Comparative Transcriptome Analysis of NaCl and KCl Stress Response in *Malus Hupehensis* Rehd. Provide Insight into the Regulation Involved in Na⁺ and K⁺ Homeostasis

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Abstract

Background: Apple is among the most widely cultivated perennial fruit crops worldwide. It is sensitive to salt stress, which seriously affects the growth and productivity of apple trees by destroying the homeostasis of Na^+ and K^+ . Previous studies focused on the molecular mechanism underlying NaCl stress. However, signaling transduction under KCl stress has not been thoroughly studied.

Results: We comprehensively analyzed the salt tolerance of *Malus hupehensis* Rehd., which is a widely used rootstock in apple orchards, by using RNA-Seq. Roots and leaves were treated with NaCl and KCl. Based on mapping analyses, a total of 762 differentially expressed genes (DEGs) related to NaCl and KCl stress in the roots and leaves were identified. The Gene Ontology (GO) terms were enriched in ion transmembrane transporter and oxidoreductase activity under NaCl and KCl stress. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways focused on the plant hormone signal transduction and mitogen-activated protein kinase signaling pathway. We also screened out 28 candidate genes from 762 DEGs and verified their expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR). All of these enriched genes were closely related to NaCl and KCl stress and take part in mediating the Na^+ and K^+ homeostasis in *M. hupehensis*.

Conclusions: This transcriptome analysis provides a valuable resource for elucidating the signaling pathway of NaCl and KCl stress and is a substantial genetic resource for discovering genes related to the NaCl and KCl stress response of *M. hupehensis*.

Background

Soil salinization is one of the most serious abiotic stress worldwide [1]. To date, more than 20% of the world's land was affected by salinization, and the trend is constantly expanding [1, 2]. Apple (*Malus domestica* Borkh.) is one of the most valuable horticultural fruit crops cultivated worldwide. It is sensitive to salt stress, which seriously affects the growth and productivity of apple trees [3]. Therefore, strategies to improve the salt tolerance of *M. hupehensis*, a widely used apple rootstock in China, should be explored. However, the studies on the physiological and molecular mechanisms of plants' response to salt stress focus on model plants, such as *Arabidopsis*, rice, and tobacco [4–6]. The defense response mechanism of *M. hupehensis* to salt stress remains unclear.

The influence of salt stress to the plants has been explored. Salt stress is usually caused by high concentrations of soluble sodium (Na^+) and chloride (Cl^-) ions in soil [7]. Salt stress causes primary damage, which are osmotic and ionic stress [8]. Salt stress can reduce soil water potential and change the cell osmotic potential, thereby resulting in physiological drought [9]. Excessive external and internal Na^+ accumulation in the cytoplasm disrupts the Na^+/K^+ homeostasis and evokes ion toxic effects [10]. Oxidative damage is usually triggered by osmotic and ionic stress. Salt stress can also produce a large amount of reactive oxygen species (ROS), including hydroxyl radicals, hydrogen peroxide, and superoxide anion accumulation in cells; this condition can severely damage plants [11, 12].

Among the three typically stresses (osmotic stress, ionic stress and oxidative damage), the most direct effect of salt stress is the destruction of Na^+ and K^+ homeostasis [13]. Cytosolic Na^+/K^+ ratio is one of the key features conferring salinity stress tolerance in plants, and this trait is often considered a potential screening tool for plant breeders [14]. Under NaCl stress, excess Na^+ can affect the uptake of potassium ions (K^+), which is caused by a cytotoxic ion that competes for the binding sites in the transport systems, thereby resulting in the deficiency of K^+ acquisition and nutrition [15–17]. K^+ has essential functions in many physiological and biochemical reactions (e.g., charge balance and osmotic adjustment) and plays an important role in maintaining enzyme activities [18, 19]. Destruction of the K^+ and Na^+ homeostasis leads to catastrophic pathologies, thereby affecting the cell survival, division, and growth [20, 21]. However, the optimal potassium concentration for enzyme activation and protein synthesis is within the range of 100 mM in plants [22]. K^+ concentrations of higher than 100 mM can destroy the K^+ and Na^+ homeostasis and induce salt stress. KCl stress also dramatically affects the growth and morphology, Chl content, oxidative stress levels, and antioxidant enzyme activities of plant [23]. Nevertheless, studies on the molecular mechanism of KCl stress are still limited.

In evolution, plants have derived sophisticated mechanisms to maintain a suitable ratio of Na^+/K^+ in the cytoplasm [16, 17, 24]. The balance is maintained in two ways. The first way is the expelling of excess Na^+ and K^+ out of the cells. The Salt Overly Sensitive (SOS) pathway was considered as the most classical approach under NaCl stress. It expels Na^+ out of the cells to maintain the balance of Na^+/K^+ ratio in the cytoplasm [25, 26]. The Na^+/H^+ antiporter SOS1 is located in the plasma membrane and acts as the decisive factor for Na^+ efflux [27, 28]. For the transport of K^+ from the cytoplasm to the outside, it is in charge of plasma membrane-located Stellar K^+ -Outward Rectifier (SKOR) and the Guard Cell Outward-Rectifying Potassium Channel proteins [29]. In addition, the *Arabidopsis thaliana* K^+ transporter 1 (AKT1) and K^+ channels KAT1 and KAT2 reportedly function as transporters of K^+ to the cells [30, 31]. The second mechanism for maintaining the cytoplasm K^+ and Na^+ homeostasis is the compartmentation of K^+ and Na^+ to the vacuole or other organelles [32]. The $\text{Na}^+(\text{K}^+)/\text{H}^+$ antiporter gene *AtNHX1* promoter activity can be adequately induced by KCl stress. *AtNHX1* and *AtNHX2* reportedly control vacuolar K^+ homeostasis to regulate growth, flower development, and reproduction [33, 34]. *AtNHX1* and *AtNHX2* are also essential for active K^+ uptake in the tonoplast for stomatal function [35]. In the current research, accelerating endocytosis under salt stress could enhance Na^+ compartmentalization in the vacuole [8]. Alternatively, the accumulation of cytosolic vesicles increases the number of Na^+/H^+ antiporters, thereby enhancing the Na^+ uptake into the vesicles and reducing Na^+ concentrations in the cytosol [36].

In woody plant apple, previous studies reported that *MdSOS1* overexpression can enhance NaCl tolerance and reduce the Na^+ content [37]. The *MdNHX1* expression is significantly induced by NaCl stress, and *MdNHX1* overexpression improves the salt tolerance in transgenic apple calli [38]. However, the current studies focus on NaCl stress, and the molecular mechanism underlying KCl stress has never been reported in *M. hupehensis*. In our study, we performed RNA-Seq to examine the transcriptomic analysis in the roots and leaves of *M. hupehensis* Rehd. seedlings under 100 mM NaCl and 100 mM KCl stress. The

differentially expressed genes and the signaling pathways responding to NaCl and KCl stress were identified. In addition, we compared the genes and pathways between the NaCl and KCl stress and found 28 key regulation and function genes. This finding would be crucial in K⁺ and Na⁺ homeostasis of the *M. hupehensis* seedlings under salt stress. This study contributes to the discovery of genes related to NaCl and KCl stress and further explores the molecular mechanisms regulating salt tolerance in *M. hupehensis*.

Results

Analysis of RNA-Seq datasets

As shown in Figure S1, the *M. hupehensis* seedlings were wilting seriously after salt treatment for one and 6 h compared with the control. The 30 libraries of RNA-Seq were shown in Table 1. After removing the low-quality reads and adapter sequences, 1,702,856,596 clean reads and 255.42G clean bases were obtained. In addition, the average of sample GC content was 48.61%. The sequencing error rate was only 0.03% of every sample, and the average Q20 and Q30 was 96.43% and 90.78%, respectively (Table S1). The clean reads mapped in *M. hupehensis* genome's ratios ranged from 32.33–84.71% among the 30 RNA-Seq libraries, and 31.54–82.68% unique reads were mapped to the reference genome (Table S2). The read1 mapped ratios were similar with read2 mapped ratios, and the positive mapped ratios were also similar with the negative mapped ratios. These results indicated that the root and leaf sample libraries data could be used for further analysis.

Table 1
Sample information of all 30 libraries in apple for RNA-Seq

Group name	Sample name	Species name	Tissues	Treatment	Treatment time (h)
CKR	CKR1	<i>Malus hupehensis</i>	Root	Control	0
	CKR2	<i>Malus hupehensis</i>	Root	Control	0
	CKR3	<i>Malus hupehensis</i>	Root	Control	0
CKL	CKL1	<i>Malus hupehensis</i>	Leaf	Control	0
	CKL2	<i>Malus hupehensis</i>	Leaf	Control	0
	CKL3	<i>Malus hupehensis</i>	Leaf	Control	0
Na1R	Na1R1	<i>Malus hupehensis</i>	Root	NaCl	1
	Na1R2	<i>Malus hupehensis</i>	Root	NaCl	1
	Na1R3	<i>Malus hupehensis</i>	Root	NaCl	1
Na6R	Na6R1	<i>Malus hupehensis</i>	Root	NaCl	6
	Na6R2	<i>Malus hupehensis</i>	Root	NaCl	6
	Na6R3	<i>Malus hupehensis</i>	Root	NaCl	6
Na1L	Na1L1	<i>Malus hupehensis</i>	Leaf	NaCl	1
	Na1L2	<i>Malus hupehensis</i>	Leaf	NaCl	1
	Na1L3	<i>Malus hupehensis</i>	Leaf	NaCl	1
Na6L	Na6L1	<i>Malus hupehensis</i>	Leaf	NaCl	6
	Na6L2	<i>Malus hupehensis</i>	Leaf	NaCl	6
	Na6L3	<i>Malus hupehensis</i>	Leaf	NaCl	6
K1R	K1R1	<i>Malus hupehensis</i>	Root	KCl	1
	K1R2	<i>Malus hupehensis</i>	Root	KCl	1
	K1R3	<i>Malus hupehensis</i>	Root	KCl	1
K6R	K6R1	<i>Malus hupehensis</i>	Root	KCl	6
	K6R2	<i>Malus hupehensis</i>	Root	KCl	6
	K6R3	<i>Malus hupehensis</i>	Root	KCl	6
K1L	K1L1	<i>Malus hupehensis</i>	Leaf	KCl	1
	K1L2	<i>Malus hupehensis</i>	Leaf	KCl	1
	K1L3	<i>Malus hupehensis</i>	Leaf	KCl	1

Group name	Sample name	Species name	Tissues	Treatment	Treatment time (h)
K6L	K6L1	<i>Malus hupehensis</i>	Leaf	KCl	6
	K6L2	<i>Malus hupehensis</i>	Leaf	KCl	6
	K6L3	<i>Malus hupehensis</i>	Leaf	KCl	6

Identification Of Degs Related To Nacl And Kcl Stress

According to FPKM values of all genes in each sample, the correlation coefficients of samples within and between groups were calculated, and a heat map was drawn. In all samples, the R^2 of almost all samples' correlation coefficients were > 0.9 (Fig. 1). This result indicated that the samples can be used to identify DEGs.

Scatter plot of the upregulated genes and downregulated genes in different tissue samples compared with control are shown in Fig. 2. In general, the upregulated DEGs were slightly more than the downregulated DEGs, except K6L and Na6L samples compared with the control (Figs. 2d and h). Moreover, the upregulated and downregulated DEGs in the roots were more than in the leaves, particularly in Na1R were 11,917 with Na1L only 4391 (Figs. 2a and c). In addition, the amount of the response genes at 6 h after KCl and NaCl treatments were more than one hour in the roots and leaves. These results indicated that the roots may be more responsive than the leaves under salt stress. DEGs quantity also increased with the extension of treatment time.

The Venn diagrams showed the distribution of similarly regulated genes in the treatment and comparison groups in the roots and leaves to further survey DEGs, which respond to different salt stress (Fig. 3). A total of 11,455, 16,516, 11,917, and 15,234 DEGs were observed in K1R, K6R, Na1R, and Na6R treatment groups, compared with the control group, respectively (Fig. 3). In the roots, 7144 DEGs were noted co-responding to NaCl stress at one and 6 h with 7528 DEGs to KCl stress. We also found 4278 DEGs collectively responded to KCl and NaCl stress at one and 6 h in the roots (Fig. 3a). In the leaves, 3009 DEGs were found responding to NaCl stress at one and 6 h, and 3830 DEGs were changed to responding to KCl stress at one and 6 h. Among these DEGs, 2295 DEGs were collectively responded to KCl and NaCl stress at one and 6 h (Fig. 3b). Moreover, 762 genes responded together to NaCl and KCl stress in the roots and leaves (Fig. 3c).

GO enrichment analysis of DEGs related to NaCl and KCl stress

To identify the DEGs functions responding to NaCl and KCl stress, we successfully analyzed the DEGs enrichment on the basis of the GO classifications. The DEGs were separated into three categories: molecular function (MF), cellular component (CC), and biological process (BP) (Tables 2 and 3). The functions of DEGs were similar between NaCl and KCl stress in the roots and leaves, indicating the similar signaling pathways responding to NaCl and KCl stress. 31 GO terms enriched in the three categories in

the roots and leaves, and they may play major roles in plant response to NaCl and KCl stress. In the category of molecular function, the GO terms were enriched in ion (such as potassium ion, metal ion, and cation) transmembrane transporter activity, ion (such as iron and calcium) binding, oxidoreductase activity, ion channel activity, hydrolase activity, and antiporter activity. In cellular component, membrane protein complex, endomembrane system, and organelle membrane were significantly enriched. The enriched biological process includes signal transduction, cation and metal ion transport, response to stress, chemical and oxidative stress, cellular component organization or biogenesis. In addition, two new GO terms (ADP binding and hydrolase activity, hydrolyzing O-glycosyl compounds) were enriched in the leaves but not in roots, indicating the unique mechanism responding to salt stress in the leaves.

Table 2
GO enrichment analysis of the consistent DEGs in roots

GO ID	GO Name	Category	K1R vs CKR DEGs number	K6R vs CKR DEGs number	Na1R vs CKR DEGs number	Na6R vs CKR DEGs number
GO:0016758	transferase activity	MF	147	231	154	204
GO:0046906	tetrapyrrole binding	MF	162	239	172	213
GO:0022891	substrate-specific transmembrane transporter activity	MF	146	201	138	182
GO:0046983	protein dimerization activity	MF	152	209	146	201
GO:0015079	potassium ion transmembrane transporter activity	MF	13	18	11	18
GO:0008233	peptidase activity	MF	150	195	149	163
GO:0016705	oxidoreductase activity	MF	124	191	129	185
GO:0046873	metal ion transmembrane transporter activity	MF	37	38	36	41
GO:0005506	iron ion binding	MF	127	191	127	176
GO:0015075	ion transmembrane transporter activity	MF	146	199	135	179
GO:0005216	ion channel activity	MF	31	50	28	51
GO:0016798	hydrolase activity, acting on glycosyl bonds	MF	171	236	179	211
GO:0020037	heme binding	MF	162	239	172	213
GO:0015297	antiporter activity	MF	40	71	37	56
GO:0022804	active transmembrane transporter activity	MF	81	134	73	106
GO:0008324	cation transmembrane transporter activity	MF	96	116	84	96
GO:0005509	calcium ion binding	MF	101	126	115	122
GO:0005198	structural molecule activity	MF	114	92	87	125

MF: Molecular Function, CC: Cellular Component, BP: Biological Process

GO ID	GO Name	Category	K1R vs CKR DEGs number	K6R vs CKR DEGs number	Na1R vs CKR DEGs number	Na6R vs CKR DEGs number
GO:0098796	membrane protein complex	CC	55	69	83	52
GO:0012505	endomembrane system	CC	45	61	44	51
GO:0005618	cell wall	CC	42	58	37	51
GO:0031090	organelle membrane	CC	41	42	41	27
GO:0007165	signal transduction	BP	148	197	159	163
GO:0006812	cation transport	BP	137	193	139	159
GO:0006950	response to stress	BP	131	210	136	175
GO:0071840	cellular component organization or biogenesis	BP	130	186	129	177
GO:0071705	nitrogen compound transport	BP	83	108	74	94
GO:0030001	metal ion transport	BP	82	111	89	101
GO:0015031	protein transport	BP	66	79	58	67
GO:0042221	response to chemical	BP	60	96	61	72
GO:0006979	response to oxidative stress	BP	48	72	57	55
MF: Molecular Function, CC: Cellular Component, BP: Biological Process						

Table 3
GO enrichment analysis of the consistent DEGs in leaves

GO ID	GO Name	Category	K1L vs CKL DEGs number	K6L vs CKL DEGs number	Na1L vs CKL DEGs number	Na6L vs CKL DEGs number
GO:0046983	protein dimerization activity	MF	110	144	82	159
GO:0046906	tetrapyrrole binding	MF	108	123	94	126
GO:0046873	metal ion transmembrane transporter activity	MF	22	20	16	20
GO:0043531	ADP binding	MF	115	58	97	76
GO:0022891	substrate-specific transmembrane transporter activity	MF	77	102	64	102
GO:0022804	active transmembrane transporter activity	MF	60	74	48	73
GO:0020037	heme binding	MF	108	123	94	126
GO:0016798	hydrolase activity, acting on glycosyl bonds	MF	119	147	91	141
GO:0016758	transferase activity, transferring hexosyl groups	MF	130	155	102	166
GO:0016705	oxidoreductase activity	MF	89	99	82	108
GO:0015297	antiporter activity	MF	27	38	25	39
GO:0015079	potassium ion transmembrane transporter activity	MF	12	7	9	6
GO:0015075	ion transmembrane transporter activity	MF	77	100	63	98
GO:0008324	cation transmembrane transporter activity	MF	42	54	31	54
GO:0008233	peptidase activity	MF	107	109	78	128
GO:0005509	calcium ion binding	MF	83	66	61	79
GO:0005506	iron ion binding	MF	92	100	61	106
GO:0005216	ion channel activity	MF	18	30	21	27
GO:0005198	structural molecule activity	MF	204	34	61	188

MF: Molecular Function, CC: Cellular Component, BP: Biological Process

GO ID	GO Name	Category	K1L vs CKL DEGs number	K6L vs CKL DEGs number	Na1L vs CKL DEGs number	Na6L vs CKL DEGs number
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	MF	110	139	86	131
GO:0098796	membrane protein complex	CC	30	39	23	43
GO:0012505	endomembrane system	CC	27	25	23	24
GO:0005618	cell wall	CC	29	37	23	35
GO:0031090	organelle membrane	CC	28	26	14	29
GO:0007165	signal transduction	BP	117	93	106	119
GO:0006812	cation transport	BP	89	99	60	96
GO:0006950	response to stress	BP	105	139	68	152
GO:0071840	cellular component organization or biogenesis	BP	98	114	48	132
GO:0071705	nitrogen compound transport	BP	51	59	38	63
GO:0030001	metal ion transport	BP	61	59	45	58
GO:0015031	protein transport	BP	40	47	31	46
GO:0042221	response to chemical	BP	68	76	54	84
GO:0006979	response to oxidative stress	BP	37	41	21	36
MF: Molecular Function, CC: Cellular Component, BP: Biological Process						

KEGG pathway enrichment analyses of DEGs related to NaCl and KCl stress

We conducted the KEGG pathway enrichment analyses of DEGs to explore the signaling pathways responding to KCl and NaCl stress. 13 KEGG pathways were identified under NaCl and KCl stress in the roots and leaves. The KEGG pathways focused on plant hormone signal transduction, carbon metabolism, biosynthesis of amino acids, ribosome, protein processing in endoplasmic reticulum, endocytosis, MAPK signaling pathway, and starch and sucrose metabolism (Tables 4 and 5).

Table 4
KEGG enrichment analysis of the DEGs in roots

KEGG ID	KEGG pathway	K1R vs CKR DEGs number	K6R vs CKR DEGs number	Na1R vs CKR DEGs number	Na6R vs CKR DEGs number
mdm04075	Plant hormone signal transduction	138	179	141	151
mdm01200	Carbon metabolism	106	148	129	122
mdm01230	Biosynthesis of amino acids	97	129	93	105
mdm03010	Ribosome	96	73	76	104
mdm04141	Protein processing in endoplasmic reticulum	92	122	81	108
mdm04626	Plant-pathogen interaction	81	107	72	96
mdm03040	Spliceosome	77	103	75	79
mdm04144	Endocytosis	76	97	60	72
mdm04016	MAPK signaling pathway - plant	73	83	75	85
mdm00940	Phenylpropanoid biosynthesis	69	97	75	87
mdm00500	Starch and sucrose metabolism	60	89	75	82
mdm03013	RNA transport	59	88	53	72
mdm04120	Ubiquitin mediated proteolysis	51	73	46	50

Table 5
KEGG enrichment analysis of the DEGs in leaves

KEGG ID	KEGG pathway	K1L vs CKL DEGs number	K6L vs CKL DEGs number	Na1L vs CKL DEGs number	Na6L vs CKL DEGs number
mdm03010	Ribosome	194	30	57	186
mdm04075	Plant hormone signal transduction	133	142	105	149
mdm01200	Carbon metabolism	91	92	37	100
mdm01230	Biosynthesis of amino acids	85	84	40	90
mdm04141	Protein processing in endoplasmic reticulum	73	70	52	63
mdm04016	MAPK signaling pathway - plant	67	63	48	70
mdm04144	Endocytosis	63	61	52	59
mdm04626	Plant-pathogen interaction	62	58	51	56
mdm03013	RNA transport	57	39	20	59
mdm00500	Starch and sucrose metabolism	51	71	40	62
mdm00940	Phenylpropanoid biosynthesis	39	50	30	50
mdm03040	Spliceosome	37	24	20	40
mdm04120	Ubiquitin mediated proteolysis	39	35	19	38

In the roots, plant hormone signal transduction pathway was the largest group with 138 and 179 DEGs enriched after KCl treatment for one and 6 h, whereas 141 and 151 DEGs were enriched after NaCl treatment for one and 6 h, respectively. This pathway was also enriched in the leaves for multiple DEGs. These results suggested that plant hormone may play an important role in response to salt stress.

Identification of novel transcripts related to NaCl and KCl stress

To identify the key genes related to the NaCl and KCl stress, we screened them out from the 762 candidate genes, which responded together to NaCl and KCl stress in the roots and leaves by RNA-Seq (Fig. 3). 28 candidate genes were identified through GO enrichment analysis and KEGG pathway enrichment analysis (Fig. 4). As shown in Fig. 4, these genes were significantly changed by NaCl and KCl stress in the roots and leaves, indicating their crucial role under salt stress. These genes were classified into six categories. The first category was ion transmembrane transporter, including *MdNHX2*, *MdCHX15*, *MdCAX5*, *MdCCX2*, *MdPOT3*, *MdPOT6*, *MdTPK1*, *MdSKOR*, *MdVIT1*, *MdABCG11*, *MdACA2*, and *MdACA13*. The second category was transcription factors, which would be important in transcriptional

regulation under salts stress, such as *MdWRKY28*, *MdNAC56*, *MdMYB108*, *MdbHLH28*, *MdbHLH162*, *MdERF019*, and *MdERF109*. The third category was hormone signal, like *MdPP2C24*, *MdPP2C51*, *MdCYP82*, and *MdPYL4*. The remaining categories were antioxidant enzymes (*MdPER19* and *MdAPXT*), MAPK signal pathway (*MdMAPKKKa*), and others (*MdHIP26* and *MdPFK2*). 17 genes were upregulated and 4 genes were downregulated by NaCl and KCl stress.

Qrt-pcr Validation Of Degs

To further confirm the candidate genes responding to NaCl and KCl stress in apple rootstock, M26 (salt-sensitive), *Malus hupehensis* (low salt-tolerance), and *Malus zumi* (high salt-tolerance) were treated with NaCl and KCl stress (Fig. S2). Under KCl stress, *M. zumi* exhibited high salt tolerance, M26 and *M. hupehensis* were suffered seriously damage in 5d and 10d, respectively (Fig. S2a). Under NaCl stress, the plants of *M. zumi* and *M. hupehensis* showed salt tolerance, but M26 showed significantly withered (Fig. S2b).

For detected the expression of candidate genes under NaCl and KCl treatments in different rootstocks, the qRT-PCR experiment was performed. The result shows that in the first category (Fig. 5), the expression of *MdNHX2*, *MdCHX15*, *MdCAX5*, *MdSKOR*, *MdPOT6*, and *MdVIT1* were induced by NaCl and KCl treatments in M26, *M. hupehensis* and *M. zumi*. Furthermore, these genes are sustainable response to salt stress in 5d and 10d. However, the expression of these genes in *M. hupehensis* and *M. zumi* were significantly higher than M26. The expression of *MdACA2* and *MdACA13* was only induced by NaCl stress in *M. hupehensis* and *M. zumi*. In addition, the *MdTPK1* expression was only induced by KCl treatment with no change under NaCl stress in M26, *M. hupehensis* and *M. zumi*.

In the second category (Fig. 6), the kinase *MdMAPKKKa*, transcription factors of *MdWRKY28*, *MdNAC56*, *MdMYB108*, *MdERF019* and *MdERF109* were induced by NaCl and KCl stress in three rootstocks, resembling with RNA-Seq. In the hormone signaling pathway (Fig. 7a), the *MdPP2C24* and *MdPP2C51* were continue significantly upregulated under NaCl and KCl treatments in M26, *M. hupehensis* and *M. zumi*. This outcome was consistent with the RNA-Seq results. The expression of *MdCYP82* was only induced by KCl stress in *M. hupehensis*. In three rootstock, *MdPYL4* was upregulated in roots but downregulated in leaves. In contrast, the antioxidant enzyme genes *MdPER19* and *MdAPXT* were induced by NaCl and KCl stress in the roots but reduced in the leaves (Fig. 7b). In accordance with RNA-Seq, the *MdHIP26*, and *MdPFK2* expression was all significantly induced by NaCl and KCl treatments in *M. hupehensis* and *M. zumi*, whereas no change in M26 (Fig. 7b).

Discussion

Increased salinity in soil has severely hampered the development of apple plantations and resulted in economic losses for orchardists [3, 38, 39]. High concentrations of NaCl in the soil could reduce soil water potential, change the cell osmotic potential [9], produce a mass of ROS [11, 12], and disrupt the uptake of

K⁺ into plant cells, adversely affecting Na⁺/K⁺ homeostasis and many metabolic pathways [8]. Excess KCl stress has a strong impact on plant growth, development, and decrease of the antioxidant enzyme activity [40]. The KCl stress could affect the Na⁺/K⁺ homeostasis and cause the ion toxicity [41]. When the stress of NaCl and KCl were at the same concentration, the KCl stress caused more serious damage to plants than the NaCl stress [42]. To date, the molecular mechanism underlying plants' response to NaCl and KCl stress has only been reported in model plants. However, such mechanism was still unclear in woody plants. In our study, we performed transcriptome sequencing under NaCl and KCl stress in *M. hupehensis* to explore the key genes, functional families, and signaling pathways related to NaCl and KCl stress.

At present, the molecular mechanism of plants responding to NaCl stress had been reported. Three primary signaling pathways (CDPK, SOS, and MAPK pathways) were considered the main ways responding to NaCl stress in plants [20]. The Ca²⁺-dependent signaling (CDPK pathway) is extensively studied, inducing the expression of DRE/CRT transcription factors and other types of *LEA-like* genes. In apple, our result indicated that the expression of DRE/CRT transcription factors *MdERF019* and *MdERF109* was also induced by NaCl stress in *M. hupehensis* leaves (Figs. 4 and 5). SOS pathway was considered an important pathway responding to NaCl stress [25, 26] High Na⁺ stress initiates a calcium signal that stimulates the SOS3–SOS2 protein kinase complex, which then activates the Na⁺/H⁺ exchange transporter SOS1 and activate or suppress the activities of other transporters involved in Na⁺ homeostasis has been reported [25, 27, 28]. In our study, most genes responding to NaCl stress in *M. hupehensis* belong to the ion transporter families, including potassium ion, metal ion, and cation transmembrane transporters. This result indicated that the ion transport would be the most important mechanism responding to NaCl stress in *M. hupehensis* (Figs. 4 and 5). In addition, the MAPK pathway regulates the production of compatible osmolytes and antioxidants and may also participate in cell cycle regulation under NaCl stress [43]. The expressions of *MdMAPKKKa* and the antioxidant enzyme genes *MdPER19* and *MdAPXT* in *M. hupehensis* were induced by NaCl stress in the roots (Fig. 5). In the woody plant pear, a RNA-Seq analysis had been conducted to explore the KEGG pathways related to NaCl stress [44]. The pathway of plant–pathogen interaction, circadian rhythm, diterpenoid biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, steroid biosynthesis, and RNA degradation were enriched under NaCl stress in pear [44]. However, our study found that the KEGG pathways focused on plant hormone signal transduction, plant–pathogen interaction, MAPK signaling pathway, RNA transport, and protein processing in the endoplasmic reticulum in *M. hupehensis* (Tables 4 and 5). This result determines the difference between the pear and apple responding to NaCl stress.

To date, the mechanism underlying the response of woody plants to KCl stress has not been reported. Our RNA-Seq research found that the GO terms focused on the ion transporter activity (ion transmembrane transporter activity, calcium ion binding, ion binding, ion channel activity, and cation transport) and response to oxidative stress (oxidoreductase activity, hydrolase activity, response to stress, and signal transduction), as shown in Tables 2 and 3. The KEGG pathway of plant hormone signal transduction, carbon metabolism, plant–pathogen interaction, endocytosis, and MAPK signaling pathway were

significantly enriched under KCl stress in the roots and leaves at one and 6 h (Tables 4 and 5). These results indicated that the ion transport, response to oxidative stress, plant hormone signal transduction, and MAPK signaling pathway are the most important mechanisms related to KCl stress in *M. hupehensis*.

The interconnected mechanism of plants in response to NaCl and KCl stress was the balance of Na^+/K^+ in the cytoplasm [16, 17, 24]. Cytosolic Na^+/K^+ ratio is one of the most important features conferring plant salt tolerance, and this trait is a potential screening tool for plant breeders [14]. We identified 28 key genes from the 762 candidate genes simultaneously related to NaCl and KCl stress to explore the crossing key genes regulating the Na^+ and K^+ homeostasis under NaCl and KCl stress (Fig. 4). The candidate genes were classified into six categories, namely, ion transporters, transcription factors, hormone signal pathway, antioxidant enzymes, MAPK signal pathway, and others.

The ion transporters were the most important families regulating the Na^+ and K^+ homeostasis. These ion transporters include Ca^{2+} , K^+ , and H^+ transporters. *AtCCX2* (cation/calcium exchanger) is directly involved in the control of Ca^{2+} fluxes between the endoplasmic reticulum and the cytosol, thereby promoting the tolerance of NaCl stress [45]. *AtACA2* (calcium-transporting ATPase) overexpression could enhance the tolerance of NaCl stress by depletion of cytosolic Ca^{2+} [46]. In our study, calcium transport genes, including *MdACA2* and *MdACA13*, were significantly upregulated under NaCl stress and changed their expression under KCl stress (Fig. 5). *MdCCX2* expression was only induced at 1 h in the leaves under NaCl stress. However, its expression was significantly changed in the roots and upregulated in the leaves under KCl stress. This result indicates the more important role of the gene under KCl stress than under NaCl stress. The *MdCCX2* function under KCl stress still needs further research. Under excess KCl stress, K^+ transporter and K^+ channel are of great importance of regulating the K^+ concentration [47]. The GO term “potassium ion transmembrane transporter activity” was enriched, and the expression of potassium transporter gene *MdPOT3*, *MdPOT6*, potassium channel gene *MdSKOR*, K^+/H^+ antiporter gene *MdNHX2*, and two-pore potassium channel gene *MdTPK1* were significantly changed under NaCl and KCl stress. SKOR was the K^+ release channel functioned to transport K^+ outside of the plasma membrane [48]. TPK1 was reported to be responsible for the K^+ compartmentation to different organelles [49]. NHX2 was K^+/H^+ exchangers essential for active K^+ uptake at the tonoplast [34]. We inferred that the enrichment of potassium ion transmembrane transporters and the change of the potassium transporter genes could regulate the cytosolic Na^+/K^+ ratio through expelling K^+ out of the cells or compartmentalizing K^+ in different organelles under NaCl and KCl stress. In addition, H^+ was an important cation involved in the resistance to salt stress. The expression of cation/proton exchanger gene *MdCAX5* and cation/ H^+ antiporter gene *MdCHX15* was significantly induced by NaCl and KCl stress in the roots and leaves. Overexpressing *GmCAX1* in *Arabidopsis* could enhance the NaCl tolerance by accumulating less Na^+ in cells [50]. Knockout *chx17* mutants accumulated less K^+ in the roots in response to salt stress and potassium starvation compared with the wild type, indicating the role of this gene in K^+ acquisition and homeostasis [51]. Our results indicated that the Ca^{2+} , K^+ , and H^+ transporters are the crucial protein families mediating the cytosolic Na^+ and K^+ homeostasis under NaCl and KCl stress.

In the long-term process of evolution, plants have derived complex signaling pathways related to NaCl and KCl stress [43, 52]. In our study, 73–85 genes were enriched in MAPK signaling pathway under NaCl and KCl stress. Our qRT-PCR results also indicated that the *MdMAPKKKa* expression was significantly induced by NaCl and KCl stress. We inferred that the MAPK signaling pathway would be the most important pathway related to NaCl and KCl stress in apple plants. In the signal transduction pathways, the transcription factors could not only receive the signal upstream but also transmit the signal to the downstream genes and played a key role in signal transduction [53]. In previous reports, overexpression of *SpERFs* [8], *MdNAC047* [3], *MdMYB44* [54], and *MdSIMYB1* [55] could significantly improve NaCl tolerance of transgenic plants. However, the key TFs responding to KCl stress had not been reported. Our results found out the key TFs related to NaCl and KCl stress, including *MdWRKY28*, *MdNAC56*, *MdMYB108*, and *MdERF109*, with the significantly increased expression under NaCl and KCl stress (Fig. 5). We hypothesized that these key TFs could receive the MAPK signals and regulate the function genes for mediating the Na⁺ and K⁺ homeostasis under NaCl and KCl stress. The relationships between the TFs, MAPKs, and the function genes under salt stress still need further research.

Different from the RNA-Seq results related to NaCl stress in pear [44], our study found that the KEGG pathways focused on plant hormone signal transduction in *M. hupehensis*. Among the plant hormones related to salt stress, ABA was the most crucial one [8]. The ABA-dependent signaling pathways were involved in mediating salt stress in various plants [8]. Previous studies reported that the ABA-dependent signal transduction was regulated by protein phosphatases [56]. Singh et al (2015) reported that PP2C, as the protein phosphatases, negatively regulated the ABA signal transduction and was induced by salt stress [56]. In our study, *MdPP2C51* and *MdPP2C24* were significantly upregulated by NaCl and KCl stress, indicating their potential role in regulating the ABA signal transduction in *M. hupehensis*. Moreover, the *MdPYL4* expression was significantly suppressed by NaCl and KCl stress in the leaves (Fig. 5). PYL, as the ABA receptor, could regulate ABA signaling by inhibiting PP2C under salt stress [57]. The NaCl and KCl stress also cause oxidative damage to plants [12], and antioxidant enzymes play an important role in alleviating ROS toxicity [58]. Our GO terms of oxidoreductase activity, hydrolase activity, response to stress, and signal transduction were significantly enriched under salt stress (Tables 2 and 3). We identified two key antioxidant enzyme genes, namely, peroxidase gene *MdPER19* and L-ascorbate peroxidase gene *MdAPXT*, which were downregulated by NaCl and KCl stress in the leaves and upregulated in the roots. Overexpression of ascorbate peroxidase *AtAPX* in tobacco enhances NaCl stress tolerance [59]. The function of *MdPER19* and *MdAPXT* under KCl stress requires further research.

Conclusion

In our study, we comprehensively analyzed the NaCl and KCl tolerance of *Malus hupehensis* Rehd. by using RNA-Seq. 28 candidate genes including ion transmembrane transporter, transcription factors, hormone signal, antioxidant enzymes and MAPK signal pathway were identified. All of these genes were closely related to NaCl and KCl stress and take part in mediating the Na⁺ and K⁺ homeostasis in *M. hupehensis*.

Methods

Plant material and salt treatment

The *M. hupehensis* seeds [which were provided from Guangli Sha's lab (Qingdao Institute of Agricultural Science)] were stratification treated at low temperature for 2 months. Seeds were germinated in the substrate at 25 °C with 60% humidity and photoperiod of 16/8 h light/dark cycle. The uniformly developed apple seedlings with four leaves were transferred into the half-strength Hoagland's nutrient solution. Then, the nutrient medium was replaced with complete nutrient solution after 1 week. For transcriptome sequencing, 60 uniformly developed seedlings with six leaves were selected and cultured in complete nutrient solution with 100 mM NaCl and 100 mM KCl, respectively. The unstressed plants were used as control (CK). The roots and leaves of the seedlings were collected at 1 and 6 h, respectively. All samples were immediately frozen in liquid nitrogen and stored at -80 °C to extract total RNA.

M. hupehensis, M26 and *Malus zumi* plants [which were provided from Guangli Sha's lab (Qingdao Institute of Agricultural Science)] were cultured on MS medium containing 0.5 mg L⁻¹ indole-3-butyric acid (IBA) and 0.5 mg L⁻¹ 6-benzylaminopurine (6-BA) at 25 °C with 60% humidity and photoperiod of 16/8 h light/dark cycle. Plants were transferred to fresh medium every 20 days. To explore the salt tolerance of *M. hupehensis*, M26 and *Malus zumi* seedlings, 60 uniformly developed plants which cultured on fresh medium 10 days were selected and cultured on MS medium containing 0.5 mg L⁻¹ indole-3-butyric acid (IBA) and 0.5 mg L⁻¹ 6-benzylaminopurine (6-BA) with 100 mM NaCl and 100 mM KCl, respectively. The phenotype of seedlings under salt stress was photographed in 5 days and 10 days. To identify the candidate genes of transcriptome analysis, the roots and leaves of *M. hupehensis*, M26 and *Malus zumi* seedlings were collected at one hour, 6 hours, 5 days and 10 days, respectively. All samples were immediately frozen in liquid nitrogen and stored at -80 °C to extract total RNA. The experiment was repeated three times.

RNA isolation

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, USA) and evaluated by 1% agarose gel electrophoresis. The RNA purity and integrity were checked using NanoPhotometer spectrophotometer (Implen, München, Germany) and the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, USA). Qubit® RNA Assay Kit in Qubi 2.0 Fluorometer (Thermo Fisher, Waltham, USA) was used to measure the RNA concentration.

Library construction and transcriptome sequencing

RNA per sample (3 µg) was used as input material for RNA sample preparation. The sequencing library was generated using the NEBNext® Ultra™ RNA Library Preparation Kit (NEB, Ipswich, USA). In addition, an index code was added to the attribute sequence for each sample [60]. Poly-T oligo-attached magnetic beads were utilized to purify mRNA from total RNA. Fragmentation was performed using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X) (NEB, Ipswich, USA).

Random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-) (Sangon, Shanghai, China) was used to synthesize first strand cDNA. DNA Polymerase I and RNase H were used to degrade RNA and second strand cDNA. NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization after adenylation of 3' ends of DNA fragments. The library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA) to select cDNA fragments of preferentially 250~300 bp. Then 3 µl USER Enzyme (NEB, Ipswich, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase (NEB, Ipswich, USA), Universal PCR primers and Index (X) Primer. Finally, PCR products were purified by using AMPure XP system and library quality was assessed on the Agilent Bioanalyzer 2100 system. There are 30 RNA-Seq libraries containing three replicates for each sample to obtain the *M. hupehensis* transcriptome under different salt treatments.

Clustering and sequencing

Index-encoded samples were clustered on the cBot Cluster Generation System by using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, library preparations were sequenced on an Illumina Hiseq platform and generated paired end reads of 125 bp/150 bp.

Quality control

Raw data in the fastq format (original reads) were first processed by an internal perl script. In this step, clean data (clean reads) were obtained by deleting the reads containing the adapter, the reads containing the poly-N, and the low quality reads from the original data. Q20, Q30, and GC content of the raw data were calculated. All downstream analyses were based on high-quality cleaning data.

RNA-Seq reads mapping

Reads were aligned to the apple genome (<https://www.rosaceae.org/species/malus/all>) by using hierarchical indexing for spliced alignment of transcripts (Hisat2 v2.0.5), which initially removed a portion of the reads according to the quality information accompanying of each read. Then, the reads were mapped to the reference genome. Hisat2 can generate a database of splice points based on it, and the gene model annotation file was better mapped than the other non spliced mapping tools.

Quantification of transcript abundance and differential expression analysis

The feature count v1.5.0-p3 was used to count the readings mapped to each gene. The fragments per kilobase of transcript per million mapped reads (FPKM) of each gene was calculated based on the length of the gene and the reading count mapped to the gene, which was used to determine the expression levels for mRNAs by calculating FPKM.

Differential expression analysis was performed using the DESeq2 R package (1.16.1) which provides statistical routines for determining differential expression in digital gene expression data by using a

model based on the negative binomial distribution [61]. Differential expression analysis of two conditions was carried out using the edgeR R package (3.18.1). The resulting P-values were fitted using Benjamini and Hochberg's approach for controlling the false discovery rate [62]. Genes with a $|\log_2(\text{fold change})| > 0$ and $\text{padj} < 0.05$ in sequence counts between libraries were considered significantly differentially expressed using DESeq2 compared with control.

GO and KEGG enrichment analysis of DEGs

GO enrichment analysis of DEGs was implemented by the cluster Profiler R package, in which the gene length bias was corrected [63]. GO terms with corrected P -value < 0.05 were considered significantly enriched by DEGs. KEGG is a database resource which for understanding high-level functions and utilities of the biological system (cell, organism and ecosystem) from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (<http://www.genome.jp/kegg/>). ClusterProfiler R package was used to test the statistical enrichment of differential expression genes in KEGG pathways [64].

Validation of RNA-Seq gene expression by using qRT-PCR

qRT-PCR analysis was performed to validate the expression levels of candidate DEGs revealed by the transcriptome data. The samples were obtained and conserved in liquid nitrogen after 100 mM NaCl and 100 mM KCl treatments for 0, 1h, 6h, 5d and 10day respectively. Total RNA was extracted with RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China), and the first-strand cDNA was synthesized from 2 μ g of total RNA using a 5X All-In-One RT MasterMix (abm, Sydney, Australia). All primers were synthesized at Sangon Biotech (Sangon, Shanghai, China) Co., Ltd. SYBR (Roche, Basel, Swiss) was used as the intercalating dye, and the *β -actin* gene of *Malus* was used as the inner reference gene. Three replicates were analyzed using a LightCycler® 480 (Roche, Basel, Swiss) instrument. The 10 μ l reactions was 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 58°C for 10 s and 72°C for 10 s, finally 72°C for 10 min. The relative expression of candidate DEGs was calculated using the $2^{-\Delta\Delta C_t}$ method. The experiment was repeated three times.

Experimental design and statistical analysis

All experiments were repeated thrice according to a completely randomized design. The data were analyzed by ANOVA followed by Fisher's least significance difference or Student's t -test analysis. Statistically significant differences were indicated by P -value < 0.05 and extremely significant differences were indicated by P -value < 0.01 . Statistical computations were conducted using SPSS (IBM, Armonk, USA).

Abbreviations

DEGs

Differentially expressed genes

GO

Gene ontology

KEGG

Kyoto encyclopedia of genes and genomes

ROS

Reactive oxygen species

SOS

Salt overly sensitive

IBA

Indole-3-butyric acid

6-BA

6-benzylaminopurine

FPKM

Transcript per million mapped reads

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data sets supporting the results of this article were included within the article and its additional files. The reads produced in this study had been deposited in the National Center for Biotechnology Information (NCBI) SRA database with accession number PRJNA588566. Access to the data was available upon publication at <http://www.ncbi.nlm.nih.gov/sra/>.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' Contributions

C. W. and Y. T. planned and designed the research. Y. L. and X. Z. performed experiments, conducted fieldwork and analyzed data etc. Y. L., X. Z., and C. W. wrote the manuscript. S. Y. and C. M. contributed to edit the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Pearson correlation of any two libraries of three repeats in the roots (a) and leaves (b). Pearson correlation was calculated using Log2 (RPKM) of expression values. CKR: the roots of control. Na1R: the roots under NaCl stress at 1 h. K1R: the roots under KCl stress at 1 h. Na6R: the roots under NaCl stress at 6 h. K6R: the roots under KCl stress at 6 h. CKL: the leaves of control. Na1L: the leaves under NaCl stress at 1 h. K1L: the leaves under KCl stress at 1 h. Na6L: the leaves under NaCl stress at 6 h. K6L: the leaves under KCl stress at 6 h.

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Figure 2

DEGs analyses between treatments and control in apple. The x-axis represents the log2fold change, and the y-axis represents the $-\log_{10}(\text{padj})$. Red dots represent the upregulated, and green dots represent the downregulated genes. (a) Na1R vs. CKR. (b) Na6R vs. CKR. (c) Na1L vs. CKL. (d) Na6L vs. CKL. (e) K1R vs. CKR. (f) K6R vs. CKR. (g) K1L vs. CKL. (h) K6L vs. CKL.

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Figure 3

Specific and common DEGs among treatments in Venn diagrams. (a) In the roots. (b) In the leaves. (c) All common DEGs in the roots and leaves.

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Figure 4

Heat map depicting log2 (fold change) of candidate differential expression genes involved in the NaCl and KCl treatments. Red indicates upregulation, and green indicates downregulation. These candidate genes were classified into six categories, namely, ion transporters, transcription factors, hormone signal pathway, antioxidant enzymes, MAPK pathway, and others.

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Ion Transporters

Figure 5

Validation of ion transporter genes with M26, *M. hupehensis* and *M. zumi* under NaCl and KCl stress at 0, 1h, 6 h, 5d and 10d, detected by qRT-PCR analysis in the roots and leaves. Each experiment was independently repeated thrice. Data are the means $\pm$ SD of triplicate experiments. Asterisks (*) indicate significant differences from the control (Student's t-test, *P<0.05, **P<0.01).

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Figure 6

Validation of MAPK pathway and transcription factor genes with M26, *M. hupehensis* and *M. zumi* under NaCl and KCl stress at 0, 1h, 6 h, 5d and 10d, detected by qRT-PCR analysis in the roots and leaves. Each experiment was independently repeated thrice. Data are the means $\pm$ SD of triplicate experiments. Asterisks (*) indicate significant differences from the control (Student's t-test, *P<0.05, **P<0.01).

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Figure 7

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