Transcriptomic analysis of differentially expressed genes in leaves and roots of two alfalfa (*Medicago sativa* L.) cultivars with different salt tolerance

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**Abstract**

**Background:** Alfalfa (*Medicago sativa* L.) production decreases under salt stress. Identification of genes associated with salt tolerance in alfalfa is essential for the development of molecular markers used for breeding and genetic improvement.

**Result:** An RNA-Seq technique was applied to identify the differentially expressed genes (DEGs) associated with salt stress in two alfalfa cultivars: salt tolerant ‘Halo’ and salt intolerant ‘Vernal’. Leaf and root tissues were sampled for RNA extraction at 0 h, 3 h, and 27 h under 12 dS m⁻¹ salt stress maintained by NaCl. The sequencing generated a total of 381 million clean sequence reads and 84.8% were mapped on to the alfalfa reference genome. A total of 237 DEGs were identified in leaves and 295 DEGs in roots of the two alfalfa cultivars. In leaf tissue, the two cultivars had a similar number of DEGs at 3 h and 27 h of salt stress, with 31 and 49 DEGs for ‘Halo’, 34 and 50 for ‘Vernal’, respectively. In root tissue, ‘Halo’ maintained 55 and 56 DEGs at 3 h and 27 h, respectively, while the number of DEGs decreased from 42 to 10 for ‘Vernal’. This differential expression pattern highlights different genetic responses of the two cultivars to salt stress at different time points. Interestingly, 28 (leaf) and 31 (root) salt responsive candidate genes were highly expressed in ‘Halo’ compared to ‘Vernal’. This differential expression pattern highlights different genetic responses of the two cultivars to salt stress at different time points. Interestingly, 28 (leaf) and 31 (root) salt responsive candidate genes were highly expressed in ‘Halo’ compared to ‘Vernal’. These candidate genes were common for leaf and root tissues. About 60% of DEGs were assigned to known gene ontology (GO) categories. The genes were involved in transmembrane protein function, photosynthesis, carbohydrate metabolism, defense against oxidative damage, cell wall modification and protection against lipid peroxidation. Ion binding was found to be a key molecular activity for salt tolerance in alfalfa under salt stress.

**Conclusion:** The identified DEGs are significant for understanding the genetic basis of salt tolerance in alfalfa. The generated genomic information is useful for molecular marker development for alfalfa genetic improvement for salt tolerance.

**Keywords:** Alfalfa, Differentially expressed genes, Salt stress, Transcriptome
Background
Alfalfa (*Medicago sativa* L.) is an important forage legume in the world. Cultivated alfalfa is an outcrossing autotetraploid (2n = 4x = 32) with a genome size of 800–1000 Mb [1]. Although alfalfa is regarded as moderately tolerant to salinity [2], alfalfa yield reduces by approximately 6–7% for each dS m⁻¹ increase above a salinity of 2 dS m⁻¹ [3]. To stabilize alfalfa production under saline regions, the development of superior salt tolerant cultivars becomes an important breeding goal. Identification of candidate genes for salt tolerance can increase the accuracy of parental selection as this trait has low heritability [4]. Salt tolerance is a complex trait controlled by multiple genes, involving different signaling pathways, osmotic tolerance, ion transport, compartmentalization of salt ions in vacuoles, the synthesis of plant hormones and photosynthesis [5].

Next-generation sequencing technologies have been used to identify candidate genes involved in salt tolerance of alfalfa. Transcriptomic studies in the 1-week old root tissue of alfalfa under salt stress found 1165 DEGs, including 86 transcription factors, which are responsible for stress tolerance, kinase, hydrolase, and oxidoreductase activities [6]. Luo et al. [7] identified 8861 DEGs in 12-day old seedlings of alfalfa under salt stress, which are responsible for ion homeostasis, antiporter, signal perception, signal transduction, transcriptional regulation, and antioxidative defense. Lei et al. [8] revealed 2237 DEGs between salt tolerant and intolerant alfalfa cultivars and found a salt tolerant alfalfa cultivar maintained relatively stable expression of genes responsible for reactive oxygen species and Ca²⁺ pathway, phytohormone biosynthesis and Na⁺/K⁺ transport under stress. Gruber et al. [9], using bulked genotypes as replications, studied transcriptomes in alfalfa and found genes responsible for numerous functions in a salt intolerant alfalfa cultivar. In recent years, genetic modification of certain genes controlling salt tolerance have also been conducted in alfalfa. Overexpression of salt responsive genes or transcription factors had improved salt tolerance in transgenic alfalfa. Such genes include *AlfSn1* [10], *AVP1* [11], *GmDREB1* [12], *SsNHX1* [13], *TaNHX2* [14], *GscBRLK* [15], *GsZFP1* [16], *OsAPX2* [17], *SeNHX1* [18], *AtNDPK2* [19], *AgcdA* [20], and *GswRKY20* [21]. These studies have advanced our understanding of the genetic control for salt tolerance in alfalfa. However, most studies mainly focused on single time point sampling of root tissue at the seedling stage after salt stress, limiting the analysis of the temporal expression of genes affecting salt tolerance.

Tissue specific protein induction is regulated during salinity stress and is unique to roots and shoots [22]. Thus, there should be tissue specific transcriptomic responses [23–25]. Although the root is the first receptor of salt stress [6, 7], leaf tissue is the main energy source for plant growth and stress tolerance during active growth and developmental stages. To advance our knowledge about the temporal gene expression in different tissues for the genetic control under salt stress between tolerant and intolerant cultivars, we conducted a RNA-Seq analysis with the objective to simultaneously analyze gene expressions of leaf and root tissues of two alfalfa cultivars with different tolerance to salinity after exposing them to 12 dS m⁻¹ of electrical conductivity salt stress for 0 h, 3 h, and 27 h. The analysis was fruitful with the identification of many unique genes conditioning salt tolerance in alfalfa.

Results
High throughput sequencing and assembly
A total of 408 million raw sequence reads were generated using the Illumina HiSeq sequencing platform. The reads were reduced to 93.5% (381 million clean reads) by removing adapter contamination and reads with length lower than 36 bp (Table 1). There were 84.8% of clean reads mapped to the alfalfa reference genome using STAR (v2.6.1a). The samples showed high percentages (78.8–92.4%) of mapping with the alfalfa reference genome except for ‘Halo’ root tissue sampled at 27 h of salt stress.

Differentially expressed genes (DEGs)
In leaf tissue, there were 237 DEGs identified between the two alfalfa cultivars. Among them, 34 DEGs were expressed at all three time points (0 h, 3 h, and 27 h) and 17 DEGs expressed after exposing to salt stress (3 h and 27 h) (Fig. 1a, b; Additional file 1: Table S1). Of these DEGs, 39, 31, and 49 DEGs were specific to ‘Halo’, and 34, 34, and 50 DEGs were specific to ‘Vernal’ at 0 h, 3 h and 27 h of salt stress, respectively (Fig. 1b). The number of DEGs in leaf tissue decreased after 3 h compared to 0 h treatment. Then, the number of DEGs increased from 3 h to 27 h of salt stress for both cultivars (Fig. 1b).

In root tissue, a total of 295 DEGs were identified between the two alfalfa cultivars. There were 33 DEGs expressed at all three time points and 5 DEGs expressed after exposing to salt stress (Fig. 2a, b; Additional file 1: Table S2). Of these DEGs, 68, 55, and 56 DEGs were specific to ‘Halo’ at 0 h, 3 h and 27 h, whereas 64, 42, and 10 DEGs were specific to ‘Vernal’, respectively (Fig. 2b). The number of DEGs in root tissue decreased after 3 h as compared to 0 h treatment for both cultivars, but the decrease was greater for ‘Vernal’ than for ‘Halo’. The main difference of DEGs between the two cultivars in the root was from 3 h to 27 h, with a 76% decrease in DEGs in ‘Vernal’ while there was almost no change for ‘Halo’ (Fig. 2b). After 27 h of salt stress in root tissue,
Table 1 Summary of Illumina sequencing data and mapped sequence reads for the assayed alfalfa samples

| Genotype | Tissue | Treatment | Biological replicate | Total reads | Mapped reads | Mapping rate (%) |
|----------|--------|-----------|----------------------|-------------|--------------|-----------------|
| Halo     | Leaf   | Control   | 1                    | 7,235,661   | 6,275,026    | 86.7            |
| Halo     | Leaf   | Control   | 2                    | 7,462,991   | 6,607,722    | 88.5            |
| Halo     | Leaf   | Control   | 3                    | 7,532,603   | 6,371,474    | 84.6            |
| Halo     | Leaf   | Control   | 4                    | 7,163,647   | 6,286,906    | 87.8            |
| Halo     | Leaf   | Stressed (3h) | 1          | 7,170,087   | 6,291,489    | 87.7            |
| Halo     | Leaf   | Stressed (3h) | 2          | 8,619,083   | 7,531,679    | 87.4            |
| Halo     | Leaf   | Stressed (3h) | 3          | 7,275,223   | 6,453,594    | 88.7            |
| Halo     | Leaf   | Stressed (3h) | 4          | 7,346,150   | 6,417,574    | 87.4            |
| Halo     | Leaf   | Stressed (27h) | 1         | 7,234,036   | 5,968,374    | 82.5            |
| Halo     | Leaf   | Stressed (27h) | 2         | 7,186,154   | 6,225,839    | 86.6            |
| Halo     | Leaf   | Stressed (27h) | 3         | 6,256,894   | 5,471,745    | 87.5            |
| Halo     | Leaf   | Stressed (27h) | 4         | 5,696,229   | 4,741,642    | 83.2            |
| Root     | Leaf   | Control   | 1                    | 7,930,008   | 6,833,311    | 86.2            |
| Root     | Leaf   | Control   | 2                    | 7,568,654   | 6,104,814    | 80.7            |
| Root     | Leaf   | Control   | 3                    | 10,017,590  | 9,054,027    | 90.4            |
| Root     | Leaf   | Control   | 4                    | 6,523,142   | 5,561,719    | 85.3            |
| Root     | Leaf   | Stressed (3h) | 1          | 9,003,316   | 7,662,274    | 85.1            |
| Root     | Leaf   | Stressed (3h) | 2          | 11,023,879  | 9,201,753    | 83.5            |
| Root     | Leaf   | Stressed (3h) | 3          | 9,647,653   | 8,529,106    | 88.4            |
| Root     | Leaf   | Stressed (3h) | 4          | 6,201,499   | 4,884,633    | 78.8            |
| Root     | Leaf   | Stressed (27h) | 1         | 6,563,910   | 4,983,668    | 75.9            |
| Root     | Leaf   | Stressed (27h) | 2         | 8,321,924   | 3,727,203    | 44.8            |
| Root     | Leaf   | Stressed (27h) | 3         | 9,424,651   | 7,313,948    | 77.6            |
| Root     | Leaf   | Stressed (27h) | 4         | 7,942,407   | 2,854,933    | 35.9            |
| Vernal   | Leaf   | Control   | 1                    | 7,085,736   | 6,147,440    | 86.8            |
| Vernal   | Leaf   | Control   | 2                    | 7,149,929   | 6,449,664    | 90.2            |
| Vernal   | Leaf   | Control   | 3                    | 8,108,009   | 7,362,827    | 90.8            |
| Vernal   | Leaf   | Control   | 4                    | 10,775,421  | 9,953,409    | 92.4            |
| Vernal   | Leaf   | Stressed (3h) | 1          | 5,372,062   | 4,901,710    | 91.2            |
| Vernal   | Leaf   | Stressed (3h) | 2          | 6,180,723   | 5,477,930    | 88.6            |
| Vernal   | Leaf   | Stressed (3h) | 3          | 7,355,464   | 6,390,995    | 86.9            |
| Vernal   | Leaf   | Stressed (3h) | 4          | 6,775,443   | 6,098,490    | 90.0            |
| Vernal   | Leaf   | Stressed (27h) | 1         | 11,398,645  | 10,388,765   | 91.1            |
| Vernal   | Leaf   | Stressed (27h) | 2         | 7,517,258   | 6,754,908    | 89.9            |
| Vernal   | Leaf   | Stressed (27h) | 3         | 8,128,644   | 7,280,110    | 89.6            |
| Vernal   | Leaf   | Stressed (27h) | 4         | 7,713,476   | 7,007,553    | 90.8            |
| Root     | Leaf   | Control   | 1                    | 11,004,685  | 9,744,748    | 88.6            |
| Root     | Leaf   | Control   | 2                    | 10,402,513  | 9,091,041    | 87.4            |
| Root     | Leaf   | Control   | 3                    | 8,070,744   | 7,001,864    | 86.8            |
| Root     | Leaf   | Control   | 4                    | 8,936,244   | 7,616,107    | 85.2            |
| Root     | Leaf   | Stressed (3h) | 1          | 8,293,827   | 7,159,751    | 86.3            |
| Root     | Leaf   | Stressed (3h) | 2          | 8,722,568   | 7,705,076    | 88.3            |
| Root     | Leaf   | Stressed (3h) | 3          | 9,769,559   | 8,854,805    | 90.6            |
| Root     | Leaf   | Stressed (3h) | 4          | 6,402,547   | 5,317,429    | 83.1            |
the number of DEGs in ‘Halo’ were five times more than that of ‘Vernal’ (Fig. 2b).

Functional annotation of DEGs
To understand what biological processes are implicated in response to salinity, we assigned the DEGs to known Gene Ontology (GO) categories. Among 237 DEGs in leaf tissue, 148 (62.4%) DEGs were assigned to three ontology classes. In ‘Halo’ leaf tissue, the most noticeable DEGs [false discovery rate (FDR) < 0.05] were “drug binding” (GO:0008144, 5), “anion binding” (GO:0043168, 8), “ion binding” (GO:0043167, 15) and “catalytic activity” (GO:0003824, 24) among molecular functions (Fig. 3a) while there was no significantly enriched functional groups from biological process and cellular component. For ‘Vernal’ leaf tissue, “cofactor binding” (GO:0048037, 7) and “oxidoreductase activity” (GO:0016491, 11) were predominant (FDR < 0.05) among molecular functions (Fig. 3b) and “oxidation-reduction process” (GO:0005114, 10) (Fig. 3c) in biological process, but there was not any significantly enriched functional groups from cellular component.

Among the 295 DEGs in root tissue, 180 (61.0%) DEGs were annotated to three gene ontology classes. In root tissue of ‘Halo’, “anion binding” (GO:0043168, 9), “ion binding” (GO:0043167, 18), “structural constituent of ribosome” (GO:0003735, 7), and “structural molecule activity” (GO:0005198, 7) among molecular functions (Fig. 4a) were noticeable, while “organonitrogen compound metabolic process” (GO:1901564, 15) was dominant among biological processes (Fig. 4b). “Ribosome” (GO:0005840, 7), “ribonucleoprotein complex” (GO:1990904, 8), “intracellular ribonucleoprotein complex” (GO:0030529, 8) were predominant in cellular components (Fig. 4c). For root tissue of ‘Vernal’, “anion binding” (GO:0043168, 9) and “drug binding” (GO:0008144, 5) (Fig. 4d) were significantly (FDR < 0.05) enriched, while no other functional group from biological processes and cellular components.

To identify pathways involved in salt tolerance, we carried out Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis of the DEGs. In total, 64 (27%) DEGs from leaf tissue and 86 (29.15%) DEGs from root tissue were assigned to 65 KEGG pathways (Table 2). In both tissues, the most significant DEGs were represented in the pathways of metabolism and biosynthesis of secondary metabolites. Of these, five pathways were common among different time points and alfalfa tissues. The highest level of enriched DEGs were in 14 pathways in leaf tissue and 6 pathways in root tissue after 27 h of salt stress. Among these pathways, the three highest enriched DEGs were involved in plant hormone signal transduction.

Candidate genes to enhance salt tolerance in alfalfa
The detected DEGs can be classified into two major groups for the candidate genes responsible for salt tolerance in alfalfa: 1) genes consistently expressed under short-term and long-term salt stress (3 h and 27 h) in ‘Halo’, and 2) the genes consistently expressed at all three time points in ‘Halo’. In the first group, there were 13 genes (11 in leaf; 2 in root) consistently expressed at both 3 h and 27 h of salt stress. While in the second group, there were 46 genes (17 in leaf, 29 in root) consistently expressed at all three time points. Thirteen candidate genes were highly expressed in both leaf and root tissues of ‘Halo’ as compared to ‘Vernal’, while 15 and 18 candidate genes revealed tissue specific expression in the leaf and root tissues of ‘Halo’, respectively (Tables 3, 4, and 5). Among the genes expressed in both tissues, MS.gene029203 (F-box/LRR-repeat protein 4) showed increasing expression with time in both leaf and root tissues of ‘Halo’, while MS.gene049294 (caffeic acid 3-O-methyltransferase) showed increasing expression with time in leaf tissue and MS.gene01091 (T-complex protein 1 subunit gamma) and MS.gene32989 (hypothetical protein TSUD_06780) showed increasing expression with time only in root tissue. Among the genes with leaf tissue specific expression, MS.gene029201 (replication

Table 1 Summary of Illumina sequencing data and mapped sequence reads for the assayed alfalfa samples (Continued)
protein A 70 kDa DNA-binding subunit C), MS.gene029206 (FAD synthetase 1, chloroplastic), and MS.gene24098 (thioredoxin-like protein CDSP32 chloroplastic-like) showed increasing expression with time. Among the genes with root tissue specific expression, MS.gene011517 (14 kDa proline-rich protein DC2.15) and MS.gene013923 (histone lysine N-methyltransferase, H3 lysine-9 specific SUVH1), had higher and consistent expression with time under salt stress.

In addition, there were also genes consistently expressed under salt stress in leaf (Additional file 1: Table S1) and root (Additional file 1: Table S2) tissues of ‘Vernal’. In ‘Vernal’, there were 21 (17 in leaf; 4 in root) genes consistently expressed at all three time points and 9 (6 in leaf; 3 in root) genes consistently expressed at both 3 h and 27 h of salt stress.

**Identification of single nucleotide polymorphisms (SNPs)**

The relative distribution of identified SNPs over alfalfa chromosome are presented in Fig. 5. A total of 74,705 SNPs were identified in this study, among which 37,527 were from ‘Halo’ and 37,178 were from ‘Vernal’. Minimum number of SNPs were found in Chr6.4 while maximum number of SNPs were detected in Chr4.4 (Fig. 5).
Discussion
This study generated a unique set of differentially expressed genes associated with salt tolerance in alfalfa. This finding is not only significant for understanding the temporal expression of genes conditioning salt tolerance in alfalfa, but also can be used to characterize alfalfa breeding material and develop molecular markers for salt tolerance selection. First, 84.8% of sequence reads were mapped onto the alfalfa reference genome. Secondly, 237 DEGs in leaf and 295 DEGs in root tissues were identified between the two alfalfa cultivars. Third, this study was able to determine candidate genes

![Figure 3](image_url)

**Fig. 3** Gene ontology (GO) analysis of differentially expressed genes in leaf tissues of two alfalfa cultivars in response to salt stress (a, salt tolerant ‘Halo’ alfalfa; b and c, salt intolerant ‘Vernal’ alfalfa). Each box shows the GO term number, p-value in parenthesis, and GO term

![Figure 4](image_url)

**Fig. 4** Gene ontology (GO) analysis of differentially expressed genes in leaf tissues of two alfalfa cultivars in response to salt stress (a, salt tolerant ‘Halo’ alfalfa; b and c, salt intolerant ‘Vernal’ alfalfa). Each box shows the GO term number, p-value in parenthesis, and GO term
Table 2 Number of differentially expressed genes (DEGs) and corresponding pathways in leaf and root of salt tolerant ‘Halo’ and salt intolerant ‘Vernal’ alfalfa cultivars at 0, 3 and 27 h of salt stress

| Pathway ID | The number of differentially expressed genes | Pathway terms |
|------------|---------------------------------------------|---------------|
| K00130     | 0 0 0 1 1 1                               | betB, gbsA; betaine-aldehyde dehydrogenase |
| K00276     | 0 0 1 0 0 0                               | AOC3, AOC2, tyrA; primary-amine oxidase |
| K00430     | 0 0 1 0 0 0                               | E1,11.1.7; peroxidase |
| K00454     | 0 0 1 0 0 0                               | LOX25; lipoxigenase |
| K00522     | 0 0 1 0 0 0                               | FTH1; ferritin heavy chain |
| K00660     | 1 1 1 1 1 1                               | metE; S-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase |
| K00915     | 0 0 0 1 0 0                               | CHS; chalcone synthase |
| K01507     | 0 0 1 1 1 1                               | IPMK, IPK2; inositol-polyphosphate multikinase |
| K01535     | 0 0 1 1 0 0                               | ppa; inorganic pyrophosphatase |
| K01623     | 1 1 1 1 1 1                               | idi, IDI; isopentenyl-diphosphate Delta-isomerase |
| K01823     | 1 1 1 1 1 1                               | ES.S.1.6; chalcone isomerase |
| K02639     | 1 1 2 1 1 0                               | pef; ferredoxin |
| K02933     | 1 0 0 1 2 1                               | psbW; photosystem II PsbW protein |
| K02991     | 0 0 1 1 1 1                               | RP-L23Ae, RPL23A; large subunit ribosomal protein L23Ae |
| K03006     | 0 0 1 0 0 0                               | RP-L3, MRPL3, rplC; large subunit ribosomal protein L3 |
| K03028     | 0 0 0 0 0 0                               | RP-L3e, RPL3; large subunit ribosomal protein L3e |
| K03071     | 0 0 1 1 1 1                               | RP-S21e, RPS21; small subunit ribosomal protein S21e |
| K03298     | 0 0 0 1 0 0                               | RP-S2e, RPS2; small subunit ribosomal protein S2e |
| K04085     | 0 0 0 0 0 0                               | RP-S3e, RPS3; small subunit ribosomal protein S3e |
| K04091     | 0 0 0 0 0 0                               | RP-S6e, RPS6; small subunit ribosomal protein S6e |
| K07323     | 2 2 2 2 2 2                               | EEF1A; elongation factor 1-alpha |
| K07328     | 0 0 0 0 0 0                               | HSPA1s; heat shock 70kDa protein 1/2/6/8 |
| K07336     | 0 0 1 0 0 0                               | CDH1; cell division cycle 20-like protein 1, cofactor of APC complex |
| K05546     | 0 0 0 1 1 0                               | GANAB; mannosyl-oligosaccharide alpha-1,3-glucosidase |
| K06617     | 0 0 0 0 1 0                               | E2.4.1.82; raffinose synthase |
| K07374     | 0 0 1 0 0 0                               | TUBA; tubulin alpha |
| K07466     | 1 1 1 1 1 0                               | RFA1, RPA1, rpa; replication factor A1 |
| K08678     | 0 0 0 1 0 0                               | UX51, uxs; UDP-glucuronate decarboxylase |
| K09495     | 1 1 1 1 1 1                               | CCT3, TRIC5; T-complex protein 1 subunit gamma |
| K09588     | 0 0 0 0 0 0                               | CYP90A1, CFD; cytochrome P450 family 90 subfamily A1 |
| K09645     | 0 0 0 1 0 0                               | CPVL; vitellogenic carboxypeptidase-like protein |
| K10534     | 0 0 0 0 1 0                               | NR; nitrate reductase (NAD(P)H) |
| K10573     | 0 0 0 0 0 1                               | UBE2A, UBC2, RADA; ubiquitin-conjugating enzyme E2 A |
| K10767     | 0 0 0 1 0 0                               | ALKBH5; mRNA N6-methyladenine demethylase |
| K11717     | 0 0 0 1 0 0                               | sulf; cysteine desulfurase / selenocysteine lyase |
| K12130     | 0 1 1 0 0 0                               | PRRS; pseudo-response regulator 5 |
| K12236     | 1 1 1 1 1 1                               | NFX1; transcriptional repressor NF-X1 |
| K12741     | 0 0 0 1 1 0                               | HNRNP1_3; heterogeneous nuclear ribonucleoprotein A1/A3 |
consistently expressed under short-term and long-term salt stress in the salt tolerant cultivar. Fourth, this study found 74,705 SNPs which are valuable marker for future alfalfa breeding for salt tolerance. Fifth, we found ‘Halo’ under salt stress maintained five times more DEGs in the root than ‘Vernal’. Finally, this study found seven candidate genes for salt tolerance (MS.gene32989, MS.gene065734, MS.gene24746, MS.gene81767, MS.gene044457, MS.gene049840, and MS.gene46459) with unknown functions, suggesting a need for further research to understand their role in salt tolerance.

Due to polyploidy and its out-crossing nature, alfalfa has encountered many challenges in genomic studies [26] as compared to self-pollinated crops such as wheat [27] and soybean (Glycine max) [28]. Thus, this transcriptomic study had considered several factors to overcome certain technical difficulties. First, identical clones were sampled at different time points from different alfalfa tissues. Unlike previous alfalfa transcriptomic studies, two technical replicates for each treatment were included to minimize technical errors. Furthermore, this study focused on both leaf and root tissues of alfalfa cultivars to capture tissue specific gene expression. These considerations seemed to be effective in capturing about 381 million high quality reads, which likely represents most of the genome of *M. sativa*. The raw reads showed a high percentage of mapping with the reference genome. These outputs should have enhanced our detection

Table 2 Number of differentially expressed genes (DEGs) and corresponding pathways in leaf and root of salt tolerant ‘Halo’ and salt intolerant ‘Vernal’ alfalfa cultivars at 0, 3 and 27 h of salt stress (Continued)

| Pathway ID | The number of differentially expressed genes | Pathway terms |
|------------|---------------------------------------------|----------------|
| HL0vsVL0   | 0 1 0 0 0 0 0 | SFRS2; splicing factor, arginine/serine-rich 2 |
| HL0vsVL0   | 0 0 1 0 1 0 | AUX1, LAX; auxin influx carrier (AUX1 LAX family) |
| VL0vsVL1   | 0 0 1 0 1 0 | SERPINB; serpin B |
| VL0vsVL3   | 0 0 1 1 1 1 | NDC1, TMEM48; nucleoporin NDC1 |
| HVL27 vs VVL27 | 0 0 1 1 1 0 | CPSF4, YTH1; cleavage and polyadenylation specificity factor subunit 4 |
| HVL27 vs VVL27 | 0 0 0 0 0 1 | SAUR; SAUR family protein |
| K14484     | 0 0 1 0 0 0 0 | TCH4; xyloglucanxyloglucosyl transferase TCH4 |
| K14504     | 0 0 0 0 0 1 1 | EMG1, NEP1; rRNA small subunit pseudouridine methyltransferase Nep1 |
| K14568     | 0 0 1 1 1 1 | KCS; 3-ketoacyl-CoA synthase |
| K14745     | 0 0 0 0 0 0 0 | SLC35D; solute carrier family 35 |
| K14746     | 0 0 1 0 1 0 | SLC45A1_2_4; solute carrier family 45, member 1/2/4 |
| K14747     | 0 0 1 0 0 0 0 | KCS; 3-ketoacyl-CoA synthase |
| K14748     | 0 0 0 0 0 0 | LUTS, CYP97A3; beta-ring hydroxylase |
| K14749     | 0 0 1 1 1 0 | SCPL-IV; serine carboxypeptidase-like clade IV |
| K14750     | 0 0 0 0 0 0 | CHID1; chitinase domain-containing protein 1 |
| K14751     | 0 0 1 0 0 0 0 | SACS; sacsin |
| K14752     | 0 0 0 0 0 0 | MSS116; ATP-dependent RNA helicase MSS116, mitochondrial |
| K14753     | 0 0 0 0 0 0 | RAB3GAP1; Rab3 GTPase-activating protein catalytic subunit |
| K14754     | 0 0 0 0 0 0 | ADH1; alcohol dehydrogenase class-P |
| K14755     | 0 0 0 0 0 0 | COPD, ARCN1, RET2; coatomer subunit delta |
| K14756     | 0 0 0 0 0 0 | exLI; expansin |
| K14757     | 0 0 0 0 0 0 | TMEM222; transmembrane protein 222 |
| K14758     | 0 0 0 0 0 0 | SAC1, SACM1L; phosphatidylinositol 4-phosphatase |
| K14759     | 0 0 0 0 0 0 | PCB1; phenylcoumaran benzyl ether reductase |
| K14760     | 0 0 0 0 0 0 | EMC10; ER membrane protein complex subunit 10 |

HL0 Halo leaf control, VL0 Vernal leaf control, HL3 Halo leaf after 3 h of salt stress, VL3 Vernal leaf after 3 h of salt stress, HL27 Halo leaf after 27 h of salt stress, VL27 Vernal leaf after 27 h of salt stress, HR0 Halo root control, VR0 Vernal root control, HR3 Halo root after 3 h of salt stress, VR3 Vernal root after 3 h of salt stress, HR27 Halo root after 27 h of salt stress, VR27 Vernal root after 27 h of salt stress
of DEGs. For example, both alfalfa cultivars showed a similar trend in the number of DEGs in leaf tissue with the increase of salt exposure time. In this study, we also selected three different time points (0 h, 3 h, and 27 h) to capture gene activation under short- and long-term salt stress. It has been established that salt responsive defense response is activated within 24 h of stress [29].

One of the main differences between the two cultivars was the number of DEGs in roots. In the root of salt tolerant alfalfa, the number of DEGs was similar between 3 h and 27 h of salt stress, but a sharp decrease was observed between 3 h and 27 h in the intolerant cultivar ‘Vernal’. We speculate that such earlier activation of salt responsive genes and maintenance of a large number of DEGs might be a key characteristic for salt tolerance in alfalfa, suggesting alfalfa tolerance is associated with up-regulation of key genes from short term salt stress. About 60% of DEGs were assigned to GO categories, while KEGG pathways for less than 30% DEGs were identified in this study. The DEGs were mainly involved in metabolic pathways as revealed by KEGG pathway analysis. Although certain pathways involved in salt tolerance may be conserved in plant species such as in halophytes, there was still variation among plant species, cultivars, and tissues [5]. This study demonstrated that transcriptional variation in adaptation to salt stress exists not only among the alfalfa cultivars but also between the different tissues. ‘Ion binding’ (GO:0043167) was significantly enriched in both leaf and root tissues of ‘Halo’, but not in ‘Vernal’ under salt stress. This suggested that the genes responsible for ‘ion binding’ should be unique for salt tolerance of ‘Halo’ alfalfa. Therefore, the tissue- and genotype-specific salt responsive genes might be useful in identification of salt tolerant genotypes in the future.

Among 13 candidate genes expressed in leaf and root tissues of ‘Halo’ under salt stress (Table 3) in this study, two genes (MS.gene013222 and MS.gene52595) are responsible for transmembrane protein function. These transmembrane proteins control gateways and selective transport of salt ions to facilitate salt tolerance in plants. Likewise, MS.gene013211, a homologous gene to ribonuclease TUDOR1, is involved in stress adaptation and highly expressed in leaf and root tissues of ‘Halo’ in our study [30]. MS.gene93979, a homologous gene to NF-X1-type zinc finger protein, is part of mechanisms that regulate growth under salt stress and was highly expressed in leaf and root tissues of ‘Halo’ in our study [31]. In addition, MS.gene029202 (E3 ubiquitin-protein ligase CIP8), MS.gene029203 (F-box/LRR-repeat protein 4), MS.gene36780 and MS.gene36960 (elongation factor 1-alpha) were highly

### Table 3 List of 13 salt responsive candidate genes simultaneously highly expressed in both leaf and root tissues of salt tolerant alfalfa cultivar ‘Halo’

| Gene ID    | Nr ID*             | log$_2$FC$^b$(Leaf) 0h| log$_2$FC$^b$(Leaf) 3h | log$_2$FC$^b$(Leaf) 27h | log$_2$FC$^b$(Root) 0h | log$_2$FC$^b$(Root) 3h | log$_2$FC$^b$(Root) 27h | Putative function                                                                 |
|------------|--------------------|------------------------|------------------------|------------------------|----------------------|------------------------|------------------------|----------------------------------------------------------------------------------|
| MS.gene01091 | XP_003593572.2     | 6.7                    | 7.3                    | 6.9                    | 8.4                  | 9.4                    | 10.3                   | T-complex protein 1 subunit gamma [Medicago truncatula (barrel medic)]            |
| MS.gene013211 | XP_003602730.1     | 7.3                    | 5.9                    | 6.7                    | 9.9                  | 8.9                    | 7.5                    | ribonuclease TUDOR1 [Medicago truncatula (barrel medic)]                           |
| MS.gene013222 | XP_003602710.1     | 5.5                    | 5.9                    | 5.5                    | 7.6                  | 6.7                    | 7.3                    | cleft lip and palate transmembrane protein 1 homolog [Medicago truncatula (barrel medic)] |
| MS.gene017955 | XP_003625216.1     | 5.5                    | 6.9                    | 5.6                    | NA                   | 9.5                    | 7.7                    | 40S ribosomal protein S20-2 [Medicago truncatula (barrel medic)]                  |
| MS.gene029200 | PNY01153.1         | 8.3                    | 7.5                    | 7.5                    | 7.4                  | 6.3                    | 7.7                    | replication factor A protein [Trifolium pratense]                                  |
| MS.gene029202 | XP_013470381.1     | 7.7                    | 8.1                    | 7.2                    | 8.2                  | 8.1                    | 8.5                    | E3 ubiquitin-protein ligase CIP8 [Medicago truncatula (barrel medic)]             |
| MS.gene029203 | XP_013470380.1     | NA                     | 6.8                    | 7.3                    | 6.8                  | 8.0                    | 8.5                    | F-box/LRR-repeat protein 4 [Medicago truncatula (barrel medic)]                   |
| MS.gene049294 | XP_003602595.1     | 4.0                    | 4.1                    | 5.3                    | 6.3                  | 5.4                    | 4.3                    | caffeic acid 3-O-methyltransferase [Medicago truncatula (barrel medic)]           |
| MS.gene32989  | GAIU34467.1        | NA                     | 6.8                    | 6.4                    | 7.0                  | 7.1                    | 7.6                    | hypothetical protein TSUD_06780 [Trifolium subterraneum]                          |
| MS.gene36780  | KEH43749.1         | 9.4                    | 8.4                    | 8.8                    | 10.4                 | 11.3                   | 8.2                    | elongation factor 1-alpha [Medicago truncatula (barrel medic)]                   |
| MS.gene36960  | AET01475.1         | 8.6                    | 8.5                    | 8.8                    | 9.8                  | 9.8                    | 8.8                    | elongation factor 1-alpha [Medicago truncatula]                                   |
| MS.gene52595  | XP_003624202.1     | 7.3                    | 8.0                    | 7.7                    | 9.5                  | 8.1                    | 7.9                    | ER membrane protein complex subunit 10 [Medicago truncatula (barrel medic)]      |
| MS.gene93979  | XP_003619874.1     | 7.7                    | 6.9                    | 7.9                    | 7.4                  | 7.4                    | 7.0                    | NF-X1-type zinc finger protein NFXL1 [Medicago truncatula (barrel medic)]         |

*Nr ID is the protein accession number in NCBI non redundant protein database
$log_2$FC stands for log Fold Change, where it is log base 2
expressed in leaf and root tissues of salt tolerant alfalfa in our study. These genes are involved in regulation of a number of biological processes including biotic and abiotic stress tolerances [32–34]. For example, MS.gene049294, which is a homologous gene of O-methyltransferase, was found to improve salt tolerance in transgenic Arabidopsis [35]. MS.gene01091, a homologous gene to the T-complex protein 1 subunit gamma, showed high expression in both root and leaf tissue and is involved in intracellular assembly and folding of various proteins [36]. MS.gene029200, a homologous gene to replication factor A protein, was highly expressed in both leaf and root tissues of ‘Halo’ in our study, which might play a role in binding, replication, repair, and recombination of DNA under stress conditions [37].

In this study, we found 15 and 18 candidate genes specific to leaf and root tissues of salt tolerance ‘Halo’ alfalfa (Tables 4, 5). In leaf tissue, nine genes showed consistent expression under salt stress, while six of them were expressed at all three time points. In our study, salt tolerant alfalfa showed an increased expression of MS.gene024018 and MS.gene24098 with putative functions of chloroplastic glutaredoxin and thioredoxin-like protein CDSP32, respectively. The two genes (MS.gene024018, MS.gene24098) were found to be important for defense against protein oxidative damage in other studies [38, 39]. This is important because salt stress results in the formation of reactive oxygen species, which damage protein, membrane lipids, and nucleic acids [40]. MS.gene63155, a homologous gene to receptor-like kinases (RLKs), are a family of transmembrane proteins, showed lowered expression with time under salt stress. This gene is involved in plant growth as well as stress response [41]. Two nucleoporin proteins (MS.gene037960 and MS.gene39381) were expressed consistently under salt stress in leaf tissue of ‘Halo’. These proteins connect cytoplasm and nucleoplasm, and are involved in abiotic stress tolerance [42].

| Gene ID          | Nr ID          | log2FC£ (Leaf) 0h 3h 27h | Putative function                                                                 |
|------------------|----------------|--------------------------|----------------------------------------------------------------------------------|
| MS.gene024018    | KHN29288.1     | NA 89 4.9                | Monothiol glutaredoxin-S14, chloroplastic [Glycine soja]                          |
| MS.gene029055    | AFK45194.1     | 5.2 7.3 5.8              | CDP-diaclyglycerol--glycerol-3-phosphate 3-phosphatidyltransferase 2 [Medicago truncatula (barrel medic)] |
| MS.gene029201    | AET03044.2     | NA 7.5 9.0               | replication protein A 70 kDa DNA-binding subunit C [Medicago truncatula (barrel medic)] |
| MS.gene029206    | XP_024628388.1 | NA 4.7 5.0               | FAD synthetase 1, chloroplastic [Medicago truncatula (barrel medic)]             |
| MS.gene037960    | XP_003559866.2 | NA 2.7 2.7               | nuclear pore complex protein NUP1 [Medicago truncatula (barrel medic)]           |
| MS.gene038586    | RHN67456.1     | NA 6.5 6.2               | putative minus-end-directed kinesin ATPase [Medicago truncatula]                  |
| MS.gene065734    | XP_013467963.1 | 6.4 9.8 6.9              | uncharacterized LOC25483798 [Medicago truncatula (barrel medic)]                  |
| MS.gene07287     | XP_003591401.1 | 8.8 11.2 10.5            | calvin cycle protein CP12-2, chloroplastic [Medicago truncatula (barrel medic)]   |
| MS.gene24098     | PNY14915.1     | 5.1 5.7 6.2              | thioredoxin-like protein CDSP32 chloroplastic-like [Trifolium pratense]            |
| MS.gene24746     | RHN68722.1     | 6.5 4.9 5.3              | hypothetical protein MtnurA17_Ch3g0116951[Medicago truncatula]                    |
| MS.gene36621     | XP_003627058.1 | NA 4.7 4.5               | stem 28 kDa glycoprotein [Medicago truncatula (barrel medic)]                    |
| MS.gene39381     | RHN38725.1     | NA 6.6 6.5               | putative nucleoporin protein Ndc1-Nup [Medicago truncatula]                       |
| MS.gene63155     | RHN41150.1     | NA 7.3 4.2               | putative protein kinase RLK-Pelle-LRR-XII-1 family [Medicago truncatula]           |
| MS.gene029206    | XP_003591401.1 | 8.8 11.2 10.5            | calvin cycle protein CP12-2, chloroplastic [Medicago truncatula (barrel medic)]   |
| MS.gene024018    | KHN29288.1     | NA 89 4.9                | Monothiol glutaredoxin-S14, chloroplastic [Glycine soja]                          |
| MS.gene029055    | AFK45194.1     | 5.2 7.3 5.8              | CDP-diaclyglycerol--glycerol-3-phosphate 3-phosphatidyltransferase 2 [Medicago truncatula (barrel medic)] |
| MS.gene029201    | AET03044.2     | NA 7.5 9.0               | replication protein A 70 kDa DNA-binding subunit C [Medicago truncatula (barrel medic)] |
| MS.gene029206    | XP_024628388.1 | NA 4.7 5.0               | FAD synthetase 1, chloroplastic [Medicago truncatula (barrel medic)]             |
| MS.gene037960    | XP_003559866.2 | NA 2.7 2.7               | nuclear pore complex protein NUP1 [Medicago truncatula (barrel medic)]           |
| MS.gene038586    | RHN67456.1     | NA 6.5 6.2               | putative minus-end-directed kinesin ATPase [Medicago truncatula]                  |
| MS.gene065734    | XP_013467963.1 | 6.4 9.8 6.9              | uncharacterized LOC25483798 [Medicago truncatula (barrel medic)]                  |
| MS.gene07287     | XP_003591401.1 | 8.8 11.2 10.5            | calvin cycle protein CP12-2, chloroplastic [Medicago truncatula (barrel medic)]   |
| MS.gene24098     | PNY14915.1     | 5.1 5.7 6.2              | thioredoxin-like protein CDSP32 chloroplastic-like [Trifolium pratense]            |
| MS.gene24746     | RHN68722.1     | 6.5 4.9 5.3              | hypothetical protein MtnurA17_Ch3g0116951[Medicago truncatula]                    |
| MS.gene36621     | XP_003627058.1 | NA 4.7 4.5               | stem 28 kDa glycoprotein [Medicago truncatula (barrel medic)]                    |
| MS.gene39381     | RHN38725.1     | NA 6.6 6.5               | putative nucleoporin protein Ndc1-Nup [Medicago truncatula]                       |
| MS.gene63155     | RHN41150.1     | NA 7.3 4.2               | putative protein kinase RLK-Pelle-LRR-XII-1 family [Medicago truncatula]           |
| MS.gene029206    | XP_003591401.1 | 8.8 11.2 10.5            | calvin cycle protein CP12-2, chloroplastic [Medicago truncatula (barrel medic)]   |

*Nr ID is the protein accession number in NCBI non redundant protein database

£log2FC stands for log Fold Change, where it is log base 2
primary pathway of carbon fixation, producing carbon compounds. CP12 facilitates the formation of a complex between glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase, thereby increasing the photosynthetic capacity of the plant [46]. MS.gene099197, a homologous gene to zeaxanthin epoxidase (ZEP), was highly expressed at all three time points in leaf tissue of ‘Halo’. It is an important enzyme in ABA biosynthesis and plays an important role in osmotic tolerance [47].

In root tissue, one (MS.gene01130) of the 18 genes detected was consistently expressed under salt stress, while the rest were expressed at all three time points in our study (Table 5). MS.gene002389, a homologous gene to secretory carrier-associated membrane proteins, is involved in membrane trafficking and found to influence accumulation of secondary cell wall components in Populus [48]. MS.gene011517, a homologous gene to 14 kDa proline-rich protein DC2.15, is involved in cell wall modification and organization [49]. The plant cell wall is not only a physical barrier between the plant and the environment but also is a responsive part of the plant to biotic and abiotic stresses. The finding of tissue specific salt tolerant candidate genes responsible for the plant cell wall is promising and underlines the need for further research on its role in response to salt stress. In addition, salt stress causes lipid peroxidation, resulting in damage of membrane lipids and eventual cell leakage. This study showed salt tolerant alfalfa had an increased expression of MS.gene0049130, a homologous gene to aldehyde dehydrogenase, responsible for oxidation of aldehydes produced during lipid peroxidation thereby detoxifying cells [50]. MS.gene095536 is a homologous gene to acyl-CoA-binding domain-containing protein 6, which is associated with phospholipid metabolism. This gene also was shown to play a role in the freezing tolerance of Arabidopsis [51]. MS.gene070486, a homologous gene to phosphatidylglycerol/phosphatidylinositol transfer proteins, plays an important role in signal transduction and facilitates lipid transfer between membranes [52]. MS.gene056386, a homologous gene to fructokinases, are important enzymes catalyzing fructose phosphorylation and are involved in plant growth and development [53]. MS.gene058673, a homologous gene to heavy-metal-associated domain-containing protein conferring tolerance to abiotic stress [54]. MS.gene073760, a homologous gene to probable E3 ubiquitin-protein ligase LOG2, which induces amino acid secretion. This is the main form of organic nitrogen in the plant [55]. MS.gene02427, a homologous gene to soluble inorganic pyrophosphatase, is tightly linked with carbohydrate

### Table 5: List of 18 salt responsive candidate genes highly expressed in root tissue of salt tolerant alfalfa cultivar ‘Halo’

| Gene ID          | Nr IDa       | log2FCb (Root) | Putative function                                                                 |
|------------------|--------------|----------------|-----------------------------------------------------------------------------------|
| MS.gene002389    | XP_003593475.1 | 7.4 8.0 7.9    | secretory carrier-associated membrane protein [Medicago truncatula (barrel medic)] |
| MS.gene011517    | XP_003608741.1 | 8.1 8.6 8.6    | 14 kDa proline-rich protein DC2.15 [Medicago truncatula (barrel medic)]           |
| MS.gene013923    | XP_003624895.1 | 6.5 6.6 6.6    | histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH1 [Medicago truncatula (barrel medic)] |
| MS.gene023013    | XP_013448530.1 | 8.0 6.0 6.2    | peptidyl-prolyl cis-trans isomerase FKBP62 [Medicago truncatula (barrel medic)]    |
| MS.gene02427     | AFK40071.1    | 6.9 5.7 5.5    | soluble inorganic pyrophosphatase PPA1 [Medicago truncatula (barrel medic)]       |
| MS.gene029223    | XP_003592714.1 | 5.9 6.7 6.1    | E3 ubiquitin ligase BIG BROTHER-related [Medicago truncatula (barrel medic)]      |
| MS.gene044457    | XP_013458006.1 | 7.3 6.4 7.0    | uncharacterized LOC25493896 [Medicago truncatula (barrel medic)]                 |
| MS.gene049130    | RDX70942.1    | 9.2 10.0 8.5   | Aldehyde dehydrogenase family 2 member C4, partial [Mucuna pruriens]              |
| MS.gene049840    | XP_024638826.1 | 7.4 7.2 8.1    | uncharacterized LOC11406476 [Medicago truncatula (barrel medic)]                 |
| MS.gene056386    | XP_013456308.1 | 8.2 8.3 6.7    | fructokinase-2 [Medicago truncatula (barrel medic)]                               |
| MS.gene058673    | PNX87529.1    | 7.9 5.5 9.5    | heavy-metal-associated domain-containing protein [Trifolium pratense]            |
| MS.gene070486    | XP_003616935.1 | 5.7 5.5 7.9    | phosphatidylglycerol/phosphatidylinositol transfer protein [Medicago truncatula (barrel medic)] |
| MS.gene073760    | XP_013468212.1 | 7.1 7.8 6.9    | probable E3 ubiquitin-protein ligase LOG2 [Medicago truncatula (barrel medic)]   |
| MS.gene03277     | XP_003608928.1 | 6.9 7.7 7.4    | betaine aldehyde dehydrogenase 1, chloroplastic [Medicago truncatula (barrel medic)] |
| MS.gene06459     | XP_013467706.1 | 8.6 9.5 7.7    | uncharacterized LOC25483559 [Medicago truncatula (barrel medic)]                 |
| MS.gene01130     | XP_004487038.1 | NA 4.4 4.1     | 605 ribosomal protein L23a-2-like [Cicer arietinum (chickpea)]                         |
| MS.gene067829    | XP_013454067.1 | 9.5 8.7 9.1    | ribosomal RNA small subunit methyltransferase nep-1 [Medicago truncatula (barrel medic)] |
| MS.gene05536     | XP_013469288.1 | 6.7 6.4 7.0    | acyl-CoA-binding domain-containing protein 6 [Medicago truncatula (barrel medic)] |

*aNr ID is the protein accession number in NCBI non redundant protein database

blog2FC stands for log Fold Change, where it is log base 2
metabolism. It plays an important role in stress adaptive responses [56]. Carbohydrate metabolism produces soluble carbohydrates that are important for salt tolerance because of its osmotic adjustment function in the root.

**Conclusion**
Our study generated a unique set of DEGs for alfalfa salt tolerance studies and breeding efforts. The information is useful for better understanding of temporal expression of genes in response to salt stress. Furthermore, GO annotation and KEGG pathway analysis of the DEGs provided insights to the different molecular and biological processes between salt tolerant and intolerant alfalfa cultivars. In particular, ‘ion binding activity’ was found as a key molecular activity specific to salt tolerant alfalfa cultivar ‘Halo’. Based on this finding, salt tolerance in alfalfa appears to be associated with consistent expression of genes for selective transport of salt ions and compounds, increasing

**Fig. 5** Distribution of SNPs identified over 32 allelic chromosomes of alfalfa (*Medicago sativa* L.) is represented with the Circos diagram. Histogram (1–70) showing distribution of SNPs per Mb bins across genome of alfalfa. The 8 alfalfa chromosomes (Chr1-8) are shown on outermost circle, middle (blue) and innermost (orange) circles represent SNPs distribution of salt intolerant ‘Vernal’ and salt tolerant ‘Halo’ alfalfa
photosynthetic capacity as well as carbohydrate metabolism, enhancing defense against oxidative damage, modification of root cell wall and protection against lipid peroxidation. The SNPs discovered in this study will be valuable for molecular marker-assisted breeding for the development of salt tolerant alfalfa.

**Methods**

**Plant material and salt treatment**

Two alfalfa cultivars, ‘Halo’ (obtained from Agriculture and Agri-Food Canada, Swift Current Research and Development Centre) and ‘Vernal’ (sourced from Dr. Biligetu’s lab, Crop Development Centre, University of Saskatchewan) were chosen for the study. Cultivar ‘Halo’ was selected for improved salinity tolerance for germination, seedling growth, and mature plant regrowth at 100 mM NaCl in the greenhouse conditions [57], and cultivar ‘Vernal’ was considered as a salinity intolerant cultivar [58, 59]. Four genotypes (biological replicates) of each cultivar were grown from seeds in the College of Agriculture and Bioresources greenhouse at the University of Saskatchewan (45 Innovation Blvd., Saskatoon, SK) for 12 weeks. Six identical clones of each biological replicate were produced by stem cuttings. Salt stress of 120 mM NaCl approximately corresponding to 12 dS m⁻¹ electrical conductivity was applied on 4 week old seedlings. Salt stress of 12 dS m⁻¹ was selected from our earlier greenhouse study where alfalfa was grown at various gradients of salt stress and alfalfa cultivars showed variation in response to salt stress at 12 dS m⁻¹, with increase in salt stress from 12 dS m⁻¹ all alfalfa cultivars showed very high mortality (Bhattarai et al., unpublished). Leaf and root samples were collected immediately before salt treatment (control, 0 h), and at 3 h and 27 h of salt treatments. The samples were immediately frozen in liquid nitrogen and then stored at −80°C for 2 weeks until total RNA extraction carried out.

**Tissue sample and RNA isolation**

About 100 mg of tissue samples were disrupted using TissueLyser II and total RNA was extracted with RLT buffer using the Qiagen RNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer’s protocol. DNase treatment was performed using the Ambion DNA-free DNase treatment and removal reagents (Life Technologies, Carlsbad, CA, USA) to remove contaminant genomic DNA from the isolated total RNA. Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) was used to measure the total RNA concentration. RNA integrity number was evaluated for 12 samples using RNA 6000 Nano labchip on 2100 Agilent Bioanalyzer (Agilent Technologies, Waldbronn, Germany) (Additional file 1: Table S3; Additional file 2: Fig. S1).

**Library preparation and sequencing**

Poly (A) RNA was purified from total RNA using Magnosphere MS150 OligodT beads according to the manufacturer’s protocol. The RNA samples were subsequently used in cDNA library preparation. Two cDNA libraries were prepared using Lexogen’s SENSE mRNA-Seq Library Prep Kit V2 (Lexogen, Vienna, Austria). To minimize technical errors, two technical replicates of each treatment were divided into two cDNA libraries. The technical replicates represented two clones of the same genotype (biological replicate) by separately extracting RNA. Thus, 96 samples (2 cultivars × 2 tissue types × 3 time points × 4 biological replicates × 2 technical replicates) were collected for the study. The cDNA libraries were sequenced using the Illumina HiSeq v4 system at the National Research Council of Canada, Saskatoon, Canada. Raw reads were deposited in the National Center for Biotechnology Information (NCBI) and received BioProject ID PRJNA657410.

**Reference-based mapping, differential gene expression analysis and annotation**

The quality of the raw sequence was assessed using the FastQC software [60]. The raw reads were cleaned by removing adapters and low-quality sequences using Trimmomatic v.0.36 based on the default setting of paired-end mode, phred 33 and threads 6 [61]. The trimmed high-quality reads of samples from the two technical replicates were merged and mapped with the alfalfa reference genome (https://doi.org/10.6084/m9.figshare.12327602.v3) [62, 63] using STAR (v2.6.1a) [64] with “quantMode” as “GeneCounts”. The obtained “ReadsPerGene” of each sample were extracted as count matrix and the differentially expressed genes were analyzed using DESeq2 package [65] where data were normalized by the median of the ratios. The threshold of padj < 0.001 and the Log fold change (Log2FC) > 2 were used to determine the significance of gene expression differences. The functional annotation of the DEGs were also extracted via searches of NR databases as available in “query.blastp.db.out” and gene ontologies were obtained via searches of the GO databases as available in “MsaGO.list.up” likewise Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog (KO) were obtained via search of KO databases as available in “query.ko” from Zeng [63]. Gene ontology analysis of the DEGs was done for biological process, cellular components, and molecular function by AgriGO v2.0 software [66]. Venn diagrams were produced using the Venny tool [67].

**Identification of single nucleotide polymorphisms (SNPs)**

SNPs calling was done using freebayes software using the bam file generated in the mapping process where at least 5 supporting observations were required to be
consider a variant [68]. To visualize the relative distribution of SNPs over chromosomes, Circos tool was used [69].

Abbreviations
ABA: Abscisic acid; AVP1: Arabidopsis type I proton-pumping pyrophosphatase; DEGs: Differentially expressed genes; dS m−2: DeceSiemers per metre; DREB: Dehydration-response element binding protein; H+H+: Na+/H+ antiporter; CBRLK: Calcium/calmodulin-binding receptor-like kinase; ZFP: Zinc finger protein; APX: Ascorbate peroxidase; NDPK2: Nucleotide diphosphate kinase 2; coDA: Choline oxidase; RLK: Receptor-like kinase; FAD: Flavin adenine dinucleotide; ZEP: Zeaxanthin epoxidase

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-03201-4.

Additional file 1. Table S1. List of 237 differentially expressed genes in leaf tissue at the control (0 h), 3 h, and 27 h of salt stress between salt tolerant ‘Haloo’ and salt intolerant ‘Vernal’ cultivars of alfalfa. Table S2. List of 295 differentially expressed genes in root tissue at the control (0 h), 3 h, and 27 h of salt stress between salt tolerant ‘Haloo’ and salt intolerant ‘Vernal’ cultivars of alfalfa. Table S3. RNA quality of 12 RNA samples determined with 2100 Agilent Bioanalyzer.

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Authors’ contributions
B.B. conceived the project; S.B., B.B. designed experiments; K.T. provided guidance on the salt stress system; B.B. prepared the study materials; S.B. performed experiments; S.B., Y.-B.F., B.B. analyzed data; S.B. wrote a first version of the manuscript and Y.-B.F., B.C., B.B., C.K., K.T. substantially contributed to the last version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Raw reads have been deposited in the National Center for Biotechnology Information (NCBI) and received BioProject ID PRJA657410. The data will be accessible with the following link: "https://www.ncbi.nlm.nih.gov/bioproject/PRJA657410".

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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