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Characterization of Plasma Membrane Proteins from Maize Roots (*Zea mays L.*) under Multiple Abiotic Stresses using LC-MS/MS Technique

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Abstract

Maize inbred plants showed tolerance when exposed to various abiotic stresses simultaneously and in combination (drought x low-N and waterlogging x low-N stress). The stressed maize plants had higher photosynthetic efficiency, shows increase in plant height, leaf area, and they also maintained high leaf relative water content in drought x low-N stress and not much reduction in morphological parameters under combined stresses. Therefore, to understand the mechanism regulating the tolerance to multiple stresses, we analyzed maize roots plasma membranes proteins of stressed plants by using LC-MS/MS techniques, the large number of proteins (295) were identified which were mainly trans- membrane proteins, low abundance proteins, and root specific proteins. Among various proteins characterized, only four proteins were selected like high-affinity Nitrate transporter, NR enzyme, PEP carboxylase, Glutamine synthetase proteins and their induction were validated by qRT-PCR approach in control and stressed plants. The qRT-PCR results exhibits that in control and stressed plants the gene of all four proteins were equally expressed. We concluded the high-affinity nitrate transporter proteins in stressed plants might represent the executive part of the protective response that plays a significant role in particular low-N stress tolerance along with NR enzyme, PEP carboxylase and glutamine synthetase. While, presence of other major proteins like kinases, stress-responsive TFs, calmodulin, aquaporins, stress-related proteins, and many more proteins and their interaction with nitrate transporter proteins and their role can be validated only after comparisons between control and treated samples based on the same peptide mass-to-charge ratios (m/z) that were acquired under the same general conditions during LC-MS/MS experiments.

Keywords: Multiple abiotic Stresses, Zea mays, LCMS/MS, Roots plasma membrane, Nrt2.1, Photosynthetic efficiency.

Introduction

Agricultural system is widely affected with various environmental factors. Recent climate prediction models indicates that rise in temperature, frequent occurrence of drought, flooding and heat waves are major constrains which are causing higher agricultural production loses [1,2]. Hence, the understanding of plant responses to various abiotic stresses is utmost crucial. Plants are sessile organism, their survival depends on coping with the environmental challenges. The abiotic stresses either singly or in combination cause significant damage to crop plants. The combination of two different stresses might have synergistic effect that may enhance tolerance of the plant or or different abiotic stresses antagonize and exaggerate the effects of each other [3]. Though

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the plant response to different stresses is highly complex and involves changes at the transcriptome, proteome and physiological levels [4]. Perhaps, plasma membranes are primary sites that received signals for the cellular level changes in protein expression and these signals transfer to the cell. Hence, plasma membranes are structural barrier through which exchange of substances and information are communicated to the extracellular environment of the cell [5]. Most of the signal and transporter proteins are embedded in the plasma membrane these integral membrane proteins contains trans-membrane domains (hydrophobic nature), they cannot be solubilized in 2DE buffers [6]. Therefore, to identify diverse arrays and a wide range of proteins, particularly basic proteins, low-abundance, small proteins and hydrophobic proteins thus LCMS/MS-based-based proteomics is a parallel method with higher efficiency [7]. Also it considered as highly sensitive, accurate method for identifying the integral membrane proteins. Maize is a staple food crops in the tropical climate, largely grown in marginal areas of rain fed system. during summer rainy season it has to face both drought and waterlogging stresses due to uneven distribution patterns of monsoon rains in the region [8]. However at the beginning of plant growth, occurrences of these two stresses may limit the photosynthetic ability of leaves and biomass gain at the vegetative stage. Further nitrogen availability is low under both these stresses, water deficit affects the N-uptake while, in waterlogging leaching and de-nitrification causes the depletion of nitrogen [9]. Since the nitrogen is the major nutrient that influences the growth of plants and roots are the main organs through which mineral nutrients are taken up. Besides, roots are the first organ that perceives abiotic stress signals, weather due to anoxia (waterlogging), cell wall remodeling under water deficit or nutrient deficiency (N or P deficiency) henceforth they are essential for plant growth, survival and fitness. it has been reported that the root is a useful tissue for proteomic research. Therefore, roots of the stressed maize plants was selected for physiological studies and the role of root plasma membrane proteins was investigated using LCMS/MS technique under various combined abiotic stress conditions.

Materials and Methods

Plant materials and stress conditions

Maize seeds were obtained from the International Maize and Wheat Improvement Center (Spanish acronym; CIMMYT®). [50-VL1018393; 51-VL0512387; 52-VL0512388; 53-VL1012838; 55-VL1018413; 56-VL0512393; 57-VL1018418; 58-VL1018419; 59-VL1018513; 60-VL1018514]. This identified inbred seeds having distinct difference in terms of tolerance/ susceptibility to single stress, like waterlogging, drought and low-N. Maize seeds were sown in earthen pots (10 cm diameter) filled with sandy loam soil. The pots were kept in a naturally lit greenhouse, with air temperature 25°-30° C and relative humidity 55-65%. Ten plants (one inbred line) were chosen with six replications per plants, each pot contains 2 plants per pot after seedling emergence. Fourteen pots for control and 40 for treatment, the nutrient applied in pots was calculated on the basis per kg soil, a full dose of phosphorous potash and zinc was mixed in the soil before sowing without any organic fertilizer. (As per agronomic recommendation is N 120 kg/ha (urea) required by maize plants) However, for Low-N (LN) treatment, 25% N was used only once (195mg N/pot), unlike in control pots normal N rates (780mg N/ pot) was given in split doses. The waterlogging stress was given 30 days after sowing, (Figure 1a, b) for up to 7 days. After completion of the stress, water was drained out from the pots by opening the holes at the bottom. Subsequently, the drought stress was given 40 days after sowing, (without recovery period) by withholding the water for a 10 days (Figure 2a, b). During the stress period soil moisture content of the pots was measured on the 7th day of stress from control and treatment pots (Figure S1 supplementary).

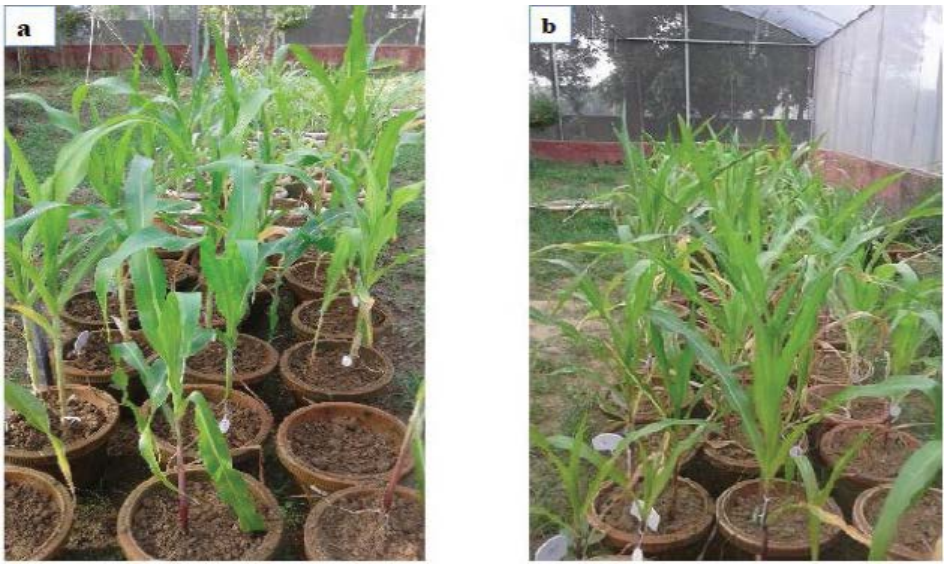

Figure 1a,b: Maize plants shows no phenotypic differentiation between control (a) and stressed (b) plants under waterlogging x low-N stress (5 days after stress). The stress symptoms are not visible in treated plants like leaf wilting, chlorosis/necrosis, lodging and white tips on the surface rooting.

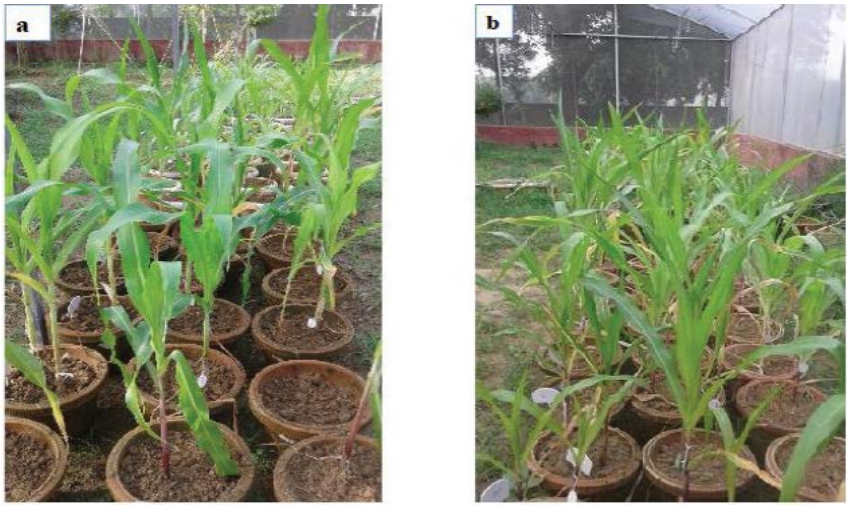

Figure 2a,b: Maize plants under drought x low-N stress control (a) and stressed (b) both the phenotypes (control and stressed) are similar.

Parameters measured under various stresses and Statistical analysis

Growth and morphological observation was recorded like, leaf area, plant height, leaf number, fresh and dry weights of shoots and roots, total fresh and total dry weights of seedlings. Physiological parameters like, Net photosynthesis rate (P_N), stomatal conductance (gs), internal CO_2 concentration (Ci), transpiration rate (E) was measured using LI-6400 (LI-COR Lincoln NE) portable closed gas exchange system. Three plants of each control and treated plants were chosen, fully expanded leaf blades were enclosed in the assimilation chamber for measuring the photosynthetic rate between 8:30 am to 10:00 am for this Same leaf was plugged for chlorophyll content determination and leaf relative water content (RWC). For all the measured parameters each pot represented one replication. A minimum of three pots were sampled for all observation, the average of three replicates were analyzed using descriptive statistics and paired T-test for each trait and data was expressed on a per plant basis. To verify the significance of the variations of all the parameters, One-way analysis of variance (ANOVA) followed by the post hoc Tukey test ($p < 0.01$) was used.

Extraction of the membrane proteins

Fresh roots samples was collected in three biological replicates and pooled. They were immediately frozen in liquid Nitrogen and stored at -80 for LCMS/MS experiment and qRT-PCR studies. However, the 3-different roots was collected and stored from control plants for qRT-PCR experiment.

Homogenate preparation and separation of membrane proteins

All extraction procedures were carried out on ice at 4°C. Fresh roots were weighed (5 g) in triplicate (biological) and the tissue was first grounded in liquid N2 then in 10 ml of cold extraction buffer (250 m Sucrose, 1 mM EDTA, 10 mM Tris HCl buffer, pH 7.2 and protease inhibitor) *(Sigma P9599*).Then homogenate was transfer to centrifuge tube and sonicated using two 10 second pulses (30 seconds in between pulses) using a probe sonication *(Bath sonication, 30 KHz frequency*), samples kept in ice bath, to minimize the sample-air interface foaming. The intact cell, nuclei and cell debris was removed by centrifugation of the homogenate at 15000 x g for 15 minutes at 4°C (the step was repeated) and the pellet was discarded. Again the supernatant was centrifuge at 100,000 x g for 1 hour at 4°C. The obtained supernatant contains the soluble proteins that were discarded. The pellet was washed by homogenization buffer and re-centrifuge at 100,000 x g, 4°C, for 1 hour. The supernatant was discard and the remaining pellet contains all of the cell's membrane fraction was kept.

Phenol/Ammonium Acetate-Methanol Precipitation of membrane proteins

Membrane pellet was suspend in 0.5 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl , 2% (v/v) 2-mercaptoethanol and 2 mM PMSF) (*Sigma-Aldrich*) and homogenize. Incubated for 10 min at 4°C and then an equal volume of Tris-saturated (7.5 pH) phenol was added. Centrifuged to separate the phases, the phenol phase was recovered and re-extract with an equal volume of extraction buffer. Proteins was precipitated from the phenol phase by adding of 5 volumes of 0.1 M ammonium acetate in methanol and incubated at -20°C for overnight. The precipitate was washed 3-times with ammonium acetate in methanol and one time with acetone, the pellet was air dried and solubilize in rehydration buffer by incubating for at least 1 hour at room temperature, with occasional vortex and then centrifuge at 100,000 x g, 4°C, for 1 hour. The supernatant was removed and save. The sample protein concentrations were quantified by Bradford's method. Bradford, using bovine serum albumin (*Fischer Scientific*) as a standard [10].

Trypsin digestion in solution samples and data analysis in LC MS/MS

The goal of this study was to comprehensive identification of integral membrane proteins. 100µg of roots protein sample was taken for digestion; the volume was made up to 100µl

with 50mM NH4HCO3. The sample was treated with 100mM DTT at 95˚C for 1 hour, followed by 250mM Iodoacidamid (IA) at room temperature in the dark for 45 min. The sample was then digested with trypsin and incubated overnight at 37˚C. The sample was vacuum dried and dissolved in 10µl of 0.1% formic acid in water. After centrifugation at 10000 x gs, the supernatant was collected into the separate tube. 1µl injection volume was used on C18 nUPLC column for separation of peptides, and then followed by analysis on the *Water Synapt G2 Q-TOF* instrument for MS and MSMS. The raw data was processed by *MassLynx 4.1 WATERS*. The individual peptides MS/MS spectra were matched to the database sequence for proteins identification *on PLGS software, WATERS*. For Protein identification Database used UNIPROT, Mass Tolerance; 50ppm and Peptide mass tolerance; 100ppm for the search to proceed. Specific modification; Carbamidomethyl and variable modification; Oxidation (M). Result based on the Scores of the matching protein masses and probable peptides was given as output.

Quantification of gene expression by Semi-quantitative RT- PCR

Total RNA was extracted from 250 mg of frozen roots tissue stored at −80˚ C of treated (after combined stress treatment) and control plants, using the TRIzol method (*as described by the manufacturer*) An aliquot of total RNA was treated with RQ1 RNase-free DNAse (*Promega*), to avoid genomic contamination and 1µl of total RNA was quantified by spectrophotometer using a Nanodrop 1000 (*Thermo Scientific, Nanodrop Products*). First cDNA was synthesized from 100 ng of total RNA and mixed with 1 µl of Oligo dT (10 µM). The reaction was incubated 5 min at 70˚C, qRT-PCR was performed with gene-specific primers corresponding to the genes encoding the identified proteins (Table S1 supplementary). The primers were designed to generate PCR products of 500-1000bp. MEP and LUG used as reference gene for normalization of internal cDNA input.

Results and Discussion

Effects of various stresses on maize plants

This studies was carried out with the purpose to understand the effects of various stresses applied simultaneously on maize inbred plants at vegetative stage and to reveal the process of tolerance by proteomic approach, specifically the low-N response. The phenotypic observations of treated plants under waterlogging x low-N stress shows no lodging, wilting, leaf necrosis, and surface rooting, instead early brace root development was observed. Subsequently in drought x low-N stress symptoms includes (Fig 1a,b) leaves drooping, yellowing, wilting and premature leaf there was no such phenotype was visible in treated plants (Fig 2a, b) Photosynthesis is among the primary process to be affected under stress condition. The decline in photosynthesis under multiple stress conditions may be due to oxidative stresses, the multiple stresses affect leaf photosynthetic machinery [11,12]. But in our studies we have found that under waterlogging x low-N stress, all treated plants shows higher photosynthetic efficiency (13.77%) relative to control plants. Whereas, in drought x low-N stress the decline in photosynthetic rate was much more compared to waterlogging x low-N stress. Although this decline in photosynthesis was not below the range 40-50µmol CO_2 $sec^{-2}mol^{-2}$ under drought Zaltev and Lidon indicated the sustenance of photosynthetic mechanism by plants under water deficit stress shows the drought tolerance capability [13]. Hence our results indicated that overall photosynthesis sustained in treated plants under multiple stresses (Figure 3a, b). Moreover, in our experiments plants were grown under low-N stress yet maintain photosynthesis. Maize plants shows the strong correlation of photosynthetic rate and leaf N to AEI (assimilation efficiency index). [14]. Also the control and treated plants have shown non-significant difference in their mean values for various growth traits, morphological, and physiological traits (Table 1). Although plants were subjected to multiple stresses yet they maintained high assimilation rate. Therefore, it appears their might be some changes during the stress or some signals were regulated to overcome the stressful conditions. Therefore to determine whether the observed rates of photosynthesis described above correlated with changes in proteins under stress conditions. The complete protein profile of treated maize roots was analyzed in detail by LCMS/MS method (in solution).

Characterization of proteins identified by LC-MS/MS technique (in solution)

The aim of the study was to understand the response of

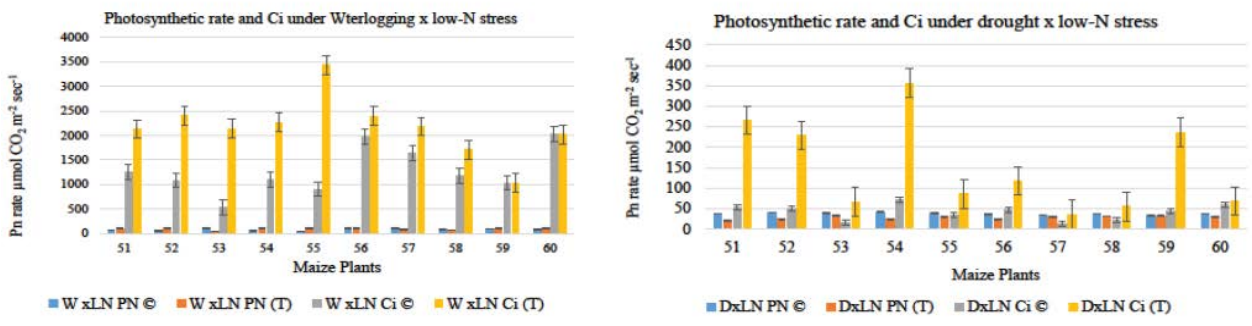

Figure 3a,b: Photosynthetic rate of control and stressed plants under waterlogging x low-N drought x low-N stresses. Mean values of Pn rate and internal CO_2 concentration (Ci) are significant at $p<0.05$.

Trait	Treatment	Waterlogging x Low-N stress			Drought x Low-N stress		
		Mean	C.D	SE(m)	Mean	C.D	SE(m)
Photosynthesis rate (µmol CO_2 m^{-2} S^{-1}	Control Treated	80.733** 93.68	24.1	8.401	38.233* 28.623	6.35	2.21
Conductance (gs)	Control Treated	0.087** 0.101	0.023	0.008	0.264** 0.142	0.107	0.037
Internal CO_2 (Ci)	Control Treated	1819.47* 1902.20	1785.61	622.433	57.267** 136.315	117.098	40.818
Transpiration rate E (mol)	Control Treated	2.428** 2.722	0.943	0.329	4.742** 2.83	N/A	0.538
Plant height (cm)	Control Treated	33.9** 31.5	N/A	3.251	29.167** 28.933	N/A	3.234
Leaf area (cm^{-2})	Control Treated	1746.54** 1146.85	N/A	334.343	1344.42* 1236.78	N/A	321.882
Leaf No. (per plant)	Control Treated	7.8* 7.0	N/A	0.699	6.633* 6.667	N/A	0.587
Fresh shoot weight (g)	Control Treatment				72.42** 45.215	N/A	12.9
Dry shoot weight (g)	Control Treatment				14.718* 9.857	N/A	3.094
Fresh root weight (mg)	Control Treated				10.12** 4.243	7.061	2.461
Dry root weight (mg)	Control Treated				2.43** 1.042	N/A	0.613
Total fresh weight (g/plant)	Control Treated				85.643* 51.215	N/A	17.887
Total dry weight (g/plant)	Control Treated				17.154** 11.442	N/A	3.481
**significant difference for p < 0/ 0.001 *significant difference for p < 0.05/ 0.01							

Table 1: The various traits measured under combined abiotic stresses in control and treated maize plants. The difference between control and treated was significant and low. Mean ± SE (m) and C.D values.

maize plants under multiple stress conditions. Maize plants subjected to combined stresses (waterlogging x low-N and drought x low-N) and the proteins were extracted from roots and analyzed by LC-MS/MS technique (in solution). In the root tissue of maize, approximately 295 proteins were identified shown in Table 2. Most of the proteins were related to diverse biological functions and has been categorized according to Bevan et al. [15]. The protein percentage was calculated by dividing the type and number of functional proteins from total no proteins present. Proteins related to nitrogen, and carbon metabolism were maximum in number (44.6%). However, some metabolism related proteins were associated with plasma membranes like, Enolase, G-3P dehydrogenase, PEP carboxylase, Nitrate Reductase enzyme. But some of the enzymes are soluble proteins, Alexandersson et al. consider them as contaminants of the plasma membrane preparation [16]. The second maximum were uncharacterized proteins (19%), calmodulin, kinases (Signal transduction), transcription factors (TF), transporter proteins, root specific proteins, cell division, translation and cell wall synthesis proteins, stress related proteins. While others were hormones, ubiquitin related proteins shown in figure 4. The concentration or abundance molecules in the sample can be detected by mass spectrometry. In our experiment, the maximum percentage coverage 95.41%, 94.58% and 75.38% is shown by NRT2.1 protein (Accession No's: Q53CL7, Q0VH26, and Q0VH25). The highest peak of the chromatogram (Figure S2 supplementary), also indicate the high expression of NRT2.1 proteins in the roots samples. Another maximum percent coverage was membrane bound Nitrate Reductase proteins (Accession No. Q4U5G4, 95.35%).

Possible role of NRT2.1 proteins, Nitrate Reductase, Phosphoenol pyruvate carboxylase and Glutamine synthetase and their validation using qRT-PCR studies in control and stressed plants under multiple stresses

The maize plants were grown under low-N stress, and we have identified the nitrate transporter protein in treated plants. The mRNA transcript of high affinity nitrate transporter in treated plants was spotted by qRT-PCR studies. Miller et al. also emphasize the regulation of HATS at the range of above 1mM concentrations (Soil low-N), Thus confirms their induction at low NO^-_3 concentration [17]. Similarly, the induction of NRT2.1 genes at very low levels of NO^-_3 (10–50 mM) was noted in *N. plumbaginifolia* and *Arabidopsis* [18,19]. The nitrate transporter transcripts was also detected in control plants (Fig 5).

The presence of two types HATS was detected in barley the one was iHATS, and other cHATS, at low NO^-_3 concentration, similar to our results the constitutive HATS (cHATS) in control plants whereas, inducible HATS (iHATS) transcripts in treated plants [20]. Moreover, possible role of NRT.2 proteins might to maintain N homeostasis in various stresses. In similar work the roots of two salt cultivars (salt-tolerant FL478 and salt-sensitive IR29 rice varieties) had

Protein Name [a]	Accession No [b]	MW(Da) [c]	pI(pH) [d]	Peptide [e]	Theoretical [f]	%Coverage [g]
Calcium Binding Proteins						
Calmodulin (fragment)	U5Q018_MAIZE	12554	4.04	24	15	100
Calmodulin (fragment)	U5Q0B5_MAIZE	11879	4.08	21	13	97
Calmodulin (fragment)	U5PZT9_MAIZE	10511	4.16	21	11	100
Calmodulin (fragment)	U5Q0D5_MAIZE	9305	4.07	16	10	100
Calmodulin (fragment)	U5Q0C3_MAIZE	12291	3.83	19	12	100
Calmodulin (fragment)	U5PZT0_MAIZE	12703	4.01	20	14	98
Calmodulin (fragment)	U5PZT0_MAIZE	12703	4.01	20	14	98
Calmodulin (fragment)	U5Q0C8_MAIZE	10553	4.16	17	11	96
Calmodulin (fragment)	U5PZQ0_MAIZE	10665	4.19	23	11	95
Calmodulin (fragment)	U5Q3A9_MAIZE	12583	4.04	26	16	100
Calmodulin-binding protein	Q41796_MAIZE	14604	5.06	21	8	69
Calcium-binding protein	Q43712_MAIZE	47983	4.28	46	45	91
Calcium transporting ATPase	A0A096PNP4_MAIZE	97157	7.59	107	56	87
Calmodulin-binding protein(F)	Q41797_MAIZE	31814	9.42	35	19	87
Calmodulin (Fragment)	U5Q0C8_MAIZE	10553	4.16	17	11	96
Calmodulin (Fragment)	U5PZQ0_MAIZE	10665	4.19	23	11	95
Annexin	Q43864_MAIZE	35237	6.91	40	33	75
Protein Kinase/Signal Transduction						
Ca^{2+}dependent protein kinase	Q41789_MAIZE	50564	4.95	51	51	93
Putative Serine/threonine -specific Protein kinase (Fragment)	Q6B7Q8_MAIZE	18932	8.45	14	13	92
Somatic embryogenesis receptor	Q8LPS5_MAIZE	66939	5.33	59	36	90
Adenosine Kinase (Fragment)	Q9XGC6_MAIZE	36009	5.04	35	23	71
CAK1AT Kinase –like Protein	B6TPK0_MAIZE	52427	4.20	53	31	99
Putative receptor protein kinase	Q93XG1_MAIZE	36134	6.44	60	34	99
SNF1-related protein kinase	P17801_MAIZE	91062	6.48	88	60	91
Putative leucine-rich repeat receptor-like protein	Q6RXY2_MAIZE	18538	8.46	20	12	92
Tousled-like kinase1(F)	K7V4X2_MAIZE	120450	5.68	95	76	81
MKK6-putative MAPKK	Q6DUC4_MAIZE	70985	7.33	79	59	81
Diacylglycerol kinase	O49975_MAIZE	39849	5.46	42	30	90
Inositol1,3,4,5,6-pentakis Phosphate 2 kinase	C0PCE8_MAIZE	77215	8.00	73	52	85
Zinc finger protein MAGPIE	A6YH14_MAIZE	48935	7.24	47	38	83
kinase CRINKLY4	Q9ZWA6_MAIZE	55791	8.29	38	32	70
CDPK-related protein kinase	Q41792_MAIZE	67356	9.23	66	50	83
ATP Sulfurylase	O48888_MAIZE	53752	9.08	65	50	89
BRASSINOSTEROIDE	Q94F62_MAIZE	68118	5.51	56	37	86
INSENSITIVE1-Associated receptor kinase Hexokinases	Q8L5G8_MAIZE	54782	6.03	66	36	96
Transcription Factors						
OCS element-binding factor1	P24068_MAIZE	16965	9.34	19	13	84
GRAS transcription factor	C0PGA9_MAIZE	63865	5.61	53	40	90
Transcription factor MYB31	Q2A702_MAIZE	31075	8.03	38	22	87
Transcription factor MYB42	Q2A700_MAIZE	28187	7.86	30	19	86

GRAS transcription factor (F)	A0A060D7Z4_MAIZE	63716	5.37	53	42	93
AP2-EREBP transcription factor	Q945C8_MAIZE	36866	4.93	22	20	85
Transcription factor MYB8	Q9XHR2_MAIZE	24223	8.84	21	15	87
Putative MYB DNA-binding domain superfamily protein	Q8RXB5_MAIZE	33493	9.45	27	25	93
PHD Transcription factor	B6U670_MAIZE	24968	7.94	26	17	88
WUSCHEL-related homeobox 9	Q8W0F1_MAIZE	22806	8.27	34	23	91
Cell Division/Translation Proteins						
Telomerase reverse transcriptase	Q1EG33_MAIZE	125900	9.65	123	90	85
Cell division protein FtsZ	Q8RMK5_MAIZE	26621	5.96	30	17	71
DNA replication licensing factor MCM3	Q9SX03_MAIZE	85155	5.89	123	77	87
Eukaryotic translation initiation factor 5A-2	Q93VP3_MAIZE	17129	5.49	23	17	94
Replication origin activator 4	Q9SX02_MAIZE	19925	8.48	29	24	97
Translation elongation factor-1 alpha	A6YDJ4_MAIZE	11013	9.43	10	9	81
Eukaryotic translation initiation factor 3 subunit A	Q9XHR2_MAIZE	111494	9.62	144	84	85
Origin recognition complex	Q945C8_MAIZE	91672	7.95	104	77	90
Zinc finger protein NUTCRACKER	Q9FFH3_MAIZE	51157	7.98	31	30	71
Protein Related To Carbon Metabolism						
Cytosolic G3-P dehydrogenase	Q43359_MAIZE	36427	6.69	39	26	83
Malate dehydrogenase	Q93XD0_MAIZE	11883	4.85	14	8	97
Enolase	P26301_MAIZE	48033	5.01	58	34	98
Trehalose-6-phosphate	K7V516_MAIZE	107148	6.31	109	67	92
Phosphoenolpyruvate carboxylase	Q9SAZ6_MAIZE	109360	5.68	123	87	93
Hexokinase	Q8L5G8_MAIZE	54782	6.03	66	36	91
Acyl CoA synthetase	B6SYY5_MAIZE	73940	6.78	82	46	81
Glucose-6-phosphate isomerase	K7V516_MAIZE	62198	7.05	64	41	95
4-hydrox-7-methoxy-3-oxo-3,4	P49235_MAIZE	64196	6.22	68	42	92
Dihydro-2H-1-4-benzoxazin Malic enzyme	O50015_MAIZE	71825	7.30	68	50	90
Diacylglycerol kinase	C0PCE8_MAIZE	77215	8.00	73	52	85
Inositol1,3,4,5,6-pentakisphosphate	A6YH14_MAIZE	48935	7.24	47	38	83
Glucose-6-phosphate isomerase cytosolic	P49105_MAIZE	62198	7.05	64	41	95
Putative inosine-uridine hydrolase	Q6PPF8_MAIZE	35123	6.15	32	22	93
Guanine nucleotide-binding protein Subunit beta	P49177_MAIZE	62198	7.05	64	41	95
1-deoxy-D-xyluose 5-P reductoisomerase 4-hydroxy-7-methoxy-3-oxo-	Q9FX27_MAIZE	51252	6.45	44	37	79
Putative dTDP-glucose4,6-dehydratase	A1X8E4_MAIZE	64644	5.88	72	46	86
Putative RUB1 conjugating enzyme	Q6PNA0_MAIZE	20642	8.77	21	14	90
Alpha-1,2-Mannosidase	K7UWA5_MAIZE	71623	6.71	66	45	89
Cis-zeatin O-glucosyltransferase	Q8RXA5_MAIZE	50711	5.33	43	30	85
Glutamate dehydrogenase	B9TST3_MAIZE	55803	5.40	62	47	88
Phospholipase D	A0A096SPA_MAIZE	91071	5.36	89	62	81
Sucrose synthase	Q8L5H0_MAIZE	91868	6.13	81	47	92
Arabinogalactan protein	Q9M715_MAIZE	27939	12.1	19	19	81
DTDP-glucose-4-epimerase	Q6QP37_MAIZE	44001	5.80	38	34	87
2C-type protein phosphatase protein-16	A0A060D93_MAIZE	39638	6.33	39	32	91
Xyloglucan endotransglucosylase	Q5JZX2_MAIZE	30766	4.49	31	18	98
6-Phosphogluconate dehydrogenase decarboxylating	O81238_MAIZE	53022	5.84	48	33	83

Putative beta glycosyltransferase	A1X8E0_MAIZE	22893	6.33	22	16	93
Uroporphyrinogen III methyltransferase	P93628_MAIZE	44939	5.52	40	30	91
Methylthioribose-1-phosphate-isomerase	B6TZD1_MAIZE	38598	5.63	41	27	98
UDP-glucose-4-epimerase	Q7XZQ2_MAIZE	22893	6.71	31	31	86
Peroxidase 2	Q9FEQ8_MAIZE	35726	5.26	46	21	97
Transpoter Proteins						
Potassium transporter	E5LFQ7_MAIZE	86673	8.96	83	51	93
Sulphate transporter	A7YF68_MAIZE	72177	9.58	72	46	87
Inorganic Phosphate transporter	Q5CC71_MAIZE	60540	8.16	79	34	95
Sodium/hydrogen exchanger	Q9ATZ9_MAIZE	101069	4.80	8	4	99
High affinity nitrate transporter	Q0VH25_MAIZE	20553	9.50	72	46	87
Mitochondrial phosphate transporter	O80413_MAIZE	38633	9.42	38	27	87
ADP, ATP carrier protein	BT6C13_MAIZE	36158	10.3	39	29	89
Stress Related Proteins						
Hypoxically induced transcript 2	O81218_MAIZE	11525	7.17	12	9	86
Senescence-associated protein DH	Q5UCF4_MAIZE	30147	8.49	14	12	78
Aquqporin TIP2-3	Q84RL6_MAIZE	25116	6.20	11	6	52
Sugar starvation induced protein	Q41855_MAIZE	25043	12.1	22	15	72
Pyrabactin resistance-like protein	C0PK92_MAIZE	20547	6.17	17	17	84
Aquaporin PIP2-4	Q9ATM6_MAIZE	30302	6.59	21	18	78
High mobility group B protein	P93047_MAIZE	15671	5.53	24	10	85
Heat shock protein 101	Q9S822_MAIZE	101069	5.76	112	77	86
Alcohol dehydrogenase class-P	P06525_MAIZE	41151	5.79	43	29	93
Submergence induced protein SI397	Q6XPW9_MAIZE	38965	6.37	35	29	90
Aquaporin PIP1-1	Q41870_MAIZE	30865	9.66	22	18	83
Aquaporin PIP2-5	Q9XF58_MAIZE	41283	7.83	24	18	98
Defense related proteins	Q41802_MAIZE	15532	8.77	12	8	55
Root Specific Proteins						
Root cap-specific protein	K7W6W9_MAIZE	41283	8.35	35	32	82
Rootless concerning crown and seminal lateral roots	A5H451_MAIZE	24796	5.62	11	11	47
Roothairless 1	Q5YLM3_MAIZE	100027	5.35	102	63	88
Protein root hair Defective 3 homolog	K7UC12_MAIZE	92200	5.99	111	74	89
Protein Shoot Gravitropism 5	F41P43_MAIZE	50108	9.19	51	36	86
CAPS-like protein 5C1	B6U300_MAIZE	16647	6.01	12	4	100
Plasma membrane instrinsic protein	Q84RL8_MAIZE	30657	9.05	31	16	97
Outer plastidial membrane protein	P42057_MAIZE	29958	8.44	23	20	95
Casparian strip membrane protein	B6T957_MAIZE	19995	9.98	17	9	93
Hormones						
Phytoene Synthase 3	B0KZ40_MAIZE	47271	8.76	61	33	92
Cytokinin dehydrogenase 1	O22213_MAIZE	64883	9.60	53	45	74
Cytokinin dehydrogenase 5	Q67YU0_MAIZE	60358	5.98	52	32	87
Cytokinin dehydrogenase 2	Q9FUJ3_MAIZE	55548	7.37	44	37	84
ABA-and ripening-inducible-like proteins	Q41730_MAIZE	18493	11.6	23	19	73
Proteins Related to Nitrogen Metabolism						
Reactive Intermediate Deaminase A	Q94JQ4_MAIZE	19803	8.74	23	17	95
Heme oxygenase-1	E5L882_MAIZE	31566	8.66	34	28	94
Ferredoxin	Q9SLP6_MAIZE	39297	8.42	54	33	86

High affinity Nitrate Transporter	Q53CL7_MAIZE	56637	7.84	64	28	95
Non-symbiotic hemoglobin	Q9FY42_MAIZE	18267	6.38	26	19	91
Glutamine synthetase root isozyme-1	P38559_MAIZE	39225	5.50	39	27	94
Cysteine synthase	P80608_MAIZE	34184	5.81	43	29	84
Glutamine S root isozyme 2	P38560_MAIZE	40068	5.50	37	26	94
Glutamine s root isozyme 4	P38562_MAIZE	38956	5.05	33	25	92
Aspartate aminotransferase	B4FUH2_MAIZE	50150	8.50	68	34	92
Amino methyl transferase	A0A096UFY3_ MAIZE	46792	7.56	63	40	97
Glutamine s root isozyme 5	P38563_MAIZE	39234	5.39	36	29	89
High affinity nitrate transporter	Q0VH26_MAIZE	21075	9.30	13	12	94
Glutamine s isozyme 3	P38561_MAIZE	39114	5.06	27	25	89
Ferredoxin –NADP reductase	Q41736_MAIZE	36352	8.21	39	30	88
Glutamate Dehydrogenases	Q43260_MAIZE	43994	6.07	50	27	90
Glutamate decarboxylase	B9TST3_MAIZE	55803	5.40	62	47	88
Asparagine synthetase	P49094_MAIZE	66535	5.78	57	43	85
Nitrate reductase [NAD(P)H]	P39871_MAIZE	26237	6.14	33	19	90
Mitochondrial phosphate transporter	O80413_MAIZE	38633	9.42	38	27	87
Cysteine proteinase	Q10716_MAIZE	40321	5.88	26	27	76
Hydroxyproline-rich Glycoproteins	Q42366_MAIZE	34409	10.3	6	4	22
D-alanine ligase	O8RVL2_MAIZE	238111	6.11	197	143	92
Putative nitrous oxide reductase	Q6VUZ8_MAIZE	24889	5.53	25	11	91
Basic leucine zipper	O22763_MAIZE	45330	5.12	51	27	93
Calpain-type cysteine protease	Q8RVL2_MAIZE	238111	6.11	197	143	92
Ubiquitin-40S ribosomal protein	P27923_MAIZE	17670	10.2	13	12	68
Putative peptidyl-prolyl cis-trans	K7UTL4_MAIZE	69487	7.37	65	45	86
Dihydrolipoamide acetyl transferase	Q41737_MAIZE	9325	9.69	12	6	89
Uroporphyrinogen III methyltransferase	P93628_MAIZE	44939	5.52	40	30	91
Methylthioribose-1-phosphate isomerase	B6TZD1_MAIZE	38598	5.63	41	27	98
Cell Wall Proteins						
3-oxoacyl-CoA reductase	Q8L9C4_MAIZE	35738	9.75	40	27	85
Laccase	Q2PAJ1_MAIZE	64538	5.62	64	38	92
Xyloglucan endotransglucosylase	Q5JZX2_MAIZE	30766	4.49	31	18	98
Actin-depolymerizing factor	Q41764_MAIZE	15889	5.29	16	11	84
Beta-tubulin	C4RS46_MAIZE	9888	4.62	6	4	95
Extensin	P14918_MAIZE	28830	10.5	3	3	4.86
Collagen alpha1-like protein	Q6PN99_MAIZE	11883	11.9	10	7	90
Tubulin alpha-1chain	P14640_MAIZE	49699	4.70	51	34	90

a) Accession number according to the UNIPROT database search for Maize proteins
b) Molecular weight in (Da),
c) pI(pH)-Isoelectric point / pH of proteins
d) Number of Peptide
e) Theoretical peptides (based on Trypsin digestion and protein sequence cleaved)
f) %Coverage of various Proteins detected in LC-MS/MS

Table 2: Plasma membrane proteins of maize roots expressed under multiple abiotic stresses and identified by LC-MS/MS technique.

Accession No	Protein Name	Starting Sequence	Gravy	pH/pI	Cellular location
Q6RXY1_MAIZE	SNF1 related protein kinase	MDGSSKGSGH	-0.352	8.32	Cytoplasm
KPRO_MAIZE	Putative receptor protein kinase ZmPK1	MPRPLAALLS	-0.208	6.92	Membrane
Q2A700_MAIZE	Transcription factor MYB42	MGRSPCCEKA	-0.472	8.03	Nucleus
TBA1_MAIZE	Tubulin alpha-1 chain	MRECISIHIG	-0.188	4.65	Cytoskeleton
A0A060D7Z4_MAIZE	GRAS transcription factor	MDTLFRSVSL	-0.343	5.50	Nucleus
A1XGH3_MAIZE	ALMT1-like protein (Malate trans)	MEIDEMESGV	0.104	8.47	Cellular M component
B4FQB2_MAIZE	AP2-EREBP transcription factor	MCGGAILAEL	-0.732	4.91	Nucleus
Q6PNA1_MAIZE	Putative ubiquitin-activating enzyme	MAGGPRRRLG	-0.153	7.32	Cellular M component
E2FB_ARATH	Transcription factor E2FB	MSEEVPQQFP	-0.672	4.38	Nucleus/cytoplasm
B4FA27_MAIZE	Alpha-galactosidase	MEAAGRLPLL	-0.228	6.36	Cell wall
K7TLQ9_MAIZE	Putative MYB DNA-binding domain	MEFIDPWDSQ	-0.382	9.53	ER PM
O81229_MAIZE	Ribosomal protein L25 (Fragment)	RPTTLKKARD	-0.676	10.62	Ribosomes
A6YDJ4_GIBIN	Translation elongation factor 1-alpha (Fragment)	KTHLNVVVIG	-0.400	9.46	
Q8RXB5_MAIZE	Origin recognition complex subunit 5	DKPSDFVAAL	-0.094	6.27	Nucleus
C0PCE8_MAIZE	Diacylglycerol kinase	MDLVGSLLLS	-0.227	8.17	PM
A6YH14_MAIZE	Inositol 1,3,4,5,6-pentakisphosphate 2-kinase	MEMDGVLQAA	-0.188	7.56	Nucleus
OCS1_MAIZE	Ocs element-binding factor 1	MSSSSLSPTA	-0.760	9.04	Nucleus
O81232_MAIZE	E3 ubiquitin-protein ligase	PRVRFCLHFE	-0.599	10.01	Nucleus
MCM33_MAIZE	DNA replication licensing factor MCM3	MEINEEAMAA	-0.344	6.30	Nucleus
B6TPK0_MAIZE	CAK1AT OS	MAIVGGGGSW	0.369	4.15	Cytosol, Nucleus
O48888_MAIZE	ATP sulfurylase	MATQAAFLAG	-0.311	9.14	Chloroplastic
Q9ZP60_MAIZE	GST7 protein	MSPPVKILGH	-0.146	5.15	Cytoplasm
Q84RL8_MAIZE	Plasma membrane intrinsic protein	MEGKEEDVR	0.402	9.08	PM and Vacuole
Q9ZP61_MAIZE	GST6 protein	MAAAAEVVLL	-0.107	5.44	Cellular component
Q9SX02_MAIZE	Replication origin activator 4 (Fragment)	MDVNEEAMAA	-0.234	8.49	Nucleus
Q53CL7_MAIZE	High affinity nitrate transporter	MAAVGAPGSS	0.369	8.01	PM and p-type Vacuole
GLNA3_MAIZE	Glutamine synthetase root isozyme 3	MACLTDLVNL	-0.424	5.01	Cytoplasm
Q0VH26_MAIZE	High affinity nitrate transporter	MARQQSVHAL	0.155	9.35	PM
Q6B7Q8_MAIZE	Putative serine/threonine-specific protein kinase	SGYLGAECQE	-0.307	8.47	---
O80413_MAIZE	Mitochondrial phosphate transporter	MALSDRSRES	0.263	9.54	Mitochondria (IM)
Q42366_MAIZE	Hydroxyproline-rich Glycoprotein (HRGP)	MGGSGRAALL	-1.321	10.62	---
K7U915_MAIZE	Phosphoserine phosphatase	MAGLISLRAG	0.018	6.29	chloroplast/cytoplasm
TIP23_MAIZE	Aquaporin TIP2-3	MVKLAFGSFR	0.864	6.67	PM
Q8RMK8_AZOBR	D-alanine:D-alanine ligase (Fragment)	KALLAPVGVR	-0.061	5.30	Cytoplasm
CYSP1_MAIZE	Cysteine proteinase 1	MAHRVLLLLS	-0.318	6.32	ES, Lysosome, vacuole
Q9ATZ9_MAIZE	Sodium/hydrogen exchanger (Fragment)	VFSEVLFFIY	0.900	4.79	Vacuolar Membrane
NIA2_MAIZE	Nitrate reductase [NAD(P)H] (Fragment)	PQKLGLPVGR	-0.473	6.60	Cytosol
Gravy index of identified plasma membrane proteins by LCMS/MS technique. The marked colored lines shows the most hydrophobic proteins. Cellular location of proteins searched from UNIPROT database					

Table 3: Gravy index of plasma membrane proteins of maize.

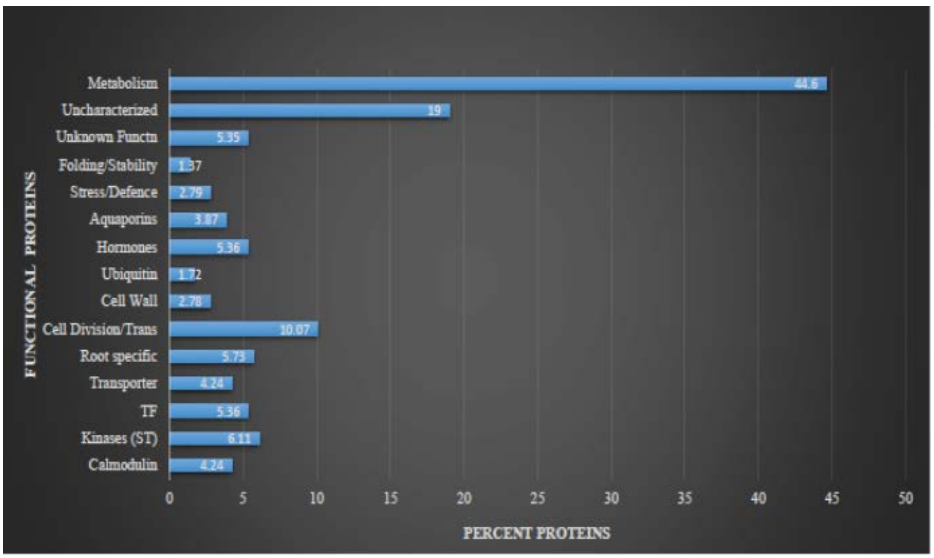

Figure 4: Functional classification of expressed proteins under combined stresses in plasma membranes of maize roots. The numbers and percentages of proteins from each functional category assigned on the basis of identified proteins. Proteins were categorized using the criteria of Bevan et al. (1998).

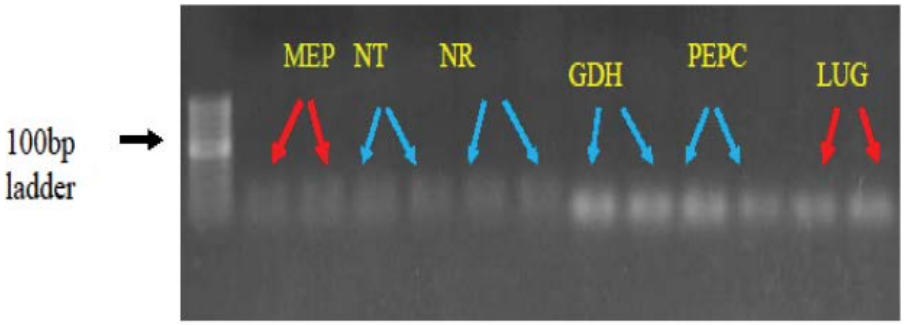

Figure 5: Transcripts of four plasma membrane proteins shows the induction of all proteins in control and stressed plants. Represented with blue arrow-Nitrate transporter (NT), Nitrate Reductase (NR), Gulamine syntetase (GDH) and Phosphenol pyruvate carboxylase (PEPC) RED arrow-MEP and LUG internal control genes of Maize.

shown significant up regulation of gene encoding nitrogen transporter in both the cultivars. Up-regulation of nitrogen transporter gene maintain the N homeostasis in tolerant cultivars, whereas, in salt sensitive cultivars up-regulated gene significantly reducing the N content [21]**.** Therefore transporter gene may have contributed to the salinity tolerant in both the cultivars. Another study shows higher abundance of transcripts related to high affinity nitrate transporters (NRT2.2, NRT2.3, NRT2.5, and NRT2.6) in tolerant genotype of barley [22]. However, soon after sensing NO^-_3 concentration in external medium plants respond by activating genes encoding NO^-_3 transport system and many enzyme systems [23]**.** In barley plants, Nitrate Reductase is the first enzyme to be involved in assimilation [24-27]. Successively, strong correlation between increased rates of NO^-_3 uptake and NR activity was observed [28,29]. Among the identified proteins by LCMS/MS was membrane bound NR in stress plants, the presence of NR transcripts in treated plant correlates with them. The transcripts of glutamine synthetase was correspondingly expressed in treated genotypes. Likewise, Li et al., detected the presences of genes of GS1-1 form and its expression in roots and confirms, the assimilation of NH_4^+ by the glutamine synthetase pathway for the amino acid synthesis. In a similar way, in treated plants transcripts of PEPC (Phosphoenolpyruvate carboxylase) was detected. [30]. A study reported the increase in levels of protein and mRNA for PEPC, by selectively increasing exogenous supply of nitrogen. Also with the steady-state level of PEPC mRNA and the major amino acids, glutamine level increased for 7 hours after nitrogen supply [31]. Thus, q RT-PCR results validates the presence of transcripts in treated and control plants. The results also verify the induction of all the four proteins in treated maize plants and their possible role in tolerance mechanism under multiple stresses. Furthermore, our results correlates with studies of Prinsi et al. They showed the incubation of maize roots for 30h in nitrogen nutrition, enhances the enzymes involved in nitrogen and carbon metabolism [32]. However, Nohzadeh et al. performed real-time PCR analysis, to investigate the correlation between mRNA and protein levels, in the roots of PM of salt-tolerant variety of rice, IR651, for three salt responsive genes (1,4-benzoquinone reductase, a putative remorin protein, and a hyper sensitive induced response protein) [33]. In their results no correlation was detected between the changes in the levels of gene and protein expression. Whereas, our results show correlation

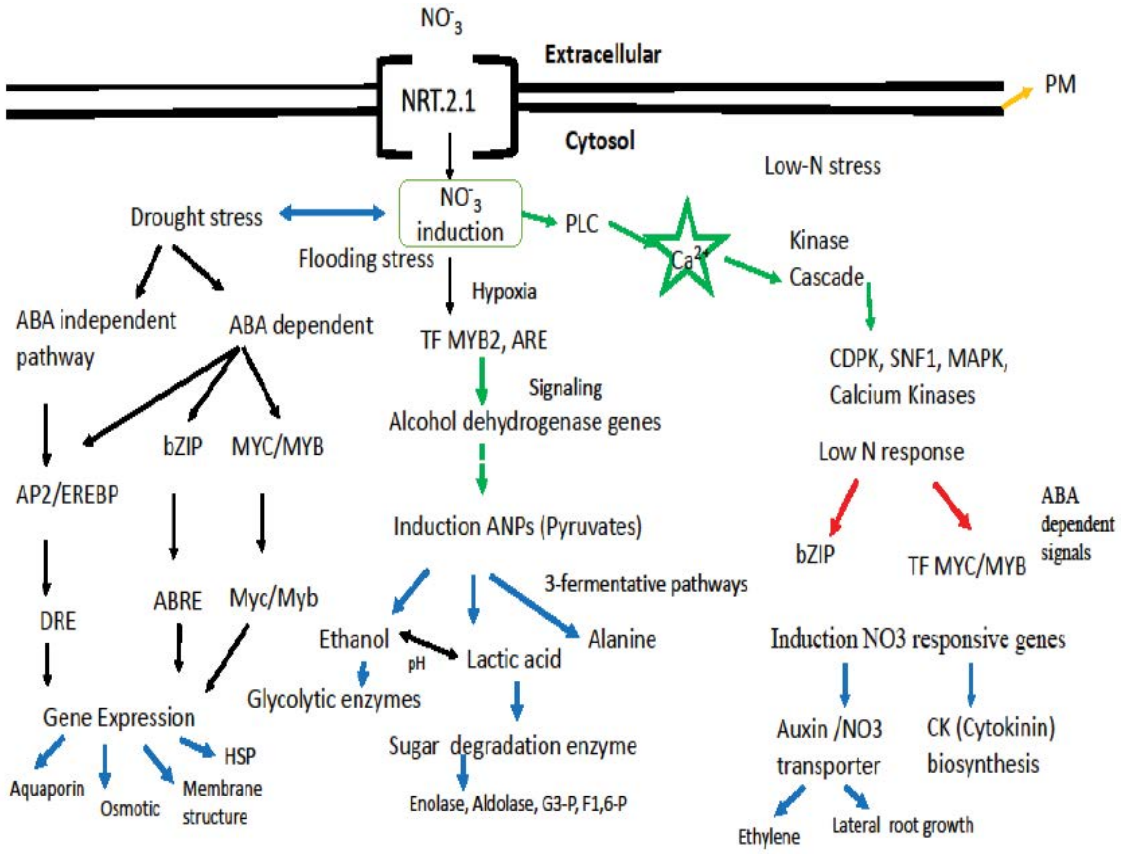

Figure 6: A Model shows the maize plant's response to multiple abiotic stresses. All the regulations occurring in the root cytosol (to make the model simple, other organelle not shown). Color arrows represent: Green- Signaling Cascade; Red – (+) ve regulation of genes and Blue-N-responsive pathways/metabolism.

between plasma membrane proteins and mRNA level. Though in our study plants were under low-N stress and deficient in nitrogen nutrition but due to the induction of nitrate transporter proteins and other 3-proteins, there is coordinated regulation of interaction of carbon and nitrogen metabolism. Subsequently maintains the homeostasis in various stresses.

Gravy Index of Plasma membrane proteins

The GRAVY is a computational program that evaluate the hydrophilic and hydrophobic properties of proteins along its amino acid sequence. The GRAVY score takes into account the size and the charge of the whole protein and ranges. The GRAVY of the maize roots plasma membrane proteins analyzed ranges from –1.32 to 0.402 [34]. Whereas, positive values referring to hydrophobic proteins. In our roots sample highly hydrophobic proteins is plasma membrane intrinsic protein (Q84RL8_MAIZE) it's Gravy score +0.4 The table 3**,** shows the Gravy index of roots plasma membrane proteins along with pI and cellular location of the proteins.

Proposed Model

Therefore, on the basis of our characterization of roots proteins data, qRT-PCR studies, and physiological status of the treated plants in response to various stresses. A model has been proposed that shows the plants response in low-N stress and their combined adversity stress adaptation strategies. The induction of Nitrate transporter proteins that enhanced and involve network of proteins to maintain homeostasis in other two stresses (waterlogging and drought) thus help to acclimatize the maize plants in various abiotic stress conditions has been shown in figure 6**.**

Conclusion

The maize plants when subjected to various abiotic stresses in combination shows tolerance. The phenotypic observations in treated plants does not exhibited any stress related symptoms. Also, statistical analysis of the mean values of various growth, morphological and physiological parameters in treated and control plants shows no significant difference. Therefore, to understand the reason of tolerance mechanism under multiple abiotic stresses, the roots plasma membrane proteins in treated plants was identify and characterized using LC-MS/MS techniques (in solution). The presence of a large number of integral hydrophilic, hydrophobic and low abundance of proteins were identified in our results. The transcriptional studies validates the role of four proteins (treated plants). Further, the role of other proteins can be validated only after comparing the control roots protein samples with the treated roots. In present context we can assume the role of other characterized proteins of stressed plants, might be due to coordinated

regulation and expression of various proteins along with induction of 'High- Affinity nitrate transporter proteins". These are involved in sensing and transporting nitrogen in low-N condition, and trigger signaling cascades which in turn activates membrane bound TFs, that initiate transcription of genes for low-N stress, waterlogging and drought stresses. Thus, maintain metabolic homeostasis and counteracting the effects of stress and ameliorate to acclimatize the plants at vegetative stage. Similarly, Scheible et al. observed the direct and indirect consequences of the nitrogen availability on the whole plant metabolism [35].

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