



# Agronomic, physiological, and molecular characterization of salt tolerant alfalfa (*Medicago sativa*)



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# Acknowledgements

**Michael Peel  
Ryan Nelson  
Kim Thorsted  
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Rokebul Anower**



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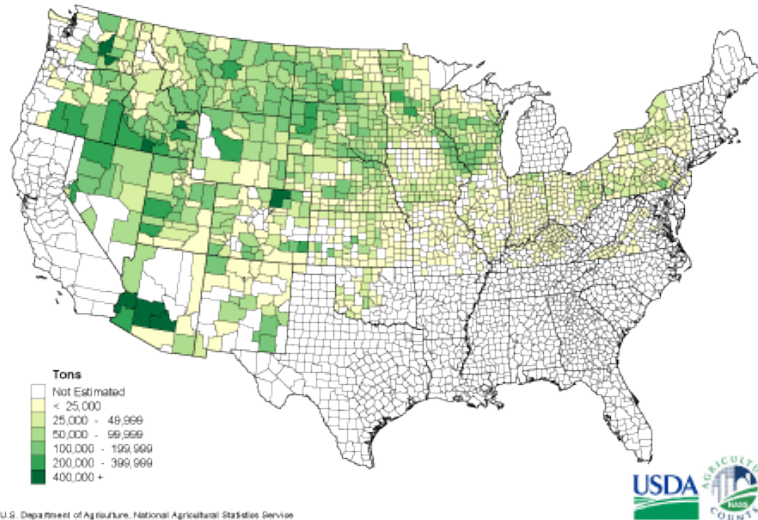
**United States  
Department of  
Agriculture**

**National Institute  
of Food and  
Agriculture**



# Alfalfa production in the US

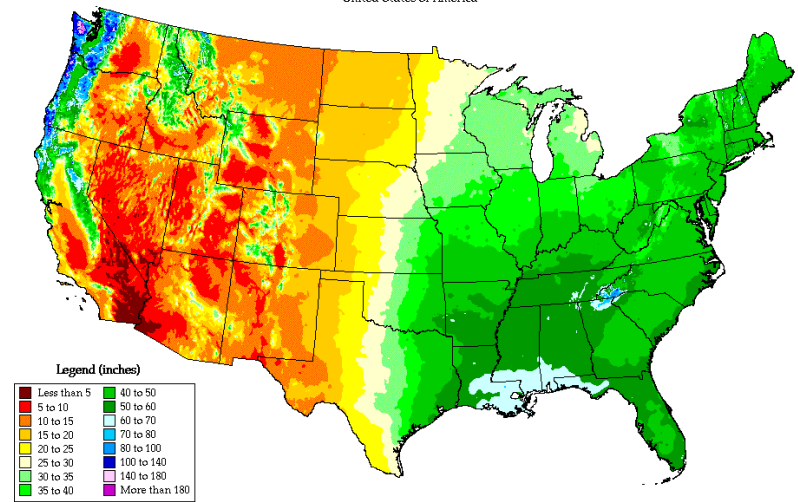
Alfalfa Hay (Dry) 2010  
Production by County  
for Selected States



U.S. Department of Agriculture, National Agricultural Statistics Service

Annual Average Precipitation

United States of America



Period: 1961-1990





# Alfalfa use in Intermountain West





# Salinity: a global problem

- Prevalent in intermountain region of the western US
- Salinity leads to reduced crop yields
- More arable land is lost to salinity than gained through forest clearing
- 1/3 of world's irrigated land is unsuitable for growing crops due to high salt levels

Frommer, et al.(1999) Science 285:1222-1223

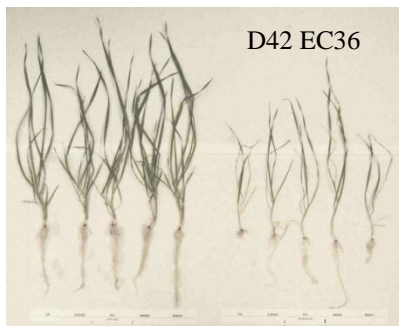






# Effect of salinity on plants

- Difficult to utilize water – drought effect
- Toxicity to plants – reduced growth, leaf burn, plant death
- Reduce availability of other ions e.g. K, Mg, N, or P





# Developing Salt Tolerant Plants

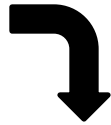
- **Important for future crop production**
- **Large variation in salt tolerance among and between species**
- **Single genes can have large effects**
- **Different species may have evolved different mechanisms of tolerance**





# Four populations of salt tolerant alfalfa

Crosse in greenhouse



Screen for survival ( $EC = 18 \text{ dS m}^{-1}$ )



Establish seedlings



Saline irrigation



3 cycles of selection

<u>Population</u>	<u>Type</u>	<u>Ploidy</u>
BC79	Falcata	Tetraploid
CkSltn	Sativa	Tetraploid
PI	Falcata	Diploid
SaltII	Sativa	Tetraploid



# Greenhouse selected alfalfa: now what?

Un-selected

Selected

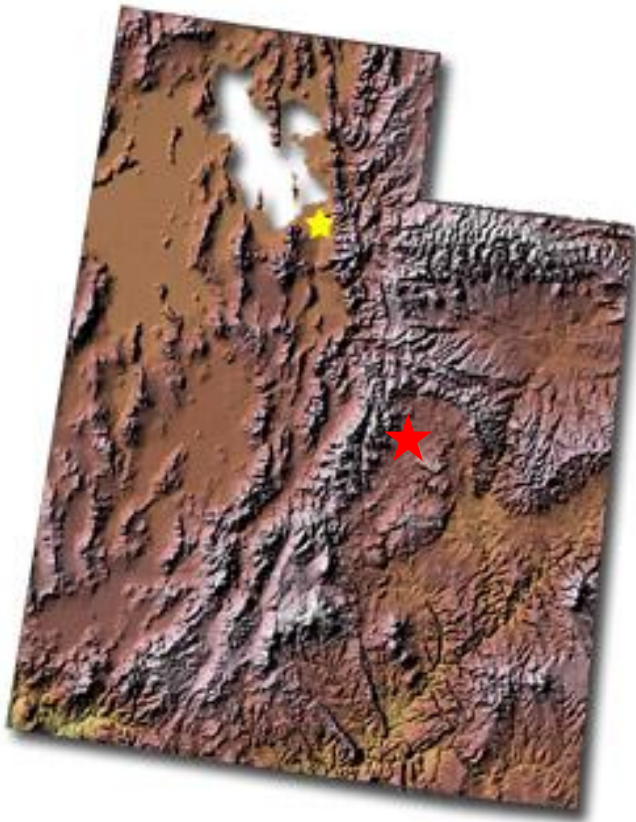


EC 18 dS m<sup>-1</sup> for 9 weeks in GH

- 1) Agronomic evaluation in saline field site
- 2) Study physiological mechanisms of tolerance
- 3) Identify molecular genetic changes



# Field evaluation



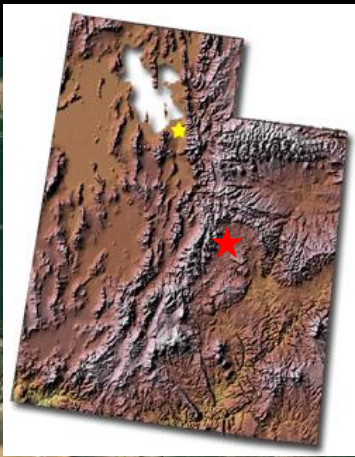
## Field site

Near Castledale, UT  
Naturally saline soil  
20+ years saline irrigation  
EC = 5.0-7.0 dS m<sup>-1</sup>

## Evaluate performance for 2 years

Morphological characteristics  
Forage yield  
Forage quality  
Salt content in plants  
Boron content in plants





© 2012 Google

Image State of Utah

Google earth









# Cloning plants from the field







# Morphological changes

Percent difference in node number, stem length and leaf to stem ratio of selected material compare to their parents.

	Node Number		Stem Length		Leaf to Stem Ratio	
	non-saline	saline	non-saline	saline	non-saline	saline
	----- % of Parent Mean -----					
BC79	89	109	87	113	113	84
SII	100	90	95	109	115	95
CkSltn	104	98	95	94	125	114

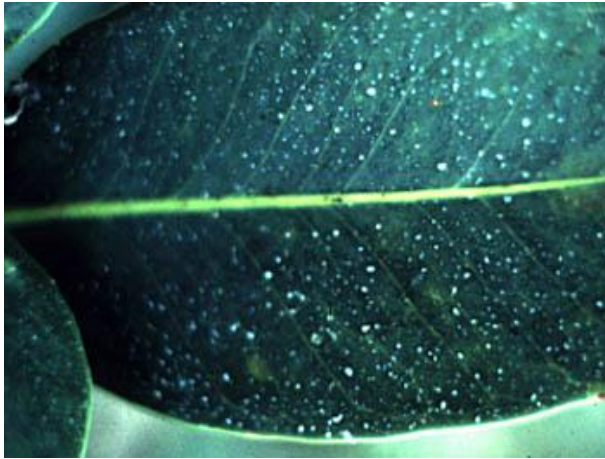


# Plant salt tolerance mechanisms

- Exclusion
  - Gated membrane channels in root cells (*HKT1*)
- Compartmentalization
  - Vacuole  $\text{Na}^+/\text{H}^+$  antiports (*NHX1*)
- Secretion
  - Plasma membrane  $\text{Na}^+/\text{H}^+$  antiports (*SOS1*)
- Osmoprotectants accumulation/detoxification (proline, trehalose, mannitol, and sorbitol; ROS)



# Physiological mechanism of salt tolerance



<http://life.bio.sunysb.edu/marinebio/mangal.html>  
Photo by Robert Twilley

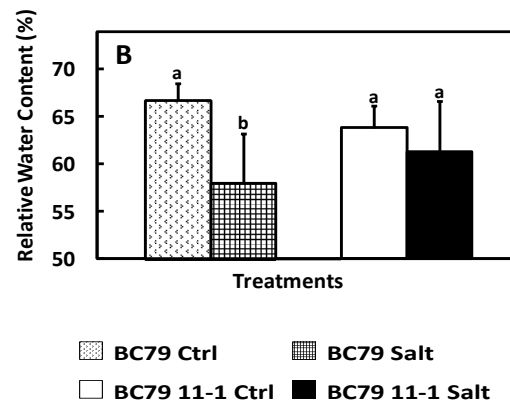
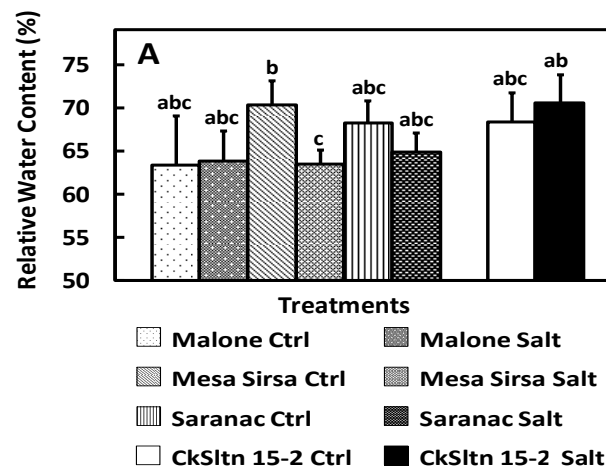
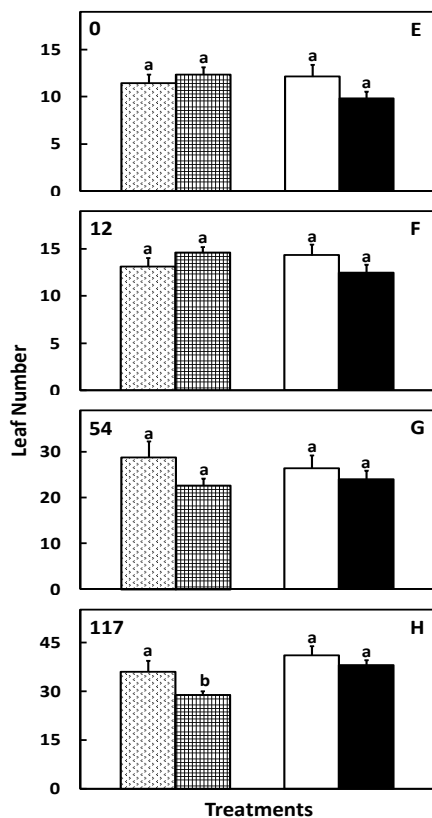
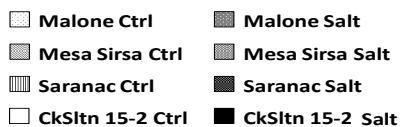
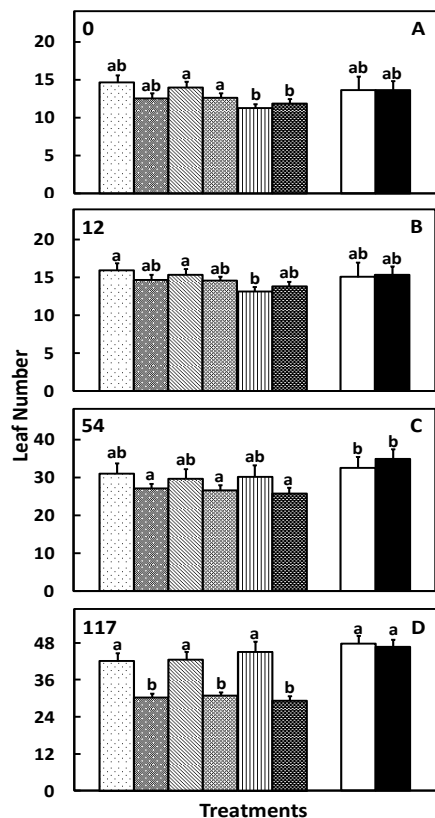
## Determine Physiological mechanisms

1. Measure growth rate and leaf senescence
2. Evaluate ion concentration in plant tissue
3. Determine salt compartmentalization in plant tissues
4. Other physiological measurements



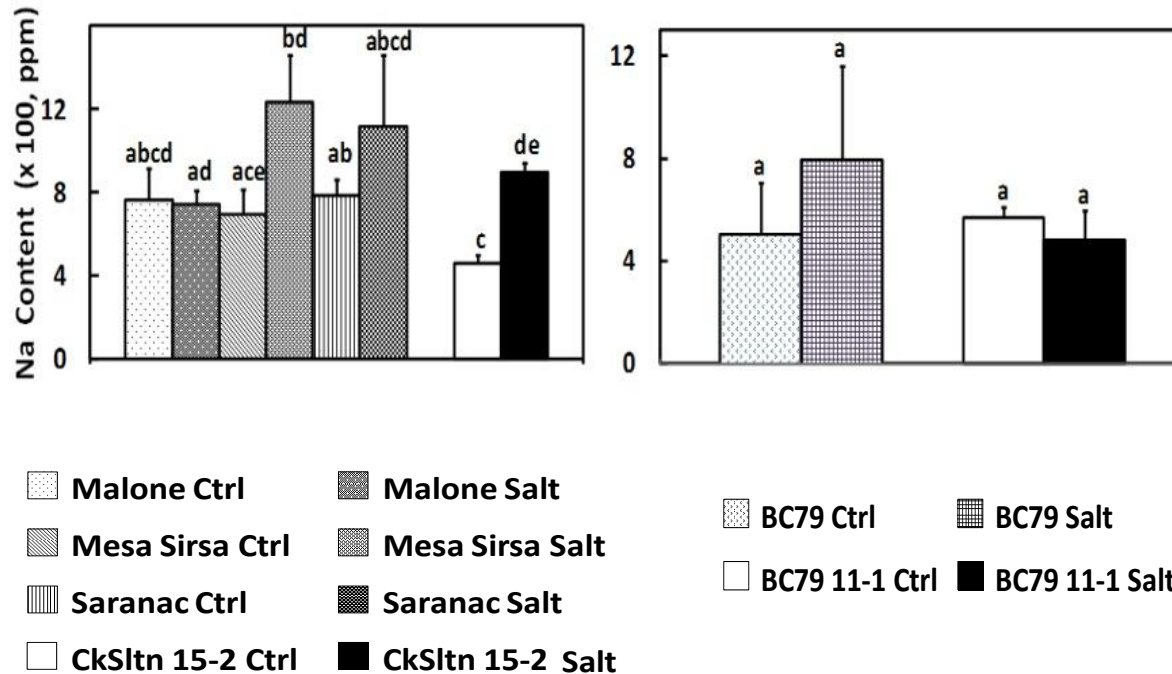


# Leaf number and RWC





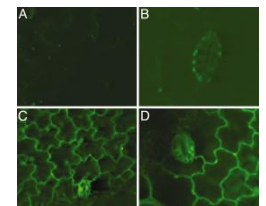
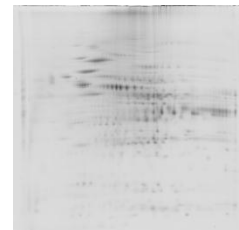
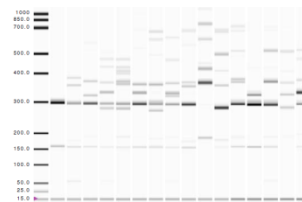
# Na<sup>+</sup> content





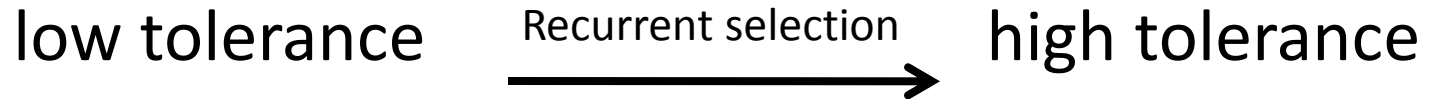
# Molecular genetic studies

1. Microarray analysis (*Medicago truncatula* GeneChip)
2. Bulk segregant analysis
3. Proteomics
4. Transformation experiments






# Selection for salt tolerance within alfalfa populations








# Selection for salt tolerance within alfalfa populations

low tolerance Recurrent selection  high tolerance

low allele frequency  
low expression Recurrent selection  high allele frequency  
high expression



# Medicago GeneChip experimental design & sampling



## 61,200 probe sets

- 32,167 *M. truncatula* genes (ESTs & chloroplast)
- 18,733 *M. truncatula* genes (Genomic predictions)
- 1,896 *M. sativa* genes (ESTs)
- 8,305 *S. meliloti* genes.(Genomic predictions)

**2 Populations      Selected & parent      2 tissues      2 treatments      3 samples**

**BC79 & CkSltn      BC79-11 & CkSltn-15      Roots & Shoots      Salt & control**

**Total = 48 GeneChips**



# Medicago GeneChip Results

## Selected BC79-11 versus non-selected BC79 under salt stress (EC=9.0 dS m<sup>-1</sup>)

Probe Set ID	Log (base 2) expression				Fold change		Target Description
	Salt-stressed		non-stressed		Salt-stressed	Non-stressed	
	BC79	BC79-11	BC79	BC79-11	BC79-11/BC79	BC79-11/BC79	
	<b>Upregulated in BC-79-11 under salt stress</b>				<b>No change when non-stressed</b>		
Mtr.11859.1.S1_s_at	4.0	7.5	6.4	6.7	11.1	1.2	NAD(P)H-quinone oxidoreductase chain 3, chloroplast
Mtr.18232.1.S1_s_at	6.8	10.2	9.6	9.8	10.7	1.2	H+ ATPase
Mtr.12676.1.S1_s_at	3.8	7.1	6.1	6.1	9.4	1.0	NADH2 dehydrogenase (ubiquinone)
Mtr.20283.1.S1_s_at	5.6	8.8	7.4	8.1	8.9	1.7	Ribosomal protein
Mtr.29143.1.S1_s_at	5.7	8.8	8.3	8.7	8.8	1.4	Chloroplast 30S ribosomal
Mtr.14609.1.S1_at	4.8	7.8	6.6	6.7	8.4	1.1	Cytochrome b/b6
Mtr.12676.1.S1_at	2.6	5.5	4.6	4.7	7.6	1.0	NADH2 dehydrogenase (ubiquinone)
Mtr.5939.1.S1_s_at	5.3	8.2	7.9	8.7	7.4	1.8	O-diphenol-O-methyl transferase
Mtr.33674.1.S1_s_at	2.1	4.9	4.3	5.0	7.3	1.6	Glutamyl-tRNA reductase 1
Mtr.14625.1.S1_at	7.7	10.6	9.7	9.8	7.2	1.1	Photosystem I assembly Ycf4
Mtr.41403.1.S1_at	1.7	4.5	4.9	4.8	6.7	1.0	Beta-ketoacyl-CoA-synthase
Mtr.40997.1.S1_s_at	7.2	9.7	9.9	9.4	6.0	0.7	Photosystem I P700 chlorophyll A apoprotein
Mtr.5656.1.S1_s_at	2.9	5.4	5.7	5.6	5.8	0.9	Vacuolar H+ATPase B subunit
Mtr.32665.1.S1_s_at	4.9	7.4	8.5	9.0	5.5	1.4	Myo-inositol-1-phosphate synthase
Mtr.8784.1.S1_s_at	2.1	4.5	4.2	4.6	5.5	1.3	pyruvate kinase-like protein
AFFX-Mtr-r2-Bs-dap-5_at	2.4	4.6	3.7	4.2	4.9	1.5	biotin acetyl-CoA-carboxylase ligase (birA)
Mtr.34428.1.S1_s_at	7.6	9.8	9.8	10.0	4.7	1.1	NADP-dependent glyceraldehydephosphate dehydrogenase
Mtr.2057.1.S1_s_at	2.9	5.1	4.9	5.4	4.7	1.4	Glutamate/malate translocator
Mtr.39594.1.S1_at	3.2	5.4	5.0	5.9	4.6	1.8	Trehalose-6-phosphate phosphatase
Mtr.8502.1.S1_s_at	3.8	6.0	5.9	5.9	4.6	1.0	Tubulin
Mtr.27751.1.S1_s_at	2.7	4.9	4.7	4.9	4.6	1.1	Tubby-like protein
Mtr.6031.1.S1_at	1.6	3.8	4.2	4.4	4.6	1.2	Lipid transfer protein
Mtr.24901.1.S1_at	2.7	4.9	3.5	2.4	4.6	0.5	Putative phospholipase
Mtr.43083.1.S1_s_at	2.4	4.5	4.5	4.7	4.4	1.2	Phosphoglucosyltransferase
Mtr.41766.1.S1_s_at	2.1	4.3	4.3	4.6	4.4	1.2	Protein kinase AFC2
Mtr.5143.1.S1_s_at	2.8	4.9	4.6	4.7	4.3	1.1	Glucosyltransferase-like protein
AFFX-r2-Bs-dap-5_at	2.6	4.7	3.4	4.1	4.3	1.7	dihydrodipicolinate reductase
Mtr.13242.1.S1_at	0.9	3.0	2.5	1.8	4.3	0.6	Magnesium chelatase
Mtr.4452.1.S1_at	1.6	3.7	3.1	3.4	4.2	1.3	COP1-Interacting Protein 7
Mtr.14644.1.S1_s_at	4.1	6.2	7.0	6.0	4.2	0.5	Photosystem I psaA and psaB
Mtr.43024.1.S1_s_at	3.1	5.2	5.4	5.6	4.1	1.2	6-phosphogluconate dehydrogenase
Mtr.26601.1.S1_s_at	2.8	4.8	6.0	5.6	4.0	0.7	NADPH oxidase
75 unknown genes					14.4-4.0	0.5-1.9	
	<b>Upregulated in BC-79-11 under salt stress</b>				<b>Upregulated when non-stressed</b>		
Mtr.39283.1.S1_at	1.6	5.3	5.1	6.1	12.7	2.0	Threonyl-tRNA synthetase
Mtr.37441.1.S1_s_at	2.8	5.6	4.2	5.8	7.0	2.9	Alcohol dehydrogenase 1
Mtr.17409.1.S1_s_at	5.0	7.7	5.2	7.9	6.4	6.4	Amino acid/polyamine transporter II
Mtr.31323.1.S1_at	3.9	6.2	5.0	6.0	5.2	2.0	Aspartic proteinase
Mtr.44991.1.S1_at	1.7	4.0	2.3	3.5	5.1	2.4	Cucumis-like serine protease
Mtr.22140.1.S1_at	2.9	5.2	3.5	5.1	4.7	2.9	glycosyltransferase
Mtr.4615.1.S1_at	2.1	4.2	1.7	4.6	4.4	7.6	Serine/threonine kinase-like protein
Mtr.33547.1.S1_at	3.8	5.9	3.5	5.3	4.2	3.6	Cellulose synthase-like protein
Mtr.11715.1.S1_at	2.7	4.8	2.7	5.1	4.1	5.4	Albumin 1 precursor
23 unknown genes					51.3-4.0	17.9-2.0	

### Shoots

• 139 probes up-regulated (>4.0-fold) in BC79-11 compared to BC79

• 107 no change when non-stressed  
32 known ontology  
75 unknown functions

• 32 up-regulated when non-stressed  
9 known ontology  
23 unknown function



# Medicago GeneChip Results

## BC79 Shoots

Probe Set ID	Log (base 2) expression				Fold change		Target Description
	Salt-stressed		non-stressed		Salt-stressed	Non-stressed	
	BC79	BC79-11	BC79	BC79-11	BC79-11/BC79	BC79-11/BC79	
Mtr.18232.1.S1_s_at	6.8	10.2	9.6	9.8	10.7	1.2	H <sup>+</sup> ATPase
Mtr.5656.1.S1_s_at	2.9	5.4	5.7	5.6	5.8	0.9	Vacuolar H <sup>+</sup> -ATPase B subunit
Mtr.11859.1.S1_s_at	4.0	7.5	6.4	6.7	11.1	1.2	NAD(P)H-quinone oxidoreductase chain 3, chloroplast
Mtr.12676.1.S1_s_at	3.8	7.1	6.1	6.1	9.4	1.0	NADH2 dehydrogenase (ubiquinone)
Mtr.12676.1.S1_at	2.6	5.5	4.6	4.7	7.6	1.0	NADH2 dehydrogenase (ubiquinone)
Mtr.34428.1.S1_s_at	7.6	9.8	9.8	10.0	4.7	1.1	NADP-dependent glyceraldehydephosphate dehydrogenase
Mtr.5143.1.S1_s_at	2.8	4.9	4.6	4.7	4.3	1.1	Glucosyltransferase-like protein
Mtr.26601.1.S1_s_at	2.8	4.8	6.0	5.6	4.0	0.7	NADPH oxidase
Mtr.39594.1.S1_at	3.2	5.4	5.0	5.9	4.6	1.8	Trehalose-6-phosphate phosphatase





# Medicago GeneChip Results

## BC79 Shoots

Probe Set ID	Log (base 2) expression				Fold change		Target Description
	Salt-stressed		non-stressed		Salt-stressed	Non-stressed	
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Mtr.11859.1.S1_s_at	4.0	7.5	6.4	6.7	11.1	1.2	NAD(P)H-quinone oxidoreductase chain 3, chloroplast
Mtr.12676.1.S1_s_at	3.8	7.1	6.1	6.1	9.4	1.0	NADH2 dehydrogenase (ubiquinone)
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# Vacuolar $\text{Na}^+/\text{H}^+$ Antiporter

Plant Mol Biol Rep (2011) 29:278–290  
DOI 10.1007/s11105-010-0224-y

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## The Vacuolar $\text{Na}^+/\text{H}^+$ Antiporter Gene *SsNHX1* from the Halophyte *Salsola soda* Confers Salt Tolerance in Transgenic Alfalfa (*Medicago sativa* L.)

Wangfeng Li · Deli Wang · Taicheng Jin · Qing Chang · Dongxu Yin · Shoumin Xu · Bao Liu · Lixia Liu

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© Springer-Verlag 2010

**Functional Plant Biology** Accepted 06 June 2012

The vacuolar  $\text{Na}^+/\text{H}^+$  Antiport Gene *TaNHX2* confer salt tolerance to transgenic alfalfa (*Medicago sativa* L.) Yanmin Zhang, Zihui Liu, Zhiyu Wen, Hongmei Zhang, Fan Yang, Xiulin Guo



# Medicago GeneChip Results

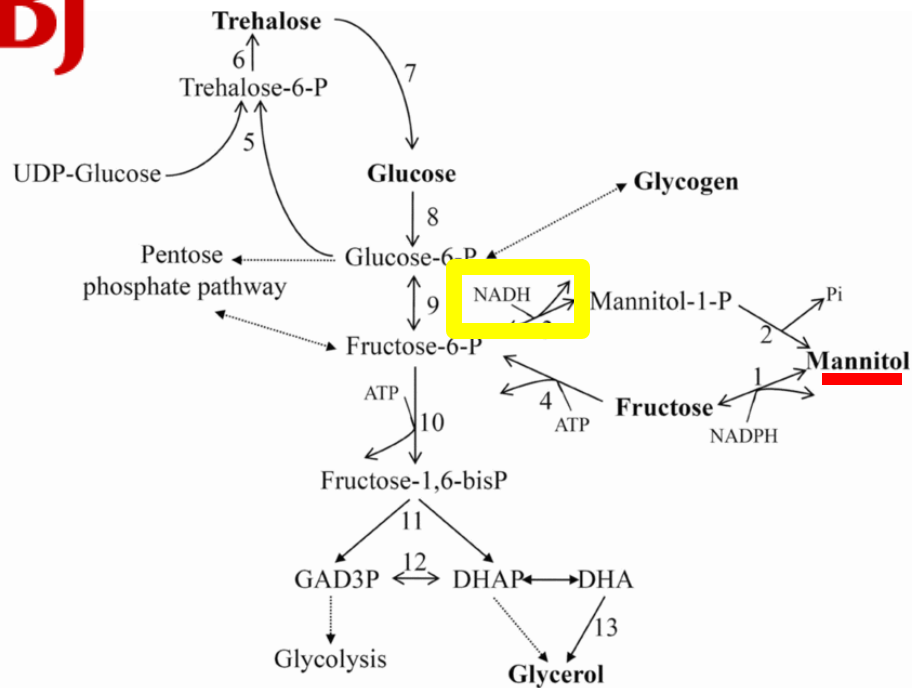
## BC79 Shoots

Probe Set ID	Log (base 2) expression				Fold change		Target Description
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	BC79	BC79-11	BC79	BC79-11	BC79-11/BC79	BC79-11/BC79	
Mtr.18232.1.S1_s_at	6.8	10.2	9.6	9.8	10.7	1.2	H+ ATPase
Mtr.5656.1.S1_s_at	2.9	5.4	5.7	5.6	5.8	0.9	Vacuolar H+-ATPase B subunit
Mtr.11859.1.S1_s_at	4.0	7.5	6.4	6.7	11.1	1.2	NAD(P)H-quinone oxidoreductase chain 3, chloroplast
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Mtr.5143.1.S1_s_at	2.8	4.9	4.6	4.7	4.3	1.1	Glucosyltransferase-like protein
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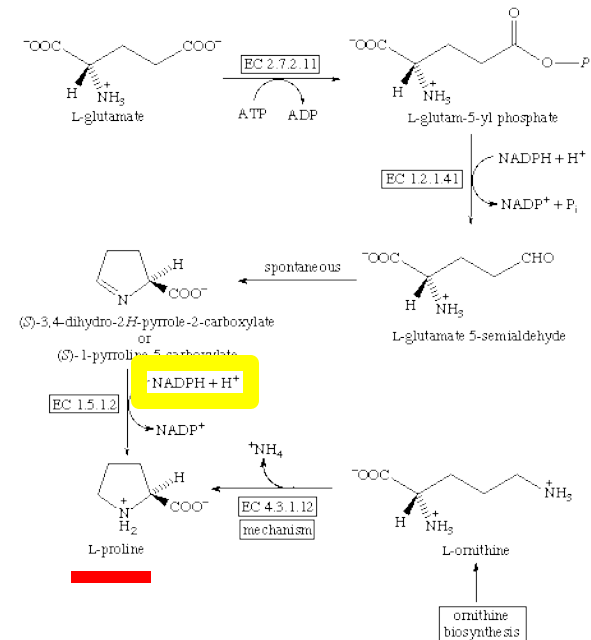
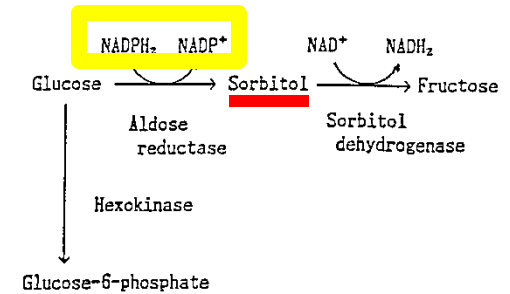


# Osmoprotectant biosynthesis

**BJ**



- |                                |                               |                                |
|--------------------------------|-------------------------------|--------------------------------|
| 1 : Mannitol dehydrogenase     | 5 : Trehalose-6-P synthase    | 9 : Phosphoglucose isomerase   |
| 2 : Mannitol-1-P phosphatase   | 6 : Trehalose-6-P phosphatase | 10 : Phosphofruktokinase       |
| 3 : Mannitol-1-P dehydrogenase | 7 : Trehalase                 | 11 : Aldolase                  |
| 4 : Fructokinase               | 8 : Glucokinase               | 12 : Triosephosphate isomerase |
|                                |                               | 13 : Glycerol dehydrogenase    |



## Plant, Cell & Environment

Expression of a celery mannose 6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a glucosyl-mannitol dimer



G. ZHIFANG, W. H. LOESCHER\*

Article first published online: 31 JAN 2003

DOI: 10.1046/j.1365-3040.2003.00958.x

Issue



Plant, Cell & Environment

Volume 26, Issue 2, pages  
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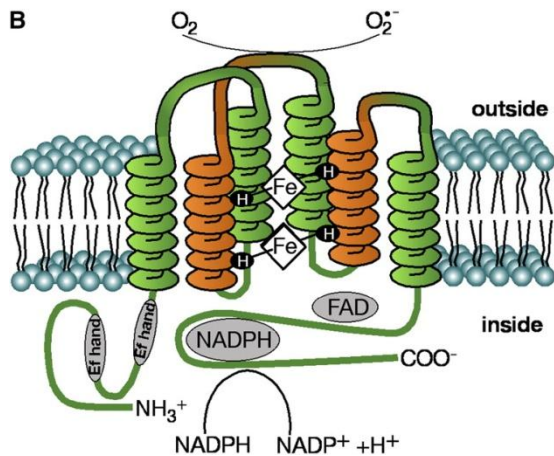
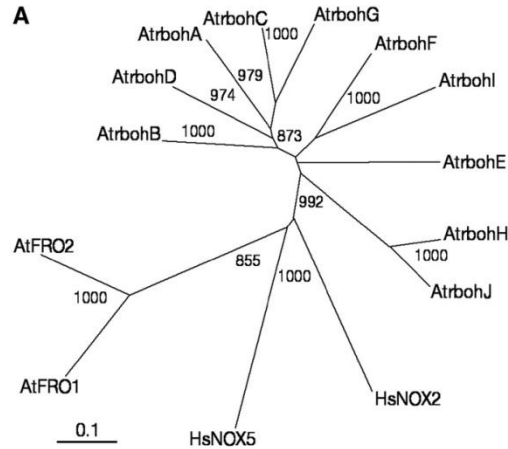
**Glucose-6-phosphate dehydrogenase-dependent hydrogen peroxide production is involved in the regulation of plasma membrane H<sup>+</sup>-ATPase and Na<sup>+</sup>/H<sup>+</sup> antiporter protein in salt-stressed callus from *Carex moorcroftii***

Jisheng Li<sup>a,b</sup>, Guichen Chen<sup>a</sup>, Xiaomin Wang<sup>b</sup>, Yanli Zhang<sup>b</sup>, Honglei Jia<sup>b</sup> and Yurong Bi<sup>a,b,\*</sup>

<sup>a</sup>Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China

<sup>b</sup>School of Life Sciences, Lanzhou University, Lanzhou 730000, China

# Reactive Oxygen Species (ROS)



Journal of Experimental Botany Advance Access published October 6, 2011

Journal of Experimental Botany, Page 1 of 13  
doi:10.1093/jxb/er280

RESEARCH PAPER

## NADPH oxidase AtrbohD and AtrbohF function in ROS-dependent regulation of Na<sup>+</sup>/K<sup>+</sup> homeostasis in *Arabidopsis* under salt stress

Liya Ma\*, Huan Zhang\*, Lirong Sun\*, Yiheng Jiao, Guozeng Zhang, Chen Miao and Fushun Hao<sup>†</sup>



Sagi M , Fluhr R Plant Physiol. 2006;141:336-340

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# Medicago GeneChip Results

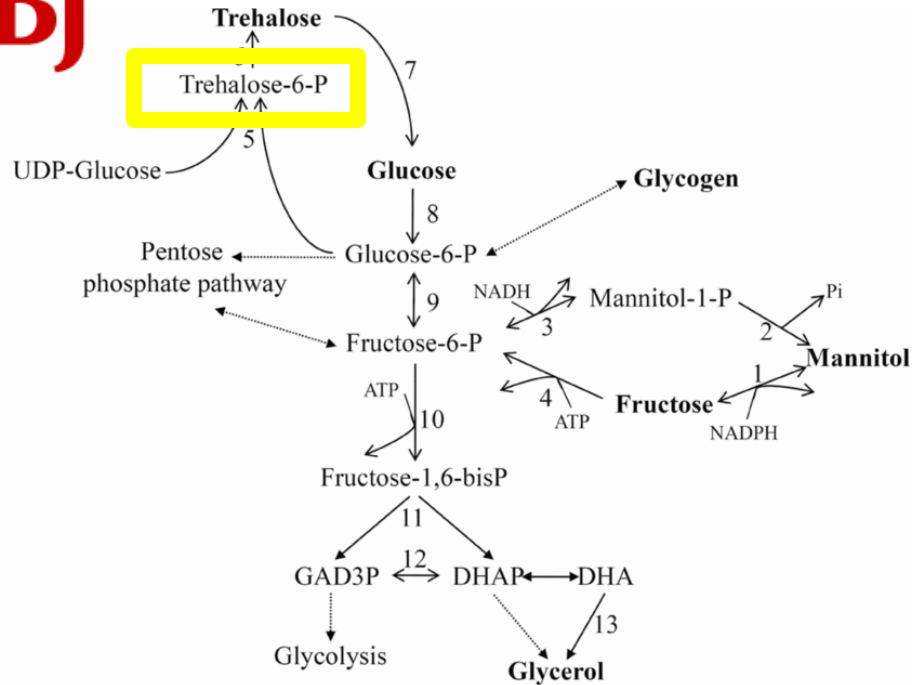
## BC79 Shoots

Probe Set ID	Log (base 2) expression				Fold change		Target Description
	Salt-stressed		non-stressed		Salt-stressed	Non-stressed	
	BC79	BC79-11	BC79	BC79-11	BC79-11/BC79	BC79-11/BC79	
Mtr.18232.1.S1_s_at	6.8	10.2	9.6	9.8	10.7	1.2	H <sup>+</sup> ATPase
Mtr.5656.1.S1_s_at	2.9	5.4	5.7	5.6	5.8	0.9	Vacuolar H <sup>+</sup> -ATPase B subunit
Mtr.11859.1.S1_s_at	4.0	7.5	6.4	6.7	11.1	1.2	NAD(P)H-quinone oxidoreductase chain 3, chloroplast
Mtr.12676.1.S1_s_at	3.8	7.1	6.1	6.1	9.4	1.0	NADH2 dehydrogenase (ubiquinone)
Mtr.12676.1.S1_at	2.6	5.5	4.6	4.7	7.6	1.0	NADH2 dehydrogenase (ubiquinone)
Mtr.34428.1.S1_s_at	7.6	9.8	9.8	10.0	4.7	1.1	NADP-dependent glyceraldehydephosphate dehydrogenase
Mtr.5143.1.S1_s_at	2.8	4.9	4.6	4.7	4.3	1.1	Glucosyltransferase-like protein
Mtr.26601.1.S1_s_at	2.8	4.8	6.0	5.6	4.0	0.7	NADPH oxidase
Mtr.39594.1.S1_at	3.2	5.4	5.0	5.9	4.6	1.8	Trehalose-6-phosphate phosphatase



# Osmoprotectant biosynthesis

**BJ**



- |                                |                               |                                |
|--------------------------------|-------------------------------|--------------------------------|
| 1 : Mannitol dehydrogenase     | 5 : Trehalose-6-P synthase    | 9 : Phosphoglucose isomerase   |
| 2 : Mannitol-1-P phosphatase   | 6 : Trehalose-6-P phosphatase | 10 : Phosphofruktokinase       |
| 3 : Mannitol-1-P dehydrogenase | 7 : Trehalase                 | 11 : Aldolase                  |
| 4 : Fructokinase               | 8 : Glucokinase               | 12 : Triosephosphate isomerase |
|                                |                               | 13 : Glycerol dehydrogenase    |



# Summary

- 1) FRRL has have developed salt-tolerant alfalfa lines for use on semi-arid rangelands  
Saline field trials completed – data alalysis continues
- 2) Physiological mechanisms of tolerant lines are under investigation  
CkSltn 15 and BC79-11 may have different mechanisms of Na<sup>+</sup> tolerance  
CkSltn 15 accumulates Na<sup>+</sup>, BC79-11 does not.
- 3) Microarray analysis identified known salt-tolerance genes in BC79 shoots  
Na<sup>+</sup> transporters (H<sup>+</sup> ATPase)  
Osmolyte synthesis/ROS (NADP dehydrogenases, trehalose-6-P)