PROTEASOME AND THE DELETED-IN-SPLIT HAND/SPLIT FOOT 1 PROTEIN SPDSS1 PARTICIPATE IN THE MODULATION OF DROUGHT STRESS TOLERANCE IN SWEET POTATO LEAVES

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國立中山大學研究生學位論文審定書

本校生物科學系研究所碩士班
研究生 馮寧寧（學號：M062010024）所提論文

蛋白酶體及 Deleted-in-Split Hand/Split Foot 1 蛋白質 SPDSS1 參與調節甘藷葉片對乾旱逆境的耐受性

Proteasome and the Deleted-in-Split Hand/Split Foot 1 Protein SPDSS1 Participate in the Modulation of Drought Stress Tolerance in Sweet Potato Leaves

於中華民國 108 年 07 月 16 日經本委員會審查並舉行口試，符合碩(博)士學位論文標準。

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Proteasome and the Deleted-in-Split Hand/Split Foot 1 Protein SPDSS1 Participate in the Modulation of Drought Stress Tolerance in Sweet Potato Leaves

Abstract

In recent year drought and soil salinization of cultivated lands under extreme climate severely result in the unbalance between agricultural crop production and demand-provision systems. Therefore, improvement of crop stress tolerance especially for drought is an important world-wide issue. Ubiquitin-Proteasome System (UPS) widely exists in plants and plays a role in association with abiotic salt stress tolerance in sweet potato. However, its physiological function under drought stress is unclear. In sweet potato, drought promoted senescence and necrosis in leaves. Significant morphological, biochemical and metabolic changes of senescence-associated markers, including morphological yellowing/necrosis, reduction of chlorophyll and F_v/F_m contents, decrease of antioxidant enzyme/activity levels, elevation of NO/H_2O_2/MDA amounts, increase of mitogen-activated protein kinase/phosphorylation levels, and enhancement of cysteine protease SPCP1 expression were observed in treated leaves on days 12 and 15 as compared to the untreated control. Drought-mediated effects were all alleviated by the proteasome inhibitor MG132, suggesting an association of proteasome activities in drought stress tolerance. In our laboratory, a cloned full-length cDNA *SPDSS1* from sweet potato leaves encodes a putative deleted in split hand/split foot 1 (*DSS1*)-like protein, which is an acid protein with 75 amino acids (228 nucleotides) and three conserved aspartic/glutamic acid-rich domains in its open reading frame and is a candidate subunit of the 19S regulatory proteasome according to the previous report in *Arabidopsis*. Drought enhanced *SPDSS1* expression level earlier than the induced leaf senescence. Exogenous SPDSS1 fusion protein accelerated drought-induced senescence/necrosis and enhanced changes of senescence-associated markers on day 9 and 12 in treated leaves, which could be inhibit and reversed by the proteasome inhibitor MG132. Based on these results we conclude that proteasome inhibitor MG132 and the 19S regulatory proteasome subunit deleted in split hand/split foot 1 (*DSS1*)-like protein SPDSS1 participate in the modulation of drought stress tolerance in sweet potato leaves. A novel physiological role of the Ubiquitin-Proteasome system in association with drought stress tolerance is also suggested.

***Keywords*:** Drought Stress, Leaf Senescence, MG132, Proteasome, Sweet Potato SPDSS1

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I dedicated this thesis for my parents, my brother and sisters who always give me supports through prayers and for always cheering me up no matter where they are.

Lastly, I hope this thesis would have positive contribution and be useful in academic research field.

Nindi Nazula Fajarini

Table of Contents

Table of Figures

Abbreviations

Acronyms	Full name
AsA	Ascorbic Acid
ABA	Abscisic acid
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
APS	Ammonium persulfate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BPB	Bromophenol Blue
BRCA2	Breast cancer 2
BHT	2-6-di-tert-butyl-4-methylphenol
BSA	Bovine serum albumin
C.C.I.	Chlorophyll content index
Ca.	Around, about, roughly, approximately
DAB	3,3′-Diaminobenzidine tetrahydrochloride
DMSO	Dimethyl sulfoxide
EDTA	Ethylendiamintetraacetat
F_v/F_m	Photochemical efficiency
GSH	Reduce glutathione
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Liquid Beef
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
NED	N-(1-Naphthyl)ethylenediamine dihydrochloride
NO	Nitric oxide
PBS	Phosphate - buffered saline
PBST	Phosphate - buffered saline – Tween 20
PCR	Polymerase Chain Reaction
RT-PCR	Reverse transcription polymerase chain reaction
ROS	Reactive oxidate species

RNS	Reactive nitrogen species
SAGs	Senescence-associated genes
SOD	Superoxide dismutase
SPAPX	Sweet potato ascorbate peroxidase
SPCAM	Sweet potato calmodulin
SPCAT1	Sweet potato catalase1
SPDSS1	Sweet potato DSS1 (deleted in split hand/split foot)
SPCP1	Sweet potato cysteine protease 1
SPCP2	Sweet potato cysteine protease 2
SPCP3	Sweet potato cysteine protease 3
SPAE	Sweet potato asparaginyl endopeptidase
SPCAT	Sweet potato catalase
SDS	Sodium dodecyl sulfate
TBA	2-thiobarbituric acid
TBE	Tris-borate-EDTA
TEP	Thiobarbituric Acid
TEMED	Tetramethylethylenediamine
UPS	Ubiquitin-proteasome system

Chapter 1. Introduction

1.1 Drought Stress and Leaf Senescence

Since the climate depend on global warming, it causes future anthropogenic emissions and natural climate variability. It generate extreme rainfall and drought which resulting environmental problem (Pratiwi et al., 2018). The extreme rainfall will increase flood and landslide. On the other hand, the extreme drought has a potential for prolonged dry season is relatively high (Mursidi A, 2017). In 2015/2016, Indonesia stricken a drought disaster causing many environmental and economy problems (Lestari et al., 2018). While droughts have the potential to affect an entire regional economy, the agricultural sector is particularly vulnerable (Climate Change Science Program 2008; Walthall et al. 2012). Therefore, climate hazards affected by drought could have an impact on agricultural production. Although researchers have documented the effects of climate changes on agriculture at different scales, past studies did not focus on adaptive changes to manage the impact of drought on crop yields (Troy et al., 2015).

In plants, drought stress induces a number of changes at the morphological, physiological and biochemical levels in all it organs (Osmond et al., 1995). Plants have evolved several strategies to cope with drought stress, including drought escape via a short life cycle or developmental plasticity, drought avoidance via enhanced water uptake and reduced water loss, as well as drought tolerance via osmotic adjustment, antioxidant capacity, and desiccation tolerance (Zhang Q, 2007). In order to resist the conditions of drought and water shortage, many plant species accumulate soluble organic compounds such as osmoregulators. This process is known as osmotic adjustment and it is considered as an important tolerance-mechanism, which allows the maintenance of cellular turgor and favors the absorption of water (White et al., 2000; Chaves et al., 2003). In this process, the biosynthesis and accumulation of nontoxic molecules of low-molecular weight in the vacuole and cytosol such as inorganic ions, soluble sugars, amino acids, proline, and glycine-betaine contribute to keep the integrity of cellular membranes and proteins, which are necessary for metabolic activities. Therefore, studies on the accumulation of these molecules have been used as biochemical and physiological indicators in the evaluation of drought tolerance in several species (Shao et al., 2006; Farooq et al., 2009).

In addition to these physio-biochemical changes mentioned above, plants also generates various secondary signals such as mitogen-activated protein kinases (Tena et al., 2001), reactive

oxygen species (ROS), calcium ions, and nitric oxide under drought stress, which may lead to alter gene expression patterns and metabolic pathways such as antioxidant enzymes, including superoxide dismutase, catalase, ascorbate-glutathione cycle-related enzymes (Noctor and Foyer, 1998; Willekens et al., 1997). Once the stress situation continues, it may finally result in altered physiological status with adverse effects (Tuteja and Sopory, 2008). Therefore, flowering plants under drought conditions altered turgor pressure and resulted in stomatal closure, reduced photosynthetic efficiency, decreased biochemical reactions, and accelerated leaf senescence (Rivero et al., 2007). In *Z. mays* L., drought induced reduction of chlorophyll content, relative water content (RWC) and soil water content, increase of reaction oxygen species, severe cell death and degradation of protein (Huang B et al., 2019). Saxena et al. (2016) clarified that abiotic stress including drought, induced the H_2O_2 generation which modulated hormone ABA homeostasis, NADPH oxidase activity, MAPK phosphorylation, signal transduction, senescence-associated gene expression, and final cell death/leaf senescence, suggesting an association of H_2O_2 with early stage signaling and late stage oxidative stress generation/damage under drought stress.

1.2 Leaf Senescence

Senescence is a normal event in the life cycle of plants (Borrás et al., 2003). It is a sequence of complex degenerative processes that are initiated at full maturity and the final developmental phase of a leaf which starts with nutrient salvage and ends with cell death (Jing et al., 2005; Tang et al., 2005). Senescence in plants is defined as the age-dependent programmed degradation and degeneration process of cells, organs or the entire organism, leading to death (Lim et al., 2007). The process of leaf senescence can be triggered by different signals such as phytohormones and environmental stresses, once the senescence program is initiated, as indicated by similar changes in gene expression, similar processes of senescence execution will be launched to achieve nutrient remobilization and ensure the completion of the life cycle of a plant (Guo & Gan, 2012; Guo, 2013).

The most remarkable events in leaf senescence are the loss of chlorophyll and the disassembly of the photosynthetic apparatus, which result in decreases in the photosynthetic energy conversion capacity and efficiency (Falqueto et al., 2009). By contrast, plant senescence at the organ level is manifested in the spectacular changes in leaf color and the subsequent death of autumn leaves (Woo et al., 2013). The first visible event during senescence is leaf yellowing,

which typically starts at the leaf margins and progresses to the interior of the leaf blade. The protein and RNA degradation parallels a loss in photosynthetic activity and the majority of the senescence processes have occurred by the time yellowing of the leaf can be seen (Buchanan-Wollaston et al., 2003). During the process of leaf senescence, macromolecule such as proteins, lipids and polysaccharides are broken down into small mobile molecules which are translocated through the vascular system to sink tissues including seeds (Li W et al., 2017).

1.3 MAPK Cascade

MAPK cascade consists of important signaling modules that convert signals generated from the receptors/sensors to cellular responses (Mishra et al., 2006). Mitogen-activated protein kinases (MAPKs) are one of the largest group of transferases, catalyzing phosphorylation of appropriate protein substrates on serine or threonine residues (Jagodzik et al., 2018). There are three signal molecules, MAPKKK, MAPKK, and MAPK associated with the MAPK cascade. The first step of signal transduction in the MAPK cascade is phosphorylation of the MAPKKK by the activated membrane receptors. Then, it in turn activates a downstream MAPKK by phosphorylation of its two serine or threonine residues in the S/T-X_5-S/T (X is any amino acid) motif of its activation loop. Once activated, the MAPKK behaves as a kinase with dual-specificity, which phosphorylates a MAPK on the threonine and tyrosine residues in the T-X-Y motif of an activation loop located between subdomains VII and VIII of its catalytic domain (Hettenhausen et al., 2014). MAPKs are serine/threonine protein kinases that activate various downstream effector proteins in the cytoplasm or nucleus, including kinases, enzymes, cytoskeletal proteins, or transcription factors (Rodriguez et al., 2010).

In plants, MAPKs locate in the cytosol and nucleus, and play important roles in the transduction of environmental cues and developmental signals leading to different cellular processes (Wang Z. et al., 2015). By regulating MAPK cascades, cells are able to respond to a range of environmental stresses such as high/low temperature, UV radiation, ozone, drought, heavy metal, wounding, insect attack, and pathogen infection (Zelicourt et al., 2016). Therefore, Arabidopsis MPK6 can be activated by drought and rapidly inactivated during rehydration in seedlings (Xu & Chua, 2012). The *mapkkk18* knockout mutants displayed hypersensitivity to drought stress. Accumulation of MAPKKK18 in transgenic Arabidopsis plants significantly enhanced drought stress tolerance (Li Y et al., 2017).

1.4 Antioxidant-related Enzymes

Reactive oxygen species (ROS), once perceived as toxic by-products, were known to cause oxidative damage in cells (Ozyigit et al, 2016). The exposure of plants to unfavorable environmental conditions increases the ROS production such as, singlet oxygen ($^1O2$), superoxide ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) (Caverzan, et al., 2012). Later, novel regulatory roles of these species were revealed in a wide range of biological processes such as cell signaling, growth, development, programmed cell death, and plant responses to various biotic/abiotic stress factors (Uzilday et al., 2014).

Enzymatic antioxidants comprise superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR). These enzymes are present in virtually all subcellular compartments. Usually, an organelle on the average is equipped more than one enzyme capable to scavenge a single ROS (Scandalios JG, 2005). In plant chloroplast, the main hydrogen peroxide-detoxification system is the ascorbate-glutathione cycle with the key enzyme APX (Asada, 1992). APX utilizes AsA as specific electron donor to reduce H_2O_2 to water. The importance of APX and ascorbate-glutathione cycle is not only restricted to chloroplasts but also in cytosol, mitochondria and peroxisomes (Noctor and Foyer, 1998; Shigeoka et al., 2002).

Expression of catalase isozymes exhibits development and tissue specificity in plants. Various abiotic stress conditions are known to trigger the ROS production such as hydrogen peroxide and the activation of its associated anti-oxidative defense reaction such as catalase. Catalase, which is an important antioxidant enzyme, has excellent enzyme kinetics and plays a key role in the removal of H_2O_2 from cells. It occurs in peroxisomes, mitochondria, cytosol and chloroplast (Ahmad P & Sharma I, 2014). The role and regulation of catalase activity as a response of plants to abiotic stress is an area worthy of further investigations (Leung D, 2018).

1.5 Senescence-associated Cysteine Proteases

Leaf senescence is a form of programmed cell death, and is believed to involve preferential expression of a specific set of “senescence-associated genes” (SAGs) (Gepstein et al., 2003). Several genes up-regulated during senescence have been identified in various species (Dang et al., 2000; Lee et al., 2001). These SAGs encode diverse proteins including RNases, proteases, lipases, proteins involved in the mobilization of nutrients and minerals, transporters, transcription factors, proteins related to translation, and antioxidant enzymes (Espinoza et al., 2007).

Protein degradation associated with senescence requires a multitude of proteases. They belong to five major classes: cysteine proteases (CPs), serine proteases (SPs), aspartate proteases (APs), metalloproteases (MPs), and threonine proteases (TPs) (Guo et al., 2004). Their specific roles in protein breakdown during leaf senescence are not well-known. Expression of CPs, however, is the class generally found in many plants during senescence (Poret et al., 2016). Therefore, the papain-like cysteine protease *SAG12* is a CP strongly induced in senescent leaves of *Brassica napus* and *Arabidopsis thaliana* (Noh and Amasino, 1999a; b), especially in plants cultivated under nitrogen limitation (Poret et al., 2016). In addition, high SAG12 protein levels are also detected in senescing leaf tissues and in fallen leaves (Desclos-Théveniau et al., 2015). There are different cysteine proteases identified in sweet potato leaves. For *SPCP1*, it is a papain-like cysteine protease and exhibited a high amino acid sequence homology with *Arabidopsis thaliana* SAG12. Its expression increased remarkably in senescent leaves (Chen et al., 2009). For *SPCP2*, it is also a papain-like cysteine protease. Its expression was enhanced in natural and ethephon-induced senescent leaves. Cysteine protease *SPCP2* is also a functional senescence-associated gene and causes altered developmental characteristics and stress responses when ectopically expressed in transgenic *Arabidopsis* plants (Chen et al., 2010b). For *SPCP3*, it belongs to a granulin-containing cysteine protease. It is a functional senescence-associated gene and significantly induced in natural and ethephon-induced senescent leaves. Ectopical expression of cysteine protease *SPCP3* also causes altered developmental characteristics and stress sensitivity in transgenic *Arabidopsis* plants (Chen et al., 2013a).

1.6 Proteasome

The Ubiquitin-Proteasome System (UPS) is one of the major protein degradation systems found in all eukaryotic cells and regulate most cellular processes, including cell division, signal transduction, and development (Finley, 2009). It is now clear that regulated protein degradation by the UPS affects virtually every cellular process. The ubiquitin protein consists of 76 amino acids. It functions likely beyond tagging proteins for degradation but acts as a versatile signaling molecule. Tagging by mono-ubiquitin and ubiquitin chains have non-proteolytic roles and signal in diverse pathways such as membrane transport, DNA repair, chromatin structure and transcription. Most proteasome-degraded proteins form a poly-ubiquitin chain in a covalent manner with ubiquitin (Pickart and Fushman, 2004).

The UPS is a huge protein complex with numerous components that act in a highly-regulated manner. It generates a chain of events which result in substrate ubiquitination and their subsequent degradation. Ubiquitination involves three types of enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme (also called ubiquitin-carrier protein), and E3 ubiquitin ligase (Finley, 2009). For ubiquitination, conjugation of ubiquitin (UB) begins with an ATP-dependent activation of the C-terminal glycine residue of the E1 molecule. Ub is then transferred as an activated moiety to an E2. The last step of substrate ubiquitination is carried out by the formation of a complex consisting of Ub-loaded E2 and a specific E3 to which the substrate protein is bound. It is then transferred from the E2 either directly to a lysine residue of the substrate when the E3 is one of the RING-finger type, or to an internal cysteine residue in the E3 when the enzyme is one of the HECT-domain type. Finally, it is conjugated to the substrate. Additional ubiquitins are then added by the same cascade, creating Ub chain (Finley, 2009). Furthermore, Ub chains can be also elongated by an additional type of ligase E4 (also termed the "Ub-chain elongation factor"). The reaction is not recognized and degraded by the proteasome until sufficient ubiquitin is attached to the target protein to form a poly-ubiquitin chain (Pickart and Fushman, 2004). Different amounts of ubiquitin have different functions.

In proteasome studies, the 26S proteasome is the catalytically active proteasome and widely exists in eukaryotes, archaea, and prokaryotic bacteria. They are mainly located in the cytoplasm and nucleus of eukaryotic cells and are responsible for the degradation of damaged proteins (Peters et al., 1994). It degrades the target proteins first into peptides containing 7-8 amino acids, then, further decomposes them into amino acids by additional enzymes (Sadanandom et al., 2012). The 26S proteasome has a high degree of similarity in eukaryotes, its structure is mainly composed of the 20S proteasome (core particle) with chymotrypsin and trypsin-like activity and the 19S proteasome (regulatory particle) with ub-dependent ATPase activity. The 19S proteasome removes and accepts ubiquitin from the labeled protein and transfer the protein portion to the 20S proteasome for degradation (Hanna and Finley, 2007).

Plant cells contain two different proteasome proteolysis mechanisms, ubiquitin-dependent and ubiquitin-independent proteasomes. The proteasome consists of 2 subunits, the 20S and the 19S subunits, in which one 20S core particle can form the 26S proteasome with two 19S regulatory particles or exist as a separate subunit to perform the degradation.

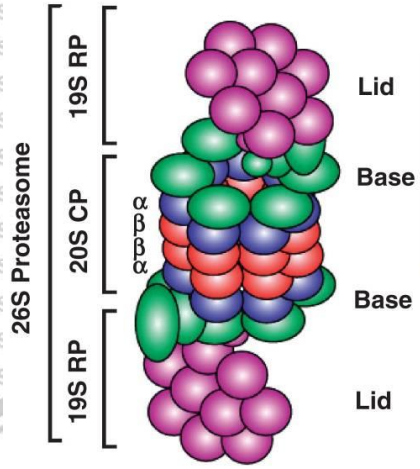


Figure 1. Schematic drawing of the proteasome subunit structure. CP, core particle (20S proteasome); RP, 19S regulatory particle consisting of the base and lid sub-complexes (adapted from Tanaka K, 2009).

1.7 Deleted in Split Hand/Split Foot 1 Protein (DSS1)

The DSS1 is a phylogenetically conserved, small acidic protein with ca. 70–90 amino acid residues in length (depending on the species) in eukaryotes. The gene was originally linked to split hand/split foot malformation (SHFM), an autosomal dominant limb developmental disorder, characterized by missing digits and fusion of the remaining digits, and therefore named DSS1 for deletion of split hand/split foot 1 (Crackower MA et al., 1996; Ignatius J et al., 1996). Its encoded protein DSS1 or its orthologs (e.g. Sem1 in Baker's yeast) is now known to be involved in many important biological and cellular processes (Pick et al., 2009), including genome stability (Li J et al., 2006), homologous recombination and DNA repair (Liu et al., 2010), cellular proliferation and neoplastic transformation (Wei et al., 2003), protein degradation (Wei et al., 2008), histone modification (Qin et al., 2009), and mRNA splicing, metabolism, and export (Ellisdon et al., 2012).

In *Saccharomyces cerevisiae*, its DSS1 orthologue has been associated with the 26S proteasome. In fission yeast *Schizosaccharomyces pombe*, its DSS1 protein has been found in association with the 19S regulatory particle of the 26S proteasome. In human, DSS1 is generally associated with the tumor suppressor protein BRCA2, and is essential for the stability of BRCA2 (Josse L et al., 2006). Therefore, depletion of the DSS1 protein leads to the increase of the BRCA2 protein degradation. In the *brca2* mutant devoid of the DSS1-binding domain (DBD), its

protein stability is unaffected by the depletion of DSS1 (Li J et al., 2006). These reports show that the DSS1 protein is evolutionarily conserved and plays a novel regulatory role of the 26S proteasome.

The DSS1 position map in proteasomes was recorded (Paraskevopoulos et al., 2014). In yeast, formation of the 19S proteasome requires the participation of SEM1 (DSS1), which is involved in the successful combination of all subunits together (Tomko and Hochstrasser, 2014). The DSS1 protein contains a highly-conserved domain: EDDEFEEF with a high Asp and Glu content (Asp-Glu acid rich region). When the conserved region is mutated, the DSS1 protein loses its ability to link with other subunits and in turn affects the ability to interact with specific proteins, such as the BRCA2 protein. The possible function of BRCA2 is likely involved in DNA repair and cell cycle regulation, and has a great correlation with the occurrence of cancer (Josse L et al., 2006). In addition, DSS1 is also involved in the degradation of tumor suppressor proteins p53. Therefore, accumulation of p53 proteins could be observed in loses-of-function mutant *hsdss1* (Wei et al., 2008).

DSS1 can also perform modification of target proteins with the reaction of DSSylation. When target proteins are under oxidative damage, the DSS1 proteins bind to the damaged proteins and promote protein DSSylation first. The DSSylated proteins are modified further through the poly-ubiquitination step and delivered to the 26S proteasome for degradation (Zhang Y et al., 2014). Therefore, it may increase cell survival rate under oxidative damage. In sweet potato, a DSS1 protein SPDSS1 cloned from leaves exhibits high amino acid sequence homology with Arabidopsis DSS1 protein ATDSS1 (GenBank Accession number NM_202361.1) (Wu, 2010). Exogenous application of SPDSS1 fusion proteins in detached leaves delayed leaf senescence induced by ethylene and abiotic high salt stress (Chou, 2016). These data suggest that 26S proteasome and the putative 19S regulatory proteasome subunit SPDSS1 may also participate in the modulation of plant responses to development and environmental stresses in sweet potato.

1.8 Sweet Potato

Sweet potato is a grass dicotyledonous plant which belongs to the family Convolvulaceae (Ali et al., 2015). Sweet potato is also one of the tuber plants that widely grown in the tropical and subtropical parts of the world. Sweet potato has many different varieties with skin colors

from almost white to brown with shades of pink, copper, magenta and purple. Even the flesh colors vary from light yellow to pink, red and orange (Giango and Giango, 2017).

Sweet potato has been widely used for food and industrial application (Ji H et al., 2015). It is one of the most important food crops in the world, ranking the seventh in annual production worldwide (FAO, 2013). Sweet potato consists of many nutrients, including carotenoids, carbohydrates, minerals (calcium, potassium, and iron), dietary fiber, protein, provitamin A, and vitamin C (Wang H et al., 1997; Shih et al., 2007). In addition, sweet potato is widely used as a useful source of starch, in animal feed, a staple food, and as a carbohydrate source for bioethanol production (Kim HS et al, 2016).

Ipomoea batatas cv. Tainong 57 is one of the main varieties cultivated in Taiwan. The leaf shape of the plant is a five-deep or three-deep-splitting trait. The color of the flesh is brownish-yellow, the stem is green or yellow-green, and the veins are lavender. In previous studies, the mechanisms of leaf senescence induced by ethylene, salt, and drought in sweet potato have been known to a certain extent, and therefore have a good research value.

1.9 Related Research of Leaf Senescence in Sweet Potato

In sweet potato, leaf senescence can be promoted by ethephon, an ethylene-releasing compound (Chen, et al., 2012a), salt stress (Chen et al., 2012b), and drought stress (Tsai T, 2016) based on the changes of senescence-associated markers, including morphological leaf yellowing, reduction of chlorophyll content and F_v/F_m level, elevation of NO/H_2O_2/ malondialdehyde (MDA) amounts, increase of membrane electrolyte leakage percentage, and enhancement of senescence-associated gene expression (Chen et al., 2012a; Afiyanti, 2015; Tsai, 2016).

For signal-related components, application of MAPK cascade inhibitor PD98059 (Chang, 2016) or calmodulin inhibitor CPZ (Chen et al., 2012a) attenuated ethephon and salt stress-induced leaf senescence. Exogenous SPMAPK (Chang, 2016) or SPCAM (Chen et al., 2012a) fusion proteins also enhanced ethephon and salt stress-mediated leaf senescence, suggesting a role of signal components SPMAPK and SPCAM in association with ethephon and salt stress-induced leaf senescence in sweet potato.

For antioxidant enzymes such as catalase (Chen et al., 2011; 2012c), superoxide dismutase and ascorbate-glutathione cycle-related enzymes (Yan, 2015), their gene expression was induced by ethephon and abiotic salt and drought stress (Afiyanti, 2015; Yan P, 2015; Tsai, 2016) and

reached the plateau at the middle stage of leaf senescence. Significant reduction of these antioxidant-related enzymes was also observed in ethephon and abiotic salt and drought stress-treated leaves at the late stage of leaf senescence (Chen et al., 2011; 2012c; Afiyanti, 2015; Yan, 2015; Tsai, 2016). Exogenous catalase SPCAT1 (Chen et al., 2012c) or ascorbate peroxidase SPAPX (Liu, 2014; Xu, 2015) fusion proteins attenuated ethephon and salt stress-induced leaf senescence in sweet potato, suggesting a role of antioxidant-related enzymes catalase SPCAT1 and ascorbate peroxidase SPAPX in association with ethephon and salt stress-induced leaf senescence in sweet potato.

For senescence-associated cysteine protease, expression of cysteine proteases *SPCP1* (Chen et al., 2009), *SPCP2* (Chen et al., 2010a), *SPCP3* (Chen et al., 2006), and *SPAE* (Chen et al., 2004) was enhanced in ethephon and NaCl stress-induced senescent leaves (Chen et al., 2012a; b). Ectopical expression of *SPCP2* (Chen et al., 2010a), *SPCP3* (Chen et al., 2013a), or *SPAE* (Chen et al., 2008) in transgenic *Arabidopsis* plants altered developmental characteristics and stress responses, including earlier flower, leaf senescence, silique development, drought stress tolerance, and salt stress tolerance. These reports suggest a role of senescence-associated cysteine proteases in association with development and stress responses in sweet potato leaves.

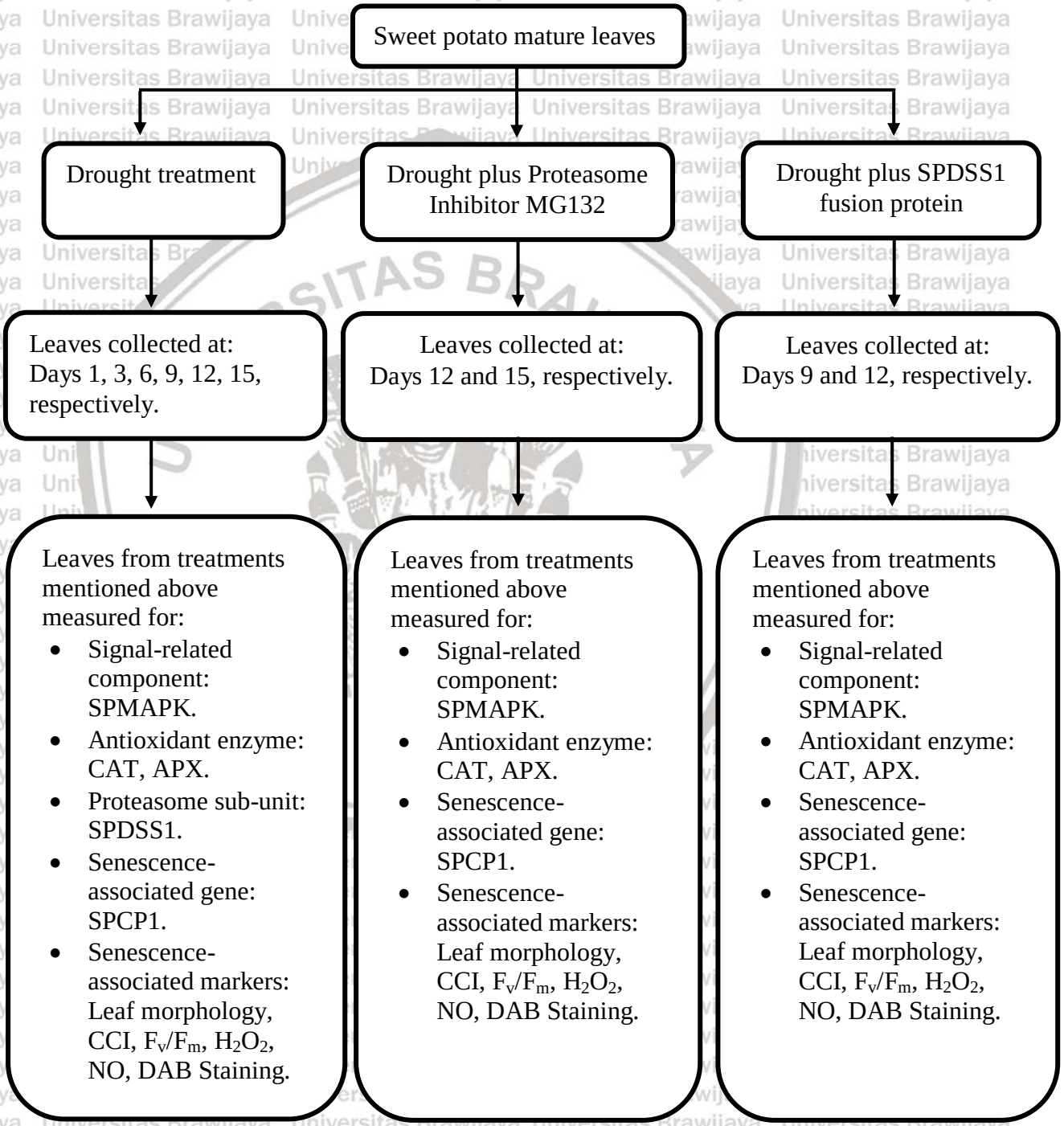
1.10 Specific Aims

In sweet potato, application of proteasome inhibitor MG132 or the putative 19S regulatory proteasome subunit SPDSS1 fusion protein attenuate ethephon and salt stress-induced lead senescence in sweet potato (Chou, 2016), suggesting a role of proteasomes and the putative 19S regulatory proteasome subunit SPDSS1 in association with ethephon and salt stress-induced leaf senescence in sweet potato. However, their possible physiological role in association with drought stress tolerance in sweet potato is still unclear. In this study, modulation of drought stress tolerance by proteasome inhibitor MG132 and the putative 19S regulatory proteasome subunit SPDSS1 fusion protein are performed in order to unravel their possible physiological role in drought stress tolerance. There are specific aims as below:

1. Whether proteasome inhibitor MG132 can modulate drought stress tolerance in sweet potato detached leaves.
2. Whether the putative 19S regulatory proteasome subunit SPDSS1 fusion protein can modulate drought stress tolerance in sweet potato detached leaves.

Chapter 2. Materials and Methods

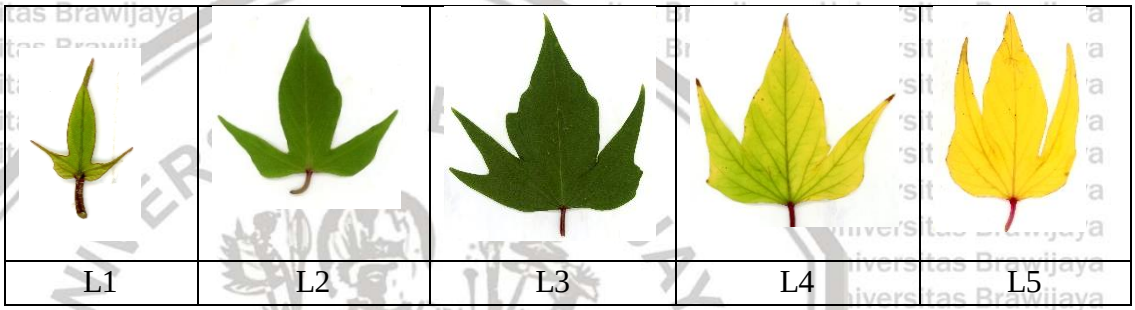
2.1 Experimental Flow Chart



2.2 Materials

2.2.1 Sweet Potato Leaves

Sprouts came out from sweet potato (*Ipomoea batatas* (L.) Lam. cv. Tainong 57) storage roots were planted in a pot and grown in an incubator with a 16 h/28°C day and 8 h/23°C night cycle. The mature leaves of plantlets on the third to the seventh positions counted from the shoot apical bud were collected and used for experiments (Chen et al., 2012a). Sweet potato leaves can be divided into L1-L5 according to the degree of maturity. L3 represents the fully mature leaves and are generally used in this research.



L1	L2	L3	L4	L5

L1: sweet potato leaves are new leaf buds, and the leaves are not yet stretched

L2: leaves have been stretched but not fully mature

L3: leaves are fully mature

L4: leaves are partially yellow

L5: leaves are completely yellow

2.2.2 Related Genes and Antibodies

There are different kinds of related genes from sweet potato leaves used in this study. They are divided into signal-related components, antioxidant-related enzymes, senescence-associated genes, and proteasome-related genes according their possible functions and shown as below:

1. Signal-related components: mitogen-activated protein kinase SPMAPK and anti-SPMAPK antibody (Shen, 2011; Chang, 2016).
2. Antioxidant-related enzymes: catalase SPCAT1 (Chen et al., 2012a)/ascorbate peroxidase SPAPX (Liu C, 2014) and anti-SPCAT1 (Afiyanti and Chen, 2014)/anti-SPAPX (Xu, 2016) antibodies.
3. Senescence-associated genes: cysteine protease SPCP1/anti-SPCP1 antibody (Chen et al., 2009).

4. Proteasome-related genes: the putative 19S regulatory proteasome subunit deleted in split hand/split foot 1 (DSS1)-like protein SPDSS1 (GU230148) (Wu, 2010; Chou, 2016).

2.2.3 Chemicals

The chemicals used in this research are list below:

Effector and Inhibitor Treatments	• Dimethyl sulfoxide/DMSO (Merck) • 10x PBS pH. 7.4 (Biomate) • MG132 (Sigma Aldrich) • Tween-20
Induction and Purification of SPDSS1 Fusion Proteins	• Ni-NTA His•Bind Resins (Merck) • IPTG (Gene mark Bio) • Kanamycin (Sigma Aldrich) • Protein Assay Dye (Bio-Rad) • Urea (Merck) • Di-sodium hydrogen phosphate (Na_2HPO_4) (Merck) • Sodium dihydrogen phosphate Dihydrate (NaH_2PO_4) (Merck) • Glycerol (Applichem)
DAB Staining	• 3,3′-Diaminobenzidine Tetrahydrochloride (DAB) (Sigma Aldrich)
H_2O_2 Measurement	• H_2O_2 solution (Sigma Aldrich) • Horseradish Peroxidase (HRP) (Sigma Aldrich) • Guaiacol (Sigma Aldrich)
Nitric Oxide Detection	• N-(1-naphthyl) ethylenediamine dihydrochloride (NED) (Merck) • Tris-HCl (Merck) • Sulfanilamide (Merck)
MDA Content Assessment	• 2-6-di-tert-butyl-4-methylphenol (BHT) (Sigma Aldrich) • 2-thiobarbituric acid (TBA) (Merck) • Thiobarbituric acid (TEP) (Merck) • N-butanol (Merck)
Catalase Activity Assay	• $FeCl_3$, K_3 [Fe $(CN)_6$] (Merck) • Glycerol (Applichem) • β-mercaptoethanol (Merck) • Bromophenol blue (Merck)

Ascorbate Peroxidase Activity Assay	• Tris base (Merck) • EDTA (Merck) • Bovine Serum Albumin (Sigma Aldrich) • L-ascorbic acid free acid (Calbiochem)
SDS-PAGE	• Coomassie Blue R250 • TEMED (Sigma Aldrich) • Glacial acid (Merck) • SDS (Calbichem)
Western Blot Hybridization	• PVDF membrane (Merck) • BCIP/NBT substrat solution (Perkin Elmer) • HRP Chemiluminescent substrat reagen kit (Invitrogen)

2.3 Methods

2.3.1 Drought Treatment

For drought treatment, the basic method is according to the report of Tsai (2016). Fully-expanded mature leaves (L3) of sweet potato were detached and placed on the surface of two dry paper towels in a plastic tray, and then sealed with aluminum foil and placed in a growth chamber at 16 h/28°C and 8 h/23°C under the dark. Samples were collected individually on days 0, 1, 3, 6, 9, 12 and 15 after treatment and analyzed for leaf morphology, chlorophyll content, F_v/F_m, DAB staining, H_2O_2/NO/MDA contents, antioxidant-related enzyme activity/protein amount (such as catalase and ascorbate peroxidase), signal-related mitogen-activated protein kinase t-SPMAPK and p-SPMAPK amount, senescence-associated cysteine protease SPCP1 level, and the putative 19S regulatory proteasome subunit deleted in split hand/split foot 1 (DSS1)-like protein SPDSS1 amount. The methods were described in detail as below.

2.3.2 Effector and Inhibitor Treatments

In order to study further whether proteasomes are involved in the drought-induced leaf senescence and necrosis in sweet potato, (1) the proteasome inhibitor MG132 pretreatment and (2) the effector SPDSS1 fusion protein application were used. The method is basically according to the report of Chou (2016) with a minor modification. For (1) MG132 pretreatment, the petioles of detached mature leaves were immersed in a 5 µl DMSO solution with or without 5 nmole proteasome inhibitor MG132 at room temperature for 2 h until complete uptake of the DMSO solution. For (2) SPDSS1 fusion protein pretreatment, the petioles of detached mature

leaves were immersed in a 25 µl $PBST_{0.01\%}$ solution with or without 25 µg SPDSS1 fusion protein at room temperature for 2 h until complete absorption of the $PBST_{0.01\%}$ solution. Then, the leaves were transferred onto the surface of two dry paper towels in a tray, and then sealed with aluminum foil and placed in a growth chamber at 16 h/28°C and 8 h/23°C under the dark. Drought alone treatment was also performed and used as the control. Samples were collected individually (1) on days 0, 12, and 15 or (2) on days 0, 9 and 12 after treatment and analyzed for leaf morphology, chlorophyll content, F_v/F_m, DAB staining, H_2O_2/NO, antioxidant-related enzyme activity/protein amount (such as catalase and ascorbate peroxidase), signal-related mitogen-activated protein kinase t-SPMAPK and p-SPMAPK amount, and senescence-associated cysteine protease SPCP1 level. The methods were described in detail as below.

For (3) reversion experiment, the petioles of detached mature leaves were immersed in a 25 µl DMSO/$PBST_{0.01\%}$ solution containing 0, 5 nmole MG132, 25 µg SPDSS1 fusion protein, and 5 nmole MG132 plus 25 µg SPDSS1 fusion protein, respectively, at room temperature for 2 h until complete uptake of the DMSO/ $PBST_{0.01\%}$ solution. Then, the leaves were transferred onto the surface of two dry paper towels in a tray, and then sealed with aluminum foil and placed in a growth chamber at 16 h/28°C and 8 h/23°C under the dark. Drought alone treatment was also performed and used as the control. Samples were collected individually (3) on days 0 and 12 after treatment and analyzed for leaf morphology, chlorophyll content, F_v/F_m, antioxidant-related enzyme activity/protein amount (such as catalase and ascorbate peroxidase), and senescence-associated cysteine protease SPCP1 level. The methods were described in detail as below.

2.3.3 Induction and Purification of SPDSS1 Fusion Proteins

The recombinant pET30a vector containing the SPDSS1 insert DNA was retransformed into BL21 (DE3) competent cells. After plating, one colony was selected from each plate for protein induction. A single colony was inoculated in 3 ml LB medium plus 50 µg/ml kanamycin and grown at 37°C at 150 rpm for 14 to 16 h. About 90 µl of the over-night cell culture was added into 9 ml LB medium with kanamycin (100 x dilution) and incubated at 37°C and 150 rpm for 3 h. When the value of $OD_{600\ nm}$ reached between 0.6 and 1.0, the cell suspension was induced with 1 mM IPTG for ca. 1, 3, and 5 h, respectively. Cells were collected from ca. 1.5 ml bacterial cell culture after centrifugation at 16,440 x*g* at room temperature for 5 minutes. The supernatant was poured off and the cell pellet was frozen quickly in liquid nitrogen and kept at -

80 °C freezer until use or directly mixed with 200 µl SDS-containing PBS buffer for direct extraction of fusion protein.

For extraction of fusion protein, the resuspended cell pellet in SDS-PBS buffer was sonicated for 5 minutes (a brief break per 30 seconds), then mingled with 40 µl 2X SDS sample buffer (0.92 gram SDS, 2 ml β-mercaptoethanol, 4 gram Glycerol, 0.3 gram "Tris" buffer, 2 ml 0.1% bromophenol blue), and boiled at 95°C for 3-5 minutes. The sample was centrifuged at 16,440 x*g* for 10 minutes, and the supernatant was used for SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Blue R250, and then de-stained with slow de-staining solution (10% methanol, 10% glacial acid).

The crude extract from IPTG-induced cell pellet was used as the source of SPDSS1 fusion protein purification via the His-tag sequence composed of 6-10 histidines. Since this amino acid is negatively charged, therefore, it can bind to the positive Ni^{2+} ions packaged in an affinity column for purification following the protocol supplied by the manufacture (Novagen). About 2 ml of buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 8.0) was evenly mixed with the cell pellet from ca. 60 ml cell culture, and frozen with liquid nitrogen, then rapidly raised the temperature with water tank 35 °C for 5 minutes in order to rupture the bacterial cell. The cell suspension was used for ultra-sonication ca. 45 minutes (a brief break per 30 seconds). After that, the total volume was increased with buffer B to ca. 60 ml and was shaked at 37 °C 120 rpm for 1 h. The sample was centrifuges at 16,440 x*g* at 4 °C for 10 minutes. The supernatant was transferred to a new centrifuge tube, then filtered through a 0.45 µm syringe filter, and finally passed the filtrate through an affinity column packaged with Ni-NTA His•Bind Resins. After the filtrate passed through, the column was washed with 50 ml each of buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 6.3), then eluted first time with buffer D (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 5.9), and the second time with buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 4.5). Clean the column and collect individual filtrate. Eluents from buffer D and E were mixed with acetone in a ratio (v:v) of 1:4, and allowed it to stand at 4°C for 45 minutes, then centrifuged at 16,440 x*g* at 4°C for 10 minutes, finally drained the supernatant and obtained the pellet of SPDSS1 fusion protein. The pellet of purified SPDSS1 fusion protein was re-dissolved in $PBST_{0.01\%}$ solution for exogenous application and reversion experiments with SPDSS1 fusion protein.

2.3.4 Leaf Morphology

Leaves from treatments mentioned above were scanned with a scanner for morphological record and comparison. Each experiment was repeated at least three times and a representative one was shown (Chen et al., 2011).

2.3.5 Chlorophyll Content Quantification

Leaves from treatments mentioned above were measured and recorded directly with a noninvasive CCM-200 Chlorophyll Content Meter. Each leaf sample was measured at least five different leaf areas, and each treatment was repeated at least three times. The data were expressed as mean ± standard error (S.E.) (Chen et al., 2012a).

2.3.6 F_v/F_m Determination

Leaves from treatments mentioned above were directly measured with a noninvasive WALZ JUNIOR-PAM Chlorophyll Fluorometer. Each leaf was measured on average at 10 different locations. Each treatment was repeated at least three times. The data were expressed as mean ± S.E. (Chen et al., 2010a).

2.3.7 DAB Staining

DAB staining method were used to qualitatively detect the H_2O_2 generation in leaves after treatments, and is basically according to our previous report (Chen et al., 2012a). Leaves from treatments mentioned above were collected individually and immersed in 1 mg/ml DAB solution (ca. 50 mg DAB in 45 ml sterile Mili-Q water with a pH 3.8 adjusted with 0.2 M HCl in order to dissolve the DAB powder) in a tray covered with aluminium foil to prevent light (since DAB is light-sensitive). The samples were gently shaking at 25°C for 90 minutes, and boiled in 95% ethanol for 10 minutes, then cooled down to room temperature and photographed. Each treatment was repeated at least three times, and a representative one was shown.

2.3.8 H_2O_2 Measurement

For quantitative measurement of H_2O_2 amount, leaves from treatments mentioned above were analyzed basically according to the method reported by Kuzniak et al. (1999). Ten leaf discs (1 cm in diameter) with fresh weight measured were incubated in 2 mL reagent mixture (50 mM phosphate buffer pH 7.0, 0.05% guaiacol and horseradish peroxidase (2.5 U mL^{-1})) for 2 h at room temperature in the dark. Four moles of H_2O_2 are required in order to form 1 M of tetraguaiacol, which has an extinction coefficient of ε = 26.6 cm^{-1} mM^{-1} at 470 nm. The absorbance in the reaction mixture was measured immediately at 470 nm and expressed as µmole

H_2O_2 g^{-1} leaf fresh weight. Each treatment was repeated at least three times. The data were expressed as mean ± S.E.

2.3.9 Nitric Oxide Detection

For quantitative measurement of NO amount, leaves from treatments mentioned above were analyzed. Ten leaf discs (1 cm in diameter) with fresh weight determined were incubated in 2 ml of 50 mM Tris buffer (pH 8.0) for 2 hr at room temperature in the dark. About 900 µl of the reaction solution were transferred into a new tube, and mixed with 100 µl solution (1% sulfanilamide and 0.1% N-1-napthylethylenediamine dihydrochloride (NED)), then held in the dark for 30 minutes. The absorbance in the reaction mixture was measured immediately at 548 nm and the concentration was converted using a standard curve (Chou, 2016).

2.3.10 MDA Content Assessment

For the establishment of MDA standard curve, 100 µl of TEP standard solution with different concentrations was mixed with 300 µl of 0.2% BHT solution and 1 ml of 0.4% TBA coloring agent (the formula is as follows). After shaking for 1 minute, the reaction is carried out as below. The solution was boiled at 90°C for 45 minutes, then transferred to an ice bath for 5 minutes, and followed by addition of an equivalent amount of n-butanol in total volume of 1,400 µl. The reaction mixture was centrifuged at 16,440 x*g* 4°C for 10 minutes, the supernatant containing the MDA standard was used for absorbance measurement at 535 nm wavelength with a spectrophotometer and a standard curve could be established.

TEP working standard solution preparation and MDA content estimation:

0.001% TEP standard solution (ml)	4.8	2.4	1.2	0.6	0.3	0.15
0.01 N HCl (ml)	5.2	7.6	8.8	9.4	9.7	9.85
Estimated MDA concentration (µM)	20	10	5	2.5	1.25	0.625

Leaves from treatments mentioned above were weighed and ground in liquid nitrogen using a mortar and pestle to fine powder. The fine powder was transferred into a centrifuge tube and mixed with 5 ml of 50 mM Tris-HCl (pH 7.5) solution for centrifugation at 16,440 x*g* 4°C for 10 minutes. About 1 ml of the supernatant was transferred to a new centrifuge tube, and mixed with 300 µl of 0.2% BHT and 1 ml of 0.4% TBA coloring agent. After shaking for 1 minute, the

solution was reacted in a 90°C water bath for 45 minutes, then placed in an ice bath for 5 minutes, and mixed with an equal volume of n-butanol (2,300 µl) for the MDA-TBA complex extraction, and finally centrifuged at 16,440 x*g* 4°C for 4 minutes to obtain the supernatant (orange). About 1 ml of the supernatant was used for the absorbance measurement at 535 nm wavelength with a spectrophotometer. The MDA contents in leaf samples were calculated according to the MDA standard curve. Each treatment was repeated at least three times. The data are expressed as mean ±S.E (Chou, 2016).

2.3.11 Catalase Activity Assay

Sweet potato leaves collected from treatments described above were used for catalase activity assay. Leaves were homogenized with mortar and pestle in liquid nitrogen into fine powder and mingled with two-fold volumes (w/v) of the extraction buffer (100 mM Tris-HCl pH 8.0, 20% glycerol and 30 mM β-mercaptoethanol) for centrifugation at 16,440 x*g* 4°C for 30 minutes. After centrifugation, the supernatant was transferred into a new centrifuge tube for protein content quantification according to the method of Bradford (1976).

For qualitative in-gel catalase activity assay with native PAGE, each sample containing ca. 20 µg total soluble proteins was mixed with 5× sample buffer without SDS (60 mM Tris-HCl pH 6.8, 50% glycerol, 28.8 mM β-mercaptoethanol and 0.1% bromophenol blue), then individually loaded into the 7.5% native-PAGE gels (0.375 M Tris-HCl pH 8.8) with a 4% stacking gel (0.125 M Tris-HCl pH 6.8) at 4°C, 80 V for 3 h. Then, the in-gel catalase activity was analyzed according to the report of Afiyanti and Chen (2014) with a slight modification. After electrophoresis, the gels were first immersed in equilibration buffer (100 mM Tris-HCl buffer pH 8.0) for 15 minutes, then the gels were soaked in 0.01% H_2O_2 solution for 10 minutes, and washed twice with Mili-Q water, and finally incubated in a solution containing both 1% $FeCl_3$ and 1% $K_3[Fe(CN)_6]$ for 5 minutes. After staining, the gels were washed again with Mili-Q water and recorded with a scanner.

For quantitative catalase activity assay, each sample containing 2 µg of total soluble protein was used. Total catalase activity was monitored with spectrophotometry according to the report of Chen et al (2011) by measuring the rate of H_2O_2 decrease in absorbance at 240 nm wavelength under room temperature. A 10 µL of supernatant sample was mixed with 990 µL of 50 mM potassium phosphate buffer pH 8.0 and 100 µL of 100 mM H_2O_2. Changes of absorbance in the reaction mixture at $A_{240\ nm}$ wavelength was monitored for 1 minutes and used to determine the

scavenging rate of hydrogen peroxide by catalases (µmole H_2O_2 consumed min^{-1} mg $protein^{-1}$). Each treatment was repeated at least three times. The data were expressed as mean ±S.E.

2.3.12 Ascorbate Peroxidase Activity Assay

Sweet potato leaves collected from treatments described above were used to determine the ascorbate peroxidase activity. Leaves were ground with mortar and pestle in liquid nitrogen into fine powder and mingled at 4 °C with 100 mM potassium phosphate buffer pH 7.0, containing 5 mM ascorbic acid (AsA) and 1 mM EDTA. After centrifugation at 16,440 x*g* 4 °C for 30 minutes, the supernatant was directly used for total ascorbate peroxidase activity assay. Protein content was quantified according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

For quantitative assay, total ascorbate peroxidase activity was determined according to the report of Amako et al. (1994). About 10 µl of supernatant was mixed with 990 µL of 50 mM potassium phosphate buffer pH 7.0 and 2.5 µL of 0.2 M ascorbic acid, and then measured the absorbance at 290 nm wavelength. After the first measurement, about 2.5 µl of 100 mM H_2O_2 was added to the reaction mixture. The reaction was started by adding H_2O_2 and the oxidation of ascorbate was determined by the decrease in the absorbance at 290 nm wavelength with a spectrophotometry. One unit of ascorbate peroxidase activity is defined as the amount of enzyme that oxidizes 1 µmole min^{-1} ascorbate under the above assay conditions. Each treatment was repeated at least three times. The data were expressed as mean ±S.E.

2.3.13 SDS-PAGE

In this experiment, Mini-PROTEAN 3 system (Bio-Rad) was used. The separating gel and the stacking gel were separately prepared (the formula is as follows):

Chemicals \ Gels	12.5 % Running Gel	4 % Stacking Gel
30% Acrylamide	4200 µl	650 µl
dH2O	3300 µl	3050 µl
1.5 M Tris-HCl (pH 8.8)	2500 µl	
0.5 M Tris-HCl (pH 6.8)		1250 µl
10% SDS	100 µl	50 µl

10% APS	50 µl	25 µl
TEMED	5 µl	5 µl

Place gels into the electrophoresis tank of Mini-PROTEAN 3 system, then pour 1x running buffer into the upper and lower tanks until it is flooded on the glass plate, and loaded the protein sample that has been mixed with 5x sample buffer and boiled at 95 °C for 5 minutes. The electrophoresis condition was set on 30 mA. After about 4 h, when the front edge of the bromophenol blue (BPB) dye was about 1.5 cm from the bottom of the glass frame, the electrophoresis was stopped, and the gel was carefully removed and washed with Mili-Q water. After adding 30 ml of Coomassie Blue R250 solution and shaked with 50 rpm at room temperature for 30 to 60 minutes, it was washed with Mili-Q water and then de-stained with a slow de-staining solution (10% methanol, 10% glacial acid) for overnight. The gel was briefly rinsed with Mili-Q water and scanned the results with a scanner.

2.3.14 Western Blot Hybridization

Sweet potato leaves collected from treatments mentioned above were used for Western blot hybridization according to the report of Chen et al. (2006). Leaves were homogenized with a mortar and pestle in liquid nitrogen and mingled with two volumes (w/v) of extraction buffer (100 mM Tris-HCl pH 8.0, 20% glycerol and 30 mM β-mercaptoethanol) at 4 °C. After centrifugation at 16,440 x*g* for 30 minutes at 4°C, the supernatant was transferred into a new centrifuge tube for protein quantification according to the report of Bradford (1976). Western blot hybridization was carried out according to the report of Chen et al. (2006). Protein samples (20 µg) extracted from leaf samples were mixed with 5x SDS sample buffer and directly boiled at 95°C for 5 minutes, except for SPDSS1 detection, prior to loading on 12.5% SDS-PAGE gel. After electrophoresis, proteins in the SDS-PAGE gel were transferred onto the PVDF membranes (Millipore) with the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The PVDF membrane after drying at 50°C for 2 h will be used for Western blot hybridization. For Western blot hybridization with anti-SPDSS1 antibody, additional steps were also applied. After protein transfer onto the PVDF membrane, it was first dried for about 45-60 minutes first and then fixed with 0.02% (v/v) glutaraldehyde for 15-20 minutes, then let it dried again for 45 minutes. The

PVDF membrane was washed briefly with Mili-Q water and immersed in methanol before Western blot hybridization with the anti-SPDSS1 antibody (Li J et al., 2006).

For detection of catalase SPCAT1/ascorbate peroxidase SPAPX/cysteine protease SPCP1 with anti-SPCAT1/anti-SPAPX/anti-SPCP1 antibodies, respectively, the substrates 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were added and reacted at 37°C for 15 minutes after the reaction of secondary Ab conjugated with alkaline phosphatase (AP) at 37°C for 1 h. The reaction was stopped with replacement of Mili-Q water. The results was finally scanned and recorded by an EPSON scanner.

For detection of mitogen-activated protein kinase SPMAPK/deleted-in-split hand/split foot 1 (DSS1)-like protein SPDSS1, the first antibody such as anti-t-SPMAPK Ab/anti-p-SPMAPK Ab/anti-SPDSS1 Ab was individually incubated with the PVDF membrane at 37°C for 1 h. After wash with $PBST_{0.05\%}$, the secondary antibody conjugated with the horse radish peroxidase (HRP) was applied and incubated at 37°C for 1 h. Finally the membrane was washed again with with $PBST_{0.05\%}$ and then immersed in phosphate buffer saline before incubation with the chemiluminescence ECL substrate (NOVEX ECL Chemiluminescence Substrate) for 5-10 minutes. The chemiluminescence image of the PVDF membrane was finally recorded by the chemiluminescence image detection machine (Gene Gnome Dedicated Chemiluminescence Imaging System).

2.3.15 Statistical Analysis

Experimental data are given as mean ± S.E. Student's *t*-test was used for comparison of two treatments. A difference was considered to be statistically significant when $P < 0.05$.

Chapter 3. Results

3.1 Drought Promote Leaf Senescence

3.1.1 Drought Promotes Changes of Senescence-Associated Markers and Cell necrosis in Leaves

Effects of drought stress on sweet potato leaf senescence/necrosis and senescence-associated markers, including morphological, biochemical, and physiological changes within a period of 15 days were conducted and analyzed (Figure 1). For leaf morphology, leaf became curling from day 6 (DR6) until day 12 (DR12) and turned gradually from dark green (DR6) into light green (DR12). On day 15 (DR15), the leaf petiole became dry and the leaf blade exhibited partial necrosis with more yellowing senescent region (Figure 1A). For chlorophyll content and F_v/F_m levels, a significant decrease of chlorophyll content was observed starting from DR9 (19.0) until DR15 (13.9) as compared to the untreated D0 control (21.8) (Figure 1B). Similar result for the F_v/F_m level was also found. A remarkable decline beginning from DR9 (0.63), through DR12 (0.33), until DR15 (0.17) as compared to the untreated D0 control (0.65) was also observed (Figure 1C).

For DAB staining, significantly higher brown areas of treated leaves DR12 and DR15 could be found as compared to the untreated D0 control which showed no remarkable brown area (Figure 2A). For H_2O_2/NO/MDA contents, the H_2O_2 amount began to increase from day 6 (DR6; 32.6 μmole g^{-1} FW leaf) and reached the plateau on day 15 (DR15; 86.3 μmole g^{-1} FW leaf) compare to the untreated D0 control (19.5 μmole g^{-1} FW leaf) (Figure 2B). Similar results were also found for NO amount. It increased starting from DR6 (20.1 μmole g^{-1} FW leaf) and gradually reached the plateau until DR15 (84.4 μmole g^{-1} FW leaf) as compare to the untreated D0 control (9.9 μmole g^{-1} FW leaf) (Figure 2C). However, MDA amount elevated beginning from DR9 (10.9 μM g^{-1} FW leaf) and gradually reached the plateau until DR15 (22.4 μM g^{-1} FW leaf) as compare to the untreated D0 control (4.7 μM g^{-1} FW leaf) (Figure 2D). Therefore, drought promotes leaf senescence/cell necrosis and changes of senescence-associated markers in sweet potato.

3.1.2 Expression and Protein Phosphorylation of Mitogen-Activated Protein Kinase SPMAPK Are Significantly Enhanced by Drought

Effects of drought stress on mitogen-activated protein kinase SPMAPK expression and protein phosphorylation in sweet potato leaves within a period of 15 days were conducted and analyzed (Figure 3). Drought slightly enhanced mitogen-activated protein kinase SPMAPK expression (Figure 3A) and protein phosphorylation (Figure 3B) at the early stage on DR1 and DR3, and then decreased on DR6. After that, it started to increase again on DR9, and reach the maximal level on DR12, and then reduced significantly to a much less undetectable level on DR15 necrotic leaves (Figure 3). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore, drought enhances mitogen-activated protein kinase SPMAPK expression and protein phosphorylation in sweet potato leaves.

3.1.3 Antioxidant Protein and Enzymatic Activity Levels are Drastically Reduced in Drought-Induced Necrotic Leaves

Effects of drought stress on antioxidant protein and enzymatic activity levels in sweet potato leaves within a period of 15 days were conducted and analyzed (Figure 4). For catalase, its in-gel activity was enhanced at the early stage on DR1 and DR3, and then started to decrease gradually from DR6 until DR15 as compared to the untreated D0 control (Figure 4A). Similar result was also found for total catalase activity. It was enhanced significantly on DR1 (21.5) and DR3 (22.2), then gradually decreased starting from DR12 (11.6) until DR15 (7.7) as compared to the untreated D0 control (17.5) (Figure 4B). Western blot hybridization also showed that the catalase SPCAT1 protein amount was slightly increased on DR1 and DR3, then started to decline gradually from DR6 until DR15 as compared to the untreated D0 control (Figure 4C). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount.

For ascorbate peroxidase, its total enzymatic activity was high at the early stage from DR1 until DR6 with no significant difference, then gradually decreased starting from DR9 (2.4) until DR15 (1.7) as compared to the untreated D0 control (3.6) (Figure 5B). Western blot hybridization showed that the ascorbate peroxidase SPAPX protein amount was enhanced at the early stage on DR1 and DR3, and then started to decline gradually from DR6 until DR15 as compared to the untreated D0 control (Figure 5B). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore, drought

reduces antioxidant protein amount and enzymatic activity level at the late stage in sweet potato leaves.

3.1.4 Expression of Senescence-Associated Cysteine Protease SPCP1 is Remarkably Elevated in Drought-Induced Necrotic Leaves

Effects of drought stress on senescence-associated cysteine protease SPCP1 expression in sweet potato leaves within a period of 15 days were conducted and analyzed (Figure 6). Western blot hybridization showed that its protein amount significantly increased on DR15 with two protein bands close to 35 kDa and 25 kDa, respectively (Figure 6). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore, drought enhances senescence-associated cysteine protease SPCP1 expression level at the late stage in sweet potato leaves.

3.2 Deleted-in-Split Hand/Split Foot 1 Protein SPDSS1 Accelerates Drought-Mediated Leaf Senescence

3.2.1 Expression of Deleted-in-Split Hand/Split Foot 1 (DSS1)-like Protein SPDSS1 Increases at the Early Stage of Drought Treatment

In our laboratory, a full-length cDNA *SPDSS1* has been cloned form ethephon-treated leaves. Its open reading frame encodes a putative deleted-in-split hand/split foot 1 (DSS1)-like protein, which contains 3 conserved domains with high aspartic and glutamic acids. The conserved Asp-Glu rich domains were shown in black in grey at the positions from amino acid residues 15 - 22 (15EDDEFEEF22), 39 - 45 (39WEDNWDD45), and 49 - 60 (49EDDFS-X-QLR-XEL60; X is any amino acid), respectively (Figure 7A) (Wu, 2010).

Effects of drought stress on sweet potato SPDSS1 expression in sweet potato leaves within a period of 15 days were performed (Figure 7B). To analyze the effect of drought treatment on the expression of deleted-in-split hand/split foot 1 (DSS1)-like protein SPDSS1, western blot hybridization was conducted. The result showed that a protein band with molecular weight near 48 kDa was detected. There was significant difference in the performance from the 1st day to the 15th day after drought treatment. On the DR1, the expression of SPDSS1 was increase compare to the untreated control (D0), but on the DR3 the expression start to gradually decreased and significantly drop at DR15 after treatment (Figure 7B). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore,

drought enhances Deleted-in-Split Hand/Split Foot 1 (DSS1)-like Protein SPDSS1 expression level at the early stage in sweet potato leaves.

The full length cDNA *SPDSS1* has been constructed with pET30a expression vector to form a recombinant vector, and induced to express by IPTG in BL21 (DE3) cells for SPDSS1 fusion protein (Figure 8). The purified SPDSS1 fusion protein was used for the following exogenous application on drought-treated leaves.

3.2.2 Exogenous SPDSS1 Fusion Protein Enhances Drought-Mediated Changes of Senescence-Associated Markers and Cell Necrosis in Leaves

Effects of SPDSS1 fusion proteins on drought-mediated leaf senescence/necrosis and senescence-associated markers were performed on days 9 and 12 (Figure 9). The $PBST_{0.01\%}$ -treated leaf samples were also performed at the same time for 9 days (PB9) and 12 days (PB12) as an additional control except drought alone (DR). For leaf morphology, leaf became curling and turned into light green on days 9 (DR9 and PB9) and 12 (DR12 and PB12) as compared to the untreated D0 control. No significant difference, whereas, was found between DR (drought alone) and PB ($PBST_{0.01\%}$) groups. Significant leaf yellowing and necrosis were found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9) and 12 (DS12) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 9A).

For chlorophyll content, a significant decrease was observed on days 9 (DR9 (11.0) and PB9 (10.4)) and 12 (DR12 (7.3) and PB12 (6.6)) as compared to the untreated D0 (20.4) control. No significant difference, whereas, was found between DR (drought alone) and PB ($PBST_{0.01\%}$) groups. Significant reduction of chlorophyll content was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9 (5.7)) and 12 (DS12 (1.7)) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 9B).

For F_v/F_m level, a significant decrease was observed on days 9 (DR9 (0.59) and PB9 (0.50)) and 12 (DR12 (0.28) and PB12 (0.23)) as compared to the untreated D0 (0.66) control. No significant difference, whereas, was found between DR (drought alone) and PB ($PBST_{0.01\%}$) groups. Significant reduction of F_v/F_m level was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9 (0.20)) and 12 (DS12 (0.07)) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 9C).

Similar results were also observed for H_2O_2 and NO contents. For H_2O_2 amount, a significant increase was observed on days 9 (DR9 (57.0 μmole g^{-1} FW leaf) and PB9 (65.7

μmole g^{-1} FW leaf)) and 12 (DR12 (64.9 μmole g^{-1} FW leaf) and PB12 (73.4 μmole g^{-1} FW leaf)) as compared to the untreated D0 (14.5 9 μmole g^{-1} FW leaf) control. No significant difference, whereas, was found between DR (drought alone) and PB ($PBST_{0.01\%}$) groups. Significant increase of H_2O_2 amount content was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9 (82.7 μmole g^{-1} FW leaf)) and 12 (DS12 (115.1 μmole g^{-1} FW leaf)) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 10A).

For NO content, a significant elevation was observed on days 9 (DR9 (47.6 μmole g^{-1} FW leaf) and PB9 (62.4 μmole g^{-1} FW leaf)) and 12 (DR12 (79.3 μmole g^{-1} FW leaf) and PB12 (81.2 μmole g^{-1} FW leaf)) as compared to the untreated D0 (12.2 μmole g^{-1} FW leaf) control. No significant difference, whereas, was found between DR (drought alone) and PB ($PBST_{0.01\%}$) groups. Significant enhancement of NO content was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9 (90.9 μmole g^{-1} FW leaf)) and 12 (DS12 (140.5 μmole g^{-1} FW leaf)) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 10B). Therefore, exogenous SPDSS1 fusion protein enhances drought-mediated changes of senescence-associated markers and leaf senescence/necrosis in sweet potato.

3.2.3 Exogenous SPDSS1 Fusion Protein Accelerates Drought-Mediated Induction and Protein Phosphorylation of Mitogen-Activated Protein Kinase SPMAPK

Effects of SPDSS1 fusion proteins on drought-mediated expression and protein phosphorylation of mitogen-activated protein kinase SPMAPK were performed. The $PBST_{0.01\%}$ -treated leaf samples were also performed at the same time as an additional control except drought alone (DR). Its expression and protein phosphorylation levels was significantly enhanced at the early stage on day 1 (DR1 and PB1), and then decreased on day 3 (DR3 and PB3) as compared to the untreated D0 control. Significant increase on day 1 (DS1) and decrease on day 3 (DS3) were found for the SPDSS1 fusion protein-pretreated group as compared to the control groups (DR1/PB1 and DR3/PB3) (Figure 11A). Its expression and protein phosphorylation levels was also significantly enhanced at the late stage on day 9 (DR9 and PB9), and then decreased on day 12 (DR12 and PB12) as compared to the untreated D0 control. Significant increase on day 9 (DS9) and decrease on day 12 (DS12) were found for the SPDSS1 fusion protein-pretreated group as compared to the control groups (DR9/PB9 and DR12/PB12) (Figure 11B). Rubisco L subunit was used as an internal control to indicate the physiological

status and the loaded protein amount. Therefore, exogenous SPDSS1 fusion protein accelerates drought-mediated changes of mitogen-activated protein kinase SPMAPK expression pattern and protein phosphorylation level during leaf senescence and necrosis.

3.2.4 Exogenous SPDSS1 Fusion Protein Enhances Drought-Mediated Decrease of Antioxidant Protein and Enzymatic Activity Levels in Leaves

Effects of SPDSS1 fusion proteins on drought-mediated reduction of antioxidant protein and enzymatic activity levels were performed on days 9 and 12 (Figure 12). The $PBST_{0.01\%}$ -treated leaf samples were also performed at the same time for 9 days (PB9) and 12 days (PB12) as an additional control except drought alone (DR). For catalase, its in-gel activity significantly decreased on days 9 (DR9 and PB9) and 12 (DR12 and PB12) as compared to the untreated D0 control. No significant difference, whereas, was found between DR (drought alone) and PB ($PBST_{0.01\%}$) groups. Significant reduction of in-gel catalase activity was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9) and 12 (DS12) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 12A).

Similar result was also found for total catalase activity. It was significantly decreased on days 9 (DR9 (11.0) and PB9 (11.1)) and 12 (DR12 (7.8) and PB12 (8.3)) as compared to the untreated D0 (25.4) control. No significant difference, whereas, was found between DR (drought alone) and PB ($PBST_{0.01\%}$) groups. Significant reduction of total catalase activity was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9 (6.1)) and 12 (DS12 (4.5)) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 12B).

Western blot hybridization also showed that the catalase SPCAT1 protein amount was slightly decreased on DR9 (DR9 and PB9) and much reduced on DR12 (DR12 and PB12) as compared to the untreated D0 control. No significant difference, whereas, was found between DR (drought alone) and PB ($PBST_{0.01\%}$) groups. Significant reduction of catalase protein level was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9) and 12 (DS12) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 12C). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount.

For ascorbate peroxidase, total enzymatic activity was gradually decreased on days 9 (DR9 (2.9) and PB9 (2.4)) and 12 (DR12 (1.4) and PB12 (1.4)) as compared to the untreated D0 (4.1) control. There is no significant difference, whereas, was found between DR (drought alone) and PB ($PBST_{0.01\%}$) groups. Significant reduction of total ascorbate peroxidase activity was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9 (1.4)) and 12 (DS12 (0.8)) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 13A). Western blot hybridization also showed that the ascorbate peroxidase SPAPX protein amount was gradually decreased from days 9 (DR9 and PB9) until DR12 (DR12 and PB12). Significant reduction of ascorbate peroxidase SPAPX protein level was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9) and 12 (DS12) in a time-dependent manner as compared to the control group (PB9 and PB12) (Figure 13B). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore, exogenous SPDSS1 fusion protein enhances drought-mediated decrease of antioxidant protein and enzymatic activity levels at the late stage in sweet potato.

3.2.5 Exogenous SPDSS1 Fusion Protein Promotes Drought-Mediated Induction of Senescence-Associated Cysteine Protease SPCP1 in Leaves

Effects of SPDSS1 fusion proteins on drought-mediated enhancement of senescence-associated cysteine protease SPCP1 expression level were performed on days 9 and 12 (Figure 14). The $PBST_{0.01\%}$ -treated leaf samples were also performed at the same time for 9 days (PB9) and 12 days (PB12) as an additional control except drought alone (DR). Western blot hybridization showed that senescence-associated cysteine protease SPCP1 expression level was enhanced on days 9 (PB9) and 12 (PB12) as compared to the untreated D0 control and the DR (DR9 and DR12) control group. Significant enhancement of senescence-associated cysteine protease SPCP1 expression level was found for the SPDSS1 fusion protein-pretreated group on days 9 (DS9) and 12 (DS12) as compared to the control group (PB9 and PB12) (Figure 14). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore, exogenous SPDSS1 fusion protein enhances drought-mediated increase of senescence-associated cysteine protease SPCP1 expression level at the late stage in sweet potato.

3.3 Proteasome Inhibitor MG132 Alleviates Drought-Mediated Leaf Senescence

3.3.1 Proteasome Inhibitor MG132 Alleviates Drought-Mediated Changes of Senescence Associated Markers and Cell Necrosis in Leaves

Effects of proteasome inhibitor MG132 on drought-mediated leaf senescence/necrosis and senescence-associated markers were performed on days 12 and 15 (Figure 15). The DMSO-treated leaf samples were also performed at the same time for 12 days (DM12) and 15 days (DM15) as an additional control except drought alone (DR). For leaf morphology, leaf became curling and turned into light green on days 12 (DR12 and DM12) and became partial leaf necrosis on day 15 (DR15 and DM15) as compared to the untreated D0 control. NO significant difference, whereas, was observed between DR (drought alone) and DM (DMSO) groups. Significant reduction of leaf yellowing and necrosis were found for the MG132-pretreated group on days 12 (MG12) and 15 (MG15) as compared to the control group (PB9 and PB12) (Figure 15A).

For chlorophyll content, a significant decrease was observed on days 12 (DR12 (10.2) and DM12 (10.1)) and 15 (DR15 (5.5) and DM15 (5.3)) as compared to the untreated D0 (22.7) control. No significant difference, whereas, was found between DR (drought alone) and DM (DMSO) groups. Significant increase of chlorophyll content was found for the MG132-pretreated group on days 12 (MG12 (14.6)) and 15 (MG15 (13.7)) as compared to the control group (DM12 and DM15) (Figure 15B).

For F_v/F_m level, a significant decrease was observed on days 12 (DR12 (0.99) and DM12 (0.44)) and 15 (DR15 (0.27) and DM15 (0.29)) as compared to the untreated D0 (0.69) control. No significant difference was found between DR (drought alone) and DM (DMSO) groups. Significant reduction of F_v/F_m level was found for the MG132-pretreated group on days 12 (MG12 (0.60)) and 15 (MG15 (0.58)) as compared to the control group (DM12 and DM15) (Figure 15C).

Similar results were also observed for H_2O_2 and NO contents. For H_2O_2 amount, a significant increase was observed on days 12 (DR12 (59.9 μmole g^{-1} FW leaf) and DM12 (61.8 μmole g^{-1} FW leaf)) and 15 (DR15 (77.0 μmole g^{-1} FW leaf) and DM15 (78.4 μmole g^{-1} FW leaf)) as compared to the untreated D0 (12.0) control. No significant difference, whereas, was found between DR (drought alone) and DM (DMSO) groups. Significant reduction of H_2O_2

amount content was found for the MG132-pretreated group on days 12 (MG12 (36.6 μmole g^{-1} FW leaf)) and 15 (MG15 (39.6 μmole g^{-1} FW leaf)) as compared to the control group (DM12 and DM15) (Figure 16A).

For NO content, a significant elevation was observed on days 12 (DR12 (63.3 μmole g^{-1} FW leaf) and DM12 (69.9 μmole g^{-1} FW leaf)) and 15 (DR15 (77.0 μmole g^{-1} FW leaf) and DM15 (78.4 μmole g^{-1} FW leaf)) as compared to the untreated D0 (9.6 μmole g^{-1} FW leaf) control. No significant difference, whereas, was found between DR (drought alone) and DM (DMSO) groups. Significant reduction of NO content was found for the MG132-pretreated group on days 12 (MG12 (38.0 μmole g^{-1} FW leaf)) and 15 (MG15 (55.2 μmole g^{-1} FW leaf)) as compared to the control group (DM12 and DM15) (Figure 16B). Therefore, proteasome inhibitor MG132 attenuates drought-mediated changes of senescence-associated markers and leaf senescence/necrosis in sweet potato.

3.3.2 Proteasome Inhibitor MG132 Mitigates Drought-Mediated Induction and Protein Phosphorylation of Mitogen-Activated Protein Kinase SPMAPK

Effects of proteasome inhibitor MG132 on drought-mediated expression and protein phosphorylation of mitogen-activated protein kinase SPMAPK were performed. The DMSO-treated leaf samples were also performed at the same time for 12 days (DM12) and 15 days (DM15) as an additional control except drought alone (DR). Its expression and protein phosphorylation levels remained high at the late stage on day 12 (DR12 and DM12), and then decreased on day 15 (DR152 and DM15) as compared to the untreated D0 control. However, significant reduction levels of mitogen-activated protein kinase SPMAPK expression and protein phosphorylation on day 12 (MG12) and 15 (MG15) were found for the MG132-pretreated group as compared to the control groups (DR12/DM12 and DR15/DM15) (Figure 17). Therefore, proteasome inhibitor MG132 mitigates drought-mediated expression and protein phosphorylation levels of mitogen-activated protein kinase SPMAPK at the late stage of leaf senescence in sweet potato.

3.3.3 Proteasome Inhibitor MG132 Delays Drought-Mediated Decrease of Antioxidant Protein and Enzymatic Activity Levels in Leaves

Effects of proteasome inhibitor MG132 on drought-mediated reduction of antioxidant protein and enzymatic activity levels were performed on days 12 and 15 (Figure 18). The

DMSO-treated leaf samples were also performed at the same time for 12 days (DM12) and 15 days (DM15) as an additional control except drought alone (DR). For catalase, its in-gel activity significantly decreased on days 12 (DR12 and DM12) and 15 (DR15 and DM15) as compared to the untreated D0 control. Significantly higher in-gel catalase activity was found for the MG132-pretreated group on days 12 (MG12) and 15 (MG15) as compared to the control group (DM12 and DM15) (Figure 18A).

Similar result was also found for total catalase activity. It was significantly decreased on days 12 (DR12 (7.5) and DM12 (6.6)) and 15 (DR15 (5.6) and DM15 (4.4)) as compared to the untreated D0 (17.0) control. Significantly higher total catalase activity was found for the MG132-pretreated group on days 12 (MG12 (11.8)) and 15 (MG15 (8.8)) as compared to the control group (DM12 and DM15) (Figure 18B). Western blot hybridization also showed that the catalase SPCAT1 protein amount gradually decreased from day 12 (DR12 and DM12) until day 15 (DR15 and DM15) as compared to the untreated D0 control. Significantly higher catalase SPCAT1 protein level was found for the MG132-pretreated group on days 12 (MG12) and 15 (MG15) as compared to the control group (DM12 and DM15) (Figure 18C). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount.

For ascorbate peroxidase, total enzymatic activity was gradually decreased on days 12 (DR12 (1.3) and DM12 (1.2)) and 15 (DR15 (1.2) and DM15 (1.1) as compared to the untreated D0 (4.2) control. Significant increase of total ascorbate peroxidase activity was found for the MG132-pretreated group on days 12 (MG12 (2.1) and 15 (MG15 (1.8) as compared to the control group (DM12 and DM15) (Figure 19A). Western blot hybridization also showed that the ascorbate peroxidase SPAPX protein amount was gradually decreased from days 12 (DR12 and DM12) until DR15 (DR15 and DM15). Significant higher ascorbate peroxidase SPAPX protein level was found for the MG132-pretreated group on days 12 (MG12) and 15 (MG15) as compared to the control group (DM12 and DM15) (Figure 19B). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore, proteasome inhibitor MG132 delays drought-mediated decrease of antioxidant protein and enzymatic activity levels at the late stage in sweet potato.

3.3.4 Proteasome Inhibitor MG132 Attenuates Drought-Mediated Induction of Senescence-Associated Cysteine Protease SPCP1 in Leaves

Effects of proteasome inhibitor MG132 on drought-mediated enhancement of senescence-associated cysteine protease SPCP1 expression level were performed on days 12 and 15 (Figure 20). The DMSO-treated leaf samples were also performed at the same time for 12 days (DM12) and 15 days (DM15) as an additional control except drought alone (DR). Western blot hybridization showed that senescence-associated cysteine protease SPCP1 expression level was significantly enhanced on day 15 (DR15 and DM15) as compared to the untreated D0 control. Significant reduction of senescence-associated cysteine protease SPCP1 expression level was found for the MG132-pretreated group on day 15 (MG15) as compared to the control group (DM15) (Figure 20). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore, proteasome inhibitor MG132 attenuates drought-mediated increase of senescence-associated cysteine protease SPCP1 expression level at the late stage in sweet potato.

3.4 Effects of SPDSS1 on Drought-Mediated Leaf Senescence Can be attenuated by Proteasome Inhibitor MG132

3.4.1 Proteasome Inhibitor MG132 Alleviates SPDSS1 Fusion Protein Effects on Drought-Mediated Changes of Senescence-Associated Markers and Cell Necrosis in Leaves

In order to investigate whether effects of the SPDSS1 fusion proteins on drought-mediated leaf senescence and necrosis can be attenuated or reversed by the proteasome inhibitor MG132, the following treatments such as D0, DR12, PB+DM12, DS12, MG12, and DS+MG12 were performed and compared on day 12 after treatment. The (PB+DMSO)-treated leaf samples were also performed at the same time for 12 day (PB+DM12) as an additional control except drought alone (DR). For leaf morphology, leaf became curling and turned into light green on DR12 and PB+DM12 as compared to the untreated D0 control. For the SPDSS1 fusion protein-pretreated sample, a further remarkable increase of leaf yellowing and necrosis levels was also observed on DS12 as compared to the PB+DM12 control. For the MG132-pretreated sample, however, significant reduction of leaf yellowing and necrosis levels was also observed on MG12 as compared to the PB+DM12 control. For the SPDSS1 fusion protein plus MG132-pretreated sample, an intermediate retention level of leaf yellowing and necrosis was found on DS+MG12 as compared to the DS12 and MG12 controls, respectively (Figure 21A).

For chlorophyll content, significant decrease was observed on DR12 (11.0) and PB+DM12 (10.7) as compared to the untreated D0 (20.7) control. For the SPDSS1 fusion protein-pretreated sample, a further remarkable decrease of chlorophyll content was also found on DS12 (6.2) as compared to the PB+DM12 control. For the MG132-pretreated sample, however, significant retention of higher chlorophyll content was observed on MG12 (17.8) as compared to the PB+DM12 control. For the SPDSS1 fusion protein plus MG132-pretreated sample, an intermediate retention level of chlorophyll content was found on DS+MG12 (19.1) as compared to the DS12 and MG12 controls (Figure 21B).

For F_v/F_m level, significant decrease was observed on DR12 (0.48) and PB+DM12 (0.48) as compared to the untreated D0 (0.68) control. For the SPDSS1 fusion protein-pretreated sample, a further remarkable decrease of F_v/F_m was also found on DS12 (0.2) as compared to the PB+DM12 control. For the MG132-pretreated sample, however, significant retention of higher F_v/F_m was observed on MG12 (0.62) as compared to the PB+DM12 control. For the SPDSS1 fusion protein plus MG132-pretreated sample, an intermediate retention level of F_v/F_m was found on DS+MG12 (0.49) as compared to the DS12 and MG12 controls (Figure 21C). Therefore, effects of SPDSS1 enhances drought-mediated changes of senescence-associated markers can be attenuated by proteasome inhibitor MG132 in sweet potato.

3.4.2 Proteasome Inhibitor MG132 Delays SPDSS1 Fusion Protein Effects on Drought-Mediated Decrease of Antioxidant Protein and Enzymatic Activity Levels in Leaves

In order to investigate whether effects of the SPDSS1 fusion proteins on drought-mediated decrease of antioxidant protein and enzymatic activity levels can be attenuated or reversed by the proteasome inhibitor MG132, the following treatments such as D0, DR12, PB+DM12, DS12, MG12, and DS+MG12 were performed and compared on day 12 after treatment. The (PB+DMSO)-treated leaf samples were also performed at the same time for 12 day (PB+DM12) as an additional control except drought alone (DR). For catalase, in-gel activity was significantly decreased on DR12 and PB+DM12 as compared to the untreated D0 control. For the SPDSS1 fusion protein-pretreated sample, a further remarkable decrease of in-gel catalase activity was also observed on DS12 as compared to the PB+DM12 control. For the MG132-pretreated sample, however, significant retention of higher in-gel catalase activity was also observed on MG12 as compared to the PB+DM12 control. For the SPDSS1 fusion protein plus MG132-

pretreated sample, an intermediate retention level of in-gel catalase activity was found on DS+MG12 as compared to the DS12 and MG12 controls, respectively (Figure 22A).

Western blot hybridization showed that the catalase SPCAT1 protein amount was significantly decreased on DR12 and PB+DM12 as compared to the untreated D0 control. For the SPDSS1 fusion protein-pretreated sample, a further remarkable decrease of the catalase SPCAT1 protein amount was also found on DS12 as compared to the PB+DM12 control. For the MG132-pretreated sample, however, significant retention of higher catalase SPCAT1 protein amount was observed on MG12 as compared to the PB+DM12 control. For the SPDSS1 fusion protein plus MG132-pretreated sample, an intermediate retention level of the catalase SPCAT1 protein amount was found on DS+MG12 as compared to the DS12 and MG12 controls, respectively (Figure 22B). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount.

Western blot hybridization also showed that the ascorbate peroxidase SPAPX protein amount was decrease on DR12 and PB+DM12 as compared to the untreated D0 control. For the SPDSS1 fusion protein-pretreated sample, a further remarkable decrease of the ascorbate peroxidase SPAPX protein amount was also found on DS12 as compared to the PB+DM12 control. For the MG132-pretreated sample, however, significant retention of higher ascorbate peroxidase SPAPX protein amount was observed on MG12 as compared to the PB+DM12 control. For the SPDSS1 fusion protein plus MG132-pretreated sample, an intermediate retention level of the ascorbate peroxidase SPAPX protein amount was found on DS+MG12 as compared to the DS12 and MG12 controls, respectively (Figure 22C). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore, effects of SPDSS1 on drought-mediated reduction of antioxidant protein and enzymatic activity levels at late stage of leaf senescence can be attenuated by proteasome inhibitor MG132 in sweet potato.

3.4.3 Proteasome Inhibitor MG132 Attenuates SPDSS1 Fusion Protein Effects on Drought-Mediated Induction of Senescence-Associated Cysteine Protease SPCP1 in Leaves

In order to investigate whether effects of the SPDSS1 fusion proteins on drought-mediated increase of senescence-associated cysteine protease SPCP1 expression level can be attenuated or reversed by the proteasome inhibitor MG132, the following treatments such as D0, DR12, PB+DM12, DS12, MG12, and DS+MG12 were performed and compared on day 12 after

treatment. The (PB+DMSO)-treated leaf samples were also performed at the same time for 12 day (PB+DM12) as an additional control except drought alone (DR). Western blot hybridization showed that the cysteine protease SPCP1 protein amount was significantly increased on DR12 and PB+DM12 as compared to the untreated D0 control. For the SPDSS1 fusion protein-pretreated sample, a further remarkable increase of the cysteine protease SPCP1 protein amount was also found on DS12 as compared to the PB+DM12 control. For the MG132-pretreated sample, however, significant reduction of cysteine protease SPCP1 protein amount was observed on MG12 as compared to the PB+DM12 control. For the SPDSS1 fusion protein plus MG132-pretreated sample, an intermediate expression level of the cysteine protease SPCP1 protein amount was found on DS+MG12 as compared to the DS12 and MG12 controls, respectively (Figure 23). Rubisco L subunit was used as an internal control to indicate the physiological status and the loaded protein amount. Therefore, effects of SPDSS1 on drought-mediated elevation of senescence-associated cysteine protease SPCP1 protein level at late stage of leaf senescence can be attenuated by proteasome inhibitor MG132 in sweet potato.

Chapter 4. Discussion

Leaf senescence is the final stage of plant developmental processes, which is stimulated by many internal and external environmental factors such as ethylene, salt and drought. Drought stress causes induces of numbers at morphological, biochemical, molecular, and physiological levels, including plant dehydration, stomatal closure, protein degradation and limited gas exchanges, followed by inhibition of metabolism and photosynthetic rate and finally plant death (Jaleel et al, 2008; Juan et al, 2009; Wang X et al., 2016). Important events in leaf senescence are the loss of chlorophyll and the demolition of photosynthetic equipment which affects the decrease in energy conversion capacity and efficiency (Falqueto et al., 2009). Similar results were also reported in sweet potato by Tsai (2016). In this study, drought also promotes changes of senescence-associated markers and cell necrosis in sweet potato leaves, including decrease of chlorophyll content and F_v/F_m (Figure 1), elevation of NO/H_2O_2/MDA amounts (Figure 2), and promotion of leaf senescence and necrosis (Figure 1). Our data are consistent with these reports.

Mitogen-activated protein kinase (MAPK) cascade performs an integral role in signal transduction from extracellular to intracellular compartments through protein phosphorylation of downstream signaling components (Kim et al., 2016). These signaling cascades are highly conserved in both animal and higher plants (Xu and Zhang, 2015). MAPK participates in diverse signaling pathways induced by abiotic stresses such as drought, high salt, low temperature, and pathogens (Zhang and Klessig, 2001; Asai et al., 2002; Droillard et al., 2004; Ding et al., 2009; Pitzschke et al., 2009). Among the MAPKs, Arabidopsis AtMPK3 and AtMPK6 can be detected by anti-pERK antibody in immunoblotting analysis (Lee and Ellis, 2007; Singh et al., 2012). MPK3 and MPK6 have also been shown to be associated with the stress-induced cell death in plant (Lee and Ellis, 2007; Colcombet and Hirt, 2008). In this study, expression and protein phosphorylation of mitogen-activated protein kinase SPMAPK are significantly enhanced by drought (Figure 3). Exogenous SPDSS1 fusion protein accelerated drought-induced leaf senescence/necrosis (Figures 9) and speed up the process of drought-mediated expression and protein phosphorylation of mitogen-activated protein kinase SPMAPK (Figure 11). Our data are consistent with these reports.

Water deficit interrupts normal cellular metabolism that results in the production of ROS and oxidative stress. Plants have evolved diverse mechanisms to keep ROS homeostasis in cells,

including antioxidative enzymes (e.g., ascorbate peroxidase and catalase) and small antioxidant compounds (e.g., glutathione and ascorbate). Catalases functions as one of the key enzymes in the scavenging of reactive oxygen species and affects the H_2O_2 homeostasis in plants (Afiyanti M and Chen HJ, 2014). Its isoforms were also detected in activity gels in *Arabidopsis* (Du et al., 2008; Zimmermann et al., 2006), tobacco (Havir et al., 1996) and sweet potato (Chen et al., 2011). In sweet potato leaves, antioxidant protein and enzymatic activity levels reduced in drought-induced necrotic leaves, including catalase (Figure 4) and ascorbate peroxidase (Figure 5). These results are consistent with the report of Chakraborty and Pradhan (2012) that catalase activity decreased in periods under drought stress. Similar results were also reported by Ye et al. (2010) that decrease of ascorbate peroxidase activity during bolting time would lead to an increase of H_2O_2, which might be used as a signal to promote leaf senescence.

A senescence-associated cysteine protease SPCP1 was isolated from senescing leaves and inducible by ethephon in sweet potato (Chen et al., 2009). In this study, expression level of senescence-associated cysteine protease SPCP1 was also enhanced significantly at the late stage in drought-induced necrotic leaves (Figure 6). Exogenous SPDSS1 fusion protein enhanced drought-induced leaf senescence/necrosis (Figure 9) and SPCP1 expression level (Figure 14). However, proteasome inhibitor MG132 delay drought-induced leaf senescence/necrosis (Figure 15) and SPCP1 expression level (Figure 20). Many drought-inducible proteases in plants have been reported, including ATP-dependent Clp protease in *H. vulgare*, cysteine proteinase in *P. vulgaris*, zinc metallo protease in *B. napus*, and aspartic proteinase in *Z. mays* (Wang X et al., 2016). In Arabidopsis, SAG12, an ortholog of SPCP1, also encodes a senescence-associated cysteine protease and participate in leaf senescence (James et al., 2018). In sweet potato, senescence-associated cysteine proteases have also been reported to participate in leaf senescence and stress tolerance (Chen et al., 2008; 2010b; 2013a). Our results are consistent with these reports and suggest that senescence-associated cysteine protease SPCP1 likely participates in drought stress-mediated leaf senescence and necrosis process.

Drought has also been shown to promote leaf senescence in sweet potato (Tsai, 2016). However, the role of proteasome in drought-induced leaf senescence/necrosis is unclear. Proteasome activity has been reported to be involved in the regulation of plant responses to many developmental processes and environmental stimuli. The most common, relatively site-specific inhibitors used in the field are epoxomicin (such as MG132) and lactacystin, all of which inhibit

β-subunits of the 20S core (Raynes R et al., 2016). MG132, also called carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, Z-LLL-CHO, is a peptide aldehyde able to inhibit different types of proteases, including serine and cysteine proteases. MG132 has also been shown to strongly inhibit multiple peptidase activities of proteasomes (Tsubuki, 1996). MG132 pretreatments attenuated drought-mediated leaf senescence/necrosis, changes of senescence-associated markers, expression and protein phosphorylation of mitogen-activated protein kinase SPMAPK, decrease of antioxidant enzyme protein amount and enzymatic activity level, and senescence-associated cysteine protease SPCP1 expression (Figures 15 – 20), suggesting a role of proteasome activity in association with drought-mediated leaf senescence and necrosis in sweet potato. In tobacco, 26S proteasome is also involved in the modulation of poly-ubiquitination proteins during plant development and environmental stress (Genschik et al., 1994). The use of proteasome inhibitors in the experiments delayed senescence in Iris flower (Pak and van Doorn, 2005), and *A. polyphaga cells* (Price CT et al., 2011). Our data are consistent with the report.

In sweet potato, the deleted-in-split hand/split foot 1 protein SPDSS1, a putative 19S regulatory proteasome subunit, exhibits a high amino acid sequence homology with *Arabidopsis* AtDSS1 protein. Exogenous SPDSS1 fusion protein enhanced drought-mediated leaf senescence/necrosis, changes of senescence-associated markers, expression and protein phosphorylation of mitogen-activated protein kinase SPMAPK, decrease of antioxidant enzyme protein amount and enzymatic activity level, and senescence-associated cysteine protease SPCP1 expression (Figures 9 - 14). However, pretreatments of proteasome inhibitor MG132 attenuated drought-mediated effects as mentioned above (Figures 15 – 20). These results further support a role of the proteasome and its subunit in association with drought-mediated leaf senescence and necrosis in sweet potato. Proteasome activity has also been demonstrated to be involved in salinity stress-induced leaf senescence in sweet potato (Chou, 2016). Proteomic studies also revealed that some protein degradation-related proteins increased in response to drought stress, including proteasomes, proteases, and peptidases. Previous studies have reported that some components in the protein degradation pathway, such as ubiquitin/26S proteasomes, small ubiquitin-like modifier (E3 SUMO) ligase, and proteases/peptidases were involved in plant drought tolerance (Gao QF et al., 2008; Yao et al., 2012; Simova-Stoilova et al., 2010). For the Ubiquitin-Proteasome System (UPS), 7 out of 11 20S proteasomes (the core regulatory particle of 26S proteasome) were increased in leaves of *P. vulgaris* (Zadraznik et al., 2013), *Hordeum*

vulgare (Ghabooli et al., 2013), *B. napus* (Koh et al., 2015), respectively. Importantly, the phosphorylation level of E3 ubiquitin ligase, which is one of the key enzymes involved in ubiquitination, exhibited significantly increased values in leaves under drought stress (Zhang et al., 2014; Hu et al., 2015). Many studies have shown that E3 ubiquitin ligases were positively related to plant drought tolerance (Seo et al., 2012; Kim et al., 2013). These findings indicate that the enhancement of the Ubiquitin-Proteasome System is important for plants to cope with drought. Our data are consistent with the report.

Zhang M et al. (2014) reported that DSS1 protein reacted with oxidized protein modulated by an unidentified ATPase to form DSS1-protein adducts, which was targeted by ubiquitination for Ubiquitin-Proteasome system (UPS) degradation. This process is called DSSylation, a novel protein modification, in which DSS1 plays as a modifier, whose attachment may render target proteins a signature leading to their subsequent ubiquitination, thereby recruits proteasome to degrade them. In sweet potato, Western blot hybridization detected a protein band with molecular weight near 48 kDa, which is much higher than the deduced MW (close to 8 to 10 kDa) (Figure 7B), suggesting a possible DSSylation of endogenous sweet potato SPDSS1 protein with an unidentified putative target protein candidate. Similar results have also been reported for human DSS1 *in vitro* (Zhang Y et al., 2014) and Arabidopsis AtDSS1 (Wei et al., 2008), such as interactions of 19S proteasome subunits RPN3/S3/DSS1 with different target protein candidates p53 tumor suppressor and BRCA2. These results suggest that sweet potato SPDSS1 may mediate alternative pathways except UPS to modulate drought-induced leaf senescence in sweet potato.

Chapter 5. Conclusion

Drought promotes leaf senescence and necrosis in sweet potato and enhances changes of senescence-associated markers, including reduction of chlorophyll content and F_v/F_m level, decrease of antioxidant protein amount and enzymatic activity level, elevation of H_2O_2/NO/MDA contents, induction and protein phosphorylation of mitogen-activated protein kinase SPMAPK, and enhancement of senescence-associated cysteine protease SPCP1 expression. Pretreatment of proteasome inhibitor MG132 attenuates drought-mediated effects as mentioned above, suggesting a novel physiological role of proteasomes in association with drought-mediated leaf senescence and necrosis. A putative 19S regulatory proteasome subunit, deleted-in-split hand/split-foot 1 (DSS1)-like protein, has been cloned from senescent leaves and is inducible by drought in senescing leaves of sweet potato. Exogenous SPDSS1 fusion protein accelerated drought-mediated effects mentioned earlier, providing further support of proteasomes with a novel physiological role in association with drought-mediated leaf senescence and necrosis in sweet potato. These data conclude for the first time that proteasome inhibitor MG132 and the deleted-in-split hand/split foot 1 protein SPDSS1 modulate drought stress tolerance in sweet potato leaves.

Chapter 6. References

Afiyanti M and Chen HJ. Catalase activity is modulated by calcium and calmodulin in detached mature leaves of sweet potato. Journal of Plant Physiology. 2014; 171 (2): 35-47.

Afiyanti M. Study the role of nitric oxide in the modulation of ethephon or NaCl-induced leaf senescence in sweet potato. Ph.D Dissertation. 2015. Department of Biological Sciences. National Sun Yat-sen University.

Ahmad P and Sharma I. (Ed): Catalase. Oxidative Damage to Plants. 2014; 131-147.

Ali S, Mohammed W and Shimelis B. Agronomic and Physicochemical Evaluation of Sweet Potato [*Ipomoea batatas* (L.) Lam.] Collections in Ethiopia. Advances in Crop Science and Technology. 2015; 3(3): 1-8.

Amako K, Chen G-X, Asada K. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiol. 1994; 35: 497–504.

Asada K. Ascorbate peroxidase - A hydrogen peroxide-scavenging enzyme in plants. Physiol Plantarum. 1992; 85: 235-241.

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J. MAP kinase signalling cascade in Arabidopsis innate immunity. Nature. 2002; 415(6875):977-983.

Borrás L, Maddonni GA and Otegui ME. Leaf senescence in maize hybrids: plant population, row spacing and kernel set effects. Field Crops Research. 2003; 82: 13-26.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 1976; 72: 248–254.

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T and Pink D. The molecular analysis of leaf senescence: a genomics approach. Plant Biotechnology Journal 1. 2003; 1(1): 3–22.

Caverzan A, Passaia G, Rosa SB, Ribeiro CW, Lazzarotto F and Margis-Pinheiro M. Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. Genetics and Molecular Biology. 2012; 35(4): 1011-1019.

Chakraborty U and Pradhan B. Drought stress-induced oxidative stress and antioxidative responses in four wheat (*Triticum aestivum* L.) varieties. Archives of Agronomy and Soil Science. 2012; 58(6): 617–630.

Chang Y. Sweet potato mitogen-activated protein kinase *SPMAPK* participates in ethephon-mediated leaf senescence. 2016. Master Thesis. Department of Biological Sciences. National Sun Yat-sen University.

Chaves MM, Maroco JP, Pereira, JS. Understanding plant responses to drought – from genes to the whole plant. Funct. Plant Biol. 2003; 30: 239-264.

Chen HJ, Hou WC, Liu JS, Yang CY, Huang DJ, and Lin YH. Molecular cloning and characterization of a cDNA encoding asparaginyl endopeptidase from sweet potato (*Ipomoea batatas* (L.) Lam) senescent leaves. Journal of experimental botany. 2004; 55: 825-835.

Chen HJ, Huang, D. J., Hou WC, Liu, J. S., and Lin, Y. H. Molecular cloning and characterization of a granulin-containing cysteine protease SPCP3 from sweet potato (*Ipomoea batatas*) senescent leaves. Journal of Plant Physiology. 2006; 163: 863-876.

Chen HJ, Wen IC, Huang GJ, Hou WC, Lin YH. Expression of sweet potato asparaginyl endopeptidase caused altered phenotypic characteristics in transgenic *Arabidopsis*. Bot Stud 2008; 49: 109-117.

Chen HJ, Huang GJ, Chen WS, Su CT, Hou WC and Lin YH. Molecular cloning and expression of a sweet potato cysteine protease SPCP1 from senescent leaves. Bot Stud. 2009; 50: 159-170.

Chen HJ, Tsai YJ, Chen WS, Huang GJ, Huang SS, and Lin YH. Ethephon-mediated effects on leaf senescence are affected by reduced glutathione and EGTA in sweet potato detached leaves. Botanical Studies. 2010a; 51: 171-181.

Chen HJ, Su CT, Lin CH, Huang GJ, and Lin YH. Expression of sweet potato cysteine protease SPCP2 altered developmental characteristics and stress responses in transgenic *Arabidopsis* plants. Journal of Plant Physiology. 2010b; 167: 838-847.

Chen HJ, Afiyanti M, Huang GJ, Huang SS, and Lin YH. Characterization of a leaf-type catalase in sweet potato (*Ipomoea batatas* Lam. (L.)). Botanical Studies. 2011; 52: 417-426.

Chen HJ, Wu SD, Huang GJ, Shen CY, Afiyanti M, Li WJ and Lin YH. Expression of a cloned sweet potato catalase SPCAT1 alleviates ethephon-mediated leaf senescence and H_2O_2 elevation. Journal of Plant Physiology. 2012a; 169: 86-97.

Chen HJ, Lin ZW, Huang GJ and Lin YH. Sweet potato calmodulin SPCAM is involved in salt stress-mediated leaf senescence, H_2O_2 elevation and senescence-associated gene expression. Journal of Plant Physioloy. 2012b; 169: 1892-1902.

Chen HJ, Wu SD, Lin ZW, Huang GJ, Lin YH. Cloning and characterization of a sweet potato calmodulin SPCAM that participates in ethephon-mediated leaf senescence, H_2O_2 elevation and senescence-associated gene expression. J Plant Physiol. 2012c; 169: 529-541.

Chen HJ, Tsai YJ, Shen CY, Tsai TN, Huang GJ, and Lin YH. Ectopic Expression of Sweet Potato Cysteine Protease SPCP3 Alters Phenotypic Traits and Enhances Drought

Stress Sensitivity in Transgenic *Arabidopsis* Plants. Journal Plant Growth Regulation. 2013a; 32: 108-121.

Chou CC. Sweet potato SPDSS1 protein and proteasome inhibitor MG132 attenuate ethephon and NaCl-induced leaf senescence. 2016. Master Thesis. Department of Biological Sciences. National Sun Yat-sen University.

Climate Change Science Program. The Effects of wlat Change on Agriculture, Land Resources, Water Resources, and Biodiversity in the United States. 2008. Washington DC: U.S. Department of Agriculture.

Colcombet J and Hirt H. Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. Biochemical Journal. 2008; 413: 217–226.

Crackower MA, Scherer SW, Rommens JM, Hui CC, Poorkaj P, Soder S, Cobben JM, Hudgins L, Evans JP, Tsui LC. Characterization of the split hand/split foot malformation locus SHFM1 at 7q21.3-q22.1 and analysis of a candidate gene for its expression during limb development. Hum. Mol. Genet. 1996; 5: 571–579.

Dang IJ, Dietrich R, Thomas H. Senescence and programmed cell death. In Biochemistry and Molecular Biology of Plants. Rockville, Maryland: American Society of Plant Physiologists, 1044-1100.

Desclos-Théveniau M, Coquet L, Jouenne T, and Etienne P. Proteomic analysis of residual proteins in blades and petioles of fallen leaves of *Brassica napus*. Plant Biol. 2015; 17: 408–418.

Ding HD, Zhang XH, Xu SC, Sun LL, Jiang MY, Zhang AY, Jin YG. Induction of protection against paraquat-induced oxidative damage by abscisic acid in maize leaves is mediated through mitogen-activated protein kinase. J Integr Plant Biol. 2009; 51:961–972.

Droillard MJ, Boudsocq M, Barbier-Brygoo H, Laurière C. Involvement of MPK4 in osmotic stress response pathways in cell suspensions and plantlets of Arabidopsis thaliana: activation by hypoosmolarity and negative role in hyperosmolarity tolerance. FEBS Lett. 2004; 574(1-3):42-48.

Du YY, Wang PC, Chen J, and Song CP. Comprehensive functional analysis of the catalase gene family in *Arabidopsis thaliana*. J Integr Plant Biol. 2008; 50(10): 1318-1326.

Ellisdon AM, Dimitrova L, Hurt E, Stewart M. Structural basis for the assembly and nucleic acid binding of the TREX-2 transcription-export complex. Nat Struct Mol Biol. 2012; 19(3): 328-36.

Espinoza C, Medina C, Somerville and Arce-Johnson P. Senescence-associated genes induced during compatible viral interactions with grapevine and *Arabidopsis*. Journal of Experimental Botany. 2007; 58(12): 3197–3212.

Falqueto AR, Cassol D, Júnior AMM, Oliveira AC and Bacarin MA. Physiological analysis of leaf senescence of two rice cultivars with different yield potential Pesq. agropec. bras, Brasília. 2009; 44(7): 695-700.

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA. Plant drought stress: effects, mechanisms and management. Agron. Sustain. Dev. 2009; 29:185-212.

Finley D. Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu. Rev. Biochem. 2009; 78: 477–513.

Food and Agriculture Organization of the United Nations (FAO). Global Production and Consumption of Roots and Tubers. Appendix 4. 2013 (online). www.fao.org/wairdocs/tac/x5791e/x5791e0q.htm

Genschik P, Marbach J, Uze M, Feuerman M, Plesse B, and Fleck J. Structure and promoter activity of a stress and developmentally regulated polyubiquitin-encoding gene of *Nicotiana tabacum*. Gene. 1994; 148: 195-202.

Gepstein S, Sabehi G, Carp M, Hajouj T, Nesher MFO, Yariv I, Dor C, Bassani M. Large-scale identification of leaf senescence-associated genes. The Plant Journal. 2003; 36: 629-642.

Ghabooli M, Khatabi B, Ahmadi FS, Sepehri M, Mirzaei M, Amirkhani A, Jorrin-Novo JV, Salekdeh GH. Proteomics study reveals the molecular mechanisms underlying water stress tolerance induced by *Piriformospora indica* in barley. J. Proteom. 2013; 94: 289–301.

Giango WC and Giango MKC. The formulation of sweet potato (*Ipomoea batatas*) bread. Academia Journal of Agricultural Research. 2017; 5(10): 271-278.

Guo Y, Cai Z and Gan S. Transcriptome of *Arabidopsis* leaf senescence. Plant Cell Environ. 2004; 27, 521–549.

Guo Y & Gan S. S. Convergence and divergence in gene expression profiles induced by leaf senescence and 27 senescence promoting hormonal, pathological and environmental stress treatments. Plant Cell Environ. 2012; 35: 644–655.

Guo Y. Towards systems biological understanding of leaf senescence. Plant Mol Biol. 2013, 82, 519–528.

Guo QF, Zhang J, Gao Q, Xing SC, Li F, Wang W. Drought tolerance through overexpression of monoubiquitin in transgenic tobacco. J. Plant Physiol. 2008; 165: 1745–1755.

Hanna J and Finley D. A proteasome for all occasions. FEBS letters. 2007; 581(15): 2854-2861.

Havir EA, Brisson LF, Zelitch I. Distribution of catalase isoforms in *Nicotiana tabacum*. Phytochemistry. 1996; 41(3):699-702.

Hettenhausen, C., Schuman, M. C., and Wu, J. MAPK signaling - a key element in plant defense response to insects. Insect Sci. 2014; 22, 157–164.

Huang B, Chen YE, Zhao YQ, Ding CB, Liao JQ, Hu C, Zhou LJ, Zhang ZW, Yuan S and Yuan M. Exogenous melatonin alleviates oxidative damages and protects photosystem II in maize seedlings under drought stress. Front. Plant Sci. 2019; 10(677): 1-16.

Ignatius J, Knuutila S, Scherer SW, Trask B, Kere J. Split hand/split foot malformation, deafness, and mental retardation with a complex cytogenetic rearrangement involving 7q21.3. J. Med. Genet. 1996; 33: 507–510.

Jagodzik P, Tajdel-Zielinska M, Ciesla A, Marczak M and Ludwikow A. Mitogen-activated protein kinase cascades in plant hormone signaling. Review Article. Front. Plant Sci. 2018; 9(1387): 1-27.

Jaleel CA, P. Manivannan, GMA Lakshmanan, M. Gomathinayagam, and R. Panneerselvam. Alterations in morphological parameters and photosynthetic pigment responses of *Catharanthus roseus* under soil water deficits. Colloids and Surfaces B: Biointerfaces. 2008; 61(2): 298–303.

James M, Poret M, Masclaux-Daubresse C, Marmagne A, Coquet L, Jouenne T, Chan P, Trouverie J, Etienne P. SAG12, a Major Cysteine Protease Involved in Nitrogen Allocation during Senescence for Seed Production in Arabidopsis thaliana. Plant Cell Physiol. 2018; 59(10): 2052-2063.

Ji H, Zhang HX, Li HT and Li YC. Analysis on the nutrition composition and antioxidant activity of different types of sweet potato cultivars. Food and Nutrition Sciences. 2015; 6: 161-167.

Jing HC, Schippers JHM, Hille J and Dijkwel PP. Ethylene-induced leaf senescence depends on age-related changes and OLD genes in *Arabidopsis*. Journal of Experimental Botany. 2005; 56(421): 2915–2923.

Jossé L, Harley ME, Pires IM, Hughes DA. Fission yeast DSS1 associates with the proteasome and is required for efficient ubiquitin-dependent proteolysis. Biochem J. 2006; 393(1): 303-309.

Juan A, González, Gallardo, Hilal, Rosa and Prado. Physiological responses of quinoa (*Chenopodium quinoa* Willd.) to drought and waterlogging stresses: drymatter partitioning. Botanical Studies. 2009; 50: 35-42.

Kim HS, Park SC, Ji CY, Park S, Jeong JC, Lee HS, and Kwak SS. Molecular characterization of biotic and abiotic stress-responsive MAP kinase genes, IbMPK3 and IbMPK6, in sweetpotato. Plant Physiology and Biochemistry. 2016; 108: 37-48.

Kim SJ, Kim WT. Suppression of *Arabidopsis* RING E3 ubiquitin ligase AtATL78 increases tolerance to cold stress and decreases tolerance to drought stress. FEBS Lett. 2013; 587: 2584–2590.

Koh J, Chen G, Yoo M,-J, Zhu N. Dufresne D, Erickson JE, Shao H, Chen S. Comparative proteomic analysis of *Brassica napus* in response to drought stress. J. Proteome Res. 2015; 14: 3068–3081.

Kuzniak E, Patykowsky J and Urbanek H. Involvement of the antioxidative system in tobacco response to fusaric acid treatment. J. Phytopathol. 1999; 147: 385–390.

Lee RH, Wang CH, Huang LT, Chen SC. Leaf senescence in rice plants: cloning and characterization of senescence up-regulated genes. Journal Exp. Botany. 2001; 52: 1117-1121.

Lee JS and Ellis BE. Arabidopsis MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. Journal of Biological Chemistry. 2007; 282: 25020–25029.

Leung D. Studies of Catalase in Plants under Abiotic Stress. Antioxidants and Antioxidant Enzymes in Higher Plants. 2018; 27-39.

Lestari DO, Sutriyono E, Sabaruddin, Iskandar I. Severe Drought Event in Indonesia Following 2015/16 El Niño/positive Indian Dipole Events. Journal of Physics: Conf. Series. 2018. 1011: 1-6.

Li J, Zou C, Bai Y, Wazer DE, Band V, and Gao Q. DSS1 is required for the stability of BRCA2. Oncogene. 2006; 25 (8): 1186-1194.

Li W, Zhang H, Li X, Zhang F, Liu C, Du Y, Gao X, Zhang Z, Zhang X, Hou Z, Zhou H, Sheng X, Wang G & Guo Y. Intergrative metabolomic and transcriptomic analyses unveil nutrient remobilization events in leaf senescence of tobacco. Scientific Reports. 2017; 7 (12126): 1-17.

Li Y, Cai H, Liu P, Wang C, Gao H, Wu C, Yan K, Zhang S, Huang J, Zheng C. Arabidopsis MAPKKK18 positively regulates drought stress resistance via downstream MAPKK3. Biochemical and Biophysical Research Communications. 2017; 484(2): 292-297.

Lim PO, Kim HJ and Nam HG. Leaf senescence. Annu. Rev. Plant Biol. 2007; 58:115-136.

Liu C. The cloned ascorbate peroxidase SPAPX mitigates and delays ethephon-induced leaf senescence in sweet potato. Master Thesis. 2014. Department of Biological Sciences. National Sun Yat-sen University.

Liu J, Doty T, Gibson B, Heyer WD. Human BRCA2 protein promotes RAD51 filament formation on RPA-covered single-stranded DNA. Nat Struct Mol Biol. 2010; 17(10): 1260-1262.

Mishra, N.S.; Tuteja, R.; Tuteja, N. Signaling through MAP kinase networks in plants. Arch. Biochem. Biophys. 2006, 452, 55–68.

Mursidi A. 2017. Management of Disaster Drought in Indonesia.

Noctor G & Foyer C. H. Ascorbate and glutathione: keeping active oxygen under control. Annual review of plant biology. 1998; 49(1): 249-279.

Noh YS, and Amasino RM. Identification of a promoter region responsible for the senescence-specific expression of SAG12. Plant Mol. Biol. 1999a; 41: 181–194.

Noh YS, and Amasino RM. Regulation of developmental senescence is conserved between *Arabidopsis* and *Brassica napus*. Plant Mol. Biol. 1999b; 41: 195–206.

Osmond CB, Grace SC. Perspectives on photoinhibition and photorespiration in the field: Quintessential inefficiencies of the light and dark reactions of photosynthesis? J. Exp. Bot. 1995; 46: 1351–1362.

Ozyigit I, Filiz E, Vatansever R, Kurtoglu KY, Koc I, Öztürk MX and Anjum NA. Identification and comparative analysis of h_2o_2-scavenging enzymes (ascorbate peroxidase and glutathione peroxidase) in selected plants employing bioinformatics approaches. Front. Plant Sci. 2016; 7(301): 1-23.

Pak C, and van Doorn WG. Delay of iris flower senescence by protease inhibitors. The New phytologist. 2005; 165: 473-480.

Paraskevopoulos K. Kriegenburg F, Tatham MH. Rösner HI, Medina B, Larsen IB, Brandstrup R, Hardwick KG, Hay RT, Kragelund BB, Petersen RH, Gordon C. DSS1 is a 26S proteasome ubiquitin receptor. Mol. Cell. 2014; 56(3): 453–461.

Peters JM, Franke WW, and Kleinschmidt JA. Distinct 19 S and 20 S subcomplexes of the 26 S proteasome and their distribution in the nucleus and the cytoplasm. The Journal of biological chemistry. 1994; 269: 7709-7718.

Pick E, Hofmann K, Glickman MH. PCI complexes: Beyond the proteasome, CSN, and eIF3 Troika. Mol Cell. 2009; 35(3): 260-264.

Pickart CM and Fushman D. Polyubiquitin chains: polymeric protein signals. Current opinion in chemical biology. 2004; 8: 610-616.

Pitzschke A, Schikora A, Hirt H. MAPK cascade signalling networks in plant defence. Curr Opin Plant Biol. 2009; 12(4):421-6.

Poret M, Chandrasekar B, van der Hoorn RAL and Avice, J.-C. Characterization of senescence-associated protease activities involved in the efficient protein remobilization during leaf senescence of winter oilseed rape. Plant Sci. 2016; 246: 139–153.

Pratiwi NAH, Karuniasa M, Suroso DSA. Exploring Historical and Projection of Drought Periods in Cirebon Regency, Indonesia. E3S Web Conference 68. 2018. 1-7.

Price CT, Al-Quadan T, Santic M, Rosenshine I, Abu KY. Host proteasomal degradation generates amino acids essential for intracellular bacterial growth. Science. 2011; 334 (6062): 1553-1557.

Qin S, Wang Q, Ray A, Wani G, Zhao Q, Bhaumik SR, Wani AA. Sem1p and Ubp6p orchestrate telomeric silencing by modulating histone H2B ubiquitination and H3 acetylation. Nucleic Acids Res. 2009; 37(6): 1843-53.

Raynes R, Pomatto LC, Davies KJ. Degradation of oxidized proteins by the proteasome: Distinguishing between the 20S, 26S, and immunoproteasome proteolytic pathways. Mol Aspects Med. 2016; 50: 41-55.

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, and Blumwald E. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proceedings of the national academy of science. 2007; 104(49): 19631-19636.

Rodriguez, M. C., Petersen, M., and Mundy, J. Mitogen-activated protein kinase signaling in plants. Annu. Rev. Plant Biol. 2010; 61, 621–649.

Sadanandom A, Bailey M, Ewan R, Lee J, and Nelis S. The ubiquitin-proteasome system: central modifier of plant signalling. New Phytologist. 2012; 196: 13-28.

Saxena I, Srikanth S, and Chen Z. Cross Talk between H_2O_2 and interacting signal molecules under plant stress response. Frontiers in plant science, 2016: 7(570): 1-43.

Scandalios JG. Oxidative stress: Molecular perception and transduction of signals triggering antioxidant gene defenses. Braz J Med Biol Res. 2005; 38: 995-1014.

Seo YS, Choi JY, Kim SJ, Kim EY, Shin JS, Kim WT. Constitutive expression of aRma1H1, a hot pepper ER-localized RING E3 ubiquitin ligase, increases tolerance to drought and salt stresses in transgenic tomato plants. Plant Cell Rep. 2012; 31: 1659–1665.

Shao HB, Chen XY, Chu LY, Zhao XN, Wu G, Yuan YB, Zhao CX, Hu ZM. Investigation on the relationship of proline with wheat anti-drought under soil water deficits. Coll. Surf. B Biointerf. 2006, 53: 113-119.

Shen CY. Molecular cloning of mitogen-activated protein kinase cDNA and study of ethylene signaling in senescent sweet potato leaves. Master Thesis. 2011. Department of Biological Sciences. National Sun Yat-sen University.

Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y and Yoshimura K. Regulation and function of ascorbate peroxidase isoenzymes. J Exp Bot. 2002; 53:1305- 1319.

Shih P, Yeh CT and Yen GC. Anthocyanins induce the activation of phase ii enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. Journal of Agricultural and Food Chemistry. 2007; 55: 9427-9435.

Simova-Stoilova L, Vaseva I, Grigorova B, Demirevska K, Feller U. Proteolytic activity and cysteine protease expression in wheat leaves under severe soil drought and recovery. Plant Physiol. Biochem. 2010; 48: 200–206.

Singh P, Kuo YC, Mishra S. The lectin receptor kinase-VI.2 is required for priming and positively regulates *Arabidopsis* patterntriggered immunity. Plant Cell. 2012; 24: 1256–1270.

Tanaka K. The proteasome: Overview of structure and functions. Proc Jpn Acad Ser B Phys Biol Sci. 2009; 85(1): 12–36.

Tang YL, Wen XG and Lu CM. Differential changes in degradation of chlorophyll-protein complexes of photosystem I and photosystem II during flag leaf senescence of rice. Plant Physiology and Biochemistry. 2005; 43: 193-201.

Tena G, Asai T, Chiu WL, and Sheen J. Plant mitogen-activated protein kinase signaling cascades. Current opinion in plant biology: 2001; 4(5): 392-400.

Tomko RJ, Jr and Hochstrasser M. The intrinsically disordered Sem1 protein functions as a molecular tether during proteasome lid biogenesis. Mol. Cell. 2014; 53: 433–443.

Troy TJ, C Kipgen and Pal. The impacts of climate extremes and irrigation on US crop yields. Environ. Res. Lett. 2015; 10:054013.

Tsai TN. Rutin alleviates ethephon, NaCl and drought stress-mediated leaf senescence in sweet potato. 2016. Master Thesis. Department of Biological Sciences. National Sun Yat-sen University.

Tsubuki S, Saito Y, Tomioka M, Ito H, Kawashima S. Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine. J Biochem. 1996; 119: 572-576.

Tuteja N and Sopory SK. Chemical signaling under abiotic stress environment in plants. Plant signaling & behavior. 2008; 3(8): 525-536.

Uzilday B, Turkan I, Ozgur R, and Sekmen AH. Strategies of ROS regulation and antioxidant defense during transition from C3 to C4 photosynthesis in the genus Flaveria under PEG-induced osmotic stress. J. Plant Physiol. 2014; 171: 65–75.

Walthall CL, Hatfield J, Backlund P, Lengnick L, Marshall E, Walsh M, Adkins S. Climate Change and Agriculture in the United States: Effects and Adaptation. 2012. Washington DC: U.S. Department of Agriculture, Technical Bulletin No. 1935.

Wang H, Cao G and Prior RL. Oxygen radical absorbing capacity of anthocyanins. Journal of agricultural and food chemistry. 1997; 45: 304-309.

Wang X, Cai X, Xu C, Wang Q, and Dai S. Drought-Responsive Mechanisms in Plant Leaves Revealed by Proteomics. Int. J. Mol. Sci. 2016; 17(1706): 1-30.

Wang Z, Jia C, Li J, Xu B, and Jin Z. Identification of six mitogen-activated protein kinase (MAPK) genes in banana (*Musa acuminata* L. AAA group, cv. Cavendish) under

infection of *Fusarium oxysporum f. sp cubense* tropical race. Acta Physiol. Plant. 2015; 37:115.

Wei SJ, Trempus CS, Cannon RE, Bortner CD, Tennant RW. Identification of Dss1 as a 12-O-tetradecanoylphorbol-13-acetate-responsive gene expressed in keratinocyte progenitor cells, with possible involvement in early skin tumorigenesis. J Biol Chem. 2003; 278(3):1758-68.

Wei SJ, Williams JG, Dang H, Darden TA, Betz BL, Humble MM, Chang FM, Trempus CS, Johnson K, Cannon RE, and Tennant RW. Identification of a specific motif of the DSS1 protein required for proteasome interaction and p53 protein degradation. Journal of molecular biology. 2008; 383: 693-712.

White DA, Turner NC, Galbraith JH. Leaf water relations and stomatal behavior of four allopatric *Eucalyptus* species planted in Mediterranean southwestern Australia. Tree Physiol. 2000, 20:1157-1165.

Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M and Van CW. Catalase is a sink for H_2O_2 and is indispensable for stress defense in C3 plants. The EMBO journal. 1997; 16(16): 4806-4816.

Woo HR, Kim HJ, Nam HG and Lim PO. Plant leaf senescence and death –regulation by multiple layers of control and implications for aging in general. Journal of Cell Science. 2013; 126(21): 4823-4833.

Wu SD. Cloning and characterization of ethephon-inducible genes from sweet potato leaves. Master Thesis. 2010. Department of Biological Sciences. National Sun Yat-sen University.

Wu X. Gene and qualitative analysis of ethephon inducible from sweet potato leaves. Master Thesis. 2010. Department of Biological Sciences. National Sun Yat-sen University.

Xu J and Chua NH. Dehydration stress activates Arabidopsis MPK6 to signal DCP1 phosphorylation. EMBO J. 2012; 31, 1975–1984.

Xu J and Zhang S. Mitogen-activated protein kinase cascades in signaling plant growth and development. Trends Plant Sci. 2015; 20(1):56-64

Xu RQ. Ascorbate peroxidase SPAPX and reduced ascorbate alleviate NaCl-mediated leaf senescence in sweet potato. Master Thesis. 2016. Department of Biological Sciences. National Sun Yat-sen University.

Yan P. Reduced glutathione mitigates ethephon or NaCl-induced leaf senescence in sweet potato: the possible mechanisms. 2015. Master Thesis. Department of Biological Sciences. National Sun Yat-sen University.

Yao X, Xiong W, Ye TT, Wu Y. Overexpression of the aspartic protease ASPG1 gene confers drought avoidance in *Arabidopsis*. J. Exp. Bot. 2012; 63: 2579–2593.

Ye ZZ, Rodriguez R, Tran A, Hoang H, de los Santos D, Brown S & Vellanoweth RL. The developmental transition to flowering represses ascorbate peroxidase activity and induces enzymatic lipid peroxidation in leaf tissue in *Arabidopsis thaliana*. Plant Science. 2000; 158: 115–127.

Zadraznik T, Hollung K, Egge-Jacobsen W, Meglic V, Sustar-Vozlic J. Differential proteomic analysis of drought stress response in leaves of common bean (*Phaseolus vulgaris* L.). J. Proteom. 2013; 78: 254–272.

Zelicourt A, Colcombet J, and Hirt H. The role of MAPK modules and ABA during abiotic stress signaling. Trends Plant Sci. 2016; 21, 677–685.

Zhang M, Lv DW, Ge P, Bian YW, Chen GX, Zhu GR, Li XH, Yan YM. Phosphoproteome analysis reveals new drought response and defense mechanisms of seedling leaves in bread wheat (*Triticum aestivum* L.). J. Proteom. 2014; 109: 290–308.

Zhang Q. Strategies for developing Green Super Rice. Proc. Natl. Acad. Sci. USA. 2007; 104: 16402–16409.

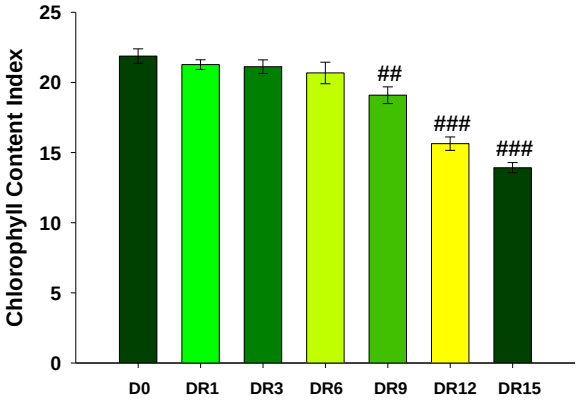
Zhang S and Klessig DF. MAPK cascades in plant defense signaling. Trends Plant Sci. 2001; 6(11): 520-527.

Zhang Y, Chang F, Huang J, Junco JJ, Maffi SK, Pridgen HI, Catano G, Dang H, Ding X, Yang F, Kim DJ, Slaga TJ, He R, and Wei SJ. DSSylation, a novel protein modification targets proteins induced by oxidative stress, and facilitates their degradation in cells. Protein Cell. 2014; 5(2): 124–140.

Zimmermann P, Heinlein C, Orendi G, Zentgraf U. Senescence-specific regulation of catalases in *Arabidopsis thaliana* (L.) Heynh. Plant Cell Environ. 2006; 29 (6):1049-1060.

A. Leaf Morphology

B. Chlorophyll Content



C. F_v/F_m

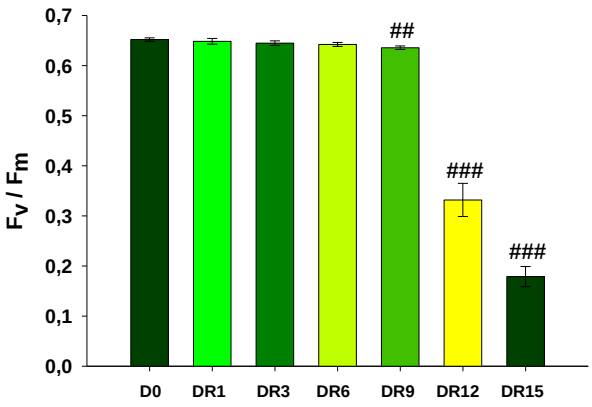
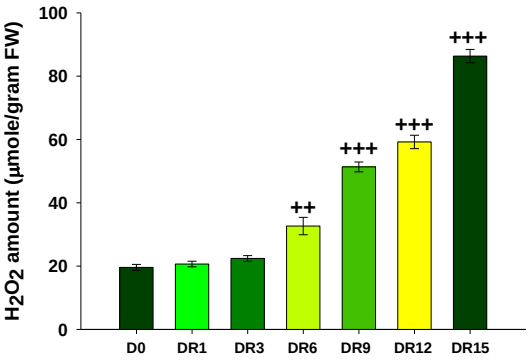


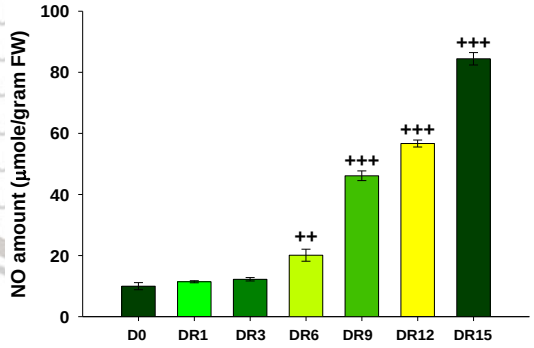
Figure 1. Effects of drought stress on leaf senescence in sweet potato. A. Leaf morphology; B. Chlorophyll content; C. F_v/F_m. Drought treatment was performed and analysed on days 1, 3, 6, 9, 12, and 15, respectively. D and DR represent control and drought treatment, respectively. **#** ($^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$) represents statistically significant as compared to the D0 control. The data were repeated at least three times and shown as mean ± standard error.

A. DAB Staining

B. H_2O_2 Level



C. NO Amount



D. MDA Content

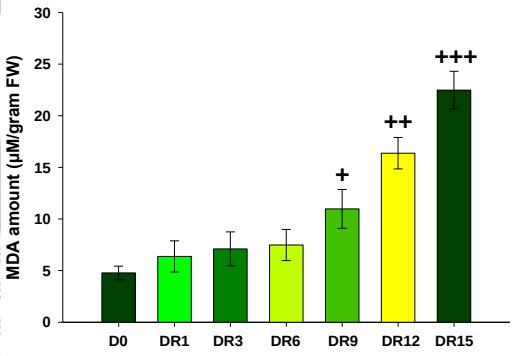
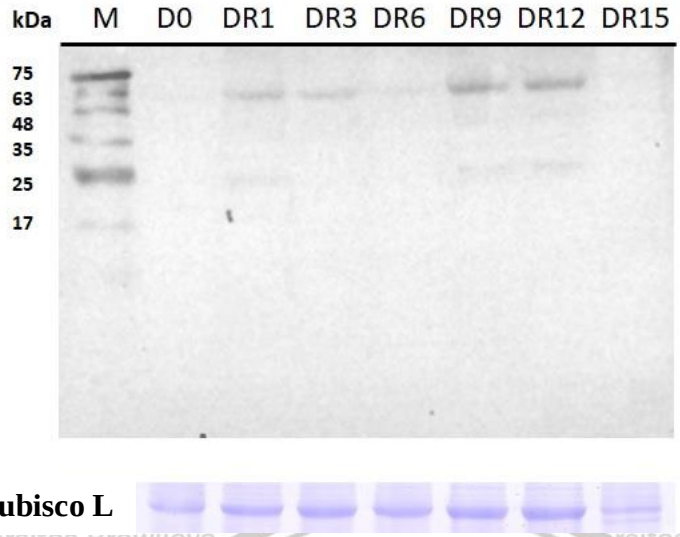


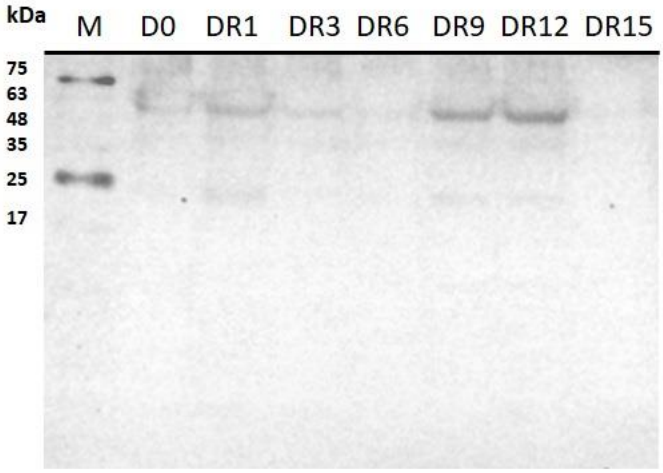
Figure 2. Effects of drought stress on H_2O_2/NO/MDA amounts in senescing leaves of sweet potato. A. DAB staining; B. H_2O_2 level; C. NO amount; D. MDA content. Drought treatment was performed and analysed on days 1, 3, 6, 9, 12, and 15, respectively. D and DR represent control and drought treatment, respectively. + ($^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$) represents statistically significant as compared to the D0 control. The data were repeated at least three times and shown as mean ± standard error.

A. Western Blot Hybridization – SPMAPK



Rubisco L

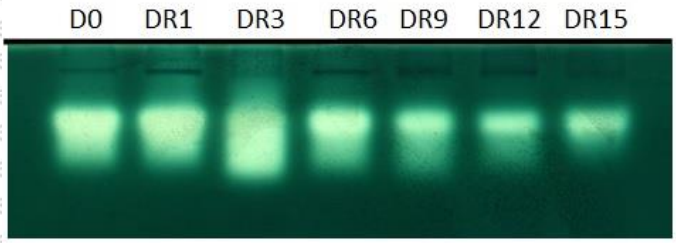
B. Western Blot Hybridization – p-SPMAPK



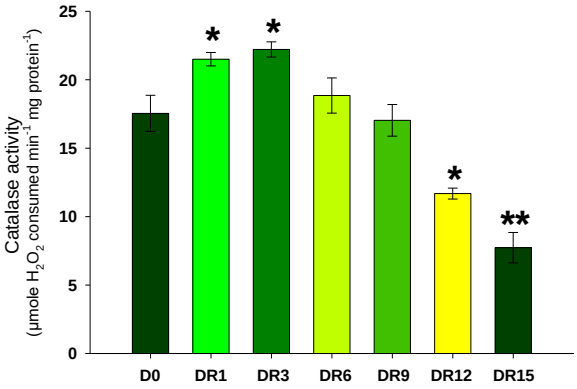
Rubisco L

Figure 3. Effects of drought stress on the expression and protein phosphorylation of mitogen-activated protein kinase SPMAPK in senescing leaves of sweet potato. A. t-SPMAPK; B. p-SPMAPK. Drought treatment was performed and analysed on days 1, 3, 6, 9, 12, and 15, respectively. D and DR represent control and drought treatment, respectively. Rubisco L represents Rubisco large subunit. The t-SPMAPK and p-SPMAPK represent SPMAPK total protein amount and protein phosphorylation level, respectively. The data were repeated at least three times and a representative one was shown.

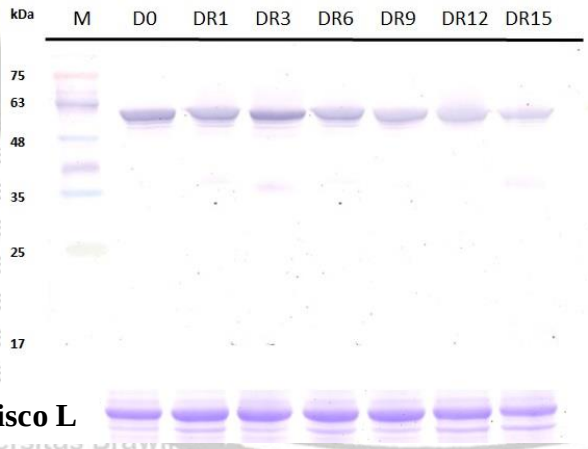
A. In Gel Catalase Activity



B. Total Catalase Activity



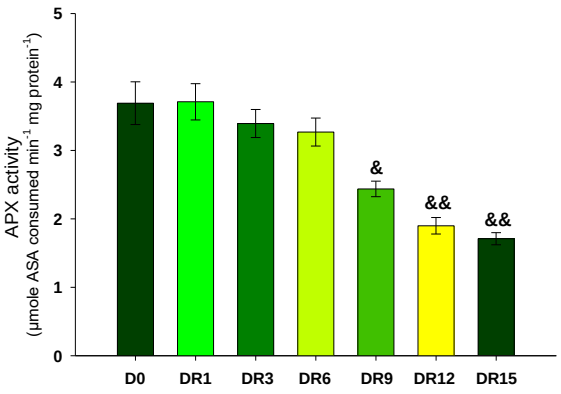
C. Western Blot Hybridization – SPCAT1



Rubisco L

Figure 4. Effects of drought stress on antioxidant protein amount and enzymatic activity level in senescing leaves of sweet potato. A. In-gel catalase activity; B. Total catalase activity; C. Western blot hybridization – SPCAT1. Drought treatment was performed and analysed on days 1, 3, 6, 9, 12, and 15, respectively. D and DR represent control and drought treatment, respectively. Rubisco L represents Rubisco large subunit. * (*$P < 0.05$; **$P < 0.01$) represents statistically significant as compared to the D0 control. The data were repeated at least three times and shown as mean ± standard error.

A. Total Ascorbate Peroxidase Activity



B. Western Blot Hybridization – SPAPX

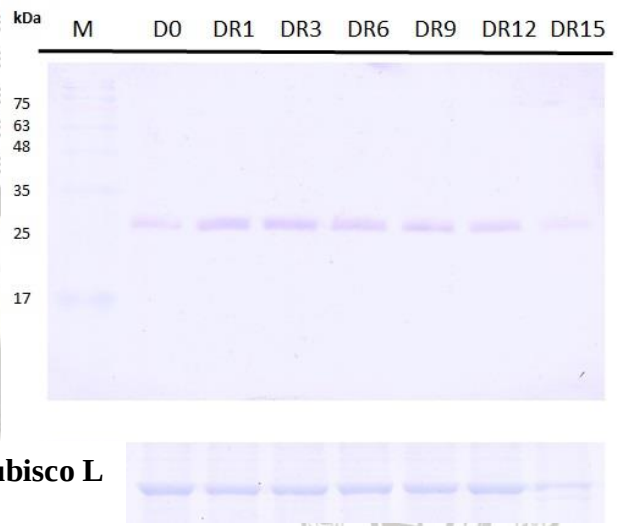


Figure 5. Effects of drought stress on antioxidant protein amount and enzymatic activity level in senescing leaves of sweet potato. A. Total ascorbate peroxidase activity; B. Western blot hybridization – SPAPX. Drought treatment was performed and analysed on days 1, 3, 6, 9, 12, and 15, respectively. D and DR represent control and drought treatment, respectively. Rubisco L represents Rubisco large subunit. & ($^{\&}P < 0.05$; $^{\&\&}P < 0.01$) represents statistically significant as compared to the D0 control. The data were repeated at least three times and shown as mean ± standard error.

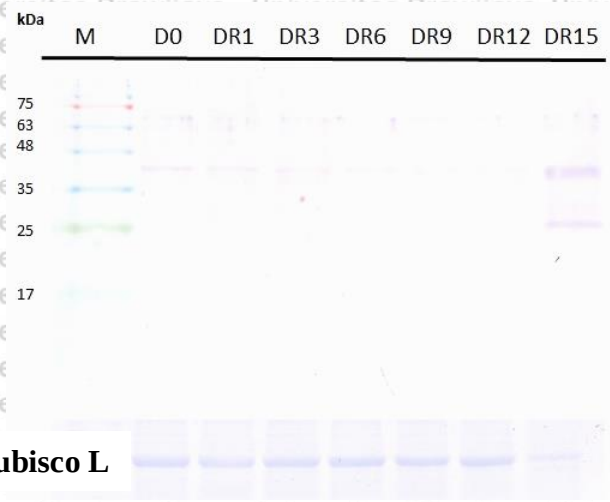
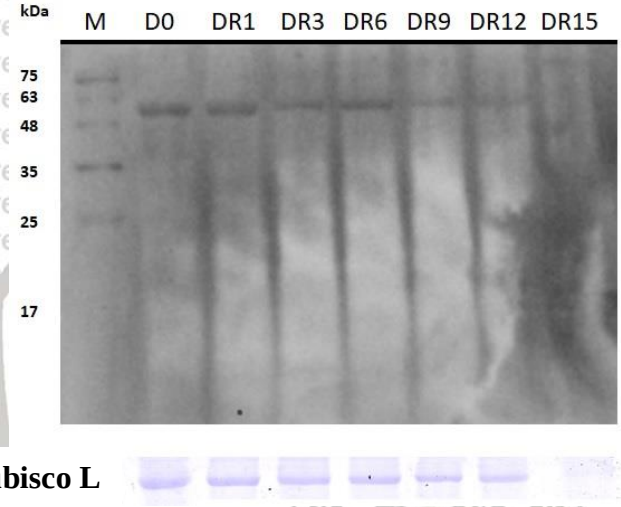


Figure 6. Effects of drought stress on the expression of senescence-associated cysteine protease SPCP1 in senescing leaves of sweet potato. Drought treatment was performed and analysed on days 1, 3, 6, 9, 12, and 15, respectively. D and DR represent control and drought treatment, respectively. Rubisco L represents Rubisco large subunit. The data were repeated at least three times and a representative one was shown.

A. Nucleotide and Amino Acid

```
   1                                                        AGCGT
   6  GGTCGCGGCCGAGGTCAACCCTTCTCTAATTCTCTCCTACGATTAGGGATTGACTATAGG
  66  ATGGCTACCGAGCAACCAAAGCCGGCGCCTGAAGACGTGAAAATGGATCTCTTCGAAGAT
        M  A  T  E  Q  P  K  P  A  P  E  D  V  K  M  D  L  F  E  D   20
 126  GACGACGAGTTTGAGGAGTTTGAAATTGACCAAGAGTGGGAGGACAAAGAGGAGGGGAAA
        D  D  E  F  E  E  F  E  I  D  Q  E  W  E  D  K  E  E  G  K   40
 186  GAAGTAACCCAGCAGTGGGAAGATGACTGGGATGATGATGATGTCAATGACGACTTCTCC
        E  V  T  Q  Q  W  E  D  D  W  D  D  D  D  V  N  D  D  F  S   60
 246  CTGCAACTTAGAAAGGAATTGGAATGTAACACTGAGAAGAAGGACTAAGCTAATACTCTG
        L  Q  L  R  K  E  L  E  C  N  T  E  K  K  D  *              75
 306  TAATACTTTGGCATCATTGCATTCCCCTCTAGAATTGATGCGTTCCTCATATATTGTACC
 366  CGGGCGGCCGCTCGAA
```

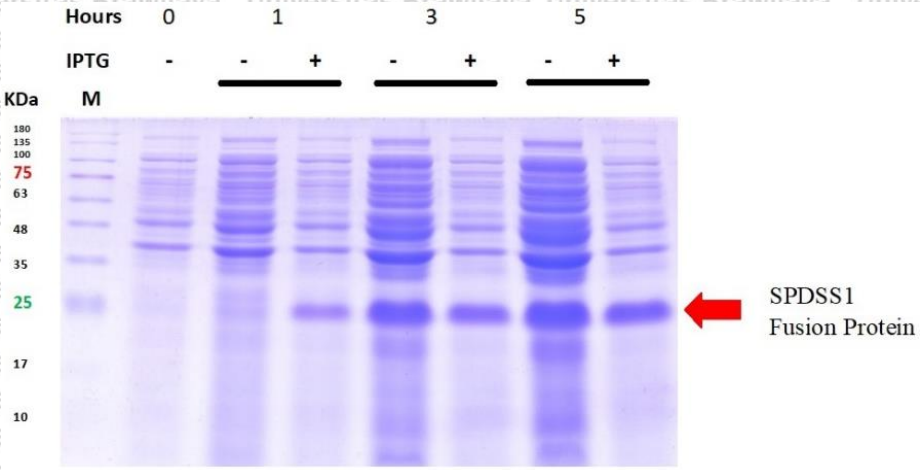
B. Expression of SPDSS1 on Drought Treatment Leaves



Rubisco L

Figure 7. Cloning and expression of sweet potato deleted-in-split hand/split foot 1 protein SPDSS1. A. SPDSS1 nucleotide and amino acid sequences; B. SPDSS1 expression under drought. The conserved aspartic/glutamic acid-rich domains of sweet potato SPDSS1 were marked and shown in black in grey at amino acid residue positions from 15 - 22 (15EDDEFEEF22), 39 - 45 (39WEDNWDD45), and 49 - 60 (49EDDFS-X-QLR-XEL60; X is any amino acid), respectively (Adapted from Wu, 2010). Drought treatment was performed and analysed on days 1, 3, 6, 9, 12, and 15, respectively. D and DR represent control and drought treatment, respectively. Rubisco L represents Rubisco large subunit. The data were repeated at least three times and a representative one was shown.

A. IPTG Induction



A. SPDSS1 Fusion Protein Purification

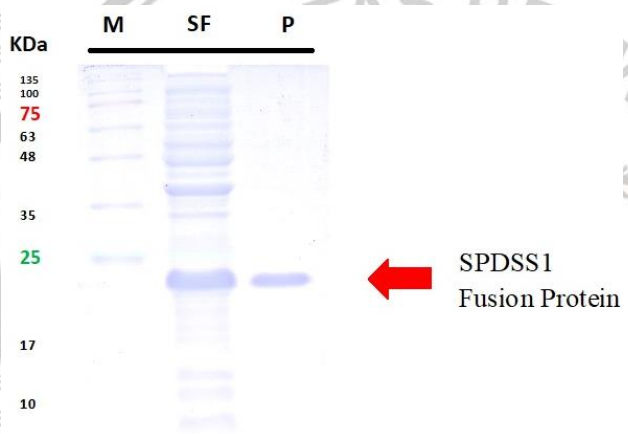
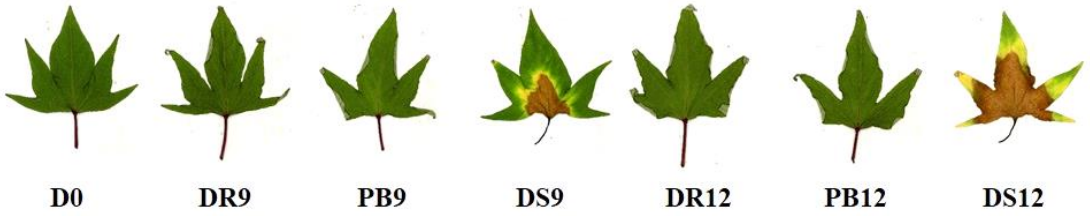
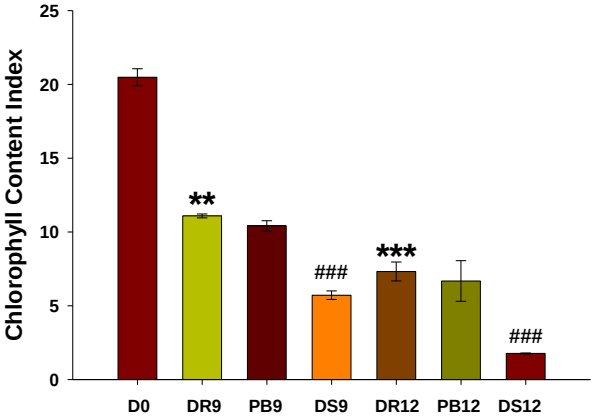


Figure 8. Induction and purification of SPDSS1 fusion protein in *E. coli* BL21 (DE3) cells. A. IPTG induction; B. SPDSS1 fusion protein purification. The induced cells were harvested and analysed with 12.5% SDS-PAGE for SPDSS1 fusion protein amounts after induction for 1, 3, and 5 h, respectively. (+) and (-) represent with or without 1 mM IPTG induction, respectively. The SPDSS1 fusion protein was purified by Ni-NTA His•Bind Resins affinity column and was analysed with 12.5% SDS-PAGE. SF: protein crude extract, P: Purified SPDSS1.

A. Leaf Morphology



B. Chlorophyll Content



C. F_v/F_m

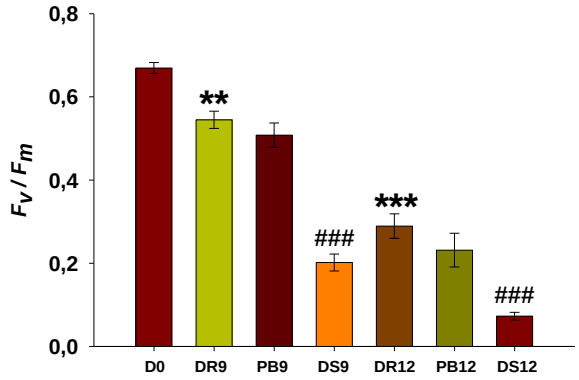
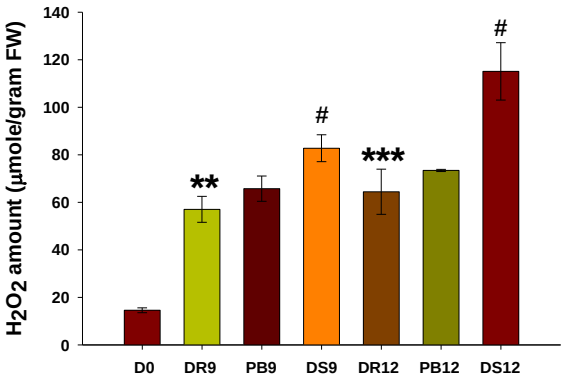


Figure 9. Effects of SPDSS1 fusion proteins on drought-induced leaf senescence in sweet potato. A. Leaf morphology; B. Chlorophyll content; C. F_v/F_m. D0 was untreated mature leaves. DR, PB and DS were drought treatment, $PBST_{0.01\%}$, and SPDSS1 fusion protein (25 μg) pretreatment for 9 days (DR9, PB9, DS9), and 12 days (DR12, PB12, DS12), respectively. * (**$P < 0.01$; ***$P < 0.001$) and # ($^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$) represent statistically significant as compared to the D0 control and PB control group, respectively. The data were repeated at least three times and shown as mean ± standard error.

A. H_2O_2 Level



B. NO Amount

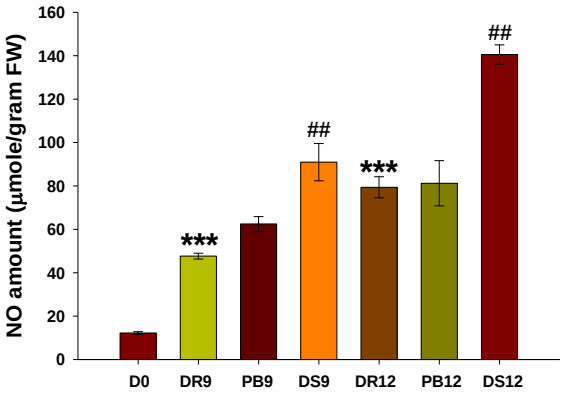
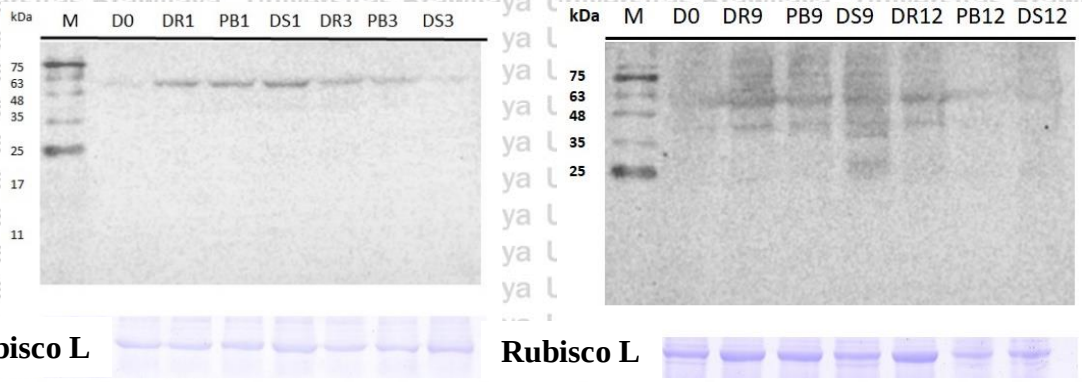


Figure 10. Effects of SPDSS1 fusion proteins on drought-induced H_2O_2/NO amounts in senescing leaves of sweet potato. A. H_2O_2 level; B. NO amount. D0 was untreated mature leaves. DR, PB and DS were drought treatment, $PBST_{0.01\%}$, and SPDSS1 fusion protein (25 µg) pretreatment for 9 days (DR9, PB9, DS9), and 12 days (DR12, PB12, DS12), respectively. * ($^{**}P < 0.01$; $^{***}P < 0.001$) and # ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$) represent statistically significant as compared to the D0 control and PB control group, respectively. The data were repeated at least three times and shown as mean ± standard error.

A. Western Blot Hybridization – SPMAPK



B. Western Blot Hybridization – p-SPMAPK

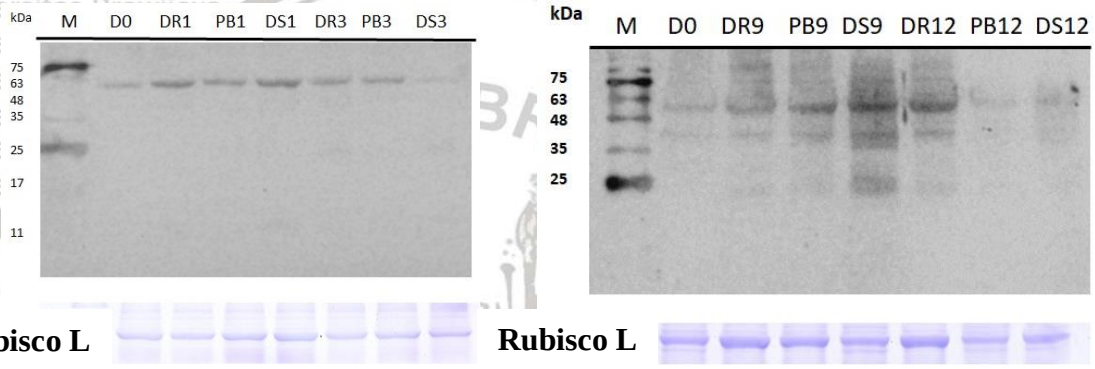
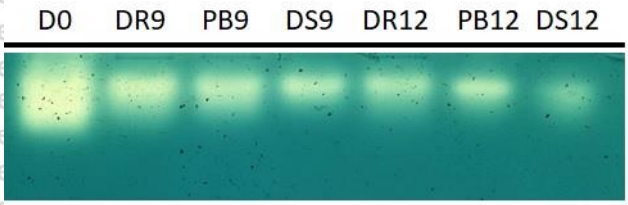
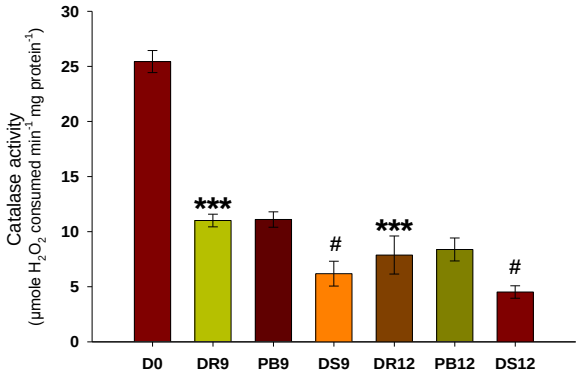


Figure 11. Effects of SPDSS1 fusion proteins on drought-induced expression and protein phosphorylation of mitogen-activated protein kinase SPMAPK in senescing leaves of sweet potato. D0 was untreated mature leaves. DR, PB and DS were drought treatment, $PBST_{0.01\%}$, and SPDSS1 fusion protein (25 µg) pretreatment for 9 days (DR9, PB9, DS9), and 12 days (DR12, PB12, DS12), respectively. Rubisco L represents Rubisco large subunit. The t-SPMAPK and p-SPMAPK represent SPMAPK total protein amount and protein phosphorylation level, respectively. The data were repeated at least three times and a representative

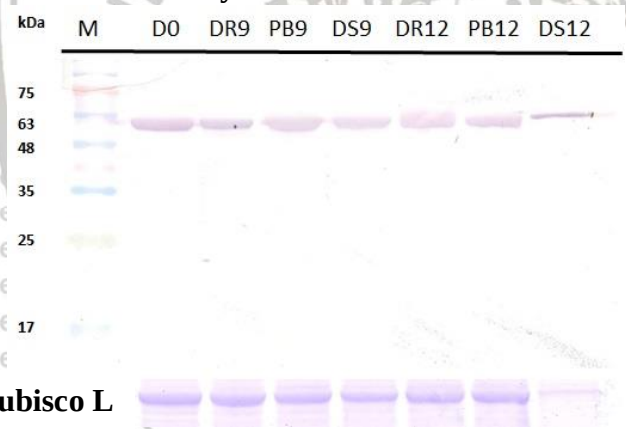
A. In Gel Catalase Activity



B. Total Catalase Activity



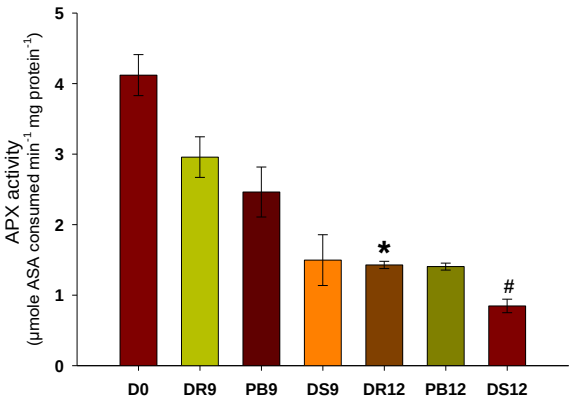
C. Western Blot Hybridization – SPCAT1



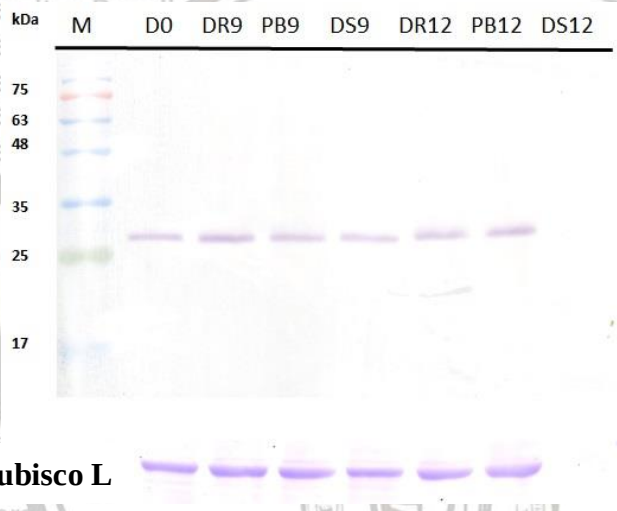
Rubisco L

Figure 12. Effects of SPDSS1 fusion proteins on drought-induced antioxidant protein amount and enzymatic activity level in senescing leaves of sweet potato. A. In-gel catalase activity; B. Total catalase activity; C. Western blot hybridization – SPCAT1. D0 was untreated mature leaves. DR, PB and DS were drought treatment, $PBST_{0.01\%}$, and SPDSS1 fusion protein (25 µg) pretreatment for 9 days (DR9, PB9, DS9), and 12 days (DR12, PB12, DS12), respectively. Rubisco L represents Rubisco large subunit. * (***$P < 0.001$) and # ($^{\#}P < 0.05$) represent statistically significant as compared to the D0 control and PB control group, respectively. The data were repeated at least three times and shown as mean ± standard error.

A. Total Ascorbate Peroxidase Activity



B. Western Blot Hybridization – SPAPX



Rubisco L

Figure 13. Effects of SPDSS1 fusion proteins on drought-induced antioxidant protein amount and enzymatic activity level in senescing leaves of sweet potato. A. In-gel ascorbate peroxidase activity; B. Total ascorbate peroxidase activity; C. Western blot hybridization – SPAPX. D0 was untreated mature leaves. DR, PB and DS were drought treatment, $PBST_{0.01\%}$, and SPDSS1 fusion protein (25 μg) pretreatment for 9 days (DR9, PB9, DS9), and 12 days (DR12, PB12, DS12), respectively. Rubisco L represents Rubisco large subunit. * ($^{*}P < 0.05$) and # ($^{\#}P < 0.05$) represent statistically significant as compared to the D0 control and PB control group, respectively. The data were repeated at least three times and shown as mean ± standard error.

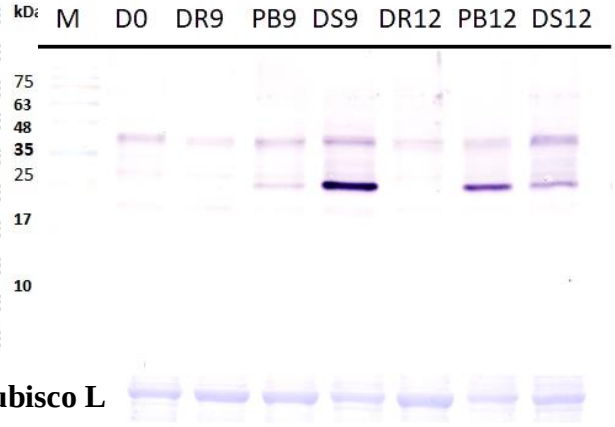
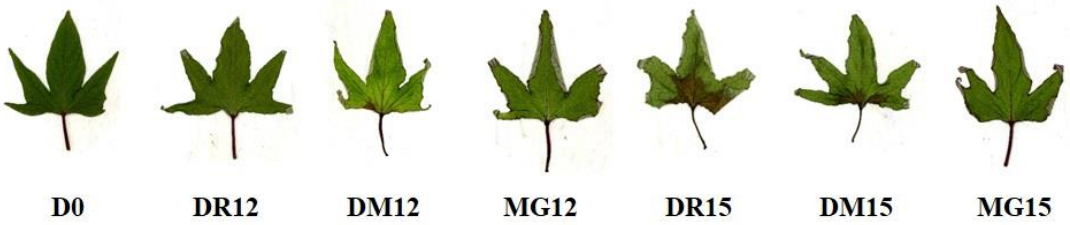
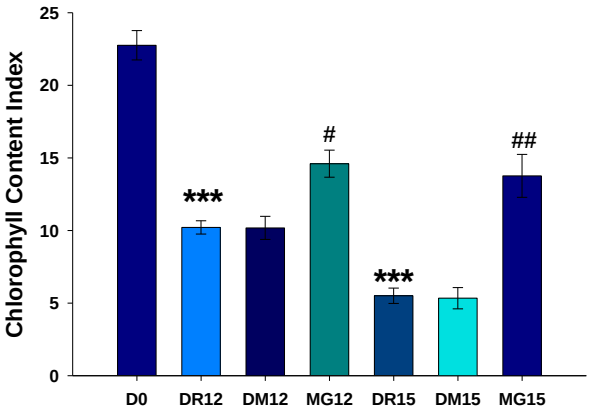


Figure 14. Effects of SPDSS1 fusion proteins on drought-induced senescence-associated cysteine protease SPCP1 level in senescing leaves of sweet potato. D0 was untreated mature leaves. DR, PB and DS were drought treatment, $PBST_{0.01\%}$, and SPDSS1 fusion protein (25 µg) pretreatment for 9 days (DR9, PB9, DS9), and 12 days (DR12, PB12, DS12), respectively. Rubisco L represents Rubisco large subunit. The data were repeated at least three times and a representative one was shown.

A. Leaf Morphology



B. Chlorophyll Content



C. F_v/F_m

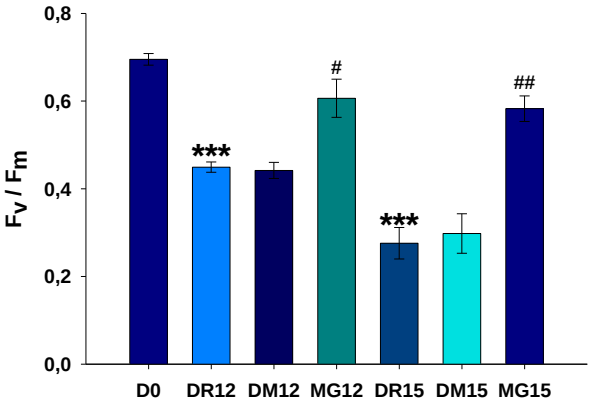
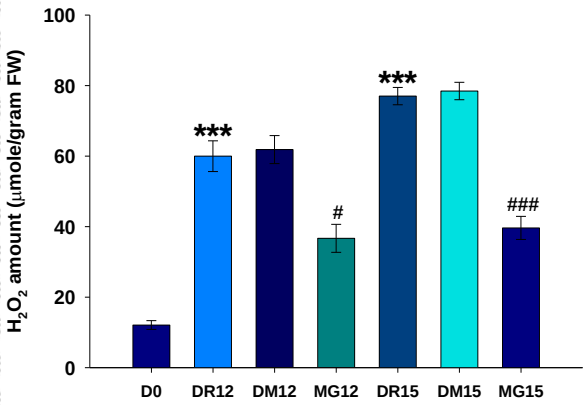


Figure 15. Effects of proteasome inhibitor MG132 on drought-induced leaf senescence in sweet potato. A. Leaf morphology; B. Chlorophyll content; C. F_v/F_m. D0 was untreated mature leaves. DR, DM and MG were drought treatment, DMSO (5 µl), and MG132 (5 nmole) pretreatment for 12 days (DR12, DM12, MG12), and 15 days (DR15, DM15, MG15), respectively. * (***$P < 0.001$) and # ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$) represent statistically significant as compared to the D0 control and DM control group, respectively. The data were repeated at least three times and shown as mean ± standard error.

A. H_2O_2 Level



B. NO Amount

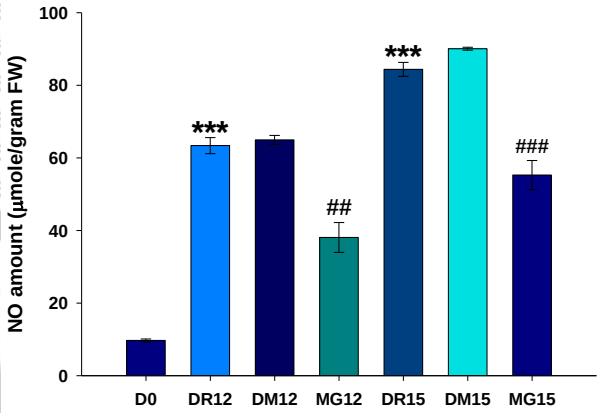
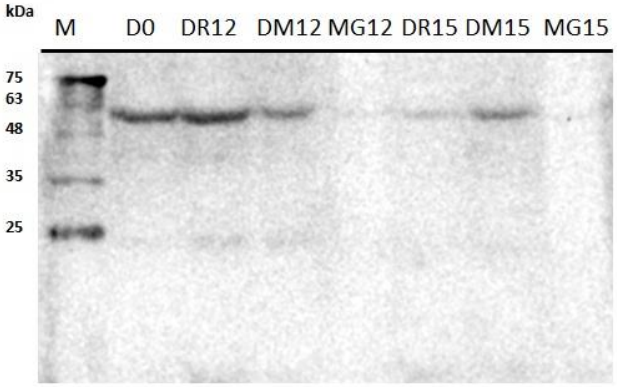


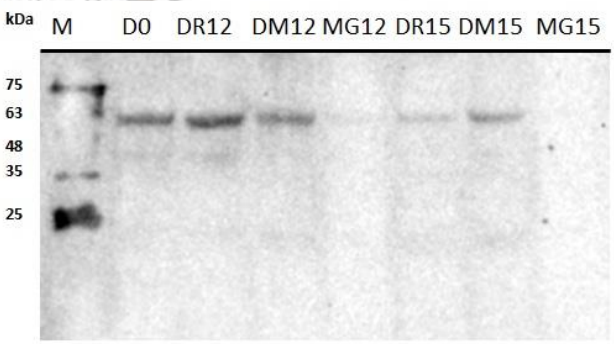
Figure 16. Effects of proteasome inhibitor MG132 on drought-induced H_2O_2/NO amounts in senescing leaves of sweet potato. A. H_2O_2 level; B. NO amount. D0 was untreated mature leaves. DR, DM and MG were drought treatment, DMSO (5 µl), and MG132 (5 nmole) pretreatment for 12 days (DR12, DM12, MG12), and 15 days (DR15, DM15, MG15), respectively. DR, DM and DS were drought treatment, $PBST_{0.01\%}$, and SPDSS1 fusion protein (25 µg) pretreatment for 9 days (DR9, PB9, DS9), and 12 days (DR12, PB12, DS12), respectively. * (***$P < 0.001$) and # ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$)) represent statistically significant as compared to the D0 control and DM control group, respectively. The data were repeated at least three times and shown as mean ± standard error.

A. Western Blot Hybridization – SPMAPK



Rubisco L

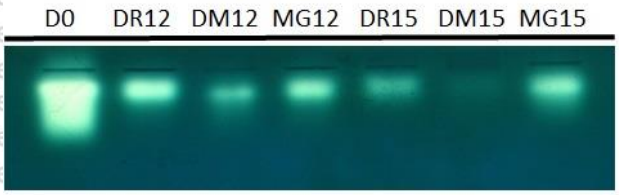
B. Western Blot Hybridization – pSPMAPK



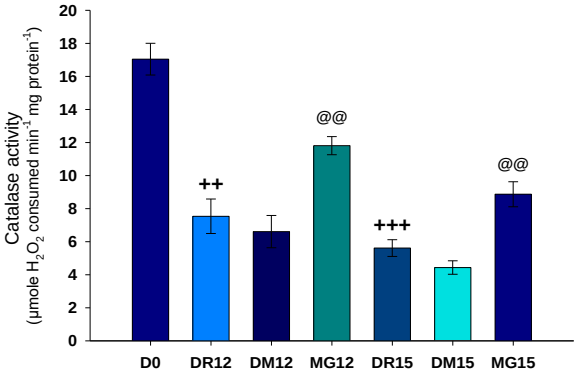
Rubisco L

Figure 17. Effects of proteasome inhibitor MG132 on drought-induced expression and protein phosphorylation of mitogen-activated protein kinase SPMAPK in senescing leaves of sweet potato. D0 was untreated mature leaves. DR, DM and MG were drought treatment, DMSO (5 µl), and MG132 (5 nmole) pretreatment for 12 days (DR12, DM12, MG12), and 15 days (DR15, DM15, MG15), respectively. Rubisco L represents Rubisco large subunit. The t-SPMAPK and p-SPMAPK represent SPMAPK total protein amount and protein phosphorylation level, respectively. The data were repeated at least three times and a representative one was shown.

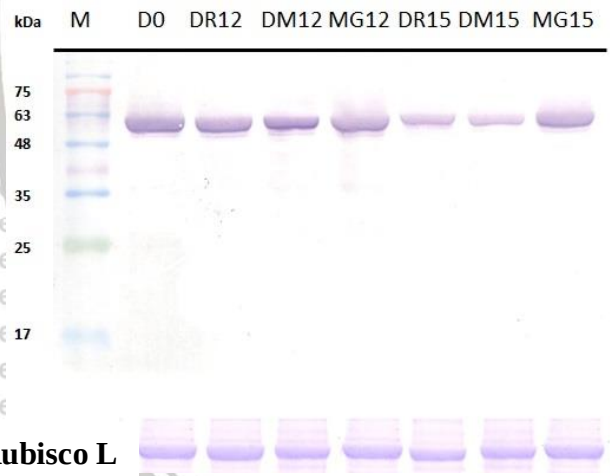
A. In Gel Catalase Activity



B. Total Catalase Activity



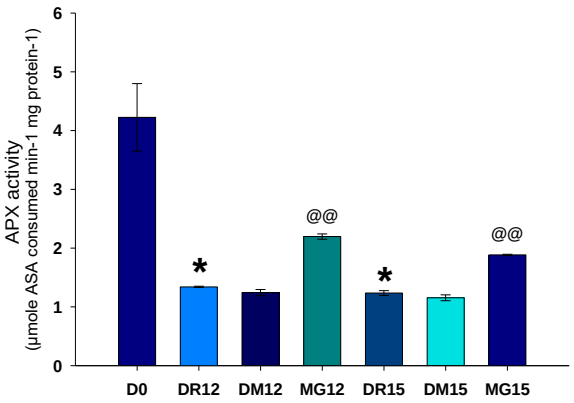
C. Western Blot Hybridization – SPCAT1



Rubisco L

Figure 18. Effects of proteasome inhibitor MG132 on drought-induced antioxidant protein amount and enzymatic activity level in senescing leaves of sweet potato. A. In-gel catalase activity; B. Total catalase activity; C. Western blot hybridization – SPCAT1. D0 was untreated mature leaves. DR, DM and MG were drought treatment, DMSO (5 µl), and MG132 (5 nmole) pretreatment for 12 (DR12, DM12, MG12), and 15 days (DR15, DM15, MG15), respectively. Rubisco L represents Rubisco large subunit. + ($^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$) and @ ($^{@@}P < 0.01$) represent statistically significant as compared to the D0 control and DM control group, respectively. The data were repeated at least three times and shown as mean ± standard error.

A. Total Ascorbate Peroxidase Activity



B. Western Blot Hybridization – SPAPX

Rubisco L

Figure 19. Effects of proteasome inhibitor MG132 on drought-induced antioxidant protein amount and enzymatic activity level in senescing leaves of sweet potato. A. Total ascorbate peroxidase activity; B. Western blot hybridization – SPAPX. D0 was untreated mature leaves. DR, DM and MG were drought treatment, DMSO (5 µl), and MG132 (5 nmole) pretreatment for 12 (DR12, DM12, MG12), and 15 days (DR15, DM15, MG15), respectively. Rubisco L represents Rubisco large subunit. * ($^{*}P < 0.05$) and @ ($^{@@}P < 0.01$) represent statistically significant as compared to the D0 control and DM control group, respectively. The data were repeated at least three times and shown as mean ± standard error.

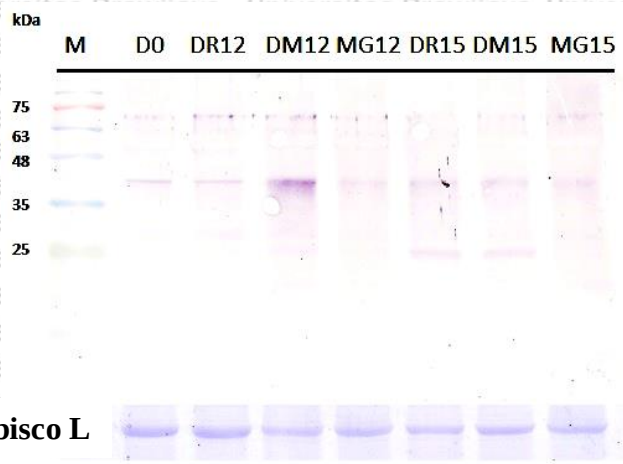
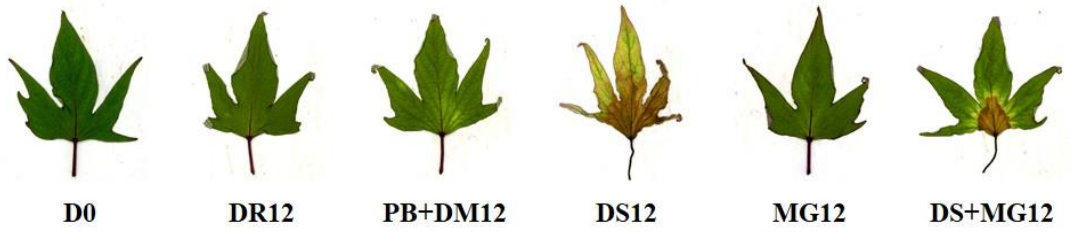
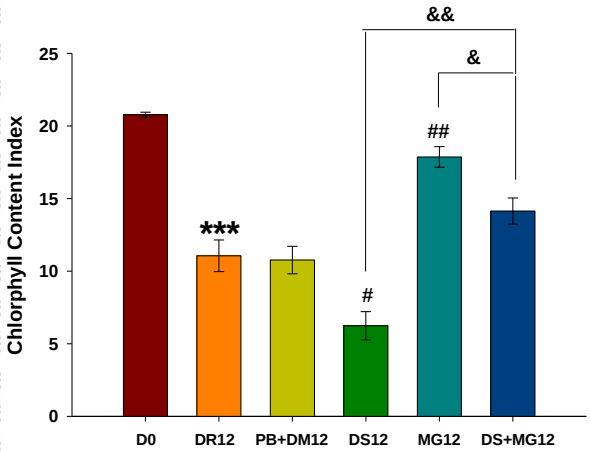


Figure 20. Effects of proteasome inhibitor MG132 on drought-induced senescence-associated cysteine protease SPCP1 expression level in senescing leaves of sweet potato. D0 was untreated mature leaves. DR, DM and MG were drought treatment, DMSO (5 µl), and MG132 (5 nmole) pretreatment for 12 days (DR12, DM12, MG12), and 15 days (DR15, DM15, MG15), respectively. Rubisco L represents Rubisco large subunit. The data were repeated at least three times and a representative one was shown.

A. Leaf Morphology



B. Chlorophyll Content



C. F_v/F_m

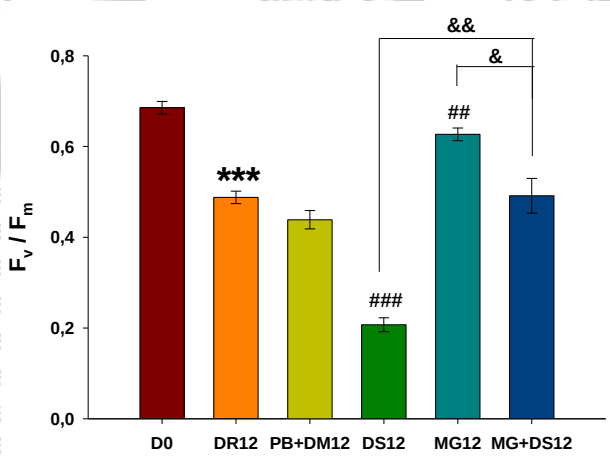
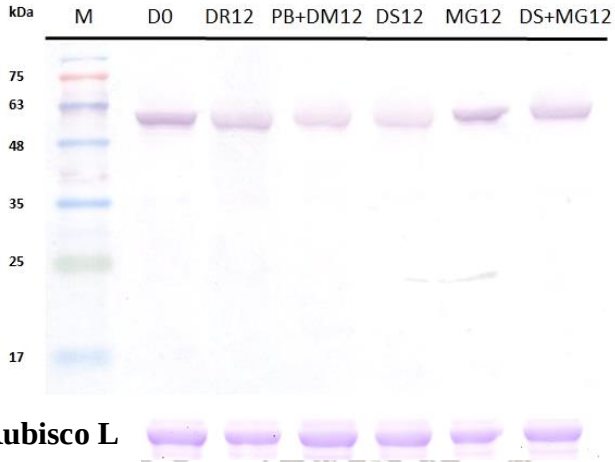


Figure 21. Proteasome inhibitor MG132 reverses the SPDSS1 fusion protein-mediated effects on drought-induced leaf senescence in sweet potato. A. Leaf morphology; B. Chlorophyll content; C. F_v/F_m. D0 was untreated mature leaves. DR, PB+DM, DS, MG and DS+MG were drought treatment, PBST + DMSO, SPDSS1 fusion protein (25 µg), MG132 (5 nmole) and SPDSS1 fusion protein (25 µg) + MG132 (5 nmole) pretreatment for 12 days (DR12, PB+DM12, DS12, MG12, DS+MG12), respectively. * (***$P < 0.001$) , # ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$)), and & ($^{\&}P < 0.05$; $^{\&\&}P < 0.01$) represent statistically significant as compared to the D0 control, PB+DM and (DS12 or MG12) control groups, respectively. The data were repeated at least three times and shown as mean ± standard error.

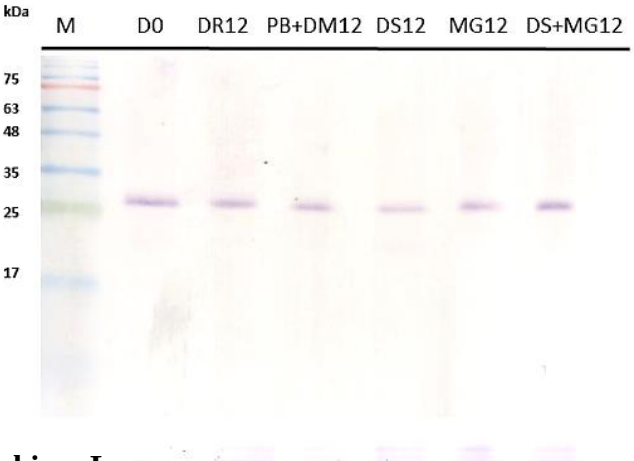
A. In Gel Catalase Activity

B. Western Blot Hybridization – SPCAT1



Rubisco L

C. Western Blot Hybridization – SPAPX



Rubisco L

Figure 22. Proteasome inhibitor MG132 reverses the SPDSS1 fusion protein-mediated effects on drought-induced antioxidant protein amount and enzymatic activity level in senescing leaves of sweet potato. A. In-gel catalase activity; B. Western blot hybridization – SPCAT1; C. Western blot hybridization – SPAPX. D0 was untreated mature leaves. DR, PB+DM, DS, MG and DS+MG were drought treatment, PBST + DMSO, SPDSS1 fusion protein (25 µg), MG132 (5 nmole) and SPDSS1 fusion protein (25 µg) + MG132 (5 nmole) pretreatment for 12 days (DR12, PB+DM12, DS12, MG12, DS+MG12), respectively. Rubisco L represents Rubisco large subunit. The data were repeated at least three times and a representative one was shown.

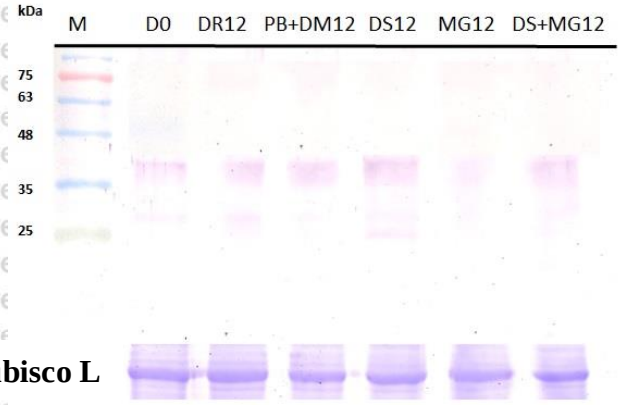


Figure 23. Proteasome inhibitor MG132 reverses the SPDSS1 fusion protein-mediated effects on drought-induced senescence-associated cysteine protease SPCP1 expression level by western blot hybridization. D0 was untreated mature leaves. DR, PB+DM, DS, MG and DS+MG were drought treatment, PBST + DMSO, SPDSS1 fusion protein (25 µg), MG132 (5 nmole) and SPDSS1 fusion protein (25 µg) + MG132 (5 nmole) pretreatment for 12 days (DR12, PB+DM12, DS12, MG12, DS+MG12), respectively. Rubisco L represents Rubisco large subunit. The data were repeated at least three times and a representative one was shown.

Figure 24 Plagiarism Certificate

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Universitas	: Universitas Brawijaya

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Proteasome and the Deleted-in-Split Hand/Split Foot 1 Protein SPDSS1 Participate in the Modulation of Drought Stress Tolerance in Sweet Potato Leaves

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NIP. 19791129 200501 2 002

Figure 25 Letter of Acceptance of Paper

Journal of Tropical
Life Science
International Journal of Theoritical, Experimental, and Applied Life Science

July 22nd, 2019

Dear Author,

We would like to congratulate you on the *acceptance* of your manuscript "Potency of Trichoderma sp. as A Bio-control Agent against Fusarium sp. Pathogen of Porang (Amorphophallus muelleri Blume) Tuber", authors: Nindi Nazula Fajarini, Rodiyati Azrianingsih, Suharjono, in Journal of Tropical Life Science.

Thank you very much for considering JTLS to publish your work.

Best regards,

NashiWidodo, Ph.D.
Editor JTLS