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Expression profiling of two stress-inducible genes encoding for miraculin-like proteins in citrus plants under insect infestation or salinity stress☆

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ABSTRACT

The expression of two genes, namely *Mir*1 and *Mir*3 and the abundance of their encoded proteins, the putative miraculin-like proteins, MLP1 and MLP3, showing similarity to the Kunitz family of protease inhibitors, were monitored in the leaves of the citrus variety, 'Clementine' after *Tetranychus urticae* infestation and elicitor treatments, or in the leaves of three other diploid citrus: 'Willow leaf', 'Cleopatra' mandarins and 'Trifoliate' orange, as well as their respective doubled diploids and the allotetraploid somatic hybrid 'FLHORAG1' under salt stress. RT-PCR and 2-DE indicated that *Mir*1 and *Mir*3 and their products were present at low-basal expression in all citrus genotypes. Both genes and products were induced in the 'Clementine' leaves infested by *T. urticae*, but a contrasting profile was observed under elicitor treatments. Under salt stress, the two genes showed an expression pattern contrasting each other and depending on the genotypes. 'Cleopatra' mandarin, 'Trifoliate' orange and 'FLHORAG1' presented overexpression of *Mir*3 and MLP3 and decreased levels of *Mir1* and MPL1. The opposite behaviour was found in 'Willow leaf' mandarin. The positive correlation of the expression profile of the two genes with that of a gene encoding a putative apoplastic cysteine protease (*CysP*) might suggest a possible interaction of the respective encoded proteins during the response to biotic stress. Under salt stress, *CysP* and Mir 1 showed a similar expression pattern but only at transcript level. The possible occurrence of post-translational *CysP* regulation is discussed.

Introduction

Citrus is one of the major fruit crops in the Mediterranean area and its productivity and fruit quality is frequently affected by biotic and abiotic stress. Among biotic stress, infestation by the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is often observed in citrus orchards. *T. urticae* produces webbing and dense colonies on the lower side of leaves, causing chlorotic spots on the upper side of leaves (Urbaneja et al., 2008). *T. urticae* may also cause fruit scarring and the decrease in the commercial value of fruit (Ansaloni et al., 2008). The impact of *T. urticae* was enhanced when associated with salt stress (Aucejo-Romero et al., 2004).

Most citrus varieties are salt-sensitive (Arbona et al., 2003), and the adaptation to soil salinity is intimately related to the use of rootstocks that promote chloride exclusion at the root level (Hussain et al., 2012). Citrus doubled diploid (4x) seedlings were shown to be more tolerant to mild salt stress than their respective diploid (2x) parents (Saleh et al., 2008). Recently, 4x 'Rangpur' lime rootstock was shown to be much more tolerant to water deficit than its respective diploid (Allario et al., 2011).

Miraculin-like proteins are a family of glycoproteins having sequence similarity to native miraculin, a family of proteins with the particular feature to switch the sour taste to sweet taste (Masuda et al., 1995). Induction of miraculin-like genes is one of the most prominent responses to stress observed in microarray experiments (Talon and Gmitter, 2008). The abundance of miraculin-like proteins have been reported to change in 'Clementine' (*Citrus clementina* Hort. ex Tan.) leaves exposed to infestation of the two spotted-spider mite *T. urticae* (Maserti et al., 2011), in Sour orange

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(*Citrus aurantium* L.) (Tanou et al., 2009) and 'Cleopatra' mandarin (*Citrus reshni*, Hort. Ex Tan.) (Podda et al., 2013) exposed to salt stress, as well as in 'Satsuma' mandarin (*Citrus unshiu*) after cold stress (Lang et al., 2005). However, the functions of miraculin-like proteins in the citrus stress response remain unclear. Miraculin-like proteins have been suggested to play a defensive role against biotic stress by limiting cell damage (Tsukuda et al., 2006) due to the high homology between their amino acid sequences and the Kunitz family of proteinase inhibitors. In accordance with this observation, miraculin-like proteins found in the leaves of rough lemon possessed trypsin inhibitor activity (Tsukuda et al., 2006). Miraculins play an important role in the regulation of different types of hypersensitive cell death (Xu et al., 2012) which will lead to the production of reactive oxygen species (ROS), one of the earliest events following pathogen infection or elicitor treatment in cultured plant cells (Grant and Loake, 2000). Other than hydroperoxide production, many other parallel processes, such as the activation of nucleases and proteinases, are involved in hypersensitive cell death execution (Montillet et al., 2005).

Proteinase inhibitors can modulate cysteine protease, which is involved in the ubiquitin-mediated protein degradation pathway (Habib and Fazili, 2007). Also, cysteine proteases were found to be involved in the response to biotic and abiotic stresses (Atkinson and Urwin, 2012). The abundance of a cysteine protease was found increased in salt stressed leaves of 'Cleopatra' mandarin (Podda et al., 2013).

The aim of this work was to identify useful genes associated with defence response to abiotic and/or biotic stress in citrus plants, and provide information on their possible function in stress response. To achieve this objective, the expression profile of two miraculin-like protein genes, namely *Mir*1 and *Mir*3 was characterized using a semi quantitative PCR analysis, in different citrus genotypes exposed to *T. urticae* infestation, elicitor treatments or salinity stress. We investigated the expression of both genes in 'Clementine' infested by *T. urticae*. The expression profile of the two genes was also characterized after methyl jasmonate and salicylic acid (SA) treatments. The rationale of this comparison is that jasmonic acid and relative compounds play a major role in promoting plant defence response to many arthropod herbivores (Howe and Jander, 2008). On the other hand, SA operates along with other phytohormones, such as jasmonic acid or ethylene as part of a signalling network playing a role in coordinating an optimal response to single or combination of pest invaders (Bostock, 2005).

In another experiment, three different seedling rootstocks and one variety, were exposed to salt stress: 'Cleopatra' mandarin is one of the most salt-tolerant citrus rootstocks and it is a good Cl^- excluder, explaining the salt-tolerant nature of this rootstock (Zekri and Parsons, 1992). According to Moya et al. (2003), if Cl^- absorption is not limited at the root level, the ions are translocated to the leaves via the transpiration stream and cause necrosis and even defoliation. 'Trifoliate' orange (*Poncirus trifoliata* L., Raf) is a close relative of *Citrus* sp. in the *Rutaceae* family and is used as rootstock to confer tolerance to Citrus Tristeza Virus (CTV) and better cold-tolerance to the citrus scion. However, 'Trifoliate' orange is considered to be salt-sensitive because it accumulates Cl^- from the soil. 'Willow leaf' mandarin is a variety known to accumulate both Na^+ and Cl^- (Mouhaya et al., 2010). It has been included in the experiment because it is one of the parents of 'FLHORAG'1 ('Willow leaf' + 'Trifoliate' orange) which is an allotetraploid somatic hybrid obtained by fusion of 'Willow leaf' and 'Trifoliate' orange protoplasts (Ollitrault et al., 2000). This genotype inherited the tendency to accumulate Cl^- in roots and leaf from its 'Trifoliate' orange parent (Mouhaya et al., 2010). Finally, to try to determine what function *Mir*1 and *Mir*3 may have in the response to mite infestation or salt stress, the expression pattern of the two genes was compared that of a *CysP* gene which encodes a cysteine protease.

The transcript profile of the three genes was compared to that of their encoding proteins, the miraculin-like protein 1 (MLP1) and miraculin-like proteins 3 (MLP3) as well as the cysteine protease (CysP) detected using two-dimensional gel electrophoresis (2-DE). The correlation between mRNA and protein level is discussed.

Materials and methods

Plant materials used to investigate Tetranychus urticae infestation

Clementine (*Citrus clementina* cv. 'Tomatera'; SRA535) plants were kindly provided by Station de Recherche SRA, San Giuliano, Corsica, France). Plants were produced by scions excised from a single mother plant and grafted on 'Trifoliate' orange cv. Pomeroy).

Plant materials for salt stress

Diploid (2x) and doubled diploid (4x) seedlings of 'Willow leaf' mandarin (*Citrus deliciosa* Ten.; SRA133), 'Trifoliate' orange (*Poncirus trifoliata* L., Raf. cv. 'Pomeroy'; SRA1074), 'Cleopatra' mandarin (*Citrus reshni* Hort. Ex Tan.; SRA948) and the allotetraploid somatic hybrid 'FLHORAG1' ('Willow leaf' + 'Trifoliate' orange, SRA951) were kindly provided by Station de Recherche Agronomique INRA-CIRAD (SRA, San Giuliano, Corsica, France). Originally, spontaneous 4x were selected at SRA among a population of seedlings from the diploid plants. The ploidy status of 2x and 4x seedlings was checked and confirmed by flow cytometry according to Aleza et al. (2011). The genetic constitution of genotypes was checked using 5 simple sequence repeat (SSR) according to Froelicher et al. (2008). All 2x and 4x seedlings presented the same SSR profiles on polyacrylamide gels as their parental tree attesting their nucellar origin.

Experimental conditions

Plants were grown in separate pots (3L) in commercially available substrate for citrus plants (1/3 sand, 1/3 soil and 1/3 compost), under greenhouse condition for two years. Plants were watered every three days and received fertilizer for citrus plants (NPK 6.5.11 – Gesal, Italy: 6% N; 5% P_2O_5; 6% K_2O; 0.01% B; 0.002% Cu; 0.02% Fe; 0.01% Mn; 0.001% Mo; 0.002% Zn, and all the trace elements were chelated with water soluble EDTA) once a week. Three months before the start of the experiments, plants were transferred into controlled growth chambers for acclimation (temperature: $28 \pm 1\,^{\circ}C$; light/dark period: 16/8; relative humidity: 60–80% and PAR: 200 mmol photons $m^{-2}\,S^{-1}$). No other plants were present in the room at the time of the experiment. *T. urticae* infestation or elicitor application experiments were performed in separate growth chambers in which the same environmental conditions were maintained.

Each experiment was performed in triplicate for control and treated plants, each of the three biological samples consisting of a pool of four leaves of the same age, same stage of development and similar leaf area. Treatments and leaf harvest were carried out around 14 p.m. The leaves were carefully washed with double distilled water and immediately frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until analysis.

Spider mite infestation

Infestation by *T. urticae* was carried out by transferring about 50 spider mites collected from naturally infested *Clementine* plants onto leaves of the experimental plants. Infested leaves were closed into nest bags to avoid mite escape. Control leaves were closed into nest bags without mites. Analysis was performed on leaves harvested at 0 h and 24 h after infestation.