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Signaling Pathways Mediating the Association between Sugarcane and Endophytic Diazotrophic Bacteria: A Genomic Approach

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Abstract

Sugarcane is a very important crop that grows associated with diazotrophic and plant hormone-producing endophytic bacteria, such as *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* and *H. rubrisubalbicans*. In this interaction, bacteria colonize the intercellular spaces and vascular tissues of most plant organs, promoting plant growth without inducing disease symptoms or nodule formation. Probably, plant genetic factors control the processes involved in plant colonization by these endophytes. The signaling pathways by which sugarcane plants can decipher bacterial signals and respond properly for a successful association are still not clearly understood. Here, we searched the sugarcane database for all expressed sequence tags (ESTs) preferentially or exclusively

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expressed in cDNA libraries constructed from sugarcane plants inoculated with *G. diazotrophicus* and *H. rubrisubalbicans*. Two such data sets of ESTs were generated in the infected libraries and ESTs from both data sets were functionally organized. For all categories, ESTs candidates to be involved in different processes of plant/bacteria signaling were identified, suggesting that the initial steps of colonization are actively controlled by the plant in the sugarcane/diazotrophic endophyte association.

Keywords: Sugarcane, diazotrophic endophytic bacteria, expressed sequence tag

1. Introduction

Sugarcane is an economically very important culture in Latin America, including Brazil. This country is one of the largest sugar producers, and is responsible for 30% (4.2 millions hectares) of the sugarcane culture in the world. In addition, the Brazilian alcohol program (Proalcohol) is an important alternative to fossil fuels, which makes the country to save approximately 260,000 barrels of petrol per day. Brazilian sugarcane varieties can grow with low addition of nitrogen fertilizers; nevertheless, this culture still consumes 240,000 ton of nitrogen per year, which represents high cost and also contributes to ground water pollution and atmospheric problems through release of NH_3 and NO_2 (Döbereiner et al., 1995).

Gluconacetobacter diazotrophicus (Döbereiner et al., 1988), *Herbaspirillum seropedicae* (Baldani et al., 1986) and *H. rubrisubalbicans* (Baldani et al., 1996) are diazotrophic and plant hormone-producing bacteria that live in an endophytic association with sugarcane. The intercellular spaces and vascular tissues of most plant organs are colonized by the bacteria, without the plant presenting any disease symptoms (Baldani et al., 1997; Reinhold-Hurek and Hurek, 1998). These diazotrophic and plant hormone-producing endophytes have been demonstrated to promote plant growth (Sevilla et al., 2001). Nevertheless, the mechanisms involved in this process have to be determined and very little is known about the role played by the plant in this association. Distinct sugarcane genotypes have different rates of Biological Nitrogen Fixation (BNF), suggesting that plant genetic factors might control the process of diazotrophic endophyte recognition, colonization and nitrogen fixation (Urquiaga et al., 1992). The understanding of this endophytic and benefic system of plant/bacteria association is essential to improve its efficiency in sugarcane, and eventually to extend it to other Graminea, such as wheat, maize and rice, which are very important food source.

Although bacteria can live inside plant tissues, the plant must keep the processes of bacterial invasion and proliferation under stringent control.

Therefore, the success of establishing an endophytic and non-pathogenic type of interaction depends on sophisticated detection and response systems that allow the plant to decipher bacteria signals and to induce the appropriate defense mechanisms and permission to bacterial invasion. The plant may perceive and respond to bacterial invasion by sending the proper signals, thus orchestrating a complex network of interactions. Data about the mechanism of signaling pathways that trigger sugarcane plant responses to be colonized by endophytic diazotrophic bacteria are not clearly understood yet. Several questions have to be addressed: which are the molecules that mediate signaling between plant and bacteria? How do sugarcane cells perceive the signals produced during the interaction and which are the physiological consequences for the plant?

Expression sequence tags (ESTs) databases have been shown to be useful tools to investigate gene expression patterns in response to changes in physiological conditions or during specific processes. Previously, we have investigated gene expression profiles of sugarcane plants colonized by diazotrophic endophytes by searching the sugarcane EST (SUCEST) database for the expression pattern of selected ESTs involved in nitrogen metabolism, plant growth, plant-microbe signaling, and early nodulin homologues as well (Nogueira et al., 2001). The data suggested that the plant may be actively involved in the interaction, and a general view of plant responses during the association was obtained.

Here, in order to precisely investigate the plant machinery implicated in signaling during the first stages of the association with the diazotrophic and plant hormone-producing endophytes, we searched the SUCEST database (http://sucest.lad.dcc.unicamp.br) for all ESTs preferentially or exclusively expressed in cDNA libraries constructed from sugarcane plants inoculated with *G. diazotrophicus* and *H. rubrisubalbicans*, designated AD1 and HR1, respectively. Two distinct data sets comprising ESTs preferentially or exclusively expressed in the AD1 and HR1 libraries (also named infected libraries) were generated and examined. All the ESTs from the two generated data sets were functionally organized based on SUCEST categorization. For all categories, we identified candidate genes to be involved in different processes of plant/bacteria signaling, which are exclusively or preferentially expressed in the infected cDNA libraries. The data suggest an active control of the initial steps of colonization by the plant in the sugarcane/diazotrophic endophyte association. This work represents a first step towards large scale genomic studies in the field.

2. Methods

The Sugarcane EST project (SUCEST) was carried out by the Brazilian ONSA consortium (Simpson and Perez, 1998) and generated one of the largest

databases of plant ESTs. SUCEST data were stored in a MySQL relational database as described (Telles et al., 2001). SQL queries were done to search for specific subsets of clusters among the total 46,031 clusters from 238,000 reads generated by the project. A complete description of the (SUCEST) database (http://sucest.lad.dcc.unicamp.br) can be found in Arruda (2001).

Although records from 37 distinct cDNA libraries were generated, only the 22 libraries with 4,500 or more sequenced records were considered in the analysis below: AM1, AM2, CL6, FL1, FL3, FL4, FL5, FL8, LB1, LB2, LR1, LV1, RT1, RT2, RT3, RZ2, RZ3, SB1, SD1, SD2, ST1 and ST3. They represent distinct tissues/organs of sugarcane plants growing under normal physiological conditions, whereas AD1 and HR1 represent the plants infected with *G. diazotrophicus* strain PAL5 or *H. rubrisubalbicans* strain HCC103, respectively. In total, 44,887 reads were used in our analysis, including 14,701 from AD1 and 9,729 from HR1. For a detailed description of each cDNA library, including number of reads sequenced and the tissue/organ that it represents, see Vettore et al. (2001). Briefly, AD1 and HR1 cDNA libraries were prepared with mRNA from the mixture of the tissues from root to shoot zone, stem and apical meristem of plantlets cultivated *in vitro*, inoculated with the endophytic bacteria. Prior to inoculation, plantlets were cultivated following a procedure kindly provided by COPERSUCAR (São Paulo, Brazil), that eliminates any kind of microorganism contamination. Cultures were maintained at 26±2°C under 16 h-light/8 h-dark photoperiod. Plant inoculations with the endophytes were performed as described by James et al. (1994). Tissues were harvested 7 days after inoculation with *G. diazotrophicus* strain PAL5 or *H. rubrisubalbicans* strain HCC103.

To determine the expression level of each EST in the different libraries, the values of absolute estimate of mRNA abundance – or the frequency estimates – in the cDNA libraries were calculated. The number of EST records (sequenced from the 5' end) corresponding to a given mRNA was divided by the total number of records (sequenced from the 5' end) in a given cDNA library.

Two EST data sets were generated with the following criteria. For an EST to be assigned as exclusively expressed in the infected cDNA libraries, it must have records only from the libraries AD1 and/or HR1, or at least two records from library AD1 only, or at least two records from library HR1 only. An EST was classified as preferentially expressed in the infected cDNA libraries when it was represented (i) at least twice in the AD1 and/or HR1 libraries and (ii) in a ratio 2 (frequency in AD1 or HR1 library/frequency in the second best represented library of non-infected tissues).

For quantitative analysis, all exclusively or preferentially expressed ESTs from AD1 or HR1 cDNA libraries were functionally organized based on SUCEST categorization, which follow the recommendations of the Munich Information Center for Protein Sequences (MIPS-GSF). The frequencies of ESTs

in each functional category were calculated by dividing the number of ESTs classified in each category by the total number of ESTs of the data set of genes, exclusively or preferentially expressed in the infected libraries. The frequencies of records were calculated dividing the total number of records of the ESTs classified in each category by the total number of records of the exclusive or preferential ESTs data sets. In the two EST data sets, the frequency of ESTs specifically found in monocotyledonous species, according to the SUCEST database criteria (Vincent et al., in preparation) was evaluated.

From the two data sets containing ESTs from all the different SUCEST categories, we have searched for ESTs possibly involved in some signaling pathways such as: ubiquitination, transcriptional control, stress/plant-microorganism interaction, sorting and cellular translocation, plant hormone and extracellular matrix.

3. Results and Discussion

The machinery used by the plant to perceive and respond to the invasion and colonization by benefic endophytic bacteria is still unexplained. In order to unravel this question, we investigated sugarcane gene expression during association with the diazotrophic and plant hormone-producer endophytes by using a genomic approach. Here, by searching the SUCEST database, we generated two EST data sets, based on the relative expression level of each EST (see Methods): ESTs (i) exclusively and (ii) preferentially expressed in the cDNA libraries of plants inoculated with *G. diazotrophicus* and *H. rubrisubalbicans*. These data sets represent an inventory of genes that are candidates to be differentially expressed during plant/bacteria association.

From the 44,887 sequenced ESTs of the SUCEST cDNA libraries used in our analysis, 450 ESTs were exclusively and 361 preferentially expressed in the infected libraries. From both EST data sets, 441 ESTs exclusively expressed and 357 ESTs preferentially expressed in the infected libraries were catalogued by their putative biological function into the SUCEST functional categories. Distribution of exclusively or preferentially expressed ESTs into the categories was compared with the distribution of the entire SUCEST database and significant changes were not found. Also, the frequencies of ESTs from AD1 and HR1 cDNA libraries in the different SUCEST categories were very similar.

Nevertheless, differences were observed between the distributions of the preferentially and exclusively expressed ESTs in the functional SUCEST categories. The data of some categories are shown in Fig. 1. The main difference was found in the "no hits" category, which is 2.6-fold more highly represented in the exclusively than in the preferentially expressed ESTs. The data suggest that the most expressed genes specific for the sugarcane/diazotrophic

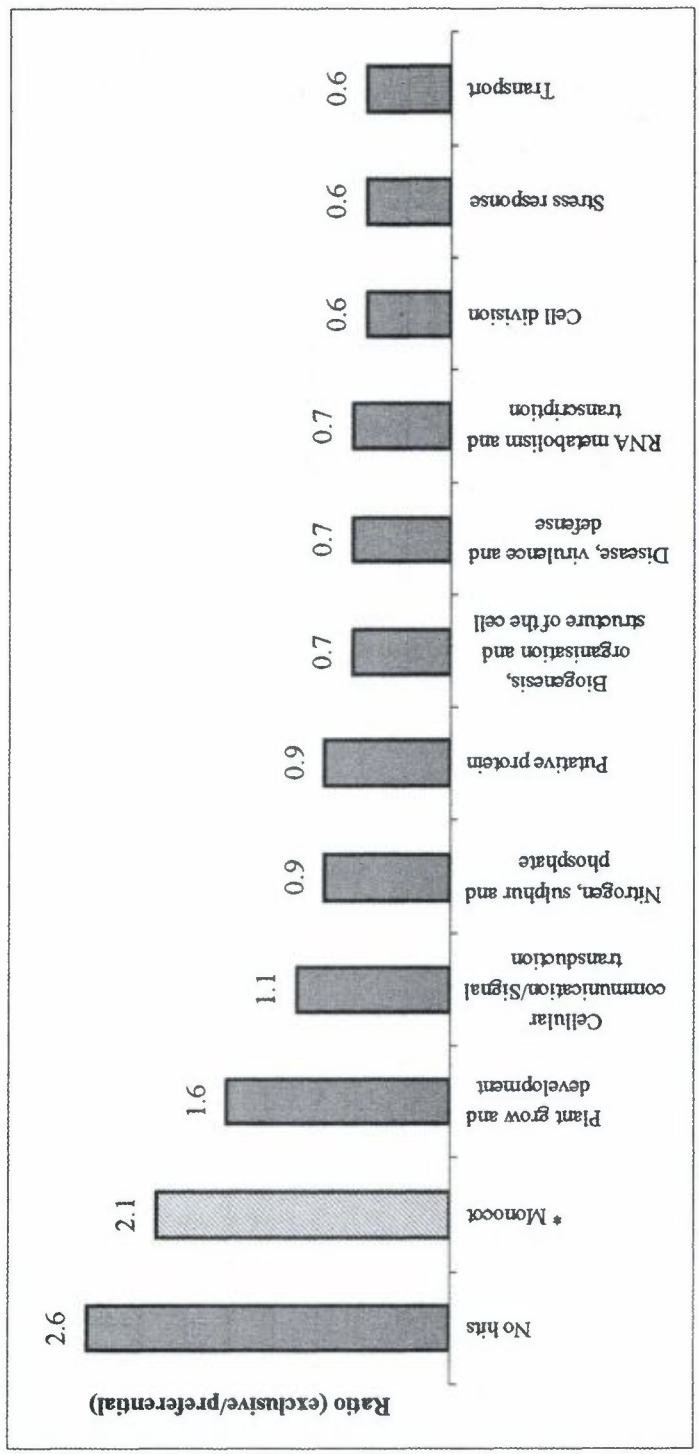

Figure 1. Ratio of the ESTs exclusively and preferentially expressed in the infected libraries distributed in different functional SUCEST categories. The ESTs exclusively or preferentially expressed in the infected libraries were classified according to their putative biological function in the SUCEST categories. For each functional category, the ratio was calculated by dividing the number of ESTs of the exclusive data set by the related numbers of the preferential data set. *Data relative to ESTs specific for monocotyledonous plants.

endophytes association can correspond to genes not described yet and of unknown function. These data support the idea that a large amount of genes and pathways related to this particular type of interaction might still be undiscovered. This assumption is also corroborated by quantification data in the two EST data sets of the frequencies of ESTs specific for monocotyledonous species, of which no clear homologues can be found in the dicotyledonous public databases. The monocot set derived from the infected libraries is larger than that of the corresponding collection found in the complete SUCEST database (data not shown). Interestingly, the monocot set derived from ESTs exclusively represented in AD1 and/or HR1 libraries is 2.1-fold higher than in the collection of ESTs that are preferentially expressed in the infected libraries (Fig. 1). This association between the diazotrophic and plant hormone-producing endophytes studied is largely distributed among gramineae species and, so far, they have been described in only a few dicotyledonous species (Jimenez-Salgado et al., 1997). Therefore, the data could imply that sugarcane and other monocot plants have developed special mechanisms to recognize and establish an efficient dialogue with the diazothrophic and plant hormone-producing endophytes studied.

Signaling in the association of sugarcane with G. diazotrophicus and H. rubrisubalbicans

Sugarcane plants are colonized by the diazotrophic endophytes throughout their life cycle. In the *in vitro* inoculation model used here, microorganism-free plants are inoculated with the diazotrophic endophytes and after 7 days they are fully colonized by the bacteria (James et al., 1994). Plant-bacteria signaling is probably the first response triggered by this system. Therefore, ESTs from all the SUCEST categories encoding proteins involved in some plant signaling pathways were extensively searched for in the data sets of exclusively and preferentially expressed ESTs. In all investigated categories, we could identify ESTs related to signaling that are candidates to be differentially expressed during the association. Some examples of ESTs related to signaling, exclusive or preferentially expressed in the infected libraries, are shown in Table 1.

Differences in EST representation were observed among the data sets of exclusively and preferentially expressed ESTs in AD1 and HR1 cDNA libraries. Nevertheless, we cannot conclude that the differences observed represent divergence among signaling processes involved in plant colonization by the two distinct endophytes. Since there are fluctuations in colonization patterns in different inoculation events (Vinagre et al., unpublished results), it is necessary to characterize in more detail the expression level of those genes during association.

Table 1. ESTs preferentially or exclusively expressed in AD1 and/or HR1 cDNA libraries, related to different plant signaling processes.

Gene	Pref AD1	Pref HR1	Pref AD1/HR1	Exc AD1	Exc HR1	Exc AD1/HR1
Extracellular matrix						
Aspartyl protease domain				1		
β-1,3-glucanase		1			1	
DUF 6 protein					1	
Hydroxyproline-rich glycoprotein					1	
Germin-like proteins		2			1	
Enoyl-CoA-hydratase	1					
Lipase	1					
WD domain			1			
Kinases			1	2	1	
Phospatase			2		1	2
Receptor-like proteins	3	6		5	7	
Plant hormone						
EIN2					1	
ACC oxidase						1
ETR1				1		
Gibberellin 7-oxidase				2	2	2
PIN1						1
AUX permease 1		3	1		1	
TIR1	1		1			
Ethylene response elong.factor EF-Ts prec.				1		
OPR1	1					
Indole-3-glycerol phosphate synthase		1				
Auxin responsive gene ARG7						1
Allene oxide synthase						1
Sorting and cellular translocation						
Actin				1		
Alpha-tubulin 1					1	
Katanin p80 subunit-like				1		
Nuclear movement protein	1					
Signal recognition particle					1	
Syntaxin-related protein				2		
Vesicle trafficking protein					1	
Dynamin-like protein ADL2		1				
Myosin-like proteins	1	1				
Endosomal protein			1			
ER lumen ptn retaining (HDEL) receptor			1			
Golgi associated protein wap41		1				
Golgi transport complex protein		1				
Vesicle-associated membrane protein						1

Table 1. Continued

Gene	Pref AD1	Pref HR1	Pref AD1/HR1	Exc AD1	Exc HR1	Exc AD1/HR1
Stress/plant-microorganism interaction						
3-hydroxy-3-methyglutaryl CoA synthase				1		
Glutathione reductase				1		
Quinone-oxidoreductase				1		
Peroxidase				2		1
Catalase		1		1		
ABC transporter	1	1				1
Immunophylin			1			1
Jacalin			1		1	
Cytochrome P450	1		1	1	1	1
Cellulose synthase			1			
Glycerol 3 phosphate dehydrogenase		1				
Cytochrome b5		1				
Malate dehydorgenase			1			
NADP-specific isocitrate dehydrogenase		1				
Blue copper-binding protein		1				
Beta-glucosidase		1				
Phosphoribosylanthranilate transferase			1			
Protochlorophyllide reductase						1
Anthranilate phosphoribosyltransferase			1			
Serine acethyltransferase			1			
Transcription control						
TGA3 transcription factor						1
Myb protein				4		1
Zinc finger protein	1	1	1	1	1	1
Homeodomain		1			1	1
AP2 domain		1	2	1	1	
CCR4-associated factor-like protein	1					
WRKY domain		1				
GRAS domain		1				
Ubiquitination						
UBP3				1		
DDI1-like, ubiquitin-like protein			1	1		
26S proteasome subunits		2		1		
RING domain proteins			1	1		1
Putative ubiquitin protein	1					

*Pref AD1 or Pref HR, ESTs which are preferentially represented in AD1 or HR1 library; Pref AD1/ HR, ESTs which are preferentially represented in AD1 and HR1 libraries; Exc AD1 or Exc HR, ESTs which are exclusively represented in AD1 or HR1 library; Exc AD1/ HR, ESTs which are exclusively represented in AD1 and HR1 libraries. **EIN2, ethylene insensitive 2; ETR1, ethylene receptor 1; ACC, 1-aminocyclopropane-1-carboxylic acid; TIR1, transport inhibitor response 1; OPR1, 12-oxophytodienoate reductase; ER, endoplasmic reticulum; UBP3, ubiquitin-specific protease 3.

Extracellular matrix

Plant bacteria colonization can rely on some interactions between the extracellular matrix (ECM) and the invading agent, and may be accompanied by signaling between the ECM and cytoplasm. Glycosylphosphatidylinositol (GPI) anchoring is probably the main modification in plants that is used to target specific proteins to the cell surface for ECM remodeling and signaling. Several plant GPI-anchored proteins (GAP) have already been identified (Borner et al., 2002). Arabinogalactan proteins, a family of extensively glycosylated hydroxyproline-rich glycoproteins that possibly function in cellular signaling at the cell surface (Showalter, 2001), were also identified as a GAP. In the data set of ESTs exclusively and preferentially expressed in the infected cDNA libraries, ESTs encoding putative GAPs – as predicted by GPI anchor and cleavage sites software (Buloz and Kronegg, 2001) – were found, such as: aspartyl protease, DUF6 protein, enoyl-CoA-hydratase, hydroxy-proline-rich glycoprotein, β-1,3-glucanase, lipase and WD protein.

In addition, germin-like protein homologues were exclusively and preferentially represented in the infected libraries. The variety of sequences and biochemical features of germins and germin-like proteins, which are glycoproteins characterized by a β-barrel core structure, indicate that they could be a class of receptors localized in the ECM and involved in physiological and developmental processes as well as pathogen and stress response (Membre et al., 2000).

Receptor-like proteins, kinases and phosphatases

The mechanisms by which plants perceive and respond to endogenous and exogenous stimuli may depend on receptor-like proteins, which are classified according to their structural domains (Baker et al., 1997). Some types of receptors were identified in the two EST data sets and they could have a function in the plant-endophytic bacteria interaction. Most of the receptors exclusively or preferentially expressed in the infected libraries are receptor-like kinases, whose role in the symbiotic signal transduction pathway has recently been described, from the perception of microbial signaling molecules to rapid symbiosis-related gene activation (Stracke et al., 2002; Vinagre et al., in preparation). In our analysis, an EST exclusively expressed in the infected libraries was found that codes for a cell wall-associated receptor kinase. This receptor has an amino-terminal domain that is tightly associated with the ECM and seems to mediate signals from the ECM to the events that are triggered by a pathogen infection (He et al., 1998).

Enzymes of the eukaryotic protein kinase superfamily catalyze the reversible transfer of the γ-phosphate from ATP to amino acid side chains of

proteins. Protein kinases function can be counteracted by the action of protein phosphatases. In plants, protein phosphorylation has been implicated in responses to many signals, including light, pathogen invasion, hormones, temperature stress, and nutrient deprivation. Activity of several plant metabolic and regulatory enzymes is also controlled by reversible phosphorylation (Stone and Walker, 1995). The involvement of phosphatases during plant-microbe interactions has already been demonstrated. Genes from the model legume *Lotus japonicus*, encoding a protein phosphatase type 2C, had enhanced expression specifically in the nodules. This gene may play a specific role in a signaling cascade at both early and late stages of nodule development (Kapranov et al., 1999). In our analysis, five ESTs encoding phosphatases and four ESTs encoding kinases exclusively or preferentially produced in the infected libraries were found and could possibly mediate signaling cascades in the studied association.

Plant hormones

A close relationship between hormone signaling elements and plant growth processes is well established. In addition, several plant/microbe signaling molecules have been described to respond to plant hormones (Mathesius et al., 2000). Among the ESTs preferentially or exclusively expressed in the infected libraries, 16 ESTs involved in plant hormone signaling, transport or biosynthetic pathways were found. Considering that inoculated plants have increased growth rate and development, several genes involved in growth hormones signaling, that are key regulators of plant cell division, expansion and elongation, are expected to be differentially expressed in the infected plants. Nevertheless, a role in mediating some of the signaling processes involved in the establishment of the association is not disregarded.

Genes involved in three plant hormones pathways – auxin, gibberellin and ethylene – were recognized. One EST, exclusively expressed in the infected library, was identified as gibberellin-7-oxidase, which belongs to the final steps of the gibberellin biosynthetic pathway (Lange, 1997). Some threshold level of endogenous auxin and gibberellin must be required for sugarcane growth and elongation following inoculation with diazothrophic bacteria.

In auxin pathways, an EST homologous to the indole-3-glycerol phosphate synthase was found that participates in the indole-3-acetic acid biosynthesis (Ouyang et al., 2000). Also, five ESTs encoding auxin 1 permease, an auxin influx carrier, were identified as exclusively or preferentially expressed in the infected libraries. A direct link between higher auxin 1 permease occurrence and production of auxin by the diazotrophic endophytes could be established because the bacteria studied here produce and release auxin in the growth

culture (Fuentes-RamÌrez et al., 1993). In addition, a homologue of the PIN auxin efflux carrier, which is essential for auxin distribution and pattern formation in plants (Reinhardt et al., 2000), is also exclusively represented in the infected libraries. Finally, two ESTs identified as transport inhibitor response 1 (TIR1) were preferentially and exclusively expressed in the infected libraries. TIR1 is the F-box protein of the SCF E3 ligase (SCFTIR1) of the ubiquitin-dependent proteolysis of auxin-negative regulators. The protein SCFTIR1 is a key complex in the auxin signaling pathway (Gray et al., 1999). One EST is homologue to the auxin responsive ARG7 gene that also takes part in the auxin signaling pathway (Gray et al., 1999).

Ethylene is a phytohormone that frequently acts as a signal in pathogen defense, wound response, and nodule formation and number (O'Donnell et al., 1996; Penninckx et al., 1996; Penmetsa and Cook, 1997). The ethylene synthesis might be regulated during the association as a homologue to 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene that catalyses the final step in ethylene biosynthesis (Zanetti et al., 2002) and is exclusively represented in the infected library. Two members of the ethylene response pathway (ETR1 and EIN2) were found to be present in the data sets of ESTs differentially expressed during the association, suggesting a possible role for this hormone in the sugarcane/diazotrophic endophytes association.

Sorting and translocation

The Golgi complex works as a processing place for newly synthesized glycoproteins and glycolipids derived from the endoplasmic reticulum and as a filtering network, separating protein and lipids destined for the plasma membrane from those to be retained in the endoplasmic reticulum (Mellman and Simons, 1992). The Golgi complex acts both on the secretory traffic and cellular growth control (Donaldson, 2000). Because movement in and out the Golgi complex requires a travel over significant distances, this complex interacts with motor and cytoskeleton proteins, including actin, myosin, tubulin, and katanin. A variety of signaling molecules are associated with the trans-Golgi network, including heterotrimeric G proteins and phosphoinositide lipids (Donaldson, 2000).

We found ESTs that encode sequences homologous to several components for protein targeting, translocation and sorting machinery, which are preferentially or exclusively represented in the infected libraries. Among them are endossomal protein, vesicle and Golgi-associated proteins, cytoskeleton proteins, and an endoplasmatic reticulum and a signal recognition particle receptors. Homologues of syntaxin, a soluble transmembrane N-ethylmaleimide-sensitive factor attachment protein receptor type-I,

located in the cytosolic face of the target membrane, play a crucial role for intracellular vesicle trafficking, fusion, and secretion (Edwardson, 1998) and are exclusively represented in the infected libraries. These results suggest that proteins of the vesicular transport pathways play an important role in regulating signal transduction events during the association of sugarcane with diazotrophic endophytes.

Transcription factors

Transcription factors are the final mediators of most signaling cascades that lead to specific protein/DNA interactions and modulation of gene expression. In plants, transcription factors are important regulatory switches that control many aspects of plant development and functioning (Shepard and Purugganan, 2002) and probably play important roles in different processes involved in the plant/diazotrophic endophytes association. ESTs coding for proteins containing domains of APETALA2 (AP2), homeobox, WRKY, zinc-finger, Myb DNA-binding and homologues of a GRAS, a TGA3 and a cinnamoyl-CoA reductase 4-associated factor 1 transcription factors as well, were found as preferentially or exclusively expressed in the infected libraries.

The most abundant transcription factors present in the infected libraries are homologues of AP2, Myb and zinc-finger genes. Signal transduction of the plant hormones jasmonate, abscisic acid, gibberellin as well as different developmental conditions and physiological adaptations might be controlled by transcription factors of the AP2 domain (Okamuro et al., 1997; Kizis et al., 2001; Memelink et al., 2001; Vahala et al., 2001). Functions of MYB proteins in plants include regulation of secondary metabolism, control of cellular morphogenesis and regulation of meristem formation, cell cycle or tolerance for various stresses (Jin and Martin, 1999; Jakoby et al., 2002). Several zinc-finger proteins are presumably involved in the regulation of pathogen defense, stress and development signaling (Jakoby et al., 2002). Therefore, these putative transcription factors may be involved in the plant-bacteria signaling, stress responses and the developmental processes occurring during the association with diazotrophic endophytic bacteria.

The putative transcription factor TGA3 is exclusively represented in the infected library. TGA3 is a member of the bZIP transcription factor family related to TGA1, which mediates root-specific and auxin-responsive expression of some genes (Miao et al., 1994). In addition, a direct link between salicylic acid-mediated signal transduction pathway inducing defense genes and the TGA3 transcription factor has recently been established in *Arabidopsis* (Zhou et al., 2000). These data could indicate that a systemic defense occurs during the association of sugarcane with the diazotrophic endophytes, together with the

developmental processes that take place in this interaction.

Several of the motifs present in the GRAS proteins indicate that they function as transcription factors. However, Richards et al. (2000) hypothesized that the GRAS proteins may be related to the protein family of signal transducers and activators of transcription, which are known as intracellular intermediaries between extracellular ligands and transcription and activation of genes. If the hypothesis was correct it would be an important aspect in the signal transduction systems occurring in the interaction between sugarcane and diazothrophic bacteria and would require particular efforts in future research.

Ubiquitination

A strong link between ubiquitination and signaling pathways in plants has recently been observed (Bachmair et al., 2001). Several important biological processes that have to be very tightly regulated, such as cell division, hormone response and circadian rhythm, are controlled through a proteolytic ubiquitin-dependent pathway (Callis and Vierstra, 2000). In this process, ubiquitin is covalently attached to certain target proteins to mark them for degradation by the proteasosome.

In the EST databases generated, several proteins involved in the ubiquitin-dependent proteolysis pathway were found as both exclusively or preferentially expressed in the infected libraries, among which one putative ubiquitin protein and two possible E3 ubiquitin protein ligase with a RING domain. E3 ubiquitin protein ligases specifies the target protein and all known E3 ligases have either a HECT or a RING finger domain. Proteins with RING domain have been shown to be sufficient to bind and mediate ubiquitination (Joazeiro and Weissman, 2000). As mentioned above, the ESTs homologous to TIR1 detected in the infected library belongs to the SCF^{TIR1}, an E3 ubiquitin ligase. Furthermore, three different subunits of the 26S proteosome were found in the preferentially expressed infected libraries. These results suggest that some genes of proteolytic pathways and the 26S proteosome are preferentially expressed during sugarcane-endophytic bacteria association, possibly regulating important processes as cell division, hormone response and other still unraveled pathways.

Stress and plant-microbe interactions

A large amount of genes involved in plant-microorganism interaction and abiotic stress responses share common signaling molecules, mainly jasmonates (Turner et al., 2002). The jasmonic acid (JA) signaling pathway involves perception of primary stress stimuli and signal transduction, inducing JA

biosynthesis. Following JA perception and the induction of the correspondent responses, JA signaling is integrated with outputs from the salicylic acid, ethylene, auxin, brassinosteroid and other signaling hormones. JA biosynthesis involves the induction of several genes for biosynthetic enzymes, including 12-oxophytodienoate-10, 11-reductase and allene oxide synthase. Homologues of these two genes are preferentially expressed in the infected libraries indicating the existence of a regulatory system for JA biosynthesis during the association. In addition, ESTs exclusively or preferentially expressed in the infected libraries that encode genes described as functioning in JA signaling responses were found in the two EST data sets, namely 12-oxophytodienoate reductase, ABC transporter, β-glucosidase, cellulose synthase, 3-hydroxy-3-methylglutaryl-coenzyme A synthase, cytochrome P450 (Schaller et al., 2000; Dammann et al., 1997; Xiong et al., 2001; Stotz et al., 2000; Ellis and Turner, 2002; Collu et al., 2001; Szekeres et al., 1996). Two ESTs homologous to members of the subgroup of the mannose-binding jacalin-related lectins were identified. Members of this gene family have been described as salt stress-induced and jasmonate-inducible proteins and as being important also for protein-carbohydrate interactions (Zhang et al., 2000; Geshi and Brandt, 1998). Furthermore, the role of plant lectins in determining host specificity in legume symbiosis has been largely discussed (Hirsch, 1999).

A critical aspect of nitrogen fixation for all diazothrophic bacteria is the fast and irreversible nitrogenase inactivation by O_2. N_2 fixation is an extremely energy-demanding process, which requires an additional supply from the aerobic respiration, and puts diazotrophic organisms at risk of inactiving nitrogenase (Dalton et al., 1998). Oxygen has been shown to regulate the *nif* gene expression of the symbiotic N_2 fixation process in *H. seropedicae* (Pedrosa et al., 2001). In plant defense responses, active oxygen species (AOS) are important and possible roles for AOS have been proposed, such as direct killing of pathogens, promotion of programmed cell death of host cells in the hypersensitive response, involvement in structural changes in the cell wall and induction of defense gene expression (Bolwell, 1999). The dynamic balance of AOS and antioxidant systems has been suggested to play a crucial role in determining how fast plants react and acclimate to changes in their environment (Mullineaux and Karpinski, 2002). The data sets of ESTs preferentially or exclusively expressed in the infected libraries contain ESTs encoding for proteins involved in generating AOS systems, e.g. peroxidases and glycolate oxidase (Bolwell, 1999; Noctor et al., 2002), antioxidant/scavengers of H_2O_2, such as catalase, peroxidase, glutathione reductase and quinone-oxidoreductase (Dalton et al., 1998; Thornalley, 2002). NADPH-regenerating systems are considered antioxidants because enhanced levels of their enzymes confer more resistance against H_2O_2/oxidative stress (Costenoble et al., 2000; Minard and McAlister-Henn, 2001). ESTs coding for

enzymes of the NADPH-regenerating system, such as glycerol 3 phosphate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase and NADP-specific isocitrate dehydrogenase, were identified in the data sets of ESTs preferentially or exclusively expressed in the infected libraries. In addition, an EST encoding protochlorophyllide reductase, an enzyme that protects plants from photooxidation that generates AOS (Sperling et al., 1997), is preferentially expressed in the infected libraries. Other genes related to the H_2O_2/redox process and involved in plant defense are represented in the data sets of ESTs preferentially or exclusively expressed in the infected libraries, such as blue copper proteins, phosphoribosylantranilate transferase, serine acetyltransferase (Nersissian et al., 1998; Conklin and Last, 1995; Blaszczyk et al., 1999; Bolwell, 1999). Finally, an EST encoding cytochrome b5, described as a candidate oxygen sensor and possibly involved in signaling of oxygen tension (Zhu et al., 2002), is preferentially expressed in the infected libraries.

When cells are exposed to high temperatures or other environmental stresses, heat shock proteins belonging to several gene families are induced. Many heat shock proteins function as chaperones and play important roles in normal growth and stress tolerance. Immnunophilins with PPIase activity catalyze slow steps in the folding of some proteins, of which an FKBP-type immunophilin accumulates in plants during stress conditions (Kurek et al., 1999). Furthermore, Nuc et al. (2001) demonstrated that cyclophilin expression is dramatically increased in root nodules during a symbiotic interaction. Clusters representing these two types of immunophilins were identified as exclusively/preferentially expressed in the infected libraries.

4. Perspectives

The computer-based analysis of ESTs exclusively or preferentially expressed in the infected libraries indicate that a great number of genes, related to distinct plant signaling pathways, are candidates to establish the sugarcane interaction with diazotrophic and plant hormone-producing endophytic bacteria. Nevertheless, as in most technologies of high-throughput gene expression analysis, e.g. microarray, we cannot infer that all the genes identified using our approach are actually regulated during the association without validating the data by using a more sensitive approach (Goto et al., 2002; Negishi et al., 2002). In general, computer-based EST expression profile data are 60–90% correlated with reverse transcription polymerase chain reaction (RT-PCR) or Northern blot results (Hwang et al., 2000; Milla et al., 2002). We are currently investigating the differential expression of selected genes from the two data sets by RT-PCR analysis to confirm the reliability of such genomic approach to study gene expression. The gene expression patterns

observed so far confirmed the differential expression found in our computer-based analysis (Vargas et al., in preparation; Vinagre et al., in preparation).

Our results suggest that an elaborated system of plant/microorganism signaling pathways is triggered when sugarcane plants are invaded and colonized by endophytic and benefic bacteria. Several of the identified genes are related to plant defense responses. Despite the benefits offered to the plant by these bacteria and the non-pathogenic aspect of the association, it is reasonable to expect that sugarcane developed recognition mechanisms to control endophytic colonization and to avoid pathogenicity, which led to defense against endophytes. Furthermore, genes preferentially or exclusively expressed in the infected libraries also exhibit a nodule-related expression in the plant association with *Rhizobium* species. In spite of the structural contrast between these two types of association, similar signaling mechanisms might act in the establishment of these two benefic categories of association. Yet, the interpretation of the results remains speculative until the function of specific genes has been established by further experiments.

Besides the functionally categorized ESTs identified, a large percentage of the ESTs exclusively expressed in the infected libraries is also represented by monocot-specific genes and genes of unknown function, suggesting that novel mechanisms of plant/microorganism signaling may still be discovered in studies on sugarcane association with diazotrophic and plant hormone-producing endophytic bacteria.

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