Pesticide Biochemistry and Physiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

A peptide derived from enzymatic digestion of globulins from amaranth shows strong affinity binding to the replication origin of Tomato yellow leaf curl virus reducing viral replication in Nicotiana benthamiana

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ABSTRACT

Tomato yellow leaf curl virus (TYLCV; genus *Begomovirus*; family *Geminiviridae*) infects mainly plants of the family Solanaceae, and the infection induces curling and chlorosis of leaves, dwarfing of the whole plant, and reduced fruit production. Alternatives for direct control of TYLCV and other geminiviruses have been reported, for example, the use of esterified whey proteins, peptide aptamer libraries or artificial zinc finger proteins. The two latter alternatives affect directly the replication of TYLCV as well as of other geminiviruses because the replication structures and sequences are highly conserved within this virus family. Because peptides and proteins offer a potential solution for virus replication control, in this study we show the isolation, biochemical characterization and antiviral activity of a peptide derived from globulins of amaranth seeds (*Amaranthus hypochondriacus*) that binds to the replication origin sequence (OriRep) of TYLCV and affects viral replication with a consequent reduction of disease symptoms in *Nicotiana benthamiana*. Aromatic peptides obtained from papain digests of extracted globulins and albumins of amaranth were tested by intrinsic fluorescent titration and localized surface resonance plasmon to analyze their binding affinity to OriRep of TYLCV. The peptide AmPep1 (molecular weight 2.076 KDa) showed the highest affinity value (Kd = 1.8 nM) for OriRep. This peptide shares a high amino acid similarity with a part of an amaranth 11S globulin, and the strong affinity of AmPep1 could be explained by the presence of tryptophan and lysine facilitating interaction with the secondary structure of OriRep. In order to evaluate the effect of the peptide on *in vitro* DNA synthesis, rolling circle amplification (RCA) was performed using as template DNA from plants infected with TYLCV or another begomovirus, pepper huasteco yellow vein virus (PHYVV), and adding AmPep1 peptide at different concentrations. The results showed a decrease in DNA synthesis of both viruses at increasing concentrations of AmPep1. To further confirm the antiviral activity of the peptide *in vivo*, AmPep1 was infiltrated into leaves of *N. benthamiana* plants previously infected with TYLCV. Plants treated with AmPep1 showed a significant decrease in virus titer compared with untreated *N. benthamiana* plants as well as reduced symptom progression due to the effect of AmPep1 curtailing TYLCV replication in the plant. The peptide also showed antiviral activity in plants infected with PHYVV. This is the first report, in which a peptide is directly used for DNA virus control in plants, supplied as exogenous application and without generation of transgenic lines.

1. Introduction

Plant diseases caused by viruses affect crops worldwide resulting in large economic losses [1]. For production of tomato (*Solanum lycopersicum*), tomato yellow leaf curl virus (TYLCV; family *Geminiviridae*; genus *Begomovirus*) is one of the most important viruses [2]. The symptoms that TYLCV induces are curling of leaves, chlorosis, dwarfing and floral abortion. TYLCV is naturally transmitted by whiteflies of the

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https://doi.org/10.1016/j.pestbp.2018.01.005
Received 27 July 2017; Received in revised form 12 January 2018; Accepted 17 January 2018

Bemisia tabaci species complex producing a systemic infection, in which the virus is translocated to the sieve elements and is spread throughout the plant [3]. The virus can accumulate in the fruit, including the seed embryo [4a,b,5], allowing this virus to be transmitted by seeds [6], which in turn increases the risk of spread of the virus.

TYLCV has a genome of a circular and single stranded (ss) DNA (viral sense, *VS*) with an approximately length of 2.8 Kb. The genome has six overlapping open reading frames (ORFs), with two ORFs on the viral sense strand (*V1* and *V2*) and the other four ORFs on the complementary DNA strand (*C1*, *C2*, *C3* and *C4*) [7]. The replication of this virus is mediated by a rolling circle replication (RCR) mechanism or recombination dependent replication (RDR). The intergenic region (IR) of the TYLCV genome contains the replication origin, which is a sequence of 25 nucleotides forming a secondary structure of "loop" shape [8], and this loop has the function to regulate replication through RCR, mainly by being target for the Rep protein (encoded by the *C1* gene), which is the initiator of this replication process [8,9]. Once the viral DNA (*VS*) enters the nucleus of the host cell, synthesis of the complementary strand (CS) occurs. Subsequently, when the Rep protein is expressed, it binds specifically to the loop type structure in the IR and nicks it leaving a free hydroxyl group for start of DNA synthesis through RCR [9]. As the new chain grows, it displaces the original *VS* strand, resulting in the production of a new *VS* strand, which will be used as a template for synthesis of the CS strand as well as for the packaging of new viral particles [10,11].

Proteins and peptides have been offering an alternative method for control of pathogens in crops, showing biological activity as inducers of systemic acquired resistance against fungal [12] and viral pathogens [13] in the experimental models of maize and tobacco, respectively. It has also been found that peptides with hydrophobic characteristics have direct antifungal activity on *Fusarium oxysporum* [14], as well as direct antibacterial activity against *Xylophilus ampelinus* and *Agrobacterium vitis* in grape plants [15]. Furthermore, it has been found that peptide extracts derived from enzymatic digestion of vegetable proteins induce growth in tomato and promote root development in maize [16].

Peptides have shown the ability to control infections by plant viruses because of their ability to bind specifically to molecular targets. Screening of aptamer peptide libraries identified peptides that showed affinity for the Rep protein of the begomovirus tomato golden mosaic virus (TGMV) and interfered with viral replication in cell cultures [17]. When two of these peptides were expressed in tomato plants infected by TYLCV or another begomovirus, tomato mottle virus (ToMoV), a decrease in symptoms development was observed in the transgenic lines [17]. Another effort for virus control with peptides has been reported using an artificial zinger protein, which has a strong affinity to the IR of the TYLCV genome [18]. However, no plant experiments were done in this study.

Cereals and pseudocereals offer a big source of bio-macromolecules, as they contain high amounts per gram-tissue of proteins, polysaccharides and lipids in comparison to other plants [19,20,21]. These molecules can be purified or partially purified and be a source of derivative molecules such as peptides and oligosaccharides, hence increasing the library of possible compounds with biological activity from natural sources. Seeds of amaranth (*Amaranthus hypochondriacus*) contain large amounts of proteins in comparison to other cereals and pseudocereal plants, making this plant a good candidate for extraction of bioactive molecules such as peptides. The albumin and globulin fraction in amaranth represents approximately 19–20% of the total protein content, and can be used directly or as peptides for the design of molecules with bio-functional activity [22,23,24].

Previous research on amaranth proteins has focused mainly on the antihypertensive, cytotoxic, antioxidant and antifungal activities, while the antiviral activity properties of these proteins in animal or plant systems are still unknown. Peptide derivatives obtained through enzymatic digestion of water-soluble proteins from amaranth keep the bioactivities mentioned above. *In vitro* experiments have shown that the bioactivity of peptides may be higher because the size of the biomolecule is smaller allowing improved uptake into the cell and interaction with cell receptors [25].

In the present work, we describe the anti-viral activity of a peptide obtained through enzymatic digestion of the globulin fraction of amaranth seeds. When the peptide solution was infiltrated into *Nicotiana benthamiana* plants infected by TYLCV, it showed antiviral activity by reducing symptoms and viral titer. The possible mechanism of action of this molecule is that AmPep1 binds with highly affinity to the virus origin of replication preventing further interaction with viral replicase and subsequent viral replication. To our knowledge, this is the first report, which uses a peptide derived directly from a plant source for direct treatment against TYLCV and applies it in an exogenous way without the generation of transgenic plants.

2. Material and methods

2.1. Cultivation of plants and bacteria

Seeds of *N. benthamiana* and *S. lycopersicum* cv. "Moneymaker" were disinfected with ethanol (70%) for one minute and then rinsed several times with sterile water. Plants were cultivated separately in pots with a mixture of perlite-vermiculite (1:1) and incubated in a growth chamber with a photoperiod of 18/6, light and darkness, respectively, at 28 °C and a relative humidity of 72%.

Cells of *Agrobacterium tumefaciens* C58C1 containing an 1.8-mer construct (1.8 genome units) of the genome for TYLCV-[EE-Imp-05-08] (Accession no. HF548826) [4b,4] cloned in pLH7000*, were grown in LB with antibiotics (rifampicin 50 μg/mL, streptomycin 300 μg/mL) for 24 h with shaking (28 °C,200 rpm). The preparation of inoculum for agroinoculation was essentially as described previously [5].

2.2. Synthetic peptide and oligonucleotides

The oligonucleotide OriRep (5'-CGTATAATATTACCGGATGGCCG CGC-3') [26] was used as viral target. This is a conserved DNA sequence of TYLCV and other geminiviruses, located in the origin of replication loop structure, which is recognized by the Rep protein to initiate replication. The short peptide RepApep (NIQGAKSSSDVKSYIDK; MW 1.84 KDa, pI 8.43), contains the domain of Rep binding to the TYLCV replication origin (sequence mentioned above) and was used as a positive control for interaction assays [8,26]. Oligonucleotide primers for rolling circle amplification (RCA) were designed to cover the complete genome of TYLCV (Table 1). For detection of systemic infection by TYLCV using standard PCR, the universal degenerate begomovirus primers AC1048 and AV548 were used [27].For TYLCV DNA

Table 1
Primers used for Rolling Circle Amplification to TYLCV. Primers cover forward and reverse sense of whole viral genome, * PTO modification for stabilization during reaction.

Primer name	Primer 5'— > 3'	%GC	Tm	Size
V2 173	TTCCTGAAT*C	40	28	10
V2 488	CAGGGCTTC*G	70	34	10
CP 519	GCCCATGTA*A	50	30	10
CP 593	CACGAGTAA*C	50	30	10
CP 694	GCAGAATCA*C	50	30	10
CP 796	ACTGGGCTC*A	60	32	10
CP 882	CCTCTGGAA*T	50	30	10
CP 1050	TAGATGCGT*A	40	28	10
C3 1105	TGAGTTTCT*G	36.36	30	10
C3 1473	GATTCACGC*A	50	30	10
C2 1247	CCAGTCTGA*G	60	32	10
C2 1608	CCTCTACGA*G	60	32	10
C1 1670	CTTCGTCTA*G	50	30	10
C1 2024	GAAGAGTGG*G	60	32	10
C1/C4 2150	AGTCCTTTG*G	50	30	10
C1/C4 2606	ATGCCTCGT*T	50	30	10