

Deep expression scrutiny of juxtaposed wild and cultivated lentil furnishes new insight into aluminium tolerance mechanism

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Abstract

Background: Aluminium (Al) stress hinders crop productivity in acidic soils. Lentil contains rich source of protein and micronutrients and cultivated in different parts of world. To enhance knowledge about Al toxicity tolerance, present study emphasizes on mechanistic analysis of genes associated with Al stress through de novo transcriptomic analysis of tolerant (L-4602), wild (ILWL-15) and sensitive (BM-4) genotypes. Result: Illumina HiSeq 2500 platform evaluated contigs ranging from 15,305 to 18,861 for all the samples with N 50 values of 1795 bp. Four annotation softwares revealed differential regulation of several genes where 30,158 genes were specifically up-regulated for combinations under Al stress conditions alone. Top up-regulated Differentially Expressed Genes (DEGs) in tolerant cultivar when compared to the sensitive one were found to be involved in protein transport as well as degradation, defences, cell growth and development. Wild v/s cultivar comparison revealed upregulation of wild DEGs that are involved in regulation of transcription in differentiating cells, pre-mRNA splicing, catalysis and protein ubiquitination. Based on assembled Unigenes, 89,722 high-quality SNPs and 39,874 SSRs were detected. Twelve selected genes were validated using qRT-PCR. KEGG pathway analysis extracted 8,757 GO annotation terms within molecular, cellular and biological processes. Pathway analysis indicated that organic acid synthesis and their transportation along with detoxification of ROS, an alternate pathway involving metacaspase-1,4,9 for programmed cell death were also significantly induced due to Al stress. Conclusion: Present study unveils the characterization of differential transcripts generated under Al stress indicating Al tolerance as a multiplex phenomenon which will directly widen crop improvement programmes for Al toxicity utilizing molecular approaches.

1. Introduction

Lentil belongs to genus *Lens* which is extremely versatile, oldest cultivated legume and is exceedingly healthy having high fibre and high protein content [1]. Due to increase in its consumption around the world, lentil production has boosted from 4.7 million tonnes to 7.6 million tonnes from 2010 to 2017 (FAOSTAT) primarily in regions of northern plains of North America and Australia [2]. Lentil production can be enhanced further if we can put a check on a major agricultural obstacle of acidic soils i.e. Aluminium toxicity. Since major part of lentil growing area is mainly affected by this abiotic stress which ultimately alters plant functioning at very early stages, therefore, there is an urgent need to tackle Al stress.

Lentils grown on soils of low pH with Al toxicity suffers from lethal effects on host plant, rhizobia and their interaction, yet it shows no initial visual symptoms on the plant, apart from slow and stunted overall growth [3] It embarks stunting of roots, especially seminal roots and death of lateral roots due to sensitivity of plants, even at micro molar concentrations of Al^{3+} . Therefore, cell division in root tip meristem is quickly inhibited [4]. Trimming of root biomass coaxed to impoverished uptake of water and nutrients. Also, Al toxicity can stimulate build-up of reactive oxygen species and callose, along with lipo-peroxidation in legume root elongation zone [3]. To scavenge and detoxify these physio-chemical traits, organic acids are induced to protect the plants under aluminium stress conditions. Ryan et al. (2001) have outlined the exudations of different organic anions such as malate, citrate or oxalate upon exposure to Al [5]. Also, it has been shown that Al tolerance can be augmented by escalating organic acid bio-production [6]. Under stress condition, operating Al tolerance mechanisms are not same in all the species, besides, in certain species, different mechanisms can operate concomitantly producing tolerance through their amalgamated effects. Even though type of tolerance mechanisms for Al toxicity is disputed, yet exclusion is widely accepted as the key mechanism involved in detoxifying Al^{3+} [4]. Thereby, to develop Al stress tolerant cultivars, an in depth understanding of divergent resistance mechanisms operating in lentil is crucial. Initial effort towards understanding these mechanisms in *Lens* species has revealed pattern II exclusion mechanism as one of the components, where secretion of citrate and malate from roots were observed which prevented binding of Al to extracellular and intracellular substances of the roots [7, 8]. Further, secretion of exudates was found to be highest in wild genotypes [7].

Wild species are evolved with the need of becoming more adaptive towards their environment and still contain valuable genes that are conserved from thousands of years. Therefore, it is essential to compare the resistance mechanism within and in between cultivars and wild accessions. Through transcriptomic profiling, differentially expressed genes and pathways connected with Al stress tolerance has been identified in wheat, maize, rice, buckwheat etc. evidencing the efficiency of RNA-Seq in molecular mechanism elucidation [6, 9–11]. In case of lentil, RNA-Seq has been utilized to develop a reference transcriptome [12] and to reveal differentially expressed genes for drought stress [13], ascochyta blight [14, 15], cold stress [16] and heat stress [17] but no such study has been conducted for Al stress so far. Since for comparative transcriptomic analysis, it is essential to select contrasting genotypes, a couple of previous studies, where different *Lens* species showing variation in morpho-physiological traits under Al stress and wilds separated from cultivars in molecular analysis [7, 8] were considered for selection of genotypes. From these studies, highly tolerant (L-4602) and sensitive cultivars (BM-4) for Al stress along with Al tolerant wild lentil (ILWL-15) genotypes were selected. Keeping in view the above-mentioned facts, selected genotypes were used in this study to i) acquaint and validate genes which are affected under Al stress via. transcriptomics and qRT-PCR, respectively ii) decipher Al stress mechanism and its pathway in lentil, iii) illuminate diverged evolutionary pathway in wild lentil under Al stress. Our findings will help in unwinding the extremely complex phenomena of Al toxicity and its resistance which will open doors for imposing appropriate strategies towards lentil improvement for Al toxicity by way of targeted breeding, genetic engineering and gene editing techniques.

2. Materials And Methods

2.1 Plant material, growth and aluminium exposure conditions

Three divergent lentil genotypes viz. L-4602, Barimasur-4 (BM-4) and ILWL-15 were used in this study [7]. First two accessions belong to *Lens culinaris*, whereas third belongs to *lamottei* species of genus *Lens*. Seeds of these genotypes were collected from National Bureau of Plant Genetic Resources (NBPGR), New Delhi. These accessions were chosen based on previous studies, where L-4602 was found to be Al tolerant breeding line developed at Indian Agricultural Research Institute (IARI), India whereas BM-4 was found to be Al sensitive cultivar, which was developed from the cross between ILL-5888 (improved landrace) and ILL-5782 (breeding line) at International Center for Agricultural Research in the Dry Areas (ICARDA), Syria [7, 18, 19]. ILWL-15 is a mineral rich wild accession which was originated in France and was reported to be moderately tolerant to Al stress [7, 20]. Growth and Al exposure conditions were provided as

per the growth response method of Singh et al. (2016) with minor modifications at National Phytotron Facility, IARI, India [7]. Seedlings emerged in hydroponic medium were placed in growth chamber that was programmed for relative humidity (%) and temperature of $55.7/68.2 \pm 2$, $27/16$ °C, respectively. Hydroponic medium containing $148 \mu\text{M}$ of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ with a pH of 4.8 was considered as treated whereas, its control was deprived of any $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ and its pH was maintained at 6.5. Roots of lentil seedlings were exposed to these two conditions for 6 h following a completely randomised design with 3 replicates each having 12 seedlings.

2.2 RNA isolation and library preparation

Total RNA was sequestered from roots of control and Al exposed samples of resistant and sensitive genotypes with nomenclature 'C' and 'T' representing 'Control' and 'Al treated' samples and 1, 2 & 3 representing genotypes L-4602, BM-4 & ILWL-15, respectively using TRIZOL reagent (Takara, Japan) where each replicate having twelve seedlings were pooled together. It was followed by RNA integrity test using Agilent Bioanalyzer 2100 and quantitation through Nanodrop. cDNA library was prepared using TruseqRNA sample prep Kit (Illumina, Inc. San Diego, CA, USA) abiding the prescribed directives. Firstly, messenger RNA (mRNA) was fragmented into shorter sequences using a magnetic bead that contained poly-T molecules. Selected small inserts were converted to cDNA using IlluminaTruSeq™ mRNA Library preparation kit. For clusters generation, pair end adapters (Illumina) were ligated to ends of selected fragments followed by cDNA enrichment by fragments having adapter molecules on both ends using polymerase chain reaction (PCR). Constituted cDNA libraries were sequenced using Illumina HiSeq 2500 platform (Illumina Inc. San Diego, CA, USA). Fragments of 120 to 200 bp with a median of 150 bp were produced from purified mRNA using TruSeq RNA fragmentation protocol. For proper selection of reads, these fragments were eluted corresponding to bead volume and incubation time at successive stages which resulted in paired end reads of 100 bp from each sample arising from total raw reads ranging between 19,500,000 to 32,185,339.

2.3 Quality control, de novo assembly and differential gene expression

Low quality reads (phred quality < 20) from raw Fastq files were filtered using FastXToolkit. Read quality after pre and post filtering was checked using FASTQC tool and the quality filtered reads were assembled using Trinity de novo assembler generating de novo contigs. In course of this process, unigenes i.e. non-redundant transcripts that cannot be extended further at their pair end and had passed redundancy exclusion course using sequence clustering software were identified. Mapping of clean reads to assembled transcripts was performed using Bowtie software. Differential gene expression was conducted using different packages like Deseq, Deseq2, Cuffdiff and EdgeR. Statistically significant differentially expressed genes (DEGs) in different comparison groups viz. 1C v/s 1T, 2C v/s 2T, 3C v/s 3T, 1T v/s 2T v/s 3T were identified based on FDR value < 0.05, respectively so that inflation caused by type-1 errors can be avoided. Most significant DEGs obtained from EdgeR package were sorted based on descending absolute \log_2 fold change. Circos plots were plotted to display comparison between contigs of different comparison groups. For graphic visualization of top significant DEGs in different comparison groups, heat maps were prepared.

2.4 Gene ontology (GO) and pathway analysis

Different Public reference databases including National Centre for Biotechnology Information (NCBI) non-redundant (nr), Swiss-Prot and (SWISSPROT), UniProt Reference Clusters (UNIREF) were used to fix unigene identity through Basic Local Alignment Search Tool (BLAST) that has sequence similarity index up to an E value < 1.0E^{-5} . Annocript program was employed for Unigene classification and assignment of GO terms to assembled transcripts. Functional categories were based on cellular components, biological processes and molecular functions. Web Gene Ontology Annotation Plot (WEGO) tool was used for constructing GO plot representing sorted transcripts. Enrichment analysis of top 50 DEGs was conducted using Shinygo database. Cluster of Orthologous Groups (COG) database was employed to achieve operational cataloguing of unigenes and their role in different metabolic pathways were deduced through Kyoto Encyclopedia of Genes and Genomes (KEGG) database. DEGs of different comparison assemblies were represented by means of numerous plots like HeatMap, Volcano Plot, Circos and MapMan (v3.51R2).

2.5 Quantitative Real Time PCR (qRT-PCR) validations

RNA initially extracted for library preparation from all the samples was also used for qRT-PCR validation of gene specific primers. cDNA was synthesized from RNA using verso cDNA kit (Thermo scientific). Amplification was done using One Step SYBR PrimeScript RT-PCR Kit II (Takara Biotechnology Co. Ltd, Dalian, Japan). Each sample was run in 4 biological replicates. Total cDNA of 100 ng was used in 10 μl reaction mixtures and CFX96 RT-PCR (Bio-Rad) was used for analysis of differential gene expression by calculating and calibrating expression levels of targeted genes by $2^{-(\Delta\Delta\text{CT})}$ method. PCR reaction of volume 20 μl was prepared using template cDNA of 6.8 μl , 2x SYBR Green mix of 10 μl , 5 μl each of forward and reverse primers and finally making the volume with nuclease free water. To normalize expression data, β tubulin was selected as reference gene for internal expression. Sequence information of primers used for qRT-PCR which were designed using primer 3 plus (<https://primer3plus.com/cgi-bin/dev/primer3plus.cgi>) is presented in Addition file 1. Table 1.

Table 1
Transcriptome profile of tolerant and sensitive genotypes under control and aluminium stress conditions

	CT (Control)					AT (Aluminium stress treatment)								
	1.1 C	1.2 C	1.3 C	2.1 C	2.2 C	2.3 C	3.1 C	3.2 C	3.3 C	1.1 T	1.2 T	1.3 T	2.1 T	2.2 T
Total number of contigs	44991	46229	45163	52160	47855	46168	28469	28428	28300	44991	44991	42795	47175	44870
Maximum length of contigs	15764	16470	15764	17511	14061	14099	15305	15305	15305	16705	18861	16569	18569	18569
Minimum length of contigs	500	500	500	500	500	500	500	500	500	500	500	500	500	500
Average contigs length	1455.6	1422.3	1466.3	1480.4	1462.8	1451.4	990.3	992.1	992.3	1477.4	1463.1	1461.7	1482.3	1471.0
Total length of contigs (bases)	44991	46229	45163	52160	47855	46168	28469	28428	28300	44991	44991	42795	47175	44870
N50	1787	1762	1766	1827	1798	1781	1050	1054	1051	1812	1799	1798	1826	1814

2.6 Identification and filtering of markers

Single Nucleotide Polymorphisms (SNPs) and Simple Sequence Repeats (SSRs) were identified using Samtoolsmpileup and MicroSatellite identification (MISA) softwares, respectively. A read depth of 20 bp was fixed to filter both heterozygous loci and false positive SNPs followed by SNP identification through Genome Analysis Tool Kit (GATK) version 3.6-0. SSRs within high quality filtered reads were identified using MISA and their corresponding primers were designed using Primer3 software. Primer attributes were fixed to a minimum length of 15 bp to a maximum of 21 bp and 18 bp was standardized as optimal primer size with a product range of 100 to 300 bp.

3. Results

3.1 RNA integrity and selection of reads

For RNA-Seq analysis, 148 μM of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ with a pH of 4.8 was used to study response of genotypes towards Al stress. RNA sequencing libraries were established from three biological replicates each for L-4602, BM-4 and ILWL-15 under control and Al treated conditions. RNA integrity number (RIN) in control and treated samples were in a satisfactory range of 6.7 to 7.9. Clean reads obtained from TruSeq fragmentation as performed in Sect. 2.2 were assembled to produce contigs in the range of 15,305 to 18,861 bp. Minimum length of contigs was fixed to 500 bp while the maximum length achieved post de novo assembly was 18,861 bp for the sample 2.1 under Al treated condition. Total number of contigs with N80, N50 and N20 were found to be 937, 1795 and 3109, respectively for overall samples. Major characteristics of reads of each sample are summarized in Table 1.

3.2 Differential gene expression annotation

Significant DEGs with FDR/q value/adjusted p value < 0.05 and \log_2 fold changes above 1.5 were found to differ for different comparison groups. Table 2 highlights the number of DEGs found for different combinations using different packages. A total of 39,515 up-regulated and 37,890 down-regulated transcripts were detected in different comparison groups using EdgeR. Sharing of contigs between different combinations along with individual contigs is presented graphically by Venn diagrams (Fig. 1). When tolerant, sensitive cultivars (L-4602; BM-4) and wild genotype (ILWL-15) were matched with their corresponding controls, 2329, 2380 and 5130 up-regulated and 1609, 1562 and 2420 down-regulated DEGs were acknowledged, respectively. Table 2 also represents total as well as significantly up and downregulated DEGs deduced from four different softwares. Circos plots representing distribution of overall DEGs for comparison group 1T-2T-3T is represented in Fig. 2.

Table 2

Significantly up and downregulated DEGs for different combinations using Cuffdiff, DESeq2, DESeq and EdgeR.

S. No.	Combination	Total Significant DEGs	Significantly Upregulated			Significantly Downregulated		
			Total	Log ₂ FC > 1.5	Log ₂ FC = inf.	Total	ABS_Log ₂ FC > 1.5	ABS_Log ₂ FC = inf.
Cuffdiff								
1.	1C-1T	11658	5940	1835	300	5718	1059	181
2.	2C-2T	10907	5203	1377	272	5704	911	83
3.	3C-3T	15492	8001	3834	2376	7491	1793	603
4.	1T-2T	2808	1413	150	32	1395	176	85
5.	1T-3T	9319	4511	973	324	4808	1300	745
6.	2T-3T	11105	5292	1321	505	5813	1819	978
DESeq2								
1.	1C-1T	14481	7135	1709	0	7346	1169	0
2.	2C-2T	14798	7313	1757	0	7485	1171	0
3.	3C-3T	19411	11285	3713	0	8126	1530	0
4.	1T-2T	2859	1438	22	0	1421	16	0
5.	1T-3T	11455	5294	429	0	6161	878	0
6.	2T-3T	15674	7558	1706	0	8116	1722	0
DESeq								
1.	1C-1T	13074	6469	2251	335	6605	1663	204
2.	2C-2T	6433	3517	2257	318	2916	1585	103
3.	3C-3T	13571	7957	4314	2482	5614	2335	931
4.	1T-2T	1314	665	286	37	649	270	44
5.	1T-3T	8485	3894	1593	377	4591	1810	1018
6.	2T-3T	12062	5763	2348	556	6299	2499	1300
EdgeR								
1.	1C-1T	14127	7196	2329	0	6931	1609	0
2.	2C-2T	14312	7304	2380	0	7008	1562	0
3.	3C-3T	18808	10624	5130	0	8184	2420	0
4.	1T-2T	2547	1260	432	0	1287	368	0
5.	1T-3T	12177	5645	1694	0	6532	2035	0
6.	2T-3T	15434	7486	2430	0	7948	2541	0

Table 3
 EdgeR data showing top 20 up-regulated DEGs for the combination tolerant sensitive treated.

ID	logFC	logCPM	PValue	FDR	Description
TRINITY_DN54283_c0_g1_i1	6.41	-0.040	4.66E-10	1.98E-06	-
TRINITY_DN74494_c0_g1_i1	6.23	-0.17	7.45E-09	1.19E-05	-
TRINITY_DN54609_c0_g1_i1	6.18	-0.20	1.49E-08	2.03E-05	-
TRINITY_DN10923_c0_g1_i1	6.13	-0.24	2.98E-08	3.16E-05	-
TRINITY_DN80888_c1_g3_i2	6.02	-0.32	1.19E-07	7.35E-05	-
TRINITY_DN67301_c0_g1_i1	5.90	-0.40	4.77E-07	0.000172	-
TRINITY_DN79682_c1_g3_i1	5.90	-0.40	4.77E-07	0.000172	Ribosomal RNA large subunit methyltransferase I
TRINITY_DN85861_c2_g4_i1	5.77	-0.48	1.91E-06	0.000476	PX domain-containing protein EREX
TRINITY_DN69746_c0_g1_i1	5.63	-0.57	7.63E-06	0.001103	Protein LIGHT-DEPENDENT SHORT HYPOCOTYLS 1
TRINITY_DN73769_c0_g1_i1	5.55	-0.62	1.53E-05	0.001686	-
TRINITY_DN84834_c0_g1_i1	5.47	-0.67	3.05E-05	0.002529	BTB/POZ domain-containing protein NPY1
TRINITY_DN83215_c4_g3_i1	5.47	-0.67	3.05E-05	0.002529	-
TRINITY_DN79925_c10_g3_i1	5.391	-0.72	3.05E-05	0.002529	ATP-dependent zinc metalloprotease FTSH 7 chloroplastic
TRINITY_DN73276_c1_g2_i1	5.39	-0.72	6.1E-05	0.003876	Transcription factor VOZ1
TRINITY_DN85829_c1_g7_i8	5.39	-0.72	6.1E-05	0.003876	-
TRINITY_DN5093_c0_g1_i1	5.20	-0.82	0.000122	0.005959	-
TRINITY_DN61154_c0_g1_i1	5.20	-0.82	0.000122	0.005959	-
TRINITY_DN70577_c0_g1_i1	5.20	-0.82	0.000122	0.005959	-
TRINITY_DN84227_c1_g1_i1	5.20	-0.82	0.000122	0.005959	-
TRINITY_DN105029_c0_g1_i1	5.10	-0.88	0.000244	0.009523	F-box protein At5g51380

Table 4

EdgeR data showing top 20 downregulated DEGs for the combination tolerant treated v/s sensitive treated.

ID	logFC	logCPM	PValue	FDR	Description SP
TRINITY_DN81385_c0_g1_i1	-6.08	-0.34	5.96E-08	4.71E-05	Zinc-finger homeodomain protein 4
TRINITY_DN79375_c1_g1_i1	-5.95	-0.42	2.38E-07	0.000121	-
TRINITY_DN37649_c0_g1_i1	-5.82	-0.51	9.54E-07	0.000289	-
TRINITY_DN58295_c0_g1_i1	-5.82	-0.51	9.54E-07	0.000289	-
TRINITY_DN19151_c0_g1_i1	-5.67	-0.60	3.81E-06	0.000712	-
TRINITY_DN65023_c1_g2_i1	-5.67	-0.60	3.81E-06	0.000712	-
TRINITY_DN65534_c0_g1_i1	-5.67	-0.60	3.81E-06	0.000712	-
TRINITY_DN61981_c0_g1_i2	-5.59	-0.65	1.53E-05	0.001686	-
TRINITY_DN80647_c0_g2_i1	-5.59	-0.65	1.53E-05	0.001686	Probable carboxylesterase 11
TRINITY_DN23470_c0_g1_i1	-5.50	-0.70	3.05E-05	0.002529	Peptidyl-prolyl cis-trans isomerase 1
TRINITY_DN81336_c0_g2_i2	-5.50	-0.70	3.05E-05	0.002529	Aldehyde dehydrogenase family 3 member F1
TRINITY_DN64108_c1_g1_i1	-5.41	-0.76	6.1E-05	0.003876	-
TRINITY_DN64247_c0_g1_i1	-5.41	-0.76	6.1E-05	0.003876	G-type lectin S-receptor-like serine/threonine-protein kinase SD1-13
TRINITY_DN69251_c0_g1_i1	-5.41	-0.76	6.1E-05	0.003876	Putative lysine-specific demethylase JM16
TRINITY_DN84985_c7_g4_i1	-5.41	-0.76	6.1E-05	0.003876	ABC transporter B family member 6
TRINITY_DN77161_c0_g1_i2	-5.41	-0.76	6.1E-05	0.003876	Protein PLANT CADMIUM RESISTANCE 8
TRINITY_DN50496_c0_g1_i1	-5.31	-0.81	0.000122	0.005959	-
TRINITY_DN84809_c0_g1_i1	-5.31	-0.81	0.000122	0.005959	-
TRINITY_DN85519_c2_g2_i1	-5.31	-0.81	0.000122	0.005959	-
TRINITY_DN50613_c0_g1_i1	-5.21533	-0.87416	0.000244	0.009523	Peptidyl serine alpha-galactosyltransferase

3.3 Differential gene expression in cultivars

Differential expressions of genes under different combination were deduced using 4 different softwares. Levels of expression varied for different softwares due to variation in their logarithmic patterns. Heatmap representing up and downregulation of DEGs for combination 1T-2T and 1T-3T has been represented in Figs. 3 and 4, respectively using Deseq2 data. However, in this study, variations in DEGs for different combination is well explained using EdgeR data. When compared between tolerant and sensitive cultivars under Al stress condition, DEGs which were most significantly up-regulated with log₂fold change > 5 were PX domain-containing protein EREX (DN85861_c2_g4_i1), Protein LIGHT-DEPENDENT SHORT HYPOCOTYLS 1 (DN69746_c0_g1_i1), BTB/POZ domain-containing protein NPY1 (DN84834_c0_g1_i1), ATP-dependent zinc metalloprotease FTSH 7 chloroplastic (DN79925_c10_g3_i1) and those with down-regulated expression with absolute log₂fold change > 5 were Zinc-finger homeodomain protein 4 (DN81385_c0_g1_i1), Probable carboxylesterase 11 (DN80647_c0_g2_i1), Peptidyl-prolyl cis-trans isomerase 1 (DN23470_c0_g1_i1), Aldehyde dehydrogenase family 3 member F1 (DN81336_c0_g2_i2), G-type lectin S-receptor-like serine/threonine-protein kinase SD1-13 (DN64247_c0_g1_i1) (Tables. 3 and 4).

Top up-regulated DEGs in Al stressed tolerant genotype when equated to its control with log₂fold change > 8 were Retrovirus-related Pol polyprotein from transposon TNT 1-94 (DN86110_c2_g1_i1), Defensin-like protein 39 (DN27225_c0_g1_i1), Transcriptional activator DEMETER (DN83628_c1_g1_i1), Threonine synthase chloroplastic (DN66404_c0_g1_i1), ABC transporter A family member 2 (DN85483_c0_g2_i1) whereas down-regulated ones with absolute log₂fold change above 7 were Retrovirus-related Pol polyprotein from transposon TNT 1-94 (DN79346_c0_g1_i1), Protein RAE1 (DN73798_c0_g1_i1), Disease resistance protein RPP5 (DN82876_c0_g1_i5), Probable serine/threonine-protein kinase PBL23 (DN39027_c0_g1_i1), Histone-lysine N-methyltransferase ATX4 (DN61581_c0_g2_i1). Similarly, top up-regulated DEGs in Al stressed sensitive genotype when paralleled to its control with log₂fold change > 8 were Auxin-responsive protein SAUR72 (DN104348_c0_g1_i1), Putative transposon Ty5-1 protein YCL074W (DN62711_c0_g1_i1), Probable polygalacturonase (DN85347_c0_g2_i3), Protein SODIUM POTASSIUM ROOT DEFECTIVE 2 (DN74237_c0_g1_i2), Protein CLEC16A homolog (DN85397_c0_g1_i14). DEGs which were down-regulated with absolute log₂fold change > 7 were Protein CHROMATIN REMODELING 25 (DN82743_c0_g1_i1), Amidophosphoribosyltransferase 2 chloroplastic (DN85963_c1_g1_i13), AMP deaminase (DN79186_c0_g4_i1), Calcium uniporter protein 2 mitochondrial (DN58813_c0_g1_i1) and UDP-galactose/UDP-glucose transporter 3 (DN93386_c0_g1_i1).

3.4 Divergence of gene expression in cultivated and wild lentil

When *Lens culinaris* cultivars (L-4602 and BM-4) were compared to *Lens lamottei* wild (ILWL-15) for differential gene expression, most of the top significant DEGs with absolute log₂fold change above 6 were found to be down-regulated. When expression profiles of L-4602 and ILWL-15 were compared, top significantly up-regulated DEGs in wild with log₂ fold change > 5 were Shaggy-related protein kinase theta (DN86312_c0_g1_i1), Polypyrimidine tract-binding protein homolog 2 (DN82814_c6_g4_i1), Retrovirus-related Pol polyprotein from transposon TNT 1-94 (DN101107_c0_g1_i1), DDB1- and CUL4-associated

factor 8 (DN24238_c0_g1_i1), Ycf49-like protein (DN36313_c0_g1_i1). Top down-regulated DEGs with absolute log₂fold change > 7 were Uncharacterized mitochondrial protein AtMg00310 (DN76784_c0_g1_i1), Protein SRC2 (DN78575_c0_g1_i1), Uncharacterized protein ycf68 (DN75327_c1_g1_i1), Probable LRR receptor-like serine/threonine-protein kinase At3g47570 (DN79472_c0_g1_i1), Serine carboxypeptidase-like 40 (DN85896_c1_g2_i7). Similarly, when expression profiles of BM-4 and ILWL-15 were compared, top significantly up-regulated DEGs in wild with log₂ fold change > 6 were Phosphatidylinositol 4-phosphate 5-kinase 1 (DN77256_c0_g1_i1), Protein SCO1 homolog 1 mitochondrial (DN78265_c0_g1_i1), Ubiquitin carboxyl-terminal hydrolase 2 (DN27270_c0_g1_i1), Fasciclin-like arabinogalactan protein 12 (DN36749_c0_g1_i1) and Subtilisin-like protease SBT2.6 (DN63737_c0_g2_i1). Similarly, top down-regulated DEGs with absolute log₂fold change > 7 were Protein TPX2 (DN78602_c0_g1_i2), Exocyst complex component EXO70B1 (DN75066_c1_g3_i1), Vacuolar protein sorting-associated protein 54 chloroplastic (DN85213_c3_g3_i4), Octanoyltransferase (DN84556_c1_g1_i1), Protein Weak Chloroplast Movement Under Blue Light 1 (DN69079_c0_g3_i1). DEGs in AI stressed wild genotype when compared to its control were found to have differential gene expression as high as log₂fold change > 11. Interestingly, all the DEGs with absolute log₂fold change above 7.2 were found to be up-regulated. Top up-regulated DEGs in this comparison group with log₂fold change > 9 were Penta tricopeptide repeat-containing protein At3g23020 (DN85953_c0_g2_i4), U-box domain-containing protein 13 (DN76791_c1_g2_i1), 60S acidic ribosomal protein P1-2 (DN13350_c1_g1_i1), Auxin response factor 6 (DN83135_c4_g2_i1) and GDSL esterase/lipase At1g29670 (DN53067_c0_g1_i1). Top down-regulated DEGs with absolute log₂fold change > 9 were Uncharacterized mitochondrial protein AtMg00810 (DN82041_c1_g1_i6), TMV resistance protein N (DN83696_c4_g1_i1), F-box protein At2g39490 (DN83919_c3_g1_i3), Putative oxidoreductase C1F5.03c (DN78853_c0_g2_i1) and Cyclin-dependent kinase F-1 (DN81491_c0_g1_i1).

3.5 Contig annotation analogous to reference database

Contigs were sequence aligned with publically available legume species databases such as *Cicer arietinum*, *Lotus japoicus*, *Medicago sativa*, *Medicago truncatula*, *Cajanus cajan* and *Glycine max* as well as publically available protein databases namely RefSeq (Reference Sequence), SWISS-PROT, PDB (Protein Database Bank), TAIR (The Arabidopsis Information Resource), TF, UNIREF100 (Uniport Reference) and GO (Gene Ontology). Total number of contigs aligned with these databases is represented in Fig. 5. Highest number of contigs was aligned with that of *Medicago truncatula* (79142) amongst the legume database whereas TAIR showed highest number of aligned databases amongst the publically available databases. Total number of unigenes which were found to be aligned with legume database such as *Medicago truncatula*, *Arabidopsis thaliana* and *Glycine max* had 1263, 7476, 20419 hits, respectively. Highest numbers of hits were found to be aligned with Genbank database (20,419).

3.6 qRT-PCR endorsement

Validation of differential gene expression was done using a total of top 12 DEGs, 4 from each comparison group viz. 1C-1T, 2C-2T and 3C-3T (Fig. 6). ABR 18, CSA-UDP, Expansin and SAUR-72 were selected for 1C-1T comparison where ABR 18 and SAUR-72 were up-regulated and CSA-UDP and Expansin were down-regulated. For 2C-2T, 30S5, Isocitrate Lyase, PCBP3 and Peroxidase-15 genes were used where all the four genes were up-regulated. CYP450E8, HSP 17.1, IRX9, UPBEAT1 were checked for 3C-3T where first two were up-regulated and rest two were downregulated. qRT-PCR expression of these genes is represented in Fig. 6. When log₂ transformed data from qRT-PCR analysis was compared with RNA-seq data, a close similarity was revealed which further endorses differential expression of genes underneath AI stress condition.

3.7 Functional allocation of DEGs

GO terms for transcripts were extracted for functional characterization of DEGs. A total of 2857, 3403 and 2872 GO annotation terms were found in combination 1T-2T, 1T-3T and 2T-3T (Fig. 7). When all the three genotypes viz. L-4602, BM-4 and ILWL-15 were matched to their respective controls, significantly enriched GO annotation classes for DEGs which were found in all the three comparison groups were in cell, organelle, membrane, protein containing complex, supramolecular complex, membrane enclosed lumen, extracellular region, cell junction and symplast within cellular component category. In molecular function group, it was for catalytic activity, transporter activity, binding, molecular function regulator, molecular transducer, transcription regulation, antioxidant and structural molecule activity. Metabolic process, biological regulation, cellular process, localization, response to stimulus, developmental process, multi-organismal process, reproductive process, signalling, biogenesis, immune system process, growth and rhythmic process were significantly enriched in biological process category. Apart from these biological processes, cell proliferation was found to be enriched only in 2C-2T and 3C-3T comparisons. Similarly, in molecular function category, obsolete signal transducer activity was found to be lacking in 1C-1T group. Also in wild, it was present only in AI treated ILWL-15 genotype and not in its control. Further enrichment analysis was also conducted separately for top 50 DEGs in all the comparison groups and GO annotation tree was prepared (Fig. 8) which revealed three major separate enrichment categories viz. Category I included 4 interconnected processes viz. developmental processes (1666 genes), Cell wall macromolecule metabolic process (144 genes), carbohydrate metabolic process (1233 genes) and anatomical structure development (1639 genes). Category II was involved with localization (2373 genes), establishment of localization (2343 genes) and vesicle mediated transport (283 genes). Category III had most of the interconnected functional processes with a total of 66,289 genes. This category included several metabolic processes including cellular nitrogen compound, protein, RNA, heterocycle, nucleobase-containing compound, cellular protein, cellular amide along with several biosynthetic processes related to cellular, cellular nitrogen compound, cellular macromolecule, aromatic compound, heterocycle, nucleobase-containing compound, RNA, Amide and Peptide together with regulations of gene expression, cellular biosynthetic process, macromolecule biosynthetic process and nucleobase-containing compound metabolic process. Also, gene expressions, response to stimulus and macromolecule modifications were found to be enriched in this category.

3.8 Pathway analysis for aluminium stress

3.8.1 Overall pathway analysis

Overall pathways involved in cultivar and wild genotype under Al stress condition was represented through up and down regulated BINs using MapMan software (Fig. 9). BINs or sub-BINs are visual representation of genes pertaining to a functional pathway. There were 6670, 7039 and 3830 data points that were mapped out of 34,151, 33,856 and 33,396 DEGs in samples 1T, 2T and 3T under Al stress conditions, respectively. Of which, 212, 241 and 212 data points were visualized in aforesaid samples under Al stress conditions, respectively. Overall, all the samples showed similar trend of visualised BINs. However, more number of prominent BINs pertaining to cell wall was found in tolerant sample when compared to sensitive and wild ones under Al stress. Number of BINs for photorespiration, nucleotide pathways also differed within tolerant and sensitive genotypes. Further, BINs related to secondary metabolite such as terpenes, flavonoids were found to be more prominent in wild sample when compared to tolerant as well as sensitive cultivars.

3.8.2 Aluminium specific pathway analysis.

Pathway analysis of top 135 DEGs in all the six comparison groups revealed 63 unique and uncharacterized Al stress related proteins. Genes which have significantly influenced pathways under Al stress conditions belonged to organic acid synthesis and exudation, phytohormone responsive genes, Al induced ROS detoxifying enzymes & alternate pathway genes along with callose synthesis genes (Fig. 10). When tolerant genotype was compared to sensitive one, genes for enzymes which were significantly induced for organic acid synthesis include pyruvate dehydrogenase (acetyl-transferring) kinase (PDK), isocitrate dehydrogenase, succinate dehydrogenase. In tolerant genotype, genes for several organic acid transporters were significantly up-regulated when compared to its control viz. ALMT 7, 9, 12, 14. When compared to sensitive genotype, MATE was found to be significantly up-regulated in tolerant genotype. In Al induced ROS detoxifying enzymes group, genes for SOD, GPX, Peroxidase N, 64, 3, 25, 42, 47, 5, 15, 16, 53, 12 etc. were observed to be up-regulated in tolerant genotype. In addition to this mechanism for detoxification of ROS, alternate pathway was also operating in tolerant genotype. This alternate pathway has commissioned Metacaspase- 1, 4, 9 to induce programmed cell death in tolerant genotype. In phytohormone signalling pathway, when tolerant genotype was compared to control, Auxin responsive protein SAUR72, Auxin efflux carrier component 3, Abscisic acid receptor PYL4, Ethylene responsive transcription factors 9, ERF095, 1B etc. were significantly up-regulated. Callose synthesis was increased in tolerant genotype in comparison to sensitive one by up-regulation of callose synthase 3, 7 and 12. When wild genotype was compared to tolerant one, genes influencing pathways which were significantly upregulated included L-ascorbate peroxidase (APX), GPX, citrate synthase and peroxidase in ROS mediated antioxidants signalling pathway. In transporters group, putative multidrug resistance protein, ALMT 7, 12 were up-regulated. For organic acids synthesis pathway, genes for enzymes which were significantly up-regulated in wild genotype included Aconitate hydratase (Aco), Acetyl Co A carboxylase, Succinate dehydrogenase, Acetyl Co A acetyl transferase, Enoyl-Co A hydratase etc.

4. Discussion

To understand Al tolerance mechanisms in lentil, present study sheds light on differential gene expression among cultivars as well as between cultivars and wild genotypes of lentil via de novo transcriptomic examination. Contigs were sequence aligned to publically available databases and functional characterization of DEGs along with pathway prediction was done. When differential gene expression among cultivars was accomplished, top DEGs which were up-regulated between tolerant and sensitive genotypes under Al stress condition were found to be involved in protein transport, protein degradation, defence, cell growth and development. Specifically, PX domain-containing protein EREX is engaged in vacuolar transport of storage proteins. It controls membrane trafficking to protein storage vacuoles (PSVs) and attaches explicitly to phosphatidylinositol 3-monophosphate [21]. Protein Light-Dependent Short Hypocotyls 1 is a plausible transcription regulator that performs as a developmental regulator by stimulating cell growth in reaction to continuous red, far-red and blue light in a phytochrome-dependent mode [22]. BTB/POZ domain-containing protein NPY1 operates as a substrate-specific adapter of an E3 ubiquitin-protein ligase complex (CUL3-RBX1-BTB) which facilitates the ubiquitination and consequent proteasomal degradation of target proteins. It regulates cotyledon development through control of PIN1 polarity and was found to be involved in root gravitropic responses in Arabidopsis [23–25]. ATP-dependent zinc metalloprotease FTSH 7 chloroplastic is a part of a complex that functions as an ATP-dependent zinc metallopeptidase. It is engaged in thylakoid formation and in exclusion of damaged D1 in the photosystem II, averting cell death under high-intensity light conditions [26, 27]. Significantly down-regulated DEGs also have different functions which were suppressed in tolerant cultivar as compared to sensitive one. Among down-regulated DEGs, Zinc-finger homeodomain protein 4 is a putative transcription factor and is implicated in the regulation of floral induction [28]. Carboxylesterase and Aldehyde dehydrogenase family member have catalytic functions whereas protein kinase SD1-13 represses disease resistance signalling pathways [29].

Top up-regulated DEGs in tolerant control v/s treated group have catalytic activity (TNT 1–94) and antifungal activity (Defensin-like protein 39). DEMETER is involved in gene imprinting and catalyzes the discharge of 5-methylcytosine from DNA by a glycosylase/lyase process. It allows expression of the maternal copy of the imprinted MEA gene before fertilization and acts by nicking the MEA promoter. This transcriptional activator is entailed for stable reproducible patterns of floral and vegetative development [30–32]. Threonine synthase chloroplastic catalyzes removal of phosphate from L-phosphohomoserine and addition of water to yield L-threonine whereas ABC transporter A family member 2 helps in nucleotide binding. DEGs whose functions were receded have roles in catalysis (TNT 1–94, ATX4), RNA and ubiquitin binding (Protein RAE1), disease resistance (Protein RPP5) and plant defence signalling (protein kinase PBL23).

When sensitive cultivar was compared to its control, topmost DEGs whose functions were elevated have roles in cell expansion, root meristem patterning, auxin-transport (SAUR72 protein). Ty5-1 protein YCL074W has been reported as a truncated part of POL protein in mutated non-functional yeast transposon [33]. Sodium Potassium Root Defective 2 is a key up-regulated protein which is involved in metal binding thereby affecting the growth of sensitive genotype under Al stress. Down-regulated DEGs has function in DNA repair and mitotic recombination (CHROMATIN REMODELING 25) [34], chloroplast biogenesis and cell division (Amido phosphoribosyl transferase 2 chloroplastic) [35]. Calcium uniporter protein 2 mitochondrial constitutes a pore-forming and calcium-conducting subunit which is involved in calcium uptake into mitochondria whereas UDP-galactose/UDP-glucose transporter 3 is an essential sugar transporter required for pollen development and embryo sac progress [36].

When wild (ILWL-15) and tolerant cultivar (L-4602) was compared, DEGs which were significantly up-regulated in wild are involved in regulation of transcription in differentiating cells; pre-mRNA splicing; catalysis and protein ubiquitination. For example, Ycf49-like protein is a part of cell membrane protein. Similarly, when expression profiles of BM-4 (sensitive cultivar) and ILWL-15 (wild) were compared, significantly up-regulated DEGs in wild have their functions in catalysis [37], cellular copper and redox homeostasis [38], processing of poly-ubiquitin precursors as well as that of ubiquitinated proteins and in resistance to the arginine analog canavanine [39], cell surface adhesion along with endopeptidase activity. As plants lack centrosomes, so they have acentrosomal microtubule arrays. Protein TPX2 which is down-regulated in wild genotype under Al stress has microtubule binding capability and regulates pro-spindle assembly during late prophase and at the onset of mitosis [40, 41]. Other down-regulated DEGs involves EXO70B1, which is encompassed in Golgi-independent membrane traffic to the vacuole and is a positive regulator of both abscisic acid (ABA)-promoted and mannitol (drought)-endorsed stomatal closure [42, 43]. Similarly, Vacuolar protein sorting-associated protein 54 chloroplastic is involved in pollen tube elongation and other polar growth [4]. Octanoyltransferase is essential for de novo plastidial protein lipoylation during seed development [45]. Protein WEAK Chloroplast Movement Under Blue Light 1 is requisite for chloroplast avoidance response under high intensity blue light. This eschewal response results in the repositioning of chloroplasts on the anticlinal side of exposed cells. It sustains the rate of chloroplast photo relocation movement via cp-actin filaments adjustments [46].

DEGs which were up-regulated in wild genotype when compared to its control involves U-box domain-containing protein 13 which acts as a E3 ubiquitin ligase and 60S acidic ribosomal protein P1-2 which is involved in protein synthesis. Another up-regulated DEG was Auxin response factor 6 which is a type of transcriptional activator that binds precisely to the DNA sequence 5'-TGTCTC-3' located in the auxin-responsive promoter elements (AuxREs). Configuration of heterodimers with Aux/IAA proteins alters their aptitude to modify initial auxin response genes expression. It synchronizes both stamen and gynoecium maturation and fosters jasmonic acid stimulation [47, 48]. Top down-regulated DEGs included TMV resistance protein N which is a disease resistance protein and Cyclin-dependent kinase F-1 that modulates CDKD-2 and CDKD-3 activities by phosphorylation of the T-loop [49]. Thought-provokingly, in all the comparison groups involving cultivars as well as wild genotypes, top DEGs were associated with down-regulation of disease resistance genes.

In present study, result for sequence alignment with databases is way ahead of Kaur et al. (2011), which suggest that advancement in the sequencing chemistry has increased the quantity and quality of acquired data that has improved de novo assembly of non-model crop plants [50]. Further, generation of huge number of molecular markers from acquired data will help in improving the development of lentil reference genomic pool i.e. knowpulse.usask.ca. Top 12 DEGs from different comparison groups when evaluated using qRT-PCR, data were validated precisely. ABR 18 and SAUR-72 was up-regulated in 1C-1T comparison group whereas CSA-UDP and Expansin were down-regulated. Study on precocious germination of cultured immature embryos of *Pisum sativum* has showed that addition of ABA increases production of ABR-18 protein and is accumulated in testa during early seed development [51]. SAUR-72 has role in regulation of cell expansion, root meristem patterning and auxin transport. In a study conducted on tissue-specific and developmentally regulated expression patterns of SAUR genes, it was found that SAUR72 was highly expressed in the steles of *Arabidopsis* roots and hypocotyls [52]. In 2C-2T comparison group all the four genes which were selected for qRT-PCR validation, viz. 30S5, Isocitrate Lyase, PCBP3 and Peroxidase-15 were up-regulated. Isocitrate lyase is an enzyme of the glyoxylate cycle which accelerates the cleavage of isocitrate to succinate and glyoxylate. *Bradyrhizobium japonicum* isocitrate lyase has been reported to have an important functional role in desiccation tolerance [53]. Poly(C)-binding proteins (PCBPs) are commonly acknowledged as RNA-binding proteins that interact in a sequence-specific manner with single-stranded poly(C). PCBP1-4 including PCB-3 proteins are concerned chiefly in innumerable posttranscriptional regulations [54], have reported that transcriptional regulation of PCBPs might itself be standardized by their localization within the cell and it can work as a signal-dependent and coordinated regulator of transcription in eukaryotic cells. In previous studies, impediment of peroxidase was linked with Al tolerance as it helps in maintaining H₂O₂ levels desirable for non-enzymatic wall loosening [55, 56]. But similar to our study, peroxidases were up-regulated during transcriptomic profiling of wheat near isogenic lines under Al stress and were reported to be connected to decreased growth rate [6]. Since, in our study this has been up-regulated in sensitive genotype under Al stress, we cannot associate it with Al stress tolerance. Instead similar to study conducted in wheat NILs, here also it might be due to reduced growth rate under Al stress. In 3C-3T comparison group, CYP45081E8, HSP 17.1, were up-regulated and IRX9, UPBEAT1 were downregulated. CYP45081E8 is a probable monooxygenase which exhibit activity with isoflavones such as formononetin, biochanin, pseudobaptigenin, daidzein, genistein, isoformononetin and prunetin, or with flavonoids including naringenin, liquiritigenin, apigenin, luteolin, or kaempferol [57]. Up-regulated HSP 17.1 is a part of small heat shock protein (HSP20) family. These genes signify the highly profuse class amid the HSPs in plants. Hsp20 genes have been reported to be connected with stress triggered by HS and other abiotic influences in soybean [58]. Down-regulated IRX9 gene encodes a putative family 43 glycosyl transferase. It was reported to be co-ordinately articulated with the cellulose synthase subunits in the course of secondary cell wall formation [59]. Cell wall examination exposed a reduction in the richness of xylan in the irx9 mutant, demonstrating that IRX9 is essential for xylan production [60, 61]. IRX9 was also identified as MUCI65 in a reverse genetic screen for Mucilage-Related genes. Despite producing only a few seeds, the irx9-1 mutant displays normal mucilage properties [62, 63]. UPBEAT1 is a transcription factor which regulates the equilibrium between cellular propagation and differentiation in root growth. It does not act via cytokinin and auxin signaling, but by curbing peroxidase expression in the elongation zone [64].

During overall pathway analysis, more number of prominent BINs pertaining to cell wall was found in tolerant sample when compared to sensitive and wild genotypes under Al stress. Aluminium ion binds to the cell wall causing increase in cell wall rigidity [65]. Thereby, it hampers the cell wall growth and cell elongation [66]. Many systems such as efflux of organic acids, signalling due to Al ion involves the role of cell wall [67]. Enhanced activity of cell wall related genes in tolerant genotype when compared to sensitive and wild ones under Al stress affirms its involvement in Al tolerance. Also, release of secondary metabolites from the root apex is well established mechanism for Al tolerance [67]. Increase in number of BINs for secondary metabolites activity in wild indicates that the Al tolerance is rendered through production of different secondary metabolites. Further, pathway analysis of top 135 DEGs in all the comparison groups have disclosed many unique and uncharacterized Al stress related proteins which are involved in organic acid synthesis and exudation, phytohormone response, Al induced ROS detoxification, callose synthesis along with genes of alternate pathway. Plants protect against Al damage by exudation of organic acids from root apices. This exudation is accompanied by release /uptake of ions to balance membrane potential. Organic anions chelate Al³⁺ to prevent it from binding to cell membrane. PDK is involved in organic acid synthesis and plays a crucial role in intermediary metabolism by acting as a negative regulator of mitochondrial dehydrogenase complex [68]. In soybean, Al triggered organic acid secretion from roots was found to be more in tolerant genotypes than the sensitive ones, although their biosynthesis was not the rate restricting step for exudation of organic acids [69]. Transporters

acts as channels for organic acid exudation. These include members of Al activated Malate transporter (ALMT) and multidrug export protein AcrE (MATE) families which encode membrane proteins that expedite organic anion efflux across cell membrane. ALMT genes are involved in organic acid transportation which were significantly up-regulated in tolerant genotype when compared to its control. These genes are classified in the Fusaric acid resistance protein like superfamily, CL0307 and it was the first Al tolerance gene to be recognized in case of plants represented by wheat [70]. ALMT 9 is a chloride channel which is activated by physiological concentration of cytosolic malate and mediates vascular malate uptake [71]. VvALMT 9 has facilitated the accumulation of malate and tartrate in the vacuole of grape berries [72]. ALMT 12 functions as quickly activating anion channel mainly for chloride and nitrate ions [73]. The action of AtALMT12 is controlled by phosphorylation through kinase Open Stomata 1, which in turn is activated by ABA under stress conditions to regulate stomata closure [74].

When tolerant and sensitive genotypes were compared, MATE was found to be significantly up-regulated in tolerant genotype. MATEs are big lineage of proteins which functions as subordinate active carriers using electrochemical gradient of other ions [67]. Furukava et al. (2007) have identified HvAACT1 gene in barley which belongs to MATE family and is responsible for citrate exudation under Al stress [75]. Al stress alters the function of plasma membrane by interfering with membrane lipids which result in increase in highly toxic ROS. To mitigate the effects of increased ROS, Al toxicity tolerance pathway involving activation of detoxifying enzymes comes into action. Many genes for Al induced ROS detoxifying enzymes e.g. SOD, Peroxidases etc. were witnessed to be up-regulated in tolerant genotype. Similar to our findings, in roots of aspen, Fe SOD genes were also found to be up-regulated under Al stress which were identified via transcriptomics [76]. Wu et al. (2017) have identified that Arabidopsis peroxidase 64 gene (AtPrx64) was up-regulated in tobacco under Al stress and it was associated with the formation of plant secondary cell wall [77]. With affirmation to our study, they also revealed that AtPrx64 is always up-regulated under Al stress suggesting that this gene plays protective role under this type of stress. AtPrx 42 and 64 were identified by microarrays to be involved in xylem secondary wall formation [78]. AtPrx53 was expressed in vascular bundles by merging of regulatory sequence with β glucuronidase and the corresponding proteins were found to be involved in lignification [79]. Also, Metacaspases were found to be induced in tolerant genotype which structures the alternative pathway for Al stress tolerance. Metacaspases are cysteine reliant proteases present in protozoa, fungi and plants that are involved in proteolytic pathway. Metacaspase-1 is a positive regulator of cell death and needs conserved caspase like- putative catalytic residues while Metacaspase-4 is positive regulator of biotic and abiotic induced programme cell death and it does not cleave caspase specific substrate [80]. According to microarray study in poplar metacaspase 9 plays a role in xylem programmed cell death [81]. Another signalling network for Al stress tolerance involves phytohormones which activates various Al tolerance genes or suppresses root growth under Al stress conditions [82]. Some phytohormone signalling pathway genes were also up-regulated in tolerant genotype. For e.g. Auxin efflux carrier components which are required for optimal auxin distribution in root tips that is vital for modulating root growth under stress conditions [83]. The impediment of root elongation by deposition of auxin is accompanied by increased production of ethylene, which regulates auxin biosynthesis and basipetal auxin shipping in root apex [84]. Yang et al. (2017) have demonstrated that up-regulation of ethylene synthesis under Al stress suppresses root growth via jasmonic acid and cytokinin responsive pathways [85]. Al stress also induces production of callose, which is a cell wall associated polysaccharide whose synthesis is not only an early sign of Al stress in plants, but it also occupies a central position in toxicity pathway that leads to hampering of root growth [86]. Al induces callose sedimentation adjacent to plasmodesmata that could obstruct cell to cell transportation and communication [87]. Callose synthesis was also increased in tolerant genotype. Callose synthase is multi subunit membrane associated enzyme complex whose activity is primarily under post-translational control but transcriptional regulation can also ensue [88]. Most of the Al tolerance pathways described above is under control of one or the other regulation system, one being sensitive to Proton rhizotoxicity 1 (Stop 1) regulation system, which in this study has significantly contributed to Al stress response in tolerant genotype by substantial upregulation of its protein. STOP 1 is zinc finger transcription factor which plays significant functions in regulating expression of downstream genes. STOP 1 like proteins are conserved among land plant species [82]. AtSTOP1 was encompassed in the positive regulation of 3 important Al resistance genes viz. AtALMT1, AtMATE, and AtALS3 in Arabidopsis. It also controls a series of potential H^+ tolerance genes [89]. Fan et al. (2015) have isolated and characterized VuSTOP 1 gene from rice bean and found that it was mainly involved in H^+ tolerance [90].

When wild genotype was compared to tolerant cultivar, ROS mediated antioxidants signalling pathway gene were significantly upregulated. APX and GPX are key antioxidant enzymes of scavenging systems which influences maximally to hydrogen peroxide detoxification. APX is one of the highly regulated enzymes [91]. In our previous studies also, we have identified significant increase in GPX activity in wild lentil genotypes [7]. For organic acids synthesis pathway, several genes were significantly up-regulated in wild genotype, one being that of Aconitate hydratase (Aco). Aco is an enzyme highly sensitive to irreversible oxidative inactivation by H_2O_2 . This enzyme catalyzes isomerization of citrate to isocitrate via cis-aconitate in the TCA cycle. Aco have been shown to be involved in regulating tolerance to oxidative stress and cell death in Arabidopsis and Nicotiana benthamiana [92].

Conclusion

Transcriptome analysis reveals several differentially expressed genes in tolerant, sensitive as well as wild lentils in response to Al stress. De novo assembly and annotation of these DEGs has helped in elucidating prominent pathways involved in rendering tolerance toward Al toxicity in tolerant and wild genotypes. DEGs involved in ROS detoxification, exudation of organic acids, callose synthesis, phytohormones signalling were identified to be involved in stress regulation. Further, MapMan plot showed that the role of cell wall and production of secondary metabolites in tolerant genotype and wild accession, respectively has an edge for Al tolerance over the sensitive genotype under Al stress conditions. Insights of these genes, pathways and identification of different markers involved in Al tolerance will help in strengthening breeding programs for Al tolerance.

Declarations

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Data Availability

Transcriptomics data reported in this article has been deposited in Sequence Read Archive (SRA) repository of NCBI database with accession number SAMN08211543.

Consent for publication

Not applicable.

Conflict of Interest

All authors state that there are no conflicts of interest.

Ethics approval

Not applicable.

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Author Contributions

D.S., M.P., P.S., P.R.S., R.S.S., formulated the experiments. D.S., C.K.S., J.T., R.S.S.T., N.S.K executed molecular and statistical data analysis. D.S., M.P., C.K.S and J.T. drafted the manuscript. D.S., C.K.S., J.T have equally contributed. All the authors have read and approved the final manuscript.

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Figures

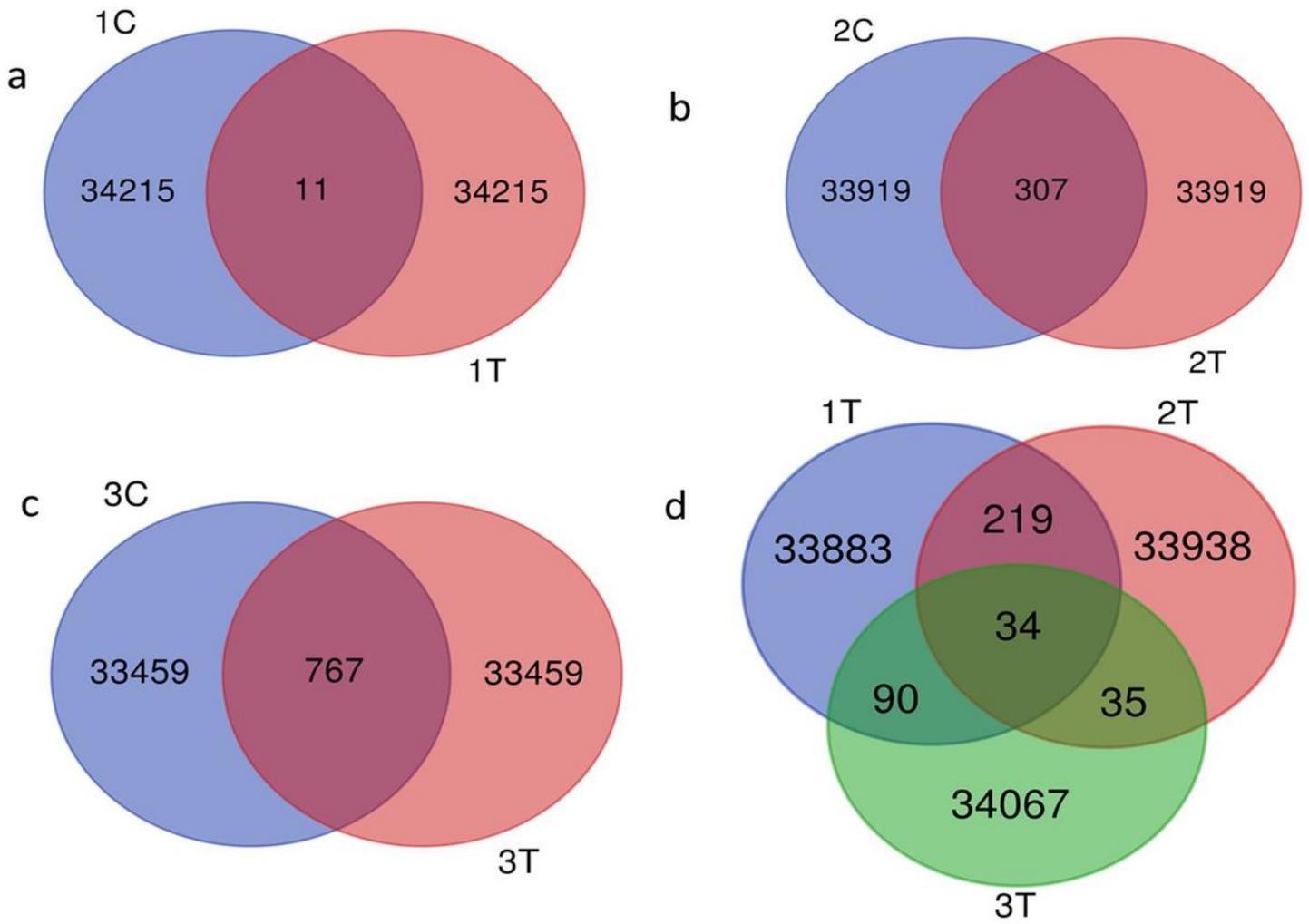


Figure 1

Number of contigs in different comparison groups.

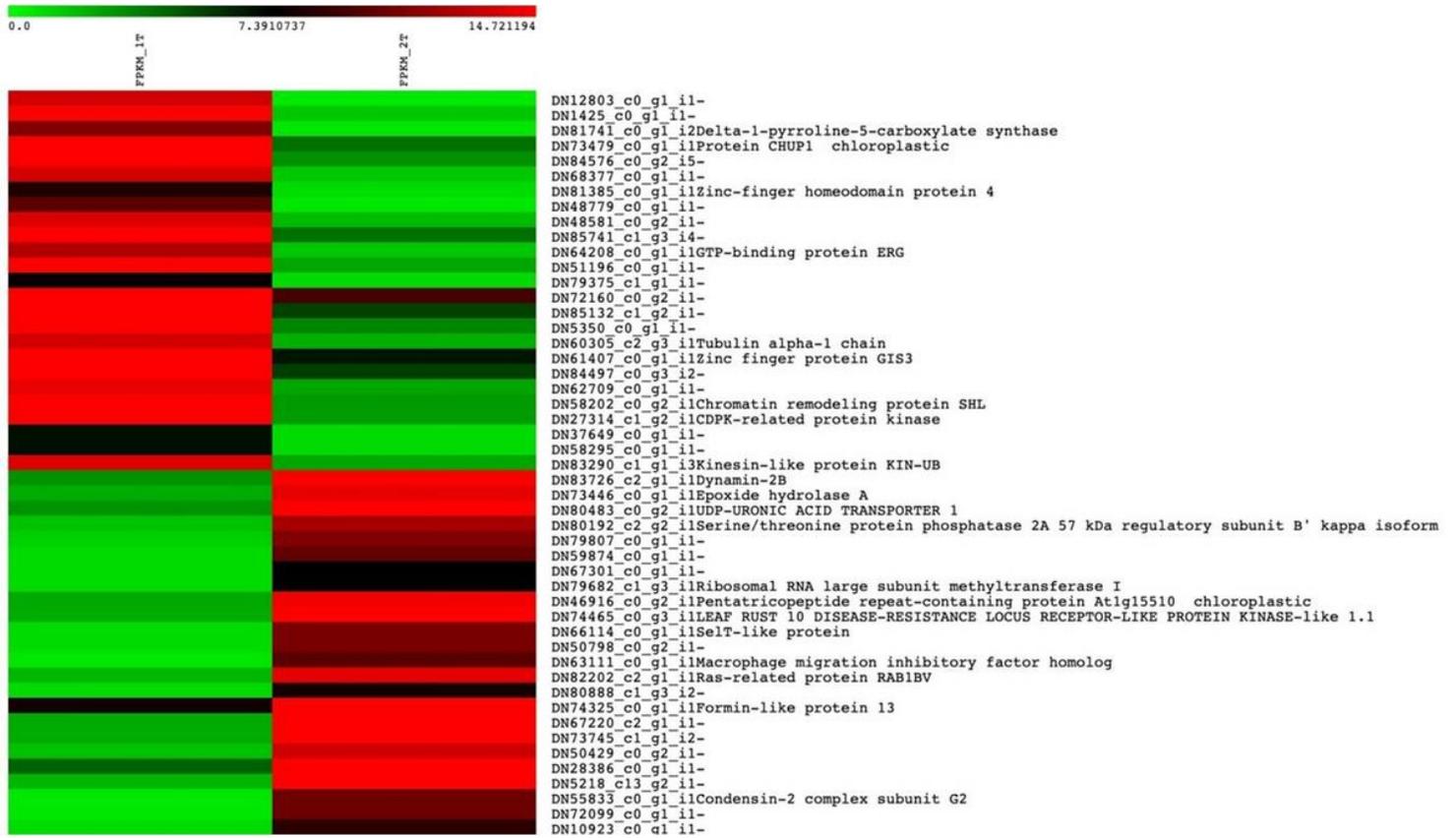


Figure 2

Top up and down regulated DEGs represented through heat map in combination 1T-2T.

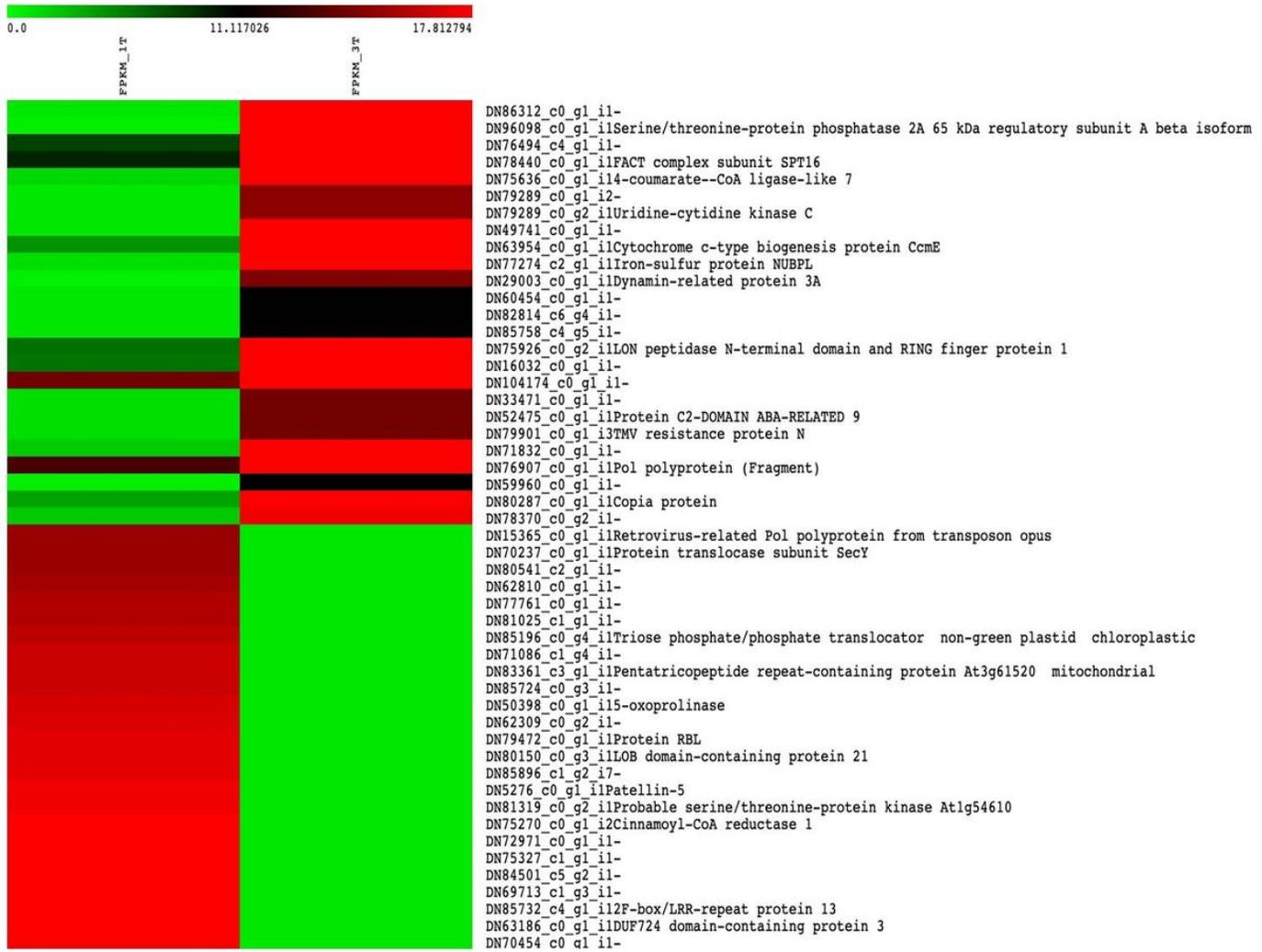


Figure 3

Top up and down regulated DEGs represented through heat map in combination 1T-3T.

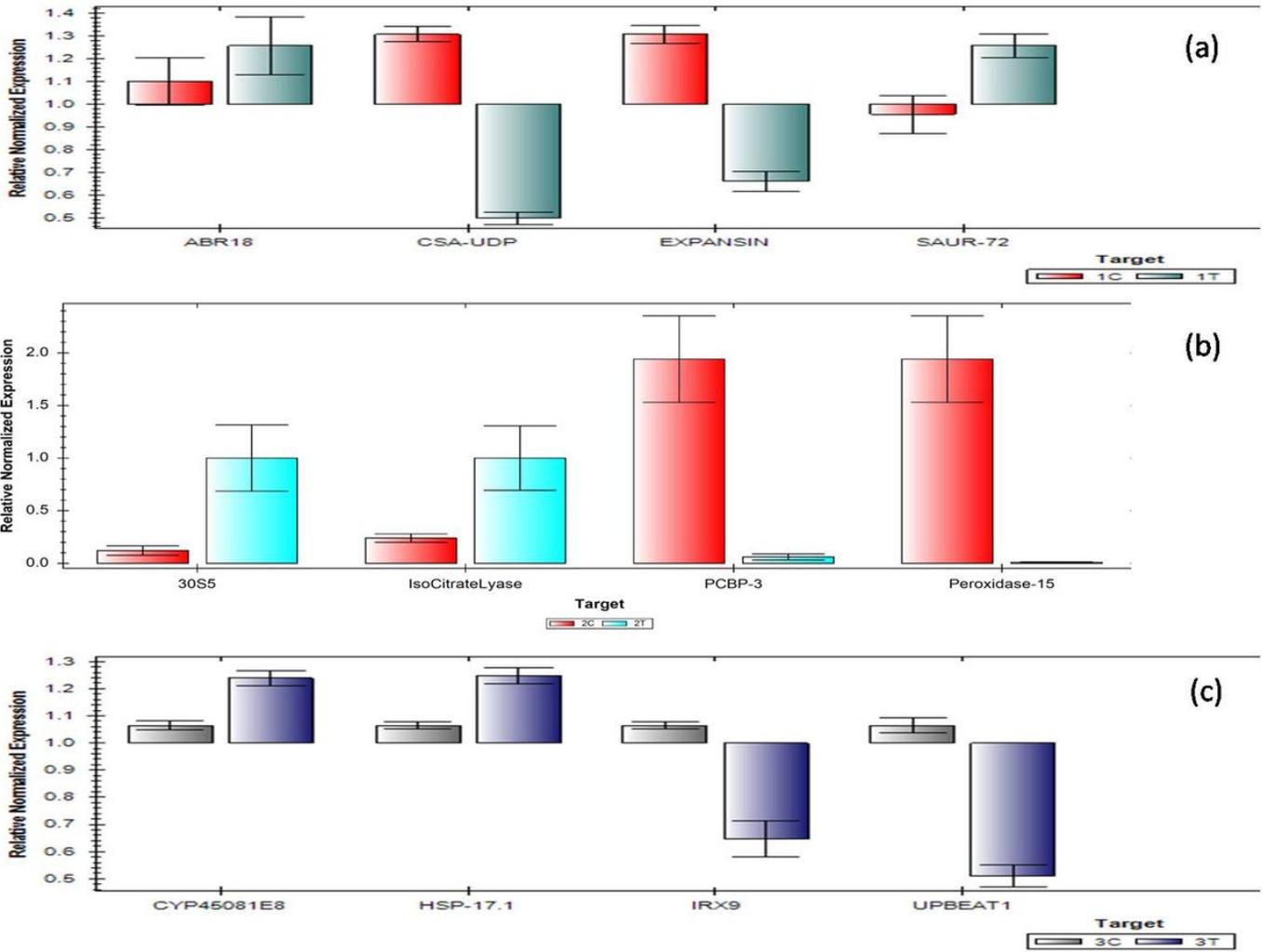


Figure 4

Real Time PCR Validation of 12 genes on 6 samples. a. 1C-1T, b. 2C-2T, c. 3C-3T

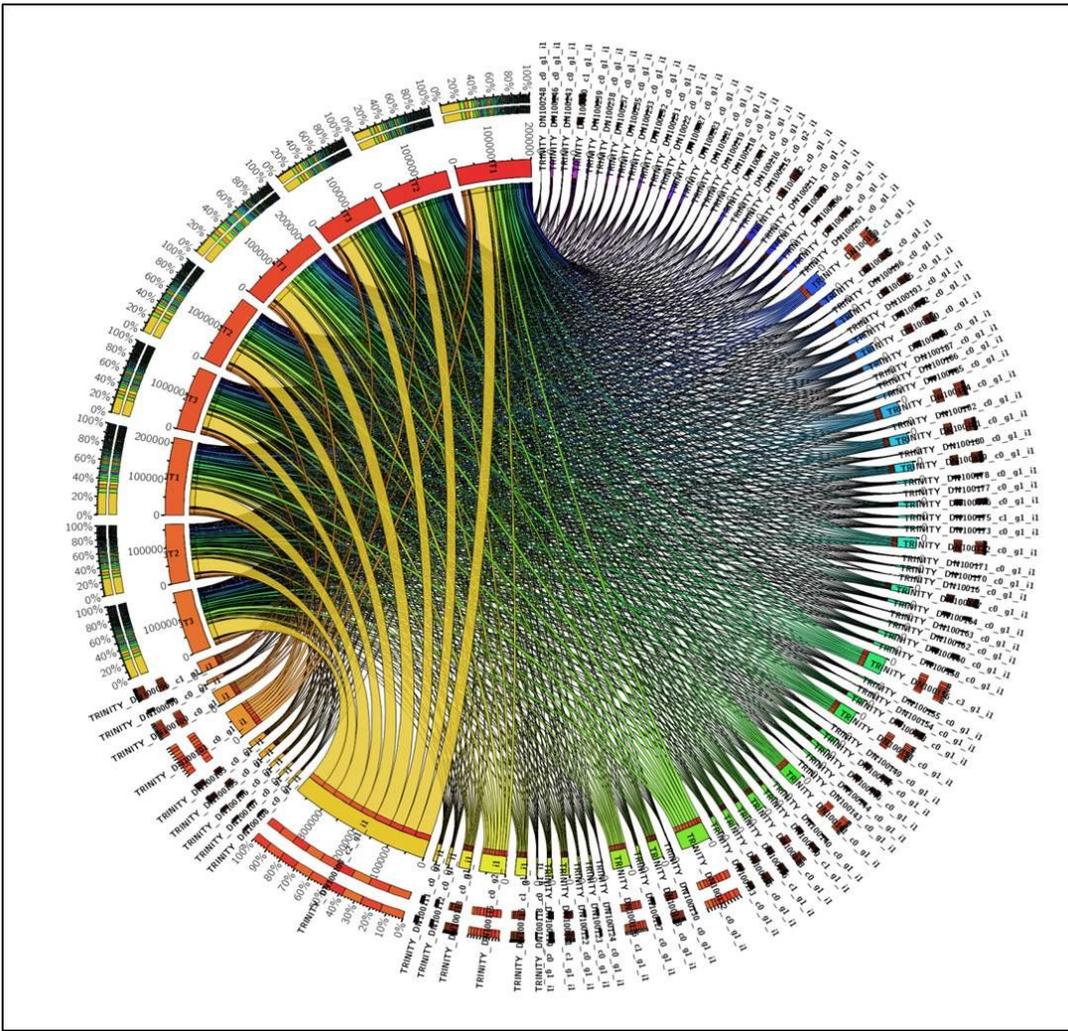


Figure 5

Circos plot representing distribution of contigs for the combination 1T-2T-3T. Ribbons represent rows & column represent IDs. The 3 outer rings are stacked bar plots that represent relative contribution of a cell to row and column totals. Expression value is expressed in colours with red colour representing lowest value & purple colour representing highest values.

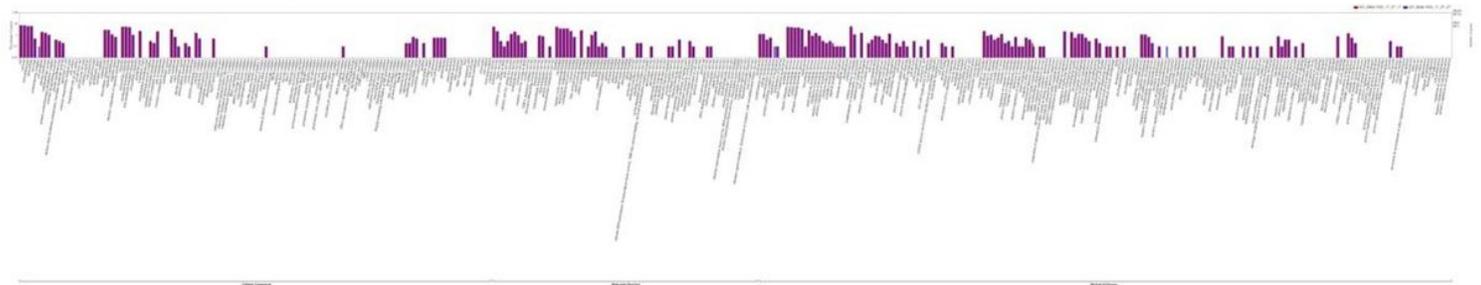


Figure 6

WEGO plot representing number of genes involved in cellular, molecular and biological functions in combination 1T-2T.

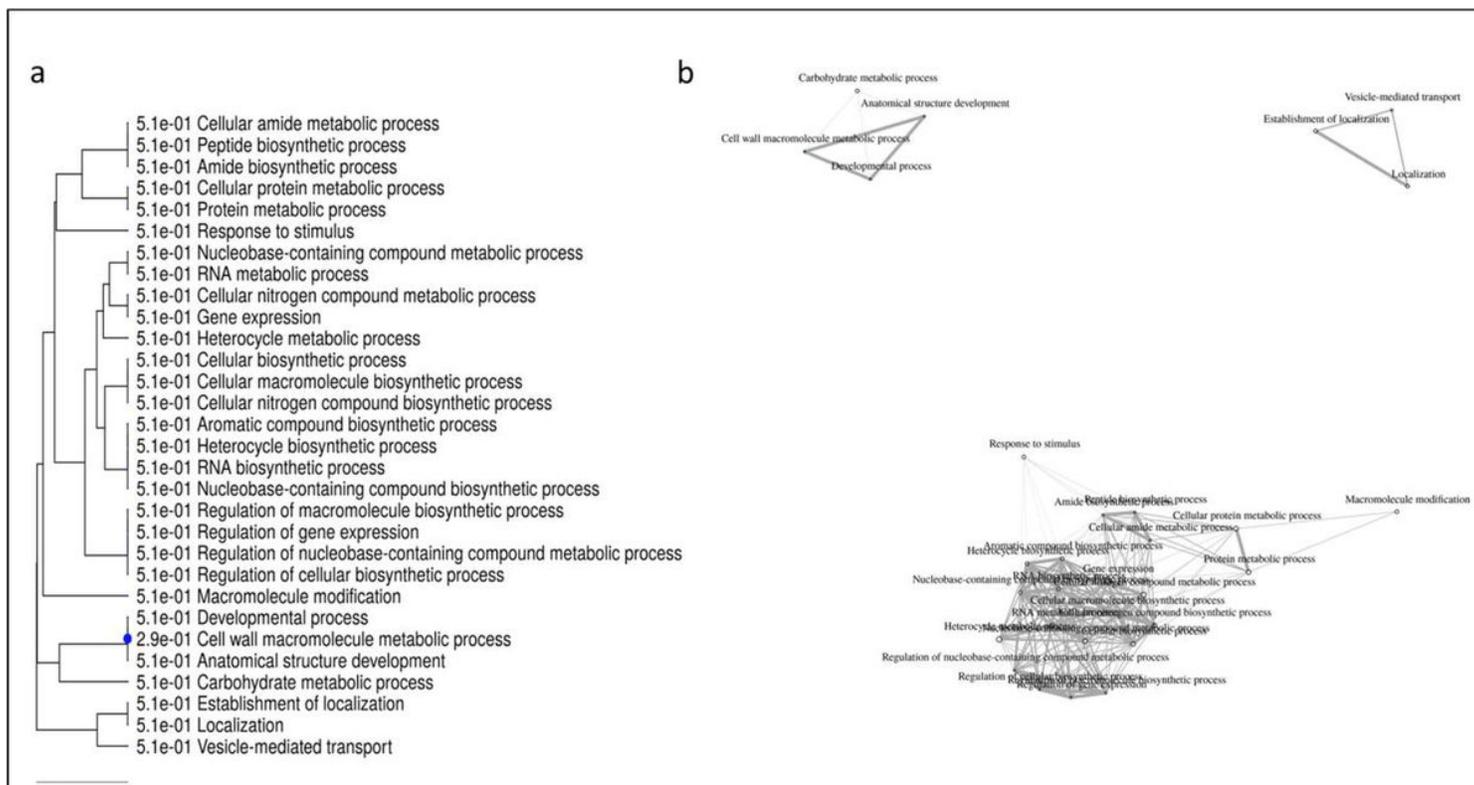


Figure 7

GO annotation tree constructed using 50 DEGs

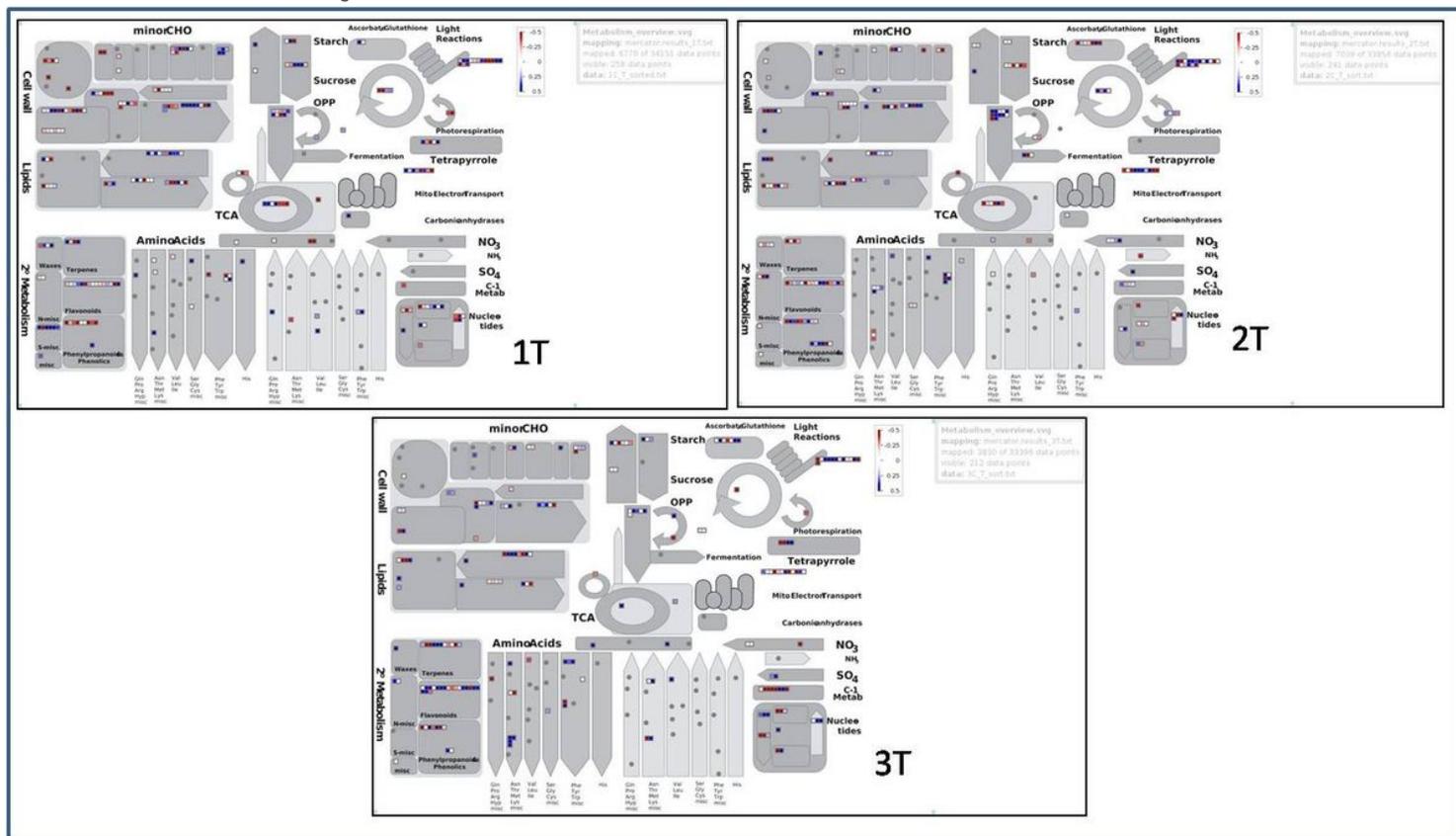


Figure 8

MapMan representation of up and down regulated BINs in tolerant and sensitive genotypes under aluminium stress conditions.

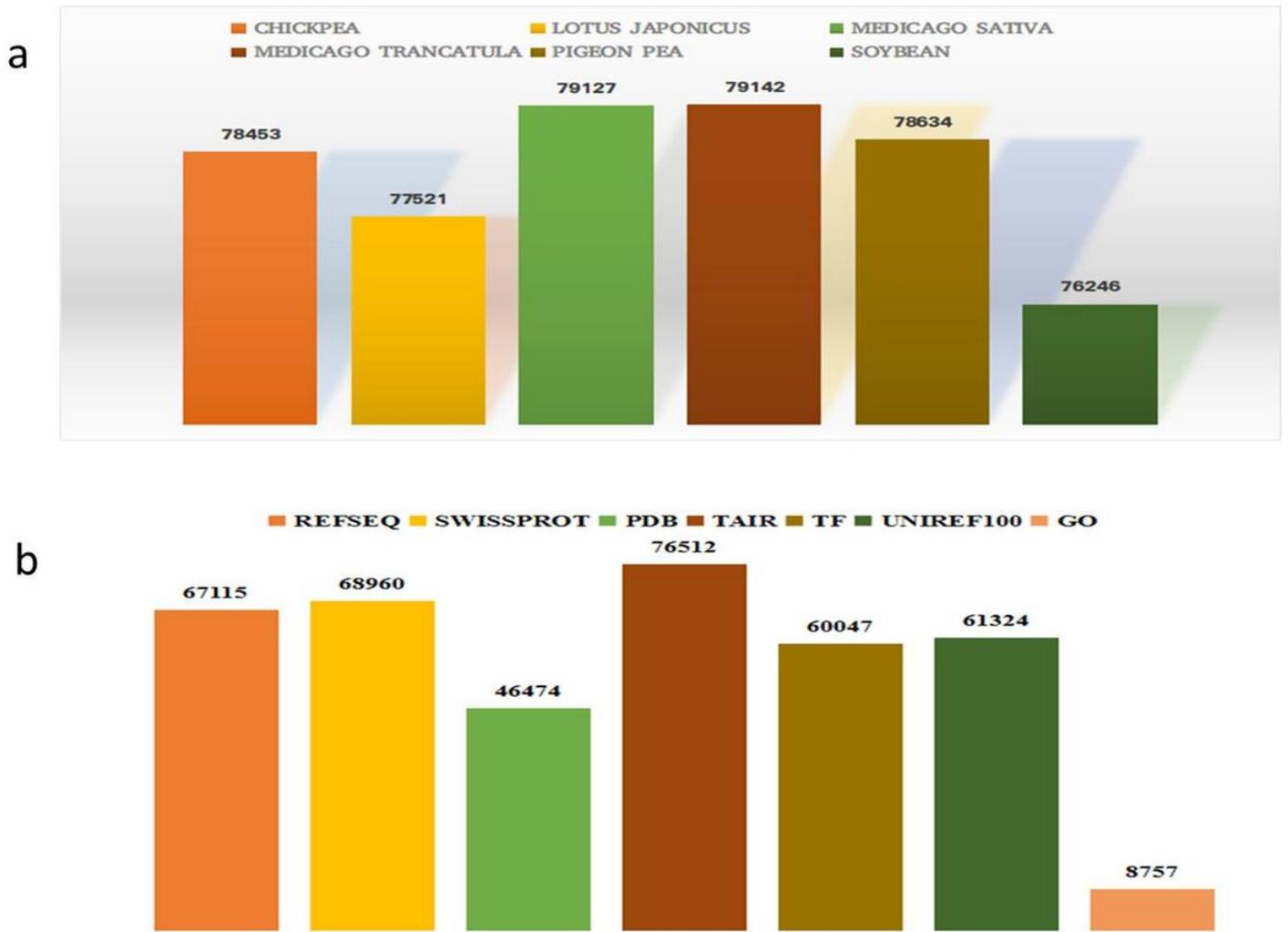


Figure 9

Blast hits of overall contigs using a. Species b. Database.

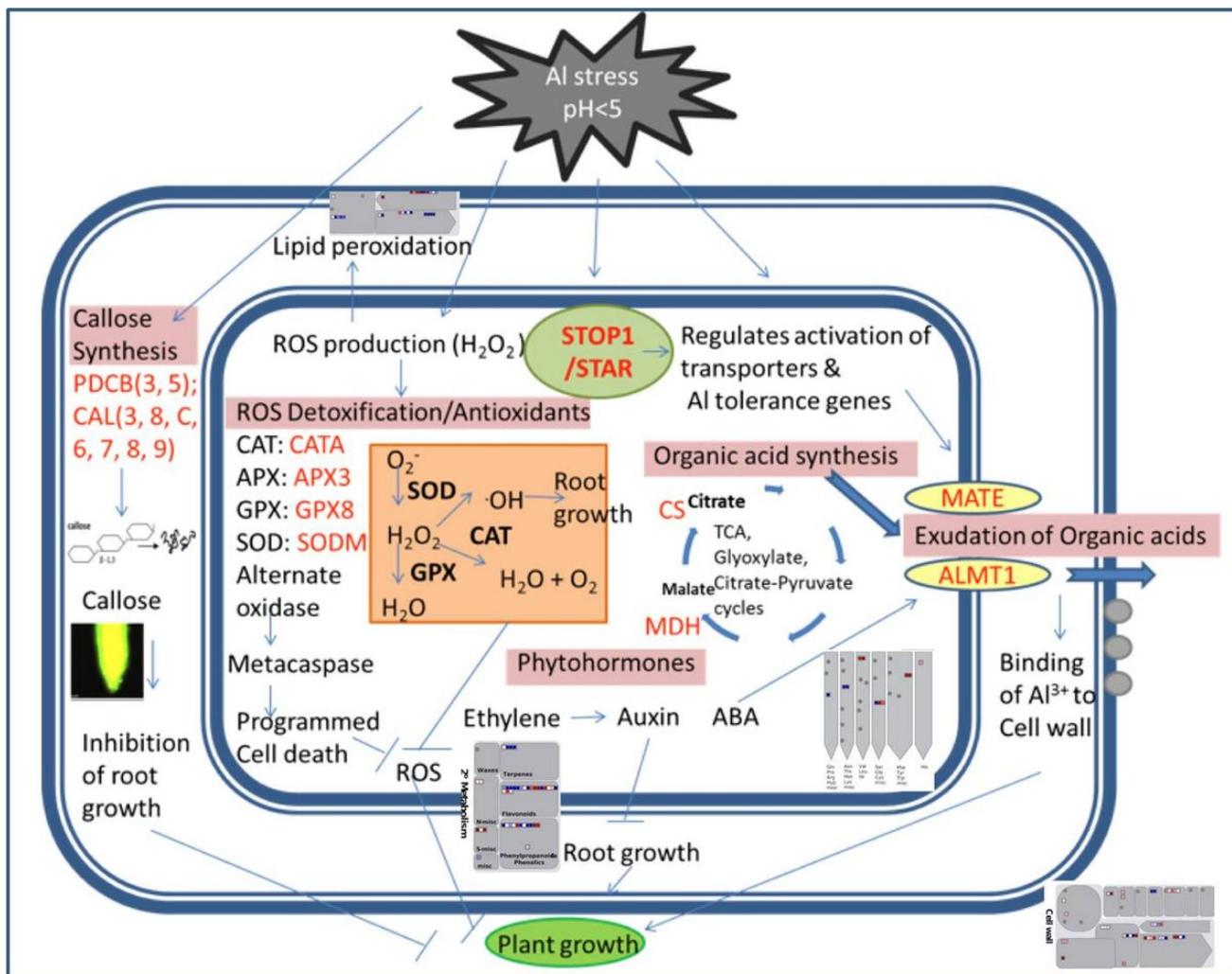


Figure 10
Representation of DEGs involved in major pathways for aluminium tolerance in lentil.

Supplementary Files

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