12

Expression of Sweet Potato Senescence-Associated Cysteine Proteases Affect Seed and Silique Development and Stress Tolerance in Transgenic *Arabidopsis*

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1. Introduction

Leaf is in general the main site of photosynthesis and acts as a carbohydrate source for nutrients to support the growth in sink organs of plants. Therefore, its longevity and senescent level may affect the photosynthesis efficiency and thus crop yield. There are endogenous and exogenous factors affecting leaf senescence, including plant growth regulators, sucrose starvation, dark, cold, drought, salt, wound, pathogen infection and insect attack (Yoshida, 2003; Lim et al., 2007). Leaf senescence is the final developmental stage of leaves and has been considered as a type of programmed cell death. During leaf senescence, it is not only a degradative process but also a recycling one. Therefore, macromolecules and organelles can be degraded into small molecules, salvaged and mobilized from the senescent cells to other sinks, such as young leaves, developing seeds, or storage tissues (Buchanan-Wollaston, 1997; Quirino et al., 2000).

In sweet potato, several morphological, biochemical and physiological changes have also be observed during leaf senescence, including leaf yellowing, decrease of chlorophyll contents, reduction of photochemical Fv/Fm, elevation of H_2O_2 amount, increase of plastoglobuli number in chloroplast, activation of senescence-associated gene expression, and finally cell death (Chen et al., 2000; Chen et al., 2003; Chen et al. 2010a). Several full-length cDNAs encoding putative isocitrate lyase, papain-like cysteine proteases and asparaginyl endopeptidase, have been cloned from senescent leaves (Chen et al., 2000, 2004, 2006, 2008, 2009, 2010b), which likely play roles in association with lipid degradation and gluconeogenesis, and protein degradation and re-mobilization. These data support the occurrence of macromolecule and organelle degradation into small molecules for recycling and re-mobilization during sweet potato leaf senescence.

During senescence, breakdown of leaf proteins by proteases provides a large pool of cellular nitrogen for recycling (Makino & Osmond, 1991). In plants, different degradation pathways

have been described and the vacuolar degradation pathway is assumed to be involved in bulk protein degradation by virtue of the resident proteases in the vacuole (Vierstra, 1996). There are two types of vacuoles described in plants: the storage vacuole and the lytic vacuole (Marty, 1999). Protein storage vacuoles are found in seed tissues and accumulate proteins that are re-mobilized and used as the main nutrient resource for germination (Senyuk et al., 1998; Schlereth et al., 2001). Most cells in vegetative tissues have lytic vacuoles, containing a wide range of proteases in an acidic environment. Substrate proteins must be transported and sequestered into these lytic vacuoles before degradation. Therefore, senescence-associated vacuoles are lytic vacuoles and involved in the degradation of imported chloroplast proteins in tobacco leaves (Martı´nez et al., 2008).

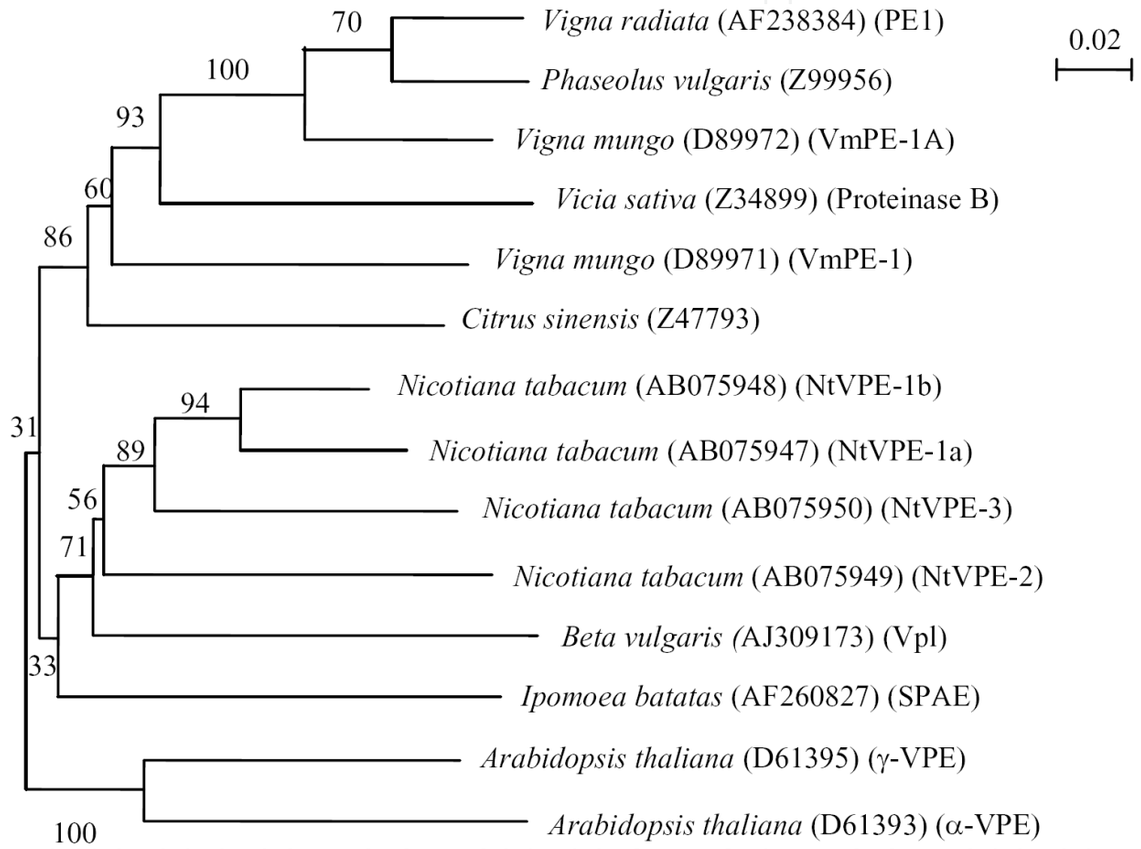


Fig. 1. Phylogenetic tree analysis of plant asparaginyl endopeptidases (Adapted and Modified from Chen et al., 2004).

The molecular mechanisms for vacuolar protein degradation and nutrient recycling pathway in senescent leaves are generally not clear. Phylogenetic tree analysis indicated that sweet potato asparaginyl endopeptidase (SPAE) exhibited high amino acid sequence identities and closely-related association with plant vacuolar processing enzymes (VPEs) or legumains, including legumain-like protease LLP of kidney bean (*Phaseolus vulgaris*), legumain-like protease VsPB2 of vetch (*Vicia sativa*), vacuolar processing enzymes of *Arabidopsis thaliana*, and asparaginyl endopeptidases VmPE-1 of *Vigna mungo* (Fig. 1). Sweet potato papain-like cysteine protease (SPCP2) also showed high amino acid sequence

identities and closely-related association with a subgroup of cysteine proteases, including *Actinidia deliciosa* CP3, *Arabidopsis thaliana* RD19, *Brassica oleracea* BoCP4, *Phaseolus vulgaris* CP2, *Solanum melongena* SmCP, *Vicia sativa* CPR2, and *Vigna mungo* SH-EP (Fig. 2). These data suggest the possible physiological roles and functions for *SPAE* and *SPCP2* related to these mentioned vacuolar processing enzymes and papain-like cysteine proteases, respectively.

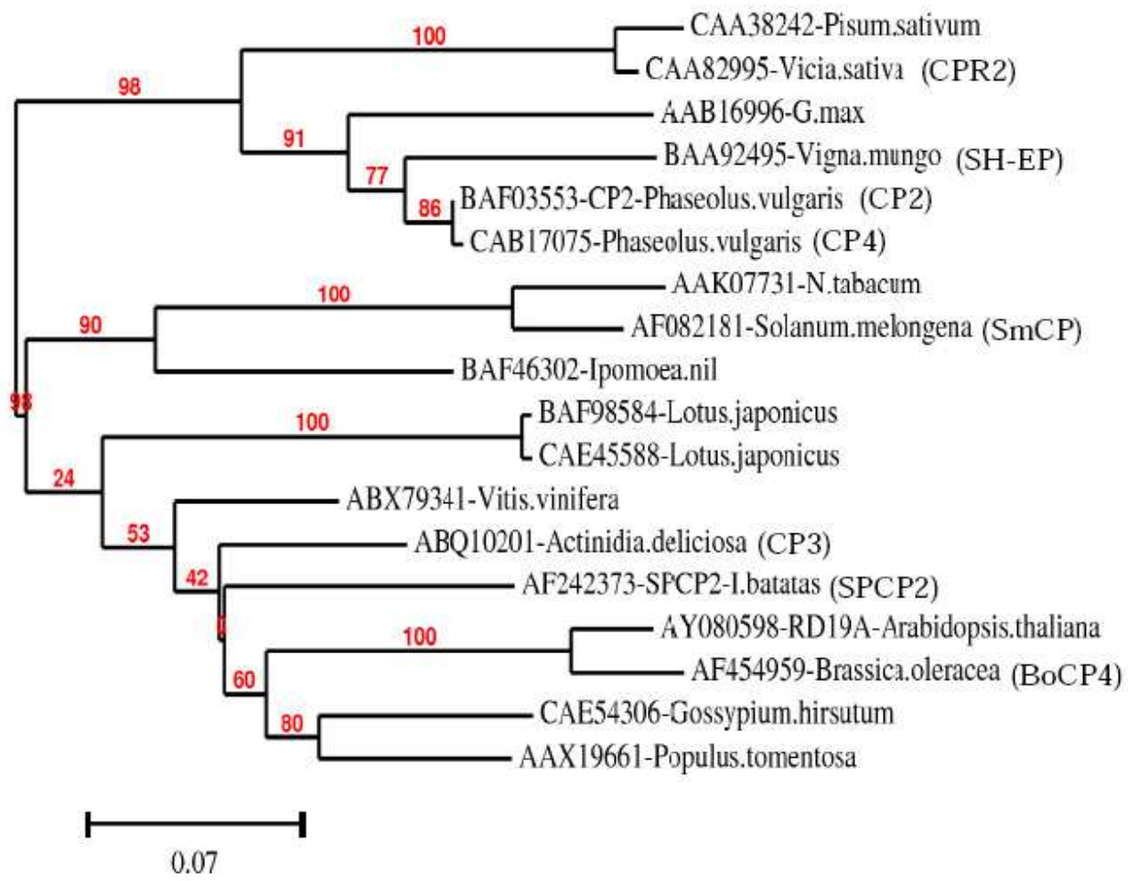


Fig. 2. Phylogenetic tree analysis of plant papain-like cysteine proteases (Adapted from Lin, 2010).

2. Association of vacuolar processing enzyme and papain-like cysteine protease with seed storage globulin protein degradation

Vacuolar processing enzyme is a novel group of cysteine endopeptidase and has recently been found in seeds. The enzyme exhibits strict cleavage specificity for the peptide bonds of seed globulin storage proteins with asparagines at the P1 position, and is called asparaginyl endopeptidase (Ishii, 1994). The substrate specificity was observed with purified asparaginyl endopeptidases from developing seeds of castor bean (Hara-Nishimura et al., 1991) and soybean (Scott et al., 1992; Hara-Nishimura et al., 1995), from mature seeds of jack bean (Abe et al., 1993), and from germinating seeds of vetch (Becker et al., 1995). Many seeds accumulate protein reserves in the storage vacuoles, and a number of these proteins

undergo proteolytic cleavage, including the 7S and 11S seed storage globulins (Müntz & Shutov, 2002). The 11S seed globulin storage proteins are synthesized as precursors and are cleaved post-translationally in storage vacuoles by an asparaginyl endopeptidase during seed development (Ishii, 1994). In castor bean and soybean seeds, vacuolar processing enzymes were found in the protein bodies and likely associated with the conversion of proproteins into their corresponding mature forms in vacuoles (Hara-Nishimura et al., 1991; Shimada et al., 1994).

Asparaginyl endopeptidases also play a role with bulk degradation and mobilization of storage proteins during seed germination and seedling growth. For example, the asparaginyl endopeptidase, which was also called "legumain-like proteinase" (LLP), was isolated from cotyledons of kidney bean (*Phaseolus vulgaris*) seedlings. It was the first proteinase ever known which *in vitro* extensively degraded native phaseolin, the major storage globulin of this grain legume (Senyuk et al., 1998). In vetch (*Vicia sativa*) seeds, the legumain-like VsPB2 and proteinase B together with additional papain-like cysteine proteinases were responsible for the bulk breakdown and mobilization of storage globulins during seed germination (Schlereth et al., 2000). In *Arabidopsis*, the seed protein profiles were compared between the wild type and a seed-type vacuolar processing enzyme β*VPE* mutant using a two dimensional gel/mass spectrometric analysis. A significant increase in accumulation of several legumin-type globulin propolypeptides was found in β*VPE* mutant seeds (Gruis et al., 2002).

For papain-like cysteine protease, the vacuolar SH-EP is synthesized in cotyledons of germinated *Vigna mungo* seeds and is responsible for the degradation of seed globulin storage proteins accumulated in protein bodies. In *Vicia faba* (vetch), globulins such as legumin and vicillin are major seed storage proteins present in the protein bodies of cotyledon, radicle, axis, and shoots. Papain-like cysteine protease such as CPR2 and CPR4 are found in cotyledon and axis of dry and imbibed seeds. Gene expression studies concluded that storage globulin mobilization in germinating vetch seeds is started by the stored cysteine proteases (CPRs), however, the bulk globulin mobilization is mediated by *de novo* synthesized CPRs (Schlereth et al., 2000; Schlereth et al., 2001; Tiedemann et al., 2001). These data suggest that papain-like cysteine proteases may play physiological roles and functions in association with seed storage globulin protein degradation and mobilization during seed germination and seedling growth. In addition to the possible physiological function with seed storage globulin protein degradation, papain-like cysteine proteases have also been implied to play a role in cope with environmental cues. For example, a dehydration-responsive papain-like cysteine protease RD19 was cloned and results showed that its expression was strongly induced under high-salt and osmotic stress conditions, which suggests a possible physiological role of RD19 in association with the regulation of plant cell osmotic potential in *Arabidopsis thaliana* (Koizumi et al., 1993; Xiong et al., 2002). In broccoli, the florets showed water loss during post-harvest storage. Gene expression of papain-like cysteine proteases BoCP4, which exhibited high amino acid sequence identity with *Arabidopsis* RD19, was also found to be dehydration-responsive and was repressed by water and sucrose (Coupe et al., 2003).

Many vacuolar enzymes are synthesized as pro-proteins and become active after proteolytically processed. In seed storage tissues, specific endoplasmic reticulum (ER)-derived compartments containing precursors of cysteine proteases have been described

(Chrispeels & Herman, 2000; Toyooka et al., 2000; Hayashi et al., 2001; Schmid et al., 2001). Germination of the seeds induces the expression and processing of those proteases into the mature active forms, which in turn participate in the degradation of cellular materials in storage tissues and provide nutrients to the growing embryo. The mechanism of asparaginyl endopeptidases (VmPE-1) and papain-like cysteine protease (SH-EP) associated with bulk seed storage globulin protein degradation has been studied in *Vigna mungo*. The vacuolar cysteine protease SH-EP is synthesized in cotyledons of germinated *Vigna mungo* seeds with an N-terminal and a C-terminal prosegments (Okamoto & Minamikawa, 1999; Okamoto et al., 1999). Okamoto & Minamikawa (1995) isolated a processing enzyme, designated VmPE-1. VmPE-1 is a member of the asparaginyl endopeptidases and is involved in the post-translational processing of SH-EP. In addition, the cleavage sites of the *in vitro* processed intermediates and the mature form of SH-EP were identical to those of SH-EP purified from germinated cotyledons of *V. mungo*. Therefore, it is proposed that the asparaginyl endopeptidase (VmPE-1)-mediated processing functions mainly in the activation of proSH-EP during seed germination (Okamoto et al., 1999). The activated SH-EP plays a major role in the degradation of seed storage proteins accumulated in cotyledonary vacuoles of *Vigna mungo* seedlings (Mitsuhashi et al., 1986). These reports demonstrate a role of asparaginyl endopeptidase associated with papain-like cysteine protease in the bulk breakdown and mobilization of storage globulins during seed germination.

3. Characterization of sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2*

Recently, similar compartments have also been described in vegetative tissues of *Arabidopsis* (Hayashi et al., 2001). These precursor protease vesicles derived from ER are plant specific compartments and contain vesicle-localized vacuolar processing enzyme (γVPE) precursor, which is critical for maturation of the vacuolar protease AtCPY. The vacuolar protease AtCPY in turn participates in the degradation of cellular components including vacuolar invertase AtFruct4 and various proteins in organs undergoing senescence in *Arabidopsis* (Rojo et al., 2003). A mechanism of senescence-induced activation of vesicle-localized vacuolar processing enzyme precursor by releasing its inactive form from the precursor protease vesicle into the acidic lumen of the vacuole is suggested. This activation triggers the processing of downstream proteases for protein degradation and recycling in senescing tissues (Rojo et al., 2003). These data suggest sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* may also play roles with functions related to protein degradation for nutrient remobilization during leaf senescence.

3.1 *SPAE*

SPAE had been cloned from senescent leaves with PCR-selective subtractive hybridization and exhibited high amino acid sequence homologies to seed vacuolar legumains/asparaginyl endopeptidases of kidney bean (*Phaseolus vulgaris*), spring vetch (*Vicia sativa*) and jack bean (*Canavalia ensiformis*) (Chen et al., 2004). The conserved catalytic residues (His and Cys) and central β-strands that supported the catalytic residues of human and mouse legumains (Chen et al., 1998) were also found in SPAE, plant legumain/asparaginyl endopeptidase, vacuolar processing enzymes, and the other cysteine proteases (Chen et al., 2004).

Asparaginyl endopeptidase *SPAE* encoded a pre-proprotein precursor, which contained a putative mature protein (325 amino acid residues) and an N-glycosylation site at its C-terminus. The deduced molecular mass of mature SPAE protein was, thus, likely between 33 and 36 kDa that detected by protein gel blot with polyclonal antibody against putative SPAE protein (Chen et al., 2004). Asparaginyl endopeptidase is an atypical cysteine endopeptidase with a reported insensitivity to the inhibitor L-3-carboxy-2,3-trans-epoxypropionyl-leucyl-amino(4-guanidino)butane (E-64) (Okamoto & Minamikawa, 1999). A cysteine protease activity band with a molecular mass near 36 kDa similar to the protein gel blot results was also detected and exhibited insensitivity to E-64 inhibitor (Chen et al., 2004). These data provide indirect evidence to support the existence of asparaginyl endopeptidase in senescent leaves.

In sweet potato, *SPAE* gene expression level is higher in dark- or ethephon-treated leaves similar to that in natural senescent leaves. Hormones such as jasmonic acid (JA) and abscisic acid (ABA) also caused the decrease of chlorophyll contents in treated leaves; whereas, did not significantly alter *SPAE* gene expression level compared to that of untreated dark control in mature green leaves within a 3-day period (Chen et al., 2004). These data suggest that *SPAE* is a senescence-associated gene and its expression in natural or induced senescent leaves is likely controlled by ethylene, but not by JA and ABA.

3.2 *SPCP2*

SPCP2 had been cloned from senescent leaves with PCR-selective subtractive hybridization. The open reading frame of *SPCP2* contained 1101 nucleotides (366 amino acids) and exhibited high amino acid sequence identities with a subgroup of vacuolar cysteine proteases including *Actinidia deliciosa* CP3, *Arabidopsis thaliana* RD19, *Brassica oleracea* BoCP4, *Phaseolus vulgaris* CP2, *Vicia sativa* CPR2, and *Vigna mungo* SH-EP (Chen et al., 2010). These data suggest an intracellular localization of SPCP2 in the vacuole. For SH-EP, a C-terminal KDEL sequence (endoplasmatic retention signal) was proved to be associated with its vacuole-targeting (Okamoto et al., 2003). However, no significant C-terminal KDEL sequence was found for SPCP2. For RD-19, a vacuolar localization was also suggested. However, it can be re-localized to nucleus in the presence of PopP2, an avirulent gene product of *R. solanacearum* (Bernoux et al., 2008; Poueymiro & Genin, 2009). Therefore, it is possible to assume that different vacuolar targeting mechanisms and signal peptides are involved and associated with different related cysteine protease genes.

SPCP2 gene expression was enhanced in natural senescent leaves and can be induced by dark, ethephon, ABA and JA (Chen et al., 2010). Buchanan-Wollaston et al. (2005) analyzed gene expression patterns and signal transduction pathways of senescence in *Arabidopsis* induced by different factors. Transcriptome analysis demonstrated that pathways such as dark, ethylene, and JA are all required for gene expression during developmental senescence. Genes associated with essential metabolic processes such as degradation of proteins and peptides and nitrogen mobilization can utilize alternative pathways for induction (Buchanan-Wallaston et al., 2005). Therefore, a possible explanation which is likely associated with multiple signal transduction pathways is suggested for the induction of sweet potato *SPCP2* gene expression by different factors, including development, dark, ABA, ethephon and JA.

4. Ectopic expression of asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* in transgenic *Arabidopsis*

Sweet potato full-length cDNAs of asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* were individually constructed in the T-DNA portion of recombinant pBI121 vector under the control of *CaMV 35S* promoter for transformation of *Arabidopsis* with *Agrobacterium*–mediated floral dip transformation method (Clough & Bent, 1998). Transgenic *Arabidopsis* plants ectopically expressing sweet potato asparaginyl endopeptidase *SPAE* (Chen et al., 2008) or papain-like cysteine protease *SPCP2* (Chen et al., 2010) were produced, identified and characterized.

4.1 Expression of sweet potato asparaginyl endopeptidase *SPAE* altered seed and silique development in transgenic *Arabidopsis*

Three transgenic *Arabidopsis* plants were isolated and identified with floral dip transformation method (Clough & Bent, 1998). Genomic PCR and protein gel blot analysis confirmed that these *Arabidopsis* plants (YP1, YP2 and YP3) were transgenic and sweet potato *SPAE* gene was expressed and properly processed into mature form with a predicted molecular mass near 36 kDa (Chen et al., 2008). Similar results have also been observed and reported for various plant vacuolar processing enzymes, including *Vigna mungo* VmPE-1 (Okamoto et al., 1999), *Arabidopsis* βVPE (Gruis et al., 2002), *Arabidopsis* γVPE (Kuroyanagi et al., 2002; Rojo et al., 2003). These data suggest that transgenic *Arabidopsis* plants may use similar mechanisms for sweet potato SPAE processing, and thus can produce mature sweet potato SPAE protein products.

Transgenic *Arabidopsis* plants exhibited earlier floral transition from vegetative growth and leaf senescence (Chen et al., 2008). Early transition of vegetative phase to reproductive phase has been considered as a type of senescence. The reasons and mechanisms that sweet potato *SPAE* gene expression can promote earlier floral transition and enhance senescence in transgenic *Arabidopsis* plants are not clear. However, Raper et al. (1988) and Rideout et al. (1992) hypothesized that floral transition is stimulated by an imbalance in the relative availability of carbohydrate and nitrogen in the shoot apical meristem. Barth et al. (2006) suggest that the flowering phenotype is likely linked to the endogenous ascorbic acid content. Degradation and removal of flowering repressor(s) by ectopic SPAE expression in transgenic *Arabidopsis* plants provides another possibility.

Expression of sweet potato *SPAE* in transgenic *Arabidopsis* plants caused altered development of seed and silique, elevated percentage of incompletely-developed siliques, and fewer silique numbers per plant than that of control (Figs. 3 and 4). The reasons for altered phenotypic characteristics in transgenic *Arabidopsis* by sweet potato SPAE expression are not clear. However, sweet potato SPAE is in close association with plant vacuolar processing enzymes of seeds from phylogenetic analysis (Chen et al., 2004). Vacuolar processing enzymes have been reported to be in association with the degradation and mobilization of globulin storage proteins during seed germination and seedling growth in *Phaseolus vulgaris* (Senyuk et al., 1998), *Vigna mungo* (Okamoto et al., 1999), *Vicia sativa* (Schlereth et al., 2000; Schlereth et al., 2001), and *Arabidopsis thaliana* (Gruis et al., 2002). In *Vigna mungo*, VmPE-1 has been demonstrated to increase in the cotyledons of germinating seeds and was involved in the post-translational processing of a vacuolar cysteine endopeptidase, designated SH-EP, which degraded seed storage proteins (Okamoto &

Minamikawa, 1999). A possible explanation that inappropriate pre-degradation of globulin-type storage protein during seed development and maturation by constitutively expressed sweet potato SPAE in transgenic *Arabidopsis* is suggested. The inappropriate pre-degradation of globulin-type storage protein may result in partial repression of seed and silique development which in turn leads to higher incompletely-developed silique percentage and lower silique numbers per plant. These data also suggest that sweet potato asparaginyl endopeptidase SPAE may have enzymatic function similar to seed vacuolar processing enzymes for protein degradation and nutrient recycling during leaf senescence.

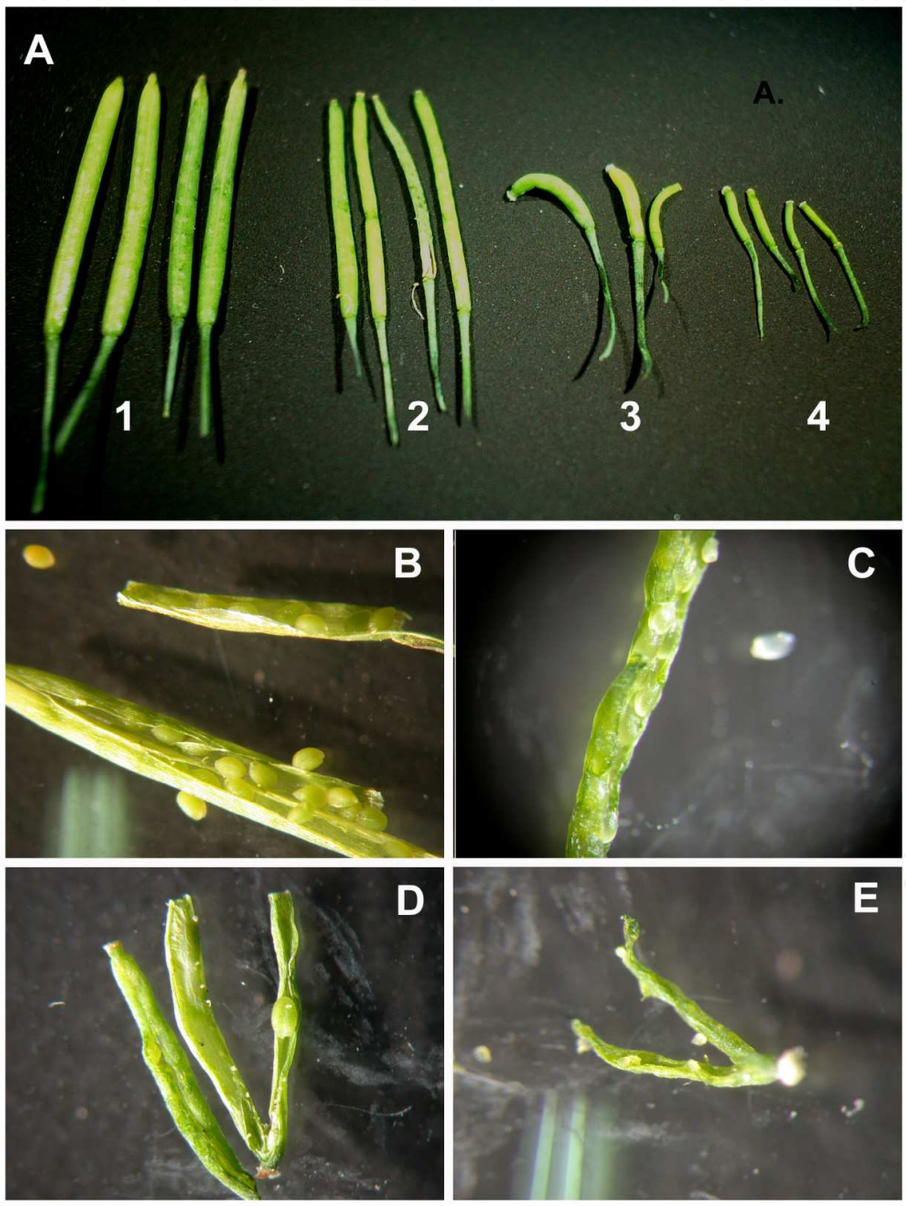
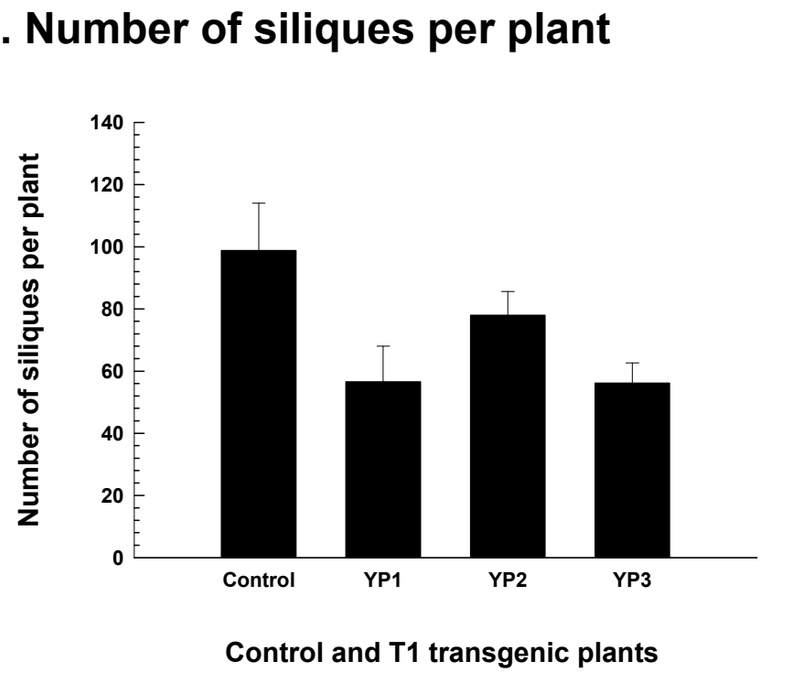


Fig. 3. Morphological classification of Arabidopsis siliques. A. Different silique types (types 1, 2, 3 and 4) classified. **B.** Dissection of type 1 silique; **C.** Dissection of type 2 silique; **D.** Dissection of type 3 silique; **E.** Dissection of type 4 silique (Adapted from Chen et al., 2008).



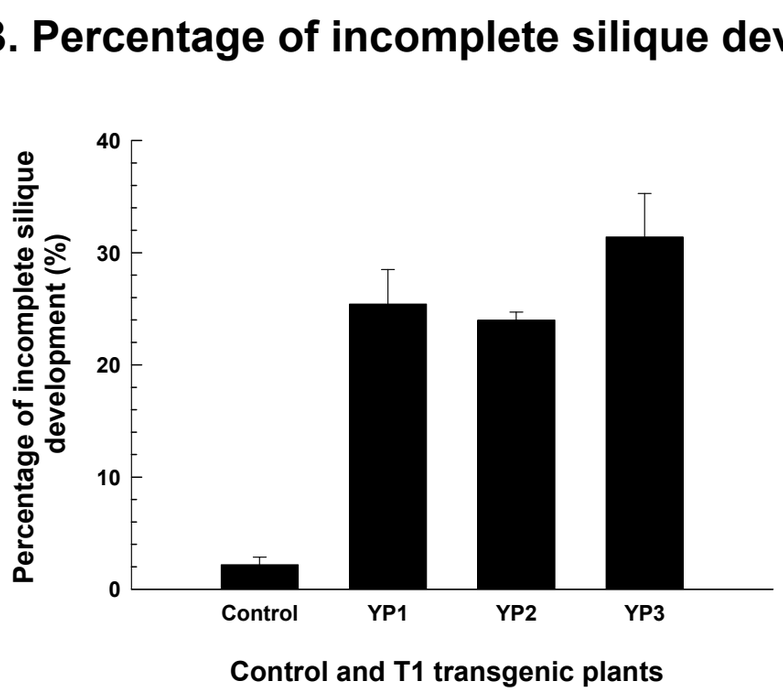


Fig. 4. Comparison of silique number per plant and incompletely-developed silique percentage among control and transgenic T1 plants ectopically expressing sweet potato *SPAE*. **A.** Comparison of silique number per plant. **B.** Comparison of incompletely-developed silique percentages. C and YP1/YP2/YP3 denote non-transformant control and transgenic *Arabidopsis* plants, respectively. The data are from the average of 5 plants per treatment and shown as mean ± S.E. (Adapted from Chen et al., 2008).

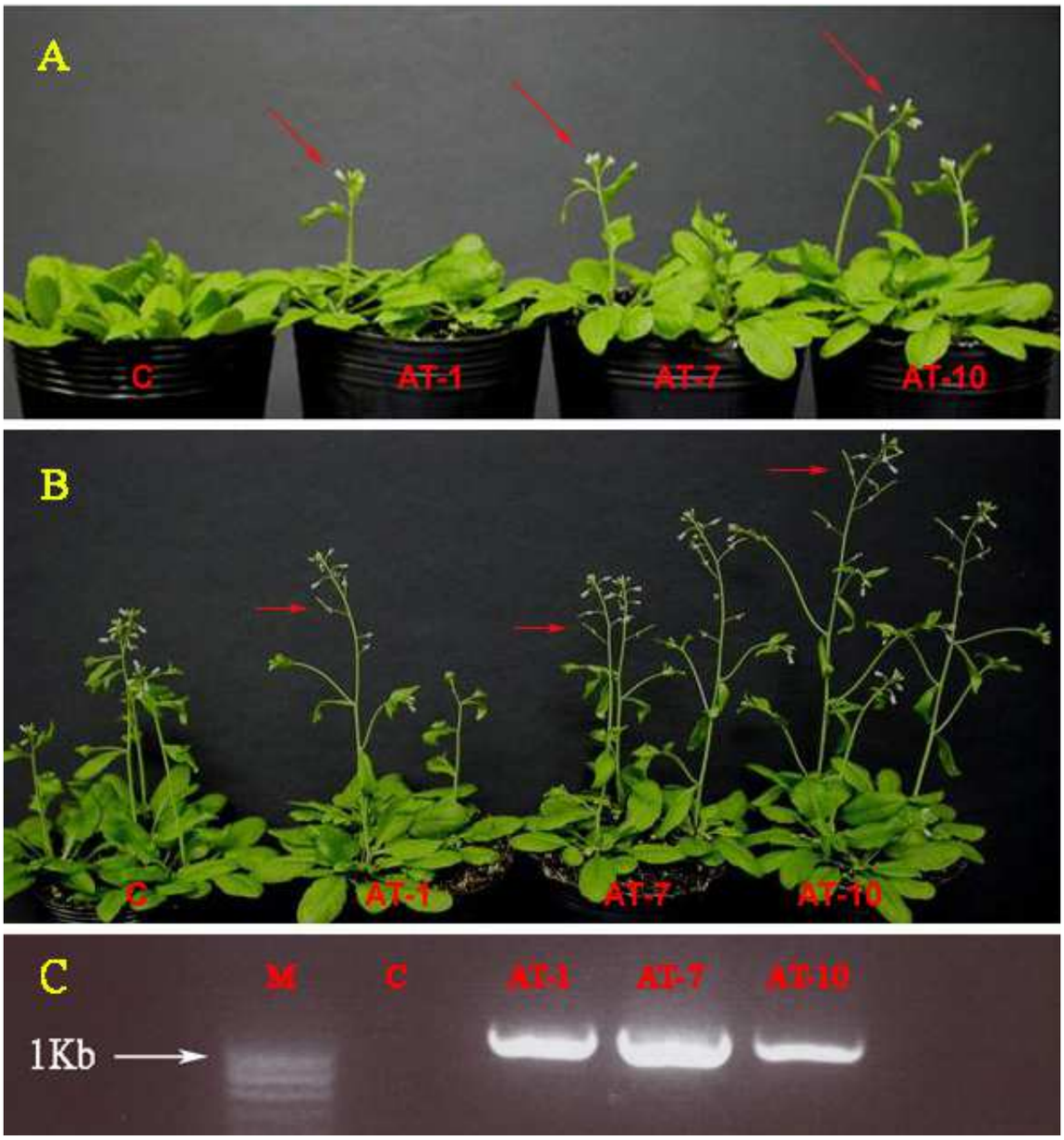


Fig. 5. Comparison of growth patterns among control and transgenic T1 Arabidopsis plants ectopically expressing sweet potato *SPCP2*. **A.** Transition from vegetative growth to flowering was observed and compared 30 days after seed germination. **B.** The appearance and size of inflorescences and siliques were observed and compared 35 days after seed germination. **C.** RT-PCR analysis of *SPCP2*. C and AT denote control and transgenic T1 *Arabidopsis* plants, respectively.

4.2 Expression of sweet potato papain-like cysteine protease *SPCP2* altered seed and silique development and enhanced stress tolerance in transgenic *Arabidopsis*

Transgenic *Arabidopsis* plants were isolated and identified with floral dip transformation method (Clough & Bent, 1998). Genomic PCR and RT-PCR analysis confirmed that the presence and expression of sweet potato papain-like cysteine protease *SPCP2* in transgenic *Arabidopsis* plants (Chen et al., 2010). Transgenic *Arabidopsis* plants also exhibited slightly earlier transition from vegetative to reproductive growth (Fig. 5). The reasons and mechanisms are not clear. However, an imbalance in the relative availability of carbohydrate and nitrogen in the shoot apical meristem (Raper et al., 1988; Rideout et al., 1992), the change of endogenous ascorbic acid content (Barth et al., 2006), and possible non-specific degradation and removal of flowering repressor(s) by ectopic SPCP2 expression are suggested.

Expression of sweet potato SPCP2 in transgenic *Arabidopsis* plants also caused elevated number of incompletely-developed silique (Fig. 6), and reduced average fresh weight per seed and lower germination percentage (Chen et al., 2010). The reasons for the altered phenotypic characteristics in transgenic *Arabidopsis* by ectopic *SPCP2* gene expression are not clear. However, SPCP2 exhibited high amino acid sequence identities with plant papain-like cysteine proteases, such as *Phaseolus vulgaris* CP2, *Vicia sativa* CPR2, and *Vigna mungo* SH-EP. These papain-like cysteine proteases together with vacuolar processing enzymes have been implicated in association with the degradation and mobilization of globulin storage proteins during seed germination and seedling growth in *Phaseolus vulgaris* (Senyuk et al., 1998), *Vigna mungo* (Okamoto et al., 1999), and *Vicia sativa* (Schlereth et al., 2000; Schlereth et al., 2001). These reports provide a possible explanation for the altered phenotypic characteristics observed in transgenic *Arabidopsis* plants, and suggest that sweet potato SPCP2 may have an enzymatic function similar to papain-like cysteine proteases, including *Vigna mungo* SH-EP and *Vicia sativa* CPR2 for protein degradation and nutrient recycling during leaf senescence.

Expression of sweet potato *SPCP2* in transgenic *Arabidopsis* plants exhibited higher salt and drought stress tolerance (Fig. 7), and contained higher relative water content than control (Fig. 8). The reasons for the altered stress responses in transgenic *Arabidopsis* by ectopic SPCP2 gene expression are not clear. However, SPCP2 exhibited high amino acid sequence identities with plant cysteine proteases, such as *Arabidopsis* RD19 and broccoli Bocp4. *Arabidopsis* RD19 was a drought-inducible cysteine protease (Koizumi et al., 1993), and belonged to osmotic stress-responsive genes (Xiong et al., 2002). Under osmotic stress such as drought, high salinity (NaCl or PEG) and ABA treatments, RD19 mRNA transcript was significantly enhanced compared to untreated control (Xiong et al., 2002). Broccoli Bocp4 exhibited high sequence identity to dehydration-responsive *Arabidopsis* RD19, and was also significantly induced in broccoli florets, which were kept in air (dry situation) but not in water or 2% sucrose solution 12 h post harvest (Coupe et al., 2003). Sweet potato cysteine protease *SPCP2* was also inducible by salt and drought stresses in detached leaves (Fig. 9), and its ectopic expression in transgenic *Arabidopsis* caused higher salt and drought resistances (Figs. 7 and 8). Our results agree with these reports and suggest a possible role of sweet potato cysteine protease *SPCP2* in osmotic stress regulation and salt/drought stress tolerance.

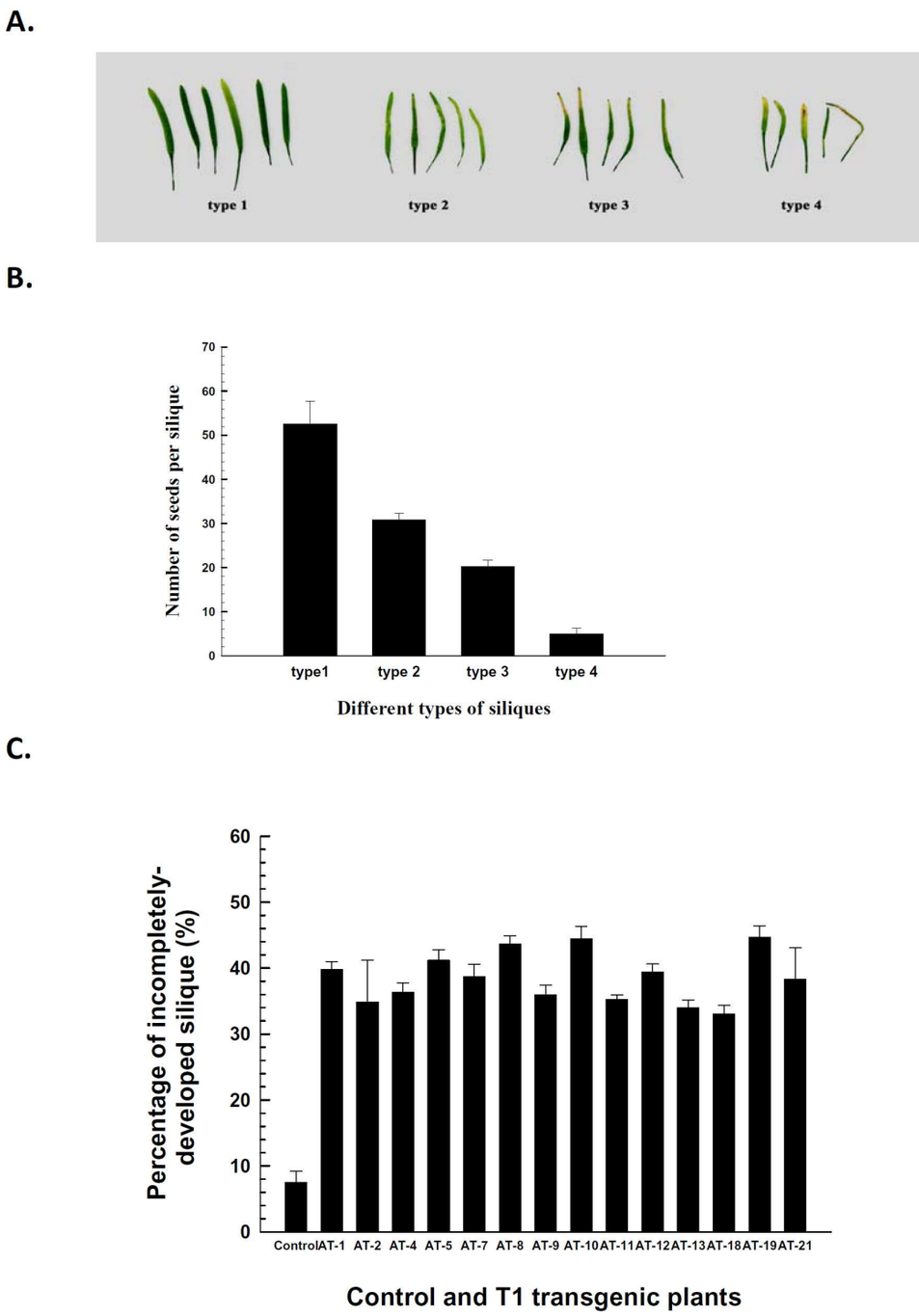


Fig. 6. Comparison of incompletely-developed silique percentages among control and transgenic T1 plants ectopically expressing sweet potato *SPCP2*. **A.** The appearance and size of different silique types (types 1, 2, 3 and 4) were observed and compared 35 days after seed germination. **B.** The average seed number of different silique type. **C.** Comparison of incompletely-developed silique percentage among control and transgenic T1 plants. C and AT denote control and transgenic T1 *Arabidopsis* plants,respectively (Adapted from Chen et al., 2010).

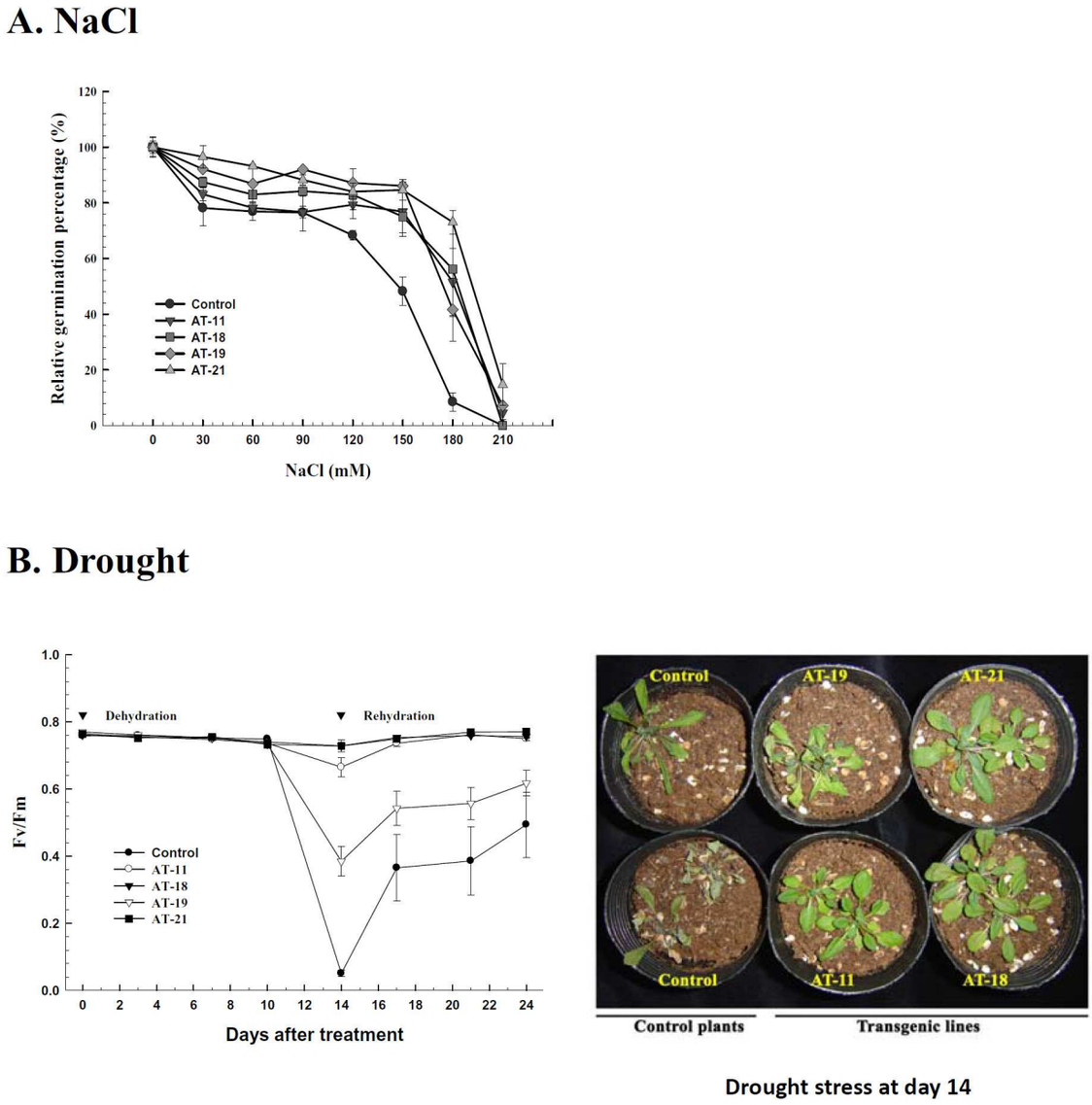


Fig. 7. Comparison of salt and drought stress tolerance among control and transgenic T1 *Arabidopsis* plants ectopically expressing sweet potato *SPCP2*. **A.** Salt. For salt treatment, seeds were germinated on half strength MS medium plus 3% sucrose and different concentrations of NaCl for ca. 2 weeks, and the relative germination percentages were recorded and compared. **B.** Drought. For drought treatment, upper panel of B is the photochemical Fv/Fm comparison among control and transgenic T1 *Arabidopsis* plants during dehydration and rehydration treatment. Lower panel of B is the morphological comparison among control and transgenic T1 *Arabidopsis* plant at day 14 after drought treatment. The data were the average of total 5 petri dishes for A or 5 seedlings per transgenic line for B, and shown as mean ± S.E. Control and AT-11/AT-18/AT-19/AT-21 denote wild type and transgenic T1 *Arabidopsis* plants, respectively. ▲ indicates the time points of dehydration and rehydration (Adapted from Chen et al., 2010).

5. Correlation of cysteine protease expression with storage protein degradation in sweet potato storage root during sprouting

In sweet potato storage root, trypsin protease inhibitors (TIs) are the main storage proteins and composed of a multiple gene family. It has been implicated that cysteine proteases are likely associated with the degradation of storage root trypsin inhibitors during sprouting (Huang et al., 2005). Therefore, expression and correlation of sweet potato asparaginyl endopeptidase SPAE and papain-like cysteine protease SPCP2 with the degradation and mobilization of the two major storage root trypsin inhibitor bands during sprouting were studied. The sprouts appeared and were visible within the first week of incubation of storage root at room temperature, whereas, degradation of trypsin inhibitors became significant in the later incubation. RT-PCR analysis of *SPAE* and *SPCP2* also demonstrated that their gene expression was significantly higher in the sprout and flesh of sprouting storage root than that of non-sprouting storage root (Fig. 10), and correlated well with the time course of degradation of the two major trypsin inhibitor bands (unpublished data). These data suggest that the asparaginyl endopeptidase SPAE and papain-like cysteine protease SPCP2 may play roles in association with storage root major trypsin inhibitor degradation during sprouting.

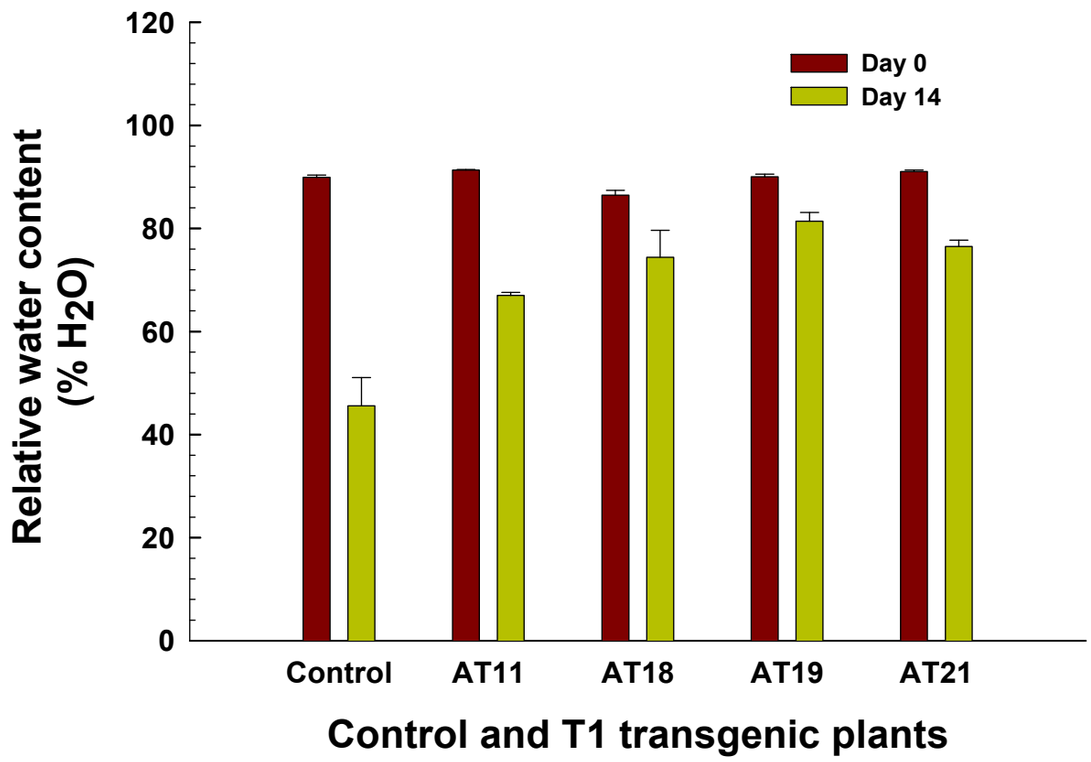


Fig. 8. Comparison of the relative water content (H2O%) between control and transgenic T1 *Arabidopsis* plants ectopically expressing sweet potato *SPCP2* at day 14 after drought treatment. The data were the average of total 5 seedlings per transgenic plants, and shown as mean ± S.E. Control and AT-11/AT-18/AT-19/AT-21 denote wild type and transgenic T1 *Arabidopsis* plants, respectively.

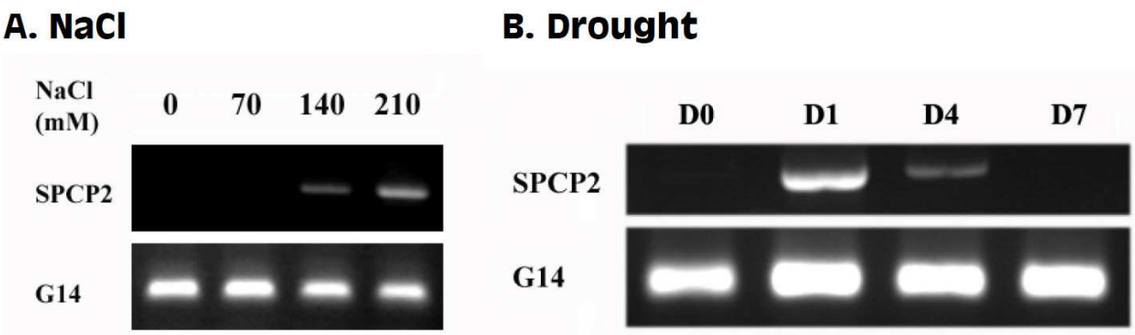


Fig. 9. Induction of sweet potato papain-like cysteine protease SPCP2 gene expression by salt and drought treatments. **A.** Salt treatment. Sweet potato detached leaves were treated with different salt concentrations (0, 70, 140 and 210 mM, respectively,) for 9 days and collected individually for RT-PCR analysis. **B.** Drought treatment. Detached sweet potato leaves were placed on dry paper tower in the dark for 0, 1, 4 and 7 days, and then collected individually for RT-PCR analysis. Sweet potato *G14* encoded a constitutively expressed metallothionein-like protein and was used as a control.

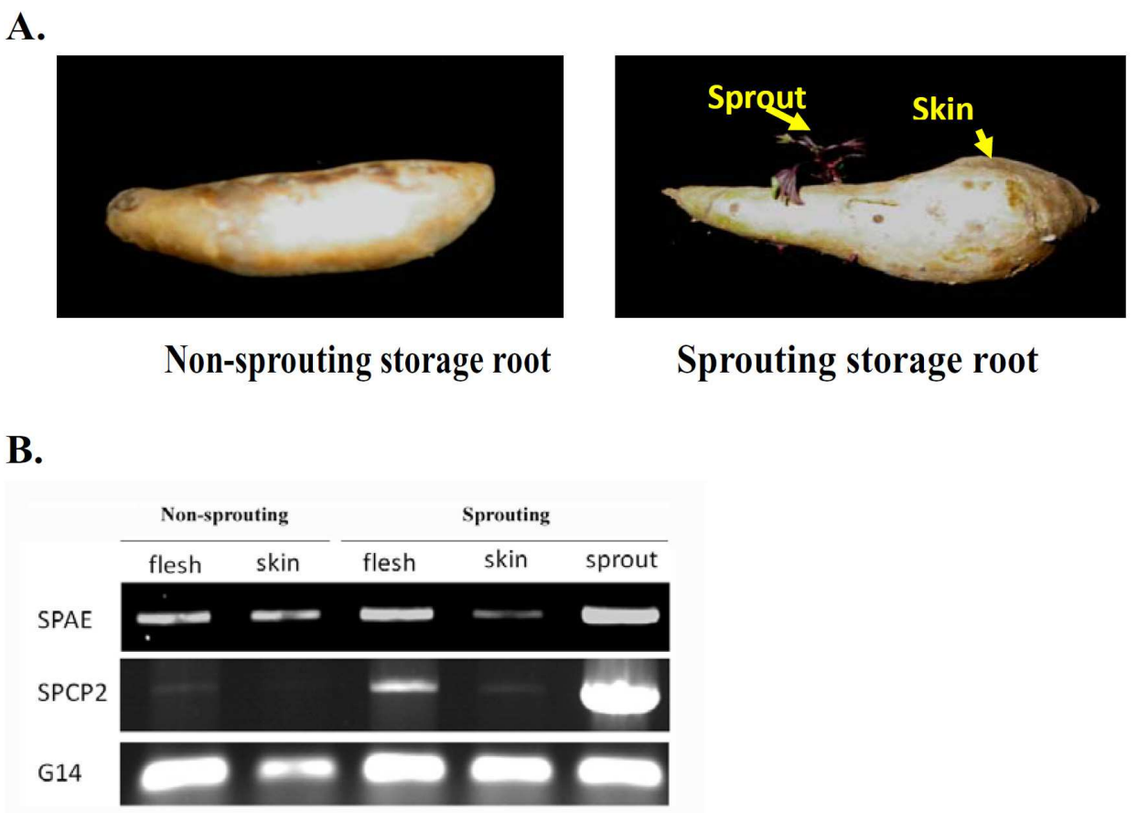


Fig. 10. Expression patterns of sweet potato asparaginyl endopeptidase SPAE and papain-like cysteine protease *SPCP2* in non-sprouting and sprouting st The orage roots. **A.** Storage root morphology. **B.** RT-PCR analysis of *SPAE* and *SPCP2*. Sweet potato *G14* encoded a constitutively expressed metallothionein-like protein and used as a control.

Sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* cDNAs have been constructed and expressed in recombinant PET vector for fusion protein production and purification. Application of the purified fusion protein to sweet potato storage root or detached leaves will be performed in the future in order to study further whether they can promote (1) the degradation of storage root major trypsin inhibitors during sprouting, (2) protein degradation and recycling during leaf senescence, or (3) stress tolerance.

6. Conclusion

Sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* are senescence-associated genes and significantly enhanced their expression in senescent leaves. Phylogenetic tree analysis shows that *SPAE* and *SPCP2* exhibit close association with vacuolar processing enzyme and papain-like cysteine protease, respectively, which are involved in seed globulin storage protein degradation. Ectopic expression of sweet potato *SPAE* and *SPCP2* in transgenic *Arabidopsis* plants also caused altered phenotypic characteristics, including abnormal seed and silique development, elevated incompletely-developed silique percentage, fewer silique numbers per plant, reduced seed germination percentage, and enhanced tolerance to drought and salt stresses. Based on these data, it can be concluded that sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* may play physiological roles in association with protein degradation and nutrient recycling during leaf senescence with enzymatic functions similar to seed globulin storage protein degradation and re-mobilization during germination and seedling growth.

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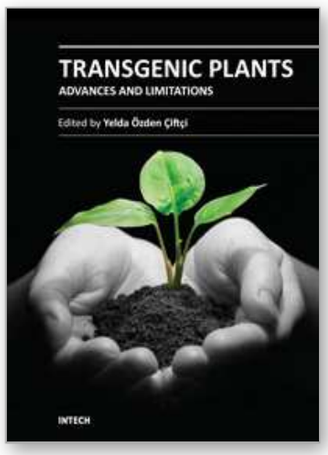

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