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Research article

Isolation and characterization of a *Solanum tuberosum* subtilisin-like protein with caspase-3 activity (*St*SBTc-3)

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ABSTRACT

Plant proteases with caspase-like enzymatic activity have been widely studied during the last decade. Previously, we have reported the presence and induction of caspase-3 like activity in the apoplast of potato leaves during *Solanum tuberosum*- *Phytophthora infestans* interaction. In this work we have purified and identified a potato extracellular protease with caspase-3 like enzymatic activity from potato leaves infected with *P. infestans*. Results obtained from the size exclusion chromatography show that the isolated protease is a monomeric enzyme with an estimated molecular weight of 70 kDa approximately. Purified protease was analyzed by MALDI-TOF MS, showing a 100% of sequence identity with the deduced amino acid sequence of a putative subtilisin-like protease from *S. tuberosum* (Solgenomics protein ID: PGSC0003DMP400018521). For this reason the isolated protease was named as *St*SBTc-3. This report constitutes the first evidence of isolation and identification of a plant subtilisin-like protease with caspase-3 like enzymatic activity. In order to elucidate the possible function of *St*SBTc-3 during plant pathogen interaction, we demonstrate that like animal caspase-3, *St*SBTc-3 is able to produce *in vitro* cytoplasm shrinkage in plant cells and to induce plant cell death. This result suggest that, *St*SBTc-3 could exert a caspase executer function during potato- *P. infestans* interaction, resulting in the restriction of the pathogen spread during plant–pathogen interaction.

1. Introduction

Plant immune system is broadly divided into two interconnected defensive layers. The first layer, called as pathogen associated molecular patterns (PAMPs)- triggered immunity (PTI) involves the recognition of conserved microbial elicitors (PAMPs) by plant pattern recognition receptors (PRRs) (Boller and Felix, 2009; Chinchilla et al., 2006; McLellan et al., 2013; Muthamilarasan and Prasad, 2013; Zipfel, 2008). The activation of PRRs results in active defense responses such as production of reactive oxygen species, callose deposition and synthesis of antimicrobial compounds (Clay et al., 2009; Reina-Pinto and Yephremov, 2009). However, there are some plant pathogen microorganisms able to attenuate PTI by the secretion of proteins (effectors) that manipulate host processes inducing the effector-triggered susceptibility (ETS) (McLellan et al., 2013; Muthamilarasan and Prasad, 2013; Hof et al., 2014; Jones and Dangl, 2006; Nimchuk et al., 2003). As a consequence of this, the second defense layer, called effector-triggered immunity (ETI), is activated. This mechanism comprises plant resistance (*R*) proteins which detect pathogen effectors, or their activity, often resulting in a localized cell death or hypersensitive response (HR) (McLellan et al., 2013; Muthamilarasan and Prasad, 2013; Hof et al., 2014; Jones and Dangl, 2006; Nimchuk et al., 2003).

Several extracellular proteases have been associated with host immunity and described during plant–pathogen interaction. Some examples are three papain-like proteases named as: 1) *Rcr*3 required for *C. fulvum* tomato resistance inducing hypersensitive reaction cell death; 2) *Nb*CathB, for *N. benthamiana* Cathepsin B, required for the development of the hypersensitive response, and 3) *St*C14, a potato defense papain-like cysteine protease (Shabab et al., 2008). Additionally, a positive correlation has been reported between the potato field resistance to *Phytophthora infestans* and the expression pattern of *St*AP1 and *St*AP3, for *Solanum tuberosum*

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aspartic proteases 1 and 3 (Guevara et al., 2005, 2002). In *Arabidopsis thaliana* susceptibility to *Pseudomonas* bacterial infection is enhanced by the knockdown of the pepsin-like protease CDR1 (constitutive disease resistance 1) (Xia et al., 2004). On the other hand, in *A. thaliana*, the overexpression of an extracellular subtilisin-like protein named as *At*SBT3.3 produce an increase of the plant disease resistance response to oomycete attack (Ramirez et al., 2013). Also, *Sl*P69B and C, two tomato subtilisin proteases, are induced in tomato after pathogen infection (Rawlings and Salvesen, 2013; Tornero et al., 1997).

During the last decade, special attention of scientists has been focused on plant proteases with caspase-like activities. Although plants have no gene orthologous to caspases in their genomes, caspase-like activities had been associated with plant programmed cell death (PCD) by the activity based protein profiling (ABPP) technology (Chichkova et al., 2010; Coffen and Wolpert, 2004; Fernandez et al., 2012; Kolodziejek and van der Hoorn, 2010). In this way, two apoplastic serine dependent proteases (subtilisin-like proteases) with caspase-6 activities and related to plant PCD have been purified and described, they are named as: saspases (Coffen and Wolpert, 2004) and phytaspases (Chichkova et al., 2010). Additionally, two caspase-like activities have been related to destructive and non-destructive vacuole mediated PCD (Hara-Nishimura and Hatsugai, 2011). Destruction mechanism is initiated by the cysteine protease named as vacuolar processing enzyme (VPE) with caspase-1 activity (Hara-Nishimura et al., 2005; Hatsugai et al., 2004). The non-destructive PCD involves the 20S proteasome subunit PBA1 with caspase-3 activity (Hara-Nishimura and Hatsugai, 2011; Hatsugai et al., 2009). On the other hand, two recombinant expressed barley legumains named as *Hv*Leg- 2 and -4 showed cysteine and caspase-like activities (Julian et al., 2013). A multifunctional role was assumed for these two cysteine peptidases, whereas *Hv*Leg-2 induces in leaves to biotic and abiotic stimuli, in seeds is induced by gibberellic acid, *Hv*Leg-4 respond in leaves to wounding and has an unknown role in the germinating seed (Julian et al., 2013). Recently, we have reported a positive correlation between apoplastic caspase-3 activity and potato field resistance to *P. infestans* infection, suggesting the induction and/or activation of apoplastic serine protease/s with caspase-3 activity during potato- *P. infestans* interaction (Fernandez et al., 2012).

In the present work, we describe the purification, identification and characterization of an apoplastic protease with caspase-3 activity from potato leaves infected with *P. infestans* (named as *St*SBTc-3). MALDI-TOF-MS identification of the isolated protein revealed that a *S. tuberosum* subtilisin like protein (Solgenomics protein ID: PGSC0003DMP400018521) is responsible of the caspase-3 activity. Additionally, we demonstrate that *St*SBTc-3 is able to induce *in vitro* cytoplasmic shrinkage and cell death on tomato cells. These results provide new evidences about the type of proteases involved in the plant defense response mechanism during potato- *P. infestans* interaction.

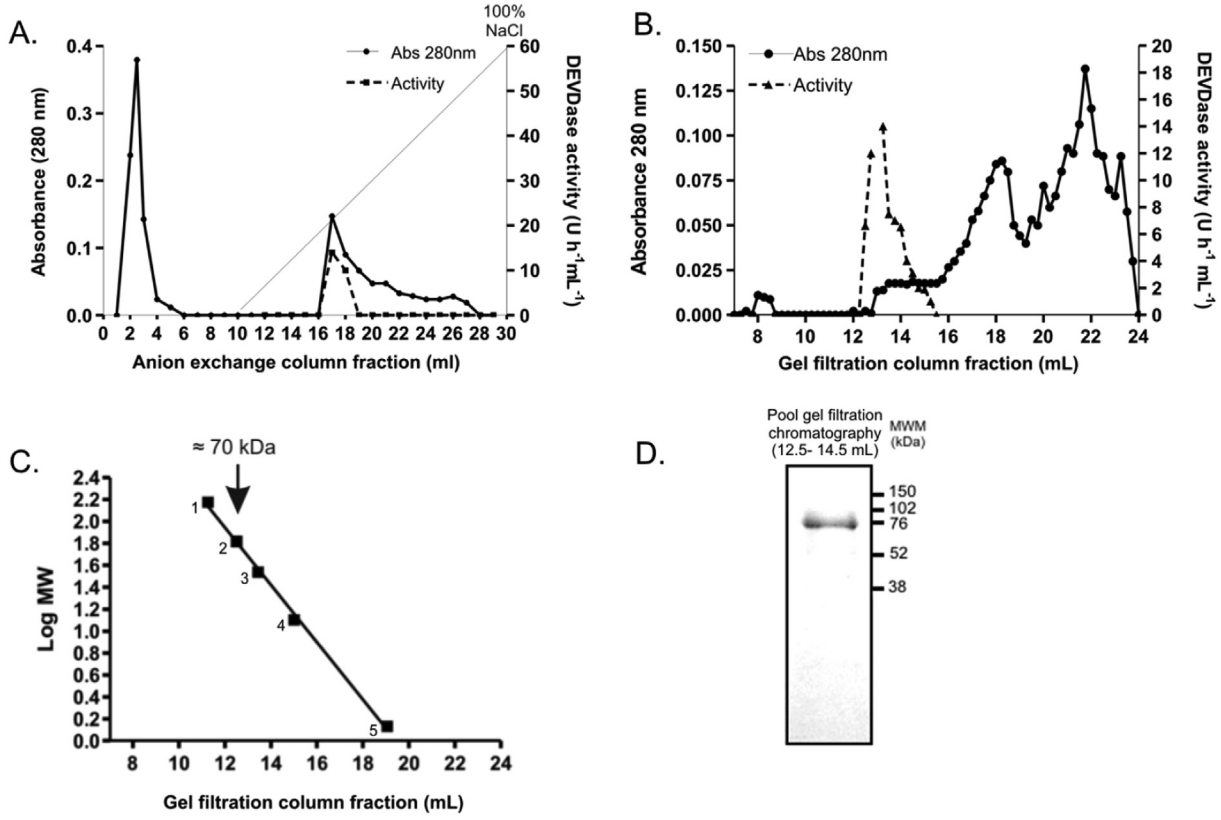

Fig. 1. Purification steps of a caspase-3 like protease from IWF of 48 h *P. infestans* infected potato leaves. IWF of 48 h infected potato leaves was subjected to anion exchange chromatography and eluted with a linear gradient of 0–500 mM NaCl (A). Fractions with caspase-3 like activity from MonoQ eluate were subjected to size exclusion chromatography (B). Molecular weight of the isolated protease in its native state was estimated using a calibration curve for the size exclusion chromatography: (1) IgG (150 kDa); (2) BSA (67 kDa); (3) Lactoalbumin (35 kDa); Cytochrome C (12.7 kDa) and Vitamin B12 (1.355 kDa). The arrow indicates logMW from the isolated protease (C). Fractions from the size exclusion chromatography with caspase-3 like activity were pooled, desalted and analyzed by 15% SDS-PAGE (D).