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Research article

Proteomic analysis of "Moncada" mandarin leaves with contrasting fruit load

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ABSTRACT

A proteomic approach was used to know more about the molecular mechanism related to *Citrus* alternate bearing. To this end, we researched protein expression differences between on-crop and off-crop "Moncada" [Clementine 'Oroval' (*Citrus clementina* Hort *ex* Tanaka) x 'Kara' mandarin (*Citrus unshiu* Marc. x *Citrus nobilis* Lou.)] mandarin leaves. This variety usually shows a remarkable behaviour in alternate production. Samples were collected in the period during which the fruit affect flowering induction. From 2D DIGE gel, 110 spots were isolated: 43 showed increased expression in the off-crop samples compared to on-crop samples, while 67 showed increased expression in the on-crop samples against off-crop samples. These spots were identified by MALDI-MS or LC-MS–MS. According to the up-expressed proteins in off-crop leaves such as proteins related to nutrient reservoir activity or to the pentose phosphate pathway, the primary metabolism was more active in off-crop trees than in on-crop trees. In contrast, the proteins up-expressed in on-crop samples such as catalase were related to the oxidoreductase activity and, therefore, the redox state seemed different for off-crop and for on-crop leaves. Other proteins with unknown functions were isolated, which could be also related to the alternate bearing and to the flowering induction.

1. Introduction

Many cultivars of *Citrus* tend to alternate bearing. It is a major problem in *Citrus* production worldwide, especially for late ripening mandarin cultivars [1]. The alternate bearing in *Citrus* is known to be due to a reduced flower production in the spring following a heavy on-crop year [2,3] this effect also depending on the time the fruit remains on the tree [4]. In addition, developing fruits exert a significant inhibitory effect on vegetative growth, reducing the number of summer/fall shoots and thereby decreasing the number of nodes that can bear flowers during the following spring [5]. This alternation in crop load makes orchard management difficult and has a negative economic impact.

The use of genetic and molecular approaches has made it possible to identify genes in leaves that regulate flower initiation and development in *Citrus* [6–8]. The isolation of *FLOWERING LOCUS T* (*FT*) and its ectopic expression conferring early flowering in *Poncirus trifoliata* and its repression by fruit load in 'Moncada' hybrid mandarin [Clementine 'Oroval' (*Citrus clementina* Hort *ex* Tanaka) x 'Kara' mandarin (*Citrus unshiu* Marc. x *Citrus nobilis* Lou.)] suggest a flowering-inducing role in *Citrus* [8,9]. All of these studies measured levels of gene expression, allowing a deeper understanding of the molecular basis of floral induction process. Besides, several proteomic Citrus studies have been recently done with different purposes [10–12]. However, there are no studies about which proteins are up-expressed at the floral induction time in *Citrus* leaves and how the presence of the fruit may affect the levels of these proteins.

In this work, we researched protein expression differences between on-crop and off-crop *Citrus* trees during the period in which the fruit affects floral induction. Moreover, we conducted an ontology analysis to know the molecular functions that these differential proteins can normally carry out and the biological processes that these proteins are involved in. As far as we know, this

Abbreviations: CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; 2D DIGE, two-dimensional difference gel electrophoresis; DTT, dithiothreitol; IPG, immobilized pH gradient; LC-MS-MS, liquid chromatography coupled with tandem mass spectrometry; MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry; pI, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; Q-TOF, quadrupole time-of-flight mass spectrometer; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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paper is the first published proteome-wide experiment on *Citrus* leaves with contrasting fruit load and therefore provides information in an undocumented area that could assist in alternated bearing control. The cultivar selected for this study was the 'Moncada' hybrid mandarin, a strong alternate bearing variety, whose mature crop is normally still on the tree during floral induction.

2. Results

2.1. Comparative proteome analysis

The aim of this work was to compare the leaf proteome from mandarin trees with contrasting crop. We identified the protein spots that were up- and down-regulated in off-crop trees comparing with on-crop trees. Therefore, leaf samples from off and on-crop trees were analyzed by 2DE. Gels were of high quality with reproducible protein patterns among replicates of the same samples (Fig. 1).

Approximately 1436 spots in gel images from samples were resolved. To assess the global differences in the expression levels between off and on-crop samples, gels were compared and quantified using the DeCyder™ Differential Analysis Software. Among the total proteins, 176 protein spots showed a significant quantitative differential accumulation (t-text < 0.05) between on and off-crop samples. 111 spots were confirmed with a good match and a sufficient volume for subsequent identification by mass spectrometry. To reliably determine quantitative changes in protein expression and therefore overcome error imposed by technical and biological variations, proteins were identified as up-regulated in off-crop samples if they were found to have an average expression level at least 1.10 higher than those of on-crop samples and as up-regulated in on-crop samples if they were found to have an average expression level at least −1.10 higher (absolute value) than those of off-crop samples. Among the 110 proteins, 43 had increased expression in the off-crop samples compared to on-crop samples (Av ratio +), while 67 showed decreased expression in the off-crop samples (Av ratio −).

2.2. Identification of differentially expressed proteins

We could manually excise the 110 proteins with a good match from a preparative 2DE gel to further identify 90 of them by MALDI-MS analysis and the other 20 proteins by LC-MS/MS analysis. Table 1 provides the spot number, the function of each protein together with the putative protein name, the accession code, the organism based on the protein has been identified, the homologue in *C. clementina* established by the database in www.phytozome.net, the homologue in *Arabidopsis thaliana* established by the database in www.arabidopsis.orgs, the values for theoretical and experimental p*I* and molecular mass, the expression ratio and *p*-value, and the MASCOT score together with the sequence coverage and peptides matched.

2.2.1. Classification of identified proteins

The identified proteins could be classified into seven groups according to its biological function: (i) primary metabolism (33 spots: 26 spots being associated with photosynthesis and carbohydrate metabolism, 4 spots related to Krebs cycle, 1 spot related to pentose phosphate pathway, and 2 spots related to nutrient reservoir activity); (ii) oxidoreductase activity (8 spots: one of them, the spot 79, up-regulated in off-crop samples, presented the highest ratio among all spots, and 5 spots were catalase, all of them up-regulated in on-crop samples, with spots 381, 384, 386 and 394 matching the same EST sequence); (iii) stress responses (3 spots, all of them up-regulated in on-crop samples); (iv) signal transduction (1 spot); (v) protein synthesis and degradation (6 spots); (vi) expansins (1 spot); (vii) other proteins (58 spots: it is the largest group, most proteins of the last group with an unknown function). The relative percentages of proteins both in on-crop leaves and in off-crop leaves appear in Fig. 2.

On the one hand, some of these spots were identified as the identical protein such as catalase, for spots 381, 384, 386 and 394 (oxidoreductase group); NADP-isocitrate dehydrogenase, for spots 515 and 516 (Krebs cycle subgroup, up-regulated in on-crop samples); RuBisCO large subunit-binding protein subunit beta chloroplast, for spots 300, 301, and 307; granule-bound starch synthase Ib precursor, for spots 327 and 328 (Fig. 3); putative cinnamoyl-CoA reductase, for spots 743, 744, and 748. The last three groups of proteins are related to primary metabolism and all of them are up-regulated in off-crop samples. On the other hand, some of the spots were identified as the same protein, but displayed different p*I* and molecular mass values and might account for isoforms or post-translationally modified forms of these proteins. Examples of the latter spots are miraculin-like protein 1 (spots 1016

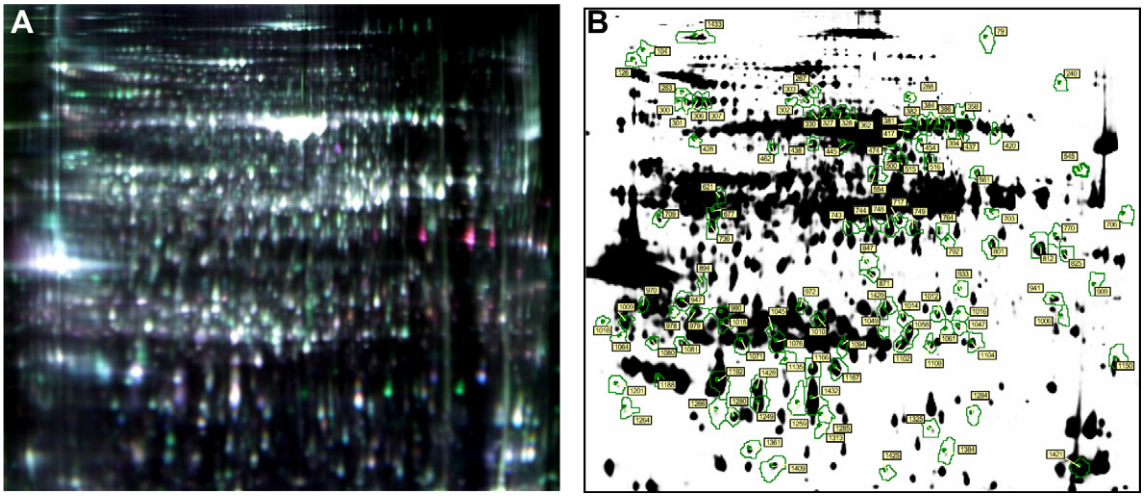

Fig. 1. Representative 2D DIGE gel of proteins extracted from "Moncada" mandarin leaves. Equal amounts (50 μg) of on-crop leaves sample (Cy5, red), off-crop leaves sample (Cy3, green) and internal standard (Cy2, blue) were loaded in the same gel. (A) Proteins up-expressed in off-crop leaves appear in green, those down-expressed in off-crop leaves appear in red and proteins unaffected appear in yellow. (B) Proteins selected for the analysis by mass spectrometry. Spot numbers correspond to the same ones indicated in Table 1.