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Differential genes of *Arabidopsis thaliana* in response to *Ustilago maydis* infection

Genes diferenciales de *Arabidopsis thaliana* en respuesta a la infección por *Ustilago maydis*

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Abstract

Arabidopsis thaliana - *Ustilago maydis* integrate the pathosystem used to study the plant-pathogen interaction, in order to know the molecular mechanisms involved in the response of the plant to the pathogenesis of *U. maydis*, a differential expression bank was constructed 72 hours after inoculation, using the subtractive hybridization technique. The fragments obtained were sequenced and subjected to bioinformatic analysis which allowed us to locate 36 different sequences with homology to *Arabidopsis thaliana* in response to *U. maydis* infection, several of them with roles in photosynthesis, reactive oxygen species, defense, and signaling among others, involved either directly or indirectly in the early response to infection. The results of this work are focused on understanding the plant-pathogen interaction and can be extrapolated to other model plants of agronomic importance.

Pathosystem, Defense, *Arabidopsis*

Resumen

Arabidopsis thaliana - *Ustilago maydis* integran el patosistema utilizado para el estudio de la interacción planta-patógeno, con la finalidad de conocer los mecanismos moleculares implicados en la respuesta de la planta a la patogénesis de *U. maydis* se construyó un banco de expresión diferencial a las 72 horas posteriores a la inoculación, mediante la técnica de hibridación sustractiva. Los fragmentos obtenidos fueron secuenciados y sometidos a un análisis bioinformático el cual permitió localizar 36 secuencias distintas con homología a *Arabidopsis thaliana* en respuesta a la infección por *U. maydis*, varios de ellos con roles de fotosíntesis, especies reactivas de oxígeno, defensa, y señalización entre otros, involucrados ya sea de manera directa o indirecta en la respuesta temprana a la infección. Los resultados de este trabajo están centrados en entender la interacción planta patógeno y pueden ser extrapolados a otras plantas modelo de importancia agronómica.

Patosistema, Defensa, *Arabidopsis*

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Introduction

In recent years *Ustilago maydis* has been a fungal model widely used in biological and genetic studies (Bolker, 2001), it requires a host to complete its sexual cycle, and is pathogenic in maize (*Zea mays* L.), its natural host causing the disease known as common bunt or "huitlacoche" (Bolker, 2001; Kahmann and Kämper, 2004).

In studies conducted by León-Ramírez et al. in 2004, the possibility of using alternative hosts to study *U. maydis* infection was raised. The *Ustilago maydis-Arabidopsis thaliana* non-natural pathosystem proved to be a useful model for the study of plant-pathogen interaction and in the analysis of virulence factors of Ustilaginales (Méndez-Morán et al., 2005), where results were achieved with a haploid strain that facilitates the analysis of the fungus in this system.

Currently, most of the work on the arabidopsis-ustilago pathosystem has focused on characterising the response genes of the fungus (Aleman-Duarte, 2009; Martínez-Soto et al, 2013) and not on the plant side, with the aim of understanding the molecular mechanisms involved in the interaction between *U. maydis* and *A. thaliana*, in the present work, a differential cDNA expression bank was constructed to analyse *A. thaliana* genes involved in the early infection of *U. maydis*, considering a time of 72 h after inoculation (hpi) of the fungus in the plant. Gene fragments obtained from an arabidopsis subtractive library were subjected to bioinformatic analysis to determine the identity of the differentially obtained sequences, with the perspective that the results obtained in this work can be extrapolated to other plant-fungus models of agronomic importance.

Materials and methods

Plant growth, fungi and growing conditions

The wild variety of *Arabidopsis thaliana Landsberg erecta* (Ler.) was used as host for *Ustilago maydis*. Seeds of *A. thaliana* were disinfected with 70 % ethyl alcohol for 5 minutes, then transferred to sodium hypochlorite solution (20 %) for 10 minutes, and washed 3 times with sterile distilled water. Seeds were sown on solid Murashigne and Skoog (MS) culture medium (0.8% v/v) and incubated at controlled temperature conditions (25°C), with a photoperiod of 16 h light and 8 h dark.

The wild-type *U. maydis* strain (*a1b1*) was grown in vitro in liquid MC medium (Hollyday, 1974) at 28°C under constant agitation for 18-24 h to obtain a concentration of $1x10^8$ sporidia/µL, cells were recovered by centrifugation and resuspended in sterile distilled water.

Inoculation

Seedlings 4 days post germination (dpg) were inoculated by placing 0.5 µL ($5X10^4$ sporidia/µL) of the $1x10^8$ sporidia/µL suspension of *U. maydis* (grown in vitro) on the epidermis of the first two true leaves. Then they were placed in growth chambers at 25°C with a photoperiod of 16 h light and 8 h dark, after 72 hpi these infected plants were collected and considered within the experiment as the problem material, and as controls 7-day-old uninoculated *A. thaliana* seedlings and the haploid culture of *U. maydis* grown in vitro were used. The material was collected in liquid nitrogen and macerated in cold mortar.

Molecular assays

RNA isolation

RNA was extracted according to the method of Sambrook et al., 2012 and in addition the PureLink Micro-to-midi Total RNA extraction protocol PureLink Micro-to-midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) was used. All samples were analysed by gel electrophoresis for integrity.

RT

From the total RNA, 1 µL of Oligo dT, 1 µL of the dNTPs mixture was added and made up to a final volume of 12 µL with sterile distilled water. It was left at 65°C for 5 min. Then 4 µL of 5x buffer, 2 µL of 0.1 M DTT and 1 µL of Superscript II reverse transcriptase enzyme (Invitrogen, Carlsbad, CA, USA) were added. Incubation was left for 50 min at 42°C and then 15 min at 70°C.

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Polymerase chain reaction (PCR)

Approximately 2 ng cDNA or 2 ng plasmid DNA were used. The amount of primers was 10 µM each, the mixture of DNTP's was 10 mM and 3 mM $MgCl_2$ in a volume of 50 µL, 5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and its 10x reaction buffer were added to the mixture, The amplification programme was as follows: initial denaturation at 94°C, for 3 min, followed by 30 cycles with denaturation at 94°C for 45 sec, alignment at temperature close to the Tm of the primers for 30 sec and polymerisation at 72°C for a time that complies with the following rule: for every 1000 bp, 1 min is required.

Subtractive hybridization

Differential banks were constructed using the "PCR-Select™ cDNA Subtraction Kit" technique (Clontech Mountain View, CA, USA) according to the supplier's specifications. The cDNA of the test sample (72 hpi seedlings), and the control cDNA (uninfected seedlings and *U. maydis* grown in vitro) were used. The test samples were separated into two populations. To one population the first adaptor (Ad1) was ligated and to the other population the second adaptor (Ad2R) was ligated. No adapters were added to the control cDNA. The Ad1 and Ad2R populations were hybridised separately to the control cDNA. The hybridisation products were then mixed and two PCR amplifications were performed to obtain only the amplification of those double-stranded cDNAs (hybrids) with different adaptors, which amplified exponentially. The products of this PCR reaction were inserted into a T/A cloning vector (pDrive, in *E. coli* cells), with ampicillin and kanamycin resistance.

Cloning and transformation of subtracted PCR products

The TOP10 F strain (Invitrogen, Carlsbad, CA, USA) was used with the conditions described by Sambrook et al. (1989), then for transformation 2 µL of the transforming DNA was added to 100 µL of TOP10 F competent cells, left on ice for 10 min, heat shocked for 2 min at 42 ºC and left on ice for 5 min, then 900 µL of LB medium was added and incubated for 1 h at 37 ºC under agitation.

Finally, the mixture obtained was spread on LB medium plates spiked with the appropriate antibiotic. The extraction of plasmid DNA from *E. coli* was performed according to the protocols described by Sambrook et al. (1989) using the alkaline lysis of Birboim and Doly (1979). When higher purity plasmid DNA was required for use in DNA sequencing, it was obtained using anion exchange columns (Qiagen, Hilden GER), according to the supplier's instructions.

Sequencing and bioinformatics analysis

Sequencing was carried out at the Centro de Investigaciones y Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Unidad IRAPUATO with an "ABI PRISM 377 DNA sequencer from Perkin Elmer" to obtain the nucleotide sequence and perform bioinformatic analysis of the sequences. The sequences obtained were analysed with the "Basic Local Alignment Search Tool" (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/) algorithm developed by the National Center for Biotechnology Information (NCBI) of the US government (Altschul et. al., 997) using the BLAST'n tools (compare a nucleotide sequence against a database containing also nucleotide sequences). The database for *Arabidopsis thaliana*: https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=3701 was used to search the sequences.

Results and discussion

Selection of positive clones from subtractive hybridization

Of these, only 181 positive clones (white) were recovered and 105 negative clones (blue) were discarded), Once the 181 positive clones were isolated, the presence of the plasmid was verified, the clones that did not present visible bands were discarded, from the 181 positive clones obtained in the subtractive library, 144 clones were selected to be sequenced, 6 of these were discarded because they did not present a sequence that could be analysed, therefore 138 sequences were submitted to bioinformatic analysis.

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Once the sequences were obtained, the adapters were searched for (TCGAGCGGCCGCCCGGGGGCAGGT [sense]; ACCTGCCCGGGGGCGGCCGCTCGA [complementary antisense] on strand 3' or AGCGTGGTCGCGGGGCCGAGGT [antisense] ACCTCGGCCGCGACCACGCT [complementary sense] on strand 5)' flanking the cloned fragments, so that the sequence belonging only to the fragment was subjected to bioinformatics analysis. The 138 clones were subjected to alignment analysis with the BLAST tool in the nucleotide mode (BLAST'n) considering the alignment significant when the error (E) was less than 1e-3. Figure 1 shows the distribution of the 106 sequences, with homology to *Arabidopsis thaliana* (76.8 %) representing 36 different genes, 22 sequences with homology to *Ustilago maydis* (15.9 %) coding for 19 different genes, and 10 more with no significant alignment (7.2 %), and 10 sequences with homology to Arabidopsis thaliana (76.8 %) representing 36 different genes.

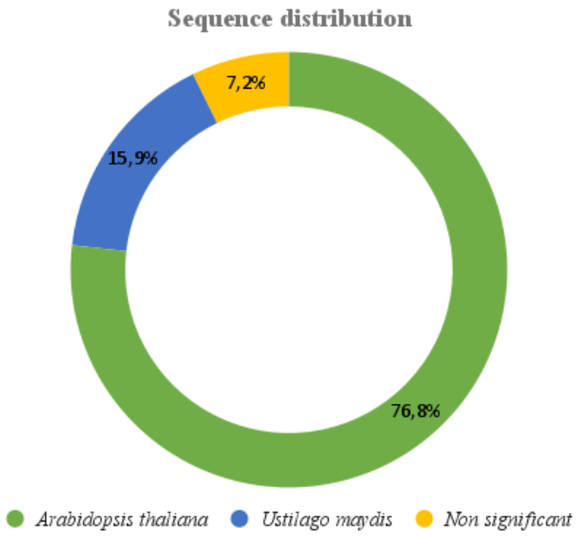

Figure 1 Sequence distribution. The number of sequences with homology to *Arabidopsis thaliana* is shown in green, sequences with identity to *Ustilago maydis* in blue and sequences with no significant alignment in yellow

Sequences with homology to Arabidopsis thaliana

Of the sequences with homology to *Arabidopsis*, 36 different sequences were obtained, which are presented in table 1 with the description at transcript level obtained from the NCBI databases, and in table 2 the annotation according to the Gene Ontology (GO) databases, which provides information on the functionality of the genes.

ID	# pb	Identity	ID Locus	Access No. NCBI	Annotation (Blast Result)
1	293	99 %	AT4G02520	NM_116486.3	Glutathione S-transferase PHI 2 (GSTF2)
2	520	100%	QBI37805.1	MK353213.1	Isolate 180404IB4 chloroplast, complete genome
3	495	99 %	AT5G60620	NM_125455.4	Glycerol-3-phosphate acyltransferase 9 (GPAT9)
4	210	100 %	36335705	NC_037304.1	Ecotype Col-0 mitochondrion, complete genome
5	490	99%	AT4G14030	NM_117478.6	Selenium-binding protein 1 (SBP1)
6	208	97%	AT3G21610	NM_113056.3	Acid phosphatase/vanadium-dependent haloperoxidase-related protein
7	196	98%	AT5G01530	NM_120231.4	Light harvesting complex photosystem II (LHCB4.1)
8	328	100%	AT2G39730	NM_179989.3	Rubisco activase (RCA)
10	689	99 %	AT1G76080	NM_106257.3	Chloroplastic drought-induced stress protein of 32 kD (CDSP32)
11	450	97 %	AT1G67090	NM_105379.4	Ribulose bisphosphate carboxylase small chain 1A (RBCS1A)
12	628	98 %	AT1G27450	NM_102509.4	Adenine phosphoribosyl transferase 1 (APT1)
13	370	99 %	AT1G79040	NM_106555.4	Photosystem II subunit R (PSBR)
14	792	99 %	AT2G25110	NM_128068.3	Stromal cell-derived factor 2-like protein precursor (SDF2)
15	81	100 %	AT4G28750	NM_119019.4	Photosystem I reaction centre subunit IV / PsaE protein (PSAE-1)
16	272	100 %	AT3G61470	NM_116012.5	Photosystem I light harvesting complex protein (LHCA2)
17	212	99 %	AT1G20620	NM_001332452.1	Catalase 3 (CAT3)
18	347	99 %	AT1G55670	NM_104443.2	Photosystem I subunit G (PSAG)
19	244	100 %	AT5G66570	NM_126055.4	PS II oxygen-evolving complex 1 (PSBO1)
20	180	82 %	9316370	XM_002880257.2	PREDICTED: Arabidopsis lyrata subsp. lyrata signal recognition particle
21	155	95 %	9299655	XM_002863533.2	PREDICTED: Arabidopsis lyrata subsp. lyrata berberine bridge enzyme-like 28
22	304	98 %	AT2G34420	NM_128994.3	Photosystem II light harvesting complex protein B1B2 (LHB1B2)
23	387	98 %	AT2G45470	NM_130109.3	FASCICLIN-like arabinogalactan protein 8 (FLA8)
24	154	100 %	AT2G45470	NM_001342565.1	Histone superfamily protein (AT4G40040)
25	135	99 %	AT5G40450	NM_001344333.1	A-kinase anchor-like protein (AT5G40450)
26	217	99 %	AT4G22690	NM_118395.3	Cytochrome P450, family 706, subfamily A, polypeptide 1 (CYP706A1)
27	145	99 %	AT1G28290	NM_102594.3	Arabinogalactan protein 31 (AGP31)
28	129	96 %	AT2G41840	NM_129748.4	Ribosomal protein S5 family protein (AT2G41840)
29	190	99 %	AT2G34430	NM_128995.3	Light-harvesting chlorophyll-protein complex II subunit B1 (LHB1B1)
30	293	93 %	AT1G78630	NM_106510.3	Ribosomal protein L13 family protein (emb1473)
31	185	98 %	AT4G34150	NM_119578.4	Calcium-dependent lipid-binding (CaLB domain) family protein (AT4G34150)
32	232	97 %	AT2G41110	NM_180013.3	Calmodulin 2 (CAM2)
33	244	100 %	AT3G50820	NM_114942.3	Photosystem II subunit O-2 (PSBO2)
34	102	100 %	At1g61670	AK117715.1	Unknown protein, complete cds
35	195	99 %	AT1G07720	NM_001331724.1	3-ketoacyl-CoA synthase 3 (KCS3)
36	400	99 %	Q5HZ38	AM489730.1	SnRK1-activating protein kinase-1 (SnAK1 gene)

Table 1 *Arabidopsis thaliana* genes obtained from subtractive hybridization

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Locus ID TAIR	Cell function annotated in Gene Ontology
AT4G02520	Cadmium ion response, cold response, oomycete response, zinc ion response, toxin catabolic process
AT5G60620	Diacylglycerol biosynthetic process, triglyceride biosynthetic process
AT4G14030	Cellular response to selenium ion, response to cadmium ion, response to hydrogen peroxide, sulphate assimilation, protein binding, selenium binding
AT3G21610	Biological processes
AT5G01530	Photosynthesis, light harvesting in photosystem I, response to light stimulus
AT2G39730	Leaf senescence, response to cold, response to jasmonic acid, response to light stimulus, ADP binding, ATP binding, ATP hydrolysis activity, enzyme regulatory activity, mrna binding, ribulose-1,5-bisphosphate carboxylase/oxygenase activating activity
AT1G76080	Bacterial defence response, heat acclimation, cellular redox homeostasis, response to oxidative stress, response to water deprivation, protein binding
AT1G67090	Photosynthesis, cold response, ribulose bisphosphate carboxylase complex assembly, copper ion binding, mrna binding, protein binding, salicylic acid binding
AT1G27450	Adenine rescue, circadian rhythm, cytokinin metabolic process, AMP rescue, adenine rescue, cytokinin metabolic process
AT1G79040	Assembly of photosystem II oxygen-evolving complex, mrna binding, protein binding
AT2G25110	Bacterial defence response, fungal defence response, pattern recognition receptor signalling pathway
AT4G28750	Process of biosynthesis of aromatic compounds, process of biosynthesis of cellular nitrogen compounds, process of biosynthesis of heterocycles, process of biosynthesis of cyclic organic compounds, response to light stimulus, response to temperature stimulus
AT3G61470	Photosynthesis, light harvesting in photosystem I, response to cold, response to high light intensity, response to light stimulus, response to low light intensity stimulus
AT1G20620	Cellular response to nitrogen starvation, cellular response to phosphate starvation, cellular response to sulphate starvation, catabolic process of hydrogen peroxide, peptidyl-cysteine S-trans-nitrosylation, response to cold, response to light stimulus, catabolic process of hydrogen peroxide, response to hydrogen peroxide, response to oxidative stress
AT1G55670	Photosynthesis, photosynthetic NADP+ reduction, photosynthetic electron transport in photosystem I, stabilisation of photosystem I, stabilisation of proteins
AT5G66570	Photoinhibition, photosynthesis, reaction to light, assembly of photosystem II, stabilisation of photosystem II, regulation of protein dephosphorylation
AT2G34420	Photosynthesis, light harvesting in photosystem II
AT2G45470.1	Meiotic cell cycle process, oxoacid metabolic process, lipid response, tissue development
AT5G40450	Organisation of lytic vacuoles molecular_function
AT4G22690	Defence response to bacteria, defence response to fungi, hormone-mediated signalling pathway, oxo acid metabolic process, regulation of defence response, response to alcohol, response to lipids, response to oxidative stress, response to water deprivation, secondary metabolic process
AT1G28290	Jasmonic acid response, rhamnogalacturonan biosynthetic process II
AT2G41840	Translation
AT2G34430	Photosynthesis, photosynthesis, light uptake in photosystem II
AT1G78630	Negative regulation of translation, translation
AT4G34150	Cellular response to hypoxia, response to cold
AT2G41110	Pollen germination, protein catabolic processing, calcium ion binding, enzyme regulatory activity, protein binding
AT3G50820	Photoinhibition, photosynthesis, reaction to light, assembly of photosystem II, stabilisation of photosystem II, regulation of protein dephosphorylation
AT1G07720	Response to cold, response to light stimulus, fatty acid biosynthetic process

Table 2 Functionality of *Arabidopsis thaliana* genes

Subtractive hybridisation was performed at 72 hpi, which is considered an early response to infection by *U. maydis* infection, we can observe that there is an induction of photosynthesis genes (photosystems I and II); some genes related to response to pathogens such as bacteria and fungi; other genes related to oxidative stress; some genes involved in signal transduction; Calvin cycle genes and several other genes related to both anabolic and catabolic metabolism, transport, biological processes, protein binding, histones, and protein of unknown function.

The arabidopsis pathosystem has been described by Mendez-Morán and collaborators in 2005, additionally, in a previous work this same pathosystem was analysed under a microarray scheme finding a set of differential genes (unpublished data), from these results it was possible to characterise the *PLA2A* gene in response to ustilago infection (Casarrubias-Castillo et al., 2021).

In plants, defensive responses are costly and therefore require internal adjustment, which may be related to reductions in growth, reproduction or storage, as well as increases in carbon assimilation to cover metabolic demands (Schwachtje and Baldwin, 2008), which can be reflected in the induction of genes related to the photosynthetic process in both photosystem I and photosystem II, and clearly the chloroplast is highly active and the induction of these genes is represented in the results (table 1). The chloroplast also provides biosynthetic functions to generate defence hormones such as salicylic acid (SA) and jasmonic acid (JA), and the reactive oxygen species (ROS) response, and this organelle is an important plant defence mediator in maize plants in response to ustilage infection (Kretschmer et al., 2017 and literature cited therein).

Increases in photosynthesis can lead to changes in the source-consumer relationship as a result of the increased demand for energy and carbon sources required for the production of defensive compounds (Schwachtje and Baldwing, 2008).

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As a result, the induction of genes related to signal transduction such as calmodulin (CAM2), protein kinases (SnAK1 and A-kinase anchor-like protein), a regulator of defence responses (CYP706A1) and a PRR receptor (SDF2), indicating that there is an initial response to the attack, and that there are genes induced both in response to JA and SA according to the functionality of several genes (table 2) and that both, in many cases, are antagonistic to each other, while ethylene signalling (ET) can be synergistic with JA signalling, both associated with resistance to necrotrophic pathogens (Pieterse et al., 2012). *U. maydis* possesses a Jsi1 effector, which triggers the response of genes related to ethylene response, probably attenuating the SA pathway that is effective against biotrophic pathogens (Darino et al., 2021).

Some JA response genes were also obtained such as the gene encoding for Arabinogalactone protein 31 (AGP31) and FASCICLIN-like arabinogalactan protein 8 (FLA8), both with a possible role in the response to pathogenic infection (Wu et al., 2020), and genes involved in the detoxification of reactive oxygen species such as Glutathione-S-transferase (GSTF2) and catalase 3 (CAT3) were also observed.

Conclusions

The subtractive hybridisation technique besides helping us to identify low abundance genes, allowed us to identify genes involved in several processes such as: photosynthesis, reactive oxygen species, defence, and signalling among others, and may be involved either directly or indirectly in the early response to infection, coupled with these results and other data confirmed by microarrays (unpublished data) what happens in this *Arabidopsis thaliana-Ustilago maydis* pathosystem occurs the same as in maize plants, the pathogen in its haploid stage keeps the plant alive and the fungus lives at the expense of its energy, reducing the size, maintaining the tumours and increasing the anthocyanin content (Méndez-Morán et al. , 2005). The results obtained in this work can be extrapolated to other plant-fungus models of agronomic importance and to understand the plant-pathogen interaction.

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