**RESEARCH ARTICLE**

**Reza Shokri Gharelo** [1]
**Ali Bandehagh** [1]

# The contribution of proteins with binding activity and specific metabolic pathways in tolerating abiotic stress by canola: An *in silico* study

***Authors' addresses:***
[1] Department of plant breeding and biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

***Correspondence:***
*Reza Shokri Gharelo*
Department of Plant Breeding and Biotechnology,
University of Tabriz, 29 Bahman Blvd., Tabriz 5166616471, East Azarbaijan, Iran.
Tel: + (98 411) 3392031
e-mail: shokrireza67@gmail.com

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**ABSTRACT**

Plants indicate different degrees of sensitivity and of tolerance upon encountering stressful conditions. In molecular level, the plant response is in a complex manner to such conditions. Obviously, the tolerant plants employ the molecular mechanisms that enable them to be tolerance/resistance. In order to investigate molecular mechanisms involved in the response of canola to stress conditions, two cultivars of canola including Sarigol (as a sensitive cultivar) and Hyola308 (as a tolerant cultivar) were *in silico* studied based on the differentially expressed proteins under abiotic stresses. The results indicated that in Hyola308, the overrepresented genes/proteins are mostly involved in response to stress, with the function of binding activity. In the case of Sarigol, the number of the genes/proteins involved in response to stress was low, while the most of its induced genes/proteins had the catalytic and antioxidant activities. Of 34 metabolic pathways, 12 pathways were common between the two cultivars, whereas numbers 17 and 5 were exclusive for Sarigol and Hyola308, respectively. Considering the tolerance of Hyola308, those unique metabolic pathways, including (1) protein processing in endoplasmic reticulum, (2) carbon fixation in photosynthetic organisms, (3) endocytosis, (4) spliceosome, and (5) fructose and mannose metabolism, could be in relation to high tolerance. PPI network illustrated interactions across differentially expressed proteins, in which 8 nodes for Sarigol and 5 nodes for Hyola308 showed high interactions, called "hub" nodes. The genes UGD2, TPI, and AT3G09440 are introduced as potential candidates to be regarded in genetic engineering of canola due to their core central roles in PPI network. In conclusion, Sarigol and Hyola308 represented some similarity and differences at the molecular level in responding to abiotic stresses, in which unique mechanisms represented by Hyola308 might be a key in tolerating abiotic stresses because of its high tolerance to these kinds of stresses.

**Key words:** Biological process, Molecular function, Protein-protein interactions, KEGG

## Introduction

Plants experience different types of stress during their lifetime, especially abiotic stress such as salt and drought. Salt and drought stress adversely affect the growth and development of plants. So plants had to evolve adaptive mechanisms to cope with environmental stresses for surviving and reproducing. Under water shortage or salt stress, plants commonly close their stomata to limit water losses. Keeping water is to maintain all ongoing biological processes in the cell. To carry out such reactions, plants alter the gene/protein express in patterns of themselves (Kaur and Gupta, 2005; Long et al., 1994; Mizoguchi et al., 2000; Osakabe et al., 2014). In the molecular level, plants respond to abiotic stresses by triggering significantly two series of proteins, one group is functional proteins including water channels, transporters, protection factors of macromolecules (LEA proteins, chaperons), proteases and second group is regulatory proteins consist of transcription factors, protein kinases, phosphatases, phospholipid metabolism, and ABA biosynthesis (Danquah et al., 2014; Hasanuzzaman et al., 2013; Rabbani, 2005; Shinozaki et al., 2003). There are many reports that indicate genetic engineering of most of such genes confers plants more tolerance under stresses. Creating improved crop plants with the better tolerance and resistance

under stress conditions have always been one of the main goals of agricultural researchers (Atkinson et al., 2013; Bhatnagar-Mathur et al., 2008; Nuruzzaman et al., 2015; Osakabe et al., 2013; Umezawa et al., 2006; Zhang et al., 2004). Strictly speaking, understanding exact and detailed knowledge of the underlying adaptive mechanisms of plants is essential to attain genetically improved crops in order to secure food production for the growing population of the world. Therefore, analyzing the relationship between different functions, biological pathways, and interaction networks of these genes and proteins is critical to further insight into the molecular mechanisms driven the plant response to stressful environments. Recently, the development of novel computational tools and algorithms has paved the way of predicting protein-protein interaction networks, biological pathways, and the molecular function of proteins (Braun et al., 2013; Conesa and Götz, 2008; M Perez-de-Castro et al., 2012; Mochida and Shinozaki, 2011). In the present study, we investigated biological pathways and protein-protein interaction (PPI) networks employed by canola cultivars under stressful conditions. It is obvious that those plants that represent an efficient performance under stressful conditions, must trigger and organize an appropriate network of genes/proteins. Determination of biological pathways and their interaction networks in tolerant and sensitive cultivars of canola could be helpful in our understanding of molecular mechanisms under unfavorable conditions and introduce candidate genes/proteins to be regarded in the genetic engineering programs of canola.

## Materials and Methods

***Input data***

We used proteins identified as differentially expressed proteins by two-dimensional gel electrophoresis technique under different degrees of salt stress from published literatures (Banaei-Asl et al., 2015; Bandeh-Hagh et al., 2008; Bandehagh et al., 2011; Gharelo Shokri et al., 2016; Motie Noparvar and Bandehagh, 2016), for Hyola 308 as a tolerant and Sarigol as a sensitive cultivar (Bandeh-Hagh et al., 2008; Bandehagh et al., 2011) for comparatively studying the differences of biological pathways, molecular functions, and protein-protein interaction networks between the tolerant and sensitive cultivars (supplementary data 1). All differentially expressed proteins were proteins that their expression changes occurred at a late stage of vegetative growth of two cultivars. All of the proteins were selected from same stage of growth because proteins from two cultivars can comparatively be studied. Proteins are final products of genes and could be used as the best indicator of biological pathways that are mostly activated and overrepresented by an organism in the different conditions of the environment. All of the collected proteins were blasted against TAIR (The *Arabidopsis thaliana* Information Resource) protein database, due to all of this protein identification was performed based on different organisms. The blast was performed using TAIR-BLAST online tools (https://www.arabidopsis.org/Blast/) with a default setting. The locus name of all proteins was used for blast and for all under studying proteins, the homologous proteins achieved. Totally, 158 number of proteins were studied that the number of 100 proteins was for Sarigol and of 48 proteins was for Hyola308. Obtained homologous proteins in *Arabidopsis* were used as entries to predict biological pathways, molecular functions, and protein-protein interaction networks for each of two cultivars and then the results were compared between two cultivars.

***Prediction of Biological process***

BiNGO (The Biological Networks Gene Ontology tool ) in Cytoscape, open source software platform, was used to study and visualize biological pathways and molecular functions of the proteins (Shannon et al., 2003). This tool calculates overrepresented Gene Ontology (GO) terms in the network and displays them as a network of significant GO terms (Maere et al., 2005). The setting of BiNGO was set with following parameters: Hypergeometric test selected for the statistical test, 0.01 selected for a significance threshold, and *Arabidopsis thaliana* selected for organism/annotation.

***Prediction of Protein-protein interaction network***

STRIN 10.0 (http://string-db.org/) is an open source online bioinformatics tool that used for predicting and studying protein-protein interaction network (PPI). The data setting was set as follows; minimum required interaction score: highest confidence (0.900), organism: *Arabidopsis thaliana* and disconnected node were hidden from the network. The most interacted proteins (hub) were determined for both Sarigol and Hyola 308. In addition, the gene counts in KEGG pathways determined and compared between Sarigol and Hyola 308 to specify unique and common pathways.

## Results and Discussion

***Molecular functions overrepresented under stressful conditions***

Comparing molecular functions significantly overrepresented in both cultivars, indicted that Hyola308 and Sarigol respond approximately with different patterns to stressful conditions (Figure 1). Proteins with catalytic activity presented in both Sarigol (86.6%) and Hyola308 (56.4%). Proteins with oxidoreductase and antioxidant activity were overrepresented by Sarigol, which may be an indicator of high production of reactive oxygen species in Sarigol. Production of reactive oxygen species are mostly observed events in the plants under abiotic stress and in the absence of efficient defensive mechanisms in the cell, it could lead

severely to oxidative damages and eventually cell death (Das

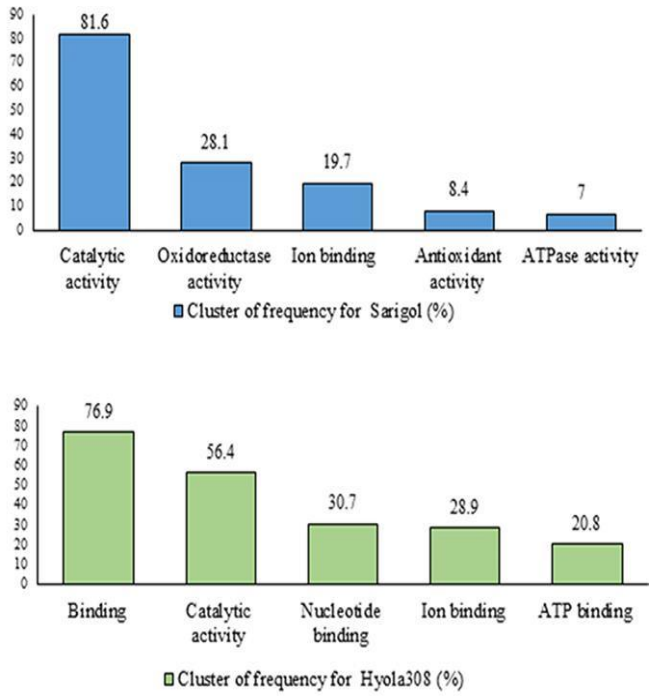


**Figure 1**. *The cluster frequency of molecular functions overrepresented by Sarigol and Hyols308 under stressful conditions. BiNGO was used with following setting: Hypergeometric test selected for statistical test, p-value of 0.01 selected for a significance threshold, and* Arabidopsis thaliana *selected for organism/annotation*

and Roychoudhury, 2014; Oukarroum et al., 2015; Sharma et al., 2012).

In contrast, proteins with binding activity (76.9%) were overrepresented in Hyola308. Considering the sensitivity of Sarigol and the tolerance of Hyola308, it could be concluded that proteins with binding activity might play a more important role in stressful conditions in canola. The major of proteins with binding activity take part in regulating the transcription and act as transcription factors. The role of these types of proteins have been characterized in regulation of the gene expression in response to external stimulus such as abiotic stress (Chen et al., 2012; Lata and Prasad, 2011; Lorković, 2009; Mizoguchi et al., 2000; Mizoi et al., 2012; Puranik et al., 2012; Shinozaki et al., 2003). The results indicated that Hyola308 employs the proteins with a binding activity which could be in association with regulation of the gene expression network. The gene expression regulation allows the cell to trigger expression of specific genes and finally specific proteins expression, that overall leads to a specific response to a particular external stress. Since Hyola308 is a tolerant crop, it seems that the major binding activity among the overreprresented molecular functions of Hyola 308 might be responsible for its tolerance.

*Biological processes overrepresented under stressful conditions*

Among overrepresented biological processes, those biological processes with significant overrepresentation and important roles under stress conditions were selected and compared between Sarigol and Hyola308 (Figure 2 and supplementary data 2). Cellular metabolic process and metabolic process were high in Sarigol than Hyola308, whereas the biological processes involved in responding to stresses were high in Hyola308.

The metabolic pathways are divided into two series of reactions, anabolic and catabolic pathways, that are complementary to each other (Reece et al., 2011). As

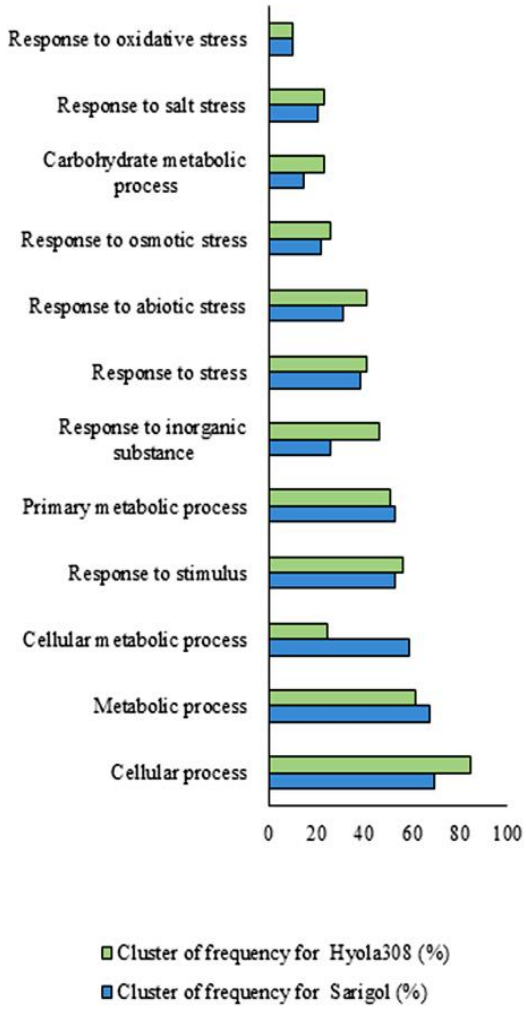


**Figure 2.** *The clusters frequency of biological processes overrepresented by two studied cultivars under stressful conditions. BiNGO was used with following setting: Hypergeometric test selected for statistical test, p-value of 0.01 selected for a significance threshold, and* Arabidopsis thaliana *selected for organism/annotation*

illustrated in Figure 1, catalytic activates are high for Sarigol under stress conditions. These reactions are an exergonic system that produce ATP and other forms of energy for the cell, however, in the absence of metabolites used for energy productions, the cell has to use own primary compounds (Araújo et al., 2011; Berg and Tymoczko). The cellular respiration, one of the catabolic reactions, is mostly occurred reactions under limited nutritional conditions same as what observed under stressful conditions. The respiration could be central to plants for surviving under stress (Clifton et al., 2005; Millar et al., 2011). However, respiration is an energy consuming reaction which in long term leads to depletion of ATPs. In the absence of ATPs and nutriation, the cell has to consume own main components, which this are manifested as the morphological decreased characteristics and the low performance (Araújo et al., 2011; Farooq et al., 2009; Tiwari et al., 2002).

On the other hand, Hayola308 overrepresented genes/proteins involved in response to stress. The stress-responsive genes/proteins are a class of genes or proteins which are elicited in response to a particular stress or in response to several stresses. The purpose of plants is definitely to protect themselves against harmful consequences of different stresses (Osakabe et al., 2014). Therefore, it could be concluded that a high number of stress-responding genes/proteins is another property that offers more tolerance to Hyola308.

*Metabolic pathways under stressful conditions*

Differentially expressed proteins in both cultivars were used to determine which of metabolic pathways are affected under stressful conditions. Of 34 determined metabolic pathways, 12 pathways were commonly observed in both Sarigol and Hyola308. 17 metabolic pathways uniquely observed in Sarigol and 5 metabolic pathways were unique in Hyola308 (Figure 3 and supplementary data 3). The results indicated that Sarigol is affected more than Hyola308 due to a number of proteins that their expression is changed in each pathway was high in Sarigol than Hyola308. Hyola308 is able to tolerate abiotic stress, so it could be concluded that the unique pathways observed in Hyola308 might have an association with the more tolerance to stress. Among the 5 pathways, a number of proteins involved in processing of proteins in the endoplasmic reticulum (9 of observed gene counts) was more than remaining pathways. The synthesis of proteins and their correct folding are couple with the processes taking place in the endoplasmic reticulum (Harding et al., 1999). Obviously, correct and efficient folding of proteins under stress conditions is crucial for their appropriate functions. Therefore, activation and efficacy of these pathways in Hyola308 could be key in tolerance and resistance against stressful conditions. It appears that Hyola308 takes strategies to protect proteins from degradation. The roles of the 5 uniquely observed pathways as to more tolerance/resistance, in other plants have been studied under different conditions of stress. Improving the carbon fixation ability of plants have been considered as a most promising strategy to enhance the ability of plants against unfavorable conditions. All reported results demonstrated the positive influence of it on the plant growth and development under different stressful conditions (Anjum et al., 2011; Ducat and Silver, 2012; Silva et al., 2010). The importance of endocytosis under a challenging condition in the plant cells have been studied and their results totally revealed that importing some substances such as biotinylated bovine serum albumin are induced under various stresses

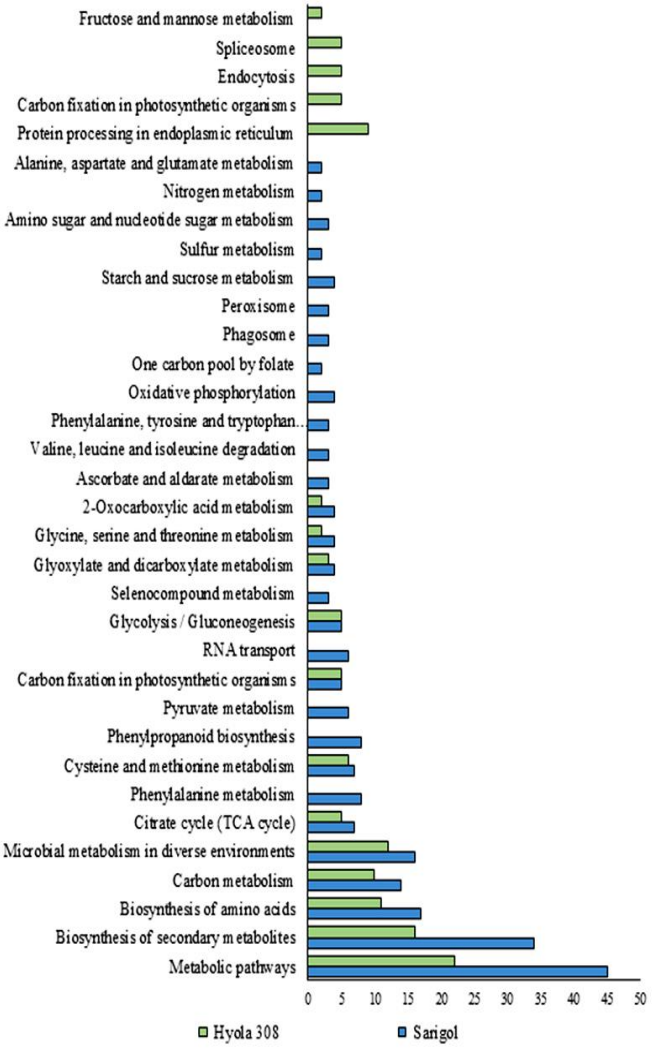


**Figure 3.** *The metabolic pathways uniquely/commonly observed in Sarigol and Hyola308 under stress conditions. Retrieved differentially expressed proteins under conditions of stress were blasted against TAIR database and used as the input in STRING10. Those metabolic pathways that proteins of interest involved were obtained from KEGG database for both cultivars separately and then compared with each other based on the number of genes involved in each pathway. Metabolic pathways with one bar indicate unique pathway for Sarigol (green bar) and Hyola308 (blue bar).*

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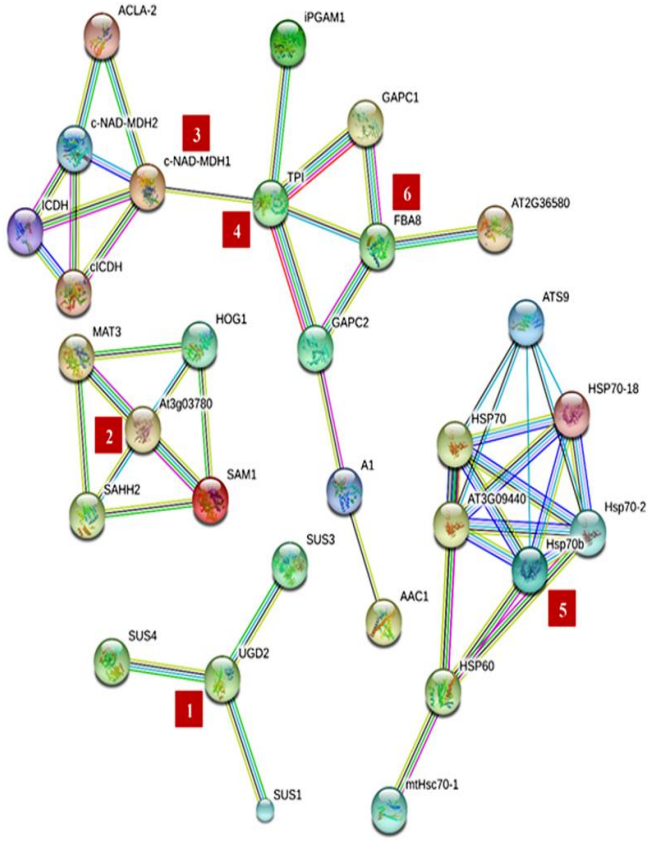


**Figure 4**. *Protein-protein interaction (PPI) network for Hyola308. STRING 10, open source online software was used. The nodes and lines indicate proteins/genes and interactions among them, respectively. Highly connected proteins with central role (hub) are numbered in red square. The numbers are correspondence with the number of hub proteins in Table 1. According to STRING 10 guideline, the color of lines indicates source of interactions as follows:*

*Curated databases, Experimentally determined, Gene neighborhood, Gene fusions, Gene co-occurrence, Textmining, Co-expression, Protein homology*

(Bahaji et al., 2003; Leborgne-Castel and Luu, 2009; Leshem et al., 2007). In *Arabidopsis*, induction of genes involved in spliceosome has been observed under salt stress, meanwhile, the role of these genes in linking the miRNA regulatory mechanisms to environmental stresses have also been revealed (Tanabe et al., 2007; Yan et al., 2012). The influence of stresses on fructose and mannose metabolism have been demonstrated in different plants (Dong et al., 2016; Li et al., 2016; Zong et al., 2013). One of the roles of fructose and mannose is in adjusting the cell osmotic pressure and regulation of reactive oxygen species (Keunen et al., 2013).

In return, Sarigol unique metabolic pathways are mostly those which are involved in the metabolism of amino acids and others compounds or the reactions that occur to defend the cell against reactive oxygen species (Figure 3). It seems that Sarigol breaks down own metabolites such as amino acids to provide energy for surviving under stressful conditions. Consuming own cellular compounds leads to the significant decrease in the dry weight of the cell. This could be observed at morphologic traits of plants as reduction in the dry weight of root, leaf, stem, and whole plant (Bandeh-Hagh et al., 2008; Bandehagh et al., 2011; Cakir, 2004; Gharelo Shokri et al., 2016; Khodary, 2004; Seemann and Critchley, 1985). One of the consequences of falling into stress conditions is the poor nutritional state within the plant cells. Under this condition, the plant cell responds to starvation, especially sugar starvation, through decreasing sugar metabolism, nitrate reduction and assimilation, and protein synthesis to conserve energy. Simultaneously, the plant cell increases their catabolic reactions including catabolism of fatty acids and amino acids. Plant do all of these changes in the regular metabolic pathways to compensate the absence of essential nutrients such as sugars (Dieuaide et al., 1992; Journet et al., 1986; Yu, 1999).

*Prediction of protein-protein interaction network*

Interaction network generated for Sarigol and Hyola308, indicated that different links present among proteins. The different interaction networks were topologically drawn for each cultivar. Five highly interacted regions (cluster) for Hyola and seven clusters for Sarigol were observed, then in each of clusters highly connected node (hub node) were determined (Table1 and Figures 4 and 5). Clusters 1 and 2 in Hyola308 and clusters 1, 3, 4, and 5 in Sarigol had links together. The hub nodes could have critical role in response of canola to stressful conditions than less connected nodes. The nodes At3g03780, c-NAD-MDH2, and FBA8 were observed in the both cultivars, but PAL4, AT1G72730, XW6, and LOS1 only observed in Sarigol (Figure 5) and UGD2, TPI, and AT3G09440 only in Hyola308 (Figure 4).

These proteins are core central proteins of the interacting network because they interact with many other proteins in each cluster. According to the results, key proteins are different in Sarigol and Hyola308, it means that almost different molecular mechanisms present in per cultivar. It is seen in the results that proteins with high connections and probably with a key role in interaction networks belongs to carbohydrate metabolism in the tolerant cultivar Hyola308, whereas it is not observed in Sarigol. This result could represent the importance of proteins involved in carbohydrate metabolism in coping with abiotic stresses. The cell carbohydrate contents vary in accordance with varying environmental factors such as light, water, and temperature. Plants substantially change their physiological and biochemical reactions to sustain metabolic process under

conditions of carbohydrates depletion (Journet et al., 1986; Rosa et al., 2009; Yu, 1999), as mentioned above. Furthermore, the protective and central role of sugars has been studied in plants under different stresses. Sugars protect the palnt cells from oxidative damages and their synthesis are increased in response to osmotic, salt, and drought stress. It has been revealed that increasing the sugar synthesis is one of the mechanisms evolved in tolerant plants to cope with environmental stresses (Djilianov et al., 2011; Keunen et al., 2013; Sperdouli and Moustakas, 2012; Van den Ende and Peshev, 2013). Taking together, in the case of Hyola308 (Table 1 and Figure 4), UDP-glucose dehydrogenase 2,

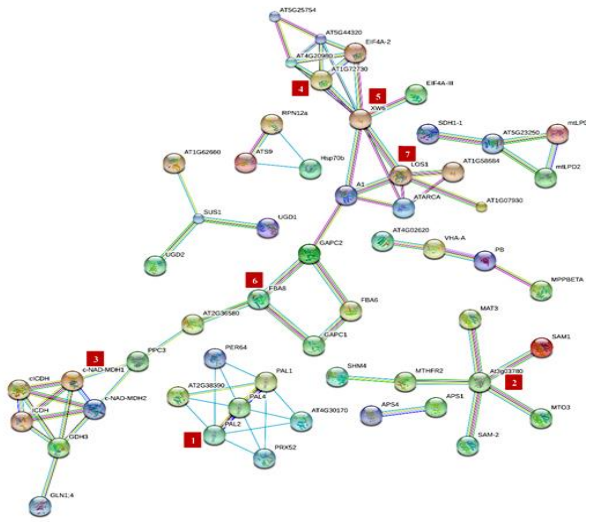

**Figure 5**. *Protein-protein interaction (PPI) network for Sarigol. STRING 10, open source online software was used. The nodes and lines indicate proteins/genes and interactions among them, respectively. Highly connected proteins with central role (hub) are numbered in red square. The numbers are correspondence with the number of hub proteins in Table 1. According to STRING 10 guideline, the color of lines indicates source of interactions as follows:*

*Curated databases, Experimentally determined, Gene neighborhood, Gene fusions, Gene co-occurrence, Textmining, Co-expression, Protein homology.*

Triose phosphate isomerase, and Fructose-bisphosphate aldolase 8 could be considered as the most potential candidate proteins for improving the canola tolerance against abiotic stresses. UDP-glucose dehydrogenase is one of the enzymes in providing a precursor for the cell wall formation and also it is a protein with regulatory role in carbon partitioning between the cell wall formation and synthesis of sucrose (Klinghammer and Tenhaken, 2007). The rigid and flexible cell wall of the plant cell is essential to balance osmotic pressure of inside the cell as well as the cell development (Zablackis et al., 1995). Triose phosphate isomerase is a key enzyme in glycolysis pathway. In *Arabidopsis*, it is required for passing through heterotrophic to autotrophic growth (Chen and Thelen, 2010). Kaur et al (Kaur et al., 2015) reported that increase in the activity level of triose phosphate isomerase under abiotic stress results in the high energy state in plants, and further prevents accumulation of methylglyoxal within the cell which is harmful to the cell under stresses. The importance of Hsp proteins and their positive roles in the plant cells have been well characterized. They carry out crucial roles in re-establishing and maintaining the integrity of proteins under various conditions of stress (Haslbeck and Vierling, 2015; Wang et al., 2004). Perez-Salamo et al (Pérez-Salamó et al., 2014) reported that estradiol-dependent induction of HSFA4A gives tolerance to *Arabidopsis* against oxidative agents and salt stress, while inactivated HSFA4A causes hypersensitivity in the plant. In another study, it was reported that transgenic *Gossypium hirsutum* with GHSP26 from *Gossypium arboreum* origin had an enhanced tolerance to drought stress (Maqbool et al., 2010). These reports further confirm the potential of the mentioned proteins to be considered in genetic engineering of canola.

**Conclusion**

The tolerance of Hyola308 to stressful conditions could attribute to its appropriate triggering of a set of genes/proteins to cope with environmental stresses. In summary, Hyola308 organized and regulated the number of genes/proteins that are mostly involved in the response to stress and function of binding activity, in contrast to Sarigol as a sensitive cultivar in which the number of genes/proteins involved in response to stress was low and the most number of its induced genes/proteins had catalytic and antioxidant activities. Five unique metabolic pathways, including protein processing in the endoplasmic reticulum, carbon fixation in photosynthetic organisms, endocytosis, spliceosome, and fructose and mannose metabolism were observed in Hyola308. The metabolic pathways uniquely observed in Hyola308 could be related to the tolerance of Hyola308. Finally, the genes UGD2, TPI, and AT3G09440 are introduced as potential candidates to be regarded in genetic engineering of canola due to their core central roles in PPI network.

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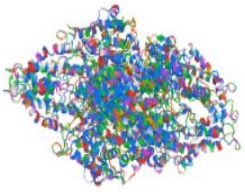
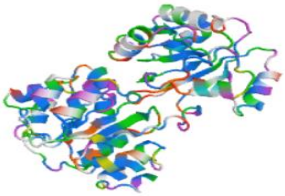
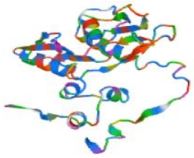
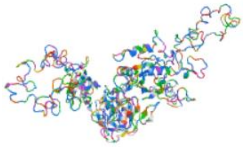
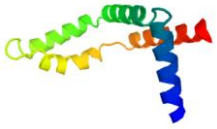
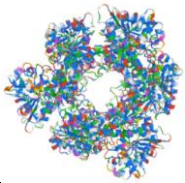
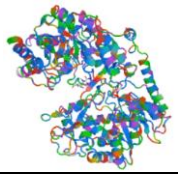
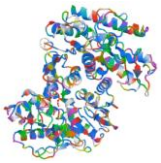
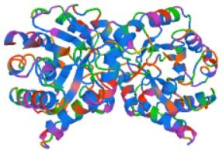
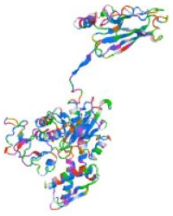
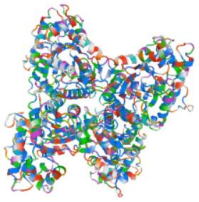
**Table 1.** *Proteins with core central role (highly interacted proteins or hub) for Sarigol and Hyola308. The 3D conformation and pathway of proteins provided from SWISS-MODEL and KEGG, respectively.*

| **Sarigol** | | | | |
|---|---|---|---|---|
| NO | Node | Description | 3D conformation | Pathway |
| 1 | PAL4 | Phenylalanine ammonia-lyase 4 |  | Phenylalanine metabolism<br>Phenylpropanoid biosynthesis<br>Metabolic pathways<br>Biosynthesis of secondary metabolites |
| 2 | At3g03780 | Methionine synthase 2 | See below | See below |
| 3 | c-NAD-MDH2 | Malate dehydrogenase | See below | See below |
| 4 | AT1G72730 | Translation initiation factor 4A-3 |  | RNA transport |
| 5 | XW6 | 40S ribosomal protein S2-1 |  | Ribosome |
| 6 | FBA8 | Fructose-bisphosphate aldolase 8 | See below | See below |
| 7 | LOS1 | Elongation factor EF-2 |  | Unidentified |
| 8 | SUS1 | Transcription and mRNA export factor |  | Starch and sucrose metabolism<br>Metabolic pathways |

| | | | | |
|---|---|---|---|---|
| **Hyola308** | | | | |
| 1 | UGD2 | UDP-glucose dehydrogenase 2 |  | Pentose and glucuronate interconversions<br>Ascorbate and aldarate metabolism<br>Starch and sucrose metabolism<br>Amino sugar and nucleotide sugar metabolism<br>Metabolic pathways |
| 2 | At3g03780 | Methionine synthase 2 |  | Spliceosome<br>Protein processing in endoplasmic reticulum<br>Endocytosis |
| 3 | c-NAD-MDH1 | Malate dehydrogenase |  | Citrate cycle (TCA cycle)<br>Cysteine and methionine metabolism<br>Pyruvate metabolism<br>Glyoxylate and dicarboxylate metabolism<br>Carbon fixation in photosynthetic organisms<br>Metabolic pathways<br>Biosynthesis of secondary metabolites<br>Carbon metabolism |
| 4 | TPI | Triose phosphate isomerase |  | Glycolysis / Gluconeogenesis<br>Fructose and mannose metabolism<br>Inositol phosphate metabolism<br>Carbon fixation in photosynthetic organisms<br>Metabolic pathways<br>Biosynthesis of secondary metabolites<br>Carbon metabolism<br>Biosynthesis of amino acids |
| 5 | AT3G09440 | Protein heat shock protein 70-3 |  | Cysteine and methionine metabolism<br>Selenocompound metabolism<br>Metabolic pathways<br>Biosynthesis of secondary metabolites<br>Biosynthesis of amino acids |
| 6 | FBA8 | Fructose-bisphosphate aldolase 8 |  | Glycolysis / Gluconeogenesis<br>Pentose phosphate pathway<br>Fructose and mannose metabolism<br>Carbon fixation in photosynthetic organisms<br>Metabolic pathways<br>Biosynthesis of secondary metabolites<br>Carbon metabolism<br>Biosynthesis of amino acids |

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