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Sweet potato cysteine proteases *SPAE* and *SPCP2* participate in sporamin degradation during storage root sprouting

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ABSTRACT

Sweet potato sporamins are trypsin inhibitors and exhibit strong resistance to digestion by pepsin, trypsin and chymotrypsin. In addition, they constitute the major storage proteins in the sweet potato and, after degradation, provide nitrogen as a nutrient for seedling regrowth in sprouting storage roots. In this report, four cysteine proteases—one asparaginyl endopeptidase (*SPAE*), two papain-like cysteine proteases (*SPCP1* and *SPCP2*), and one granulin-containing cysteine protease (*SPCP3*)—were studied to determine their association with sporamin degradation in sprouting storage roots. Sporamin degradation became significant in the flesh of storage roots starting from week 4 after sprouting and this correlated with expression levels of *SPAE* and *SPCP2*, but not of *SPCP1* and *SPCP3*. In the outer flesh near the skin, sporamin degradation was more evident and occurred earlier than in the inner flesh of storage roots. Degradation of sporamins in the outer flesh was inversely correlated with the distance of the storage root from the sprout. Exogenous application of SPAE and *SPCP2*, but not *SPCP3*, fusion proteins to crude extracts of the outer flesh (*i.e.*, extracted from a depth of 0.3 cm and within 2 cm of one-week-old sprouts) promoted *in vitro* sporamin degradation in a dose-dependent manner. Pre-treatment of SPAE and *SPCP2* fusion proteins at 95 °C for 5 min prior to their application to the crude extracts reduced sporamin degradation. These data show that sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* participate in sporamin degradation during storage root sprouting.

1. Introduction

Sweet potato is a gamopetalous dicot that belongs to the order Polemoniales and the family Convolvulaceae (Sihachakr et al., 1997). It is also an important food crop in the tropics and some temperate zones, and has been grown in Taiwan since it was first imported in the 17th century. The leaves and storage roots are the edible portions. The nutritional content of sweet potato is similar to that of cereal and potato. It is also rich in the vitamin B complex, vitamin C, β-carotenoids, calcium, multiple other minerals (Yang et al., 1975; Hattori et al., 1985), total phenolic and flavonoid compounds (Huang et al., 2004c), and dietary fiber (Eastwood and Kritchevsky, 2005; Anderson et al., 2009).

Sweet potato sporamin is the most abundant protein in storage roots. It is encoded by a gene family of more than 10 different nuclear genes, which consist of closely related polypeptides with molecular masses near 22 kDa (*ca.* 21–23 kDa) (Maeshima et al., 1985; Hattori et al., 1989). Sporamin consists of approximately 80% soluble proteins (Maeshima et al., 1985; Shewry, 2003) and is mainly localized in the vacuole (Hattori et al., 1988) of sweet potato storage roots. Unlike many plant vacuolar proteins, sporamin is not glycosylated (Maeshima et al., 1985). It is synthesized as a preproprotein: its mRNA is translated by membrane-bound polysomes into a larger precursor (Hattori et al., 1985) through the activity of an N-terminal signal peptide and a pro-segment sequence (Murakami et al., 1986; Hattori et al., 1989) that are removed during transport into the endoplasmic reticulum and subsequent protein maturation (Hattori et al., 1987; Matsuoka et al., 1990; Nakamura and Matsuoka, 1993).

Maeshima et al. (1985) reported the purification of two major sporamins (sporamin A and sporamin B) with similar molecular

Abbreviations: DTT, dithiothreitol; RT-PCR, reverse transcription-polymerase chain reaction.

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masses using SDS-PAGE, amino acid compositions, immunological properties, and peptide maps. When separated on SDS-PAGE without the reduction of disulfide bonds by dithiothreitol (DTT) or β-mercaptoethanol, sporamins A and B migrate to different positions with molecular masses close to 31 kDa and 22 kDa, respectively. Molecular evidence supporting the existence of two major sporamin subfamilies originated from the direct cloning and sequencing of their corresponding cDNAs (Hattori et al., 1985; Murakami et al., 1986; Hattori et al., 1989). In total, 22 and 27 cDNAs corresponding to sporamins A and B, respectively, have been cloned and sequenced (Hattori et al., 1989). An approximate 94–98% amino acid sequence similarity has been observed among the members within the same subfamily, and a similarity of about 82–84% has been found between the members of different subfamilies (Hattori et al., 1989). Sporamin A without reduction of disulfide bonds displays a molecular mass near 31 kDa, which drops to about 22 kDa (similar to the molecular mass of sporamin B) following the reduction of disulfide bonds (Maeshima et al., 1985; Shewry, 2003).

Sweet potato sporamin typically exhibits trypsin inhibitor activity (Yeh et al., 1997a; Hou et al., 2001). The possible physiological roles and functions of sweet potato sporamins have been reported in association with defense against insect attack, pathogen infection, and abiotic stress tolerance (Senthilkumar and Yeh, 2012). Compared with a non-transformant control, transgenic tobacco plants ectopically expressing sweet potato sporamin show higher resistance against leaf damage caused by *Spodoptera litura* (Yeh et al., 1997b) and *Helicoverpa armigera* insect larvae (Senthilkumar et al., 2010), bacterial soft rot disease caused by *Erwinia carotovora*, damping-off disease due to *Pythium aphanidermatum* infection (Senthilkumar et al., 2010), and abiotic salt/osmotic stress (Chen et al., 2014). Similar results have also been observed for transgenic cauliflower plants with ectopic expression of sweet potato sporamin against leaf damage caused by *S. litura* and *Plutella xylostella* insect larvae (Ding et al., 1998).

Sweet potato sporamins also function as the major storage proteins and provide nutritional resources for seedling growth in sprouting storage roots (Lin and Tsu, 1987). Therefore, sporamin degradation provides nitrogen as a nutrient for the regrowth of sprouts from storage roots. Sporamins typically exhibit resistance to protease digestion, including pepsin, trypsin, and chymotrypsin (Maloney et al., 2014). Specific mechanisms and proteases have been reported in association with sporamin degradation. Sporamins with a molecular mass of approximately 38 kDa from the sweet potato cv. Tainong 57 are resistant to protease degradation (Huang et al., 2005, 2009). With the addition of DTT, however, they are reduced to the 22-kDa sporamin and become sensitive to protease digestion (Huang et al., 2004a,b, 2005, 2009). A cloned sweet potato aspartic protease SPAP fusion protein has been found to promote *in vitro* degradation of sporamins with the addition of DTT (Huang et al., 2009). Other research has focused on thioredoxin *h2*, the cDNA of which has recently been cloned (Huang et al., 2004a). Association of the cloned NADPH/thioredoxin system with the reduction and *in vitro* degradation of sporamins during storage root sprouting has also been demonstrated (Huang et al., 2004a,b, 2005). In addition, a cloned papain-like cysteine protease *SPCPRPP* from a sweet potato storage root also specifically promotes *in vitro* degradation of the reduced 22-kDa sporamin in the presence of DTT or the NADPH/thioredoxin system (Huang et al., 2005). Therefore, reduction of sporamins resulting from DTT or the NADPH/thioredoxin system seems to be necessary and sufficient prior to their degradation by particular proteases.

We have previously reported the cloning and characterization of several ethephon-inducible cysteine proteases, including asparaginyl endopeptidase *SPAE* (Chen et al., 2004), papain-like cysteine proteases *SPCP1* (Chen et al., 2009) and *SPCP2* (Chen et al., 2010), and granulin-containing cysteine protease *SPCP3* (Chen et al., 2006). Transgenic *Arabidopsis* plants expressing sweet potato papain-like cysteine protease *SPCP2* (Chen et al., 2010) or granulin-containing cysteine protease *SPCP3* (Chen et al., 2013) possess enhanced resistance (*SPCP2*) or sensitivity (*SPCP3*) to abiotic drought stress. Ectopic expression of asparaginyl endopeptidase *SPAE* (Chen et al., 2008) and papain-like cysteine protease *SPCP2* (Chen et al., 2010), but not granulin-containing cysteine protease *SPCP3* (Chen et al., 2013), alter the seed and silique development in such transgenic *Arabidopsis* plants. In this report, we determined whether these cysteine proteases participate in the degradation of sweet potato sporamins in sprouting storage roots.

2. Materials and methods

2.1. Storage root treatment

Storage roots of sweet potato (*Ipomoea batatas* (L.) Lam.) were incubated in a growth chamber with a 28 °C/16-h day and 23 °C/8-h night cycle, and individually collected and recorded on weeks 0–5 after sprouting for analysis of (1) storage root morphology, (2) temporal gene expression of cysteine proteases *SPAE*, *SPCP1*, *SPCP2* and *SPCP3*, and (3) total soluble protein and sporamin contents in the flesh of storage roots. For temporal gene expression, each sample was further divided into the sprout, skin and flesh portions for total RNA extraction and RT-PCR analysis of cysteine proteases (*SPAE*, *SPCP1*, *SPCP2*, and *SPCP3*) (Chen et al., 2012) and metallothionein-like protein *G14* (an internal control) (Chen et al., 2003). For total soluble protein and sporamin contents in the flesh of storage roots, the skin of each sample was peeled off, and the flesh was further divided into the outer and inner flesh corresponding to the portions exterior and interior to 70% of the radius, respectively. Samples from the treatments above were ground in liquid nitrogen using mortars and pestles, and the powder of each sample was mixed in a 1:2 ratio (w/v) with an extraction buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1% w/v ascorbate, and 0.5 M sucrose). The mixtures were centrifuged at 13,000 × *g*, 4 °C for 20 min, after which the supernatants were transferred to new centrifuge tubes. The total soluble protein and sporamin contents of the (1) whole, (2) outer, and (3) inner flesh of the storage roots during sprouting were analyzed using the Bradford method (1976), SDS-PAGE (Chen et al., 2012), and ImageJ software.

To determine the effect of distance from the sprout on the total soluble protein and sporamin contents in the outer flesh of storage roots, samples were collected on week 1 after sprouting. The skin and sprout portions of each sample were peeled and removed, and the outer flesh (*ca.* 0.3 cm thick) was individually collected at certain distances (0–0.5, 0.5–1.0, 1.0–2.0, and >2 cm) away from the one-week-old sprout. The total soluble protein and sporamin contents of each outer flesh sample were analyzed and compared using the Bradford method (1976), SDS-PAGE (Chen et al., 2012), and ImageJ software.

2.2. Induction, overexpression, and purification of cysteine protease (SPAE, SPCP2 and SPCP3) fusion proteins from E. coli BL21 (DE3)

The recombinant PET-32 Xa/LIC vectors (Novagen) harboring different cysteine proteases (*SPAE*, *SPCP2* and *SPCP3*) were previously constructed and transferred into *Escherichia coli* BL21 (DE3) competent cells for fusion protein production (Chen et al., 2004, 2006; Huang et al., 2005). Transformed BL21 (DE3) cells (after induction with 1 mM IPTG for 5 h) were collected and centrifuged at 10,000 x *g* for 10 min and re-suspended in phosphate buffer saline (PBS; 137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4 pH 7.4) for sonication. Fusion proteins were extracted from