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The antimicrobial peptide snakin-2 is upregulated in the defense response of tomatoes (*Solanum lycopersicum*) as part of the jasmonate-dependent signaling pathway

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ABSTRACT

Antimicrobial peptides (AMPs) are produced by all living organisms and play an important role in innate immunity because they are readily available and non-specific against invading pathogenic microorganisms. Snakin-2 (SN2) from tomato is a short, cationic peptide that forms lethal pores in biomembranes of microbes. In plant cells, SN2 is produced as a prepeptide with a signal sequence for ER targeting and an acidic region to decrease toxicity in the producing organism. Gene expression analysis by qRT-PCR in tomato plants demonstrated that SN2 is constitutively expressed, mostly in leaves and flowers. After fungal infection, wounding, or external application of phytohormones (such as methyl jasmonate, MeJa) operating in the JA-dependent defense response, a systemic reaction with an elevated expression of the SN2 gene is triggered in all parts of tomato plants. Abiotic stress factors like extreme temperatures or dehydration do not affect SN2 expression. Upon wounding, the expression of SN2 and LoxD are strongly enhanced in tomato fruits. Furthermore, we provide evidence that the protein level of bioactive SN2 is also increased upon application of methyl jasmonate in tomato seedlings.

1. Introduction

Tomatoes (*Solanum lycopersicum*) are one of the most important crops worldwide and belong to the Solanaceae family, together with potatoes, peppers, and eggplant. It is fundamental to understand their physiology to cultivate healthy and productive tomato plants. Disease resistance and plant defenses are important areas of plant science (Liu et al., 2015; Reymond and Farmer, 1998). Many signaling pathways are connected in the complex network of innate plant immunity. Response to pathogen infection is stimulated by a series of complex signaling events, including identification of microbial or pathogen-associated molecular patterns (MAMPs or PAMPs), i.e., resulting in PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006).

Downstream of PTI and ETI activation, plant hormones play a central role in the innate immune system. Jasmonic acid (JA) and salicylic acid (SA) are acknowledged as major defense hormones; however, other hormones like abscisic acid, ethylene, auxins, gibberellins, brassinosteroids, cytokinins, and nitric oxide regulate the signaling network of host defense as well (Pieterse et al., 2012). Abscisic acid promotes JA signaling, but negatively regulates the SA response pathway and is likely to affect cross-talk between JA and SA. The gaseous hormone ethylene can act both negatively and positively on plant defense responses and often operates synergistically with JA to activate defense-related genes after pathogen infection (Bari and Jones, 2009). In addition, JA increases the expression of genes coding for enzymes that produce defense molecules such as secondary metabolites, like terpenoids, flavonoids, alkaloids, and other antimicrobial compounds (Bolouri Moghaddam et al., 2015; Halitschke and Baldwin, 2004).

Within this intricate network, a permanent antimicrobial defense is provided by antimicrobial peptides (AMPs) operating directly against pathogenic cells, including bacteria, fungi, and viruses (Zasloff, 2002). AMPs can be divided into several classes depending on overall charge, mode of action, secondary structure, and presence of disulfide bonds (Jenssen et al., 2006). Besides their direct antimicrobial activity, AMPs act in defense signaling pathways involving reactive oxygen species (ROS), mitogen-activated kinases (MAPK), sugar signaling, and hormone cross-talk (Bolouri Moghaddam et al., 2015).

Abbreviations: ABA, abscisic acid; AMP, antimicrobial peptide; COI1, coronatine insensitive 1; Cq, quantification cycle; EF1, elongation factor 1α; ETI, effector triggered immunity; GA, gibberellic acid; JA, jasmonic acid; LoxD, lipoxygenase D; MAMP, microbial-associated molecular patterns; MAPK, mitogen-activated kinase; MeJa, methyl jasmonate; PAMP, pathogen-associated molecular patterns; PTI, PAMP-triggered immunity; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; SA, salicylic acid; SN2, snakin-2.

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The snakin AMP family is characterized by six disulphide bonds in the C-terminus of the peptide, generated by 12 cysteines in highly conserved positions, which are essential both for secondary structure and biological activity (Harris et al., 2014; Nahirnak et al., 2012b). Two members of the snakin peptide family occur in tomato plants (snakin-1, SN1 and snakin-2, SN2); they share AMP characteristics like small molecular weight, cationic net charge, and six disulfide bonds in the mature peptide (Balaji and Smart, 2012). The homologue proteins from potato (*Solanum tuberosum*) as well as their gene expression levels were characterized previously (Berrocal-Lobo et al., 2002; Segura et al., 1999). The snakins act most likely through pore formation in the biomembrane of pathogenic cells, but the exact mode of action needs further investigation (Jenssen et al., 2006).

In this study, gene expression analysis of SN2 by qRT-PCR was executed *in planta*. It was recently shown that recombinantly produced SN2 exhibits strong antimicrobial activity against bacteria and fungi and causes agglomeration of microbial cells (Herbel et al., 2015). It seems that SN2 targets the phospholipid membrane of pathogenic bacteria or fungi non-specifically and causes agglomeration of cells in a dose-dependent manner which is supposed to arrest a further distribution of the pathogen inside the plant (Herbel et al., 2015).

In this study, SN2 gene expression was analyzed by qRT-PCR in tomato seedlings and plants in different growth stages upon treatment with several plant hormones, wounding, fungal infection, or abiotic stress factors. The aim was to explore the distribution of SN2 in tomato plants and find out whether its expression responds to biotic and abiotic stress conditions. Antimicrobial activity of protein extracts derived from hormone-treated seedlings was also examined.

2. Materials and methods

2.1. Bioinformatic calculations

The different forms of the SN2 peptide were analyzed using online calculators. Amino acid sequences of SN2 (GenBank: ADR32106.1) were copied from the NCBI website (http://www.ncbi.nlm.nih.gov/). For calculation of molecular weight and pI, the ExPASyCompute pI/Mw tool (http://web.expasy.org/compute_pi/) (Artimo et al., 2012), and for calculation of the net charge, the Protein Calculator v3.4 (http://protcalc.sourceforge.net/) were used.

2.2. Media, fungal strains, and tomato plant material

Tomato seeds (*Solanum lycopersicum*, cultivar Moneymaker) were purchased from Bingenheimer Saatgut AG (Echzell-Bingenheim, Germany). Parts of an adult tomato plant (cultivar Sungrape) were donated from market garden Lenz (Gärtnerei Lenz, Heidelberg, Germany), and tomato fruits (cultivar Philovita) were purchased there. *Fusarium solani* was used to induce defense responses upon fungal infection. The yeast *Saccharomyces cerevisiae* was used to demonstrate an agglomerating effect of tomato seedling extracts. Both fungi grew in SAB (Sabouraud dextrose) liquid broth (2% mycological peptone, 4% D(+)-glucose), and 10^5 cells per mL were used for infection and agglomeration studies, respectively.

2.3. Growth conditions of tomato plants

Tomato seedlings were cultivated in petri dishes on MS agar (Murashige and Skoog, 1962). Initially, seeds were surface-sterilized by incubation in 70% ethanol for 10 min, followed by incubation in 5% sodium hypochlorite for 25 min and rinsing five times in sterile water. Nine seeds per plate were positioned in the lower third of the dish, and the plates were placed onto a windowsill or in a dark box. The dishes were tilted at a 45° angle to allow upright growth of the seedlings for eight days. To obtain tomato plants, seedlings were planted into potting soil and grown in a greenhouse for three months. Afterwards, the plants were transferred into the garden for three more months to produce tomato fruits. Fruits, leaves, flowers, and parts of the stems of adult plants were harvested, and approximately 200 mg tissue was transferred into a 2 mL tube, immediately frozen in liquid nitrogen, and stored at −80 °C.

2.4. Treatments of seedlings and plants

Tomato seedlings were treated with the following phytohormones: MeJa (methyl jasmonate, 5 mM), SA (salicylic acid, 3 mM), ABA (abscisic acid, 3 mM), GA (gibberellic acid, 3 mM). Hormones were applied by coating the entire seedling with the corresponding hormone solution. For analyzing fungal infection, 10 μL of a spore solution of *Fusarium solani* (10^5 cells per mL) were applied to each seedling. Wounding was carried out by gently slicing seedlings using a scalpel. Samples were taken after 0 h, 1 h, 3 h, 6 h, and 24 h and were frozen immediately in liquid nitrogen and stored at −80 °C. Twelve-week-old plants were treated with 5 mM MeJa, wounding at the third leaf (counted from below), or water stressed along with wounding. Samples were taken 3 h after the treatment and compared with samples from untreated control seedlings. In order to compare gene expression values in varying plant organs, samples from adult plants (six months old) were taken from different parts of garden plants without any special treatment.

2.5. Primer design and control PCR reaction for qRT-PCR

The primers for quantitative real-time PCR were designed for the SN2 gene (accession number HQ008860) in this study. The forward primer spans intron 1 of the SN2 gene to ensure that only cDNA (complementary DNA) and no genomic DNA (gDNA) will be amplified. The reverse primer binds in exon 3 of the SN2 gene. A control primer pair (SN2c) was designed to execute a control PCR reaction with every cDNA sample to verify that all gDNA was digested. Therefore, the forward control primer was designed to bind in exon 1 and the reverse control primer in intron 1. If the control PCR reaction (initial denaturation: 95 °C for 5 min; 30-times repetition of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 30 s; final elongation: 72 °C for 4 min) shows no amplification, the sample was devoid of gDNA. If amplification occurred, there was gDNA remaining in the sample as in extracted RNA samples, which were used as positive controls. The primers for EF1 (elongation factor 1a), COI1 (coronatine insensitive 1), and LoxD (lipoxygenase D) were used previously (Lovdal and Lillo, 2009; Song et al., 2014) and are presented in Table 1.

2.6. RNA isolation, reverse transcription and quantitative real-time PCR

For RNA isolation, 100 mg of frozen plant sample were ruptured using the mixer mill MM400 (Retsch GmbH, Haan, Germany) 3-times for 30 s at 30 Hz, and the samples were frozen in liquid nitrogen between the milling steps to avoid thawing. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, and integrity was checked on a 1% agarose gel. The concentration was measured spectrophotometrically, and purity was checked with 260 nm/280 nm and 260 nm/230 nm quotients. For reverse transcription, 1 μg of RNA was used (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany) to produce cDNA. For quantitative real-time PCR (qRT-PCR), the cDNA was 10-fold-diluted in nuclease-free water. The SensiFAST™ SYBR No-ROX Kit (Bioline GmbH, Luckenwalde,