensel *et al.* (2002) identified wheat endosperm proteins at 10dpa and 36dpa using MALDI-TOF and LC-MS-MS and identified a total of 250 spots. MALDI-TOF was used to identify 30% endosperm proteins but to identify the major 80% of salt soluble proteins which could not be identified by peptide mass mapping using MALDI-TOF the authors developed nano-LC-MS-MS. Therefore as the sensitivity of LC-MS-MS is more authors proposed MALDI-TOF in first stage of sample screening and LC-ESI-MS-MS for samples which were not detected by the first technique.

2.13 MS data analysis

The softwares used for MS/MS peptide identification help in data acquisition and analysis of MS results. These include SEQUEST, Mascot, X!Tandem, X!!Tandem, Phenyx, OMSSA (Open Mass Spectrometry Search Algorithm), MyriMatch, greylag, ByOnic, InsPecT, SIMS and MassWiz. De-novo sequencing algorithms include DeNoS, PEAKS and Lutefisk.

2.13.1 Mascot database

Mascot is an identification program available from Matrix Science. It performs mass spectrometry data analysis through a statistical evaluation of matches between observed and projected peptide fragments rather than cross correlation. The different searching methods in Mascot include peptide mass fingerprint, sequence query and MS/MS ion search. Mascot search can be restricted to a particular species using the taxonomy filter. A default significance level of 5% is used for the search. A mass spectrum of the peptide mixture resulting from the digestion of a protein by an enzyme provides a fingerprint. Mascot searches the database on the basis of the protein molecular mass observed on a 2DE gel. In sequence query peptide mass data are combined with amino acid sequence, composition and fragment ion data. The sequence information has to be given in standard one letter code. The sequence information is preceded by a prefix with a one letter code indicating the sequence type. An example has been shown in Table 2.5 and the query depicted matches peptide with sequence ACDEFGHI. The composite information consists of a number followed by the corresponding amino acid between square brackets and an asterisk. The example shows a peptide containing 2 histidines, no methionines, 3 acidic residues (glutamic or aspartic acid) and at least 1 lysine. On the other hand ion

Table 2.5: Sequence query information codes for Mascot search

Sequence Information	Prefix	Meaning	Example
	b-	>N-C	Seq(b-DEFG)
	y-	C->N sequence	Seq(y-GFED)
	*-	Orientation unknown	Seq(*-DEFG)
	n-	N terminal sequence	Seq(n-ACDE)
	c-	C terminal sequence	Seq(c-FGHI)
Composite Information	comp(2[H]0[M]3[DE]*[K])		
Ion information	Prefix	Meaning	Example
	b-	b series ions	ions(b-m_1:i_1,m_2:i_2,m_n:i_n)
	y-	y series ions	ions(y-m_1,m_2, …,m_n)
			ions(m_1:i_1,m_2:i_2, ,m_n:i_n)

information includes a prefix indicating the type of ion series the m/z values belongs to. Therefore, sequence tag qualifier consists of the observed mass of the first peak of an identified sequence ladder, a stretch of interpreted amino acid sequence, and the observed mass of the final peak of the ladder. An error tolerant tag finds a match by adjusting peptide mass by fitting in the two possibilities on the N-terminal side or the C-terminal side. As in error tolerant tag as the constraint on the peptide mass is dropped, if one tag is error tolerant, then any other tags for the same query are also treated as error tolerant, even if they have been entered as standard tags. Hence, it is not possible to mix ions qualifiers and sequence tags. The MS/MS ions search accepts data in a number of different peak list formats. In this either a single MS/MS spectrum or a multi-dimensional LC-MS/MS run containing data from many thousands of peptides can be searched. The search parameters include taxonomy, database, enzymes, mass tolerances, charge and instrument.

An enzyme of low specificity, which digests proteins to a mixture of very short peptides should be avoided because almost any given 3 or 4 residue peptide will be found in many database entries. Generally trypsin is used as it produces longer peptides and therefore inceases the specificity. A minimum of variable modifications should be used for a sensitive search. An error tolerant search is a better way to find rare modifications than selecting them as variable modifications. In an MS/MS search, the peptide mass tolerance determines the number of candidate peptides tested for a match which affects the significance threshold score, but not affect the ions score. The match is same match whether there is 1 candiate or 10,000 candidates. The number of candidates simply determines whether the match is significant or not. The instrument setting determines the set of ion series that are considered when trying to find a match. Most of the instruments are very similar and only when the data come from an ECD (electron capture dissociation) or ETD (electron transfer dissociation) experiment does instrument selection becomes critical as that produce c and z ions, not found in most of the other instrument types.

Chapter III
Materials and Methods

3.1 Plant material

3.1.1 Development of NILs

The plant material consisted of a set of bread wheat (*Triticum aestivum* L.) Near Isogenic Lines (NILs) and cultivar PBW 343 as control. The NILs were derived from a cross between wheat cultivars PBW 343, amber coloured and PHS susceptible and SPR8198, red coloured and PHS tolerant with PBW 343 as the recurrent parent (Garg *et al.*, 2007). The NILs were graded on a scale of 0-9 on the basis of PHS tolerance. Out of 5 NILs i.e. PHST 0, PHST 2, PHST 3, PHST 6 and PHST 9 two were selected for proteomic studies. PHST0 has red seed colour and is PHS tolerant, while PHST9 has amber coloured seed and is PHS susceptible like the parent PBW 343.

3.1.2 Collection and storage of seeds

The seeds were collected at three stages of development. The stages were selected according to the cereal decimal code (Zadoks *et al.*, 1974). These included soft dough stage (stage85), hard dough stage (stage87) and dry caryopses (stage95). Individual packets were made for PBW 343, PHST 9 and PHST 0 and the material was stored at -80°C till further use.

3.1.3 Characterization of NILs

Two NILs were tested for PHS according to Kulwal *et al.* (2004). NILs near physiological maturity were harvested and spikes were soaked overnight in water. In the morning they were placed on a wet sand bed and covered with moist jute bag. Care was taken to keep the jute bag moist throughout the experiment so the spikes were sprinkled with water every 3–4 hours. The level of PHS tolerance was monitored for sprouting daily in the ten day experiment.

3.1.4 Characterization of *T. aestivum* landraces

A total of 25 landraces of wheat were tested for PHS tolerance. The details of the IITR landraces has been given in Annexure III. The landraces were collected from hilly regions of

Uttarakhand and showed variability in there tolerance to PHS. Their tolerance to PHS was tested using moist jute bag experiment (Kulwal *et al.*, 2004).

3.2 Germination test

Ten seeds each of the two NILs and PBW 343 were used for the test. Both the seed material kept at room temperature and that stored at -80°C were used for the experiment. The seeds stored in both the conditions were sterilized with 10% solution of sodium hypochlorite. Sterilization was done to avoid fungal growth during the experiment duration. Two petri plates each for PBW 343, PHST 9 and PHST 0 were used. In one petri plate seeds stored at room temperature were used for germination test and in other seeds stored at -80°C were used. Same germination conditions were used for all the seeds. The filter paper was moistened with milliQ water and care was taken not to let the filter paper dry.

3.3 Effect of growth regulators on seed germination

The selected NILs along with parent PBW 343 were analysed for effect of selected exogenous hormones abscisic acid (ABA) and gibberellic acid (GA_3). The concentration of hormones used ranged from 10ppm to 100ppm for ABA and 100-500ppm for GA_3. The seeds of PBW 343, NILs PHST 0 and PHST 9 were sterilized with 10% sodium hypochlorite and grown on petri plates with three layers of Whatmann no.1 filter paper. In general the solutions were not added more than twice for a period of ten days. Germination was monitored on a daily basis and data was recorded. In the experimental part in which the seed material had to be supplied with different concentrations of ABA or GA milliQ water was not used and the seeds were only moistened with respective ABA or GA solutions prepared in milliQ water. The petri plates were kept sealed with parafilm to avoid contamination and it also helped prevent drying of the seed material. The extent of sprouting and germination was monitored and recorded daily. All the experiments were given the same treatment with same volume of milli Q water and respective ABA or GA solutions added at same time intervals. The experiment was repeated three times for normalization of results.

The experiment was performed for the whole seeds as well as embryonic end half seeds. The filter papers were then moistened with the respective concentrations of the phytohormones as described above. The petriplates were kept covered with 12 hours of illumination. The germination rates were recorded daily for ten days.

3.4 Scanning electron microscopy

To study the comparative seed anatomy between PHS tolerant (PHST0) and susceptible (PBW343 and PHST9) lines, scanning electron microscopy (SEM) was done. Wheat seeds were soaked in Formaldehyde-acetic acid-alcohol (FAA) fixative consist of 60% absolute ethanol, 4% formaldehyde, 5% glacial acetic acid & 15% of milliQ water for 24 hours. Thereafter, seeds were washed with 2.5% gluteraldehyde for 2 hours, 50% gluteraldehyde for 5 minutes, 70% gluteraldehyde for 30 minutes (2 times) and 90% gluteraldehyde for 30 minutes (2 times) respectively. Seeds were then dehydrated in absolute alcohol for 30 minutes and then cut transversally and fixed on metal plate. Fixed seed transverse sections were bombarded with gold particles in vacuum (BAL-TEC SCD 005 sputter coater) and analyzed on Scanning electron microscope (FEI Quanta 200F). At least three sections were studied for all the three lines.

3.5 Isolation and purification of genomic DNA from leaf tissues

DNA was extracted from young leaves of PBW 343, NILs PHST 0 and PHST 9 and ten selected landraces by using CTAB method described by Murray and Thomson (1980).

3.5.1 DNA extraction buffer

The composition of DNA extraction buffer is as described below.

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

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

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

3.5.2 DNA isolation and purification reagents

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

3.5.3 DNA isolation protocol

About 5-7g of young, healthy and disease free leaves from each plant were collected and kept in the plastic bags on ice. Leaves were frozen in liquid nitrogen and crushed to fine powder using autoclaved and pre-chilled mortar and pestle. The powder was transferred to 50ml Oakridge tubes containing pre-warmed (65°C) DNA extraction buffer (15ml for approximately 3g of leaves). It was gently mixed and incubated in 65°C water bath for 1 hour, mixing briefly every 15 minutes. Equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) solution was added to the samples followed by gentle mixing for 15 minutes to ensure emulsification of phases. The samples were centrifuged at 10,000rpm for 20 minutes at 25°C. Supernatants were transferred to the falcon tubes with the help of pipettes. Equal volume of ice cold propon-2-ol was added and left overnight at 4°C for complete precipitation of DNA. DNA was spooled out using large bore pipette tips into the 1.5ml microcentrifuge tubes. It was centrifuged at 8000rpm to get a pellet of DNA. Supernatant was discarded and pellet was washed with 400µl 70% ethanol followed by centrifugation at 8000rpm for 5 minutes. Ethanol was drained out, pellets were air dried and resuspended in 500µl TE buffer. Subsequently RNase treatment at final concentration of 100µg/mL was done at 37°C for 1 hour. The DNA was re-extracted with fresh chloroform: isoamyl alcohol followed by reprecipitation with ethanol and pelleting by centrifugation (8000rpm, 4°C). Pellet was collected, air dried (37°C) for few hours and dissolved in appropriate volume of 1X TE. For DNA quantification, spectrophotometric readings of the DNA samples were taken at wavelengths 260nm and 280nm. Ratio of

OD260/OD280 was checked to be around 1.8 as a measure of DNA purity. At wavelength 260nm, the concentrations of DNA (OD260x 50x dilution factor) were determined and subsequently samples were diluted to 50ng/µl concentration. Electrophoresis (Sambrook and Russell, 2001) was carried out for the qualitative and quantitative analysis in 0.8% agarose gel with 0.5µg/ml ethidium bromide (10mg/ml) in 1X TAE.

3.6 SSR marker analysis

SSR markers mapped on chromosome 3A of wheat flanking a QTL for PHST (Kulwal *et al.*, 2004) were used for validation of NILs and recurrent parent and ten selected IITR landraces. This QTL present on chromosome 3AL explained 78% of phenotypic variation (Kulwal *et al.*, 2004). The remaining seven SSR markers were mapped on chromosome 3AL between wmc153

Table 3.1: Chromosome location and primer sequences of various SSR primers used for validation of QTL for PHST in wheat

SSR Marker Name	Chromos -ome location	Tm (°C)	SSR marker Forward Primer	SSR marker Reverse Primer
wmc 153	3A	60	ATGAGGACTCGAAGCTTGGC	CTGAGCTTTTGCGCGTTGAC
gwm 155	3A	60	CAATCATTTCCCCCTCCC	AATCATTGGAAATCCATATGCC
cfa 2193	3A	60	ACATGTGATGTGCGGTCATT	TCCTCAGAACCCCATTCTTG
wmc559	3A	60	ACACCACGAATGATGTGCCA	ACGACGCCATGTATGCAGAA
wmc215	3A	61	CATGCATGGTTGCAAGCAAAAG	CATCCCGGTGCAACATCTGAAA
wmc169	3A	61	TACCCGAATCTGGAAAATCAAT	TGGAAGCTTGCTAACTTTGGAG
wmc388	3A	60	TGTGCGGAATGATTCAATCTGT	GGCCATTAGACTGCAATGGTTT
wmc594	3A	51	CCCCTCACTGCCG	ATATCCATATAGTACTCGCAC
barc57	3A	55	GCGACCACCTCAGCCAACTTATTATGT	GCGGGGAGGCACATTCATAGGAGT
barc170	4A	50	CGCTTGACTTTGAATGGCTGAACA	CGCCCACTTTTTACCTAATCCTTTTGAA
gwm 269	4A	60	TGCATATAAACAGTCACACACCC	TTTGAGCTCCAAAGTGAGTTAGC
wmc89	4A	51	ATGTCCACGTGCTAGGGAGGTA	TTGCCTCCCAAGACGAAATAAC
wmc 420	4A	51	ATCGTCAACAAAATCTGAAGTG	TTACTTTTGCTGAGAAAACCCT
zxq118	4A	56	CTGACTGATATACGGCAATC	ATGTGATTGGTTGATCAAGCG

and gwm155 on genetic map of Somers *et al.* (2004). In case of chromosome 4AL, two SSR markers barc170 and gwm269 were the flanking QTL markers explaining 21% phenotypic variation (Mares *et al.*, 2005). Other two SSR markers wmc89 and wmc420 were selected from Somers *et al.* (2004) located near the flanking QTL markers on chromosome 4. The marker zxq118 was selected from Zhang *et al.* (2008b). In addition to PHST0 and PBW343, Ten wheat landraces were selected for SSR studies. A total of 14 SSR markers were used for the study. Out of these 9 SSR markers were specific to chromosome 3 and 5 were specific for chromosome 4 of wheat. The details of SSR markers are given in Table 3.1. The selection of SSR markers was based on major QTLs reported for dormancy (Kulwal *et al.*, 2004; Mares *et al.*, 2005; Zhang *et al.*, 2008b). Polymorphism for SSR markers was checked on 10% polyacrylamide gel or 2% super fine agarose and visualized using ethidium bromide.

3.6.1 Composition of PCR master mix

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

3.6.2 PCR conditions

The PCR was carried on Eppendorf Thermocycler with following conditions:

Initial denaturation at 94°C for 4 minutes; 35 cycles of - denaturation at 94°C for 1 minutes and annealing at 50-68°C depending upon the primer T_m for 1 minute; extension at 72°C for 1 minute; Final extension at 72°C for 7 minutes. The conditions for SSR amplification were kept as above unless otherwise mentioned as in case of zxq118. The PCR amplification conditions for the primer were 94°C for 3 minutes, 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds, repeat cycling 30-35 times, followed by 72°C for 5 minutes final extension (Zhang *et al.*, 2008).

3.6.3 Resolution of the amplified SSR product

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

3.7 RNA isolation

RNA from seeds collected at soft dough stage 85 was isolated using the hot phenol method. The composition of 50ml extraction buffer is as follows:

1M Tris-Hcl (pH 8.0)	5ml
8M LiCl (lithium chloride)	0.625ml
0.5M EDTA (pH 8.0)	1.0ml
SDS	0.5g
DEPC treated water	43.2ml

The procedure used for RNA extraction was described by Köhrer and Domdey (1991). Equal volume of phenol (pH 4.7) was added to the extraction buffer i.e. to a final concentration of 1:1 and the mixture was heated to 80°C for at least half an hour. The tissue was ground in liquid nitrogen and 500µl of extraction buffer with phenol was added followed by homogenization by vigorous vortexing. Then half volume of chloroform was added to the samples followed by vortexing. 1.5µl microfuge tubes were then centrifuged at room temperature for 30 minutes at 4000rpm and the supernatant was transferred to a fresh tube. This was followed by addition of one-third volume of 8M lithium chloride to the supernatant. The above solution was precipitated on ice for two hours. After precipitation the 1.5ml microfuge tubes were centrifuged at 10,000 rpm for 30 minutes at 4°C. Then the supernatant was discarded and RNA pellet was washed with 3ml of 2M LiCl and again cetrifuged at 10,000rpm for 5 minutes at room temperature. RNA pellet was then washed with 3ml of 70% DEPC treated ethanol

followed by centrifugation at 10,000rpm for 5 minutes at room temperature. After centrifugation ethanol was discarded and pellet was air dried. Finally RNA was dissolved in 2ml of DEPC treated water. RNA solution was centrifuged at 10,000rpm for 10 minutes at 4°C and supernatant transferred to a new tube. Then 210µl of sodium acetate (3M, pH 5.2) and 5750µl of 100% ethanol were added at room temperature. RNA was precipitated at –80°C for 20 minutes and centrifuged at 10,000rpm for 15 minutes at 4°C. The pellet was washed with 1ml 70% ethanol at room temperature followed by centrifugation at 10,000rpm for 5 minutes at 4°C. The pellet was air dried and RNA was dissolved in 500µl of DEPC treated water.

3.7.1 RNA quantification

RNA was quantified using UV visible spectrophotometer. 1ml of DEPC water was used as blank. 2µl of RNA solution was added to 998µl of DEPC water and 260nm/280nm and 260nm/230nm readings were taken.

3.7.2 cDNA synthesis

For cDNA synthesis 1µg of RNA, 4µl autoclaved milliQ water and 1µl oligodT were mixed. This mixture was kept at 65°C for 10 minutes and 2 minutes at room temperature. Then the mixture was pulse centrifuged. 10µl of reaction mixture was used with the composition as 2.5X RNase inhibitor 0.25µl, 10X DTT 1.0µl, 5X reverse transcriptase buffer 4.0µl, dNTP 2.0µl, reverse transcriptase enzyme 0.3µl and milliQ water 2.45µl. The PCR profile for cDNA preparation was 42°C for 1 hour and 95°C for 2 minutes. cDNA was stored at -20°C till further use.

3.8 Transcriptome study

The expression of dihydro flavonol 4 reductase (DFR), myb10 and protein disulfide isomerase (PDI) were studied using gene specific primers. RNA was extracted from soft dough stage85 of PBW 343 and NILs PHST 9 and PHST 0 and after cDNA synthesis, Reverse Transcriptase PCR (RT-PCR) was done.

3.8.1 Primer designing for RT-PCR

Primers were designed from the mRNA sequences for two genes i.e. PDI (protein disulfide isomerase) and myb10. PDI was chosen for RT-PCR because it was identified in endosperm protein 2-DE for PHST0. Primer sequences for DFR (dihydro flavonol reductase) were taken from Himi and Noda (2004). The primer sequences of the three gene specific primers have been given in Table 3.2.

Table 3.2: Gene specific primers used for RT-PCR of various genes responsible for PHST in wheat

Gene/ Transcription factor	Tm (°C)	Forward Primer	Reverse Primer
Myb10	55	CACTGGCGAATCGAACATAG	TCAGGGTGGTGTTCCAGTAG
DFR	58	TAGTATTCATGCAGCAGCAGT	AGAACAAGCCATTGCTGGAG
PDI	57	CCACATTGTTGGTGTCTTCAC	CAACAACGAGCTCATCAAA

3.8.2 RT-PCR

RT-PCR was done by using 2µl of cDNA each for the three gene specific primers. The master mix composition used for RT-PCR was same as that used for genomic DNA PCR except that cDNA is used instead of DNA. The PCR profile used was 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 40 seconds, 72°C for 45 seconds and 72°C for 4 minutes. The profile for DFR was kept same as described by Himi and Noda (2004).

3.9 Protein extraction

All the chemicals were electrophoresis/biological grade (Sigma Aldrich) and Milli Q pure water (18Ω) was used during study. All experiments were carried out at 4°C unless other temperature is stated and sterile latex gloves were used throughout to avoid contamination

3.9.1 Protein extraction from whole seed

Protein extraction was performed according to Hurkman and Tanaka (2007) using urea buffer. Briefly, the composition of buffer was 2M urea, 10% glycerol, 65mM DTT (to be added just before extraction) and 20mM Tris, pH 8.0. With urea buffer the extraction was not carried out at 4°C as urea crystallizes at low temperature, so all extractions were done at room temperature.

The seeds were crushed to a fine powder in liquid nitrogen and urea buffer was added as 4ml for 1g seed. The mixture was then incubated for one hour with constant shaking at room temperature. The insoluble material was centrifuged at 15°C at 5000g for 10 minutes. The supernatant was collected in fresh 2.0ml eppendorf tubes and four times ice cold acetone was added to each tube and the contents were mixed well. The 2.0ml eppendorf tubes were left at -20°C overnight. Following day 2.0ml eppendorf tubes were centrifuged at 15°C at 5000g for 10 minutes. The pellet obtained was washed with cold acetone and the pellet was broken into particles with the help of micropipette and vortexing. The eppendorf tubes were left at -20°C for 20 minutes and centrifuged at 5000g at 15°C for 20 minutes. Two acetone washings were given. The washed pellet was left to air dry before solubilization in lysis buffer. Lysis buffer was same as rehydration buffer except that it did not contain ampholytes. After solubilization the protein extracted was stored in 40µl aliquots at -20°C till further use.

3.9.2 Protein extraction from embryos

The embryos were excised from mature seeds and immature seeds on ice. The excised embryos were then frozen in liquid nitrogen and stored at -80°C. The buffer composition is as described below-

1. Tris extraction buffer: The composition is as described below.

For 10ml- 500µl 1M Tris, pH 7.5(50mM)
20µl 0.5M EDTA, pH 8.0 (1mM)
40µl 0.5M DTT (2mM)

2. Phenol extraction buffer: The composition is as described below.

For 20ml- 4.8g Sucrose (0.7M)
1.2g Tris (0.5M)
0.6ml 1N HCl (30mN)
2.0ml 0.5M EDTA (50mM)
0.15g Potassium chloride(0.1M)
0.4ml 2-mercaptoethanol 2%(v/v)

For both the buffers final volume was made with milliQ water.

Thirty embryos were homogenised in 0.5ml of ice cold Tris extraction buffer and transferred to 2.0ml eppendorf tubes. The homogenizer was washed with 0.2ml of Tris extraction buffer. Therefore, the final volume of the extract was 0.7ml. The eppendorf tubes were then centrifuged at 14,000rpm for 10 minutes at 4°C and the pellet obtained was washed with Tris extraction buffer. After washing 0.7ml of phenol extraction buffer was added and eppendorf tubes were stored in ice for 10 minutes. Then equal volume of phenol extraction buffer was added and the tubes were shaken at room temperature for 10 minutes. This was followed by centrifugation at 14,000rpm for 10 minutes at 4°C. The upper phenol phase was collected in separate tube and equal volume of phenol extraction buffer was added and tubes were shaken at room temperature for 10 minutes. The tubes were again centrifuged at 14000rpm for 10 minutes at 4°C. Upper phenol phase was collected and 5 volumes of 0.1M of methanolic ammonium acetate was added. The eppendorf tubes were stored at -20°C for 1.5 hours followed by centrifugation at 10,000rpm for 10 minutes at 4°C. The protein pellet obtained was washed 5 times with methanolic ammonium acetate and once with acetone. The protein pellet was then vacuum dried and solubilised in lysis buffer. The embryo protein obtained was stored in -20°C in 20µl aliquots till further use.

3.10 Bradford protein assay for protein determination

The Bradford protein assay is a spectroscopic analytical methods used to determine the total protein concentration of a sample (Bradford, 1976). This method is based on the absorption shift from 470nm to 595nm in the Coommassie brilliant blue G-250 dye when it binds to protein. Upon addition of sample, the dye binds to protein, resulting in a colour change from green to blue. For that reason, the Bradford method is also known as colorimetric protein assay as the protein concentration increases, the colour of the test sample becomes darker. The CBB G-250 dye binds to protein through an electrostatic attraction of the dye's sulfonic groups, theoretically to arginine, lysine, and histidine residues. Additionally, the dye also binds weakly

to the tyrosine, tryptophan, and phenylalanine via van der Waals forces and hydrophobic interactions. Bradford assay has a linear dyanmic range, generally from 2µg/ml to 120µg/ml, it is usually necessary to make dilutions of sample before analysis. As analytical rule, at least five protein standards are needed to determine the protein concentration of a test sample. Thus, it is important to generate a standard curve using a protein of known concentration.

In this study Bovine serum albumin (BSA) was used as a standard protein to compare with sample protein concentration. For Bradford analysis 1mg/ml stock of BSA standard was prepared by dissolving 1mg BSA in 1ml of lysis buffer. 1ml Quartz cuvettes were used for absorbance measurement in UV-visible spectrophotometer at 595nm. 1ml of Bradford reagent was mixed with appropriate amount of standard or BSA and allowed to incubate at room temperature for 5 minutes. Absorbance of each sample was measured at 595nm using a UV-visible spectrophotometer. The standard curve was established by plotting the absorbance at 595nm versus concentration of protein in BSA standard samples. The best straight line was determined in the form of "y=mx + b" where y is absorbance reading at 595nm and x is protein concentration. At the end, this equation was used to calculate the concentration of the protein sample based on the measured absorbance.

3.11 Protein solubilization with rehydration buffer

The rehydration buffer consists of urea, CHAPS, DTT, and Ampholyte pH 3-10. The stock solution was prepared by adding 4.2g of urea, and 0.2g of CHAPS in milliQ water to a final solution volume of 10ml. The solution was gently heated at 37°C and the solution should not be heated beyond this temperature range to prevent urea breakdown. After the dissolution of above components the buffer was stored at 4°C. A pinch of DTT and 1.25µl of Ampholyte (pH 3-10) were added as DTT and ampholyte must be added fresh. Appropriate amount of protein is added in the rehydration buffer. For 11cm IPG strips total volume of rehydration buffer to be used in BIORAD Protean-II IEF cell is 285µl. So, if 20µl protein (equivalent to 90µg concentration) has to be used then 165µl of rehydration buffer along with DDT and

ampholytes were used to make total volume to 185µl. The rehydration buffer mixture was spread on rehydration tray and the IPG strip was placed on it without trapping bubbles. After half an hour the IPG strip was overlaid with 2-3ml of mineral oil. IPG strip was rehydrated for 12-16 hours before proceeding to IEF.

3.12 2-DE of Proteins

3.12.1 Isoelectric Focussing

The first dimension or isoelectric focussing (IEF) was carried out in IPG strips (11 cm, pH 3-10, linear gradient, from Bio-Rad) by using a Protean IEF Cell (Bio-Rad Inc. USA). The focussing tray was selected corresponding to the IPG strip length chosen for the experiment and it must be clean and dry before IEF. Paper wicks were placed onto the electrodes and wetted with approximately 8µl milliQ water. In this work, IEF experiments were carried out in parallel for proteins of PBW 343 and PHST 0 under same experimental conditions. Same amount of protein (90µg) was loaded onto strips to compare up- and down-regulated proteins in susceptible and tolerant plant samples. The rehydrated IPG strips were transferred to clean focussing tray with gel side down and overlaid with 2-3ml mineral oil to minimize evaporation and urea crystallization. Care was taken not to trap any bubbles as they could interfere with IEF. Throughout focussing a constant temperature of 20°C was maintained inside Protean IEF cell. IEF voltage settings for 11cm IPG strips were programmed as follows:

Phase 1, linear gradient up to 250 Volts in 30 minutes; Phase 2, linear gradient up to 8000 Volts in 3 hours; Phase 3, rapid gradient up to 8000 Volts at 20,000 Volts hours. Working temperature was set to 20°C in IEF. Total focussing time was 6-8 hours without rehydration step. After the IEF run was complete, strips were removed from IEF focussing tray and mineral oil was drained. The focussed strips were placed at -80°C or immediately equilibrated with equilibrium buffers for second dimension of the experiment.

3.12.2 Equilibration of IPG strips

The strips containing the focussed proteins were equilibrated in equilibration buffers which contain SDS for transforming the focussed proteins into SDS-protein complexes. Besides DS, equilibrium buffer I had Tris-HCl pH 8.8, glycerol, urea, DTT, while equilibrium buffer II had iodoacetamide instead of DTT.

Composition of equilibration buffer I: 1.81g urea (6M), 1.25ml Tris-HCl pH 8.8 (0.375M), 0.1g SDS (2%), 1ml glycerol (20%), and 0.1g of DTT (2%). The final volume was adjusted to 5ml with milliQ water for one strip. DTT is added to equilibration buffer as it is necessary for cleavage of sulfhydryl bonds between cysteine residues within a protein and acts as a reductant.

Composition of equilibrium buffer II: 1.81g urea (6M), 1.25ml of Tris-HCl pH 8.8 (0.375M), 0.1g SDS (2%), 1ml glycerol (20%), and 0.125g iodoacetamide (2.5%). The final volume was adjusted to 5ml with milliQ water for one strip. Iodoacetamide was used as an alkylating agent for reduced sulfhydryl groups in the protein and as a scavenger of the excess reductant.

After preparation of equilibrium buffers, strips were laid gel side up into the disposable equilibrium tray and treated with equilibrium buffer I for 15 minutes with gentle shaking. After 15 minutes, strips were taken from one well of tray and placed into another dry well and treated with equilibrium buffer II for 15 minutes with gentle shaking.

3.12.3 SDS-PAGE

After removing strip from equilibrium buffers, strip was rinsed with 1X Tris-Glycine-SDS (TGS) running buffer before attaching to the polyacrylamide gel .

Composition of 5X Tris-Glycine-SDS (TGS) running buffer: 15.1g of Tris base and 94g of glycine were dissolved in 900 ml of water. Then, 50 ml of 10% (w/v) SDS solution was added and the final volume was adjusted to 1000ml with milliQ water. It was stored at room temperature till further use.

To prepare 100ml of 1X TGS buffer, 20ml of 5X TGS buffer was diluted to a final volume of 100ml with ultra pure water. The graduated cylinder was filled to a 100ml with 1X TGS buffer

and any bubbles on the surface of the buffer were removed by the help of plastic pasteur pipette. The equilibrated strip was dipped in 1X TGS buffer for 15 seconds. Therafter, The strip was transferred on 12% SDS-PAGE for second dimension separation of proteins according to their molecular weight differences.

3.12.4 Separation in second dimension

Second dimension SDS-PAGE was carried out in PROTEAN II xi Cell (Bio-Rad Inc. USA). 12% polyacrylamide gels containing 1.5M Tris-HCl buffer, pH 8.8, 10%SDS, 10% ammonium per sulfate, TEMED were used and the running buffer was composed of 1X TGS buffer. Glass plate sizes are 16 x 20 cm for inner plate and 18.3 x 20 cm for outer plate. 30% acrylamide mixture was prepared by dissolving 29.0g of acrylamide and 1.0g of N, N′ methylenebisacrylamide in 60ml of milliQ water. The total volume was adjusted to 100ml with milliQ water. This solution can be stored at 4°C in dark bottle. The acrylamide mix was not stored for more than a month as acrylamide and bisacrylamide are slowly converted to acrylic acid and bisacrylic acid. 1.5M Tris-HCl, pH 8.8 was prepared by dissolving 3.634g of Tris-base in 15ml of milliQ water and adjusting pH to 8.8 with concentrated HCl and making final volume of 20ml with milliQ water. It was stored at 4°C. 10% SDS was prepared by dissolving 1.0g of SDS in milliQ water to a final volume of 10ml. 10% ammonium per sulfate (APS) was prepared by dissolving 1.0g of APS in milliQ water to a final volume of 1.0ml. This solution should be prepared just before use. 12% polyacrylamide gel was prepared by mixing 16.5ml of water, 20.0ml of 30% acrylamide mixture, 12.5ml of 1.5M Tris-HCl (pH 8.8), 500µl of 10% SDS, 500µl of 10% ammonium per sulfate, 20µl of TEMED to a final volume of 50ml. The mixture was then poured between two glass plates and left for one to two hours to polymerize. The casted SDS-polyacrylamide gel was kept for at least one day at 4°C prior to electrophoresis in order to reduce protein modifications caused by free acrylamide or reagents involved in the polymerization process. Equilibrated IPG strip was placed onto the polyacrylamide slabs and sealed with 1ml of 0.5% (w/v) low melting agarose (0.5% agarose in 1X Tris-Glycine-SDS

with traces of bromophenol blue). There must not be any air bubbles between the IPG strip and the resolving gel interface. Once the overlay had solidified, the gels were placed in a buffer tank. The reservoirs were half filled with pre cooled 1X TGS running buffer and electrophoresis was begun. Electrophoresis run was made at constant current at 16 mA/gel for 45 minutes and followed by constant voltage at 150 Volts for at least 3 hours until the blue dye front reached the bottom of the gel.

3.13 Staining and destaining of gel

After electrophoresis was finished, the gel was taken out from two glass plates carefully into a large tray for staining. Two methods were used for gel staining.

3.13.1 Silver staining

The procedure for silver staining is as described-

Fixing solution- 50ml methanol + 40ml milliQ water + 10ml glacial acetic acid. The gels were soaked in this solution overnight. The gels were then washed three times with milliQ water for 5 minutes each.

Hypo solution- 40mg of hypo (sodium thiosulfate) was dissolved in 200ml of milliQ water. The gel was soaked in hypo solution for one minute and then washed three times with milliQ water.

Silver nitrate (or staining) solution- 0.4g of silver nitrate was dissolved in 200ml of milliQ water and 75µl of formaldehyde was added (prior to use). Gels were kept in staining solution for half an hour in dark. Thereafter, three quick washes were given with milliQ water.

Developing solution- 4g of sodium carbonate was dissolved in 200ml of milliQ water and the solution was chilled. Just before use 100µl of formaldehyde was added and the solution was used to develop protein spots.

Stopping solution- 1.4g EDTA or 10% acetic acid solution in 200ml of milliQ water was used to stop the staining process.

The stained gels were dried and stored.

3.13.2 Commassie staining

Initially the 2-DE gels were fixed using fixing solution. The composition of the fixing solution was same as described in section 3.12.1. For preparation of staining solution 0.25g of CBB R-250 was added to 100ml fixing solution (50ml methanol: 40ml milliQ water: 10ml glacial acetic acid). Care was taken to dissolve CBB R-250 in 2ml methanol before adding it to fixing solution to avoid formation of clumps as the dye has increased solubility in methanol. The staining solution was then filtered through Whatmann no.1 filter paper and stored in amber bottle at 4°C.

3.14 Imaging and data analysis of gel

After staining and destaining steps, the photograph of the gel was taken using BIORAD densitometer. PD Quest 8.0.1 advanced version image analysis software by BIORAD was used

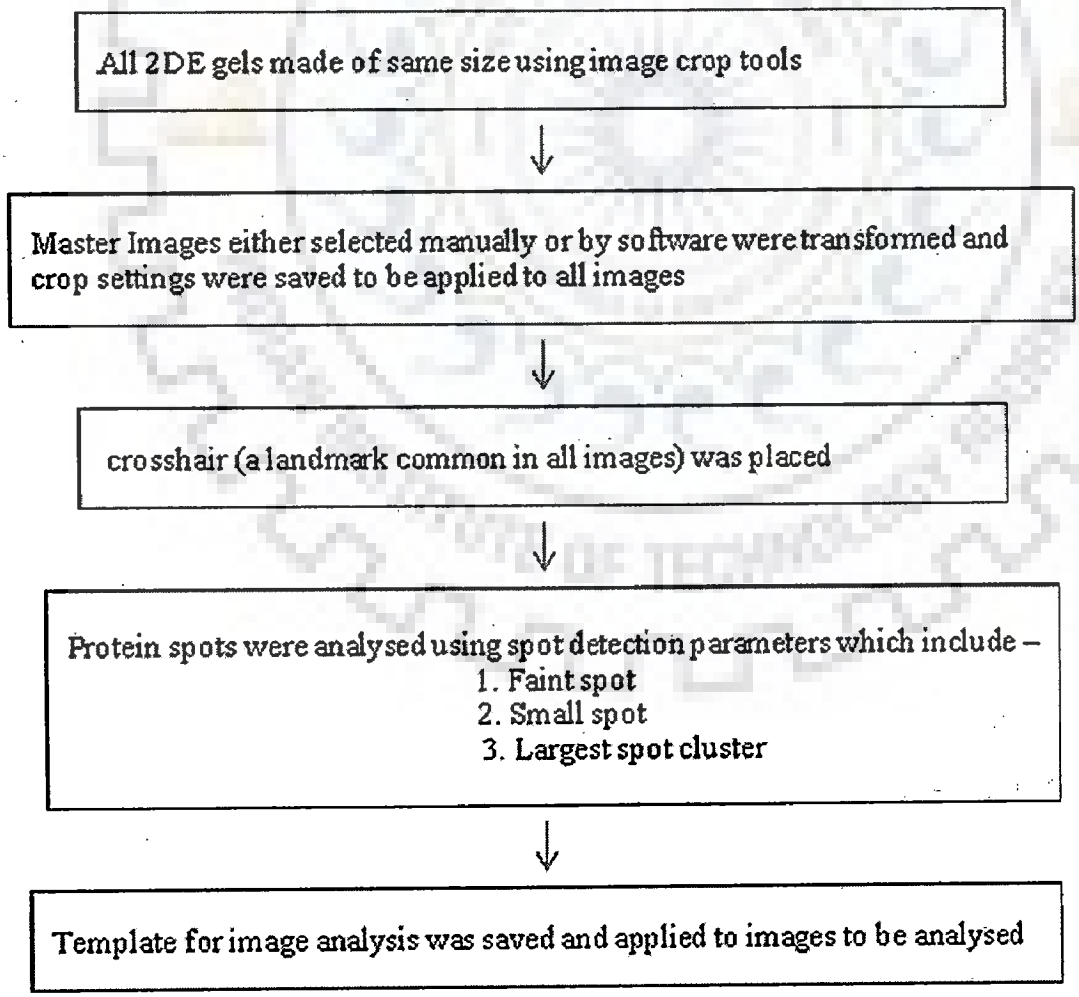


Fig 3.1: Flow diagram showing various steps for image detection by PD Quest image analysis software

for image analysis. Prior to image analysis all the raw 2DE gels were grouped. Template for image analysis was designed which included background subtraction and specification of spot detection parameters. The various steps for image analysis have been shown in Fig 3.2.

3.15 Reduction, alkylation and tryptic digestion of excised protein spots

Protein identification using nanoLC-ESI-MS/MS was done on contractual basis by The Centre for Genomic Application (TCGA, Okhla, New Delhi). The MS system consisted of an Agilent 1100 nano LC system. Protein spots were excised from the gel and in-gel digested with trypsin.

Spot cutting- The procedure included the following steps.

1. Band excision- The protein gel was rinsed two times for 10 minutes each with milliQ water. Then it was transferred on alcohol wiped transparency and the spot/band of interest were cut using a clean scalpel or razor blade. The spots were then transferred into clean eppendorf tubes.

2. Destaining of gel pieces : The composition of the solution used for destaining gel pieces was either 15mM potassium ferricyanide or 50mM sodium thiosulphate. For silver stained spots gel pieces were washed once with HPLC grade water and then washed twice with destaining solution for 10-15 minutes with continuous vortexing. Then again the gel pieces were washed twice with HPLC grade water for 10-20 minutes. This was followed by washing with destaining solution-I for 5 minutes.

3. Shrinking of gel pieces- The gel pieces or spots were shrunk or dehydrated by adding acetonitrile followed by incubation for 5 minutes at room temperature. Then acetonitrile was removed by air drying the gel pieces for 5 minutes.

4. Reduction and alkylation- Reduction solution comprised of 10mM DTT in 100mM ammonium bicarbonate. The reduction solution was added to the eppendorf tubes containing gel pieces followed by incubation at 56°C for 30 minutes. The tubes were left at room temperature for 5 minutes and the reduction solution was discarded. Then alkylation buffer containing 50mM iodoacetamide in 10mM of ammonium bicarbonate was added to gel pieces followed by incubation in dark at room temperature for 30 minutes. Then the alkylation buffer

was discarded and the gel pieces were washed by adding destaining solution-II (15mM potassium ferricyanide/50mM potassium thiosulphate) and vortexed for 10 minutes. The washing step was repeated twice.

6. The gel pieces were shrunk by adding acetonitrile followed by incubation at room temperature for 5 minutes followed by air drying for 5 minutes to remove acetonitrile.

Trypsin digestion- The gel pieces were swollen by adding 20ng/µl of trypsin solution to eppendorf tube depending on the size of gel piece. This was followed by incubation at room temperature for 10 minutes to allow the gel pieces to swell and absorb trypsin solution. The eppendorf tubes were then incubated at 37°C for 16 hours.

Peptide extraction- To extract the peptides after the overnight incubation peptide extraction solution was added according to the gel volume. The composition of peptide extraction solution was 1% TFA in ultra pure water. The tubes were then sonicated in water bath for 5 minutes.

3.16 MS (AGILENT LC-MS)

A 6µl solution of the tryptic digested sample was used for analysis with LC-MS/MS (Agilent, Palo Alto, CA, USA). The nano LC (nLC) was performed with Agilent 1100 NanoLC-1100 system combined with a microwell-plate sampler and thermostatted column compartment for preconcentration (LC Packings, Agilent). The samples were loaded on the column (Zorbax 300SB-C18, 150 mm X 75 µm, 3.5 µm) using a pre-concentration step in a micro pre-column cartridge (Zorbax 300SB-C18, 5 mm X 300 µm, 5 µm). Six microliters of the sample was loaded on the pre-column at a flow rate of 5µl/minute. After 5 minutes the pre-column was connected with the separating column. Then multistep gradient was started. The running profile for multigradient was 3% for 5 minutes, 15% for 5-8 minutes, 45% for 8-50 minutes, 90% for 50-55 minutes, 90% for 55-70 minutes and 3% for 71 minutes. The buffers used were 0.1% HCOOH in water which was solution A and 0.1% HCOOH in 90% acetonitrile which was solution B. The run took 85 minutes to complete. A LC/MSD Trap XCT with a nano-electrospary interface (Agilent) operated in the positive ion mode was used for MS. Ionization

(1.5 kV ionization potential) was performed with a liquid junction and a non coated capillary probe (New Objective, Cambridge, USA). Calibration of the instrument was performed using the standard Agilent tune mix. Peptide ions were analyzed by the data-dependent method which included full MS scan. The scan sequence consisted of one full MS scan followed by 4 MS/MS scans of the most abundant ions. Data was analyzed using Agilent Ion trap Analysis software version 5.2 and proteins were identified by database search against the Mascot database 3.17

3.17 MS data analysis using bioinformatics

Bioinformatics was used for bin mapping ESTs on wheat chromosome 3A identified using SSR marker sequences and protein mRNA sequences. For bioinformatics NCBI and Graingenes databases were used. This was done to identify the chromosome bins 3AL carrying the dormancy gene. For SSR markers, bin mapping was specifically done for QTL flanking markers as these can indicate the location of the dormancy gene in the bin. The group3 chromosome bin map of wheat was used for bin mapping (Munkvold *et al.*, 2004).The proteins identified by LC-ESI-MS/MS were also searched on NCBI for coding sequences and blastn was done with mapped wheat ESTs on graingenes. The ESTs belonging to 3Al, 3BL and 3DL were shortlisted from the list of EST matches. The tables regarding respective EST matches for SSR markers and selected proteins have been given in results (table4.5 and 4.6).

Chapter IV
Results

4.1 Seed colour and 1000 grain weight analysis

The seed colour was tested visually in light. Fig 4.1 indicates that PBW 343 and PHST 9 had amber coloured and PHST 0 had bold and red coloured seeds. During NIL development SPR8198 with red seed colour and PHS was used as the donor parent (Garg *et al.*, 2007). The red seed colour of PHST0 with high PHST indicates that PHST may be linked to red seed colour. The photographs were taken against white background to avoid any colour interference due to background. In addition to seed colour, 1000 grain weight analysis was also recorded. 1000 grain weight of PBW 343 was 38.5g, 36.8g for PHST 9 and 40.0g for PHST 0. This is also visible in Fig 4.1 where the seeds of PHST 9 look thinner in comparison to PBW 343 and PHST 0.

Fig 4.1: Seed Colour difference in *T. aestivum* cultivar PBW 343 and its NILs PHST 9 and PHST 0

4.2 Preharvest sprouting test

Five dry and physiologically mature spikes of the three lines were used for PHS tolerance test. Parent PBW 343 and NIL PHST 9 showed symptoms of sprouting on the second day of the experiment and by the end of ten days there was profuse germination in both the lines with proper development of shoots and roots (Fig 4.2a). On the

contrary NIL PHST 0 did not show any symptoms of sprouting even till ten days of exposure to moist and damp conditions (Fig 4.2b). These observations show the tolerance of NIL PHST 0 to preharvest sprouting.

4.3 Maintenance of dormancy

The seed material at stage 95 was tested for dormancy by imbibing the seeds on moist Whatmann no.1 filter paper. This germination test was performed on seed material kept at -80°C and that stored at room temperature. PBW 343 and PHST 9 sprouted on the second day in both the lines kept at -80°C and that at room temperature. While PHST 0 stored at the room temperature sprouted on 2^{nd} day and that stored at -80°C did not germinate till 10^{th} day. PHST in wheat lasts for not more than two months after harvest under room temperature. But storage of seeds at -80°C helped in maintenance of dormancy for about a year.

4.4 Comparison of seed structure using SEM

Transverse sections of PBW 343, PHST 9 and PHST 0 were cut to study the seed structure and are shown in Fig 4.3. It was observed that the thickness between epidermis and aleurone layer varied among the three lines. The scale used in the study was that 100μM is equivalent to 6.2cm. Using this scale the length of epidermis, aleurone, middle layer between epidermis and aleurone and total length of epidermis and aleurone layer were calculated. The results for the same are mentioned in Table 4.1. The PHS tolerant NIL PHST 0 had approximately 1.3 times thicker epidermis, 1.5 times intermediate layer thickness and 1.1 times aleurone layer thickness as compared to PBW 343 and NIL PHST 9. These results suggest that as the seed coat of PHST 0 is thicker than PBW 343 and PHST 9, so it might be resistant to penetration of water. This is just a preliminary study, so PHS dormant and susceptible lines like NIL need to be studied using SEM to come to a valid conclusion.

Fig 4.1a: Preharvest sprouting test for PBW 343 and PHST 9

Fig 4.2b: Preharvest sprouting test for PBW 343 and PHST 0

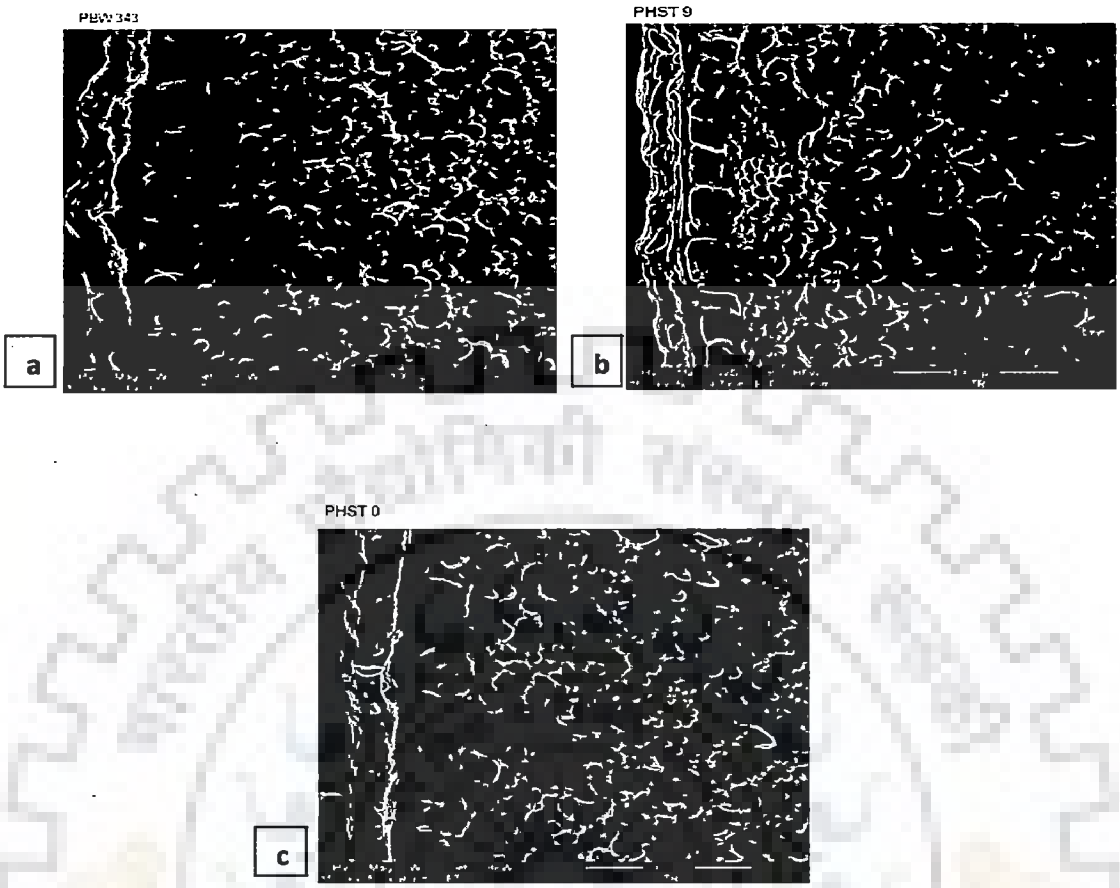


Fig 4.3: SEM images of TS of mature seeds of *T. aestivum* a) cultivar PBW 343 and its NILs b) PHST 9 and c) PHST 0. AL stands for aleurone layer and ED stands for epidermis. Arrow indicates the space between AL and ED

Table 4.1: Thickness of seed layers observed by SEM (TS) of seeds of wheat cultivar PBW343 and NILs

Cultivar name	**Epidermis thickness**	**Intermediate thickness**	**Aleurone thickness**	**Epidermis + Aleurone thickness**
PBW 343	24.19-32.25µm	8.06µm	32.25-38.70µm	56.45- 64.5µm
PHST 9	24.19- 30.6µm	8.06µm	30.64- 37.09µm	48.38-53.22µm
PHST 0	27.4- 40.32µm	12.90µm	40.32-41.93µm	72.5-80.64µm

Fig 4.4: Responses of PBW343, PHST9 and PHST0 upon treatment with different ABA concentrations. a) control, b) 60ppm ABA and c) 100ppm ABA

Fig 4.5: Response of PBW 343, PHST 9 and PHST 0 upon treatment with different GA concentrations. a) control, b) 200ppm GA and c) 500ppm GA

4.5 Effect of exogenously added phytohormones

The hormonal analysis included both ABA and GA_3 treatments which are important in governing dormancy and germination. Varying dosages of both the hormones were used in different experiments.

4.5.1 ABA treatment

In the control, germination was normal with PBW 343 and PHST 9 sprouting second day after moistening seeds, while PHST 0 did not sprout till ten days for which the experiment was set up. The results showed that ABA treatment did not inhibit germination in PBW 343 and PHST 9 as these lines germinated normally even at 100ppm ABA concentration indicating that PBW 343 and PHST 9 are ABA insensitive while PHST 0 is ABA sensitive. Fig 4.4 shows germination of PBW 343, PHST 9 and PHST 0 upon treatment with different concentrations of ABA. There was no germination in PHST 0 upto 5 days whereas almost all the seeds sprouted and germinated in PBW 343 and PHST 9 at all the ABA concentrations indicating that PHS susceptible lines are insensitive to ABA.

After ripened half seeds from embryo side of the three lines were studied for their germination behaviour using various concentrations of ABA. By cutting the seeds into half the germination of dormant line PHST0 could be stimulated thereby, allowing ABA dose dependent inhibition of germination studies. In half seed experiment the germination of PBW 343 and PHST 9 was only slightly delayed indicating that PBW 343 and PHST 9 were ABA insensitive. On the contrary PHST 0 half seeds stored at room temperature germinated on third day. The radical emergence was also monitored and the radicles of PHST 0 were found to be the smallest. The results for the analysis are shown in Table 4.2.

Table 4.2: Germination status of *T. aestivum* cultivar PBW 343 and its NILs at different concentrations of ABA

Plant name	**Abscissic acid (ppm)**	**Days for germination**				
PBW 343		**1st day**	**2nd day**	**3rd day**	**4th day**	**5th day**
	10	All sprouted	4 seeds germinated	6 seeds germinated	7 seeds germinated	All ten germinated
	30	All sprouted	4 seeds germinated	6 seeds germinated	7 seeds germinated	All 10 germinated
	60	4 seeds sprouted	6 seeds sprouted	6 seeds sprouted	7 seeds germination	9 seeds germinated
	100	4 seeds sprouted	5 seeds sprouted	7 seeds sprouted	7seeds germinated	8 seeds germinated
PHST 9		**1st day**	**2nd day**	**3rd day**	**4th day**	**5th day**
	10	All sprouted	4 seeds germinated	6 seeds germinated	7 seeds germinated	All ten germinated
	30	All sprouted	4 seeds germinated	6 seeds germinated	7 seeds germinated	All 10 germinated
	60	4 seeds sprouted	6 seeds sprouted	6 seeds sprouted	7 seeds germination	9 seeds germinated
	100	3 seeds sprouted	6 seeds sprouted	7 seeds sprouted	7seeds germinated	8 seeds germinated
PHST 0		**1st day**	**2nd day**	**3rd day**	**4th day**	**5th day**
	10	none sprouted	none sprouted	none sprouted	none sprouted	none sprouted
	30	none sprouted	none sprouted	none sprouted	none sprouted	none sprouted
	60	none sprouted	none sprouted	none sprouted	none sprouted	none sprouted
	100	none sprouted	none sprouted	none sprouted	none sprouted	none sprouted

Table 4.3: Germination status of *T. aestivum* cultivar PBW 343 and its NILs at different concentrations of GA

	Gibberellic acid (ppm)	**Days for germination**				
PBW343		**1st day**	**2nd day**	**3rd day**	**4th day**	**5th day**
	100	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
	200	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
	300	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
	400	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
	500	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
PHST 9		**1st day**	**2nd day**	**3rd day**	**4th day**	**5th day**
	100	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
	200	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
	300	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
	400	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
	500	All 10 sprouted	All 10 germinated	All 10 germinated	All 10 germinated	All 10 germinated
PHST 0		**1st day**	**2nd day**	**3rd day**	**4th day**	**5th day**
	100	None sprouted	1 seed sprouted	2 seeds sprouted	3 seeds sprouted	5 seeds sprouted
	200	1 seed sprouted	2 seeds sprouted	4 seeds sprouted	5 seeds sprouted	7 seeds sprouted
	300	2 seed sprouted	3 seeds sprouted	4 seeds sprouted	6 seeds sprouted	8 seeds sprouted
	400	2 seeds sprouted	3 seeds sprouted	5 seeds sprouted	7 seeds sprouted	8 seeds sprouted
	500	3 seeds sprouted	4 seeds sprouted	5 seeds sprouted	6 seeds sprouted	8 seeds sprouted

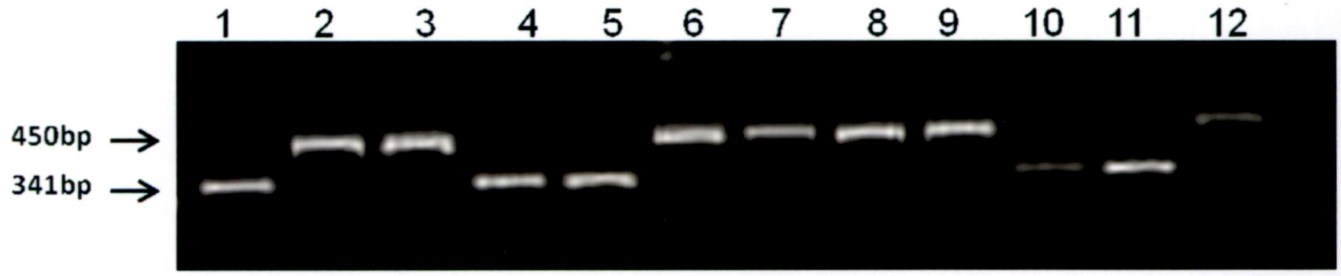
4.5.2 Application of gibberellic acid

The experimental set up for GA_3 was same as ABA set up. The analysis was performed to check the sensitivity of the three lines especially PHST 0 to exogenous application of GA_3. In both PBW 343 and PHST 9 the germination was the same as control germination at all concentrations of GA ranging from 100ppm to 500ppm. In PHST 0 maximum germination took place at 500ppm concentration and minimum at 100ppm concentration. Fig 4.5 shows responses of PBW 343, PHST 9 and PHST 0 after treatment with different concentrations of GA_3. The results obtained are shown in Table 4.3.

4.6 Validation of QTL for PHS tolerance using linked SSR markers

Group 4 PHS alleles were validated in PBW 343, PHST 0 and selected landraces using 4AL linked SSR marker zxq118. The amplification conditions used for zxq118 were according to Zang *et al.* (2008b). PBW 343, PHST 0, IITR20, IITR25, IITR103, IITR23 and IITR96 showed 450 base pair allele. Among these PHST 0, IITR20, IITR25 and IITR103 were red grained and PHS tolerant, PBW 343 was amber grained and PHS susceptible and IITR96 and IITR23 were amber grained and PHS tolerant. On the contrary IITR15, IITR31, IITR73, IITR26 and IITR65 gave 340 base pair allele. Out of these IITR15, IITR31 and IITR73 were red grained and PHS tolerant, IITR26 was amber and PHS tolerant and IITR65 was amber and PHS susceptible. This marker has been reported to be closely linked with PHS tolerance/dormancy in Australian wheat (Zhang *et al.*, 2008b). In our lines the resistance allele size of 341bp was observed in IITR65 also which is PHS susceptible and a different allele size of 450bp was observed in PHST0 and other red grained and PHS tolerant lines (Fig 4.6a). The possible reason for this observation could be that the dormancy gene on chromosome 4AL in red grained wheat was a different allele. In addition to zxq118 a total of thirteen SSR markers specific to chromosome 3AL and 4AL were used for the study. The list of all the SSR markers along with respective sequences is given in Table 3.1. These selected QTL flanking SSRs were validated in NILs and landraces. We validated another 4AL SSR marker gwm269 in NILs and IITR landraces.

a) zxq118



b) gwm269

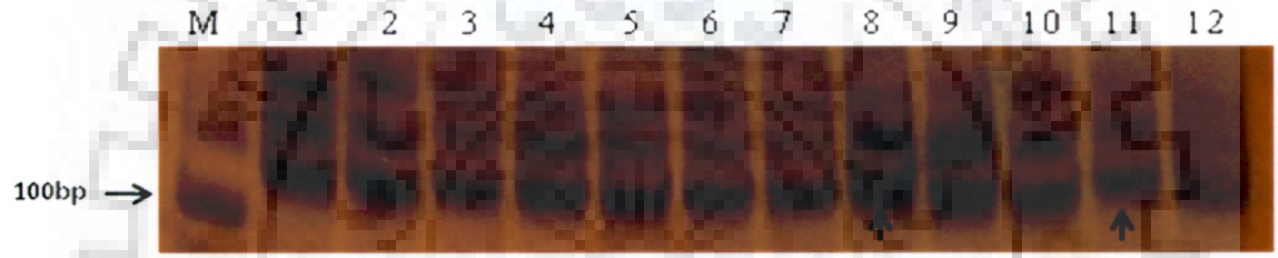


Fig 4.6: Validation of QTL for PHST on 4AL in NILs and wheat landraces using linked SSR markers a) zxq118 and b) gwm269. For zxq118 the PCR product was run on 2% SFR and for gwm269 PCR product was run on 10% PAGE. Lane1: IITR 15, Lane2: IITR 20, Lane3: IITR 25, Lane4: IITR 31, Lane5: IITR 73, Lane6: IITR 103, Lane 7: PHST 0, Lane8: PBW 343, Lane9: IITR 23, Lane10: IITR 26, Lane11: IITR 65, Lane12: IITR 96. The expected product size have been indicated using arrow

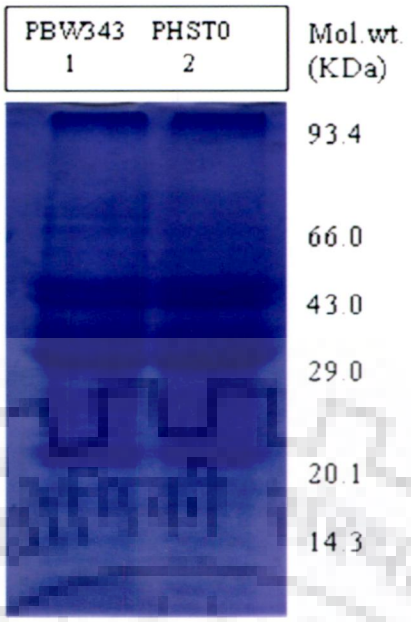


Fig 4.7: One dimensional SDS-PAGE of endosperm proteins. Lane1-25µg PBW343 and Lane2-25µg PHST0 protein. Molecular weights are indicated on right hand.

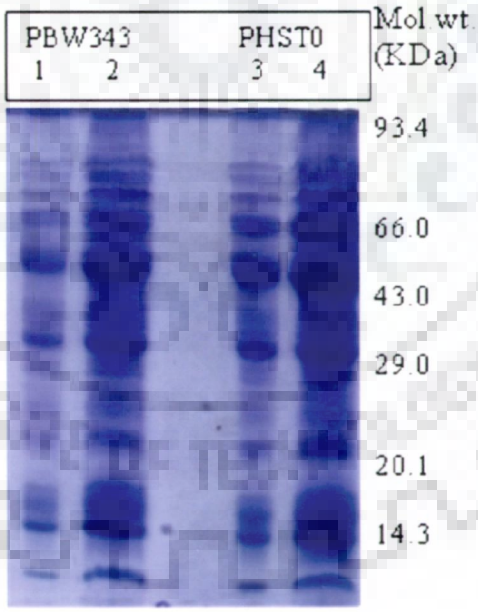


Fig 4.8: One dimensional SDS-PAGE of embryo proteins in PBW343 and NIL PHST0. Lane1-5µg PBW343 protein, Lane2- 25µg PBW343 protein, Lane3- 5µg PHST0 protein and Lane4- 25µg PHST0 protein

The marker could differentiate between PHS tolerant and susceptible amber landraces (Fig 4.6b). The allele size of six red seed colour landraces (IITR15, IITR20, IITR25, IITR31, IITR73, and IITR103), PHST0, amber PHS tolerant landraces (IITR23, IITR26, and IITR96) was similar and that of amber susceptible landrace IITR65 and PBW343 was similar. Therefore gwm269 differentiated between amber PHS tolerant and PHS susceptible lines. The amplification conditions for the primer were as mentioned on graingenes. PCR product was run on 10% PAGE and the gel was silver stained to visualize polymorphism, as the difference between the alleles was less. In this study we tried to validate chromosome 3AL QTL, *QPhs.ccsu-3A.1* flanking markers wmc153 and gwm155 in NILs and IITR landraces. In addition to these two flanking markers we used 7 other SSR markers lying between wmc153 and gwm155 in genetic map by Somers *et al.* (2004). But we could not validate any of these markers in our population as wmc153 and gwm155 were monomorphic while other SSR markers were not able to differentiate between PHS tolerant and susceptible varieties.

4.7 1D SDS-PAGE of proteins

Prior to 2-DE, proteins extracted using respective protein extraction methods were analysed using 1D SDS-PAGE. This was done to check the quality of extracted proteins and also to check any differences, if present, in protein profile at this level. The gels were stained with CBB-R250 to visualize the proteins. The 1D SDS-PAGE pictures of endosperm proteins and embryo proteins of PBW 343 and PHST 0 are given in Fig 4.7 and Fig 4.8 respectively. It was also observed that negligible differences could be detected between PBW 343 and PHST 0 on 1D SDS-PAGE which could be attribute to their similar background.

4.8 Protein analysis using 2-DE

Comparative 2-DE was done to study the differences in protein profiles of PBW 343 and NIL PHST 0. 2-DE was done for three stages selected for study including soft dough stage (stage 85), hard dough stage (stage 87) and dry caryopses stage (stage 95). The hard dough and dry caryopses stages were used for proteomic study using embryos and endosperm while soft

dough stage was used for the transcript expression studies. Proteomic analysis of the embryo specific and endosperm specific proteins was done separately. The extracted proteins from the above mentioned stages were checked for protein concentration using Bradford reagent. On 2DE gels the proteome maps of PBW 343 and its NIL PHST 0 showed a broad distribution of spots along the pI range from 3.0 to 10.0 and a mass nearly from 14.0 KDa to 100.0 KDa (Fig 4.9, 4.10 and 4.11). There were visible quantitative and qualitative differences between PHS susceptible (PHSS) PBW 343 and its PHS tolerant (PHST) NIL PHST 0 in low, middle and high molecular mass regions. As already mentioned comparative 2-DE was done to check the differences between protein patterns of PHSS PBW 343 and PHST NIL PHST 0 and also to identify novel proteins which were expressed only in the tolerant line. Fig 4.9 shows the comparative 2-DE profile of endosperm proteins of PBW 343 and PHST 0. Five regions have been highlighted on the endosperm proteins 2-DE gel. The higher expression in PHST 0 except for box b in which PBW 343 is showing a higher expression. 2-DE of immature embryo has been shown in Fig 4.10. The boxes numbered a to f show difference in protein expression of PBW 343 and PHST 0 while numbered circles show proteins specific to each line. Encircled protein spots show the proteins present in one cultivar and absent in other. Therefore, protein encircled in spot no.1 was present only in PBW 343 while protein spot nos. from 2 to 7 are specific to PHST 0. Fig 4.11 shows the mature embryo (stage 87) comparative 2-DE of PBW 343 and PHST 0. As evident, PHST 0 had more number of expressed proteins than PBW 343. Differential protein expression has been highlighted in boxes which have been marked from a to i in both 2-DE gels. Differential protein spots present only in PHST 0 have been numbered from 1 to 7. Out of these protein spots no. 3, 5, 6 and 7 have been analysed using LC-ESI-MS/MS. The major differences were detected in the mature embryo proteins of PHST 0 and PBW 343. Differences were also observed in the expression patterns of various proteins between the two experimental lines. The protein profiles of mature and immature embryos was very similar except for few proteins which are present in mature embryo 2-DE. The whole

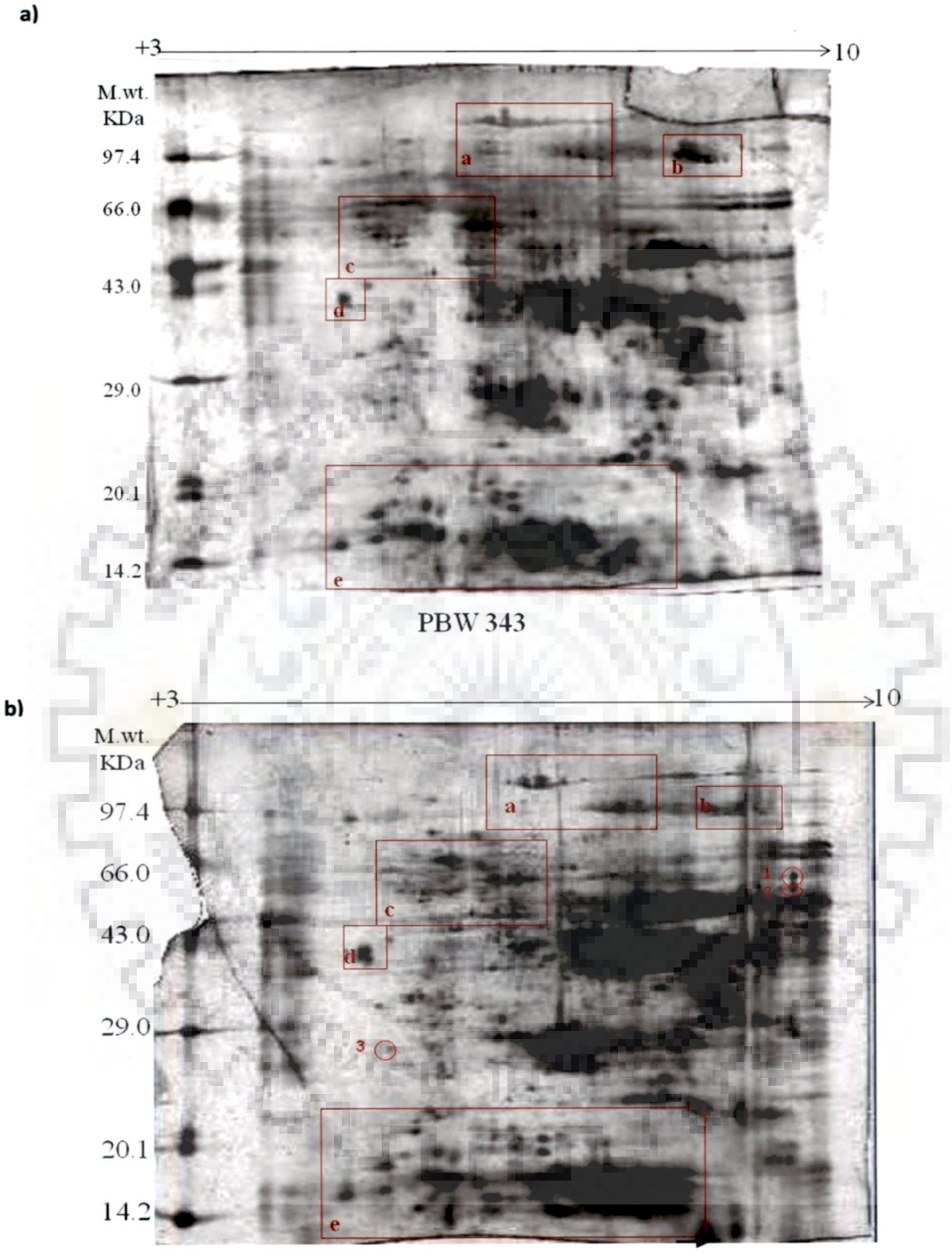
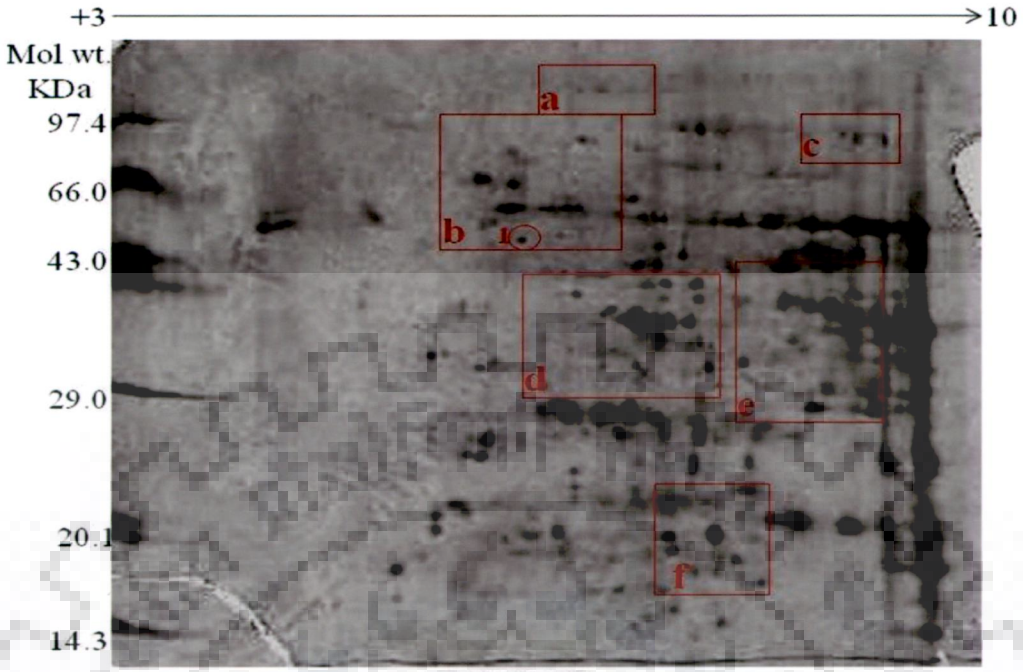


Fig 4.9: 2–DE endosperm protein profiles of a) PBW 343 and b) PHST 0. Molecular weights are indicated on left hand side. Gels are silver stained. Rectangular boxes a-e indicate differences in protein expression between PBW 343 and PHST 0, while encircled spots numbered 1-3 show differential spots. pH range of IPG stripi used is given as 3→10

a)



b)

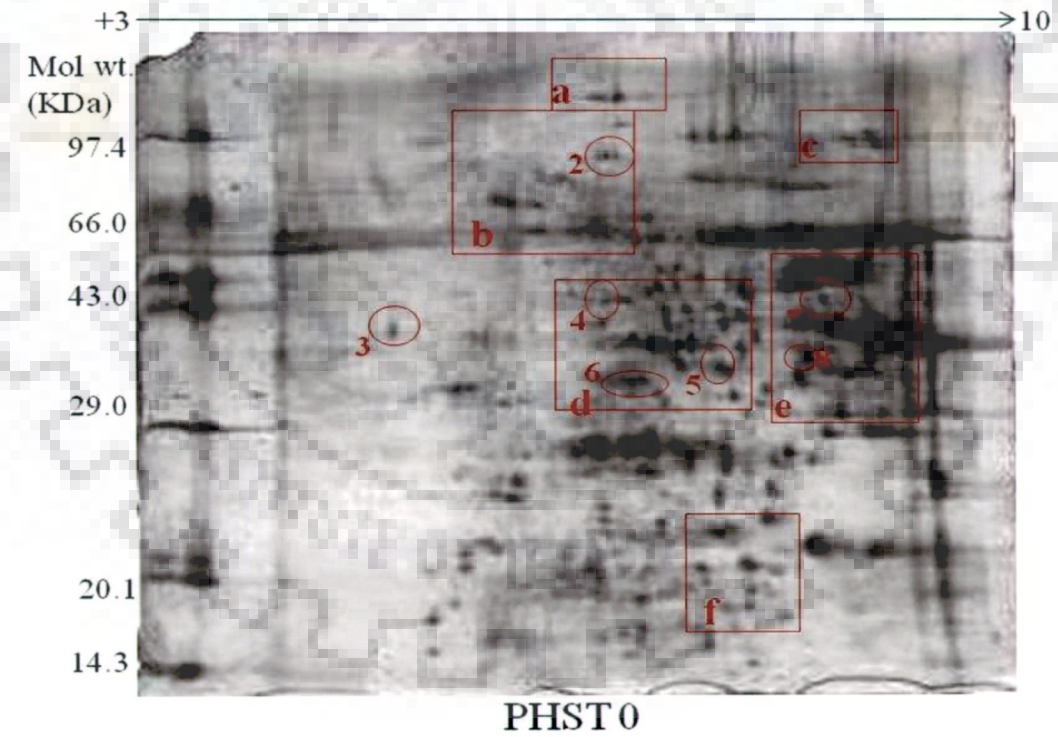


Fig 4.10: 2-DE immature embryo protein profiles of a) PBW 343 and b) PHST 0. Molecular weights are indicated on left hand side. Gels are silver stained. Rectangular boxes a-f indicate differences in protein expression between PBW 343 and PHST 0, while encircled spots numbered 1-8 show differential spots present only in PHST 0. pH range of IPG strip used is given as 3 →10

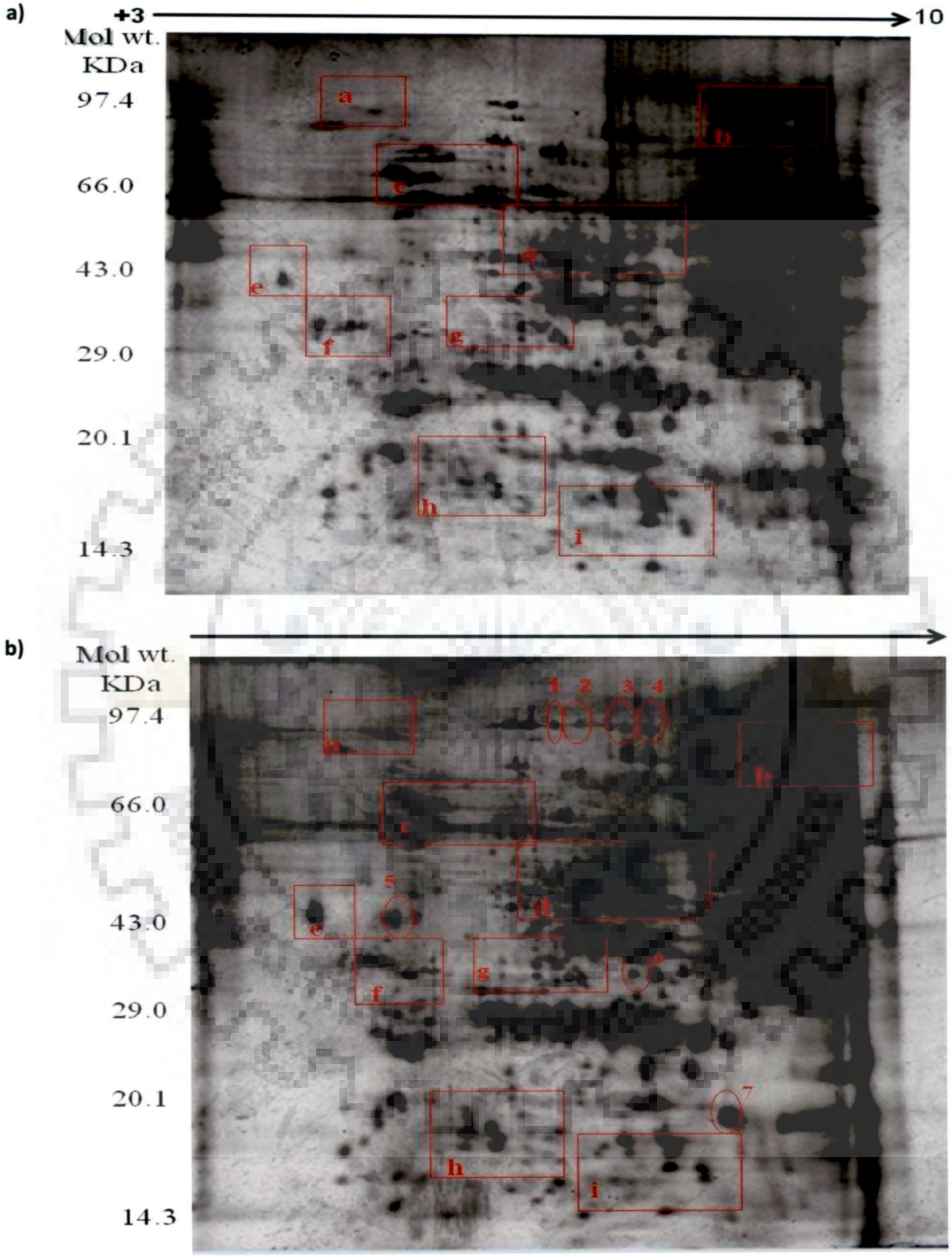


Fig 4.11: 2-DE mature embryo protein profiles of a) PBW 343 and b) PHST 0. Molecular weights are indicated on left hand side. Gels are silver stained. Rectangular boxes a-i indicate differences in protein expression between PBW 343 and PHST 0, while encircled spots numbered 1-7 show differential spots present only in PHST 0. pH range of IPG strip used is given as 3 →10

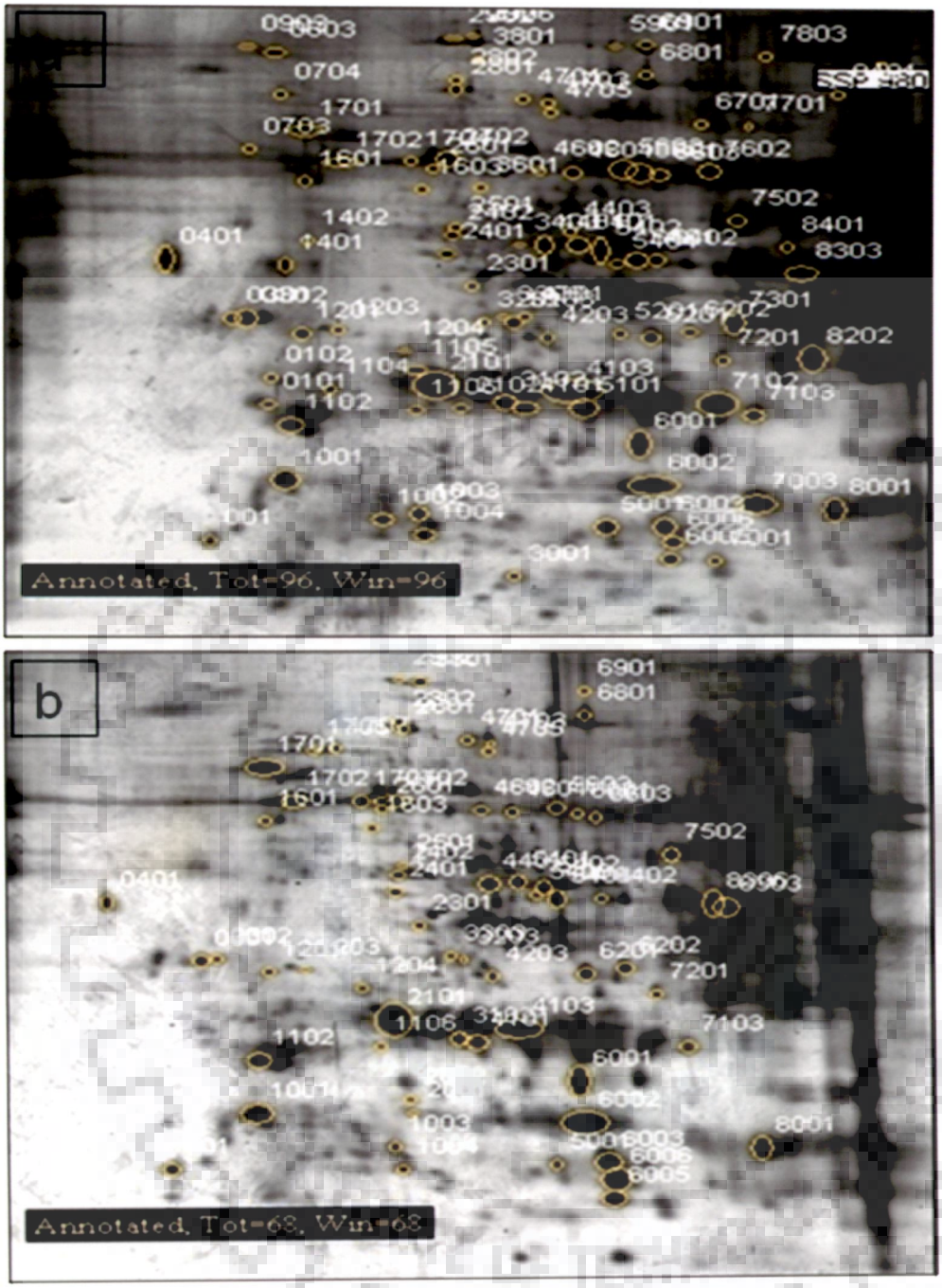


Fig 4.12a: Protein spot annotation by PDQuest for mature and immature embryo. a) 2-DE of mature embryo of PHST 0 and b) 2-DE of mature embryo of PBW 343

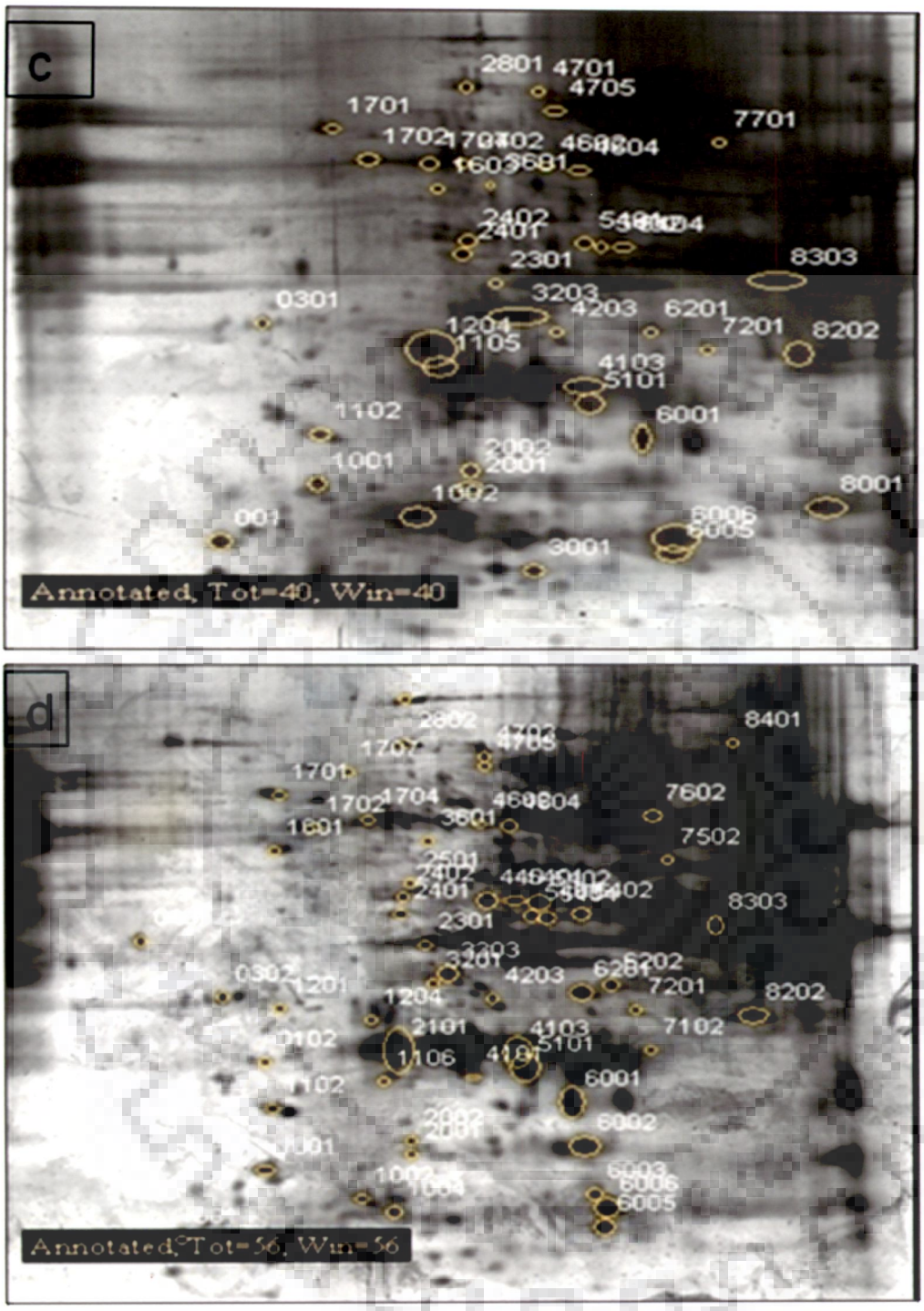
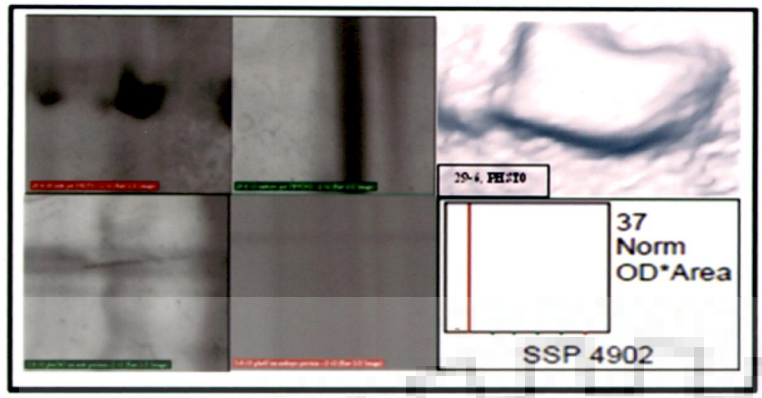
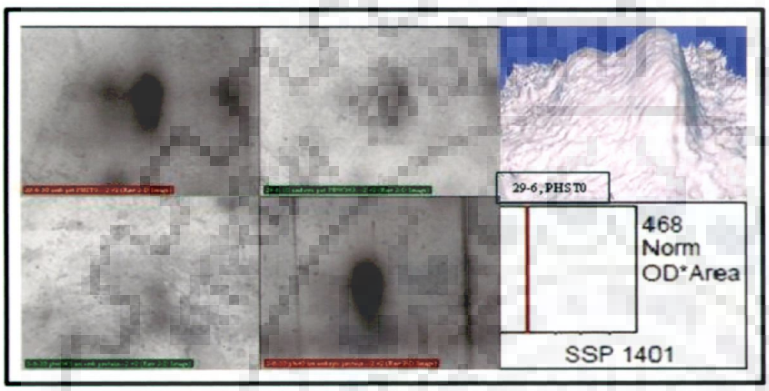


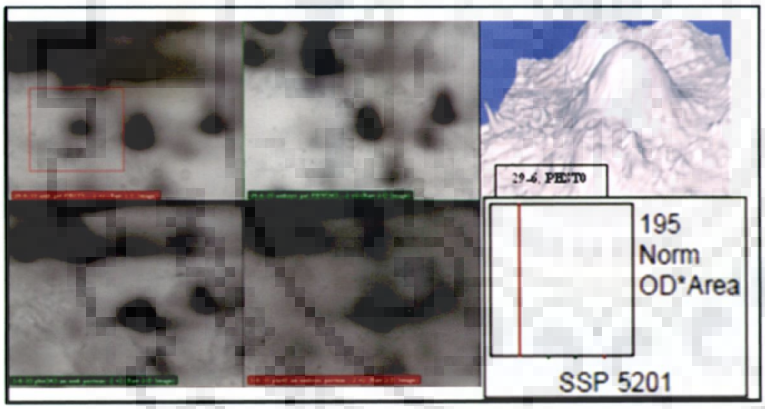
Fig 4.12b: Protein spot annotation by PDQuest for mature and immature embryo. c) 2-DE of immature embryo of PHST 0 and d) 2-DE of immature embryo of PBW 343



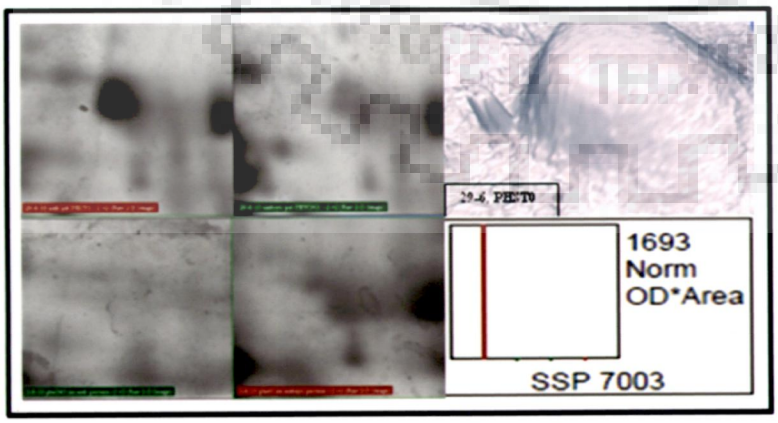
a) Spot3



b) Spot 5



c) Spot6



d) Spot7

Fig 4.13: Comparison of mature embryo and immature embryo proteins of PBW 343 and PHST 0 for respective protein spots. The 3D of protein and SSP numbers are also shown

Fig 4.14: Protein spots annotation by PDQuest of mature endosperm for PBW 343 and PHST 0. a) 2-DE of mature endosperm (stage 95) of PHST 0 and b) 2-DE of mature endosperm (stage 95) of PBW 343

experiment including extraction and 2-DE was repeated at least twice in the laboratory and similar proteome patterns were observed every time.

4.9 Gel analysis using PDQuest advanced software

2-DE gels were compared using PDQuest software to know the differences between embryo proteins and endosperm proteins. The 2-DE gels for mature and immature proteins of PBW 343 and PHST 0 were compared and the protein spots were annotated on the respective gels using the software. As seen in Fig 4.12a and 4.12b, maximum proteins spots were present in mature embryo protein gel of PHST 0. Therefore, in mature embryo 2-DE of PHST0 96 spots were annotated and in mature embryo 2-DE of PBW343 68 protein spots were annotated. The number of protein spots annotated in immature embryo 2-DE gels of PHST0 and PBW343 were 40 and 56 respectively. All the protein spots could not be annotated as there would have been overlapping of SSP (sample spot protein) numbers and visualization would have been difficult. Fig 4.13 shows the difference in expression in the four gels (mature and immature embryo) for the protein spots for which LC-MS/MS was done. Upon comparison it was found that protein spots are either absent or if present the expression is low. The 3D of the protein spot has also been shown. Comparative 3D of the protein spots could not be presented, as the protein spot with lower expression could not be detected by the software. The selected pQTLs with SSP numbers for the proteins expressed in endosperm of PHST 0 and PBW 343 have been shown in Annexure II. The proteins spots that were unique to either gels have not been shown as only differences had to be highlighted between the four embryo protein gels. Protein spot annotation was also done for endosperm protein gels of PBW 343 and PHST 0 2-DE gels. The annotated spots on 2-DE gel of endosperm proteins have been shown in Fig 4.14. The pQTLs showing differences in mature and immature embryo protein expression across the 2-DE gels have been shown in Annexure I. For mature endosperm gel of PHST 0

and PBW 343, 215 and 210 protein spots each were detected. In contrast for immature endosperm 200 and 196 protein spots were detected for PHST 0 and PBW 343 respectively.

4.10 LC-ESI-MS/MS analysis

LC-ESI-MS/MS was done to identify the differential protein spots from 2-DE gels.

4.10.1 LC-ESI-MS/MS of endosperm differential protein spots

LC-MS/MS was done for three differential protein spots for endosperm proteins from PHST 0. These were designated as PH-ENDO-1, PH-ENDO-2 and PH-ENDO-3. All the three differntial spots were selected from PHST 0. PH-ENDO-1 and PH-ENDO-2 were identified as Low molecular weight glutenin (LMW) proteins. The fragmentation patterns of the peptides corresponding to PH-ENDO-1 has been given in Fig 4.15. In PH-ENDO-1 two peptides were identified based on probability based mowse score. First peptide was identified as Low molecular weight glutenin subunit LMW-M1 from *Triticum monococcum* with Q2PQJ6_TRIMO sequence identity and had a probability score of 126. The other peptide was identified as LMW glutenin subunit TD130 from *Aegilops tauschii* and had a probability score of 58. The first peptide identified for PH-ENDO-1 had VFLQQQCIPVAMQR peptide sequence and the second peptide was SQMLQQSICHVMQQQCCQQLR. Both these peptides were matching the same LMW glutenin protein. For PH-ENDO-2 three peptides were identified as Q3LGB3_WHEAT which is Low molecular weight glutenin subunit from *Triticum aestivum*, Q2PQJ6_TRIMO which is Low molecular weight glutenin subunit LMW-M1 from *Triticum monococcum* and Q3LUL9_GOSHI Translation elongation factor 1A-8 from *Gossypium hirsutum*. The probability scores for the three peptides were 67 for first and 54 each for the other two. However, the third peptide identified as that from *G.hirsutum* was not further analysed inspite of a score of 54. The peptide in Q3LGB3_WHEAT had the sequence VFLQQQCSPVAMPQSLAR while Q2PQJ6_TRIMO had the peptide sequence as VFLQQQCIPVAMQR. The MS fragmentation patterns for the identified peptides have been

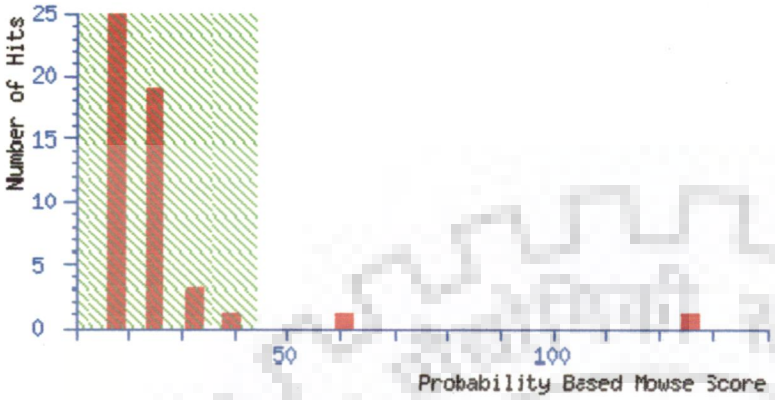
given in Fig 4.16. The third endosperm protein spot analysed by LC-MS-MS was PH-ENDO-3. Three peptide fragments identified were Q4W247_WHEAT which was protein disulfide isomerase precursor (EC 5.3.4.1) from *T. aestivum*, Q1X8N5_PRUAR which was enolase (Fragment) from *Prunus armeniaca* (Apricot) and T03984 adenylosuccinate synthase (EC 6.3.4.4) from maize. The peptide fragmentation patteren for protein disulfide isomerise (PDI) is given in Fig 4.17. The individual scores for the three peptides were 76, 51 and 49. The peptide sequence identified as PDI was APEDATYLEDGK. However, only PDI was further analysed in this case as the other two were not significant in comparison to wheat.

4.10.2 LC-MS/MS for mature embryo proteins

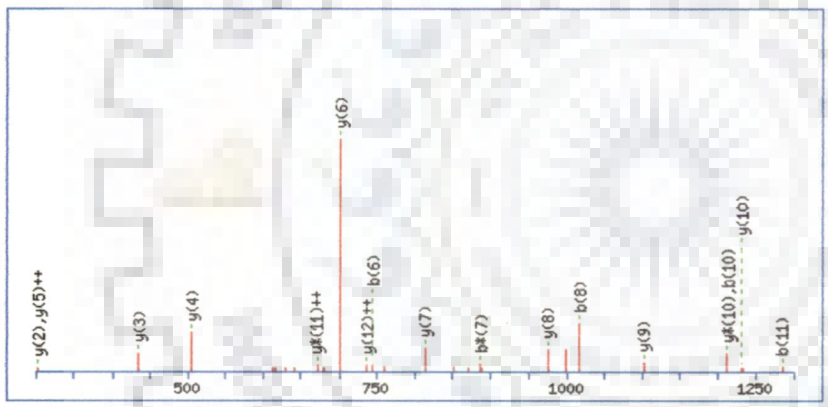
LC-MS/MS analysis was done for differential protein spots from mature embryo protein 2DE. The selection was based on visual observation of the 2DE gels. The same has been shown in Fig 4.12a and 4.12b. The LC-ESI-MS/MS results of endosperm and embryo proteins of NIL PHST0 have been summarized in Table 4.4. LC-MS/MS was done for four differential spots namely spot no.3, spot no.5, spot no.6 and spot no.7. All these differential spots were chosen from 2-DE gels of PHST 0. There were two peptide matches for spot no.3 after LC-MS/MS data analysis using Mascot. The peptide ELQESSLEACR was identified as HMW glutenin subunit 1By9 from *T. aestivum* with gi|22090 as protein accession number. The probability score for the protein was 51.The MS fragmentation pattern for the same is shown in Fig 4.18. The second protein hit was for an unknown protein from gymnosperms with protein accession as gi|172046673 and a probability score of 50. The peptide sequence for the protein was VLDELTADR. The MS fragmentation pattern for the protein which is given in Fig 4.18. For spot no.5 the protein identified was serine carboxy peptidase with gi|443481 as protein identity. The probability score for the same was 61. There were two peptide fragments matching this protein with the peptide sequences as VEPSGHAADR and TAHDSYAFLAK respectively with

PH-ENDO-1

Database : MSDB 20070501 (3239079 sequences; 1079594700 residues)
Taxonomy : Viridiplantae (Green Plants) (247880 sequences)
Significant hits: Q2PQJ6_TRIMO Low molecular weight glutenin subunit LMW-M1.- *Triticum monococcum*
Mascot score : 126 for Q2PQJ6_TRIMO



MS/MS Fragmentation of **VFLQQQCIPVAMQR**
Found in **Q2PQJ6_TRIMO**, Low molecular weight glutenin subunit LMW-M1.- *Triticum monococcum* (Einkorn wheat) (Small spelt).



MS/MS Fragmentation of **SQMLQQSICHVMQQQCCQQLR**
Found in **Q2PQJ6_TRIMO**, Low molecular weight glutenin subunit LMW-M1.- *Triticum monococcum* (Einkorn wheat) (Small spelt).

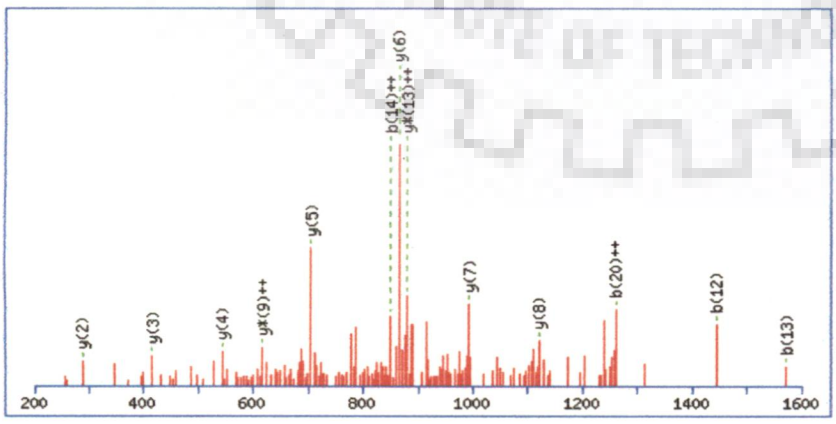
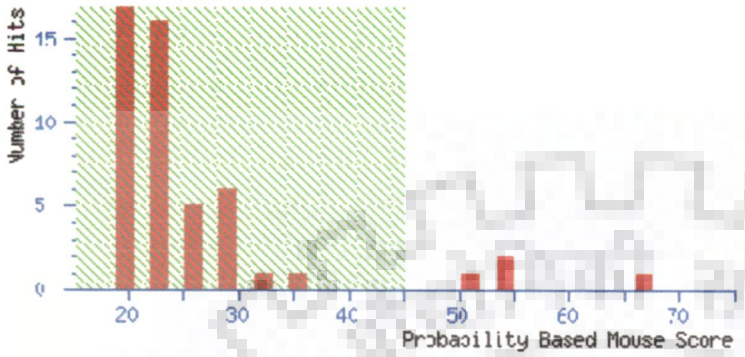


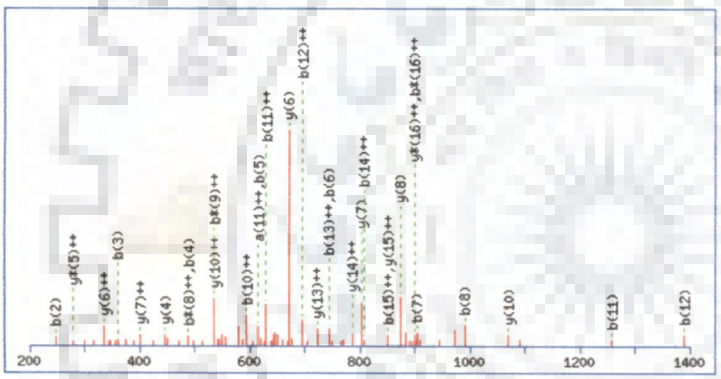
Fig 4.15: LC-ESI-MS/MS for endosperm protein PH-ENDO-1

PH-ENDO-2

Database : MSDB 20070501 (3239079 sequences; 1079594700 residues)
Taxonomy : Viridiplantae (Green Plants) (247880 sequences)
Significant hits : Q3LGB3_WHEAT Low-molecular-weight glutenin subunit (Fragment).- *Triticum aestivum*.
Q2PQJ6_TRIMO Low molecular weight glutenin subunit LMW-M1.- *Triticum monococcum*
Mascot score: 67 for Q3LGB3_WHEAT; 54 for Q2PQJ6_TRIMO



MS/MS Fragmentation of **VFLQQQCSPVAMPQSLAR**
Found in **Q3LGB3_WHEAT**, Low-molecular-weight glutenin subunit (Fragment).- *Triticum aestivum* (Wheat).



MS/MS Fragmentation of **VFLQQQCIPVAMQR**
Found in **Q2PQJ6_TRIMO**, Low molecular weight glutenin subunit LMW-M1.- *Triticum monococcum*

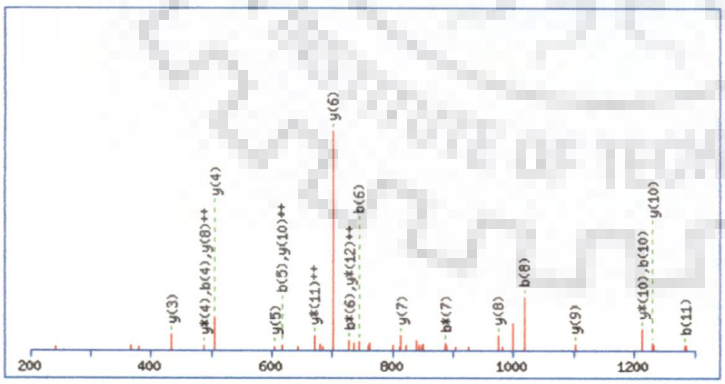
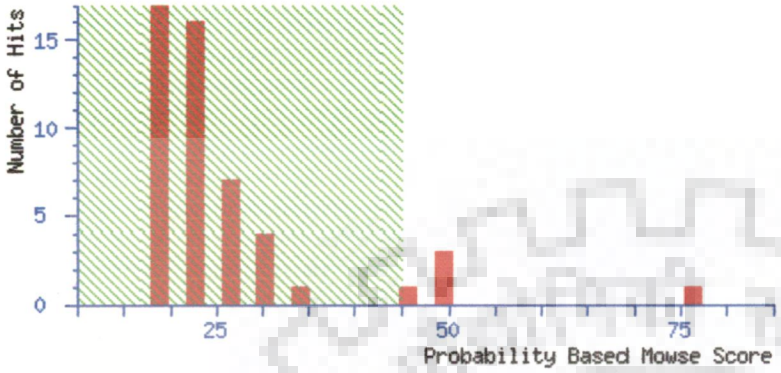


Fig 4.16: LC-ESI-MS/MS for endosperm protein PH-ENDO-2

PH-ENDO-3

Database : MSDB 20070501 (3239079 sequences; 1079594700 residues)
Taxonomy : Viridiplantae (Green Plants) (247880 sequences)
Significant hits : Q4W247_WHEAT Protein disulfide isomerase precursor (EC 5.3.4.1) (Wheat).
Mascot score : 76 for Q4W247_WHEAT



MS/MS Fragmentation of APEDATYLEDGK
Found in Q4W247_WHEAT, Protein disulfide isomerase precursor (EC 5.3.4.1).- *Triticum aestivum* (Wheat).

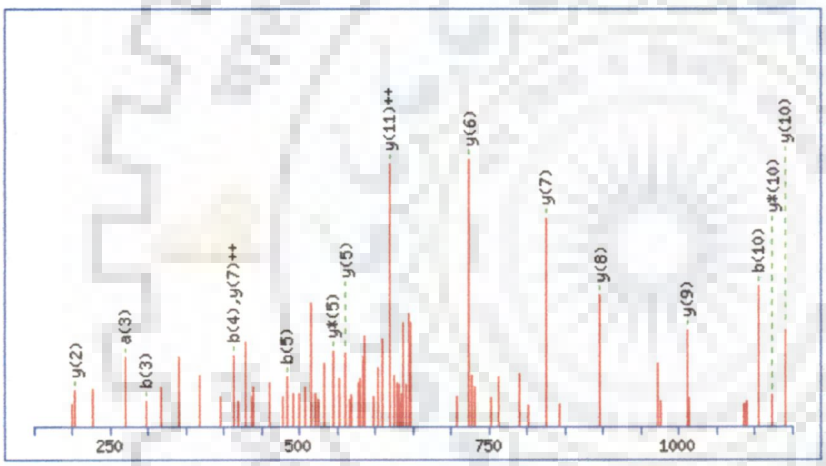
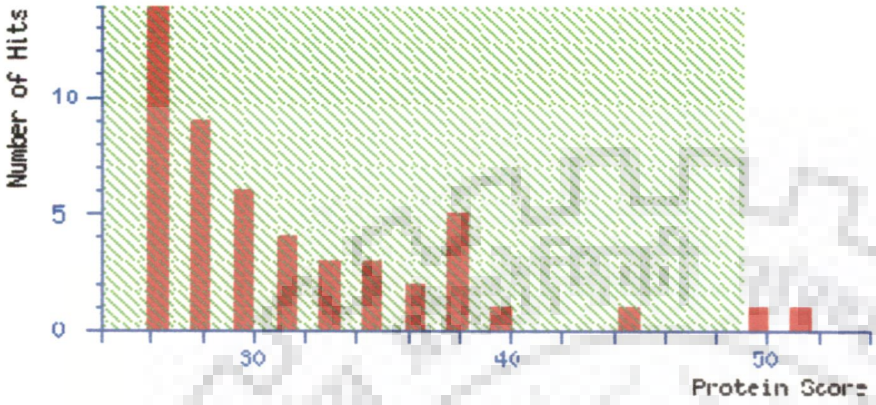


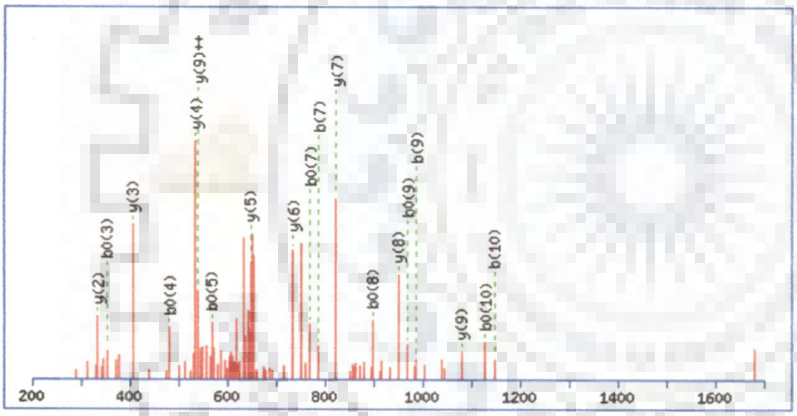
Fig 4.17: LC-ESI-MS/MS for endosperm protein PH-ENDO-3

PH-EMB--3

Database : NCBInr 20110304 (13254464 sequences; 4535100807 residues)
Taxonomy : Viridiplantae (Green Plants) (855441 sequences)
Protein hits : gi22090 HMW glutenin protein subunit By9 (*Triticum aestivum*)
gi172046673 RecName: Full unknown protein
Mascot score : 51 for gi22090 ; 50 for gi172046673



MS/MS Fragmentation of **ELQESSLEACR**
Found in gi|22090, HMW glutenin subunit 1By9 (*Triticum aestivum*)



MS/MS Fragmentation of **VLDELTADR**
Found in gi|172046673, RecName: Full=Unknown protein 1

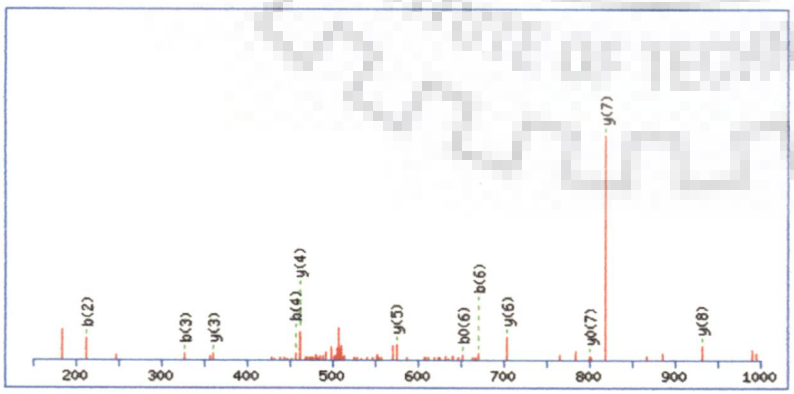
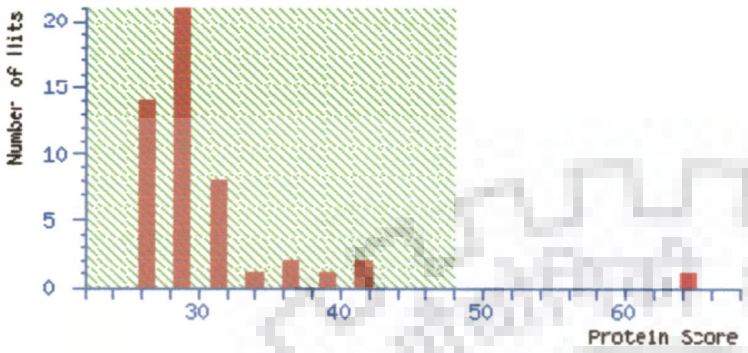


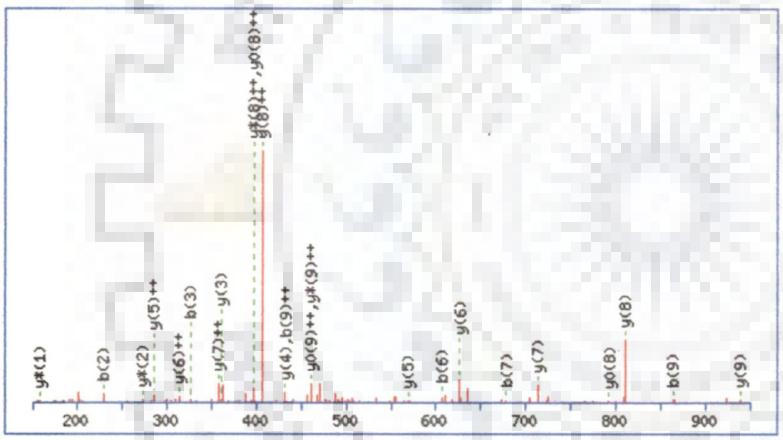
Fig 4.18: LC-ESI-MS/MS for embryo protein PH-EMB-3

PH-EMB-5

Database : NCBInr 20110304 (13254464 sequences; 4535100807 residues)
Taxonomy : Viridiplantae (Green Plants) (855441 sequences)
Protein hits : gi|443481 Chain A refined atomic model 0f wheat serine carboxypeptidase Ii at 2.2 angstroms
Mascot score : 64 for gi|443481



MS/MS Fragmentation of **VEPSGHAADR**
Found in **gi|443481**, Chain A, Refined Atomic Model Of Wheat Serine Carboxypeptidase Ii At 2.2-Angstroms Resolution



MS/MS Fragmentation of **TAHDSYAFLAK**
Found in **gi|443481**, Chain A, Refined Atomic Model Of Wheat Serine Carboxypeptidase Ii At 2.2-Angstroms Resolution

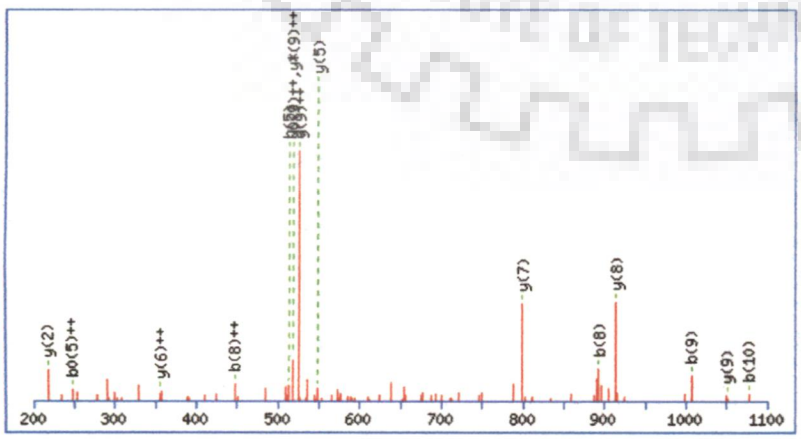
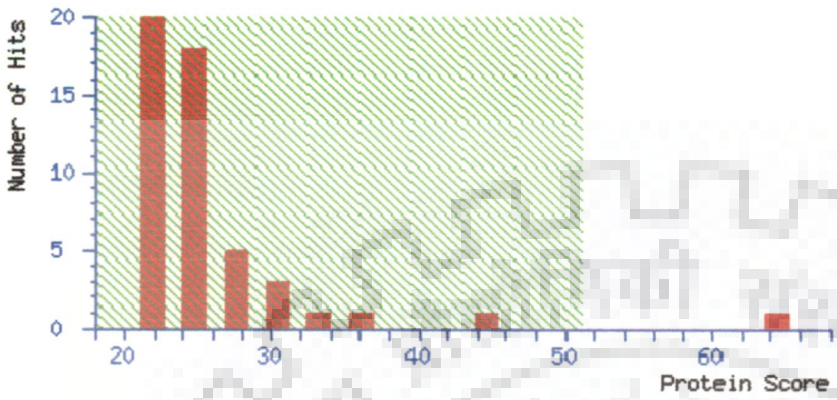


Fig 4.19: LC-ESI-MS/MS for endosperm protein PH-EMB-5

PH-EMB-6

Database : NCBInr 20110304 (13254464 sequences; 4535100807 residues)
Taxonomy : Viridiplantae (Green Plants) (855441 sequences)
Protein hits : gi|115439929 Os01g0749000 (*Oryza sativa* japonica group)
Mascot score : 64 for gi|115439929



MS/MS Fragmentation of **LIGVEYIVSR**
Found in **gi|115439929**, Os01g0749000 [*Oryza sativa* Japonica Group]

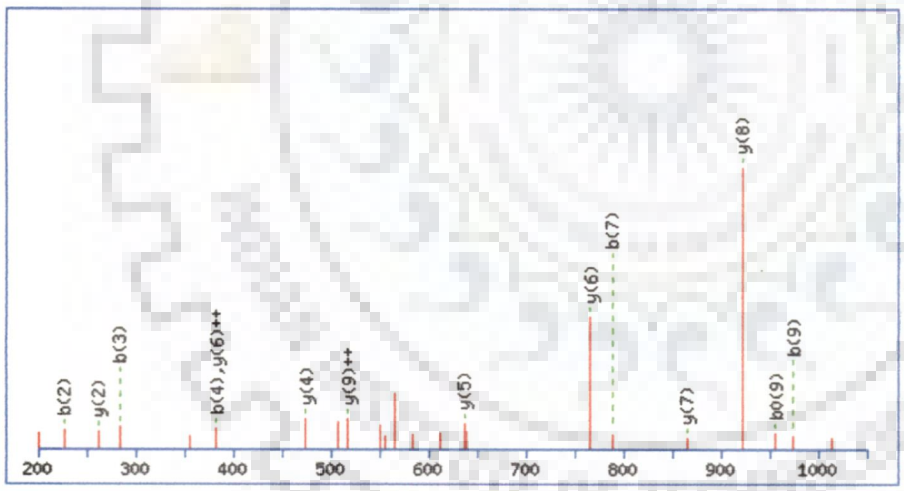
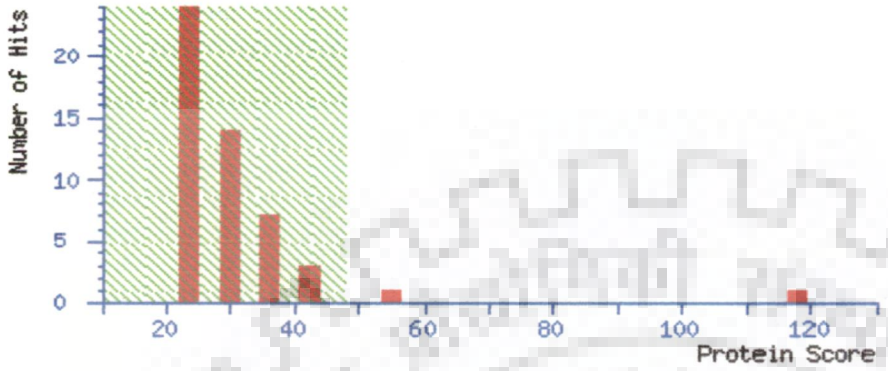


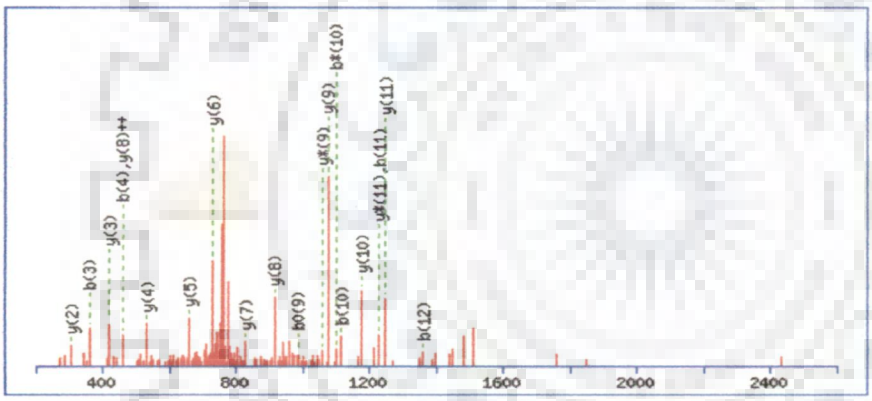
Fig 4.20: LC-ESI-MS/MS for embryo protein PH-EMB-6

PH-EMB-7

Database :NCBInr 20110304 (13254464 sequences; 4535100807 residues)
Taxonomy : Viridiplante (Green Plants) (855441 sequences)
Protein hits : gi89143120 putative avenin like precursor (*Triticum aestivum*)
gi255538634 cytochrome P450, putative (*Ricinus communis*)
Mascot score : 118 for gi89143120 ; 54 for gi255538634



MS/MS Fragmentation of CQAVCSVAQIIMR
Found in gi|89143120, putative avenin-like a precursor [*Triticum aestivum*]



MS/MS Fragmentation of QQCCQPLAQISEQAR
Found in gi|89143120, putative avenin-like a precursor [*Triticum aestivum*]

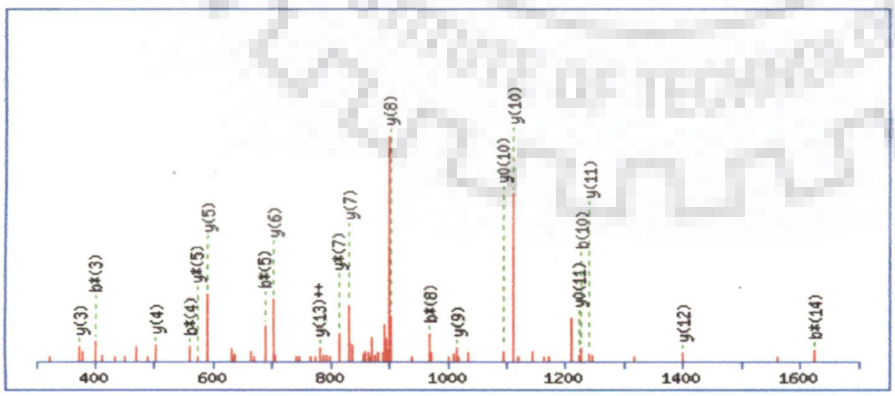


Fig 4.21: LC-ESI-MS/MS for embryo protein PH-EMB-7

Table 4.4: LC-ESI-MS/MS results of endosperm and embryo proteins of NIL PHST0

S.No.	Species	Accession no.	MASCOT score	Observed mol.wt.(KDa)/pI	Theoretical mol.wt. (KDa)/pI	Matched peptides	Peptide sequences
Endosperm proteins							
1. Spot1	Low molecular weight glutenin subunit *Triticum monococcum*	Q2PQJ6_TRIMO	126>44	35.0/8.0	43.0/ 8.22	2	VFLQQQCIPVAMQR SQMLQQSICHVMQQQCCQQLR
	LMW glutenin subunit TD130 *Aegilops tauschii*	Q2V722_AEGTA	58>44			2	VNVPLYR TTTSVPFGVGAGVGAY
2. Spot2	Low-molecular-weight glutenin subunit (Fragment) *Triticum aestivum*	Q3LGB3_WHEAT	67>44	29.0/7.5	43.0/ 8.22	1	VFLQQQCSPVAMPQSLAR
	Low molecular weight glutenin subunit LMW-M1 *Triticum monococcum*	Q2PQJ6_TRIMO	54>44			1	VFLQQQCIPVAMQR
	Translation elongation factor 1A-8 *Gossypium hirsutum*	Q3LUL9_GOSHI	54>44			2	QTVAVGVIK LPLQDVYK
3. Spot3	Protein disulfide isomerase precursor (EC 5.3.4.1) *Triticum aestivum*	Q4W247_WHEAT	76>44	32.0/7.3	56.8/4.99	1	APEDATYLEDGK
	Enolase (Fragment) *Prunus armeniaca*	Q1X8N5_PRUAR	51>44			1	SCNALLLK

	adenylosuccinate synthase (EC 6.3.4.4) maize	T03984	49>44			1	LVDVLAPR
	OSJNBb0004A17.4 protein (H0624F09.11 protein) *Oryza sativa*		49>44			1	.APEYLAMDEK
Embryo proteins							
4. Spot 3	HMW glutenin subunit 1By9 *Triticum aestivum*	gi|22090	51>44	97.4/6.3	76.0/8.64		ELQESSLEACR
	Full=Unknown protein 1	gi|172046673	50>44				VLDELTADR
5. spot5	Chain A, Refined Atomic Model Of Wheat Serine Carboxypeptidase Ii At 2.2-Angstroms Resolution	gi|443481	64>44	30.1/5.1	28.8/4.91	2	VEPSGHAADR TAHDSYAFLAK
6. spot6	Os01g0749000 [Oryza sativa Japonica Group]	gi|115439929	64>44	29.0/7.0	28.2/7.79	1	LIGVEYIVSR
	Os10g0125700 [Oryza sativa Japonica Group]	gi|115480968	46>44			1	LLQELSKFQVNR
7. Spot7	putative avenin-like a precursor *Triticum aestivum*	gi|89143120	118>44	20.1/7.6	19.2/8.42	2	CQAVCSVAQIIMR QQCCQPLAQISEQAR
	cytochrome P450, putative *Ricinus communis*	gi|255538634	55>44				LNDLSLDLIDAK

peptide sequence was LIGVEYIVSR and the MS fragmentation for the same is given in Fig 4.20. The protein identified in spot no.6 was further studied using bioinformatics which have been described in later sections. For spot no.7 there were two protein hits. The first protein hit with probability score as 118 was avenin like precursor in *T. aestivum* and the peptide sequences for the same were CQAVCSVAQIIMR and QQCCQPLAQISEQAR. The MS fragmentation for both the peptides is given in Fig 4.21. The second protein identified was cytochrome P450 from *Ricinus communis* with gi|255538634 as protein identity and the probability score was 55. The peptide sequence for the same was LNDLSLDLIDAK. The protein from *R. communis* was not further analysed as it was not relevant in our context.

4.11 Transcriptome analysis

RNA was extracted from seed development stage 85 using hot phenol method for plant RNA extraction (K□hrer and Domdey 1991). Gene specific primers were designed for two transcription factors including DFR and myb10 and one enzyme protein disulfide isomerise. RNA studies using these three primers were done on PBW 343, PHST 9 and PHST 0. Fig 4.22 shows the quality of RNA used for the study and the RNA concentration varied from 1.5µg/5µl in PBW 343 to 3.0µg/5µl in PHST 9 and 2.8µg/5µl in PHST 0. The profile of the three gene specific primers is shown in Fig 4.23. For transcription factor myb10 the predicted product size was 239 base pairs. There is slight difference in the expression in PBW 343, PHST 9 and PHST 0. Primer PDI (protein disulfide isomerase) was designed on the basis of the LC-MS/MS results to check the difference in its expression level in PBW 343, PHST 9 and PHST 0. The expected product size for the primer was 190 base pairs as predicted by PRIMER3 software but the product obtained was approximately 200 base pairs. The primer sequence for DFR-A gene was taken from Himi and Noda (2004). DFR-A gene specific primer was selected as in PBW 343, PHST 9 and PHST 0 PHS tolerance is related to chromosome 3AL as demonstrated by SSR marker zxq118. In this case the product size of 890 base pair was observed, though non-

specific amplification of more than 1000 base pairs was observed in all the three experimental lines. The annealing temperature was selected after testing all the three gene specific primers in gradient PCR. Finally the annealing temperature selected for the three primers was 58°C. The PCR profile included initial denaturation at 94°C for 3 minutes, 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 45 seconds and final extension at 72°C for 5 minutes with steps 2 to 4 repeated for 35 cycles. The house keeping gene to study the mRNA expression in all the three primers used was E1fα which is a transcription factor. The amplification conditions for E1fα were same as that for PDI, myb10 and DFR.

4.12 Bioinformatic analysis

4.12.1 Bin map identification for selected SSR markers

The SSR marker sequences for flanking QTL SSR markers were bin mapped on chromosome 3AL and 4AL. The flanking markers for major 3AL QTL *QPhs.ccsu-3A.1* (Kulwal *et al.*, 2005), wmc153 and gwm155 were mapped on 3AL bin map. The two SSR markers mapped on two chromosome 3AL bins viz., 3AL3-0.42-0.78 and 3AL5-0.63-1.00. ESTs mapping on bin 0.42-0.78 were BE590549, BF473016, BE442875 for gwm155 and BE490613 for wmc153. Flanking marker of another QTL reported on 3AS by Liu *et al.* (2008), barc57 was also bin mapped. The chromosome bins included 3AL3-0.42-0.78, 3AL5-0.78-1.00 and C-3AL3-0.42 for EST BF483618, BF473732 and BE494345 respectively. In addition to chromosome 3AL QTL the flanking markers for major chromosome 4AL QTLs were also checked for their respective chromosome bins. The four SSR markers on chromosome 4A selected for the study included barc170 and gwm 269 which flank the major PHS QTL in chromosome 4AL (Mares *et al.*, 2005). Chromosome bins were identified using sequences of these two SSR markers. Barc170 mapped to 3AL bins C-3AL3-0.42 and 3AL5-0.78-1.00 and the ESTs had identities were BE500510 and BE591738 respectively, while gwm269 did not map to any bin on

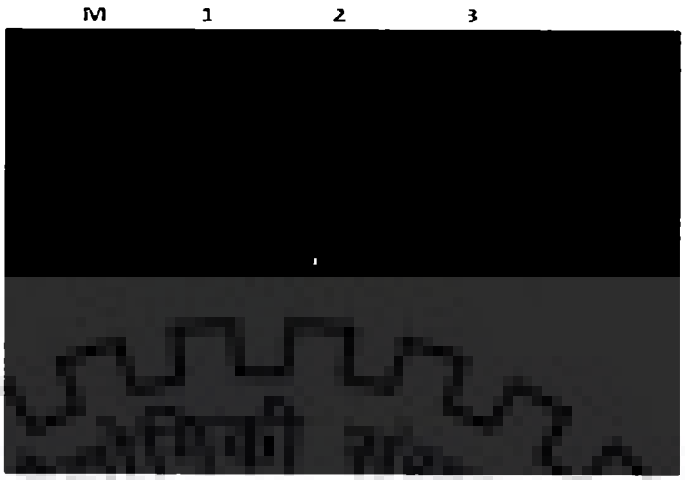


Fig 4.22: 1% agarose gel showing RNA quality. M indicates 1Kb DNA ladder, Lane1- PBW 343, Lane2- PHST 9 and Lane3- PHST 0

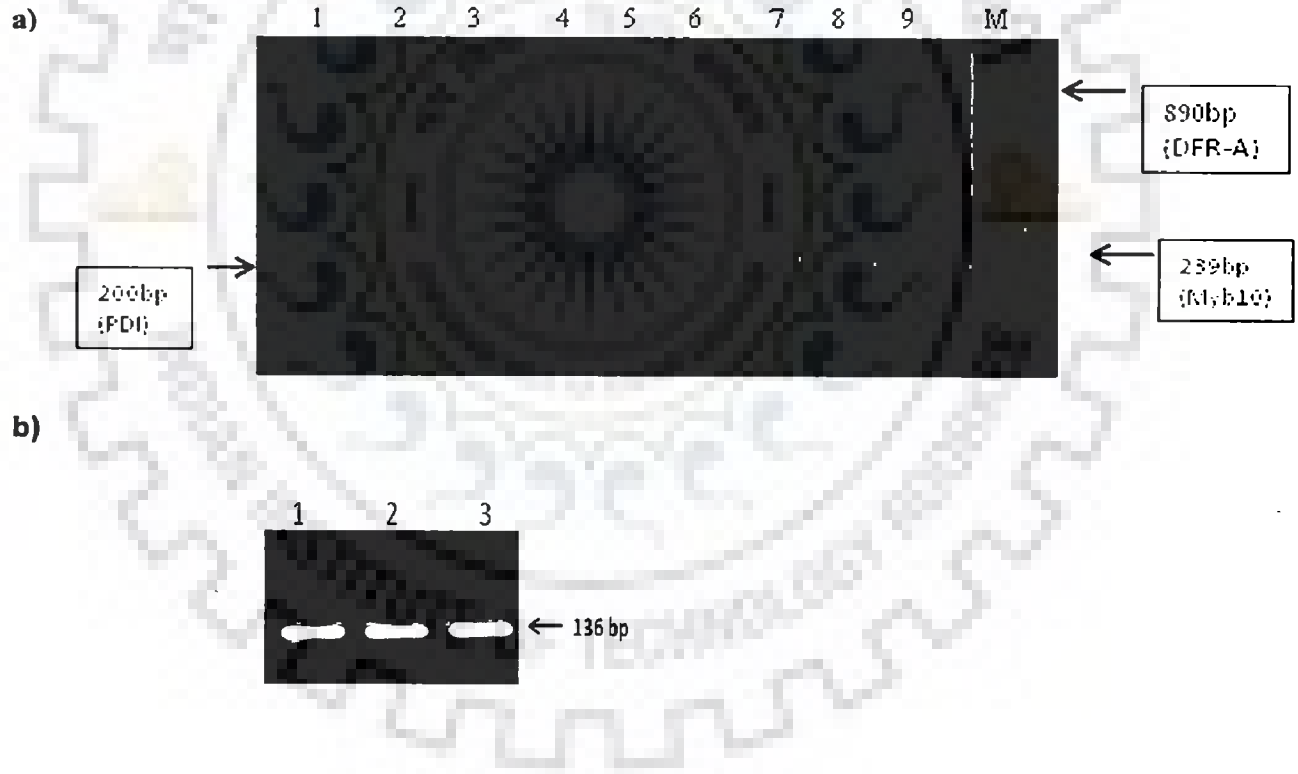


Fig 4.23: a) 1% agarose gel showing RT-PCR for three gene specific primers, PDI, myb10 and DFR-A. Lanes1, 4, 7 – PBW 343, Lanes2, 5, 8 - PHST 9, Lanes3, 6, 9 – PHST 0, Lane 10- 1Kb marker; b) House keeping gene, E1fα Lane1- PBW 343, Lane2- PHST 9, Lane3- PHST 0

chromosome 3AL or 4AL. SSR markers wmc420 and wmc89 which lied between barc170 and gwm269 were also bin mapped. So wmc420 was mapped on 3AL bin 3AL3-0.42-0.78 and 4AL bins 4AL4-0.80-1.00 and 4AL13-0.59-0.66. The ESTs were BE497784 and BE495175 for 3AL and BE404705, BE494382 and BE489999 for chromosome 4AL respectively. Chromosome bins on both chromosome 3AL and 4AL were identified for wmc89. ESTs BE497740 and BE498761 mapped to bins 3AL5-0.78-1.00 and C3AL3-0.42 respectively, while BE517802 mapped to chromosome bin 4AL4-0.80-1.00 respectively. 4AL SSR marker zxq118 (Zhang *et al.*, 2008b) mapped to both 3AL and 4AL bins i.e. 3AL3-0.42.0.78 and 4AL12-0.43-0.59 respectively. The mapping of this marker to both 3AL and 4AL bins can be justified as it differentiated between group 3 and group 4 alleles in NILs and landraces. SSR marker wmc89 bin mapped only to chromosome 3AL and not to chromosome 4AL while the QTL flanking marker barc170 mapped only to chromosome 3AL and not to 4AL although it was detected on 4AL QTL. The details about bin mapping have been summarized in Table 4.5.

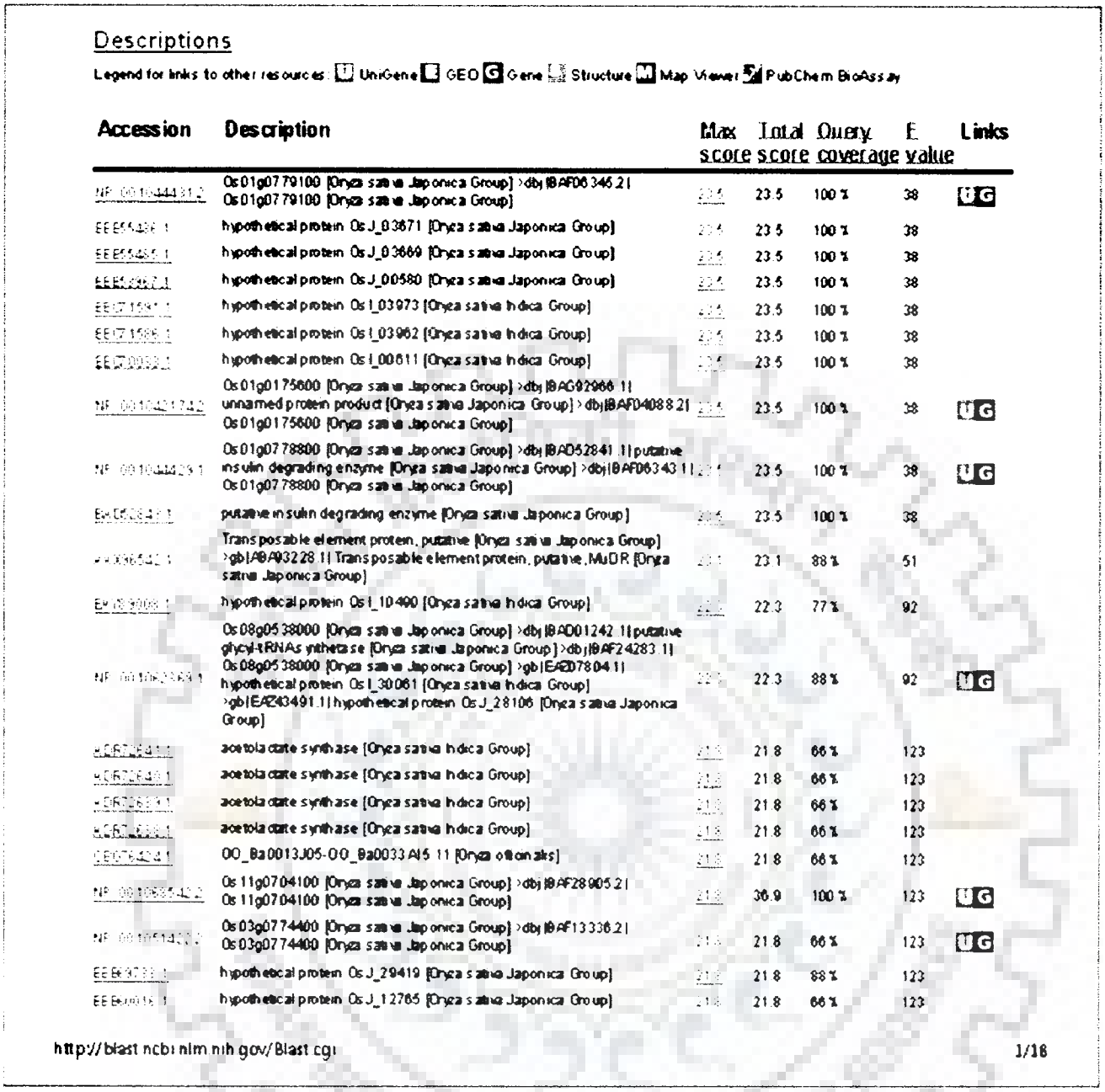
4.12.2 Protein identification and bin mapping

Selected spots were digested with trypsin and processed for LC-MS/MS analysis. The peaks list obtained for the protein spot i.e. PMF spectra was analyzed with the aid of Mascot search engine for Viridiplantae database. Mascot search results were further searched in NCBI database using blastp or tblastn to get the related or identical sequences. Blast was also performed with the mapped wheat EST's in graingenes to find similarity between the query and the reported 3AL bins. For spot no.3 identified in immature embryo protein 2-DE, probability score of 51 and 50 were obtained for HMW glutenin subunit 1By9 (*T. aestivum*, accession no. 22090) and unknown protein1 (accession no. 172046673) respectively. HMW glutenin is a storage protein in wheat while unknown protein was searched on NCBI using its accession number. This protein was found to be a small peptide sequence from a gymnosperm. Blastp results for the peptide are shown in Fig 4.24. This peptide has a 100% match to our peptide and

had the sequence VLDELTADR. The peptide blasted with *Oryza sativa* sequences and the sequences showing 100% coverage were selected for further study. Hence, nine *Oryza* sequences were selected for further study. The mRNA sequences of these nine selected protein sequences were further blasted with wheat mapped ESTs on graingenes and mRNA sequences of three were found to blast on group 3 chromosomes of wheat. Sequence Os01g0779100

Table 4.5: Wheat chromosome bins for selected SSR markers

SSR markers for chromosome 3AL	ESTs	Chromosome bins	SSR markers for chromosome 4AL	ESTs	Chromosome bins
gwm155	BE590549	3AL5-0.78-1.00 3BL7-0.63-1.00 3DL3-0.81-1.00	barc170	BE500510	C-3AL3-0.42 C-3BL2-0.22 C-3DL2-0.27
	BM138503	3BS8-0.78-1.00		BE591738	3AL5-0.78-1.00 3DL
	BF484790	3DS6-0.55-1.00	wmc420	BE497784	3AL3-0.42-0.78 3DL2-0.27-0.81 3BL
	BG262657	C-3BS1-0.33		BE404705	4AL13-0.59-0.66
	BF473016	3BS9-0.57-1.00 3DS3-0.24-1.00 3AL3-0.42-0.78		BE494382	4AL4-0.80-1.00
	BE442875	3AL5-0.78-1.00 3BL7-0.63-1.00 3DL3-0.81-1.00		BE489999	4AL4-0.80-1.00
	BE403326	3BS1-0.33-0.57		BE495175	3BL7-0.63-1.00 3DL2-0.27-0.81
wmc153	BE490613	3AL3-0.42-0.78 3BL7-0.63-1.00 3BL2-0.22-0.50 3DL2-0.27-0.81	wmc89	BE517802	4AL4-0.80-1.00
barc57	BF483618	3AL3-0.42-0.78 3DL2-0.27-0.81 3BL7-0.63-1.00		BE497740	3AL5-0.78-1.00 3BL7-0.63-1.00 3DL3-0.81-1.00
	BF473732	3AL5-0.78-1.00 3DL3-0.81-1.00		BE498761	C3AL3-0.42 C3BL2-0.22 C3DL2-0.27
	BE494345	C-3AL3-0.42 3BL2-0.22-0.50 3DL2-0.27-0.81	zxq118	BG604766	3AL3-0.42-0.78 3BL2-0.22-0.50 3DL2-0.27-0.81
				BF293573	4AL12-0.43-0.59



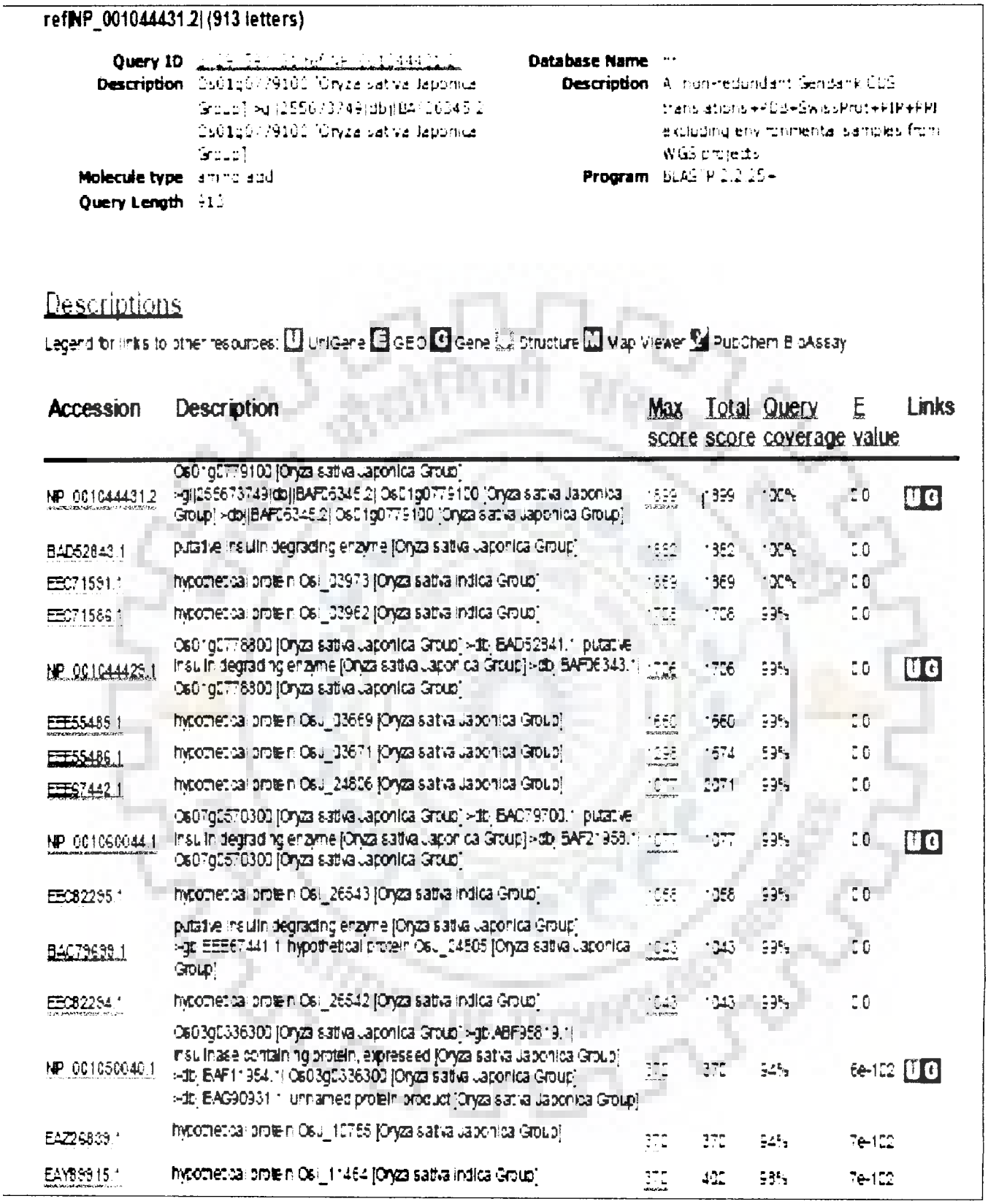
Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

Accession	Description	Max score	Total score	Query coverage	E value	Links
NP_001044312.2	Os01g0779100 [Oryza sativa Japonica Group] >dbj|BAF06346.2| Os01g0779100 [Oryza sativa Japonica Group]	23.5	23.5	100 %	38	U G
EEE55436.1	hypothetical protein OsJ_03671 [Oryza sativa Japonica Group]	23.5	23.5	100 %	38	
EEE55435.1	hypothetical protein OsJ_03669 [Oryza sativa Japonica Group]	23.5	23.5	100 %	38	
EEE54267.1	hypothetical protein OsJ_00580 [Oryza sativa Japonica Group]	23.5	23.5	100 %	38	
EEC71581.1	hypothetical protein OsI_03973 [Oryza sativa Indica Group]	23.5	23.5	100 %	38	
EEC71582.1	hypothetical protein OsI_03982 [Oryza sativa Indica Group]	23.5	23.5	100 %	38	
EEC70552.1	hypothetical protein OsI_00611 [Oryza sativa Indica Group]	23.5	23.5	100 %	38	
NP_001042174.2	Os01g0175600 [Oryza sativa Japonica Group] >dbj|BAG92966.1| unnamed protein product [Oryza sativa Japonica Group] >dbj|BAF04088.2| Os01g0175600 [Oryza sativa Japonica Group]	23.5	23.5	100 %	38	U G
NP_001044326.1	Os01g0778800 [Oryza sativa Japonica Group] >dbj|BAD52841.1| putative insulin degrading enzyme [Oryza sativa Japonica Group] >dbj|BAF06343.1| Os01g0778800 [Oryza sativa Japonica Group]	23.5	23.5	100 %	38	U G
EAZ05284.1	putative insulin degrading enzyme [Oryza sativa Japonica Group]	23.5	23.5	100 %	38	
EAY93542.1	Transposable element protein, putative [Oryza sativa Japonica Group] >gb|ABA93228.1| Transposable element protein, putative, MuDR [Oryza sativa Japonica Group]	23.1	23.1	88 %	51	
EAZ39099.1	hypothetical protein OsI_10490 [Oryza sativa Indica Group]	22.3	22.3	77 %	92	
NP_001063349.1	Os08g0538000 [Oryza sativa Japonica Group] >dbj|BAD01242.1| putative glycyl-tRNA synthetase [Oryza sativa Japonica Group] >dbj|BAF24283.1| Os08g0538000 [Oryza sativa Japonica Group] >gb|EAZ07804.1| hypothetical protein OsI_30061 [Oryza sativa Indica Group] >gb|EAZ43491.1| hypothetical protein OsJ_28106 [Oryza sativa Japonica Group]	22.3	22.3	88 %	92	U G
ACG27641.1	acetolactate synthase [Oryza sativa Indica Group]	21.8	21.8	66 %	123	
ACG27640.1	acetolactate synthase [Oryza sativa Indica Group]	21.8	21.8	66 %	123	
ACG27639.1	acetolactate synthase [Oryza sativa Indica Group]	21.8	21.8	66 %	123	
ACG27638.1	acetolactate synthase [Oryza sativa Indica Group]	21.8	21.8	66 %	123	
CAE05424.1	OO_Ba0013J05-OO_Ba0033A15.11 [Oryza officinalis]	21.8	21.8	66 %	123	
NP_001068542.2	Os11g0704100 [Oryza sativa Japonica Group] >dbj|BAF28905.2| Os11g0704100 [Oryza sativa Japonica Group]	21.8	36.9	100 %	123	U G
NP_001051433.1	Os03g0774400 [Oryza sativa Japonica Group] >dbj|BAF13336.2| Os03g0774400 [Oryza sativa Japonica Group]	21.8	21.8	66 %	123	U G
EEE63738.1	hypothetical protein OsJ_29419 [Oryza sativa Japonica Group]	21.8	21.8	88 %	123	
EEE60015.1	hypothetical protein OsJ_12765 [Oryza sativa Japonica Group]	21.8	21.8	66 %	123	

http://blast.ncbi.nlm.nih.gov/Blast.cgi 1/18

ig 4.24: Blastp for peptide sequence of hypothetical gymnosperm protein (accession no. 172046673) lentified for spot3 from 2-DE of PHST 0



ref|NP_001044431.2| (913 letters)

Query ID [redacted]
Description Os01g0779100 [Oryza sativa Japonica Group] >gi|255673749|db|BAF06345.2| Os01g0779100 [Oryza sativa Japonica Group]
Molecule type amino acid
Query Length 913

Database Name nr
Description All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
Program BLASTP 2.2.25+

Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer P PubChem BioAssay

Accession	Description	Max score	Total score	Query coverage	E value	Links
NP_001044431.2	Os01g0779100 [Oryza sativa Japonica Group] >gi|255673749|db|BAF06345.2| Os01g0779100 [Oryza sativa Japonica Group] >db|BAF06345.2| Os01g0779100 [Oryza sativa Japonica Group]	1899	1899	100%	0.0	U G
BAD52843.1	putative insulin degrading enzyme [Oryza sativa Japonica Group]	1862	1862	100%	0.0	
EEC71591.1	hypothetical protein OsI_03973 [Oryza sativa Indica Group]	1859	1859	100%	0.0	
EEC71586.1	hypothetical protein OsI_03962 [Oryza sativa Indica Group]	1726	1726	93%	0.0	
NP_001044429.1	Os01g0778800 [Oryza sativa Japonica Group] >db|BAD52841.1| putative insulin degrading enzyme [Oryza sativa Japonica Group] >db|BAF06343.1| Os01g0778800 [Oryza sativa Japonica Group]	1726	1726	93%	0.0	U G
EEE55485.1	hypothetical protein OsJ_03669 [Oryza sativa Japonica Group]	1660	1660	93%	0.0	
EEE55486.1	hypothetical protein OsJ_03671 [Oryza sativa Japonica Group]	1674	1674	93%	0.0	
EEE67442.1	hypothetical protein OsJ_24806 [Oryza sativa Japonica Group]	2071	2071	93%	0.0	
NP_001060044.1	Os07g0570300 [Oryza sativa Japonica Group] >db|BAC79703.1| putative insulin degrading enzyme [Oryza sativa Japonica Group] >db|BAF21958.1| Os07g0570300 [Oryza sativa Japonica Group]	1977	1977	93%	0.0	U G
EEC82295.1	hypothetical protein OsI_26543 [Oryza sativa Indica Group]	1958	1958	93%	0.0	
BAC79699.1	putative insulin degrading enzyme [Oryza sativa Japonica Group] >gb|EEE67441.1| hypothetical protein OsJ_24505 [Oryza sativa Japonica Group]	1943	1943	93%	0.0	
EEC82294.1	hypothetical protein OsI_26542 [Oryza sativa Indica Group]	1943	1943	93%	0.0	
NP_001060040.1	Os03g0336300 [Oryza sativa Japonica Group] >gb|ABF95819.1| insulinase containing protein, expressed [Oryza sativa Japonica Group] >db|BAF13954.1| Os03g0336300 [Oryza sativa Japonica Group] >db|BAG90931.1| unnamed protein product [Oryza sativa Japonica Group]	370	370	54%	6e-102	U G
EAZ26839.1	hypothetical protein OsJ_10755 [Oryza sativa Japonica Group]	370	370	54%	7e-102	
EAY89915.1	hypothetical protein OsI_11464 [Oryza sativa Indica Group]	370	422	93%	7e-102	

ig 4.25: Blastp for Os01g0779100, a putative protein identified from blastp of hypothetical gymnosperm rotein

blasted on EST BE497979 and BF478742 with 3AL5-0.78-1.00, C-3BL10-0.50, 3DL2-0.42-0.78 and 3AL3-0.42-0.78 as respective chromosome bins. Blastp results for Os01g0779100 have been shown in Fig 4.25. The second sequence Os01g0175600 blasted on EST BF474720 with 3AL5-0.78-1.00 and 3BL7-0.63-1.00 chromosome bins. The third sequence was Os01g0778800 which were mapped on two ESTs BE497979 and BF478742. The chromosome bin location of these ESTs was 3AL5-0.78-1.00, C-3BL10-0.50, 3DL2-0.27-0.81 and 3AL-0.42-0.78 respectively. It is important to view that two ESTs BE497979 and BF478742 were common for Os01g0779100 and Os01g0778800. LC-ESI-MS/MS study of spot no.5 identified in 2-DE of immature protein identified it as wheat serine carboxypeptiase (SCP) (accession no. 443481). SCP are protein hydrolyzing enzymes and are classified as SCP I, II, III. In rice there are 71 SCPs. The third protein spot studied from immature embryo proteins 2-DE was number 6 which was identified as Os01g0749000 (*O. sativa*). Blastp was done and three other proteins matched the same set of peptides which were OsJ_03454 (*O.sativa*), unknown protein (*Zea mays*) and hypothetical protein SORBIDRAFT_03g034600 (*Sorghum bicolor*). All these proteins had a probability score of 64. Os01g0749000 belongs to DUF1264 superfamily and is an embryo specific protein. The function of this protein is not known. The protein sequence yielded a predicted protein from *Hordeum vulgare* (accession no. BAK04681.1) along with other organism like *Sorghum*, *Vitis*, *R. Communis* etc. The sequence of Os01g0749000 (accession no. 115439929) was searched against wheat EST database. The ESTs bin mapped on group 3 chromosomes were BE607045, BE517750, BF483299, BE591590, BE497740 and BE442531. There was no significant EST found matching exclusively on group 4 chromosomes. EST BE607045, BE483299, BE442531 were from chromosome 3AL. EST BE607045 belonged to a unigene cluster for DFR gene. Another protein identified with

Basic Local Alignment Search Tool

Formatting Results - VJTWPYBE014

dbj|BAF25991.1| (1401 letters)

Query ID [illegible]
Description Os10g0125700 [Oryza sativa Japonica Group] >gb|AAK52518.1|AC079128_1 Putative bacterial blight resistance protein Xa1-like protein [Oryza sativa Japonica Group] >gb|AAM08734.1|AC025098_1 Putative bacterial blight resistance protein Xa1-like protein [Oryza sativa Japonica Group] >gb|AAP51954.1| NB-ARC domain containing protein, expressed [Oryza sativa Japonica Group] >gb|11368696|dbj|BAF25991.1| Os10g0125700 [Oryza sativa Japonica Group] >gb|125573898|gb|EAZ15172.1| hypothetical protein OsJ_30590 [Oryza sativa Japonica Group]
Molecule type amino acid
Query Length 1401

Database Name nr
Description All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
Program BLASTP 2.2.25+

		score	score	coverage	value	
NP_001064077.1	Os10g0125700 [Oryza sativa Japonica Group] >gb|AAK52518.1|AC079128_1 Putative bacterial blight resistance protein Xa1-like protein [Oryza sativa Japonica Group] >gb|AAM08734.1|AC025098_1 Putative bacterial blight resistance protein Xa1-like protein [Oryza sativa Japonica Group] >gb|AAP51954.1| NB-ARC domain containing protein, expressed [Oryza sativa Japonica Group] >dbj|BAF25991.1| Os10g0125700 [Oryza sativa Japonica Group] >gb|EAZ15172.1| hypothetical protein OsJ_30590 [Oryza sativa Japonica Group]	2899	2899	100%	0.0	G
EAY83662.1	hypothetical protein OsI_38887 [Oryza sativa Indica Group]	1293	1570	77%	0.0	
EEC66508.1	hypothetical protein OsI_32622 [Oryza sativa Indica Group]	979	1308	50%	0.0	
EAZ22496.1	hypothetical protein OsJ_06159 [Oryza sativa Japonica Group]	782	782	97%	0.0	
XP_002451904.1	hypothetical protein SORBIDRAFT_04g009750 [Sorghum bicolor] >gb|EES04880.1| hypothetical protein SORBIDRAFT_04g009750 [Sorghum bicolor]	770	770	99%	0.0	G
NP_001046492.1	Os02g0262800 [Oryza sativa Japonica Group] >dbj|BAD28895.1| putative bacterial blight-resistance protein Xa1 [Oryza sativa Japonica Group] >dbj|BAD29495.1| putative bacterial blight-resistance protein Xa1 [Oryza sativa Japonica Group] >dbj|BAF08406.1| Os02g0262800 [Oryza sativa Japonica Group]	769	769	97%	0.0	UG
BAK03994.1	predicted protein [Hordeum vulgare subsp. vulgare]	763	763	97%	0.0	
ABU54405.1	RGA-1 [Triticum aestivum] >gb|ABU54408.1| Lr1-like protein [Triticum aestivum]	713	713	97%	0.0	

Fig 4.26: Blastp of Os01g0749000 identified from LC-ESI-MS/MS of spot6 from 2-DE of PHST 0. A hypothetical protein from *Oryza* and RGA1 like protein from *T. aestivum* were identified

ref|NP_177681.2| (206 letters)

Query ID [illegible]
Description DNA binding / transcription factor [Arabidopsis thaliana] >gi|48428164|sp|Q9LQZ2.1|DRE2D_ARATH RecName: Full=Dehydration-responsive element-binding protein 2D; Short=Protein DREB2D >gi|9369375|gb|AAF87124.1|AC006434_20 F10A5.29 [Arabidopsis thaliana] >gi|51969946|dbj|BAD43665.1| transcription factor DREB2A like protein [Arabidopsis thaliana]
Molecule type amino acid
Query Length 206

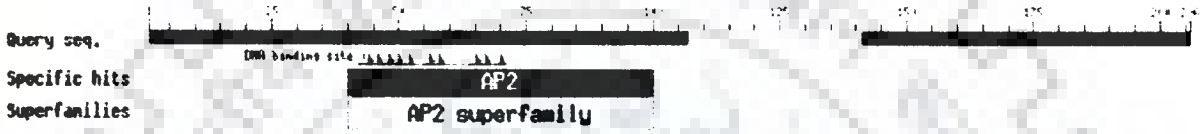
Database Name nr
Description All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
Program BLASTP 2.2.25+ [illegible]

Other reports: [illegible]

▼ Graphic Summary

▼ [illegible]

Putative conserved domains have been detected, click on the image below for detailed results.



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Links
[illegible]	DNA binding / transcription factor [Arabidopsis thaliana] >gi	[illegible]	427	100%	5e-118	U G
[illegible]	At1g75490 [Arabidopsis thaliana]	[illegible]	409	95%	1e-112	
[illegible]	hypothetical protein ARALYDRAFT_476698 [Arabidopsis lyra	[illegible]	372	95%	2e-101	G
[illegible]	Dehydration-responsive element-binding protein 2G, putativ	[illegible]	170	96%	9e-41	G
[illegible]	JHL18I08.6 [Jatropha curcas]	[illegible]	163	92%	1e-38	
[illegible]	PREDICTED: hypothetical protein [Vitis vinifera]	[illegible]	163	95%	1e-38	U G M
[illegible]	AP2/ERF domain-containing transcription factor [Populus tri	[illegible]	157	80%	7e-37	U G
[illegible]	putative DRE-binding protein 5 [Populus trichocarpa]	[illegible]	157	80%	7e-37	
[illegible]	AP2/ERF domain-containing transcription factor [Populus tri	[illegible]	150	82%	9e-35	U G
[illegible]	hypothetical protein VITISV_037056 [Vitis vinifera]	[illegible]	150	54%	1e-34	
[illegible]	PREDICTED: hypothetical protein [Vitis vinifera]	[illegible]	146	78%	1e-33	U G M
[illegible]	hypothetical protein VITISV_032355 [Vitis vinifera]	[illegible]	142	71%	1e-32	
[illegible]	unnamed protein product [Vitis vinifera]	[illegible]	141	85%	5e-32	
[illegible]	hypothetical protein ARALYDRAFT_909855 [Arabidopsis lyra	[illegible]	140	42%	8e-32	G
[illegible]	hypothetical protein RCOM_0610630 [Ricinus communis] >g	[illegible]	139	60%	3e-31	G
[illegible]	hypothetical protein ARALYDRAFT_903325 [Arabidopsis lyra	[illegible]	138	73%	4e-31	G
[illegible]	AP2 domain-containing transcription factor, putative [Arabi	[illegible]	137	39%	7e-31	U G
[illegible]	Dehydration-responsive element-binding protein 2C, putativ	[illegible]	135	60%	4e-30	G
[illegible]	AP2 domain-containing transcription factor, putative [DRE2	[illegible]	134	73%	5e-30	U G
[illegible]	dehydration responsive element binding protein [Solanum ly	[illegible]	132	42%	4e-29	G
[illegible]	AP2 domain transcription factor [Arabidopsis thaliana]	[illegible]	131	73%	5e-29	
[illegible]	hypothetical protein SORBIDRAFT_07g023575 [Sorghum bic	[illegible]	131	35%	6e-29	G
[illegible]	dehydration responsive element binding protein [Buchloe da	[illegible]	130	37%	7e-29	
[illegible]	hypothetical protein SELMODRAFT_38502 [Selaginella moell	[illegible]	130	36%	9e-29	U G
[illegible]	DREB-like protein 1 [Zoysia japonica] >gb|ACX37096.1| DRI	[illegible]	128	37%	4e-28	

Fig 4.27: Blastp of (spot6) *A. thaliana* protein (accession no.79381684) identified DREB like proteins

Table 4.6: Chromosome bins for genes coding selected proteins

Serial No.	Protein spot no.	Sequence i.d./ organism	BLASTp	ESTs	Chromosome bin
1.	3 IME	172046673/gymnosperm	Os01g0779100	BE497979	3AL5-0.78-1.00 C-3BL10-0.50 3DL2-0.27-0.80
				BF478742	3AL3-0.42-0.78
			Os01g0175600	BF474720	3AL5-0.78-1.00 3BL7-0.63-1.00
			Os01g0778800	BE497979	3AL5-0.78-1.00 C-3BL10-0.50 3DL2-0.27-0.81
				BF478742	3AL3-0.42-0.78
2.	6IME	Os01g0749000	Os01g0749000	BE607045	3AL3-0.42-0.78 C-3DL2-0.27
				BE517750/ BE591590	C-3AL3-0.42 C-3BL2-0.22 C-3DL2-0.27
				BF483299/ BE442531	3AL5-0.78-1.00
				BE497740	3AL5-0.78-1.00 3BL7-0.63-1.00 3DL3-0.81-1.00

MASCOT probability score of 46 for spot no.6 was Os10g0125700. This protein is a putative blight resistance protein in *Oryza*. Blastp was run for the protein on NCBI and a RGA-1 like protein in *T. aestivum* (accession no. ABU54405.1) was identified which showed 97% homology to this protein. Blastn was done for CDS (conserved domain sequence) of RGA-1 and various different proteins ranging from *rga* to *Lr* (leaf rust) sequences were identified with varying homology (Fig 4.26). It is important to discuss here about a protein identified with low

probability value of 30. The protein was a DNA binding/ transcription factor (accession no.79381684) from *A. thaliana*. Upon blastp AP2 domain proteins were identified like DREB (dehydration responsive element binding protein). DREB transcription factor 6 (accession no. AY781361.1) showed a high homology of 83% with the above mentioned *A. thaliana* protein (Fig 4.27). Spot no.7 from immature embryo 2-DE had probability scores of 118 and 55 for putative avenin like precursor (*T. aestivum* accession no. 89143120) and putative cytochrome P450 (*Ricinus communis* accession no. 255538634). No further study was done on avenin as it is a storage protein. The results for chromosome bin mapping have been summarized in Table 4.6.

Chapter V
Discussion

CHAPTER-5

To avoid frequent yield losses and deterioration of processing quality in wheat due to PHS (pre harvest sprouting), it would be desirable to develop wheat varieties tolerant to PHS. The QTL of PHST in wheat have been mapped on almost all wheat chromosomes (Anderson *et al.,* 1993; Roy *et al.,* 1999; Zanetti *et al.,* 2000; Mares *et al.,* 2001; Kato *et al.*, 2001; Groos *et al.*,2002; Mrva & Mares, 2002; Osa *et al.,* 2003; Mares *et al.,* 2005; Torada *et al.,* 2005; Mori *et al.,* 2005; Kulwal *et al.*,2005; Zhang *et al.,* 2008b; Liu *et al.,* 2008 and Liu and Bai, 2010). Hence, it would be desirable to identify a major QTL stable across environments, with tightly linked flanking markers to facilitate marker assisted breeding. Development of NILs for such QTL is very crucial for fine mapping, proteomics, characterization and cloning of genes for a quantitative trait. In this study a NIL highly tolerant, to PHS due to a major QTL in an elite wheat cultivar PBW343 has been used to study the proteomics and expression for PHS tolerance.

5.1 Seed colour

Seed colour plays an important role in governing dormancy in wheat, with red coloured grain being dormant. Wheat grain colour is controlled by R genes on the end region of the long arms of wheat chromosomes 3A, 3B and 3D (McIntosh *et al.,* 1998) with each R gene i.e. R1 on 3D, R2 on 3A, or R3 on 3B contributing to dormancy. Red seed colour in wheat is related to seed dormancy as R loci is 30cm away from dormancy gene in wheat (Bailey *et al.*, 1999). The plant material used in our study consisted of PBW 343, PHS susceptible (PHSS) with amber seed colour and NIL PHST 0, PHS tolerant (PHST) with red seed colour (Fig 4.1). We tested these lines in our lab for PHS tolerance and concluded that NIL PHST 0 which had red seed colour, showed high tolerance to PHS like the donor parent SPR8198, while PBW343 with amber seed colour demonstrated high PHSS suggesting that PHST may be either due to red seed colour or closely associated with it. The major QTL controlling PHST in a population developed from SPR8198 (Garg *et al.*, 2007) was mapped on chromosome 3AL in an interval between SSR

markers wmc155 and gwm153 (Kulwal *et al.*, 1994). The graphical genotypes of introgression lines of SPR5 (PHST) and PBW343 (PHSS) were studied and SSR profile of wmc153 and gwm155 were found to be associated with PHST (Garg et al., 2007). In addition the gene for red seed colour (R gene) is also present on long arm of group 3 chromosomes, further indicating the association between red seed colour and PHST. Therefore, red seed colour has been shown to affect grain dormancy and resistance to PHS (Flintham *et al.*, 2000; Warner *et al.*, 2000; Himi *et al.*, 2002). Sharma *et al.* (1994) using SPR8198 in a backcross programme reported that PHST in SPR8198 was controlled by a single dominant gene. They were able to recover a highly tolerant PHS derivative in the background of wheat cultivar WL711, highly susceptible to PHS suggesting that the red seed colour maybe only tightly linked to PHST and may not have pleiotropic effect for PHST.

5.2 Preservation of dormancy

Sprouting tolerance in wheat is lost between two months after harvesting. Dormancy is dissipated by after ripening in dry storage at room temperature in a similar manner for the red and white lines. We therefore preserved PHS tolerance after harvesting by storing seeds of NIL PHST0 at -86°C. It was interesting to observe that the PHST in NIL PHST0 was preserved for around two years. The preserved seeds of PHST0 were dormant and did not germinate till ten days after imbibition. Hence, it can be said that under room storage conditions, with high metabolic activity the factor responsible for PHST might be degraded. To the best of my knowledge this is the first report of prolonged maintenance of PHST under deep freezer storage conditions. To further elucidate, the plant material stored at -86°C was used for phytohormone analysis (Fig 4.4 and 4.5) in our study.

5.3 Effect of Phytohormones

PHST and seed germination have been reported to be due to intricate balance and antagonism between phytohormones ABA and GAs. Exogenous application of ABA and GA_3 at different concentrations during *in vitro* germination of seeds of PBW 343 and PHST 0 stored at -86°C

indicated that PBW 343 was insensitive to ABA, while PHST 0 was ABA sensitive. Concentrations as high as 100ppm ABA did not alter the response of PBW343 which continued to demonstrate ABA insensitivity (Fig 4.4). On the contrary PHST0 was found to be Gibbrellic acid (GA_3) insensitive and concentration as high as 500ppm GA had only limited effect on inducing germination in NIL PHST0 (Fig 4.5). These results strongly suggest that PHS susceptible lines are insensitive to ABA and GA is not able to break tolerance of PHST lines. ABA being the hormone imparting dormancy was more of interest than GA as GA plays a role in germination after breaking seed dormancy. Mutant studies have shown that ABA insensitivity is imparted either due to loss of function or deficiency of ABA biosynthetic hormone or deficiency in a transcription factor (Kormneef *et al.,* 1982; Xiong *et al.,* 2001; Leon-kloosterziel *et al.,* 1996; Llorente *et al.,* 2000; Rook *et al.,* 2001; Xiong *et al.,* 2001; Robertson, 1955; McCarty *et al.,* 1991; Neill *et al.,* 1986; Schwartz *et al.*, 1997 Suzuki *et al.,* 2008 and Fang *et al.,* 2008). Walker-Simmons (1987) have described that ABA sensitivity is a more important factor than endogenous ABA content for regulation of wheat seed dormancy. Embryonic ABA levels of each cultivar were similar with the sprouting-susceptible cultivar having about a 25% lower ABA level than that of the sprouting-resistant cultivar. Larger differences between the cultivars were noted in sensitivity to ABA, as measured by capability of ABA to block embryonic germination. It was also shown that ABA inhibited embryonic germination much more effectively in the sprouting tolerant cultivar (Walker-Simmons, 1987). It has been demonstrated by Ried and Walker-Simmons (1990) and Wang *et al.* (1995) that dormant embryos have ability to re synthesize ABA during imbibition. Noda *et al.* (2002) have also suggested that sensitivity of wheat embryos to germination inhibition by ABA is controlled primarily by a gene(s) located on the long arm of chromosome 4A.

5.4 QTL studies for chromosome 3A and 4A

The QTL *Phs.ccsu-3A.1* controlling 78% of the variance in the ITMI (international triticeae mapping initiative) mapping population derived from W7984 (synthetic wheat) X Opata85

(Kulwal *et al.*, 2004) was validated in PHST0 NIL (Garg *et al.*, 2007) within the marker interval wmc153 and gwm155 was associated with red seed colour, which is not desirable for *chapatti* making quality. It will therefore be desirable to identify some other PHS tolerant sources with amber seed colour. A major QTL on 4AL for sprouting tolerance has also been reported in several Australian wheat germplasm with amber seed colour (Mares *et al.*, 2001; Kato *et al.*, 2001; Torada *et al.*, 2005; Zhang *et al.*, 2008). A list of wheat landraces collected from remote hilly areas of Uttarakhand with amber seed had high level of PHS tolerance has been given in Annexure I). We tried to validate barc170 and gwm269, the QTL flanking markers on 4AL explaining 21% of phenotypic variation (Mares *et al.*, 2005) in our study. SSR marker zxq118 has been reported to be chromosome 4AL specific and has been mapped between barc170 and gwm269 on chromosome 4A by Zhang *et al.* (2008). The molecular marker zxq118 reported to be linked to PHST on 4AL was used to validate the QTL in ten selected landraces, PBW 343 and its NILs. The allele of zxq118 associated with PHST was present in PHST landraces both with red (IITR15, IITR31, IITR73) and amber (IITR26) seed colour, whereas it was absent in PBW 343 and PHST0 indicating that PHST in PHST 0 could be only due to QTL on chromosome 3AL. The landraces with 4AL QTL can be effectively used for allele which confers PHST through marker assisted breeding with amber seed colour (Zhang *et al.*, 2008). Another 4A QTL flanking marker gwm269 differentiated between amber PHS tolerant and amber PHS susceptible IITR landraces in our study. The QTL flanking markers barc170 and gwm269 have also been validated by Christopher *et al.* (2008) for resistance to PHS. 3AL QTL flanking markers wmc 153 and gwm 155 have been validated in a NIL population by Garg *et al.* (2007). In our plant material comprising of IITR landraces, PBW343 and NILs wmc153 and gwm 155 were monomorphic and therefore could not be validated. Other then these three SSR markers we also tried to validate ten more SSR makers lying between the flanking markers for both 3AL and 4AL were selected from genetic maps of Somers *et al.* (2004). But none of these SSR markers could be validated in NILs or IITR

landraces or differentiate between PHST or PHSS lines. Moreover as PHST0 is ABA sensitive so that might be the reason that we could validate chromosome group 4A SSR markers and not group 3A SSR markers. As the flanking markers for QTL *Phs.ccsu-3A.1* could not be validated in PBW343 and NIL PHST0 further efforts are required to develop markers for efficient marker assisted selection.

5.5 Study of differential proteins

1D SDS-PAGE was done for PBW343 and NIL PHST0, but no differences could be found in one dimension between PBW343 and PHST0. This indicates that PBW343 derived NIL PHST0 was very similar to it. Therefore to study differences at the proteome level we proceeded to 2-DE for both PBW343 and PHST0. 2-DE was done for endosperm proteins, mature embryo proteins and immature embryo proteins. In 2-DE gels number of distinct and variable protein spots with both qualitative and quantitative differences could be observed which might be associated with PHST. For unique proteins present in PHST0 and absent in PBW343, LC-ESI-MS/MS was done. Therefore, a total of seven protein spots were analyzed by LC-ESI-MS/MS, three of which were from endosperm and four from mature embryo 2-DE gels.

5.5.1 Endosperm proteins

Three protein spots were analysed using LC-ESI-MS/MS for endosperm proteins 2-DE gel of PHST 0.The spots were selected from PHST0 as these were unique to the NIL as they were absent from PBW 343. PH-END-1 and PH-END-2 were LMW glutenin proteins which are the chief storage proteins in wheat. END-3 was identified as protein disulfide isomerase protein (PDI). Though PDI is a seed storage protein but it plays a role in ABA responses indirectly. PDI locus of wheat is reported to be syntenic to esp2 locus of rice (Johnson *et al.*, 2006). PDI gene family encodes several PDI and PDI-like proteins containing thioredoxin domains and controlling diversified metabolic functions, including disulfide bond formation and isomerisation during protein folding. The flow diagram of the possible role of PDI is given in Fig 5.1. PDI genes have been mapped on group 4 chromosomes and show close association

with GERMIN genes. Physical mapping led to localisation of wheat PDI genes to chromosomal bins on chromosome 4AL, 4BS and 4DS (Johnson *et al.*, 2006). PDI is responsible for arrangement of disulphide bonds which determines the tertiary and quaternary

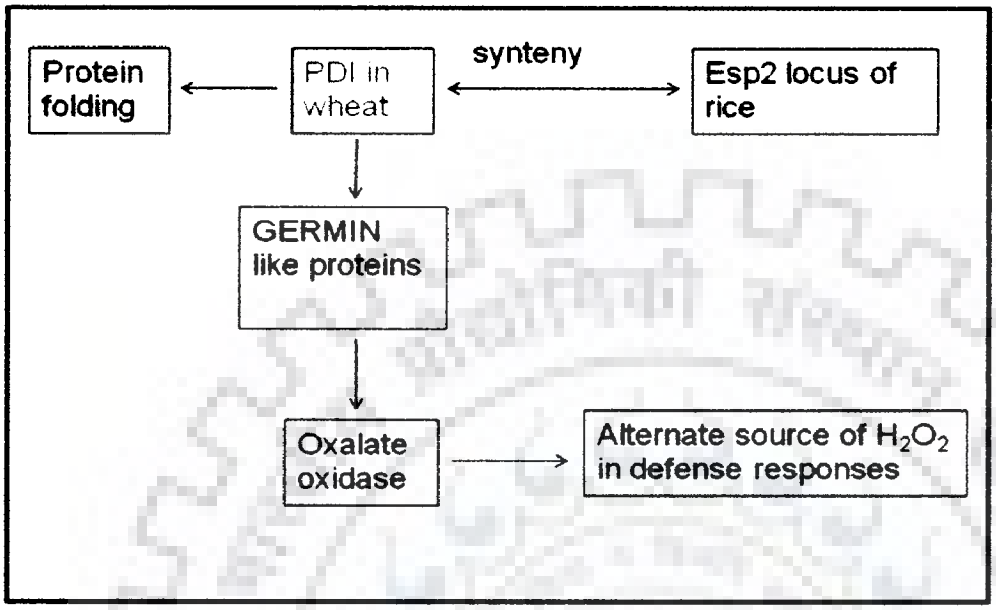


Fig 5.1: Flow diagram showing different functions of PDI

structures of storage proteins (Shewry *et al.*, 2003). This implicates the role of enzyme as a potentially important factor in the developing wheat endosperm. Six germin subfamilies (GER1-6) were characterised by Druka *et al.*, (2002) with varying enzymatic activities e.g. OXO action in GER1 and SOD activity in GER2 (Banerjee and Maiti, 2010), GER4 and GER5 sub-families. Germins possess oxalate oxidase activity which enzymatically releases hydrogen peroxide (H_2O_2) from oxalate. H_2O_2 generated can participate in signalling cascades triggering defence responses. Therefore, germin family of proteins has a role in plant defense (Lane, 1994) via oxalate oxidase activity. So, PDI is a protein folding enzyme found specifically in endosperm, but on further review it was found that PDI genes show close association with germin genes which are responsible for disease or pathogen responses.

5.5.2 Embryo proteins

From 2-DE gel of embryo specific protein spots were analysed using LC-ESI-MS/MS. The proteins identified were HMW glutenin and an unknown gymnosperm sub cellular protein

(172046673) for spot3, second protein identified was wheat serine carboxy peptidase for spot5, two unknown proteins from *O.sativa* were identified for spot6 and spot7 was identified as avenin like protein and cytochrome P450 from *R.communis*.

HMW glutenins are seed storage proteins in wheat but their presence in embryo was intriguing as these are endosperm specific. The glutenin/gliadin ratio has been reported to be higher in tolerant varieties during drought stress by Guóth *et al.* (2009). A high level of embryo ABA and *Vp-1* gene product are both required in early maturation phase for germination suppression and also for accumulation of storage globulins encoded by the gene Glbl (Globulin1) in maize. It has been suggested that ABA influences storage protein accumulation by initiating synthesis, suppressing degradation, and inhibiting precocious germination (Rivin and Grudt, 1991). Abscisic acid has been reported to be responsible for maintenance of maturation and protein synthesis of developing alfalfa embryos. In other words, ABA prevented germination and maintained storage protein synthesis (Xu and Bewley, 1995). In our case NIL PHST 0 was ABA sensitive while PBW 343 was ABA insensitive, so the occurance of HMW glutenin can be explained taking into account the above observations.

Serine carboxy peptidase (SCP) is an exopeptidase which releases free amino acids from the C terminal ends of proteins. In wheat five types of SCPs (SCP-I to V) have been identified. In a study conducted in *Arabidopsis* sleepy-1 (*sly-1*) mutants, it has been shown that serine carboxy peptidase S10 (SCS10) protein shows higher expression in after ripened *sly1-2* than in *ga1-2* in the absence of GA than in the presence of GA (Ariizumi and Steber, 2007). SLY-1 is a GA signalling gene and mutations in this gene lead to increased seed dormancy. In our case, SCP was identified in NIL PHST0 and PHST0 is GA insensitive, as inferred after application of concentrations as high as 500ppm exogenously. So our observation is not in conjunction with Ariizumi and Steber (2007).

Another evidence that can be said in support of SCP in PHST0 has been shown in Fig 5.2. In another study Brassinosteroid supressor1 (*BRS1*) has been reported to encode a secretory SCP

and regulate brassinosteroid insensitive 1(BRI1), a brassinosteroid receptor gene, signalling in *Arabidopsis*. Using activation tagging of weak *bri1* allele (*bri1-5*), a new locus *brs-1D* (*bri1supressor dominant*) was identified which was predicted to encoded secretory carboxypeptidase (Li *et al.*, 2001). In another study, bri 1-5 enhanced (BEN1) gene was identified to encodes a dihydroflavonol 4-reductase (DFR) like protein regulating brassinosteroid (BR) levels in Arabidopsis. BEN1 is a paralog for DFR and anthocyanidin reductase (BAN). Both DFR and BAN are involved in flavonoid synthesis and so it was proposed that BEN1 could also indirectly modulate BR levels through flavonoids (Yuan

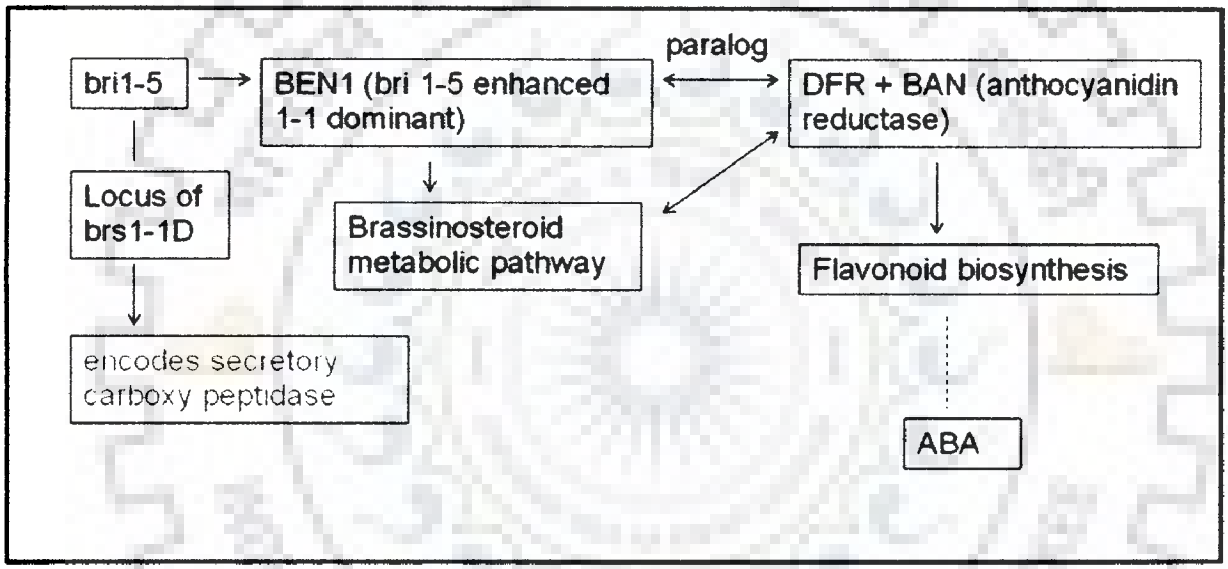


Fig 5.2: The proposed hypothesis for role of brassinosteroid in ABA metabolic pathway

et al., 2007). The proposed hypothesis for role of BR in flavonoid biosynthesis and possible relation with ABA has been depicted in Fig 5.2. In wheat, flavonoids are responsible for the production of red pigment phlobaphene which produces of red seed colour. BRs have also been proposed to regulate grain filling in rice by stimulating the flow of assimilates from source to sink (Wu *et al.*, 2008). SCP is related to BR signalling in *Arabidopsis* and BR is responsible for controlling seed size and weight in rice. Similarly, in our case also BR might be responsible for increased seed size and weight of PHST0. The seeds for PHST0 are bolder and bigger than either PBW343 or PHST9 (Fig 4.1). 24-epibrassinolide (EBR) has been proposed to function in reducing water stress in tomato by elevating endogenous ABA concentration. This suggests

that BRs might function upstream of ABA to regulate stomatal conductance and reduce stress (Yuan *et al.*, 2010). ABA levels are elevated in most of the stress conditions and BR might possibly play a role in this elevation.

For spot number6 two unknown proteins from *O.sativa* were identified. First protein identified was Os01g0749000 which matched OsJ_03454 (*O.sativa*), unknown protein (*Zea mays*) and hypothetical protein SORBIDRAFT_03g034600 (*Sorghum bicolor*) upon Blastp. Function of all these proteins is unknown. EST bin mapping was done for Os01g0749000 and EST BE607045 was found to belong to DFR unigene cluster and the EST was also bin mapped to chromosome 3AL. Himi *et al.* (2005b) reported that the expression of flavonoid biosynthesis genes, CHS, CHI, F3H and DFR were not activated in white grained wheat. The authors concluded that *R* (for grain colour) and *Rc* (gene for coleoptiles colour) genes were involved in the upregulation of flavonoid synthesis genes, but had different modes of gene activation. In our study the protein was detected in PHST0 which has red seed colour. Another protein identified for spot no.6 was Os10g0125700. This protein is a putative blight resistance protein in *oryza* but upon Blastp *RGA-1* (repressor of GA1-3 1) like protein in *T.aestivum* (accession no. ABU54405.1) was identified which showed 97% homology to this protein. *RGA* and *GAI* are negative regulators of GA signal transduction pathway in *Arabidopsis*. *RGA* and *GAI* have partially redundant functions in maintaining repressive state of GA signalling pathway but *RGA* plays a more dominant role (Dill and Sun, 2001). In our case the protein was identified in PHST0 and it showed GA insensitivity which can be attributed to *RGA-1*.

The other protein identified for spot6 with a lower probability score of 30 was a DNA binding/ transcription factor (accession no.79381684) from *A.thaliana*. Upon blastp DREB was identified which is an *AP2/ERF* domain protein. DREB proteins are mainly involved in plant response to abiotic stresses such as cold, drought or high salinity as well as in ABA signalling. In *Arabidopsis*, DREBs generally function in ABA independent manner except C-repeat binding factor (CBF4) which is ABA dependent (Kobayashi *et al.*, 2008). Therefore, DREBs

control the expression of stress-responsive genes via ABA-independent/dependent pathways under both abiotic and biotic stress (Agarwal *et al.*, 2006). In our study DREB would have been identified as the transcription factor might be required in drought tolerance during seed maturity as during physiological maturity, the seed moisture content reduces from 35-45% to 12-9%. Therefore, to conclude we had identified three proteins/ transcription factors like *DFR*, *RGA-1* and *DREB* for the same protein spot from mature embryo (stage 95) of PHST0. Hence, this protein spot might be the putative candidate gene/ gene product for dormancy in bread wheat.

5.6 Transcriptome analysis

Transcriptome studies were conducted using gene specific primers for PDI, DFR and myb. As PDI is responsible for protein folding, so it was expected to be present in all the lines. But it was identified using LC-ESI-MS/MS only in PHST0, which might point towards its varied role other than protein folding. For PDI the product size was 200bp and a slight difference in the transcript was observed between the three experimental lines with PHST0 transcript level being higher than the other two. PDI genes have been cloned and located on group4 homeologous chromosomes (Ciaffi *et al.*, 2006). In case of gene specific primer of myb10 a product size of 239bp was obtained. In myb10 also the transcript level of PHST0 was higher than PBW343 and PHST9. The primer sequences for gene specific primer of DFR-A gene were taken from Himi and Noda (2004). The expected product size of 890bp was obtained in PHST0, while it was absent from PBW343 and PHST9. Wheat DFR genes have been cloned and located on group3 homeologous chromosomes (Himi and Noda, 2004).

Therefore, though we could not validate any of the 3AL SSR markers but expression of DFR was validated in PHST0. The proteomic study, transcriptomic study and bioinformatic analysis have revealed that chromosome 3AL undoubtedly harbours PHS tolerance though more work needs to be done in same regard.

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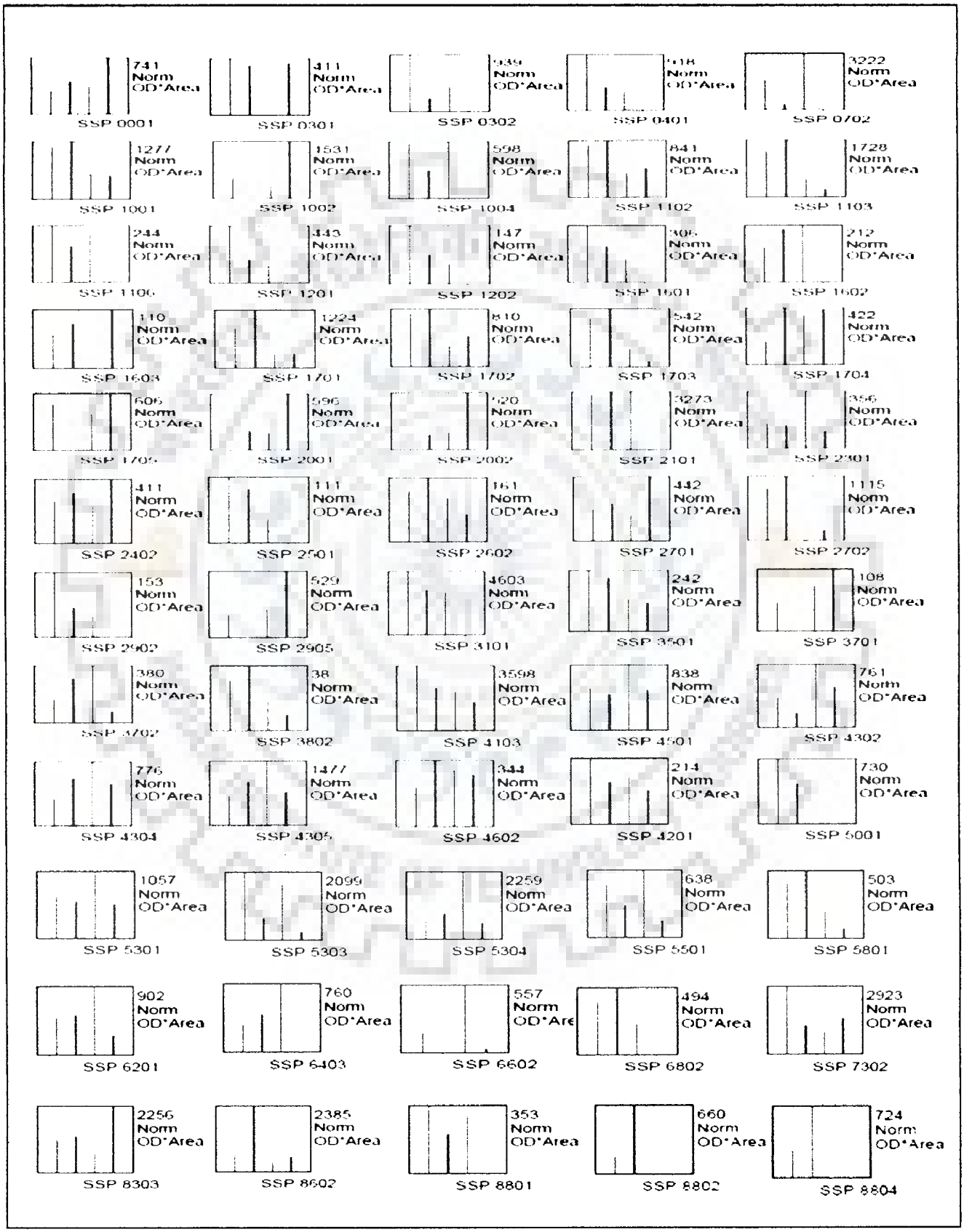
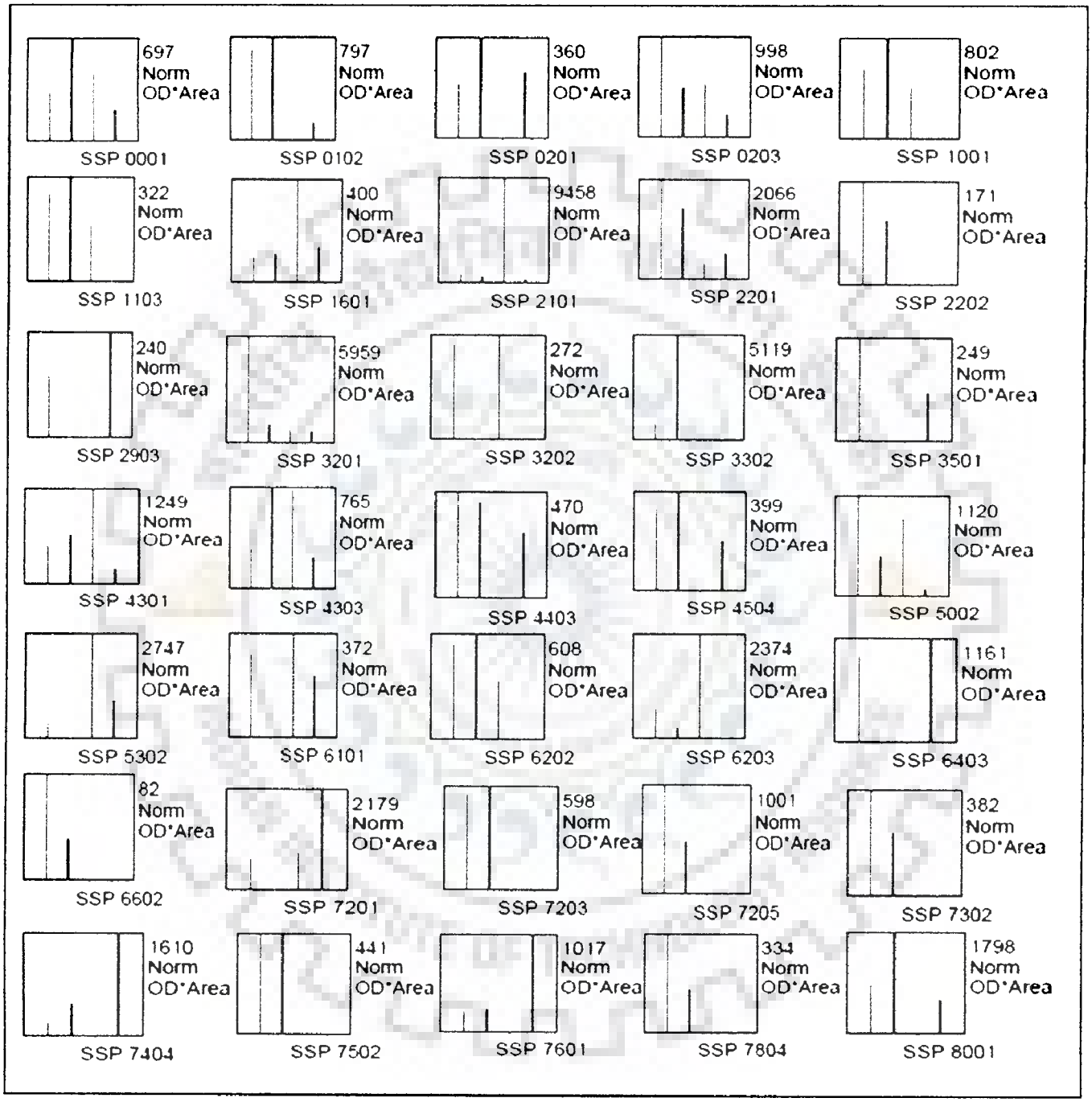
ANNEXURE I

Differences in protein expression of selected proteins from comparative 2-DE of mature and immature embryo from PBW343 and PHST0



ANNEXURE II

Differences in protein expression of selected proteins from comparative 2-DE of endosperm PBW343 and PHST0



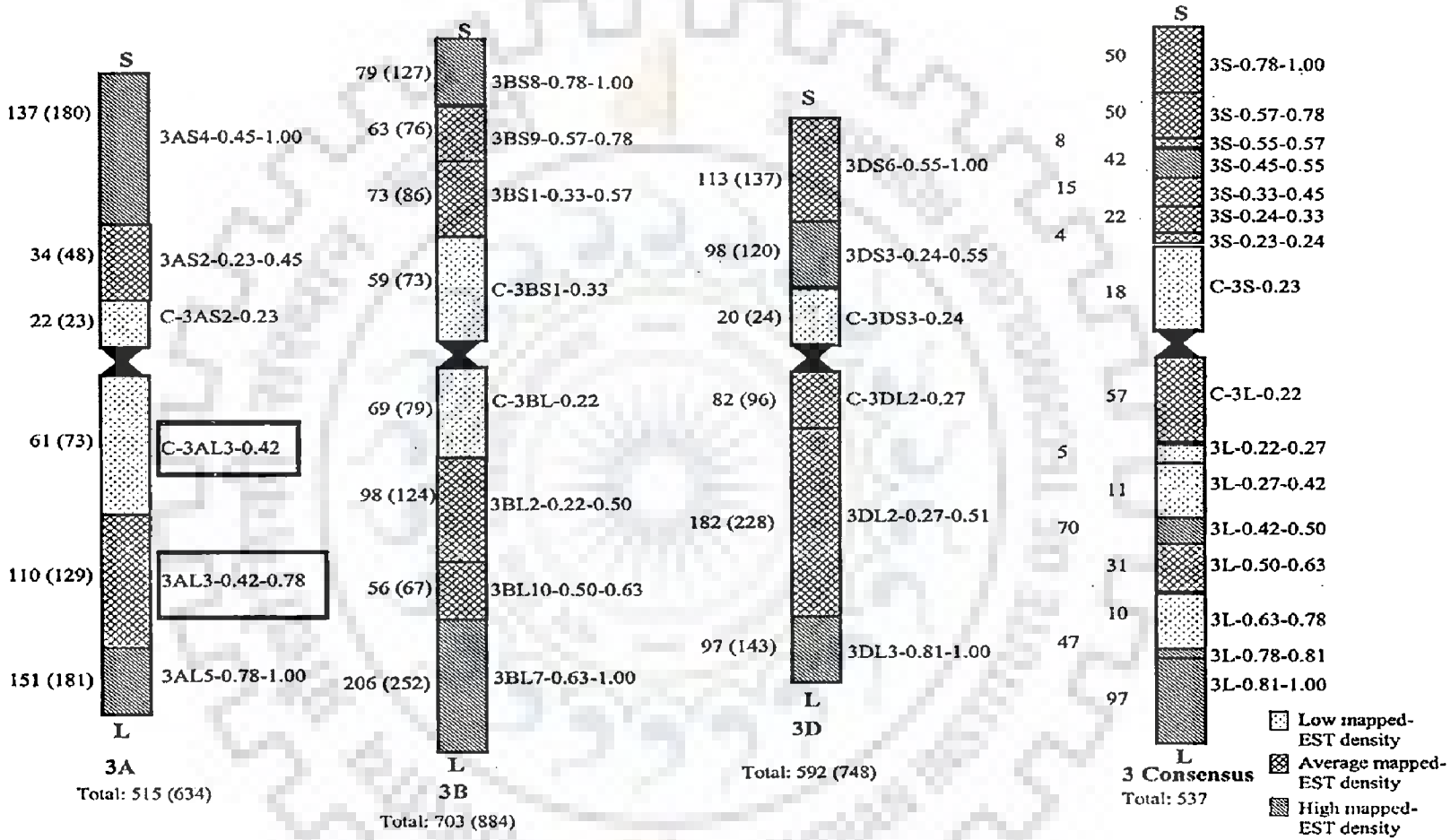
ANNEXURE III

List of IITR PHST wheat landraces

Lane	IITR No.	Seed Colour	Spike Character	Ist data recording after 4 days	IInd data recording after 6 days	IIIrd data recording after 8 days	IVth day data recording after 10 days	Vth day recording after 12 days	Total number germinated seeds in five spikes	No. of germinated seeds/ spike
1	IITR 8	Amber	Awn	-	+	+	++	++	94	18.8
2	IITR 9	Amber	Awnless	-	+	+	++	++	98	19.6
3	IITR 11	Amber	Awnless	-	+	++	++	+++	104	20.8
4	IITR 13	Red	Awnless	-	+	+	++	++	58	11.6
5	IITR 15	Red	Awnless	-	-	-	+	+	10	5
6	IITR 17	Amber	Awn	-	+	+	++	++	87	17.4
7	IITR 18	Red	Awnless	-	-	-	+	+	52	10.4
8	IITR 19	Red	Awn	-	+	+	++	++	18	4.5
9	IITR 20	Red	Awn	-	-	-	+	+	14	2.8
10	IITR 21	Amber	Awn	-	+	++	++	++	63	12.6
11	IITR 22	Amber	Awnless	-	+	+	++	+++	105	21
12	**IITR 23**	**Amber**	**Awn**	-	-	+	+	+	**32**	**6.4**
13	IITR 24	Red	Awn	-	-	-	+	+	18	4.5
14	IITR 25	Red	Awn	-	-	-	+	+	15	3
15	IITR 26	Amber	Awn	-	-	-	+	+	40	8
16	IITR 27	Red	Awn	-		+	+	+	40	8
17	IITR 28	Amber	Awn	-	+	+	++	++	80	16
18	IITR 29	Red	Awnless	-	-	-	+	+	45	9
19	**IITR 30**	**Amber**	**Awnless**	-	-	+	++	++	**64**	**12.8**
20	IITR 31	Red	Awn	-	-	-	-	+	23	4.6
21	IITR 33	Red	Awn	-	-	+	+	+	38	7.6
22	IITR 34	Amber	Awnless	-	+	++	++	+++	122	24.4
23	IITR 65	Amber	Awn	-	++	+++	+++	++++	170	34
24	IITR 66	Red	Awn	-	-	+	+	++	67	13.4
25	IITR 67	Red	Awn	-	+	+	++	++	102	20.4
26	IITR 69	Amber	Awnless	-	+	+	++	++	63	12.6
27	IITR 70	Red	Awn	-	-	-	+	+	41	8.2
28	IITR 71	Red	Awn	-		+	++	++	76	15.2

Lane	IITR No.	Seed Colour	Spike Character	Ist data recording after 4 days	IInd data recording after 6 days	IIIrd data recording after 8 days	IVth day data recording after 10 days	Vth day recording after 12 days	Total number germinated seeds in five spikes	No. of germinated seeds/ spike
29	IITR 72	Amber	Awnless	-	+	+	++	+++	106	21.2
30	IITR 73	Red	Awn	-	-	-	+	+	32	6.4
31	IITR 74	Red	Awn	-	+	+	++	++	98	19.6
32	IITR 75	Red	Awn	-	-	+	++	++	59	11.8
33	IITR 76	Red	Awn	-	-	+	+	+	49	9.8
34	IITR 79	Red	Awn	-	+	+	++	++	61	12.2
35	IITR 82	Red	Awnless	-	+	+	+	+	35	7
36	IITR 83	Red	Awn	-	+	+	++	++	65	13
37	**IITR 88**	**Amber**	**Awn**	-	-	+	++	++	**59**	**11.8**
38	IITR 89	Red	Awn	-	-	+	++	++	55	11
39	IITR 91	Red	Awn	-	+	+	++	++	66	13.2
40	IITR 92	Red	Awn	-	+	+	++	++	55	11
41	IITR 93	Red	Awn	-	-	+	+	+	37	7.4
42	IITR 95	Red	Awn	-	-	+	+	+	52	10.4
43	**IITR 96**	**Amber**	**Awn**	-	-	-	+	+	**50**	**5**
44	IITR 102	Red	Awn	-	-	-	+	+	47	9.4
45	IITR 103	Red	Awnless	-	-	-	-	+	29	5.8

Annexure V



Graphical representation of mapped wheat ESTs on group3 chromosomes of wheat and consensus chromosome bin map. The SSR markers on chromosome 3AL and identified proteins were located on two boxed bins as shown in the figure.

LIST OF PUBLICATIONS

1. Genomics and Proteomics of Preharvest sprouting tolerance in wheat. Naincy Girdharwal, Renu Deswal, H.S. Dhaliwal, G.S. Randhawa and R. Prasad (manuscript under preparation)

Conferences:

1. Naincy Girdharwal, Renu Deswal, H.S. Dhaliwal, G.S. Randhawa and R. Prasad Dissection of Preharvest sprouting tolerance in wheat using Proteomic and Genomic approaches. *World Congress on Biotechnology* 21-23 march, 2011, Hyderabad

2. Naincy Girdharwal, Renu Deswal, H.S. Dhaliwal, G.S. Randhawa and R. Prasad. Proteomics of Preharvest sprouting in wheat. *The Indian proteomics conference* 3-5 april, 2011, JNU, Delhi