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Two metallocarboxypeptidase inhibitors are implicated in tomato fruit development and regulated by the Inner No Outer transcription factor

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ABSTRACT

The *TCMP-1* and *TCMP-2* genes of tomato code for metallocarboxypeptidase inhibitors and show sequential, tightly regulated expression patterns during flower and fruit development. In particular, *TCMP-1* is highly expressed in flower buds before anthesis, while *TCMP-2* in ripe fruits. Their expression pattern suggests that they might play a role in fruit development. Here, to investigate their function, we altered their endogenous levels by generating transgenic plants harbouring a chimeric gene expressing the *TCMP-1* coding sequence under the control of the *TCMP-2* promoter. The expression of the transgene caused an earlier fruit setting with no visible phenotypic effects on plant and fruit growth. The altered *TCMP-1* regulation determines an increased level of *TCMP-1* in the fruit and unexpected changes in the levels of both *TCMPs* in flower buds before anthesis, suggesting a mechanism of transcriptional cross-regulation. We *in silico* analysed *TCMPs* promoter regions for the presence of common *cis* acting elements related to ovary/fruit development and we found that both promoters contain putative binding sites for INNER NO OUTER (INO), a transcription factor implicated in ovule development. By chromatin immunoprecipitation, we proved that INO binds to *TCMP-1* and *TCMP-2* promoters, thereby representing a candidate regulatory factor for coordinated control of *TCMPs*.

1. Introduction

Protease inhibitors (PIs) are small proteins, widely spread among plants, animals, and microorganisms that control the activity of the corresponding proteases [1]. In plants, PIs are particularly abundant in reproductive and storage organs such as seeds and tubers [2,3]. Many of these inhibitors are implicated in the plant defence responses against herbivores and pathogens [2,4–6]. PIs are produced either constitutively or in response to damage such as mechanical wounding or insect lesion [7]. Two metallocarboxypeptidase inhibitors, possessing a cystine-knot structure, were identified in solanaceous plants: the potato carboxypeptidase inhibitor (PCI) and its homologue from tomato, named TCMP-1 [8,9]. PCI, a 39 amino acids-long protein, accumulates in potato tubers as well as in wounded potato leaves [7,10]. *TCMP-1*, a cDNA clone isolated from an ovary library, encodes a mature protein of 37 amino acids [11,12]. The precursor contains at N-terminal region a signal peptide for entry into the secretory system and an 8-amino acid carboxyterminal extension [11,12]. The *TCMP-1* is highly expressed in flower buds collected before anthesis (1.0–1.1 cm long); at anthesis its expression decreases, reaching the lowest level after fertilization (4–5 days after anthesis) [9]. In very young fruits and red fruits, the mRNA level increases again [9]. TCMP-1 protein accumulates later in comparison to the mRNA, with the highest level in flowers collected 4–5 days after anthesis [9]. In leaves *TCMP-1* is expressed at very low level, however it is induced by about 100-fold in response to wounding [2,12]. The role of TCMP-1 in plant is still unknown, even though based on its wound-responsiveness, it has been considered a defensive peptide [13].

In 1989, the differential screening of a cDNA library constructed from ripe tomato fruit allowed the isolation of a cDNA (clone 2A11) coding for a second metallocarboxypeptidase inhibitor of the cystine-knot type [14], referred to as *TCMP-2* [9]. *TCMP-2* gene is poorly expressed before anthesis, while after anthesis its expression gradually increases reaching the highest level in the fruit. It is interesting to note that *TCMP-2* mRNA in ripe tomato fruits represents about 1% of the total poly(A^+) mRNA [14]. *TCMP-2* expression is apparently absent in

Abbreviations: TCMPs, tomato metallocarboxypeptidase inhibitors; PCI, potato carboxypeptidase inhibitor; VEGF, vascular epidermal growth factor; INO, Inner No Outer; ChIP, chromatin immunoprecipitation

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roots, stems, and leaves [14].

The spatiotemporal expression patterns of TCMPs suggest a role for these genes in flower/fruit development. In addition, the sequential patterns of the two mRNAs might be indicative of a tight and co-ordinated regulation.

The two mature TCMPs are 32% identical [9] and are characterized by a cystine-knot structural motif resulting from three intra-molecular disulphide bonds [15]. This particular scaffold confers to the cystine-knot proteins a stable structure and a conformational rigidity that are probably responsible for the resistance of TCMPs against drastic treatment conditions such as extreme pH, chemical and thermal denaturation, and proteolytic attack [16–19]. TCMP-1 and TCMP-2 are highly similar in structure to the potato PCI [7,20]. Both TCMPs and PCI share structural homology also with some mammalian growth factors, in particular with the epidermal growth factor (EGF) and vascular epidermal growth factor (VEGF), and are bioactive in mammalian cells [9,21,22]. PCI has been demonstrated to compete with EGF for the binding to EGF receptor, inhibiting its activation [21]. TCMPs inhibit angiogenesis both *in vitro* in human umbilical vascular cells and *in vivo* in zebrafish by affecting the VEGF receptor activation [9,22]. These findings indicate the capacity of the cystine-knot metallocarboxypeptidase inhibitors to interfere with growth factor signalling pathways in animal cells.

In this investigation, to gain insight into the functional role of TCMPs in fruit development, we produced a genetic construct (*pTCMP2:TCMP-1*) containing the entire coding region of the *TCMP-1* gene under the regulation of the *TCMP-2* promoter, known to be preferentially expressed in green and ripe fruits. By the analysis of tomato plants transformed with the *pTCMP2:TCMP-1* construct, we show for the first time that TCMPs are implicated in tomato fruit development and we uncover the first hints of the regulation of TCMPs expression.

2. Materials and methods

2.1. Plant material and growth conditions

UC82 tomato (*Solanum lycopersicum*) plants, a typical cultivar used by the processing industry, were grown in a greenhouse during the springtime in two consecutive years (2016 and 2017). After germination, plantlets at the third-fourth true leaf were transplanted in pots (25 cm diameter) and randomly distributed on the benches. The following traits were recorded: number of fruits at different times, weight of ripe fruits, and number of seeds.

2.2. Generation of pTCMP2:TCMP-1 and pTCMP2:GUS transgenic lines

A 1378 bp-long region upstream of the tomato T*CMP-2* translation start codon (GenBank accession number X13743) was used to control the expression of the two genetic constructs. The promoter sequence was amplified with the following forward (F) and reverse (R) primers: F, 5′-GAATTCCTCGAGCCCTTTAAAAAGTAT-3′; R, 5′-GGTACCAATGG TTTTGGATTAATTGCT-3′. Either a 234 bp-long DNA fragment corresponding to the coding sequence of TCMP-1 precursor (X59282.1), amplified with the following primers: F, 5′-GGTACCATGGCACAAAAA TTTACT-3′; R, 5′-GGATCCTTATCACACGCCTATGGCCATGGC-3′, or β-glucuronidase (GUS) gene (S69414.1; see [22]) were placed downstream of the *TCMP-2* promoter. The sequence of the gene for the nopaline synthase (NOS) of *Agrobacterium tumefaciens* was used as terminator regulatory sequence. The two resulting constructs, named *pTCMP2:TCMP-1* and *pTCMP2:GUS*, were cloned into a derivative of pBin19 binary vector [23] and recombinant plasmids were introduced in *A. tumefaciens* strain GV2260. For the genetic transformation of tomato, cotyledons from germinated seedlings were cut and incubated in a suspension of agrobacteria in the presence of 200 μM acetosyringone. Plant regeneration was performed according to a well set up protocol [24]. Histochemical detection of GUS was carried out as previously described [25].

2.3. Southern blot and PCR analysis

1.1.2 Genomic DNA was isolated from 0.5–1 g of frozen leaves using the “Illustra Nucleon PhytoPure” kit (GE Healthcare) according to the manufacturer's instructions. DNA (20 μg) was digested with 70 U of *Hind*III, electrophoresed on a 0.8% agarose gel at 4.5 V cm^{-1}, and transferred on positively charged Hybond N^+ membrane (GE Healthcare). DNA probe for the analysis, spanning the entire coding sequence for TCMP-1 precursor and the NOS terminator sequence was obtained by PCR with the following forward (F) and reverse (R) primers: F 5′-GGTACCATGGCACAAAAATTTACT-3′ and R 5′-AAGCTTGATCTAGTAACATAGATGACACCG −3′. The probe was labelled with [^{32}P]dCTP by random priming using “Prime-It II Random Primer Labeling Kit”(Stratagene). Unincorporated nucleotides were removed with the “Illustra AutoSeq G-50 Dye Terminator Removal Kit” (GE Healthcare). The membrane was hybridized overnight at 42 °C in ULTRAhyb buffer (Ambion) in the presence of 10^6 cpm ml^{-1} of labelled probe. After hybridization, the membrane was washed in 2X SSC containing 0.1% SDS at 42 °C. Autoradiography was then performed using Kodak XAR-5 films.

2.4. SDS-PAGE and western blot assays

Total protein samples were isolated from ripe fruits using the CelLytic reagent (Sigma-Aldrich).

For TCMPs analysis, 100 μg of total proteins were loaded on 15% Tris-Tricine SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). Membrane was incubated overnight at 4 °C with primary polyclonal antibodies produced in rabbit (PRIMM s.r.l.) using as an antigen the purified recombinant TCMP-1 [9]. For INO analysis, 30 μg of total proteins were loaded on 12% Tris-Glycine SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). Membrane was incubated over night at 4 °C with 2 μg of affinity purified polyclonal antibody produced in rabbit (GenScript USA Inc.) using as immunogen the sequence KDETDDANKKNSNSC (14 amino acid-long INO sequence plus an extra “C” to simplify the conjugation with keyhole limpet hemocyanin). Equal amount of protein loading was verified by staining the membrane with Ponceau Red S solution.

Then, membranes were treated with monoclonal horseradish peroxidase-conjugated anti-rabbit IgG (1:5000) at 25 °C for 1 h. Proteins were detected with an enhanced chemiluminescent reagent.

2.5. Quantitative real time PCR (qRT-PCR) and reverse-transcriptase-PCR (RT-PCR)

Total RNA was isolated using NucleoSpin RNA Plant (Macherey-Nagel) starting from 100 mg of frozen pooled tissues. After DNase treatment, 1 μg of total RNA was reverse transcribed using ImProm-II Reverse Transcription System (Promega).

For qRT-PCR analysis, cDNA was amplified using SYBR Green qPCR Supermix-UDG (Invitrogen) on the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). For each determination of mRNA level, three cDNA samples derived from three independent RNA extractions were analysed. Melting curve analysis was performed to identify non-specific PCR products and primer dimers. The $2^{-\Delta\Delta Ct}$ method was used to show the difference between the target gene and actin gene as internal control [26]. Transcript levels of genes coding for *TCMP-1*, *TCMP-2*, and actin were determined with the following primers: *tcmp-1F*, 5′-GCTGCTCAAGATGTGATGGC-3′; *tcmp-1r*, 5′-TCCAACAGGCCTG ACAGAAC-3′; *tcmp-2f*, 5′-ACGAACCTTGCAGCTCAAAC-3′; *tcmp-2r* 5′-GCAACAGGTTGCATGTACGG-3′; actin F, 5′-CCCGTTCAGCAGTGGT GGT-3′, actin r, 5′- TACGAGGGTTATGCTTTGCC-3′.

For the expression analysis of the *pTCMP2:GUS* gene construct by RT-PCR, the first 891 nts of the GUS coding sequence were amplified