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# Heterosis investigation of sunflower (*Helianthus annuus* L.) by two-dimensional electrophoresis

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**Abstract**

Sunflower breeding program aimed to produce F1 hybrid with high seed and oil yield. In this study, two sunflower inbred lines and their hybrid were used to investigate molecular mechanism of heterosis through proteomic approach. A completely randomized design was conducted to study the 13 morphological characters. Two-dimensional electrophoresis along with CBB staining was applied for hybrid and its parental lines. Comparison of hybrid with parental lines discovered a total of 153 proteins spots, of which 14 spots indicated non-additive changes. Comparing hybrid with parental lines B330 indicated 176 protein spots, of which 17 spots showed significant changes in hybrid. Comparison of two-dimensioned gels between hybrid and R46 line appeared 187 protein spots, of which 11 protein spots showed significant expression changes. Classification of up-regulated proteins in hybrid in metabolism and photosynthesis might be the molecular reason of hybrid vigor and growth compare with the parent. Other changed proteins were grouped in energy, cell detoxification, signal transduction and defense/disease.

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## Introduction

Heterosis or hybrid vigor, describes the superiority performance in terms of vigor, size, yield, speed of development, fertility and resistance to disease and to insect pests, of a $F_1$-hybrid over its homozygous parental inbred line (Shull *et al.*, 1952). Different genetic models have been supposed to explain heterosis, comprising, dominance, over dominance, and epistasis (Birchler *et al.*, 2003). Exhaustive estimation of the loci contributing to hybrid vigor is mostly not available due to the limited resolution of classic genetic analysis in tracking multiple loci (Birchler *et al.*, 2003; Jones, 1917; Yao, *et al.*, 2005; Lippman and Zamir, 2007). One of the assignments in modern genetic analysis is to describe the relationships between molecular genetic markers and their physiological performance. Gene expressions or gene interactions, is the final function of a plant, which indirectly correlate with gene locus. Therefore, the description of dominance or epistasis seems asymmetric upon molecular levels (Yu *et al.*, 1997; Canovas *et al.*, 2004). Several studies in maize, rice, and Arabidopsis analyzed heterosis on the level of the genome, transcriptome and proteome applying a diversification of molecular tools, recently (Hochholdinger and Hoecker, 2007). To appoint correlations between polymorphism of individual protein amounts indications, and hybrid vigor for agronomic traits, two-dimensional gel electrophoresis was recently employed (Song *et al.*, 2007a, 2009b; Hoecker *et al.*, 2008; Markon *et al.*, 2010; Dahal *et al.*, 2012). Recently, in the studies on maize embryo proteome, In total, the number of differentially expressed protein spots between hybrid and its parental were 134 and 191, respectively, among which 47.01% (63/134) and 34.55% (66/191) protein spots displayed non-additively expressed pattern (Gue *et al.*, 2013). Sunflower is one of the three crop species along with soybean and rapeseed which account for approximately 78% of the world vegetable oil. Utilization of heterosis has allowed sunflower to become one of the major oilseed in many countries of Eastern and Western Europe, Russia and South America and is an important crop in the USA, Australia, South Africa, China, India and Turkey (Miller, 1998). In sunflower investigations demonstrated that heterotic hybrids showed higher level of head diameter, days to 50% flowering, days to maturity, plant height leaf area, per plant in F1 generation (Igbal Khan *et al.*, 1993: Ahmad *et al.*, 2005: Sayyar Khan *et al.*, 2003). Previously study on heterosis of sunflower by two-dimensional electrophoresis, is not reported..In this study, we report further research into the differences in proteome profiles of leaves between hybrid and its parents at the end of vegetative stage. The proteomic analysis in this study reveals that the proteins involved in photosynthesis, metabolism, energy, signal transduction, cell structure and disease/defense. The molecular insights provided by this study might help to better understand the possible molecular networks involved in sunflower heterosis.

## Material and method

*Plant materials*

One highly heterotic hybrid B330 / R46 and its inbred parental lines were used for this study which was obtained from the Research Center of Agriculture and Natural Resources of Khoy. Plants were grown in the greenhouse in plastic pots in replicated trial completely randomized design with three replications for two dimensional electrophoresis and four replicate experiments were performed to measure morphological traits. At the end of vegetative stage, leave collected at the time of agronomic traits measurement and were immediately immersed in liquid nitrogen and stored at -80℃ until used for protein extraction.

*Aerial parts and root heterosis measurement*

Aerial and root parts of hybrid and parents harvested and dried in an oven at 70℃ for 74 h for dry weight determination. eight aerial parts and five root traits characterized including plant fresh weight (PFW), plant height (PH), stem diameter (SD), total leaf number (TLN), leaf area (LA), leaf fresh weight (LFW), plant dry weight (PDW), leaf dry weight (LDW), root fresh weight (RFW), root length (RL), root volume (RV) and root dry weight (RDW). The

mid-parent heterosis (MPH) and best-parent heterosis (BPH) were calculated. Statistical analysis of the differences in aerial and root part traits were performed by using F-test.

*Extraction of sunflower leaf proteins and quantitation*

Total protein extracts were isolated from approximately 0/5 g of frozen leaf per biological replicate and suspended the fine powder in cold acetone containing 10% TCA and 0/07% 2-Mercapthoetanol. The resultant powder dissolved in lysis buffer containing 7 M Urea, 2 M thiourea, 2% CHAPS, 60 mM DDT and 1% ampholyte (pH:3-10). Protein concentration was determined by Bradford assay (Bradford, 1976).

*2-DE and image analysis*

IEF of proteins performed with 140 µg of protein extract using 11 cm tube gels and 3 mm diameter (O'Farrell, 1975). IEF gel solution consisted of 8 M urea, 3.5% polyacrylamide, and 2% NP-40, 2% ampholines (pH 3.5–10.0 and pH 5.0–8.0), ammonium persulfate and TEMED. The voltage settings of the IEF were a 200 V gradient for 30 min, a 400 V gradient for 16 h and a 600 V for 1h. Proteins in the tube gels separated on the basis of their IF. The second dimension SDS-PAGE gels, tube gels were subjected to the second dimension electrophoresis after transferring onto a 15% acrylamide separating gel and 5% acrylamide stacking gel. After electrophoresis, we stained proteins with Coomassie Brilliant Blue G-250. The resulting gel images were performed with PDQuest software (BioRad). The pI and Mr of each protein were determined using 2D-PAGE markers (Bio-Rad, Hercules, CA, USA). After normalization, a one-way ANOVA model and Student's t-test were used to identify the differentially expressed protein spots between hybrid and parental lines and hybrid with each parental lines, respectively ($p<0.05$, absolutely intense difference). Protein identification was obtained from MSDB, NCBI and SwissProt protein database.

**Result**

*Analysis of variance*

Analysis of variance indicated that the differences between the inbred parental lines and hybrid for eight morphological traits were statistically significant (Table 1). The mid-parent and best parent heterosis values were estimated for eight significant traits (Table2).

**Table 1.** Mean squares for analysis of variance for different characters in *Helianthus annuus* L.

| Column1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 | Column 7 | Column 8 | Column 9 |
|---|---|---|---|---|---|---|---|---|
| S.V | DF | PH (cm) | SD (mm) | TLN | PFW (mg) | LFW (mg) | LA ($cm^2$) | RL |
| Genotype | 2 | $1500.6^{**}$ | $3.30^{**}$ | $53.08^{**}$ | $1693.4^{Ns}$ | $107.2^{NS}$ | $94.4^{NS}$ | $28.4^{NS}$ |
| Error | 9 | 43.3 | 0.34 | 3.06 | 495.82 | 50.61 | 69.23 | 12.26 |
| C.V (%) | | 8.81 | 8.69 | 9.8 | 38.7 | 46.97 | 89.11 | 24.14 |

| Column1 | Column2 | Column3 | Column4 | Column5 | Column6 | Column7 | Column8 |
|---|---|---|---|---|---|---|---|
| S.V | DF | RFW (mg) | RD (mm) | RV ($cm^2$) | PDW | RDW | LDW |
| Genotype | 2 | $82.21^{**}$ | $1.15^{NS}$ | $62.15^{**}$ | $78.94^{**}$ | $0.58^{**}$ | $5.37^{*}$ |
| Error | 9 | 8.78 | 0.51 | 5.49 | 2.49 | 0.05 | 0.83 |
| C.V (%) | | 27.95 | 11.29 | 24.14 | 17.3 | 24.49 | 32.67 |

*Analysis of proteome*

Proteome analysis, is a clearly method to identifying the genes activity. The connection of proteome analysis with genome sequence data, is an important strategy for functional genomic (Komatsu and Tanaka, 2004). According to Song *et al.* (2007) model, when comparing the patterns of differentially expressed protein spots between hybrid and its parents, both quantitative and qualitative differences could be observed. The quantitative differences can be grouped into four classes: (i) up-regulated in hybrid (URH), expression in hybrid is higher than in both female and male parents; (ii) down-regulated in hybrid (DRH), expression in hybrid is lower than in

two parents; (iii) high-dominant in hybrid (HDH), expression in hybrid is equal to the highly expressed parent; and (iv) low-dominant in hybrid (LDH), expression in hybrid is equal to the lowly expressed parent (figure 1). Among the 14 differentially expressed protein spots, 5, 2, 4 and 3 spots showed URH, DRH, HDH, and LDH expression pattern respectively. Gel analysis between hybrid and each parental line were done separately. 176 protein spots compared to hybrid with superior parental line (B330) and 187 protein spots compared to hybrid with another parental line, R46. T-test was used for the detection of protein spot with significant expression between hybrid and its parental lines separately. 17 protein spots (9.66%) of 176 protein spots which appeared between hybrid and B330 parental line and 11 (5.88%) protein spots of 187 protein spots which appeared between hybrid and R46 parental line, were accumulated non-additive Proteins. Among the 17 significantly protein spots between hybrid and B330 parental line, 12 and 5 protein spots showed up-regulated and down-regulated respectively. Among 11 significantly protein spots between hybrid and R46 parental line, 10 and 1 protein spots exhibited up-regulated and down-regulated respectively (figure 2, 3). Tables 3, 4 and 5 show the detail of modified proteins between hybrid and parental line, hybrid and B330 parental line, and hybrid and R46 parental line. These proteins or isoforms were classified into seven functional classes, including metabolism (13 spots, phosphoglycerate kinase, ketol-acid reductoisomerase (chloroplastic-like), Sucrose synthase, putative malate dehydrogenase, putative cytoplasmic NADP-malic enzyme, cytosolic malate dehydrogenase, Dihydroflavonol reductase, fructose-bisphosphate aldolase, 2-phospho-D-glycerate hydroylase), photosynthesis (3 spots, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit), disease and defense (one spot, bifinctional polymaxin resistance protein ArnA-like), cell detoxification (two spots, glutathione transferase), Phosphate dehydrogenase B, Enolase, Ferodoxin-NADP reductase (leaf isomerase, chloroplastic-like isoform 1), cell Structure (two spots, tubulin alpha-3 chain, actin), signal transduction (one spot, cupin family protein) and two are spot unknown (figure 4).

**Table 2.** Estimates of heterosis in respect of hybrid B330 × R46.

| Colum 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 | Column 7 | Column 8 | Column 9 |
|---|---|---|---|---|---|---|---|---|
| Cross | PH | SD | TLN | RFW | RV | PDW | RDW | LDW |
| | Heterosis of F1 over | Heterosis of $F_1$ over | Heterosis of $F_1$ over | Heterosis of $F_1$ over | Heterosis of $F_1$ over | Heterosis of $F_1$ over | Heterosis of $F_1$ over | Heterosis of $F_1$ over |
| B330 × R46 | M.P B.P | MP | M.P B.P | M.P B.P | M.P B.P | M.P B.P | M.P B.P | M.P B.P |
| | $0.52^{**}$ $0.47^{**}$ | $0.12^{*}$ | $0.32^{**}$ $0.17^{**}$ | $0.87^{**}$ $0.51^{*}$ | $0.85^{**}$ $0.58^{*}$ | $1.13^{**}$ $0.88^{**}$ | $0.89^{**}$ $0.59^{*}$ | $0.9^{**}$ $0.65^{*}$ |

*, * = Significant at 5 and 1 %, respectively, NS= Non significant M.P = Mid Parent, B.P = Better Parent.

**Discussion**

*Morphological traits*

Morphological differences between hybrid and its parental line in eight traits was significant and mid-parent heterosis showed high percentage in all eight characters. Better-parent heterosis was observed in seven traits of eight. These results indicate the existence of genetic differences between hybrid and its parental lines and hybrid superiority of morphological characteristics toward mid-parent. In a study of Iqbal Khan *et al.* (1993), on sunflower hybrid and parental lines showed positive significant combination of heterosis from 30% to 29.64% toward parental lines. The result of this study approved initial studies of Pathak *et al.* (1983), Borodu-lina (1982) and Shrinivasa (1982) based on acceptable heterosis in plant height of $F_1$ generation. For leaf trait, Sayyar Khan *et al.* (2003) observed high significant differences between hybrids ad their parental lines in sunflower.

**Table 3.** Profile of significant changes in protein expression of hybrid and its parental lines.

| Column 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 |
|---|---|---|---|---|---|
| Spot number | Accession number | Protein name | Functions | Experimental Mr/pI | Calculated Mr/pI |
| 1 | - | Phosphoglycerate kinas | Metabolism | 47-5.8 | 50-6.1 |
| 2 | Q9ZRR5.1 | Tubulin alpha-3 chain | Cell structure | 47-5.3 | 50.38-4.89 |
| 3 | gi\|226509468\|refLNP_001147040.1 | Cupin family protein | Signal transduction | 55.43-6.34 | 56.77-6.1 |
| 4 | P17614.1 | ATP synthase subunit beta, mitochondrial | Energy | 58-5.7 | 59.99-5.95 |
| 5 | XP_003540396.1 | Ketol-acid reductoisomerase,chloroplastic-like | Metabolism | 65-6.8 | 63.69-6.85 |
| 6 | Q01390.1 | Sucrose synthase | Metabolism | 100-6 | 92.34-6.5 |
| 7 | - | Ribulose-1.5-bisphosphate carboxylase/ oxygenase large subunit | Photosynthesis | 30.1-7.3 | 58.8-5.9 |
| 8 | Q6F361_ORYSA | Putative malate dehydrogenase | Metabolism | 40-6.4 | 35.41-8.22 |
| 9 | XP_003538161.1 | Bifunctional polymaxin resistance protein ArnA-like | Disease/defense | 41-5.8 | 43.68-5.83 |
| 10 | AAK83073.1 | Putative cytosolic NADP- malic enzyme | Metabolism | Jun-63 | 65-5.73 |
| 11 | CAC10208.8 | Cytosolic malate dehydrogenase | Metabolism | 34-5.8 | 35.82-5.92 |
| 12 | AAK68820.1 | Similar to dihydroflavonol reductase | Secondary Metabolism | 42-5.6 | 44.18-5.58 |
| 13 | - | Ribulose-1.5-bisphosphate carboxylase/ oxygenase large subunit | Photosynthesis | 35/9-5.9 | 50-7.7 |
| 14 | gi\|9965319 | Actin | Cell structure | 44-5.34 | 41.66-5.31 |

**Table 4.** Profile of significant changes in protein expression of hybrid and B330 parental line.

| Column 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column 6 |
|---|---|---|---|---|---|
| Spot number | Accession number | Protein name | Functions | Experimental Mr/pI | Calculated Mr/pI |
| 1 | - | Phosphoglycerate kinase | Metabolism | 47-5.8 | 50-6.1 |
| 2 | Q9ZRR5.1 | Tubulin alpha-3 chain | Cell structure | 47-5.3 | 50.38-4.89 |
| 3 | gi\|226509468\|refLNP_001147040.1 | Cupin family protein | Signal transduction | 55.43-6.34 | 56.77-6.1 |
| 4 | P17614.1 | ATP synthase subunit beta, mitochondrial | Energy | 58-5.7 | 59.99-5.59 |
| 5 | XP_003540396.1 | Ketol-acid reductoisomerase,chloroplastic-like | Metabolism | 65-6.8 | 63.69-6.85 |
| 6 | Q01390.1 | Sucrose synthase | Metabolism | 100-6 | 92.43-6.5 |
| 7 | - | Ribulose-1.5-bisphosphate carboxylase/ oxygenase large subunit | Photosynthesis | 30.1-7.3 | 58.8-5.9 |
| 8 | Q6F361_ORYSA | Putative malate dehydrogenase | Metabolism | 40-6.4 | 35.41-8.22 |
| 9 | gi\|20663 | Glyceraldehyde-3-phosphate dehydrogenase B | Energy | 42.5-5.8 | 48.07-7.57 |
| 10 | AAQ77240.1 | Enolase | Energy | 50-6.2 | 48.31-6.17 |
| 11 | EAAWGLAR SAAYYQQGAR | Fructose-bisphosphate aldolase precursor | Metabolism | 37.6-6.2 | 38-5.5 |
| 12 | - | Unknown | - | - | - |
| 13 | XP_003537636.1 | Ferodoxin-NADP reductase, leaf isomerase, chloroplastic-like isoform 1 | Energy | 41-6.7 | 40.8-8.7 |
| 14 | gi\|2288969\|emb\|I-CAA73369.1 | Glutathione transferase | Cell detoxification | 26.19-6.30 | 26.44-6.21 |
| 15 | - | 2-phospho-D-glycerate hydroylase | Metabolism | 32.6-6.6 | 51.6-5.3 |
| 16 | - | Probable fructose-bisphosphate aldolase precursor | Metabolism | 39.6-6.2 | 42.8-6.4 |
| 17 | AEP71393.1 | Glyceraldehyde-3-phosphate dehydrogenase | Energy | 21-6.6 | 21.04-7 |

**Table 5.** Profile of significant changes in protein expression of hybrid and R46 parental line.

| Colum 1 | Column 2 | Column 3 | Column 4 | Column 5 | Column6 |
|---|---|---|---|---|---|
| Spot number | Accession number | Protein name | Functions | Experimental Mr/pI | Calculated Mr/pI |
| 1 | - | Phosphoglycerate kinase | Metabolism | 47-5.8 | 50-6.1 |
| 2 | Q9ZRR5.1 | Tubulin alpha-3 chain | Cell structure | 47-5.3 | 50.38-4.89 |
| 3 | gi\|226509468\|refL NP_001147040.1 | Cupin family protein | Signal transduction | 55.43-6.34 | 56.77-6.1 |
| 4 | P17614.1 | ATP synthase subunit beta, mitochondrial | Energy | 58-5.7 | 59.99-5.95 |
| 5 | - | Carbonic anhydrase | Metabolism | 28.3-6.3 | 35.9-5.8 |
| 6 | CAA76746 | Rubisco large subunit | Photosynthesis | 50-6.5 | 54-6.3 |
| 7 | Q8RW02_WHEAT | Glutathione transferase | Cell detoxification | 27-6.4 | 24.99-6.35 |
| 8 | BAG09374.1 | Peroxisomal hydroxypyruvate reductase | Metabolism | Jul-44 | 42.39-7.01 |
| 9 | XP_003538161.1 | Bifunctional polymaxin resistance protein ArnA-like | Disease/defense | 41-5.8 | 43.685.83 |
| 10 | AAK83073.1 | Putative cytosolic NADP-malic enzyme | Metabolism | Jun-63 | 65-5.73 |
| 11 | | - Unknown | - | - | - |

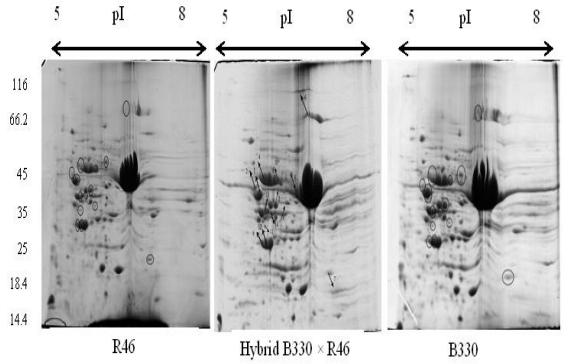


**Fig. 1.** Reference 2-D map of leaf differential proteins between Hybrid B330 × R46 and its parents at end of vegetative stage. Protein spots that were accumulated non-additively in hybrids compared with their parental inbred lines are numbered on the map.

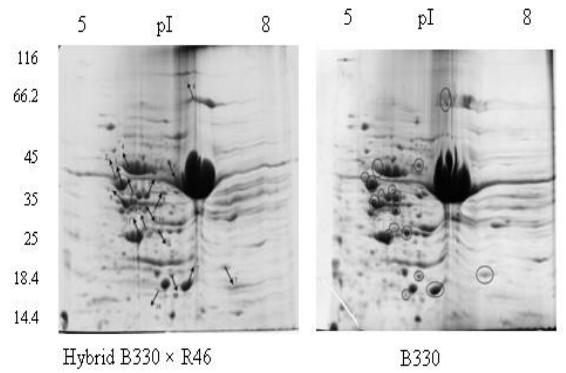


**Fig. 2.** Two-dimensional electrophoresis pattern of leaf proteome in hybrid and parent B330 in sunflower which are stained by CBB.

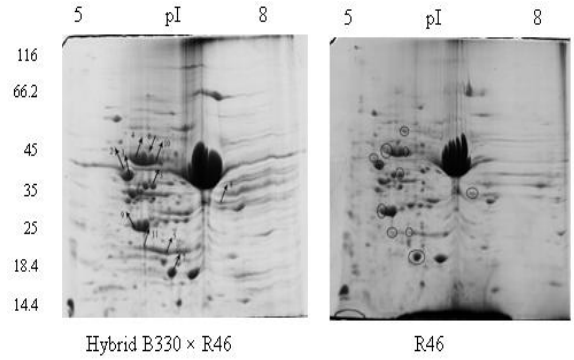


**Fig. 3.** Two- dimensional electrophoresis pattern of leaf proteome in hybrid and parent R46 in sunflower which are stained by CBB.

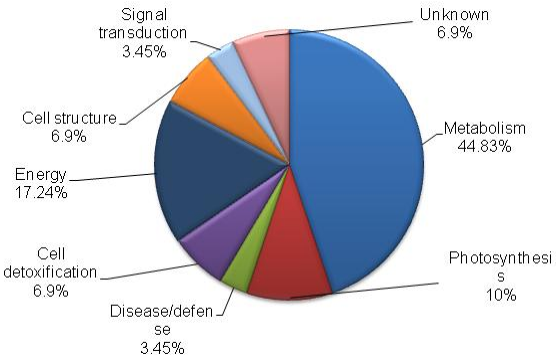


**Fig. 4.** Functional classification of 29 identified proteins and isoforms differentially expressed in

leaves between hybrid and its parental line and hybrid by parental line separately.

*Characteristics of the leaf reference proteome map of sunflower (Helianthus annuus L.)*

In this study, experimental data demonstrated that at least 14 protein spots in the hybrid $F_1$ leaves were differentially accumulated as compared to the inbred parental lines, on the other hand, 17 protein spots in the hybrid $F_1$ and 11 protein spots in the hybrid $F_1$ were differentially as compared separately to the B330 an R46 parental line, respectively. Leaves play an essential role in photosynthesis as the major sources of organic carbon (Song *et al.,* 2009b). Among the 42 protein spots in three analyses of gel images, 16 spots are involved in photosynthesis and carbon metabolism. Phosphoglycerate kinase (Spot 1) is involved in metabolism pathway. In the Calvin cycle, phosphoglycerate kinase catalyze 3-phosphoglycerate into 1, 3-phosphoglycerate (Zhang *et al.,* 2012) were specifically identified as HDH pattern and up-regulated in hybrid in comparing with R46, B330 parental lines separately. Spots 6, 8 expressed HDH and LDH patterns, in addition spot 8 showed up-regulated in hybrid comparing by B330 parental line. These spots are probably related to sucrose synthase and malate dehydrogenase respectively. Sucrose synthase, catalyzes the reversible modification of sucrose and UDP to UDP-glucose and fructose in vitro, is an important enzyme in sucrose metabolism (Martin *et al.,* 1993). Malate dehydrogenase catalyzes oxaloacetate and malate to NAD and NADH coenzymes (Minarik *et al.*, 2002). Spot 5 is probably related to Ketol-acid reductoisomerase which showed LDH pattern and down-regulated in hybrid comparing with B330 parental line. Spots 10 and 12 indicated URH pattern in addition spot 10 expressed up-regulated in hybrid in comparing with R46 parental line which these spots involved in NADP-malic cytoplasmic enzyme and Dihydroflavonol reductase respectively. NADP-malic catalyzes malate and $NADP^+$ to pyruvate, NADPH and $Co_2$. It appears that the metabolism of malate in guard cells of stomata can be effective in the plant growth during drought period and water saving at irrigation time (Laporte *et al.,* 2002). Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), (Spot 13) expressed URH model. Rubisco is the major photosynthetic enzyme in plants, and its activity in vivo is regulated by Rubisco activase (Martin *et al.,* 1993). Spot 11 related to fructose bisphosphate aldolase protein (FBA), which is a glycolytic and Calvin cycle enzyme and expressed up-regulated in hybrid comparing to B330 parental line and high expression of this protein may contribute to the observed vigorous growth in hybrid. Spot 7 showed DRH comparing to mid-parent value and parent B330, also spots 15 and 16 showed down-regulated in hybrid toward B330 parental line and related proteins are 2-phospho-D-glycerate hydroxylase and fructose-bisphosphate dehydrogenase respectively. Carbonic anhydrase and peroxisomal hydroxypyruvate reductase proteins which related to spots 5 and 8 respectively, expressed up-regulated in hybrid toward R46 parental line. Carbonic anhydrase prepared Co2 for Rubisco (Badger and Price 1994) and help to Rubisco carboxylation by regulation of Co2 concentration in chloroplast (Merewitz *et al.,* 2011). ATP synthase subunit beta (mitochondrial), (Spot 4) expressed HDH model toward mid-parent and up-regulated in hybrid as compared to parental lines. ATP synthase enzyme has main role to provide energy in cloroplast and mitochondry (Tezara *et al.,* 1999). Spots 9, 10 and 17 revealed up-regulated in hybrid in comparision with B330 parental line. The related proteins are, Glyceraldehyde-3-phosphate dehydrogenase B, Enolase and Glyceraldehyde-3-phosphate dehydrogenase, respectively. Enolase catalyzes the reversible dehydration of 2-phosphoglycerate to phosphoenol pyruvate. Phosphate of pyruvate has high negative energy which provides the transfer of phosphate to ADP (Dennis and Blakeley, 2000). Glyceraldehyde-3-phosphate dehydrogenase has the role of oxidoreductase and involve in primary metabolism of carbon and glycolysis (Fang *et al.,* 2011). Tubulin alpha-3-chain (spot 2) revealed URH pattern toward mid-parent value and up-regulated in hybrid toward B330, R46 parental lines separately. Tubulins are the monomers of microtubules and their polymerization

need energy in the form of ATP and are related traits with root growth.

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