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PROTEOMICS STUDY OF PRE-HARVEST SPROUTING IN WHEAT

BY

PRAMOD KHADKA

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

Specialization in Biology

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2016

PROTEOMICS STUDY OF PRE-HARVEST SPROUTING IN WHEAT

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

ABA	Abscisic acid
ADH	Alcohol dehydrogenase
ASI	Amylase/subtilisin inhibitors
ATP	Adenosine triphosphate
DEP	Differentially expressed proteins
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
FDR	False discovery rate
GA	Gibberellic acid
GABA	Gamma-butyric acid
GST	Glutathione S-transferase
HCA	Hierarchical clustering analysis
HCL	Hydrochloric acid
HSP	Heat shock protein

ICAT	Isotope-coded affinity tags
ICPL	Isotope-coded protein labeling
IEF	Isoelectric focusing
iTRAQ	Isobaric tag for relative and absolute quantitation
LC	Liquid chromatography
LEA	Late embryogenesis abundant
LOX	Lipoxygenases
MALDI	Matrix-assisted laser desorption/ionization
MFT	Mother of flowering time
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
PAGE	Polyacrylamide gel electrophoresis
PDI	Protein disulfide isomerase
PHS	Pre-harvest sprouting
PPI	Protein-protein interaction

QTL	Quantitative trait loci
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SILAC	Stable isotope labeling by amino acids in cell culture
SHMT	Serine hydroxymethyltransferases
TCA cycle	Tricarboxylic acid cycle
TMT	Tandem mass tag
USDA	The United States Department of Agriculture
UTP	Uridine triphosphate

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ABSTRACT

PROTEOMICS STUDY OF PRE-HARVEST SPROUTING IN WHEAT

PRAMOD KHADKA

2016

Pre-harvest sprouting (PHS) of wheat (*Triticum aestivum* L.) is a condition characterized by the early germination of spikes during moist environmental conditions. PHS lowers yield, degrades the quality of grain and thus limits the profits of wheat producers groups. During this investigation, proteomics studies of PHS-resistant and PHS-susceptible wheat embryos were conducted at different imbibition time periods via a cutting-edge technology called iTRAQ. Proteomic analysis revealed that 190 differentially expressed proteins might be involved in various cellular functions, such as carbohydrate metabolism, nitrogen metabolism, stress response, redox regulation, ATP synthesis, and protein translation, during this untimely germination of the wheat embryo. Hierarchical clustering analysis revealed the expression pattern of proteins in each of the resistant and susceptible germplasm and relative abundance of respective proteins between the two germplasm. Expression of stress-related and inhibitors proteins was found to be important in maintaining seed dormancy in resistant germplasm; whereas over-expression of energy metabolism related proteins was observed in PHS-susceptible germplasm for the production of energy required for seedling growth. ABA appeared to be involved in seed dormancy, directly or indirectly by controlling the expression of several LEA and EMB-1 proteins. A higher level of ROS production was observed in PHS-susceptible germplasm. Through bioinformatics analysis, a Thioredoxin h protein

was found to be a central player in controlling PHS in wheat and synthesis of methionine was found to be major metabolic control of the seedling establishment.

Chapter 1

1. Review of Literature

1.1. Wheat

Wheat (*Triticum aestivum* L.) is one the most important crop on earth. It is grown in most countries except in hot, humid tropical regions. Wheat is the most widely grown crop in the world (17% area) and provides 21% of the food calories for more than 4.5 billion people throughout the world (Von Braun 2007). Wheat can be grounded into flour which is a key ingredient in foods such as bread, noodles, and tortillas due to its unique viscoelastic properties. Wheat is an important source of carbohydrates, proteins, minerals and vitamins and one-fifth of the calories consumed by humans around the world are derived from wheat products. Due to its high nutritional value, good storing and transporting ability, wheat is considered as an important food crop.

Wheat belongs to family Poaceae (Gramineae) which includes major crop plants such barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), rye (*Secale cereale* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.). Morphological, cytogenetic and molecular studies have shown that common wheat originated from the natural hybridization of three different wild diploid grasses belonging to *Triticum* and *Aegilops* genera approximately 8,000 years ago. Wheat is allohexaploid plant which consists of 21 pairs of chromosomes ($2n = 6x = 42$, AABBDD genomes) (Mayer 2014) and has an estimated genome size of 16,700 Mb/1C (Singh 2008) with an estimate of 94,000 to 96,000 genes in its genome (Brenchley, et al. 2012).

1.1.1. Wheat Production in the United States

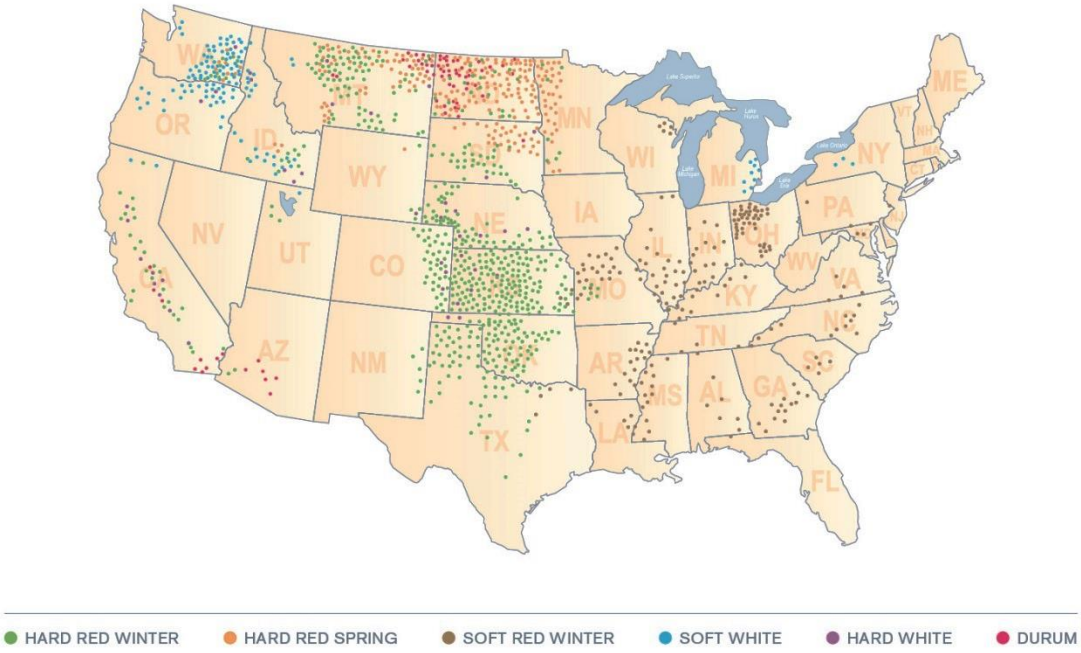

Figure 1.1. Wheat production areas in the United States (Source: National Association of Wheat Growers, 2013).

The United States is one of the largest producer of wheat in the world after China, European Union, and India. In the US, almost every state is involved in agricultural wheat production (Figure 1.1). According to the USDA in the year 2014, 26,630.335 million bushels of wheat was harvested worldwide and 2,025.651 million bushels of wheat was harvested in the US alone. In year 2014/2015, 113.89 million acres of land was used for wheat plantation in the United States. Wheat varieties grown in the United States are classified as "winter wheat" or "spring wheat," depending on the season in which they are planted. About 70-80 percent of total US production is occupied by winter wheat. These varieties are usually sown in fall and harvested in early to mid-

summer of the following year. During winter, young plants become dormant and resume further growth during the subsequent spring season. Winter in Northern Plains are harsh, so spring wheat and durum wheat are planted in springtime and harvested in late summer or fall of the same year. Based on kernel color, hardiness and uses, the US wheat is further divided into six major classes (Table 1.1).

Table 1.1. Different classes of wheat grown in the US (Kadariya, 2014)

Class	Name	Description	Uses
I	Hard Winter Red Wheat	About 40% of all of the wheat grown in the United States is hard winter red wheat. Mostly grown in the Plains states as well as the northern states.	It is used for making pan bread Asian noodles, hard rolls, flat bread, general purpose flour, and as an improver for blending.
II	Hard Spring Red Wheat	It comprises about 24% of the wheat grown in the United States. Mostly grown in Northern plains.	Specialty bread, hearth bread, rolls, croissants, bagels, pizza crust, and to improve flour blends.
III	Soft Winter Red Wheat	It comprises about 25% of the wheat grown in the United States. Mainly grown in the eastern states.	Cakes, cookies and crackers.
IV	Hard Winter White Wheat	This is the newest class of the US wheat and comprises of only about 1% of the wheat grown.	It is great for making Asian noodles, whole wheat, pan bread and flat bread.
V	Soft Spring White Wheat	Generally grown in a few eastern states and in the Pacific Northwest and California. Accounts for about 7% of the wheat grown in the United States.	Great for making for cakes and pastries.
VI	Durum Wheat	It comprises only about 3% of the US wheat is durum, mostly grown in North Dakota.	For making pasta products, couscous and Mediterranean bread.

According to the USDA, South Dakota is the fourth largest producer of wheat in the US. In the year 2014, 131,260,000 bushels of wheat was harvested in South Dakota which consists of 59,400,000 bushels of winter wheat and 71,860,000 bushels of spring wheat.

1.1.2. Wheat kernel morphology

The wheat kernel is one seeded fruit known as caryopsis. Wheat kernels are generally oval shaped, although some kernels have diverse shapes ranging from almost spherical to long, narrow and flattened shapes. The grain is usually between 5 and 9 mm in length, and weighs between 35 and 50 mg. The wheat kernel has a crease that extends almost to the center of the kernel (Evers and Millar 2002).

In general, the wheat seed contains three major parts: the seed coat, the endosperm and the embryo (Figure 1.2). The seed coat is composed of dead cells and serve as a barrier between the embryo and outer environment (Bewley J. D. 1994). Generally, a wheat kernel contains approximately 84% endosperm, 6.5 % aleurone, 4.5 % pericarp, 2.5 % seed coat and 2.5 % embryo. The true seed coat is the testa and it is thought to be responsible for permitting water to enter inside the wheat embryo. Endosperms consists of two parts, aleurone, and endosperm The aleurone layer encloses the endosperm and it is made up of large thick walled cells filled with functional proteins and nutritional components (Evers and Millar 2002). The fully developed endosperm reserves both carbohydrate and protein. The endosperm consists of large cells which stores starch granules. These starch granules are surrounded by thin layer of adherent proteins. The embryo is capable of developing a new plant, so it is considered as the most

important part in grain. The scutellum is shield like structure which lies between endosperm and embryonic axis.

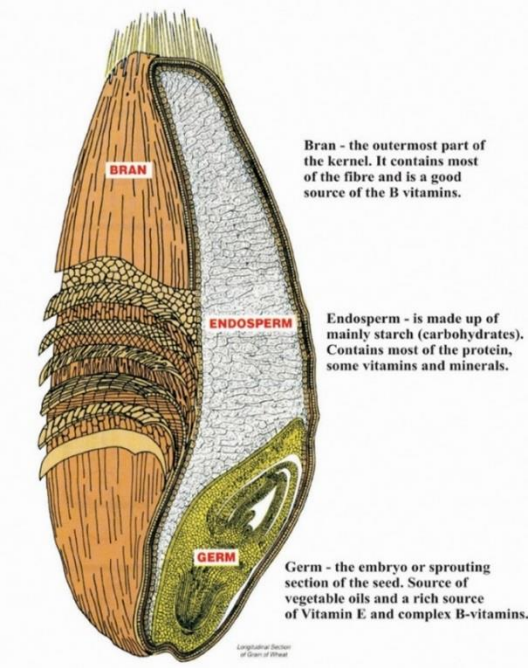

Figure 1.2. Anatomy of wheat kernel (Source: http://www.wheatfoods.org/).

1.1.3. Germination of wheat kernel

Seed germination is a physiological process, which results in the emergence of the embryo from the seed coverings (Bewley, et al. 2006). As the seed germinates it begins to develop and eventually turn into a mature plant. Germination usually begins with water uptake by the seed and ends with the emergence of the embryonic axis through the structures surrounding it (Bewley J. D. 1994). Absorption of water within kernel increases the hormonal activity. Increased Gibberellic acid (GA_3) will cause the release of hydrolytic enzymes, α-amylase (De Laethauwer, et al. 2013). The dissolved GA_3 turns on certain genes in aleuronic cells leading to their transcription. The mRNA thus

produced is transported to the cytoplasm, where ribosomes begin the process of making amylase. The amylase protein starts the enzymatic cleavage of starch present in the aleurone into sugar molecules. The released sugar is transported to the embryo where it acts as fuel for the growth of embryo (Figure 1.3), which results in the emergence of the radical from the seed coat (Gao, et al. 2013).

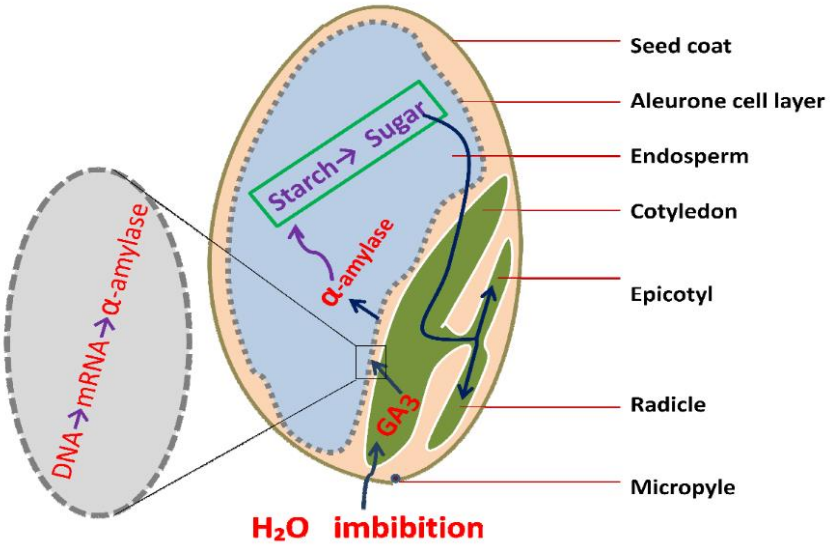

Figure 1.3. Germination process of wheat (Koning, 1994).

1.2. Pre-harvest sprouting

Pre-harvest sprouting (PHS) is precocious germination of grains within the wheat spikes before harvest (Derera, 1989). Environmental conditions like high temperatures and drought during grain filling have a significant effect on the expression of sprouting in wheat (Biddulph, et al. 2005). PHS occurs when physiologically mature grain is exposed to rain and high humidity before harvest and these environmental conditions lead to the absorbance of moisture by the wheat kernel from the air (Thomason et al., 2009).

In general, pre-harvest sprouting affects all crops including wheat. PHS is known to cause a huge amount of financial loss to cereal producers. It is recognized as one of the main factors, which downgrades the bread-making quality of the wheat (Imtiaz, et al. 2008). Many regions in the world including USA, Canada, Australia and Europe are affected by sprout damage in wheat. Sprouted grain can be difficult to thresh resulting in harvest losses and sprouted grain is a factor resulting in a reduced economic return for the producer. Due to its sporadic occurrence through certain years, it is difficult to estimate economic losses caused by PHS to producers (Derera, 1989). Direct annual losses caused by PHS worldwide can reach up to the US $1 billion (Bewley, et al. 2006).

1.2.1. Effects of PHS on wheat grain quality

The wheat kernel is mainly composed of carbohydrates and proteins. PHS results in premature germination of the embryo in the wheat kernels on the head (Figure 1.4) while still in the field (Groos, et al. 2002). For the germination of the new plant, series of physiological changes occurs in kernels to produce required energy and nutrients. So, sprouted wheat produces enzymes such as amylases, proteases, and lipases which break down starch, protein and oil, respectively (Simsek, et al. 2014b). The action of these enzymes causes major anatomical as well as physiochemical changes in the morphology of the seed. When the wheat kernel gets wet, it becomes bleached and has a soft mealy texture. Hormonal activity within the kernel increases and cause the release of hydrolytic enzymes such as α-amylase. This α-amylase hydrolyze the carbohydrate reserves and these are used by growing embryo. Along mealy texture, reduced test weight, lower milling yield, and flour with reduced falling numbers are symptoms of PHS damage (Derera, 1989).

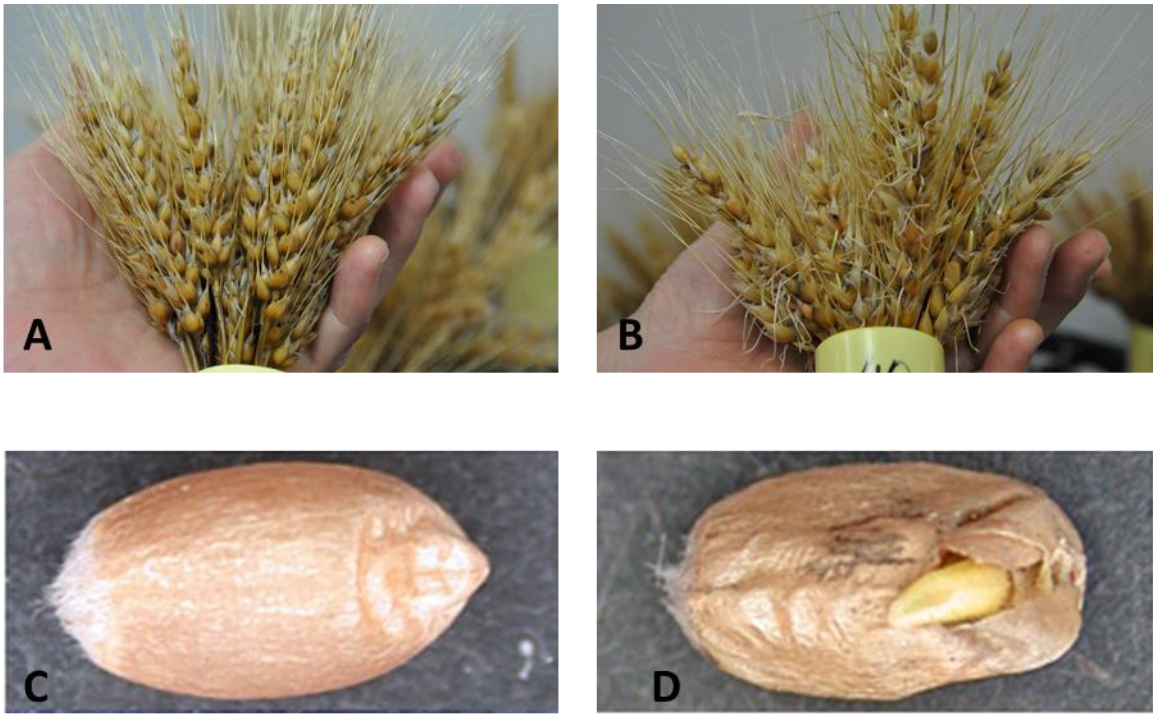

Figure 1.4. Physical damage caused by PHS; A: Spike of PHS-resistant germplasm; B: Spike of PHS-susceptible germplasm; C: Grain of PHS-resistant germplasm; D: Grain of PHS-susceptible germplasm.

1.2.1.1. Starch degradation

Starch accounts for the about 70 – 80 % of the total dry weight of wheat grain (Simsek, et al. 2014a). It is also very important for determining the processing and eating quality of various products from wheat. Starch consists of two components: amylose and amylopectin. The typical levels of amylose and amylopectin are 25-28% and 72-75%, respectively in wheat flour (Colonna & Bule′on, 1992).

Amylases are the enzymes that hydrolyze starch. Three major types of amylase based on their mode of action are endo-amylases (α-amylases), exo-amylases (β-amylase, glucoamylases, α-glucosidases), and debranching enzymes (iso-amylases and limit

dextrinase). The presence of amylase in wheat provides enough evidence of starch degradation during maturation, storage and processing of food. 30% of total proteins synthesized during germination is α-amylase (Mohamed, et al. 2009). As α-amylase degrades starch granules, PHS has a profound effect on starch properties.

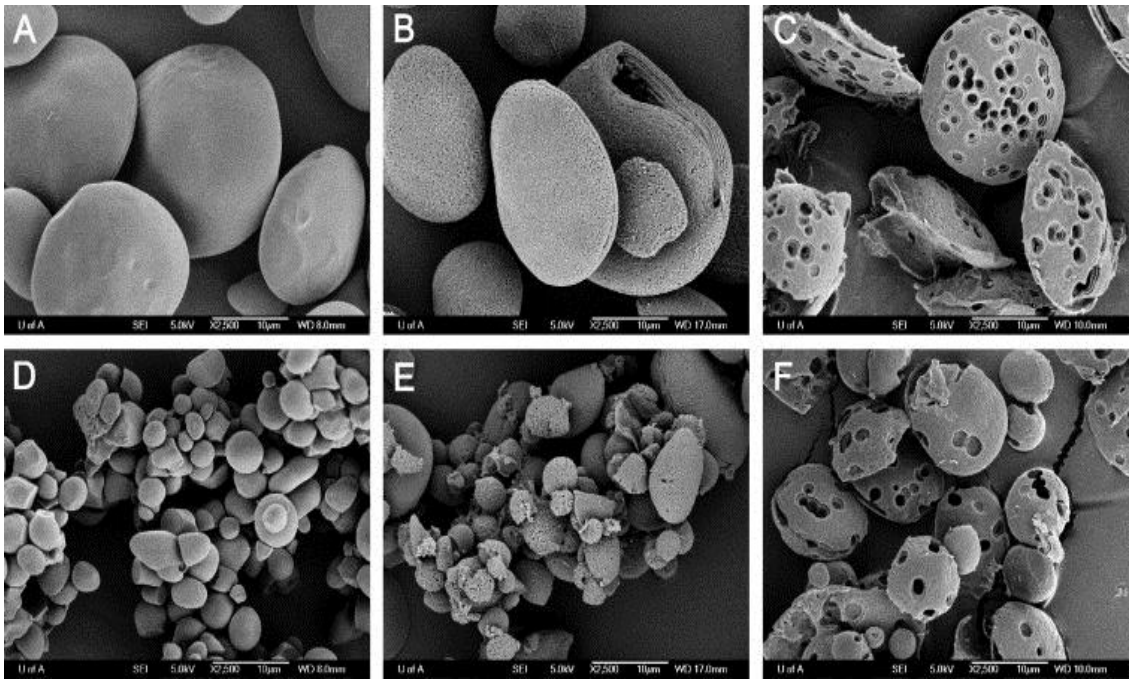

Figure 1.5. Scanning electron micrographs of large (A–C) and small (D–F) wheat starch granules hydrolyzed (Naguleswaran et al. 2012).

Scanning electron microscopy (SEM) studies (Figure 1.5) have suggested that the enzymatic hydrolysis of starch granules starts from the surface, generating pits, enlarging existing pore size and penetrating into an interior granule. This produces a honeycomb-like structure which has reduced gelation and pasting ability (Naguleswaran, et al. 2012).

Many studies have revealed that PHS damage results in significant changes in physicochemical properties of the starch. Due to higher activity of α-amylase, PHS

results in reduced resistance of starch granules to swelling and this results in lowered paste viscosity in PHS-susceptible wheat varieties (Simsek, et al. 2014a). Thus, the hydrolytic activity of α-amylases during PHS causes structural changes in the endosperm by degradation of starch granules leading to inferior grain quality.

1.2.2. Protein degradation

The protein content of wheat grains may vary between 10% - 18% of the total dry matter (Šramková, Gregová, et al. 2009). Wheat storage proteins are a rich reservoir of nitrogen, sulfur and carbon required for the growth of wheat seedlings (Shafqat 2013). Chemically, wheat proteins can be separated into two groups: the high molecular weight insoluble gluten and low molecular weight soluble proteins (Simsek, et al. 2014b). The soluble group consists of albumins, globulins, and peptides, and can be dissolved in aqueous mediums. The insoluble proteins represent 80-85% of wheat storage proteins. Gluten proteins have maximum value in terms of food processing and quality (Shewry, et al. 2002). Wheat proteins quantity and quality are critical factors in determining the quality of wheat and quality of bread flour. Gluten proteins composition is believed to have a high correlation with dough strength and baking quality (Simsek, et al. 2014b). When mixing flour with water, gluten proteins enable the formation of a cohesive viscoelastic dough which is capable of holding gas produced during fermentation and oven-rise, resulting in the typical fixed open foam structure of bread after baking (Veraverbeke and Delcour 2002).

PHS affects the rheological properties of wheat due to increased proteolytic activity that hydrolyzes storage proteins rapidly (Shafqat 2013). Proteolytic enzymes break high molecular weight proteins into smaller fractions resulting in decreased

elasticity and strength of dough (Capocchi, et al. 2000). The increased proteolytic enzyme activity in sprouted wheat results in increased amount of free asparagine which is a precursor of acrylamide formed during bread baking. Studies have shown that acrylamide has potential risks of carcinogenic activity in human (Tareke, et al. 2002). All the studies suggest that elevated endoprotease activity in sprouted wheat causes degradation of proteins which reduces the wheat quality and leads towards economic losses to the producers (Simsek, et al. 2014b).

1.2.2.1. Fiber degradation

Wheat is an important source of dietary fiber. Dietary fiber contains lignin and polysaccharide components of plants which are indigestible by enzymes present in the human gastrointestinal tract. Several researchers have reported the beneficial effects of fiber consumption in protection against heart disease and cancer, regulation of glucose absorption and prevention of constipation (Šramková, Kraic, et al. 2009). The bran consists of the pericarp, testa, and hyaline and aleurone layers. Arabinoxylans is a major component of wheat endosperm cell walls (Šramková, Gregová, et al. 2009). As arabinoxylan is present in high amount in wheat, it affects wheat grain and wholemeal flour functionality during processing and bread making (Shafqat 2013).They improve dough handling properties and stability spring and loaf volume.

In germinating wheat, arabinoxylans degrading enzymes are also produced causing structural changes in the cell wall components which affect arabinoxylans' physiochemical properties in solution and their impact on food systems (Courtin and Delcour 2001).

1.2.3. Effect of PHS on end-product quality of wheat

Numerous studies have also shown the PHS has a negative impact on the quality parameters of different products of wheat such as noodles, Arabic flatbreads (Edwards et al., 1989), bread, cookies, pies (Lorenz et al., 1983). Effects of PHS on wheat quality depend directly upon the types of product to be produced and the processing methods to be used. Bread baked from sprout damaged wheat has a decreased volume, compact interior, and a dark crust. Flour milled from the endosperm of sprouted wheat produces bread that is porous, sticky, and has low loaf volume (Mansour, 1993). As starch is degraded by α-amylase during mixing and fermentation, the water holding capacity of starch is also reduced. It leads to a sticky dough, which causes handling problems, a more open coarse crumb structure and gummy crumb (Fu, et al. 2014). The extreme stickiness of dough causes requirement of extra special handling which can disrupt the bakery operations (Paulsen and Auld, 2004). Bread loaves made from sprouted wheat are often grayish in color (Fu, et al. 2014).

Sprout damage affects both quality and processing of different types of noodles and pasta. According to many pasta processors, high levels of sprout damage cause production problems such as uneven extrusion, strand stretching, and irregularities in drying, that is, checking or cracking of strands during storage. Most importantly the pasta made cannot withstand overcooking and becomes soft or mushy. Sprouting raises alkaline activity in the kernels which can increase discoloration of the noodles up to five times more than the normal kernel (Singh 2008). As the appearance of noodles and pasta is the first critical factors considered by consumers to evaluate the quality, this increased

discoloration can make noodles and pasta unattractive and undesirable (Hatcher and Symons 2000).

Pre-harvest sprouting causes damage in three-folds: 1) loss in wheat yield, 2) reduction in test weight of wheat, and 3) low-quality products of wheat. The loss in yield and reduced test weight directly impact the profit of the farmers, while the reduction in end-products causes great loss to milling and cereal companies. Studies revealed that if the wheat kernel contains more than 4% of the damage it is classified as not suitable for the human consumption and price could be reduced by 20%–50% (Simsek, et al. 2014a). These damaged grains are usually fed to animals so farmers get discounted price which leads to substantial economic loss.

1.3. Factors affecting pre-harvest sprouting

Pre-harvest sprouting is a complex phenomenon which is affected by various factors. The major factors affecting PHS include environment conditions, seed dormancy, seed coat permeability and color, α-amylase activity, endogenous hormones levels, functional proteins, genes, and quantitative trait loci (QTLs) (Gao, et al. 2013). The roles of various factors have been described in details in the following text.

1.3.1. Role of seed dormancy

Seed dormancy can be defined as the temporary arrest of seed germination under the favorable conditions (Gubler, et al. 2005). It is the type of adaptive strategy developed my some species to survive in adverse environmental conditions. In other words, we can say that dormancy provides a strategy for seeds to inhibit germination in order to reduce the risk of premature death of new plant in unfavorable environmental

conditions (Bewley, et al. 2006). For the process of germination, many factors such as availability of water, air, temperature, light conditions and certain chemicals play a crucial role. In some conditions, the seed may still fail to germinate even with the presence of all required conditions. Such types of seed are known as dormant. Dormancy induction, maintenance, and release in the seed are closely related to PHS. In the case of wheat, seed dormancy is considered as the most important factor for resistance against PHS.

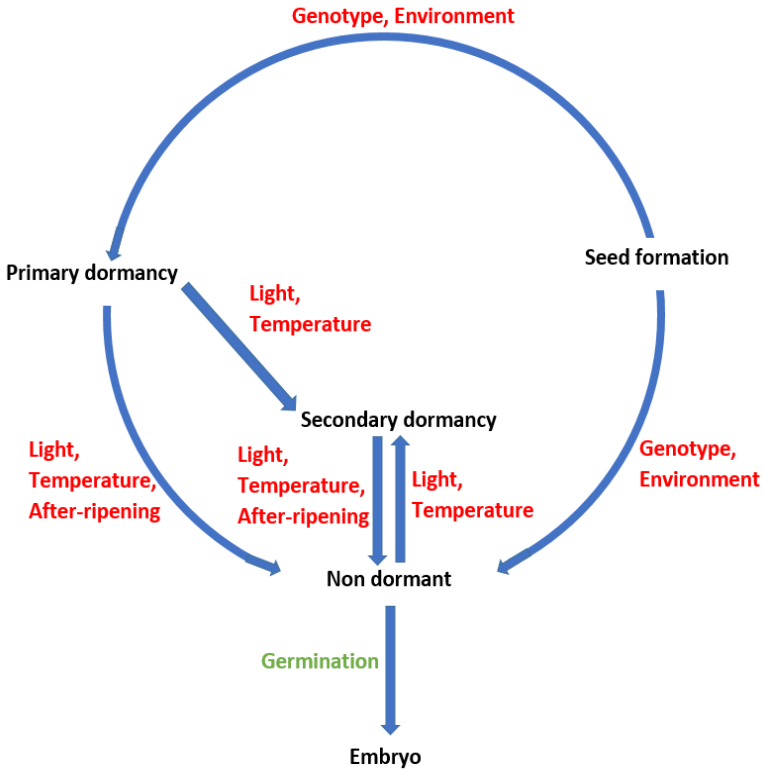

Figure 1.6. Induction of primary and secondary dormancy (Kermode, 2005).

On the basis of the timing of development, dormancy can be divided as primary and secondary dormancy. Primary dormancy of seed is initiated during seed maturity and it depends on both environmental and genetic factors. Usually, seeds dispersed from dormant state mother plant develops primary dormancy (Bewley 1997). Secondary

dormancy is usually initiated in mature seeds which have already lost their primary dormancy (Gubler, et al. 2005). Secondary dormancy may be developed due to prolonged inhibition of germination by unfavorable factors which may be either internal or external (Dahal 2012).

Mechanism of seed dormancy can also be classified as seed-coat imposed dormancy and embryo related dormancy. Seed-coat imposed dormancy is the type of dormancy which is imposed by embryo surrounding structures such as endosperm, pericarp or seed coat and other floral organs (Dahal 2012). Studies revealed that embryos were still viable and were able to germinate when the seed coat was removed and embryos were provided favorable conditions for germination (Kadariya 2014). Mainly cereals, conifers, and dicots have developed seed-coat imposed dormancy (Kermode 2005). Seed-coat is usually hard and waxy which restricts the absorption of water and gases required for seed germination.

Seed-coat also stores some germination inhibitors such as flavonoids (Debeaujon, et al. 2007) which may delay the germination, can lead to seed-coat imposed dormancy (Bewley J. D. 1994) . The cells of seed coat decide the resistance of the seed towards PHS. If the epidermal cells of the seed coat are loosely arranged, the seed will be easily permeable for the exchange of water and gases, thus resulting in the susceptibility to PHS while, the tight arrangement of the epidermal cells in seed coat ensures resistance to PHS (Gao, et al. 2013).

Another type of dormancy is known as embryo related dormancy is intrinsic to the embryo and is not related to other tissues surrounding the embryo. Many researchers found that isolated embryo was not able to germinate even they provided favorable

conditions for the germination (Dahal 2012). This type of dormancy is developed mainly due to the presence of growth inhibitors and absence of growth regulators. This type of dormancy is released when the seeds are stored for a long time and moisture content in the embryo reduces to a certain level, a phenomenon known as after-ripening (Bewley 1997). Embryo related dormancy is found in Rosacea and in wild species of oat (Kermode 2005).

Seed dormancy is maximum when the seeds are physiologically mature. When seeds enter desiccation process, the dormancy is slowly released. Longer the seeds remain in the desiccated state, seeds become less dormant and more prone to the germination. As internal seed dormancy level decreases, seed can quickly pass the dormancy threshold during the early stage of desiccation. At this stage, PHS can occur when the environmental conditions are favorable (Obroucheva and Antipova 2000). Also, loss of primary dormancy is responsible for pre-harvest sprouting in wheat.

1.3.2. Roles of abscisic acid and gibberellic acid

Abscisic acid (ABA) and gibberellic acid (GA) are two plant hormones which are directly related with seed germination and dormancy. These both are related with "physiological dormancy", a reversible type of dormancy located in plant embryo. Though both ABA and GA are linked functionally but they act antagonistically in the expression of dormancy and germination (Bewley 1997). Many studies have revealed that ABA is important in dormancy induction during seed development while GA is important in promoting germination in non-dormant seeds (Pisipati 2008).

Abscisic acid is directly related to initiation and maintenance of seed dormancy and prevention of precocious hydrolysis of stored starch granules (Dahal 2012). ABA

content increases during seed development and it regulates main processes involved in maintaining the dormancy of seed (Bewley 1997). External application of ABA has shown to suppress the embryonic germination. In developing grain, the concentration of ABA is higher in embryo than in endosperm. Immature seed is rich in ABA and the amount of ABA present in the grain decreases when it reaches maturation (Pisipati 2008). The level of ABA is critical for both dormant and non-dormant seeds because ABA maintains embryos in a developmental mode during early seed development until they are fully matured and have accumulated sufficient amount of reserves required for the successful germination and subsequent seedling establishment (Kermode 2005). Pre-harvest sprout damage is catalyzed by α-amylase by breaking the starch accompanied by the proteolysis of the grain proteins (Walker-Simmons 1987). ABA is found to suppress the activity of the α-amylase as well as its synthesis by inhibiting the GA3- enhanced α-amylase synthesis in aleuronic cells (Dahal 2012).

During the course of development, seeds change their sensitivity to ABA. A study done by Walker-Simmons (1987) revealed that there is a positive correlation was observed between wheat embryo sensitivity to ABA level and, resistance to germinating seed development and dormancy after maturation. The study also showed the differences in sensitivity to ABA in developing embryos between sprouting resistant and susceptible cultivars. Embryos from the resistant cultivars continued to exhibit sensitivity towards ABA even after reaching desiccation but susceptible varieties failed to do that.

Gibberellic acid is a bioactive growth regulator which is associated with plant growths and diverse developmental functions such as seed germination (Singh 2008). GA play a vital role in dormancy release. During embryo development, GA levels are usually

high but when seeds reach maturity most GA are deactivated (Ogawa, et al. 2003). The level of GA again increases at the onset of germination just prior to radicle formation. GA is not involved in the control of seed dormancy but it plays an important role in germination by (i) increasing embryo growth potential , and (ii) weakening the tissues surrounding radicle and reducing the mechanical barrier provided by seed coat (Bewley 1997; Pisipati 2008). When GA is applied, it can switch off the inhibitory effect of ABA and promote germination in seeds. An excessive amount of GA stimulates the degradation of GA signaling repressor protein known as DELLA proteins, via ubiquitin-proteasome pathway (Silverstone, et al. 2001). This process now stimulates the downstream events of germination of seed. When GA is released, it triggers aleurone cells to secrete hydrolytic enzymes such as α-amylases, which supplies the endosperm reserves to support the germination process. GA released from the embryo can also trigger several responses such as gene induction, down-regulation, and up-regulation of secretory responses.

1.3.3. Role of amylase

The α-amylase enzyme is widely found in plants and plays various roles in many physiological processes in plants, including hydrolysis of α-1, 4-glycosidic bond present in the saccharides. There are two major types of alpha-amylases in wheat, *α-AMY-1* located on homologous chromosome 6 and *α-AMY-2* located in chromosome 7 (Gale, et al. 1983). GA3 could regulate the expression level of α-amylase-1 and α-amylase-2 in plants (Marchylo, et al. 1984). The activity of α-amylase increases quickly as enough water is absorbed by seed and significant difference in activity was observed between the resistant and susceptible varieties of wheat to PHS (Gao, et al. 2013). The α-amylase-1

isozymes are found in more abundance in first days (1-2) of germination while α-amylase-2 isozymes increased after 3 days of germination (Sargeant 1980). The combination of α-amylase/subtilisin inhibitors (ASI) and α-amylase-1 were able to reduce the catalytic activity of α-amylase and increase the resistance of barley against PHS (Yuan, et al. 2004).

1.3.4. Role of environment

Environmental factors including temperature, rainfall and high relative humidity play a role in the expression of sprouting and dormancy in wheat. The change in environmental conditions during the grain filling and maturation period can have significant impacts on dormancy expression. Temperature is one of the most important environmental factors that influences the induction and expression of seed dormancy. In wheat, low temperatures during grain filling increases seed dormancy but as the grain reaches its maturity, low temperatures during imbibition reduces expression of the dormancy and helps in germination (Nyachiro, et al. 2002). Mares (1993) found that rainfall during the 20 days prior to harvest accounted for almost 85% of the variation in dormancy. He found that seeds which received more rain were highly susceptible to sprouting. Rainfall and high humidity during the grain ripening stage were found to decrease grain drying and dormancy level while, water stress levels and high temperatures were found to increase grain drying rates and dormancy (Lunn, et al. 2002).

Environmental stresses such as temperature variation, moisture content, and salinity are also known to trigger the synthesis of ABA in plants (Biddulph, et al. 2005) which impacts the dormancy in mature grain. The sensitivity of embryos towards ABA was observed during high temperature and drought conditions as compared to low

temperatures and irrigated conditions. ABA biosynthesis and genes regulating catabolism of GA was found to be increased during winter season resulting in increased dormancy while increased ABA catabolism and GA synthesis was observed in spring season which resulted in the release of dormancy in Arabidopsis seeds (Footitt, et al. 2011). Effect of seed coat color on dormancy are also depending upon environmental conditions during the development of the wheat seed. No significant difference in dormancy between red and white wheat was observed under dry conditions, whereas the red lines were more dormant at maturity making them more resistant to sprouting than white genotypes (Torada and Amano 2002).

1.3.5. Genes controlling PHS

Pre-harvest sprouting is a complex trait and is controlled by genotypes, environments and interaction between these factors (Marzougui, et al. 2012). PHS in wheat is regulated by both embryonic and coat-imposed pathways controlled by separate genetic systems (Himi, et al. 2002). Seed coat color is associated with seed dormancy and PHS-susceptibility is associated with white grain color while resistance is associated with red grain color (Torada and Amano 2002). Red wheat genotypes showed consistently higher falling number than the white wheat genotypes (Rasul, et al. 2012) indicating that in general red wheat genotypes are comparatively PHS-resistant than white wheat. The color of the seed is usually determined by *R* (Red grain color) genes and is heritable to the offspring (Gao, et al. 2013). Three *R1* genes (*R-A1*, *R-B1* and *R-D1*) control seed coat red color and dominant alleles of *R* genes (present in each chromosomes 3A, 3B, and 3D) promote biosynthesis of red phlobaphenes and have a pleiotropic effect on dormancy. Wheat *R1* gene increases seed dormancy by increasing

the sensitivity of embryo towards ABA (Himi, et al. 2002) and it encodes MYB-type transcription factor (Himi and Noda 2005). Multiple-parental mating systems study revealed that both additive and dominance effects are responsible for PHS resistance (Kadariya, et al. 2011).

In maize, transcription factor VIVIPAROUS-1 encoded by *Vp-1* gene is known to play an important role in induction and maintenance of dormancy (McCarty, et al. 1991). Inactivation of *Vp-1* gene in maize leads to disruption of embryo maturation and promotes germination of embryos while still attached in the cob (vivipary). Vp-1 homologues genes were mapped in at 30cM from *R* locus in the long arm of group 3 chromosomes of wheat and identified as *taVp 1* gene (Bailey, et al. 1999).

ABA-insensitive 3 (ABI3) and *GA-insensitive (GAI*) genes identified in Arabidopsis mutants are key players during the germination process (Koornneef, et al. 2002). *ABI3* orthologs, *GAI* orthologs and *reduced height 3 (RHT3)* (Flintham and Gale 1982), has been identified in wheat and are reported to have similar functions. *Delay of germination 1 (DOG 1)* in Arabidopsis (Bentsink, et al. 2006), *seed dormancy 4* (*SDR4*) in rice (Sugimoto, et al. 2010) and *TaPHS1,* a wheat homolog of *mother of flowering time* (*MFT*) on short arm of chromosome 3A (Liu, et al. 2013) are some of the recently cloned genes related to seed dormancy and germination. Down-regulation of thioredoxin gene (Trx h9) was found to reduce pre-harvest sprouting in wheat (Ren, et al. 2012).

1.4. Proteomics studies in plants

Genomic studies have provided valuable information about the structure and function of a gene in living organisms. Knowledge about the complete DNA sequence is

helpful to understand the biology of organisms but many biological processes can be studied only at the protein level. Proteomics is defined as the systematic analysis of the proteome, the protein complement of genome (Pandey and Mann 2000), which permits quantitative and qualitative estimation of number of proteins that specifically impact cellular biochemistry, and therefore give precise investigation of cellular state or system changes during growth, development, and response to environmental factors (Chen and Harmon 2006). "Expression proteome" of a cell consists of an entire set of proteins expressed in a cell while the "functional proteome" examines the protein-protein interactions on a genome-wide scale which attempt to study functional pathways (Kocher and Superti-Furga 2007). Therefore, a comprehensive protein analysis can provide a unique global perspective on how these molecules interact and cooperate to create and maintain a working biological system.

There are a wide range of methods, reagents, instrumentation and data analysis tools available to design a proteomics experiment (Pandey and Mann 2000). A Standard proteomics approach consists of four basic stages: 1) sample preparation, 2) Sample extraction/fractionation/purification, 3) mass spectrometry analysis, and 4) data analysis. Optimizations can be done in each step to ensure that most useful and instructive data is gained. Variations can be done during the steps of extraction, separation, and labeling. Although experimental design and sample preparation are equally important but labeling or modifications of proteins and their separation plays a vital role for successful proteomics experiment.

1.4.1. Gel-based proteomics

Traditionally, the main analytical techniques used in proteomics study have been gel electrophoresis. Electrophoresis can be used to separate the macromolecules, especially proteins, according to their size, charge and conformation (Smithies 2012). A gel usually formed by cross-linked polymerization of acrylamide and N,N'-Methylenbisacrylamide is suitable supporting medium for electrophoresis (Raymond and Weintraub 1959). Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) is one of the most used techniques for the identification of the proteins due to its low cost and adequate resolution. SDS-PAGE separates the protein molecules according to their molecular mass. One-dimensional SDS-PAGE, when coupled with appropriate software, can be used for plant finger-printing (Supek, et al. 2008). Though SDS-PAGE combined with band cutting, trypsin digestion, and Liquid chromatography (LC) separation remains to be widely used proteomics approach (de Godoy, et al. 2006), the resolution of SDS-PAGE is insufficient in terms of large scale proteome research.

The two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separates the proteins in two different steps, first in-gel Isoelectric Focusing (IEF) of proteins to separate them according to their isoelectric point (*pI*), and then SDS-PAGE to separate proteins according to their molecular mass (O'Farrell 1975; Gorg, et al. 2004). Due to its two-dimensional separation, it has a better resolving power and is, therefore, suitable for the analysis of complex samples. Coomassie brilliant blue and silver nitrate are two of the most generally used stains for the visualization of the proteins in the gel. Coomassie brilliant blue binds to basic and aromatic amino acids of proteins and can detect the protein at Nanogram level (De St. Groth, et al. 1963) whereas silver nitrate can bind

covalently cross-linked to the proteins when used with formaldehyde and can detect protein at Picogram level (Rabilloud 1990).

The difference gel electrophoresis (DIGE) is another modification of 2D-PAGE which differentiates two protein samples in a single gel. This is achieved by labeling the samples with different color fluorescent dyes and running simultaneously on the same gel. Most commonly dyes used in DIGE are cyanine dyes known as Cy2, Cy3 and Cy5 dyes which react with free amino groups (amino terminus and amino groups of lysine residues) of the proteins (Unlu, et al. 1997). After the electrophoretic separation, the intensities of fluorescence originating from three different samples are quantified by a digital fluorescence scanner.

Two-dimensional gel electrophoresis provides several advantages by allowing us to obtain a final analytical image which is quantitative and reproducible but there are some disadvantages associated with these methods. Undoubtedly, the resolution of proteins having higher molecular mass will increase, but it will decrease the resolution of low molecular weight proteins present in the sample. Despite numerous optimization techniques used during electrophoresis, all the proteins present in a given sample will not be revealed, thus leading to loss of information. It was shown that there was a loss of more than 50% protein yields (Zhou, et al. 2005). Most of the times one spot in the gel can contain more than one protein, which can cause the quantification of the individual proteins more challenging. Thus, the analysis of protein by electrophoresis is limited to the study of the most abundant proteins which makes it unsuitable to a high throughput screening.

1.4.2. Mass spectrometry (MS)

Mass spectrometry has emerged as a very powerful experimental tool for proteome analysis due to its sensitivity and ability to identify a large number of proteins present in the complex mixtures. In proteomics, mass spectrometers are used to identify proteins, detect their covalent modifications including post-translational modifications and glycosylation, and characterize and control the quality of recombinant proteins (Mann, et al. 2001). MS is used by coupling with 2D-DIGE to detect proteins and peptides from spots of interest in the gel. For a mass spectroscopy based proteomics, the spots are picked and digested by trypsin followed by fractionation of the peptide by liquid chromatography (LC). The variable level of laser shots is applied to the sample in Mass Spectrometer to convert the peptides into ions for the identification of proteins via Tandem MS method.

1.4.3. Gel-free proteomics

Apart from the quantification of the proteins intact in gel after electrophoresis, the peptide-based quantification of proteins by MS is continuously increasing. In this process, proteins are directly submitted for enzymatic digestion and the mixture of resulted peptides are separated and analyzed by MS. These approaches are less sample consuming and provide the most accurate protein identification. Currently, two approaches are used for the quantification of peptides, namely, the label-free quantification and stable-isotope labeling quantification. Both the quantification methods are relative i.e., they determine the relative abundance of the corresponding peptides by the ratio of the intensity of ions in each sample (Deracinois, et al. 2013). In the case of label-free quantification, the samples to be compared are prepared separately and

analyzed individually by MS/MS. This technique relies on two methods, (i) the comparison of mass spectra and (ii) the comparison of spectral counting (Bondarenko, et al. 2002; Wang, et al. 2008). The isotope labeling quantification is based on characteristic labeling of proteins or peptides. These approaches utilize the fact that the peptides tagged with different stable isotopes exhibit same chromatographic and ionization properties, then will be detected in the same spectrum but can be distinguished from each other by a mass shift caused by the isotope used for tagging (Abdallah, et al. 2012; Deracinois, et al. 2013). Two types of labeling are used in this method, the first type is metabolic labeling in which labeling is carried out during protein synthesis through the introduction of the label in growth medium and second type is chemical or enzymatic labeling applied later in the proteomics experiment through a chemical reaction (Schulze and Usadel 2010). Metabolic labeling includes Stable isotope labeling by amino acids in cell culture (SILAC) and $^{14}N/^{15}N$ labeling whereas chemical labeling includes Proteolytic labeling, Isotope-coded affinity tags (ICAT), Isotope-coded protein labeling (ICPL), Tandem mass tag (TMT), and Isobaric tags for relative and absolute quantification (iTRAQ).

1.4.3.1. Isobaric tags for relative and absolute quantification (iTRAQ)

iTRAQ labeling is based on the covalent labeling of amino groups of peptides which allows simultaneous identification of protein and their relative quantification (Deracinois, et al. 2013). The chemical label consists of a reporter chemical group, a balance chemical group and a chemical group reactive on primary amines. Each label consist of a peptide reactive group (NHS ester) and an isobaric tag of 145 Da (for iTRAQ-4plex) or 305 Da (for iTRAQ-8plex) which contains a balancer group (carbonyl

group having mass range 28-31 Da for iTRAQ-4plex and 184-192 Da for iTRAQ-8plex) and a reporter group (based on N-methyl piperazine) which is joined by a fragmentation site (Figure 1.7). There are eight chemically identical isobaric tags named: 113,114, 115, 116, 117, 118, 119, and 121 which have overall same mass (Ross, et al. 2004; Abdallah, et al. 2012). The peptide reactive group specifically attaches to free primary amino groups-N-termini and ε-amino groups of lysine residues.

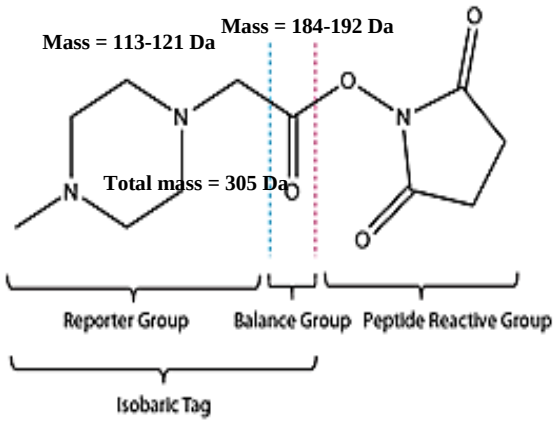

Figure 1.7. Schematic representation of iTRAQ reagents (adapted from Broad Institute).

Each sample to be analyzed is digested by trypsin and labeled with a single iTRAQ label which is then mixed for tandem mass analysis. The labeled peptides are separated by two-dimensional LC. The separated fractions are then subsequently analyzed by using MS and tandem mass spectrometry (MS/MS). Same peptides from each sample appear as a single peak in MS spectrum due to the isobaric nature of these reagents. After collision induced dissociation, the balancer group dissociates from the iTRAQ reagent (Aggarwal, et al. 2006). Intensities of the peaks after MS/MS scans derived from the 8-plex iTRAQ reporter ions detected in the 113-121 m/z region is used for the identification and relative quantification of the proteins. Data acquired is always

compared to a reference sample, and the quantity of each peptide is expressed as a ratio relative to the reference sample (Bantscheff, et al. 2007). One of the major advantages of iTRAQ is that it allows parallel proteomic analysis of eight different samples with high sensitivity which conserves a significant amount of time. Also, the peptide sequence coverage of iTRAQ labeled peptides detected during MS analysis are similar to those obtained during other MS-based approaches which confirm that there is no negative effect of reagent in the fragmentation (Aggarwal, et al. 2006).

1.5. Proteomics studies of seed germination and pre-harvest sprouting

Proteomics is an important approach to studying the pre-harvest sprouting mechanism because it gives an ultimate account of differential gene expression during this process. By studying the changing patterns of protein expression during seed development can give a clearer overview of the events during the process. Proteomics of germination and dormancy process has been studied in lot of plants including *Arabidopsis* (Gallardo, et al. 2002a), rye (*Secale cereale*) (Masojć and Kosmala 2012; Masojć, et al. 2013), rice (*Oryza sativa*) (Yang, et al. 2007; Kim, et al. 2009), Norway maple (*Acer platanoides*) (Staszak and Pawłowski 2014), Mung bean (*Vigna radiata*) (Ghosh and Pal 2012), *Castanea crenata* (Nomura, et al. 2007), barley (*Hordeum vulgare*) (Finnie, et al. 2002), and wheat (Kamal, et al. 2009; Mak, et al. 2009; Shin, et al. 2009; He, et al. 2015). Proteins involved in metabolism, especially those involved in the carbohydrate metabolic pathways including glycolysis, TCA, fermentation, gluconeogenesis, glyoxylate cycle, and pentose phosphate pathway were present in higher abundance in germinating rice seeds (Yang, et al. 2007). These changes indicate that germination requires large amounts of energy and nutrition which is provided by the

seed itself. Increased amount of β-amylase, a major enzyme involved in carbohydrate degradation and ATP synthase indicates that the role of energy production in essential for germination. Down-regulation of the storage proteins during germination suggests that storage proteins are degraded by proteases to produce amino acids, peptides, which are used as a precursor for the synthesis of new proteins required for the germination (He, et al. 2015). The role of both ABA and GA in Arabidopsis seed germination was discussed by using proteomics approach (Gallardo, et al. 2001). Reactive oxygen species (ROS) are produced by the plant in response to biotic and abiotic stresses. Increased level of proteins related to stress response such as superoxide dismutase and ascorbate peroxidase in mung bean cotyledons suggests that adaptation of seeds against stress is important for successful seed germination (Ghosh and Pal 2012). Proteins involved in cytoskeleton formation including tubulins were up-regulated in the embryo of developing tomato seeds suggesting that cell is frequently progressing toward mitosis (de Castro, et al. 1995). Proteins involved in protein degradation, protein folding, cytoskeletal activities, and energy metabolism-related enzymes were observed in higher abundance during the first three days of germination of wheat embryo while β-amylase, protease inhibitors, alcohol dehydrogenase, peroxidases, and ADP-glucose phosphorylase showed decrease in abundance during the time period (Mak, et al. 2009).

Some of the researchers have focused their research on comparing the proteome between PHS-susceptible and PHS-resistant lines. A Higher level of dimeric alpha-amylase inhibitor and xylanase inhibitor in PHS-resistant lines suggests that accumulation of these particular defense proteins is important for the PHS-resistance in rye (Masojć, et al. 2013). The rate of protein degradation also plays a role in premature

germination. Masojć, et al. (2013) hypothesized that the different rates of protein degradation due to proteasome activity and a higher level of glutathione s transferase can induce PHS in rye. Proteins related with PHS in both wheat and rye include peroxiredoxin, xylanase inhibitor, RNA binding protein, and heat shock proteins (Kamal, et al. 2009; Bykova, et al. 2011; Masojć, et al. 2013)

1.6. Conclusion

Wheat is one of the most widely grown crops in the world. Pre-harvest sprouting is known as the condition of in-spike germination of physiologically mature grain before harvest due to unfavorable environmental conditions including rainfall and high moisture content in the air. This results in significant amount of lost in yield and affects the nutritional and functional quality of wheat flour. Understanding the mechanism behind PHS has been done in the past by using conventional methods but using newer approaches including proteomics helps to increase the knowledge about PHS at molecular and cellular level. Because proteins are the translated version of genes, proteomic analysis is a useful tool that can be used to envision and contrast mixtures of proteins expressed in a specific process and to gain crucial information about individual proteins involved in a specific biological process. The analysis of the proteome changes in response to development, disease, or environment is the ultimate goal of comparative proteomics. As embryo plays an important role during seed germination, so protein profile analysis of the embryo of dormant and non-dormant seeds may be helpful to understand the complex mechanism of this process. Although different proteomics studies have been done on pre-harvest sprouting, those investigations were done by using conventional gel-based techniques. iTRAQ analysis is more reliable than those

conventional methods and it will help us to identify a sufficient number of proteins to predict a pathway and conduct protein-protein interaction analysis. This study focuses on comparing the differentially expressed proteins in the embryo of PHS-resistant and susceptible germplasm and identifying key players at molecular levels.

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Chapter 2

2. Proteomics study of pre-harvest sprouting in wheat

2.1. Introduction

Wheat (*Triticum aestivum* L.) is the most widely grown (17% area) crop in the world and provides 21% of the food calories and 20% of the protein for more than 4.5 billion people throughout the world (Von Braun 2007). Many kinds of wheat cultivars are grown and used for the production of commercial foods such as bread, noodles, biscuits, pasta, and cakes.

Pre-harvest sprouting (PHS) is the germination of the premature seeds within the grain head before harvest (Kadariya 2014; Shu, et al. 2015). It occurs when physiologically mature grain is exposed to rain and high humidity before harvest that leads to the absorbance of moisture by the wheat kernel from the air (Thomason 2009). After absorbance of moisture, the kernel is bleached and its texture turns "mealy" (Bassoi, et al. 2006). The exposure of wheat kernels to moist conditions at ripening stage triggers a sequence of physiological processes, including the release of various plant hormones such as gibberellic acid (GA) (Bassoi, et al. 2006; Imtiaz, et al. 2008). GA released by soaked kernel promotes the synthesis and secretion of hydrolytic enzymes including α-amylase and proteases (Gale and Lenton 1987; Yu 2012). This increase in the amount of hydrolytic enzymes causes carbohydrates and proteins reserves in grains to be hydrolyzed. Due to the degradation of carbohydrate and protein reserves, the yield of wheat is reduced and the affected crop is unsuitable for processing. Ultimately the flour quality is downgraded which results in sticky crumb, compact interiors and undesirable

color in baking products (Derera, et al. 1977; Edwards, et al. 1989). Noodles made from the sprouted wheat flour results in decreased elasticity and increased discoloration as compared to noodles made from sound wheat flour (Hatcher and Symons 2000). PHS thus restricts production and end-use applications of wheat which results in financial losses of almost $1 billion dollars worldwide to growers, millers, and bakers (Bewley, et al. 2006).

PHS is a complex trait which is dependent on several factors such as spike and plant morphology, environmental conditions during seed maturation, the presence of inhibitors (ABA) or regulators (GA) of germination, the level of α-amylase activity in wheat kernels, genes and others (Gubler, et al. 2005; Bykova, et al. 2011; Yücel, et al. 2011). Viviparous -1 family genes are found to be associated with PHS-resistance in wheat and maize (Chang, et al. 2011) whereas R allele genes which are associated with seed coat color is associated with PHS-resistance in wheat (Flintham, et al. 2002; Hristov, et al. 2012). Several genomics studies have been carried out in cereal species which concluded that the numerous quantitative trait loci (QTL) are involved in regulation of dormancy in plants (Gale, et al. 2002; Masojć, et al. 2013). In wheat, QTL for PHS-resistance are distributed over all 21 chromosomes (Zhang, et al. 2014) and QTL that demonstrate major effects on PHS-resistance are found on chromosomes 2B, 3A, 4A, 6B and 7D (Munkvold, et al. 2004; Mares, et al. 2005; Liu, et al. 2011; Cabral, et al. 2014).

Genomic studies have provided the blueprint of PHS mechanism in wheat, but a study of spatial and temporal expressions, functions and interactions of gene products is necessary for the validation purpose (Eldakak, et al. 2013). Information carried by genetic material (DNA) is translated to proteins via mRNA, so proteomics can be an

important tool to understand the global genes expression profiles and their function in response to developmental or environmental stimuli (Wang, et al. 2015). Recent advances in proteomics have provided an important tool to compare a mixture of proteins and identify the role of specific proteins in biological processes (Liu, et al. 2015). Proteomics studies of PHS-resistant and PHS-susceptible kernels allows the identification of differentially expressed PHS-related genes, even if they are clustered with other loci (Bykova, et al. 2011; Masojć and Kosmala 2012). These studies were performed by using 2D-DIGE but these 2D-gel based techniques have several limitations such as low identification rate of proteins, low reproducibility, and difficulty in separation of hydrophobic proteins. Isobaric tag for relative and absolute quantitation (iTRAQ) has emerged as a powerful technique to perform quantitative proteome analysis and it allows identification of more proteins and can provide reliable quantitative information as compared to the traditional techniques (Karp, et al. 2010; Unwin, et al. 2010; Ma, et al. 2014). The embryo is known to play a crucial role during seed germination in plants (He and Yang 2013), so protein profile expression analysis of PHS-resistant and susceptible cultivars may be helpful in understanding the complex mechanism of this process in molecular level. This study focuses on finding the key protein that controls PHS mechanism and discovering genes controlling those proteins in wheat.

2.2. Materials and methods

2.2.1. Plant materials

Two Korean winter wheat cultivars (Figure 2.1) were grown at the National Institute of Crop Science, Rural Development Administration (RDA). First one is

Baegjoong, a white winter wheat which is susceptible to pre-harvest sprouting (showing moderate rate of pre-harvest sprouting, 23.9%) and another one is Sukang, a red winter wheat which is tolerant to sprouting (showing low rate of pre-harvest sprouting, 0.2%) (ChidSoo, et al. 2008; ChlulSoo, et al. 2009).

Figure 2.1. Korean wheat cultivars used in the study; A: PHS-susceptible, Baegjoong; B: PHS-resistant, Sukang.

2.2.2. Study design and overview

The objective of the study was to compare the difference in proteins expressed in wheat embryo of two wheat germplasm (Baegjoong and Sukang) over a time course spanning 48 hours (different time points are 0 hrs, 12 hrs, 24 hrs and 48 hrs) where seeds were imbibed in water for 48 hrs to trigger sprouting. Samples were named A, B, C and D for Sukang 0 hrs, 12 hrs, 24 hrs and 48 hrs of treatment and E, F, G, H for Baegjoong 0 hrs, 12 hrs, 24 hrs and 48 hrs of treatment respectively. Wheat embryos of 4 different time points were collected and processed for proteomics analysis. The ratio of the protein expression in the same cultivar at the different time periods and the ratio of protein

expression at the same time period in different cultivars were used for the proteomics study.

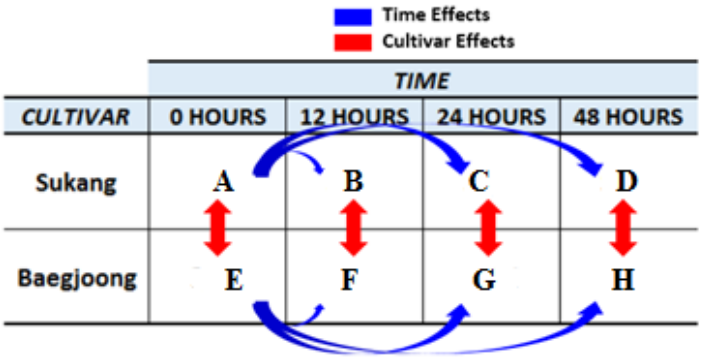

Figure 2.2. Cultivar and treatment for sample collection.

2.2.3. Protein isolation from dissected embryos

The wheat embryos from freshly harvested and 48 hrs imbibition treated seeds (samples A-H) were isolated and were snap frozen and grounded to a very fine powder using mortar and pestle under liquid nitrogen and stored at -80 °C until used. Total protein was isolated according to the modified phenol-based procedure of Hurkman and Tanaka (Hurkman and Tanaka 1986). Isolated embryos (0.1g) were suspended in 4mL of homogenization buffer (0.9 M Sucrose, 10mM EDTA, 0.4% 2-mercaptoethanol, 100mM Tris-HCL [pH 8.8] and equal volume of Tris saturated phenol. The mixture was transferred to a 50mL polypropylene tube, mixed and incubated for 30 min at 4 °C. Centrifugation for 15 min at 5000 rpm 4 °C was done and the phenol phase was collected after that. Proteins were then precipitated overnight with 5 volumes of ice-cold 0.1 M ammonium acetate in methanol at -20 °C. Centrifugation at 5000 rpm for 10 min was done and protein pellet was washed thoroughly twice in 20mL of 0.1 M ammonium

acetate in 100% methanol. The protein pellet was washed twice with ice-cold 80% acetone with a final wash in 70% ethanol. The protein was air dried and stored at -80 °C until further processing.

2.2.4. iTRAQ labeling and MS/MS

The embryo protein pellet samples were re-suspended in 0.1% SDS, 500mM triethylammonium bicarbonate [pH 8.5], and 5 M urea and then subjected to sonication on ice. Protein concentrations were determined by a modified Bradford assay (BioRad, Hercules, CA, USA). The iTRAQ labeling, protein identifications, were performed at the Applied Biomics. The samples (40µg protein in 0.05% SDS and 1.5 M urea) were reduced, alkylated with methyl methanethiosulfate, and trypsin-digested. After digestion, samples were labeled with iTRAQ reagents (8-plex kit, Applied Biosystems, USA) following manufacturer's guidelines.

Table 2.1. Samples labeling for iTRAQ analysis

Label	113	114	115	116	117	118	119	121
Sample	A	B	C	D	E	F	G	H

After labeling embryo proteins, the peptides were mixed and vacuum dried. Labeled peptides were applied to an OASIS® medium cation exchange (MCX) extraction cartridge (Waters Corporation, Milford, MA, USA) to remove trypsin, excess hydrolyzed iTRAQ reagents and for buffer exchange. Strong cation exchange (SCX) fractionation was done for all labeled peptides (Lund, et al. 2007). Peptides selected from fractionation

were subjected to LC separation and spotted onto a MALDI target using Tempo ™ LC MALDI system. MS data were acquired on a 4800 MALDI TOF/TOF as described previously (Smith, et al. 2009).

2.2.5. iTRAQ data analysis

Protein pilot v3.0 software, which utilizes the Paragon™ scoring (Shilov, et al. 2007) and Progroup™ protein grouping algorithms (ABI/MDS-Sciex, Toronto, Canada) was used to analyze MS/MS spectral data. Searches were made against NCBI non-redundant whole protein database. The search parameters included iTRAQ 8-plex peptide label, quantitation mode, trypsin enzyme, cysteine methyl methanethiosulfonate alkylation reagent, thorough search mode, biological modifications (includes >220 post-translation and artifact modifications) and minimum detected protein threshold of 10% instrument element defines resistance, MS and MS/MS, 0.15 Da and 0.4 Da, respectively. Proteomics System Performance Evaluation Pipeline (PSPEP) (Tang, et al. 2008) algorithm was incorporated into ProteinPilot to estimate the False Discovery Rate (FDR). Confidence score of at least 95%, the Unused score greater than 1.3 and a global FDR estimation lower than 5% were the parameters used for the identification of a protein.

2.2.6. Bioinformatics analysis

Proteins were examined using Uniprot database (Consortium 2007) and more information about protein function was retrieved. Information of proteins which were involved in metabolic pathways were retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG database) (Kanehisa, et al. 2010). Hierarchical clustering was done by using Gene Cluster 3.0 software and visualized in Java TreeView software. Analysis of protein-protein interaction (PPI) was followed by three steps: (1) Protein sequence of all

differentially expressed proteins were run for BLAST in NCBI against non-redundant protein sequence (nr) database for Arabidopsis homologs; (2) All accession numbers retrieved from BLAST were collected for PPI using STRING database version 9.1 (Franceschini, et al. 2013); and (3) Primary interaction retrieved from STRING database was portrayed in Cytoscape 3.3.0 software (Cline, et al. 2007) along with categorization of their functions and expression patterns.

2.3. Results

2.3.1. Protein expression profiles during PHS

Our iTRAQ-based quantitative proteome characterization revealed the proteins involved in premature seed germination in wheat. A global profiling of quantitative proteome was obtained from the embryos at 0 hrs, 12 hrs, 24 hrs and 48 hrs time period using the biological replicates detected 306 different proteins (Appendix 1). A 1.5-fold cut-off was used to implicate significant changes in the abundance of differentially expressed proteins (DEPs) during pre-harvest sprouting. Of 306 non-redundant proteins identified, 190 showed more than 1.5-fold changes in protein expression in at least one of the ten comparisons and therefore identified as DEPs (Appendix 2). Total of 114 proteins were found to be differentially expressed in at least one of the time effect comparison in Sukang, out of which 60 were found to be up-regulated in at least one of the time period, 57 were found to be down-regulated at least once. In the case of Baegjoong, 117 proteins were found to be expressed significantly out of which 72 were found to be down-regulated and 65 were found to be up-regulated at least once in the time period. A total of

176 proteins were found to be in significantly different abundance between two cultivars at same time period at least once.

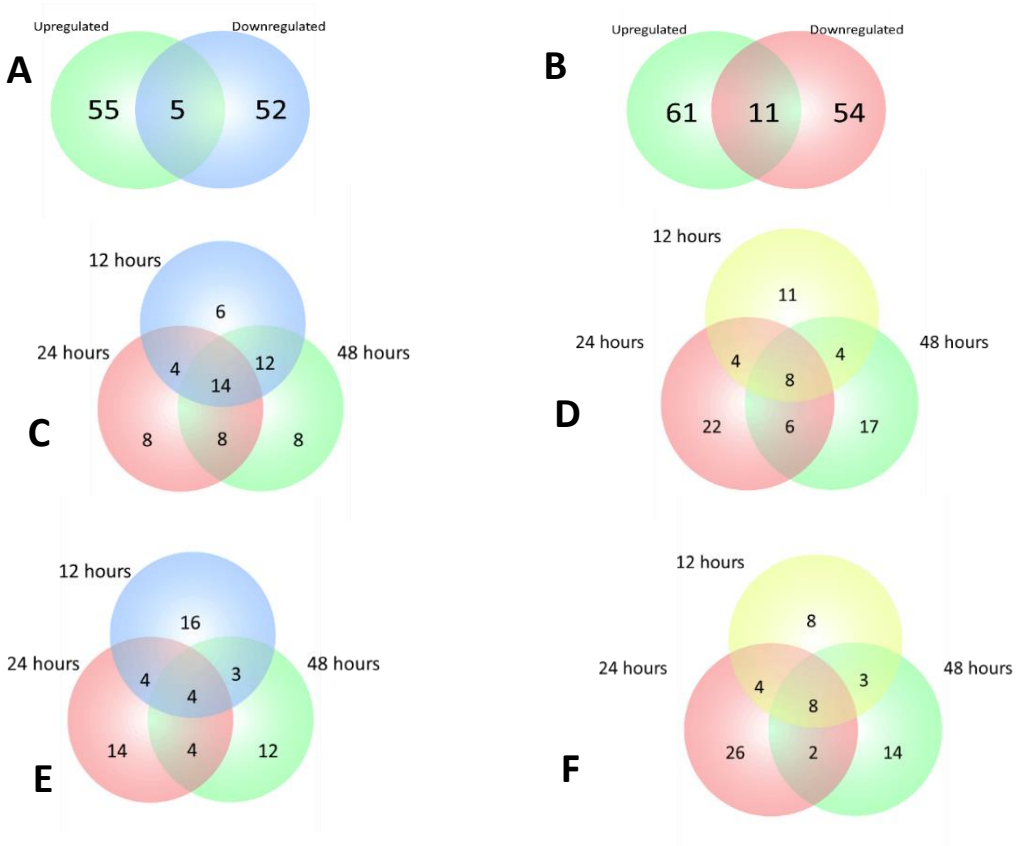

Figure 2.3. Venn diagrams of differently expressed proteins. **A**: Proteins differently expressed in Sukang; **B**: Proteins differently expressed in Baegjoong; **C**: The number of DEPs upregulated in Sukang; **D**: The number of DEPs upregulated in Baegjoong; **E**: The number of DEPs down-regulated in Sukang; **F**: The number of DEPs downregulated in Baegjoong.

2.3.2. Differential protein expression during PHS

Hierarchical clustering analysis (HCA) was performed to display the dynamic expression patterns of the proteins (Figure 2.4). The log-transformed expression ratios of

the proteins identified by iTRAQ were used. Gene cluster 3.0 software with Euclidean distance similarity metric and average linkage method were used as parameters. Java Treeview software was used to visualize the clusters.

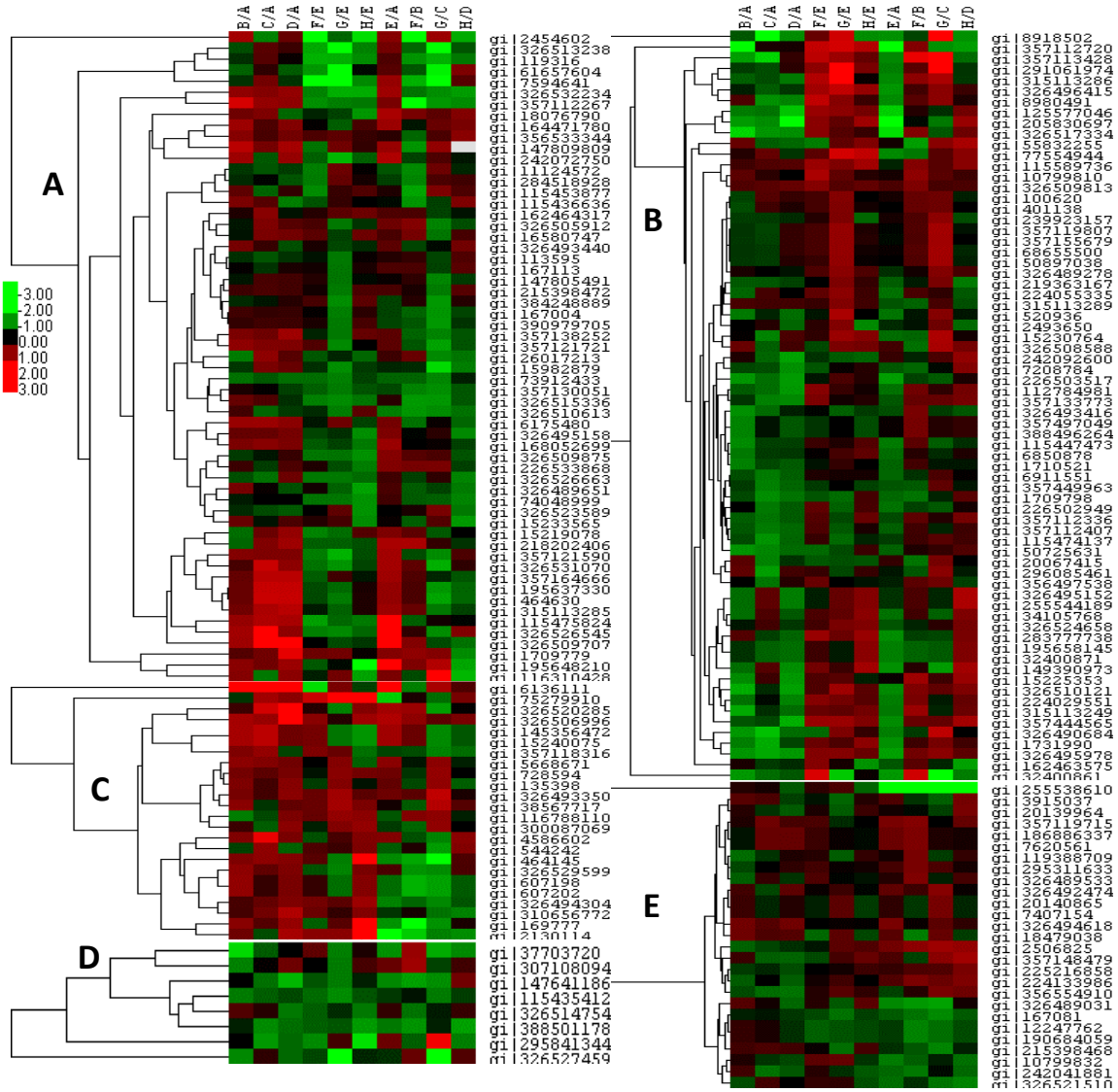

Figure 2.4. Heat map of proteins expressed during the experiment. Ten different experimental groups are shown horizontally and protein GI number vertically. **A**: Proteins up-regulated in Sukang; **B**: Proteins up-regulated in Baegjoong; **C**: Proteins up-regulated in both Sukang and Baegjoong; **D**: Proteins down-regulated in both Sukang and Baegjoong: **E**: Protein not regulated in either genotype but different in abundance among various time periods.

2.3.3. Functional classification of identified proteins

According to molecular functions listed on the UniProt and Gene Ontology website, the 190 DEPs were classified into eleven functional categories. These thirteen functional categories in which proteins were involved in are Carbohydrate metabolism, Nitrogen metabolism, Stress-induced, ATP synthesis, Lipid metabolism, Redox regulation, Transporters, Storage protein, Protein biosynthesis/Degradation, Nucleic acid binding, Inhibitors, Cytoskeleton and Unknown function.

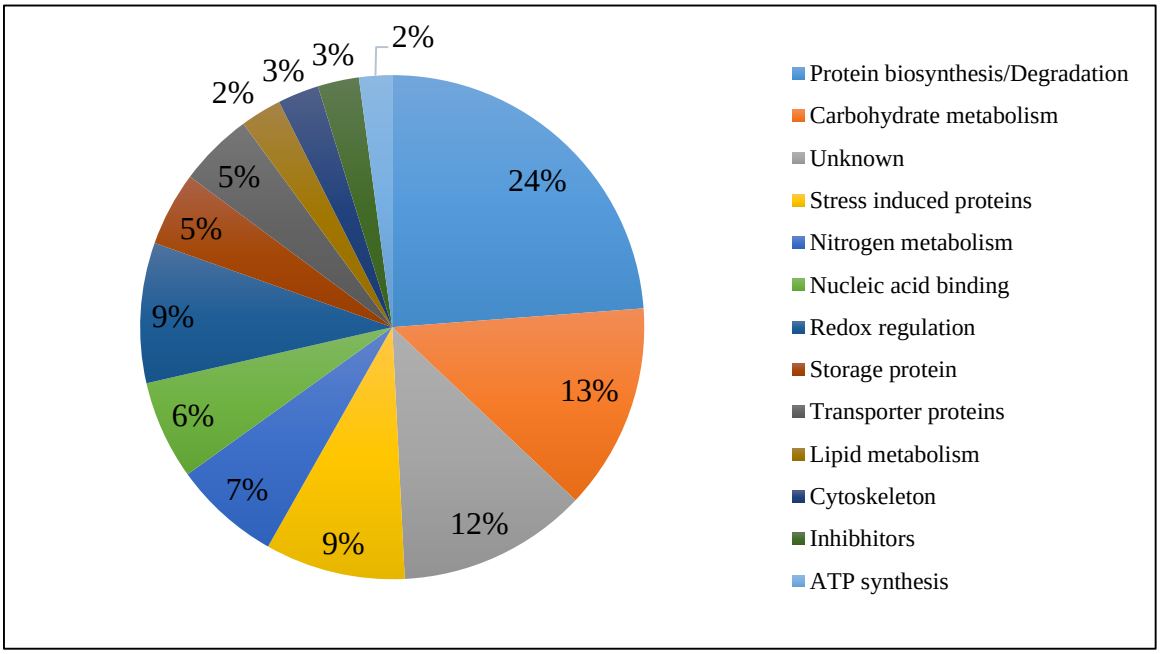

Figure 2.5. Functional categorization of the differentially expressed proteins expressed during pre-harvest sprouting.

2.3.4. Protein-protein interaction analysis

Protein-protein interactions have a key role in cellular level. Catalysis of the different metabolic pathway, regulation of transcription or post-transcription are few

examples of protein-protein interaction. To find the interaction between differentially expressed proteins during pre-harvest sprouting we used STRING database (http://string-db.org/). We found 60 homolog proteins in Arabidopsis (Appendix 3), which were differentially expressed in at least 2 time periods in PHS-resistant or -susceptible cultivar.

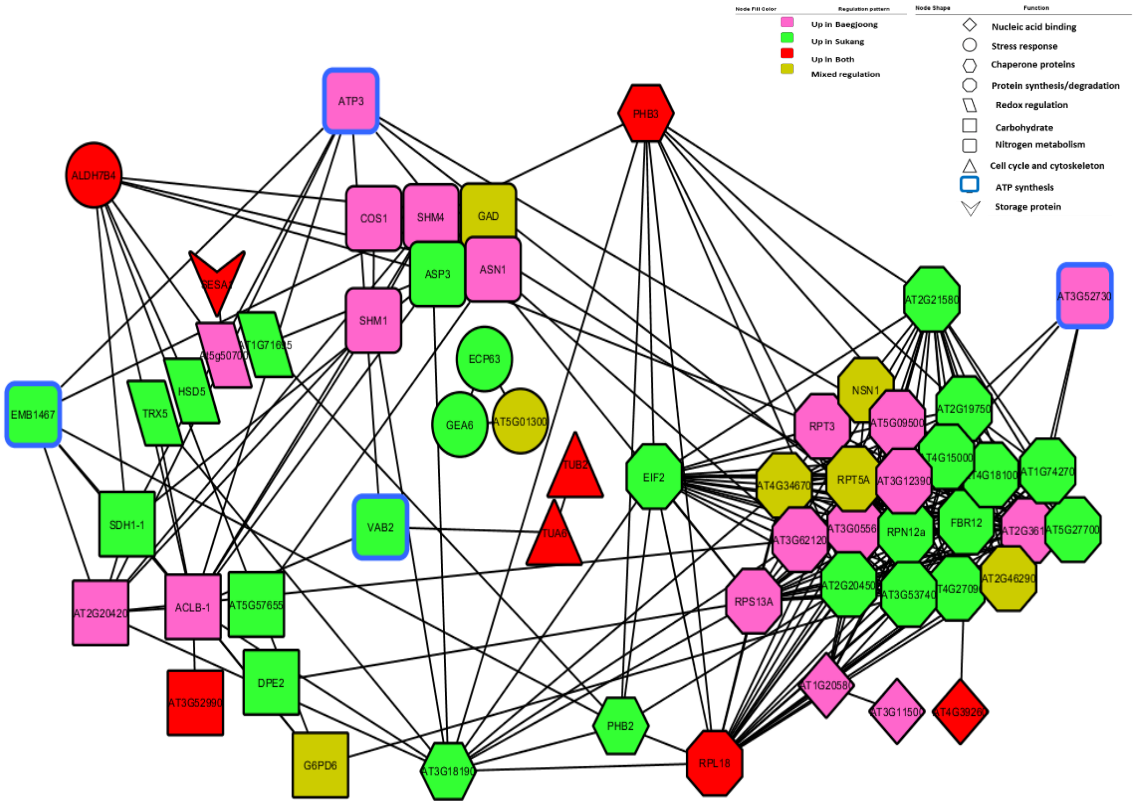

Figure 2.6. Protein-protein interaction map for Arabidopsis homologs of differentially expressed proteins (at least in 2 time periods) in PHS-resistant or PHS-susceptible cultivar. Interaction data was derived from STRING database, and visualized in Cytoscape 3.3.0 with discrete mapping of protein interaction and their function.

Most of the stress related proteins and chaperone proteins were seen to be interacting with other groups of proteins. Three chaperone proteins, PHB2, PHB3, and

AT3G18190 are involved in a variety of cellular processes including response to stress and cell division. Most of the ribosomal proteins have a higher number of interactions but that was among themselves. Among ribosomal proteins, EIF2 has more than six interactions in total and it was interacting with chaperone proteins and stress related proteins. ATP3 protein, which is related to ATP production and ALDH7B4 which is involved in stress response has also more than 8 interactions and looks like central connecting elements in the network. Most of the proteins having more interactions were related to stress response, ATP synthesis, redox regulation and carbohydrate metabolism. This interaction suggests that up-regulation or down-regulation of these central proteins in the network may affect the expression pattern of other proteins present in the network. As stress related proteins seem to be the key proteins in the network, so the differential expression of these proteins may trigger a signal in breaking the dormancy in wheat.

2.4. Discussion

2.4.1. Metabolism and energy supply

Glycolysis, tricarboxylic acid (TCA) cycle, and the mitochondrial electron transport chain are essential steps for energy production for various cellular functions. Ten proteins responsible for the starch metabolism were identified. Sucrose synthase enzyme was also identified as DEP and is up-regulated in 24 hrs sample in Baegjoong. In developing seeds, sucrose synthase is responsible for the accumulation of starch by hydrolyzing sucrose into fructose and UDP-glucose (Ghosh and Pal 2012). UGPase (UTP:glucose 1-phosphate uridylyltransferase) was found to be up-regulated in both cultivars and it was more accumulated in the PHS-susceptible cultivar. Depending on a

metabolic status of the tissue, UGPase is involved in sucrose synthesis or breakdown. Involvement of UGPase in sucrose degradation was also observed in developing barley endosperm (Eimert, et al. 1996). Beta-amylase enzyme, which is a major enzyme for the degradation of starch is up-regulated in both of the cultivars. In the beginning, it remained steady in Baegjoong and showed up-regulation of more than 3 folds in 48 hrs time period. In germinating seeds, beta-amylase is slowly accumulated and is significantly increased during 1-4 days of germination (Yamasaki 2003) and it is usually produced in aleurone layer of seed where the degradation of the starch usually takes place. Han, et al. (2014) concluded that in germinating rice embryo, starch accumulation was more in early stages followed by the rapid degradation after 24 hrs time periods. The up-regulation of the beta amylase enzyme at 48 hours in Baegjoong indicates that the more carbohydrate metabolism occurs in the later periods of imbibition.

By the process of synthesis and degradation of sucrose, the glucose could be transformed into glucose phosphate. The glucose phosphate then enters glycolysis and TCA cycle. The breakdown of carbohydrate molecules by glycolysis is important during germination. The ATP produced by glycolysis is an important energy source in the absence of photosynthesis in seedlings (Andre and Benning 2007). As glucose is responsible for delaying germination in some plants like *Arabidopsis thaliana* (Dekkers, et al. 2004) the breakdown of glucose seems to be vital for germination of seeds. Totally, 6 proteins that are involved in glycolysis cycle were detected in this experiment. Most of the proteins were accumulated more in Baegjoong. Two proteins including Phosphoglucomutase and Pyruvate kinase were accumulated more in the initial stage and then remained steadily accumulated in all of the time periods in Baegjoong. Andre and

Benning (2007) observed that seeds of mutant Arabidopsis deficient in plastidic pyruvate kinase were unable to perform metabolism of storage oil and utilize externally applied sucrose for hypocotyl elongation in the dark conditions. Pyruvate dehydrogenase E1 component was observed to be up-regulated in all of the time periods in PHS-susceptible wheat cultivar and have been accumulated in more abundance as compared to the resistant cultivar. The pyruvate dehydrogenase complex is a multi-enzyme complex which catalyzes the oxidative decarboxylation of pyruvate to yield acetyl-CoA and NADH (Tovar-Mendez, et al. 2003). This protein links two primary metabolic pathways, glycolysis, and TCA cycle and is responsible for control of metabolic flow in the organisms (Luethy, et al. 2001).

The anaerobic respiratory pathway, fermentation, was found to be existed in the PHS-susceptible cultivar. This was supported by the more accumulation of alcohol dehydrogenase in Baegjoong in 12 hrs. Alcohol dehydrogenase (ADH) enzyme catalyzes the two-step reaction of alcoholic fermentation to produce ethanol. In the scarcity of oxygen, TCA cycle will be negatively affected and in that case, fermentation pathway may help to provide ATPs for the energy production (He, et al. 2011). ADH is involved in coleoptile growth under oxygen-limiting conditions and deficiency of ADH negatively impacts the growth of coleoptile (He, et al. 2015).

The final product of glycolysis, pyruvate is transferred into mitochondria and is used as the substrate for TCA cycle (He, et al. 2011). In our study, we identified 8 differentially expressed proteins (DEPs) related with TCA cycle. Most of the DEPs related with TCA cycle were up-regulated in Baegjoong cultivar. Succinate dehydrogenase, a complex enzyme bound to the inner mitochondrial membrane is

responsible for the oxidation of succinate to fumarate was found to be up-regulated in 12 hrs sample and then down-regulated in 24 hrs and 48 hrs samples of Baegjoong in this experiment. NAD-dependent isocitrate dehydrogenase (NAD-IDH) is up-regulated in 24 hrs sample of Baegjoong, and this protein is a key enzyme which catalyzes the oxidative carboxylation of isocitrate to 2-oxoglutarate, NADH, and CO_2. Up-regulation of these TCA related enzymes in the initial stage in PHS-susceptible cultivar implies that TCA cycle is very active during the early embryo development stages, mainly providing energy for the embryo development.

In our study, we observed that Glucose-6-phosphate dehydrogenase showed abnormal up-regulation in the PHS-susceptible wheat cultivar. This protein was up-regulated in 24 hrs time period in Baegjoong and was accumulated 25 folds compared to Sukang at same time period. Glucose-6-phosphate dehydrogenase along with 6-phosphate gluconate dehydrogenase catalyze the initial steps of the pentose-phosphate pathway. Swamy and Sandhyarani (1986) observed that the activity of the enzymes participating in pentose phosphate pathway was sharply increased in embryonic axis of non-dormant lines of peanut seeds from 24 hrs to 96 hrs of germination and concluded that Pentose-phosphate pathway plays crucial role in breakage of dormancy in peanut seeds and Glucose-6-phosphate dehydrogenase plays a key role in control of the pentose phosphate pathway. Roberts (1977) has suggested that pentose-phosphate pathway controls germination in certain graminaceous seeds by increasing glucose catabolism. The release of dormancy in seeds demands an increase in glucose oxidation by the pentose shunt for forming particular metabolites needed to complete reactions such as protein synthesis and this reaction requires NADPH which is produced in pentose phosphate pathway (Neish

1960; Kovacs and Simpson 1976). NADPH also reduces proteins with disulfide bridges of the thioredoxin type by activating NADPH-dependent enzyme thioredoxin reductases. These reduced proteins may activate enzymes that are necessary for germination in non-dormant lines (Taylorson 2012).The activity of Glucose-6-phosphate along with 6-phosphate gluconate dehydrogenase was found to be increased in susceptible seeds while both were decreased in dormant seeds of wild oats during germination test (Kovacs and Simpson 1976).

Lipoxygenases (LOX) are iron-containing dioxygenases that catalyze the oxygenation of polyunsaturated fatty acids and is widely distributed in plants and animals (Holtman, et al. 1996). PHS-resistant lines showed less accumulation of the LOX in all of the time periods as compared to the susceptible lines in the experiment. Though it is unclear that how LOX plays a role in breaking the dormancy but it is observed that it helps in the mobilization of storage lipids in cucumber and soybean seedlings (Feußner and Kindl 1992).

ATP synthesis is an essential part which plays a pivotal role in energy transduction in living cells (He, et al. 2015). In our study, we identified that 3 ATP synthase alpha subunit and 2 mitochondrial electron transfer subunit were accumulated more than 1.5 folds in PHS-susceptible cultivar throughout the experiment. This indicates that energy metabolism continuously increased the process of germination in Baegjoong. Other proteins which were involved in oxidative phosphorylation process in Mitochondria such as NADH-ubiquinone oxidoreductase 75 kDa subunit was also found to be accumulated more in the PHS-susceptible cultivar. Overexpression of these proteins

suggests that whole mitochondrial ATP synthesis in seed embryos may contribute to breaking the dormancy prematurely in the PHS-susceptible germplasm.

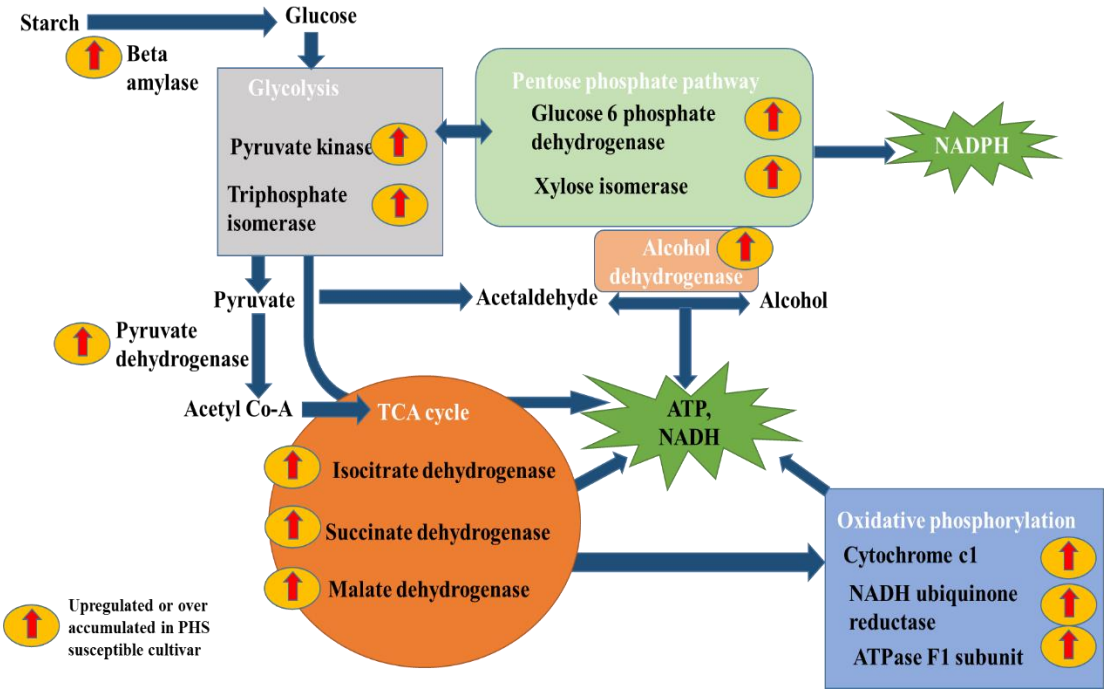

Figure 2.7. Proteins involved in energy metabolism. Most of the proteins were found to be upregulated in Baegjoong.

Carbohydrates are generally stored in the form of sucrose in wheat embryos and are used as fuel for the germination. The up-regulation of energy production (Glycolysis, TCA) related proteins (Figure 2.7) and more accumulation of these proteins in PHS-susceptible germplasm was a specific feature detected in this proteomics study. Kovacs and Simpson (1976) hypothesized that the pentose phosphate pathway and glycolysis-tricarboxylic acid pathways are involved in the control of seed dormancy. Up-regulation of most of the energy metabolism related proteins and more accumulation of those proteins in Baegjoong suggests that elevated synthesis of the proteins involved in energy

metabolism may be required to initiate storage protein mobilization and to achieve the high rates of germination in the PHS-susceptible cultivar.

2.4.2. Nitrogen metabolism

Thirteen proteins involved in Nitrogen related metabolism were observed in this study. Glutamate decarboxylase was up-regulated in 48 hrs sample of PHS-susceptible germplasm. Glutamate carboxylase is known to produce gamma butyric acid (GABA) by decarboxylation of L-glutamic acid. Inatomi and Slaughter (1971) reported steady growth in glutamate decarboxylase activity during the germination of barley seeds. They reported that the activity of glutamate decarboxylase was steady during the embryo soaking but once the growth began the activity rose rapidly. The accumulation of Glutamate decarboxylase indicates the increase in GABA-shunt pathway which is responsible for providing carbons for oxidation in TCA cycle is an important step required for germination of seeds (Oh and Choi 2001). Another protein putative aminotransferase was found to be up-regulated in PHS-susceptible cultivar in all of the time periods. Asparagine synthetase catalyzes the asparagine biosynthesis in plants using glutamine as nitrogen donor. This protein is known to play an important role in nitrogen re-allocation during germination of legume plant seeds (Rognes 1970). Overexpression of the asparagine aspartate synthetase resulted in a change in seed nitrogen content along with the increase of free amino acids in Arabidopsis (Lam, et al. 2003). So up-regulation of this protein in wheat may be important for the growth of the seedlings.

One carbon metabolism (C1) is a major cellular event that occurs during germination and subsequent post-germination growth. This process is mediated by tetrahydrofolate coenzymes and C1 transfer reactions result in the synthesis of purines,

metabolism of amino acids, biogenesis of mitochondrial and chloroplastic proteins and methionine synthesis (Jabrin, et al. 2003). Serine hydroxymethyltransferases (SHMTs) are important enzymes that participate in cellular C1 metabolism and in the photorespiratory conversion of glycine into serine (Besson, et al. 1995; Douce and Neuburger 1999). In our study, we identified two isoforms of SHMTs, which showed up-regulation in all of the time periods of PHS-susceptible cultivar and down-regulation in PHS-resistant. Methionine synthase is another important enzyme involved in methionine cycle (Figure 2.8) which is a part of C1 metabolism. It catalyzes the formation of L-methionine from L-homocysteine, which is the last step of methionine synthesis. In plants, methionine functions not only as building blocks of protein but it also acts as the precursor of S-Adenosyl methionine which is the primary methyl group donor and precursor of polyamines, lignin, pectin and the ripening plant hormone ethylene . As ethylene plays a crucial role in plant growth and development (Rodriguez-Gacio and Matilla 2001) these precursors enzymes involved in the biosynthesis of methionine may play an important role in the control of seed germination. The accumulation of methionine synthase in Arabidopsis seeds was highest after 1 day of imbibition in water which indicated that methionine synthase along with S-adenosylmethionine synthetase is fundamental components which control the metabolism in the transition from an inactive to a highly active state during seed germination (Gallardo, et al. 2002a). Ethylene is also involved in regulating the expression of cysteine proteinase genes and it's protein complex which results in the removal of seed dormancy (Borghetti, et al. 2002). In our study, we identified 4 methionine synthase enzyme which was up-regulated in 24 hrs sample of the PHS-susceptible wheat cultivar. They have up-regulated almost 2 folds in

PHS-susceptible cultivar during 24 hrs of imbibition. Most of the embryonic cells are arrested in G1-phase of cell cycle and high rate of DNA synthesis is required for seeds to switch from quiescent to proliferating stage during imbibition (Gallardo, et al. 2002b) and the C1 metabolic pathway seems to be important for synthesis of purines and pyrimidines required for DNA synthesis and subsequent growth of imbibed seeds

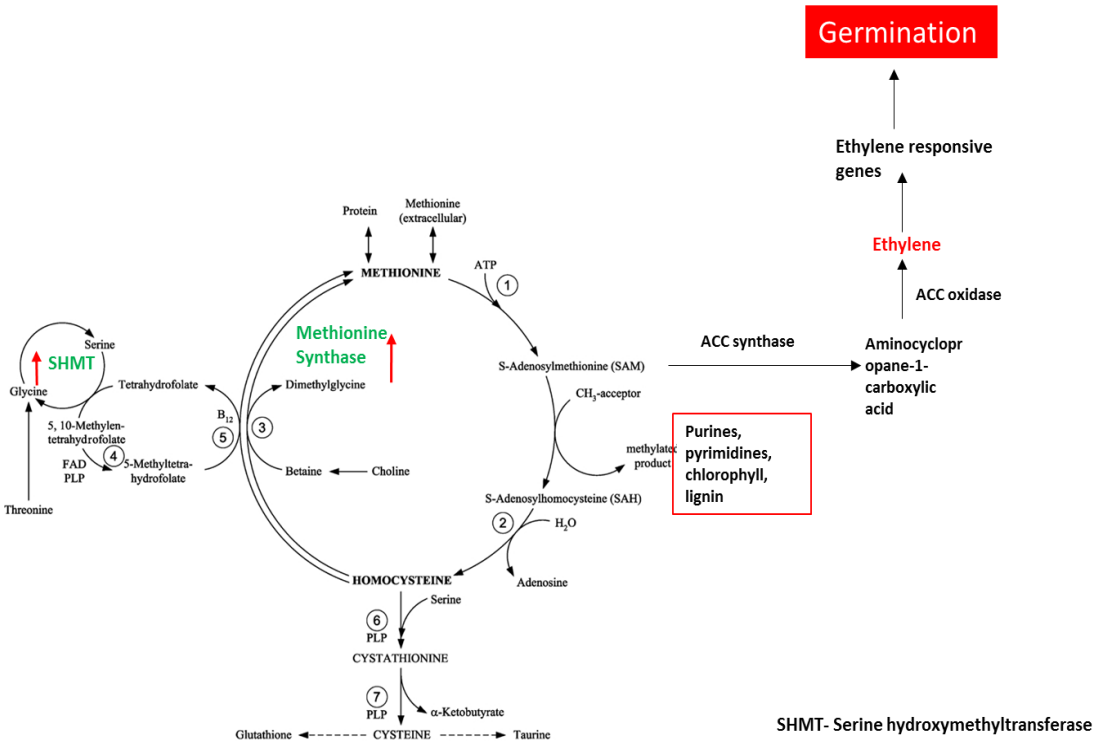

Figure 2.8. The role of methionine biosynthesis in seed germination. Red arrow indicates the proteins that were found to be upregulated in PHS-susceptible germplasm.

2.4.3. Stress induced proteins

A number of stress/defense related proteins are expressed during wheat grain development. The main functions of these proteins are to protect the seed from various biotic and abiotic stresses like drought, salt, osmotic stress, extreme temperatures, and

several pathogenic microorganisms. This study revealed that 17 stress/defense related DEPs during the process of germination. Some of the proteins were labeled as late embryogenesis abundant (LEA) proteins and heat shock proteins (Hsp) which are exclusively synthesized during seed development. In our study, we identified 5 LEA including Dehydrin and Early methionine labeled polypeptide which was found to be down-regulated in the PHS-susceptible cultivar. Early methionine labeled polypeptide and Glycine-rich protein peptide is embryo specific in nature and both plays role in maintaining the minimum level of hydration to prevent the denaturation of cytoplasmic components. Kalaiselvi and Manickam (1999) have reported the role of this protein during imbibition by controlling water uptake and characterized this protein as an abscisic acid responsive protein. Early methionine labeled protein was in higher amount in susceptible cultivar before imbibition but is sharply down-regulated after imbibition. Early methionine labeled protein is directly linked with ABA, an inhibitor of germination in plants. According to Williamson and Quatrano (1988), the level of Early methionine labeled protein sequence is replaced by germination-specific sequences during germination. In the absence of ABA, there was no accumulation of early methionine labeled protein at detectable levels in the wheat embryo (Williamson, et al. 1985) . As the level of this protein is regulated by the amount of endogenous ABA present inside the embryo, we can conclude that this protein represents the amount of ABA present inside the embryo. As the germination proceeds, the ABA is down-regulated in PHS-susceptible cultivar but ABA remains same or up-regulated in PHS-resistant cultivar to inhibit the process. Another protein that is directly linked with the ABA is EMB-1 protein which is usually found in developing embryo of the carrot (Shiota, et al. 2004). EMB-1 is thought

to be related to the proteins which act as hydration agent to protect the cellular components of the embryo from desiccation when seeds become dormant (Wurtele, et al. 1993). In our study, we found that there was steadily down-regulation of EMB-1 protein in the PHS-susceptible cultivar. Dehydrin has a specific role in calcium binding which has a significant role on signaling process and regulation of secondary messenger transmission (Ma, et al. 2014). Dehydrin was found to be up-regulated by ABA and decrease in quantity was observed during dormancy breaking and germination of Norway maple seeds (Pawłowski 2009). Gene expression analysis of seed dormancy breaking in wild oat showed that GA treatment causes the reduction in transcripts level of LEA, whereas ABA treatment increased transcripts level of LEA (Li and Foley 1995). All of the evidence support the fact that LEA proteins are directly regulated by ABA to inhibit germination in various plants and up-regulation of these proteins in PHS-resistant line also confirms that this is a regulatory machinery used by resistant wheat seeds to protect themselves from pre-harvest sprouting under unfavorable conditions.

The involvement of heat shock proteins (HSP) in assembly and degradation of protein complexes suggests that they can play an important role in diverse cellular processes such as stress response and protein metabolism (Neuwald, et al. 1999). Six HSPs including member of HSP70 and HSP90 was identified in our study. One HSP which is a member of HSP70 chaperone family was identified which was accumulated in all time periods in susceptible cultivar, suggesting that these HSPs have protective function during seed maturation and throughout germination (Mak, et al. 2009). One uncharacterized protein was found to be up-regulated in PHS-susceptible cultivar which

was induced by brassinosteroid. Brassinosteroid is found to enhance the seed germination by inhibiting the activity of ABA on seed germination (Zhang, et al. 2009).

2.4.4. Inhibitors

In our study, we identified 5 inhibitors that were differentially expressed in our experiment. Sugang had increased the level of Trypsin inhibitor CMx precursor and Defensin protein as compared to PHS-susceptible Baegjoong. Trypsin inhibitor is known to play a role in inhibition of alpha-amylase activity and protection of storage proteins from exogenous proteases released from fungi and insects (Ghosh and Pal 2012). Defensin protein is known to have diverse function including inhibition of protein synthesis and inhibition of alpha-amylase activity (Odintsova, et al. 2007). The resistance to PHS in various cereals including rye can be reduced when the seed coat is subjected to rupture by pests during the course of development (Masojć, et al. 2013). More than 2-fold difference in accumulation of these proteins in PHS-resistant cultivar was observed in our study so this suggests that these two proteins are important for PHS-susceptibility in wheat. Serpins are another class of proteins identified in this study. Serpins proteins are also known to play a role in inhibition of proteinases, which play a role in growth, development of plant and stress responses in the plant. For the protection of storage proteins from rapid digestion, Serpins are usually accumulated in the germinating wheat. Previous research has shown that serpin was highly expressed under salt and cold stresses (Lampl, et al. 2010). Masojć, et al. (2013) observed more than 2 fold accumulation of serpin in PHS-susceptible rye and concluded that serpin could be a candidate gene for regulation of PHS. As serpin is highly accumulated in PHS-susceptible cultivar, defense and inhibitor proteins seem to be important for PHS susceptibility in wheat.

2.4.5. Redox Regulation

When plants are exposed to biotic or abiotic stresses, it can induce the production of reactive oxygen species (ROS) (Figure 2.9) which includes superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), or singlet oxygen ($_1O^2$) are important molecules in plant biological processes. These ROS are produced in various processes including mitochondrial electron transfer chain reaction (Gomes and Garcia 2013).These ROS are known to play role in endosperm weakening, seed reserves mobilization, and programmed cell death in aleurone layer (El-Maarouf-Bouteau and Bailly 2008).

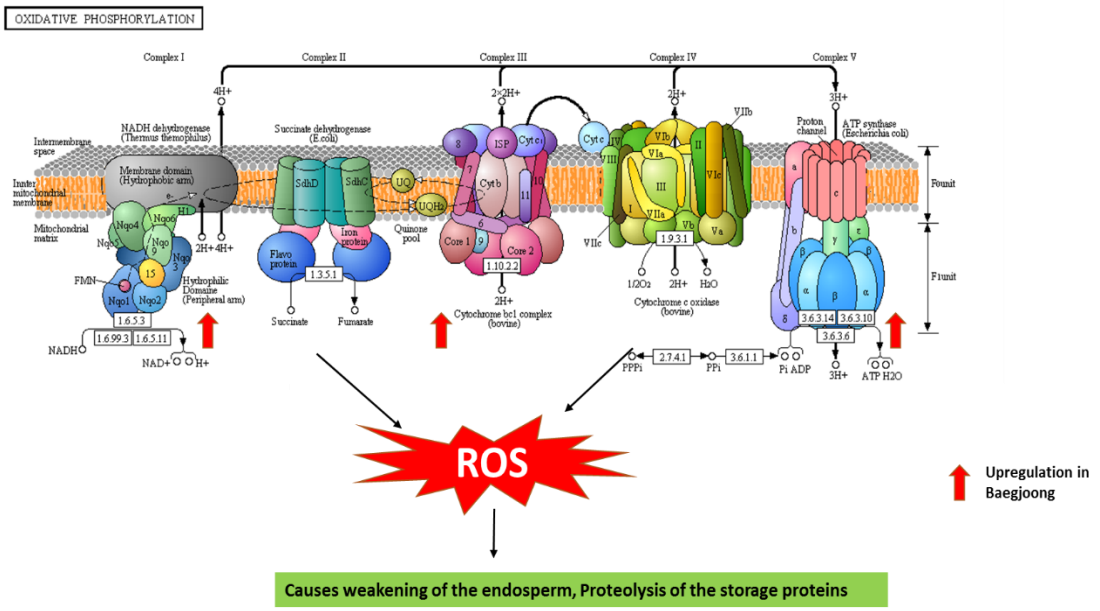

Figure 2.9. Reactive oxygen production during oxidative phosphorylation.

Leymarie, et al. (2012) observed ROS accumulation in germinating seeds of *Arabidopsis thaliana* and concluded that ROS plays a role in gibberellin signaling to break dormancy in seeds. In our study, along with the ATPase molecules, we identified two isoforms of cytochrome c1 complex involved in mitochondrial electron transfer chain were upregulated in the PHS-susceptible cultivar.

In response to these toxic materials, the plant produces some defense proteins including superoxide dismutase, catalases, thioredoxin (Ma, et al. 2014). A higher level of anti-oxidant protection is needed to PHS-susceptible lines during the germination as compared to the PHS-resistant line. ROS-scavenging enzymes may play a protective role during premature germination (Masojć, et al. 2013). Glyoxalase I is responsible for Lactoglutathione lyase activity which causes the detoxification of methylglyoxal, a highly toxic electrophilic glycolytic by-product that inactivates both proteins and nucleic acids (Liu, et al. 2015). Lower expression of glyoxalase I in transgenic tobacco plant caused the accumulation of methylglyoxal resulting in the inhibition of seed germination (Yadav, et al. 2005), thus increased the level of glyoxalase I in wheat may be responsible for breaking the dormancy. Four proteins were identified which were related to glyoxalase domain and found to be accumulated in all of the time periods except 24 hrs imbibition time.

Peroxidases, which catalyzes oxidoreduction between hydrogen peroxide and various reductants (Hiraga, et al. 2001) was also identified in this study. We found that all the peroxidases were up-regulated in PHS-resistant cultivar. Peroxidase activity (Figure 2.10) is directly related to the oxygen uptake capability of the seed and the level of oxygen availability is the major factor determining the germination of rice embryo (Navasero, et al. 1975). Lower activity of peroxidase present in the hull of rice seed increased amylase and dehydrogenase activity resulting in the breakage of dormancy (Seshu and Dadlani 1991). Gaspar, et al. (1977) hypothesized that the low peroxidase activity is linked with alpha amylase activity and germination of wheat. Sufficiently low peroxidase activity in the embryo along with minimum auxin level is responsible for

activating alpha amylase synthesis in the endosperm which helps in the germination of wheat.

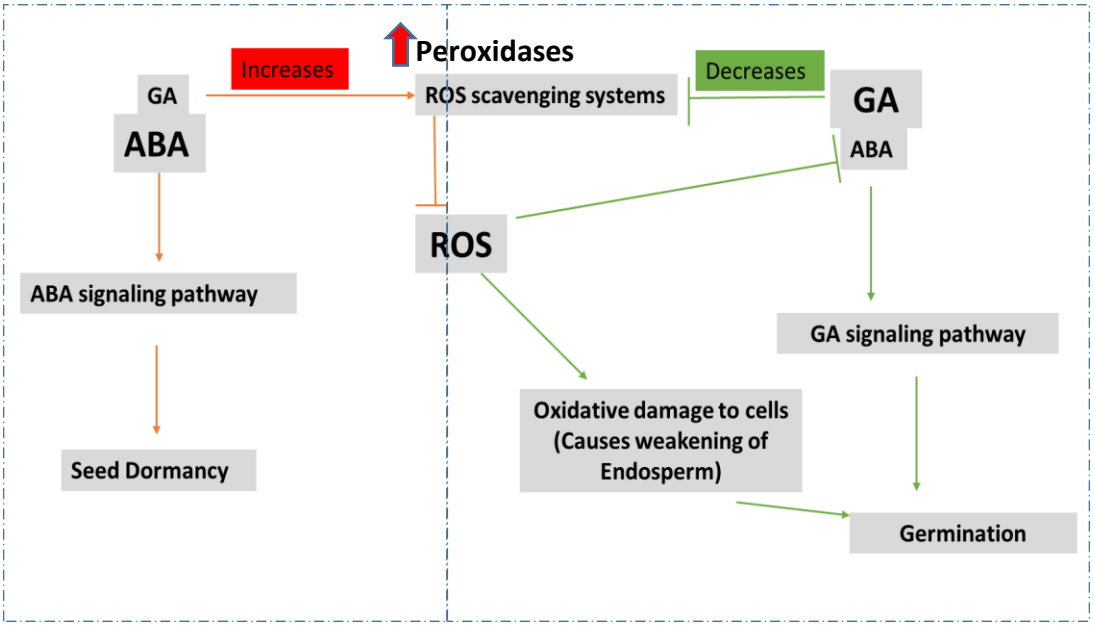

Figure 2.10. Predicted model for the role of ROS and ROS scavenging enzymes in dormancy release and germination. (Modified from (El-Maarouf-Bouteau and Bailly 2008)). Peroxidase was found to be upregualted in PHS-resistant germplasm.

Peroxiredoxin is an antioxidant which is expressed in the aleurone and embryo layer during the late developmental phase of seeds and is encoded by dormancy related gene Per1 in barley seeds and is involved in the protection of aleurone and embryo cells against free radical damage during imbibition of dormant seeds (Stacy, et al. 1996). Peroxiredoxin helps to prevent the radical attack of lipids, enzymes and provides cells with efficient machinery to detoxify oxides like hydrogen peroxide, alkyl hydroperoxides. Under stressed conditions, peroxiredoxin is involved in inhibition of the germination (Wood, et al. 2003). When wheat seeds were treated with ABA, the up-

regulation of peroxiredoxin was observed, suggesting the role in maintaining the dormancy of wheat (Bykova, et al. 2011). Some authors believe that peroxiredoxin is not directly involved in maintaining the dormancy of wheat but it helps to inhibit the germination of wheat under stressed conditions (Haslekås, et al. 2003). In our study, we observed that two form of 1-Cys peroxiredoxin and peroxiredoxin were accumulated more in the resistant cultivar.

In our study, we found that Thioredoxin h has been up-regulated in 12 hrs and 24 hrs in PHS- susceptible cultivar. Thioredoxin h is a redox-active compound found in all almost all organisms. During the process of seed germination, thioredoxin h is known to break the intramolecular disulphide bonds of storage proteins, increase amino acid metabolism by up-regulating glutamate dehydrogenase, glutamate oxaloacetic transaminase, and glutamic pyruvic transaminase (Guo, et al. 2013) and reducing oxidized protein which leads to increased solubility and mobilization of carbon and nitrogen for growth of new seedling (Wong, et al. 2004). The overexpression of thioredoxin h in the endosperm of the barley (*Hordeum vulgare*) accelerated the germination along with the release of starch hydrolyzing enzyme and reduction of the storage protein (Guo, et al. 2007). Thioredoxin h, when overexpressed in the barley endosperm is believed to communicate directly with the embryo and the aleurone layer, to accelerate the germination process and appearance of alpha-amylase. It is also possible that thioredoxin h enhances the synthesis of GA in the embryo (Wong, et al. 2002). Thioredoxin gene was found to be targeting diverse group of proteins involved in various functions such as carbon metabolism, cell wall synthesis, protein biogenesis and degradation, signal transduction (Figure 2.11) in germinating seeds of *Medicago*

trancatula seeds (Alkhalfioui, et al. 2007) and in wheat seeds (Wong, et al. 2004). The down-regulation of the thioredoxin h can assist the seeds to maintain their dormancy during adverse conditions. Down-regulation of thioredoxin h9 gene in barley delayed the expression of the target proteins which led to the suppression of pre-harvest sprouting (Li, et al. 2009). The antisense thioredoxin h gene when incorporated in the transgenic wheat, weakened the metabolism of wheat seeds by decreasing the amount of proteins which were involved in the metabolism process. This leads to the inhibition of the germination process, eventually protecting the seed from pre- harvest sprouting (Guo, et al. 2007).

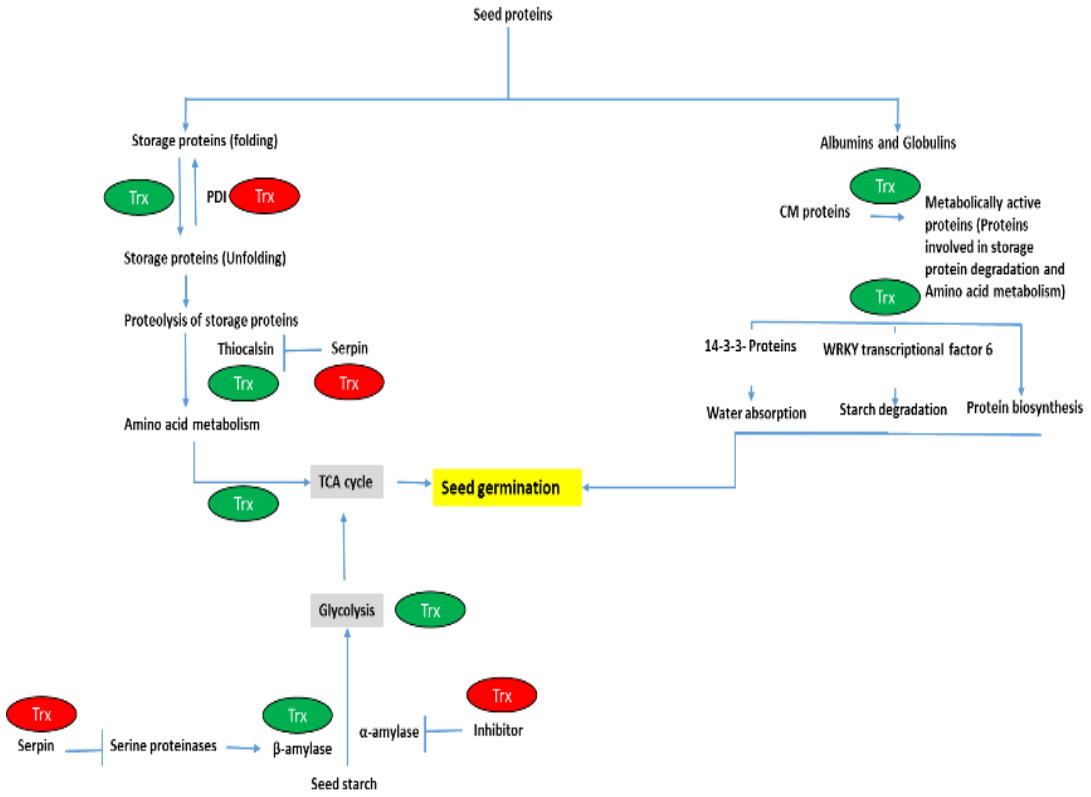

Figure 2.11. Hypothetical model showing the role of Thioredoxin in breaking the dormancy and germination of seeds. Green represents process upregulated by Trx and Red represent process downregulated by Trx (Adapted from (Guo, et al. 2013)).

Glutathione S-transferases (GSTs) are induced by several biotic and abiotic factors including heat shock, salt stress and hormonal treatments such as ethylene, auxin and abscisic acid (Gong, et al. 2005). GSTs have diverse functions including oxidative stress resistance, detoxification (Edwards and Dixon 2005). Two isoforms of GSTs were identified in our study, which showed less accumulation at 12 hrs time period in PHS-susceptible cultivar and one isoform showed more accumulation in 24 hrs time period in the susceptible cultivar. The role of GSTs in breaking the bud dormancy has been described in various woody plants including oak (Ueno, et al. 2013) and Japanese chestnut (Nomura, et al. 2007). The higher activity of GST in the dormant seed as compared to the germinating seeds was reported in *Trifolium alexandrinum* (Ragaa Reda Hamed 2015). A Higher level of glutathione transferase activity seems to be responsible for the initiation of PHS in rye (Masojć, et al. 2013) and same can also be true for wheat.

2.4.6. Storage proteins

Germination and the growth of seedling need a large amount of nutrition and energy, which is provided by the components stored by seed itself (Yang, et al. 2007). Storage proteins are the classes of enzymes do not have enzymatic function and mainly store amino acids for the growing seedling (Murray 1979). Wheat storage proteins are a rich source of carbon, nitrogen, and sulfur which are required for the growth of wheat embryo (Shafqat 2013). During the period of germination, these proteins are degraded by proteases and they are further hydrolyzed to amino acids. These amino acids are mobilized to the embryonic axis where it supports the growth and provide the energy (Ramakrishna 2007). Nine storage proteins were identified in this study and all of these storage proteins showed down-regulation in PHS- susceptible cultivar at 12 hrs and 24

hrs time period. Down-regulation of storage proteins and up-regulation of protein catabolism related proteins was observed during the germination of rice seeds (He and Yang 2013). In our study we identified the proteases like carboxypeptidase, Aspartic proteinase were up-regulated in the PHS-susceptible cultivar. Oil storage protein Oleosin, 15kDa softness protein, embryo globulin were found to be down-regulated in the PHS-susceptible cultivar. The proteolysis of 3 isoforms of globulin protein along with embryo globulin was identified in our study. Generally, globulin proteins accumulate during seed maturation before dormancy but it is consumed during the germination of seed. Up-regulation of the globulin protein is controlled by ABA in maize before dormancy (Ueno, et al. 2013). The up-regulation of Globulin protein in PHS-resistant wheat line suggests that the activity of ABA is higher resulting in the dormancy while down-regulation of globulin in PHS-susceptible suggests that the amount of ABA is lower resulting in the release of dormancy.

Oleosins are lipid-associated proteins and present in seed and pollen as oil storage vesicles (Crowe, et al. 2000). They functions as docking sites for lipases during mobilization of triglyceride stores upon germination(Bowman, et al. 1988). Oleosin gene expression is regulated by ABA with the help of ABA insensitive3 (ABI3) (Crowe, et al. 2000) and it was observed that ABA up-regulated the transcripts of oleosin in embryo and endosperm of *Arabidopsis thaliana* (Penfield, et al. 2006). Low accumulation of this protein in PHS-susceptible cultivar indicates the lower activity of ABA and release of dormancy in wheat kernels.

2.4.7. Cytoskeleton and cell cycle regulation

Five proteins involved in cytoskeleton structure construction and cell cycle were found to be up-regulated in both cultivars but the accumulation was higher in Baegjoong and all of them showed more accumulation in 24 hrs time period. Profilin was observed in higher amount in the germinating embryo of tomato seeds and found to be increased during the germination process. In Arabidopsis, profilins are known to play a role in cell elongation and cell shape maintenance (Ramachandran, et al. 2000). β-tubulin was observed to be up-regulated in PHS-susceptible line and remained steadily accumulated in the embryo during the process of germination. The expression of β-tubulin is required for the passage through the cell cycle. The amount of β-tubulin was observed to be increased in germinating embryo of tomato seeds within 48 hrs of imbibition and this suggested that beta tubulin is probably related to the progression of cell cycle towards mitosis, which might occur during the visible germination of the seedlings (de Castro, et al. 1995). β-tubulin accumulation leads to cell expansion, division and growth of the radicle through seed coat in *Acer plantanoids* seeds (Pawłowski, et al. 2004) and in axillary buds of Norway maple during dormancy breaking (Bergervoet, et al. 1999). So the accumulation of β-tubulin can be an indicator of dormancy release process in seeds. The construction of microtubule may help in the formation of plant structures during the rapid growth of the seedling (Kim, et al. 2009). Up-regulation and accumulation of the cytoskeleton-related proteins in PHS-susceptible cultivar suggests that cell division and growth was activated after imbibition.

2.4.8. Nucleic acid binding proteins

Nucleic acid binding protein play role in the various molecular process such as replication of DNA, transcription and post-transcription regulation of RNAs in living organisms. We found that more than 11 proteins were identified in both the cultivars and showed a different regulation pattern. Glycine-rich RNA-binding proteins are involved in post-transcriptional regulation of gene expression in plants under stress conditions and they were found to be down-regulated by application of GA and up-regulated by ABA in Norway maple leaves tree seeds (Pawłowski 2009). In beech (*Fagus sylvatica*) seeds, the expression of these ABA-responsive glycine-rich proteins corresponds with the level of seed dormancy (Nicolás, et al. 1997). ABA-responsive cDNA of dormancy-related gene *GRPF1*, which encodes glycine-rich RNA binding protein was found to be associated with the degree of dormancy and decline of these genes caused the release of seed dormancy in beech seeds (Mortensen, et al. 2004). In our study, we identified, that there was up-regulation of these proteins in 12 hrs and 48 hrs of the time period in PHS-resistant cultivar, so this may suggest that these proteins are also involved in maintaining seed dormancy in wheat seeds too. Another protein which is a nascent polypeptide-associated complex (NAC) which acts as a protector of nascent chains from premature interaction with other cellular proteins is known to play a role in transcription regulation and mitochondrial protein import. This protein was found to be down-regulated in all of the time periods of PHS-resistant germplasm. The application of GA causes the down-regulation of this protein in beech seeds (Pawłowski 2007).

2.4.9. Protein biosynthesis, folding and degradation

In total, 45 proteins that are related to protein biosynthesis, initiation, folding, and degradation were identified in this study. Twenty nine proteins were involved in ribosome biosynthesis, 6 in the initiation of translation and 10 proteins were involved in protein assembly and degradation. Almost all of the proteins involved in protein biosynthesis showed up-regulation in at least one time period in PHS-susceptible line and were steadily accumulated. Whenever the dry grains get contact with water, rapid assembly of ribosomes and mRNAs takes place which initiates the synthesis of other protein components (Mory, et al. 1972).

The ubiquitin-proteasome group including 26s proteasome subunits were observed to be accumulated more in 48 hrs time period in PHS-susceptible line. The ubiquitin-proteasome system is known to regulate plant development and cell division by regulating different cellular signals (Moon, et al. 2004). High accumulation of this proteasome may be involved in degradation of proteins involved in proteins which are used during cell division and cell structure construction (Kim, et al. 2009). We can hypothesize that proteasome-mediated protein degradation could be an essential pathway for premature germination of wheat seedlings.

2.5. Conclusions

Our results demonstrated that iTRAQ based quantitative proteome analysis is a powerful technique for investigating proteins involved in pre-harvest sprouting in wheat. Our proteomics study finds 190 differentially expressed proteins during pre-harvest sprouting. Several proteins showed contrasting expression pattern and few showed

similar expression pattern in PHS-resistant and PHS-susceptible germplasm. DEPs involved in cellular metabolism along with protein degradation and ROS production displayed significant up-regulated pattern in PHS-susceptible cultivar while proteins involved in stress response and inactivation of proteases showed up-regulated pattern in PHS-resistant germplasm. One protein thioredoxin has been seen to controlling the whole mechanism of PHS in wheat. Protein-protein interaction analysis also revealed some key protein controlling the PHS mechanism. Our results have provided comprehensive proteome insights into PHS mechanism and increased our understanding of the molecular mechanism involved.

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Appendices

Appendix 1. List of all the proteins identified by iTRAQ as DEPs

S.N.	Protein GI #	Protein name
1	gi|100620	sucrose synthase (EC 2.4.1.13) - barley (fragment)
2	gi|10799810	cold-regulated protein [Hordeum vulgare subsp. vulgare]
3	gi|10799832	ribosomal protein L11-like [Nicotiana tabacum]
4	gi|11124572	triosephosphat-isomerase [Triticum aestivum]
5	gi|112784981	11-beta-hydroxysteroid dehydrogenase-like protein [Triticum aestivum]
6	gi|113595	RecName: Full=Aldose reductase; Short=AR; AltName: Full=Aldehyde reductase
7	gi|115435412	Os01g0226400 [Oryza sativa Japonica Group]
8	gi|115436636	Os01g0375000 [Oryza sativa Japonica Group]
9	gi|115447473	Os02g0634900 [Oryza sativa Japonica Group]
10	gi|115453877	Os03g0577000 [Oryza sativa Japonica Group]
11	gi|115474137	Os07g0683900 [Oryza sativa Japonica Group]
12	gi|115475824	Os08g0308100 [Oryza sativa Japonica Group]
13	gi|115589736	serine hydroxymethyltransferase [Triticum monococcum]
14	gi|116310428	H0305E08.6 [Oryza sativa Indica Group]
15	gi|116788110	unknown [Picea sitchensis]
16	gi|119316	RecName: Full=EMB-1 protein
17	gi|119388709	alcohol dehydrogenase ADH1 [Triticum monococcum subsp. aegilopoides]
18	gi|12247762	1-Cys peroxiredoxin [Triticum durum]
19	gi|125577046	hypothetical protein OsJ_33805 [Oryza sativa Japonica Group]
20	gi|135398	RecName: Full=Tubulin alpha-1 chain; AltName: Full=Alpha-1-tubulin
21	gi|145356472	predicted protein [Ostreococcus lucimarinus CCE9901]
22	gi|147641186	RecName: Full=Defensin Tm-AMP-D1.2
23	gi|147805491	hypothetical protein VITISV_020895 [Vitis vinifera]

24	gi|147809809	hypothetical protein VITISV_006493 [Vitis vinifera]
25	gi|149390973	60S ribosomal protein l3 [Oryza sativa Indica Group]
26	gi|15219078	histone H2A protein 9 [Arabidopsis thaliana]
27	gi|15225353	Succinyl-CoA ligase [GDP-forming] subunit beta [Arabidopsis thaliana]
28	gi|15230764	ATP-citrate lyase B-1 [Arabidopsis thaliana]
29	gi|15233565	40S ribosomal protein S30 [Arabidopsis thaliana]
30	gi|15240075	succinate dehydrogenase [ubiquinone] flavoprotein subunit 1 [Arabidopsis thaliana]
31	gi|15982879	AT4g39730/T19P19_120 [Arabidopsis thaliana]
32	gi|162463575	LOC732740 [Zea mays]
33	gi|162464317	putative RH2 protein [Zea mays]
34	gi|164471780	aspartate aminotransferase [Triticum aestivum]
35	gi|16580747	glyoxalase I [Oryza sativa Japonica Group]
36	gi|167004	embryo globulin [Hordeum vulgare subsp. vulgare]
37	gi|167081	peroxidase BP 1 [Hordeum vulgare]
38	gi|167113	aldose reductase-related protein [Bromus inermis]
39	gi|168035593	predicted protein [Physcomitrella patens subsp. patens]
40	gi|168052699	26S proteasome regulatory complex, ATPase RPT6 [Physcomitrella patens subsp. patens]
41	gi|169777	beta-amylase [Oryza sativa Japonica Group]
42	gi|1709779	RecName: Full=Profilin-1
43	gi|1709798	RecName: Full=26S protease regulatory subunit 6B homolog
44	gi|1710521	RecName: Full=60S ribosomal protein L24
45	gi|1731990	serine carboxypeptidase II, CP-MII [Hordeum vulgare subsp. vulgare]
46	gi|18076790	phosphoglucomutase [Triticum aestivum]
47	gi|18479038	glutathione transferase [Hordeum vulgare subsp. vulgare]
48	gi|186886337	ATPase F1 alpha subunit [Patosia clandestina]
49	gi|190684059	peroxiredoxin [Triticum aestivum]
50	gi|195637330	60 ribosomal protein L14 [Zea mays]

51	gi|195648210	NADH-ubiquinone oxidoreductase 75 kDa subunit [Zea mays]
52	gi|195658145	60S ribosomal protein L23 [Zea mays]
53	gi|20067415	glutathione transferase [Triticum aestivum]
54	gi|20139964	RecName: Full=40S ribosomal protein S7
55	gi|20140865	RecName: Full=Translationally-controlled tumor protein homolog; Short=TCTP; AltName: Full=HTP
56	gi|205830697	RecName: Full=Unknown protein 18
57	gi|2130114	trypsin inhibitor CMx precursor - wheat
58	gi|215398468	globulin 3C [Triticum aestivum]
59	gi|215398472	globulin 3B [Triticum aestivum]
60	gi|218202406	hypothetical protein OsI_31924 [Oryza sativa Indica Group]
61	gi|219363167	guanine nucleotide-binding protein beta subunit-like protein [Zea mays]
62	gi|224029551	unknown [Zea mays]
63	gi|224055335	predicted protein [Populus trichocarpa]
64	gi|224133986	predicted protein [Populus trichocarpa]
65	gi|225216858	26S protease regulatory subunit S10B [Oryza nivara]
66	gi|226502949	60S ribosomal protein L5-1 [Zea mays]
67	gi|226503517	6,7-dimethyl-8-ribityllumazine synthase [Zea mays]
68	gi|226533868	heat shock protein 70 [Triticum aestivum]
69	gi|239923157	lipoxygenase 2 [Triticum aestivum]
70	gi|242072750	hypothetical protein SORBIDRAFT_06g013980 [Sorghum bicolor]
71	gi|242092600	hypothetical protein SORBIDRAFT_10g008820 [Sorghum bicolor]
72	gi|242041881	hypothetical protein SORBIDRAFT_01g043980 [Sorghum bicolor]
73	gi|2454602	Barperm1 [Hordeum vulgare subsp. vulgare]
74	gi|2493650	RecName: Full=RuBisCO large subunit-binding protein subunit beta, chloroplastic; AltName: Full=60 kDa chaperonin subunit beta; AltName: Full=CPN-60 beta
75	gi|2506825	RecName: Full=Linoleate 9S-lipoxygenase 1; AltName: Full=Lipoxygenase 1

76	gi|255538610	deoxyuridine 5'-triphosphate nucleotidohydrolase, putative [Ricinus communis]
77	gi|255544189	pyruvate kinase, putative [Ricinus communis]
78	gi|26017213	cold regulated protein [Triticum aestivum]
79	gi|283777738	26S protease regulatory subunit-like protein [Lolium perenne]
80	gi|284518928	ozone-responsive stress-related protein [Triticum aestivum]
81	gi|291061974	putative pyruvate dehydrogenase E1 component alpha subunit [Triticum aestivum]
82	gi|295311633	ATPase subunit 1 [Citrullus lanatus]
83	gi|295841344	benzoxazinone:UDP-Glc glucosyltransferase [Triticum aestivum]
84	gi|296085461	unnamed protein product [Vitis vinifera]
85	gi|300087069	aldehyde dehydrogenase 7b [Triticum aestivum]
86	gi|307108094	hypothetical protein CHLNCDRAFT_144796 [Chlorella variabilis]
87	gi|310656772	putative oleosin [Triticum aestivum]
88	gi|315113249	Chain A, Localization Of The Large Subunit Ribosomal Proteins Into A 5.5 A Cryo-Em Map Of Triticum Aestivum Translating 80s Ribosome
89	gi|315113285	Chain k, Localization Of The Large Subunit Ribosomal Proteins Into A 5.5 A Cryo-Em Map Of Triticum Aestivum Translating 80s Ribosome
90	gi|315113286	Chain p, Localization Of The Large Subunit Ribosomal Proteins Into A 5.5 A Cryo-Em Map Of Triticum Aestivum Translating 80s Ribosome
91	gi|315113289	Chain t, Localization Of The Large Subunit Ribosomal Proteins Into A 5.5 A Cryo-Em Map Of Triticum Aestivum Translating 80s Ribosome
92	gi|32400861	40S ribosomal protein, partial [Triticum aestivum]
93	gi|32400871	ribosomal Pr 117, partial [Triticum aestivum]
94	gi|326489031	predicted protein [Hordeum vulgare subsp. vulgare]
95	gi|326489278	predicted protein [Hordeum vulgare subsp. vulgare]
96	gi|326489533	predicted protein [Hordeum vulgare subsp. vulgare]
97	gi|326489651	predicted protein [Hordeum vulgare subsp. vulgare]
98	gi|326490684	predicted protein [Hordeum vulgare subsp. vulgare]

99	gi|326492474	predicted protein [Hordeum vulgare subsp. vulgare]
100	gi|326493350	predicted protein [Hordeum vulgare subsp. vulgare]
101	gi|326493416	predicted protein [Hordeum vulgare subsp. vulgare]
102	gi|326493440	predicted protein [Hordeum vulgare subsp. vulgare]
103	gi|326494304	predicted protein [Hordeum vulgare subsp. vulgare]
104	gi|326494618	predicted protein [Hordeum vulgare subsp. vulgare]
105	gi|326495152	predicted protein [Hordeum vulgare subsp. vulgare]
106	gi|326495158	predicted protein [Hordeum vulgare subsp. vulgare]
107	gi|326495978	predicted protein [Hordeum vulgare subsp. vulgare]
108	gi|326496415	predicted protein [Hordeum vulgare subsp. vulgare]
109	gi|326505912	predicted protein [Hordeum vulgare subsp. vulgare]
110	gi|326506996	predicted protein [Hordeum vulgare subsp. vulgare]
111	gi|326508588	predicted protein [Hordeum vulgare subsp. vulgare]
112	gi|326509707	predicted protein [Hordeum vulgare subsp. vulgare]
113	gi|326509813	predicted protein [Hordeum vulgare subsp. vulgare]
114	gi|326509875	predicted protein [Hordeum vulgare subsp. vulgare]
115	gi|326510121	predicted protein [Hordeum vulgare subsp. vulgare]
116	gi|326510613	predicted protein [Hordeum vulgare subsp. vulgare]
117	gi|326513238	predicted protein [Hordeum vulgare subsp. vulgare]
118	gi|326514754	predicted protein [Hordeum vulgare subsp. vulgare]
119	gi|326515336	predicted protein [Hordeum vulgare subsp. vulgare]
120	gi|326517334	predicted protein [Hordeum vulgare subsp. vulgare]
121	gi|326520285	predicted protein [Hordeum vulgare subsp. vulgare]
122	gi|326521510	predicted protein [Hordeum vulgare subsp. vulgare]
123	gi|326523589	predicted protein [Hordeum vulgare subsp. vulgare]
124	gi|326524658	predicted protein [Hordeum vulgare subsp. vulgare]
125	gi|326526545	predicted protein [Hordeum vulgare subsp. vulgare]
126	gi|326526663	predicted protein [Hordeum vulgare subsp. vulgare]
127	gi|326527459	predicted protein [Hordeum vulgare subsp. vulgare]
128	gi|326529599	predicted protein [Hordeum vulgare subsp. vulgare]
129	gi|326531070	predicted protein [Hordeum vulgare subsp. vulgare]

130	gi|326532234	predicted protein [Hordeum vulgare subsp. vulgare]
131	gi|34105768	ribosomal protein L3 [Triticum aestivum]
132	gi|356497538	PREDICTED: U-box domain-containing protein 35-like [Glycine max]
133	gi|356533344	PREDICTED: histone H4-like [Glycine max]
134	gi|356554910	PREDICTED: LOW QUALITY PROTEIN: ubiquitin-60S ribosomal protein L40-like [Glycine max]
135	gi|357112267	PREDICTED: 40S ribosomal protein S21-like [Brachypodium distachyon]
136	gi|357112336	PREDICTED: vicilin-like antimicrobial peptides 2-2-like [Brachypodium distachyon]
137	gi|357112407	PREDICTED: uncharacterized protein LOC100835762 [Brachypodium distachyon]
138	gi|357112720	PREDICTED: asparagine synthetase [glutamine-hydrolyzing]-like [Brachypodium distachyon]
139	gi|357113428	PREDICTED: probable mitochondrial-processing peptidase subunit beta-like [Brachypodium distachyon]
140	gi|357118316	PREDICTED: annexin D1-like [Brachypodium distachyon]
141	gi|357119715	PREDICTED: uncharacterized protein LOC100832830 [Brachypodium distachyon]
142	gi|357121590	PREDICTED: 4-alpha-glucanotransferase DPE2-like [Brachypodium distachyon]
143	gi|357121721	PREDICTED: hydroxysteroid 11-beta-dehydrogenase 1-like protein B-like [Brachypodium distachyon]
144	gi|357119807	PREDICTED: 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase-like [Brachypodium distachyon]
145	gi|357130051	PREDICTED: peroxidase 12-like [Brachypodium distachyon]
146	gi|357133773	PREDICTED: cytochrome b-c1 complex subunit 9-like [Brachypodium distachyon]
147	gi|357138252	PREDICTED: 60S ribosomal protein L35a-3-like isoform 1 [Brachypodium distachyon]
148	gi|357148479	PREDICTED: T-complex protein 1 subunit eta-like [Brachypodium distachyon]

149	gi|357155679	PREDICTED: 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase-like [Brachypodium distachyon]
150	gi|357164666	PREDICTED: 60S ribosomal protein L14-1-like [Brachypodium distachyon]
151	gi|357444565	Bifunctional polymyxin resistance arnA protein [Medicago truncatula]
152	gi|357449963	Heat shock protein [Medicago truncatula]
153	gi|357497049	Cytochrome c1 heme protein [Medicago truncatula]
154	gi|37703720	putative aminotransferase AGD2 [Oryza sativa Japonica Group]
155	gi|384248889	hypothetical protein COCSUDRAFT_37026 [Coccomyxa subellipsoidea C-169]
156	gi|38567717	B1358B12.15 [Oryza sativa Japonica Group]
157	gi|388496264	unknown [Lotus japonicus]
158	gi|388501178	unknown [Lotus japonicus]
159	gi|390979705	globulin-3A [Triticum aestivum]
160	gi|3915037	RecName: Full=Sucrose synthase 2; AltName: Full=Sucrose-UDP glucosyltransferase 2
161	gi|401138	RecName: Full=Sucrose synthase 1; AltName: Full=Sucrose-UDP glucosyltransferase 1
162	gi|4586602	pyruvate kinase [Cicer arietinum]
163	gi|464145	beta-amylase [Hordeum vulgare subsp. vulgare]
164	gi|464630	RecName: Full=60S ribosomal protein L27
165	gi|50725631	putative 40S RIBOSOMAL PROTEIN S13 [Oryza sativa Japonica Group]
166	gi|50897038	methionine synthase [Hordeum vulgare subsp. vulgare]
167	gi|520936	gamma-TIP-like protein [Hordeum vulgare subsp. vulgare]
168	gi|544242	RecName: Full=Endoplasmin homolog; AltName: Full=Glucose-regulated protein 94 homolog; Short=GRP-94 homolog; Flags: Precursor
169	gi|55832255	putative glutamate decarboxylase [Hordeum vulgare]
170	gi|5668671	Beta-tubulin [Zinnia elegans]
171	gi|607198	15kDa grain softness protein, partial [Triticum aestivum]
172	gi|607202	15kDa grain softness protein [Triticum aestivum]

173	gi|6136111	RecName: Full=UTP--glucose-1-phosphate uridylyltransferase; AltName: Full=UDP-glucose pyrophosphorylase; Short=UDPGP; Short=UGPase
174	gi|61657604	dehydrin [Triticum durum]
175	gi|6175480	RecName: Full=Xylose isomerase
176	gi|6850878	ribosomal protein S27 [Arabidopsis thaliana]
177	gi|68655500	methionine synthase 2 enzyme [Hordeum vulgare subsp. vulgare]
178	gi|6911551	heat shock protein 70 [Cucumis sativus]
179	gi|7208784	60S ribosomal protein L6 [Cicer arietinum]
180	gi|728594	glycine rich protein, RNA binding protein [Hordeum vulgare subsp. vulgare]
181	gi|73912433	aspartic proteinase [Triticum aestivum]
182	gi|74048999	eukaryotic translation initiation factor 5A1 [Triticum aestivum]
183	gi|7407154	human tumor protein-like protein [Hordeum vulgare]
184	gi|75279910	RecName: Full=Serpin-Z1B; AltName: Full=TriaeZ1b; AltName: Full=WSZ1b; AltName: Full=WZS2
185	gi|7594641	Early-methionine-labeled polypeptide [Secale cereale]
186	gi|7620561	F1 ATPase alpha subunit [Gnetum ula]
187	gi|77554944	Bifunctional aminoacyl-tRNA synthetase, putative, expressed [Oryza sativa Japonica Group]
188	gi|8918502	glucose-6-phosphate dehydrogenase [Triticum aestivum]
189	gi|8980491	thioredoxin h [Triticum aestivum]
190	gi|242037055	hypothetical protein SORBIDRAFT_01g048270 [Sorghum bicolor]
191	gi|226528260	uncharacterized protein LOC100273170 [Zea mays]
192	gi|242058321	hypothetical protein SORBIDRAFT_03g030950 [Sorghum bicolor]
193	gi|242062930	hypothetical protein SORBIDRAFT_04g031810 [Sorghum bicolor]
194	gi|242090681	hypothetical protein SORBIDRAFT_09g021660 [Sorghum bicolor]
195	gi|2443757	cyclophilin [Arabidopsis thaliana]

196	gi|2511541	DNA-binding protein GBP16 [Oryza sativa Japonica Group]
197	gi|253783729	glyceraldehyde-3-phosphate dehydrogenase [Triticum aestivum]
198	gi|256708473	thioredoxin-dependent peroxidase [Leymus chinensis]
199	gi|27461579	ATPase F1 alpha subunit, partial (mitochondrion) [Ecdeiocolea monostachya]
200	gi|28172907	cytosolic 3-phosphoglycerate kinase [Aegilops tauschii subsp. tauschii]
201	gi|28192421	dehydroascorbate reductase [Triticum aestivum]
202	gi|294462212	unknown [Picea sitchensis]
203	gi|224966968	glyceraldehyde-3-phosphate dehydrogenase [Ipomoea nil]
204	gi|195658029	lipoprotein [Zea mays]
205	gi|20067417	glutathione transferase [Triticum aestivum]
206	gi|20467367	ATPase beta subunit [Ephedra viridis]
207	gi|218184502	hypothetical protein OsI_33537 [Oryza sativa Indica Group]
208	gi|224059642	predicted protein [Populus trichocarpa]
209	gi|224098390	predicted protein [Populus trichocarpa]
210	gi|22607	14-3-3 protein homologue [Hordeum vulgare subsp. vulgare]
211	gi|226316439	fructose-bisphosphate aldolase [Secale cereale]
212	gi|226316441	fructose-bisphosphate aldolase [Triticum aestivum]
213	gi|226495599	40S ribosomal protein SA [Zea mays]
214	gi|226497596	LOC100281932 [Zea mays]
215	gi|226499592	LOC100282460 [Zea mays]
216	gi|115440881	Os01g0834500 [Oryza sativa Japonica Group]
217	gi|115459800	Os04g0551800 [Oryza sativa Japonica Group]
218	gi|115468394	Os06g0538000 [Oryza sativa Japonica Group]
219	gi|118484047	unknown [Populus trichocarpa]
220	gi|118484894	unknown [Populus trichocarpa]
221	gi|122022	RecName: Full=Histone H2B.1
222	gi|122044864	RecName: Full=Histone H2B.2

223	gi|129916	RecName: Full=Phosphoglycerate kinase, cytosolic
224	gi|13925731	cyclophilin A-1 [Triticum aestivum]
225	gi|14717940	ATP synthase beta subunit [Ardisia crenata]
226	gi|148508784	glyceraldehyde-3-phosphate dehydrogenase [Triticum aestivum]
227	gi|162457723	luminal-binding protein 2 precursor [Zea mays]
228	gi|1498388	actin, partial [Zea mays]
229	gi|168016452	predicted protein [Physcomitrella patens subsp. patens]
230	gi|195622050	40S ribosomal protein SA [Zea mays]
231	gi|195635409	histone H4 [Zea mays]
232	gi|300808467	poly(ADP-ribose) polymerase [Hordeum vulgare subsp. vulgare]
233	gi|301666340	translocase of inner membrane 17 [Triticum aestivum]
234	gi|302595830	RecName: Full=Ubiquitin-40S ribosomal protein S27a; Contains: RecName: Full=Ubiquitin; Contains: RecName: Full=40S ribosomal protein S27a; Flags: Precursor
235	gi|315113298	Chain K, Localization Of The Large Subunit Ribosomal Proteins Into A 5.5 A Cryo-Em Map Of Triticum Aestivum Translating 80s Ribosome
236	gi|32401367	cyc07 [Triticum aestivum]
237	gi|32478662	cytosolic glyceraldehyde-3-phosphate dehydrogenase [Triticum aestivum]
238	gi|326487540	predicted protein [Hordeum vulgare subsp. vulgare]
239	gi|326487628	predicted protein [Hordeum vulgare subsp. vulgare]
240	gi|326488061	predicted protein [Hordeum vulgare subsp. vulgare]
241	gi|326488131	predicted protein [Hordeum vulgare subsp. vulgare]
242	gi|326488173	predicted protein [Hordeum vulgare subsp. vulgare]
243	gi|326493636	predicted protein [Hordeum vulgare subsp. vulgare]
244	gi|326493772	predicted protein [Hordeum vulgare subsp. vulgare]
245	gi|326494674	predicted protein [Hordeum vulgare subsp. vulgare]
246	gi|326497111	predicted protein [Hordeum vulgare subsp. vulgare]
247	gi|326497219	predicted protein [Hordeum vulgare subsp. vulgare]
248	gi|326497973	predicted protein [Hordeum vulgare subsp. vulgare]
249	gi|326499075	predicted protein [Hordeum vulgare subsp. vulgare]

250	gi|326501362	predicted protein [Hordeum vulgare subsp. vulgare]
251	gi|326501462	predicted protein [Hordeum vulgare subsp. vulgare]
252	gi|326502266	predicted protein [Hordeum vulgare subsp. vulgare]
253	gi|326510251	predicted protein [Hordeum vulgare subsp. vulgare]
254	gi|326511289	predicted protein [Hordeum vulgare subsp. vulgare]
255	gi|326511321	predicted protein [Hordeum vulgare subsp. vulgare]
256	gi|326513840	predicted protein [Hordeum vulgare subsp. vulgare]
257	gi|326513948	predicted protein [Hordeum vulgare subsp. vulgare]
258	gi|326490934	predicted protein [Hordeum vulgare subsp. vulgare]
259	gi|326491885	predicted protein [Hordeum vulgare subsp. vulgare]
260	gi|326491897	predicted protein [Hordeum vulgare subsp. vulgare]
261	gi|326519769	predicted protein [Hordeum vulgare subsp. vulgare]
262	gi|326527541	predicted protein [Hordeum vulgare subsp. vulgare]
263	gi|326529469	predicted protein [Hordeum vulgare subsp. vulgare]
264	gi|326531902	predicted protein [Hordeum vulgare subsp. vulgare]
265	gi|3309243	aconitase-iron regulated protein 1 [Citrus limon]
266	gi|332713695	elongation factor 1-alpha [Deschampsia antarctica]
267	gi|33318663	ATP synthase beta subunit [Dioon purpusii]
268	gi|34538473	caleosin 1 [Hordeum vulgare]
269	gi|34582341	RecName: Full=ATP synthase subunit beta, chloroplastic; AltName: Full=ATP synthase F1 sector subunit beta; AltName: Full=F-ATPase subunit beta
270	gi|357110922	PREDICTED: 60S ribosomal protein L9-like [Brachypodium distachyon]
271	gi|357111367	PREDICTED: 60S ribosomal protein L4-1-like [Brachypodium distachyon]
272	gi|357113738	PREDICTED: 40S ribosomal protein SA-like [Brachypodium distachyon]
273	gi|357121199	PREDICTED: UDP-arabinopyranose mutase 1-like [Brachypodium distachyon]
274	gi|357124822	PREDICTED: 40S ribosomal protein S2-4-like [Brachypodium distachyon]
275	gi|357125156	PREDICTED: 60S ribosomal protein L7-2-like isoform 1 [Brachypodium distachyon]

276	gi|357126908	PREDICTED: 40S ribosomal protein S10-like [Brachypodium distachyon]
277	gi|357134623	PREDICTED: 40S ribosomal protein S4-like [Brachypodium distachyon]
278	gi|357134729	PREDICTED: glucose and ribitol dehydrogenase homolog [Brachypodium distachyon]
279	gi|357140576	PREDICTED: 60S ribosomal protein L6-like [Brachypodium distachyon]
280	gi|357160854	PREDICTED: heat shock cognate 70 kDa protein 2-like [Brachypodium distachyon]
281	gi|363814563	uncharacterized protein LOC100778713 [Glycine max]
282	gi|37780996	putative 40S ribosomal protein S5, partial [Vitis vinifera]
283	gi|380697316	GAPDH, partial [Oncidium hybrid cultivar]
284	gi|399414	RecName: Full=Elongation factor 1-alpha; Short=EF-1-alpha
285	gi|4158232	reversibly glycosylated polypeptide [Triticum aestivum]
286	gi|417745	RecName: Full=Adenosylhomocysteinase; Short=AdoHcyase; AltName: Full=S-adenosyl-L-homocysteine hydrolase
287	gi|449450860	PREDICTED: elongation factor 2-like [Cucumis sativus]
288	gi|51038130	putative embryo-specific protein Ose731 [Oryza sativa Japonica Group]
289	gi|525291	ATP synthase beta subunit [Triticum aestivum]
290	gi|585783	RecName: Full=GTP-binding nuclear protein Ran/TC4
291	gi|6017812	ATP synthase beta subunit [Myrothamnus flabellifolia]
292	gi|6682246	putative 40S ribosomal protein S23 [Arabidopsis thaliana]
293	gi|48716271	putative fibrillarin [Oryza sativa Japonica Group]
294	gi|49328013	putative 60S ribosomal protein L37a [Oryza sativa Japonica Group]
295	gi|49343245	cytosolic malate dehydrogenase [Triticum aestivum]
296	gi|50058579	F1-ATPase alpha subunit [Iseia luxurians]
297	gi|68655466	putative S-adenosylhomocystein hydrolase 2 [Hordeum vulgare subsp. vulgare]
298	gi|7431022	glucose and ribitol dehydrogenase homolog - barley

299	gi|75336483	RecName: Full=ATP synthase subunit beta, chloroplastic; AltName: Full=ATP synthase F1 sector subunit beta; AltName: Full=F-ATPase subunit beta
300	gi|7708452	ATP synthase beta subunit [Irvingia malayana]
301	gi|81176509	atp1 [Triticum aestivum]
302	gi|944842	ATP/ADP carrier protein [Triticum turgidum]
303	gi|94502565	ATPase subunit 1 [Zea mays subsp. mays]
304	gi|108708022	Cupin family protein, expressed [Oryza sativa Japonica Group]
305	gi|89280711	ATP synthase F0 subunit 1 [Oryza sativa Indica Group]
306	gi|110270498	heat shock protein 90 [Triticum aestivum]

Appendix 2. Proteins identified as DEPs (showing relative expression and functional categorization)

GI #	Uniprot ID	Protein Name	Protein abundance ratio									
			B/A	C/A	D/A	F/E	G/E	H/E	E/A	F/B	G/C	H/D
1. Stress induced proteins (17)												
gi|326495158	F2CS54	predicted protein [Hordeum vulgare subsp. vulgare]	1.359	1.362	1.525	0.862	0.918	0.524	1.595	0.986	1.077	0.559
gi|6911551	Q9M4E7	heat shock protein 70 [Cucumis sativus]	0.825	0.635	0.762	1.056	1.04	1.036	0.728	0.908	1.195	1.011
gi|357449963	G7IQ96	Heat shock protein [Medicago truncatula]	0.89	0.603	0.738	0.889	0.999	0.868	0.799	0.778	1.326	0.96
gi|544242	P36183	RecName: Full=Endoplasmin homolog; AltName: Full=Glucose-regulated protein 94 homolog; Short=GRP-94 homolog; Flags: Precursor	0.865	1.905	0.748	1.208	1.123	1.595	0.775	1.055	0.458	1.687
gi|226533868	C3V133	heat shock protein 70 [Triticum aestivum]	1.124	0.88	1.207	1.056	0.824	0.623	1.663	1.522	1.56	0.875
gi|10799810	F2DAA1	cold-regulated protein [Hordeum vulgare subsp. vulgare]	1.298	1.084	0.996	2.442	1.328	1.07	1.034	1.894	1.268	1.133
gi|26017213	Q8H0B8	cold regulated protein [Triticum aestivum]	0.628	1.906	1.263	0.795	0.929	0.725	1.132	1.396	0.552	0.663
gi|284518928	D3K1B4	ozone-responsive stress-related protein [Triticum aestivum]	0.925	0.989	0.811	0.628	1.346	1.036	0.874	0.578	1.192	1.14
gi|326513238	F2EHN7	predicted protein [Hordeum vulgare subsp. vulgare]	0.825	1.274	1.122	0.413	0.209	0.597	1.482	0.724	0.244	0.805
gi|300087069	D9IFB7	aldehyde dehydrogenase 7b [Triticum aestivum]	1.126	0.764	1.805	0.95	1.67	1.994	0.891	0.733	1.951	1.005
gi|147805491	A5B2N0	hypothetical protein VITISV_020895 [Vitis vinifera]	0.964	1.18	1.156	1.06	0.662	0.983	1.022	1.094	0.574	0.887
gi|2454602	O22462	Barperm1 [Hordeum vulgare subsp. vulgare]	2.333	0.594	1.368	0.253	0.734	0.311	1.746	0.184	2.16	0.405
gi|326526545	F2DQB8	predicted protein [Hordeum vulgare subsp. vulgare]	2.29	4.358	1.898	0.606	0.547	0.727	4.241	1.093	0.532	1.656
gi|119316	P17639	RecName: Full=EMB-1 protein	0.914	1.238	1.018	0.381	0.392	0.536	1.212	0.492	0.384	0.651
gi|326532234	F2ECH4	predicted protein [Hordeum vulgare subsp. vulgare]	1.929	1.691	2.001	0.583	0.391	0.399	2.245	0.661	0.52	0.457
gi|7594641	Q9LD94	Early-methionine-labelled polypeptide [Secale cereale]	0.912	1.505	0.906	0.174	0.157	0.548	2.052	0.382	1	1.267
gi|61657604	Q5CAQ2	dehydrin [Triticum durum]	0.748	1.093	0.776	0.483	0.255	0.976	1.255	0.79	0.293	1.61
2. Redox regulation (17)												

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gi|167113	Q39284	aldose reductase-related protein [Bromus inermis]	1.014	0.902	1.049	1.031	0.654	1.05	1.206	1.194	0.87	1.271
gi|113595	P23901	RecName: Full=Aldose reductase; Short=AR; AltName: Full=Aldehyde reductase	0.914	0.805	0.913	0.97	0.645	1.024	1.066	1.102	0.856	1.221
gi|8980491	Q9LDX4	thioredoxin h [Triticum aestivum]	1.198	0.515	0.589	2.838	1.746	1.122	0.543	1.254	1.846	1.056
gi|12247762	Q9AXH7	1-Cys peroxiredoxin [Triticum durum]	1.105	1.044	0.914	0.757	0.809	0.847	0.889	0.593	0.689	0.841
gi|190684059	D0PRB4	peroxiredoxin [Triticum aestivum]	1.105	1.044	0.914	0.757	0.809	0.847	0.889	0.593	0.689	0.841
gi|357130051	I1HE64	PREDICTED: peroxidase 12-like [Brachypodium distachyon]	1.046	1.016	0.874	0.753	0.633	0.809	0.847	0.593	0.528	0.799
gi|326515336	F2E8A9	predicted protein [Hordeum vulgare subsp. vulgare]	1.357	1.21	0.914	0.608	0.54	0.656	1.085	0.473	0.484	0.794
gi|326521510	F2DZ09	predicted protein [Hordeum vulgare subsp. vulgare]	0.822	1.158	1.003	1.095	1.09	0.815	0.694	0.918	0.667	0.587
gi|167081	Q40069	peroxidase BP 1 [Hordeum vulgare]	0.952	0.967	0.896	0.845	0.678	0.877	0.776	0.671	0.546	0.776
gi|242072750	C5YEU0	hypothetical protein SORBIDRAFT_06g013980 [Sorghum bicolor]	2.18	0.788	2.039	0.755	0.372	0.751	2.561	0.864	1.21	0.963
gi|326489533	F2E325	predicted protein [Hordeum vulgare subsp. vulgare]	0.763	1.107	0.905	1.213	0.989	0.974	1.062	1.644	0.949	1.166
gi|16580747	Q948T6	glyoxalase I [Oryza sativa Japonica Group]	0.974	1.573	1.482	1.288	0.871	1.434	1.184	1.524	0.657	1.169
gi|326493416	F2CQP8	predicted protein [Hordeum vulgare subsp. vulgare]	0.512	0.709	0.947	0.991	0.757	0.891	0.822	1.551	0.879	0.789
gi|326520285	F2EJ79	predicted protein [Hordeum vulgare subsp. vulgare]	1.133	3.094	4.01	1.068	0.599	1.709	2.32	2.129	0.45	1.009
gi|326506996	F2DKF4	predicted protein [Hordeum vulgare subsp. vulgare]	2.344	2.105	3.779	1.789	1.076	2.248	2.549	1.896	1.305	1.547
gi|18479038	Q8VWW3	glutathione transferase [Hordeum vulgare subsp. vulgare]	1.449	1.204	1.246	0.718	1.294	0.851	1.267	0.612	1.364	0.882
gi|20067415	Q8RW04	glutathione transferase [Triticum aestivum]	1.275	0.596	0.912	0.786	0.821	0.808	1.107	0.664	1.528	1.001
3. Inhibhitors (5)												
gi|75279910	P93693	RecName: Full=Serpin-Z1B; AltName: Full=TriaeZ1b; AltName: Full=WSZ1b; AltName: Full=WZS2	0.905	2.288	1.834	2.958	4.889	8.345	0.328	1.046	0.703	1.525
gi|2130114		trypsin inhibitor CMx precursor - wheat	1.446	0.824	2.389	1.791	1.806	4.5	0.276	0.333	0.605	0.53
gi|147641186	P84964	RecName: Full=Defensin Tm-AMP-D1.2	1.003	0.682	0.373	0.804	0.632	0.951	0.564	0.441	0.523	1.467
gi|326489278	F2DB00	predicted protein [Hordeum vulgare subsp. vulgare]	1.046	1.006	0.974	1.202	1.831	1.211	0.919	1.029	1.675	1.165

gi|357112336		PREDICTED: vicilin-like antimicrobial peptides 2-2-like [Brachypodium distachyon]	0.768	0.665	0.781	1.231	0.844	1.379	0.837	1.307	1.064	1.509
4. Carbohydrate metabolism (25)												
A. Sugar/Starch Metabolism (10)												
gi|100620		sucrose synthase (EC 2.4.1.13) - barley (fragment)	0.866	1.24	1.116	1.166	1.71	0.999	1.04	1.364	1.435	0.95
gi|357121590	I1GRF8	PREDICTED: 4-alpha-glucanotransferase DPE2-like [Brachypodium distachyon]	1.699	1.628	1.61	0.627	0.38	0.824	2.308	0.83	0.539	1.206
gi|3915037	O24301	RecName: Full=Sucrose synthase 2; AltName: Full=Sucrose-UDP glucosyltransferase 2	1.257	1.195	0.876	0.968	0.738	1.066	1.217	0.913	0.752	1.512
gi|401138	P31922	RecName: Full=Sucrose synthase 1; AltName: Full=Sucrose-UDP glucosyltransferase 1	0.83	1.148	1.053	1.162	1.646	1.032	0.998	1.361	1.433	0.998
gi|6175480	Q40082	RecName: Full=Xylose isomerase	2.08	2.155	1.847	0.964	1.452	0.865	1.511	0.682	1.019	0.722
gi|169777	Q42989	beta-amylase [Oryza sativa Japonica Group]	1.764	1.236	2.032	0.753	1.263	4.216	0.495	0.206	0.506	1.047
gi|464145	P16098	beta-amylase [Hordeum vulgare subsp. vulgare]	1.62	1.879	1.702	1.264	0.62	3.692	0.545	0.414	0.18	1.206
gi|8918502	Q9LRJ1	glucose-6-phosphate dehydrogenase [Triticum aestivum]	0.79	0.06	0.54	1.37	3.04	0.53	0.51	0.86	25.05	0.52
gi|295841344	D5MTD9	benzoxazinone:UDP-Glc glucosyltransferase [Triticum aestivum]	0.998	0.481	0.718	0.552	1.154	0.309	1.413	0.762	3.396	0.62
gi|6136111	Q43772	RecName: Full=UTP--glucose-1-phosphate uridylyltransferase; AltName: Full=UDP-glucose pyrophosphorylase; Short=UDPGP; Short=UGPase	3.893	5.089	5.72	0.311	1.561	0.87	7.844	0.61	2.41	1.217
B. Glycolysis (7)												
gi|11124572	Q9FS79	triosephosphat-isomerase [Triticum aestivum]	0.892	0.806	0.884	0.617	1.431	1.009	0.804	0.542	1.429	0.936
gi|18076790	Q8VX48	phosphoglucomutase [Triticum aestivum]	1.451	1.618	0.715	0.938	0.863	0.485	2.432	1.532	1.299	1.682
gi|242092600	C5Z7K8	hypothetical protein SORBIDRAFT_10g008820 [Sorghum bicolor]	1.053	0.735	0.431	0.909	0.781	0.743	1.116	0.938	1.188	1.963
gi|291061974	D4P3E7	putative pyruvate dehydrogenase E1 component alpha subunit [Triticum aestivum]	0.883	0.833	1.08	1.864	5.176	1.546	0.617	1.269	3.838	0.902
gi|326495152	F2CS51	predicted protein [Hordeum vulgare subsp. vulgare]	0.702	1.313	0.625	0.948	1.353	1.697	0.908	1.194	0.936	2.515
gi|4586602	Q9SXU6	pyruvate kinase [Cicer arietinum]	1.643	3.349	1.546	0.819	1.173	1.653	1.679	0.815	0.589	1.832
gi|255544189	B9RGK5	pyruvate kinase, putative [Ricinus communis]	0.702	1.313	0.625	0.948	1.353	1.697	0.908	1.194	0.936	2.515

C. Fermentation (1)												
gi|119388709	A9U8F7	alcohol dehydrogenase ADH1 [Triticum monococcum subsp. aegilopoides]	**0.702**	**0.9**	**1.052**	**1.074**	**0.767**	**1.099**	**1.032**	**1.538**	**0.88**	**1.1**
D. TCA Cycle (7)												
gi|145356472	A4SAL7	predicted protein [Ostreococcus lucimarinus CCE9901]	**2.375**	**2.753**	**1.434**	**1.508**	**0.66**	**0.491**	**2.28**	**1.409**	**0.547**	**0.796**
gi|15240075	O82663	succinate dehydrogenase [ubiquinone] flavoprotein subunit 1 [Arabidopsis thaliana]	**2.375**	**2.753**	**1.434**	**1.508**	**0.66**	**0.491**	**2.28**	**1.409**	**0.547**	**0.796**
gi|15225353	O82662	Succinyl-CoA ligase [GDP-forming] subunit beta [Arabidopsis thaliana]	**0.542**	**1.133**	**0.479**	**1.215**	**1.255**	**0.95**	**1.024**	**2.237**	**1.136**	**2.075**
gi|326493350	F2CQL5	predicted protein [Hordeum vulgare subsp. vulgare]	**1.523**	**1.155**	**1.806**	**1.396**	**2.262**	**1.286**	**1.443**	**1.288**	**2.829**	**1.048**
gi|326523589	F2DCZ4	predicted protein [Hordeum vulgare subsp. vulgare]	**0.925**	**0.839**	**0.786**	**0.999**	**1.315**	**0.499**	**0.992**	**1.043**	**1.556**	**0.643**
gi|15230764	Q9C522	ATP-citrate lyase B-1 [Arabidopsis thaliana]	**1.009**	**0.591**	**1.191**	**1.091**	**2.118**	**1.721**	**0.698**	**0.736**	**2.508**	**1.03**
gi|326493440	F2CQR0	predicted protein [Hordeum vulgare subsp. vulgare]	**1.53**	**1.117**	**0.817**	**1.045**	**0.849**	**0.926**	**1.29**	**0.857**	**0.982**	**1.492**
5. Lipid Metabolism (5)												
gi|239923157	C6K7G3	lipoxygenase 2 [Triticum aestivum]	**0.879**	**0.888**	**0.928**	**1.061**	**1.603**	**0.728**	**1.034**	**1.216**	**1.87**	**0.829**
gi|2506825	P29114	RecName: Full=Linoleate 9S-lipoxygenase 1; AltName: Full=Lipoxygenase 1	**0.746**	**0.862**	**0.919**	**0.967**	**1.287**	**1.222**	**1.478**	**1.866**	**2.208**	**2.005**
gi|326509875	F2CWD2	predicted protein [Hordeum vulgare subsp. vulgare]	**0.885**	**0.966**	**1.117**	**0.658**	**0.667**	**0.513**	**1.73**	**1.254**	**1.196**	**0.81**
gi|357119715	I1GPU2	PREDICTED: uncharacterized protein LOC100832830 [Brachypodium distachyon]	**1.057**	**1.449**	**1.173**	**1.192**	**0.923**	**1.002**	**1.555**	**1.709**	**0.992**	**1.357**
gi|225216858	Q401N7	aspartic proteinase [Triticum aestivum]	**0.677**	**0.783**	**0.689**	**0.695**	**0.607**	**0.863**	**0.744**	**0.743**	**0.577**	**0.95**
6. Nitrogen Metabolism (13)												
gi|164471780	B0FRH4	aspartate aminotransferase [Triticum aestivum]	**2.21**	**1.198**	**1.688**	**1.009**	**0.768**	**1.18**	**2.169**	**0.965**	**1.392**	**1.547**
gi|357112720	I1H6K4	PREDICTED: asparagine synthetase [glutamine-hydrolyzing]-like [Brachypodium distachyon]	**0.288**	**1.042**	**1.04**	**2.894**	**3.095**	**2.628**	**0.173**	**1.688**	**0.514**	**0.445**
gi|37703720	Q10MQ2	putative aminotransferase AGD2 [Oryza sativa Japonica Group]	**0.316**	**0.773**	**0.994**	**1.318**	**0.609**	**1.046**	**0.512**	**2.079**	**0.403**	**0.549**
gi|55832255	Q5EXM3	putative glutamate decarboxylase [Hordeum vulgare]	**1.387**	**0.769**	**0.716**	**0.624**	**1.328**	**1.642**	**1.029**	**0.451**	**1.778**	**2.409**

gi|115589736	A6XMY5	serine hydroxymethyltransferase [Triticum monococcum]	1.144	1.227	1.24	2.113	1.63	2.307	0.941	1.694	1.252	1.787
gi|125577046	A3CB05	hypothetical protein OsJ_33805 [Oryza sativa Japonica Group]	0.628	0.669	0.343	2.099	1.867	1.516	0.328	1.067	0.917	1.479
gi|357119807	I1GPV3	PREDICTED: 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase-like [Brachypodium distachyon]	0.9	0.862	1.112	1.169	2.027	1.054	0.985	1.221	2.317	0.971
gi|357155679		PREDICTED: 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase-like [Brachypodium distachyon]	0.895	0.864	1.112	1.169	1.99	1.069	0.976	1.202	2.25	0.996
gi|50897038	Q6BCT3	methionine synthase [Hordeum vulgare subsp. vulgare]	0.894	0.88	1.07	1.114	1.967	1.024	1.005	1.22	2.251	0.951
gi|68655500	F2DLU9	methionine synthase 2 enzyme [Hordeum vulgare subsp. vulgare]	0.905	0.89	1.112	1.169	2.02	1.028	1.022	1.241	2.322	0.933
gi|226503517	B6SNG5	6,7-dimethyl-8-ribityllumazine synthase [Zea mays]	0.488	0.687	0.467	0.964	1.189	1.062	0.451	0.866	0.781	1.045
gi|326489031	F2E2C7	predicted protein [Hordeum vulgare subsp. vulgare]	1.24	1.025	0.723	1.075	0.786	1.286	0.565	0.477	0.434	1.026
gi|255538610	B9R7S7	deoxyuridine 5'-triphosphate nucleotidohydrolase, putative [Ricinus communis]	1.059	1.039	0.784	1.091	1.197	0.733	0.132	0.132	0.152	0.126
7. Storage Proteins (9)												
gi|167004	Q03678	embryo globulin [Hordeum vulgare subsp. vulgare]	1.145	1.095	1.121	1.009	0.617	1.085	0.866	0.743	0.488	0.856
gi|215398468	B7U6L3	globulin 3C [Triticum aestivum]	1.142	1.19	1.149	1.02	0.69	1.16	0.92	0.8	0.58	0.95
gi|215398472	B7U6L5	globulin 3B [Triticum aestivum]	1.139	1.195	1.152	1.03	0.635	1.193	1.183	1.042	0.629	1.252
gi|390979705	I6QQ39	globulin-3A [Triticum aestivum]	1.099	1.144	1.133	1.042	0.641	1.172	0.858	0.789	0.478	0.905
gi|242041881	C5WUN6	hypothetical protein SORBIDRAFT_01g043980 [Sorghum bicolor]	1.148	0.673	0.799	1.075	0.917	0.788	0.715	0.652	0.976	0.72
gi|310656772	I3NM41	putative oleosin [Triticum aestivum]	1.167	1.478	2.564	1.421	1.095	1.675	0.903	1.04	0.67	0.594
gi|607198	Q43657	15kDa grain softness protein, partial [Triticum aestivum]	1.966	1.107	1.553	1.109	0.69	1.694	0.7	0.384	0.437	0.779
gi|607202	Q43659	15kDa grain softness protein [Triticum aestivum]	1.966	1.107	1.553	1.109	0.69	1.694	0.7	0.384	0.437	0.779
gi|326529599	F2EBM4	predicted protein [Hordeum vulgare subsp. vulgare]	1.883	1.322	1.604	0.809	0.588	1.565	1.197	0.501	0.533	1.192
8. ATP synthesis (4)												

gi|186886337	B2LHU2	ATPase F1 alpha subunit [Patosia clandestina]	0.971	1.37	1.363	1.123	1.023	0.981	1.415	1.595	1.058	1.039
gi|195648210	B6U2J0	NADH-ubiquinone oxidoreductase 75 kDa subunit [Zea mays]	1.824	1.458	2.153	0.783	0.982	0.229	3.894	1.627	2.626	0.423
gi|295311633	D5I3B4	ATPase subunit 1 [Citrullus lanatus]	0.85	0.99	1.09	1.16	1.02	1.03	1.16	1.54	1.19	1.04
gi|7620561	Q9MM39	F1 ATPase alpha subunit [Gnetum ula]	0.971	1.37	1.363	1.123	1.023	0.981	1.415	1.595	1.058	1.039
9. Transporter proteins (9)												
gi|357133773	I1HJM2	PREDICTED: cytochrome b-c1 complex subunit 9-like [Brachypodium distachyon]	0.901	0.604	0.565	1.591	1.026	1.062	0.793	1.363	1.347	1.521
gi|357497049	G7KN99	Cytochrome c1 heme protein [Medicago truncatula]	0.589	0.972	0.79	0.904	1.005	0.831	1.023	1.529	1.059	1.097
gi|116788110	A9NVU9	unknown [Picea sitchensis]	0.613	0.847	1.588	1.354	2.001	1.861	0.796	1.713	1.885	0.952
gi|296085461	D7TFC0	unnamed protein product [Vitis vinifera]	1.325	0.449	1.152	1.119	0.85	1.118	0.964	0.793	1.829	0.955
gi|326508588	F2DL45	predicted protein [Hordeum vulgare subsp. vulgare]	1.39	0.755	1.156	0.848	1.578	1.486	1.155	0.686	2.417	1.515
gi|520936	D2KZ38	gamma-TIP-like protein [Hordeum vulgare subsp. vulgare]	0.641	0.943	0.694	0.963	2.452	0.993	0.659	0.966	1.717	0.963
gi|326509813	F2CWA1	predicted protein [Hordeum vulgare subsp. vulgare]	1.224	1.269	1.362	1.718	1.244	1.337	1.193	1.631	1.171	1.195
gi|326510121	F2CWQ6	predicted protein [Hordeum vulgare subsp. vulgare]	0.398	0.922	0.438	1.212	2.224	1.275	0.549	1.628	1.325	1.629
gi|388496264	I3S7F6	unknown [Lotus japonicus]	0.589	0.972	0.79	0.904	1.005	0.831	1.023	1.529	1.059	1.097
10. Cytoskeleton (5)												
gi|135398	P14640	RecName: Full=Tubulin alpha-1 chain; AltName: Full=Alpha-1-tubulin	1.597	1.151	1.74	0.879	1.685	0.974	1.336	0.843	1.957	0.763
gi|1709779	P52184	RecName: Full=Profilin-1	1.228	1.468	3.28	1.43	1.473	1.022	1.7	1.929	1.708	0.541
gi|5668671	Q9STC9	Beta-tubulin [Zinnia elegans]	1.855	1.682	1.469	1.006	1.704	0.747	1.831	0.967	1.857	0.95
gi|7407154	Q9M5G3	human tumor protein-like protein [Hordeum vulgare]	1.124	0.934	1.062	0.947	1.297	0.887	1.094	0.897	1.521	0.932
gi|20140865		RecName: Full=Translationally-controlled tumor protein homolog; Short=TCTP; AltName: Full=HTP	1.124	0.934	1.062	0.947	1.297	0.887	1.094	0.897	1.521	0.932
11. Nucleic acid binding (12)												
gi|162464317	Q2MJJ9	putative RH2 protein [Zea mays]	1.05	1.78	0.97	1.07	1.28	1.43	1.21	1.20	0.87	0.98
gi|326505912	F2D7X4	predicted protein [Hordeum vulgare subsp. vulgare]	0.97	1.61	1.25	1.18	0.65	0.91	1.44	1.71	0.58	0.83

gi|356533344	I1L934	PREDICTED: histone H4-like [Glycine max]	1.75	1.18	1.49	1.21	1.31	1.12	1.08	0.72	1.20	1.81
gi|168035593	A9SV98	predicted protein [Physcomitrella patens subsp. patens]	---	---	---	0.22	1.27	---	---	0.21	1.51	0.92
gi|326510613	F2CXF2	predicted protein [Hordeum vulgare subsp. vulgare]	1.15	0.72	0.89	0.93	0.62	1.29	0.62	0.49	0.54	0.68
gi|326517334	F2DY62	predicted protein [Hordeum vulgare subsp. vulgare]	0.32	0.49	0.52	1.76	1.24	1.12	0.31	1.67	0.79	1.43
gi|728594	Q40052	glycine rich protein, RNA binding protein [Hordeum vulgare subsp. vulgare]	1.50	1.30	1.52	0.97	1.80	1.49	1.44	0.91	2.00	1.00
gi|15219078	Q9C944	histone H2A protein 9 [Arabidopsis thaliana]	0.693	1.414	1.997	0.583	0.693	1.233	1.597	1.309	0.783	1.006
gi|115435412	Q5NAF6	Os01g0226400 [Oryza sativa Japonica Group]	0.636	0.769	0.844	0.707	0.541	0.814	0.899	0.973	0.633	0.884
gi|307108094	E1ZD10	hypothetical protein CHLNCDRAFT_144796 [Chlorella variabilis]	0.6	1.01	1.438	1.006	0.635	1.17	1.373	2.244	0.865	1.14
gi|115447473	B4FKM1	guanine nucleotide-binding protein beta subunit-like protein [Zea mays]	0.79	0.79	0.87	1.01	1.60	1.11	0.76	0.95	1.54	0.63
gi|326495978	F2D690	predicted protein [Hordeum vulgare subsp. vulgare]	0.487	0.412	0.481	1.688	1.73	1.394	0.439	1.481	1.846	1.298
12. Protein Biosynthesis/Degradation (45)												
A. Ribosomal protein (29)												
gi|77554944	Q2QS12	Bifunctional aminoacyl-tRNA synthetase, putative, expressed [Oryza sativa Japonica Group]	1.063	1.357	0.9	1.282	3.098	2.87	0.549	0.645	1.254	1.787
gi|10799832	Q9FSF6	ribosomal protein L11-like [Nicotiana tabacum]	1.052	0.904	0.852	1.296	1.359	0.903	0.632	0.758	0.951	0.684
gi|115453877	Q75G91	Os03g0577000 [Oryza sativa Japonica Group]	1.308	0.743	1.097	0.538	1.121	1.223	1.056	0.423	1.595	1.202
gi|149390973	A6MZT4	60S ribosomal protein l3 [Oryza sativa Indica Group]	0.695	1.245	0.523	1.493	1.124	2.32	0.512	1.072	0.463	2.317
gi|195637330	B6TM00	60 ribosomal protein L14 [Zea mays]	1.366	2.935	2.904	0.836	0.71	1.045	2.007	1.197	0.486	0.737
gi|195658145	B6UGQ7	60S ribosomal protein L23 [Zea mays]	0.908	0.746	0.695	1.137	0.955	1.82	0.609	0.743	0.781	1.628
gi|20139964	Q9ZNS1	RecName: Full=40S ribosomal protein S7	1.074	0.908	0.776	1.047	0.77	1.13	1.033	0.981	0.877	1.536
gi|218202406	B8BDC7	hypothetical protein OsI_31924 [Oryza sativa Indica Group]	0.726	1.656	2.221	0.759	0.757	0.817	2.43	2.473	1.112	0.913
gi|32400861	Q7X9K6	40S ribosomal protein, partial [Triticum aestivum]	0.48	0.698	0.795	3.169	0.338	1.019	0.477	3.069	0.232	0.624
gi|32400871	Q7X9K1	ribosomal Pr 117, partial [Triticum aestivum]	0.908	0.746	0.695	1.137	0.955	1.82	0.609	0.743	0.781	1.628
gi|326492474	F2E3U8	predicted protein [Hordeum vulgare subsp. vulgare]	1.158	0.853	1.306	1.017	1.125	0.986	1.323	1.133	1.746	1.02

gi|326496415	F2D0U3	predicted protein [Hordeum vulgare subsp. vulgare]	0.95	0.77	0.897	2.3	2.863	2.031	0.489	1.153	1.821	1.129
gi|326524658	F2EA93	predicted protein [Hordeum vulgare subsp. vulgare]	0.96	1.156	0.893	1.152	1.551	1.659	0.806	0.942	1.082	1.527
gi|326531070	F2EC14	predicted protein [Hordeum vulgare subsp. vulgare]	1.169	2.949	1.884	0.675	0.493	0.858	2.338	1.316	0.392	1.086
gi|34105768	Q6V959	ribosomal protein L3 [Triticum aestivum]	0.689	1.21	0.783	1.191	1.326	1.843	0.68	1.146	0.746	1.633
gi|357112267	I1H5K3	PREDICTED: 40S ribosomal protein S21-like [Brachypodium distachyon]	3.102	2.009	1.921	0.517	0.619	0.651	1.553	0.252	0.479	0.537
gi|357138252	I1IFL2	PREDICTED: 60S ribosomal protein L35a-3-like isoform 1 [Brachypodium distachyon]	1.591	1.437	2.229	0.937	0.651	1.362	1.293	0.742	0.587	0.806
gi|357164666	I1IZM4	PREDICTED: 60S ribosomal protein L14-1-like [Brachypodium distachyon]	1.345	2.722	2.728	0.765	1.142	0.965	2.213	1.227	0.929	0.799
gi|464630	Q05462	RecName: Full=60S ribosomal protein L27	1.369	2.967	2.93	0.848	0.637	1.058	1.975	1.192	0.424	0.728
gi|6850878	Q9M2F1	ribosomal protein S27 [Arabidopsis thaliana]	0.647	0.886	0.896	1.01	1.165	1.321	0.703	1.068	0.926	1.057
gi|7208784	Q9M3Z0	60S ribosomal protein L6 [Cicer arietinum]	0.853	0.822	0.516	0.8	1.019	1.069	0.792	0.723	0.983	1.676
gi|115436636	Q5ZCV4	Os01g0375000 [Oryza sativa Japonica Group]	1.587	1.249	0.615	0.809	1.11	0.999	0.983	0.488	0.874	1.63
gi|1710521	P50888	RecName: Full=60S ribosomal protein L24	0.604	0.843	0.778	0.968	1.099	0.996	0.74	1.154	0.965	0.967
gi|326494304	F2D1K5	predicted protein [Hordeum vulgare subsp. vulgare]	1.175	1.344	1.634	1.502	1.288	2.204	0.585	0.728	0.561	0.805
gi|326509707	F2CW48	predicted protein [Hordeum vulgare subsp. vulgare]	2.215	3.836	5.159	1.001	0.842	0.896	3.823	1.684	0.84	0.677
gi|388501178	I3SEG3	unknown [Lotus japonicus]	0.951	0.545	0.694	0.758	0.798	0.466	0.647	0.502	0.949	0.444
gi|50725631	Q69UI2	putative 40S RIBOSOMAL PROTEIN S13 [Oryza sativa Japonica Group]	0.493	0.57	0.705	0.71	0.838	1.125	0.75	1.051	1.105	1.221
gi|357113428	Q0WWR7	40S ribosomal protein S30 [Arabidopsis thaliana]	1.385	0.97	1.195	1.066	1.166	0.536	1.024	0.768	1.233	0.469
gi|226502949		60S ribosomal protein L5-1 [Zea mays]	1.005	0.645	0.776	1.331	0.75	1.175	0.673	0.868	0.783	1.04
B. Initiation (6)												
gi|115475824	Q32SG2	LOC732740 [Zea mays]	1.047	0.887	0.928	1.721	1.039	0.497	0.795	1.273	0.932	0.434
gi|326490684	A5ALZ4	hypothetical protein VITISV_006493 [Vitis vinifera]	2.72	1.52	2.37	0.67	1.46	1.10	2.06	0.49	1.99	---
gi|15233565	Q3S4I1	eukaryotic translation initiation factor 5A1 [Triticum aestivum]	0.953	0.997	1.005	0.662	0.657	0.506	1.147	0.777	0.756	0.589

gi|384248889	Q6YS69	Os08g0308100 [Oryza sativa Japonica Group]	2.053	2.556	2.419	0.439	0.72	0.641	3.656	0.762	1.032	0.988
gi|73912433	F2D4I8	predicted protein [Hordeum vulgare subsp. vulgare]	0.482	0.352	0.699	0.712	1.599	1.106	0.619	0.891	2.814	1
gi|2493650	F2DXB7	predicted protein [Hordeum vulgare subsp. vulgare]	1.218	1.069	0.649	0.766	0.928	0.644	1.099	0.673	0.955	1.114
C. Protein folding and degradation (10)												
gi|168052699	C0J9X5	26S protease regulatory subunit S10B [Oryza nivara]	0.888	0.934	0.83	0.992	1.03	1.137	1.109	1.206	1.224	1.55
gi|1709798	D3G8A3	26S protease regulatory subunit-like protein [Lolium perenne]	1.237	0.875	0.744	2.084	1.406	2.104	0.531	0.872	0.855	1.533
gi|1731990	I1H862	PREDICTED: probable mitochondrial-processing peptidase subunit beta-like [Brachypodium distachyon]	0.445	0.318	1.095	2.738	2.164	1.348	0.556	3.334	3.792	0.699
gi|283777738	Q43831	RecName: Full=RuBisCO large subunit-binding protein subunit beta, chloroplastic; AltName: Full=60 kDa chaperonin subunit beta; AltName: Full=CPN-60 beta	1.001	1.076	0.731	1.077	1.67	0.549	0.924	0.968	1.436	0.708
gi|219363167	I0YVF3	hypothetical protein COCSUDRAFT_37026 [Coccomyxa subellipsoidea C-169]	0.926	0.962	1.072	1.175	0.529	1.298	0.866	1.072	0.477	1.07
gi|162463575	P54778	RecName: Full=26S protease regulatory subunit 6B homolog	0.843	0.532	0.669	0.806	0.698	1.162	0.715	0.667	0.94	1.268
gi|147809809	F2CT80	serine carboxypeptidase II, CP-MII [Hordeum vulgare subsp. vulgare]	0.619	0.403	0.861	1.402	1.198	1.876	0.514	1.135	1.53	1.143
gi|357148479	I1HXE7	PREDICTED: T-complex protein 1 subunit eta-like [Brachypodium distachyon]	1.126	0.762	0.742	0.843	1.161	1.374	1.191	0.868	1.818	2.25
gi|74048999	A9TJJ6	26S proteasome regulatory complex, ATPase RPT6 [Physcomitrella patens subsp. patens]	1.584	1.797	1.576	0.776	0.87	0.641	2.111	1.008	1.023	0.876
gi|326514754	Q6H852	Os02g0634900 [Oryza sativa Japonica Group]	0.544	0.874	0.875	1.129	1.053	1.237	0.635	1.284	0.767	0.916
13. Unknown (23)												
gi|115474137	Q6Z4N6	Os07g0683900 [Oryza sativa Japonica Group]	0.838	0.5	0.738	1.114	0.714	0.94	0.878	1.137	1.253	1.14
gi|116310428	Q01IK5	H0305E08.6 [Oryza sativa Indica Group]	1.721	0.647	1.836	0.945	1.474	0.429	1.57	0.839	3.579	0.374
gi|15982879	Q93ZG8	AT4g39730/T19P19_120 [Arabidopsis thaliana]	0.896	1.567	1.379	0.915	0.525	0.891	0.932	0.928	0.313	0.615
gi|224029551	C0PF85	unknown [Zea mays]	0.686	0.981	0.592	1.746	1.495	1.255	0.625	1.55	0.954	1.353
gi|224055335		predicted protein [Populus trichocarpa]	0.734	1.161	1.003	1.311	1.857	1.133	0.681	1.186	1.091	0.785
gi|224133986		predicted protein [Populus trichocarpa]	0.773	1	0.769	1.029	1.141	0.998	1.224	1.587	1.399	1.621

gi|315113249		Chain A, Localization Of The Large Subunit Ribosomal Proteins Into A 5.5 A Cryo-Em Map Of Triticum Aestivum Translating 80s Ribosome	0.413	0.915	0.703	1.377	1.319	1.489	0.652	2.118	0.94	1.41
gi|315113285		Chain k, Localization Of The Large Subunit Ribosomal Proteins Into A 5.5 A Cryo-Em Map Of Triticum Aestivum Translating 80s Ribosome	1.466	2.486	2.697	0.954	0.745	1.057	2.173	1.378	0.652	0.869
gi|315113286		Chain p, Localization Of The Large Subunit Ribosomal Proteins Into A 5.5 A Cryo-Em Map Of Triticum Aestivum Translating 80s Ribosome	0.758	0.833	0.652	2.603	3.663	1.02	0.559	1.87	2.46	0.893
gi|315113289		Chain t, Localization Of The Large Subunit Ribosomal Proteins Into A 5.5 A Cryo-Em Map Of Triticum Aestivum Translating 80s Ribosome	1.181	1.151	1.152	1.117	1.662	1.14	0.808	0.744	1.169	0.816
gi|326494618	F2DH57	predicted protein [Hordeum vulgare subsp. vulgare]	1.475	1.238	1.464	1	1.02	1.009	1.55	1.023	1.279	1.089
gi|326526663	F2E048	predicted protein [Hordeum vulgare subsp. vulgare]	0.819	0.676	1.422	0.644	0.644	0.705	1.147	0.877	1.094	0.58
gi|326527459	F2EKY2	predicted protein [Hordeum vulgare subsp. vulgare]	0.555	1.091	0.716	0.85	0.157	0.936	0.918	1.369	0.132	1.225
gi|356497538		PREDICTED: U-box domain-containing protein 35-like [Glycine max]	0.998	0.657	1.075	1.392	1.008	0.971	0.946	1.286	1.454	0.872
gi|356554910		PREDICTED: LOW QUALITY PROTEIN: ubiquitin-60S ribosomal protein L40-like [Glycine max]	0.836	0.699	0.67	1.067	1.248	1.093	0.864	1.074	1.543	1.436
gi|357112407	I1H5X4	PREDICTED: uncharacterized protein LOC100835762 [Brachypodium distachyon]	0.789	0.636	0.719	1.217	0.784	1.05	0.763	1.146	0.941	1.136
gi|357118316		PREDICTED: annexin D1-like [Brachypodium distachyon]	1.625	2.004	0.859	1.606	1.365	0.686	0.861	0.829	0.587	0.701
gi|357121721		PREDICTED: hydroxysteroid 11-beta-dehydrogenase 1-like protein B-like [Brachypodium distachyon]	1.921	1.639	1.621	1.219	0.547	1.002	1.29	0.797	0.431	0.813
gi|357444565		Bifunctional polymyxin resistance arnA protein [Medicago truncatula]	0.804	0.855	0.389	2.11	2.142	1.658	0.536	1.371	1.344	2.332
gi|38567717	Q6MWE2	B1358B12.15 [Oryza sativa Japonica Group]	1.803	0.9	1.339	1.395	2.336	1.582	1.027	0.774	2.667	1.238
gi|112784981	Q0GJJ2	11-beta-hydroxysteroid dehydrogenase-like protein [Triticum aestivum]	0.778	0.692	0.478	1.734	1.118	1.026	0.677	1.469	1.094	1.482
gi|326489651	F2E384	predicted protein [Hordeum vulgare subsp. vulgare]	1.524	1.017	0.818	1.029	0.745	0.431	1.179	0.775	0.864	0.633
gi|205830697		RecName: Full=Unknown protein 18	0.473	0.541	0.286	1.809	1.325	2.231	0.262	0.977	0.643	2.087

Appendix 3. Arabidopsis homologs of proteins (similarity derived from NCBI BLAST)

S.N.	Name of proteins	Accession	TAIR Symbol	Percentage similarity
1	aldehyde dehydrogenase 7B4 [Arabidopsis thaliana]	NP_175812.1	ALDH7B4	80%
2	Nascent polypeptide-associated complex subunit alpha-like protein 1 [Arabidopsis thaliana]	NP_187845.1	AT3G12390	85%
3	chaperonin subunit, putative [Arabidopsis thaliana]	AAM66101.1	AT3G18190	79%
4	putative phosphatidylethanolamine-binding protein [Arabidopsis thaliana]	NP_195750.1	AT5G01300	57%
5	LEA protein in group 3 [Arabidopsis thaliana]	BAA11017.1	ECP63	44%
6	Em-like protein GEA6 [Arabidopsis thaliana]	NP_181546.1	GEA6	73%
7	peroxidase 12 [Arabidopsis thaliana]	NP_177313.1	AT1G71695	59%
8	thioredoxin H5 [Arabidopsis thaliana]	NP_175128.1	TRX5	59%
9	ATP-citrate lyase B-1 [Arabidopsis thaliana]	NP_187317.1	ACLB-1	100%
10	Succinyl-CoA ligase [GDP-forming] subunit beta [Arabidopsis thaliana]	NP_179632.1	AT2G20420	100%
11	pyruvate kinase [Arabidopsis thaliana]	NP_001078275.1	AT3G52990	79%
12	xylose isomerase [Arabidopsis thaliana]	NP_568861.3	AT5G57655	76%

13	4-alpha-glucanotransferase DPE2 [Arabidopsis thaliana]	NP_181616.3	DPE2	60%
14	glucose-6-phosphate dehydrogenase 6 [Arabidopsis thaliana]	NP_198892.1	G6PD6	76%
15	succinate dehydrogenase [ubiquinone] flavoprotein subunit 1 [Arabidopsis thaliana]	NP_201477.1	SDH1-1	100%
16	hydroxysteroid dehydrogenase 1 [Arabidopsis thaliana]	NP_568742.1	At5g50700	49%
17	hydroxysteroid dehydrogenase 5 [Arabidopsis thaliana]	NP_192740.1	HSD5	55%
18	aspartic proteinase A1 [Arabidopsis thaliana]	NP_172655.1	APA1	70%
19	asparagine synthetase [glutamine-hydrolyzing] [Arabidopsis thaliana]	NP_190318.1	ASN1	73%
20	aspartate aminotransferase 3 [Arabidopsis thaliana]	NP_196713.1	ASP3	83%
21	6,7-dimethyl-8-ribityllumazine synthase [Arabidopsis thaliana]	NP_181933.1	COS1	76%
22	glutamate decarboxylase 1 [Arabidopsis thaliana]	NP_197235.1	GAD	69%
23	serine transhydroxymethyltransferase 1 [Arabidopsis thaliana]	NP_195506.1	SHM1	86%
24	serine hydroxymethyltransferase 4 [Arabidopsis thaliana]	NP_193129.1	SHM4	80%
25	seed storage albumin 1 [Arabidopsis thaliana]	NP_194444.1	SESA1	34%

26	mitochondrial F1-ATPase, gamma subunit [Arabidopsis thaliana]	BAE98585.1	ATP3	76%
27	NADH-ubiquinone oxidoreductase subunit [Arabidopsis thaliana]	NP_568550.1	EMB1467	78%
28	V-type proton ATPase subunit B2 [Arabidopsis thaliana]	NP_001190954.1	VAB2	96%
29	putative ubiquinol-cytochrome c reductase subunit 9 [Arabidopsis thaliana]	NP_190841.1	AT3G52730	76%
30	ubulin alpha-6 chain [Arabidopsis thaliana]	NP_193232.1	TUA6	96%
31	tubulin beta-2/beta-3 chain [Arabidopsis thaliana]	NP_568959.1	TUB2	97%
32	small nuclear ribonucleoprotein, putative [Arabidopsis thaliana]	AAM63846.1	AT1G20580	78%
33	small nuclear ribonucleoprotein G [Arabidopsis thaliana]	NP_187757.1	AT3G11500	89%
34	glycine-rich RNA-binding protein 8 [Arabidopsis thaliana]	NP_849524.1	AT4G39260	74%
35	60S ribosomal protein L35a-3 [Arabidopsis thaliana]	NP_177567.1	AT1G74270	82%
36	40S ribosomal protein S30 [Arabidopsis thaliana]	NP_194668.1	AT2G19750	100%
37	60S ribosomal protein L14-1 [Arabidopsis thaliana]	NP_179635.1	AT2G20450	84%
38	40S ribosomal protein S25-2 [Arabidopsis thaliana]	NP_179752.1	AT2G21580	83%
39	60S ribosomal protein L40-1 [Arabidopsis thaliana]	NP_565836.1	AT2G36170	96%

40	transducin/WD40 repeat-like superfamily protein [Arabidopsis thaliana]	NP_182152.2	AT2G46290	77%
41	60S ribosomal protein L22-2 [Arabidopsis thaliana]	NP_187207.1	AT3G05560	80%
42	60S ribosomal protein L36-2 [Arabidopsis thaliana]	NP_850697.1	AT3G53740	81%
43	class II aaRS and biotin synthetases superfamily protein [Arabidopsis thaliana]	NP_191771.1	AT3G62120	75%
44	60S ribosomal protein L27-3 [Arabidopsis thaliana]	NP_193236.1	AT4G15000	84%
45	60S ribosomal protein L32-1 [Arabidopsis thaliana]	NP_193544.1	AT4G18100	86%
46	60S ribosomal protein L14-2 [Arabidopsis thaliana]	NP_194439.1	AT4G27090	82%
47	AT4G34670 [Arabidopsis thaliana]	BAH57033.1	AT4G34670	84%
48	40S ribosomal protein S15-3 [Arabidopsis thaliana]	NP_196512.1	AT5G09500	85%
49	At5g27700 [Arabidopsis thaliana]	ABK32166.1	AT5G27700	78%
50	26S proteasome regulatory subunit [Arabidopsis thaliana]	AAL32634.1	EIF2	68%
51	eukaryotic translation initiation factor 5A-2 [Arabidopsis thaliana]	NP_173985.1	FBR12	82%
52	nucleolar GTP-binding protein NSN1 [Arabidopsis thaliana]	NP_187361.1	NSN1	64%
53	cytoplasmic ribosomal protein L18 [Arabidopsis thaliana]	AAA69928.1	RPL18	82%

54	26S proteasome non-ATPase regulatory subunit RPN12A [Arabidopsis thaliana]	NP_176633.1	RPN12a	53%
55	40S ribosomal protein S13-2 [Arabidopsis thaliana]	NP_567151.1	RPS13A	86%
56	regulatory particle triple-A ATPase 3 [Arabidopsis thaliana]	NP_200637.1	RPT3	91%
57	regulatory particle triple-A ATPase 5A [Arabidopsis thaliana]	NP_187204.1	RPT5A	91%
58	serine carboxypeptidase-like 27 [Arabidopsis thaliana]	NP_187456.1	SCPL27	66%
59	prohibitin 2 [Arabidopsis thaliana]	NP_171882.1	PHB2	78%
60	prohibitin 3 [Arabidopsis thaliana]	NP_198893.1	PHB3	72%