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Sweet potato *SPAP1* is a typical aspartic protease and participates in ethephon-mediated leaf senescence

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ABSTRACT

Plant aspartic proteases are generally divided into three categories: typical, nucellin-like, and atypical aspartic proteases based on their gene and protein structures. In this report, a full-length cDNA *SPAP1* was cloned from sweet potato leaves, which contained 1515 nucleotides (504 amino acids) and exhibited high amino acid sequence identity (*ca.* 51–72%) with plant typical aspartic proteases, including tomato LeAspP, potato StAsp, and wheat WAP2. SPAP1 also contained conserved DTG and DSG amino acid residues within its catalytic domain and plant specific insert (PSI) at the C-terminus. The cDNA corresponding to the mature protein (starting from the 66th to 311th amino acid residues) without PSI domain was constructed with pET30a expression vector for fusion protein and antibody production. RT-PCR and protein blot hybridization showed that *SPAP1* expression level was the highest in L3 mature leaves, then gradually declined until L5 completely yellow leaves. Ethephon, an ethylene-releasing compound, also enhanced *SPAP1* expression at the time much earlier than the onset of leaf senescence. Exogenous application of SPAP1 fusion protein promoted ethephon-induced leaf senescence, which could be abolished by pre-treatment of SPAP1 fusion protein with (a) 95 °C for 5 min, (b) aspartic protease inhibitor pepstatin A, and (c) anti-SPAP1 antibody, respectively. Exogenous SPAP1 fusion protein, whereas, did not significantly affect leaf senescence under dark. These data conclude that sweet potato SPAP1 is a functional typical aspartic protease and participates in ethephon-mediated leaf senescence. The SPAP1-promoted leaf senescence and its activity are likely not associated with the PSI domain. Interaction of ethephon-inducible components for effective SPAP1 promotion on leaf senescence is also suggested.

Introduction

Aspartic protease is widely distributed in living organisms and divided into three categories: typical, nucellin-like, and atypical aspartic proteases in plants (Faro and Gal, 2005; Chen et al., 2009b). The typical aspartic proteases contain an extra protein domain of *ca.* 50–100 amino acid residues known as plant specific insert (PSI) within the C-terminus, which is usually removed during protein maturation. The PSI domain showed no sequence homology with animal or microbial aspartic proteases (Runeberg-Roos and Tormakangas, 1991; Asakura et al., 1995), but exhibited high similarity with saposin-like proteins (Munford et al., 1995; Mutlu and Gal, 1999). The nucellin-like aspartic proteases encode proteins similar to nucellin, which was first detected in barley nucellar cells and did not contain PSI domain at the C-terminus (Chen and Foolad, 1997; Bi et al., 2005). Atypical aspartic proteases display intermediate features between the typical and nucellin-like sequences (Faro and Gal, 2005).

The typical aspartic proteases have been detected, purified and cloned from monocots, dicots and gymnosperms. Gene and protein structural analysis reveals that precursor protein contains a signal peptide and prosegment at the N-terminus, conserved catalytic domain in the middle, and PSI domain within the C-terminus

Abbreviations: CCI, chlorophyll content index; DAB, diaminobenzidine; MES, 2-(N-morpholino)ethanesulphonic acid; PSI, plant specific insert; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

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(Runeberg-Roos and Tormakangas, 1991; Asakura et al., 1995; Mutlu and Gal, 1999). Most aspartic proteases contain two conserved catalytic aspartic acid residues within the DTG/DSG motifs of the active sites. They are active in acidic pH and are specifically inhibited by aspartic protease inhibitor pepstatin A. Aspartic protease is synthesized as a single polypeptide chain of preproenzyme, which is sequentially processed first into proenzyme, then intermediate form, and finally two-chain mature enzyme during activation by autoproteolytic processing under low pH (Glathe et al., 1998; Ramalho-Santos et al., 1998; Mutlu and Gal, 1999; Domingos et al., 2000; Park et al., 2001).

The PSI domain contains six conserved cysteines, several hydrophobic residues and a consensus glycosylation site. During maturation, the PSI is processed and cleaved off from the enzyme (Simões and Faro, 2004). The physiological function of PSI is not clear. Plant aspartic proteases are known to reside in storage vacuoles. The vacuolar targeting of the precursor protein in the secretory pathway has been proposed in moss (Schaaf et al., 2004). Transient expression in tobacco protoplasts also demonstrated a role of barley phytepsin PSI in vacuolar sorting, and deletion of PSI resulted in secretion of the truncated form (Kervinen et al., 1999; Mutlu and Gal, 1999; Törmäkangas et al., 2001). In soybean, the PSI-deleted soyAP1, but not soyAP2 was also transported to the vacuole (Terauchi et al., 2006). In potato, the PSI domain of StAsp exhibited antimicrobial activity against the spore germination of two potato pathogens (*Fusarium solani* and *Phytophthora infestans*) and the cell growth of gram-positive bacteria (Mendieta et al., 2006; Munõz et al., 2010; Bryksa et al., 2011). Therefore, the PSI domains of various aspartic proteases may play diverse roles in different species.

The typical aspartic proteases have been reported in association with plant growth and development (Mutlu and Gal, 1999; Simões and Faro, 2004). The first plant aspartic protease was isolated from barley and exhibited higher expression level in the developing embryos and leaves (Runeberg-Roos and Tormakangas, 1991; Törmäkangas and Kervinen, 1994). Barley phytepsin (previously called HvAP) could process prolectin *in vitro* (Runeberg-Roos et al., 1994), and also highly expressed during autolysis of developing tracheary elements and sieve cells (Runeberg-Roos et al., 1998). In rice, oryzasin exhibited a milk-clotting activity (Asakura et al., 1997) and was remarkably expressed during seed development and germination (Asakura et al., 1995). In wheat, aspartic proteases purified from germinating seeds could digest gluten storage protein *in vitro* (Tamura et al., 2007). Sweet potato SPAP cloned from storage root was able to digest *in vitro* trypsin inhibitors, the major storage proteins in storage root (Huang et al., 2009). Cardosins from cardoon flowers exhibited milk-clotting activity for cheese manufacture (Veríssimo et al., 1996; Frazão et al., 1999). Cardosin A accumulated mainly in protein storage vacuoles of the stigmatic papillae. Cardosin B localized in the extracellular matrix of stylar transmitting tissue, and correlated with the programmed cell death events in the nucellus (Vieira et al., 2001).

Plant typical aspartic proteases are also associated with environmental stress responses (Mutlu and Gal, 1999; Simões and Faro, 2004). The tropical carnivorous plant *Nepenthes* accumulated acidic fluid with aspartic proteases in its trapping organs, which were involved in the digestion and utilization of insect proteins as a nitrogen source (An et al., 2002). Tomato LeAspP has been cloned from leaves and was inducible by wounding, jasmonate and systemin (Schaller and Ryan, 1996). Potato StAsp accumulated in leaves after *P. infestans* infection, and correlated with differential field resistance in cultivars to this pathogen (Guevara et al., 2005). In common bean, PvAP1 was up-regulated in leaves under water stress, which occurred earlier and stronger in the drought-susceptible cultivar (Contour-Ansel et al., 2010). Pineapple AcAP1 from fruit flesh showed a positive correlation with resistance to blackheart development under postharvest chilling stress (Raimbault et al., 2013).

Leaf senescence is the final stage of leaf development and affects photosynthesis efficiency and plant productivity (Lim et al., 2007). Endogenous and exogenous factors, including plant growth regulators, wounding, drought, salt, pathogen infection and insect attack have been reported to influence leaf senescence. Among these factors, ethylene has been considered as a key regulator of leaf senescence (Lim et al., 2007). Although many typical aspartic proteases have been previously cloned and characterized from diverse plant species, however, conclusive reports related to their physiological roles and functions are scarce. Researches about ethylene-inducible plant typical aspartic protease and its association with senescence and plant productivity are just at the beginning. In sweet potato, ethephon-promoted leaf senescence has been previously demonstrated (Chen et al., 2000). Significant changes, including leaf morphology, chlorophyll content reduction, Fv/Fm decline, H_2O_2 elevation, senescence-associated gene expression have been observed (Chen et al., 2003, 2004, 2006, 2009a, 2010b, 2011, 2012b, 2012c, 2013a; Afiyanti and Chen, 2014). Therefore, these parameters were compared and used as indicators of leaf senescence. Transgenic *Arabidopsis* plants ectopically expressing different type of cysteine proteases (*SPCP2*, *SPCP3* and *SPAE*, respectively) altered plant developmental characteristics and drought/salt stress resistance (Chen et al., 2008, 2010a, 2013b). In this report, a typical aspartic protease *SPAP1* has been cloned and characterized. Its association with ethephon-induced leaf senescence was also studied.

Materials and methods

Plant materials

The storage roots of sweet potato (*Ipomoea batatas* (L.) Lam.) were grown in a growth chamber at 28 °C/16 h day and 23 °C/8 h night cycle. Plantlets sprouted from the storage roots provided detached leaves for (1) temporal and spatial expression of aspartic protease *SPAP1*, (2) dark and ethephon treatments, (3) exogenous SPAP1 fusion protein treatment, and (4) repression of exogenous SPAP1 fusion protein effect by anti-SPAP1 antibody, pepstatin A, and boiled (95 °C for 5 min) treatments. Leaves were classified from L1 to L5 according to their size and developmental stage (Chen et al., 2012c). L1 was folded and unopened, whereas, L2 was open but not fully expanded leaves. L3 was fully-expanded mature leaves, however, L4 and L5 were partially and completely yellow senescent leaves, respectively. L3 was mainly collected from the leaf positions between 3 and 7 counted downward from the shoot apex. Samples from treatments mentioned above were individually collected for analysis of leaf morphology, chlorophyll content, Fv/Fm, diaminobenzidine (DAB) staining, H_2O_2 amount, RT-PCR and protein blot hybridization.

PCR-based subtractive hybridization and RACE PCR

Sweet potato L3 mature leaves were detached and treated with 1 mM ethephon for 6 and 24 h, then the samples were combined together for total RNA extraction, mRNA purification, PCR-based subtractive hybridization, and RACE PCR basically according to the report of Chen et al. (2003). Dark-treated leaves were used as a control. The mRNA was purified from total RNA with a purification kit (Promega), and the differentially expressed cDNAs was cloned with a PCR-selective subtractive hybridization kit (Clontech) and pGEM-T easy vector (Promega). The RACE PCR method with the Marathon cDNA amplification kit (Clontech) was used to isolate the 5′ and 3′ ends of the target aspartic protease cDNAs according to the protocols provided by the manufacturer. Finally, a primer pair (5′ primer: ATCTTCATCATTT GTGCAAGGTCAAGGAAA; 3′ primer: GAGAAGGCCATCATGCAGCTTCAGC AAAGC) was used to