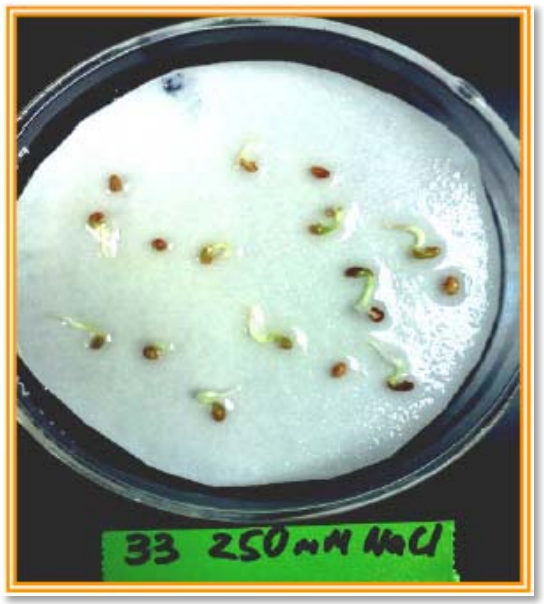
Alfalfa Research in Molecular Plant Pathology Laboratory, Beltsville Agricultural Research Center

Lev G. Nemchinov and Olga A. Postnikova

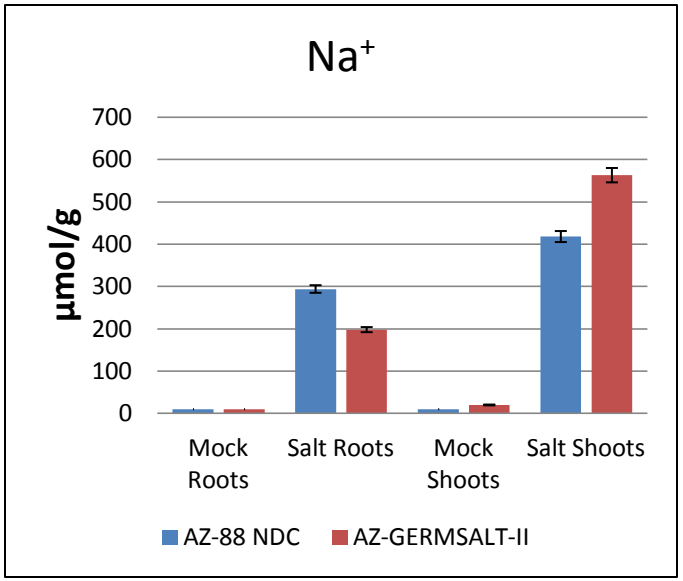
I. Analysis of Alfalfa Root Transcriptome in Response to Salinity Stress

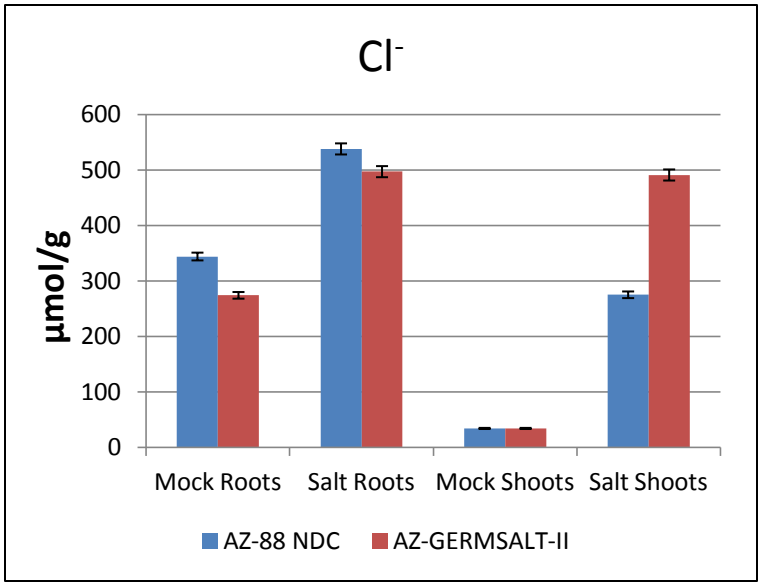
Seed germination and concentration of ions in two alfalfa accessions used for NGS

AZ-88NDC, a salt-susceptible accession



AZ-GERM SALT-II, salt tolerant at the germination stage





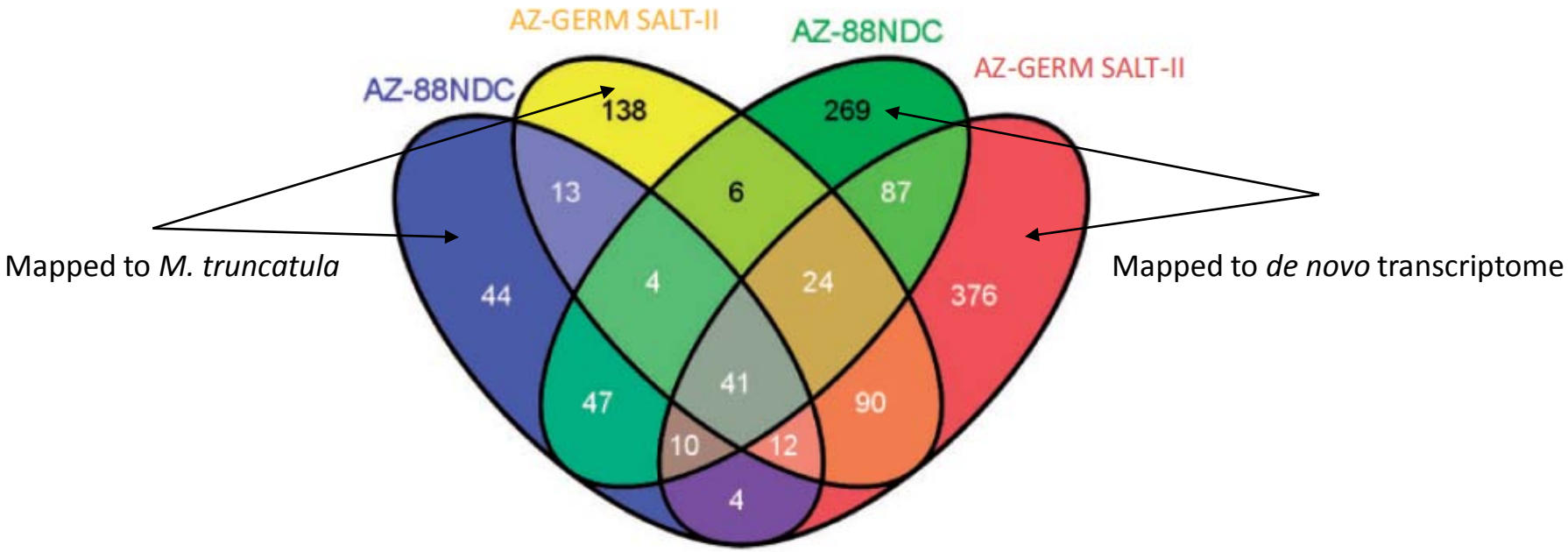
Comparison of the root transctiptome between the two alfalfa accessions

Table 1 De novo assembly statistics

	AZ-88NDC	AZ-GERM SALT-II
Short reads	184,596,972	183,022,614
TCs	60,290	61,874
Non-redundant blastx 1E-10	19,625	19,942
M. truncatula blastx 1E-10	12,620	13,613

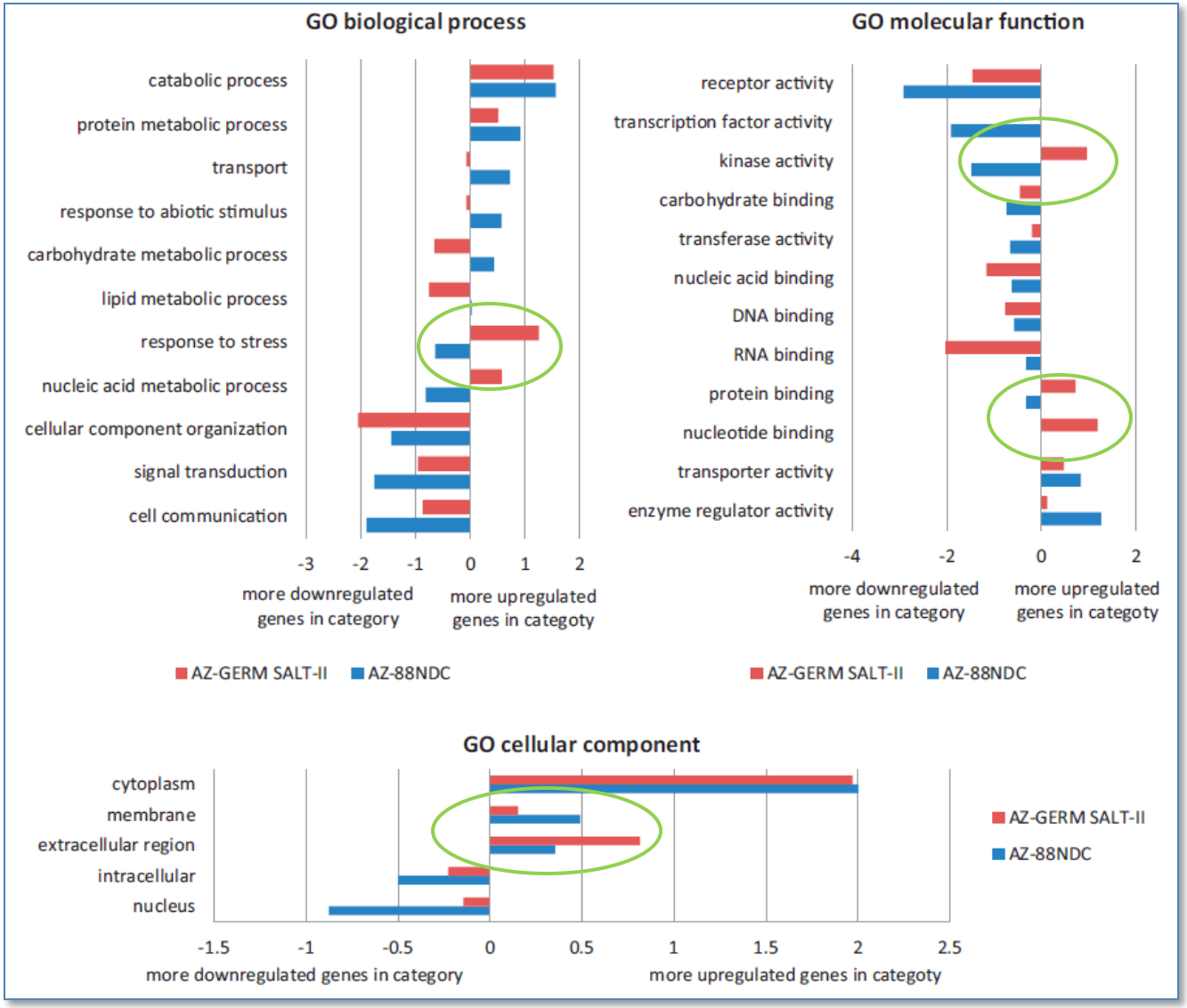
Table 2 The number of up- and down-regulated DEGs in each line

	AZ-88NDC	AZ-GERM SALT-II
Up-regulated	288	468
Down-regulated	273	337



A Venn diagram depicting the number of statistically significant (>2-fold) DEGs when the *de novo* transcriptome (**green and red ovals**, FDR <0.025) or the *M. truncatula* 3.5 data release (**blue and yellow ovals**, FDR <1E-04) were used as a reference for row reads mapping.

Distribution of the differentially expressed genes among functional Gene Ontology (GO) categories



The bars represents the log2-transformed ratio between category portions in up-regulated and down-regulated gene sets. A ratio greater than zero indicates that there were more up-regulated genes in the category

Transcription factors (TFs) differentially expressed under salt stress condition

Table 4 Transcription factors differentially expressed in the two genotypes under salinity stress

Transcription factor family	AZ-88NDC	AZ-GERM SALT-II
MYB	3	10
AP2/EREBP	5	8
bZIP	5	6
HB, Homeobox	2	4
bHLH	3	4
GRAS	0	3
CCAAT	3	3
C3H zinc finger	2	3
AS2	3	3
C2H2 zinc finger	2	3
ARR	0	2
MADS	2	1
JUMONJI	0	1
G2-like, GARP	2	0
WRKY	1	0
TCP	1	0
NAC	1	0
Other	6	10
Total	41	61

Eighty six TFs responsive to salt treatment were found in both accessions that represented 12% of all unique TFs identified in the assembled transcriptome (726 TFs).

Validation of NGS data by qPCR

PCP
PLANT & CELL PHYSIOLOGY

O. A. Postnikova *et al.*

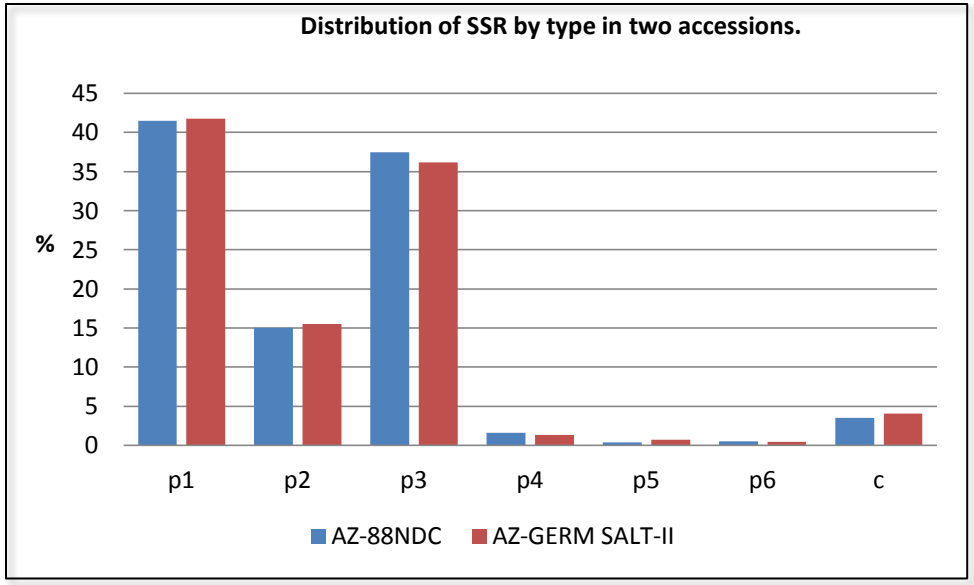
Table 3 Validation of NGS data by qRT-PCR

Gene ID	AZ-88NDC		Gene ID	AZ-GERM SALT-II	
	Log2-fold change			Log2-fold change	
	NGS	qRT-PCR		NGS	qRT-PCR
Medtr1g075030.1	4.79	4.08	Medtr2g060880.1	6.54	3.15
Medtr3g031650.1	3.69	2.43	Medtr5g043550.1	4.06	2.30
Medtr4g098570.1	3.04	1.80	Medtr2g010590.1	3.42	1.57
Medtr5g043550.1	2.83	1.89	Medtr3g114530.1	3.13	2.66
Medtr3g114530.1	2.71	2.00	Medtr1g073170.1	2.90	0.93
Medtr1g073170.1	2.43	3.06	Medtr3g005420.1	2.81	3.25
Medtr8g020630.1	1.25	3.21	Medtr3g031650.1	2.23	1.16
Medtr4g098850.1	−1.59	−1.76	Medtr3g103960.1	−1.95	−1.76
Medtr3g103960.1	−1.63	−2.51	Medtr3g071740.1	−2.17	−1.23
Medtr5g024020.1	−1.95	−1.08	Medtr2g020710.1	−2.92	−1.47
Medtr1g012710.1	−2.06	−1.90	Medtr1g093600.1	−3.03	−1.17
Medtr3g071740.1	−2.76	−2.34	Medtr5g024020.1	−3.06	−0.99
Medtr1g093600.1	−3.26	−1.02	Medtr1g012710.1	−3.36	−0.52
Medtr2g020710.1	−3.81	−2.86	Medtr4g098850.1	−3.83	−1.62

Pearson correlation coefficient $r = 0.84$

Simple Sequence Repeats identified in two accessions

SSR type	Number of SSR		% of SSR	
	AZ-88NDC	AZ-GERM SALT-II	AZ-88NDC	AZ-GERM SALT-II
p1, mononucleotide repeats	1885	2051	41.50	41.76
p2, dinucleotide repeats	683	762	15.04	15.52
p3, trinucleotide repeats	1702	1778	37.47	36.20
p4, tetranucleotide repeats	72	64	1.59	1.30
p5, pentanucleotide repeats	16	36	0.35	0.73
p6, hexanucleotide repeats	23	21	0.51	0.43
c, variable-number repeats	161	199	3.54	4.05
all SSR	4542	4911	100	100
In annotated genes	3240	3526		
In unigenes	2156	2323		
Common genes with SSR: 1296				



Candidate genes responsible for adaptation to salinity in alfalfa

- **Receptor–like kinases** : hyperosmotic sensors
- **Calmodulin-like proteins and transcription activators**: Ca^{2+} signaling; salt stress sensed by Ca^{2+} channels
- **MYB, AP2/EREBP, GRAS transcription factors**: tolerance responses
- **ABC transporters, nitrate transporters**: transporting stress-related secondary metabolites; nitrate assimilation
- **Albumins**: osmoprotectants, reduce osmotic stress
- **Remorins**: stabilization of the damaged plasma membrane

Conclusions, root transcriptome under salinity stress:

- Salt-responsive genes identified
- Gene candidates with roles in adaptation to salinity proposed
- Polymorphic simple sequence repeats (SSR) identified

PCP
PLANT & CELL PHYSIOLOGY

Analysis of the Alfalfa Root Transcriptome in Response to Salinity Stress

Olga A. Postnikova, Jonathan Shao and Lev G. Nemchinov*

USDA/ARS, Plant Sciences Institute, Molecular Plant Pathology Laboratory, 10300 Baltimore Avenue, Beltsville, MD 20705, USA
*Corresponding author: E-mail, Lev.Nemchinov@ars.usda.gov
(Received November 6, 2012; Accepted April 4, 2013)

Salinity is one of the major abiotic factors affecting alfalfa productivity. Identifying genes that control this complex trait will provide critical insights for alfalfa breeding programs. To date, no studies have been published on a deep sequencing-based profiling of the alfalfa transcriptome in response to salinity stress. Observations gathered through research on reference genomes may not always be applicable to alfalfa. In this work, Illumina RNA-sequencing was performed in two alfalfa genotypes contrasting in salt tolerance, in order to estimate a broad spectrum of genes affected by salt stress. A total of 367,619,586 short reads were generated from cDNA libraries originated from roots of both lines. More than 60,000 tentative consensus sequences (TCs) were obtained and, among them, 74.5% had a significant similarity to proteins in the NCBI database. Mining of simple sequence repeats (SSRs) from all TCs revealed 6,496 SSRs belonging to 3,183 annotated unigenes. Bioinformatics analysis showed that the expression of 1,165 genes, including 86 transcription factors (TFs), was significantly altered under salt stress. About 40% of differentially expressed genes were assigned to known gene ontology (GO) categories using Arabidopsis GO. A random check of differentially expressed genes by quantitative real-time PCR confirmed the bioinformatic analysis of the RNA-seq data. A number of salt-responsive genes in both tested genotypes were identified and assigned to functional classes, and gene candidates with roles in the adaptation to salinity were proposed. Alfalfa-specific data on salt-responsive genes obtained in this work will be useful in understanding the molecular mechanisms of salinity tolerance in alfalfa.

Keywords: *Medicago sativa* • Next-generation sequencing • Root transcriptome • Salt stress.

Abbreviations: bHLH, basic helix–loop–helix; CDS, coding sequences; DEG, differentially expressed gene; DFR, dihydroflavonol reductase; FDR, false discovery rate; GO, gene ontology; MS, Murashige and Skoog; NSG, next-generation sequencing; ORF, open reading frame; qRT-PCR, quantitative real-time PCR; SSR, simple sequence repeat; TF, transcription factor; TCs, tentative consensus sequences.

DDBJ accession number: PRJDB749.

Introduction

More than one-third of all irrigated lands contain high levels of salts. Salinization of non-irrigated agricultural soils is also a serious issue that leads to lower yields and reduces the ability of crops to take up water. Plant adaptation to salinity is thought to be of three different types (Munns and Tester 2008): tolerance to osmotic shock mediated by calcium signaling; exclusion of salt ions that inhibit metabolic processes inside the cell by transporter proteins and tolerance to already accumulated sodium ions by their internal distribution; and compartmentation away from the cytosol through the operation of a vacuolar Na^+/H^+ antiport (Apse et al. 1999). While the physiological reactions underlying these three tolerance mechanisms are well known, understanding of molecular responses to high salinity is often limited to individual components of the model system *Arabidopsis thaliana*, a salt-sensitive species (Ren et al. 2010).

Alfalfa (*Medicago sativa*) is the most extensively cultivated forage legume in the world and the fourth most widely grown crop in the USA, being planted on >23 million acres in all 50 states. Although alfalfa is considered a moderately salt-tolerant species as compared to other legumes (Munns and Tester 2008), salinity stress is among the most problematic environmental factors limiting alfalfa production. Thus, increased salt tolerance in alfalfa has great economic potential (Ottman 1999, Peel et al. 2004).

Salinity research in alfalfa had benefited from a variety of approaches, such as selection of germplasm resources for increased salt tolerance at different growth stages, identification and selection for salt tolerance via physiological traits and molecular markers, regeneration from salt-tolerant cell lines, in vitro selection and electrophysiological studies of traits associated with salt tolerance and genetic engineering through transgene expression (Rumbaugh and Pendery 1990, Winicov and Bastola 1999, Winicov 2000, Djilianov et al. 2003, Smethurst et al. 2008, Monirifar and Barghi 2009, Soltani et al. 2012).

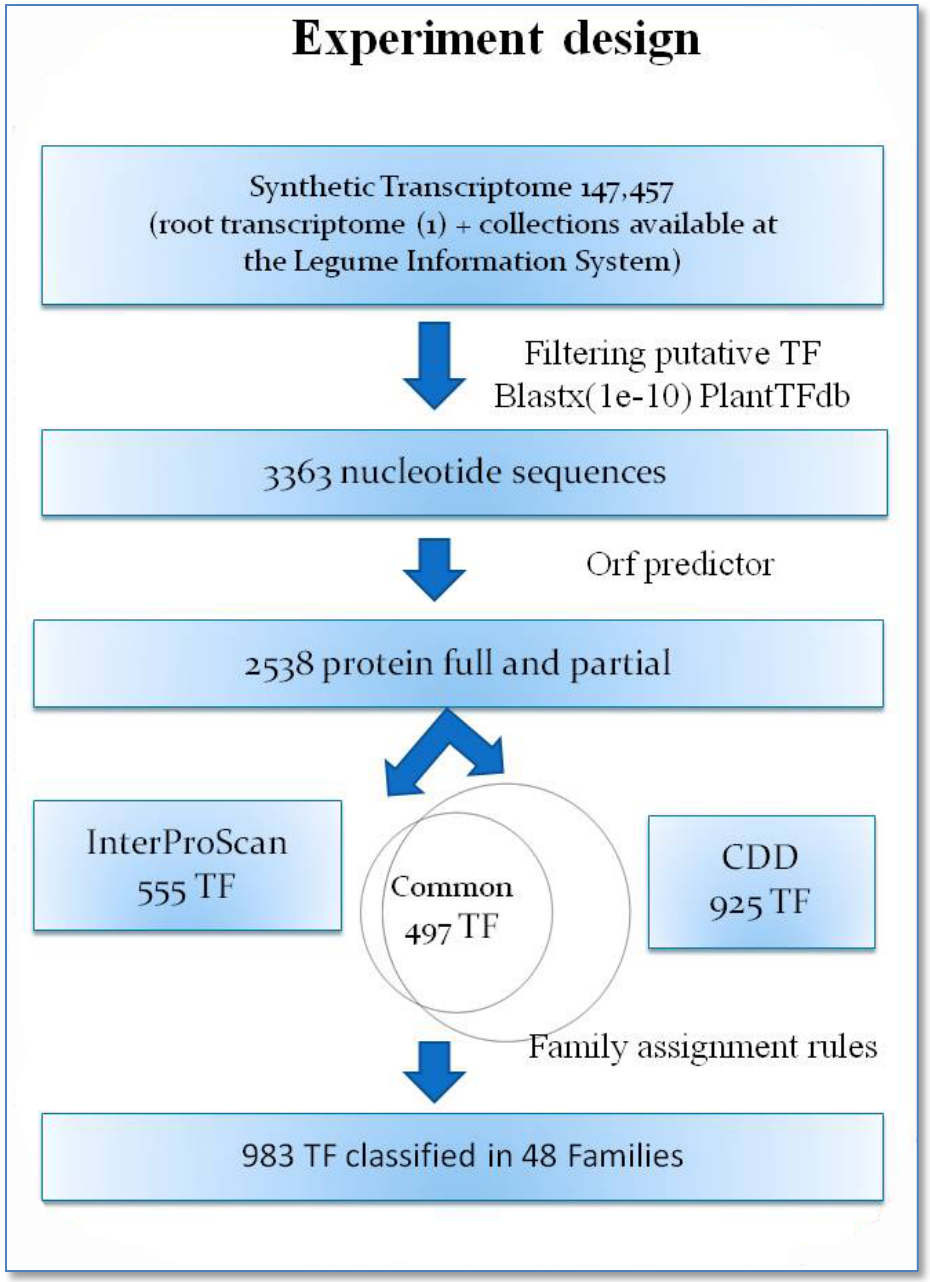
A number of individual genes whose expression is induced by salt stress in alfalfa have recently been isolated and

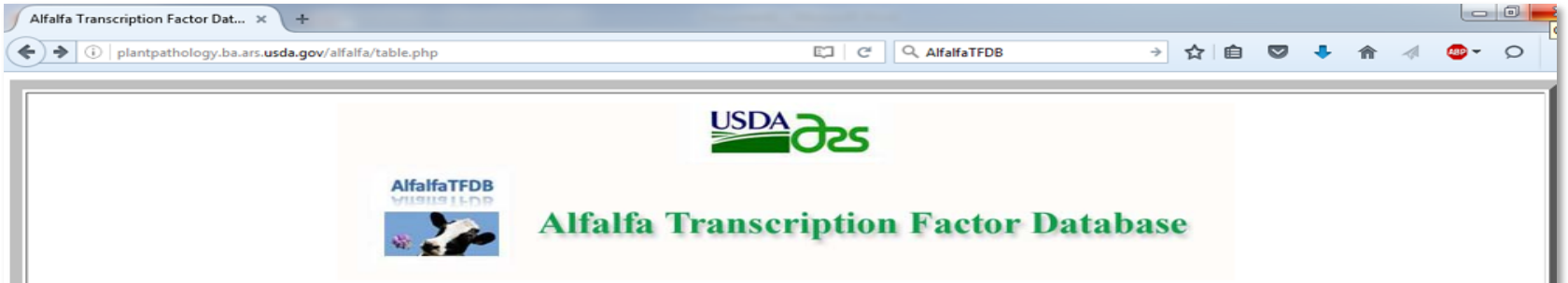
Plant Cell Physiol. 54(7): 1041–1055 (2013) doi:10.1093/pcp/pct056, available online at www.pcp.oxfordjournals.org
Published by Oxford University Press on behalf of Japanese Society of Plant Physiologists.

Regular Paper

II. *In silico* identification of transcription factors in *Medicago sativa* using available transcriptomic resources

Computational identification of transcription factors in *Medicago sativa*





AlfalfaTFDB was generated by an *in silico* analysis of transcriptome data obtained in the Nemchinov lab and by using publicly available data at the Legume Information System. Redundancies were removed via CD-HIT tool. Analyses and annotations were performed using InterProScan, a protein domain identifier that combines different protein signature recognition methods and by CDD, a conserved domain search for the annotation of protein sequences with the location of conserved domain footprints. Transcriptome-wide mining enabled prediction of 983 TFs classified into 47 families:

Transcription factors in Medicago sativa, computationally predicted by transcriptomic analysis
(browse by family)

AP2 (10)	ARF (20)	B3 (26)	BBR-BPC (4)
BES (6)	bHLH (106)	bZIP (62)	C2H2 (25)
C3H (26)	CAMTA (5)	CO-like (2)	CPP (6)
DBB (15)	DOF (29)	E2F_BP (6)	EIL (3)
ERF (96)	FAR (40)	GATA (24)	GeBP (4)
GRAS (43)	GRF (5)	HB-other (34)	HB-phd (2)
HD-ZIP (22)	HSF (22)	LBD (27)	LSD (4)
MADS (19)	MYB (8)	MYB-rel (40)	NAC (67)
NF-X (3)	NF-Y (25)	Nin-like (6)	RAV (2)
S1Fa-like (2)	SAP (2)	SBP (14)	SRS (6)
TALE (7)	TCP (13)	Trihelix (10)	Whirly (3)
WRKY (71)	YABBY (2)	ZF-HD (9)	

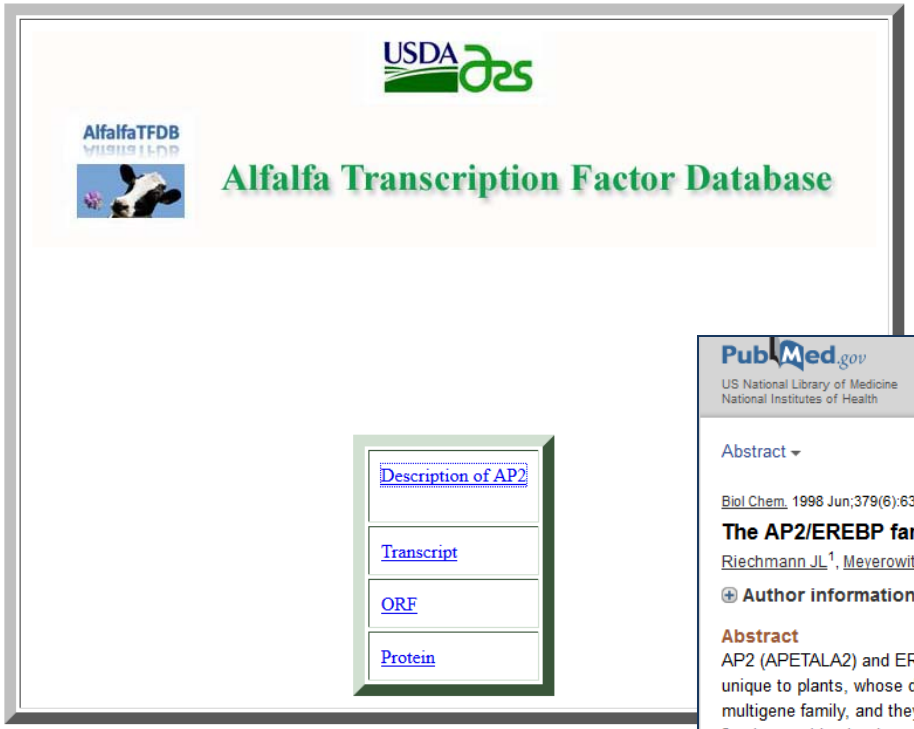
Retrieve all alfalfa transcription factor datasets
All Transcripts Search

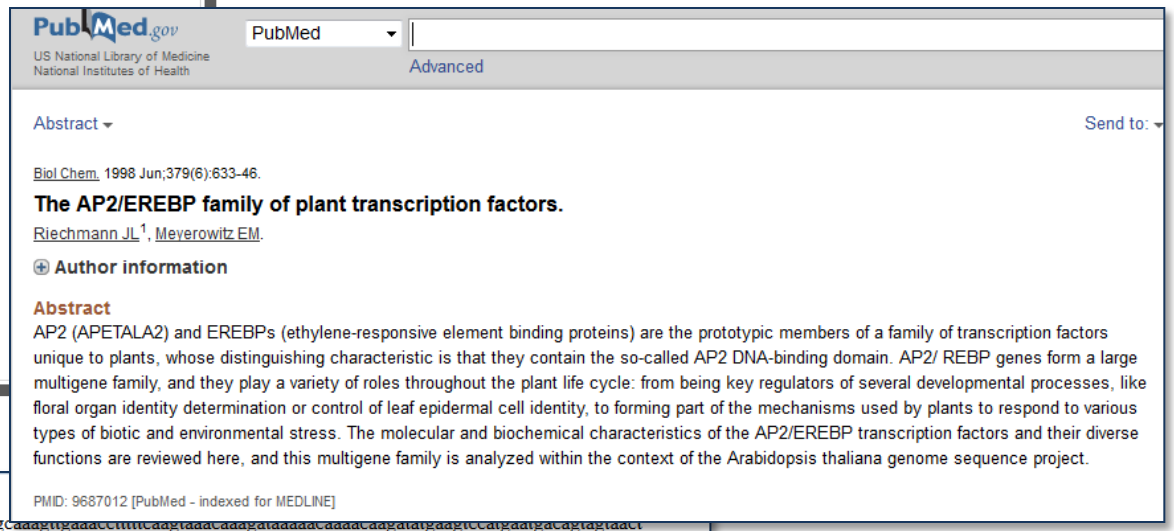
Return to Alfalfa Transcription Factor Resource Center

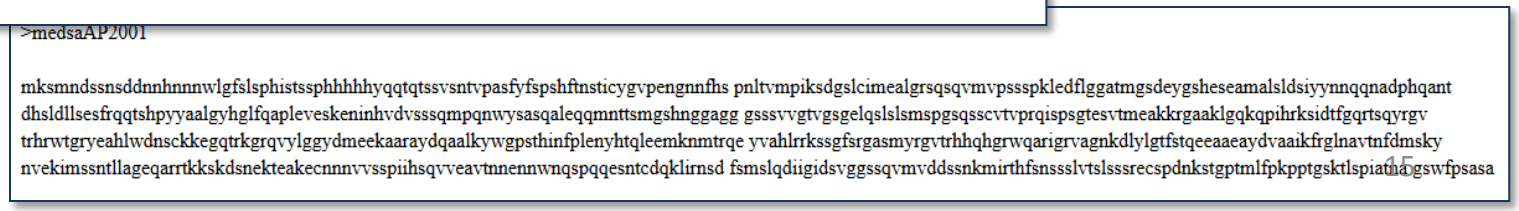
Reference:

Postnikova OA, Shao, J and Nemchinov LG (2014). *In silico* identification of transcription factors in *Medicago sativa* using available transcriptomic resources. Molecular Genetics and Genomics.link
DOI 10.1007/s00438-014-0823-7, Epub 2014 Feb 21

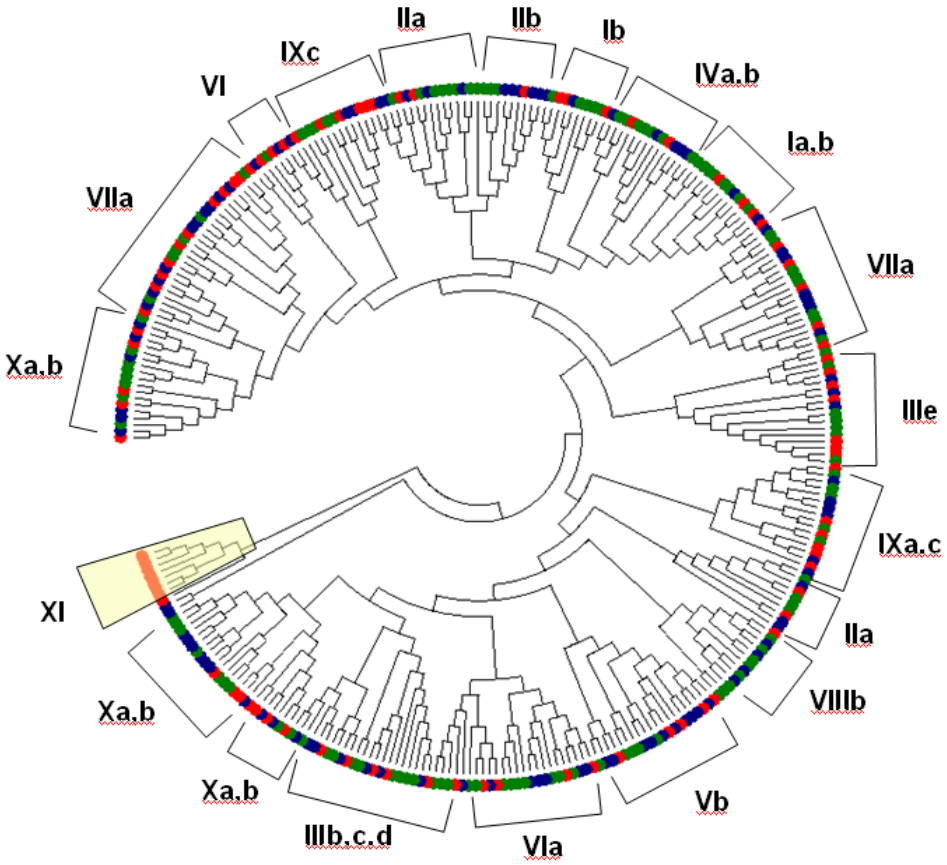
AlfalfaTFDB



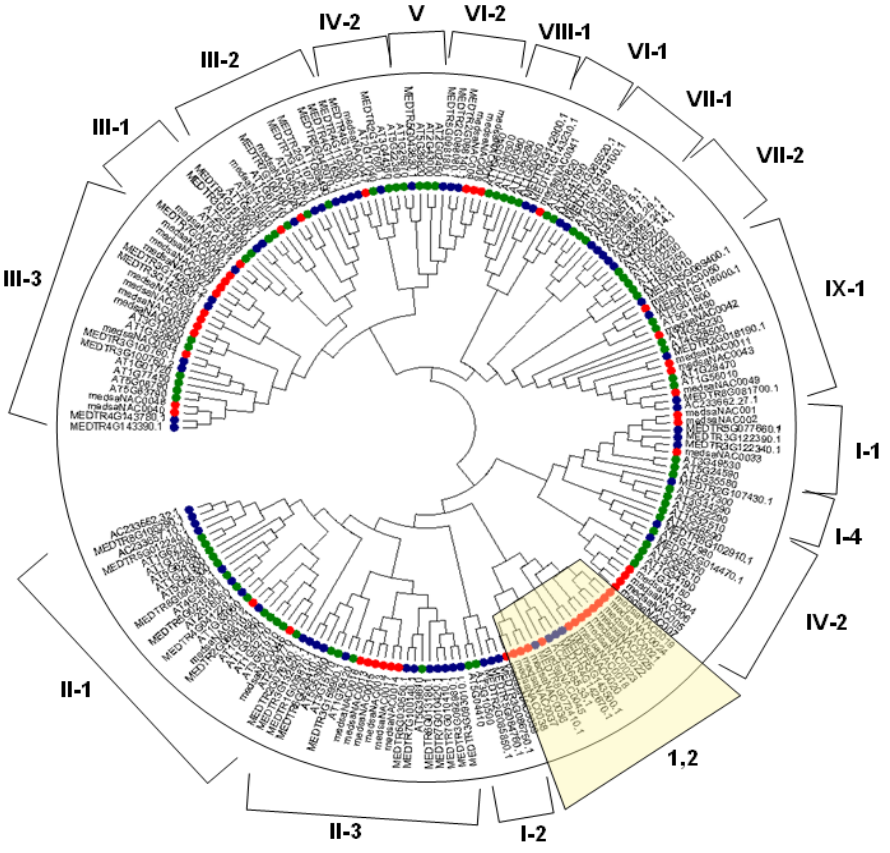




Phylogenetic analysis of the ERF (A) and NAC (B) transcription factors



A



B

A. thaliana (green circles), *M. truncatula* (blue circles) and *M. sativa* (red circles). Tentative locations of the previously described *A. thaliana* subfamilies are indicated above the trees in Roman numerals. Novel clades found in alfalfa highlighted in yellow.

Conclusions, identification of TFs in alfalfa:

Predicted 983 TFs along with their sequence features and phylogenies

Assembled an open-access database AlfalfaTFDB

Revealed diversity in the composition of major TF families

Mol Genet Genomics (2014) 289:457–468
DOI 10.1007/s00438-014-0823-7

ORIGINAL PAPER

In silico identification of transcription factors in *Medicago sativa* using available transcriptomic resources

Olga A. Postnikova · Jonathan Shao · Lev G. Nemchinov

Received: 27 September 2013 / Accepted: 30 January 2014 / Published online: 21 February 2014

Abstract Transcription factors (TFs) are proteins that govern organismal development and response to the environment by regulating gene expression. Information on the amount and diversity of TFs within individual plant species is critical for understanding of their biological roles and evolutionary history across the plant kingdom. Currently, only scattered information on separate TFs is available for alfalfa, the most extensively cultivated forage legume in the world. In the meantime, several large transcriptomic resources that can be used to identify and characterize alfalfa TF genes are freely accessible online. In this study, we have performed an in silico analysis of transcriptome data generated in our laboratory and publicly acquirable from other sources to reveal and systematize alfalfa transcription factors. Transcriptome-wide mining enabled prediction of 983 TFs along with their sequence features and putative phylogenies of the largest families. All data were assembled into a simple open-access database named AlfalfaTFDB (http://plantpathology.ba.ars.usda.gov/alfalfatfdb.html). Transcriptomic analysis used in this work represents an effective approach for the identification of TF genes in plants with incomplete genomes, such as alfalfa. Integrated TF repertoires of *Medicago sativa* will provide an important tool for studying regulation of gene expression in other complex non-model species of agricultural significance.

Keywords Alfalfa · *Medicago sativa* · Transcription factors · Phylogeny

Electronic supplementary material The online version of this article (doi:10.1007/s00438-014-0823-7) contains supplementary material, which is available to authorized users.

O. A. Postnikova · J. Shao · L. G. Nemchinov (✉)
Molecular Plant Pathology Laboratory, Beltsville Agricultural Research Center, United States Department of Agriculture, 10300 Baltimore Avenue, Beltsville, MD 20705, USA
e-mail: lev.nemchinov@ars.usda.gov

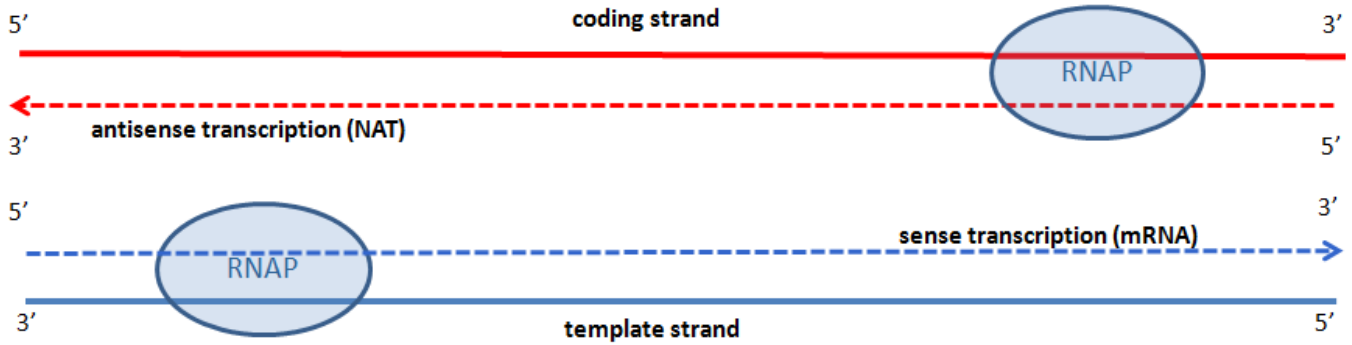
Introduction

Transcription factors (TFs) are at the center of the complex system controlling cellular growth, differentiation, genetic responses to the environment, organismal development and evolution. The amount and diversity of TFs directly correlate with complexity of organisms (De Mendoza et al. 2013). Knowledge of specific TF repertoires in the individual plant species is essential to gain insights into the role and evolution of TFs across the plant kingdom.

Alfalfa is one of the most widely planted forage legume in the world (Li and Brummer 2012). At the moment, no complete genomic sequence is publicly available for alfalfa and its functional genomics is in the developing stage. Detailed knowledge of the expression and regulation of genes associated with alfalfa development and stress tolerance will accelerate conventional breeding programs.

Currently, more than 134,000 TF classified into 58 families have been discovered in the plant kingdom (49 species) based on their signature DNA-binding domains (DBDs) (http://planttfdb.cbi.pku.edu.cn/; Zhang et al. 2011). A model species, *Arabidopsis thaliana*, has 2,296 identified TFs. As of today, 8,043 TFs have been identified in three legume species with the sequenced genomes: *Medicago truncatula*, *Glycine max* and *Lotus japonicus*. The vast majority of them remain to be characterized (Udvardi et al. 2007; Libault et al. 2009). The most comprehensive plant TF database PlantTFDB does not carry any information on *Medicago sativa*, although it lists 1663 TFs in closely related species with a sequenced genome, *M. truncatula*. Whereas these two species share a high degree of sequence

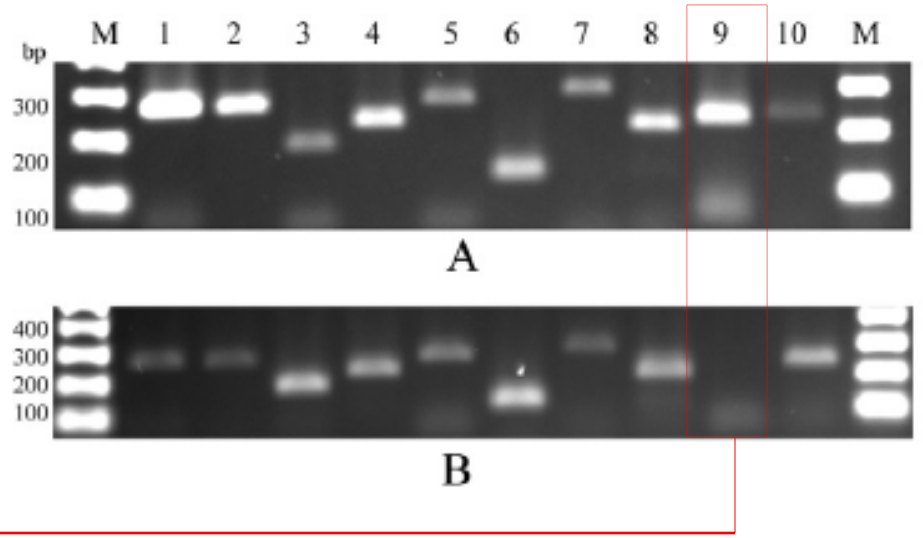
III. Natural antisense transcripts associated with salinity response in alfalfa



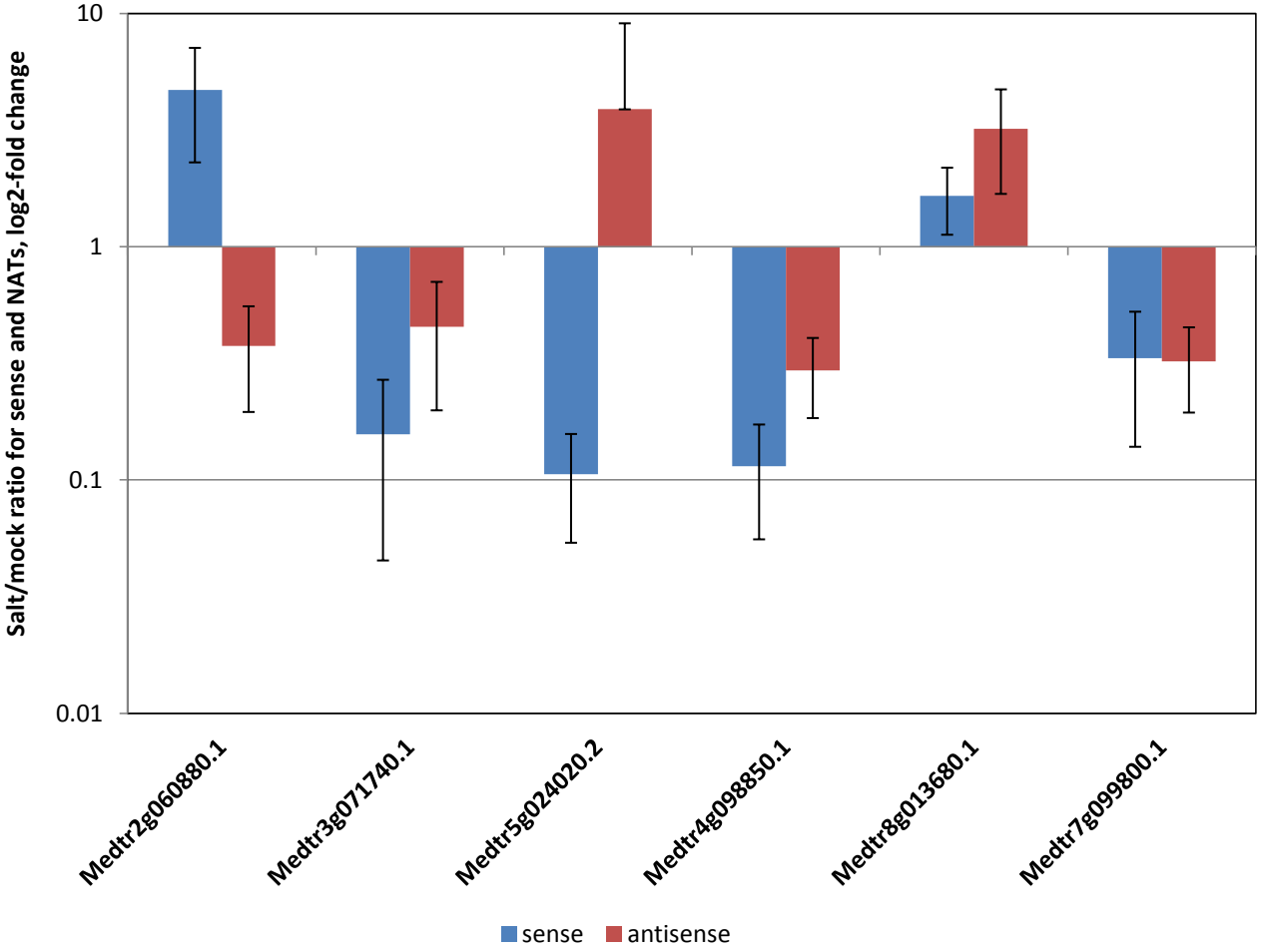
Strand-specific RT-PCR using nested primers with 10 genes that are differentially expressed under salinity stress

Table 1. Genes differentially expressed under salt conditions in AZ-88NDC (salt-sensitive) and AZ-GERM SALT-II (salt-tolerant) alfalfa lines. Plus (+) and minus (–) symbols indicate presence or absence of the natural antisense transcripts (NATs) in salt-tolerant line, as detected by reverse transcription polymerase chain reaction.

	Gene ID	Descriptions	NATs
1	Medtr2g060880.1	C2H2 zinc finger protein	+
2	Medtr3g071740.1	Abscisic acid receptor PYL6	+
3	Medtr8g020630.1	Germin-like protein 9	+
4	Medtr5g024020.1	Seed lipoxygenase	+
5	Medtr4g098850.1	Inositol-145-trisphosphate5-phosphatase-like protein	+
6	Medtr4g021350.1	Aldose reductase	+
7	Medtr8g013680.1	Aquaporin TIP2-1	+
8	Medtr7g099800.1	K(+)/H(+) antiporter	+
9	Medtr1g093100.1	Xyloglucan-specific endoglucanase inhibitor protein	–
10	Medtr3g070880.1	Zinc finger CCCH domain-containing protein	+



Expression profiling of six sense/antisense pairs using qPCR



1, Medtr2g060880.1, C2H2 zinc finger protein; 2, Medtr3g071740.1, abscisic acid receptor PYL6; 3,Medtr5g024020.2, seed lipoxygenase; 4, Medtr4g098850.1, inositol-145-trisphosphate5-phosphatase-likeprotein; 5, Medtr8g013680.1, aquaporin TIP2-1; 6, Medtr7g099800.1, K(+)/H(+) antiporter

Natural antisense transcripts with possible roles in regulating transcription of salt-responsive genes in alfalfa

- **C2H2 zinc finger protein**: transcription activator, stress responses
- **Abscisic acid (ABA) receptor**: adaptation to osmotic stress
- **Seed lipoxygenase**: scavenging of ROS
- **Inositol-145-trisphosphate5-phosphatase-likeprotein (IP_3)**: activation of stress responses via Ca^{2+}-regulated TFs
- **Aquaporin TIP2-1**: membrane channel protein; plant-water relations, abiotic stressor
- **K(+)/H(+) antiporter**: ion transport processes; turgor maintenance

Conclusions, NATs in alfalfa:

- Detected NATs associated with salt-responsive genes
- Detected NATs that changed their expression in response to salinity
- Proposed gene regulation by NATs in alfalfa

Natural Antisense Transcripts Associated with Salinity Response in Alfalfa

O. A. Postnikova and L. G. Nemchinov*

Abstract

Natural antisense transcripts (NATs) are long noncoding RNAs (lncRNAs) complementary to the messenger (sense) RNA (Wang et al., 2014). Many of them are involved in regulation of their own sense transcripts thus playing pivotal biological roles in all processes of organismal development and responses to the environment. In our previous study, we have identified a number of differentially expressed genes (DEGs) in alfalfa plants (*Medicago sativa* L.) subjected to salinity stress (Postnikova et al., 2013). In this work, we selected several experimentally validated DEGs identified in response to salt and analyzed them for the presence of NAT pairs. The majority of the examined DEGs encoded NATs. Expression of some NAT pairs changed in response to salinity, suggesting their involvement in regulating the responses of alfalfa to salt.

BOTH DNA strands can be transcribed (Werner, 2013). Until recently, natural antisense transcripts of protein coding genes were considered transcriptional noise without a functional role (Struhl, 2007). Development of next-generation sequencing and gene-silencing technologies has changed this opinion, revealing extensive antisense transcription in both eukaryotes and prokaryotes (Pelechano and Steinmetz, 2013). A growing number of NATs are found to be functional, although a mechanism of their action in most cases is far from being understood (Arthanari et al., 2014).

Natural antisense transcripts are ubiquitous in plants; according to Wang et al. (2014), 70% of *Arabidopsis thaliana* (L.) Heynh. mRNAs produce long noncoding NATs. They play various important roles in plant cells, presumably through transcriptional and posttranscriptional gene regulation (Borsani et al., 2005; Pelechano and Steinmetz, 2013). No information is currently available on NATs and other lncRNAs in alfalfa. Our interests lie in the identification of NATs and lncRNAs and an understanding of regulatory roles they play in this most extensively cultivated forage legume. Earlier, while studying the molecular mechanisms of salt tolerance in alfalfa using RNA sequencing, we described many DEGs associated with response to salt stress (Postnikova et al., 2013). In this work, we have examined if a subset of those genes is able to generate natural antisense transcripts

Published in The Plant Genome 8
doi: 10.3835/plantgenome2014.09.0057

5585 Guilford Rd., Madison, WI 53711 USA
An open-access publication

USDA–ARS, Beltsville Agricultural Research Center, Molecular Plant Pathology Lab., 10300 Baltimore Ave., Beltsville, MD 20705. Received 26 Sept. 2014. Accepted 9 Feb. 2015. *Corresponding author (lev.nemchinov@ars.usda.gov).

Abbreviations: cDNA, complementary DNA; DEG, differentially expressed gene; lncRNA, long noncoding RNA; NAT, natural antisense transcript; PCR, polymerase chain reaction; RT-qPCR, reverse transcription–quantitative polymerase chain reaction; RT-PCR, reverse-transcription PCR.

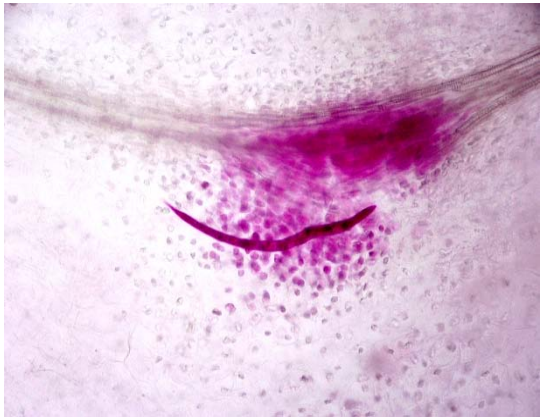
IV. Transcriptome Analysis of Resistant and Susceptible Alfalfa Cultivars Infected With Root-Knot Nematode *Meloidogyne Incognita*

Root-knot nematode (RKN) *Meloidogyne incognita*

- ***Meloidogyne incognita*** or southern root-knot nematode is a major pest worldwide able to infect almost every cultivated crop, including alfalfa

- Little information is available on molecular mechanisms of defense responses in alfalfa against RKN

- Prior to this work, no studies have been published on global gene expression profiling in alfalfa infected with RKN or on any other plant parasitic nematode



Alfalfa cultivars used in the study

Check cultivar resistant to *M. Incognita:* Moapa 69

Check cultivar susceptible to *M. incognita:* Lahontan

Source:

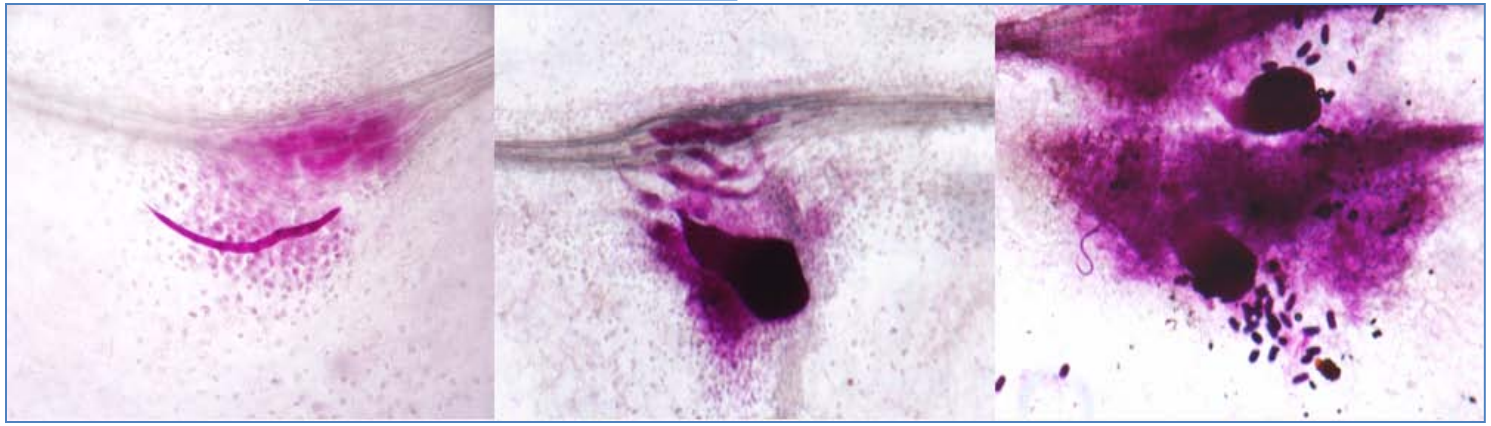
G. D. Griffin, R. N. Peaden and W. J. Knipe. 1991. Root-Knot Nematode Resistance. Standard Tests to Characterize Alfalfa Cultivars, third addition (amended 2004). North American Alfalfa Improvement Conference. https://www.naaic.org/resource/stdtests.php

M. incognita on alfalfa excised root culture:

since nematodes infect the roots, the most important changes in gene expression are expected to be in the roots.

A

B



1 **2** **3**

A. Alfalfa excised root culture, five weeks old. **B.** *M. incognita* on alfalfa excised root culture, four weeks post inoculation. **1,** infective juveniles. **2,** female nematode and infective juveniles. **3,** sedentary females surrounded by laid eggs.

Differentially expressed transcripts (DETs) found in resistant Moapa 69 and susceptible (Lahontan) cultivars

DETs	TCs	Mt-related TCs	Unique TCs	Common
Lahontan	1143 ←	923	712 ←	51
Moapa	319 ←	246	217 ←	51
Basal ratio	2350	1968	1433	

TCs: total number of differentially expressed tentative consensus sequences (TCs)

Mt-related: DETs that were found to be related to *Medicago truncatula* by BLASTp

Unique TCs: non-redundant DETs orthologous to *M. truncatula*

Common: DETs differentially expressed in both cultivars

Basal ratio: DETs found under normal conditions, ratio cv. Moapa/cv.Lahontan

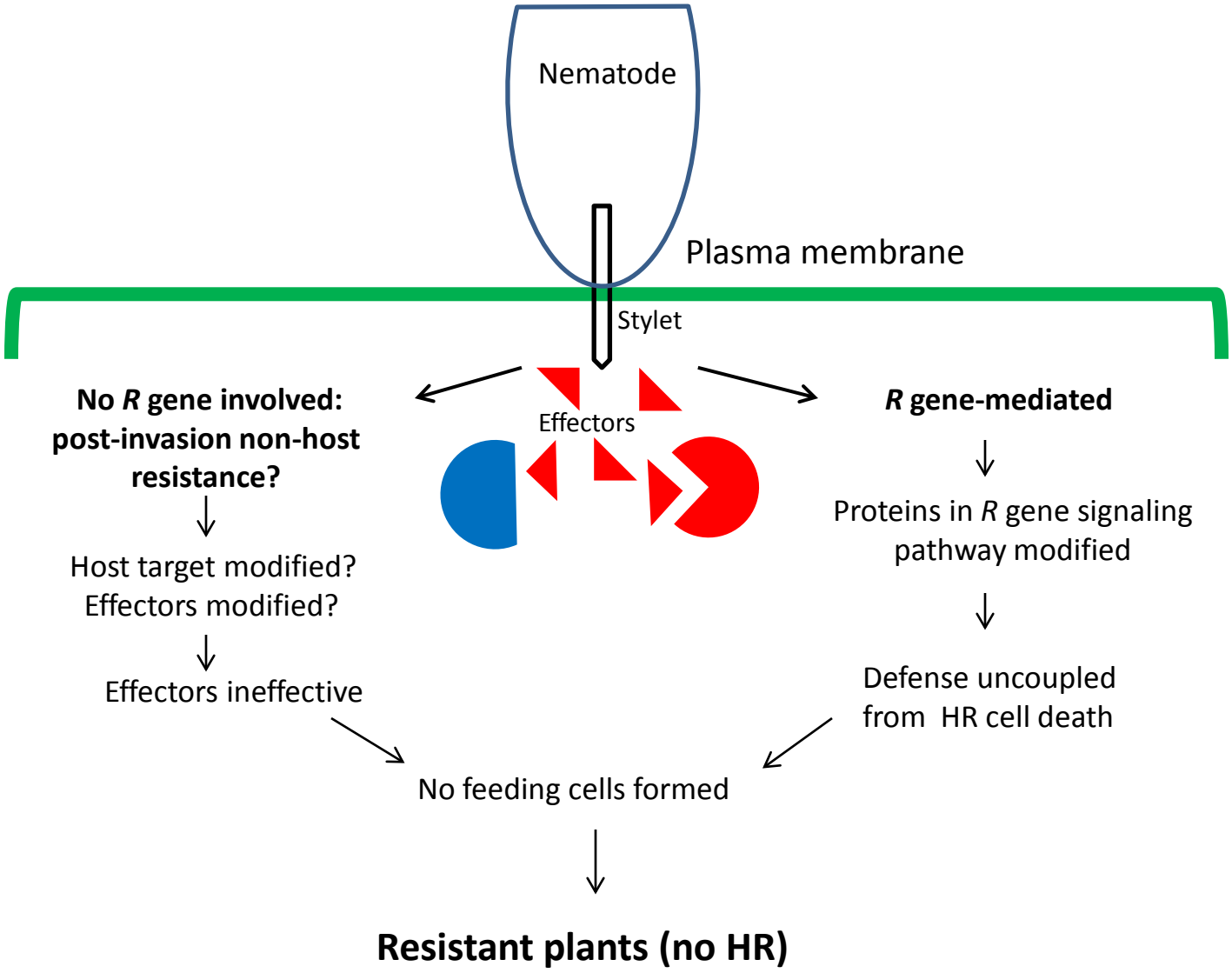
The number of DEGs in susceptible cv. exceeds those in resistant cv. by three-to-one margin: successful infection was established in the susceptible cultivar but was mostly aborted in the resistant cultivar

A short list of candidate genes responsible for the unique resistance interactions between cv. Moapa 69 and RKN

M. truncatula ID	Description
Medtr2g096970.1	putativeproteinkinaseAPK1A[Trifoliumpratense]
Medtr3g054080.1	CDK-activatingkinase[Medicagosativasubsp.xvaria]
Medtr3g056585.1	LRRandNB-ARCdomaindiseaseresistanceprotein
Medtr5g087320.1	receptor-likeprotein|LC|chr5:37825611-37822549
Medtr7g105720.1	hypotheticalprotein|LC|chr7:42885505-42883613
Medtr0277s0020.3	diseaseresistanceprotein(TIR-NBS-LRRclass)

Highlighted in green: up-regulated orthologs of genes induced in resistant tomato and soybean in response to RKN. (Schaff et al., Plant Physiol 2007 144:1079-92; Beneventi et al., BMC Genomics 201314:322).
Highlighted in yellow: common DEGs up-regulated in resistant cultivar and down-regulated in susceptible cv.

Tentative mechanisms of resistance against RKN in *Medicago sativa*



Conclusions, transcriptome of alfalfa infected with RKN:

- Identified genes responsive to nematode infection
- Proposed candidate genes that contribute to protection against *M. incognita*
- Proposed mechanism of resistance against *M. incognita* in alfalfa

PLOS | ONE

RESEARCH ARTICLE

Transcriptome Analysis of Resistant and Susceptible Alfalfa Cultivars Infected With Root-Knot Nematode *Meloidogyne incognita*

Olga A. Postnikova[1], Maria Hult[2], Jonathan Shao[1], Andrea Skantar[2], Lev G. Nemchinov[1]*

1 USDA-ARS, Beltsville Agricultural Research Center, Molecular Plant Pathology Laboratory, Beltsville, Maryland, United States of America, **2** USDA-ARS, Beltsville Agricultural Research Center, Nematology Laboratory, Beltsville, Maryland, United States of America

* Lev.Nemchinov@ars.usda.gov

OPEN ACCESS

Citation: Postnikova OA, Hult M, Shao J, Skantar A, Nemchinov LG (2015) Transcriptome Analysis of Resistant and Susceptible Alfalfa Cultivars Infected With Root-Knot Nematode Meloidogyne incognita. PLoS ONE 10(2): e0118269. doi:10.1371/journal.pone.0118269

Academic Editor: John Jones, James Hutton Institute, UNITED KINGDOM

Received: September 30, 2014

Accepted: January 12, 2015

Published: February 24, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the United States Department of Agriculture, Agricultural Research Service. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

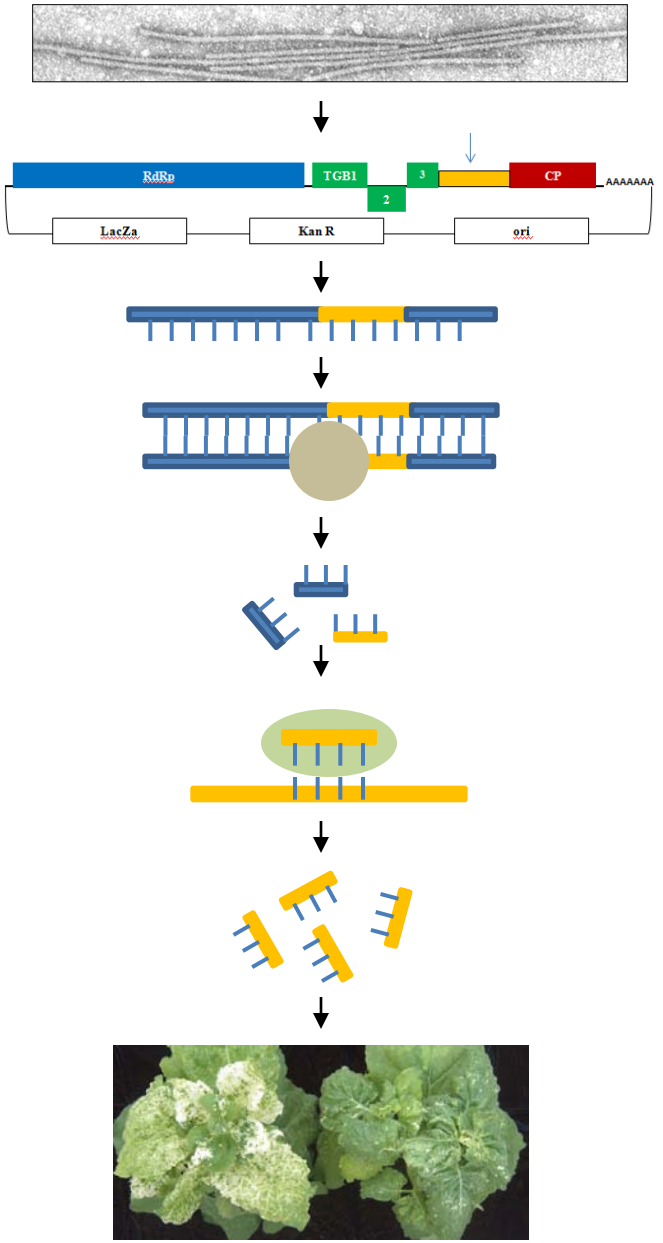
Abstract

Nematodes are one of the major limiting factors in alfalfa production. Root-knot nematodes (RKN, *Meloidogyne* spp.) are widely distributed and economically important sedentary endoparasites of agricultural crops and they may inflict significant damage to alfalfa fields. As of today, no studies have been published on global gene expression profiling in alfalfa infected with RKN or any other plant parasitic nematode. Very little information is available about molecular mechanisms that contribute to pathogenesis and defense responses in alfalfa against these pests and specifically against RKN. In this work, we performed root transcriptome analysis of resistant (cv. Moapa 69) and susceptible (cv. Lahontan) alfalfa cultivars infected with RKN *Meloidogyne incognita*, widespread root-knot nematode species and a major pest worldwide. A total of 1,701,622,580 pair-end reads were generated on an Illumina Hi-Seq 2000 platform from the roots of both cultivars and assembled into 45,595 and 47,590 transcripts in cvs Moapa 69 and Lahontan, respectively. Bioinformatic analysis revealed a number of common and unique genes that were differentially expressed in susceptible and resistant lines as a result of nematode infection. Although the susceptible cultivar showed a more pronounced defense response to the infection, feeding sites were successfully established in its roots. Characteristically, basal gene expression levels under normal conditions differed between the two cultivars as well, which may confer advantage to one of the genotypes toward resistance to nematodes. Differentially expressed genes were subsequently assigned to known Gene Ontology categories to predict their functional roles and associated biological processes. Real-time PCR validated expression changes in genes arbitrarily selected for experimental confirmation. Candidate genes that contribute to protection against *M. incognita* in alfalfa were proposed and alfalfa-nematode interactions with respect to resistance are discussed.

V. Development of VIGS (virus-induced gene silencing) technology for functional genomics studies in alfalfa

Virus-induced gene silencing (VIGS): a powerful tool for functional genomics

- VIGS vector with a target gene insert
- Virus transcripts with a target gene
- **DICER** enzyme cleaves dsRNA intermediate into short double-stranded RNA fragments, siRNA
- **RISC** (RNA-induced silencing complex) directs siRNA to host complementary messenger RNA (mRNA) transcripts and cleaves them
- **mRNA degradation**
- **Gene silencing**

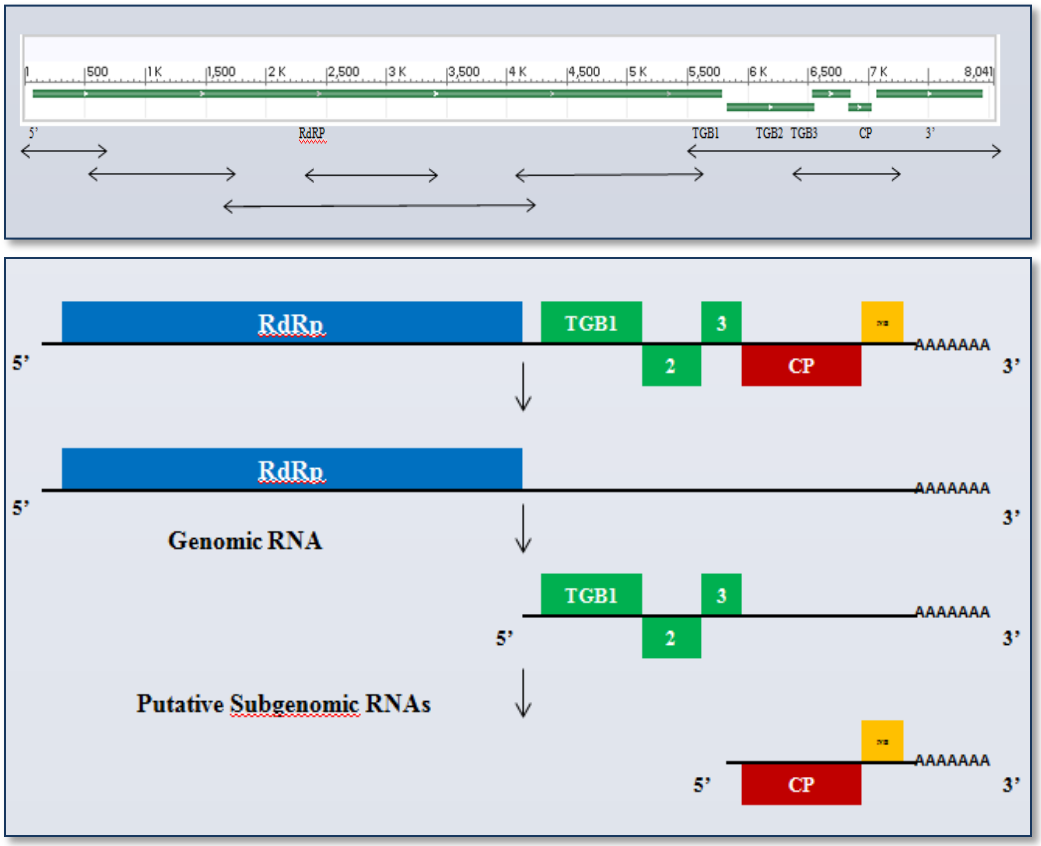


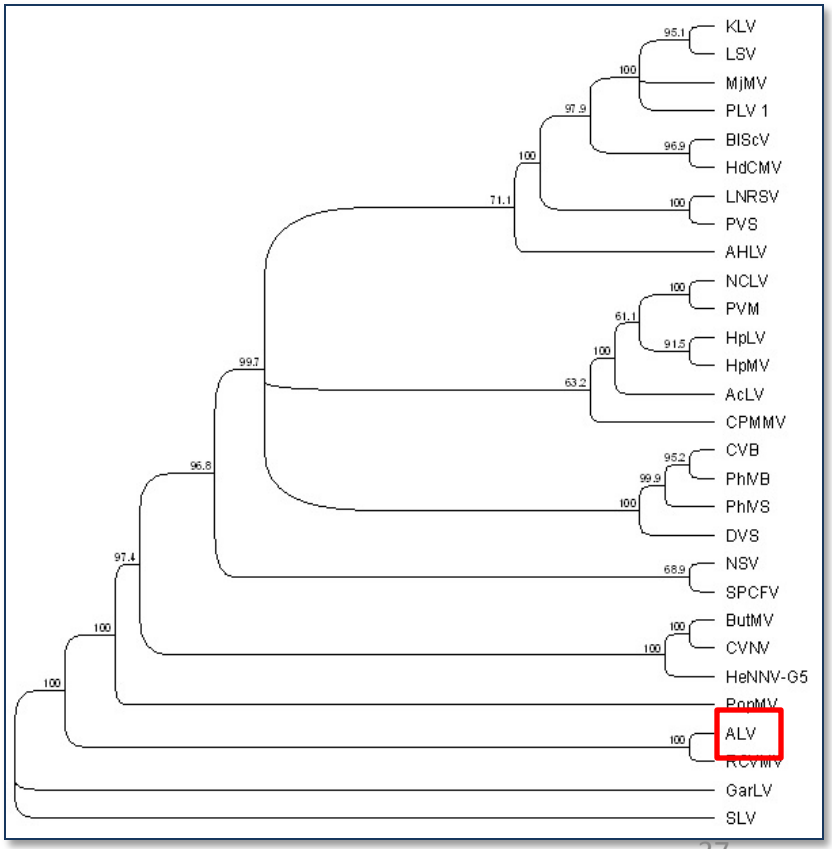
Alfalfa latent virus

- *Alfalfa latent virus* (ALV) is a member of the carlavirus group and occurs symptomlessly in alfalfa
- In the United States it is prevalent in Nebraska and Wisconsin
- The virus is recognized as a strain of *Pea streak virus (PeSV)*
- No complete genomic sequence of PeSV or ALV was available prior to this work

Alfalfa latent virus, cont.

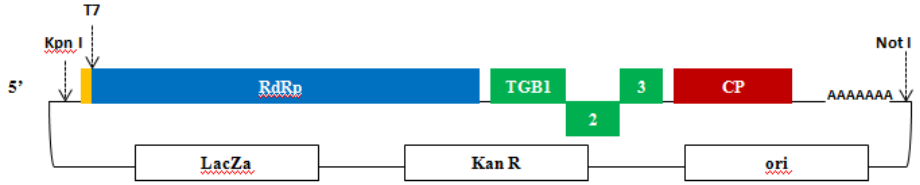
- Achieved the first complete genome sequence of ALV
- Determined genome structure of the virus
- Established phylogenetic relationship of ALV with other carlaviruses



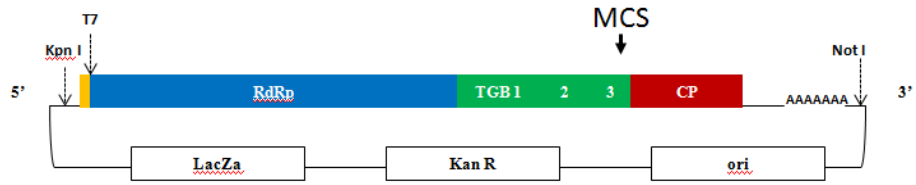


Development of VIGS vector based on *Alfalfa latent virus*

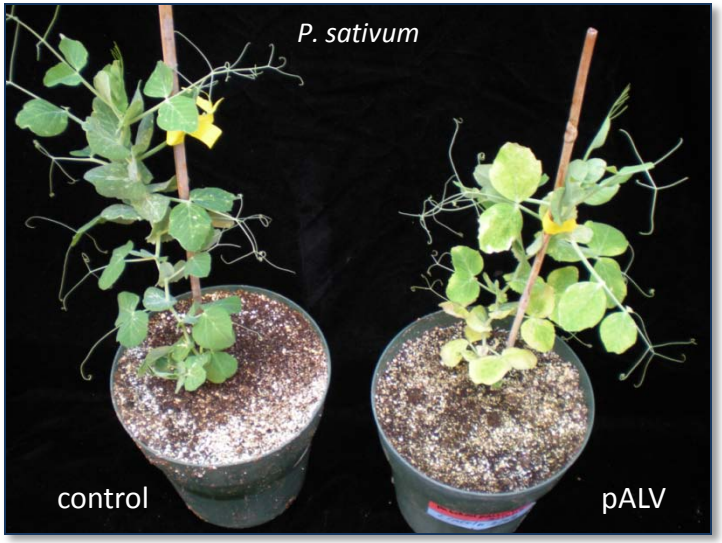
- Obtained a full-length ALV cDNA clone
- Engineered ALV-based vector
- RNA transcripts generated from pALV and ALV/MCS plasmids were infectious

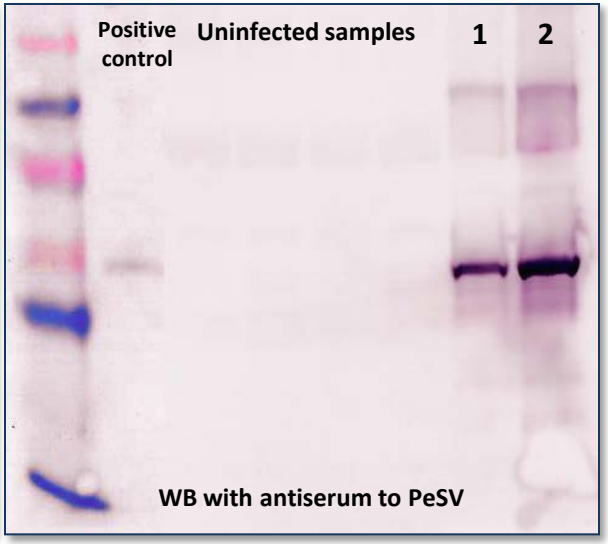


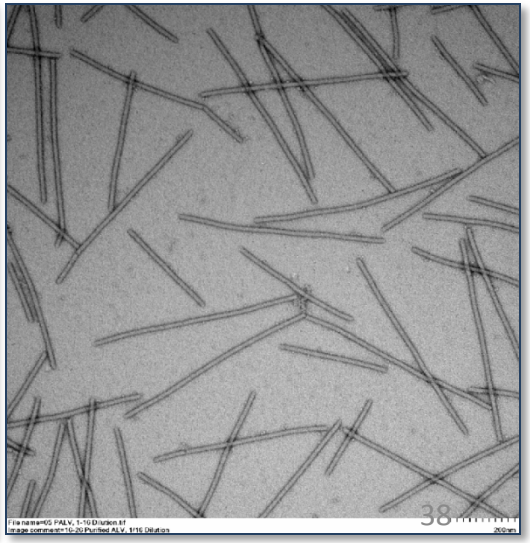
1



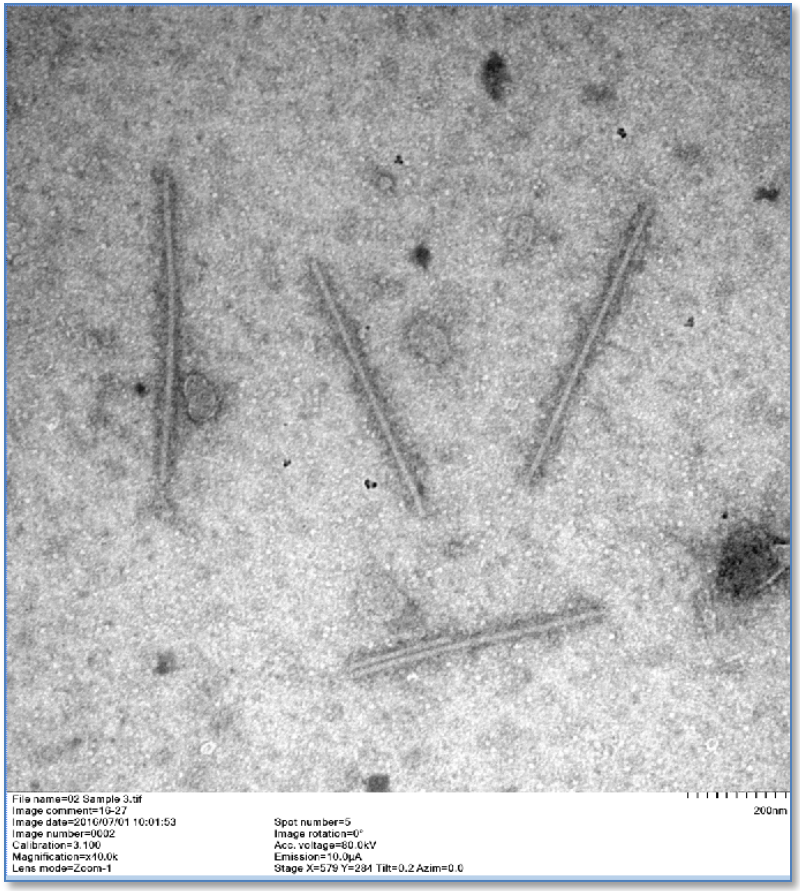
2







ALV derived from the cloned viral cDNA was infectious in alfalfa



Conclusions, VIGS in alfalfa:

- Obtained first complete genomic sequence of *Pea streak virus,* ALV strain
- Constructed a full-length infectious cDNA clone of ALV
- Engineered ALV-based virus vector

genomeA
Journals.ASM.org

Complete Genome Sequence of the *Alfalfa latent virus*

Lev G. Nemchinov, Jonathan Shao, Olga A. Postnikova

USDA-ARS, Beltsville Agricultural Research Center, Molecular Plant Pathology Laboratory, Beltsville, Maryland, USA

The first complete genome sequence of the *Alfalfa latent carlavirus* (ALV) was obtained by primer walking and Illumina RNA sequencing. The virus differs substantially from the Czech ALV isolate and the *Pea streak virus* isolate from Wisconsin. The absence of a clear nucleic acid-binding protein indicates ALV divergence from other carlaviruses.

Received 20 February 2015 **Accepted** 4 March 2015 **Published** 16 April 2015

Citation Nemchinov LG, Shao J, Postnikova OA. 2015. Complete genome sequence of the *Alfalfa latent virus*. Genome Announc 3(2):e00250-15. doi:10.1128/genomeA.00250-15.

Address correspondence to Lev G. Nemchinov, lev.nemchinov@ars.usda.gov.

Alfalfa latent virus (ALV) is a member of the carlavirus group and occurs symptomlessly in alfalfa (*Medicago sativa*) (1). In the United States it is prevalent in Nebraska and Wisconsin (http://www.dpvweb.net/dpv/showdpv.php?dpvno=211). The virus is recognized as a strain of *Pea streak virus* (PeSV) (2–4). No complete genomic sequence of PeSV or ALV was available prior to this report.

ALV-infected tissues were obtained from ATCC (PV-264 isolate from Lancaster County, NE, USA). Total RNA was extracted with the TRIzol protocol and used in primer walking, employing SuperScript RT-PCR system (Life Technologies) for long templates. The 5′ end of the genome was amplified with the RACE system (Life Technologies). PCR fragments were sequenced and assembled into contigs, and the complete genome consensus sequence was generated.

Independently, total RNA purified with the RNeasy mini kit (Qiagen) was used as a template for Illumina RNA sequencing. Paired-end libraries (470 million reads) were cleaned by removing host plant DNA. Using Bowtie2 (5), the reads were mapped to the *Medicago truncatula* genome (Mt4.0v1), mitochondrial DNA, and plastid DNA. The 200 million reads that did not map were assembled with Trinity (6) into 73,574 contigs and queried against known plant viruses using BLASTn (7). A single contig was selected that had a negative *E* value with other plant viruses. It was 99.8% identical to the PCR-derived ALV sequence. The consensus sequence of 8,041 nucleotides (nt) was produced and used for further analysis.

The ALV genome contains five tentative open reading frames encoding a viral replicase (RdRp) (65 to 5,791 nt, 1,909 aa), triple gene block protein 1 (TGB1) (5,823 to 6,557 nt, 245 aa), TGB2 (6,535 to 6,849 nt, 105 aa), TGB3 (6,831 to 7,031 nt, 67 aa), and capsid protein (CP) (7,068 to 7,952 nt, 295 aa). The ALV does not appear to encode a clear 3′ proximal nucleotide acid-binding protein (NABP) that is typical for carlaviruses.

On the nucleotide level, ALV has 99%, 76%, and 79% identity with the partial sequences of the same ALV isolate PV-264 (presumably a variant, GenBank: AY037925), the Czech ALV isolate (HM107774.1), and the Wisconsin PeSV isolate (AF354652), respectively. The next closest species from the genus *Carlavirus*, *Shallot latent virus*, is 48.5% identical to the ALV (PASC tool [8]).

On the amino acid level (BLASTp), ALV RdRp was 91% identical to the partial RdRP sequence of HM107774.1 and ~43% to 50% identical to the RdRP of other carlaviruses. Identity scores for the TGB proteins were as follows: TGB1, 96% to AY037925, 80% to AF354652, 72% to HM107774.1, and ~34% to 45% to other carlaviruses; TGB2, 100% to AY037925, 84% to AF354652, 75% to HM107774.1, and ~42% to 52% to other carlaviruses; TGB3, 95% to AY037925, 65% to AF354652, 62% to HM107774.1, and ~44% to 50% to other carlaviruses. The CP was 99% identical to AY037925, 96% to HM107774.1, 95% to AF354652, and 45% to 57% to other carlaviruses. We propose that the sequenced ALV isolate from Nebraska differs substantially from the Czech ALV and Wisconsin PeSV isolates. The absence of clear NABP indicates that ALV is divergent from other members of the genus *Carlavirus*.

Nucleotide sequence accession number. The sequence has been deposited in GenBank under the accession number KP784454.

ACKNOWLEDGMENTS

This research was financially supported by the USDA-ARS.

We thank John Hammond for his insightful comments.

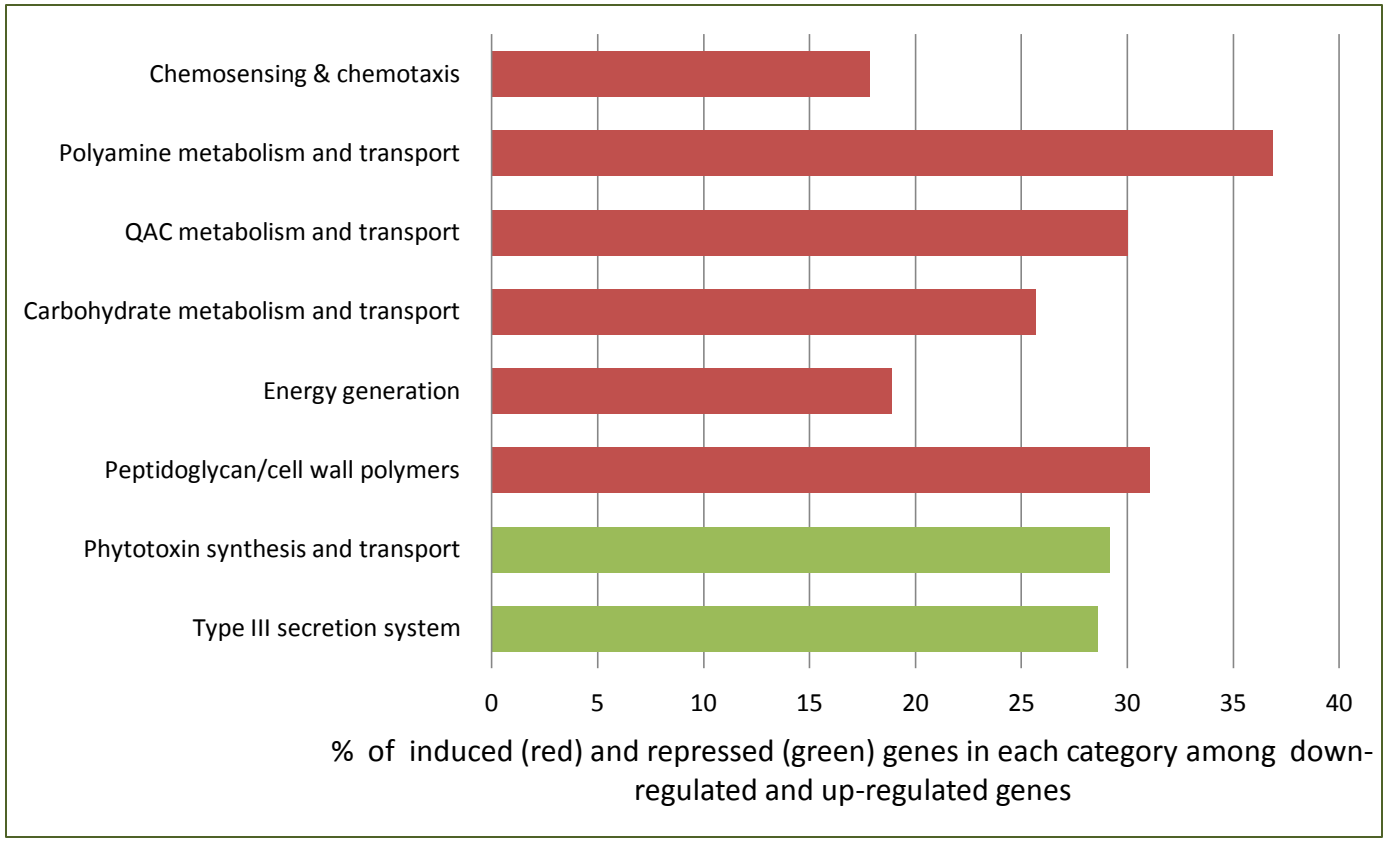
REFERENCES

1. Veerisetty V, Brakke MK. 1977. Alfalfa latent virus, a naturally occurring carlavirus in alfalfa. Phytopathology 67:1202–1206. http://dx.doi.org/10.1094/Phyto-67-1202.
2. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (ed). 2012. Virus taxonomy: classification and nomenclature of viruses: ninth report of the International Committee on Taxonomy of Viruses. Elsevier Academic, San Diego, CA.
3. Hampton RO. 1981. Evidence suggesting identity between alfalfa latent and pea streak viruses. Phytopathology 71:223.
4. Edwardson GR, Christie RG. 1991. Pea streak virus, p 108–111. In CRC handbook of viruses infecting legumes. CRC Press, Boca Raton, FL.
5. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359. http://dx.doi.org/10.1038/nmeth.1923.
6. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A. 2011. Full-length transcriptome assembly from RNA-seq data without a reference genome. Nat Biotechnol 29:644–652. http://dx.doi.org/10.1038/nbt.1883.
7. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403–410. http://dx.doi.org/10.1016/S0022-2836(05)80360-2.
8. Bao Y, Chetvernin V, Tatusova T. 2014. Improvements to pairwise sequence comparison (PASC): a genome-based web tool for virus classification. Arch Virol 159:3293–3304. http://dx.doi.org/10.1007/s00705-014-2197-x.

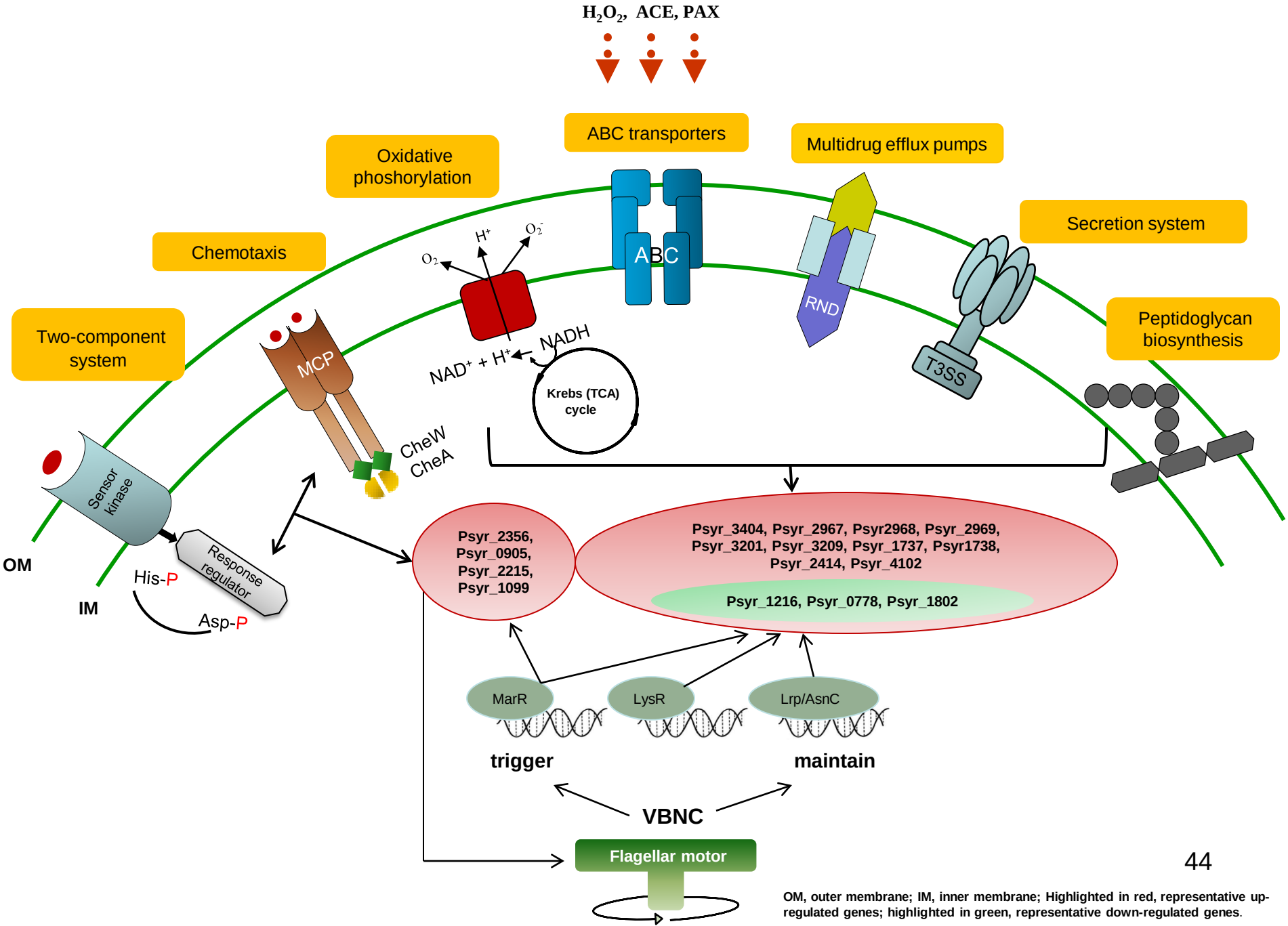
VI. Gene expression profiling in *Pseudomonas syringae* pv *syringae*

- Bacterial stem blight of alfalfa caused by *P. syringae* pv. *syringae* occurs in the central and western U.S. and yield losses can be as high as 50% of the first harvest
- To obtain more information on the pathogen, we performed a large scale transcriptome profiling of *P. syringae* pv. *syringae*
- Focused on viable but nonculturable (VBNC) state of bacteria, a possible bacterial survival strategy in response to various stresses

Overrepresented functional categories identified in VBNC cells of *P. syringae* pv. *syringae* using gene representation analysis



VBNC state in *P. syringae* pv. *syringae*: proposed network of biochemical reactions



OM, outer membrane; IM, inner membrane; Highlighted in red, representative up-regulated genes; highlighted in green, representative down-regulated genes.

Conclusions, expression profiling in *P.syringae* pv *syringae* :

- Identified bacterial genes and pathways associated with VBNC
- Proposed mechanisms that trigger VBNC in *P. syringae pv. syringae*

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 December 2015
doi: 10.3389/fmicb.2015.01419

Gene Expression Profiling in Viable but Nonculturable (VBNC) Cells of *Pseudomonas syringae* pv. *syringae*

*Olga A. Postnikova, Jonathan Shao, Norton M. Mock, Con J. Baker and Lev G. Nemchinov**

Molecular Plant Pathology Laboratory, Beltsville Agricultural Research Center, United States Department of Agriculture, Agricultural Research Service, Beltsville, MD, USA

Pseudomonas syringae infects diverse crop plants and comprises at least 50 different pathovar strains with different host ranges. More information on the physiological and molecular effects of the host inhibitory environment on the pathogen is needed to develop resistant cultivars. Recently, we reported an *in vitro* model system that mimics the redox pulse associated with the oxidative burst in plant cells inoculated with *Pseudomonas syringae* pv. *syringae*. Using this system, we demonstrated that oxidation of acetosyringone, a major extracellular phenolic compound induced in some plants in response to bacteria, rendered *Pseudomonas syringae* pv. *syringae* to a "viable but nonculturable" (VBNC) state. Here we performed a large scale transcriptome profiling of *P. s.* pv. *syringae* in the VBNC state induced by acetosyringone treatment and identified bacterial genes and pathways presumably associated with this condition. The findings offer insight into what events occur when bacterial pathogens are first encountered and host defense responses are triggered. The acquired knowledge will improve our understanding of the molecular mechanisms of stress tolerance. We believe that this is the first work on global gene expression profiling of VBNC cells in plant pathogenic bacteria.

Keywords: VBNC, plant pathogenic bacteria, *P. syringae* pv. *syringae*, RNA-seq, global gene expression profiling

OPEN ACCESS

Edited by:
Eric Altermann, AgResearch, New Zealand

Reviewed by:
Qingping Zhong, South China Agricultural University, China
Thandavarayan Ramamurthy, Translational Health Science and Technology Institute, India

****Correspondence:***
Lev G. Nemchinov
lev.nemchinov@ars.usda.gov

Specialty section:
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 October 2015*
Accepted: *30 November 2015*
Published: *18 December 2015*

Citation:
Postnikova OA, Shao J, Mock NM, Baker CJ and Nemchinov LG (2015) Gene Expression Profiling in Viable but Nonculturable (VBNC) Cells of Pseudomonas syringae pv. syringae. Front. Microbiol. 6:1419. doi: 10.3389/fmicb.2015.01419

INTRODUCTION

Pseudomonas syringae infects many crop plants and is also widely spread in non-agricultural niches (Morris et al., 2008, 2013). At least 50 different pathovar strains have been described based on pathogenicity toward different hosts (https://microbewiki.kenyon.edu/index.php/Pseudomonas_syringae; http://pseudomonas-syringae.org/). It is one of the most studied bacterial pathogens. Three complete annotated genomes of *P. syringae* (*P. syringae* pv. *syringae* B728a, *P. syringae* pv. *tomato* DC3000, and *P. syringae* pv. *phaseolicola* 1448A) and multiple draft genome sequences are currently available (Buell et al., 2003; Creasy et al., 2005; Feil et al., 2005; Joardar et al., 2005; Baltrus et al., 2011).

One of our objectives is related to understanding molecular mechanisms of stress tolerance in alfalfa (*Medicago sativa*), the most widely grown forage crop in the world (Postnikova et al., 2013, 2015). It was recently reported that bacterial stem blight of alfalfa caused by *P. syringae* pv. *syringae* ALF3 occurs sporadically in the central and western U.S. and yield losses from the pathogen can be as high as 50% of the first harvest (Samac et al., 2014). More information on the physiological and molecular events of the host inhibitory environment on the pathogen is needed to develop resistant cultivars.

Summary

- Identified genes-candidates that are involved in controlling complex trait of salt tolerance in alfalfa
- Revealed and systematized alfalfa transcription factors
- Discovered natural antisense transcripts (NATs) differentially expressed under salinity stress
- Identified candidate genes that contribute to protection against RKN *M. incognita* in alfalfa and proposed mechanism of resistance against the RKN
- Obtained the first nucleotide sequence of Alfalfa latent virus (ALV), its complete infectious cDNA clone and engineered ALV-based virus vector
- Performed large scale transcriptome profiling of *P. syringae* pv. *syringae* and identified candidate genes and pathways involved in bacterial resistance to stress

Relevance & concerns

- This is primarily a fundamental research project intended to improve our basic understanding of stress tolerance in alfalfa
- The project does have a practical side: identification of gene candidates will be followed by experimental confirmation of their roles; development of molecular markers based on the discovered SSRs or SNPs is one of the project's priorities
- From our point of view, each of the research directions mentioned here has a great potential for alfalfa improvement but needs further development and investment
- This is a grossly underfunded project

Acknowledgements

Olga Postnikova, Postdoctoral Fellow, Molecular Plant Pathology Lab, BARC

Jonathan Shao, Bioinformatician, Molecular Plant Pathology Lab, BARC

Maria Hult, Biologist, Nematology Lab, BARC

Andrea Skantar, Research Molecular Biologist, Nematology Lab, BARC

C. Jacyn Baker, Research Plant Pathologist**,** Molecular Plant Pathology Lab, BARC