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Ethephon-mediated effects on leaf senescence are affected by reduced glutathione and EGTA in sweet potato detached leaves

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ABSTRACT. In this report several senescence-associated markers were used to study the ethephon-mediated effects on leaf senescence in detached sweet potato leaves. The chlorophyll contents and Fv/Fm values were drastically reduced, however, H_2O_2 contents detected with diaminobenzidine (DAB) staining and a papain-like cysteine protease SPCP1 expression were significantly enhanced in ethephon-treated leaves compared to untreated dark control. In the presence of reduced glutathione, EGTA or cycloheximide, the reduction of chlorophyll contents and Fv/Fm values were alleviated, however, the induction or enhancement of H_2O_2 contents and cysteine protease SPCP1 expression were repressed. Both calcium ionophore A23187 and glutathione synthase inhibitor, L-buthionine sulfoximide (BSO), remarkably induced SPCP1 expression in detached leaves, and the induction was also repressed by EGTA and reduced glutathione, respectively. The time effective for cycloheximide repression of SPCP1 expression was ca. 6 to 12 hours after ethephon treatment. In conclusion, ethephon-mediated effects on leaf senescence and gene expression in detached sweet potato leaves are significantly repressed by reduced glutathione, EGTA, and cycloheximide, respectively. These data suggest a possible involvement of oxidative stress, external calcium influx, and *de novo* synthesized proteins in association with ethephon signaling leading to leaf senescence and gene expression in sweet potato detached leaves.

Keywords: Cysteine protease; EGTA; Ethephon; Glutathione; Leaf senescence; Sweet potato.

INTRODUCTION

Leaf is the main place of photosynthesis and serves as a source of carbohydrate for sink nutrients in plants. Its longevity and senescence thus affect the photosynthesis efficiency and crop yield. Leaf senescence is influenced by endogenous and exogenous factors, including plant growth regulators, starvation, wound, and environmental stresses (Yoshida, 2003; Lim et al., 2007). Leaf senescence is the final stage of development and has been considered as a type of programmed cell death (Lim et al., 2007). Leaf cells undergo highly coordinated changes in structure, metabolism, and gene expression during senescence in a defined order. Breakdown of chloroplast is the earliest and most significant change in cell components (Makino and Osmond, 1991). The carbon assimilation is metabolically replaced by catabolism of chlorophyll and macromolecules such as proteins, membrane lipids, and RNA (Lim et al., 2007).

Ethylene plays a key role in leaf senescence and its signaling is an area of intensive studies with molecular genetics, molecular biology, and biochemistry. Previous reports demonstrate that the main pathway for ethylene biosynthesis comes from methionine, which is first converted to *S*-adenosyl methionine (SAM), then 1-aminocyclopropane-1-carboxylic acid (ACC), and finally ethylene in three consecutive reactions catalyzed by the enzymes of SAM synthetase, ACC synthase (ACS) and ACC oxidase (ACO), respectively (Bleecker and Kende, 2000). The ACC synthase and ACC oxidase constitute multi-gene families in diverse plant species and show differential regulation in response to a wide range of environmental and developmental stimuli (Wang et al., 2002). Elevated oxidative stresses caused by environmental stimuli, including ozone, UV-B, and wounding has been demonstrated to

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enhance ethylene production via ACC synthase and ACC oxidase (Wang et al., 2002). In ozone treatment, ethylene also enhanced reactive oxygen species (ROS) generation, which in turn leads to cell death (Wang et al., 2002). In sweet potato, a wound-inducible ipomoelin (IPO) gene expression can be induced by ethylene (Chen et al., 2008b), but was completely repressed by diphenylene iodonium, an inhibitor of NADPH oxidase (Jih et al., 2003). These data suggest that elevated oxidative stress may play important role in ethylene biosynthesis, ethylene signaling, and ethylene-mediated effects. Examples concerning the role of elevated oxidative stress have been reported. It may function as a signal molecule of signal transduction pathways leading to gene expression and regulation (Hung et al., 2005), can target directly to particular proteins especially with active thiol groups, which in turn transmit the signal to the next players in the signal transduction pathways (Hancock et al., 2006), and can interfere biochemical and physiological metabolisms and finally causes cell death (Wang et al., 2002; Vahala et al., 2003; Koehl et al., 2007).

In higher plants, the main antioxidants, including glutathione and ascorbate, are important redox signalling components (Vranová et al., 2002; Meyer and Hell, 2005; Shao et al., 2008). The cellular glutathione redox buffer is assumed to be part of signal transduction pathways transmitting developmental and environmental signals, and therefore, is important in the regulation of gene expression and metabolism. Glutathione, as the most abundant low-molecular weight thiol in the cellular redox system, is used for detoxification of reactive oxygen species and transmission of redox signals. Detoxification of H_2O_2 via the glutathione-ascorbate system leads to a transient change in the degree of oxidation of the cellular glutathione pool, and thus a change in the glutathione redox potential. The deviation of glutathione potential due to either depletion of reduced glutathione or increase of oxidized form can be used for fine tuning the activity of targeted proteins. Therefore, glutathione homeostasis and redox signalling can be integrated together (Meyer, 2008).

Zhao et al. (2007) demonstrate that ethylene activates a plasma membrane Ca^{2+}-permeable channel in tobacco suspension cells with patch-clamp technique and confocal microscopy. In tobacco, an ethylene-up-regulated gene *NtER1*, which encoded a calmodulin-binding peptide, was cloned and act as a trigger for senescence and death. Calmodulin binds to NtER1 with high affinity in a calcium-dependent manner (Yang and Poovaiah, 2000). In sweet potato, ipomoelin (*IPO*) gene expression was induced by ethylene and the induction was repressed in the presence of EGTA (Ouaked et al., 2003; Chen et al., 2008b). These data clearly demonstrate the involvement of Ca^{2+} signaling in ethylene action.

Sweet potato (*Ipomoea batatas* (Lam.) is an important food crop in the tropics and subtropics including Taiwan. its storage roots and leaves are the edible portions, and contain plenty of vitamin B complex, vitamin C, β-carotenoids, multiple minerals and high calcium (Yang et al., 1975; Hattori et al., 1985). Several medicative effects of sweet potato have been reported previously, including accelerated excretion of toxins and carcinogens, antioxidant activities of trypsin inhibitor (Hou et al., 2001; Huang et al., 2007a and 2007b), inhibition of angiotensin converting enzyme activity (Hou et al., 2003; Huang et al., 2006), reduction of hypertension in diabetic mice, and growth inhibition and induction of apoptosis in NB4 promyelocytic leukemia cells (Huang et al., 2007c). In addition, ethephon, an ethylene-releasing compound, can induce leaf senescence and senescence-associated gene expression in detached sweet potato leaves (Chen et al., 2000; 2003; 2006). Several senescence-associated cysteine proteases have been ectopically expressed in transgenic *Arabidopsis* plants and caused altered developmental characteristics (Chen et al., 2004; 2008a) and stress responses (unpublished data). These results suggest the importance of sweet potato either in biomedicine or agriculture. Ethylene effect on leaf senescence and gene expression is an intensively-studied area in plants, however, its signaling most remains unclear in sweet potato. We have previously isolated a cysteine protease *SPCP1* from sweet potato senescent leaves. The nucleotide and deduced amino acid sequences of *SPCP1* exhibited high sequence identity with *Arabidopsis* cysteine protease *SAG12*. *SPCP1* gene expression was regulated by developmental and environmental cues, and was induced in naturally and ethephon-induced senescent leaves (Chen et al., 2009). In this manuscript, chlorophyll contents, Fv/Fm values, H_2O_2 amounts, and cysteine protease SPCP1 expression were used to investigate the possible components, such as elevated oxidative stress and external calcium influx in ethylene signaling leading to senescence in sweet potato detached leaves.

MATERIALS AND METHODS

Plant materials

The storage roots of sweet potato (*Ipomoea batatas* (L.) Lam.) were grown in a growth chamber, and plantlets from the storage roots were used as materials. Mature green leaves near the top of stems were detached for experiments, treating with ethephon, reduced glutathione, EGTA, cycloheximide, L-buthionine sulfoximide, and calcium ionophore A23187.

Ethephon and effector treatments

Detached mature leaves were placed on a wet paper towel containing 3 mM 2-(N-morpholino)ethanesulp honic acid (MES) buffer pH 5.8, and kept at 28°C in the dark. Ethephon, an ethylene-releasing compound, and effectors, such as EGTA, reduced glutathione, and cycloheximide were also included in the 3 mM MES buffer for treatments. Detached mature leaves were treated with 1 mM ethephon for 0, 1, 2, and 3 days, respectively. For effector treatments, (a) 1 mM ethephon plus 5 mM

EGTA pretreatment, (b) 1 mM ethephon plus 0.75 mM reduced glutathione pretreatment, (c) 1 mM ethephon plus 20 μg/ml cycloheximide pretreatment, and (d) 1 mM ethephon plus 20 μg/ml cycloheximide treatment at the time intervals of 30 min earlier, at the same time, or 1, 3, 6, and 12 h, respectively, after 1 mM ethephon addition. For EGTA and reduced glutathione pretreatment, individual chemical was added into MES buffer ca. 30 minutes prior to ethephon treatment. Leaves were kept at 28°C in the dark for 3 days, then were individually collected and analyzed for leaf morphology, chlorophyll content, Fv/Fm value, H_2O_2 amount, and cysteine protease SPCP1 expression.

Treatments with calcium ionophore A23187 and glutathione synthesis inhibitor L-buthionine sulfoximide

For calcium ionophore A23187, detached mature leaves were treated with 100 μM A23187 in the presence or absence of 5 mM EGTA for 3 days. Detached leaves were also treated with 1 mM ethephon in the presence or absence of 5 mM EGTA as a positive and a negative control, respectively. The EGTA compound was added into 3 mM MES buffer ca. 30 min prior to A23187 or ethephon addition. For L-buthionine sulfoximide, an endogenous glutathione or homoglutathione synthesis inhibitor, detached mature leaves were treated with 2 mM L-buthionine sulfoximide in the presence or absence of 0.75 mM reduced glutathione for 3 days. Detached leaves were also treated with 1 mM ethephon in the presence or absence of 0.75 mM reduced glutathione as a positive and a negative controls, respectively. Reduced glutathione was added into MES buffer ca. 30 minutes prior to L-buthionine sulfoximide or ethephon addition. Leaves were kept at 28°C in the dark for 3 days, and then harvested for protein gel blot hybridization.

Measurement of pigments

For quantitative analysis of pigment contents, leaves from treatments mentioned above were measured and recorded directly with non-invasive CCM-200 Chlorophyll Content Meter. Each leaf sample were measured at least 5 different leaf areas, and each treatment was repeated three times.

Measurement of Fv/Fm

For quantitative analysis of Fv/Fm values, leaves from treatments mentioned above were measured and recorded with non-invasive Chlorophyll Fluorometer (WALZ JUNIOR-PAN). The Fv/Fm value was used to determine the maximum quantum efficiency of photosystem II (PSII) primary photochemistry. In healthy leaves, this value is close to 0.8 and independently of the plant species. Chlorophyll fluorescence is very useful to study the environmental stress effects on plants since photosynthesis is often reduced in plants experiencing adverse conditions, including water deficit, high salt, nutrient deficiency, polluting agents, temperature and pathogen attack. Therefore, the Fv/Fm values were measured, recorded and compared among control and treated samples. Each leaf sample was measured at least 3 different leaf areas, and each treatment was repeated three times.

DAB staining

DAB staining method was used to detect the H_2O_2 generation in leaves after treatments, and was basically according to the method described by Hu et al. (2005). Leaves from treatments mentioned above were collected separately and stained with 1 mg/ml diaminobenzidine (DAB) solution pH 3.8 at 37°C for 2 h. After DAB staining, leaves were boiled in ethanol for 10 min, then cooled down to room temperature and photographed.

Protein gel blot hybridization

Polyclonal antibody against putative sweet potato cysteine protease SPCP1 has been previously produced from rabbit and was used for protein gel blot hybridization according to the report of Chen et al. (2009). Samples from different treatments mentioned above were used for total protein extraction and protein gel blot hybridization. About 0.5 g leaf sample was ground with mortar and pestle in liquid N_2 and the powder was extracted in a 1:10 (FW: V) ratio with extraction buffer containing 10 mM Tris-HCl and 1 mM EDTA pH 6.8. The mixture was centrifuged at 13,000 x g, 4°C for 10 min, then, the supernatant was transferred to a new centrifuge tube. About the same total protein amount (ca. 5 μg) from individual sample was mixed with equal volume of 5x sample buffer (60 mM Tris-HCl pH 6.8, 50% glycerol, 2% SDS, 28.8 mM 2-mercaptoethanol, 0.1% bromophenol blue), and boiled at 95°C for 5 min, then performed a 12.5% SDS-PAGE. After electrophoresis, proteins were transferred onto PVDF membrane (Millipore), and was reacted in order with primary polyclonal antibody produced previously against the putative SPCP1 protein (Chen et al., 2009), then alkaline phosphatase-conjugated, goat-antirabbit secondary antibody, and finally Nitro blue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) substrates (Sigma). Once the band corresponding to the putative SPCP1 protein appeared, the reaction was stopped with replacement of mini-Q water.

RESULTS

Ethephon-mediated effects on leaf senescence, senescence-associated markers and SPCP1 expression

Ethephon-mediated effects on leaf senescence, chlorophyll content, Fv/Fm value, H_2O_2 amount, and cysteine protease SPCP1 expression were studied. Sweet potato detached leaves senesced earlier in ethephon treatment compared to dark control within a three-day period. The leaves began to turn visible yellowing at day 2, and became almost completely yellow at day 3 (Figure

1A). Significant increase of H_2O_2 amount was observed at day 3 in ethephon treatment compared to dark control (Figure 1B). The chlorophyll content of detached leaves drastically decreased in ethephon treatment, and was about 17% that of D0 control at day 3. However, the chlorophyll content of dark control was not significantly varied and was about 89% that of D0 at day 3 (Figure 2A). The Fv/Fm value was also remarkably less and was about 38% that of D0 at day 3 in ethephon treatment. However, the Fv/Fm value of dark control was not significantly varied and was about 86% that of D0 control (Figure 2B). Cysteine protease SPCP1 expression was significantly enhanced from day 2 in ethephon treatment compared to untreated dark control (Figure 2C). These results clearly demonstrate that ethephon treatment can elevate H_2O_2 amount, reduce chlorophyll and Fv/Fm contents, induce cysteine protease SPCP1 expression, and promote leaf senescence in sweet potato detached leaves.

Ethephon-mediated effects were repressed by reduced glutathione

Reduced glutathione influence on ethephon-mediated induction of leaf senescence was studied. Sweet potato detached leaves senesced much earlier and almost turned yellow in ethephon treatment compared to dark control. However, ethephon-mediated effects were alleviated by reduced glutathione pretreatment. The degree of leaf senescence and H_2O_2 production at day 3 were drastically less in reduced glutathione pretreatment (Figure 3). The chlorophyll content of detached leaves at day 3 was about 14% and 36% that of D0 control for ethephon and ethephon plus reduced glutathione, respectively (Figure 4A). The Fv/Fm value also significantly decreased in ethephon treatment and was about 32% that of D0 control at day 3. However, reduced glutathione delayed the ethephon-mediated Fv/Fm reduction and was about 62% that of D0 control at day 3 (Figure 4B). Cysteine protease SPCP1 expression was enhanced in ethephon treatment compared to untreated dark control, and the induction at day 3 was repressed by reduced glutathione pretreatment (Figure 4C). These results clearly demonstrate that ethephon-mediated effects were significantly repressed by reduced glutathione pretreatment, and suggest that intracellular glutathione content may be important and involved in ethephon-mediated effects on leaf senescence and gene expression. Therefore, L-buthionine sulfoximide, which functions as an endogenous glutathione biosynthesis inhibitor, was used to induce SPCP1 expression, and the

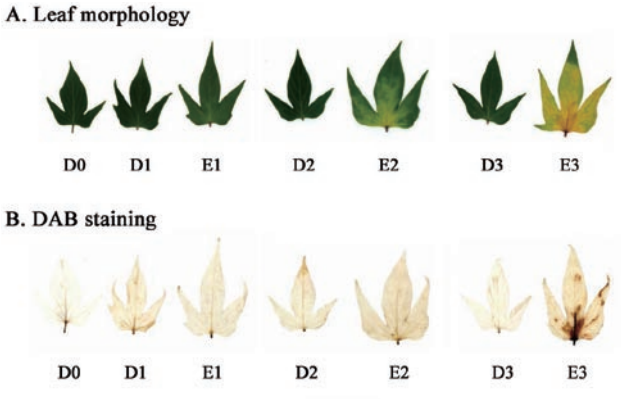

Figure 1. Effects of ethephon on leaf senescence and oxidative stress. (A) Leaf morphology; (B) H_2O_2 detection with DAB staining in detached sweet potato leaves. Detached leaves were treated with 1 mM ethephon for 0, 1, 2 and 3 days, respectively. D and E denote dark and ethephon treatments, respectively. The experiments were performed three times and a representative one was shown.

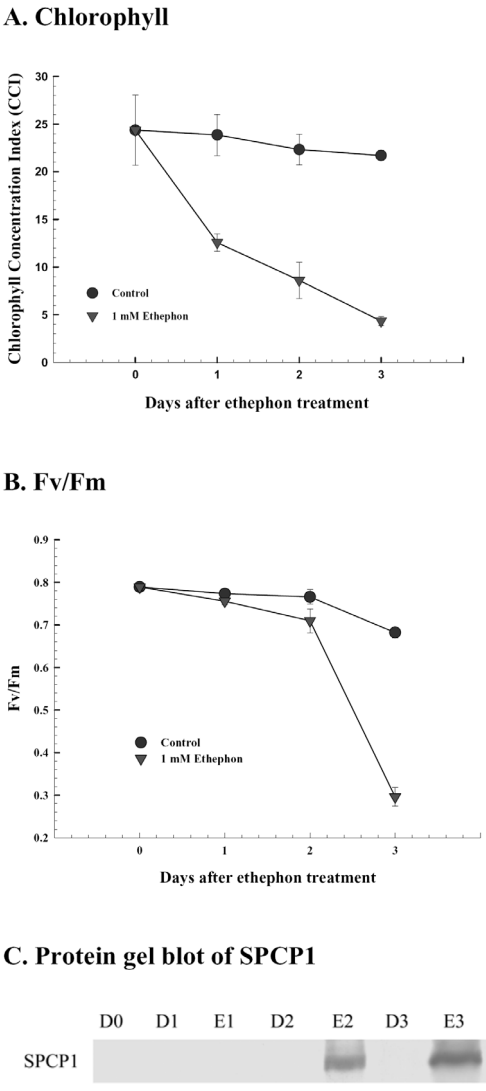

Figure 2. Effects of ethephon on chlorophyll content, Fv/Fm value, and cysteine protease SPCP1 expression in detached sweet potato leaves. (A) Chlorophyll content; (B) Fv/Fm value; (C) Protein gel blot of SPCP1 expression. Detached leaves were treated with 1 mM ethephon for 0, 1, 2 and 3 days, respectively. D and E denote dark and ethephon treatments, respectively. Protein gel blot was performed with polyclonal antibody raised previously against putative SPCP1 protein. The experiments were performed three times and a representative one was shown.

induction was also repressed by exogenously applied reduced glutathione (Figure 4C). These data provide further evidence to support the possible involvement of H_2O_2 generated by ethephon for SPCP1 induction and leaf senescence.

Ethephon-mediated effects were repressed by EGTA

EGTA influence on ethephon-mediated induction of leaf senescence was studied. Qualitative results exhibited that sweet potato detached leaves senesced earlier and almost became yellow in ethephon treatment compared to dark control. However, ethephon-mediated effects were slowed down by EGTA pretreatment. The degree of leaf senescence and H_2O_2 production at day 3 were much less by EGTA pretreatment (Figure 5). Quantitative results also showed that the chlorophyll content of detached leaves at day 3 was about 9% and 16% that of D0 control in ethephon and ethephon plus EGTA pretreatment, respectively (Figure 6A). The Fv/Fm value also significantly decreased in ethephon treatment and was about 44% that of D0 control at day 3. However, EGTA delayed the ethephon-mediated Fv/Fm reduction and Fv/Fm value was about 56% that of D0 control at

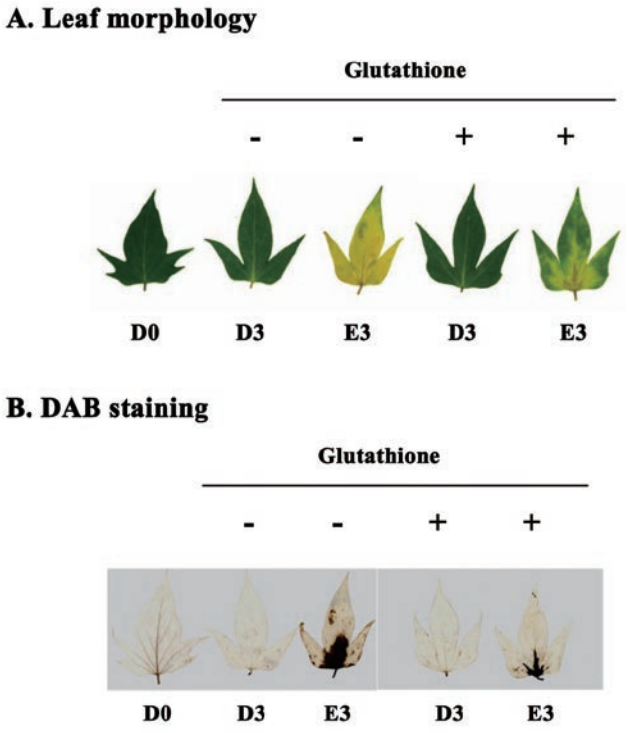

Figure 3. Reduced glutathione influence on ethephon-mediated effects of leaf senescence and oxidative stress in detached sweet potato leaves. (A) Leaf morphology; (B) H_2O_2 detection with DAB staining in detached sweet potato leaves. Detached leaves were pretreated with or without 0.75 mM reduced glutathione for ca. 30 min prior to 1 mM ethephon treatment. D3 and E3 denote dark and ethephon treatments, respectively, for 3 days. The experiments were performed three times and a representative one was shown.

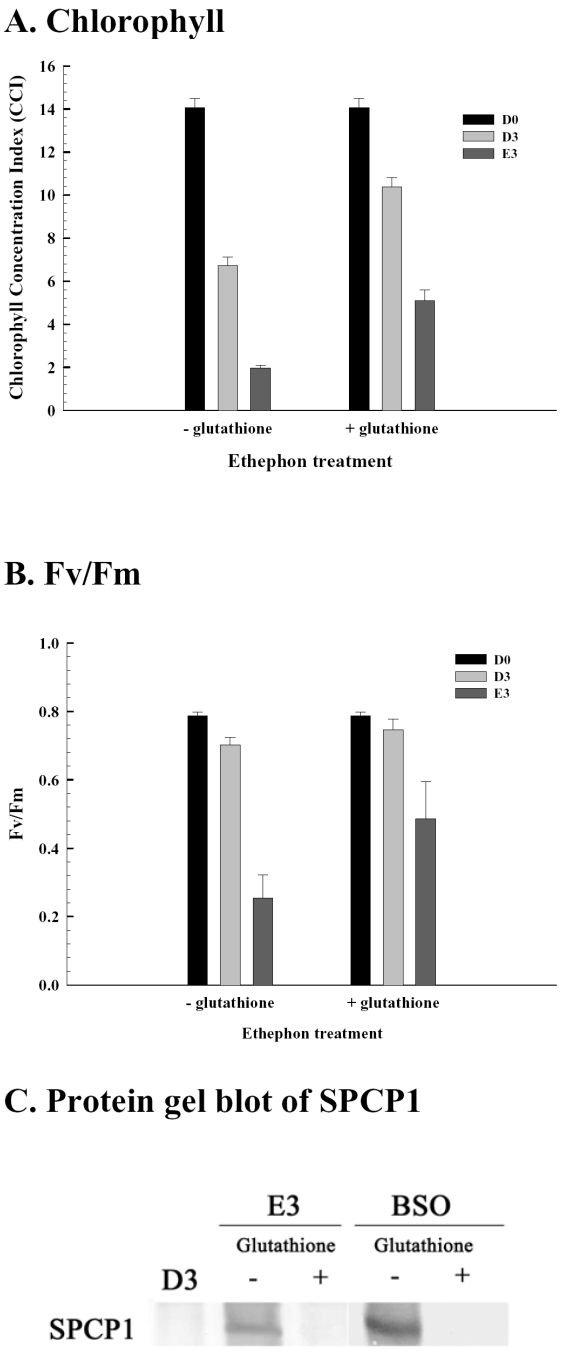

Figure 4. Reduced glutathione influence on ethephon-mediated effects of chlorophyll content, Fv/Fm value, and cysteine protease SPCP1 expression in detached sweet potato leaves. (A) Chlorophyll content; (B) Fv/Fm value; (C) Protein gel blot of SPCP1 expression. Detached leaves were pretreated with or without 0.75 mM reduced glutathione for ca. 30 min prior to 1 mM ethephon treatment. Detached leaves were also treated with L-buthionine sulfoximide in the presence or absence of reduced glutathione for 3 days. D3, E3 and BSO denote dark, ethephon and L-buthionine sulfoximide treatment, respectively, for 3 days. Protein gel blot was performed with polyclonal antibody raised previously against putative SPCP1 protein. The experiments were performed three times and a representative one was shown.

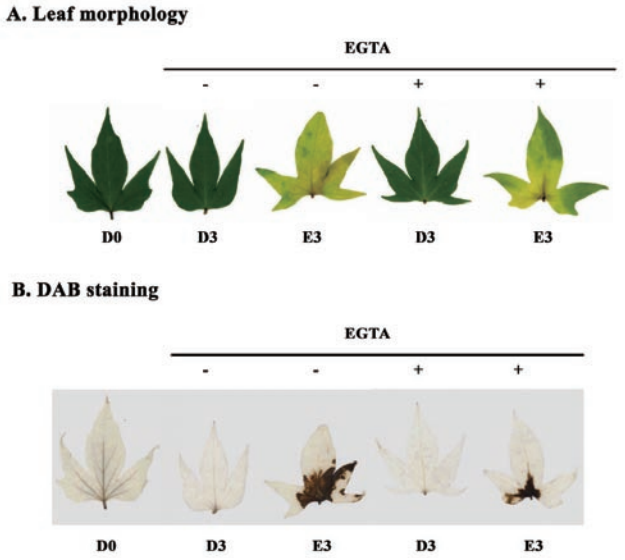

Figure 5. EGTA influence on ethephon-mediated effects of leaf senescence and oxidative stress in detached sweet potato leaves. (A) Leaf morphology; (B) H_2O_2 detection with DAB staining. Detached leaves were pretreated with 5 mM EGTA for ca. 30 min prior to 1 mM ethephon treatment. D3 and E3 denote dark and ethephon treatment, respectively, for 3 days. The experiments were performed three times and a representative one was shown.

day 3 (Figure 6B). Cysteine protease SPCP1 expression was enhanced in ethephon treatment compared to untreated dark control, and the induction at day 3 was repressed by EGTA pretreatment (Figure 6C). These results clearly demonstrate that ethephon-mediated effects are significantly repressed by EGTA pretreatment, and suggest that external calcium influx may be important and involved in ethephon-mediated effects on leaf senescence and gene expression. Therefore, calcium ionophore A23187, which functions as a calcium channel, was used to induce SPCP1 expression, and the induction was also repressed by exogenously applied EGTA (Figure 6C). These data provide further evidence to support the possible involvement of external calcium influx generated by ethephon for SPCP1 induction and leaf senescence.

Ethephon-mediated effects were repressed by cycloheximide

Cycloheximide influence on ethephon-mediated induction of leaf senescence was studied. Qualitative results exhibited that sweet potato detached leaves senesced earlier and almost turned yellow in ethephon treatment compared to dark control. However, ethephon-mediated effects were alleviated by cycloheximide pretreatment. The degree of leaf senescence and H_2O_2 production at day 3 were much less in cycloheximide pretreatment (Figure 7). Quantitative results at day 3 also showed that the chlorophyll content of detached leaves was about 11% that of D0 control in ethephon treatment, however, was ca. 25% that of D0 control in ethephon plus cycloheximide pretreatment (Figure 8A). The Fv/Fm value also significantly decreased in ethephon treatment and was about 45% that of D0 control at day 3. However, cycloheximide delayed the ethephon-mediated Fv/Fm reduction and Fv/Fm value was about 65% that of D0 control at day 3 (Figure 8B). Cysteine protease SPCP1 expression was enhanced in ethephon treatment compared to untreated dark control, and the induction at day 3 was repressed by cycloheximide pretreatment (Figure 8C). These results clearly demonstrate that ethephon-mediated effects are significantly repressed by cycloheximide pretreatment, and suggest that *de novo* synthesized proteins play important roles and are required in ethephon-mediated effects on leaf senescence and gene expression.

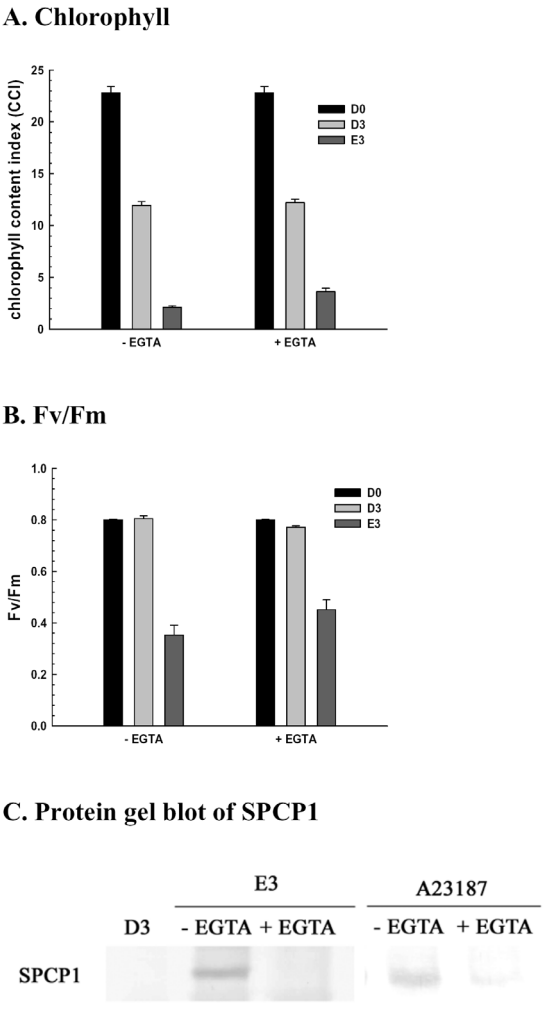

Figure 6. EGTA influence on ethephon-mediated effects of chlorophyll content, Fv/Fm value, and cysteine protease SPCP1 expression in detached sweet potato leaves. (A) Chlorophyll content; (B) Fv/Fm value; (C) Protein gel blot of SPCP1 expression. Detached leaves were pretreated with 5 mM EGTA for ca. 30 min prior to 1 mM ethephon treatment. Detached leaves were also treated with calcium ionophore A23187 in the presence or absence of EGTA for 3days. D3 and E3 denote dark and ethephon treatment, respectively, for 3 days. Protein gel blot was performed with polyclonal antibody raised previously against putative SPCP1 protein. The experiments were performed three times and a representative one was shown.

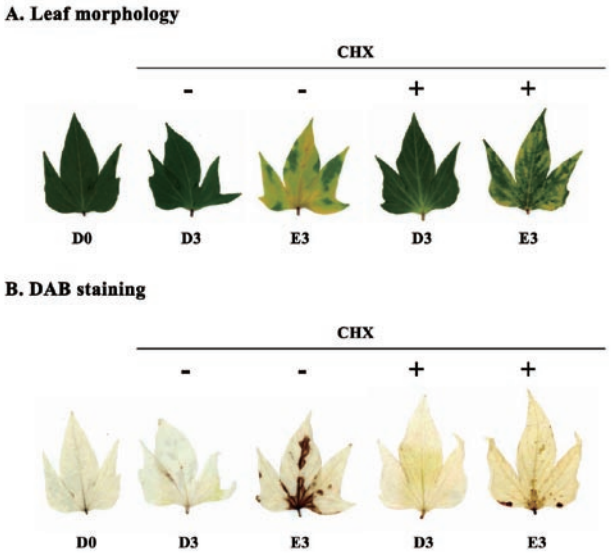

Figure 7. Cycloheximide influence on ethephon-mediated effects of leaf senescence and oxidative stress in detached sweet potato leaves. (A) Leaf morphology; (B) H_2O_2 detection with DAB staining. Detached leaves were pretreated with 20 μg/ml cycloheximide for ca. 30 min prior to 1 mM ethephon treatment. CHX, D3 and E3 denote cycloheximide, dark, and ethephon treatment, respectively, for 3 days.

Time course studies showed that effective repression of ethephon-induced cysteine protease SPCP1 expression by cycloheximide was within the first 6 to 12 hours after ethephon addition (Figure 8C).

DISCUSSION

Ethylene signaling in leaf senescence is intensively studied in many plant species, however, is a new area in sweet potato. Ethephon, an ethylene-releasing compound, caused reduction of chlorophyll content and Fv/Fm, elevation of H_2O_2 amount, cysteine protease SPCP1 expression, and leaf senescence in detached sweet potato leaves (Figures 1 and 2). In oat, ethylene promoted the deterioration of chloroplasts isolated from seeding primary leaves, and significantly reduced the chlorophyll content and PSI and PSII photosynthetic activities (Choe and Whang, 1986). In sweet potato, the wound-inducible ipomoelin (*IPO*) gene expression was induced by ethephon (Chen et al., 2008b). In tobacco cell suspension culture, ethylene is required for elicitin-induced oxidative burst (Koehl et al., 2007). Our data agree with these reports and demonstrate the importance of ethylene leading to the changes of senescence-associated markers and leaf senescence in sweet potato detached leaves.

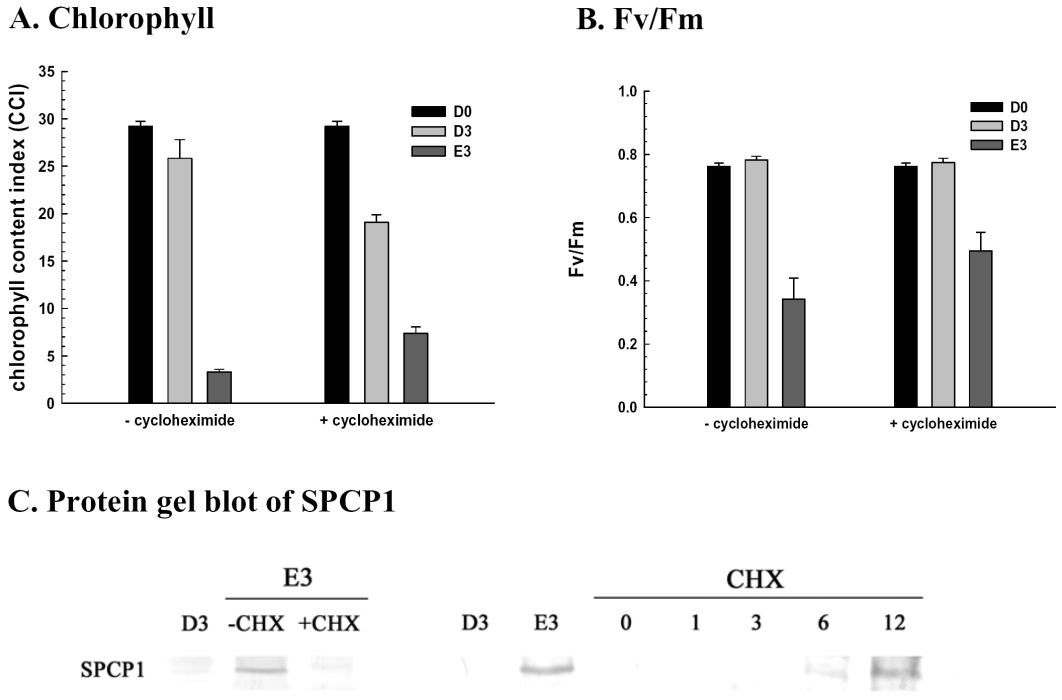

Figure 8. Cycloheximide influence on ethephon-mediated effects of chlorophyll content, Fv/Fm value, and cysteine protease SPCP1 induction in detached sweet potato leaves. (A) Chlorophyll content; (B) Fv/Fm value; (C) Protein gel blot of SPCP1 expression. Detached leaves were pretreated with 20 μg/ml cycloheximide for ca. 30 min prior to 1 mM ethephon treatment (A, B, and C left panel). Detached leaves were also treated with 20 μg/ml cycloheximide at different time intervals, including 30 min prior to, at the same time as, or 1, 3, 6, and 12 h, respectively, after the addition of 1 mM ethephon (C right panel). CHX, D3 and E3 denote cycloheximide, dark and ethephon treatment, respectively, for 3 days. Protein gel blot was performed with polyclonal antibody raised previously against putative SPCP1 protein. The experiments were performed three times and a representative one was shown.

Ethephon-mediated effects on leaf senescence and senescence-associated markers in sweet potato detached leaves were significantly repressed by exogenously applied reduced glutathione (Figures 3 and 4). In tobacco cell suspension culture, ethylene is required for elicitin-induced oxidative burst (Koehl et al., 2007). In *Capsicum* plants, H_2O_2 acts downstream from ethylene in *in vitro* abscission signaling of leaves (Sakamoto et al., 2008). Chen et al. (2008b) reported that sweet potato wound-inducible ipomoelin (*IPO*) gene expression was induced by ethephon. *IPO* gene expression was completely repressed by diphenylene iodonium, an inhibitor of NADPH oxidase which caused the elevation of intracellular oxidative stress, such as H_2O_2 (Jih et al., 2003). Our results agree with these reports and suggest that oxidative stress level elevated by ethephon plays an important role in the ethylene signalling leading to the changes of senescence-associated markers and leaf senescence. Therefore, reduced glutathione, which scavenges H_2O_2 produced in ethephon-treated leaves, significantly delays leaf senescence, represses SPCP1 expression, decreases chlorophyll content and Fv/Fm value (Figure 3A).

Sweet potato cysteine protease *SPCP1* exhibited high amino acid sequence identity with *Arabidopsis* SAG12, and its expression was significantly enhanced during leaf senescence (Chen et al., 2009). L-buthionine sulfoximide (BSO), a highly specific inhibitor of endogenous glutathione biosynthesis (Griffith, 1982), also induced SPCP1 expression and its induction was repressed by reduced glutathione (Figure 4). In higher plants, the main antioxidants, including glutathione and ascorbate, are important redox signalling components and play crucial roles in scavenging reactive oxygen species and regulation of gene expression associated with plant growth, development, and biotic/abiotic stress responses (Vranová et al., 2002; Meyer and Hell, 2005; Shao et al., 2008). In *Arabidopsis*, a redox-sensitive green fluorescence protein (roGFP) are expressed in the cytosol and used as a quantitative biosensor to monitor the change of glutathione redox potential in living plant cells with confocal microscopy (Meyer et al., 2007; Schwarzlander et al., 2008). Meyer et al. (2007) reported that exogenous addition of L-buthionine sulfoximide (BSO) increased the intracellular oxidized roGFP, which indicated the increase of endogenous oxidized glutathione GSSG level. However, exogenous application of reduced glutathione decreased BSO-induced elevation of the intracellular oxidized roGFP, which indicated the increase of endogenous reduced glutathione GSH level. Our data agree with these reports and provide further evidence to support the importance of endogenous glutathione content and oxidative stress level such as H_2O_2 in the ethephon-mediated effects on leaf senescence, SPCP1 expression, and senescence-associated markers.

Ethephon-mediated effects on leaf senescence, SPCP1 expression, and senescence-associated markers were also repressed by exogenously applied EGTA (Figures 5 and 6). Calcium ionophore A23187, which cause external calcium influx, induced SPCP1 expression and the induction was repressed by EGTA (Figure 6C). In cabbage, acceleration of leaf disc senescence by high calcium was observed, and the senescent leaf discs contained less chlorophyll contents in treatments with 250 mM calcium chloride compared to that of untreated control. The acceleration of senescence in cabbage leaf discs by supraoptimal calcium concentration is likely associated with elevated enzymatic degradation of membrane lipids (Chéour et al., 1992). Zhao et al. (2007) demonstrated that ethephon activated a plasma membrane Ca^{2+}-permeable channel in tobacco suspension cells with patch-clamp technique and confocal microscopy. In tobacco, an ethylene-up-regulated gene *NtER1*, which contained a 25-mer peptide corresponding to calmodulin-binding region, was cloned. The senescing leaves and petals had significantly increased *NtER1* induction as compared with young leaves and petals. Gel mobility-shift assay showed that the peptide of *NtER1* formed a stable complex with Calmodulin only in the presence of Ca^{2+}, but not EGTA (Yang and Poovaiah, 2000). These data demonstrate the involvement of Ca^{2+}/Calmodulin-mediated signaling in ethylene action. In sweet potato, ipomoelin (*IPO*) gene expression was induced by ethylene and the induction was repressed in the presence of EGTA. The application of PD98059, a mitogen-activated protein kinase kinase (MAPKK) inhibitor, did not prevent Ca^{2+} influx induced by ethylene, but inhibited the *IPO* gene expression stimulated by staurosporine (STA), a protein kinase inhibitor (Ouaked et al., 2003; Chen et al., 2008c). These data suggest that calcium influx and elevation of cytosolic Ca^{2+} by ethylene may stimulate protein phosphatase and MAPKK, which finally activates *IPO* gene expression. Our data agree with these reports and suggest the importance of external calcium influx in ethylene signalling leading to the induction of SPCP1 expression, leaf senescence and senescence-associated markers.

The relationship between external calcium influx and internal elevated oxidative stress in ethylene signaling is complex and most remains unclear. Previous reports demonstrate that elevated oxidative stresses caused by environmental stimuli, including ozone, UV-B, and wounding enhance ethylene production via ACC synthase and ACC oxidase (Wang et al., 2002). These data suggest that oxidative stress may function upstream to regulate ethylene biosynthesis. Therefore, transgenic potato overexpressing a chloroplastic Cu/ZnSOD gene of lily results in elevated H_2O_2 and in turn triggers ethylene biosynthesis (Kim et al., 2008). In tobacco cell suspension culture, an elicitor, quercinin, induced ethylene biosynthesis and H_2O_2 formation. Ethylene at low concentrations proved to be necessary for induction and maintenance of H_2O_2 production in tobacco cells treated with quercinin. However, ethylene biosynthesis inhibitor α-amino-oxy-acetic acid (AOA) and $CoCl_2$ decreased or inhibited the quercinin-induced oxidative burst (Koehl et al., 2007). In ozone treatment, ethylene also can enhance reactive oxygen species (ROS) generation, which in turn leads to ethylene biosynthesis and cell

death (Wang et al., 2002). These data suggest that oxidative burst may also function downstream of ethylene, and can be affected by ethylene *de novo* synthesis and calcium influx. Therefore, repression of ethephon-induced oxidative stress elevation by EGTA in detached sweet potato leaves (Figure 5B) agrees with these reports, and suggest a possible explanation for EGTA repression of ethephon-induced oxidative stress elevation mediated by calcium influx. The relationship of external calcium influx and elevated oxidative stress in ethylene signaling is complex and most still remains inconclusive in sweet potato leaf senescence.

Induction of SPCP1 expression, leaf senescence and senescence-associated markers were all repressed by exogenous cycloheximide within the first 6 to 12 h in ethephon treatment (Figures 7 and 8), and suggest the requirement of *de novo* synthesized proteins for ethephon effects. The reasons for the requirement of *de novo* synthesized proteins are unclear. However, genes associated with ethephon-mediated effects described above likely require the primary response gene products within the first 6 to 12 hours after treatment for activation. In *Arabidopsis*, EIN2 is a positive regulator of ethylene response and regulates a transcriptional cascade initiated by EIN3 and EIL1, two members of a small family of DNA-binding proteins. EIN3 activates ethylene responses by binding to the EIN3-binding site (EBS) in the promoter of ERF1, a transcriptional activator that binds to the GCC-box in the promoters of several ethylene-responsive genes (Kendrick and Chang, 2008). Therefore, expression of primary response genes such as ERF1 transcriptional activator are required for ethylene responsive genes, and affected by cycloheximide. For auxin signaling, transcriptional activation of early genes, which encode short-lived nuclear transcription factors, is required for activation or repression of secondary response genes has also been reported (Abel et al., 1994). In conclusion, ethephon-mediated induction of SPCP1 expression, leaf senescence and senescence-associated markers in sweet potato detached leaves requires external Ca^{2+} influx, elevated oxidative stress, and *de novo* synthesized proteins. "How are the oxidative stress, external Ca^{2+} influx, and *de novo* synthesized proteins weaved together in the ethylene signaling pathways leading to leaf senescence and gene induction in sweet potato leaf" awaits further investigation.

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Ethephon 誘導之分離的甘藷葉片老化受還原態的 glutathione 及EGTA 影響

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本研究利用甘藷分離的葉片探討 ethephon 誘導老化過程幾個相關標幟的變化。於 ethephon 誘導的老化葉片其葉綠素含量及 Fv/Fm 值顯著減少，然而其 H_2O_2 含量及半胱胺酸蛋白酶 (SPCP1) 表現顯著比對照組增加。於添加還原態的 glutathione、EGTA 或 cycloheximide 前處理下，ethephon 誘導的老化葉片其葉綠素含量及 Fv/Fm 值的減少顯著趨緩，然而其升高的 H_2O_2 含量及增加的 SPCP1 表現量顯著受抑制。Ethephon 誘導增加 SPCP1 表現量於還原態的 glutathione、EGTA 及 cycloheximide (CHX) 存在下顯著受到抑制。切下的葉片處理 calcium ionophore A23187 及內生 glutathione 合成抑制劑 L-buthionine-sulfoximide (BSO) 也會增加 SPCP1 的表現，此增加的表現量亦分別受 EGTA 及還原態的 glutathione 抑制。Cycloheximide 有效抑制 ethephon 誘導 SPCP1 表現的時間約在 ethephon 加入後 6 至 12 小時內。依據這些實驗數據結論 ethephon 誘導甘藷葉片老化及 SPCP1 的表現顯著受到還原態的 glutathione、EGTA、及 cycloheximide 的抑制，這些結果也建議 ethephon 誘導葉片老化及基因表現時可能與細胞外的鈣離子、氧化逆境、及新合成的蛋白質有關。

關鍵詞：半胱胺酸蛋白酶；EGTA；ethephon；穀胱甘肽；甘藷；葉片老化。