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A L-type lectin gene is involved in the response to hormonal treatment and water deficit in Volkamer lemon

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ABSTRACT

Combination of biotic and abiotic stress is a major challenge for crop and fruit production. Thus, identification of genes involved in cross-response to abiotic and biotic stress is of great importance for breeding superior genotypes. Lectins are glycan-binding proteins with a functions in the developmental processes as well as in the response to biotic and abiotic stress. In this work, a lectin like gene, namely *ClLectin1,* was characterized in Volkamer lemon and its expression was studied in plants exposed to either water stress, hormonal elicitors (JA, SA, ABA) or wounding to understand whether this gene may have a function in the response to multiple stress combination. Results showed that *ClLectin1* has 100% homology with a L-type lectin gene from *C. sinensis* and the *in silico* study of the 5′UTR region showed the presence of *cis*-responsive elements to SA, DRE2 and ABA. *ClLectin1* was rapidly induced by hormonal treatments and wounding, at local and systemic levels, suggesting an involvement in defence signalling pathways and a possible role as fast detection biomarker of biotic stress. On the other hand, the induction of *ClLectin1* by water stress pointed out a role of the gene in the response to drought. The simultaneous response of *ClLectin1* expression to water stress and SA treatment could be further investigated to assess whether a moderate drought stress may be useful to improve citrus performance by stimulating the SA-dependent response to biotic stress.

1. Introduction

Global climate change is expected to increase abiotic stresses, such as drought, salinity, extreme temperature, which are among the most challenging threats to crop plants (Huang et al., 2016). On the other hand, changes of abiotic factors may lead to the spread of plant pathogens and pests in several regions of the world (Bebber et al., 2014). The simultaneous exposure to abiotic and biotic stress triggers specific physiological and molecular responses. Recent evidences demonstrated that drought might positively or negatively affect the performance of exposed plants against biotic stress (Fujita et al., 2006; Rejeb et al., 2014). Hormonal signalling cascades, including abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) play an important role in the molecular mechanisms that optimize adaptive plant responses to abiotic and biotic stresses, by modulating the expression of numerous genes involved in plant response to complex environments (Howe and Jander, 2008; Robert-Seilaniantz et al., 2011; Nguyen et al., 2016). Therefore, the identification of genes involved in the response to multiple stress may be useful to understand adaptive mechanisms and ultimately to develop superior crops resilient to environmental challenges (Dangl et al., 2013; Suzuki et al., 2014).

Citrus are an economic valuable fruit crop worldwide (Liu et al., 2012), frequently exposed to a variety of abiotic and biotic stresses, which can act either alone or often in combination, affecting growth and development and ultimately causing reduced yield and fruit quality (Iglesias et al., 2007). To maintain a sustainable citrus industry, it is

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necessary to better understand the gene expression profiling of the existing genotypes as well as to breed new cultivars with tolerance against both abiotic and biotic stress in order to meet the increasing demands of the current competing market (Gong and Liu, 2013).

Lectins are a class of proteins characterized by at least one non-catalytic site allowing them to specifically recognize and bind glycosylated macromolecules such as glycolipids, proteoglycans, polysaccharides and free sugars. The characteristic of binding carbohydrates allows this class of proteins to accomplish their activities both at the internal and external cell levels (Rudiger and Gabius, 2001), acting in various developmental processes by interacting with storage proteins or enzymes. Lectins have a well-recognized role in the responses to biotic stresses, acting as cell-surface and intracellular receptors in the defence system developed by plants against pathogens and herbivorous insects (Lannoo and Van Damme, 2014). Induction by abiotic stresses including temperature, water-stress and salinity has also been reported (Jiang et al., 2010) making them potential candidates in cross-talk mechanisms between abiotic and biotic stresses.

Citrus plant genome encodes a large number of lectins (Terol et al., 2007, 2008) and their response to abiotic and biotic stress have been reported in different citrus genotypes (Freitas-Astúa et al., 2007; Nwugo et al., 2013; Rawat et al., 2015; Maserti et al., 2011; Podda et al., 2013). However, to our knowledge, no information is available on the response of lectin gene to abiotic or hormonal elicitation in the same citrus genotype.

Therefore, the aims of this work was to study the expression of a lectin-like gene in Volkamer lemon (*C. limonia*) exposed to water stress, hormonal treatment or wounding to assess how the gene is regulated by different elicitors in the same citrus genotype. To this purpose, seedlings of Volkamer lemon were exposed to a water-stress cycle. Furthermore, as phytohormones play well-known role in the response to environmental stimulus (Huang et al., 2008) or artificial wounding simulating insect attack (Major and Constabel, 2007), Volkamer lemon seedlings were also exposed to hormonal treatments with JA, SA or ABA. A lectin–like gene, namely *ClLectin1* was characterized and its expression was monitored by semi quantitative RT-PCR (sqRT-PCR) in control and treated plants.

2. Material and methods

2.1. Plant material, growth conditions and water stress experiments

Six-month-old seedlings of Volkamer lemon (*Citrus limonia* Osb.) were grown in the growth chamber of the Institute for Sustainable Plant Protection in Florence under the following controlled conditions: photoperiod of 16 h, with day/night of 25–32 °C/18–20 °C and relative humidity varying daily between 50 and 80%. The photon flux density at leaf level was 300–350 μM $m^{-2} s^{-1}$, supplied mainly with cool lights. The plants were regularly irrigated and fertilizated with Bayfolan (Bayer) (NPK 5–7–8 + microelements) every 15 days. The day before the beginning of the water-stress experiment, plants were fully irrigated and the excess water was allowed to drain overnight. After draining, the pots were weighed to determine the weight at pot water capacity. Each pot was then enclosed in a plastic bag that was tied around the stem to prevent soil evaporation. Water deficit development was followed and parameterized measuring the pot weight and calculating the fraction of transpirable soil water (FTSW) (Sinclair and Ludlow, 1986; Brilli et al., 2013). The experiment was ended when stomatal conductance approached zero (10% FTSW); then water was again provided to all the plants to pot capacity. Full details of the growth conditions are given in a previous paper (Santana-Vieira et al., 2016). Two leaves from four independent plants were harvested for each experimental condition (well-watered; water- stressed) at 100%, 50% and 10% of FTSW, and after 24 h from water stress relief (Supplementary Table S1).

2.2. Hormonal and wounding treatment

Hormonal and wounding treatments were performed on well-watered Volkamer lemon plants by spraying the upper and lower leaf lamina of tagged branches with aqueous solution of 1% (v/v) glycerol supplemented with either 100 μM jasmonic acid (JA) (Sigma 14631), 1 mM salicylic acid (SA) (Sigma S5922) or 100 μM abscisic acid (ABA) (Sigma A1049) as reported by Del Carratore et al. (2011). Leaves of control plants were treated with aqueous solution containing 1% (v/v) glycerol. Wounding was performed by rubbing the upper and lower leaf lamina with autoclaved carborundum (Geonatura, Spain). JA or SA were applied continuosly for 9 days and then the treatment was stopped. Two leaves were harvested at 0 h, 6 h, 24 h and 9 days of continuous application, and at 24 h, 48 h, 72 h after the end of the application. After ABA or wounding treatments, leaves were sampled at 0 h and 24 h (Supplementary Table S1). In the hormonal and wounding treatments, leaves were collected both from tagged-treated branches and distal, not-treated branches of four independent plants to evaluate both local and systemic responses.

2.3. Genomic DNA extraction, RNA extraction and sqRT-PCR analysis

To extract genomic DNA, approximately 40 mg of leaf tissue were chilled and powdered in liquid nitrogen following the manufacturer's protocol of the DNA purification kit (Promega). To isolate the 5′UTR fragment of L-type lectin gene, a PCR was performed on genomic DNA using the couple of primers reported in supplementary Table S3. In order to design the primers for sqRT-PCR, the sequences of peptides found to be increased in abundance in *C. clementina* infested by *Tetranychus urticae* (Maserti et al., 2011) and in *C. reshni* under salt stress (Podda et al., 2013) were blasted to find the putative lectin-related protein (Q9FQ12); the entire amino acid sequence was then translated into nucleotide sequence using NCBI BLAST (Supplementary, Table S2). The web based Primer3Plus tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) was used to design the primers (Supplementary Table S3). Total RNA extraction and cDNA synthesis were performed as already reported in Podda et al. (2014), modifying the protocol of the Taqman Gene Expression Cells-to-CT TM Kit (Applied Biosystems). After preliminary tests to select the most suitable reference gene, the cyclophilin gene (gene bank: AB981058.1) was used as reference for relative quantification. PCR was performed at the following condition: 94 °C for 5 min; then for cyclophilin 33 cycles at 94 °C for 30 s, 58 °C for 50 s, and 72 °C for 60s; for *ClLectin1* 35 cycles 94 °C for 30s, 60 °C for 50 s, 72 °C for 60 s; followed by a final extension at 72 °C for 7 min for both genes. Each sample was amplified in duplicate reactions (technical replicates). Three independent biological and two technical replicates were used. PCR products were separated by 1.5% agarose gel electrophoresis and stained with SYBR safe DNA gel stain (Invitrogen). Gel images were acquired with a GEL DOC XR+ system (BioRad) and bands quantification was performed with the Image Lab Software (BioRad). Bands area are expressed in arbitrary units which is represented by the pixels measured in the band boundaries. To ensure that the primer was amplifying the targeted genes, the amplicons were excised and purified from the agarose gels, using the Wizard® SV Gel and PCR Clean-Up System (Promega), and then sequenced (Supplementary Fig. S1).

2.4. Statistical and sequence analysis

The statistical significance of the differences observed among the experimental treatments was determined by ANOVA and Tukey's test using STATISTICA software (StatSoft, Italy) at the probability level of $P < 0.05$. The sequence analysis of Volkamer lemon lectin gene was performed by TIGR (http://blast.jcvi.org/euk-blast/plantablast.cgi) and NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez) databases. BLAST nucleotide collection was used to align our experimentally