cDNA-AFLP ANALYSIS OF GENE EXPRESSION IN RED RASPBERRY (*Rubus idaeus* L.) DURING WATER STRESS

ANALIZA EXPRESIEI GENICE LA ZMEUR (*Rubus idaeus* L.) ÎN CONDIȚII DE STRES HIDRIC PRIN TEHNICA cDNA-AFLP

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Abstract. *Drought stress has negative effects on plant growth and productivity. A cDNA-AFLP (amplified fragment length polymorphism) analysis was performed to identify differential gene expression in response to drought stress in red raspberry (Rubus idaeus L.) plants. The expression profile was compared between well-watered control plants (100±10% field capacity) and stressed plants (40±5% field capacity) cultured in greenhouse conditions. Screening with 64 primer combinations identified multiple transcript-derived fragments (TDFs) that are differentially expressed in drought stress conditions. The differences between control and stressed plants were qualitative when TDFs were either present or absent or quantitative when TDFs showed different levels of expression. The results show that cDNA-AFLP is a valuable technique for studying expression patterns of genes involved in sensitivity/tolerance mechanisms to drought stress in red raspberry.*

Key words: cDNA-AFLP, water stress, *Rubus idaeus*.

Rezumat. *Stresul hidric are efecte negative asupra creşterii şi productivităţii plantelor. Analiza cDNA-AFLP a fost efectuată la zmeur (Rubus idaeus L.) pentru a identifica modificări în expresia genică induse de stresul hidric. Profilul genic a fost comparat între plante bine hidratate (100±10% din capacitatea de câmp) care au fost folosite în calitate de control şi plante stresate hidric (40±5% din capacitatea de camp), ambele categorii fiind cultivate în condiţii de seră. Screeningul efectuat cu 64 combinatii de primeri a identificat multiple fragmente de transcripte (TDFs) care sunt exprimate diferenţial în condiţii de stres hidric. Diferenţele între plantele control şi cele stresate au fost, fie de ordin calitativ, cand fragmentele TDF erau prezente sau absente, fie de ordin cantitativ cand aceste fragmente arătau nivele de expresie diferite. Rezultatele obţinute demonstrează că cDNA-AFLP este o tehnică valoroasă pentru studierea paternelor de expresie a genelor implicate în mecanismele de senzitivitate/toleranţă la stresul hidric la zmeur.*

Cuvinte cheie: cDNA-AFLP, stres hidric, *Rubus idaeus*.

INTRODUCTION

According to the World Atlas of Desertification, UNEP (United Nations Environment Programme), dry lands cover 40% of the world's land surface (more than five billion ha), and they are the habitat and source of livelihood for about 1 billion people. With the growth of population and the development of modern agro-industry and the other industries, extension of the dry land surfaces becomes a growing concern all over the world. This concern is reflected in the Convention

on Biological Diversity's dry lands work program, and in the establishment of the UN Convention to Combat Desertification (Xiao-Cheng Jiang et al., 2004). To mitigate the effects of drought, it is very important first to promote the effective and efficient use of existing plant resources, second to investigate the physiological processes and mechanisms that help plants to acclimatize to the water deficiency conditions. It is assumed that molecules and compounds such as proline, glycine betaine and soluble sugars etc. synthesized and accumulated during desiccation play an important role in the protection of the plants from stress (Pan et al., 2000). However, at this time the molecular basis for these protective mechanisms is almost unknown. In this experiment, the screening of the differentially expressed genes in water deficit conditions was accomplished by means of the high reproductive technique cDNA-AFLP. By this method we identified in stressed plants multiple polymorphic transcript-derived fragments (TDFs) that highlight specific plant reactions on molecular level.

MATERIAL AND METHODS

Plant material

Raspberry (*Rubus idaeus* L.) plants were grown in the greenhouse of the "V. Adavachi" Research Station (University of Agricultural Sciences and Veterinary Medicine of Iasi). When plants have reached maximum development they have undergone stress conditions by reducing soil moisture to 40±5%. The control plants were normally irrigated. Raspberry leaves were collected from mature greenhouse-grown plants and were immediately frozen in liquid nitrogen and stored at –80 ^{o}C.

RNA extraction

Total RNA was isolated from raspberry very fine grinded leaves, in liquid nitrogen, with the SpectrumPlantTotal RNA Kit (Sigma) according to the manufacturer's protocol. RNA quality was checked using Bioanalyzer 2100 and RNA 6000 Nano Kit (Aggilent Technologies) that allow visualization of 18S and 28S subunits and calculation of RNA Integrity Number (RIN).

First-strand cDNA synthesis

The first-strand cDNA synthesis was accomplished with SuperScript II Reverse Transcriptase (RT) Kit (Invitrogen) that is an engineered version of MMLV RT with reduced RNase H activity and increased thermal stability.

Second-strand cDNA synthesis

The second-strand cDNA synthesis was performed with SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) and an oligo(dT) primer (Promega) according to manufacturer protocols.

Restriction Endonuclease Digestion

The restriction fragments were obtained by digestion with two restriction endonucleases: **EcoR I** and **Mse I**. EcoR I has a 6-bp recognition site, and Mse I has a 4-bp recognition site. When used together, these enzymes generate small DNA fragments that will amplify well and are in the optimal size range (<1 kb) for separation on denaturing polyacrylamide gels. To digest the cDNA template, we used the AFLP Analysis System I (Invitrogen) following the instructions provided by the manufacturer with some modifications.

Ligation of Adapters

After heat inactivation of the restriction endonucleases, the cDNA fragments are ligated to EcoR I and Mse I adapters to generate DNA template for amplification. These common adapter sequences flanking variable cDNA sequences serve as

primer binding sites on these restriction fragments. Using this strategy, it is possible to amplify many DNA fragments without having prior sequence knowledge.

Amplification Reactions

The cDNA-AFLP reactions were performed twice, with RNAs from independent samples, using the standard AFLP protocol. Duplicate samples gave similar banding patterns, indicating that the amplification was specific.

PCR was performed in two consecutive steps. In the first step, called preamplification, cDNAs are amplified with AFLP primers each having one selective nucleotide. The resulted PCR products were diluted and used as template for the selective amplification with EcoR I (E) and Mse I (M) selective primers, each containing three selective nucleotides. We tested a number of 64 E-M primer combinations

Separation of Amplified Fragments

Products from the selective amplification were separated on a 4% agarose gel. The banding pattern was analyzed using a fluorescence/chemiluminescence gel documentation system (ECL3/UVP) for the identification of differentially expressed fragments (TDFs).

RESULTS AND DISCUSSION

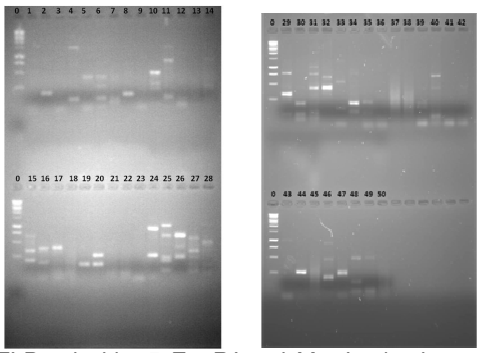
Plant growth and productivity are affected by various abiotic stresses such as drought, heat, cold, etc. The mechanism of drought tolerance is one of the most important research topics in horticultural sciences as drought stress decreases worldwide fruits and berries production.

Plant responses to dehydration involve many different proteins that have specific roles such as proteins for the biosynthesis of osmolytes, transport proteins, proteins involved in degradation and repair processes etc (Campalans et al. 1999). However, the molecular responses involved in drought tolerance remain largely unknown, especially in woody plants. Although many genes induced by dehydration have been identified in various plant species, there is little evidence for the involvement of a specific gene in desiccation protection. Tolerance to abiotic stresses, and in particular to drought stress, is a complex mechanism involving many different genes.

In our work we used cDNA-AFLP technique to compare gene expression profiles of red raspberry (*Rubus idaeus* L.) cultivar 'Opal' in response to drought stress and well-watered conditions. The cDNA-AFLP technique gives highly reliable expression profiles (Milioni et al., 2002).

Screening with *EcoR* I and *Mse* I selective primers revealed many of transcript-derived fragments (TDFs) per each primer combination (picture 1) in both stressed and control plants.

Approximately 400 transcript-derived fragments (TDFs) were obtained and analyzed. About 92 TDFs were differentially expressed in stress conditions. Among these fragments, a number of 11 TDFs increased in response to water stress. We also found 2 TDFs that decreased in water stressed plants. Further characterization of these transcribed sequences will indicate potential candidate genes that might be involved in drought stress.



Picture 1. A cDNA-AFLP gel with 25 *EcoR* I and *Mse* I selective primer pairs: 0 – ladder; 1, 2 - E-ACC / M-CAG; 3, 4 - E-ACC / M-CAC; 5, 6 - E-ACC / M-CAT; 7, 8 - E-ACC / M-CTT; 9, 10 - E-ACC / M-CTG; 11, 12 - E-ACT / M-CAA; 13, 14 - E-ACT / M-CAC; 15, 16 - E-ACT / M-CTA; 17, 18 - E-ACT / M-CAT; 19, 20 - E-ACT / M-CTC; 21, 22 - E-ACT / M-CTG; 23, 24 - E-ACT / M-CAG; 25, 26 - E-AAG / M-CAG; 27, 28 - E-AAG / M-CAA; 29, 30 - E-AAG / M-CAC; 31, 32 - E-AAG / M-CAT; 33, 34 - E-AAG / M-CTC; 35, 36 - E-AAG / M-CTT; 37, 38 - E-AGG / M-CAT; 39, 40 - E-AGG / M-CAA; 41, 42 - E-AGG / M-CTA; 43, 44 - E-AGG / M-CTC; 45, 46 - E-AGG / M-CTT; 47, 48 - E-ACA / M-CTA; 49, 50 - E-ACA / M-CAT. Even numbers – stressed plants, odd numbers – control.

CONCLUSIONS

Screening with 64 primer combinations identified multiple transcript-derived fragments (TDFs) that are differentially expressed in drought stress conditions. The differences between control and stressed plants were qualitative when TDFs were either present or absent or quantitative when TDFs showed different levels of expression. The results show that cDNA-AFLP is a valuable technique for studying expression patterns of genes involved in sensitivity/tolerance mechanisms to drought stress in red raspberry.

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