



In silico identification of NaCl responsive genes in apple

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Abstract

Salinity is one of the major abiotic stresses, limiting the productivity of agricultural crops. The present research designed to find and evaluate sodium chloride-responding genes in *Malus domestica* which have homologs in *Arabidopsis thaliana*. We found in the literature that there are 112 *Arabidopsis* proteins categorized in 28 osmotic stress responsive groups and performed a comparative analysis against *Malus domestica* database in GDR and eventually could identify 212 *Malus domestica* sequences. These genes were identified according to the high similarity percentage, high consequently alignment score, low E-value and the pattern sequence coverage. The analysis indicated a high number of sequences and diverse genes from all mentioned categories in apple. One of the most frequent ones came up with unknown function. Analyzing these sequences with an unknown function lead to the annotation of 45 *Malus domestica* sequences. By collecting a bunch of genes probably having a function in salt or osmotic stress responses in apple, we could have a better understanding about how a plant in Rosaceae can survive in salt stress condition.

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Introduction

Salinity is one of the most important factors restraining the yield of agricultural crops affecting adversely germination, plant vigor and crop yield (Munns and Tester, 2008). Salinity which is mostly caused by NaCl is a soil circumstance described by a high concentration of soluble salts. Soils are classified as saline when the EC is 4 dS/m or more (Anonymous, 2015), which is equivalent to approximately 40mM NaCl and generates an osmotic pressure of approximately 0.2 MPa.

Osmotic stress conditions trigger various physiological reactions including defense and metabolic pathways with a role in survival and growth. Salinity tolerance is caused by the genes that confine the amount of salt uptake from the soil and its transport through the plant, put ions in balance in and out of the cells, and control leaf development and the time senescence begins (Munns, 2005).

Apple is one of the 20 most productive crops in the world when measured by tonnage (Anonymous, 2016). This important horticultural crop is prone to yield and growth reductions in saline soils (Ferree and Warrington, 2003). Soil remediation cannot merely alleviate the salinity issue and breeding more tolerant crops would address more efficiently this economic disaster. Through identification of putative salt stress tolerance-involved genes in apple, further information about the response mechanisms for osmotic stresses in Rosaceae would be concluded and perhaps valuable criteria for crop improvement challenges would be provided.

Material and methods

Identification of genes

The genes involved in salt uptake and transport of *Arabidopsis* were obtained from the studies of Apse *et al.* (1999), Gaxiola *et al.* (2001), Laurie *et al.* (2002), Shi *et al.* (2003), Liu *et al.* (2000), Liu *et al.* (1998) and Seki *et al.* (2002).

Identification of protein sequences

The protein sequences of these NaCl-inducible genes were obtained from the NCBI database, at FASTA

format, and the locus tags of each gene was used as query sequences.

Identification of genes function

A brief description of each gene was obtained from the NCBI database. This information helped us to categorize genes, and revealed the function of some unknown genes that their function had already identified.

After this step, the protein sequences of *Arabidopsis thaliana* (as a model plant) compared with apple, in order to look for similar sequences against the *Malus domestica* database, using the BLASTp in the Genome Database for Rosaceae (GDR) website.

Result and discussion

In *Arabidopsis*, Seki *et al.* (2002) recognized 103 coding genes out of 7,000 genes and categorized them in 27 functional groups with a more than five-fold increased expression in response to osmotic stress. We used these genes and the genes from other references (see Materials and Methods) to find putatively involved genes in salinity tolerance in apple.

Arabidopsis thaliana is a leading genetic model system for discovering the molecular and biochemical basis of many important biological processes in plants, including the responses of a plant to environmental conditions such as salinity (Taiz and Zeiger, 2010).

Malus domestica is one of the most appreciated horticultural fruit crops in the world. Apple is liable to yield and growth cutbacks in saline soils (Ferree and Warrington, 2003). The draft apple genome has been recently published, in which the majority of the genes have not yet been functionally annotated (Velasco *et al.*, 2010). According to this record, apple has about 57000 genes, more genes than the human genome (about 30000).

Similar sequences often originate from the same ancestral sequence (Claverie and Notredam, 2007). This means that similar sequences possibly have the

same ancestor, sharing a similar structure, and a similar function. This principle is true even for the sequences from very different organisms (Claverie and Notredam, 2007). Two very similar proteins or gene sequences are called homologs if 25 percent of the amino acid in two sequences be identical, the length of protein sequences be at least 100 amino

acids and the E-value in comparison of two sequences be less than 10^{-4} (Claverie and Notredam, 2007). According to these, there were only 8 seed sequences that didn't have homology in apple genome database (Table1). According to similarity percentage (positives), the highest frequency of gene sequences was in the range of %70-%80 (Fig. 1).

Table 1. Eight seed sequences that didn't have homogeneity in apple genome database.

Query sequences	Positives	Identities	E-value	Gap percentage	Alignment score
<i>At1g42990</i>	60	44	0.014	-	37.7
<i>At2g18050</i>	66	51	2e-29	6	125
<i>At4g02380</i>	65	57	7e-20	8	92.8
<i>At1g73920</i>	41	26	1e-22	11	106
<i>At1g15430</i>	54	39	9e-40	16	160
<i>At1g55280</i>	65	50	5e-28	3	122
<i>At4g38060</i>	58	44	3e-15	11	78
<i>At1g76650</i>	60	42	2e-30	6	128

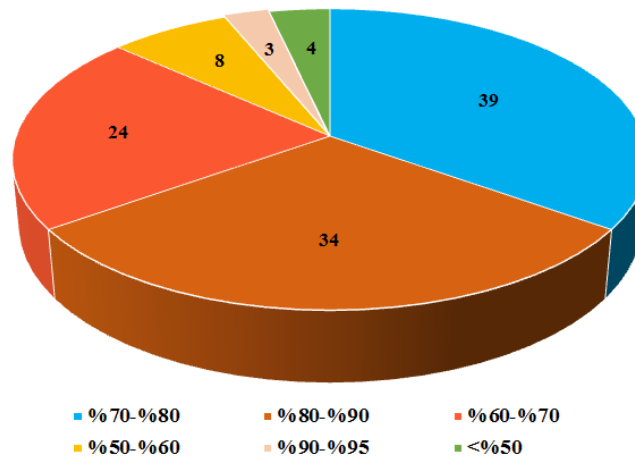


Fig. 1. The frequency of gene sequences in different similarity percentage.

The genes with unknown function were among the most appearing categories in expression analyses in terms of osmotic stress response in *Arabidopsis* which have got great attention from scientists as new candidate genes for crop improvement. The role of 25 genes in this category were identified based on the NCBI database (Table 2).

The identified genes involved in transcription regulation were from the gene families of bZIP, Zinc finger, WRKY, NAC, MYB, Homeodomain and DREB/ERF. It was a surprising result that there was no bZIP homolog in apple. This transcription factor function in many pathways such as osmotic stress

tolerance and has been identified in many plants. The DREB/ERF protein family had the highest number of sequences in comparison to the other transcription factors. This result was predictable, because 144 out of about 1,600 transcription factors found in *Arabidopsis thaliana* are members of the DREB/ERF-like family (Dietz *et al.*, 2010).

Zinc finger proteins are small structural motifs coordinated by one or more zinc ions to stabilize the fold (Krishna *et al.*, 2003). Zinc fingers proteins can variably bind DNA, RNA, proteins, or other small, useful molecules, based on their binding specificity (Ravasi *et al.*, 2003).

Table 2. Identification of some unknown sequences.

Query sequences	Positives	E-value	Alignment score	Description
<i>At1g02660</i>	70	0	743	Alpha/beta-hydrolases super family protein
<i>At1g11360</i>	77	7e-84	307	Alpha hydrolases-like protein
<i>At1g17380</i>	46	4e-23	105	Jasmonate-zim-domain protein 5
<i>At1g19180</i>	58	2e-48	189	Jasmonate-zim-domain protein 1
<i>At1g55280</i>	65	5e-28	122	Lipase/lipoxygenase, PLAT/LH2 family protein
<i>At1g63010</i>	85	0	1008	Major facilitator super family with SPX domain
<i>At1g76650</i>	60	2e-30	128	Calcium-binding protein CML38
<i>At2g21620</i>	78	4e-65	244	Dessication responsive protein
<i>At2g26560</i>	79	e-155	544	Phospholipase A 2A
<i>At2g40140</i>	65	0	653	Zinc finger CCH domain-containing protein
<i>At2g41190</i>	74	e-175	613	Trans membrane amino acid transporter family
<i>At2g41640</i>	70	e-167	587	Glycosyl transferase family 61 protein
<i>At3g61060</i>	80	1e-99	360	Phloem protein 2-A13
<i>At4g25670</i>	64	4e-47	184	Conserved peptide upstream ORF 12
<i>At4g27520</i>	54	3e-60	229	Early nodulin-like protein 2
<i>At4g30650</i>	88	3e-24	107	Putative low temperature and salt responsive
<i>At4g33050</i>	74	e-166	582	Calmodulin-binding protein
<i>At4g36040</i>	64	9e-35	143	Chaperon protein
<i>At4g37390</i>	85	0	919	Indole-3-acetic acid-amino synthetase GH3.2
<i>At5g09440</i>	72	1e-95	346	Protein exordium like 4
<i>At5g22290</i>	70	2e-68	256	Membrane-tethered transcription factor
<i>At5g42050</i>	68	e-140	375	Development and cell death domain protein
<i>At5g43260</i>	89	1e-44	175	Chaperon protein dnaJ-like protein
<i>At5g50100</i>	72	3e-71	265	Putative thiol-disulfide oxidoreductase DCC
<i>At5g63160</i>	76	e-118	422	BTB and TAZ domain protein 1

It is obvious that all of the analyzed categories can contribute to an increase in salt tolerance, however potential roles of some them seem more relevant. The genes that control salt uptake and transport have an important role in salt tolerance (Munns, 2005). *NHX1*, *AVP1*, *HKT1*, *SOS1*, *SOS2* and *SOS3* are the analyzed gene sequences in this group (Table S1). All the sequences in this group had a high alignment score, a low E-value and a high similarity. These genes have a function in Na⁺ transport. Because of negative electrical potential in plant cells (about -180 mV), the regulation of Na⁺ uptake looks more difficult and energy-demanding than Cl⁻. The prevention of Na⁺ uptake from the apoplast demands more ion selectivity and energy costs in comparison to the avoidance of Cl⁻ uptake: whenever the cytosolic Cl⁻ concentration is very low, Cl⁻ would enter a cell passively, otherwise not. Moreover, genetic variance of salt tolerance in plants shows a correlation with their capacity in limiting the rate of Na⁺ transport to leaves (Munns, 2005).

HKT1 has a role in limiting root-to-shoot movement of Na through unloading Na from the xylem (Sunarpi *et al.*, 2005).

Rus *et al.* (2006) demonstrated that HKT1 has a function primarily in the root to regulate shoot Na, and Davenport *et al.* (2007) could illustrate the role of HKT1 in Na efflux from the root xylem by using radioactive Na (²³Na). Interestingly, HKT1 had only one homolog in apple (Table S1).

Active ion transportation takes place through symporters and antiporters that can transport ions against an electrochemical potential gradient. The difference in electrochemical potential of a coupled solute, usually H⁺, can drive this transportation. The NHX family of antiporters (Na⁺/H⁺exchangers) are selective for Na⁺, while the rest being highly selective for an ion other than Na⁺ (Munns, 2005).

Overexpression of *NHX1*, a tonoplast membrane protein that pumps Na⁺ into the vacuole, has increased salt tolerance in many plants (Zhang and Blumwald, 2001; Davenport, 2007; Chen *et al.*, 2008). Li *et al.* (2017) expressed constitutively RtNHX1 from a recretohalophyte *Reaumuria trigyna* in *Arabidopsis* and could improve salt tolerance in transgenic *Arabidopsis* plants. The query sequence of NHX1 resulted in three counterparts in apple (Table S1).

Two types of tonoplast H⁺ electrogenic pumps have been reported: 1) a multi-subunit V-type (vacuolar type) ATPase and 2) the AVP1 H⁺-pyrophosphatase (PPase) respectively having ATP or pyrophosphate (PPi) as their substrates (Gaxiola *et al.*, 2007; Paez-Valencia *et al.*, 2011). The overexpression of AtAVP1 which encodes a type I vacuolar H⁺-pyrophosphatase,

has increased salt tolerance in transgenic *Arabidopsis* (Gaxiola *et al.*, 2001), rice (*Oryza sativa*) (Zhao *et al.*, 2006), alfalfa (*Medicago sativa*) (Bao *et al.*, 2009), creeping bentgrass (*Agrostis stolonifera*) (Li *et al.*, 2010), cotton (*Gossypium hirsutum*) (Pasapula *et al.*, 2011), peanut (*Arachis hypogaea*) (Qin *et al.*, 2013). AVP1 had five homologs in apple (Table S1).

Table S1. Identified candidates among abiotic stress responsive gene categories in Apple.

Category	Locus tag	Positives (%)	Identity (%)	Gaps (%)	E-Value	Alignment score	Number of sequences
bZIP Transcription Factor	<i>At1g42990</i>	60	44	-	0.014	37.7	1
	<i>At3g10740</i>	81	69	2	0	951	1
	<i>At3g06500</i>	78	66	5	0	910	1
Carbohydrate Metabolism	<i>At3g60130</i>	72	57	1	e-158	556	1
	<i>At5g18670</i>	76	59	3	e-180	627	1
	<i>At3g04240</i>	91	83	0	0	1650	2
	<i>At2g43820</i>	69	55	7	e-138	490	1
	<i>At3g53180</i>	80	67	0	0	1164	1
Cellular Metabolism	<i>At3g45300</i>	87	80	7	e-180	629	1
	<i>At2g39210</i>	81	70	1	0	829	1
	<i>At1g68620</i>	66	49	13	1e-92	337	1
	<i>At1g03220</i>	77	63	2	e-50	530	1
Cellular Structure Organization and Biogenesis	<i>At3g10720</i>	88	76	-	e-243	440	1
	<i>At5g62350</i>	74	54	5	e-55	211	1
	<i>At5g20230</i>	50	39	11	2e-33	139	2
Cytochrome P450	<i>At2g34500</i>	79	67	2	0	672	1
	<i>At3g26220</i>	68	47	3	e-133	473	2
Detoxification Enzyme	<i>At2g31570</i>	87	74	-	5e-68	253	1
	<i>At2g29450</i>	70	48	2	3e-51	198	2
	<i>At5g44070</i>	80	64	2	0	664	2
DNA Nucleus DREB/ERF Transcription Factor	<i>At2g18050</i>	66	51	6	2e-29	125	3
	<i>At1g22190</i>	71	62	14	e-160	230	3
Ethylene Biosynthesis	<i>At4g17500</i>	69	58	12	5e-78	288	3
	<i>At5g43450</i>	70	53	1	e-110	394	5
Fatty Acid Metabolism	<i>At1g17020</i>	77	59	2	e-121	431	2
	<i>At1g73480</i>	70	57	10	e-129	460	2
	<i>At4g09760</i>	83	64	0	e-136	483	1
	<i>At1g73920</i>	41	26	11	1e-22	106	1
Ferritin	<i>At1g07720</i>	81	69	2	0	673	2
	<i>At5g01600</i>	81	68	3	4e-90	328	3
Heat Shock Protein	<i>At3g46230</i>	86	73	2	6e-64	239	7
	<i>At1g16030</i>	90	82	-	0	1077	2
Homeodomain Transcription Factor	<i>At2g35940</i>	64	52	17	e-175	708	3
	<i>At5g54170</i>	64	48	5	e-116	417	2
Membrane Protein	<i>At1g30360</i>	81	66	1	0	983	2
	<i>At1g01060</i>	45	34	28	7e-84	309	1
MYB Transcription Factor	<i>At5g63790</i>	70	62	16	4e-86	315	1
	<i>At4g27410</i>	81	72	6	e-107	369	2
NAC Transcription Factor	<i>At2g47180</i>	85	78	4	e-162	567	2
	<i>At1g09350</i>	82	73	5	e-144	509	2
	<i>At1g60470</i>	77	70	4	e-145	510	3
	<i>At3g57520</i>	84	75	5	0	748	2
Osmoprotectant	<i>At5g20830</i>	91	81	0	0	1366	4
	<i>At4g15530</i>	87	78	1	0	1511	1
Photosynthesis							

Category	Locus tag	Positives (%)	Identity (%)	Gaps (%)	E-Value	Alignment score	Number of sequences
Plant Defense	<i>At3g55430</i>	73	56	4	e-141	499	3
	<i>At4g13580</i>	81	71	1	2e-95	345	2
	<i>At2g40000</i>	71	53	8	e-145	511	2
	<i>At5g06860</i>	77	64	0	e-120	429	2
Protein degradation	<i>At1g47128</i>	82	68	3	0	684	3
Protein Kinase	<i>At2g31880</i>	79	63	1	0	803	1
Protein Phosphatase	<i>At5g25110</i>	84	72	0	0	635	3
	<i>At4g26080</i>	70	60	11	e-113	407	7
Reproductive development	<i>At3g11410</i>	72	60	10	e-134	476	4
	<i>At5g56750</i>	82	73	8	e-143	504	2
Secondary Metabolism	<i>At3g22370</i>	86	73	1	e-124	441	4
Senescence-related	<i>At2g38240</i>	75	60	1	e-126	448	2
	<i>At5g13170</i>	71	55	7	1e-82	303	3
Transport Protein Ion channel Carrier	<i>At1g58360</i>	81	66	3	0	684	3
	<i>At1g08930</i>	68	48	3	e-121	483	2
	<i>At5g20380</i>	77	67	5	0	695	2
	<i>At2g22500</i>	84	71	3	e-127	452	4
	<i>At5g22290</i>	70	52	8	2e-68	256	1
	<i>At1g11210</i>	47	32	15	3e-26	116	1
	<i>At1g15430</i>	54	39	16	9e-40	160	1
	<i>At1g55280</i>	65	50	3	5e-28	122	1
	<i>At1g63720</i>	65	54	8	2e-56	216	1
	<i>At1g69890</i>	72	57	4	5e-89	324	1
	<i>At1g76600</i>	64	47	16	2e-42	169	1
	<i>At2g26560</i>	79	69	0	e-155	544	5
	<i>At2g32240</i>	69	50	6	0	1256	2
	<i>At2g38820</i>	67	48	10	5e-80	295	1
	<i>At2g41190</i>	74	60	5	e-175	613	1
	Unknown Protein	<i>At3g17800</i>	78	64	3	e-147	516
<i>At4g21570</i>		88	74	0	e-132	469	1
<i>At4g25670</i>		64	53	13	4e-47	184	1
<i>At4g27520</i>		54	40	10	3e-60	229	3
<i>At4g30650</i>		88	80	-	3e-24	107	1
<i>At4g38060</i>		58	44	11	3e-15	78	1
<i>At5g02020</i>		55	48	20	2e-29	125	1
<i>At5g42050</i>		68	58	11	e-140	375	1
<i>At5g50100</i>		72	60	5	3e-71	265	4
<i>At3g61060</i>		80	69	1	1e-99	360	1
<i>At4g37390</i>		85	76	1	0	919	3
<i>At5g63160</i>		76	61	3	e-118	422	1
<i>At5g43260</i>		89	82	-	1e-44	175	1
<i>At1g76650</i>		60	42	6	2e-30	128	1
<i>At1g29395</i>		78	59	4	3e-59	225	3
<i>At2g40140</i>		66	53	15	0	653	2
<i>At4g36040</i>	64	50	8	9e-35	143	2	
<i>At4g33050</i>	74	61	7	e-166	582	2	
<i>At5g09440</i>	72	61	8	1e-95	346	2	
<i>At1g17380</i>	46	32	19	4e-23	105	1	
<i>At1g02660</i>	70	54	8	0	743	3	
<i>At2g21620</i>	78	66	11	4e-65	244	1	
<i>At1g27760</i>	81	64	1	e-161	565	3	
<i>At1g19180</i>	58	44	11	2e-48	189	1	
<i>At1g63010</i>	85	75	3	0	1008	2	
<i>At2g41640</i>	70	58	10	e-167	587	2	
<i>At1g11360</i>	77	66	8	7e-84	307	2	
Water Channel Protein	<i>At2g37180</i>	79	69	2	e-101	366	1
WRKY Transcription Factor	<i>At2g30250</i>	61	45	12	3e-91	332	2
	<i>At5g13080</i>	92	82	-	4e-46	180	2
Zinc Finger Transcription Factor	<i>At2g19580</i>	80	64	5	e-112	400	1
	<i>At5g59820</i>	57	46	19	1e-130	129	1
	<i>At2g31380</i>	70	57	5	5e-72	268	1

Category	Locus tag	Positives (%)	Identity (%)	Gaps (%)	E-Value	Alignment score	Number of sequences
Salt Uptake and transport	<i>At5g27150</i>	89	79	0	0	867	3
	<i>At1g15690</i>	94	88	0	0	1092	2
	<i>At4g10310</i>	68	53	4	e-142	501	1
	<i>At2g01980</i>	69	58	10	0	1272	2
	<i>At5g35410</i>	85	75	2	0	644	2
	<i>At5g24270</i>	88	76	2	1e-84	310	2

*The information is for best hits

*The number of sequences column shows the number of other hits

There is another pathway called SOS which contains three proteins cooperating to enhance the efflux of Na⁺ from cells in response to salinity. These three proteins of SOS pathway, SOS1, SOS2 and SOS3 have an important function in responding to NaCl oversupply in *A. thaliana* (Zhu *et al.*, 2000). The first noted reaction to an increase in Na⁺ around the roots is an upsurge in cytosolic free Ca²⁺; the extracellular addition of Na⁺ could trigger the flux of Ca²⁺ into the cytosol across the plasma membrane as well as the tonoplast (Kiegle *et al.*, 2000; Moore *et al.*, 2002; Tracy *et al.*, 2008). Increase in cytosolic Ca²⁺, sensed by SOS3, a Ca²⁺ binding protein, binds Ca²⁺ initiating SOS3 binding directly with SOS2 (Halfter *et al.*, 2000). SOS2 is a protein kinase which can phosphorylate other proteins. Binding of SOS3 to SOS2 activates this phosphorylase activity and then the SOS2/SOS3 complex phosphorylates SOS1 in the plasma membrane (Quintero *et al.*, 2002). This phosphorylation increases the Na⁺ pumping activity of SOS1 which in turn increases the efflux of Na⁺ from cells. The SOS1 and SOS2 genes are upregulated after exposure to NaCl to enhance further the Na⁺ efflux (Shi *et al.*, 2002).

Conclusion

Many of the osmotic stress responsive proteins were data-mined from the literature on *Arabidopsis thaliana* and comparatively analyzed against *Malus domestica* database. Some 212 *Malus domestica* sequences were identified according to the high similarity percentage, high consequently alignment

score, low E-value and the pattern sequence coverage. The genes found in this *in silico* discovery belonged to diverse functional categories, however, those with unknown function in apple were the most frequent (45 sequences). We hope this information may pave the way for identification and functional characterization of the osmotic stress tolerance related genes in apple.

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