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Research Article

Transcriptome analysis of tobacco in response to *Ralstonia solanacearum* infection

Cobra Moslemkhani[1], Javad Mozafari[2*], Masoud Shams-Bakhsh[3] and Ebrahim Mohammadi Goltapeh[3]

1. Seed and Plant Certification and Registration Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.
2. Seed and Plant Improvement Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.
3. Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran.

Abstract: One of the best strategies to control bacterial wilt caused by *Ralstonia solanacearum* (Smith) is generally based on breeding resistant cultivars. The information obtained from the expression of plant defense genes will provide new insight for improving plant resistance against pathogens. This study was to identify inducible genes under defense no death (DND) reaction of tobacco (*Nicotiana tabacum*)-*R. solanacearum* interaction using cDNA-AFLP technique. In this assay five different primer combinations were used. Out of 1320 Transcript derived fragments (TDF) that were detected, 101 fragments were identified as differentially expressed genes in 0, 24, 48 and 72 hours post inoculation. Most of the differentially expressed genes were obtained 48 hours post inoculation. Following sequencing, most of sequenced TDFs showed homology to known genes interfering in signaling, regulation and defense functions. DND phenotype in tobacco has some similarities specially in signaling process with mechanism associated with induction of the hypersensitive reaction and it is distinct from general defense mechanisms.

Keyword: Tobacco, *Ralstonia solanacearum*, transcriptome, interaction

Introduction

The phytopathogenic bacterium *Ralstonia solanacearum* is a devastating, soil-borne pathogen that causes lethal wilting of plants in over 200 species (Hayward, 1991; Buddenhagen and Kelman, 1964). The strategy to control bacterial wilt is generally, based on breeding resistant cultivars, while other preventive methods are difficult to apply (Carputo and Barone, 2005).

During plant-pathogen interactions gene expressions are altered and defense mechanisms activated or suppressed. When Plants are exposed to pathogens attack, compatible interaction occurs via colonizing susceptible plant with pathogen and under incompatible interaction defense mechanisms stimulate and prevent pathogen entry, establishment and growth in the plant (Kamoun, 2001; Thordal-Christensen, 2003; Daurelio *et al*., 2009 a, b).

Elicitation of hypersensitive reaction (HR) as incompatible reaction occurs by leaf infiltration with rich bacterial suspension and rapid necrosis of plant cells in infection site has been observed 12-24 hours post inoculation (Carney *et al*., 1990; Klement, 1982). In compatible interaction, bacteria multiply for a prolonged period and

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* **Corresponding author**, e-mail: j.mozafari@yahoo.com
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water soaked lesions appear nearly 3 days after inoculation (Lozano and Sequeira, 1970). Race 3 of *R. solanacearum* isolates induce defense no death reaction but don't induce HR on tobacco (OEPP/EPPO, 1990). The bacteria unable to spread into adjacent tissues and cause chlorotic reaction. The factors responsible for this resistance may be different from HR (Lozano and Sequeira, 1970). Race 3 of *R. solanacearum* isolates is the prevalent race in potato production sites in Iran (Nouri *et al*., 2009). The level of resistance in cultivated potato varieties is low and crossing of Solanum species is time-consuming and hard, therefore the development of resistant genotypes has become a challenge for potato breeders (Carputo *et al*., 2009).

Generally, investigation on tobacco molecular responses against *R. solanacearum* may be assisted by transferring desirable defense genes to *S. tuberosum* cultivars using genetic engineering methods.

Defense mechanisms and induced responses in the various host and non host/*R. solanacearum* interactions have been studied previously (Dahal *et al*., 2009; Schacht *et al*., 2011; Kiba *et al*., 2007; Godiard *et al*., 1990; Gao *et al*., 2009; Esposito *et al*., 2008).

Natural invasion of *R. solanacearum* occurs through the root system and after heavy colonization of the vascular tissues, wilting symptoms appear (Vasse *et al*., 1995). Leaf infiltration technique, is more accurate in comparison to root inoculation and has been suggested for studying molecular aspects of Tobacco /*R. solanacearum* interactions (Kiba *et al*., 2007)

Transcriptome analysis of incompatible response (non-host or avirulent) in tobacco and *R. solanacearum* (race 3) interaction will provide new insights for improving plant resistance against pathogens and for effective control of bacterial wilt disease. Information on the defense mechanisms and genes involved in plant-pathogen interactions would be highly desirable and necessary. This study is conducted to identify inducible genes in interaction of tobacco against *R. solanacearum* by leaf-infiltration using cDNA-AFLP method.

Materials and Methods

Plant material and bacterial inoculation procedure

Nicotiana tabacum cv. Samsun (Seed obtained from Seed and Plant Improvement Institute, Iran), were grown in pots containing a mixture of vermiculite-peat moss (3:1) under controlled greenhouse conditions at 25 °C and 10000 lux with a photoperiod of 16h. Forty days old tobacco plants were used for leaf infiltration. The bacterial strain (SH_{12}) used in this study was *R. solanacearum* race3/biovar2 isolated from potato in Khuzestan province. Culture of bacteria were routinely grown (24h, 28 ºC) on yeast peptone glucose agar (YPGA) medium. Bacterial suspension of 10^8 CFU/ml (0.1 at OD_{600}) from overnight bacterial culture was pressure infiltrated into the abaxial side of the leaves using a syringe without needle. Inoculated regions were harvested at 0, 24, 48 and 72 hours post inoculation (hpi). Water infiltrated leaves were used as control at the first point time. Collected tissues were frozen in liquid nitrogen immediately after harvesting. Samples were prepared in four replicates.

RNA isolation and cDNA synthesis

Total RNA was extracted from infected and non-infected tobacco using RNeasy® mini kit (Qiagen), according to the manufacturer's protocol. The mRNA was purified from 200µg of total RNA using oligotex mRNA purification kit according to the manufacturer's instructions (Qiagen). The quantity and quality of total RNA and mRNA were examined on 1% agarose gel. First strand cDNA was synthesized using first strand cDNA synthesis kit according to the manufacturer's protocol (Fermentas).

Double stranded cDNA was synthesized by adding, 20 µl first-strand cDNA reaction to a master mix consisting of 7.5 µl of 10× cDNAII buffer, 25 U of DNA Polymerase I (Promega), 2.25 U of RNase H (Promega), and 1.25 µl dNTPs (10 mM) in a final volume of 80 µl, and incubating for 2h at 16 °C. The quality of double-stranded cDNA was determined by

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electrophoresis on a 1% agarose gel. The remaining sample was purified by extraction with phenol/chloroform/isoamyl alchohol (1:1:24) and then ethanol precipitated.

cDNA-AFLP analysis

A cDNA-AFLP analysis was performed as described by Bachem *et al*. (1998). Transcript derived fragment (TDFs) were obtained after digestion of the dscDNA with 5U of *Mse*I and *EcoR*I (invitogen). *EcoR*I and *Mse*I adaptors were then ligated to the digested cDNA.Ten µL of the ligation mix and *Mse*I and *EcoR*I primers without selective nucleotide were used for pre-selective amplification. In pre-amplification assay, PCR conditions in volume of 25 µl were as follow: 3 µl template cDNA (ligation product), 0.2 mM dNTP (10 mM), 1x PCR buffer, 1.5 mM MgC_{l2}, 1 U *Taq* polymerase and 0.4 µM of each primer (Mse0 and Eco0). The temperature profile for PCR amplification was 94 °C pre-denaturation for 3 min followed by 15 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. After pre-amplification the mixture was diluted 50 fold and 3 µl was used for selective amplification with 5 primer combinations (MseAT/EcoCT, MseAT/EcoAC, MseCC/EcoCT, MseCC/EcoAC, MseAC/ EcoAC). PCR conditions were the same as pre-amplification assay. Touchdown PCR conditions for selective amplification were as follows: 94 °C pre-denaturation for 1 min followed by denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 30 s. (12 cycles, scale down of 0.7 °C per cycle), 30 s denaturation at 94 °C, 30 s annealing at 56 °C, 60 s extension at 72 °C (23 cycle) and final extension at 72 °C for 5 min. Selective amplification products were resolved on a 6% denaturing polyacrylamide gel (Sequi-Gen GT sequencing cell of Bio-Rad) and running for 2.5h at 105 W and 50 °C. Polyacrylamide gel was visualized by silver staining (Bassam *et al*., 1991). TDFs of interest were excised from the gel. Each gel slice was immerged in 50 µl of sterile water and incubated overnight at 4 °C in order to allow the DNA fragments to diffuse. After centrifugation, the extracts were reamplified using AFLP selective primers under the same PCR conditions. PCR product was purified by Fermentas DNA extraction kit according to the manufacturer's protocol then purified PCR products were sequenced. The nucleotide sequences were analyzed for homology against in Gen-Bank non-redundant database using Basic Local Alignment Search Tool (Blast) Program. Sequences were manually assigned to functional categories based on the analysis of scientific literature.

Result

Tobacco leaf reaction following bacterial infiltration

Isolate SH_{12} induced severe chlorosis in the infiltrated area 48 h after infiltration (Table 1). The degree of chlorosis increased up to 72 h post inoculation but size of the lesion remained constant.

Table 1 Difference between true hypersensitive reaction (HR) induced by *Erwinia amylovora* and defense no death (DND) induced by *Ralstonia solanacearum*.

Symptoms	HR	DND
Restricted pathogen growth	+	+
Spot color	brown	yellow
Appearance discoloration	24 hpi	48 hpi

hpi: hours post inoculation.

cDNA-AFLP analysis

The cDNA-AFLP strategy was successfully used to characterize differential gene expression under tobacco incompatible reaction against race 3 of *R. solanacearum* infiltration. The leaves were evaluated at different times after infection including 0, 24, 48 and 72 hour post inoculation (hpi). For five primer combinations, 1320 TDFs were visualized as bands, 50-800 bp in size. To determine reproducibility of these profiles, the experiments were repeated twice (Fig. 1).

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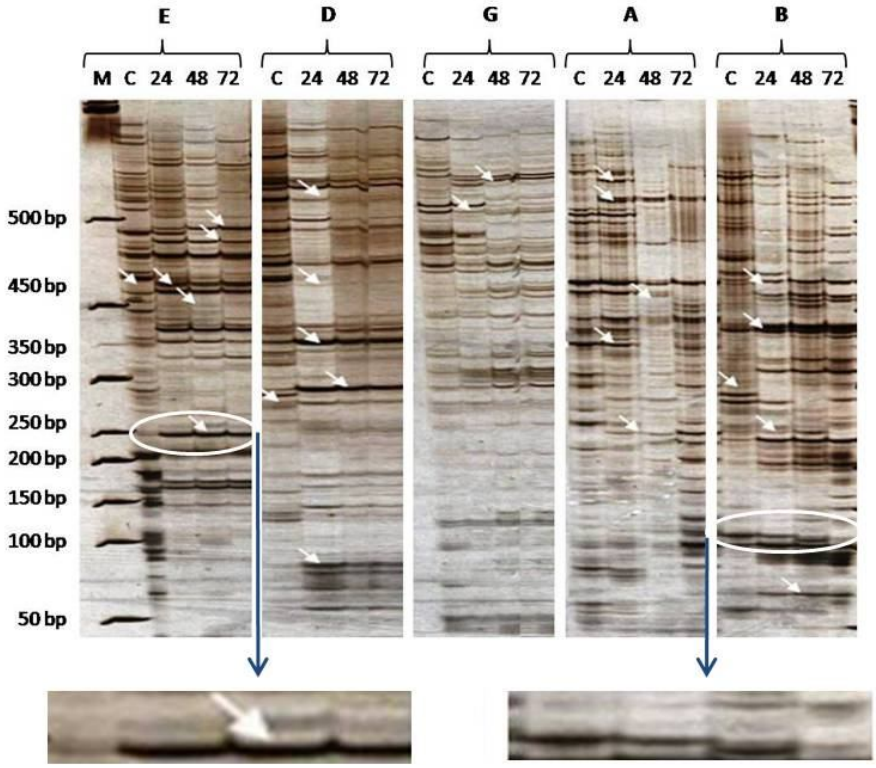

Figure 1 cDNA-AFLP display using five primers combination (E, D, G, A and B) from water infiltrated leaves (lane c), pathogen infiltrated leaves that harvested at 24, 48 and 72 hours post inoculation (hpi). Arrows represent differential bands and magnifications show different expression patterns in control sample compared to pathogen infiltrated leaves.

From 1320 TDFs, 101 genes showed significantly different expression pattern in response to bacteria infiltration. Twenty four hours after infiltration many polymorphic fragments could be evidenced and most of them were up-regulated compared to control plant. 19.2% of cDNA-AFLP fragments corresponded to up regulated transcripts and 9.3% to down regulated transcripts (Fig. 2). Twenty of these cDNA fragments were excised from the gel and sequences were determined. The recovery from acrylamide gel and re-amplification was tricky step and thus we were not able to get a clean sequence for all cDNA fragments. Out of 20 sequences we obtained 11 clean sequences from stress responsive transcripts that showed a length between 79-159 bp (Table 2).

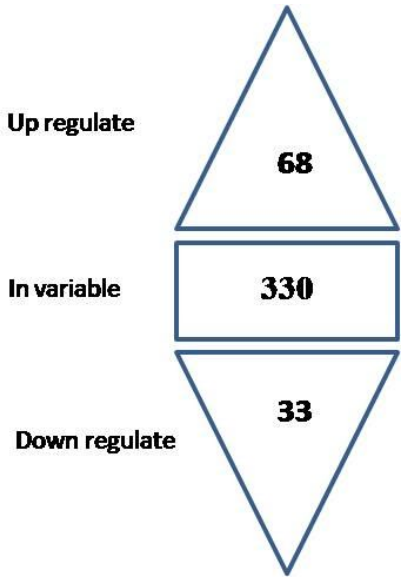

Figure 2 Overall results of cDNA-AFLP analysis in tobacco-*Ralstonia solanacearum*.

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Table 2 Functional annotation of transcript derived fragments (TDFs) obtained by cDNA-AFLP in tobacco-*Ralstonia solanacearum* interaction.

TDF	Length (bp)	Annotation	E-value
TE1	154	Hypothetical protein	6e-49
TE2	138	Ascorbate peroxidase	1e-27
TE3	101	Radical induced cell death1 (RCD1)-like cell differentiation protein	8e-40
TE4	127	Plastid lipid associated protein	6e-54
TE5	159	Predicted protein Wax2 like	8e-35
TE6	154	Metal-dependent protein hydrolase	9e-50
TE8	159	Sesquiterpene synthase 1	3e-62
TE9	79	GEM-like protein 4	7e-17
TF10	117	Avr9/cf-9 rapidly elicited protein 140	6e-50
TF11	115	ABA response elements	3e-41
TF13	105	putative ML domain protein	6e-43

Bioinformatics' analyses were performed and TDFs classified in two groups with functional annotation and unknown function. The major function of deregulated genes was signaling, regulation and defense.

Discussion

Restriction of pathogen to its site of penetration is one of the plant defense strategies against pathogen. Defense No Death (DND) phenotype is induced in tobacco by race 3 of *Ralstonia solanacearum* (OEEP/EPPO, 2004). A cDNA-AFLP approach was used to study transcriptome variation during *R. solanacearum*-tobacco interaction. This revealed the presence of metabolites generally expressed under stress conditions and which could be involved in the plant–pathogen incompatible reaction. Infiltration of suspension of *R. solanacearum* to tobacco leaves induced a condition similar to other stresses especially oxidative stress.

Generally when bacteria are introduced into plant tissues, defense gene activation may be more important than hypersensitive cell death in order to control pathogen growth (Jakobeck and Lindgran, 1993; Klement *et al*., 2003).

Ascorbate peroxidase (APX) (TE2) was shown to be induced under incompatible tobacco and *R. solanacearum* interaction. APX is a reactive oxygen species (ROSs) scavenging enzyme involved in regulation of intracellular ROS levels by reduction of H_2O_2.Excess amounts of H_2O_2 are known to cause oxidative damages to the host cells. H_2O_2 usually acts as signaling molecules in programmed cell death (PCD) under environmental stresses as well as in response to pathogen invasions (Qin *et al*., 2008; Mittler *et al*., 2004).

Reactive oxygen species, such as H_2O_2 play a central role in the activation of pathogen-induced PCD in plants. Findings suggest pathogen-induced PCD in tobacco is accompanied by the suppression of APX expression and reduction in cell capability to scavenge H_2O_2 so accumulation of H_2O_2 and the acceleration of PCD occurs (Mittler *et al*., 1998). In DND reaction in this study we observed that up regulation in APX expression and H_2O_2 levels wasn't sufficient for cell death. Level and timing of H_2O_2 production in tobacco cells are critical points for APX regulation (de Pinto *et al*., 2006) and it seems that level of H_2O_2 production increases before appearance of necrosis symptom, so antioxidant defense systems such as APX activity protect plant against damage of oxidative stress (Sarowar *et al*., 2005).

Abscisic acid (ABA) response elements (TF11) are significant in plant response against abiotic and biotic stresses and ROS production (Mahalingam *et al*., 2003; Sarowar *et al*., 2005; Pei *et al*., 2000). Over production of ABA response elements in the promoters of differentially expressed genes in stress conditions may reflect crosstalk among stress signaling pathway meditated by messengers like H_2O_2 (Mahalingam *et al*., 2003).

Putative ML domain protein (TF13) belonging to the ML domain family is implicated in lipid recognition, particularly in the recognition of pathogen related products. These domain proteins are predicted to mediate diverse biological functions through interaction with specific lipids. (Marchler-Bauer *et al*., 2011).

The Radical-induced Cell Death1 (TE3) protein is a key regulator of several ROS and abiotic stress-related responses and it interacts

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with several transcription factors (Jaspers *et al*., 2010). It displayed an enhanced cell death phenotype during incompatible interaction with the bacterial pathogen *Pseudomonas syringae* strain DC3000 (Overmyer *et al*. 2000a, b). Plants lacking this protein function display pleiotropic phenotypes including sensitivity to apoplastic ROS and salt, ultraviolet B and early flowering and senescence (Jaspers *et al*., 2010).

Avr9/Cf-9 rapidly elicited (TF10) genes are predicted to encode regulatory proteins, including protein kinases and transcription factors (Baulcombe, 1999). Many of the Avr9/Cf-9 rapidly elicited genes encode putative signaling components and thus may play pivotal roles in the initial development of the defense response (Rowland *et al*., 2005).

Wax2 (TE5) has a metabolic function associated with both cuticle membrane and wax synthesis (Chen *et al*., 2003). Waxes are major constituents of the cuticle and play important roles in plant defenses against bacterial and fungal pathogens (Jenks *et al*., 1994). Cutin and cuticular wax provide a physical barrier to pathogen ingress as the primary line of defense (Riederer, 2006).

Sesquiterpene synthases (TE8) are protein families which catalyze the conversion of sesquiterpene compounds in various plant species according to the demand and the environment (Kessler and Baldwin, 2001).

Induced sesquiterpenes can also form secondary chemical defenses (Bohlmann *et al*., 1998) and be involved in plant reproductive systems by protecting against any attacks of insects, bacteria and fungi (Dorothea *et al*., 2005). 12-15 copies of sesquiterpene synthase are found in the tobacco genome. It is also possible that these genes are regulated in response to the environment (Facchini and Chappell, 1992).

Our data support the hypothesis that DND phenotype in tobacco has some similarity especially in signaling process to more specific mechanism associated with induction of the hypersensitive reaction and it is distinct from general defense mechanisms.

Finally understanding the molecular basis of *R. solanacearum*-plant interactions by isolation and characterization of genes which are regulated in compatible or incompatible combinations could be useful for control of *R. solanacearum* by genetic engineering and breeding resistant cultivars. The bacterial wilt resistance is generally under polygenic control, while the control of resistance in *A. thaliana* is monogenic (Ishihara *et al*., 2012). Research shows that interfamily transfer of R genes can provide a new strategy to develop pathogen-resistant crops (Narusaka *et al*., 2013) and Quantitative resistance information could be used for generating markers for the breeding of resistant cultivars.

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آنالیز ترانسکریپتوم توتون در واکنش به آلودگی *Ralstonia solanacearum*

کبری مسلم‌خانی[1]، جواد مظفری[2*]، مسعود شمس‌بخش[3] و ابراهیم محمدی گل‌تپه[4]

۱- مؤسسه تحقیقات ثبت و گواهی بذر و نهال، سازمان تحقیقات، آموزش و ترویج کشاورزی، کرج، ایران.
۲- مؤسسه تحقیقات اصلاح و تهیه نهال و بذر، سازمان تحقیقات، آموزش و ترویج کشاورزی، کرج، ایران.
۳- گروه بیماری‌شناسی گیاهی، دانشکده کشاورزی، دانشگاه تربیت مدرس، تهران، ایران.
* پست الکترونیکی نویسنده مسئول مکاتبه: j.mozafari@yahoo.com
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چکیده: یکی از بهترین استراتژی‌ها برای کنترل بیماری پژمردگی باکتریایی با عامل *Ralstonia solanacearum* استفاده از ارقام مقاوم اصلاح شده است. کسب اطلاعات در زمینه بیان ژن‌های دفاعی باعث ایجاد نگرش جدید برای اصلاح مقاومت گیاه علیه پاتوژن‌ها می‌شود. تحقیق حاضر با بهره‌گیری از روش cDNA-AFLP به مطالعه ژن‌های القاء شده در واکنش دفاعی از نوع DND (Defense No Death) در گیاه توتون علیه *R. solanacearum* پرداخته است. در این روش با استفاده از پنج ترکیب پرایمری ۱۳۲۰ قطعه مشتق از رونوشت (TDF) مشاهده شد که از بین آن‌ها ۱۰۱ قطعه در زمان‌های صفر، ۲۴، ۴۸ و ۷۲ ساعت پس از آلوده‌سازی بیان متفاوت نشان دادند. بیش‌ترین تفاوت بیان ژن‌ها در ۴۸ ساعت پس از آلوده‌سازی مشخص شد. پس از توالی‌یابی، TDFهای توالی‌یابی شده بیش‌ترین شباهت را با ژن‌های دخیل در پیام‌رسانی، تنظیم و دفاعی داشته‌اند. القاء ژن‌ها در فنوتیپ DND در توتون خصوصاً در فرایند پیام‌رسانی شباهت با واکنش فوق حساسیت دارد و متفاوت از مکانیسم دفاعی عمومی در گیاه است.

واژگان کلیدی: توتون، *Ralstonia solanacearum*، ترانسکریپتوم و تعامل

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