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Early proteomic changes in pear (*Pyrus communis* L.) calli induced by co-culture on microcallus suspension of incompatible quince (*Cydonia oblonga* Mill.)

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ABSTRACT

The pear (*Pyrus communis* L.)/quince (*Cydonia oblonga* Mill.) graft incompatibility is associated to biochemical and morphological alterations impairing the establishment of a functional union between the two partners, in which the callus at graft interface plays pivotal roles. This work was aimed to study the early effects evoked by factor(s) released by quince cells on the metabolism of pear callus. Using two-dimensional difference gel electrophoresis, the study analysed the changes between the proteome of pear callus co-cultured on suspensions of pear microcalli (homologous combination) or of incompatible quince microcalli (heterologous combination) by an *in vitro* system. This approach revealed changes in the abundance of proteins involved in several metabolic pathways and cell processes. In pear callus in heterologous combination, the less abundance of α-amylase and enolase and the increase of enzymes involved in amino acid and pyrimidine recycling indicate an alteration of the carbohydrate usage and a concomitant upsurge of salvage pathways. Moreover, the higher accumulation of a polyphenol oxidase suggests a participation of the flavonoid metabolism in the protection from the stress evoked by the proximity of quince cells. At the same time, this stress condition resulted associated with a reduction of proteins involved in the cell translation machinery and vesicle traffic, supporting that in heterologous combination the pear callus undergoes a physiological status less favourable for proliferation and differentiation. Overall, the work provides new information about the pear/quince incompatibility, highlighting as it involves a network of different metabolic responses.

1. Introduction

Grafting is a horticultural technique largely used for fruit tree propagation in order to combine advantageous traits from productive aerial part (scion) and rootstock into a single plant (Hartmann et al., 2002). The Europe pear (*Pyrus communis* L.) scions are commonly grafted on different clonal quince rootstocks (*Cydonia oblonga* Mill.) to regulate plant size and to reduce juvenile period (Browning and Watkins, 1991). However, important pear cultivars show graft incompatibility towards several common genotypes of quince rootstocks (Musacchi et al., 2000; Tomaz et al., 2009). For instance, the cultivars 'Beurré Bosc', 'Clapp's Favorite' and 'Epine du Mas' show this trait (Musacchi et al., 1997; Ermel et al., 1999). In particular, pear/quince combinations are subjected to localized graft incompatibility for which the typical symptoms are vascular discontinuity, cell necrosis and cambium involution at the graft interface (Andrew and Marquez, 1993; Mosse, 1962).

The establishment of a functional graft requires a defined sequence of structural events. The proliferation of a callus bridge from both scion and stock tissues brings to the initial adhesion between the two partners, increasing their cohesion by a dictyosome-mediated secretion of cell wall precursors. From this interstitial callus new cambium and, finally, new secondary xylem and phloem vessels are then differentiated, reconstituting the

Abbreviations: 2-D DIGE, two-dimensional difference gel electrophoresis; IEF, isoelectric focusing; LC-ESI-MS/MS, liquid chromatography tandem mass spectrometry; PVC, pre-vacuolar compartment; TGN/EE, trans-Golgi network/early endosome.

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vascular continuity between scion and rootstock (Andrew and Marquez, 1993). It is evident that the proliferation/differentiation potentialities of the interstitial callus cells as well as their chemical nature play fundamental roles in the development of the graft union (Pina and Errea, 2005). Graft incompatibility is ascribed to cellular and/or physiological intolerance between rootstock and scion (Andrew and Marquez, 1993). Feucht (1988) defined graft-incompatibility as a premature senescence of the tree due to physiological and biochemical changes. Further environmental stress and diseases can shorten the lifespan of incompatible combinations. The pear/quince incompatibility is generally attributed to the production by quince of cyanogenic glycosides, such as prunasin. These compounds in pear tissues are hydrolysed by β-glucosidases with the release of toxic cyanides, provoking cell necrosis and vascular degeneration at graft interface (Gur et al., 1968).

Several studies show that in *in vitro* micrografts of shoots, internodes or calli it is possible to observe effects on growth (Moore, 1986) as well as histological (Espen et al., 2005; Musacchi et al., 2004; Piagnani et al., 2006), ultrastructural (Errea et al., 2001; Moore 1984; Pina et al., 2012), molecular (Nocito et al., 2010; Pina and Errea, 2008a,b; Pirovano et al., 2002) and (bio)chemical (Errea et al., 2001; Musacchi et al., 1997; Nocito et al., 2010; Pina and Errea, 2008b) responses that are specifically influenced by the graft (in)compatibility between the two partners. By studying the inhibitory effect of quince metabolite(s) toward the growth of pear cells in suspension culture, Moore (1984) provided evidences that the pear/quince incompatibility does not require the direct cellular contact between callus cells. Recently, to study molecular and biochemical interactions between undifferentiated cells, it was proposed a novel *in vitro* system by which pear calli are co-cultured upon a suspension of homologous/heterologous microcalli onto a membrane raft that allows the exchange of low molecular weight metabolites (Nocito et al., 2010). It was shown that the proximity of incompatible (quince) microcallus suspension has severe negative impact on the growth rate and physiological status of pear calli, which manifest clear symptoms of oxidative stress and senescence (Nocito et al., 2010). This oxidative stress appears related to a general impairment of both mitochondrial respiration and alternative oxidation pathway, suggesting that other factors, beyond cyanides, are involved (Nocito et al., 2010).

Currently, proteomics is contributing in the study of plant responses to (a)biotic stress, disclosing networks among metabolic pathways, enzymes, non-enzymatic and regulative proteins (Vanderschuren et al., 2013). In particular, the application of the two-dimensional difference gel electrophoresis (2-D DIGE) allows a very high sensibility and a good reliability in spot quantification (Capaldi Arruda et al., 2011).

Taking advantage of the release of the *Pyrus bretschneideri* Rehd. genome (Wu et al., 2013) for protein identification by mass spectrometry, in this work we combined this *in vitro* system and the 2-D DIGE technology to study the proteomic differences between pear calli co-cultured in compatible (pear/pear) and incompatible (pear/quince) combinations in order to improve the knowledge about the metabolic factors intervening during the early incompatible responses.

2. Materials and methods

2.1. Plant material

Callus proliferation and microcallus suspension cultures from *in vitro* shoots of pear (*Pyrus communis* L., cv. Beurré Bosc; B) and incompatible quince (*Cydonia oblonga* Mill., East Malling clone C; EMC) were obtained as previously described (Nocito et al., 2010). Similarly, the same set up for the co-culture of callus masses upon membrane floating on microcalli suspensions (Nocito et al., 2010) was applied in order to obtained pear callus masses growth in compatible (B/B) and incompatible (B/EMC) combinations. After a 7-day period of co-culture in darkness, the pear callus masses were collected, rinsed with distilled water, blotted with paper towels, weighted and immediately frozen in liquid N_2 and stored at −80 °C for further uses. Three biological replicates were obtained for each combination.

2.2. Protein extraction

Pear calli were finely powdered in liquid N_2 by mortar and pestle, added with 1.5% (w/w) polyvinylpolypyrrolidone and resuspended in 4 volumes (v/w) of extraction buffer [700 mM sucrose, 500 mM Tris-HCl pH 8, 10 mM EDTA disodium salt, 4 mM ascorbic acid, 4 mM Pefabloc ® SC (Fluka), 1 mM phenylmethanesulfonylfluoride, 1 μM leupeptin, 0.4% (v/v) β-mercaptoethanol, 0.2% (v/v) Triton X-100]. After shaking for 30 min at 4 °C, the samples were centrifuged at 12,000 *g* for 20 min at 4 °C. The supernatants were then subjected to a very delicate sonication at 4 °C (10 s for three times). Finally, the protein fraction was purified according to the phenol extraction procedure described by Hurkman and Tanaka (1986). The pellet was dried and dissolved in labelling buffer [7 M urea, 2 M thiourea, 30 mM Tris-HCl pH 8, 3% (w/v) CHAPS detergent, 1% (v/v) NP-40].

2.3. 2-D DIGE

Sample protein concentration was determined by 2-D Quant Kit according to the manufacturer's instructions (GE Healthcare). Internal standard was constituted as a pool of equal portion of each sample (three biological replicates for each combination, $n = 6$). The protein samples were treated following the minimal labelling procedure with the CyDye DIGE dyes (GE Healthcare), using the Cy3 and Cy5 to label the internal standard and individual samples, respectively. Briefly, sample aliquots corresponding to 50 μg of proteins were incubated with 400 pmol of CyDye for 30 min, at 4 °C in the dark. The reaction was then quenched by the addition of 1 μl of 10 mM lysine and incubation for 10 min, at 4 °C in the dark. Finally, the protein samples were prepared for the following isoelectric focusing (IEF) by the addition of DTT and IPG buffer pH 4–7 (GE Healthcare) to the final concentration of 50 mg ml^{-1} and 2% (v/v), respectively. IEF was done loading 50 μg of Cy3-standard with 50 μg of Cy5-sample on pH 4–7, 24 cm IPG strip (GE Healthcare), previously rehydrated in over-night [7 M urea, 2 M thiourea, 3% (w/v) CHAPS detergent, 1% (v/v) NP-40, 10 mg ml^{-1} DTT, 0.5% (v/v) IPG Buffer pH 4–7]. IEF run was conducted in an Ettan IPGphor (GE Healthcare) at 20 °C with a limit of 50 μA per strip, applying the following protocol: 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 3 h at 8000 V in gradient, 16 h at 8000 V. Strip equilibration and SDS-PAGE on 12.5% polyacrylamide gels were done as previously described (Prinsi et al., 2009). Each biological sample was analysed twice, obtaining six gels per combination ($n = 6$). Gel images were acquired at 100 μm resolution with a Typhoon 9200 (GE Healthcare) laser scanner, set at 532 nm and 633 nm for the detection of the Cy3-standard and Cy5-samples, respectively. Gel matching (improved with manual matching), spot quantification and statistical analysis were performed by the DeCyder software (version 5.01, GE Healthcare). Spot quantification was expressed as standardized log abundances ($\log_{10}$ of the ratio of the spot normalized volume). As indicated by Marengo et al. (2005), spots detected in all maps ($n = 12$) showing a fold change of at least ±1.20 between B/EMC and B/B combinations and statistically significant according to the Student's *t*-test ($p < 0.05$) were selected for the following characterization by liquid chromatography tandem mass spectrometry