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碩士論文

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Master Thesis

阿拉伯芥中的 SCE1 和 COP1 會透過泛素化與類小泛素化調節轉錄因子 ERF1 在光照與黑暗下的蛋白質穩定性

SCE1 and COP1 regulate the stability of ERF1 through SUMOylation and ubiquitination under light-dark cycle in Arabidopsis

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在碩士班這短短的兩年時間，最終濃縮成了這一本論文精華，能完成這本論文，要感謝的人很多，首先是我的指導老師-梅君老師，作為老師的第一屆學生，得到了很多不一樣的關心與照顧。剛進實驗室初來乍到，對於環境或是實驗都很不熟悉，老師很熱情的帶著我們做實驗，把所學到的經驗以及知識不藏私的傳授給我們，更感謝老師總是在我遇到瓶頸以及低潮時適時的拉我一把，最後讓我可以順利的完成每一次的報告以及這本論文，也在這兩年期間不斷地讓我去嘗試不一樣的實驗方法，即使是失敗了，仍然很認真的看待每一次的結果討論。在最終論文口試的部分也很感謝各個口試委員的提問以及建議，提出了很多我沒有考慮到的盲點，才能讓這篇論文更完整。也要謝謝生化組的每一位老師們在每一次的 seminar 和 master day 提出的問題，這些都讓我成長了很多，也讓我學會了科學邏輯思考能力的重要性。

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中文摘要

ETHYLENE RESPONSE FACTOR 1 (ERF1)是阿拉伯芥當中參與在生物性與非生物性逆境中，一個極為重要的轉錄因子，並且主要調控乙烯訊息傳遞路徑。先前已有研究指出 ERF1 蛋白在黑暗中會受到 UBIQUITIN-CONJUGATING ENZYME 18 (UBC18)的泛素化調控，經過酵素的合作，會負責標記目標蛋白使其透過 proteasome 的途徑降解而趨於不穩定。在本研究中，我們發現 SUMO-CONJUGATING ENZYME 1 (SCE1)與 ERF1 有交互作用。同時另一個在阿拉伯芥當中扮演 E3 ubiquitin ligase 的蛋白 CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1)，在黑暗下會藉由調控 ERF1 的 ubiquitination 而使其降解，反之在光照下 SCE1 會透過 SUMOylation 使 ERF1 蛋白趨於穩定。我們更進一步發現，當 ERF1 蛋白的 SUMOylation 位點被突變 ($ERF1^{4KR}$) 後，ubiquitination 和 SUMOylation 的修飾都有減少的現象，並且在植物當中我們也觀察到在進入黑暗後 $ERF1^{4KR}$ 沒有被降解，間接證實了對於 ERF1，ubiquitination 和 SUMOylation 存在著光照條件之間的競爭關係。不僅如此，我們也看到 *sce1* 突變株不管在鹽處理或是乾旱下，側根數量以及植株存活率都較 WT 來的低。同時我們也發現 *sce1* 突變株在黑暗處理下，ERF1 的下游基因 *P5CS1* 以及 *OSM34* 的表現量都較 WT 更低，表示 SCE1 可以藉由調控 ERF1 的穩定性正向地參與在植物逆境反應中。綜上所述，我們的研究證實了一項新的調控 ERF1 蛋白穩定性的分子機制，以及 SCE1 在逆境訊息傳遞當中扮演的重要角色。

關鍵字：阿拉伯芥、ERF1、SCE1、COP1、泛素化、類小泛素化、蛋白質穩定性、鹽逆境

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ABSTRACT

ETHYLENE RESPONSE FACTOR 1 (ERF1) is an important transcription factor which involves in biotic and abiotic stress, and plays a major role in ethylene signaling. Previous studies have shown that ERF1 is unstable in the dark and its degradation is mediated by UBIQUITIN-CONJUGATING ENZYME 18. Here, we demonstrated that SUMO-CONJUGATING ENZYME 1 (SCE1) can physically interact with ERF1 in plants. CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) is an E3 ubiquitin ligase that target substrate protein for proteosome degradation pathway. *In vitro* and *in vivo* ubiquitination and SUMOylation assays suggest that COP1 mediates ERF1 ubiquitination in the dark while SCE1 mediates ERF1 SUMOylation in the light. Moreover, *in vitro* ubiquitination assay showed that the SUMOylation sites-mutated ERF1 (ERF1-4KR) led to less ubiquitination compared to wild-type ERF1, suggesting that ubiquitination of ERF1 might compete with its SUMOylation on the same residues. Our drought- and salt-stress analyses also suggest that SCE1 plays a positive role in stress response. *sce1* mutants showed less tolerant phenotype under both drought and high salinity. The induction of ERF1's downstream genes such as *P5CS1* and *OSM34* are negatively regulated by SCE1 under light/dark cycle. Collectively, this study reveals the molecular mechanism regulating ERF1's stability and light-stress signaling crosstalk.

Keywords: Arabidopsis, ERF1, SCE1, COP1, ubiquitination, SUMOylation, protein stability, salt stress

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Chapter 1 Introduction

Plants suffer from environmental stresses on a daily basis throughout their life cycles. Continuous global climate changes are resulting in more and more severe stresses to plants in the past few decades. Abiotic stresses such as drought, high salinity, heat and cold cause major damage to plant growth and reduction on crop yield. In order to survive, plants possess unique and sophisticated gene regulatory networks in response to different stresses. In these stress response systems, upstream transcription factors would activate stress-responsive downstream genes to turn on stress defense in plants. Understanding these regulatory systems can provide us more novel and precise genetic approaches for generation of resilient crops in the future.

1.1 The abiotic stress signaling pathways in plants

In times of global climate change, plants undergo severe and various abiotic stresses more than ever. To offer specific strategies for genetic improvement in stress-resistant crop production, understanding how plants respond to these stresses becomes important. Accumulating studies are revealing how stress signaling pathways work and exploring new molecular mechanisms underlying the stress signaling pathways. In response to all kinds of internal and external stimulations, plants employ special ways to regulate their growth and development (Walters et al., 2009). Phytohormones, a various group of signaling molecules which is found in small amounts in a plant cell, are showed to mediate these responses. Generally, stress tolerance is an intricate phenomenon since plants could undergo diverse stresses at the

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same time during their development.

To date, phytohormone-abscisic acid (ABA) is reported to play a pivotal role and is known as a required messenger in the adaptive response of plants to abiotic stresses. As several stresses induce ABA synthesis, it is commonly regarded as a plant stress hormone (Mahanja et al., 2005; Swamy et al., 1999). Main abiotic environmental factors such as drought, high salinity and temperature stresses cause responsive genes expression that can be roughly divided into two groups: ABA-dependent and ABA-independent genes (Yamaguchi-Shinozaki and Shinozaki, 2015).

In ABA-dependent pathway, many drought-related genes contain a conserved ABA-responsive element (ABRE) in their promoter regions. Both ABRE-binding proteins (AREB) or ABRE-binding factors (ABF), which contain basic-domain leucine zipper (bZIP), mainly regulate the expressions of these responsive genes (Uno et al., 2000; Fujita et al., 2009). In addition, the transcription factors such as MYC, MYB and RD26/NAC were also reported to activate those target genes in ABA-dependent pathway (Abe et al., 2003; Fujita et al., 2004).

Although various drought-responsive genes participate in ABA signaling pathway, some of them are not induced by ABA treatment (Shinozaki, 2000). The drought responsive element (DRE) and C-repeat (CRT) cis-acting elements are usually in the promoters of these genes and are regulated by the combination of DRE-binding protein (DREB) or C-repeat-binding factor (CBF) transcription factors. These activators that contain an APETALA (AP2) DNA-binding domain, are crucial to ABA-independent gene expression under drought stress (Tran et al.,2004; Sakuma et al., 2006). Some other transcription factors like MYB/MYC, WRKY and NAC transcription factors are also well known to play major roles in the ABA-independent signaling (Abe et al., 1997; Hu et al., 2006).

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In addition to drought stress, salinization is another threat among the most damaging stresses to the crops on Earth. Salinity leads to ion toxicity, hyperosmotic and oxidative stress (Zhu, 2002). Redundant sodium ion can cause plant growth retardation and cellular damage, thus the Na^+ transporters in the membrane are vital to reduce the stress effects (El Mahi et al., 2019).

SALT-OVERLY-SENESITIVE 1 (SOS1) encodes a Na^+/H^+ antiporter in the plasma membrane, and plays an important role in regulating long-distance Na^+ transportation from root to shoot (Shi et al., 2002; El Mahi et al., 2019). Salt stress signals are induced by the Ca^{2+}-dependent SOS signaling pathway and would promote SOS1 activity. Ca^{2+}-binding protein SOS3 activates the protein kinase SOS2 which results in protein kinase complex formation. SOS1 proteins are then phosphorylated and activated via releasing its auto-inhibition (Qiu et al., 2002; Zhu, 2003; Quintero et al., 2011). Therefore, SOS1 mediates the exportation of Na^+ to maintain the cellular homeostasis.

1.2 AP2/ERF family transcription factors in Arabidopsis

The AP2 and ethylene-responsive element-binding factor (ERF) gene family is one of the largest plant-specific transcription factor gene families. According to the differences in binding sequence of AP2/ERF DNA-binding domain (BD), this superfamily is categorized into five subfamilies, consisting of AP2, related to ABI3/VP (RAV), ERF, DREB and soloist. AP2 family genes contain several repeated AP2 domains, while RAV family genes have an AP2 and a B3 domain. ERF and DREB family genes usually possess a single AP2 domain, but were divided into two

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subfamilies due to the amino acid residue sequence (Nakano et al., 2006). Additionally, a small part of the transcription factor with a low similarity from AP2 domain is called soloist (Sakuma et al., 2002).

AP2 genes are often thought to be vital to flowering regulation and seed development (Jofuku et al., 1994). They encode transcription factors which are recognized with a novel DNA binding motif named as AP2 domain. Two highly conserved sequences observed in AP2 domain are YRG element and RAYD element. YRG element is comprised of 19-22 amino acids, which is highly basic and has a conserved YRG amino acid motif. RAYD element consists of 42-43 amino acids and has a highly conserved 18-amino acid core region which is supposed to form an amphipathic α-helix (Okamuro et al., 1997). Function of YRG element is considered to be DNA binding, whereas RAYD element might be capable of mediating the association between protein and DNA (Okamuro et al., 1997).

ERF domain is very similar to AP2 domain. The highly conserved motif was found existing in four DNA-binding proteins, ERF1, ERF2, ERF3 and ERF4 in the earlier studies. In fact, both AP2 and ERF domain are believed to function in binding specifically to both GCC-box and DRE cis-acting element in the promoter sequence of ethylene responsive genes (Ohme-Takagi and Shinshi, 1995; Cheng et al., 2013). The structure of AP2/ERF domain consists of three anti-parallel β-strands and one α-helix, with the β-strands binding to the GCC-box in a major groove of the double helix. (Allen et al., 1998).

The AP2/ERF transcription factors (AP2/ERFs) regulate the expression of genes which participate in various biological processes, such as growth, development, stress responses and hormone by controlling both transcription and post-transcription through a variety of mechanisms (Dietz et al., 2010; Gibbs et al., 2015). These AP2/ERFs

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proteins have also been considered to be regulated by phytohormones for improving plant survival under stress conditions. For these transcription factors to regulate their downstream genes, they prefer binding to several conserved DNA sequences (Nakano et al., 2006). Moreover, the preferences are similar among species including Arabidopsis, tobacco (Park et al., 2001), maize (Liu et al., 2013), wheat (Gao et al., 2018) and rice (Wan et al., 2011).

1.3 The background of ERF1

ERF1 is a well-known ethylene-responsive transcription factor which plays an important role in ethylene signaling. It is directly mediated by ETHYLENE-INSENSITIVE 3 (EIN3) (Solano et al., 1998) and functions as a key integrator of ethylene (ET) and jasmonic acid (JA) pathways and participates in the regulation of defense response genes, such as *BASIC-CHITINASE* and *PLANT DEFENSIN 1.2 (PDF1.2)* (Lorenzo et al., 2003). ERF1 also targets several stress-associated genes under diverse abiotic stimulation such as salt, drought and heat stress (Cheng et al., 2013). In addition to its vital roles in various stress responses, ERF1 also regulates ethylene-related developmental processes. In the root development, ERF1 functions as an ethylene-induced repressor via directly modulating ANTHRANILATE SYNTHASE α1 (ASA1) expression (Mao et al., 2016).

Like other AP2/ERF transcription factors, ERF1 possesses a conserved DNA-binding domain that consists of approximately 60 amino acids (Ohme-Takagi and Shinshi, 1995), which forms an interface of one α-helix and three anti-parallel β-strands that binds to both GCC-box and DRE (Wang et al., 2009; Cheng et al., 2013). The

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AP2/ERF domain interacts with GCC-box through seven amino acid residues in β-strands including Arg29, Arg31, W33, Glu39, Arg41, Arg49 and Trp51 (Allen et al., 1998; Wang et al., 2009).

In other plant species, the homologs of ERF1 also play vital roles in both biotic and abiotic stress responses. In *Lotus japonicus*, the closest homolog of Arabidopsis *ERF1* acts as a positive regulator in the early process of *Mesorhizobium loti* nodulation (Asamizu et al., 2008). *In* wheat (*Triticum aestivum* L.), TaERF1 not only is induced by drought, salt, cold stresses, and phytohormones such as ET, salicylic acid (SA) and exogenous ABA, but also participates in regulating the infection of *Bluemeria graminis f. sp. Tritici* (Xu et al., 2007). Furthermore, TaERF1 directly binds to both GCC-box and CRT/DRE element in order to upregulate the expression of *PATHOGENESIS RELATED* (*PR*) and *COLD-REGULATED/RESPONSIVE TO HYDRATION* (*COR/RD*) genes. Therefore, *TaERF1* gene functions as an important member for biotic and abiotic signaling pathways and integrates various stress signals via encoding a multifunctional factor (Xu et al., 2007).

Our previous study reported that the expression of Arabidopsis *ERF1* is rapidly induced by high salinity and drought treatment (Cheng et al., 2013). Moreover, transgenic plants overexpressing *ERF1* exhibits a more tolerant phenotype in drought, salt and heat stress compared to wild-type plants (WT). Cheng et al. also reported that ERF1 regulates different sets of stress-related genes under specific stress signals through unknown mechanism (Cheng et al., 2013). One possible mechanism may be the involvement of the mediator complex that was reported by Çevik et al. (2012). MEDIATOR 25 interacts with both ERF1 and MYC2 to form a mediator complex that functions as an integrative hub by regulating both ET- and JA- associated gene expression. The study by Cheng et al. suggests a new role of ERF1 in abiotic stress

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response in addition to its well-known role in biotic stress.

Besides the correlation in the stress signaling pathway, ERF1 is also reported to be regulated by light signaling. ERF1 protein is stable under light and becomes labile in the dark condition (Zhong et al., 2014; Cheng et al., 2017). Cheng et al. reported that an E2 ubiquitin ligase, UBIQUITIN-CONJUGATING ENZYME 18 (UBC18) mediates the ubiquitination of ERF1 and regulates its protein stability (Cheng et al., 2017).

1.4 Post-translational modification-ubiquitination

Post-translational modification (PTM) of protein is one of the most rapid and earliest plant responses during environmental changes, so understanding the mechanisms and dynamics of PTM is very important in plant science. Ubiquitination is one of the most investigated PTM, which is involved in the regulation of various aspects in plants including abiotic stress, immunity, and hormone signaling (Lee and Kim, 2011; Marino et al., 2012; Kelley and Estelle, 2012). Ubiquitination mainly regulates these physiological functions by mediating protein stability. The molecular mechanism of ubiquitination is that the ubiquitin thioester bond is transferred from E1 activating enzyme to E2 conjugating enzyme with a cysteine residue in the ubiquitin conjugating domain (Ramadan et al., 2015). Depending on the substrate specificity, E3 ligases will recruit the relating-conjugated E2 enzyme and substrate to mark the ubiquitin onto target protein (Iconomou and Saunders, 2016).

Ubiquitin is a highly conserved protein which is similar to the other kingdoms of eukaryotic organisms. It contains seven lysine residues which could attach multiple itself in a process known as polyubiquitination (Yau and Rape, 2016). Meanwhile,

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there's also another form called monoubiquitination that mostly functions as a traffic signal for the target protein transporting into vacuoles or lysosomes that sometimes results in degradation (Hicke et al., 2003; Schwihla and Korbei, 2020).

The most well-known characteristic of ubiquitination is modulating proteins via marking them with ubiquitin for degradation by 26S proteasome. Over the last two decades, ubiquitin-26S proteosome system (UPS) has drawn the plant research community's attentions due to its important role in biotic and abiotic stress responses. The UPS optionally select the key components for degradation in response to a given stimulus of stress, and acts as positive or negative regulators in plant hormones synthesis, gene expressions, protein interactions and many other physiological functions.

The different types of poly-ubiquitinated proteins are often categorized by selective degradation, which can decide the destiny of the protein (Yau and Rape, 2016). Nevertheless, the major specificity of the UPS selection is dedicated to the large group of ubiquitin E3 ligases. Based on the structural compositions and the conjugating process of activated ubiquitin moieties, the plant's E3 ligases can be classified into three primary groups, including HOMOLOGOUS TO THE E6-AP CARBOXYL TERMINUS (HECT), PLANT U-BOX (PUB) and REALLY INTERESTING NEW GENE (RING) (Azevedo et al., 2001; Mazzucotelli et al., 2006; Metzger et al., 2012).

Among them, RING E3 ligases are considered to be one of the most complicated and interesting groups of protein encoded by plant genomes, due to the composition of both mono-subunit and multi-subunit E3 enzymes. For the mono-subunit ligase, it possesses a RING motif for the interaction between E2 conjugating enzyme and its substrate. However, the multi-subunit ligase can be further divided into two groups, including cullin-RING ubiquitin ligase (CRL) (Guo et al., 2013) and

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anaphase-promoting complexes (APCs) (Heyman and De Veylder, 2012). Moreover, CRL E3 ligases can also be classified into three main subgroups according to the substrate receptors: (1) SKP1-CUL1-F-box (SCF) complexes which acts as the substrate receptors by F-box proteins (Zheng et al., 2002), (2) Broad-complex, Tramtrack, and Bric-à-brac (BTB)-Cul3a/b complexes that recognize substrates with its BTB proteins (Chaharbakhshi and Jennifer, 2016), and (3) DDB1-binding/WD40-Cul4 complexes that directly ubiquitinate the substrates by the DDB1 binding/WD40 proteins (Hua and Vierstra, 2011).

CONSTITUTIVE PHOTOMORPHOGENIC 1(COP1) functions as a master negative regulator of photomorphogenesis in light signaling (Deng et al., 1991). It is considered to be an E3 ligase that belongs to the DDB1-binding/WD40-Cul4 complexes (Chen et al., 2010), and promotes the degradation of multiple substrates to proteasome (Lau and Deng, 2012; Han et al., 2020). In particular, COP1's E3 ligase activity is decided by the interaction between SUPPRESSOR OF PHYA-105 (SPA) proteins (Hoecker and Quail, 2001; Zhu et al., 2015). Under light exposure, photoreceptors co-localize with SPA1 and prevent the interaction with COP1 to the COP1/SPA complex for inhibiting COP1 (Lu et al., 2015; Sheerin et al., 2015). Nevertheless, the COP1/SPA complex facilitates HY5 or other positive transcription factors degradation in order to depress photomorphogenesis in the nucleus in the dark (Hoecker, 2017). In general, COP1 is consumed from nucleus in the light but displays abundant in nucleus during darkness (Subramanian et al., 2004; Pacín et al., 2014).

1.5 Post-translational modification-SUMOylation

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Another PTM which we investigated in this study is SUMOylation, conjugating the molecules of Small Ubiquitin-related Modifier (SUMO) onto substrate proteins. In plant cells, SUMOylation is an important PTM that mediates protein stability, interaction and subcellular localization of transcription factors (Han et al., 2021). This type of PTM regulates the covalent binding of SUMO molecules through the C-terminal glycine residues onto the accessible lysine residues of protein substrates (Pichler et al., 2017). SUMOylation is driven by E1-activating enzyme complex with ATP and the activated SUMO protein is transferred to E2-conjugating enzyme then finally facilitated to substrates via an E3 ligase. Some substrates might also be poly-SUMOylated which is assisted by E4 ligases such as (PROTEIN INHIBITOR OF ACTIVATED STAT LIKE 1) PIAL1 and PIAL2. On the other hand, SUMO can be released from targeted substrate with isopeptide bond cleavage by SUMO-specific protease in order to act as a switch for substrate protein and to uptake SUMO for the subsequent cycles (Yates et al., 2016).

So far, the most identified SUMOylation targets are found locating in the nucleus in plants, indicating that this modification predominantly occurs in the nucleus. The transcription factor complex formation mediated by SUMOylation was reported to participate in modulating interaction with regulatory factors in many signaling pathways including hormone signaling. For instance, the protein stability of RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1B)-a negative regulator in ABA response, is improved by SUMO conjugation and its interaction with RAP2.6, an AP2/ERF family protein, was strengthened through altering its DNA elements affinity (Guo and Sun, 2017).

Since SUMOylation is one kind of ubiquitin-like modification and is similar to ubiquitination in enzyme cascades, they compete on the same lysine residues in some

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cases. Previous studies have suggested that SUMO might serve as an antagonist against ubiquitination by increasing the protein stability during transcriptional regulation. For instance, DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A (DREB2A) and HEAT-SHOCK TRANSCRIPTION FACTOR A2 (HSFA2) are SUMOylated under heat stress condition. However, under normal condition, DREB2A interacts with a complex of ubiquitin ligase, BTB/POZ AND MATH DOMAIN PROTIENS (BPMs), and is targeted for degradation through 26S proteasome. In this case, heat stress leads the SUMOylation of DREB2A, which inhibits the interaction with BPM2 for the increased protein stability (Wang et al., 2020). Similarly, BASIC-REGION LEUCINE ZIPPER 23 (bZIP23) is a drought-responsive transcription factor that can be SUMOylated in rice. bZIP23 increases its protein stability through SUMOylation while the SUMO protease OVERLY TOLERANT TO SALT 1 (OTS1) promotes its degradation by reducing the SUMOylation level (Srivastava et al., 2017).

SUMOylation and ubiquitination often happen on the same transcription factor, thus regulating the protein stability or activities cooperatively or competitively. The level of different modifications can be dynamic under some specific conditions during stress responses or hormonal signal transduction. For example, the ABA-related bZIP transcription factor, ABA INSENSITIVE 5 (ABI5), is ubiquitinated for degradation (Liu and Stone, 2010) but is protected by SAP AND MIZ DOMAIN-CONTAINING LIGASE 1 (SIZI) through SUMOylation (Miura et al., 2009). Another example participating in cold stress signaling is INDUCER OF CBF EXPRESSION 1 (ICE1). ICE1 protein is stabilized by SIZI while degraded by HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1)-mediated ubiquitination. SUMOylation improves the transcription regulation of ICE1 in cold tolerance (Miura et al., 2007; Miura and Hasegawa, 2010).

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SUMOylation not only is involved in stress responses, but is also involved in many biological processes including photomorphogenesis. COP1 mediates the degradation of ELONGATED HYPOCOTYL 5 (HY5), a crucial transcription factor in light signal transduction, but SIZI binds to COP1 and mediates its SUMOylation in nuclear speckles in order to improve COP1 activity in plant cells. The stabilized COP1 then degrades HY5 and inhibits photomorphogenesis in the dark (Lin et al., 2016).

1.6 Experimental strategy and goals

In this study, our goal is to understand the mechanisms that mediate ERF1 protein stability under light-dark cycle and stress responses. To understand whether ERF1 is mediated by both SUMOylation and ubiquitination, we investigated the physical interaction of ERF1 with the SUMO E2 enzyme, SCE1, and the ubiquitin E3 ligase COP1 by performing both *in vitro* and *in vivo* Co-IP assays. We also employed both gain-of-function and loss-of-function analyses to demonstrate whether ERF1 protein stability could be regulated by both SCE1 and COP1 under light-dark cycle. More importantly, we want to understand whether ERF1 could be SUMOylated and ubiquitinated by SCE1 and COP1, respectively. Moreover, we observed the phenotype under salt and drought stress and analyzed the downstream gene expression of *ERF1* to verify whether SCE1 participates in stress signals through ERF1. Taken together, our study extends the knowledge of mechanism that regulates ERF1's stability under light-dark cycles and modulates stress responses in the future.

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Chapter 2 Materials and Methods

2.1 Plant materials

2.1.1 Arabidopsis wild type (WT)

The ecotype of *Arabidopsis thaliana* we used as wild type is Columbia (Col-0).

2.1.2 *sce1* mutants

The two SALK T-DNA insertion lines of *SCE1* were named as *sce1-4* (SALK_066164) and *sce1-7* (SALK_022200) according to Saracco et al., 2007, were obtained from ABRC (Arabidopsis Biological Resource Center). The genotypes of mutants were screened by polymerase chain reaction (PCR) of genomic DNA with gene-specific primers to map the T-DNA insertion sites. For analysis of *sce1-4*, the following forward and reverse primers were used: LP1, 5'-TCCACGGCTCTATGTGTAAGC-3' and RP1, 5'-TTTCCGACCATTCTGTTTGAC-3. For analysis of *sce1-7*, the following forward and reverse primers were used: LP2, 5'-AATCTTCCACGGCTCTATGTG-3' and RP2, 5'-AGTAAATGGGCCCAGATATGG-3. The gene-specific primers above were used to perform PCR with LBb1.3 primer (5'-ATTTTGCCGATTTCGGAAC-3') for homozygous line selection. The resulting PCR fragments were sequenced to map the T-DNA insertion site.

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2.1.3 *ERF1* overexpression transgenic lines

The *ERF1*-overexpressing transgenic lines were generated in Col-0, *sce1-4*, *sce1-7* and *cop1-6* backgrounds. *ERF1* coding sequence was constructed into pEarlyGate103 vector and then was transformed into *Agrobacterium* GV3101. The floral dipping method was used to transform the plants through the integration of the transferred strand into the host chromosome randomly (Nester, 2015). The T_1 plants were screened by herbicide Basta spraying. The survived plants were then analyzed by PCR using genomic DNA with gene- and vector-specific primers for the T-DNA insertion of *ERF1-GFP*. The following forward and reverse primers were used: F, 5'-GGCTTTAGCCTACGATCAAGCTGCTTT-3' and R, 5'- ACGTATCCCTCAGGCAT GGCG-3'. The T_2 plants were again screened on 1/2 Murashige and Skoog (MS) plates which contains 20 μM Basta for homozygous lines. The total protein extracts from homozygous lines were checked by Western blot for ERF1 protein expression. The T_3 plants were used for experiments. The $ERF1^{4KR}$-overexpressing transgenic were generated in Col-0 background and were screened by the same way.

The *ERF1* gene expression of each transgenic lines were analyzed by quantitative RT-PCR and were used in the experiments based on the fairly *ERF1* overexpression (Appendix 1).

2.2 Plant growth conditions

The plants were grown in 16: 8 h, light: dark photoperiod at 22℃. For normal growth conditions, seeds were surface-sterilized with 20% bleach containing 0.1%

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Tween 20 for 10 min, and germinated on 1/2 MS medium (pH 5.7) with 1% phytoagar. Seedlings were grown under a 16: 8 h, light: dark photoperiod at 22°C at a light intensity of 100-150 μmol m^{-2} s^{-1}.

2.3 Methods

2.3.1 *In Vitro* Co-immunoprecipitation Assays

For *in vitro* co-immunoprecipitation assay, Myc-SCE1 was used as bait protein to precipitate GST-ERF1. Bacterial extracts expressing GST-ERF1 were purified with glutathione agarose resin (Thermo Fisher, Cat. No: 16101) as described in the manufacturer's protocol. Cell lysates of Myc-SCE1 from tobacco leaves were mixed with 25 μl Dynabeads Protein A (Invitrogen, Cat. No: 10001D) which conjugated with 2 μg anti-myc (Sigma, Cat. No: C3956), and incubated with GST-ERF1 for 2 h at 4°C. The samples were boiled and analyzed by Western blot onto PVDF membrane. Anti-c-myc antibody (1:1000 dilution; Millipore) and anti-GST-HRP conjugation (1:10000 dilution; Cytiva) were used to detect bait and prey proteins.

For another *in vitro* co-immunoprecipitation assays, tobacco leaves were co-transfected with *Agrobacterium* GV3101 that contained ERF1-GFP and Myc-SCE1 which were constructed in pEarlyGate103 and pEarlyGate203 respectively. The plants were collected with 4 h illumination after 48 h incubation in normal growth condition. Total proteins were extracted with IP buffer and cell lysates were incubated with 15 μl Dynabeads Protein A which was bound with 0.1 μg anti-GFP antibody (Abcam, Cat. No: ab6556), and incubated for 2 h at 4°C. The immuno-precipitated proteins were

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denatured with 1x SDS loading buffer and boiled for 10 min. Samples were loaded on an 12% SDS–PAGE gel, blotted onto PVDF membranes and probed with anti-c-myc (1:1000 dilution; Millipore) and anti-GFP (1:200 dilution; Santa Cruz) antibodies.

2.3.2 Genomic DNA extraction from Arabidopsis

Leaves of 3-w-old plants were homogenized and suspended with 330 μl extraction buffer (200 mM Tris-Cl at pH 8.0, 250 mM NaCl, 25 mM EDTA and 0.5% SDS). Samples were centrifuged at 16,000 x g for 5 min. Three-hundred μl supernatant was collected and mixed with 300 μl isopropanol gently. The mixture was frozen in -20°C for an hour in order to precipitate the genomic DNA. Samples were then centrifuged at 16,000 x g for 10 min and the supernatant was decanted. Five-hundred μl 75% ethanol was added for washing the salt away. Next, the samples were centrifuged at 16,000 x g for another 5 min. After removing the ethanol, the pellet was dried and dissolved with 30 μl sterilized water.

2.3.3 Relative gene expression

2.3.3.1 RNA isolation from Arabidopsis

Ten-d-old seedlings were homogenized and suspended with 1 ml TRI reagent® (Molecular Research Center, Inc.) vigorously. After standing for 10 min, the samples were added with 200 μl chloroform and vortexed for 10 sec. After standing for another 5 min, and the samples were centrifuged at 16,000 x g for 10 min. Five-hundred μl of

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the aqueous phase on the top layer was collected carefully and mixed gently with the same volume of isopropanol. After standing for 10 min, the samples were centrifuged at 16,000 x g for 10 min in 4°C. The supernatant was then discarded and 1 ml 75% ethanol was added to remove the salt. Next, the samples were centrifuged and supernatant removed before the pellet was dried. The pellet was dissolved with 30 μl DEPC (diethyl pyrocarbonate) -treated water when becoming translucent. RNA concentration was measured using NanoDrop spectrophotometer for further analyses (NanoDrop, ND-1000).

2.3.3.2 cDNA synthesis

The reverse transcription was performed using HiScript II One Step RT-PCR Kit (Vazyme). Three μg RNA was added for one reaction together with 4x gDNA wiper Mix. After a 2 min incubation in 42 °C, 5x HiScript II qRT SuperMix II was further added into the samples for reverse transcription and incubated for 15 min in 50°C. Due to the reaction volume (10 μl), About 300 ng/μl cDNA was obtained in 10 μl reaction volume. The resulted cDNA is then ready for the use in qPCR.

2.3.3.3 Real-time PCR

The cDNA samples were diluted into 30 ng/μl for the template added in qRT-PCR. The qRT-PCR was performed with iQ™ SYBR® Green Supermix (Bio-Rad) according to the manufacturer's instructions and the reagents were added in the following figure.

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The reagent components of qPCR

Reagent	Volume
iQ™ SYBR® Green Supermix (2x)	10 µl
Forward primer	0.6 µl
Reverse primer	0.6 µl
cDNA template	1 µl
ddH_2O	7.8 µl
Total	**20 µl**

For the RT-PCR, gene-specific primers listed in Table 1 were used to detect mRNA levels. Quantitative PCR was conducted using CFX96 Real-Time System (Bio-Rad). UBQ10 (At4g05320) or PP2A (At1g13320) was used as an internal control for normalization of the expression data. The programs which were used was shown in the following figure.

The programs of qPCR

Step 1	95°C	3 min
Step 2 (40 cycles)	95°C	10 sec
	55°C	30 sec
Step 3	95°C	10 sec
Step 4	65°C	5 sec

2.3.4 Protein extraction and western blot analysis

For individual sample, total protein was extracted in IP buffer containing 50 mM Tris-Cl at pH 8.0, 150 mM NaCl, 0.1% Tergitol, 10% Glycerol, 1 mM PMSF, 0.05 mM Bortizomib, 5 mM DTT and 1x complete protease inhibitor (Roche). Samples were

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centrifuged at 12,000 x g for 10 min at 4°C. The resulting supernatant was then boiled for 10 min with 6x SDS buffer. Forty μl of supernatant of individual samples was loaded on an 10% SDS–PAGE gels. The total protein was blotted onto PVDF membranes and probed with anti-GFP (1:500 dilution; Santa Cruz) or anti-SCE1 (1:3000 dilution; Agrisera). The blots were stripped and re-probed with anti-RPT5 (1:6000 dilution; ENZO Life Sciences) and anti-TUB (1:5000 dilution; Sigma) as loading control.

2.3.5 Bimolecular fluorescence complementation (BiFC)

2.3.5.1 Construction and preparation of plasmids

ERF1 full-length coding sequence was cloned into pEarlyGate202-cYFP vector and SCE1 full-length coding sequence was cloned into the pEarlyGate201-nYFP vector. The plasmid extraction was performed by Presto^{TM} Mini Plasmid Kit (Geneaid) according to the manufacturer's instructions.

2.3.5.2 Protoplast isolation and plasmid transformation

Three-w-old Arabidopsis were used for the experiment. The well-expanded leaves were chosen for cutting 0.5-1 mm leaf strips from the middle leaf vein without crushing the tissue. The leaf strips were immediately transferred into 30 ml enzyme solution (0.4 M mannitol, 20 mM KCl, 20 mM MES (pH 5.7), 1% cellulase R10, 0.25% macerozyme R10, 10 mM $CaCl_2$, 5 mM β-ME and 0.1% BSA) and under 30-35 rpm shaking for 3 h until the solution turned green. After enzyme digestion, the solution was filtered

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through a 100 μM nylon mesh gently. The released protoplasts were counted under microscope using hemacytometer. The protoplasts were collected by centrifugation at 100 x g for 3 min in a round-bottomed tube, and the supernatant was removed as much as possible. The protoplasts were then washed by resuspension with pre-cooled W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES (pH 5.7) and 5 mM glucose) and the washing step was repeated for another 2 times. The protoplasts were chilled on ice for 30 min and diluted them into 2×10^5/ml with W5 solution. Next, the solution was replaced with 200 μl MMg solution (0.4 M mannitol, 15 mM $MgCl_2$ and 4 mM MES (pH 5.7)) by spinning down and resuspending the protoplasts. The prepared YN and YC plasmids were added equally for 10-20 μg (up to 20 μl) into protoplasts that contained MMg solution and mixed with 200 μl PEG solution (40% PEG 4000, 0.2 M mannitol and 0.1 M $CaCl_2$). After incubating on ice for 10 min, the transformed protoplasts were washed with 800 ml W5 solution for 3 times. Ultimately, the protoplasts were resuspended in 1 ml W5 solution and were transferred into a 6-well plate which was pre-coated with 1% BSA. The protoplasts were incubated at room temperature for 12-16 h before detecting the cellular images of yellow fluorescent protein (YFP) fluorescence with a laser scanning fluorescence microscope (Olympus, BX53, 492 Taipei, Taiwan).

2.3.6 *In vitro* SUMOylation and Ubiquitination

2.3.6.1 Protein purification

The coding sequence of ERF1 was PCR-amplified and ligated into pGEX-4T-1.

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The constructed plasmid was then transformed into *Escherichia coli* BL21 (DE3) competent cells. The transformed *E. coli* cells were grown in 600 ml LB to OD 0.6. Then 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used for protein induction at 16℃ for 16 h. The cells were centrifuged at 8,000g for 15 min and resuspended with 25 ml binding buffer (20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 0.1% Tergitol, 1 mM PMSF, 5 mM DTT and 1x complete protease inhibitor (Roche)). The cells were lysed by sonication using a typical sonication cycle (Total time: 4 min., 15 sec ‘‘on’’, 45 sec ‘‘off’’), and centrifuged at 12000g for 15 min in order to separate the cell debris and proteins. GST-ERF1 was purified from total extracts using 200 μl Pierce™ Glutathione Agarose (Thermo) through 2 h incubation at 4℃. The agarose resin was precipitated by centrifugation at 700g for 2 min and washed for 3 times with 1 ml binding buffer. Then 100 μl elution buffer (50 mM Tris-Cl, 150 mM NaCl and 10 mM reduced glutathione, pH 8.0) was added and mixed gently among the resin. The agarose was kept on ice for 10 min and the supernatant which contained GST-ERF1 was collected. The elution step was repeated for another 2 times and then SDS-PAGE analysis was performed to measure the protein amount of GST-ERF1.

For MBP-SCE1 and MBP-COP1, the coding sequences were respectively PCR amplified and constructed into pMAL-p4x-1 and pVP13. The protein production and extraction methods were similar as described above, only Amylose Resin (New England BioLabs) and 10 mM maltose were used for MBP protein purification.

2.3.6.2 *In vitro* SUMOylation assay

In brief, 8 μg of His-SUMO1 (UL-715; Boston Biochem), 100 ng of E1 (SAE1&UBA2, E-315; Boston Biochem), 500 ng of MBP-SCE1 and 40 ng of

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GST-ERF1 were incubated in reaction buffer (20 mM HEPES at pH 7.5, 5 mM $MgCl_2$ and 2 mM ATP) at 30℃ for 3 h. The western blot was measured with anti-His (1:8000 dilution; Bioman) and anti-GST-HRP conjugation (1:10000 dilution; Cytiva).

2.3.6.3 *In vitro* ubiquitination assay

The ubiquitination assay was performed as described previously (Saijo et al., 2003) with minor modifications. 5 μg of FLAG-ubiquitin (U-120; Boston Biochem), 25 ng of E1 (UBE1, E-305; Boston Biochem), 25 ng of E2 (UbcH5b/UBE2D2, E2-622; Boston Biochem), 500 ng of MBP-COP1 and 40 ng of GST-ERF1 were incubated in reaction buffer (50 mM Tris-Cl at pH 7.5, 5 mM $MgCl_2$, 2 mM ATP and 2 mM DTT) at 30℃ for 2 h. MBP-COP1 was pretreated with 20 mM $ZnCl_2$ for 45 min at 22℃ before adding to the reaction. Western blot analysis was performed with anti-FLAG (1:200 dilution; Santa Cruz) and anti-GST-HRP (1:10000 dilution; Cytiva) detection.

2.3.7 Salt stress tolerance test

Seven-d-old seedlings were grown on 1/2 MS medium under normal condition and were transferred to 150 mM NaCl containing plate. After 10 d of salt stress, seedlings were observed and measured for the survival rate and lateral root numbers. The survival rate was calculated by dividing the percentage of green leaf number by the percentage of total leaf number. For the lateral root numbers, the roots which extend horizontally from the primary roots were calculated.

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Chapter 3 Results

3.1 ERF1 interacts with SCE1 *in vitro*

In our previous study, SCE1 was identified as an interacting partner of ERF1 through yeast two-hybrid and BiFC analysis (Appendix 2). To prevent the false positive signal of yeast-two-hybrid and further investigate the relationship between ERF1 and SCE1, we performed *in vitro* co-immunoprecipitation (Co-IP) assays to confirm their protein-protein interaction. For *in vitro* Co-IP assay, we conducted a semi-*in vitro* Co-IP assay in which the ERF1 fusion protein was expressed in *E. coli* and SCE1 fusion protein was expressed in tobacco leaves. We first cloned the coding sequences of *ERF1* and *SCE1* into pGEX-4T-1 and pEarlyGate203, respectively. Myc-SCE1 fusion protein was first purified from tobacco leaves using α-myc antibody and then incubated with purified GST-ERF1 protein which is expressed from *E. coli*. The semi-*in vitro* Co-IP assay shows that GST-ERF1 could be immunoprecipitated by Myc-SCE1 (Fig. 1). Furthermore, *another* Co-IP assay is conducted by expressing both ERF1-GFP and Myc-SCE1 proteins in tobacco leaves. ERF1-GFP fusion protein was immunoprecipitated using α-GFP antibody. The result also shows that ERF1-GFP could interact with Myc-SCE1 (Fig. 2).

3.2 Screening of the T-DNA insertion mutants of *SCE1*

To understand whether SCE1 functions in abiotic stress response, we first obtained *SCE1* T-DNA insertion lines from Arabidopsis Biological Resource Center, Ohio States

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University. The two SALK T-DNA insertion lines of *SCE1* were named as *sce1-4* (SALK_066164) and *sce1-7* (SALK_022200) according to Saracco et al., 2007. The gene-specific primers were performed and the resulting PCR fragments were sequenced in order to map the T-DNA insertion site. After analyzing the sequenced-PCR amplified products, we found that the T-DNA insertions both happened on *SCE1* promoter in *sce1-4* and *sce1-7* (Fig. 3A).

The *SCE1* gene expression in the mutant lines were further verified using quantitative real-time PCR. Seven-day-old seedlings grown under normal condition were used for total RNA extraction. As shown in Figure 3B, both *sce1-4* and *sce1-7* showed down-regulation of *SCE1* expression.

3.3 SCE1 facilitates ERF1 protein stabilization

To further investigate the biological function of SCE1-ERF1 interaction, we examined whether ERF1 protein level is altered in *SCE1* knockdown mutants. *35S:ERF1-GFP-His* overexpression lines (*ERF1OE*) were generated in wild-type (WT), *sce1-4* and *sce1-7* backgrounds. Four-d-old etiolated seedlings were first illuminated for 4 h (L) and incubated in the dark for another 2 h (D). As shown in Figure 4, ERF1 became unstable after 4 h dark incubation in all backgrounds. However, ERF1 is even less stable both under light and dark condition in the two *SCE1* knockdown mutants, suggesting that ERF1 protein stability is positively regulated by SCE1.

3.4 SCE1 facilitates the SUMOylation of ERF1 in the light

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Next, since SCE1 is an important E2 ligase which mediates SUMOylation process, we asked whether SCE1 mediates ERF1 stability via SUMOylation. We first immunoprecipitated the ERF1-GFP fusion protein with GFP antibody from 4-d-old etiolated seedlings that were either illuminated for 8 h (L) or incubated in the dark (D). We found a strong SUMO1 signal that conjugated to ERF1 in the light-treated sample, whereas in the dark-treated sample, SUMOylation strongly decreased (Fig. 5). Furthermore, the *sce1* mutants showed less SUMOylation of ERF1 compared with WT under light condition (Fig. 5). These results suggest that SCE1 modulates ERF1 SUMOylation in a light-dependent manner. However, the data showed the ERF1 SUMOylation also happens in the dark but plays a dominant role in the light.

3.5 ERF1 shows stronger interaction activity with SCE1 in the light

Since our data showed that ERF1 SUMOylation is regulated by SCE1 in a light-dependent manner, we want to confirm whether ERF1 also displays stronger interaction with SCE1 in the light. The bimolecular fluorescence complementation (BiFC) system was conducted to investigate their interactions in the plant cells. Full-length coding sequences of SCE1 and ERF1 were respectively fused with N terminal of the yellow fluorescent protein (1-155, YN) and C terminal of YFP (156-239, YC). Two plasmids were then co-transformed into Arabidopsis protoplasts and the YFP fluorescence signals were further detected. The results showed that ERF1 mainly interacts with SCE1 under the light condition (Fig. 6). In the transformed protoplast incubated in the dark for 2 h (D), SCE1-YN and ERF1-YC failed to yield YFP signal

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(Fig 6). In summary, SCE1 interacts with ERF1 mainly in the light condition and both ERF1 and SCE1 co-localized in the nucleus of plant cell.

3.6 COP1 promotes ERF1 protein degradation

Since COP1 is an E3 ubiquitin ligase, which is a master negative regulator of photomorphogenesis by targeting many transcription factors. Thus, we first verified the interaction between COP1 and ERF1 through pull-down and Co-IP assay (Appendix 3). Next, we wondered whether COP1 also mediates ERF1 degradation under darkness. To study whether COP1 mediates ERF1 in the dark, we overexpressed ERF1-GFP in both WT and *cop1-6* mutant and compare its protein level during light to dark transition. Four-d-old etiolated seedlings were first illuminated for 4 h and then incubated in the dark for 2 and 4 h. We found that in *cop1-6* background, ERF1 maintained its protein abundance, whereas in WT background, the level of ERF1 was significantly decreased at 2h and was almost vanished at 4h under darkness (Fig. 7). This result suggested that COP1 mediates ERF1 degradation under darkness.

3.7 COP1 promotes the ubiquitination of ERF1 under darkness

To further understand whether COP1 also mediates ERF1 through ubiquitination, we performed *in vivo* ubiquitination assay under both light and dark condition. Four-d-old etiolated seedlings of *35S:ERF1-GFP/Col-0* and *35S:ERF1-GFP/cop1-6* were used for total protein isolation. After ERF1-GFP was immunoprecipitated by GFP

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antibody, ubiquitinated ERF1 was detected. The result showed that ERF1 was modified by multiple ubiquitins in the WT background. When we normalized the ubiquitination intensity with ERF1-GFP protein abundance, much less ubiquitination level was detected in *cop1-6* background (Fig. 8, left panel). We also examined the ERF1 ubiquitination level after light treatment. As shown on the right panel in Fig. 8, the ubiquitination level of ERF1 decreased after 4 h light incubation. Taken together, our data suggested that COP1 mediates ERF1 degradation via ubiquitination under darkness.

3.8 SUMOylation site analysis and purification of SUMO-site mutated ERF1

To further elucidate the regulation of ERF1 ubiquitination and SUMOylation, we analyzed the potential SUMOylation sites of ERF1 using GPS-SUMO (Zhao et al., 2014) and JASSA (http://www.jassa.fr/index.php?m=jassa). The SUMO protein target site on the substrate is reported to locate mostly on a short consensus sequence ψKXD/E (Seeler and Dejean, 2003). Four lysine residues were predicted to be the SUMOylation sites on ERF1, including K77, K177, K180 and K190 (Fig. 9).

3.9 K77, K177, K180 and K190 might be the SUMOylation sites of ERF1

In order to examine whether the four lysine residues play a role in ERF1

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SUMOylation, we carried out *in vitro* SUMOylation assay using both GST-ERF1^{WT} and GST-ERF1^{4KR} (quadruple mutant with the replacement of 4 lysine residues into arginine). The recombinant proteins were expressed from *E. coli* and co-incubated with other essential proteins that participate in SUMOylation pathway, including E1 activating enzyme SUMO-ACTIVATING ENZYME SUBUNIT 1 (SAE1) & UBIQUITIN-ACTIVATING ENZYME E1-LIKE (UBA2), E2 conjugating enzyme SCE1 and SUMO1 protein. In agreement with the *in vivo* SUMOylation experiment, both GST-ERF1 and SUMO1-conjugated ERF1 could be detected in our Western blot analyses (Fig. 10). Moreover, SUMOylated ERF1 almost vanished when using GST-ERF1^{4KR} as the substrate, suggesting that these four lysine residues might serve as the SUMOylation sites in ERF1 (Fig. 10).

3.10 Ubiquitination competes with SUMOylation on the same lysine residues of ERF1

In mammalian cells, several studies have claimed that SUMOylation and ubiquitination mostly compete on the same lysine residues and often remains opposite regulations in various kinds of transcription factors through mediating the protein stability (Desterro et al., 1998; Lamsoul et al., 2005). Since COP1 regulates ERF1 through ubiquitination and serve as an opposite role of protein stabilization against SUMOylation, we wanted to ask whether the ubiquitination sites are the same as the SUMOylated sites on ERF1. In a similar way, we conducted *in vitro* ubiquitination assay using both GST-ERF1^{WT} and GST-ERF1^{4KR}. The enzymes that the assay required were added respectively, which are E1 activating enzyme UBIQUITIN ACTIVATING

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ENZYME E1 (UBE1), E2 conjugating enzyme UBIQUITIN-CONJUGATING ENZYME H5B/UBIQUITIN CONJUGATING ENZYME E2 D2 (UbcH5b/UBE2D2), E3 ligase-COP1 and ubiquitin. Ubiquitinated ERF1 were detected using both anti-GST antibody for ERF1 and anti-Flag antibody for ubiquitin. Consistent with the result shown in Figure 8, COP1 also participates in ERF1 ubiquitination *in vitro*. Interestingly, when using GST-ERF1^{4KR} as the substrate, the ubiquitination level decreased to similar level as COP1 only, which is about the basal level of E3 ubiquitination (Fig. 11). This result indicated that K77, K177, K180 and K190 also serve as the ubiquitination sites for ERF1, suggesting that ubiquitination of ERF1 might compete with its SUMOylation. K77, K177, K180 and K190 may serve the major ubiquitination sites for COP1.

3.11 ERF1^{4KR} is more stable than ERF1^{WT} in the dark

In order to understand the ERF1^{4KR} protein stability under different light conditions in plants, we also generated *35S:ERF1^{4KR}-GFP-His/Col-0* transgenic lines. Four-d-old etiolated seedlings were first illuminated for 4 h and then incubated in the dark for 2 and 4 h. The results showed that *35S:ERF1-GFP-His/Col-0*was significantly decreased at 2 h and was almost vanished at 4 h under darkness, whereas the site-mutated ERF1 (ERF1^{4KR}) maintained its protein abundance (Fig. 12). This result suggested that despite ERF1^{4KR} was not able to be stabilized through SUMOylation, it also cannot be degraded via ubiquitination in the dark due to the same recognition lysine residues on ERF1.

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3.12 *sce1-4* and *sce1-7* mutants are more sensitive to salt stress compared to WT

Our previous study reported that ERF1 is involved in multiple abiotic stress-responsive genes regulation, and the plant overexpressing ERF1 possesses better tolerance to drought and salt stresses (Cheng et al., 2013). Since SCE1 might positively regulate ERF1's stability via SUMOylation, we wonder if SCE1 also plays a positive role in abiotic stress response. We used both *sce1-4* and *sce1-7* mutants in our salt stress tests. We observed the root elongation and lateral root numbers in response to prolonged periods of high salinity. Seedlings were grown on 1/2 MS medium for 7 d and then transplanted to 150 mM NaCl containing plate. After 10 d of salt stress, WT had more lateral root and survived better than *sce1* mutants, suggesting that SCE1 positively regulates salt stress response (Fig. 13). We also conducted drought and salt stress tests for plants grown in the soil. As shown in appendix 4, *sce1-4* and *sce1-7* exhibited a more sensitive phenotype in response to both drought and high salinity stress. These results suggest that SCE1 positively regulates drought and salt stress responses.

3.13 SCE1 mediates the ERF1 downstream gene expression

To further verify whether SCE1 regulates abiotic stress responses through ERF1 signaling, we examined whether SCE1 affects the expression of ERF1's downstream genes. We selected two of the downstream genes that are representative in salt stress, including *DELTA-1-PYRROLINE-5-CARBOXYLATE SYNTHASE* (*P5CS1*) and

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OSMOTIN 34 (*OSM34*). To examine their gene expression, WT, *ERF1OE*, *sce1* mutants and *ERF1OE/sce1-7* were grown for 14 d under normal condition. The samples were collected after dark incubation for different time periods. The results showed that the expressions of *P5CS1* and *OSM34* gradually decreased in every line since ERF1 is degraded under darkness. Notably, *P5CS1* and *OSM34* gene expression are significantly enhanced in ERF1 overexpression line but decreased to basal level in *ERF1OE/sce1-7* plants (Fig. 14). However, in *sce1-7* mutant background, the expression level of *P5CS1* and *OSM34* were not altered compared with those in the WT background, suggesting that these two genes might be regulated by other components in *sce1* mutants. Our data suggest a role of SCE1 in stress response by regulating some but not all ERF1's downstream genes expressions.

3.14 Protein level of SCE1 is regulated under light/dark cycle and ACC treatment

To examine whether there are other pathways regulating the protein stability of SCE1 under light/dark cycle and ethylene signaling, we detected SCE1 protein level under different conditions. As shown in Figure 15, SCE1 became less stable when transferring from light to dark. We further tested whether ethylene treatment would also affect the stability of SCE1, since ERF1 is mainly induce by ethylene. We treated WT plants with ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) for different time periods. SCE1 remained about the same after ACC treatment (Fig. 15). These data suggest that SCE1 protein level is regulated through light signaling.

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Chapter 4 Discussion

4.1 SCE1 interacts with ERF1 in the light but not in the dark

Using both *in vitro* and *in vivo* Co-IP assays, we have demonstrated that SCE1 could physically interact with ERF1 (Fig. 1 and 2). The evidence that they interact *in planta* strongly suggests that ERF1 might be a target substrate of SUMOylation by SCE1. We also provide evidence that SCE1 mediates the SUMOylation of ERF1 in the light, whereas COP1 mediates the ubiquitination of ERF1 in the dark, thereby promoting ERF1 fluctuation under light/dark cycle (Fig. 5 and 8). According to our preliminary data (Appendix 5), SCE1 localized both in the cytosol and in the nucleus in a plant cell. However, whether SCE1 could shuttle between cytosol and nucleus similarly to COP1 is still unknown. Since ERF1 is a transcription factor and is reported to localize in the nucleus, it is expected that SCE1 might interact with ERF1 in the nucleus. To further understand whether these PTM changes are resulted from changes in physical interaction, we performed BiFC assay to examine the SCE1-ERF1 interaction under both light and dark conditions. As shown in Fig. 6, the combination of SCE1-YN and ERF1-YC gives YFP signals in the nucleus only in the light condition, while no YFP signal was observed in the dark condition. This result suggests that SCE1 interacts with ERF1 in the light but not in the dark. There are three possible explanations for this phenomenon: (1) SCE1 might shuttle to the cytosol under dark condition, (2) SCE1 protein might be regulated by other components and becomes unstable in the dark, (3) COP1 competes with SCE1 to interact with ERF1 in the nucleus under dark condition.

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We have examined the protein stability of SCE1 in the light-to-dark condition. As shown in Fig. 15, SCE1 slightly became unstable when we transferred the seedlings from light to dark for up to 4 h. This indicates that other components are involved in regulating SCE1's protein stability. Further investigations, such as subcellular localization and *in vivo* Co-IP assay are needed to elucidate the underlying mechanism.

4.2 SCE1 and COP1 mediate ERF1 stability under light/dark cycle

Protein SUMOylation can be accomplished either in an E3-dependent or an E3-independent manner (Knipscheer et al., 2008). In 2017, Guo and Sun reported that RACK1B could be sufficiently SUMOylated by E1 (SUMO-activating enzyme) and SCE1 without an E3 ligase. ERF1 might also be stabilized under light condition through E3-independent SUMOylation as observed in this study. SUMOylation of ERF1 was detected in the *in vitro* SUMOylation assay without adding E3 ligase (Fig. 10). Moreover, SCE1 could directly interact with ERF1 in the nucleus (Fig. 6), indicating that ERF1 could be SUMOylated by SCE1 in an E3-independent manner. On the other hand, in 2017, Cheng has already reported that UBC18 mediates ERF1 ubiquitination also through direct interaction with ERF1 (Cheng et al., 2017). However, the need for a specific E3 ligase for ERF1 ubiquitination cannot be ruled out because there was no *in vitro* evidence that UBC18 functions as an E3-independent E2 for ERF1 ubiquitination. In this study, we demonstrated that COP1 mediates the ubiquitination of ERF1 both *in vivo* and *in vitro* (Fig. 8 and 11), suggesting that UBC18 and COP1 might be the E2 and E3 ligases for ERF1 ubiquitination in Arabidopsis. An *in vitro* ubiquitination assay

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using both UBC18 and COP1 can further confirm this assumption.

In the previous studies, ERF1 was identified as positive regulator in both biotic and abiotic stress response, and its stability contributes to the light-dark oscillation of proline biosynthesis and many stress-responsive genes (Cheng et al., 2013; Cheng et al., 2017). *ERF1* gene expression is highly induced by abiotic stress but has almost no expression under normal conditions. Its induction level is not altered by the light-dark cycle (Cheng et al., 2017). This means that ERF1's interactions between SCE1 and COP1 play the major role in regulating ERF1's stability. It is well accepted that COP1 shuttles into the nucleus and forms the COP1/SPA E3 ligase complex which actively ubiquitinates many transcription factors such as HY5, HY5 HOMOLOG (HYH), LONG HYPOCOTYL IN FAR-RED 1 (HFR1), HECATEs (HECs), and B-BOX ZINC-FINGER PROTEIN FAMILY (BBX), among many others, and mediates their degradation through the 26S proteasome pathway (Xu et al., 2015; Lin et al., 2018; Han et al., 2020; Kathare et al., 2020; Song et al., 2020). It is possible that also through this kind of nucleus transition under darkness, COP1 mediates the light-dark oscillation of ERF1. Even though SCE1's shuttle between cytosol and nucleus according to light conditions was not reported so far, its protein stability might be regulated by light-to-dark transition. SCE1 protein abundance decreased under dark incubation for 1-4 h (Fig. 15). The reason why ERF1 is less SUMOylated and more ubiquitinated in the dark condition might due to less SCE1 protein abundance and more COP1 protein abundance in the nucleus (Fig. 15 and Appendix 6). This result also indicates that there might be other components regulating SCE1 protein stability in the light signaling pathway.

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4.3 SCE1 is involved in stress response through ERF1

SCE1 has been reported to be involved in various stress responses, including ABA-regulated abiotic stress responses and plant immunity against viral and bacterial infection (Nurdiani et al., 2018; Guo and Sun, 2017; Skelly et al., 2019; Rodrigues Oblessuc et al., 2019). In the abiotic stress analyses, we showed that SCE1 also plays roles in stress responses by regulating ERF1's stability. In the drought and salt stress tests, *sce1-4* and *sce1-7* mutants showed much less stress tolerance due to lower ERF1 protein abundance and decreased expression of ERF1 target genes (Fig. 13). These results suggest that SCE1 positively regulates the abiotic stress response by sustaining ERF1 stability. We also examined whether the protein stability of SCE1 is regulated through ethylene signaling, the major inducer of ERF1. SCE1 protein abundance slightly increased under ACC treatment, indicating that there are other components promoting stress responses through SCE1-ERF1 module.

4.4 SUMOylation and Ubiquitination might compete the same lysine site

SUMOylation and ubiquitination often function antagonistically because of their similar mode of action and binding properties (Kerscher et al., 2006). Ubiquitination might compete with SUMOylation through competing the same lysine residues on the substrates. For example, DREB2A suppresses its interaction with BPM2, a ubiquitin ligase component, consequently increasing DREB2A protein stability under high temperature (Wang et al., 2020). SUMOylation of RACK1B also enhances its stability

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and prevents its ubiquitination-mediated degradation in ABA response (Guo and Sun, 2017). Here, we showed that ERF1 might be both SUMOylated and ubiquitinated through the same lysine residues, K77, K177, K180 and K190 (Fig. 10-12). Moreover, SUMOylation of ERF1 is enhanced under light condition whereas its ubiquitination is enhanced under dark condition. These data suggest that ubiquitination of ERF1 might compete with its SUMOylation. To further understand the PTM of ERF1, we examined the protein stability of $ERF1^{4KR}$ under both light and dark conditions. As shown in Fig. 12, $ERF1^{4KR}$ remained stable under dark condition, suggesting that the ubiquitination site-mutated ERF1 could not be degraded in the dark ERF1 protein stability is mainly regulated by UPS system. The fact that ERF1 is labile in the dark also indicates that it might act as a positive regulator in light signaling pathway and plays the role of central hub for ethylene-light signaling crosstalk.

4.5 Conclusion

In previous reports, ERF1 was identified as the central hub between JA and ET signaling, and plays a positive role in both biotic and abiotic stress responses (Solano et al., 1998; Berrocal-Lobo et al., 2002; Lorenzo et al., 2003; Cheng et al., 2013). Recently, it was also shown that UBC18 mediates the ubiquitination of ERF1 and promotes ERF1 degradation under darkness through 26S proteasome pathway (Cheng et al., 2017). Here, we demonstrated that SCE1 directly interacts with ERF1 (Fig. 1 and 2). Moreover, SCE1 mediates the SUMOylation of ERF1 in the light, whereas COP1 mediates the ubiquitination of ERF1 in the dark, thereby promoting ERF1 fluctuation under light/dark cycle (Fig. 5 and 8). Consistently, the ERF1 target genes expressions were

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down-regulated in *sce1* mutant background (Fig. 14). Thus, SCE1 is functioning positively to regulate salt stress responses (Fig. 13). Based on our current and previous data, we propose a model that summarizes our findings as shown in Fig. 16. In the light condition, SCE1 interacts with ERF1 and facilitates its SUMOylation and stabilization, and thereby promoting stress response. In the dark, ERF1 is associated with UBC18 (Cheng et al., 2017) and COP1 complex, and is degraded through the 26S proteasome pathway. Taken together, our study reveals a novel mechanism by which SCE1-ERF1-COP1 module fine-tunes light-stress signaling crosstalk, which is possibly an important pathway of plant stress tolerance that is mediated through the light..

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FIGRURES AND TABLES

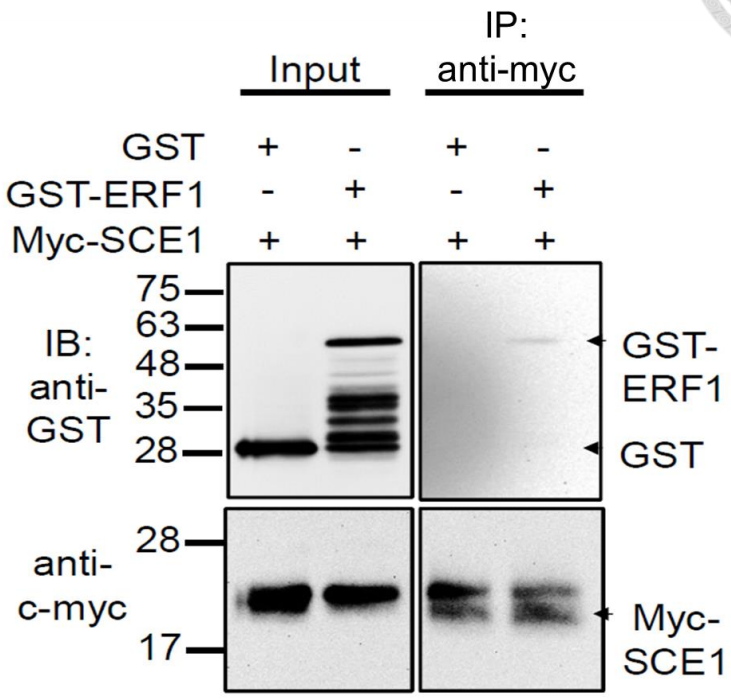

Figure 1. ***In vitro*** **Co-IP assay of ERF1 and SCE1.**

In vitro Co-IP assay shows that ERF1 can interact with SCE1. Cell lysates containing Myc-SCE1 proteins were incubated with anti-myc antibody to immunoprecipitate SCE1. The left panels show the input and the right panels show the immunoprecipitated proteins. Lower panels are the immunoblots probed with anti-c-myc antibodies. Upper panels show the immunoblots probed with anti-GST antibody. GST only (lane 1) and GST-ERF1 (lane 2) proteins were respectively incubated with Myc-SCE1.

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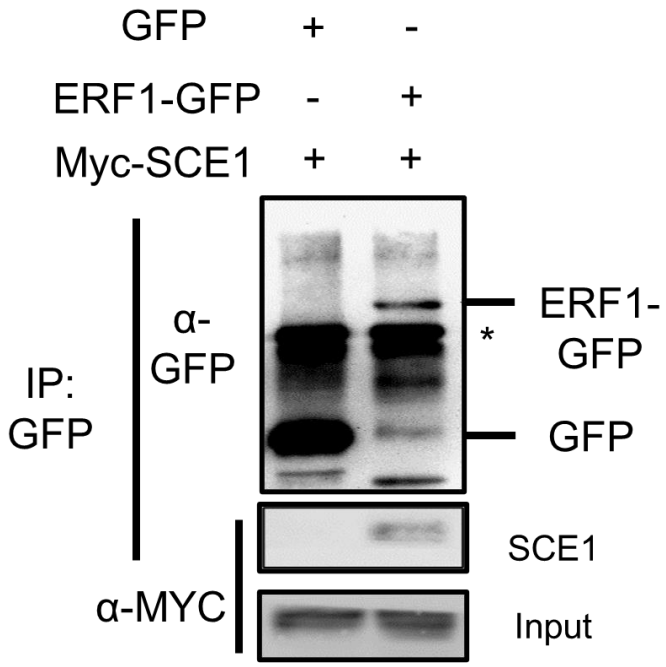

Figure 2. Co-IP assay of ERF1 and SCE1.

Co-IP assay showed ERF1-GFP interacts with Myc-SCE1. GFP only (lane 1) and GFP-ERF1 (lane 2) were separately co-transfected into Tobacco with Myc-SCE1. The immunoprecipitation was performed with anti-GFP antibodies and the proteins were detected with the antibodies as indicated.

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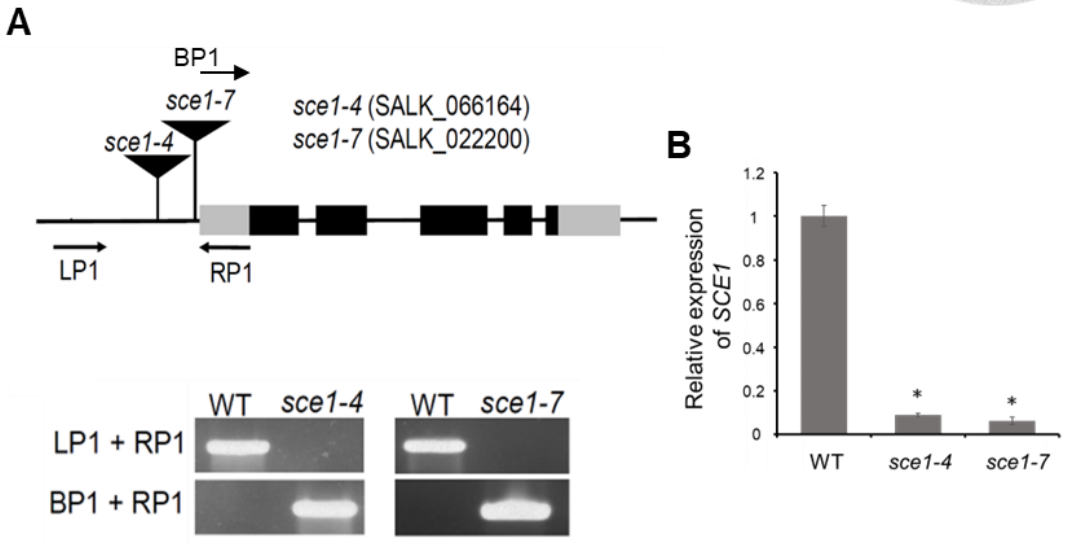

Figure 3. Identification of *sce1-4 and sce1-7* T-DNA insertion mutants.

(A) The gene diagram of *SCE1*. Boxes in black and grey represented exons and untranslated region (UTR) respectively, while the lines between exons represented introns. The T-DNA insertions were depicted as black arrowheads. The arrows showed the genotyping PCR-used primers. The lower panel showed genotype analyses of *sce1-4* and *sce1-7* homozygous mutants using PCR.

(B) Expression analyses of *SCE1* in *sce1-4* and *sce1-7* mutants by real-time quantitative RT-PCR. RT-PCR suggested that both *sce1-4* and *sce1-7* were knockdown mutants. *UBQ10* was used as an internal control and the bars indicate the SE (Student's *t* test; *, $P < 0.05$).

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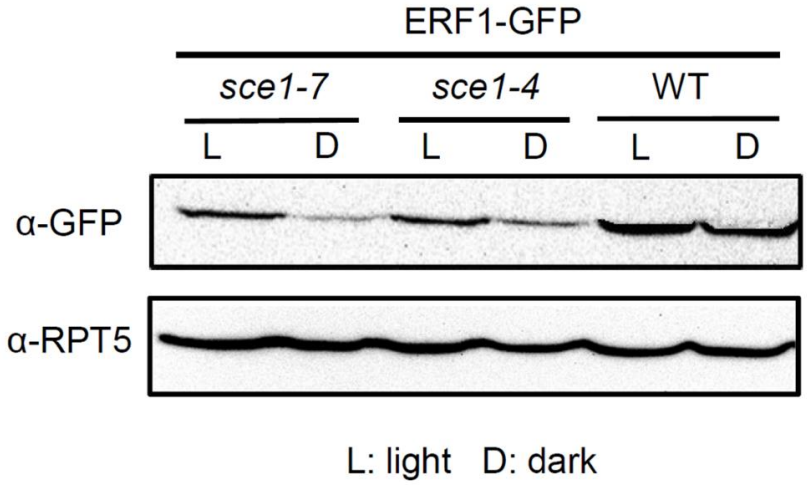

Figure 4. ERF1 protein level is regulated by SCE1 under light and dark condition.

Immunoblot analysis showing ERF1 protein degradation in 4-d-old etiolated seedlings of Col-0 (WT), *sce1-4* and *sce1-7* mutant. The seedlings were first illuminated for 4 h (WL) and then keep in the dark for another 2 h. RPT5 was used as loading control.

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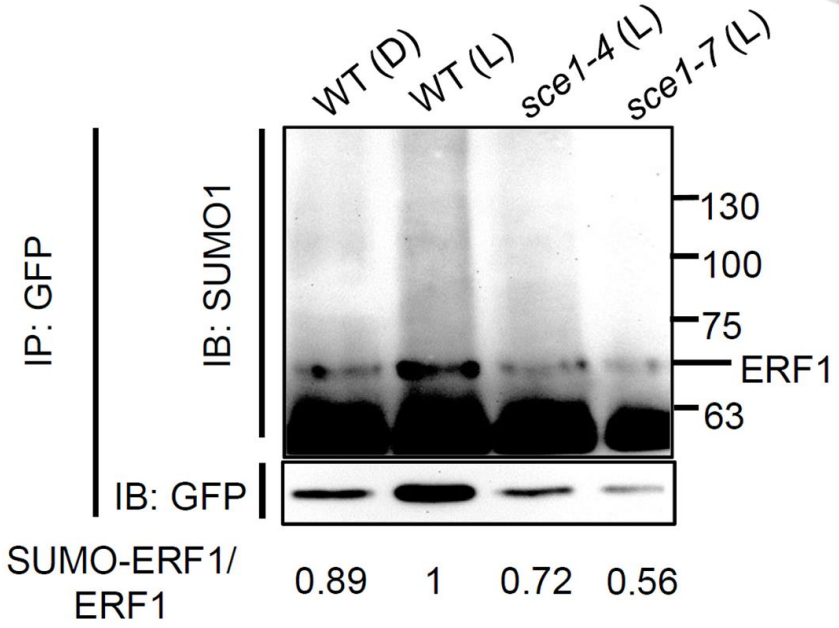

Figure 5. SUMOylation of ERF1 is mediated by SCE1.

Immunoblots analyses showing the relative ERF1 SUMOylation status in response to dark (D), light (L), and in sce1 mutants compared with the WT. Four-d-old etiolated seedlings were either kept in the dark or illuminated for 8 h and then immunoprecipitated with anti-GFP (rabbit) antibody after protein extraction. The immunoprecipitated samples were then separated on 6.5% SDS-PAGE gels and probed with anti-GFP (mouse) or anti-SUMO1 antibodies. The SUMOylation levels were quantified by ImageJ and the numbers underneath represent the relative levels of SUMO-conjugated ERF1 over ERF1 only.

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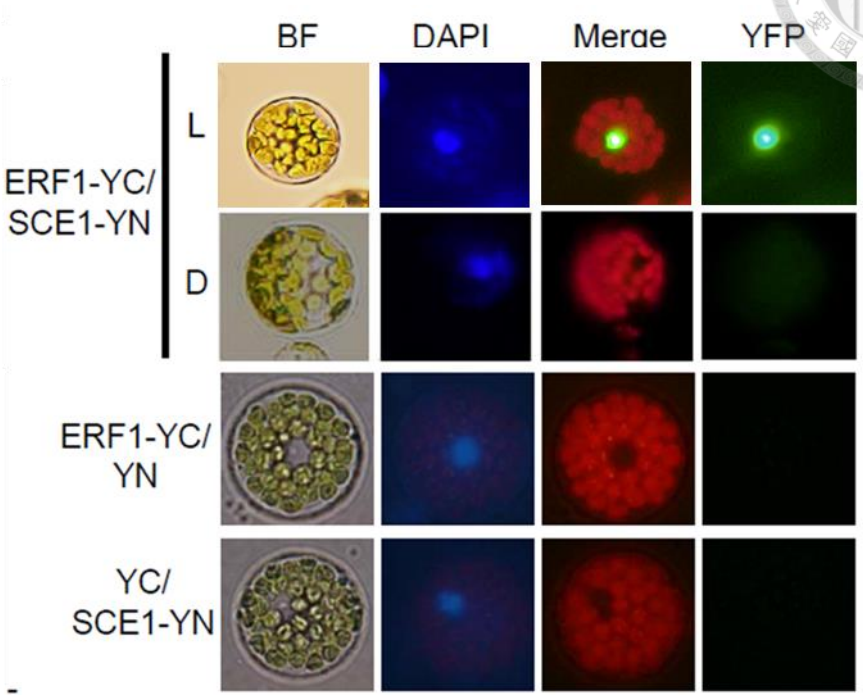

Figure 6. BiFC assay of SCE1 and ERF1 under light-to-dark condition.

BiFC assay verified the interaction between ERF1 and SCE1 interactions. The constructs indicated on the left were co-transformed into Arabidopsis protoplasts. L: 2 h light illumination before observation; D: 2 h dark incubation before observation. Bright field images (BF), Chl, 4', 6'-diaminophenylindole (DAPI; for nuclear staining), and yellow fluorescent protein (YFP) fluorescence were shown in each type of transformation combination.

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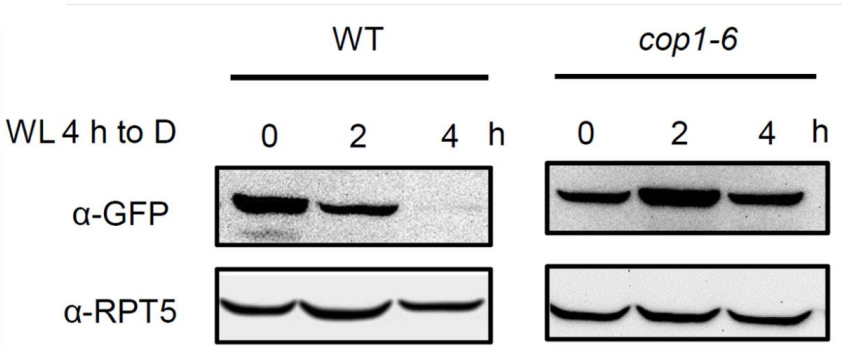

Figure 7. ERF1 protein level is regulated by COP1 in the dark.

Immunoblot analysis showing ERF1 protein degradation in 4-d-old etiolated seedlings of WT and *cop1-6* mutant. The seedlings were first treated with light irradiation (WL) for 4 h for ERF1 expression and then keep in the dark for another 2 and 4 h. RPT5 was used as loading control.

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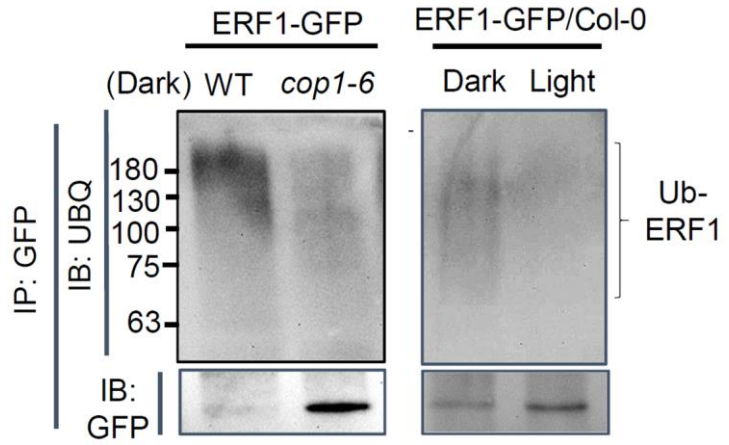

Figure 8. Ubiquitination of ERF1 is mediated by COP1.

Immunoblots analyses showing the relative ERF1 ubiquitination status in response to dark (right) and in *cop1-6* mutants compared with the WT. Total proteins were extracted from 4-d-old etiolated seedlings and then immunoprecipitated with anti-GFP (rabbit) antibody. The immunoprecipitated samples were then separated on 6.5% SDS-PAGE gels and probed with anti-GFP (mouse) or anti-UBQ antibodies.

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  1-50 MDPFLIQSPF SGFSPEYSIG SSPDSFSSSS SNNYSLPFNE NDSEEMFLYG
 51-100 LIEQSTQQTY IDSDSQDLPI KSVSSRKSEK SYRGVRRRPW GKFAAEIRDS
101-150 TRNGIRVWLG TFESAEEAAL AYDQAAFSMR GSSAILNFSA ERVQESLSEI
151-200 KYTYEDGCSP VVALKRKHSM RRRMTNKKTK DSDFDHRSVK LDNVVVFEDL
201-218 GEQYLEELLG SSENSGTW
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Figure 9. Prediction of SUMOylation sites in ERF1.

Amino acid sequence of ERF1. The underlined four lysine residues were predicted to be the potential SUMOylation sites.

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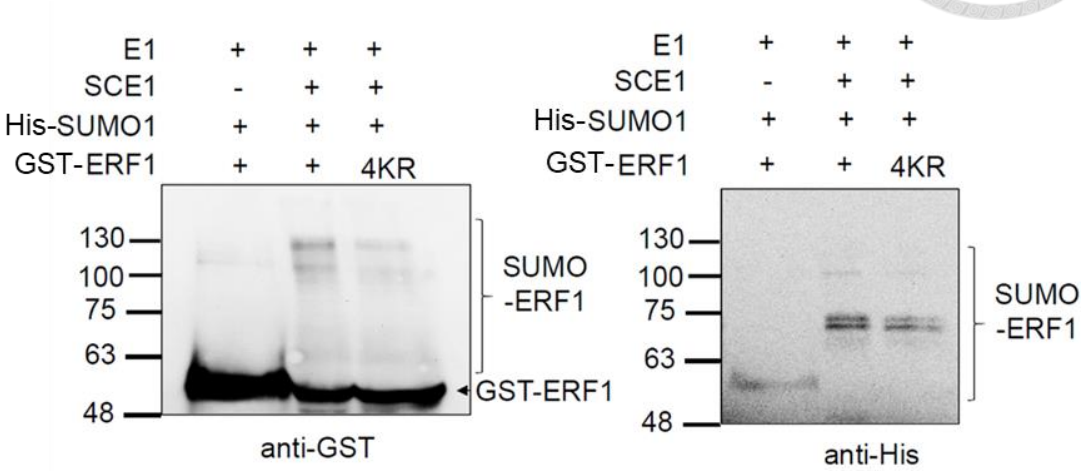

Figure 10. SCE1 promotes SUMOylation of ERF1 *in vitro*.

In vitro SUMOylation assay was performed for both ERF1 and SUMOylation site-mutated ERF1 (4KR). His-tagged SUMO1 was used. Left panel showed the immunoblot which was probed with anti-GST antibody for ERF1 detection. Right panel showed the immunoblot which was probed with anti-His for SUMO1 detection. Arrows indicate GST-ERF1.

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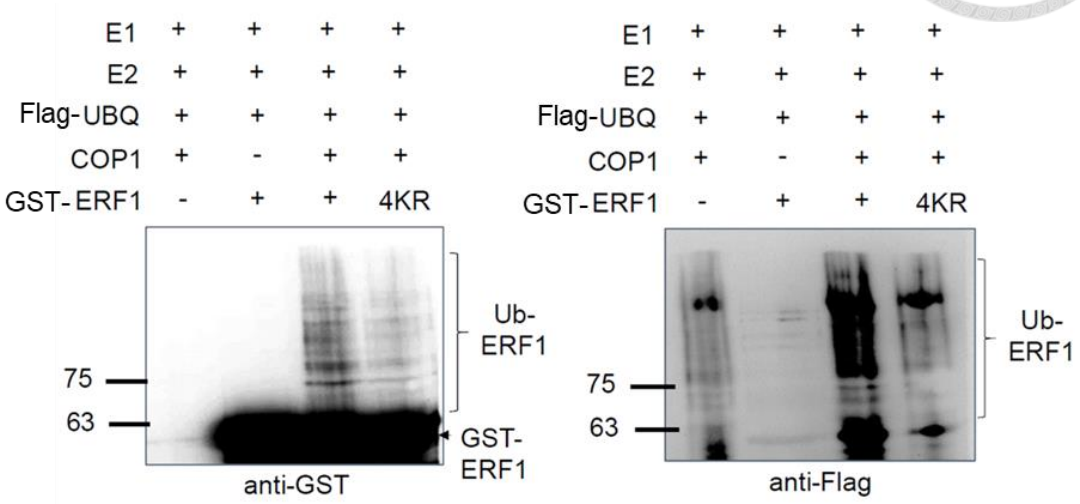

Figure 11. COP1 promotes ubiquitination of ERF1 *in vitro*.

In vitro ubiquitination assay was performed for both ERF1 and SUMOylation site-mutated ERF1 (4KR). Flag-tagged ubiquitin was used. Left panel showed the immunoblot which was detected by anti-GST antibody for ERF1. Right panel showed the immunoblot probed with anti-Flag for ubiquitin. Arrows indicate GST-ERF1.

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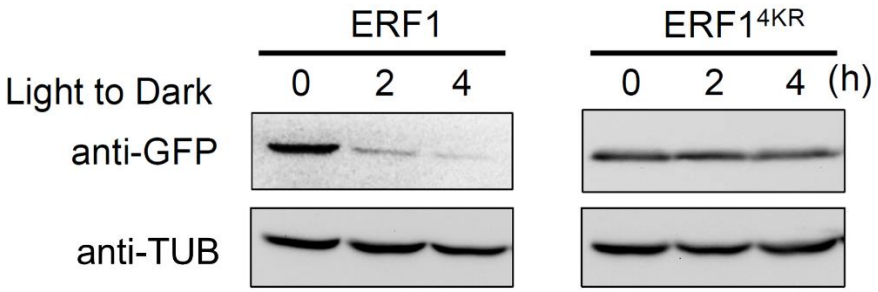

Figure 12. ERF1^{4KR} maintains the ERF1 protein level.

Immunoblot analysis showed the SUMOylation site-mutated ERF1 protein stability in the light-to-dark transition. The seedlings were first illuminated for 4 h and then incubated in the dark for up to 4 h. The samples were collected at the indicated time following dark incubation. Anti-GFP and anti-TUB were used for detecting ERF1 and as loading control, respectively.

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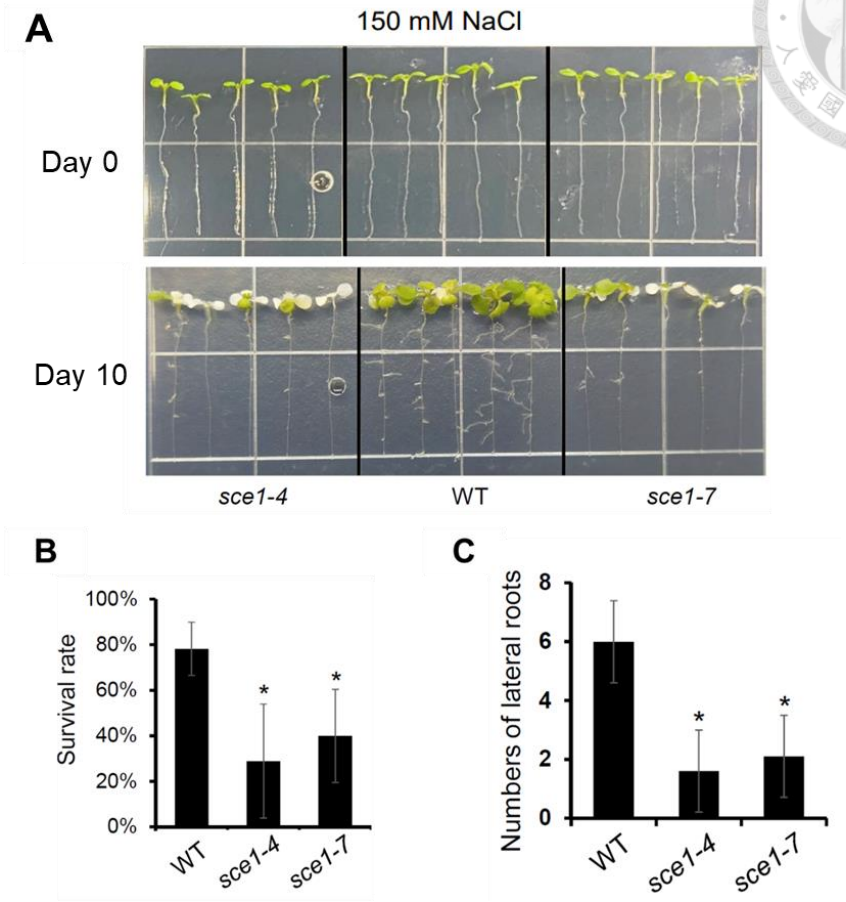

Figure 13. Phenotypic analysis of *sce1-4* and *sce1-7* mutants in response to salt stress.

(A) Salt-sensitive phenotypes of *sce1-4* and *sce1-7* mutants. Plants were grown on 1/2 MS agar plate for 7 d and then transferred to 1/2 MS plate containing 150 mM NaCl. The experiment was repeated more than 3 times with similar results. Numbers of lateral roots and (C) survival rates of WT, *sce1-4* and *sce1-7* plants under salt stress. The results are averages of three replicates. Error bars indicate SE (Student's *t* test; *, $P < 0.05$).

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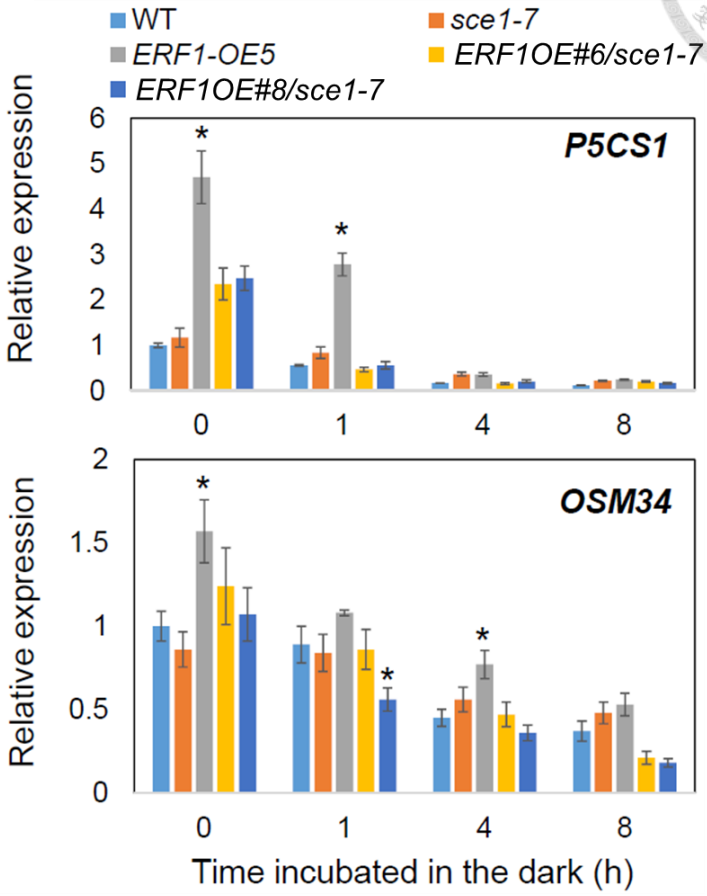

Figure 14. Gene expression of *P5CS1* and *OSM34* in the dark.

Expression patterns of stress-responsive genes *P5CS1* and *OSM34* in WT, *sce1-7*, *ERF1* overexpression lines (*OE5*) and *ERF1* overexpression line in *sce1-7* mutant background (#6 and #8). Two-w-old seedlings were first illuminated with light for 4 h and then incubated in the dark for up to 8 h. *PP2A* was used as internal control. Error bars indicate SE (Student's *t* test; *, P < 0.1).

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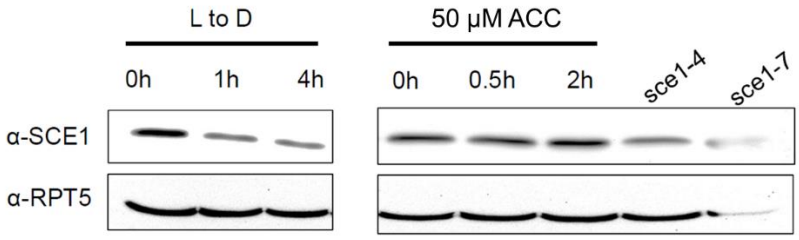

Figure 15. SCE1 is mediated by light and ACC treatment.

Immunoblot analysis showed the protein abundance of SCE1 in different conditions. Two-w-old seedlings were first illuminated for 4 h and then the samples were collected at the indicated times under light-to-dark transition or ACC treatment (50 μM). RPT5 was used as loading control.

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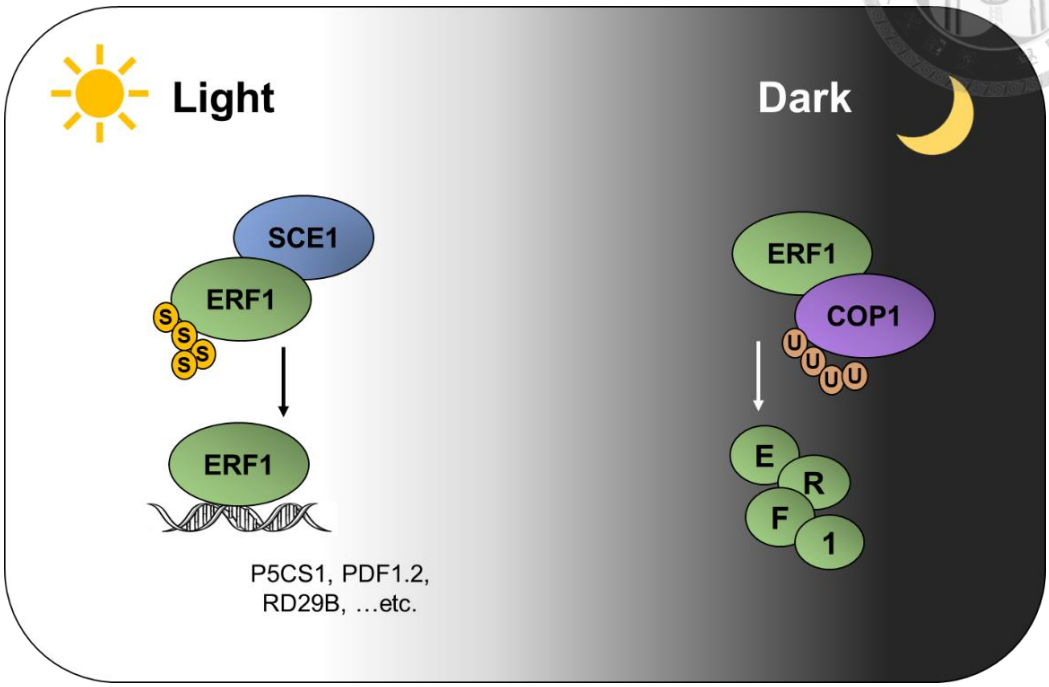

Figure 16. A proposed model showing SCE1- and COP1-mediated regulation of ERF1 in light and stress response.

Upon light exposure, SCE1 accumulates in the nucleus and interacts with ERF1. This interaction triggers the rapid light-induced SUMOylation of ERF1. The SUMOylated form of ERF1 is more stable and can bind to the promoter region of stress-responsive target genes and activate their expression to promote stress response. In the dark condition, COP1 accumulates in the nucleus and interacts with ERF1. ERF1 is then recruited to the COP1-SPA1 complex for rapid ubiquitination and subsequent degradation through the 26S proteasome pathway.

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Table 1. List of primers for qPCR

Gene	AGI code	Primer name and sequence (5'->3')
P5CS1	At2g39800	P5CS1-qPCR-F: CAACCATGAGTACTGTGCCAAGGC P5CS1-qPCR-R: CCACTTGGCGAAGGAATAGCTCTG
OSM34	At4g11650	OSM34-qPCR-F: GCAGAGATGCCCTGACGCTTAC OSM34-qPCR-R: CTCCTCGGTGACCATCTTGATCG
PP2A	At1g69960	PP2A-qPCR-F: TATCGGATGACGATTCTTCGTGCAG PP2A-qPCR-R: GCTTGGTCGACTATCGGAATGAGAG
SCE1	At3g57870	SCE1-qPCR-F: ATGGCTAGTGGAATCGCT SCE1-qPCR-R: TTAGACAAGAGCAGGATA
ERF1	At3g23240	ERF1-qPCR-F: GCGGAGAGAGTTCAAGAGTC ERF1-qPCR-R: TTCGTCTTCTTATTGGTCATTCTC
UBQ10	At4g05320	UBQ10-qPCR-F: ACTGGGAAAACTATCACTTTG UBQ10-qPCR-R: TCGGCCAAAGTTCTGCCATCT

doi:10.6342/NTU202203192

Table 2. List of primers for other usages

For cloning

Gene	Vector	Primer name and sequence (5'->3')
ERF1	pENTR/D-TOPO	ERF1-F: TTCACCATGGATCCATTTTTAATTCAGTCCCC ERF1-R-XhoI: CCGCTCGAGCCAAGTCCCACTATTTTC
	pGEX-4T-1	ERF1-F-EcoRI: CGGCCGGAATTCATGGATCCATTTTTA ERF1-R-XhoI: CCGCTCGAGCCAAGTCCCACTATTTTC
SCE1	pENTR/D-TOPO	SCE1-F: TTCACCATGGCTAGTGGAATCGCT SCE1-R-XhoI: CCGCTCGAGTTAGACAAGAGCAGGATA
	pMAL-p4x-1	SCE1-F-EcoRI: CCGGAATTCATGGCTAGTGGAATCGCT SCE1-R-SalI: ACGCGTCGACTTAGACAAGAGCAGGATA

For transgenic line screening

Gene	AGI code	Primer name and sequence (5'->3')
ERF1	At3g23240	ERF1-pEarlygate103-F:GGCTTTAGCCTACGATCAAGCTGCTTT ERF1-pEarlygate103-R: ACGTATCCCTCAGGCATGGCG

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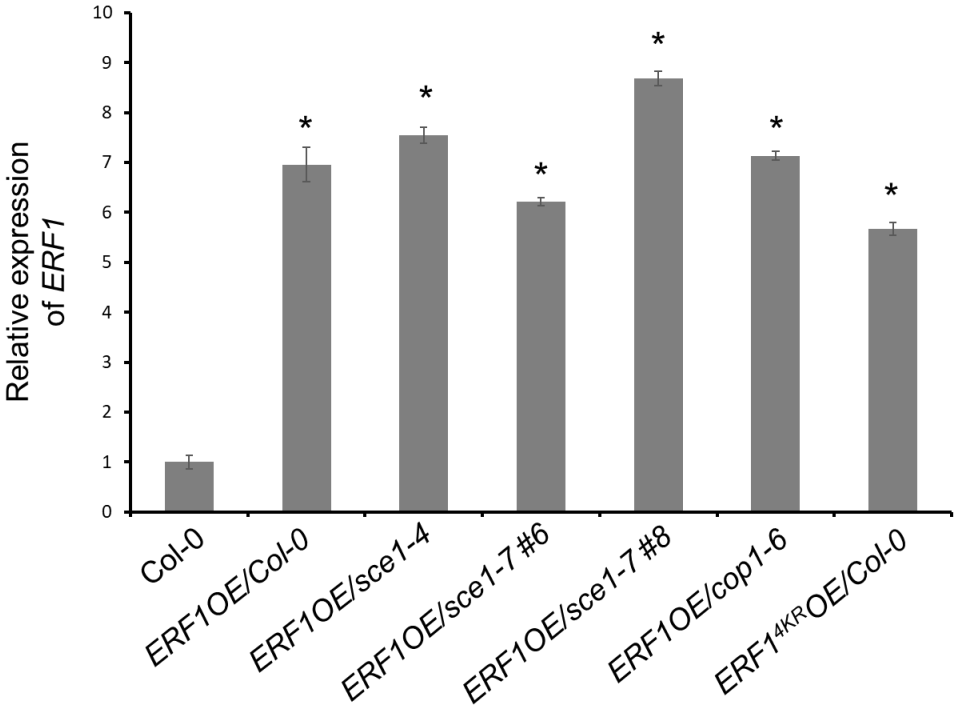
APPENDIX



Appendix 1. *ERF1* gene expression of transgenic lines.

Expression analyses of *ERF1*/*ERF1*^{4KR} in WT, *sce1-4*, *sce1-7, cop1-6* mutants by real-time quantitative RT-PCR. *UBQ10* was used as an internal control and the bars indicate the SE (Student's *t* test; *, $P < 0.05$).

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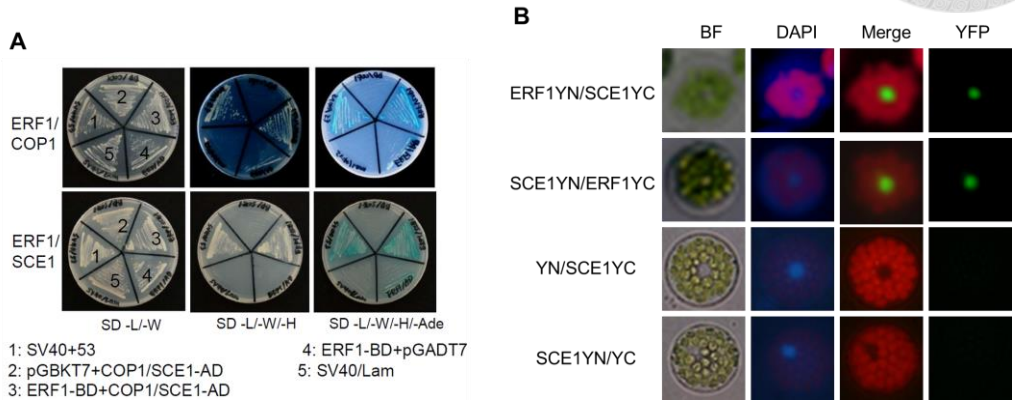

Appendix 2. Y2H and BiFC assay of SCE1 and ERF1.

(A) Yeast two-hybrid assay showed the interaction between ERF1 and SCE1. ERF1-BD and SCE1-AD were co-transformed into yeast cells which grew on the selective medium and exhibited β-galactosidase activity. (Wen-Chieh Kuo. 2013 thesis)

(B) Bimolecular fluorescence complementation assay showed the interaction between ERF1 and SCE1. ERF1-YN and SCE1-YC were co-transformed into Arabidopsis protoplasts. Bright field images (BF), Chl, 4', 6'-diaminophenylindole (DAPI; for nuclear staining), and yellow fluorescent protein (YFP) fluorescence were shown for each type of transformation combination. (Wen-Chieh Kuo. 2013 thesis)

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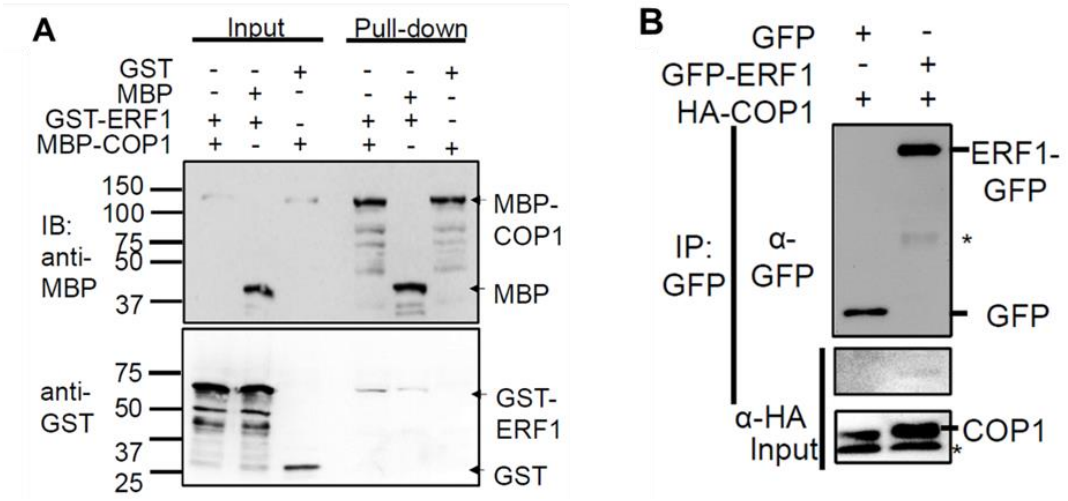

Appendix 3. Pull-down and Co-IP assay of COP1 and ERF1.

(A) Pull-down assay showed the interaction between ERF1 and COP1. Cell lysates containing GST only or GST-ERF1 proteins were incubated with the amylose resin conjugated with MBP only or MBP-COP1. (Zi-Bin Huang. 2022 thesis)

(B) Co-IP assay showed the interaction between ERF1 and COP1. Co-IP was carried out using the anti-GFP antibody and then probed with anti-GFP and anti-HA antibodies. The input and pellet fractions were indicated. (Zi-Bin Huang. 2022 thesis)

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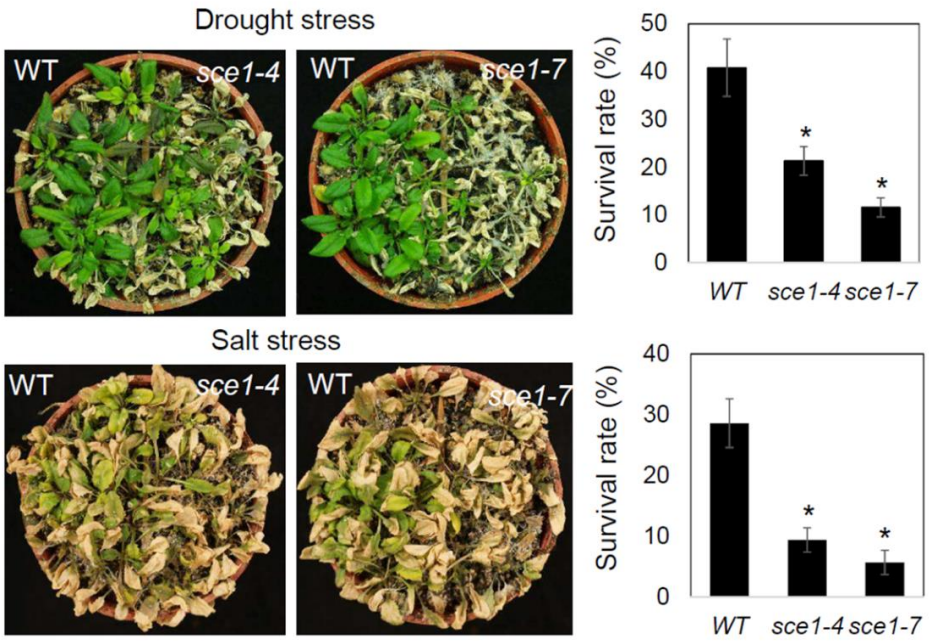
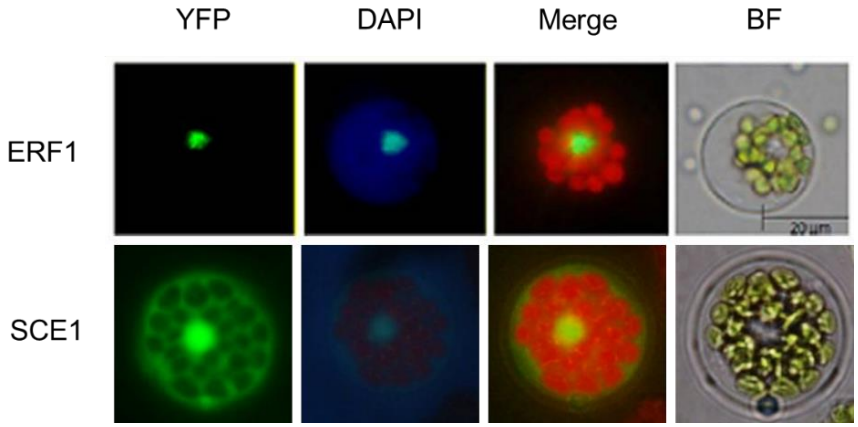


Appendix 4. Survival analysis of *sce1-4* and *sce1-7* mutants in response to salt stress.

Drought- and salt stress-sensitive phenotypes of *sce1-4* and *sce1-7* mutants were grown in soil compared with that of the Col-0 (WT). The results are averages of three replicates. Survival rates of WT, *sce1-4* and *sce1-7* plants are shown on the side (Student's *t* test; *, $P < 0.05$). (I-Ming Wang. 2015 thesis)

doi:10.6342/NTU202203192



Appendix 5. Subcellular localization of ERF1 and SCE1

Bimolecular fluorescence complementation assay showed the subcellular localization of ERF1 and SCE1. ERF1-GFP and SCE1-GFP were transformed into Arabidopsis protoplasts respectively. Yellow fluorescent protein (YFP) fluorescence, Chl, 4', 6'-diaminophenylindole (DAPI; for nuclear staining), and bright field images (BF) were shown for each type of transformation combination. (Wen-Chieh Kuo. 2013 thesis)

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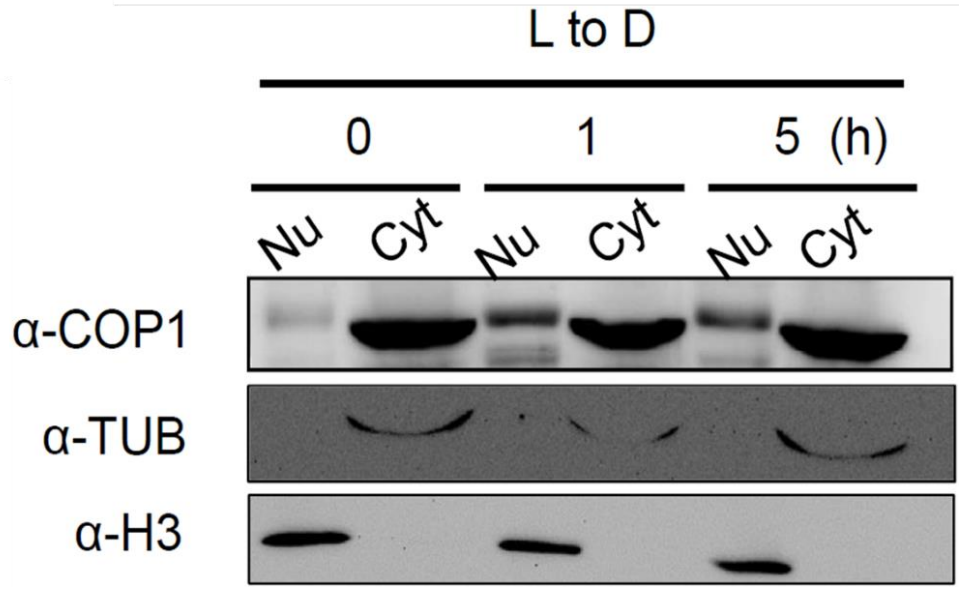

Appendix 6. COP1 protein abundance nuclear localization under light-to-dark transition.

The amount of COP1 levels in the nucleus or cytoplasm in WT plants under light-to-dark transition for a time period as indicated. Tubulin and histone H3 were used as loading controls for cytosolic and nuclear protein, respectively. (Zi-Bin Huang. 2022 thesis)

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論文口試問答集與討論建議

問答集:

1. Q: 在最後一張 model 圖中，左下角列出的下游基因只有 *P5CS1* 有做分析，其餘兩個卻沒有做，而你在 introduction 有提到 ERF1 會轉錄這麼多的下游基因，當初是如何挑選的並且有想要再嘗試其他基因嗎?(許富鈞老師)

   A: 首先我們會選擇這兩個基因是因為根據前面的 ChIP assay 發現這些列出來的都是 ERF1 的下游基因，我們是依據 ERF1 binding 的分數最高的這兩個基因 *P5CS1* 和 *OSM34* 去做測試，如老師剛剛所提到的 *RD29B* 和 *PDF1.2*，我們之後也會去嘗試更多這樣的基因。

2. Q: 在 *In vitro* SUMOylation 當中，看的出來 ERF1 和 $ERF1^{4KR}$ 之間的 SUMOylation 是有顯著差異的，只不過在 $ERF1^{4KR}$ 的部分他還是有 SUMO 的訊號，是不是因為你預測到的這四個位點可能是錯的?(許富鈞老師)

   A: 當初我們用特定網站在預測 SUMOylation 位點的時候，有分別篩選到 11 個位點，其中包含了像是特定胺基酸序列上面的 lysine，除了有用這樣 consensus sequence 的特性去找，但其實有很多的研究也有發現並不是所有的蛋白質都會依照這樣的規則，所以依照之前研究發現的 site，我們跑出了這樣的結果之後，選出了分數最高的這四個位點。老師剛剛提到說為甚麼 $ERF1^{4KR}$ 還是有 SUMOylation 訊號，我認為可能是因為也是有其他 lysine 去影響到的。

3. Q: 在蛋白質的層次有看到黑暗跟光照會調控 ERF1，尤其是在黑暗下 ERF1 會被降解，但是後面在處理鹽逆境的植物卻沒有分光照和黑暗，你要如何把生理的 stress tolerance 連結到 ERF1 的蛋白質穩定性?(洪傳揚老師)

   A: 因為蛋白質的層次就是幾個小時內發生的水平上差異，如果要跟鹽逆境這樣長時間的 tolerance 相比，我認為可能沒有辦法這麼直接的用光照跟黑暗去討論這麼長時間的鹽逆境，我們可能只能透過蛋白質的層次去發現它在光照或黑暗，ERF1 的蛋白質會受到調控，這樣的調控他可能會間接的去影響到植物整體對抗鹽逆境的表現。

4. Q: Ubiquitination 結果圖的訊號感覺較模糊，有沒有辦法分辨出一個清楚的條

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帶，是因為跑膠的條件需要改變還是有甚麼其他原因嗎?(洪傳揚老師)

A: 因為參考了 2013 年發表 ERF1 ubiquitination 的研究，他們的圖也是呈現這樣 smear 的訊號，我們認為可能是因為 polyubiquitination 的現象，所以在論文裡面也會有這樣類似的訊號。

5. Q: 圖 12 的 ERF1 蛋白質下降的水平比起圖 7 下降的量差了很多，有甚麼特殊的原因嗎?(洪傳揚老師)

   A: 我們認為可能是植物裡面的個體差異，因為當初我們只想看到 ERF1 漸漸降低的現象，所以可能就沒有去探討他下降的水平是不是要跟之前的 pattern 相似。

6. Q: 論問的前半段的 SUMOylation 跟 ubiquitination 雖然已經是一個很漂亮的故事，你認為要怎麼去突顯這個故事的 physiological significance 以及應用的層面，並且如何與植物的生理做結合?(洪傳揚老師)

   A: 因為目前我們發現到的這些東西都是在長日照的環境下，如果說在黑暗下這樣的植物會比較不抗逆境，換成在短日照植物當中是比較不利的，因為他的黑夜是比日照還要長的，可能就可以應用在這樣短日照的作物當中。

7. Q: 在實驗裡面用到很多的轉植株,你有做相關的 ERF1 基因表現量測試嗎?(常怡雍老師)

   A: 這些轉植株篩選到之後，我們有做一個 ERF1 的基因表現量分析，確定他們之間的表現量都是差不多的，才挑選這些轉植株去做後續的實驗，這部分的數據我之後會補在 appendix 當中。

8. Q: 在圖 1 的蛋白質交互作用實驗當中，ERF1 跟 SCE1 分別用菸草和大腸桿菌這樣不同的表現系統是有甚麼原因嗎?(常怡雍老師)

   A: 當初是有去嘗試兩個 protein 都用大腸桿菌去純化，但是我們發現他們不會 interaction，我們認為是因為大腸桿菌這樣的生物所產生的 SCE1 沒有辦法跟 ERF1 交互作用，可能是因為大腸桿菌跟植物體本身的修飾作用就有差異，因此 SCE1 需要在植物的系統裡面才能表現出正常的功能，這是我們初步的猜測。

9. Q: 在圖 4 結論的部分有提到 *sce1* mutant 在光照底下沒有辦法使 ERF1 穩定，所以 ERF1 在光照和黑暗下應該差不多，可是看起來好像還是在光照下比

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較穩定，這是不是代表了其實 ERF1 是不需要 SCE1 的?(常怡雍老師)

A: 我們認為在黑暗下可能有其他的東西在調控，導致我們看到的表現量就變少了，但因為當初我們就只純粹要跟 WT 做 ERF1 的比較，所以沒有想到老師提出的顧慮。

10. Q: 圖 5 的 ERF1 SUMOylation 也是呈現 smear 的狀態，有沒有可能他有特定大小的訊號?(常怡雍老師)

    A: 在一些 paper 裡面有發現 SUMOylation 是有一個特定的位置，但也有些是呈現 smear 的狀態，這主要是看這些 SUMO protein 接在這樣的 substrate 上是什麼樣的形式，所以有很多種不同的可能性。

11. Q: 圖 5 的 SUMO 1 抗體在 75 到 63 kDa 這樣的範圍，你有提到是 non-SUMOylated 的訊號，為甚麼還是會被 SUMO 1 的抗體所辨認呢?(常怡雍老師)

    A: 因為上下這兩張不同抗體的 membrane 其實是同一張，這一張 membrane 第一次是使用 GFP 的抗體，接著再去 strip 之後換成 SUMO 1 抗體，所以我們認為可能是因為沒有 strip 乾淨的關係。

12. Q: 圖 12 所用到的 ERF1^{4KR} 轉植株有沒有像 ERF1 去做 phenotype 的測試?(常怡雍老師)

    A: 因為 ERF1^{4KR} 的轉植株最近才篩選到的，這張圖是在印論文之前把他趕出來放上去的，所以這之後可以把他加到我們的 future work。

13. Q: 你所篩選到的 *sce1* mutant 的 SCE1 基因表現量非常低，但圖 15 用到的 SCE1 抗體為甚麼在 *sce1* mutant 中的偵測訊號還是這麼強?(常怡雍老師)

    A: 那時候我們沒有考慮到這個問題，只是單純的去找到 SCE1 的蛋白質會出現在哪一個位置，這部分會回去再想想。

建議:

1. ***sce1*** mutant 有比較不耐鹽的 phenotype，但是就 ERF1 OE 在 *sce1* mutant 下，*P5CS1* 跟 *OSM34* 卻沒有基因表現的差異，卻又提出 SCE1 會對下游影響的結論，感覺很像訊息傳遞只有到 ERF1 而已，所以建議你可以再多測試幾個基因，讓這個結論更有說服力。(許富鈞老師)

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2. 需要再補放各大量表現株的 ERF1 基因表現量，這樣對於下游基因表現的結果才更有可信度。（許富鈞老師）
3. 在 PPT 裡的 future work，好像不是每一個部分在論文裡的 discussion 都有提到，建議可以放進去，說服力就會高出很多。（許富鈞老師）
4. 中文標題和英文標題每一個字所表達的意思需要一致，至於論文內文的標點符號和小細節，需要再仔細的檢查一遍，還有其中像是 introduction 或 appendix 的數據，citation 都需要標註清楚。（許富鈞老師）
5. 結果的第一段需要把動機再加強，ACC 處理的實驗也需要把動機寫清楚。（許富鈞老師）
6. 基因表現量的圖表部分統計出來的顯著差異要標清楚是哪兩個比較出來的結果。（許富鈞老師）
7. *sce1* mutant 的 T-DNA 兩個都插在 promoter 上，sce1-4 距離起始密碼子 ATG 蠻遠的，卻可以 knockdown 這麼多的基因表現，希望可以在材料方法的部分補上這兩個突變株的 T-DNA insertion 各自距離 ATG 的長度。（洪傳揚老師）
8. 各個實驗的 protein level 建議可以做量化圖，在說明上會更有說服力。（洪傳揚老師）
9. 圖 6 的 BiFC 所呈現的 DAPI 染色有出現葉綠體的螢光色，可能需要再挑選其他只有染出細胞核的細胞再放上來。（洪傳揚老師）
10. 圖 7 的 western 圖可以將背景顏色調到相似會更有說服力。（洪傳揚老師）
11. 圖 2 的 Co-IP assay 是否真的可以稱作為 *in vivo*，這個需要去進一步確認其他的 paper 是否也這樣表示。（常怡雍老師）
12. 圖 3 的 panel C 需要把 Y 軸標上。（常怡雍老師）
13. 圖 5 若要比較光照和黑暗間的差異，其實應該也要多處理 *sce1* mutant 在黑暗下的 SUMOylation 組別去比較。（常怡雍老師）
14. Appendix 1 的 Y2H 的盤子拍照的時候沒有對齊，很容易解讀錯誤，建議可以更改旋轉圖片以更改。（常怡雍老師）
15. 圖 10 的 SUMOylation 除了被標註的大範圍 smear 訊號，在 100 kDa 有一個明顯的單一訊號，建議可以特別標註他可能是 SUMOylated 的 ERF1。（常怡雍老師）

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16. 在材料方法的部分建議可以提及 *sce1* mutant 的命名是根據 2003 年的某一個作者團隊。(常怡雍老師)
17. 若之後有 ERF1 的 T-DNA insertion 突變株，可以利用這樣的材料去看 ERF1 跟 E2 或是 E3 ligase 的 interaction，也可以做 genetic epistasis 的探討。(常怡雍老師)
18. 中文摘要部分的語句要再修改的更通順。(楊健志老師)

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