cDNA ARRAYS AND PCR-SELECT APPLIED FOR CHARACTERIZATION OF AGRICULTURAL FEATURES IN PLANTS

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ABSTRACT

Identification of genes activated or inhibited during the analyzed process is the base for plant biotechnology. PCR-select (suppression subtraction hybridization) – technique of isolation of genes specific for a process of interest – was applied for analysis of symbiotic interactions between *Lupinus angustifolius* and nitrogen fixing bacteria.

The subtraction, with RNA from the early stages of symbiosis of *Lupinus angustifolius* – nitrogen fixing bacteria as a tester and RNA from uninfected roots as a driver, enriched the pool of cDNA molecules with ones specific for symbiosis. ENOD40 and enolase were used to evaluate efficiency of PCR-select. The pool of subtracted cDNA molecules was cloned and a set of 768 randomly picked up clones was organized in an array. SNF (Symbiotic Nitrogen Fixation)-specific genes (cDNAs) were identified with differential hybridization using cDNA array.

Eighteen marker cDNAs representing genes of well characterized expression pattern and/or function (carbon and nitrogen metabolism, SNF development, cytoskeleton) were cloned in advance and used as references in expression profiling.

Key words: Symbiotic Nitrogen Fixation, *Lupinus angustifolius*, nodule, PCR-select, suppression subtractive hybridization, cDNA, gene expression

Abbreviations: SNF – symbiotic nitrogen fixation, dpi – days post infection, UTR – untranslated region, 20 x SSC – 0.3 M tri-sodium citrate, 3 M sodium chloride.

INTRODUCTION

Symbiosis between legumes and nitrogen fixing bacteria involves three stages: partner recognition, nodule organogenesis, and nitrogen fixation in the mature nodule. Symbiotic nitrogen fixation has generated interest not only because of its enormous agricultural significance, but also because of its

potential usefulness as a model of molecular signal exchange, signal transduction, and organogenesis.

The plant responds to symbiotic bacteria by modulating the expression of several hundred genes (Fedorova et al., 2002). The first step is the mutual recognition of the symbiotic partners. Plant flavonoids activate bacterial genes responsible for the synthesis of specific lipo-chito-oligosaccharides which act as bacterial signal molecules (Bassam et al., 1988; Peters et al., 1986). These bacterial signal molecules are recognized by plant receptors such as NFR1, NFR5 and SYMRK, which is the first step for successful bacterial colonization of root hair (Radutoiu et al., 2003; Madsen et al., 2003; Stracke et al., 2002). The bacteria are then surrounded by plant cell wall, forming an infection thread which progresses toward a group of cortex cells. These cells begin to divide and differentiate. A second group of mitotically active cells is formed in the pericycle opposite the cortex cells. Mitotic activity in the two groups leads to the formation of a nodule. This new organ consists of cortex, a vascular bundle, epidermis and central tissue which are different than those seen in uninfected roots. In the indeterminate nodules of *Lupinus angustifolius*, the meristem is located in the distal region of the central tissue. In the infection zone, young central tissue cells are infected by the bacteria. In the fixation zone, nitrogen is fixed in plant cells harboring numerous bacteroids.

Symbiosis requires the modulation the expression of many plant genes, including the activation of SNF (symbiotic nitrogen fixation specific) genes and the repression of some other genes. Some SNF gene products have been identified, such leghemoglobin, which regulates oxygen level in the nodule, or nodulin-45, whose function is not yet known (Konieczny et al., 1987; Stróżycki et al., 2000; Macknight et al., 1995; Sikorski and Rożek, 1994).

In this paper, we describe the use of PCR-select in isolating SNF genes which are active in nodule formation in *L. angustifolius* 5 dpi (days post infection), when the root cortex cells differentiate into nodule specific tissues. PCR-select is a suppression subtraction hybridization technique for isolating genes responsible for a particular process (Diatchenko et al., 1996).

MATERIAL AND METHODS

Preparation of plant material

Lupinus angustifolius L. var. 'Emir' plants were grown in two-liter pots with perlite with a 16 h photocycle at 23°C. Procedures for seed sterilization, seeding, and preparing nutrient solutions are described elsewhere (Strozycki et al., 2003). The nutrient solution used for plants grown symbiotically did not contain potassium nitrate.

Plants were inoculated when they were seven days old and had four leaves with a 10 ml of a seven-day-old culture of *Bradyrhizobium* sp. (*Lupinus*)

WM9 grown on YMB medium (Vincent, 1970). The absorbance at 550 nm of the inoculant was 1.2.

SNF early stage analysis was performed on root sections 2.0 to 4.0 cm long cut off 2.0 cm below the hypocotyls, and at least 2 cm above the root tips. Root sections were frozen in liquid nitrogen within two minutes of collection.

mRNA isolation

The root sectors from five plants frozen in liquid nitrogen were ground in a mortar cooled with liquid nitrogen. 100 to 200 mg of ground plant tissue was used for a single RNA isolation with the RNeasy Plant Mini Kit (Qiagen) yielding 30 to 70 µg of total RNA. 500 µg of total RNA was used for mRNA isolation with the Oligotex mRNA Midi Kit (Qiagen).

PCR-select and cDNA library preparation

2 µg of mRNA was used for cDNA synthesis. Two-step subtraction followed by PCR amplification was performed using the Clontech PCR-select cDNA subtraction Kit (BD Bioscience). Two parallel sets of cDNA were prepared:

- SNF-forward: driver RNA from uninfected root was subtracted from tester RNA from infected roots in the early stages of symbiosis (5 dpi)
- SNF-reverse: driver RNA from infected roots in the early stages of symbiosis (5 dpi) was subtracted from tester RNA from uninfected root (Diatchenko et al., 1996).

The products of the SNF-forward and SNF-reverse subtractions were amplified using two-step PCR in accordance with the procedure recommended by the manufacturer.

The amplified product of the SNF-forward subtraction was cloned into the pCR2.1 vector with the TA cloning kit (Invitrogen). Complementation testing showed that 75% of the 5,000 transformants were recombinants. The cDNA library consisted of 768 randomly selected recombinants stored in eight microtiter plates designated MB201 to MB208.

PCR amplification

Single PCR amplification of the library clone was carried out in a final volume of 100 µl using Taq DNA Polymerase (Fermentas) and using 4 µl of template. The template was prepared by suspending a single colony in 25 µl of water and incubating at for 10 minutes at 100°C, and then cooling and shaking. The template was kept on ice and centrifuged just before use. Two universal primers were used:

- JP203 (CGGCCAGTGAATTGTAATACG), and
- JP204 (ATGACCATGATTACGCCAAGC) (200 nm).

ENOD40 and enolase cDNA-hybridization probes were amplified by the same method, except that 1 ng of cDNA was used as a template and two gene-specific primers were used. The products were purified with the QIAquick PCR purification kit (Qiagen).

Analytical PCR was carried out to measure adaptor ligation efficiency. The method was the same as described above except that the reaction volume was 25 µl, 1/200 µl of adaptor ligation reaction was used as a template, and two enolase targeted primers were used:

- ENOL-F-6 (CCATGCAGGTCAGAGCGTCT) and
- ENOL-R-17 (CATTAAACAGCCTTTAAATATAGG).

PCR conditions and primer targeting adaptors were as recommended in the PCR-select protocol (Clontech).

Probe labeling

The final product of PCR-select (pooled dsDNA) was purified with the QIAquick PCR purification kit (Qiagen), digested with *Rsa* I (Fermentas) in order to cut off the adapters ligated during the PCR-select process, and again purified with the QIAquick PCR purification kit.

100 ng of DNA was labeled with alfa [P^{32}] dCTP (3000 Ci/mmol, 10 mCi/ml) (ICN) using the HexaLabel DNA Labeling Kit (Fermentas). Labeling was carried out in accordance with the procedure recommended by the manufacturer, except that the incubation at 37ºC was extended to 30 minutes.

The labeled dsDNA was purified using the QIAquick PCR purification kit. Around 20% of the radioactivity was incorporated into the cDNA under these conditions. The activity of the probes was 18-20 x 10^6 cpm. The same protocol was used to label cDNA from the ENOD40 and enolase cDNA-hybridizations except that digestion with *Rsa* I was omitted.

cDNA array production and hybridization

cDNA arrays were produced with Nunc 96 PIN Replication System by spotting 0.1 µl of DNA prep on a Hybon N^+ membrane (Amersham). The volume of DNA used for spotting was between 20 and 25 µl. Source plates had U-shape bottoms.

The arrays were denatured neutralized spotted surface up three times for ten minutes each on Whatman No. 3 filter paper soaked in a solution containing 0.5 N NaOH and 1.5 M NaCl. They were then neutralized spotted surface up three times for ten minutes each on Whatman No. 3 filter paper soaked in a solution containing 1 M Tris/HCl pH 7.5 and 1.5 M NaCl. The arrays were then dried at room temperature overnight and baked for two hours at 80°C.

The arrays were pre-hybridized at 68°C for six to ten hours in a solution containing 5 x SSC, 5 x Denhardt solution, 0.5% sodium dodecyl sulfate, and 0.1 mg/ml salmon sperm DNA (Jordan, 2001).

The arrays were hybridized for 48 h by adding a denatured ss cDNA or ds cDNA probe directly to the pre-hybridization solution. Finally, the arrays were washed for one hour with pre-warmed (68°C) buffer containing 0.1 x SSC, 0.1% sodium dodecyl sulfate using 2 x 200 ml of the buffer per array.

The arrays were analyzed with an image plate, a Typhoon 8600 phosphorimager, and ImageQuant software.

Reverse transcription and cloning of selected genes cDNA

Reverse transcription was carried out using the RevertAid First Strand cDNA synthesis kit (Fermentas). 2 µg of total RNA was used as a template. Instead of using (dT)18, one of the following primers was used:

- JP207: CGCCTGAATTCTAGATAGGTACCGTCGACG(T)16 (1.5 µM); or
- a gene specific primer (1 µM). PCR amplification was carried out in a final volume of 40 µl using 0.5 to 5 µl of the reverse transcription reaction as a template. Two primers were used:
- an arbitrary 3' end primer, JP205: CGCCTGAATTCTAGATAG (200 nM), and
- a gene specific primer (200 nM).

The final concentration of magnesium chloride (including the template) was 1.75 mM. The first four cycles were carried out without the primer JP205. The arbitrary primer JP205 was changed for gene specific primer in the PCR mix when the gene specific primer was used for cDNA first strand synthesis.

The amplicons were ligated and cloned using the TA Cloning Kit (Ivitrogen), in accordance with the procedure described above for cDNA library preparation.

RESULTS

Cloning cDNA for enolase, ENOD40 and other markers genes

The cDNA of all marker genes except for ENOD40 and enolase was cloned using pooled RNA as a template. Pooled RNA from two early stages of SNF was used (5 dpi and 12 dpi).

ENOD40 and enolase, markers important for PCR-select efficiency analysis, were cloned using 5 dpi RNA as a template. ENOD40 is a gene specific for SNF and is expressed from one day after infection until the nodule matures (Minami et al., 1996; Yang et al., 1993). Two rounds of nested PCR amplification were necessary to synthesize the first strand of ENOD40. A degenerated gene-specific primer was used:

- M11_E40R: CCTAC(TC)T(AT)CTCATCTGCA.

The cloned fragment is 416 bp long and corresponds to the region from positions 123 to 502 of the 551 nucleotide long *L. luteus* ENOD40B homolog (AF352375), with a 95% identity on the nucleotide level.

Enolase, the second marker for PCR-select efficiency analysis, is a gene expressed on high level in uninfected roots and during SNF (van Ghelue et al., 1996). The cloned fragment is 349 bp long with a 189 bp region 87% identical to the coding sequence of *G. max* enolase (AY496909) and 160-bp 3' end untranslated region.

Table 1. Hybridization of marker clones with probes: A – reverse transcriptase – synthesized, complex probes (copy of transcriptome), B – PCR-select probes. Relative hybridization for each maker was calculated as ratio of hybridization intensity of given marker to actin. Ratio of relative hybridization level obtained with "SNF 5 dpi" probe to "uninfected root" probe is presented in column A. Column B presents ratio of relative hybridization intensity obtained with "SNF-forward" PCR-select probe to relative hybridization intensity obtained with "SNF-reverse" PCR-select probe

Clone	Annotation	A Reverse transcriptase-synthesized complex probe; SNF : Uninf. root	B PCR-select probe; SNF-forward : SNF-reverse
Nitrogen metabolism			
LA10_01	Aspartate aminotransferase	9.19	1.60
LA10_02	Asparagine synthetase	1.75	0.76 : 0.00
LA10_03	Uricase	1.23	0.79
LA10_04	Glutamine synthetase	2.37	0.08
Carbon metabolism			
LA10_20	Enolase - phosphopyruvate hydratase	1.84	0.87
LA10_21	Phosphoenolpyruvate carboxylase	1.61	0.93
LA10_22	Sucrose synthase	1.54	1.25
Nodule development			
LA10_40	ENOD40	6.50	0.48 : 0.00
LA10_41	Nodulin 45	1.53	1.60
LA10_42	Nodulin 26	1.32	2.10
Quantification and RNA quality markers			
LA10_60	18 rRNA	1.62	0.45
LA10_61	26 rRNA	1.67	1.20
LA10_62	Phytoene desaturase	1.66	2.37
LA10_64	Actin	1	1
Stress related			
LA10_80	AUX/IAA induced protein	1.55	1.68
LA10_81	Chalcone synthase	0.77	0.73
LA10_82	Pathogen induced oxygenase	1.48	1.02

cDNA for other markers genes, except for phytoene desaturese, was cloned using the 3' end RACE technique as for enolase. The cloned fragments of the marker genes possess at least 100 nt of coding sequence and 3' end UTR. The cloned fragment of phytoene desaturase corresponds to the 1251 bp long fragment of the soybean phytoene desaturease (M64704) coding sequence. The cloned marker genes are listed in Table 1.

PCR-select and cDNA library enriched in SNF 5 dpi specific genes

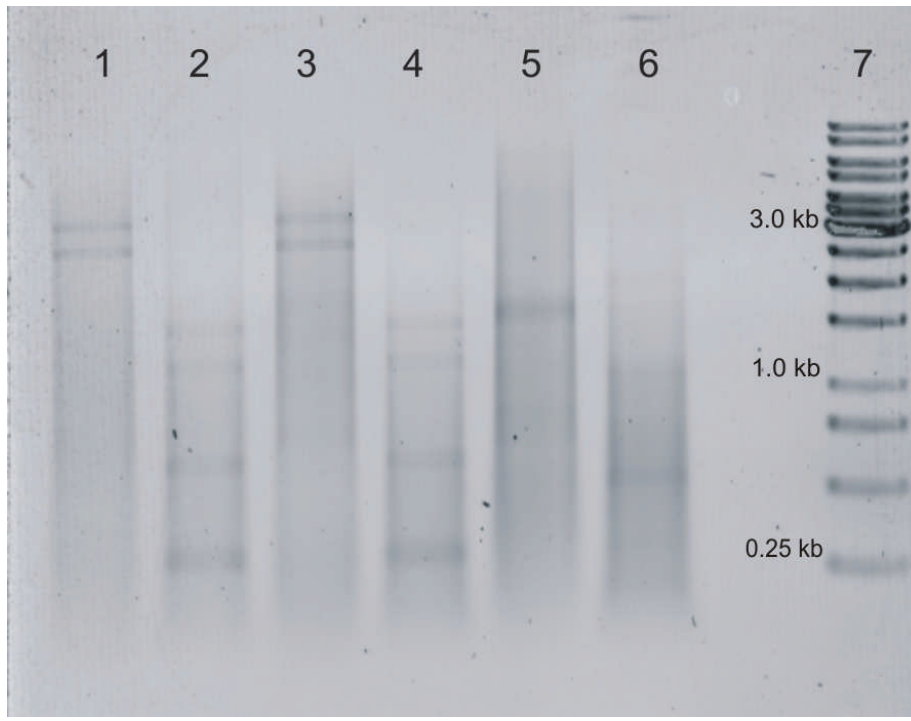

Figure 1. Digestion of cDNA with *Rsa* I

cDNA from SNF 5 dpi: lane 1 – before *Rsa* I digestion, lane 2 – after *Rsa* I digestion; cDNA from uninfected root: lane 3 – before *Rsa* I digestion, lane 4 – after *Rsa* I digestion; human muscle cDNA (control): lane 5 – before *Rsa* I digestion, lane 6 – after *Rsa* I digestion; Fermentas 1 kb ladder – lane 7.

The following tests were performed to check the efficiency of SNF specific cDNA selection: control of digestion of cDNA with *Rsa* I, adaptor ligation efficiency, subtraction efficiency. The progress of *Rsa* I digestion was analyzed by running in parallel digested and undigested DNA (Fig. 1). The enolase-specific primers ENOL-F-6 and ENOL-R-17 were projected to amplify short fragment without an *Rsa* I site suitable for evaluation of adaptor ligation efficiency. The amount of product amplified with the two enolase specific primers was similar to the amount of product obtained with one

primer targeted adaptor and one enolase-specific primer. The quantification is based on post-electrophoresis analysis of the stained gel with a Typhoon 8600 fluorescence scanner and ImageQuant 5.1 software (Fig. 2). The ratio of the two products was between 0.8 and 1.0. The final check of efficiency of two-step subtraction and PCR suppression depended on hybridization of the pooled cDNA samples, both original and PCR-select generated, with the enolase and ENOD40 probes. The enolase fragment used as the probe for PCR-select efficiency analysis was amplified with the primers ENOL-F-6 and ENOL-R-17. The ENOD40 fragment 323 bp long was amplified with the following primers:

- JP4-08: ACCAAGGGATATGCATACA, and
- JP4_09: GGCTTCCTACTTACTCATC.

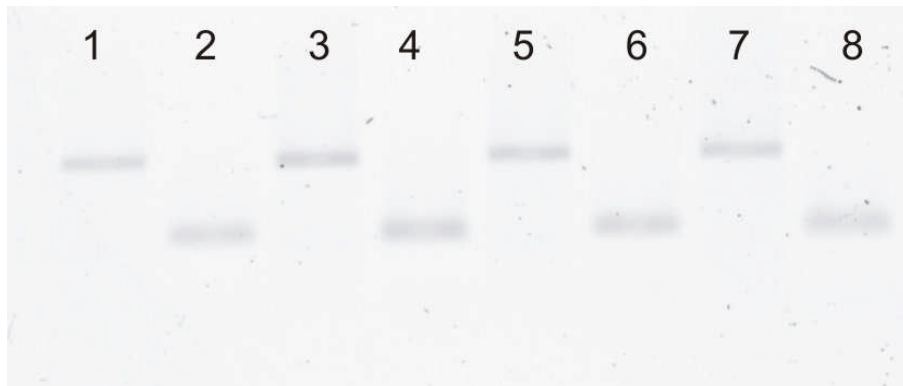

Figure 2. Efficiency of adaptor ligation

SNF 5 dpi cDNA ligated with Adaptor 1: lane 1 – PCR amplification with enolase targeted primer and adaptor targeted primer, lane 2 – PCR amplification with two enolase targeted primers; SNF 5 dpi ligated with Adaptor 2R: lane 3 – PCR amplification with enolase targeted primer and adaptor targeted primer, lane 4 – PCR amplification with two enolase targeted primers; uninfected root cDNA ligated with Adaptor 1: lane 5 – PCR amplification with enolase targeted primer and adaptor targeted primer, lane 6 – PCR amplification with two enolase targeted primers; uninfected root cDNA ligated with Adaptor 2R: lane 7 – PCR amplification with enolase targeted primer and adaptor targeted primer, lane 8 – PCR amplification with two enolase targeted primers.

The ratio of hybridization intensity of the two probes with pooled cDNA before and after the PCR-select process showed that the pooled cDNA was 95 times enriched in SNF specific cDNA. The ratio of ENOD40 to enolase before PCR-select in SNF cDNA was 0.08, whereas after the PCR-select, the ratio was 7.6 in SNF-forward pool of cDNA. The differences between the original pool of mRNA and PCR-select generated pool of cDNA were also revealed by hybridization with cDNA of marker genes (Tab. 1).

The pool of cDNA enriched in transcripts specific for SNF 5 dpi (SNF-forward PCR-select) was cloned and five clones were sequenced (Tab. 2). Subsequently, 768 clones were selected at random from among 3,700 recombinants and were organized without sequencing on eight microplates designated MB 201 to MB 208. The efficiency of the whole process (colony growing, PCR amplification and concentration of amplicones) was higher than 90% per plate. The average length of the insert was 650 bp.

Table 2. SNF-forward PCR select clones

Sample of "SNF-forward" PCR select library from *L. angustifolius* 5 dpi – five clones pick up by random and characterized with single sequencing reactions. The homologues of the highest E value from non redundant database (nr homologues) and est database (EST homolgues) of Genbank are shown.

Clone name	Nr homologues	EST homologues
LA04_01	gi|21907993; *L. corniculatus* genomic DNA, ch. 5; E = 2e-36	gi|6913360; G. max; SYMBIOSIS-RELATED; E = e-103
LA04_02	gi|414549; *A. thaliana*; cytosolic triose phosphate isomerase; E = 5e-04	gi|27458675; 1211_F -P proteoid roots 12 and 14 DAE *Lupinus albus*; E v. = e-140
LA04_03	no hits	gi|27459719; 984_F -P proteoid roots 12 and 14 DAE *Lupinus albus* cDNA clone 984; E = e-105
LA04_04	gi|3738258; *Populus nigra* mRNA for cytosolic phosphoglycerate kinase 2; E = 1e-88	gi|19347371; G. max; PHOSPHOGLYCERATE KINASE, CYTOSOLIC; E = e-106
LA04_05	no hits	no hits

Legend of tables and figures

Screening the library for SNF specific genes

The nylon arrays, each with 384 clones from four microplates, were produced by spotting in duplicate. Two filter arrays covering the whole library, the first with clones from plates MB 201 to MB 204 and the second one with clones from plates MB 205 to MB 208, were composed of 1536 (96 x 2 x 4 x 2) spots. The mean difference between two spots of the same DNA preps was 6.9% as evaluated by comparing hybridization intensity for fifty randomly chosen spots pairs.

Two hybridizations were performed with each set of filters covering the whole library. In the first hybridization, the PCR-select SNF forward probe was used, in which cDNA molecules common for root and SNF were subtracted from the pooled SNF cDNA. In the second hybridization, the PCR-select SNF reverse probe was used, in which cDNA molecules common for both systems were subtracted from the pooled cDNA from uninfected root. Comparison of the results from both hybridizations identified 24 SNF specific

candidate cDNA's. The relative hybridization intensity was twice as high for the SNF- forward probe then for the SNF-reverse probe. The relative hybridization intensity is a ratio of the mean hybridization intensity of given clone to the sum of the mean hybridization intensities of all clones.

DISCUSSION

Two cDNA markers were important for PCR-select efficiency evaluation. The first marker represents a gene which is expressed at a high level in both uninfected roots and in roots at an early stage of SNF (5 dpi). The second marker represents an SNF specific gene. The enolase transcript abundant in SNF 5 dpi and in uninfected root was cloned with 3' end RACE technique. The cloned fragment corresponded to the 3' end UTR and fragment of the enolase coding sequence.

The high expression level of enolase is correlated with its essential role in glycolysis and gluconeogenesis. The activity of the gene does not change in the early stages of the plant response to the symbiotic bacteria, even though gene expression was modulated in aerobic stress and also in different tissues of the mature nitrogen fixing nodule (van Ghelue et al., 1996; Lal et al., 1998).

ENOD40 is expressed at lower level, especially in the early stages of SNF, and the gene specific primer M11-E40R had to be used for synthesis of the cDNA first strand followed by two rounds of PCR amplification with nested forward primers. The cloned fragment lacked 49 nt of the 3' end compared to its closest homologue, ENOD40B from *L. luteus*.

Hybridization of marker cDNA with complex probes was used here to characterize the probes and pooled cDNA generated with PCR-select (Tab. 1). The pool of ss-cDNA molecules synthesized with reverse transcriptase also contained cDNA of ribosomal RNA, the result of oligo (dT)18 primer annealing to polyA-rich stretches in ribosomal RNA. A similar level of efficiency of copying the rRNA on DNA during the labeling of the probes was observed in numerous experiments (reverse Northern, expressed sequence tag libraries). Hybridization of markers with complex probes synthesized with reverse transcriptase revealed three SNF up-regulated or activated genes: ENOD40, aspartate aminotransferase and glutamate transferase. Nodulin 26 and nodulin 45 are known to be SNF specific, although their expression starts at a later stage of SNF (Macknight et al., 1995; Miao and Verma, 1993). At 5 dpi, the hybridization signals for the two nodulins were low. The amounts of cDNA corresponding to the markers in PCR-select probes- reverse and forward – were different. Enrichment in ENOD40 revealed a complete lack of signal for the gene in the hybridization with the SNF-reverse probe, as for asparagine synthetase. Phytoene desaturase and nodulin 26 were represented in the SNF-forward pool of cDNA at higher level than in the SNF-reverse pool. The possibility that nodulin 26 is expressed at 5 dpi will be investigated together with the analysis of candidate

clones. The role of phytoene desaturase in SNF has not been elucidated. Another explanation for the fact that phytoene desaturese increased hybridization intensivity is cross-hybridization with cDNA for other desaturases.

The ratio of ENOD40 cDNA molecules to enolase cDNA molecules increased after PCR-select as a result of two step hybridization with an excess of the driver (uninfected root) RNA and subsequent PCR amplification suppressing by-products: ds-cDNA molecules with the same adapters on both strands. The controls performed during PCR-select confirmed that the digestion with *Rsa* I was efficient. Short, blunt-ended fragments were generated, and more then 80% of the fragments were ligated with adaptors. Before preparing a collection of hundreds of clones, five randomly selected clones were sequenced (Tab. 2). Four of them were similar to sequences of legume plants in the EST database of GeneBank. The one clone without any homologues in the database could represent and untranslated region of mRNA, the most specific part of a transcript or a new gene. The set of five clones represents a small sample of the DNA pool, but it confirmed that that the pool represented different genes and that most of cDNA molecules at least partially corresponded to coding regions.

Twenty-four cDNA clones for which the hybridization intensity was twice as high with the PCR-select SNF-forward probe than with the SNF-reverse probe were isolated. The array hybridization with complex ss cDNA probes (a copy of transcriptome) did not reveal any differences in expression between uninfected root and 5 dpi. Probably, the PCR-select isolated clones are expressed differently and at a such low level that inverted Northern is not sensitive enough to detect them. The candidate clones will be investigated using subtraction probes, virtual northern or real time PCR.

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ZASTOSOWANIE MACIERZY cDNA I SELEKCYJNEGO PCR DLA SCHARAKTERYZOWANIA CECH UŻYTKOWYCH ROŚLIN

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S T R E S Z C Z E N I E

Poznanie genów, których ekspresja ulega zmianie w trakcie badanego procesu, jest istotne dla zrozumienia tego procesu i dla jego wykorzystania w biotechnologii roślin. “PCR-select” (subtrakcyjna hybrydyzacja połączona z supresją) – technika izolacji genów ulegających ekspresji na niskim, i bardzo niskim, poziomie została zastosowana do analizy symbiozy pomiędzy *Lupinus angustifolius* i bakteriami wiążącymi azot.

Subtrakcja, w której od puli mRNA z wczesnych faz symbiozy (*tester*) odjęto mRNA z korzeni nieinfekowanych bakteriami wiążącymi azot (d*river*), wzbogaciła pulę cząsteczek cDNA w cDNA specyficzne dla oddziaływań rośliny z bakteriami symbiotycznymi. Otrzymane w wyniku subtrakcji cząsteczki cDNA sklonowano, a z 768 losowo wybranych klonów cDNA przygotowano macierz. cDNA reprezentujące geny specyficzne lub o podwyższonej ekspresji w trakcie symbiozy zidentyfikowano poprzez różnicową hybrydyzację macierzy cDNA.

W celu interpretacji wyników hybrydyzacji na macierz naniesiono również cDNA osiemnastu markerowych genów o dobrze znanym profilu ekspresji i/lub funkcji (metabolizm węgla, azotu, rozwój symbiozy).

Słowa kluczowe: symbiotyczne wiązanie azotu, *Lupinus angustifolius*, PCR, subtrakcyjna hybrydyzacja, supresja, cDNA, ekspresja genów