BIOLOGIA PLANTARUM XX (X): XXX-XXX, 20XX

BRIEF COMMUNICATION

Early transcription of defence-related genes in *Platanus* × *acerifolia* leaves following treatment with cerato-platanin

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Abstract

The protein elicitor cerato-platanin (CP) is known to induce defence-related responses in various plants. Some of these responses occur very quickly. In the present work, transcriptional changes caused by CP in leaves from *Platanus* × *acerifolia* (Aiton) Willd. were studied. With a cDNA microarray, 131 differentially regulated transcripts were identified as responsive to CP after 24 h of treatment. Eighty-six of these were cold- or ozone-modulated transcripts, thus revealing a significant overlap between genes responsive to CP and to cold/ozone stress. The transcriptional changes caused by CP were compared with the CP-orthologous protein Pop1 in a time-course analysis performed after 3, 6, 12, and 24 h of treatment by real-time RT-PCR on five defence-related genes. Despite some differences, CP and Pop1 were both able to induce early transcriptional changes (*WRKY* was overexpressed after only 3 h) confirming that pathogen-associated molecular patterns (PAMPs) act very quickly on gene transcription.

Additional key words: cerato-populin, cold stress, ozone, plane tree, poplar, RT-PCR.

Plants can defend themselves from potential pathogenic microorganisms by two-level defence system. At a first level, plants recognise microbial molecules evolutionarily stable which are commonly known as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs); at a second level, if the pathogen overcomes the first layer of plant defences, a specific interaction between plant resistance proteins and pathogen effectors occurs (Jones and Dangl 2006). Few PAMPs have been described in detail until now, and most of these are microbial cell wall components. In fungi, an extensively studied example is represented by chitin (Schwessinger and Zipfel 2008). Perception of PAMPs by plant receptors causes very rapid responses. Among these, a substantial transcriptional reprogramming occurs (Anderson *et al.* 2010). Cerato-platanin (CP) is a small non-catalytic protein for which the current understanding of the experimental evidences suggests that it is a PAMP.

Cerato-platanin (CP) is produced by the ascomycete *Ceratocystis platani* responsible for canker stain disease of plane trees, and belongs to the family, which takes its name from this protein, the "cerato-platanin family" (Pfam PF07249) (Pazzagli *et al.* 1999). CP is located in the cell wall of *C. platani* and is also abundantly excreted outside the fungal cells (Boddi *et al.* 2004, Scala *et al.* 2004, Bernardi *et al.* 2011). The structure of the protein and the pattern of its gene regulation suggest that the primary function of CP in the fungal life may be similar to that of expansins (De Oliveira *et al.* 2011, Baccelli *et al.* 2012).

When applied on host or non-host plants, CP elicits defence-related responses; in *Platanus* × *acerifolia* leaves it causes production of hydrogen peroxide, nitric oxide, phytoalexins, and phenolic compounds, causes over-expression of defence-related genes, localised resistance, and cell plasmolysis as a result of programmed cell death (Pazzagli *et al.* 1999, Scala *et al.* 2004, Bennici *et al.* 2005, Lombardi *et al.* 2010). Content of phytoalexins begin to rise between 12 and 24 h after treatment with

Received 28 February 2012, *accepted* 2 November 2012.
Abbreviations: CP - cerato-platanin; PAMP - pathogen-associated molecular pattern; Pop1 - cerato-populin; RT-PCR - reverse transcriptase - polymerase chain reaction.
Acknowledgments: The work was supported by the Ministero Italiano dell'Università e della Ricerca Scientifica, Progetti di Ricerca di Interesse Nazionale 2009.
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CP; cyto-histological responses occur after 24 h; at the same time, the germination of *C. platani* conidia on the leaf surface is prevented (Bennici *et al.* 2005, Fontana *et al.* 2008). As CP-induced defence-related genes were identified from *P. acerifolia* leaves treated for 48 h (Baccelli *et al.* 2008, Fontana *et al.* 2008), the present study is focused on the transcriptional changes occurring during the first 24 h of treatment.

Due to the fact that the *Platanus* genome has not yet been sequenced and few plane tree genes are available in GenBank, in order to increase the number of genes to be analysed, we designed a cDNA microarray containing both plane tree and poplar transcripts. Poplar is a species genetically related to plane tree, and differentially regulated transcripts from poplar leaves stressed with ozone and cold were available at the Department of Crop Biology of the University of Pisa. The cDNA microarray was used to identify differentially regulated genes after 24 h of treatment with CP on the basis of the existence of a significant overlap among genes expressed in response to different stresses (Shinozaki and Yamaguchi-Shinozaki 2000, Rabbani *et al.* 2003, Francia *et al.* 2007, Orsini *et al.* 2010). Five defence-related genes were further studied after 3, 6, 12, and 24 h of treatment by real-time reverse transcriptase - polymerase chain reaction (RT-PCR) comparing the eliciting activity of CP with cerato-populin (Pop1), a CP-orthologous protein from *C. populicola*, with PAMP activity on plane tree (Comparini *et al.* 2009).

A total of 318 cDNA sequences (library clones), grouped as follows, were spotted on microarray glass slides by *MicroCRIBI* (University of Padoa, Italy): 56 poplar clones induced by ozone (Rizzo *et al.* 2007); 162 poplar clones induced by cold (Maestrini *et al.* 2009); and 100 plane tree clones induced by CP after 48 h of treatment and isolated in previous works (Baccelli *et al.* 2008, Fontana *et al.* 2008). The spots area was 15480 × 15480 μm, and the array was made up of 16 squares, each made up of 13 rows and 10 columns. Each spot was 85 μm diameter and 220 μm spaced. Each probe was deposited in 4 replicates.

For the microarray analysis, total RNA was extracted from detached *P. acerifolia* leaves treated according to Fontana *et al.* (2008) with minor modifications. Different leaves were treated with 10 drops (1 mm^3) containing 150 μM CP (treated sample) or sterile distilled water as a control sample, and incubated in a moist chamber for 24 h. Cetyltrimethylammonium bromide (CTAB) buffer was used to extract total RNA from leaves according to Fontana *et al.* (2008) without performing the CsCl gradient, and amplification grade *DNase I* (*Sigma-Aldrich*, St Louis, MO, USA) was used to remove DNA contamination. Total RNA was reverse transcribed, labelled, and subjected to the hybridization steps as described by Trainotti *et al.* (2007). The cDNA microarray was scanned by *MicroCRIBI* service with a two-channel confocal microarray scanner (*ScanArray® Lite*, *Perkin Elmer*, MA, USA) using its dedicated software (*ScanArray Express 3.0.0*). Raw data was normalized by using total algorithm and the spots with intensity close to background noise were eliminated. Differentially expressed genes were obtained using *One Class SAM* (significance analysis of microarray, by Tusher *et al.* 2001) with false discovery rate (FDR) 0 %. A functional classification of the differentially regulated genes was made according to Fontana *et al.* (2008).

In order to validate the microarray result and to study the gene regulation induced by CP and Pop1 in early time-points, real-time quantitative RT-PCRs (qRT-PCRs) were performed on RNA extracted from *P. acerifolia* leaves treated for 3, 6, 12, and 24 h with 150 μM CP, Pop1, or water, as described above. RNA was transcribed into cDNA (400 ng per sample) as described by Bernardi *et al.* (2011) and transcripts codifying well known defence- or stress-related genes were analysed: β-1,3-glucanase (pathogenesis-related protein 2, PR-2), thaumatin protein (PR-5), and lipid transfer protein (PR-14) (Van Loon *et al.* 2006); and the transcription factor WRKY (Eulgem 2005) and the ThiF family protein, also known as ubiquitin activating enzyme, whose importance in the plant innate immunity has been recently reported (Balaji *et al.* 2011).

For WRKY and ThiF, whose cDNAs spotted on the microarray belonged to poplar, the orthologous partial sequences from *P. acerifolia* were isolated (GenBank acc. Nos. HE651262 and HE651263). The same RNA used to perform the microarray analysis was used to validate the result whereas three different biological replicates were used to compare CP and Pop1. The following gene-specific primers were used:
PR2-F 5'-CCTTGTTTACAGCCCCATCAG-3',
PR2-R 5'- GAACCACCCGCCTTCTCAA-3';
PR14-F 5'-CACTCAACAACGACGCTAAAACC-3',
PR14-R 5'-CGGACTCTCACCACATCTCATC-3';
PR5-F 5'-CGGTGCTTACGGCAATCC-3',
PR5-R 5'-GGGCATGCGTTCTTGAAGA-3';
ThiF-F 5'-GGATCTGGTGCTGAGTTGTGTAGA-3',
Thif-R 5'-GCTCATTGCAAGCCTGGTTT-3';
WRKY-F 5'-TTGCCCAGTCAAGAAGAAGGTT-3',
WRKY-R 5'-GAGAAGGGTGTGGGTGGTTGT-3'.
The *18S* rRNA gene was used as endogenous control for its transcriptional stability (Fontana *et al.* 2008, Bernardi *et al.* 2011). The gene-specific primers for *18S* were:
18S-F 5'-GGCGGATGTTGCTTTTAGGA-3',
18S-R 5'-TTCAGCCTTGCGACCATACTC-3'.
The amplifications of the target gene and the endogenous control were run in triplicate on the same plate in separate tubes. Reactions (20 mm^3) were carried out with 20 ng of cDNA as template, primers 200 nM each, and 1× *Fast SYBR Green® Master Mix* (*Applied Biosystems*, Foster City, CA, USA) following the manufacturer's instructions. PCRs were run in a *StepOne™* real-time PCR System (*Applied Biosystems*) using the recommended thermal-cycling conditions for the fast mode (hold 95 °C, 20 s; 40 cycles 95 °C, 3 s; 60 °C, 30 s). The size of the amplification products was checked on agarose gel. Before the quantification, a validation experiment was performed to ensure that the amplification efficiencies of

the target and the reference genes were approximately equal. The relative amount of target in each sample was determined using the comparative C_T method as described in the *ABI PRISM 7700* sequence detection system user bulletin #2 (*Applied Biosystems*). By this method, the amount of target normalized to the endogenous reference and relative to a calibrator sample is given by the arithmetic formula $2^{-\Delta\Delta Ct}$ (Ct, threshold cycle). The obtained $2^{-\Delta\Delta Ct}$ values (or fold-change values) were expressed as Log_2, so that the relative amount of target in the treated samples (CP- or Pop1-treated) acquired positive values for the up-regulated transcripts and negative values for those down-regulated, compared to the respective water-treated control samples which had Log_2 values = 0 (not shown in the tables).

Out of 318 cDNA sequences present on the microarray, 131 turned out to be differentially regulated in *P. acerifolia* leaves treated with CP for 24 h: 71 transcripts were up-regulated and 60 were down-regulated (Fig. 1*A*,*B*). Out of the 131 modulated transcripts, 45 were plane tree clones and 86 were poplar clones (transcripts list available at www.wuala.com/IvanBaccelli/Documenti/Supplement.doc/) and thus the cross-species hybridization allowed identification of 86 new sequences of *P. acerifolia* differentially regulated by CP. Given the origin of the poplar clones, the result revealed a significant overlap between genes responsive to different stresses. Well known defence- or stress-related genes were identified as responsive to CP and to cold/ozone stress like those codifying metallothioneins (Choi *et al.* 1996), WRKY (Eulgem 2005), and ThiF (Balaji *et al.* 2011). Of these genes, 35 were up-regulated and 51 down-regulated by CP.

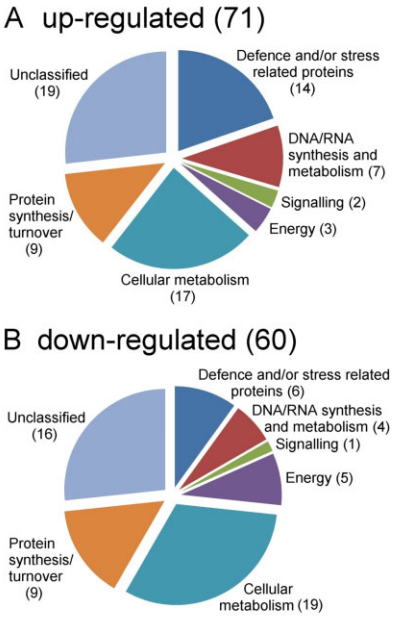

Fig. 1. Microarray result. Functional classification of the cDNA sequences differentially regulated in *P. acerifolia* leaves treated with CP for 24 h: up-regulated *(A)* and down-regulated *(B)* transcripts.

Table 1. Comparison between gene expression (relative values) in CP-treated leaves obtained by microarray analysis and real-time RT-PCR on five selected transcripts: PR-2 (pathogenesis-related protein 2, β-1,3 glucanase), PR-5 (thaumatin), PR-14 (lipid transfer protein), ThiF family protein, and transcription factor WRKY. Relative expression values are reported as Log_2 fold change. Negative values mean down-regulation. Water treated leaves were used as control ($Log_2 = 0$, not shown).

Accession	Gene	Microarray	qRT-PCR
AM260509	*PR-2*	0.2	2.5
FN821454	*PR-5*	0	-0.1
AM286249	*PR-14*	-1.1	-1.4
HE651263	*ThiF*	1.2	0.7
HE651262	*WRKY*	0.3	0.4

Table 2. Expression analysis performed with real-time RT-PCR in *P. acerifolia* leaves treated for 3, 6, 12, or 24 h with cerato-platanin (CP) or cerato-populin (Pop1). Five defence-related genes were analyzed: *PR-2*, *PR-5*, *PR-14*, *ThiF*, and *WRKY*. Relative expression values are reported as Log_2 fold change. Negative values mean down-regulation. Means ± SD, $n = 3$, * - significant difference at $P < 0.05$ between treated and control leaves (unpaired *t*-test performed using *GraphPad InStat v. 3.05* (*GraphPad Software*, San Diego, CA, USA).

Gene	Elicitor	3 h	6 h	12 h	24 h
PR-2	CP	2.5 ± 1.0*	-0.1 ± 0.4	0.2 ± 0.3	4.7 ± 2.1*
	Pop1	0.6 ± 0.9	0.8 ± 0.0*	0.5 ± 0.6	2.2 ± 0.6*
PR-5	CP	0.0 ± 0.0	2.4 ± 0.7*	0.4 ± 0.7	-0.5 ± 0.8
	Pop1	0.9 ± 0.3*	2.0 ± 1.0*	0.7 ± 0.3*	1.0 ± 0.3*
PR-14	CP	-0.1 ± 0.4	-2.1 ± 0.8*	-0.2 ± 0.3	-2.1 ± 1.0*
	Pop1	-0.4 ± 0.7	-2.5 ± 1.1*	-0.2 ± 0.8	-0.5 ± 0.8
ThiF	CP	-0.4 ± 0.1*	0.8 ± 0.5*	-0.1 ± 0.4	0.4 ± 0.2*
	Pop1	0.4 ± 0.0*	0.6 ± 0.0*	0.2 ± 0.0*	-0.6 ± 0.3*
WRKY	CP	1.6 ± 1.0*	0.7 ± 0.4*	0.6 ± 0.0*	1.5 ± 0.9*
	Pop1	1.9 ± 0.2*	0.7 ± 0.1*	0.5 ± 0.3*	0.3 ± 0.1*

Regarding the plane tree clones spotted on the microarray, these represented transcripts already known to be up-regulated by CP, but after a longer treatment (48 h). Interestingly, out of 100 clones present on the microarray, 55 were not modulated in the present study, 9 were down-regulated, and only 36 were up-regulated. This result showed unequivocally that the gene modulation undergoes remarkable changes over time after treatment with CP.

The microarray result was confirmed by qRT-PCRs performed on defence-related genes: three plane tree clones (*PR-2*, *PR-14* and *PR-5*) and two poplar clones (*ThiF* and *WRKY*). One of these (*PR-5*) was selected as it did not show modulation. Although with some differences, qPCR data was consistent with that of microarray. This also occurred for *ThiF* and *WRKY*, thus confirming the possibility of cross-species hybridization (Table 1).

The same transcripts used to validate the microarray result were also analysed after 3, 6, 12, and 24 h of treatment. Since it is known that a significant overlap exists in transcriptional regulation by different PAMPs (Schwessinger and Zipfel 2008), both CP and Pop1 were used to separately treat plane tree leaves at the different time-points. In a previous work, the ability of Pop1 to induce production of phenolic compounds in plane tree leaves after 24 h of treatment had been reported (Comparini *et al.* 2009). Both CP and Pop1 were able to cause very early changes at the level of gene transcription in plane tree leaves confirming that the transcriptional changes induced by PAMPs occur during the first hours after their perception (Schwessinger and Zipfel 2008, Anderson *et al.* 2010). Six hours was the treatment time where the modulation induced by the proteins was the most similar, with *PR-5*, *ThiF*, and *WRKY* that were up-regulated, and *PR-14* that was down-regulated (Table 2). However, after only 3 h, *WRKY* was induced by both CP and Pop1, *PR-2* was induced only by CP; and *PR-5* and *ThiF* were induced only by Pop1. Thus, the result showed a different timing in gene induction between CP and Pop1. Unlike *PR-2* and *PR-5*, *PR-14* was never up-regulated during the first 24 h of treatment, either by CP or by Pop1, although Fontana *et al.* (2008) reported an up-regulation by CP after 48 h. However, down-regulations of lipid transfer proteins under stress are also reported in other studies (Carvalho *et al.* 2006).

Overall, the different modulation pattern shown by CP and Pop1 over time was in accordance with preliminary data by Lombardi *et al.* (2010) which found differences between CP and Pop1 in timing of activation of defence responses. A difference in timing and amplitude of gene induction could be due to differences in the 3D structure of the two proteins and might be helpful in the future in order to define a structure-function relationship.

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