Different gene expression for Creeping-rooted alfalfa based on DNA analysis as a rehabilitation material for inner Mongolia

Graduate School of Forestry, Beijing Forestry University, Wang Xiaona
Present address: Research faculty of Agriculture, Hokkaido University
Institute of Grassland and Ecology, Beijing Forestry University, Wang Tiemei
Institute of Grassland and Ecology, Beijing Forestry University, Lu Xinshi

Introduction

Alfalfa is the highest value planting and legume forages all over the world. Creeping-rooted character in alfalfa was originated from yellow flowered (*Medicago falcate* L.) which can originate a shoot from the root system, and provide the plant with a rhizome-like ability to regenerate in stands. This habit of spreading by means of root proliferation provides alfalfa with a form of vegetative spread and multiplication, which confers to the plant greater resistance to abiotic and biotic stresses, and greater power of recovery from injury than ordinary growth habit*(1)*. Creeping-rooted can make the plant to form a wide root-net which enhances the cold and drought resistance. The most important point was that in arid region alfalfa was easy survival than trees at first to restraining soil erosion and to preserve the ecological environment and promote the development of animal husbandry (*2*) (Fig.2 A).

Creeping-rooted character was comprehensively studied from morphology to physiology*(3,4)*. The research of alfalfa root ester enzyme and endogenous hormones change showed that the intumescent part of root was related with the increase of GA_3. How about the molecular mechanism? Which kind of gene control this character? In order to make gene pyramiding breeding for cultivate new species for arid region we conducted this research.

In recent years, functional genomics has made rapid development to analysis the gene expression under different development period and physiological state. Reveals the regulation of plant growth and development is one of the main components about functional genomics research *(5)*.

Based on the PCR Sequence-Related Amplified Polymorphism (SRAP) was proposed by LI and Quiros as a novel molecular-marker method in 2001 *(6)*. It was used for DNA and cDNA analysis similar with AFLP markers.

Differently expressed genes (DEGs) were detected more uniformly with SRAP-cDNA compared with ESTs got from cDNA library. Secondly because most product of SRAP with little overlap, objective fragment can be sequenced directly use the universal primer after recovery. Experiments show that cDNA-SRAP performed efficiently on gene expression of Brassica through comparing species and varieties. It explained cDNA-SRAP method was available for DEGs analysis in plant *(7)*.

In order to find the mechanism of creeping-rooted alfalfa and lay the groundwork for further molecular breeding, DEGs of creeping-rooted alfalfa was analyzed in mRNA level conducted with cDNA-SRAP by GA_3 inducement.

Fig.1 The different stage of creeping rooted bud devolopement
A: Creeping rooted swellon stage; B: Creeping rooted bud stage;
C: Creeping rooted shoots stage

Fig.2 Sample used for the experiment
A: Experiment plots of Beijing Shunyi; B: Creeping-rooted plant;
C: Treated plant with GA_3 in greenhouse

Materials and methods

1. Plants materials

The alfalfa species of *Medicago sativa* L. named BL-101 was used for this experiment which transplanted to Beijing Shunyi from Inner Mongolia in 2007.

Creeping-rooted(CR) and Noncreeping-rooted(NCR) plant were selected in the same strains and potted to the greenhouse on Aipril 10th 2010, respectively (Creeping-rooted occurrence period).We treated the non-creeping-rooted (NCR) plant with a phyto-hormone GA_3 (200mg/L/d) for one week. Then take the root sample separately from creeping- rooted (CR) and non-creeping-rooted (NCR) plant for the further study (Fig2 B).

2. Main reagent and instrument

Trizol, M-MLV (RNase H), dNTP, Oligo (dT) 15, primer(Table.1),PCR Thermal Cycler (Biometra T-Gradient Thermoblock), Electro- phoresis apparatus (DYY-6C).

3. cDNA Synthesis

Total RNA were extracted from CR and NCR used Trizol

王 晓娜（北京林業大学大学院、現所属：北海道大学農学研究院、札幌 060-8589）・卢欣石（北京林業大学林学院・草地資源生態研究所、北京 100083、中華人民共和国）

(guanidinium isothiocy anate alcoholphenyl chloro- form) and then reverse transcription to cDNA used M-MLV (RNase H), cDNA was stored as PCR template for SRAP.

4. cDNA-SRAP Amplification and SDS-PAGE

15 pair sequence were selected as up and down primer for PCR reaction (Table.1). Amplification system:

10×PCR buffer	2.5μL（Mg^{2+}）
dNTP	2μL（0.20mmol/L）
Taq DNA polymerase	0.3μL（1.50U）
up、down primer	1μL（0.40μmol/L）
cDNA template	1μL
Total	25μL

Amplification reaction condition as follows: 94℃ 5min, 1 cycle; 5 cycle for 94℃ 1min, 35℃ 1min,72℃ 1min; 35 cycle for 94℃ 1min, 50℃ 1min,72℃ 1min; 72℃ 7min as annealing temperature, stored in 4℃.

The product of PCR were amplified with 6% of the modified SDS-PAGE used 15 pair primer, first step 200 V voltage, 300 mA electricity for 20 min, second step 300 V, 300 mA constant for 50 min.

5. Amplified fragment recovered and sequenced

After the SDS-DAGE, amplified fragment were recovered and transferred to 0.5 mL eppendorf tube use surgery blade before the gel got dry. Add 20μl TE stay at 60 ℃ for 10 hours. Next inserted into expression plasmid pEAST-T1 make clone use nutrient medium for sequencing.

Table.1 Primer sequences used in cDNA-SRAP amplification of *Medicago sativa* L.

Primer	Sequence（5'-3'）	Primer	Sequence（5'-3'）
F12-r15	F:5'-CGAATCTTAGCCGGAGC-3' R:5'-CGCACGTCCGTAATTCCA-3'	Me4-em4	F:5'-TGAGTCCAAACCGGACC-3' R:5'-GACTGCGTACGAATTTGA-3'
F14-r14	F:5'-CGAATCTTAGCCGGAAT-3' R:5'-CGCACGTCCGTAATTAAC-3'	F12-r9	F:5'-CGAATCTTAGCCGGAGC-3' R:5'-GACACCGTACGAATTTGA-3'
F16-em1	F:5'-GATCCAGTTACCGGCAC-3' R:5'-GACTGCGTACGAATTAAT-3'	F9-em5	F:5'-GTAGCACAAGCCGGACC-3' R:5'-GACTGCGTACGAATTAAC-3'
Me1-r14	F:5'-TGAGTCCAAACCGGATA-3' R:5'-CGCACGTCCGTAATTAAC-3'	F16-r15	F:5'-GATCCAGTTACCGGCAC-3' R:5'-CGCACGTCCGTAATTCCA-3'
Me2-em4	F:5'-TGAGTCCAAACCGGAGC-3' R:5'-GACTGCGTACGAATTTGA-3'	Me2-r9	F:5'-TGAGTCCAAACCGGAGC-3' R:5'-GACACCGTACGAATTTGA-3'
F16-em5	F:5'-GATCCAGTTACCGGCAC-3' R:5'-GACTGCGTACGAATTAAC-3'	F7-r9	F:5'-GTAGCACAAGCCGGAGC-3' R:5'-GACACCGTACGAATTTGA-3'
F16-r14	F:5'-GATCCAGTTACCGGCAC-3' R:5'-CGCACGTCCGTAATTAAC-3'	F9-r9	F:5'-GTAGCACAAGCCGGACC-3' R:5'-GACACCGTACGAATTTGA-3'
Me2-em5	F:5'-TGAGTCCAAACCGGAGC-3' R:5'-GACTGCGTACGAATTAAC-3'		

Results and Discussion

1. RNA Extraction and cDNA synthesis

Electrophoresis pattern displayed, 28S and 18S band is brightand clear, no trailing phenomenon, and 28S brightness is twice than 18S band, the result of quantitative analysis showed that we got the high quality (Fig.3)

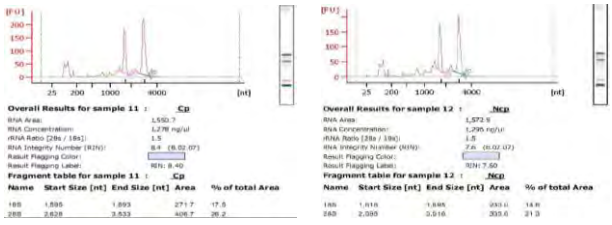

Fig.3 Quantitative analysis for total RNA of CR and NCR

2. cDNA-SRAP Amplification and SDS-PAGE

We used the cDNA for SRAP amplification (Fig.4), 15primer for each sample (CR and NCR). The result of SDS-PAGE showed that differently amplified fragment appeared from the CR and NCR sample with same primer (Fig.5).

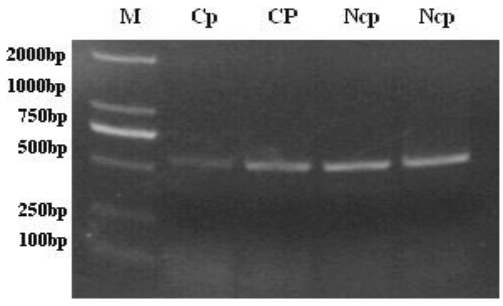

Fig.4 The electrophoresis pattern of cDNA

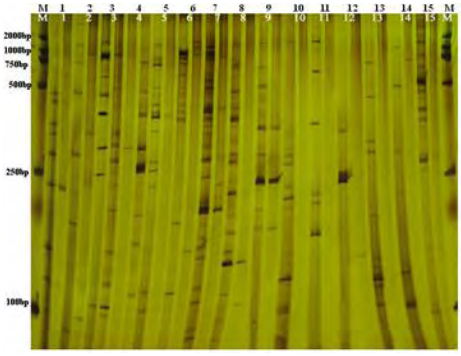

Fig.5 The electrophoresis pattern of cDNA-SRAP

Note: CR and NCR used with one same primer from left to right

3. Recovery sequencing

76 discrepant bands were found after cDNA-SRAP amplification, 42 amplified fragments were inserted into expression plasmid Peasy-T1 after recovering which displayed stably in CR sample. Amplified fragments were cloned used LB culture medium for sequencing (Fig.6). By homology analysis in NCBI and compared with *Mediago* Genome (http://www.medicagohapmap.org/?genome) cor -responding homologous genes of those fragments were found (Table 3).

From Table2, we got the results that among the 42 stable differentially expressed genes 9 were the unknown function gene, the other known function genes mainly includes AP-2 complex subunit beta-1, zinc finger protein, thioredoxin h7, MYB transcription factor MYB49, xyloglucan galactosyltrans-ferase.

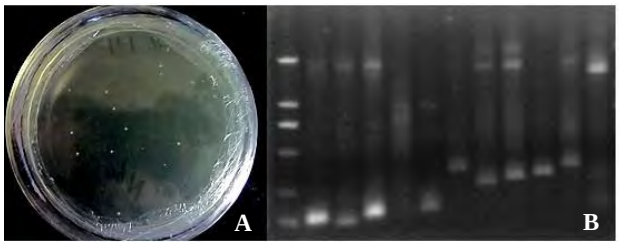

Fig.6 Amplified fragments cloned on LB culture medium

A: Train and clone of recovered fragment;

B: The E. coli liquid electrophoresis result.

Table.2 Classification of gene function

Gene Function Classification of gene function	Gene Amount The number of gene
Disease/defence	4
Energy	8
Transcription	2
Signal transduction	1
Protein synthesis	10
Cell structure	1
unnamed protein product	5
unknown	9
No hit	2

AP2/EREBP transcription factors included zinc finger protein structure domain *(8)*. In addition, AP-2 complex subunit beta-1 was found in the differences expressed genes. It explained that under the inducement of GA_3, AP2 transcription factors expressed. Transcriptome were compared with RNA-seq, one of the unigene gnl|UG|Mtr#S5430627 was found and confirmed that it was RAV transcription factor. (unpublished data). As the subfamily of AP2 transcription factor, RAV effect with plant meristem. We preliminary confirmed that AP2 was one of the related genes involved in development of creeping-rootedness.

Conclusion

We got the conclusion that in the GA_3 may induced the expression of AP2 transcription factors , the gene expression of heat shock protein and related hydrolysis enzyme explained that under the stimulation of external factors, signal transduction gene responded which started the expression of transcription factors, further combined with protein, at the same time metabolism became more active due to the ATP hydrolyzed by carbohydrate, enhanced the meristematic ability of root, and then the creeping-rootness occurred.

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Table.3 Homology comparison analysis of sequenced gene

No.	Accession	Size(bp)	Description	E-value
1	NM_125046.2	757	6-phosphofructokinase [Arabidopsis thaliana]	0
2	CBI21313.3	1520	unnamed protein product [Vitis vinifera]	40.56091607
3	ABO28522.1	506	kinesin-related protein [Gossypium hirsutum]	18.90668297
4	D2XNR1.1	1428	RecName: Full=Flotillin-like protein 4	0
5	XP_002510980.1	658	zinc finger protein, putative [Ricinus communis]	1.00E-06
6	ACZ37071.1	659	thioredoxin h7 [Medicago truncatula]	3.00E-70
7	ACU23644.1	645	unknown [Glycine max]	2.00E-30
8	--	530	--	8.00E-04
9	XP_003597891.1	710	Longin-like [Medicago truncatula]	6.00E-102
10	XP_003593643.1	828	Heat shock protein DnaJ [Medicago truncatula]	2.00E-95
11	XP_002521957.1	728	ethylene receptor, putative [Ricinus communis]	4.00E-102
12	XP_002298129.1	850	2-oxoglutarate-dependent dioxygenase [Populus trichocarpa]	4.00E-123
13	ACN28162.1	722	unknown [Zea mays]	5.00E-40
14	CBI15756.3	848	unnamed protein product [Vitis vinifera]	1.00E-95
15	XP_002514582.1	836	inositol or phosphatidylinositol kinase, putative [Ricinus communis]	4.00E-144
16	XP_003604318.1	916	hypothetical protein MTR_4g009410 [Medicago truncatula]	5.00E-13
17	BAC55228.1	814	serine palmitoyltransferase [Lotus japonicus]	5.00E-75
18	ACU18078.1	770	unknown [Glycine max]	2.00E-132
19	ACJ84355.1	916	unknown [Medicago truncatula]	2.00E-166
20	ACU19735.1	805	unknown [Glycine max]	2.00E-51
21	XP_003615315.1	601	unnamed protein product [Vitis vinifera]	2.00E-81
22	XP_002885050.1	846	integral membrane single C2 domain protein [Arabidopsis lyrata subsp. lyrata]	2.00E-53
23	--	787	--	0.67533401
24	CAC29435.1	645	P-type H+-ATPase [Vicia faba]	1.00E-75
25	XP_003607731.1	795	NAC protein [Medicago sativa]	2.00E-134

26	CBI27142.3	727	unnamed protein product [Vitis vinifera]	2.00E-21
27	ACJ83987.1	892	unknown [Medicago truncatula]	4.00E-139
28	XP_002521539.1	680	metal ion binding protein, putative [Ricinus communis]	4.00E-76
29	XP_003607308.1	732	AP-2 complex subunit beta-1, putative [Ricinus communis]	2.00E-123
30	XP_003626112.1	857	Protein of unknown function [Medicago truncatula]	4.00E-157
31	AAU90320.1	680	Putative mandelonitrile lyase, related [Solanum demissum]	1.00E-57
32	XP_002519648.1	819	Poly(rC)-binding protein, putative [Ricinus communis]	8.00E-52
33	ACJ84988.1	906	unknown [Medicago truncatula]	2.00E-167
34	ACU20166.1	757	unknown [Glycine max]	20.36082973
35	ACJ85512.1	922	unknown [Medicago truncatula]	128.5485836
36	BAA02108.1	532	GTP-binding protein [Pisum sativum]	1.35E-07
37	ABR28337.1	657	MYB transcription factor MYB49 [Medicago truncatula]	22.24644796
38	XP_002526536.1	753	Xyloglucan galactosyltransferase KATAMARI1, putative [Ricinus communis]	34.58553177
39	ACY08857.1	795	xyloglucan galactosyltransferase [Eucalyptus grandis]	86.90996547
40	XP_002526536.1	753	vesicle-associated membrane protein 725 [Zea mays]	2.00E-26
41	BAA02108.1	532	GTP-binding protein [Pisum sativum]	1.35E-07
42	ACY08857.1	795	xyloglucan galactosyltransferase [Eucalyptus grandis]	3.18E-14

References

(1) Bouton, J.H., Smith, S.R., Jr., Wood, D.T., Hoveland, C.S. and Brummer, E.C. (1991) Registration of'Alfagraz' alfalfa. [J]. Crop Sci., 31: 479.

(2) Gao Zhengsheng, Wang Pei. et al. (1994) Change of Esterase Isozyme and Endogenous Hormone in Root System and Ontogenesis of Creeping- Rooted Character in Alfalfa. Acta Agrestia Sinica 1(02): 1-11.

(3) Pecetti L, Piano E.(2002) Penetrance of creeping-rooted ness clonal progenies of Lucerne and observations on underground morphology of plants differing for this character. Euphytica,128：35-45.

(4) Heinrichs D H, Morley FH (1960) Inhritance of resistance to winter injure and it's correlation with creeping-rootedness in alfalfa.Can. J.Plant.Sci.,40:487-489

(5) Steinmetz LM. and Davis RW. (2004) Maximizing the potential of functional genomics . Nat Rev Genet 5(3): 190-201.

(6) Li G ., Quiros C F. (2001) Sequence related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor Appl Genet 103: 455-461.

(7) GUI Qin,WANG Jialu, WU Xiaoming (2007) Application of SRAP-cDNA method in analysis of differential gene expression in plants. Jianbo1 Chinese J. Oil Crop Sciences 29(4): 497-502.

(8) Bi Yongxian, Meng Lingbo, and Chen Shen. (2009) The Plant salt resistance research of molecular biology and of proteomics. WORLD HEALTH DIGEST, 6(21): 118-119.